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New chemotherapy approaches based on mucus-penetrating nanoparticles as carriers of paclitaxel

Trabajo presentado por Dña.Patricia Calleja González para obtener el Grado de Doctor

Fdo. Patricia Calleja González

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El presente trabajo, titulado "**New chemotherapy approaches based on mucus-penetrating nanoparticles as carriers of paclitaxel**", presentado por DÑA. PATRICIA CALLEJA GONZÁLEZ para optar al grado de Doctor en Farmacia, ha sido realizado bajo nuestra dirección en el Departamento de Farmacia y Tecnología Farmacéutica de la Universidad de Navarra. Estimamos que puede ser presentado al tribunal que lo ha de juzgar.

Y para que así conste, firman la presente:

Fdo.: Dr. Juan M. Irache Garreta Fdo.: Dra. Socorro Espuelas Millán

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Albert Einstein

“Cuando creíamos que teníamos todas las respuestas, de pronto, cambiaron todas las preguntas”

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TABLE OF CONTENTS

ABBREVIATIONS

AUC	Area under the curve
CD	β-Cyclodextrin
Cl	Clearance
C_{max}	Peak concentration
CrEL	Cremophor EL®
CsA	Cyclosporin A
CYP450	Cytochrome P450
CYP3A4	Cytochrome P450 isoform 3A4
DHA	Docosahexaenoic acid
DCX	Docetaxel
DDS	Drug delivery system
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detection
EMA	European medicines agency
F	Bioavailability
FDA	Food and Drug Administration
g.i.	Gastrointestinal tract
HPLC	High performance liquid chromatography
HPCD	2-Hydroxypropyl-β-cyclodextrin
i.p.	Intraperitoneal
i.v.	Intravenous administration
MeCD	Methylated β-cyclodextrin
MDR	Multidrug resistance
MRT	Mean residence time
MTD	Maximum tolerated dose
NCI	National Cancer Institute

NHS	National health system
NP	Nanoparticle
NSCLC	Non-small cell lung cancer
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffered saline
PCL	Poly(ε-caprolactone)
PDI	Polydispersity index
PEG	Poly(ethylene glycol)
PLA	Poly lactic acid
PLGA	Poly (lactic-co-glycolic) acid
Pgp	P-glycoprotein
PTX	Paclitaxel
PTX-CD-NP	Paclitaxel-β-cyclodextrin complex loaded in poly(anhydride) nanoparticles
PTX-CD-PEG-NP	Paclitaxel- β-cyclodextrin complex in pegylated nanoparticles
PTX-HPCD-NP	Paclitaxel-2-hydroxypropyl-β-cyclodextrin in poly(anhydride) nanoparticles
PTX-PEG-NP	Paclitaxel loaded in pegylated nanoparticles
PVC	Polyvinylchloride
SEM	Scanning electron microscopy
SEOM	Sociedad española de oncología médica
SMEDDS	Self-microemulsifying drug delivery systems
$t_{1/2}$	Half-life in the terminal phase
T_{max}	Time to reach peak concentration
TPGS	D-α-tocopheryl-poly(ethylene glycol) succinate
V	Volume of distribution
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

ABREVIATURAS

AUC	Área bajo la curva
CD	β-Ciclodextrina
Cl	Aclaramiento
C_{max}	Concentración máxima
CrEL	Cremophor EL®
CYP450	Citocromo P450
CYP3A4	Citocromo P450 isoforma 3A4
CsA	Ciclosporina A
DHA	Ácido docosahexaenoico
DCX	Docetaxel
DDS	Sistemas de liberación de fármacos
EDTA	Ácido etilenediaminotetraacético
ELSD	Evaporative light scattering detection
EMA	Agencia europea del medicamento
F	Biodisponibilidad
FDA	Food and drug administration
g.i.	Tracto gastrointestinal
HPCD	2-hidroxipropil-β-ciclodextrina
HPLC	Cromatografía líquida de alta resolución
i.p.	Intraperitoneal
i.v.	Administración intravenosa
MeCD	β-Ciclodextrina metilada
MDR	Resistencia a fármacos
MRT	Tiempo medio de residencia
MTD	Dosis máxima tolerada
NCI	Instituto nacional del cáncer (USA)

NHS	Sistema nacional de salud (Reino Unido)
NP	Nanopartícula
NSCLC	Cáncer de pulmón no microcítico
P_{app}	Coeficiente de permeabilidad aparente
PBS	Buffer fosfato salino
PCL	Poli(ε-caprolactona)
PDI	Índice de polidispersión
PEG	Poli(etilenglicol)
PLA	Ácido poliláctico
PLGA	Ácido poli(lactic-co-glicólico)
Pgp	P-glicoproteína
PTX	Paclitaxel
PTX-CD-NP	Nanopartículas de poli(anhídrido) con complejos de paclitaxel-β-ciclodextrina
PTX-CD-PEG-NP	Nanopartículas pegiladas con complejos de paclitaxel-β-ciclodextrina
PTX-HPCD-NP	Nanopartículas de poli(anhídrido) con complejos de paclitaxel-2-hidroxipropil- β-ciclodextrina
PTX-PEG-NP	Nanopartículas pegiladas con paclitaxel
PVC	Policloruro de vinilo
SEM	Microscopía electrónica de barrido
SEOM	Sociedad española de oncología médica
SMEDDS	Sistemas de autoemulsionables para liberación de fármacos
$t_{1/2}$	Semivida biológica de la fase terminal
T_{max}	Tiempo para alcanzar la concentración máxima
TPGS	D-α-tocoferil-poli(etilen glicol) succinato
V	Volumen de distribución
VEGF	Factor de crecimiento endotelial vascular
WHO/OMS	Organización Mundial de la Salud

CHAPTER 1

INTRODUCTION

1. Cancer

Cancer is one of the most important diseases in the world for its incidence, prevalence and mortality. It is the second leading cause of death in many developed countries closely following heart diseases, and it is therefore a priority health problem.

In the United States of America (USA), one out of four deaths is due to cancer in both sexes and these statistics remain similar in Europe. In fact, cancer causes around 6 million deaths every year or 12% of deaths worldwide according to the World Health Organization (WHO). The most common cancer types are prostate (25-30%), lung (15%) and colorectal (10-15%) in males and breast (29-31%), lung (12-14%) and uterus/ovarian (5%) in females. More than 10 million people are diagnosed with cancer every year. So, by 2020 15 million new cases are estimated to be occurring every year. In 2010, about 1,530,000 new cancer cases and almost 570,000 deaths were calculated by the Cancer Statistics Review in the USA [1]. In the United States, approximately 16 million new cancer cases have been diagnosed since 1990. Statistics from National Cancer Institute (NCI) in USA stated that men have a little less than 1 in 2 lifetime risk of developing cancer while women have a little more than 1 in 3, in the US [2]. That implies that 30% of all Americans will develop some kind of cancer in their lifetimes. In Europe statistics have mentioned that one in three men and one in four women will be diagnosed with cancer during their lifetime. Each year the incidence of cancer increases in many developed countries. However, the mortality associated with this disease has been decreasing, reflecting the advances in early diagnosis and treatment.

In a recent report published by the European Cancer Observatory the incidence of cancer in Europe in the year 2010 was around 2,500,000 of cases with a mortality of around 1,230,000 patients [3]. With an estimated 3.2 million new cases (53% occurring in men, 47% in women) and 1.7 million deaths (56% in men, 44% in women) each year, cancer remains an important public health problem in Europe and the ageing of the European population will cause these numbers to continue to increase even if age-specific rates remain constant [3]. Focusing on Spain, the Spanish Society of Medical Oncology (SEOM) recently published a prospective report with estimations related to cancer for the year 2015. In this report, the overall incidence of cancer scheduled for the Spanish population in 2015 is 222,000 people (137,000 men and 85,000 women), being the most frequent type colorectal cancer, followed by lung and breast cancer. Cancer has resulted in the leading cause of death in men and the second in women, which has led to 26% of the deaths in the Spanish population. However, the mortality risk associated with cancer has decreased considerably from 1990 to 2007.

Cancer is characterized by the uncontrolled growth, invasion and sometimes, metastasis of abnormal cells that disrupt body tissue, metabolism, etc. and tend to spread locally or to distant parts of the body [4]. Normal cells divide and grow at a controlled rate. In contrast, cancer initiates as a change during replication in the genes of a single normal cell in any part of the body. Nonetheless, all the changes in genes during this cell replication will not turn the cell into a cancer cell. Some errors might remain unchanged and will not affect the normal processes in the cell. On the other hand, if the changes in genes affect either an essential function or tend to accumulate at a high rate, the set of instructions in the genes is changed and the cell becomes abnormal. Cancer is actually due to the accumulation of many such errors in replication. Life-threatening cancer develops gradually as a result of a complex mixture of factors such as complex interactions of viruses, genetic background, immune response and exposure to other risk factors which may favor the development of cancer.

Although our current understanding of what causes cancer is not complete, we now know enough to prevent at least one-third of all cancers and the treatment of such disease is in continuous investigation with important breakthroughs in the last decades.

1.1. Cancer treatment

Cancer treatment is based on three main pillars: 1) surgical excision, 2) radiation therapy and 3) chemotherapy. The value of these approaches depends on the tumor type and the development stage of the disease as well as the patient's general state. Chemotherapy, alone or in combination with other forms of therapy, is basically considered as the major approach for localized and metastasized cancer.

Chemotherapeutic agents can be divided into 3 categories: cytotoxic, biological and hormonal drugs. Firstly, the cytotoxic agents are the traditional drugs that work damaging cancer cells by interfering with DNA and/or inhibiting cell division. However, the main drawback of these anticancer drugs is that they present no specificity, that is, they kill healthy cells as well as cancer ones. Cytotoxic agents include alkylating agents (i.e. cyclophosphamide) [5, 6], antimetabolites (i.e. 5-fluoruracil (5-FU)) [7] and plant alkaloids (i.e. paclitaxel) [8]. As biological agents, monoclonal antibodies and cancer vaccines can be included and could be considered as targeted agents [9-12]. This therapy is also known as immunotherapy since it uses the body's immune system to fight against cancer. Finally, hormonal drugs are considered to interfere with hormone dependent pathways which are related to development or growth of cells. This kind of drugs is commonly used in the treatment of breast and prostate cancers. Some examples of hormonal agents are tamoxifen

or aromatase inhibitors (exemestane or anastrozole) [13-15]. Examples of chemotherapeutic agents are shown in **table 1**.

Table 1. Some examples of chemotherapeutic agents used in clinics, classified by their mechanism of action.

Chemotherapeutic agents	Mechanism of action	Examples
Cytotoxic agents	Interference with DNA and/or inhibition of cell division	**Alkylating agents:** cyclophosphamide **Antimetabolites:** 5-fluoruracil, methotrexate. **Plant alkaloids:** paclitaxel, docetaxel, vinblastine, irinotecan.
Biological agents	Designed to boost the immune system, either directly or indirectly	**Monoclonal antibodies:** Bevacizumab (Avastin®), Cetuximab (Erbitux®) **Interferons and interleukins** **Cancer vaccines**
Hormonal drugs	Interference with hormone dependent pathways	**Aromatase inhibitors**: exemestane, anastrozole **Selective estrogen receptor modulator:** Tamoxifen, Raloxifen

Oncology is one of the few areas of medicine where most patients are treated intravenously (i.v.) rather than receiving oral medication. Initially, the goal was to administer the maximum tolerated dose (MTD) of drug to optimize cell killing in a single episode [16]. The intravenous route is the most direct route and drugs administered like this avoid absorption sites since the entire drug is available in the bloodstream and therefore, the bioavailability is complete and immediate. After such administration, patients undergo several weeks with no drug therapy at all to allow bone marrow recovery. In all these treatments, patients have to suffer from the inconvenience of being treated in the hospital where specially qualified staff is required. In addition, this route is the most hazardous because potentially high concentration of the drug is delivered to normal tissue [17]. Furthermore, i.v. chemotherapy needs hospitalization, nursing and palliative care treatment in most cases. Although ambulatory pumps and new equipment has enabled home-based treatment, the i.v. administration remains inconvenient for patients. It is painful, it can lead to severe side effects (especially hematological) and thrombosis and in the long term it could be associated with infection [18]. In addition, patients' daily life is clearly influenced by the medication schedule.

Over the past years, quality of life has become of importance in the palliative setting and in oncology [19]. It is particularly important to allow a more comfortable life to patients during the final stage or in long-term treatments. In this context, it is where the new trend in chemotherapy

has risen. In the recent years, there has been an increasing interest in developing oral formulations either of the traditional chemotherapeutic agents or newly designed ones in order to facilitate chronic treatments since the oral route has been described as the preferred route of administration by patients [20]. The increase in oral chemotherapy for the treatment of cancer offers patients a more convenient, less invasive treatment option and ease of administration, particularly in chronic-like treatments and palliative care where prolonged drug exposure is desirable [21]. However, oncologists have a major concern regarding the oral treatment in cancer, mainly related to patient's adherence to the treatment and to the unpredictable bioavailability that drugs may present compared to i.v. administration [21].

1.2. Oral chemotherapy

Oral chemotherapeutic drugs have been available for the past decades including numerous agents such as chlorambucil, cyclophosphamide, methotrexate and 6-mercaptopurine. However, in the past decade there has been an expansion in the development of oral anticancer agents available.

Nowadays, 10% of the chemotherapy treatments are provided to patients as oral formulation. However, the National Comprehensive Cancer Network (NCCN) in the USA predicted some years ago that by the year 2013 this percentage would jump to 25% [22]. Between the years 2005 and 2007, over a dozen new oral cancer chemotherapy agents were approved by the Food and Drug Administration (FDA) [23, 24]. **Table 2** shows numerous oral anticancer therapeutic agents approved by the FDA over the past years.

The first drug approved in the USA for its oral use against cancer was mercaptopurine (Purinethol®) in 1953 in order to treat several types of neoplasms. Since this first approval, the number of oral anticancer drugs has been increasing significantly. In the last five years the therapeutic tendency has been characterized by a steady increase in the availability of oral anticancer drugs with more than 20 oral cytotoxic drugs currently approved for clinical use in USA and Europe. Moreover, one quarter of all the anticancer agents under development is oral drugs [25]. In this regards, there is growing consensus that oral therapies should replace i.v. alternatives as long as they can demonstrate, at least, equivalent efficacy and that tolerability is not compromised. 82% of US oncologists interviewed in a survey stated that their key consideration in selecting an oral chemotherapy agent was efficacy.

Currently, more than 20 oral cytotoxic agents are available **(table 2)**. So, from a clinical point of view why is there still so much reliability on the intravenous route as choice for anticancer therapies? The main reason for this is that oral formulations of anticancer agents have been available for relatively little time and therefore, are still considered as new formulations. Another possible reason could be based on the general assumption or belief that chemotherapy should be administered by means of the intravenous route. However, this trend is changing recently since new alternative drug delivery systems are being studied as well in order to facilitate the oral delivery.

At first glance, oral chemotherapy appears to provide only benefits. With the oral administration of drugs the painful and more inconvenient aspects of the i.v. access disappear: patients spend more time at home and cancer patients are also in a position of greater autonomy in which they have greater control over their treatments because they are the ones administering the drugs [23].

There is no question that oral chemotherapy offers many advantages which promote the well-being of cancer patients and their families. Yet, oral administration of anticancer agents entails other concerns regarding compliance/adherence, absorption and variable pharmacokinetics.

Table 2. Oral chemotherapeutic agents approved by the Food and Drug Administration (FDA) in the USA.

Drug	Brand Name	FDA Approval	Indication	Marketing Company
Mercaptopurine	Purinethol (tablet)	1953	Acute lymphatic leukemia	Teva
Methotrexate sodium	Methotrexate (tablet)	1953	Breast, head and neck, small cell lung cancers, rheumatoid arthritis	Dava Pharm Inc
Busulfan	Myleran (tablet)	1954	Chronic myelogenous (myeloid, myelocytic, granulocytic) leukemias	Aspen Global
Cyclophosphamide	Cytoxan (tablet: discontinued)	1959	Leukemia, cutaneous T-cell lymphoma, breast, lung, ovarian cancers and myeloma	Baxter Healthcare
Thioguanine	Tabloid (tablet)	1966	Acute nonlymphocytic leukemias	GSK
Capecitabine	Xeloda (tablet)	1998	Advanced breast cancer resistant to paclitaxel in combination with anthracyclines	Hoffman La Roche
Temozolomide	Temodar (capsule)	1999	Newly diagnosed glioblastoma multiforme	Merck
Etoposide	Etoposide (capsule)	2001	Prostate cancer, Kaposi's sarcoma, small cell lung cancer and lymphoma	Mylan
Imatinib mesylate	Gleevec (tablet)	2003	Chronic myeloid leukemia and inoperable or metastatic malignant gastrointestinal tumors	Novartis
Gefinitib	Iressa (tablet)	2003	Non-small-cell lung cancer (NSCLC)	Astra Zeneca
Erlotinib	Tarceva (tablet)	2004	Monotherapy in advanced or metastatic NSCLC after failure of at least 1 prior chemotherapeutic regimen	OSI Pharm
Sorafenib tosylate	Nexavar (tablet)	2005	Advanced renal cell carcinoma	Bayer Healthcare
Sutinib malate	Sutent (capsule)	2006	Gastrointestinal tumor after disease progression or intolerance to imatinib.	CPPI CV
Dasatinib	Sprycel (tablet)	2006	Chronic myeloid leukemia; Philadelphia chromosome-positive acute lymphoblastic leukemia	Bristol-Myers-Squibb
Topotecan hydrochloride	Hycamtin (capsule)	2007	In combination with cisplatin, for the treatment of cervix carcinoma	GSK

Data obtained from Curtiss at al. [26] and www.fda.gov (GSK: GlaxoSmithKline)

1.2.1. Misconceptions about oral chemotherapy

Generally, the main misconceptions associated with the oral administration of anticancer agents are related to side effects and toxicity as well as efficacy. Investigations and reports on clinical aspects regarding oral chemotherapy suggest that many patients and their families believe that oral chemotherapy has minimal, if any, side effects. In fact, although some oral cytotoxic agents may be reasonably well or even better tolerated than the i.v. regimens, all have side effects. Comparative studies between oral and intravenous agents, both of which have similar mechanisms of action, suggest that side effect profiles may be somewhat more favorable with oral chemotherapy [24, 27]. In this context, some patients could incorrectly assume that oral chemotherapy is not “real” treatment, more like taking a vitamin or other types of drugs considered as minors [22]. Patients must understand that oral chemotherapeutic agents have side effects that are similar to the intravenous therapies. In fact, the side effects appearing in oral chemotherapy should be monitored in the same way as for the intravenous therapies

Still, another misconception from cancer patients is that oral chemotherapy is less effective than the intravenous therapy. In a survey of patients starting oral chemotherapy for metastatic breast cancer, Catania and coworkers observed that approximately 10% of patients thought that oral chemotherapy was the last effort in their treatment. They perceived the treatment as suboptimal [28].

In order to prevent these misconceptions taken by patients and their families when explained the oral chemotherapeutic regimens, it is imperative that patient education should be incorporated into the prescription practice and the therapeutic team should emphasize that oral cancer therapy must be taken seriously. Patients must be informed of all the effects the drugs may present and in the same manner, the potential benefits. Not all the oral chemotherapy under all circumstances will yield benefit just as not all the intravenous chemotherapy will yield benefit either.

1.2.2. Adherence to treatment

Compliance has been defined as “the degree or extent of conformity to the recommendations about day-to-day treatment by the provider with respect to the timing, dosage and frequency” [29]. Compliance is also called adherence because the latter term is generally believed to have a less pejorative and less judgmental connotation. In any case, adherence is distinguished from persistence, which is defined as the duration of time from the initiation to discontinuation of therapy [25]. Adherence can be described as the concordance or obedience of

the patient to the medical advice received in relation to the administration of the medicines. In the same way, compliance is a related term that also applies [30].

From a clinician point of view a major concern related to the oral administration of any kind of drug is the patient adherence to the treatment. The optimal adherence occurs when a patient follows the prescribed regimen exactly [30]. This is; if the patient does not miss any dose, no extra doses are taken and no doses are taken in the wrong quantity or at the incorrect time. On the opposite side, another concern can be related with the over-adherence when patients are taking too much of a medication.

In all cases, the concern from the medical point of view is real and little information is available. However, the hypotheses for non-adherence are mainly associated with individual characteristics, features of the disease and the treatment regimen and aspects of the medical care. **Figure 1** depicts a model of adherence where factors such as personal aspects and treatment factors could interact. In some cases, the overuse of drugs has been associated with confusion by patients about what to take and even, with the thought that extra medication could induce a better therapeutic effect. Studies have shown that adherence is related to socio-demographic characteristics, type of regimen (especially side effects and duration) and characteristics of the illness (symptoms and seriousness). The main factors described to influence on adherence have mainly been: complex treatment regimens, behavioral changes, inadequate supervision, poor communication with health care providers, patient's dissatisfaction with care, personal health beliefs and personal situation of patient. Providing patients with a good educational background on when and how to take their medications is of paramount importance.

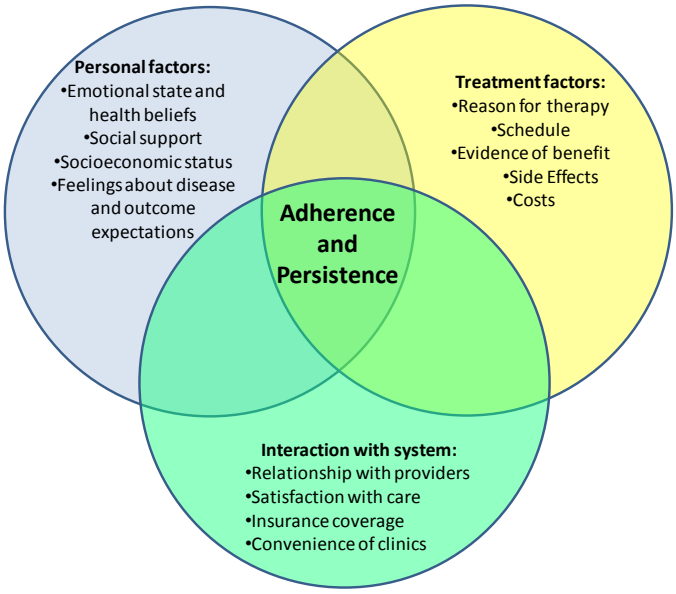

Figure 1. Factors influencing adherence to treatment from a patient's perspective.

The majority of oncologists consider oral chemotherapy advantageous but not all the patients are good candidates for oral treatment. In trials, approximately 50% of the patients will discontinue taking the medication within 6 months of the initiation [31]. Unfortunately, there is currently no well established mechanism to assess adherence. Monitoring techniques are not well determined but they can be classified as direct or indirect methods. In the direct methods, the simplest is to directly observe therapy, which is very easy for i.v. administration but it is rather limited for the oral route [22]. The alternative is the pharmacokinetic measurement. However, this measurement can also be manipulated by the patient.

Amongst the indirect methods, questioning the patient about adherence, following a patient diary, pill counts, or electronic medication monitors are the most commented. However, patient self-reporting may sometimes be unreliable and rather inaccurate [4, 22]. On the other hand, the main problems with electronic methods are that there is no proof that the tablet has been taken since the act of opening a pill counter does not necessarily mean that the patient really took the medication. In addition, these systems are expensive [31].

Uncertainty about patients' ability to adhere to recommended prescribed treatments can create therapeutic dilemma for the physician. In these cases, the physician cannot distinguish between lack of responsive to treatment due to true chemotherapy resistance or non-adherence. Besides, the non-adherence also results in an increase of costs for the healthcare system; not only for the changes in treatments but also for the higher visits to clinicians.

1.2.3. Economic issues

During several clinical trials on oral chemotherapy, pharmaco-economic analyses were carried out to evaluate the cost effectiveness of new oral treatments compared to the i.v. administration. To date, these analyses have been performed for certain drugs, such as capecitabine [32], capecitabine/cisplatin [33], ibandronate [34, 35] and tegafur-uracil [36].

In the United Kingdom, Cassidy et al [32] conducted a study in which oral capecitabine was compared to i.v. 5-fluoruracyl (5-FU). They evaluated the total costs of the treatments taking into account all the direct costs to the National Health System (NHS) such as: cost of chemotherapeutic drugs, costs of visit for drug administration, cost of hospital use, costs of clinician visits related to adverse effects and treatment and costs of ambulance trips. They added the social costs too and the total costs for the oral capecitabine were around £3500 while for the treatment with 5-FU it was £8,500. With these results, they denominated oral capecitabine as a dominant treatment

strategy from an economical point of view since the cost savings were around £5,500 per patient. The same study was conducted in the USA considering similar parameters and the results showed again that the cost savings for oral capecitabine against 5-FU were around 2000$ per patient and treatment [27].

In Spain, a comparison of costs between the association of oral cisplatin/capecitabine and i.v. cisplatin/5-FU was conducted in patients suffering gastric cancer. The oral regimen was estimated to reduce annual drug costs in 1,333€ per person approximately *versus* the i.v. administration. Furthermore, considering the administration of the drugs and the costs related to the side effects derived, the annual costs reached 2,800€ for the oral cisplatin/capecitabine against 4,000€ for the i.v. cisplatin/5-FU treatment [33]. Other studies confirmed these preliminary results and supported the benefit of oral chemotherapy from an economical point of view.

Orally administered chemotherapy provides a valuable option for treating cancer patients because of its advantages, including ease of administration, convenience for patient and reduced need for hospitalization. The increase in the use of oral chemotherapeutic agents affects many relevant aspects in clinical practice and requires a particular consideration of patient and physician perspectives, possible differences in activity, toxicity and quality of life, management and costs between i.v. and oral anticancer agents. Its administration also invokes safety issues that require further acknowledgement. Thus, patients need full information about efficacy and toxicity of oral treatments [21, 25].

As stated by the Spanish Society of Medical Oncology, the responsibility of safe and effective delivery of anticancer oral therapy can only be guaranteed within the heart of multidisciplinary teams who have undergone thorough training in all aspects of comprehensive cancer care and who are governed by strict protocols, with wide therapeutic experience and are coordinated by medical oncologists [37]. As more oral agents become available, more effort should be done to accomplish the goal of the treatment and facilitate the oral regimen in the anticancer therapy.

1.3. Metronomic chemotherapy

Anti-cancer drugs were originally developed in order to directly kill tumor cells. Initially, traditional chemotherapy has been mainly focused on administrating the maximum tolerated dose (MTD) of the anticancer agent without causing life-threatening levels of toxicity in order to kill the maximum number of cancer cells, because this schedule has the most potent effect against a cancer patient. These high doses of drugs are compensated with several weeks with no

administration at all. However, this traditional trend of anticancer therapy has changed in the past recent years to the opposite: the interest of the therapy is to administer lower doses of the drugs but sustained in time. Thus, this new concept of chemotherapy is based on chronifying regimens rather than attempting high doses.

Metronomic chemotherapy refers to the close, regular administration of a chemotherapeutic agent at relatively low (non-toxic) doses, over prolonged periods with no extended drug-free breaks [16]. It can be seen as a form of long-term maintenance chemotherapy that can be used on its own, or perhaps, more importantly, combined over long periods of time with biologic targeted therapies, especially antiangiogenic drugs. Furthermore, metronomic delivery or frequent administration of cytotoxic drugs at doses much lower than MTD may have antiangiogenic properties [16]. This metronomic regimen could maximize the growth-limiting effects on the tumor vasculature.

The advantages of metronomic therapies include reduced acute toxicities such as high grade myelosuppression, hair loss, vomiting, nausea, damage to the intestinal mucosa, etc; reduced costs when using off patent chemotherapeutic drugs and increase convenience when using oral drugs which can be taken at home [38]. This recent concept in cancer treatment has promoted the thought that “the more frequent the better” and that” less is more”. This shift in therapy has, in some way, been pushed by the results obtained with the traditional treatment regimen based on MTD, which has not provided the kind of survival benefits expected [39]. In addition, this traditional approach is also very expensive and highly toxic. In contrast, the so-called metronomic therapy with a more frequent administration of at least one chemotherapeutic agent has shown clear benefits in several randomized phase III clinical trials [40, 41]. Unfortunately, there is no “minimum-dose” standard defined for chemical drugs or ionizing radiation in medical literature. This is due mainly to the multiphase dose response in living organisms. **Figure 2** shows a schematic representation of different therapeutic regimens used in chemotherapy.

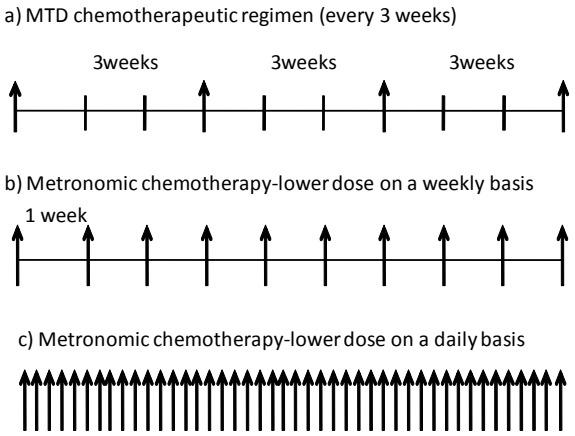

Figure 2. Different chemotherapy regimens. a) Standard chemotherapy regimen based on maximum tolerated dose (MTD) followed by a long drug-free period. b) and c) examples of metronomic based chemotherapeutic regimens where chemotherapy agents are administered more regularly with no drug-free interruptions.

With the results from the initial clinical trials based on metronomic approaches, the new trend in clinics has been to administer weekly or even daily treatment. Indeed, it is becoming more common to administer taxanes, paclitaxel and docetaxel, and many other cytotoxic drugs to patients with certain types of cancer, such as breast, ovarian, lung cancers, on a weekly schedule [42, 43]. Frustrated with conventional MTD outcomes, now physicians are increasingly resorting to the frequent repetition of low-dose chemotherapy administered regularly for a longer time. It is expected that low-dose strategy may also produce better outcomes in patients suffering with chronic diseases [44].

1.3.1. Mechanism of metronomic therapies

Metronomic therapy is thought to work primarily through antiangiogenic mechanisms and has the property to kill resistant cancer cells and/or to inhibit tumor growth while significantly reducing undesirable toxic side effects [45]. During angiogenesis, new endothelial cells are extremely fragile as they multiply and migrate to a tumor in response to chemical signals secreted by tumor cells, and eventually form tubular structures to give rise to new vessels. Endothelial cells are the cells that line the blood vessels. The endothelial cells that form the lining of newly formed blood vessels are the targets for this type of therapy. Angiogenesis, or the formation of new blood vessels, is necessary for tumors to grow and to spread. Stopping this process has been shown to be an effective way to stop tumor growth and metastasis.

The strategy in therapy is to attack the cancer-feeding endothelial vasculatures as well as the solid tumor itself. The endothelial cells engaged in angiogenesis are extremely sensitive to killing by

cytotoxic drugs, much more than most cancer cells. Thus, when low-dose chemotherapy is administered on a daily schedule, the continual death of endothelial cells attempting to form new blood vessels can substantially disrupt the angiogenic process, slowing it down notably. There is a considerable body of evidence that even very low, non-toxic doses of chemotherapy drugs, when delivered frequently for a prolonged period of time can retard tumor blood vessel growth by destroying cells. Simply put, if you starve the blood supply to the tumor, it is deprived of necessary oxygen and nutrients needed to survive and subsequently, tumor cells will die by apoptosis.

Gasparini has conducted scientific debate on the strategy, drug development and clinical indications for antiangiogenetic agents and their scheduling [46-48]. Since it is clear that endothelial cells in the angiogenic site proliferate rapidly and these cells are susceptible to anticancer drugs. Browder and coworkers considered that an appropriate schedule of chemotherapy damages angiogenic endothelial cells as well as tumor cells [49], and that the treatment-free period in conventional chemotherapy also permits the growth of angiogenic endothelial cells and supports tumor cell re-growth. Based on this idea, they developed a novel administration schedule, called an antiangiogenic schedule and targeting rapid growing endothelial cells, in which anti-cancer drugs are administer in a lower dose at shorter intervals than in the conventional schedule. This antiangiogenic schedule showed apoptosis of endothelial cells in tumor bed and suppressed tumor growth more efficiently than the conventional schedule. The antiangiogenic schedule also suppressed drug-resistant tumor cell growth in solid tumor-bearing mice. Moreover, when this schedule was combined with the treatment of an angiogenesis inhibitor, even a drug-resistant tumor was efficiently eradicated [16]. These results suggest that the antiangiogenic schedule, the metronomic administration schedule, has a potent anti-tumor effect by killing endothelial cells in the tumor bed with reduced side effects.

On the other hand, one of the particular merits of this metronomic approach centers on cancer drug resistance. Whereas conventional, high-dose chemotherapy tends to select tumor cells that are resistant to the drugs used, metronomic chemotherapy targets normal endothelial cells that do not grow resistant to the drugs. In other words, metronomic chemotherapy keeps on working when conventional therapy fails. Tumors may be able to adapt to a degree by increasing their production of pro-angiogenic factors that promote endothelial cells survival. This explains why cancers, which initially revert in response to metronomic therapy sometimes, grow back despite continuing therapy.

1.3.2. Examples of metronomic approaches with different chemotherapeutic agents

1. Metronomic therapy based on cyclophosphamide

Cyclophosphamide has been widely used in metronomic scheduling since it was the first anticancer agent to show interesting antiangiogenic properties in a murine model of cyclophosphamide-resistant tumors [16]. Metronomic dosage of oral cyclophosphamide (20 mg/kg/day) combined with injected irinotecan (10 mg/kg, twice a week), cisplatin (1 mg/kg, twice a week) or paclitaxel (1 mg/kg, three times a week) in mice demonstrated a safe profile, though it was not very effective in this mouse model [50]. However, the use of low-dose oral cyclophosphamide was recommended as a potential strategy against tumor progression in platinum-resistant patients after standard chemotherapy and with a poor performance status [51]. Man and collaborators had reported the effectiveness and safety of the chemotherapeutic drug, cyclophosphamide at low doses, used on human breast or ectopic colon cancer xenografts in nude or SCID mice. They conjectured that other drugs at low doses are likely to produce similar beneficial effects with less toxicity [52].

2. Metronomic therapy based on cisplatin

A lower dose of cisplatin (1 mg/kg, twice weekly) was used in combination with other cytotoxic agents in mice with proved safety and efficiency [50]. Since platinum was usually included in the MTD chemotherapy regimen (100-200 mg/m^2, 2-6 hours, every 3-4 weeks) as the first line adjuvant therapy for ovarian cancer, there are limited reports about metronomic regimens. However, metronomic cisplatin has still revealed antiangiogenic and anti-tumor effects in a rat model and has been used with other types of cancers, such as colon or lung cancers [53].

3. Metronomic therapy based on the use of taxanes

The usual dosage of paclitaxel as an adjuvant chemotherapy for epithelial ovarian cancer is 135 mg/m^2 every 3 weeks. Lower-dose paclitaxel (1 mg/kg, three times a week) was used in a metronomic combination in a mice ovarian cancer model, with minimal toxicity and a potential effect. [50] In another interesting work, metronomic taxanes (0.5 mg/kg, twice weekly), alone or in combination with an oral dual EGF and VEGF inhibitor, were used in an orthotopic mouse model of ovarian cancer and also showed promising effectiveness [54].

4. Metronomic therapy based on topotecan

Topotecan in ovarian cancer is usually administered at a dose of 1.5 mg/m^2 by infusion for more than 30 minutes on 5 consecutive days every 5 weeks (MTD regimen). A continuous low dose of irinotecan (10 mg/kg) was proved to be safe and effective in a mice model of ovarian cancer [50]. In another study, a metronomic regimen of topotecan (1 mg ± 0.5 mg once every day, four times a

day) showed promising results in an advanced ovarian cancer model [55]. In addition, oral topotecan (25 mg/kg twice a day or 100 mg/kg/day) has shown a positive effect against ovarian cancer in mice especially when combined with an antiangiogenic drug. The main problem observed was that severe side effects like hematologic toxicity and diarrhea still occurred in patients with advanced ovarian cancer. The appropriate dose and schedule of this agent are still to be studied.

In this context, many assays are being developed in clinical phases in order to attempt metronomic scheduling in cancer treatments as opposed to the traditional MTD regimens. Some examples are stated in **table 3**.

Table 3. Metronomic chemotherapy in adult patients with various cancer types in clinical assays, previously treated with conventional chemotherapy.

Cancer type	Metronomic Protocol	Response Rate (RR) and Clinical Benefit (CB) (%)	Refs
Recurrent ovarian cancer	Cyclophosphamide (oral, daily) and bezacizumab (every 2 weeks)	RR: 44% CB: 67%	[56, 57]
Hormone-refractory prostate cancer	Cyclophosphamide (oral, daily) and dexamethasone (oral, daily)	RR: 64% CB: 70%	[58-60]
Progressive multiple myeloma	Cyclophosphamide (oral, daily) and prednisone (oral, daily)	RR: 69%	[61]
Progressive, locally advanced or metastatic renal-cell carcinoma	Capecitabine (oral, twice daily), Gemcitabine (i.v., weekly) and Sorafenib (oral, twice daily)	RR: 50% CB: 93%	[62, 63]
Metastatic melanoma	Paclitaxel (i.v. for 3 days, weekly) and Celecoxib (oral, twice daily)	RR: 5% CB: 35%	[64]
Metastatic or locally advanced neuroendocrine carcinoma	5-Fluoruracil (i.v., daily) and long-acting release octreotide (monthly)	RR: 24% CB: 93%	[65]

i.v.: intravenous

Virtually all current chemotherapy regimens have, as a fundamental strategy, the goal of killing maximal numbers of tumor cells. Usually, this is achieved through application of the highest

drug dose that results in acceptable patient toxicity. More recently, metronomic therapy has been proposed as an alternative strategy. Low-dose metronomic therapy is a new concept with claims of better effects, few to no side effects, and lower costs. This approach uses smaller doses of drugs given in shorter, regular intervals or continuously to reduce toxicity and increase the antiangiogenic effects of cytotoxic drugs. This strategy maintains fixed dosing schedules and retains maximum tumor cell death. Clearly, the ideal cancer therapy is one that identifies and successfully attacks the key fitness parameters in tumor subpopulations, so that, despite the tumor heterogeneity and evolutionary capacity, complete eradication is obtained.

Metronomic chemotherapy is expected to radically change the practice of medicine related to chronic diseases in the future. Hence, this research concerning continuous administration of low dose chemotherapy reveals compelling evidence of its effectiveness and superior clinical results in cancer patients and therefore, the use of low-dose chemotherapy for advanced cancers is on the rise for its reported effectiveness, alleged little to no toxicity and relative economic benefits.

2. Immunotherapy and chemotherapy

Chemotherapy is still the treatment of choice for most cancers. It is well-known that cancer cells are capable of developing mechanisms of evasion that prevent the immune system from working correctly. This immune escape is a crucial feature of cancer progression about which little is known [66].

Cancer cells express certain antigens in the surface thanks to which the recognition by the immune system is impeded and therefore, cancer cells can replicate easily without control and even, migrate to different tissues [66]. Tumor immunity is defined as the ability of the immune system to reject a tumor. Cancer cells are able to elude immune response by the host and therefore, grow in an uncontrolled way. In fact, tumors can establish immunoregulatory networks that can favor the immune tolerance. Ultimately, this process can lead to immune equilibrium or tolerance, which is a key step to tumor survival, growth, local invasion and metastasis [67].

Some investigators speak about the capacity of tumors to create a selective pressure in the tumor microenvironment which can ultimately “edit” the immune response, referring to this as “immunoediting” [68]. Schreiber and colleagues described “immunoediting” in several stages. First, it involves a response by the immune system against the blossoming tumor; this step has been named as “immunosurveillance”. This stage is characterized by no clinical evidences of disease and in some cases; the immune system may be effective at eradicating the initial tumor. The second

stage begins when tumor cells are capable of colonizing sites in the tissue microenvironment. Tumor cells can divide although the immune system is still capable of restraining the tumor. This phase is called “immune equilibrium”. The third and final stage begins when tumor cells evolve to a state in which they can effectively evade, suppress and overcome control by the immune system. It is the so-called “immune escape”. At this point, tumors can progress and clinical signs become more apparent. So from this perspective, understanding the mechanism of immune escape is critical to understand cancer patho-physiology and its relation with clinical significance.

Figure 3 represents the fundamental characteristics of cancer with immune escape as a hallmark. It includes the characteristics which are considered as intrinsic and extrinsic to cancer cells. In this context, intrinsic characteristics involve the cancer cell itself while extrinsic involves not only cancer cells but also the tumor microenvironment.

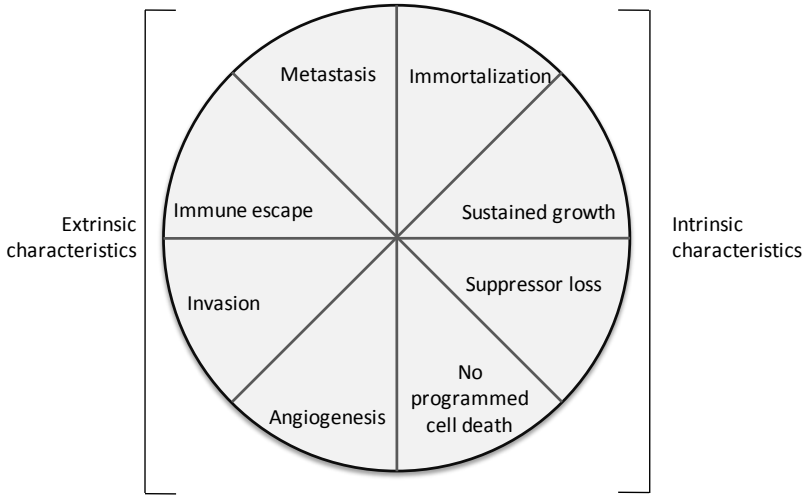

Figure 3. Main characteristics of cancer cells, with special relevance of the immune escape property.

In the last decades, significant advances have been done in understanding how tumors escape the immune system [69]. While most of the proposed immunotherapies have been focused on stimulating immune effector cells, such approaches may be prevented by escape mechanisms involving active suppression networks fostered by the tumor or by surrounding cells under the influence of the tumor. An implication of these advances is that disrupting immune suppression may activate the immune system and subsequently conduct immunity against tumor cells. The investigation of disruption of the immunosuppressive mechanisms offers a new target for therapeutic intervention. Small molecules have been recognized as inhibitors of selective enzymes implicated in the immunosuppressive mechanisms. These strategies offer a promising therapeutic

target and are included in the immunotherapy treatments. Nevertheless, immunotherapy is a less conventional form of therapy and as it can be said for chemotherapy, it is rarely curative [70].

2.1. Immunotherapy in cancer

Immunotherapy is an alternative anticancer treatment rather recently described and promising [71]. The immunotherapy treatment is aimed at directing the host immune response against tumor cells (without harming healthy cells) to achieve repair, stimulation or amplification of the immune mechanisms involved in the arrest and tumor dissemination. Some forms of immunotherapy are already part of the standard treatment against certain cancers, while others are under preclinical development or are undergoing clinical trials. The greatest benefits may arise by combining various methods of immunotherapy, with additive or synergistic effects, with the usual therapeutic strategies.

The areas of development in immunotherapy include: identification of tumor antigens, development of monoclonal antibodies with anticancer activity, cell therapy with activated lymphocytes, use of cytokines with antitumor capacity and therapeutic vaccination with dendritic cells. The mentioned strategies work on different levels at the immune system:

1. Activating immune response thanks to the correct presentation of tumor-associated antigens.
2. Maintaining and amplifying an already started immune response.
3. Eliminating mechanisms that could limit the immune response: inhibitory receptors or T cell regulatory cells.

The use and development of monoclonal antibodies has been widely studied for the past recent years and great advances have been made in this area. Many examples of monoclonal antibodies have already been commercialized and are currently used in clinics against different types of cancers, alone or in combination with chemotherapeutic agents. Such monoclonal antibodies include Bevacizumab (Avastin®), Trastuzumab (Herceptin®) or Cetuximab (Erbitux®), approved by the FDA. In contrast, the search for therapeutic vaccination strategy against cancer is not as advanced. Some preliminary works have been published in this regard, i.e. the use of dendritic cell vaccination combined with specific enzyme inhibitor.

From a clinical standpoint, combining an immunomodulatory agent with conventional chemotherapeutic agents represents logical strategy. A new challenge in immunotherapy is to

develop strategies that can effectively overcome tumor-induced immunosuppression and evasion, while safely augmentating antitumor responses [72].

2.2. Indoleamine-2,3-dioxygenase

Indoleamine-2,3-dioxygenase, commonly known as IDO, is a cytosolic heme-containing enzyme that catalyzes the initial step in tryptophan catabolism along the kynurenine pathway [73].This tryptophan catabolic pathway **(figure 4)** is initiated by the oxidative reaction of tryptophan leading to *N*-formylkynurenine and subsequently, other enzymes are in charge of the degradation of the products rendering nicotinamide adenine dinucleotide (NAD+) or picolinic acid [74]. The first step in this kynurenine pathway is rate-limiting and 3 possible enzymes have been described as responsible for the initial degradation. These enzymes are: indoleamine-2,3-dioxygenase (IDO), indoleamine-2,3-dioxygenase 2 (IDO2) and tryptophan-2,3-dioxygenase (TDO), depending on the tissue and cell type [75]. Both IDO and IDO2 are rather similar in their amino acid structure and differ from the TDO enzyme despite catalyzing the same reaction of tryptophan to N-formyl-kynurenine. In humans, the kynurenine pathway catabolizes over 90% of tryptophan.

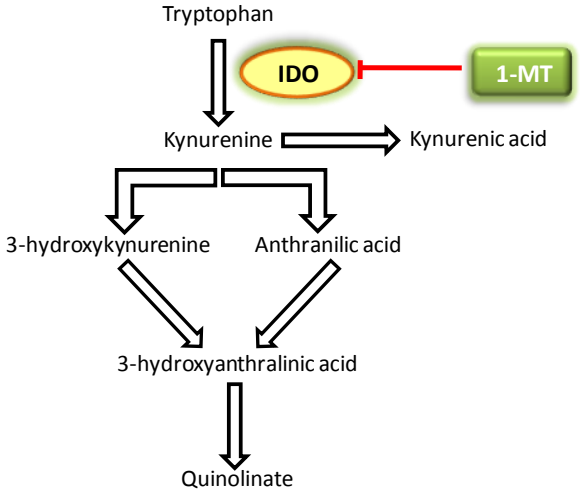

Figure 4. Kynurenine pathway of tryptophan metabolism.

2.2.1. Expression of IDO

The isolation of IDO, originally called D-tryptophan pyrrolase, was reported in 1963 [76]. The enzymes in this family are well conserved amongst different species. However, they are expressed differently in tissues. While TDO is expressed in liver, IDO is expressed ubiquitously and at higher levels in lung, gastrointestinal tract, epidymis and thymus [77]. IDO enzyme has 2 isoforms named IDO and IDO2. Although the two isoforms of IDO are expressed in the same tissues, they appear in

different cell types suggesting they carry out different functions and are not redundant. Additionally, their expression responds to different stimuli. Tryptophan catabolic enzymes, TDO, IDO and IDO2, have different inducers and are antigenically dissimilar sharing little homology in the amino acid sequence [78]. Despite the fact that IDO and IDO2 proteins do not share a high degree of homology, critical amino acids for the catalytic activity determined by crystallographic studies are all conserved amongst the 2 isoforms [79].

2.2.2. Biological function

IDO is expressed intracellularly in a constitutive or inducible manner in placenta, lung, small and large intestine, colon, spleen, liver, kidneys, stomach and brain. It can be induced by dendritic cells, monocytes, macrophages, epithelial cells, fibroblasts, vascular smooth muscle and endothelial cells as well as certain tumor cell lines [80]. In 1998, Munn and Mellor revolutionized the model of IDO-mediated tryptophan breakdown. They discovered that IDO expression at the fetal-maternal interface was crucial to prevent fetal rejection in pregnant mice [81].

Once expressed, IDO depletes tryptophan from local environments and promotes the formation of kynurenine pathway metabolites. This clearly underscores the biological roles of IDO related to host innate immune defence and immune control. IDO expression is also increased *in vivo* in response to various infectious agents (i.e. influenza infection in lung) suggesting that IDO represents an *in vivo* anti-microbial mechanism, although this has yet not been established.

2.2.3. Which tumors express IDO?

Several studies have been performed using IDO-transfected cell lines to evidence the role of this enzyme in immunosuppression [82]. Indeed, they have demonstrated that IDO is expressed not only in tumor cells but in presumably tumor associated antigen-presenting cells (APCs) in the tumor infiltrating microenvironment, in the tumor stroma and in tumor-draining lymph nodes [83, 84]. The relevant host APCs that might express IDO would be tumor-derived dendritic cells.

In parallel, research has focused on screening different tumor types to evidence the presence of IDO. Thanks to different samples of human tumors obtained from cancer patients, Uyttenhove and colleagues studied the expression of IDO by immunohistochemistry [82]. They concluded that many human tumor cell lines express the mRNA of INDO, the IDO-encoding gene, constitutively. However, the proportion of IDO-positive tumor cells was not as extended. The highest proportions (>50%) were observed in prostatic, pancreatic and colorectal cancers; in less proportion (10-50%)

they observed gastric carcinoma and glioblastomas; and in the lowest proportion cervical, endometrial, ovarian, breast, bladder, lymphomas and non-small cell lung carcinomas (<10%).

2.2.4.IDO induced tumor immune escape

Recently, Uttenhoye and coworkers [82] examined in their work different human cancer tissues (lung, prostate, gastric, pancreatic, etc) and found that these tissues contained a significant proportion of tumor cells expressing IDO. These findings were consistent with the work by Munn and coworkers who observed that the IDO transfection rendered tumor cell lines with immunosuppressive characteristics *in vitro* [85]. All together, these data suggest that IDO might have a role in enabling tumors to escape the immune system, implying that IDO could be an attractive new target for cancer immunotherapy.

2.3. IDO as a target for therapeutic intervention

IDO has evolved from a single tryptophan-catabolizing enzyme to an important immune regulator and an important player in tumor immunosurveillance. Evidence suggests that IDO represents an important immune regulatory enzyme under diverse physiological and pathological conditions *in vivo* and it is of considerable medical importance. It represents an ideal target for therapeutic intervention. Inhibition of inappropriate IDO activity in tumors *in vivo* may attenuate the ability of tumors to evade the immune surveillance and promote clearance. Indeed, drugs that inhibit IDO can act as effective adjuvants for tumor immunotherapy. Such effect has been observed in mice [86]. Several studies have evidenced that IDO inhibition with small inhibitors can exert anti-tumor activity. On the other hand, strategies that enhance IDO activity during autoimmune or inflammatory diseases may be beneficial via inhibition of undesirable T cell activities.

2.4. IDO inhibitors and chemotherapy

As mentioned above, the inhibition of IDO has been described as an interesting strategy in several malignancies such as cancer in order to potentiate traditional treatments. Agents that interfere with tumoral immune tolerance may be useful to prevent or treat cancer. The most common and studied IDO inhibitor is 1-methyl-tryptophan (1-MT) but recently, other studies have been published mentioning other inhibitors which will be here presented **(figure 5)**. A recent review [87] summarizes the range of compounds that have been tested as IDO inhibitors. Strikingly, almost all IDO inhibitors, whether competitive or noncompetitive, retain the indole ring of the natural substrate, tryptophan.

1-Methyl-tryptophan: It is the best known and more studied IDO inhibitor. 1-MT, a competitive IDO inhibitor used in therapeutic trials, also exists as two isomers: D-1MT and L-1MT. Initial evidence emerged in 2002 that 1-MT could slow down the growth of mouse lung carcinoma cells engrafted in a host [83]. Similar results were obtained as part of an investigation to assess the ramifications of IDO over-expression that was detected in a wide range of human tumors. In this work, Uttenhoye et al. [82] evaluated the immunosuppressive effect of IDO and defended that this effect can be substantially reversed, that is, tumor growth could be inhibited, by concomitant administration of the IDO inhibitor 1-MT. The antitumor effect of 1-MT was found to be completely dependent on the presence of an intact immune system, supporting the hypothesis that IDO functions by disabling the host immune response [88].

However, regarding 1-MT, the major question discussed currently in the scientific environment is which of the 2 stereoisomers, D or L, present the best efficacy profile and is more effective. Most studies in the literature have used the natural racemic mixture of 1-MT. Yet, in some biological systems, D-1-MT seemed to be a more active form. Hou et al. [89] used recombinant human IDO in their assays to evaluate the ability of the stereoisomers to inhibit the kynurenine pathway. They found that L-1-MT functioned as competitive inhibitor while D-1-MT was much less effective. In other studies, similar results were obtained using a murine MC57 tumor cell line. In all cases, the L-isomer was superior to D-1-MT at inhibiting tryptophan metabolism. IDO has a tenfold higher affinity for the L-isomer than the D-isomer. Furthermore, *in vivo* administration of D-1MT or its racemic mixture, 1-methyl-D,L-tryptophan, was proven to block the immunosuppressive effect of IDO [86, 90, 91]. The DL mixture demonstrated to be synergistic with a number of commonly used chemotherapeutic agents [86]. This synergistic effect is immune mediated, and it may reflect increased antigen presentation following chemotherapy and/or depletion of regulatory T cells. Compared to the DL mixture, the D isomer appears to be at least as effective *in vivo*. The D isomer has been used in a number of recent human and mouse studies [80, 86, 90, 92-94].

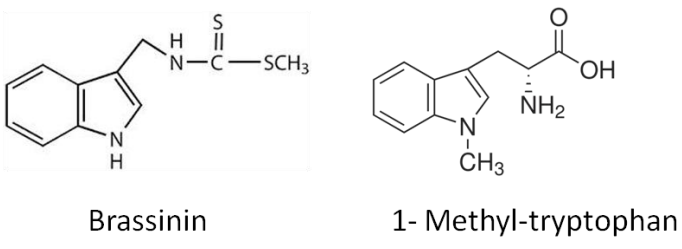

Figure 5. Chemical structures of Brassinin and 1-Methyl-tryptophan, example inhibitor molecules of indoleamine-2,3-dioxygenase (IDO) enzyme.

Brassinins: Brassinin ([3-(S-methyldithiocarbamoyl) aminomethyl indole]), first isolated from Chinese cabbage inoculated with *Pseudomonas chichorii* [95] belongs to a group of sulfur-containing, tryptophan-derived phytoalexins that are unique to crucifers [96]. Brassinin inhibits apparently the formation of carcinogen-induced pre-neoplastic lesions in rodents and to suppress papilloma formation in the classical two-stage skin carcinogenesis model. But the mechanisms underlying its anticancer properties are unknown. Recently, it has shown IDO inhibition too [95]. In this work by Banerjee and coworkers, they tested natural brassinin and a synthetic derivative, 5-bromo-brassinin. Although the lead compound brassinin was a relatively poor inhibitor, synthetic derivatives have been developed which appear to be more active *in vitro* than 1-MT. In this work, brassinin and 5-bromo-brassinin were administered by oral bolus dosing at 400 mg/kg twice a day. Delivered in this manner, neither compound alone demonstrated significant single agent activity. Therefore, both of these compounds were combined with chemotherapy to elicit regression of breast tumor models in MMTV-Neu mice. Furthermore, growth of highly aggressive melanoma isograft tumors was suppressed by single agent treatment with 5-Br-brassinin. The natural product brassinin thus provides the structural basis for a new class of compounds with *in vivo* anticancer activity that is mediated through the inhibition of IDO.

Other examples: Although small molecule IDO inhibitors exist, there is a lack of high-affinity compounds with *in vivo* efficacy. Recently, agents have been discovered with inhibitory activities in the nanomolar range in contrast to 1-MT. Annulin B, an extract from a marine hydroid [97], is one of these compounds and its key pharmacophore is naphtoquinone. In addition, new synthetic inhibitors based on this substance have been developed [98], including pyranonaphtoquinones, which are the most potent compounds described so far. Another extremely effective natural product is exiguamine A, a substance extracted from a marine sponge [99], which combines structural features found in 1-MT and annulin C. Synthetic analogues of exiguamine A have been prepared and evaluated for their ability to inhibit IDO [100]. More recently, the crystal structure of IDO was elucidated [79]. This discovery will pave the way for computer-guided design and the synthesis of novel potent inhibitors, highly efficient alternatives to 1-MT.

Such findings re-iterate that we do not fully understand the function of 1-MT *in vivo*, and we have not started to fully comprehend the effects of altering molecules known to regulate the immune response (significantly perturbed in patients with cancer) [101]. Because targeting tumoral immune tolerance is a unique approach to cancer treatment, the use of IDO inhibitors in combination with other types of agents may represent the best opportunity to simultaneously attack tumors on multiple fronts. In this context, future directions in anticancer therapy could be

focused on the development of combined therapies based on stimulating the host immune system while administrating chemotherapy to treat directly the tumor itself.

3. Paclitaxel

Taxanes are an important class of anticancer agents with a unique mechanism of action. Paclitaxel (PTX) and docetaxel (DCX) are the two main representatives of this family of anticancer agents. Paclitaxel is a natural product extracted from the Pacific yew tree (*Taxus brevifolia* sp.) of the *Taxaceae* family. It was first isolated by Wani et al. [102] in the 1960s as part of a screening program of the National Cancer Institute in the USA to obtain new cytotoxic agents from natural products. But it was not until the early 70s that its structure was elucidated and the molecule was obtained in a pure form. However, the importance of paclitaxel was not recognized until sometime later, in the late 1970s, since it was difficult to obtain. At this time, Horwitz at al. [103] discovered the particular mechanism of action of the anticancer agent. By the year 1983, clinical trials with the new anticancer agent begun and the drug development continued. Thus, since 1993, paclitaxel has been one of the most used chemotherapeutic agents in clinic.

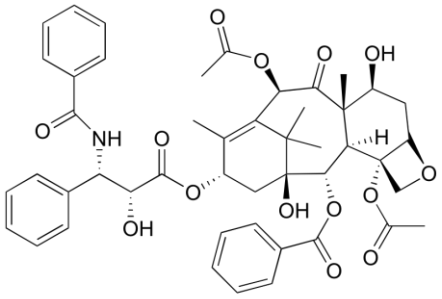

Figure 6. Paclitaxel, a natural diterpenoid extracted from the bark of the Pacific yew tree, *Taxus brevifolia.*

2.1. Physicochemical characteristics

Paclitaxel is a diterpenoid pseudoalkaloid (**figure 6**) with a molecular formula $C_{47}H_{51}NO_{14}$, corresponding to a molecular weight of 853 Da. It appears as a white to off-white crystalline powder. It is highly lipophilic, insoluble in water (0.03 mg/ml) and with a melting point between 216-217 °C [104]. It has a complex chemical structure with a characteristic taxane ring (**figure 7**), responsible for the unique and distinctive antitumor activity [105].

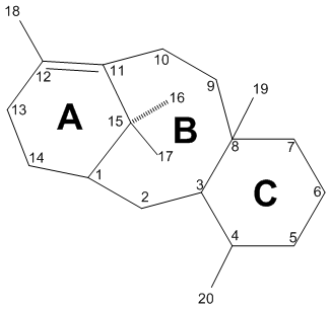

Figure 7. The characteristic taxane ring of the structure of paclitaxel, responsible for its unique mechanism of action.

2.2. Mechanism of action

Paclitaxel is classified as anticancer agent due to its cytotoxic activity against a wide variety of cancer types. It has a particular mechanism of action that differs from that of other currently available anticancer drugs. Until Schiff at al. [103] outlined the mechanism of action of paclitaxel in 1979, little was known about the antineoplastic nature of the taxanes. Today, more is understood regarding the mechanism of action of the taxanes; however, there are many aspects yet to be discovered.

Paclitaxel interacts with the microtubules during cell replication. Microtubules are cylindrical structures formed by polymers of tubulin which are in dynamic equilibrium with tubulin heterodimers composed of α- and β-subunits. Despite their involvement in the formation of the mitotic spindle that enables cell division, microtubules have other important functions, including maintenance of shape, motility, signal transmission and intracellular transport [106]. Unlike other antimicrotubule drugs, such as vinca alkaloids, which induce the disassembly of microtubules, paclitaxel promotes the polymerization of the microtubules, while it increases the number and mass of the microtubules (**figure 8**) [107].

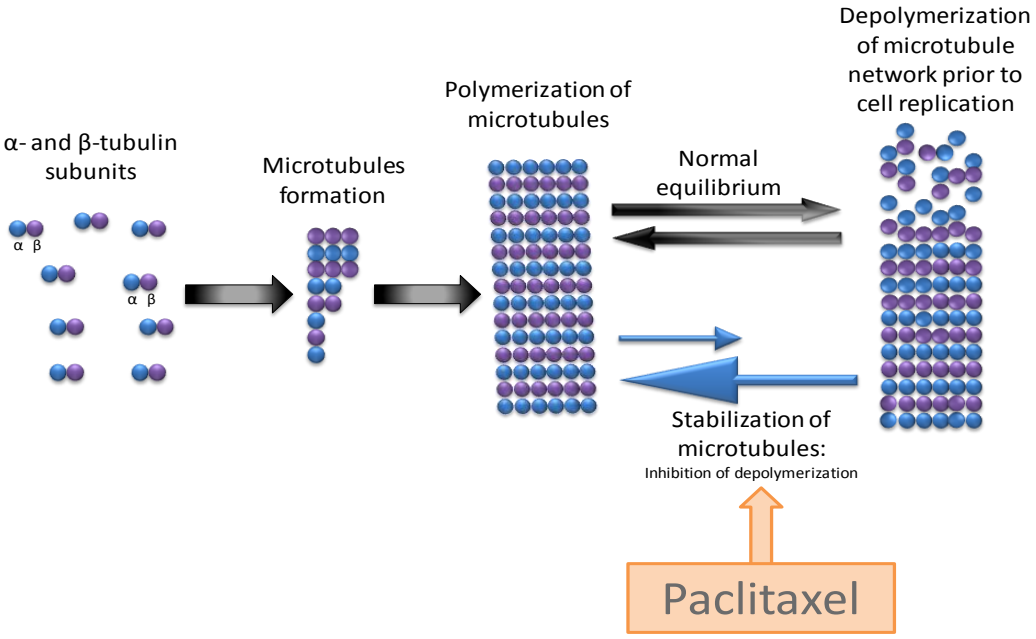

Figure 8. Mechanism of action of paclitaxel in the β-subunit of the microtubules during cell replication preventing the polymerization.

Taxanes bind poorly to the soluble tubulin itself but instead bind directly to tubulin along the length of the microtubules. The binding site for paclitaxel is in the β-subunit mainly located in the inside face of the microtubule; specifically it binds to the N-terminal 31 amino acids of such subunit producing abnormal microtubule bundles [108]. Therefore, the microtubules polymerized by paclitaxel are very stable becoming dysfunctional. This fact results in a suppression of the dynamic equilibrium in the cell stopping the cell cycle in the pre-mitotic G_2 and/or mitotic (M) phases preventing it from dividing and eventually, the cell dies by apoptosis (f**igure 9**) [109, 110].

In addition, paclitaxel induces apoptosis in a wide variety of cancer cells, independently of the state p53 (p53 is the protein that regulates cell death) [111]. This fact implies that paclitaxel is capable of acting in more than 60% of human cancers, even if p53 is mutated [112].

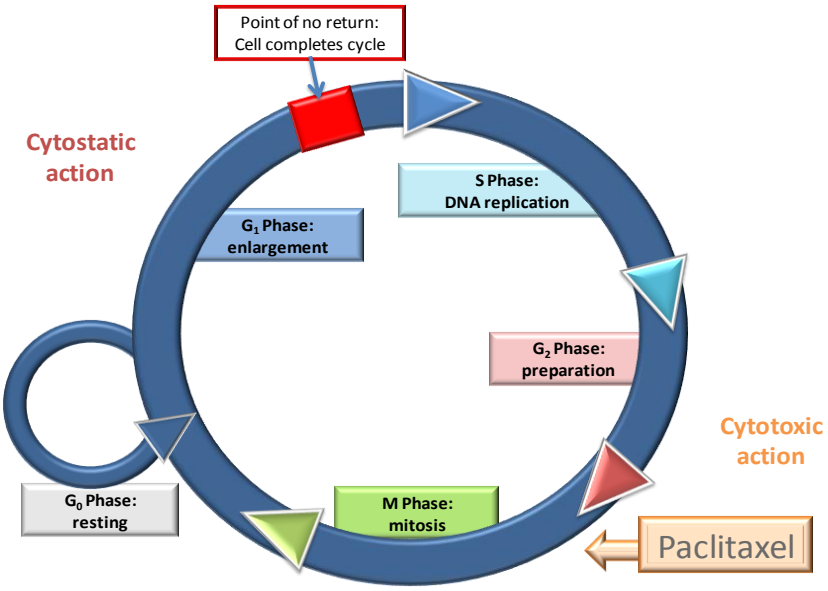

Figure 9. Paclitaxel, as representative of cytotoxic drug, stops cell cycle in phase G_2 or M, during cell replication.

2.3. Extraction and synthesis

In spite of the interest of paclitaxel as antitumor agent, its use has been restricted because of the availability limitations arising from the difficulties of obtaining the active. It is interesting to point out that the concentration of active in the bark of the tree is very low (0.007% of dry weight) and that the extraction process is rather expensive. In this context, 300 trees must be sacrificed to achieve 1 kg of paclitaxel. In addition, the yew tree is a tree with a very slow growth and for the tree to be in the optimal conditions for exploitation 70 years must go by [113]. The availability of the drug to meet the growing demands appeared to be one of the limiting factors. So, new strategies had to be designed to fulfill the demand and increase the capacity of the production processes.

The first attempt was the total synthesis of the drug. Nevertheless, this resulted in a complex chemical procedure due to the structural complexity of the molecule [114-116]. Although total synthesis of paclitaxel was achieved [116], the total chemical synthesis on an industrial scale is very difficult and may not be feasible commercially [114].

Another approach and the most successful one was the semi-synthesis of paclitaxel from a precursor extracted from the needles and twigs of more abundant yew trees, such as *Taxus baccata*. This precursor is 10-deacetylbaccatin III (**figure 10**). These semi-synthetic procedures attained yields of around 75%.

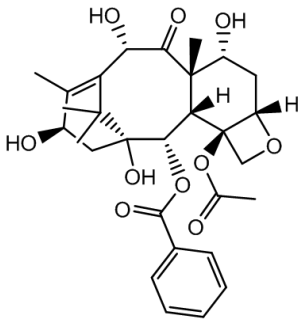

Figure 10. 10-deacetylbaccatin III, the precursor for the chemical synthesis of paclitaxel, obtained from the needles of *Taxus baccata*.

In addition, modifications to the structure were evaluated in order to develop analogues which could display a different activity or a more potent efficacy profile. However, the limiting factor is the construction of the taxane framework which became a challenge for synthetic chemists. Studies indicated that an intact taxane ring and an ester side-chain are essential for the cytotoxic activity of the anticancer drug [117]. So, the synthesis of the drug in the laboratory and the attainment of synthetic derivatives meant a challenge which was overcome when docetaxel, a more potent analogue of paclitaxel was obtained. Docetaxel was developed to circumvent the issues of solubility and availability of paclitaxel. It is produced semisynthetically from 10-deacetylbaccatin III and became of relevant importance since it was the first time there was a renewable source for the production of a taxane [118].

Today paclitaxel is produced through a semisynthetic process, chemically altering the precursor molecule to form the drug in the laboratory. Paclitaxel is also produced by an endophytic fungus associated with *Taxus brevifolia, Taxomyces andreanae* [119]. The amount of paclitaxel produced from this organism is small, but genetic engineering may prove to enhance this production. Currently this process is not utilized with commercially available paclitaxel though.

2.4. Clinical pharmacology and pharmacokinetics

Paclitaxel has a low therapeutic index and the therapeutic response is widely associated with the toxic side effects which appear in patients receiving the treatment [113]. The generally standard/accepted dose of paclitaxel is 135-175 mg/m^2 given as 3 or 24-hour infusion.

The clinical pharmacokinetics have been described as a biphasic plasma curve [120]. The initial rapid decline represents distribution to the central compartment and elimination of the drug and the later phase is due to the efflux of the drug to the peripheral compartment [121]. However,

pharmacokinetics of paclitaxel shows wide variability. More than 90% of the drug binds rapidly and extensively to plasma proteins following intravenous administration [122]. The highest concentration of paclitaxel following a 6-hour infusion in rats was found in lung, liver, kidney and spleen [107, 122].

Most of the drug is eliminated by feces and less than 10% of the administered dose is excreted in urine in the unchanged form indicating an important non-renal clearance [123]. Paclitaxel is mainly metabolized by the cytochrome P450 (CYP450), mainly by 2 isoforms: CYP2C8 and CYP3A4. The first is responsible for the formation of the 6α-hydroxylpaclitaxel, present in the liver and the other for the formation of 3' p-hydroxypaclitaxel, present at intestinal level [124-126]. So, biliary excretion is the main elimination pathway for paclitaxel and its metabolites [127]. On the other hand, the oral bioavailability of the drug is very low which has been described to be between 2-8% depending on the administered dose, in any case, lower than 10% [128].

2.5. Antitumor activity

Paclitaxel has been proved active against a wide variety of cancers in therapeutics including ovarian, breast, non-small cell lung cancers, Kaposi's sarcoma and head and neck carcinomas:

1. **Ovarian carcinoma**: Paclitaxel was first approved by the Food and Drug Administration (FDA) in the USA in 1992 to treat women with epithelial ovarian cancer. In first line treatment, paclitaxel is combined with cisplatin in patients with advanced ovarian cancer. In second line, it has also been approved in patients suffering from metastatic ovarian cancer when standard therapy based on platinum derivatives has failed [129].
2. **Breast cancer**: Paclitaxel is used in therapy as adjuvant therapy against breast cancer in patients that have already undergone a combined therapy with anthracycline and cyclophosphamide [129]. Besides, it is used as single therapy in the treatment of metastatic breast cancer when previous therapy has failed or patients are not candidates for anthracycline therapy.
3. **Non-small cell lung carcinoma (NSCLC):** The use of paclitaxel against lung cancer was approved in cases where no possibility of surgery or radiation therapy is available, always combined with cisplatin. In these cases, paclitaxel demonstrated to increase the life expectancy in almost 2 months more [130].
4. **Kaposi's sarcoma:** Paclitaxel is used against Kaposi's sarcoma associated with AIDS in patients in whom the anthracycline therapy has not been successful [131-133].

5. **Head and neck cancer:** Paclitaxel has demonstrated activity in patients with locally recurrent and metastatic squamous-cell carcinoma of head and neck who have received no prior chemotherapy [134]. The role of different dosage levels of paclitaxel and the efficacy of different schedules with other active drugs is currently being investigated.

2.6. Drawbacks of current taxane formulations

Apart from the problems associated with its availability and synthesis, there are two main concerns with this drug: aqueous solubility and oral bioavailability.

1. **Solubility**: Paclitaxel is poorly soluble in water but it can be dissolved in organic solvents. Since the paclitaxel molecule has no ionisable groups from a pharmaceutical interesting point of view, it is difficult to enhance its solubility. Therefore, for its clinical use, paclitaxel has been formulated and commercialized with a mixture of ethanol and Cremophor EL® (CrEL) (1:1, v/v) as vehicles. Cremophor EL® is a non-ionic surfactant obtained from the reaction of castor oil with ethylene oxide. It is widely used as excipient in many pharmaceutical applications [135]. Nonetheless, this excipient presents certain problems for its use. On one hand, Cremophor EL® has been described as responsible for the severe and acute hypersensitivity reactions described in patients receiving paclitaxel intravenously, characterized by dyspnea, flushing, rash, hypotension, tachycardia and hypersensitivity [136]. Despite premedication, consisting of high-dose corticosteroids (dexamethasone) and H_1 and H_2 antagonists (diphenhydramine and cimetidine/ranitidine or famotidine), minor reactions can still occur in 44% of patients and even life-threatening reactions in 3% [122, 137]. Mostly, these reactions occur within the first two courses of paclitaxel and can be prevented by reducing the infusion rates. Additionally, the doses of the required premedication can interact with the metabolism of paclitaxel since the H_2 antagonists inhibit the cytochrome P450 in liver [138]. Another side effect derived from the administration of paclitaxel to patients and related to the presence of Cremophor EL® in the formulation is the neurotoxicity. Patients receiving the treatment have presented peripheral neuropathy. It is not clear whether this neurotoxic effect is directly caused by Cremophor EL® or by the drug itself. Thus, studies in rats treated with CrEL-free paclitaxel did not display these symptoms since no paclitaxel was detectable in the peripheral nervous system [139]. On the other hand, regarding administration in clinics, CrEL is incompatible with the polyvinylchloride (PVC) equipment commonly used. So, the use of PVC-free equipment is mandatory since CrEL is known to leach plasticizers from infusion bags and tubing sets which can cause severe hepatic toxicity [135, 140]. Finally, various studies have shown that CrEL alters the pharmacokinetics of many drugs, amongst

these, paclitaxel. CrEL has been described as responsible for the non-linear pharmacokinetic profile observed in plasma after the administration of the commercial formulation [141]. More recently, it has been proposed that the effect of CrEL on the pharmacokinetics of the anticancer drug could be associated with the fact that CrEL forms micelles where the drug is encapsulated causing changes in cellular partitioning and in blood-plasma concentration ratios of paclitaxel [142].

2. **Oral bioavailability:** Another limitation of paclitaxel is that it has a low oral bioavailability due to the pre-systemic metabolism it suffers and to its affinity for P-glycoprotein (Pgp). Paclitaxel is metabolized in the intestine by the cytochrome P450. On the other hand, it is substrate of the P-glycoprotein (Pgp). This transporter belongs to the ATP-binding cassette family and plays an important role in preventing the absorption of toxic compounds and certain anticancer agents. In fact, Pgp is situated in the apical side of the intestinal membrane as well as in the kidney and liver acting as a pump eliminating xenobiotics through the intestinal lumen, bile and urine [143]. In addition, Pgp displays a similar localization as other metabolizing enzymes in charge of the degradation of a wide variety of drugs and chemicals. So, the presence of these mechanisms prevents the orally administered drugs from being absorbed and therefore, acting at their target site.

2.7. Commercially available formulations of paclitaxel

Nowadays, in clinics in addition to the first developed formulation of paclitaxel, Taxol®, there are several alternatives.

1. **Taxol® and the generics of paclitaxel**: Taxol® was the first commercialized medication containing paclitaxel as active ingredient. The formulation contains Cremophor EL® and ethanol as vehicles in order to solubilize the anticancer drug. The administration of such drugs is by means of the intravenous route.
2. **Paxene®:** It is a paclitaxel formulation which was approved by the European Medicines Agency (EMA) in 1999 to treat ovarian and non-small cell lung cancers and Kaposi's sarcoma but the commercialization of this medicine was withdrawn in Europe in 2010. It was a concentrate for solution for intravenous administration with a drug concentration of 6 mg/ml, containing Cremophor EL® and ethanol as vehicles. The marketing authorized company decided to voluntarily withdraw the product for commercial reasons in 2010. It was only marketed in France.
3. **Abraxane™:** Paclitaxel protein-bound particles (*nab* paclitaxel; ABI-007) is a novel formulation of paclitaxel that does not employ the Cremophor EL® as solvent. This formulation was approved by the FDA in 2005 and commercialized as intravenous alternative to Taxol®. Abraxane was approved

in Europe in 2008 to treat metastatic breast cancer in adults who have not responded to the first line treatment or for patients to whom the anthracycline alternative is not suitable. It is prepared by high-pressure homogenization of paclitaxel in the presence of human serum albumin at a concentration of 3-4%, similar to the blood concentration of albumin, resulting in a nanoparticle colloidal suspension [144, 145]. The nanoparticle suspension displays a mean particle diameter of 130-150 nm, thus eliminating the need of any solvent. Furthermore, the absence of CrEL eliminates the need for steroid premedication and alleviates the danger of leaching plasticizers from infusion bags and tubing.

2.8. Alternative formulations of taxanes in clinical development

New alternatives are being developed with the aim of eliminating Cremophor EL® from the formulation. The interest lies in finding a vehicle better tolerated, ideally with no side effects and possibly improving the efficacy of paclitaxel or to overcome the drug resistance which many patients present when undergoing a long-term therapy based on taxanes. Another strategy in clinical development that has demonstrated promising preclinical results is the synthesis of analogues of paclitaxel. The aim in many cases is to bypass the toxicity of conventional taxane formulations and to maintain or ideally improve the clinical antitumor activity. Many researchers are seeking the development of a compound with a good bioavailability and the absence of unmanageable side-effects. Two trends can be distinguished in the development of alternatives: i) analogues and ii) new formulations for paclitaxel.

1. **Analogues of paclitaxel:** among these:

1.1. **Taxoprexin®**: is a novel compound formed by covalently linking the natural fatty acid, docosahexaenoic acid (DHA), to paclitaxel. It is also known as DHA-paclitaxel. This conjugate was designed to function as a prodrug and to accumulate preferentially in tumor tissue [146, 147]. The clinical preparation of DHA-paclitaxel is formulated in a vehicle containing 80% less Cremophor EL® and ethanol than the standard formulation of paclitaxel. This agent can be reconstituted in dextrose 5% to a maximum concentration of 8 mg/ml and administered intravenously over 2 h every 21 days [147]. Owing to the presence of CrEL, steroid and antihistamine premedications, as well non-PVC tubing and in-line filtration systems, are required for drug administration. Several clinical trials have been completed or are under development using Taxoprexin® against several types of cancers, such as advanced eye melanoma, advanced skin melanoma, advanced lung cancer in combination with cisplatin or in metastatic prostate cancer.

1.2. **Opaxio® (formerly known as Xyotax)**: also known as CT-2103 or paclitaxel polyglumex. It is formulated to enhance the solubility of the anticancer agent, increase the tumor permeability, minimize normal tissue exposure and prevent multidrug resistance (MDR) efflux pumps via pinocytotic tumoral uptake [148]. It was designed to improve the delivery of PTX to tumor tissue while protecting normal tissue from toxic side effects. Xyotax is a macromolecule consisting of a biodegradable, water-soluble polymer of glutamic acid, a naturally occurring amino acid, linked to paclitaxel. Unlike standard formulations of paclitaxel, the clinical preparation of paclitaxel polyglumex does not contain CrEL owing to the ability of polyglutamic acid to render highly soluble hydrophobic molecules. Paclitaxel polyglumex has been evaluated in phase I/II studies in combination with platinum chemotherapeutic agents in tumors refractory to conventional therapy.

1.3. **BMS-184476:** is characterized by the substitution of a 7-methylthiomethyl ether group for the 7-hydroxyl group present on paclitaxel [149]. This substitution increases the solubility of the drug and allows formulating the drug with 80% less CrEL per milligram of drug, thus potentially eliminating the premedication required in the standard formulations [150]. It demonstrated interesting properties in preclinical and early clinical models, suggesting an advantage over standard taxanes [150, 151]. In various *in vitro* and *in vivo* tumor models, BMS-184476 has shown improved potency over either paclitaxel or docetaxel and has the ability to overcome paclitaxel resistance in some models [150].

1.4. **DJ-927:** also known as tesetaxel, is designed to overcome drug resistance and improve the clinical efficacy of the standard formulations of paclitaxel. Preclinically, it has shown marked efficacy *in vitro* and *in vivo* in various tumor cell lines displaying higher intracellular concentrations in Pgp expressing tumors and enhanced potency compared to paclitaxel and docetaxel [152]. Most of the clinical trials approved for this novel paclitaxel analogue are on the recruitment phase, although a couple have already been conducted as second line treatment in locally advanced or metastatic colorectal adenocarcinomas. In December 2010, an orphan designation for tesetaxel was granted by the European Commission to Genta Development Ltd, United Kingdom, for the treatment of gastric cancer.

1.5. **Ortataxel:** also known as BAY 59-8862 or IDN 5109, has displayed an improved toxicity profile as well as enlarged spectrum of antitumor activity in a large number of human tumor xenografts after intravenous administration [153, 154]. These results prompted researchers to investigate its bioavailability and cytotoxic activity after oral administration [155, 156]. Preliminary assays demonstrated an oral bioavailability of approximately 50%, owing to the inability of Pgp to recognize this molecule as substrate and therefore, displaying interesting levels of drug in plasma

after oral administration. In fact, oral ortataxel exhibited similar antitumor potency in several tumor models compared to the intravenous formulation of paclitaxel [155, 157]. In addition, phase II clinical studies have been completed using this novel agent intravenously in solid tumors [158].

2. Newly developed formulations:

2.1. Genexol-PM: this newly developed formulation of paclitaxel is based on the use of polymeric micelles obtained from a biodegradable block copolymer to evade the use of CrEL as vehicle. *In vitro* this formulation demonstrated comparable cytotoxicity to commercial Taxol®, against many human cancers, including ovarian, breast, non-small cell lung and colon cancer cell lines. Moreover, the antitumor efficacy of polymeric micellar paclitaxel *in vivo* exceeded that of Taxol® in several tumor cell lines [159-161]. It is an intravenous formulation developed with no CrEL and therefore, it permits a higher administration of paclitaxel without associated increase in toxicities. Phase I studies estimated the maximum tolerated dose (MTD) in 390 mg/m^2 with the dose limiting toxicities mainly being neuropathy, myalgia and neutropenia [162]. Currently, phase II studies are underway for patients with breast, ovarian and previously treated bladder cancer, either alone or in combination with carboplatin or capecitabine.

2.2. OncoGel™: is a new experimental drug delivery system containing no CrEL, which allows the slow continuous release of paclitaxel from a gel over a long period of time. It was designed for local delivery of paclitaxel to solid tumors and provide targeted cytotoxicity without the systemic toxicities associated with conventional systemic paclitaxel delivery. OncoGel™ provides a depot for the continuous release of paclitaxel directly to the tumor and surrounding tissue for 6 weeks. Once released from OncoGel™, the high tissue-binding affinity of PTX continues to localize the drug near the injection site, maintaining high local concentrations. OncoGel™ has demonstrated an excellent safety profile after administration to solid tumors. Dose-limiting toxicities in both non-clinical and clinical studies are local and systemic toxicities have not been noted. The lack of systemic toxicity is a reasonable finding considering the negligible levels of systemic paclitaxel noted following OncoGel™ administration. Phase I and II clinical studies are being carried out as a dose escalation study in patients with recurrent glioblastoma multiforme [163].

2.3. Tocosol/S-8184: is a paclitaxel vitamin E-TPGS emulsion for injection, formulated CrEL free. Vitamin E-TPGS has been described as Pgp inhibitor [164] and therefore, its addition to the formulation not only facilitates the administration but it minimizes toxicity. Phase I studies were performed in a wide range of tumors, many of which were taxane resistant and it resulted in a safer profile for Tocosol in these conditions than the standard available formulation [165]. Phase II evaluation of the

novel alternative was well-tolerated and demonstrated promising efficacy when administered weekly to patients with metastatic or locally advanced refractory transitional cell carcinoma of the urothelium, NSCLC and ovarian cancers [166, 167]. The most common side effects reported in these clinical studies were neutropenia and anemia. However, no severe neuropathy was reported.

2.4. **Paclimer™:** is an injectable biopolymer-based formulation of paclitaxel consisting of the encapsulation of the anticancer drug in polymeric microspheres. Unlike traditional systemic chemotherapeutic regimens, Paclimer™ microspheres have been designed to provide site-specific and controlled delivery of paclitaxel. It incorporates paclitaxel in especially designed biodegradable microspheres composed of a polyphosphoester polymer. Once administered, it is designed to slowly dissolve over an extended period of time to provide controlled and continuous delivery of paclitaxel directly at the tumor site. Preclinical studies in mice offered promising results in enhancing local therapy [168, 169]. Phase I clinical studies have been developed for patients with recurrent or persistent ovarian cancer or primary peritoneal cancers and with central nervous system malignancies, unresectable NSCLC or advanced prostate cancer [170]. Intraperitoneal (i.p.) administration of paclitaxel microspheres was well tolerated without defining MTD. The low but persistent detection of plasma paclitaxel indicated that paclitaxel continued to be released for at least 8 weeks after i.p. treatment. The finding of significant peritoneal abnormalities, including the presence of residual polymer filaments, months after i.p. Paclimer™ treatment suggested that the polymer preparation used in Paclimer™ degraded slowly [170].

2.9. Strategies to increase the oral bioavailability of paclitaxel

For paclitaxel, the importance of developing an improved drug delivery system (DDS) is obvious from the problems encountered in therapy and in addition, focusing on the oral route it would imply a more comfortable and suitable treatment for patients suffering from cancer. However, it is evident that not all drugs can be administered orally due to the influence of various factors on their pharmacokinetic profile, such as physico-chemical properties, pharmaceutical factors, and physiological factors of the gastrointestinal system. The presence of P-glycoprotein on the enterocyte surface limits oral bioavailability. As mentioned previously, paclitaxel is pumped out by Pgp which also limits the oral uptake of paclitaxel and mediates the direct excretion of the drug from the systemic circulation into the intestinal lumen [127]. Therefore, the strategy to enhance the bioavailability of paclitaxel by oral administration is very interesting and requires novel formulations. The urge to develop an oral alternative formulation for paclitaxel is reflected in all the different strategies published over the past recent years. However, not many of these approaches

have managed to pass to clinical development and are mainly in the preclinical stage of development.

The firstly developed strategy which passed onto trials with patients was the co-administration with Pgp inhibitors. The approaches published in literature include co-solvency, pro-drug strategy, combination with cyclodextrins, emulsification, micelles, lipid based carriers (liposomes and solid lipid nanoparticles) and polymeric carriers (nanoparticles).

A. Co-administration of paclitaxel with Pgp inhibitors.

A selective and potent Pgp inhibitor would pave the way to oral chemotherapy for drugs such as taxanes (docetaxel and paclitaxel) [171]. Pgp, located at the intestinal epithelium, actively excretes the anticancer drugs to the intestinal lumen preventing their absorption and subsequently, their anticancer efficacy when orally administered. Initially, the most common and known Pgp inhibitors were verapamil and cyclosporin A (CsA). These two are the most extensively characterized inhibitors and were the first multidrug resistance-reversal agents used in clinical trials [172-174]. However, the usefulness of these drugs is limited because plasma concentrations required to reverse multidrug resistance could result in cardiac toxicity and immunosuppressive activity [175]. Cyclosporin A is an efficacious inhibitor of Pgp and one of the first agents used to modulate Pgp. The main advantages of CsA are that it is commercially available and it has demonstrated to potentially inhibit the metabolism of paclitaxel, since CsA is also metabolized by the same cytochrome P450 isoform as paclitaxel. In fact, Pgp inhibition with cyclosporin A increased the oral bioavailability of paclitaxel in a phase I clinical trials in humans [176-178]. On the other hand, considerable efforts have been directed towards the development of compounds that can inhibit Pgp without undesired toxicological effects [179]. Thus, many different compounds have been identified as Pgp inhibitors and new more potent inhibitor molecules have been developed and have been tested to increase the oral uptake of paclitaxel and therefore, its oral bioavailability. Such examples are curcumin, a safe dietary supplement from *Curcuma longa*, widely used in Asian cooking with many properties such as anticancer, anti-inflammatory or anti-oxidant properties [180], valspodar/ PSC833, a no-immunosuppressive CsA analogue [181], elacridar/GF120918 [181] and tariquidar/XR9576 [182, 183] other more potent CsA analogues, KR-30031, a verapamil analogue with Pgp inhibiting effect but with no cardiovascular adverse effects [175] or flavonoids such as quercetin or silibinin [184, 185]. Importantly, these newly developed modulators have not demonstrated severe side effects as it occurs with CsA or verapamil and may therefore be better candidates for clinical use, especially for repeated administrations.

B. Drug Delivery Systems (DDS): The drug delivery systems under research include:

1. Prodrug strategy.

Prodrugs are therapeutically inactive derivatives of therapeutically active drugs. Prodrugs undergo metabolism (bioconversion) by hydrolysis or enzymatic degradation to produce therapeutically active drugs in biological environments [113]. It is an interesting strategy for drugs with solubility problems. Paclitaxel is not soluble in water and lacks of functional groups that would allow the formation of salts to enhance the solubility. Therefore, the prodrug approach to improve solubility of PTX is gaining attention. In this context, a interesting molecule used has been poly(ethylene glycol) (PEG). PEG is an amphiphilic molecule known to increase the solubility of conjugates of hydrophobic compounds. The combination of PEG with PTX was proved by Choi et al. [186] by obtaining a water-soluble pegylated PTX prodrug compound which could be spontaneously decomposed into PTX [187]. This prodrug was assayed in rats after oral administration and the pharmacokinetic profile was evaluated obtaining a remarkable increase in the area under the curve (AUC) and in the absolute bioavailability of the prodrug [186]. Another prodrug strategy recently published combined PTX with cathepsin B, a lysosomal cysteine protease [188]. In this work, they evaluated *in vitro* the cytotoxic capacity of this novel conjugate displaying a significantly better antitumor effect than Taxol®. It presented high water solubility, no toxic surfactant nor organic solvent included in the formulation and a high therapeutic index.

2. Co-solvency.

The solvents used in combination to increase the solubility of certain drugs which are insoluble in water are known as co-solvents [104]. Commonly, water miscible co-solvents are used as the method to formulate intravenous non-water soluble drugs. Additionally, this strategy has also been used in order to facilitate the oral administration of drugs. In this area, an important work has been done when formulating PTX since the vehicle included in the commercial formulation, CrEL, has been described as responsible for the toxic side effects. So, alternative vehicles to CrEL have been studied in order to avoid its use. Several approaches have been done including D-α-tocopheryl poly(ethylene glycol) 400 succinate (TPGS 400), and vitamin E-TPGS 1000 [189, 190]. TPGS 1000 and 400 are amphiphilic molecules that differ in the length of the PEG moiety and the molecular weight. The presence of TPGS improved the solubility of PTX by micellar solubilization and its oral bioavailability. Besides, these surfactants have been described as inhibitors of Pgp, so their use in the oral route could enhance the absorption of the drug [191]. In these works, the increase in the oral bioavailability of PTX was due to the combination of increased solubility and inhibition of Pgp thanks to the presence of TPGS.

3. Paclitaxel-polymer conjugates.

Natural and synthetic polymers are used widely as components of medical devices, for example, as rate-controlling coatings, as hydrogels for the topical administration of drugs, in tablets and capsules for oral administration and controlled release systems. However, it has only been during the last decade that the first polymer-based therapeutics emerged as clinically accepted medicines for drug administration. Typically, polymer-drug conjugates are given intravenously. Nevertheless, new conjugates have arisen lately intended for the oral route too. Ringsdorf had the idea of polymer–anticancer-drug conjugates in 1975 [192] and this work was continued by Duncan and colleagues [193] designing the first synthetic polymer–drug conjugates to progress to clinical trial. The clinical aims of polymer–drug conjugation are to achieve improved drug targeting to the tumor, to reduce drug toxicity (by limiting access to the sites of toxicity) and to overcome the mechanisms of drug resistance [194]. First generation conjugates sought to improve the therapeutic index of drugs already in routine clinical use (as examples doxorubicin, paclitaxel, camptothecin) enabling easier formulation and patient administration. Almost all polymer–drug conjugates that have been clinically tested rely on increased tumor vascular permeability for tumor targeting. Many different polymers have been selected to conjugate with anticancer agents. In the case of paclitaxel, a drug-polymer conjugate containing glutamic acid is under clinical trials, Opaxio®, as described in section 2.8.

Research in this field is focusing on the use of biodegradable polymers such as low molecular weight chitosan [195], which is a biocompatible and biodegradable polymer widely used in the pharmaceutical context. It presents mucoadhesive properties within the gut and it is non toxic [196, 197]. Lee at al. [195] developed a new platform for oral delivery of PTX using a chemical conjugate system comprised of PTX and low molecular weight chitosan. After oral administration, this conjugate form of PTX was absorbed in the small intestine and effectively inhibited tumor growth with an efficacy comparable to the clinically available injected form, but with much lower toxicity. The strong antitumor activity of the chitosan-PTX conjugate after oral administration would be attributable to its greater water solubility, prolonged retention in the g.i. tract and ability to bypass Pgp efflux pumps and CYP450-dependent metabolism in intestine and liver.

Other research groups have focused on other types of polymers such as thiolated polymers. These polymers are ideal candidates for non-invasive drug delivery due to their mucoadhesive [198], permeation enhancing [199] and enzyme inhibitory [200] properties. In addition, since they present a high molecular weight, thiolated polymers are not absorbed from the gut and therefore, they do not present systemic side effects [201]. Thiolated polymers can be easily conjugated with hydrophobic drugs in order to attempt their oral administration. Thus, Iqbal and colleagues [202] evaluated the conjugation of poly(acrylic acid)(PAA)-cysteine with PTX both *in vitro* in Caco-2 cells

and *in vivo* in Sprague Dawley rats. The administration of oral formulations containing paclitaxel and PAA-cysteine conjugates resulted in a further enhanced paclitaxel plasma concentration and improved bioavailability, compared to the paclitaxel solution given orally.

4. Paclitaxel-cyclodextrin complexes.

Cyclodextrins are crystalline cyclic oligosaccharides containing six, seven or eight (α-1,4) linked D-glucopyranose units with amphiphilic properties and a shape of a truncated cone or "bucket"[203]. According to the number of α-D-glucopyranose units, they are named: α-cyclodextrin possessing six units, β-cyclodextrin possessing seven units and γ-cyclodextrin possessing eight units. The β-cyclodextrin family is the most commonly used cyclodextrin. Regarding their structure, the external surface is hydrophilic whereas the internal cavity is lipophilic. This central moiety is formed by the skeletal carbons, hydrogen atoms and glycosidic oxygen atoms of the glucose structure conferring the lipophilic characteristics [204]. On the other hand, the external surface is hydrophilic due to the presence of secondary hydroxyl groups at the wide edge of the structure and primary hydroxyl groups at the narrow edge. These natural cyclodextrins form total or partial inclusion complexes with active molecules. These combinations cyclodextrin-drug alter the water solubility, stability, diminish side effects and promote compatibility of drugs with other drugs or excipients, as well as ameliorating patient compliance by taste masking [205, 206]. Today, cyclodextrins are considered as useful excipients widely spread in pharmaceutical applications.

Among other reasons, cyclodextrins may be used as complexing agents since they alter the water solubility and therefore, stability of poorly water-soluble drugs. Thus, by the oral route, the effect of cyclodextrins may improve the oral bioavailability of lipophilic compounds. Cyclodextrins interact with specific components of the membranes but are rarely absorbed from the gastrointestinal tract [207]. Additionally they have been reported to act as inhibitors of Pgp [208, 209] and CYP450 [210]. Different hydrophilic derivatives interact with the cholesterol units of the membrane resulting in a depletion of these domains and therefore, decrease the ATPase activity and subsequently, the action of the efflux pumps [211]. A schematic representation of this effect is shown in **figure 11**.

In this context and taking advantage of this property, Fenyvesi and co-workers have carried out different experiments to study the effects of different cyclodextrins combined with Taxol® on a Caco-2 model. They were capable of removing cholesterol from the structure of the membrane and change its physicochemical properties, permeability and fluidity and in addition, modulate the action of the Pgp efflux pump, resulting in a higher permeability for Taxol® [208]. Regarding the effect on CYP450, Ishikawa and collaborators reported the interactions of HPCD and MeCD, on

different hepatic isoforms of CYP450 [210]. These two important properties of cyclodextrins; the complex formation with hydrophobic molecules and the inhibition of Pgp and CYP450; are the pillars for the election of these excipients in order to increase the oral bioavailability of paclitaxel and many other hydrophobic drugs.

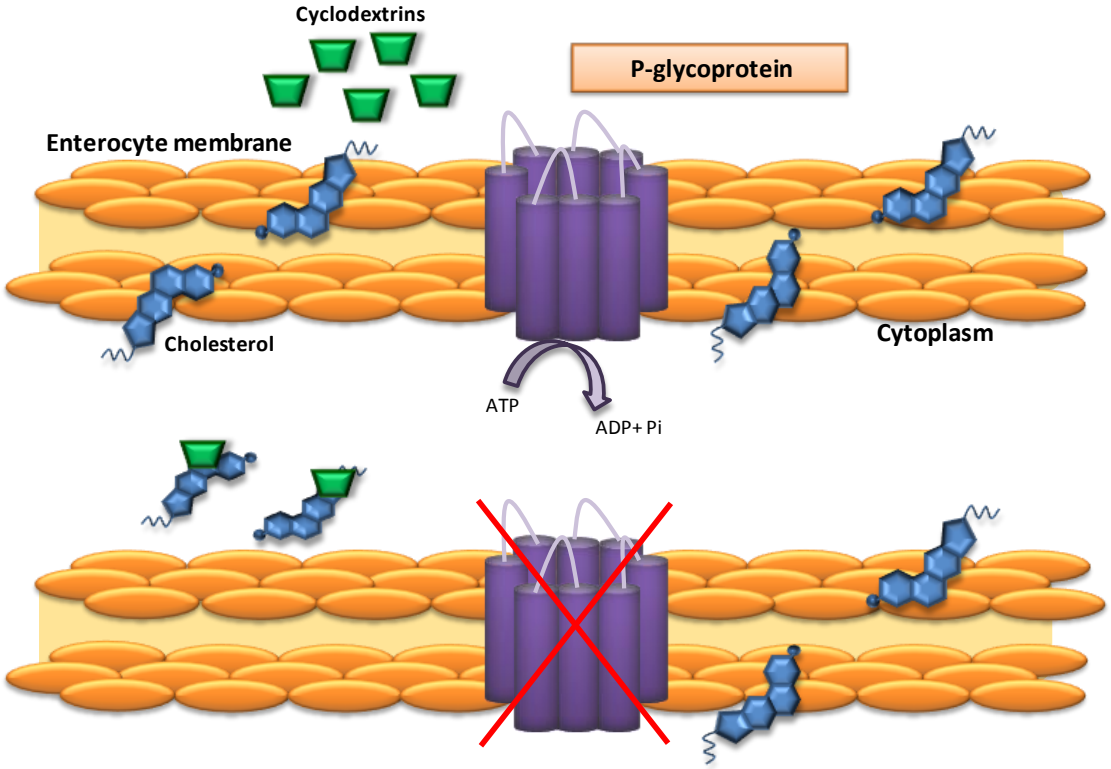

Figure 11. Schematic representation of the mechanism by which cyclodextrins (CDs) inhibit P-glycoprotein efflux pump.

5. Polymeric micelles.

Over the last decade, polymeric micelles have been investigated as potential delivery systems for therapeutic compounds [212, 213]. Polymeric micelles are nanometric core-shell structures formed by amphiphilic polymers [214] which can provide a controlled and targeted delivery of encapsulated drugs. These devices possess a hydrophobic core which acts as reservoir for lipophilic molecules, surrounded by a hydrophilic corona. This corona confers aqueous solubility and steric stability to the set [215]. In this context, polymers that spontaneously self-assemble into stable micellar aggregates in aqueous media provide promising colloidal systems [216].

Amphiphilic copolymers used in drug delivery contain either a polyester or a poly(amino acid) derivatives as the hydrophobic segment. These polyesters are biodegradable and approved by the FDA. Examples are poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA) or they may be non-degradable such as Pluronic or poloxamer family. Pluronics are non-toxic and frequently used as pharmaceutical excipients. They have been widely used as polymers in drug

delivery since they can easily incorporate into membranes, translocate into cells and affect several cellular functions. As a result, Pluronics cause sensitization of tumors to various anticancer agents and enhance drug transport across the intestinal barrier [217]. Besides, Pluronics have been described as Pgp inhibitors by causing fluidization of the lipid bilayer [218, 219]. Various polymeric micellar systems have been investigated to improve the oral delivery of hydrophobic molecules. In the case of paclitaxel, Yoncheva and coworkers developed stabilized Pluronic micelles loaded with the anticancer agent and evaluated the formulations *in vivo*. Their micelles were stabilized by cross-linking to prevent disaggregation upon dissolution in biological fluids [220]. Micelles were able to prolong the gastrointestinal transit by apparently penetrating deeper in the intestinal mucosa and therefore, PTX could be absorbed displaying more maintained plasma levels.

6. Self-microemulsifying drug delivery systems (SMEDDS).

SMEDDS are isotropic mixtures of oils and surfactants which can be dispersed in the gastrointestinal lumen to form microemulsions (transparent dispersed systems with oil droplet size of less than 30 nm) or fine opaque emulsions (either submicrometer emulsions with oil droplet size of 50–200 nm or coarse emulsions with oil droplet size larger than 500 nm) upon dilution with water or gastrointestinal fluids [221]. Furthermore, the commercial success of the self-microemulsifying drug delivery system (SMEDDS) formulation cyclosporin A (Neoral®), ritonavir (Norvir®) and saquinavir (Fortovase®) have raised the interest in such promising systems to improve the oral bioavailability of lipophilic drugs.

A novel SMEDDS comprised vitamin E as an oil phase, deoxycholic acid sodium salt, TPGS and Cremophor RH 40 as surfactants to increase the solubility of paclitaxel was developed with or without concomitant use of Pgp inhibitors in order to enhance the oral bioavailability of the cytotoxic agent. Compared to orally administered Taxol®, the oral bioavailability of PTX-SMEDDS increased by 29-53% at various doses. The surfactants might moderately inhibit Pgp leading to improvement in the oral absorption of the anticancer drug. In addition, when co-administered with verapamil, higher bioavailability and much longer therapeutic levels were observed. These findings indicate that SMEDDS is a promising delivery system for the efficient oral administration and enhancement of PTX, especially when co-administered with an effective Pgp inhibitor [222].

Another proposed work has been that of Oostendorp and coworkers [223]. The SMEDDS were administered with Pgp inhibitor cyclosporin A to laboratory animals to enhance the oral bioavailability of PTX. Their results revealed that the bioavailability and the systemic exposure to paclitaxel after a single oral administration of their SMEDDS co-administered with a Pgp inhibitor were comparable to the standard Cremophor EL®:ethanol formulations. In line with these works,

other works have been published regarding the oral administration of PTX such as supersaturable SEDDS of paclitaxel [224-226].

7. Nanoparticles.

In the past years, the use of biodegradable nanoparticle systems has aroused great interest for its potential to enhance the biopharmaceutical properties of many biologically active substances, particularly in the cancer area. Nanoparticles are colloidal systems of solid particles with sizes ranging from 10 to 1,000 nanometers [227]. Depending on the preparation method, nanospheres or nanocapsules can be distinguished. Nanocapsules are vesicular-like systems in which the active substance in located in the interior of the cavity surrounded by a unique polymeric coating. In contrast, nanospheres are matrix systems in which the drug is uniformly dispersed. [228], see **figure 12**.

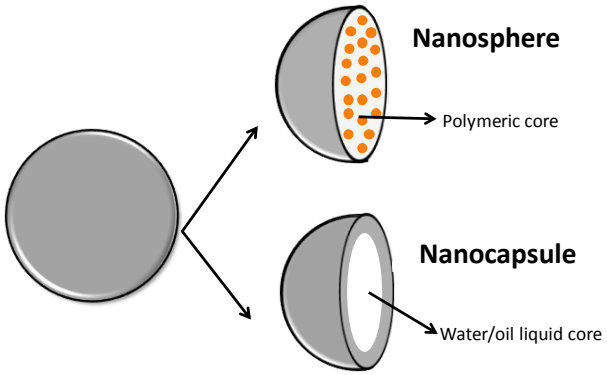

Figure 12. Differences between nanospheres and nanocapsules

The therapeutic applications of nanoparticles have been attributed to their distribution in the body which depends on the physico-chemical properties (mainly size and surface properties). Nanoparticles aim to enhance the therapeutic index of an encapsulated drug by increasing and sustaining the delivery of the drug in close contact with or inside the cell. They can be easily manipulated to increase their half-life and therefore, increase the accumulation of nanoparticles at tumor or specific sites. Nanoparticles also have the ability to permeate through certain tissues, adding to their drug targeting potential. Nanoparticle drug delivery can be either an active or a passive process. Passive delivery refers to their transport by passive diffusion. In contrast, active targeting involves delivery to a specific site based on molecular recognition by a ligand attached, generally to the surface of the carrier which can interact with a receptor at the target site.

All these benefits have been utilized in several published works regarding nanoparticle-based drug delivery. In many cases, well known chemotherapeutic agents (i.e. paclitaxel, doxorubicin,

docetaxel) have been combined with nanoparticles and efficacy profiles have been obtained compared to free drug. In all cases, there was an important increase in the therapeutic index and hopefully some will find their way to clinical applications in a near future. However, before nanomaterials can be used in cancer treatments in clinics, issues of biodistribution and toxicity must be addressed.

Nanoparticles for drug delivery include numerous architectural designs in terms of size, shape and materials. Dendrimers, liposomes, solid lipid nanoparticles, polymeric nanoparticles, fullerenes and nanotubes can be included in this classification. However, in this work only those nanocarriers used to enhance the oral bioavailability of paclitaxel are described.

7.1. Lipid-based nanoparticles (LNC).

LNC are biomimetic carriers that mimic lipoproteins, designed to encapsulate lipophilic drugs, such as PTX, without using organic solvents [229]. They are prepared by a solvent-free, soft-energy phase-inversion procedure and present a great stability (with physical stability up to 18 months) and their sizes range from 20 to 100 nm. They have generally an oily core, corresponding to medium-chain triglycerides surrounded by a membrane made from a mixture of lecithin and a surfactant. Structurally, the lipophilic drug, PTX, is solubilised into the central lipid core, which is surrounded by a membrane of lecithins and pegylated hydroxystearate [230]. The formulation is based on the phase-inversion temperature phenomenon of an emulsion leading to lipid nanocapsule formation with good mono-dispersion [231]. At human body temperature, the core of the LNC is liquid, whereas the membrane is rigid. Moreover, LNCs prepared by the phase-inversion process have demonstrated Pgp inhibiting properties thanks to their ingredients, especially Solutol®, key excipient in the composition of LNC. This surfactant has demonstrated ability to block Pgp-related drug efflux with a very low level of *in vitro* toxicity [232]. They can also be grafted with ligands for the purpose of actively targeting [233].

The interesting possibilities displayed for the use of LNCs allow their use in many therapeutic applications, not only for drug delivery, cancer diagnosis and therapy, but also for gene and cell therapy. This issue can also be addressed by the entrapment of PTX in the LNCs [234, 235]. Indeed, by loading paclitaxel into the oily core of LNCs, its mean plasmatic concentration appeared to rise 3 times higher compared to the conventional formulation (Taxol®) and 1.5 times higher than the co-administration of Taxol® and verapamil. In this context, Pandita et al. have also investigated the entrapment of PTX in solid lipid nanoparticles to enhance its oral uptake at the intestinal level [236]. In their study, the oral administration of LNCs produced a significant 10-fold increase in the oral bioavailability of the drug when compared to paclitaxel solution. In addition, the toxicity studies performed by these research groups confirmed the relatively safe nature of the

nanocarriers. Thus, such drug/carrier particulate systems provide an attractive and exciting drug delivery approach for highly potent drug substances that are usually unsuitable for oral use.

7.2. Niosomes.

Niosomes are non-ionic surfactant-based liposomes and are formed from the self-assembly of nonionic amphiphiles in aqueous media resulting in closed bilayer structures. Niosomes have been prepared from different classes of nonionic surfactants, for example, sorbitan monoesters (Span 20, 40, 60, and 80) [237, 238] or polyoxyethlene sorbitan monoesters (Tween 20, 60, 61, 80)[239]. Cholesterol is generally added giving rigidity and orientation order to the bilayer [240]. Recently, Bayindir et al [241] published their work combining these non-ionic surfactant-based liposomes with paclitaxel for the oral administration of the anticancer drug. In their work, they successfully developed the loaded niosomes using different surfactants and performed *in vitro* release studies under sink conditions.

7.3. Polymeric nanoparticles.

Polymeric nanoparticles offer a promising means of drug delivery for chemotherapeutic drugs with enhanced efficacy, reduced toxicity, controlled and long-term release rates, prolonged bioactivity, increased patient compliance due to less frequency of administration, and the ability to co-deliver multiple drugs with synergistic effects at the same site [242]. Polymers used are biocompatible and biodegradable, either synthesized or natural, which are subject to FDA approval. The drug can either be dispersed in the polymeric matrix, or conjugated/attached to the polymer molecules. The drug release mechanism can be diffusion, polymer matrix swelling, polymer erosion and degradation [243]. However, there is a great development in polymer science with better properties and characteristics. Many polymers have been proposed for the preparation of nanoparticles. Some of the synthetic polymers are: poly(lactic-co-glycolic) (PLGA) [244], poly(ε-caprolactone) [245] and poly(anhydride) or copolymer of methyl-vinyl-ether and maleic anhydride [246, 247]. Amongst the natural polymers, the most used are: albumin, polysaccharides such as chitosan [248], and β-casein [249].

Example works for the development of oral formulations of PTX based on polymeric nanoparticles are here described. Fonseca et al. encapsulated the cytotoxic agent in PLGA nanoparticles They performed *in vitro* release studies in phosphate buffered saline (PBS) incubating the formulations at 37°C in a horizontal shaker. The particles exhibited a biphasic pattern release, illustrated by an early fast release during the first 24 hours, followed by a slower and constant release. Then, *in vitro* antitumor activity of the drug-loaded nanoparticles was determined in a small cell lung cancer cell line comparing their results with the commercial Taxol®, observing that

the cytotoxicity results depended on the time and concentrations of the study. In fact, for longer incubation periods, a higher number of cells were arrested in phases G_2 and M of the cell cycle implying a higher activity of PTX [244]. On the other hand, Sharma et al. reported the preparation of paclitaxel containing polyvinylpyrrolidone nanoparticles crosslinked with N, N-methylene bisacrylamide (MBA) and determined their antitumor effect in murine melanoma. The *in vivo* efficacy of these nanoparticles, when measured using reduction in tumor volume and increased survival time as parameters, was significantly greater compared to an equivalent concentration of free Taxol® [250].

Another example of polymeric nanoparticles is the combination of PTX with poly(anhydride) nanoparticles [246, 247]. Poly(anhydride) is a synthetic biodegradable polymer commonly used in many pharmaceutical applications, recognized as safe and with a very low toxicity. In addition, poly(anhydride) nanoparticles display interesting bioadhesive properties with the gastrointestinal mucosa that could be beneficial to enhance the absorption at the gastrointestinal level [251, 252]. Agüeros and Zabaleta have successfully encapsulated the anticancer drug in poly(anhydride) nanoparticles by combining the drug with PEG or by forming complexes with cyclodextrins. They performed pharmacokinetic experiments *in vivo* in rats with an increase in the oral bioavailability of paclitaxel (relative oral bioavailability around 80%). Their results demonstrated that the oral administration of the drug was feasible by combining the bioadhesive properties of poly(anhydride) nanoparticles and the inhibitor effect of both cyclodextrins and PEG on Pgp and cytochrome P450 [208].

Natural polymers have not been as widely used as synthetic ones. However, Abraxane®, paclitaxel-bound albumin nanoparticle formulations, has been approved for its use in clinics since it has demonstrated a good pharmacokinetic profile and fewer side effects than Taxol®, since it has no CrEL. Nonetheless, research is being carried out with other natural molecules. Such is the case of β-casein. Recently, a research group has encapsulated PTX in β-casein nanoparticles. Casein is a natural protein occurring in milk and derivatives, with amphiphilic properties [249]. In their work, they evaluated the efficacy of these casein nanoparticles to entrap different anticancer agents rendering β-casein nanoparticles as excellent potential vehicle for targeting gastric diseases, including gastric cancer.

Table 4. Summary of the main strategies to increase the oral bioavailability of paclitaxel published lately.

	Examples	Commentaries	References
Prodrugs	PTX-PEG	Increased AUC and bioavailability (6.3%) compared with oral Taxol® (1.6%) Improved antitumor effect; Inhibition of Pgp and cytochrome P450 *in vivo*	[186]
	PTX-Glucose	Selective targeting to glucose transporters Lower cytotoxic effect than PTX alone. Lower toxicity *in vitro*	[253]
	PTX-Polyvinyl acetate phthalate	Oral evaluation in patients. Oral bioavailability: 13%. When combined with cyclosporin A increase up to 26%	[254]
Cosolvency	Change Cremophor EL® for vitamin E-TPGS 1000	Increased oral bioavailability: 30%	[189]
Polymer conjugates	Chitosan (low MW)-PTX	Mucoadhesive properties Oral bioavailability: 42% Significant inhibition of tumor growth *in vitro*	[195]
	Poly(acrylic) acid-cysteine-PTX	Increased intestinal permeability (P_{app}= 8.7x10^{-6} cm/s) *in vitro* Increase in AUC, bioavailability increased 5-6-fold (75-95%) compared to Taxol® (F=16%)	[202]
PTX-CD complexes	Modified CD: 2,6-dimethyl-β-CD; mono-6-*O*-maltosyl-β-CD; hydroxyethyl-β-CD	Increased aqueous solubility Increased *in vitro* antitumor activity	[255]
	Randomly methylated CD	Increased intestinal permeability *in vitro*	[209]
SMEDDS	Based on vitamin E-TPGS	Oral bioavailability: 29%. When associated to verapamil bioavailability up to 53%	[222]
	Associated to hydroxypropyl methyl cellulose	PTX loading= 60mg/g 10-fold increase in C_{max} and oral F=9.5% compared to oral PTX (F= 2%)	[224]]
SMEOF	Composed of vitamin E-TPGS; Tyloxapol; Sodium Desoxycholate	Oral bioavailability: 5%. When co-administered with cyclosporin A, bioavailability up to 20%	[223]

Cont. Table 4. Summary of the main strategies to increase the oral bioavailability of paclitaxel published lately.

	Examples	Commentaries	References
Polymeric micelles	Pluronic-based micelles	Increased AUC and MRT Oral bioavailability of 90%	[220]]
	Deoxycholic acid-chitosan based micelles	High drug loading (up to 32%) AUC 3-fold higher than oral Taxol®, lower clearance and MRT=13h	[256]
Niosomes	Composed of different surfactants (Tween, Span), cholesterol and dicetyl phosphate	Encapsulation efficiency depending on surfactants: 12-96% Slow release in vitro. Gastrointestinal stability	[241]
Lipid nanocapsules (LNC)	Composed of Solutol®, Lipoid® and Captex®	Paclitaxel loading: 2mg/g Increased AUC. Similar results for LNC than for the combination of Taxol® orally administered with verapamil	[234]
	Containing stearylamine, soya lecithin and Poloxamer 188	Paclitaxel loading: 31.5% (w/w) Increased C_{max} and longer circulation time in bloodstream Drug mainly accumulated in liver, lung, kidney , spleen and brain	[236]
Polymeric nanoparticles	PLGA nanoparticles	Encapsulation efficiency= 12-96% *In vitro* slow release and stability in the gastrointestinal tract	[244]
	Poly(anhydride) nanoparticles	Paclitaxel encapsulated as complex with cyclodextrins Drug loading up to 17% Increased intestinal permeability Oral bioavailability up to 80% in rats depending on the type of cyclodextrin	[246]
	Pegylated poly(anhydride) nanoparticles	Drug loading up to 15% Increased intestinal permeability Oral bioavailability up to 80% in rats depending on the MW of PEG	[247]
	β-casein	Casein presents gastric digestibility and possible targeting interesting for stomach tumors	[249]

AUC: Area under the curve; CD: cyclodextrin; C_{max}: peak concentration; F: bioavailability; MRT: mean residence time; PEG: poly(ethylen glycol); Pgp: P-glycoprotein; PLGA: Poly(lactic-co-glycolic) acid; PTX: paclitaxel; SMEOF: self-microemulsifying oily formulation; vit E-TPGS: D-α-tocopheryl polyethylene glycol 1000 succinat

References

[1] A. Jemal, Cancer Statistics, 2010 (vol 60, pg 277, 2010), Cancer Journal for Clinicians, 61 (2010) 134-134.
[2] R. Siegel, D. Naishadham, A. Jemal, Cancer Statistics, 2012, Cancer Journal for Clinicians, 62 (2012) 10-29.
[3] J. Ferlay, D.M. Parkin, E. Steliarova-Foucher, Estimates of cancer incidence and mortality in Europe in 2008, European Journal of Cancer, 46 (2010) 765-781.
[4] S.N. Weingart, J. Flug, D. Brouillard, L. Morway, A. Partridge, S. Bartel, L.N. Shulman, M. Connor, Oral chemotherapy safety practices at US cancer centres: questionnaire survey, British Medical Journal, 334 (2007) 407-409.
[5] T. Helleday, E. Petermann, C. Lundin, B. Hodgson, R.A. Sharma, DNA repair pathways as targets for cancer therapy, Nature Reviews Cancer, 8 (2008) 193-204.
[6] W.P. Roos, B. Kaina, DNA damage-induced cell death by apoptosis, Trends in Molecular Medicine, 12 (2006) 440-450.
[7] S.B. Kaye, New antimetabolites in cancer chemotherapy and their clinical impact, British Journal of Cancer, 78 (1998) 1-7.
[8] A.B. da Rocha, R.M. Lopes, G. Schwartsmann, Natural products in anticancer therapy, Current Opinion in Pharmacology, 1 (2001) 364-369.
[9] J.M. Reichert, V.E. Valge-Archer, Outlook - Development trends for monoclonal antibody cancer therapeutics, Nature Reviews Drug Discovery, 6 (2007) 349-356.
[10] D. Schrama, R.A. Reisfeld, J.C. Becker, Antibody targeted drugs as cancer therapeutics, Nature Reviews Drug Discovery, 5 (2006) 147-159.
[11] P.K. Srivastava, Therapeutic cancer vaccines, Current Opinion in Immunology, 18 (2006) 201-205.
[12] G.P. Adams, L.M. Weiner, Monoclonal antibody therapy of cancer, Nature Biotechnology, 23 (2005) 1147-1157.
[13] R.W. Brueggemeier, J.C. Hackett, E.S. Diaz-Cruz, Aromatase inhibitors in the treatment of breast cancer, Endocrine Reviews, 26 (2005) 331-345.
[14] C.K. Osborne, Drug therapy - Tamoxifen in the treatment of breast cancer, New England Journal of Medicine, 339 (1998) 1609-1618.
[15] I.E. Smith, M. Dowsett, Drug therapy: Aromatase inhibitors in breast cancer, New England Journal of Medicine, 348 (2003) 2431-2442.
[16] T. Browder, C.E. Butterfield, B.M. Kraling, B. Shi, B. Marshall, M.S. O'Reilly, J. Folkman, Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer, Cancer Research, 60 (2000) 1878-1886.
[17] J.M.M. Terwogt, J.H.M. Schellens, W.W.T. Huinink, J.H. Beijnen, Clinical pharmacology of anticancer agents in relation to formulations and administration routes, Cancer Treatment Reviews, 25 (1999) 83-101.
[18] M. Borner, W. Scheithauer, C. Twelves, J. Maroun, H. Wilke, Answering patients' needs: Oral alternatives to intravenous therapy, Oncologist, 6 (2001) 12-16.
[19] S. Kaasa, J.H. Loge, Quality-of-life assessment in palliative care, Lancet Oncology, 3 (2002) 175-182.
[20] G. Liu, E. Franssen, M.I. Fitch, E. Warner, Patient preferences for oral versus intravenous palliative chemotherapy, Journal of Clinical Oncology, 15 (1997) 110-115.
[21] V.J. O'Neill, C.J. Twelves, Oral cancer treatment: developments in chemotherapy and beyond, British Journal of Cancer, 87 (2002) 933-937.
[22] S.N. Weingart, P.B. Bach, K. Eng, S.A. Johnson, T.M. Kuzel, T.S. Langbaum, R.D. Leedy, R.J. Muller, L.N. Newcomer, S. O'Brien, D. Reinke, M. Rubino, R.S. Walters, NCCN Task Force Report: Oral chemotherapy, SO - J Natl Compr Canc Netw. 2008 Mar;6 Suppl 3:S1-14., (2008).

[23] M. Gornas, C. Szczylik, Oral treatment of metastatic breast cancer with capecitabine: what influences the decision-making process?, European Journal of Cancer Care, 19 (2010) 131-136.
[24] T.R. Halfdanarson, A. Jatoi, Oral Cancer Chemotherapy: The Critical Interplay Between Patient Education and Patient Safety, Current Oncology Reports, 12 (2010) 247-252.
[25] G.L. Banna, E. Collova, V. Gebbia, H. Lipari, P. Giuffrida, S. Cavallaro, R. Condorelli, C. Buscarino, P. Tralongo, F. Ferrau, Anticancer oral therapy: Emerging related issues, Cancer Treatment Reviews, 36 (2010) 595-605.
[26] F.R. Curtiss, Pharmacy benefit spending on oral chemotherapy drugs, Journal of Managed Care Pharmacy, 12 (2006) 570-577.
[27] C.J. Twelves, Xeloda (R) in adjuvant colon cancer therapy (X-ACT) trial: Overview of efficacy, safety, and cost-effectiveness, Clinical Colorectal Cancer, 6 (2006) 278-287.
[28] C. Catania, F. Didier, M.E. Leon, A. Sbanotto, L. Mariani, F. Nole, E. Leida, A. Rocca, T. De Pas, A. Goldhirsch, Perception that oral anticancer treatments are less efficacious: development of a questionnaire to assess the possible prejudices of patients with cancer, Breast Cancer Research and Treatment, 92 (2005) 265-272.
[29] J.A. Cramer, A. Roy, A. Burrell, C.J. Fairchild, M.J. Fuldeore, D.A. Ollendorf, P.K. Wong, Medication compliance and persistence: Terminology and definitions, Value in Health, 11 (2008) 44-47.
[30] K. Ruddy, E. Mayer, A. Partridge, Patient Adherence and Persistence With Oral Anticancer Treatment, Ca-a Cancer Journal for Clinicians, 59 (2009) 56-66.
[31] A.H. Partridge, J. Avorn, P.S. Wang, E.P. Winer, Adherence to therapy with oral antineoplastic agents, Journal of the National Cancer Institute, 94 (2002) 652-661.
[32] J. Cassidy, Y. Douillard, C. Twelves, J.J. McKendrick, W. Scheithauer, I. Bustova, P.G. Johnston, L. Lesniewski-Kmak, S. Jelic, G. Fountzilas, F. Coxon, E. Diaz-Rubio, T.S. Maughan, A. Malzyner, O. Bertetto, A. Beham, A. Figer, P. Dufour, K.K. Patel, W. Cowell, L.P. Garrison, Pharmacoeconomic analysis of adjuvant oral capecitabine vs intravenous 5-FU/LV in Dukes' C colon cancer: the X-ACT trial, British Journal of Cancer, 94 (2006) 1122-1129.
[33] S. Irshad, N. Maisey, Considerations when choosing oral chemotherapy: identifying and responding to patient need, European Journal of Cancer Care, 19 (2010) 5-11.
[34] E. De Cock, J. Hutton, P. Canney, J.J. Body, P. Barrett-Lee, M.P. Neary, G. Lewis, Cost-effectiveness of oral ibandronate compared with intravenous (i.v.) zoledronic acid or i.v. generic pamidronate in breast cancer patients with metastatic bone disease undergoing i.v. chemotherapy, Supportive Care in Cancer, 13 (2005) 975-986.
[35] E. De Cock, J. Hutton, P. Canney, J.J. Body, P. Barrett-Lee, M.P. Neary, G. Lewis, Cost-effectiveness of oral ibandronate versus IV zoledronic acid or IV pamidronate for bone metastases in patients receiving oral hormonal therapy for breast cancer in the United Kingdom, Clinical Therapeutics, 27 (2005) 1295-1310.
[36] C. Eng, H.L. Kindler, R.L. Schilsky, Oral fluoropyrimidine treatment of colorectal cancer, Clinical colorectal cancer, 1 (2001) 95-103.
[37] R. Colomer, E. Alba, A. Gonzalez-Martin, L. Paz-Ares, M. Martin, A. Llombart, A. Rodriguez Lescure, J. Salvador, J. Albanell, D. Isla, M. Lomas, C.A. Rodriguez, J.M. Trigo, J.R. Germa, J. Bellmunt, J. Tabernero, R. Rosell, E. Aranda, R. Cubedo, J. Baselga, Treatment of cancer with oral drugs: a position statement by the Spanish Society of Medical Oncology (SEOM), Annals of Oncology, 21 (2010) 196-198.
[38] R.S. Kerbel, Improving conventional or low dose metronomic chemotherapy with targeted antiangiogenic drugs, Cancer research and treatment : official journal of Korean Cancer Association, 39 (2007) 150-159.
[39] R.S. Kerbel, B.A. Kamen, The anti-angiogenic basis of metronomic chemotherapy, Nature Reviews Cancer, 4 (2004) 423-436.
[40] M.L. Citron, D.A. Berry, C. Cirrincione, C. Hudis, E.P. Winer, W.J. Gradishar, N.E. Davidson, S. Martino, R. Livingston, J.N. Ingle, E.A. Perez, J. Carpenter, D. Hurd, J.F. Holland, B.L. Smith, C.I. Sartor, E.H. Leung, J. Abrams, R.L. Schilsky, H.B. Muss, L. Norton, Randomized trial of dose-dense versus

conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: First report of intergroup trial C9741/cancer and leukemia group B trial 9741, Journal of Clinical Oncology, 21 (2003) 1431-1439.
[41] R.S. Tuma, Dosing study seen as victory for clinical trials, mathematical models, Journal of the National Cancer Institute, 95 (2003) 254-255.
[42] T. Aihara, Y. Kim, Y. Takatsuka, Phase II study of weekly docetaxel in patients with metastatic breast cancer, Annals of Oncology, 13 (2002) 286-292.
[43] J. Lokich, Phase I clinical trial of weekly combined paclitaxel plus docetaxel in patients with solid tumors, Cancer, 89 (2000) 2309-2314.
[44] V. Brower, Less is more - Research into anti-angiogenic therapies for treating cancer has finally had its first breakthroughs. But it may also influence the way in which classical chemotherapy is used for cancer treatment, Embo Reports, 4 (2003) 831-834.
[45] N. Andre, L. Padovani, A. Verschuur, Metronomic Chemotherapy: Back to the future!, Drug News & Perspectives, 23 (2010) 143-151.
[46] G. Gasparini, Antiangiogenic drugs as a novel anticancer therapeutic strategy - Which are the more promising agents? What are the clinical developments and indications?, Critical Reviews in Oncology Hematology, 26 (1997) 147-162.
[47] G. Gasparini, Metronomic scheduling: the future of chemotherapy?, Lancet Oncology, 2 (2001) 733-740.
[48] J. Satti, The emerging low-dose therapy for advanced cancers, Dose-Response, 7 (2009) 208-220.
[49] S. Rafii, D. Lyden, R. Benezra, K. Hattori, B. Heissig, Vascular and haematopoietic stem cells: Novel targets for anti-angiogenesis therapy?, Nature Reviews Cancer, 2 (2002) 826-835.
[50] K. Hashimoto, S. Man, P. Xu, W. Cruz-Munoz, T. Tang, R. Kumar, R.S. Kerbel, Potent Preclinical Impact of Metronomic Low-Dose Oral Topotecan Combined with the Antiangiogenic Drug Pazopanib for the Treatment of Ovarian Cancer, Molecular Cancer Therapeutics, 9 (2010) 996-1006.
[51] R. Samaritani, G. Corrado, E. Vizza, C. Sbiroli, Cyclophosphamide "metronomic" chemotherapy for palliative treatment of a young patient with advanced epithelial ovarian cancer, Bmc Cancer, 7 (2007).
[52] S. Man, G. Bocci, G. Francia, S.K. Green, S. Jothy, D. Hanahan, P. Bohlen, D.J. Hicklin, G. Bergers, R.S. Kerbel, Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water, Cancer Research, 62 (2002) 2731-2735.
[53] P. Albertsson, B. Lennernas, K. Norrby, On metronomic chemotherapy: Modulation of angiogenesis mediated by VEGF-A, Acta Oncologica, 45 (2006) 144-155.
[54] A.A. Kamat, T.J. Kim, C.N. Landen, Jr., C. Lu, L.Y. Han, Y.G. Lin, W.M. Merritt, P.H. Thaker, D.M. Gershenson, F.Z. Bischoff, J.V. Heymach, R.B. Jaffe, R.L. Coleman, A.K. Sood, Metronomic chemotherapy enhances the efficacy of antivascular therapy in ovarian cancer, Cancer Research, 67 (2007) 281-288.
[55] W.M. Merritt, C.G. Danes, M.M.K. Shahzad, Y.G. Lin, A.A. Kamat, L.Y. Han, W.A. Spannuth, A.M. Nick, L.S. Mangala, R.L. Stone, H.S. Kim, D.M. Gershenson, R.B. Jaffe, R.L. Coleman, J. Chandra, A.K. Sood, Anti-angiogenic properties of metronomic topotecan in ovarian carcinoma, Cancer Biology & Therapy, 8 (2009) 1596-1603.
[56] A.A. Garcia, H. Hirte, G. Fleming, D. Yang, D.D. Tsao-Wei, L. Roman, S. Groshen, S. Swenson, F. Markland, D. Gandara, S. Scudder, R. Morgan, H. Chen, H.-J. Lenz, A.M. Oza, Phase II clinical trial of bevacizumab and low-dose metronomic oral cyclophosphamide in recurrent ovarian cancer: A trial of the California, Chicago, and princess Margaret hospital phase II consortia, Journal of Clinical Oncology, 26 (2008) 76-82.
[57] J.M. Jurado, A. Sanchez, B. Pajares, E. Perez, L. Alonso, E. Alba, Combined oral cyclophosphamide and bevacizumab in heavily pre-treated ovarian cancer, Clinical Translational Oncology, 10 (2008) 583-586.
[58] A. Fontana, L. Galli, A. Fioravanti, P. Orlandi, C. Galli, L. Landi, S. Bursi, G. Allegrini, E. Fontana, R. Di Marsico, A. Antonuzzo, M. D'Arcangelo, R. Danesi, M. Del Tacca, A. Falcone, G. Bocci, Clinical and

pharmacodynamic evaluation of metronomic cyclophosphamide, celecoxib, and dexamethasone in advanced hormone-refractory prostate cancer, Clin Cancer Res. , 15 (2009) 4954-4962.
[59] L.M. Glode, B. Albaha, F. Crighton, E.D. Crawford, R. Kerbel, Metronomic therapy with cyclophosphamide and dexamethasone for prostate carcinoma, SO - Cancer. 2003 Oct 15;98(8):1643-8., (2003).
[60] R. Lord, S. Nair, A. Schache, J. Spicer, N. Somaihah, V. Khoo, H. Pandha, Low dose metronomic oral cyclophosphamide for hormone resistant prostate cancer: A phase II study, Journal of Urology, 177 (2007) 2136-2140.
[61] O. deWeerdt, N.W. van de Donk, G. Veth, A.C. Bloem, A. Hagenbeek, H.M. Lokhorst, Continuous low-dose cyclophosphamide-prednisone is effective and well tolerated in patients with advanced multiple myeloma, Neth J Med. , 59 (2001) 50-56.
[62] J. Bellmunt, J. Manuel Trigo, E. Calvo, J. Carles, J.L. Perez-Gracia, J. Rubio, J. Antonio Virizuela, R. Lopez, M. Lazaro, J. Albanell, Activity of a multitargeted chemo-switch regimen (sorafenib, gemcitabine, and metronomic capecitabine) in metastatic renal-cell carcinoma: a phase 2 study (SOGUG-02-06), Lancet Oncology, 11 (2010) 350-357.
[63] M.K. Krzyzanowska., I.F. Tannock, G. Lockwood, J. Knox, M. Moore, G.A. Bjarnason, A phase II trial of continuous low-dose oral cyclophosphamide and celecoxib in patients with renal cell carcinoma, Cancer Chemotherapy and Pharmacology, 60 (2007) 135-141.
[64] M.P. Brizzi, A. Berruti, A. Ferrero, E. Milanesi, M. Volante, F. Castiglione, N. Birocco, S. Bombaci, D. Perroni, B. Ferretti, O. Alabiso, L. Ciuffreda, O. Bertetto, M. Papotti, L. Dogliotti, Continuous 5-fluorouracil infusion plus long acting octreotide in advanced well-differentiated neuroendocrine carcinomas. A phase II trial of the Piemonte oncology network, BMC Cancer. 2009 Nov 3;9:388., (2009).
[65] R.S. Bhatt, J. Merchan, R. Parker, H.-K. Wu, L. Zhang, V. Seery, J.V. Heymach, M.B. Atkins, D. McDermott, V.P. Sukhatme, A Phase 2 Pilot Trial of Low-Dose, Continuous Infusion, or "Metronomic" Paclitaxel and Oral Celecoxib in Patients With Metastatic Melanoma, Cancer, 116 (2010) 1751-1756.
[66] L. Zitvogel, L. Apetoh, F. Ghiringhelli, G. Kroemer, Immunological aspects of cancer chemotherapy, Nat Rev Immunol, 8 (2008) 59-73.
[67] J.B. Katz, A.J. Muller, G.C. Prendergast, Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape, Immunol Rev, 222 (2008) 206-221.
[68] G.P. Dunn, L.J. Old, R.D. Schreiber, The immunobiology of cancer immunosurveillance and immunoediting, Immunity, 21 (2004) 137-148.
[69] W. Zou, Immunosuppressive networks in the tumour environment and their therapeutic relevance, Nat Rev Cancer, 5 (2005) 263-274.
[70] R.A. Lake, B.W. Robinson, Immunotherapy and chemotherapy--a practical partnership, Nat Rev Cancer, 5 (2005) 397-405.
[71] T. Parvez, Cancer treatment: what's ahead?, J Coll Physicians Surg Pak, 15 (2005) 738-745.
[72] L. Jia, K. Schweikart, J. Tomaszewski, J.G. Page, P.E. Noker, S.A. Buhrow, J.M. Reid, M.M. Ames, D.H. Munn, Toxicology and pharmacokinetics of 1-methyl-d-tryptophan: absence of toxicity due to saturating absorption, Food Chem Toxicol, 46 (2008) 203-211.
[73] M. Zamanakou, A.E. Germenis, V. Karanikas, Tumor immune escape mediated by indoleamine 2,3-dioxygenase, Immunol Lett, 111 (2007) 69-75.
[74] H.J. Ball, H.J. Yuasa, C.J. Austin, S. Weiser, N.H. Hunt, Indoleamine 2,3-dioxygenase-2; a new enzyme in the kynurenine pathway, Int J Biochem Cell Biol, 41 (2009) 467-471.
[75] M.F. Murray, The human indoleamine 2,3-dioxygenase gene and related human genes, Curr Drug Metab, 8 (2007) 197-200.
[76] K. Higuchi, O. Hayaishi, Enzymic formation of D-kynurenine from D-tryptophan, Arch Biochem Biophys, 120 (1967) 397-403.
[77] H.J. Ball, A. Sanchez-Perez, S. Weiser, C.J. Austin, F. Astelbauer, J. Miu, J.A. McQuillan, R. Stocker, L.S. Jermiin, N.H. Hunt, Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice, Gene, 396 (2007) 203-213.

[78] Y. Watanabe, R. Yoshida, M. Sono, O. Hayaishi, Immunohistochemical localization of indoleamine 2,3-dioxygenase in the argyrophilic cells of rabbit duodenum and thyroid gland, J Histochem Cytochem, 29 (1981) 623-632.
[79] H. Sugimoto, S. Oda, T. Otsuki, T. Hino, T. Yoshida, Y. Shiro, Crystal structure of human indoleamine 2,3-dioxygenase: catalytic mechanism of O2 incorporation by a heme-containing dioxygenase, Proc Natl Acad Sci U S A, 103 (2006) 2611-2616.
[80] A.L. Mellor, D.H. Munn, IDO expression by dendritic cells: tolerance and tryptophan catabolism, Nat Rev Immunol, 4 (2004) 762-774.
[81] D.H. Munn, M. Zhou, J.T. Attwood, I. Bondarev, S.J. Conway, B. Marshall, C. Brown, A.L. Mellor, Prevention of allogeneic fetal rejection by tryptophan catabolism, Science, 281 (1998) 1191-1193.
[82] C. Uyttenhove, L. Pilotte, I. Theate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, B.J. Van den Eynde, Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase, Nat Med, 9 (2003) 1269-1274.
[83] M. Friberg, R. Jennings, M. Alsarraj, S. Dessureault, A. Cantor, M. Extermann, A.L. Mellor, D.H. Munn, S.J. Antonia, Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection, Int J Cancer, 101 (2002) 151-155.
[84] S. Astigiano, B. Morandi, R. Costa, L. Mastracci, A. D'Agostino, G.B. Ratto, G. Melioli, G. Frumento, Eosinophil granulocytes account for indoleamine 2,3-dioxygenase-mediated immune escape in human non-small cell lung cancer, Neoplasia, 7 (2005) 390-396.
[85] A.L. Mellor, D.B. Keskin, T. Johnson, P. Chandler, D.H. Munn, Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses, J Immunol, 168 (2002) 3771-3776.
[86] A.J. Muller, J.B. DuHadaway, P.S. Donover, E. Sutanto-Ward, G.C. Prendergast, Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy, Nat Med, 11 (2005) 312-319.
[87] A.J. Muller, W.P. Malachowski, G.C. Prendergast, Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors, Expert Opin Ther Targets, 9 (2005) 831-849.
[88] D.H. Munn, A.L. Mellor, IDO and tolerance to tumors, Trends Mol Med, 10 (2004) 15-18.
[89] D.Y. Hou, A.J. Muller, M.D. Sharma, J. DuHadaway, T. Banerjee, M. Johnson, A.L. Mellor, G.C. Prendergast, D.H. Munn, Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses, Cancer Res, 67 (2007) 792-801.
[90] D.H. Munn, M.D. Sharma, D. Hou, B. Baban, J.R. Lee, S.J. Antonia, J.L. Messina, P. Chandler, P.A. Koni, A.L. Mellor, Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes, J Clin Invest, 114 (2004) 280-290.
[91] R. Potula, L. Poluektova, B. Knipe, J. Chrastil, D. Heilman, H. Dou, O. Takikawa, D.H. Munn, H.E. Gendelman, Y. Persidsky, Inhibition of indoleamine 2,3-dioxygenase (IDO) enhances elimination of virus-infected macrophages in an animal model of HIV-1 encephalitis, Blood, 106 (2005) 2382-2390.
[92] B. Baban, P. Chandler, D. McCool, B. Marshall, D.H. Munn, A.L. Mellor, Indoleamine 2,3-dioxygenase expression is restricted to fetal trophoblast giant cells during murine gestation and is maternal genome specific, J Reprod Immunol, 61 (2004) 67-77.
[93] D.H. Munn, Indoleamine 2,3-dioxygenase, tumor-induced tolerance and counter-regulation, Curr Opin Immunol, 18 (2006) 220-225.
[94] D.H. Munn, M.D. Sharma, B. Baban, H.P. Harding, Y. Zhang, D. Ron, A.L. Mellor, GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase, Immunity, 22 (2005) 633-642.
[95] T. Banerjee, J.B. Duhadaway, P. Gaspari, E. Sutanto-Ward, D.H. Munn, A.L. Mellor, W.P. Malachowski, G.C. Prendergast, A.J. Muller, A key in vivo antitumor mechanism of action of natural product-based brassinins is inhibition of indoleamine 2,3-dioxygenase, Oncogene 27 (2008) 2851-2857. Epub 2007 Nov 2819.

[96] R. Mezencev, J. Mojzis, M. Pilatova, P. Kutschy, Antiproliferative and cancer chemopreventive activity of phytoalexins: focus on indole phytoalexins from crucifers, Neoplasma, 50 (2003) 239-245.
[97] A. Pereira, E. Vottero, M. Roberge, A.G. Mauk, R.J. Andersen, Indoleamine 2,3-dioxygenase inhibitors from the Northeastern Pacific Marine Hydroid Garveia annulata, J Nat Prod, 69 (2006) 1496-1499.
[98] S. Kumar, D. Jaller, B. Patel, J.M. LaLonde, J.B. DuHadaway, W.P. Malachowski, G.C. Prendergast, A.J. Muller, Structure based development of phenylimidazole-derived inhibitors of indoleamine 2,3-dioxygenase, J Med Chem, 51 (2008) 4968-4977.
[99] H.C. Brastianos, E. Vottero, B.O. Patrick, R. Van Soest, T. Matainaho, A.G. Mauk, R.J. Andersen, Exiguamine A, an indoleamine-2,3-dioxygenase (IDO) inhibitor isolated from the marine sponge Neopetrosia exigua, J Am Chem Soc, 128 (2006) 16046-16047.
[100] G. Carr, M.K. Chung, A.G. Mauk, R.J. Andersen, Synthesis of indoleamine 2,3-dioxygenase inhibitory analogues of the sponge alkaloid exiguamine A, J Med Chem 51 (2008) 2634-2637. Epub 2008 Apr 2638.
[101] S. Lob, A. Konigsrainer, H.G. Rammensee, G. Opelz, P. Terness, Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees?, Nat Rev Cancer, 9 (2009) 445-452.
[102] M.C. Wani, H.L. Taylor, M.E. Wall, P. Coggon, A.T. McPhail, Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia, J Am Chem Soc 93 (1971) 2325-2327.
[103] P.B. Schiff, J. Fant, S.B. Horwitz, Promotion of microtubule assembly in vitro by Taxol, Nature, 277 (1979) 665-667.
[104] A.K. Singla, A. Garg, D. Aggarwal, Paclitaxel and its formulations, International Journal of Pharmaceutics, 235 (2002) 179-192.
[105] M. Wall, M.C. Wani, Camptothecin and taxol: from discovery to clinic, J Ethnopharmacol. , 51 (1996) 239-253.
[106] L. Wilson, Microtubules as drug receptors-Pharmacological properties of microtubule protein, Annals of the New York Academy of Sciences, 253 (1975) 213-231.
[107] E.K. Rowinsky, R.C. Donehower, Drug-Therapy-Paclitaxel (Taxol), New England Journal of Medicine, 332 (1995) 1004-1014.
[108] E. Nogales, S.G. Wolf, I.A. Khan, R.F. Luduena, K.H. Downing, Structure of tubulin at 6.5 angstrom and location of the Taxol-binding site, Nature, 375 (1995) 424-427.
[109] M.A. Jordan, K. Wendell, S. Gardiner, W.B. Derry, H. Copp, L. Wilson, Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death, Cancer Research, 56 (1996) 816-825.
[110] A.M.C. Yvon, P. Wadsworth, M.A. Jordan, Taxol suppresses dynamics of individual microtubules in living human tumor cells, Molecular Biology of the Cell, 10 (1999) 947-959.
[111] A.F. Wahl, K.L. Donaldson, C. Fairchild, F.Y.F. Lee, S.A. Foster, G.W. Demers, D.A. Galloway, Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis, Nature Medicine, 2 (1996) 72-79.
[112] T.H. Wang, H.S. Wang, p53, apoptosis and human cancers, Journal of the Formosan Medical Association, 95 (1996) 509-522.
[113] R. Panchagnula, Pharmaceutical aspects of paclitaxel, International Journal of Pharmaceutics, 172 (1998) 1-15.
[114] P. Jenkins, Taxol branches out, Chemistry in Britain, 32 (1996) 43-46.
[115] K.C. Nicolaou, W.M. Dai, R.K. Guy, Chemistry and biology of Taxol, Angewandte Chemie-International Edition in English, 33 (1994) 15-44.
[116] K.C. Nicolaou, Z. Yang, J.J. Liu, H. Ueno, P.G. Nantermet, R.K. Guy, C.F. Claiborne, J. Renaud, E.A. Couladouros, K. Paulvannan, E.J. Sorensen, Total synthesis of Taxol, Nature, 367 (1994) 630-634.
[117] D.G.I. Kingston, Taxol-The chemistry and structure-activity-relationship of a novel anticancer agent, Trends in Biotechnology, 12 (1994) 222-227.

[118] J. Verweij, M. Clavel, B. Chevalier, PACLITAXEL (TAXOL(TM)) AND DOCETAXEL (TAXOTERE(TM)) - NOT SIMPLY 2 OF A KIND, Annals of Oncology, 5 (1994) 495-505.
[119] A. Stierle, G. Strobel, D. Stierle, Taxol and taxane production by Taxomyces-andrenae, an endophytic fungus of Pacific Yew, Science, 260 (1993) 214-216.
[120] P.H. Wiernik, E.L. Schwartz, J.J. Strauman, J.P. Dutcher, R.B. Lipton, E. Paietta, Phase-I clinical and pharmacokinetics study of Taxol, Cancer Research, 47 (1987) 2486-2493.
[121] T. Brown, K. Havlin, G. Weiss, J. Cagnola, J. Koeller, J. Kuhn, J. Rizzo, J. Craig, J. Phillips, D. Vonhoff, A phase I trial of Taxol give by a 6-hour intravenous-infusion, Journal of Clinical Oncology, 9 (1991) 1261-1267.
[122] E.K. Rowinsky, R.C. Donehower, The clinical-pharmacology of paclitaxel (Taxol(R)), Seminars in Oncology, 20 (1993) 16-25.
[123] J. Rizzo, C. Riley, D. Vonhoff, J. Kuhn, J. Phillips, T. Brown, Analysis of anticancer drugs in bilogical-fluids-Determination of Taxol with application to clinical phramacokinetics, Journal of Pharmaceutical and Biomedical Analysis, 8 (1990) 159-164.
[124] M. Nakajima, Y. Fujiki, S. Kyo, T. Kanaya, M. Nakamura, Y. Maida, M. Tanaka, M. Inoue, T. Yokoi, Pharmacokinetics of paclitaxel in ovarian cancer patients and genetic polymorphisms of CYP2C8, CYP3A4, and MDR1, Journal of Clinical Pharmacology, 45 (2005) 674-682.
[125] T. Walle, Assays of CYP2C8- and CYP3A4-mediated metabolism of taxol in vivo and in vitro, Cytochrome P450, Pt B, 272 (1996) 145-151.
[126] A. Henningsson, S. Marsh, W.J. Loos, M.O. Karlsson, A. Garsa, K. Mross, S. Mielke, L. Vigano, A. Locatelli, J. Verweij, A. Sparreboom, H.L. McLeod, Association of CYP2C8, CYP3A4, CYP3A5, and ABCB1 polymorphisms with the pharmacokinetics of paclitaxel, Clinical Cancer Research, 11 (2005) 8097-8104.
[127] A. Sparreboom, J. vanAsperen, U. Mayer, A.H. Schinkel, J.W. Smit, D.K.F. Meijer, P. Borst, W.J. Nooijen, J.H. Beijnen, O. vanTellingen, Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine, Proceedings of the National Academy of Sciences of the United States of America, 94 (1997) 2031-2035.
[128] M.M. Malingre, J.H. Beijnen, J.H.M. Schellens, Oral delivery of taxanes, Investigational New Drugs, 19 (2001) 155-162.
[129] B. Melichar, R. Hyspler, E. Dragounova, J. Dvorak, H. Kalabova, A. Ticha, Gastrointestinal permeability in ovarian cancer and breast cancer patients treated with paclitaxel and platinum, Bmc Cancer, 7 (2007).
[130] P. Bonomi, K.M. Kim, D. Fairclough, D. Cella, J. Kugler, E. Rowinsky, M. Jiroutek, D. Johnson, Comparison of survival and quality of life in advanced non-small-cell lung cancer patients treated with two dose levels of paclitaxel combined with cisplatin versus etoposide with cisplatin: Results of an eastern cooperative oncology group trial, Journal of Clinical Oncology, 18 (2000) 623-631.
[131] E.B. Baskan, S. Tunali, S.B. Adim, M. Kiyici, R. Ali, Treatment of advanced classic Kaposi's sarcoma with weekly low-dose paclitaxel therapy, International Journal of Dermatology, 45 (2006) 1441-1443.
[132] T. Dhillon, J. Stebbing, M. Bower, Paclitaxel for AIDS-associated Kaposi's sarcoma, Expert Review of Anticancer Therapy, 5 (2005) 215-219.
[133] T. Vanni, E. Sprinz, M.W. Machado, R.d.C. Santana, B.A.L. Fonseca, G. Schwartsmann, Systemic treatment of AIDS-related Kaposi sarcoma: Current status and perspectives, Cancer Treatment Reviews, 32 (2006) 445-455.
[134] A.A. Forastiere, Use of paclitaxel (taxol(R)) in squamous-cell carcinoma of head and neck, Seminars in Oncology, 20 (1993) 56-60.
[135] H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom, Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation, European Journal of Cancer, 37 (2001) 1590-1598.
[136] D. Friedland, G. Gorman, J. Treat, Hypersensitivity reactions from Taxol and Etoposide, Journal of the National Cancer Institute, 85 (1993) 2036-2036.

[137] R.B. Weiss, R.C. Donehower, P.H. Wiernik, T. Ohnuma, R.J. Gralla, D.L. Trump, J.R. Baker, D.A. Vanecho, D.D. Vonhoff, B. Leylandjones, Hypersensitivity reactions from Taxol, Journal of Clinical Oncology, 8 (1990) 1263-1268.
[138] R.W. Klecker, C.A. Jamisdow, M.J. Egorin, K. Erkmen, R.J. Parker, R. Stevens, J.M. Collin, Effect of cimetidine, probenecid, and ketoconazole on the distribution, biliary-secretion and metabolism of[H-3] Taxol in the Sprague-Dawley rat, Drug Metabolism and Disposition, 22 (1994) 254-258.
[139] G.J. Lesser, S.A. Grossman, S. Eller, E.K. Rowinsky, THE DISTRIBUTION OF SYSTEMICALLY ADMINISTERED [H-3] PACLITAXEL IN RATS - A QUANTITATIVE AUTORADIOGRAPHIC STUDY, Cancer Chemotherapy and Pharmacology, 37 (1995) 173-178.
[140] L.A. Trissel, Q.Y. Xu, J. Kwan, J.F. Martinez, Compatibility of paclitaxel injection vehicle with intravenous administration and extension sets, American Journal of Hospital Pharmacy, 51 (1994) 2804-2810.
[141] A. Sparreboom, O. vanTellingen, W.J. Nooijen, J.H. Beijnen, Nonlinear pharmacokinetics of paclitaxel in mice results from the pharmaceutical vehicle Cremophor EL, Cancer Research, 56 (1996) 2112-2115.
[142] A. Sparreboom, L. van Zuylen, E. Brouwer, W.J. Loos, P. de Bruijn, B. Gelderblom, M. Pillay, K. Nooter, G. Stoter, J. Verweij, Cremophor EL-mediated alteration of paclitaxel distribution in human blood: Clinical pharmacokinetic implications, Cancer Research, 59 (1999) 1454-1457.
[143] L.M.S. Chan, S. Lowes, B.H. Hirst, The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability, European Journal of Pharmaceutical Sciences, 21 (2004) 25-51.
[144] N.K. Ibrahim, N. Desai, S. Legha, P. Soon-Shiong, R.L. Theriault, E. Rivera, B. Esmaeli, S.E. Ring, A. Bedikian, G.N. Hortobagyi, J.A. Ellerhorst, Phase I and pharmacokinetic study of ABI-007, a cremophor-free, protein-stabilized, nanoparticle formulation of paclitaxel, Clinical Cancer Research, 8 (2002) 1038-1044.
[145] B. Nuijen, M. Bouma, J.H.M. Schellens, J.H. Beijnen, Progress in the development of alternative pharmaceutical formulations of taxanes, Investigational New Drugs, 19 (2001) 143-153.
[146] A. Sparreboom, A.C. Wolff, J. Verweij, Y. Zabelina, D.M. van Zomeren, G.L. McIntire, C.S. Swindell, R.C. Donehower, S.D. Baker, Disposition of docosahexaenoic acid-paclitaxel, a novel taxane, in blood: In vitro and clinical pharmacokinetic studies, Clinical Cancer Research, 9 (2003) 151-159.
[147] A.C. Wolff, R.C. Donehower, M.K. Carducci, M.A. Carducci, J.R. Brahmer, Y. Zabelina, M.O. Bradley, F.H. Anthony, C.S. Swindell, P.A. Witman, N.L. Webb, S.D. Baker, Phase I study of docosahexaenoic acid-paclitaxel: a taxane-fatty acid conjugate with a unique pharmacology and toxicity profile, Clinical Cancer Research, 9 (2003) 3589-3597.
[148] C. Li, Poly(L-glutamic acid) - anticancer drug conjugates, Advanced Drug Delivery Reviews, 54 (2002) 695-713.
[149] T.J. Altstadt, C.R. Fairchild, J. Golik, K.A. Johnston, J.F. Kadow, F.Y. Lee, B.H. Long, W.C. Rose, D.M. Vyas, H. Wong, M.J. Wu, M.D. Wittman, Synthesis and antitumor activity of novel C-7 paclitaxel ethers: Discovery of BMS-184476, Journal of Medicinal Chemistry, 44 (2001) 4577-4583.
[150] W.C. Rose, C. Fairchild, F.Y.F. Lee, Preclinical antitumor activity of two novel taxanes, Cancer Chemotherapy and Pharmacology, 47 (2001) 97-105.
[151] J.S. Kim, G.P. Amorino, H. Pyo, Q.W. Cao, J.O. Price, H. Choy, The novel taxane analogs, BMS-184476 and BMS-188797, potentiate the effects of radiation therapy in vitro and in vivo against human lung cancer cells, International Journal of Radiation Oncology Biology Physics, 51 (2001) 525-534.
[152] M. Shionoya, T. Jimbo, M. Kitagawa, T. Soga, A. Tohgo, DJ-927, a novel oral taxane, overcomes P-glycoprotein-mediated multidrug resistance in vitro and in vivo, Cancer Science, 94 (2003) 459-466.
[153] G. Cassinelli, C. Lanzi, R. Supino, G. Pratesi, V. Zuco, D. Laccabue, G. Cuccuru, E. Bombardelli, F. Zunino, Cellular bases of the antitumor activity of the novel taxane IDN 5109 (BAY59-8862) on hormone-refractory prostate cancer, Clinical Cancer Research, 8 (2002) 2647-2654.

[154] D. Polizzi, G. Pratesi, M. Tortoreto, R. Supino, A. Riva, E. Bombardelli, F. Zunino, A novel taxane with improved tolerability and therapeutic activity in a panel of human tumor xenografts, Cancer Research, 59 (1999) 1036-1040.
[155] D. Polizzi, G. Pratesi, S. Monestiroli, M. Tortoreto, F. Zunino, E. Bombardelli, A. Riva, P. Morazzoni, T. Colombo, M. D'Incalci, M. Zucchetti, Oral efficacy and bioavailability of a novel taxane, Clinical Cancer Research, 6 (2000) 2070-2074.
[156] M.I. Nicoletti, T. Colombo, C. Rossi, C. Monardo, S. Stura, M. Zucchetti, A. Riva, P. Morazzoni, M.B. Donati, E. Bombardelli, M. D'Incalci, R. Giavazzi, IDN5109, a taxane with oral bioavailability and potent antitumor activity, Cancer Research, 60 (2000) 842-846.
[157] K. Tonkin, H. Hurwitz, C. Lathia, M. Gallentine, D. Voliotis, E. Hobdy, A phase I and pharmacokinetic study of an oral novel taxane BAY 59-8862 given daily for 5 days every 3 weeks, Breast Cancer Research and Treatment, 82 (2003) S86-S86.
[158] J.S. Gurtler, J. Von Pawel, C.H. Spiridonidis, F. Grossi, J.L. Larriba, M. Moscovici, E. Markovitz, D. Voliotis, M. Gottfried, An uncontrolled phase II study evaluating anti-tumor efficacy and safety of ortataxel (BAY 59-8862) in patients with taxane-resistant non-small cell lung cancer, Journal of Clinical Oncology, 22 (2004) 650S-650S.
[159] S.C. Kim, D.W. Kim, Y.H. Shim, J.S. Bang, H.S. Oh, S.W. Kim, M.H. Seo, In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy, Journal of Controlled Release, 72 (2001) 191-202.
[160] X.C. Zhang, H.M. Burt, D. VonHoff, D. Dexter, G. Mangold, D. Degen, A.M. Oktaba, W.L. Hunter, An investigation of the antitumour activity and biodistribution of polymeric micellar paclitaxel, Cancer Chemotherapy and Pharmacology, 40 (1997) 81-86.
[161] X.C. Zhang, H.M. Burt, G. Mangold, D. Dexter, D. VonHoff, L. Mayer, W.L. Hunter, Anti-tumor efficacy and biodistribution of intravenous polymeric micellar paclitaxel, Anti-Cancer Drugs, 8 (1997) 696-701.
[162] T.Y. Kim, D.W. Kim, J.Y. Chung, S.G. Shin, S.C. Kim, D.S. Heo, N.K. Kim, Y.J. Bang, Phase I and pharmacokinetic study of Genexol-PM, a cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced malignancies, Clinical Cancer Research, 10 (2004) 3708-3716.
[163] N.L. Elstad, K.D. Fowers, OncoGel (ReGel/paclitaxel) - Clinical applications for a novel paclitaxel delivery system, Advanced Drug Delivery Reviews, 61 (2009) 785-794.
[164] E.-M. Collnot, C. Baldes, U.F. Schaefer, K.J. Edgar, M.F. Wempe, C.-M. Lehr, Vitamin E TPGS P-Glycoprotein Inhibition Mechanism: Influence on Conformational Flexibility, Intracellular ATP Levels, and Role of Time and Site of Access, Molecular Pharmaceutics, 7 (2010) 642-651.
[165] N. Bogdanova, A. Lissianskaya, A. Gorelov, V. Moiseyenko, A. Golubev, P. Weiden, TOCOSOL (TM) Paclitaxel (Paclitaxel injectable emulsion): Phase 1-2 study of weekly administration in patients with non-small cell lung, ovarian, urothelial transitional cell or colorectal carcinoma, Clinical Cancer Research, 9 (2003) 6136S-6137S.
[166] N. Bogdanova, N. Karaseva, N. Ognerubov, O. Golubeva, P. Weiden, Paclitaxel injectable emulsion: Phase 2a study of weekly administration in patients with non-small cell lung cancer (NSCLC), Journal of Clinical Oncology, 22 (2004) 649S-649S.
[167] A. Gorelov, S. Gorelov, P. Karlov, O. Golubeva, M. Stewart, Paclitaxel injectable emulsion: Phase 2a study of weekly administration in patients with metastatic or locally advanced unresectable or recurrent urothelial transitional cell cancer (TCC), Journal of Clinical Oncology, 22 (2004) 403S-403S.
[168] E. Harper, W.B. Dang, R.G. Lapidus, R.I. Garver, Enhanced efficacy of a novel controlled release paclitaxel formulation (PACLIMER Delivery System) for local-regional therapy of lung cancer tumor nodules in mice, Clinical Cancer Research, 5 (1999) 4242-4248.
[169] R.G. Lapidus, W.B. Dang, D.M. Rosen, A.M. Gady, Y. Zabelinka, R. O'Meally, T.L. DeWeese, S.R. Denmeade, Anti-tumor effect of combination therapy with intratumoral controlled-release paclitaxel (PACLIMER (R) Microspheres) and radiation, Prostate, 58 (2004) 291-298.

[170] D.K. Armstrong, G.F. Fleming, M. Markman, H.H. Bailey, A phase I trial of intraperitoneal sustained-release paclitaxel microspheres (Paclimer((R))) in recurrent ovarian cancer: A Gynecologic Oncology Group study, Gynecologic Oncology, 103 (2006) 391-396.
[171] J.-O. Kwak, S.H. Lee, G.S. Lee, M.S. Kim, Y.-G. Ahn, J.H. Lee, S.W. Kim, K.H. Kim, M.G. Lee, Selective inhibition of MDR1 (ABCB1) by HM30181 increases oral bioavailability and therapeutic efficacy of paclitaxel, European Journal of Pharmacology, 627 (2010) 92-98.
[172] G.A. Fisher, B.I. Sikic, Clinical-studies with modulators of multidrug-resistance, Hematology-Oncology Clinics of North America, 9 (1995) 363-382.
[173] M.M. Gottesman, I. Pastan, Clinical trials of agents that reverse multidrug-resistance, Journal of Clinical Oncology, 7 (1989) 409-411.
[174] R.F. Ozols, R.E. Cunnion, R.W. Klecker, T.C. Hamilton, Y. Ostchega, J.E. Parrillo, R.C. Young, Verapamil and adriamycin in the treatment of drug-resistant ovarian-cancer patients, Journal of Clinical Oncology, 5 (1987) 641-647.
[175] J.S. Woo, C.H. Lee, C.K. Shim, S.J. Hwang, Enhanced oral bioavailability of paclitaxel by coadministration of the P-glycoprotein inhibitor KR30031, Pharmaceutical Research, 20 (2003) 24-30.
[176] M.M. Malingre, J.H. Beijnen, H. Rosing, F.J. Koopman, O. van Tellingen, K. Duchin, W.W.T. Huinink, M. Swart, J. Lieverst, J.H.M. Schellens, The effect of different doses of cyclosporin A on the systemic exposure of orally administered paclitaxel, Anti-Cancer Drugs, 12 (2001) 351-358.
[177] M.M. Malingre, W.W.T. Huinink, K. Duchin, J.H.M. Schellens, J.H. Beijnen, Pharmacokinetics of oral cyclosporin A when co-administered to enhance the oral absorption of paclitaxel, Anti-Cancer Drugs, 12 (2001) 591-593.
[178] C.D. Britten, S.D. Baker, L.J. Denis, T. Johnson, R. Drengler, L.L. Siu, K. Duchin, J. Kuhn, E.K. Rowinsky, Oral paclitaxel and concurrent cyclosporin A: Targeting clinically relevant systemic exposure to paclitaxel, Clinical Cancer Research, 6 (2000) 3459-3468.
[179] J. vanAsperen, O. vanTellingen, A. Sparreboom, A.H. Schinkel, P. Borst, W.J. Nooijen, J.H. Beijnen, Enhanced oral bioavailability of paclitaxel in mice treated with the P-glycoprotein blocker SDZ PSC 833, British Journal of Cancer, 76 (1997) 1181-1183.
[180] S. Ganta, H. Devalapally, M. Amiji, Curcumin Enhances Oral Bioavailability and Anti-Tumor Therapeutic Efficacy of Paclitaxel upon Administration in Nanoemulsion Formulation, Journal of Pharmaceutical Sciences, 99 (2010) 4630-4641.
[181] H.A. Bardelmeijer, M. Ouwehand, J.H. Beijnen, J.H.M. Schellens, O. van Tellingen, Efficacy of novel P-glycoprotein inhibitors to increase the oral uptake of paclitaxel in mice, Investigational New Drugs, 22 (2004) 219-229.
[182] M. Kuehnle, M. Egger, C. Mueller, A. Mahringer, G. Bernhardt, G. Fricker, B. Koenig, A. Buschauer, Potent and Selective Inhibitors of Breast Cancer Resistance Protein (ABCG2) Derived from the p-Glycoprotein (ABCB1) Modulator Tariquidar, Journal of Medicinal Chemistry, 52 (2009) 1190-1197.
[183] J. Walker, C. Martin, R. Callaghan, Inhibition of P-glycoprotein function by XR9576 in a solid tumour model can restore anticancer drug efficacy, European Journal of Cancer, 40 (2004) 594-605.
[184] J.S. Choi, H.K. Choi, S.C. Shin, Enhanced bioavailability of paclitaxel after oral coadministration with flavone in rats, International Journal of Pharmaceutics, 275 (2004) 165-170.
[185] J.S. Choi, B.W. Jo, Y.C. Kim, Enhanced paclitaxel bioavailability after oral administration of paclitaxel or prodrug to rats pretreated with quercetin, European Journal of Pharmaceutics and Biopharmaceutics, 57 (2004) 313-318.
[186] J.S. Choi, B.W. Jo, Enhanced paclitaxel bioavailability after oral administration of pegylated paclitaxel prodrug for oral delivery in rats, International Journal of Pharmaceutics, 280 (2004) 221-227.
[187] B.W. Jo, M. Hess, M. Zahres, Self-diffusion of poly (ethylene oxide) - modified paclitaxel in dilute aqueous solutions, Materials Research Innovations, 7 (2003) 178-182.
[188] L. Liang, S.-W. Lin, W. Dai, J.-K. Lu, T.-Y. Yang, Y. Xiang, Y. Zhang, R.-T. Li, Q. Zhang, Novel cathepsin B-sensitive paclitaxel conjugate: Higher water solubility, better efficacy and lower toxicity, Journal of Controlled Release, 160 (2012) 618-629.

[189] M.V.S. Varma, R. Panchagnula, Enhanced oral paclitaxel absorption with vitamin E-TPGS: Effect on solubility and permeability in vitro, in situ and in vivo, European Journal of Pharmaceutical Sciences, 25 (2005) 445-453.
[190] P.-Y. Ho, T.-K. Yeh, H.-T. Yao, H.-L. Lin, H.-Y. Wu, Y.-K. Lo, Y.-W. Chang, T.-H. Chiang, S.H.W. Wu, Y.-S. Chao, C.-T. Chen, Enhanced oral bioavailability of paclitaxel by D-alpha-tocopheryl polyethylene glycol 400 succinate in mice, International Journal of Pharmaceutics, 359 (2008) 174-181.
[191] J.M. Dintaman, J.A. Silverman, Inhibition of P-glycoprotein by D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS), Pharmaceutical Research, 16 (1999) 1550-1556.
[192] H. Ringsdorf, Structure and properties of pharmacologically active polymers, Journal of Polymer Science Part C-Polymer Symposium, (1975) 135-153.
[193] R. Duncan, Polymer conjugates as anticancer nanomedicines, Nature Reviews Cancer, 6 (2006) 688-701.
[194] M.J. Vicent, H. Ringsdorf, R. Duncan, Polymer therapeutics: Clinical applications and challenges for development Preface, Advanced Drug Delivery Reviews, 61 (2009) 1117-1120.
[195] E. Lee, J. Lee, I.H. Lee, M. Yu, H. Kim, S.Y. Chae, S. Jon, Conjugated chitosan as a novel platform for oral delivery of paclitaxel, J Med Chem. , 23 (2008) 6442-6449.
[196] M. Thanou, J.C. Verhoef, H.E. Junginger, Oral drug absorption enhancement by chitosan and its derivatives, Advanced Drug Delivery Reviews, 52 (2001) 117-126.
[197] M. Kumar, R.A.A. Muzzarelli, C. Muzzarelli, H. Sashiwa, A.J. Domb, Chitosan chemistry and pharmaceutical perspectives, Chemical Reviews, 104 (2004) 6017-6084.
[198] A. Bernkop-Schnurch, V. Schwarz, S. Steininger, Polymers with thiol groups: A new generation of mucoadhesive polymers?, Pharmaceutical Research, 16 (1999) 876-881.
[199] A.E. Clausen, A. Bernkop-Schnurch, In vitro evaluation of the permeation-enhancing effect of thiolated polycarbophil, Journal of Pharmaceutical Sciences, 89 (2000) 1253-1261.
[200] C.E. Kast, D. Guggi, N. Langoth, A. Bernkop-Schnurch, Development and in vivo evaluation of an oral delivery system for low molecular weight heparin based on thiolated polycarbophil, Pharmaceutical Research, 20 (2003) 931-936.
[201] M.K. Marschutz, A. Bernkop-Schnurch, Thiolated polymers: self-crosslinking properties of thiolated 450 kDa poly(acrylic acid) and their influence on mucoadhesion, European Journal of Pharmaceutical Sciences, 15 (2002) 387-394.
[202] J. Iqbal, F. Sarti, G. Perera, A. Bernkop-Schnuerch, Development and in vivo evaluation of an oral drug delivery system for paclitaxel, Biomaterials, 32 (2011) 170-175.
[203] P. Calleja, J. Huarte, M. Agueros, L. Ruiz-Gaton, S. Espuelas, J.M. Irache, Molecular buckets: cyclodextrins for oral cancer therapy, Therapeutic delivery, 3 (2012) 43-57.
[204] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins .1. Drug solubilization and stabilization, Journal of Pharmaceutical Sciences, 85 (1996) 1017-1025.
[205] D. Duchene, D. Wouessidjewe, Industrial uses of cyclodextrins and their derivatives, Journal of Coordination Chemistry, 27 (1992) 223-236.
[206] D.O. Thompson, Cyclodextrins - Enabling excipients: Their present and future use in pharmaceuticals, Critical Reviews in Therapeutic Drug Carrier Systems, 14 (1997) 1-104.
[207] T. Irie, K. Uekama, Pharmaceutical applications of cyclodextrins .3. Toxicological issues and safety evaluation, Journal of Pharmaceutical Sciences, 86 (1997) 147-162.
[208] F. Fenyvesi, E. Fenyvesi, L. Szente, K. Goda, Z. Bacso, I. Bacskay, J. Varadi, T. Kiss, E. Molnar, T. Janaky, G. Szabo, Jr., M. Vecsernyes, P-glycoprotein inhibition by membrane cholesterol modulation, European Journal of Pharmaceutical Sciences, 34 (2008) 236-242.
[209] F. Fenyvesi, T. Kiss, E. Fenyvesi, L. Szente, S. Veszelka, M.A. Deli, J. Varadi, P. Feher, Z. Ujhelyi, A. Tosaki, M. Vecsernyes, I. Bacskay, Randomly Methylated beta-Cyclodextrin Derivatives Enhance Taxol Permeability Through Human Intestinal Epithelial Caco-2 Cell Monolayer, Journal of Pharmaceutical Sciences, 100 (2008) 4734-4744.

[210] M. Ishikawa, H. Yoshi, T. Furuta, Interaction of modified cyclodextrins with cytochrome P-450, Bioscience Biotechnology and Biochemistry, 69 (2005) 246-248.
[211] A. Garrigues, A.E. Escargueil, S. Orlowski, The multidrug transporter, P-glycoprotein, actively mediates cholesterol redistribution in the cell membrane, Proceedings of the National Academy of Sciences of the United States of America, 99 (2002) 10347-10352.
[212] N. Nishiyama, K. Kataoka, Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery, Pharmacology & Therapeutics, 112 (2006) 630-648.
[213] G. Gaucher, P. Satturwar, M.-C. Jones, A. Furtos, J.-C. Leroux, Polymeric micelles for oral drug delivery, European Journal of Pharmaceutics and Biopharmaceutics, 76 (2010) 147-158.
[214] S.R. Croy, G.S. Kwon, Polymeric micelles for drug delivery, Current Pharmaceutical Design, 12 (2006) 4669-4684.
[215] G. Gaucher, M.H. Dufresne, V.P. Sant, N. Kang, D. Maysinger, J.C. Leroux, Block copolymer micelles: preparation, characterization and application in drug delivery, Journal of Controlled Release, 109 (2005) 169-188.
[216] L. Bromberg, Polymeric micelles in oral chemotherapy, Journal of Controlled Release, 128 (2008) 99-112.
[217] E.V. Batrakova, A.V. Kabanov, Pluronic block copolymers: Evolution of drug delivery concept from inert nanocarriers to biological response modifiers, Journal of Controlled Release, 130 (2008) 98-106.
[218] T. Bansal, N. Akhtar, M. Jaggi, R.K. Khar, S. Talegaonkar, Novel formulation approaches for optimising delivery of anticancer drugs based on P-glycoprotein modulation, Drug Discov Today, 14 (2009) 1067-1074.
[219] Y.L. Lo, Relationships between the hydrophilic-lipophilic balance values of pharmaceutical excipients and their multidrug resistance modulating effect in Caco-2 cells and rat intestines, J Control Release, 90 (2003) 37-48.
[220] K. Yoncheva, P. Calleja, M. Agueros, P. Petrov, I. Miladinova, C. Tsvetanov, J.M. Irache, Stabilized micelles as delivery vehicles for paclitaxel, Int J Pharm, 436 (2012) 258-264.
[221] T. Gershanik, S. Benita, Self-dispersing lipid formulations for improving oral absorption of lipophilic drugs, Eur J Pharm Biopharm, 50 (2000) 179-188.
[222] S. Yang, R.N. Gursoy, G. Lambert, S. Benita, Enhanced oral absorption of paclitaxel in a novel self-microemulsifying drug delivery system with or without concomitant use of P-glycoprotein inhibitors, Pharm Res, 21 (2004) 261-270.
[223] R.L. Oostendorp, T. Buckle, G. Lambert, J.S. Garrigue, J.H. Beijnen, J.H.M. Schellens, O. van Tellingen, Paclitaxel in self-micro emulsifying formulations: oral bioavailability study in mice, Investigational New Drugs, 29 (2011) 768-776.
[224] P. Gao, B.D. Rush, W.P. Pfund, T. Huang, J.M. Bauer, W. Morozowich, M.S. Kuo, M.J. Hageman, Development of a supersaturable SEDDS (S-SEDDS) formulation of paclitaxel with improved oral bioavailability, J Pharm Sci, 92 (2003) 2386-2398.
[225] N. Gursoy, J.S. Garrigue, A. Razafindratsita, G. Lambert, S. Benita, Excipient effects on in vitro cytotoxicity of a novel paclitaxel self-emulsifying drug delivery system, J Pharm Sci, 92 (2003) 2411-2418.
[226] A. Chaurasiya, A.K. Singh, G.K. Jain, M.H. Warsi, E. Sublet, F.J. Ahmad, G. Borchard, R.K. Khar, Dual approach utilizing self microemulsifying technique and novel P-gp inhibitor for effective delivery of taxanes, J Microencapsul, 29 (2012) 583-595.
[227] B. Haley, E. Frenkel, Nanoparticles for drug delivery in cancer treatment, Urol Oncol, 26 (2008) 57-64.
[228] T. Kubik, K. Bogunia-Kubik, M. Sugisaka, Nanotechnology on duty in medical applications, Curr Pharm Biotechnol, 6 (2005) 17-33.
[229] N.T. Huynh, C. Passirani, P. Saulnier, J.P. Benoit, Lipid nanocapsules: A new platform for nanomedicine, International Journal of Pharmaceutics, 379 (2009) 201-209.

[230] A. Vonarbourg, C. Passirani, P. Saulnier, P. Simard, J.C. Leroux, J.P. Benoit, Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake, Journal of Biomedical Materials Research Part A, 78A (2006) 620-628.
[231] B. Heurtault, P. Saulnier, B. Pech, J.E. Proust, J.P. Benoit, A novel phase inversion-based process for the preparation of lipid nanocarriers, Pharm Res, 19 (2002) 875-880.
[232] J.S. Coon, W. Knudson, K. Clodfelter, B. Lu, R.S. Weinstein, Solutol HS 15, nontoxic polyoxyethylene esters of 12-hydroxystearic acid, reverses multidrug resistance, Cancer Res, 51 (1991) 897-902.
[233] I. Brigger, C. Dubernet, P. Couvreur, Nanoparticles in cancer therapy and diagnosis, Adv Drug Deliv Rev, 54 (2002) 631-651.
[234] S. Peltier, J.M. Oger, F. Lagarce, W. Couet, J.P. Benoit, Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules, Pharm Res, 23 (2006) 1243-1250.
[235] J. Hureaux, F. Lagarce, F. Gagnadoux, M.C. Rousselet, V. Moal, T. Urban, J.P. Benoit, Toxicological study and efficacy of blank and paclitaxel-loaded lipid nanocapsules after i.v. administration in mice, Pharm Res, 27 (2010) 421-430.
[236] D. Pandita, A. Ahuja, V. Lather, B. Benjamin, T. Dutta, T. Velpandian, R.K. Khar, Development of lipid-based nanoparticles for enhancing the oral bioavailability of paclitaxel, SO - AAPS PharmSciTech. 2011 Jun;12(2):712-22. Epub 2011 Jun 3., (2011).
[237] I.F. Uchegbu, J.A. Double, J.A. Turton, A.T. Florence, Distribution, metabolism and tumoricidal activity of doxorubicin administered in sorbitan monostearate (Span 60) niosomes in the mouse, Pharm Res, 12 (1995) 1019-1024.
[238] J. Varshosaz, A. Pardakhty, V.I. Hajhashemi, A.R. Najafabadi, Development and physical characterization of sorbitan monoester niosomes for insulin oral delivery, Drug Deliv, 10 (2003) 251-262.
[239] G.K. Pillai, M.L. Salim, Enhanced inhibition of platelet aggregation in-vitro by niosome-encapsulated indomethacin, Int J Pharm, 193 (1999) 123-127.
[240] A. Girigoswami, S. Das, S. De, Fluorescence and dynamic light scattering studies of niosomes-membrane mimetic systems, Spectrochim Acta A Mol Biomol Spectrosc, 64 (2006) 859-866.
[241] Z.S. Bayindir, N. Yuksel, Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery, J Pharm Sci, 99 (2010) 2049-2060.
[242] N.R. Jabir, S. Tabrez, G.M. Ashraf, S. Shakil, G.A. Damanhouri, M.A. Kamal, Nanotechnology-based approaches in anticancer research, Int J Nanomedicine, 7 (2012) 4391-4408.
[243] S.S. Feng, L. Mu, K.Y. Win, G. Huang, Nanoparticles of biodegradable polymers for clinical administration of paclitaxel, Curr Med Chem, 11 (2004) 413-424.
[244] C. Fonseca, S. Simoes, R. Gaspar, Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity, J Control Release, 83 (2002) 273-286.
[245] Z. Zhu, C. Xie, Q. Liu, X. Zhen, X. Zheng, W. Wu, R. Li, Y. Ding, X. Jiang, B. Liu, The effect of hydrophilic chain length and iRGD on drug delivery from poly(epsilon-caprolactone)-poly(N-vinylpyrrolidone) nanoparticles, Biomaterials, 32 (2011) 9525-9535.
[246] M. Agueros, V. Zabaleta, S. Espuelas, M.A. Campanero, J.M. Irache, Increased oral bioavailability of paclitaxel by its encapsulation through complex formation with cyclodextrins in poly(anhydride) nanoparticles, J Control Release, 145 (2010) 2-8.
[247] V. Zabaleta, G. Ponchel, H. Salman, M. Agueros, C. Vauthier, J.M. Irache, Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: permeability and pharmacokinetic study, Eur J Pharm Biopharm, 81 (2012) 514-523.
[248] P.P. Lv, Y.F. Ma, R. Yu, H. Yue, D.Z. Ni, W. Wei, G.H. Ma, Targeted delivery of insoluble cargo (paclitaxel) by PEGylated chitosan nanoparticles grafted with Arg-Gly-Asp (RGD), Mol Pharm, 9 (2012) 1736-1747.
[249] A. Shapira, Y.G. Assaraf, D. Epstein, Y.D. Livney, Beta-casein nanoparticles as an oral delivery system for chemotherapeutic drugs: impact of drug structure and properties on co-assembly, Pharm Res, 27 (2010) 2175-2186.

[250] D. Sharma, T.P. Chelvi, J. Kaur, K. Chakravorty, T.K. De, A. Maitra, R. Ralhan, Novel Taxol formulation: polyvinylpyrrolidone nanoparticle-encapsulated Taxol for drug delivery in cancer therapy, Oncol Res, 8 (1996) 281-286.
[251] M. Agueros, P. Areses, M.A. Campanero, H. Salman, G. Quincoces, I. Penuelas, J.M. Irache, Bioadhesive properties and biodistribution of cyclodextrin-poly(anhydride) nanoparticles, Eur J Pharm Sci, 37 (2009) 231-240.
[252] P. Arbos, M. Wirth, M.A. Arangoa, F. Gabor, J.M. Irache, Gantrez AN as a new polymer for the preparation of ligand-nanoparticle conjugates, J Control Release, 83 (2002) 321-330.
[253] Y.-S. Lin, R. Tungpradit, S. Sinchaikul, F.-M. An, D.-Z. Liu, S. Phutrakul, S.-T. Chen, Targeting the Delivery of Glycan-Based Paclitaxel Prodrugs to Cancer Cells via Glucose Transporters, Journal of Medicinal Chemistry, 51 (2008) 7428-7441.
[254] S.A. Veltkamp, C. Alderden-Los, A. Sharma, H. Rosing, J.H. Beijnen, J.H.M. Schellens, A pharmacokinetic and safety study of a novel polymeric paclitaxel formulation for oral application, Cancer Chemotherapy and Pharmacology, 59 (2007) 43-50.
[255] H. Hamada, K. Ishihara, N. Masuoka, K. Mikuni, N. Nakajima, Enhancement of water-solubility and bioactivity of paclitaxel using modified cyclodextrins, Journal of Bioscience and Bioengineering, 102 (2006) 369-371.
[256] H. Li, M. Huo, J. Zhou, Y. Dai, Y. Deng, X. Shi, J. Masoud, Enhanced Oral Absorption of Paclitaxel in N-Deoxycholic Acid-N, O-Hydroxyethyl Chitosan Micellar System, Journal of Pharmaceutical Sciences, 99 (2010) 4543-4553.

CHAPTER 2

OBJECTIVES

OBJECTIVES

The **general objetive** of the project was to evaluate the capability of cetain types of polymeric nanoparticles to improve the oral bioavailability and antitumor efficacy of paclitaxel *in vivo*. Additionally, for this last purpose, the combination of these nanoparticles with 1-methyl-tryptophan as immunomodulator was also studied.

Thus, this general objective was divided into the following **partial objectives**:

1. To prepare and evaluate the intestinal permeability *ex vivo* in rat and assess the pharmacokinetics of paclitaxel when encapsulated in poly(anhydride) nanocarriers.

2. To study the pharmacokinetics, organ distribution and antitumor efficacy of paclitaxel-loaded nanoparticles *in vivo* in a murine model of subcutaneous tumor using Lewis Lung carcinoma cell line (3LL).

3. To evaluate the potential benefit of combining an indoleamine-2,3-dioxygenase inhibitor, 1-methyl-tryptophan, and orally administered paclitaxel encapsulated in poly(anhydride) nanoparticles in a non-small cell lung cancer tumor model (3LL) in C57BL/6J female mice.

CHAPTER 3

Preparation and evaluation of the intestinal permeability and pharmacokinetics of paclitaxel-cyclodextrin complexes encapsulated in mucus-penetrating nanoparticles

Abstract

The oral route is the preferred route of administration by patients when receiving chronic treatments (i.e. cancer) since they gain autonomy and no dependency on hospital life. However, few oral chemotherapeutic agents are available currently. So, there is an increasing interest in developing numerous strategies to increase the oral bioavailability of typical anticancer agents, such as paclitaxel. We based our work on the formulation of paclitaxel in poly(anhydride) nanoparticles complexed with β-cyclodextrin. A second attempt was to combine the paclitaxel-cyclodextrin complexes with poly(ethylene glycol) to increase the drug loading in the poly(anhydride) nanoparticles. The presented strategies increased the apparent permeability of the cytotoxic agent in the Ussing chambers through rat intestine *ex vivo*, enhancing the permeability of the drug around 10-15 times compared to the commercial formulation, Taxol®. In addition, when pharmacokinetic studies were performed in C57BL/6J female mice, the relative oral bioavailability of paclitaxel increased up to 60-80% when administered orally depending on the presence of poly(ethylene glycol) in the poly(anhydride) nanoparticles. Overall, the encapsulation of paclitaxel in poly(anhydride) nanoparticles enhanced the oral uptake of the drug by increasing the intestinal permeability and subsequently, the drug plasma levels.

Introduction

Oral chemotherapy is an attractive approach for cancer treatment because of its convenience, safety and ease of administration, especially in chronic regimens [1, 2]. In several studies, patients stated that the oral route is the preferred route of administration. Patients claimed that the oral administered medication interfered less with their daily life and they felt more freedom which could be translated into an increase in their quality of life [3, 4].

Many anticancer agents are administered by means of the intravenous route since they present a low oral bioavailability. Only 10% of the cancer treatments are provided as oral formulations. It is estimated that this percentage should increase to 25% in the close future [5]. This poor oral availability is mainly due to certain physicochemical properties of the drugs (solubility, lipophilicity, pK_a), physiological factors (intestinal transit time, gastrointestinal pH, absorption mechanisms) and other aspects associated with the dosage forms. An example drug suffering from this drawback is paclitaxel (PTX).

Paclitaxel, a natural diterpenoid obtained from the bark of the Pacific yew tree (*Taxus brevifolia sp*.), is one of the most used anticancer agents in clinics nowadays. It has proven to be useful against several types of cancers such as breast, refractory ovarian and non-small cell lung cancers [6]. PTX works by promoting the stabilization of microtubules inhibiting cell proliferation and finally inducing apoptosis [7]. It presents three main limitations to its use in clinics. Firstly, the availability and cost of the drug remain important issues. In fact, to obtain 1 gram of paclitaxel or its precursors such as baccatin III and 10-deacetylbaccatin III a large amount of the bark of the yew is needed [8, 9]. That is why the semi-synthesis of analogues such as docetaxel is currently under research in order to solve this problem. Secondly, the main adverse effects encountered with the administration of the commercially available formulations of paclitaxel, (Taxol® or its generic paclitaxel injection formulations), are the severe hypersensitivity reactions, hematologic toxicity (mainly neutropenia) and neurotoxicity appearing as peripheral neuropathy. Hence, it has a low therapeutic index.

Finally, because paclitaxel presents a very low aqueous solubility (0.3 µg/ml), the use of typical pharmaceutical solvents is limited. In fact, the commercially available formulations of PTX are formulated with ethanol and Cremophor EL® (1:1, v/v) as vehicles. Cremophor EL® is a polyethoxylated castor oil often related to hypersensitivity reactions that patients present when treated, requiring premedication with antihistamines (H_2) and corticosteroids**.** Moreover, apart from the lipophilicity, the oral administration of PTX is also hampered due to the fact that it is

substrate of the P-glycoprotein (Pgp) and it is metabolized by the cytochrome P450 (isoform CYP3A4), both highly expressed in the gut [10].

In order to overcome these drawbacks and attempt the oral administration of the anticancer agent, numerous strategies have been reported and are under investigation. These include the synthesis of taxane analogues (i.e. ortotaxel, larotaxel dihydrate) [7, 11], the co-administration with Pgp and CYP3A4 selective inhibitors, i.e. cyclosporin A or its analogues [12, 13], conjugation with low molecular weight chitosan [14], the combination with cyclodextrins [15], self-emulsifying drug delivery systems (SEDDS) [16], nanoemulsions [17], micelles [18-20], lipid nanoparticles and biodegradable polymeric nanoparticles [10, 21-23]. Amongst nanoparticles, an interesting strategy recently published was the combination of the poly(anhydride) nanoparticles and the complexes formed by cyclodextrins and paclitaxel [21, 24, 25]. Poly(anhydride) nanoparticles are interesting delivery systems since they are able to establish bioadhesive interactions with the gut mucosa [26]. In addition, the surface of the nanoparticles can be easily modified by incubation with certain ligands to alter the surface. However, the main problem encountered is the limitation to encapsulate lipophilic molecules such as PTX. Two strategies have been proposed to improve the loading of the anticancer drug: a) use of cyclodextrins [21, 25] and b) use of poly(ethylene glycols) (PEG) [27]. Furthermore, both types of pharmaceutical excipients have been described as inhibitors of the Pgp efflux pump and of cytochrome P450 [28-30] promoting the absorption of the drug at the gastrointestinal mucosa.

Therefore, the aim of this work was, in a first step, to investigate the effect of PEG on the loading of paclitaxel-cyclodextrin complexes in poly(anhydride) nanoparticles. In a second step, the intestinal permeability and pharmacokinetic profiles of paclitaxel when formulated in these poly(anhydride) nanoparticles were evaluated in C57BL/6J female mice.

Materials and methods

1. Reagents

Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) (PMV/MA) [Gantrez® AN 119; MW 200,000] was purchased from ISP (Barcelona, Spain). Paclitaxel (USP 26, grade >99.5%) and docetaxel (99.0%) were supplied by 21CECpharm (London, UK). Taxol® obtained from Bristol-Myers-Squibb (NY, USA). Phosphate buffered saline (PBS), verapamil, glutamine, glycine, β-cyclodextrin (CD) and 2-hydroxylpropyl-β-cyclodextrin (HPCD) were obtained from Sigma Aldrich (Germany) and disodium edetate (EDTA) and poly(ethylene glycol) 2000 (PEG2000) were provided

by Fluka (Switzerland). Acetone, ethanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Deionised reagent water (18.2 MΩ resistivity) was prepared by a water purification system (Wasserlab, Spain). All reagents and chemicals used were of analytical grade.

2. Preparation of PTX-cyclodextrin complexes

The preparation of inclusion complexes of paclitaxel and cyclodextrins (HPCD or CD) was performed, by the evaporation method, in the molar ratio 1:1 as described previously [21, 25]. On one hand, 10 mg paclitaxel were dissolved in 2 ml ethanol. This solution was then added to 8 ml water, containing the oligosaccharide. After agitation of the mixture for 72h, ethanol was evaporated under reduced pressure (Büchi R-144, Switzerland) and the resulting suspensions filtered through a 0.45µm membrane filter. Finally, the obtained clear solution was evaporated under vacuum at a temperature of 50°C in the rotary evaporator in order to obtain a solid dry residue.

3. Preparation of nanoparticles

Paclitaxel-cyclodextrin inclusion complexes (PTX-HPCD or PTX-CD) were encapsulated in poly(anhydride) nanoparticles by a solvent displacement method as described previously. [21, 25] Pegylated nanoparticles with PEG2000 were prepared following the method published by Zabaleta et al.[27] with minor modifications.

3.1. Preparation of poly(anhydride) nanoparticles loaded with PTX-cyclodextrin complexes

Paclitaxel (8.8 mg as inclusion complex with either HPCD or CD) was dispersed in 5 ml of acetone containing 100 mg poly(anhydride) previously dissolved. The mixture was magnetically stirred for 30 min at room temperature. Then, nanoparticles were formed by the addition of an ethanol/water mixture (1:1, v/v). After elimination of the organic solvents, the resulting suspensions were purified by centrifugation 27,000x*g*, for 20 min. The supernatants were removed and the pellets resuspended in water. Finally, the formulations were frozen and freeze-dried (Genesis 12EL, Virtis, USA) using sucrose (5% w/w) as cryoprotector.

For the identification of the different formulations the following abbreviations were used: PTX-HPCD-NP (nanoparticles containing the PTX-HPCD inclusion complex) and PTX-CD-NP (nanoparticles containing the PTX-CD inclusion complex).

3.2. Preparation of pegylated nanoparticles loaded with PTX-CD (PTX-CD-PEG-NP)

In this case, paclitaxel (8.6 mg as inclusion complex with CD) was dispersed in 5 ml of acetone containing 100 mg poly(anhydride) and 12.5 mg PEG2000 previously dissolved. The mixture was magnetically stirred for 30 min at room temperature. Afterwards, the nanoparticles were formed by the addition of a mixture of ethanol and water (1:1, v/v). The resulting suspension was purified by centrifugation and finally, freeze-dried using sucrose (5%) as cryoprotector.

3.3. Preparation of pegylated nanoparticles loaded with PTX (PTX-PEG-NP)

Ten mg PTX were incubated with 100 mg Gantrez® AN and 12.5 mg PEG2000 under magnetic stirring for 30 minutes in 5 ml acetone. Then, nanoparticles were formed by the addition of 10 ml of ethanol followed by the addition of 10 ml of an aqueous solution containing glycine (50 mg) and disodium edetate (20 mg). The organic solvents were eliminated by evaporation under reduced pressure and the nanoparticle suspensions purified in Vivaspin tubes (300,000 MWCO, Sartorius Group, Germany) at 3,000x*g* for 20 min. The pellets were resuspended in water and the purification step was repeated twice. Finally, the formulations were frozen and then freeze-dried using sucrose (5%) as cryoprotector.

4. Characterization of the different nanoparticles

4.1. Physicochemical characterization

The mean hydrodynamic diameter of the nanoparticles and the zeta potential were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments Ltd., Worcestershire, UK). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1:10) and measured at 25°C by dynamic light scattering angle of 90°C. The zeta potential was determined as follows: 200 µL of the samples were diluted in 2 ml of a 0.1 mM KCl solution. On the other hand, the morphology of the nanoparticles was assessed by scanning electron microscopy (SEM) and electron cryomicroscopy (cryo-EM) techniques. The yield of the process was calculated by gravimetry as described previously [26].

4.2. Paclitaxel content in nanoparticles

The amount of paclitaxel loaded into nanoparticles was quantified by HPLC-UV [21] in an Agilent model 1100 series LC and a diode-array detector set at 228 nm. The chromatographic system was equipped with a reversed-phase 150 mm x 3 mm C18 Phenomenex Gemini column

(particle size 5 µm) and a precolumn. The mobile phase, pumped at 0.5 ml/min, was a mixture of phosphate buffer (0.01 M, pH 2) and acetonitrile (50:50, v/v). The column was placed at 30°C and the injection volume was 100 µL. Docetaxel (DCX) was used as internal standard. Calibration curves were designed over the range of 80–7000 ng/ml (r^2 >0.999). The limit of quantification was calculated to be 80 ng/ml.

For analysis, nanoparticles were solubilized with acetonitrile (1:5, v/v). Samples were transferred into auto-sampler vials, capped and placed in the HPLC auto-sampler. Each sample was assayed in triplicate and results were expressed as the amount of PTX (in µg) per mg nanoparticles.

5. *In vitro* release study

Release experiments were conducted under sink conditions at 37°C using simulated gastric (SGF; pH 1.2; pepsin 0.32% w/v) and intestinal (SIF; pH 6.8; pancreatin 1% w/v) fluids containing 1% of polysorbate 80 (Tween 80) as solubilising agent for PTX. The studies were performed under agitation in a Vortemp 56™ Shaking Incubator (Labnet International Inc., NJ, USA) after the dispersion of the nanoparticles in the appropriate medium.

For each time point, 17 µg of PTX formulated in nanoparticles were resuspended in 1 ml of the corresponding simulated fluid. The different formulations were kept in the SGF for 2 hours and for 20 hours in SIF. At different time points, sample tubes were collected and centrifuged at 27,000x*g* for 20 minutes. The amount of PTX released from the formulations was quantified by HPLC (calibration curves of free PTX in supernatants obtained from SGF and SIF, r^2 >0.999).

6. *Ex vivo* studies in Ussing chambers

The studies were carried out after approval by the responsible Ethical Committee of the University of Paris-Sud (agreement number A092-019-01) in strict accordance with the European legislation in animal experiments.

6.1. Permeation assays

Ussing chambers were used to determine the permeability of fresh rat intestinal tissue to paclitaxel formulated in nanoparticles or commercial Taxol®. The methodology and terminology used was previously described [31]. Jejunum portions from fresh small intestine of sacrificed male Wistar rats (200-250 g; Charles River, Paris) were excised, rinsed with chilled NaCl 0.9% and cut into segments of 2-3 cm length, discarding sections containing Peyer's patches. The selected portions

were cut along the mesenteric border and mounted in Ussing chambers (apparent intestinal surface of 1 cm^2) while bathed with PBS at pH 7.4 containing glutamine 0.2 M.

Each compartment (donor and receptor) was filled with 4 ml PBS, maintained at 37°C and continuously oxygenated with O_2/CO_2 (95/5%). In the receptor compartment, 0.1% (w/v) HPCD was also included as solubilising agent for paclitaxel. A 30-minute equilibration time was allowed to achieve steady-state electrophysiological conditions. After equilibration, 2 mg PTX loaded in nanoparticle formulation or as Taxol® were diluted in 300 µl PBS and added to the donor compartment. Four tissue portions of four different animals were assessed for each formulation and experiments were repeated on different days.

Aliquots of 200 µl were withdrawn from the receptor chambers every 30 minutes up to 2 hours and the volume was immediately replaced by fresh medium pre-equilibrated at the experimental temperature (37°C). In addition, at the beginning and end of the experiments samples (200 µl) were also obtained from the donor compartments, in order to monitor any changes in this chamber during the experiment and to safeguard mass balance. All samples were immediately frozen and stored at -20°C until analysis. PTX amount was quantified by HPLC-UV as described above.

The cumulative amounts of drug permeated in the mucosal-to-serosal (M to S; absorptive transport, or apical to basolateral) and serosal-to-mucosal (S to M, excretory transport or basolateral to apical) directions were calculated. In addition, the influence of the presence of verapamil (selective Pgp inhibitor) was evaluated by adding 0.2 mM of the calcium-channel blocker to the PBS solution to the donor chamber.

For the comparison between the different formulations, the following parameters were calculated: apparent permeability coefficient (P_{app}) and the absorption enhancement ratio (R). The apparent permeability coefficient was calculated by the following equation:

$$Papp = \frac{dQ}{dt}\, x\, \frac{1}{A\, C_0} \quad [eq.1]$$

where *dQ/dt* is the flux of paclitaxel from the donor to receiver compartment, C_0 is the initial concentration of PTX in the donor chamber and *A* is the surface of the tissue membrane (1 cm^2). In order to standardize calculations, the P_{app} values were estimated between 30 and 90 minutes after the addition of the formulation in study [32].

The absorption enhancement ratio (R) was calculated from the P_{app} values:

$$R = \frac{P_{app}\ (sample)}{P_{app}\ (control)} \quad [eq.2]$$

where P_{app} *(sample)* is the apparent permeability of jejunum to PTX when included in the tested nanoparticle formulation and P_{app} *(control)* is the apparent permeability to the drug when included in the reference formulation (Taxol®).

6.2. Measurement of electrical parameters

During all experiments, electrical parameters were recorded to determine tissue viability. Transmucosal potential difference (PD) was continuously recorded between two KCl saturated agar bridges connected to a MDVC-2C voltage clamp (Titis Bussines Corporation, France) via calomel electrodes filled with saturated KCl solution. Potential difference was short-circuited through the experiment by a short-circuit current (I_{sc}) via agar bridges placed in each half-cell and adapted to platinum electrodes connected to an automatic voltage clamp.

After a 30-minute equilibration, only tissues presenting a transmucosal difference of potential higher than $2x10^{-3}$ V were maintained for study. As a further test of viability of the tissues, bumetamide DMSO stock solution (0.01M) was added in the serosal compartment at the end of the experiment. Bumetamide is a specific inhibitor of the [$Na^+K^+2Cl^-$] co-transporter and it decreases the secretion by cells and therefore, I_{sc} also decreases [33]. If no such decrease was observed in I_{sc}, damages in tissues could be suspected and samples discarded.

7. *In vivo* pharmacokinetic studies in C57BL/6J mice

7.1. Pharmacokinetic studies

Pharmacokinetic studies were performed on C57BL/6J female mice (20-22 g) obtained from Harlan (Barcelona, Spain). Studies were conducted in accordance with the ethical guidelines and policies for investigations in laboratory animals approved by the Ethical Committee for Animal Experimentation of the University of Navarra (protocol number 147-11) in accordance with the European legislation on animal experiments (86/609/EU).

Before the experiment, animals were adaptively fed for 1 week with free access to food and drinking water (22±2°C; 12-h light and 12-h dark cycles; 50-60% relative humidity). Previous to the oral administration of the formulations, animals were fasted overnight to avoid interference with the absorption, allowing free access to water.

For the pharmacokinetic study, mice were randomly divided into 6 groups (n=9) based on the times of blood sampling. Each time point corresponded to 3 animals. The experimental groups were: (a) PTX-HPCD-NP, (b) PTX-CD-NP, (c) PTX-PEG-NP and (d) PTX-CD-PEG-NP. As controls, one group of animals received Taxol® intravenously and another group was treated with the commercial formulation orally. Each animal received the equivalent amount of PTX to a dose of 10 mg/Kg body weight either orally with a blunt needle via the esophagus into the stomach or intravenously via tail vein.

Blood samples were collected at set times after administration (0, 10 min, 30 min, 1, 1.5, 3, 6, 8, 24, 48 and 72 hours). EDTA was used as anticoagulant agent. Blood volume was recovered intraperitoneally with an equal volume of normal saline solution pre-heated at body temperature. Samples were immediately placed on ice and centrifuged at 2,500x*g* for 10 minutes. Plasma was separated into clean tubes and kept frozen at -20°C until HPLC analysis.

7.2. Determination of PTX plasma concentration by HPLC-UV

The amount of paclitaxel was determined in plasma by HPLC-UV [21]. Calibration curves were used for the conversion of the PTX/DCX chromatographic area to concentration. Calibrator and quality control samples were prepared by adding appropriate volumes of standard PTX in ethanol solution to drug free plasma. Calibration curves were designed over the range 80 to 3200 ng/ml (r^2>0.999). An aliquot (100 µl) of plasma was mixed with 25 µl of internal standard solution (docetaxel, 4 µg/ml in ethanol, previously evaporated). After vortex mixing, liquid–liquid extraction was accomplished by adding 4 ml of tert-buthylmethylether following vortex gentle agitation (1 min). The mixture was centrifuged for 10 min at 3000*xg*, and then, the organic layer was transferred to a clean tube and evaporated until dry (Savant, Barcelona, Spain). Finally, the residue was dissolved in 125 µl of reconstitution solution (acetonitrile–phosphate buffer 0.01 M pH=2; 50:50, v/v) and transferred to auto-sampler vials, capped and placed in the HPLC auto-sampler. A hundred microlitre-aliquot of each sample was injected onto the HPLC column. The limit of quantification was calculated to be 80 ng/ml with a relative standard deviation of 5.2%.

7.3. Pharmacokinetic data analysis

The pharmacokinetic analysis of plasma concentration plotted against time data, obtained after administration of the different PTX formulations, was analyzed using a non-compartmental model with the WinNonlin 5.2 software (Pharsight Corporation, USA). The following parameters were estimated: the maximal plasmatic concentration (C_{max}), time in which the maximum concentration is reached (T_{max}), the area under the concentration-time curve from time 0 to∞

(AUC), the mean residence time (MRT), the clearance (Cl), the volume of distribution (V) and half-life in the terminal phase ($t_{1/2}$).

Furthermore, the relative oral bioavailability, F (%), of paclitaxel was estimated by the following equation:

$$F(\%) = \frac{AUC\ oral}{AUC\ i.v.}\ x\ 100 \quad [eq.3]$$

where *AUC i.v.* and *AUC oral* corresponded to the areas under the curve for the intravenous or oral administrations, respectively.

8. Statistical analysis

Data are expressed as the mean ± S.D. of at least three experiments. The non parametric Kruskall-Wallis followed by Mann-Whitney U-test was used to investigate statistical differences. In all cases, $p<0.05$ was considered to be statistically significant. All data processing was performed using GraphPad Prism 4.0 statistical software (GraphPad Software, USA).

3.Results

3.1. Preparation and characterization of poly(anhydride) nanoparticles

Table 1 summarizes the main physicochemical properties of the different poly(anhydride) nanoparticles containing paclitaxel. When nanoparticles were loaded with the paclitaxel-cyclodextrin complexes, the mean size of the resulting carriers was higher than when paclitaxel was formulated with poly(ethylene glycol).

Herein, pegylation of nanoparticles decreased the mean size of the resulting nanoparticles. Thus, PTX-CD-PEG-NP displayed a mean diameter about 25% lower than PTX-CD-NP (**table 1**). Nevertheless, in all cases, the polydispersity index (PDI) was lower than 0.2 (not shown), which implies a homogeneous formulation. Concerning the zeta potential of nanoparticles, all formulations were formed by particulates with negative surface charges. However, again, when nanoparticles were pegylated with PEG2000, the zeta potential appeared to be slightly more negative, around -55 mV. For the formulation with just the PTX-CD complexes, the surface charge was -48 mV. Furthermore, the yield of the process was calculated to be between 60 and 75% for the PEG and cyclodextrin containing formulations, respectively.

Table 1. Physicochemical characterization of the different poly(anhydride) nanoparticles. Data expressed as mean ± S.D. (n=4).

Formulations	Size (nm)	Zeta Potential (mV)	PTX loading (µg PTX/mg NP)	Yield (%)
PTX-HPCD-NP	298 ± 9	-47 ± 1	151 ± 5	65 ± 5
PTX-CD-NP	265 ± 9	-48 ±4	46 ± 5***	76 ± 8
PTX-CD-PEG-NP	215 ± 4	-56 ± 2	65 ± 6*	62 ± 5
PTX-PEG-NP	190 ± 5	-55 ± 2	110 ± 4	60 ± 4

PTX-HPCD-NP: paclitaxel complexed with 2-hydroxyl-propyl-β-cyclodextrin and loaded in poly(anhydride) nanoparticles; PTX-CD-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles; PTX-PEG-NP: paclitaxel loaded in poly(anhydride) nanoparticles combined with PEG2000; PTX-CD-PEG-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles combined with PEG2000. *p< 0.05: PTX-CD-PEG-NP vs. PTX-HPCD-NP; ***p<0.001 (U-Mann-Whitney test) PTX-CD-NP vs. PTX-HPCD-NP.

Regarding the drug loading, the amount of PTX encapsulated in the nanoparticles varied according to the excipient used. On one hand, looking at the amount of drug encapsulated when PTX was complexed with HPCD, this drug loading (150 µg/mg nanoparticles) was approximately 3 times higher than when PTX was complexed with β-cyclodextrin and loaded in nanoparticles (46 µg/mg nanoparticles).

On the other hand, when PEG was incorporated to the formulation the amount of PTX encapsulated was around 110 µg/mg NP and 2.5-fold higher drug loading than for PTX-CD-NP. When PTX was complexed with β-cyclodextrin and loaded in pegylated nanoparticles, there was around a 40% increase in the drug loading, from 46 µg/mg nanoparticles to 65 µg/mg nanoparticles.

Figure 1 shows the microphotographs obtained by SEM and cryo-EM of the nanoparticles. Figure 1A corresponds to SEM image of the PTX-HPCD-NP, figure 1B corresponds to cryo-EM image of PTX-CD-NP, and figures 1C and 1D belong to SEM images of PTX-CD-PEG-NP and PTX-PEG-NP, respectively. In all cases, the apparent sizes of nanoparticles were similar to the obtained values by photon correlation spectroscopy and nanoparticles displayed spherical shapes. It is interesting to highlight that the formulations that contained cyclodextrins (figures 1A, 1B and 1C) presented more irregular and rougher surfaces while the nanoparticles formulated with PEG (figures 1C and 1D) showed more diffuse surfaces in the photographs.

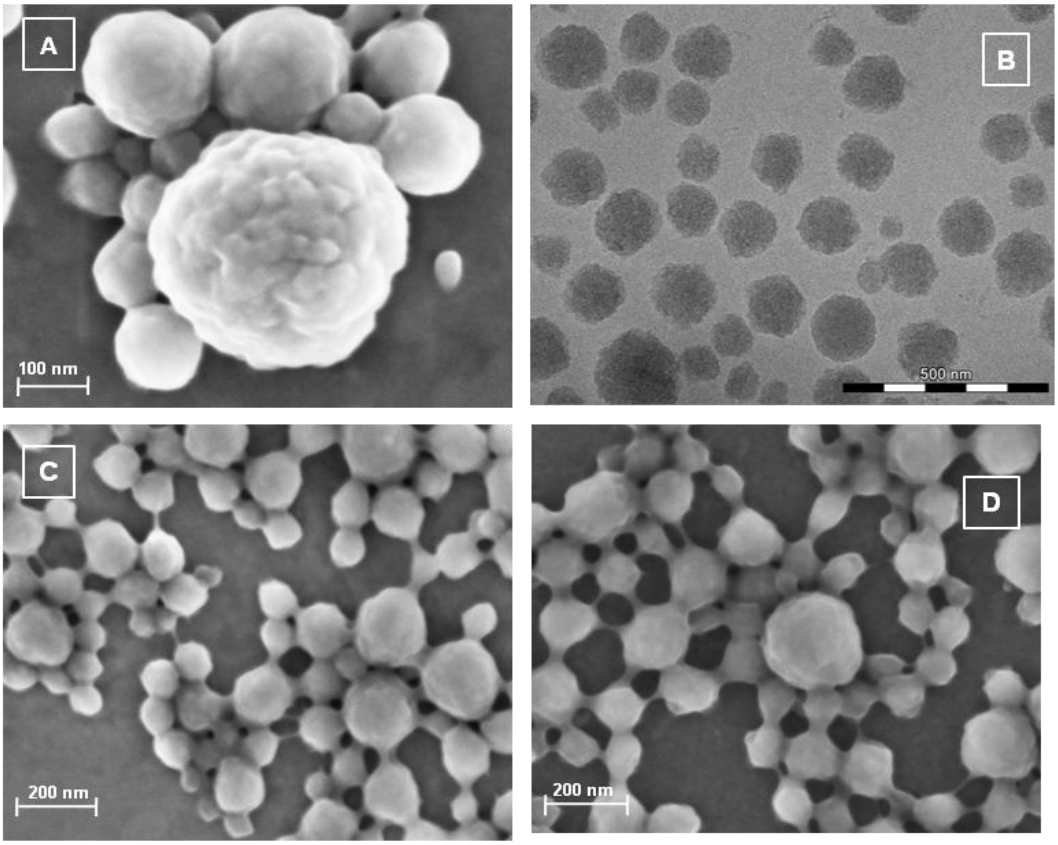

Figure 1. Scanning electron microscopy (SEM) and electron cryomicroscopy (cryo-EM) images of the different poly(anhydride) nanoparticles loaded with paclitaxel. A) SEM image of PTX-HPCD-NP: paclitaxel complexed with 2-hydroxylpropyl-β-cyclodextrin loaded in poly(anhydride) nanoparticles; B) Cryo-electron microscopy (Cryo-EM) image of PTX-CD-NP: paclitaxel complexed with β-cyclodextrin loaded in poly(anhydride) nanoparticles; C) SEM image of PTX-CD-PEG-NP: paclitaxel complexed with β-cyclodextrin loaded in poly(anhydride) nanoparticles combined with PEG2000; D) SEM image of PTX-PEG-NP: paclitaxel loaded in poly(anhydride) nanoparticles combined with PEG2000.

3.2. *In vitro* release study

PTX release kinetics from nanoparticles was evaluated in two different media: simulated gastric and intestinal fluids with polysorbate 80 as solubilizing agent for paclitaxel. **Figure 2** represents the release profiles of PTX from the different assayed formulations as cumulative percentage of drug released as a function of time. For all the evaluated formulations, there was no release of the drug when nanoparticles were dispersed in SGF. In contrast, as it can be seen, when nanoparticles were dispersed in the SIF, the release behavior of paclitaxel from the poly(anhydride) nanoparticles exhibited a biphasic pattern. There was an initial rapid release for PTX-PEG-NP (60% released) followed by a more sustained release up to 18 hours. For the formulations containing cyclodextrins (PTX-CD-NP and PTX-CD-PEG-NP), there was a first slow release of the drug

maintained for 6 hours. After 6 hours, there was a higher release rate prolonged for the next 10-12 hours.

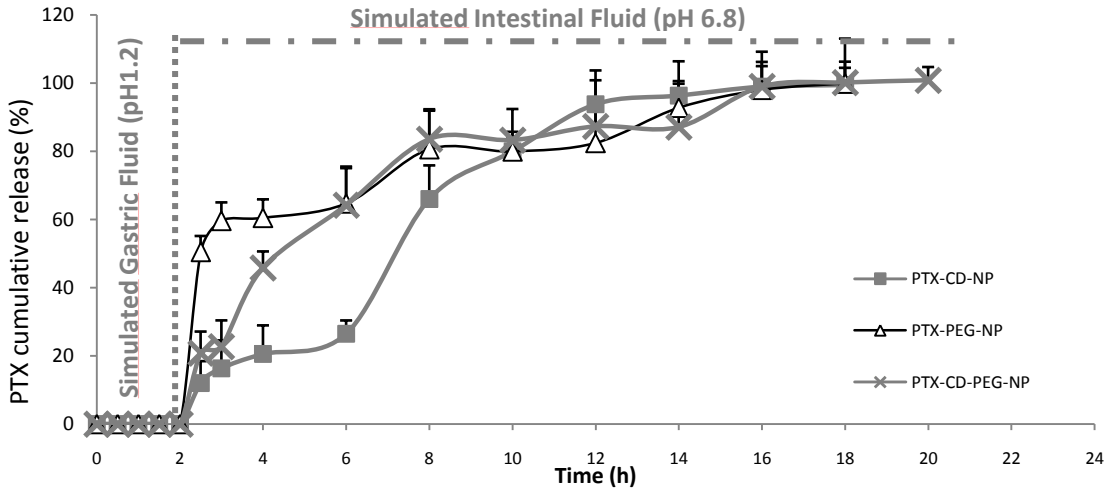

Figure 2. Paclitaxel release profile from the poly(anhydride) nanoparticles formulations after incubation in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) at 37°C. Data represented as mean ± S.D. (n=4). PTX-CD-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles; PTX-PEG-NP: paclitaxel loaded in poly(anhydride) nanoparticles combined with PEG2000; PTX-CD-PEG-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles combined with PEG2000.

Focusing on the time to achieve the 50% release of paclitaxel from the nanoparticles, differences were observed. For pegylated nanoparticles, 30 minutes of incubation in SIF appeared to be enough to promote the release of 50% of the loaded drug. However, when the nanoparticles contained CD, this time lapse appeared to be longer. Thus, for the paclitaxel-cyclodextrin complexes loaded in the nanoparticles (PTX-CD-NP), the time to achieve the 50% release was around 5 hours of incubation in the intestinal simulated fluid. For the combined strategy incorporating cyclodextrin and PEG in the nanoparticles (PTX-CD-PEG-NP), the release rate appeared to increase because just 2 hours of incubation in the SIF were necessary to release 50% of the loaded drug. Interestingly, at 18-20 hours, PTX was completely released to the medium in all the assayed formulations.

3.3. *Ex vivo* Ussing chambers

The transport and permeability of PTX when loaded in nanoparticles through the intestinal mucosa of rats was investigated *ex vivo* by the Ussing chamber technique. In the M-S direction, when PTX was studied in the commercially available formulation, Taxol®, the apparent permeability coefficient (P_{app}) was very low ($1.77x10^{-6}$ cm/s). However, when paclitaxel was encapsulated in nanoparticles, the intestinal permeability for PTX increased almost 7, 9 and 12-fold for PTX-CD-NP,

PTX-CD-PEG-NP and PTX-PEG-NP, respectively. Comparing between nanoparticles, the highest permeability values were obtained for the poly(ethylene glycol) containing formulation, PTX-PEG-NP, (P_{app}= 20.6x10^{-6} cm/s). Interestingly, for the two formulations containing the PTX-CD complexes, there were different values of permeability (P_{app} PTX-CD-NP= 11.4x10^{-6} cm/s *vs*. P_{app} PTX-CD-PEG-NP= 15.1x10^{-6} cm/s).

The absorption enhancement ratios (R) are summarized in **figure 3** where the variation in absorption for the different formulations is plotted after 2-hour experiment in the Ussing chambers for all the experimental conditions evaluated.

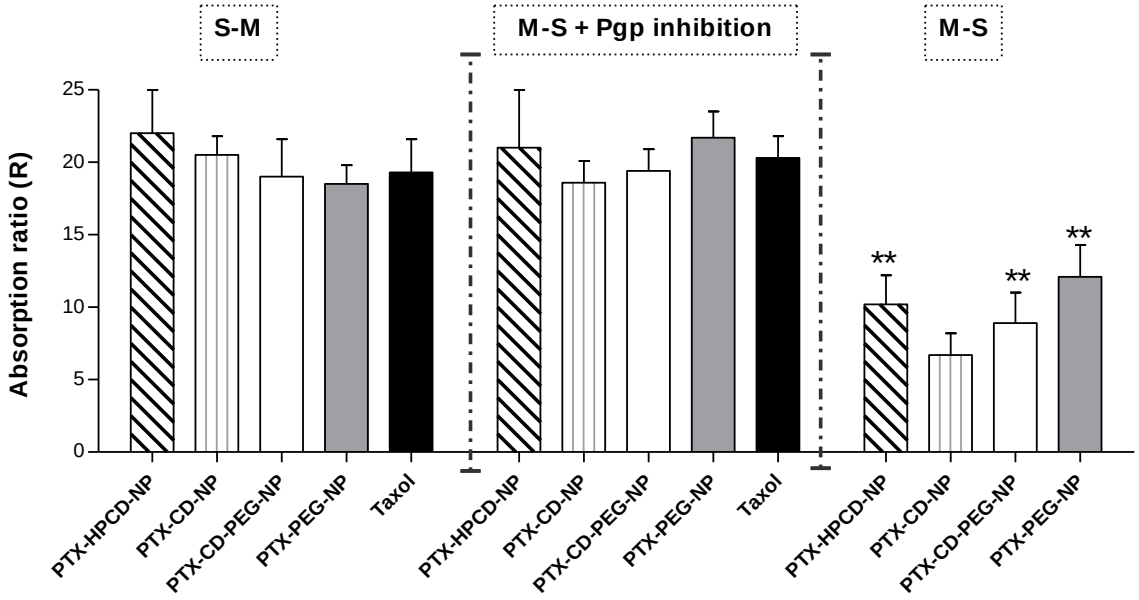

Figure 3. Comparative values for the calculated absorption ratios (R) for the different poly(anhydride) nanoparticles incorporating paclitaxel and the commercially available Taxol®. Absorption ratio for Taxol®=1. ** p< 0.01 PTX-CD-NP vs. PTX-HPCD-NP, PTX-CD-PEG-NP and PTX-PEG-NP. (Mann Whitney U-test)

When the apparent intestinal permeability was evaluated on the excretory direction, this is serosal-to-mucosal direction (S-M); the values of drug permeability were much higher than those obtained in the absorptive direction (M-S). Surprisingly, there were no statistical differences in the permeability values between the studied formulations: Taxol® and nanoparticles. Thus, for Taxol®, the absorption enhancement ratio (R) was about 18 times higher than when evaluated in the mucosal-to-serosal direction. Similarly, when PTX was formulated in poly(anhydride) nanoparticles, the P_{app} values were found to be about around 32x10^{-6} cm/s and the absorption ratios were close to 20, in all cases (see **figure 3**). On the other hand, when the intestinal permeability of PTX was assessed in the presence of verapamil in the medium, the values of P_{app} increased compared to the

mucosal-to-serosal transport. The absorption ratio for commercial Taxol® and for the different formulations rose to 20 approximately, in all cases **(figure 3)**.

Figure 4 represents the percentage of the dose of PTX absorbed through the jejunum portions in the Ussing chambers plotted against time for the absorptive direction (M-S). When the experiments were carried out with the nanoparticle formulations, there was a prolonged increase of the amount of PTX absorbed through the intestine for the first 90 minutes. In contrast, after 60 minutes a slower absorption profile was observed, becoming rather stable. At the end of the study, the amount of PTX in the receptor chamber for the nanoparticle formulations was 5 to 8 times higher than for the commercial Taxol®. However, significantly higher levels of PTX present in the receptor chamber were observed for pegylated containing formulations than for PTX-CD-NP ($p<0.05$).

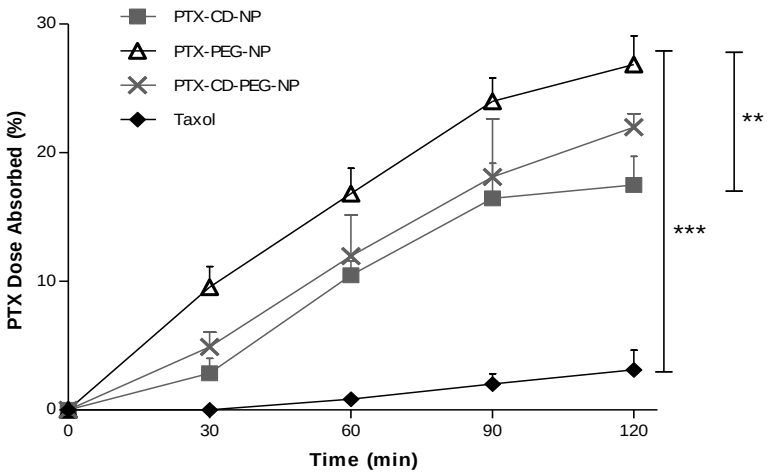

Figure 4. Percentage of the dose of paclitaxel absorbed across the rat intestinal segments mounted in Ussing hambers plotted against time for mucosal-to-serosal transport (absorptive direction). Data are expressed as mean ± S.D. (n=4). *** p< 0.001 all nanoparticle formulations *vs.* commercial Taxol® and PTX-CD-NP *vs.* PTX-PEG-NP ; **p< 0.01 PTX-CD-NP vs. PTX-PEG-NP and PTX-CD-PEG-NP

3.4. Pharmacokinetic studies in C57BL/6J mice

The plasma concentration profiles for the commercial Taxol® after a single intravenous injection (dose 10mg/kg) are presented in **figure 5.** Data were analyzed by a non-compartmental model. The paclitaxel plasma concentration decreased rapidly in a biphasic way. The peak plasma concentration (C_{max}) of PTX was about 65 µg/ml. The values obtained for AUC and half-life ($t_{1/2}$) were 71 µg h/ml and 3.1 hours, respectively. The mean residence time (MRT) was 2.22 hours **(table 2)**.

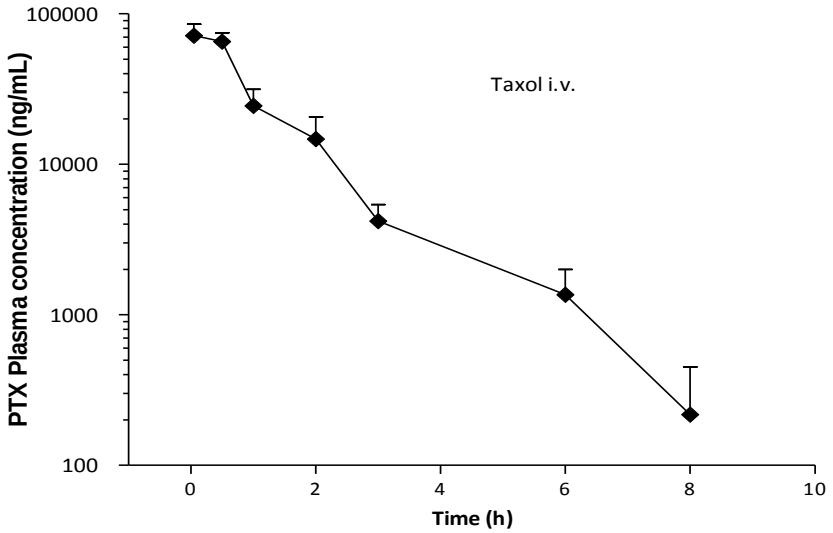

Figure 5. Mean (± S.D.) concentration-time profile of PTX in plasma of female C57BL mice after a single intravenous dose (10 mg/kg) of the commercially available formulation Taxol®. Data expressed as mean ± S.D. (n=3).

Figure 6 shows the plasma concentration versus time profile after oral administration of paclitaxel (single dose of 10 mg/kg in female C57BL/6J mice) included in the different nanoparticle formulations. While oral administered Taxol® showed no detectable plasma levels in mice, the poly(anhydride) formulations offered sustained plasma curves characterized by increasing amounts of PTX for the first 2 hours (T_{max}) reaching the maximum concentration (C_{max}) followed by another phase of slow and prolonged decline of the plasma levels until at least 24 hours. At the end, the elimination phase was characterized by a rapid decline of the plasma levels.

Comparing the different nanoparticle formulations, the paclitaxel plasma levels reached with either PTX–PEG-NP or PTX–HPCD-NP or PTX-CD-PEG-NP were found to be between 1.5 and 2-fold higher than for PTX–CD NP. In fact, PTX-PEG-NP displayed the highest plasmatic levels of PTX and, more importantly, remained within the detection range for at least 72 hours post-administration. On the contrary, both PTX-HPCD-NP and PTX-CD-NP displayed paclitaxel plasma levels for at least 24 h whereas the pegylation of nanoparticles loaded with PTX-CD complexes offered high plasma levels for at least 48 h. In addition, for this formulation (PTX-CD-PEG-NP), the second phase of the plasma curve followed a similar trend than the one obtained for PTX-PEG-NP.

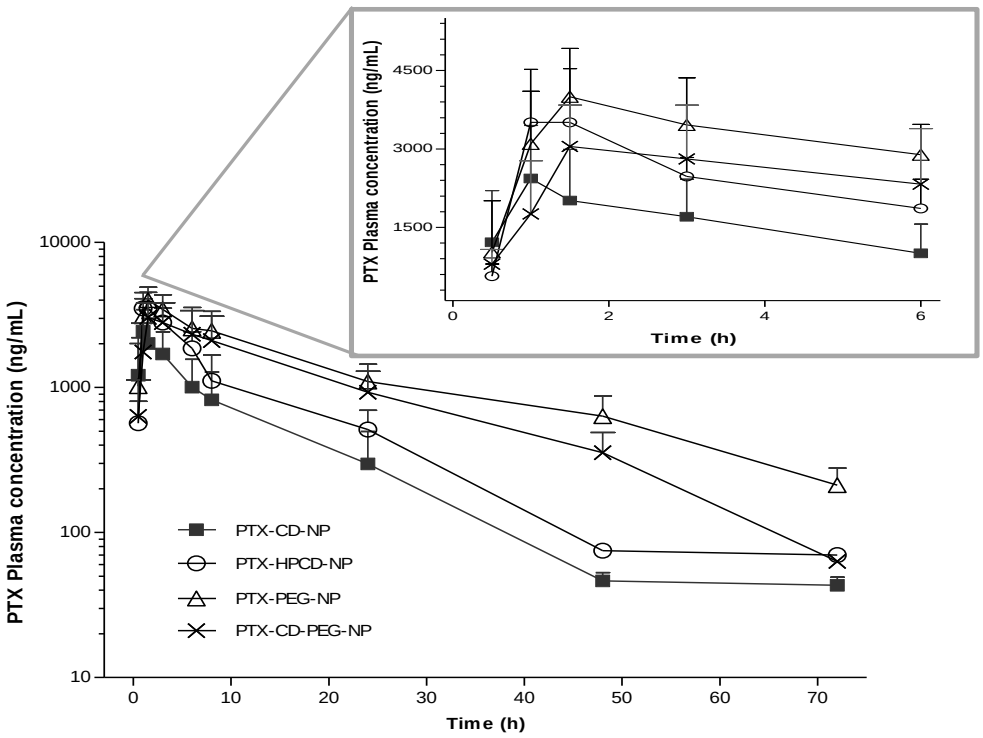

Figure 6. Paclitaxel plasma concentration vs. time after the oral administration of nanoparticle formulations as a single dose of 10 mg/kg body weight. Data represent mean ± S.D. (n=3). PTX-HPCD-NP: paclitaxel complexed with 2-hydroxypropyl-β-cyclodextrin and loaded with poly(anhydride) nanoparticles; PTX-CD-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles; PTX-PEG-NP: paclitaxel loaded in poly(anhydride) nanoparticles combined with PEG2000; PTX-CD-PEG-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles combined with PEG2000.

Table 2 summarizes the pharmacokinetic parameters estimated with a non-compartmental analysis of the experimental data obtained after the administration of the different paclitaxel formulations to mice.

The AUC of PTX in nanoparticles varied depending on the formulation. Thus, for PTX-HPCD-NP and PTX-PEG-NP, AUC was around 52-56 µg h/ml. Likewise, for the nanoparticles containing just the PTX-CD complexes and the formulation ith this inclusion complex and PEG (PTX-CD-PEG-NP), AUC was around 44-48 µg h/ml, this value was 1.3 times lower. In all, the AUC values of poly(anhydride) nanoparticles were significantly higher than those of orally administered Taxol® since no detectable levels were obtained. The peak plasma concentration (C_{max}) of PTX in the poly(anhydride) nanoparticles was between 1.8 and 4 µg/ml and the T_{max} in all cases was similar (about 1.5 hours). These maximum plasma concentrations for the poly(anhydride) nanoparticles were around 16-35 times lower than that of the paclitaxel (Taxol®) when intravenously administered.

Table 2. Pharmacokinetic parameters estimated after a single dose of 10 mg/kg body weight of paclitaxel formulated in nanoparticles or in the commercial Taxol®, either orally or intravenously administered.

	Route	AUC (µg h/ml)	C max (µg/l)	T max (h)	$t_{½\,z}$ (h)	Cl (l/h)	V (l)	MRT (h)	Fr (%)
Taxol®	i.v.	71.1 ± 2.1	64.7 ± 1	0.03	3.1 ± 0.3	2.7 ± 0.8	6.9 ± 1	2 ± 0.2	100
Taxol®	p.o.	ND	ND	ND	ND	ND	ND	ND	ND
PTX-HPCD-NP	p.o.	51.1 ± 1.7	3.5 ± 1	1.0	10.1 ± 1	0.7 ± 0.2	8.1 ± 0.3	21 ± 0.3	72
PTX-CD-NP	p.o.	43.7 ± 2.1	2.4 ± 1	1.0	9.7 ± 0.3	0.5 ± 0.4	8.9 ± 0.3	22 ± 1.2	61
PTX-CD-PEG-N	p.o.	48.4 ± 2.8	3.1 ± 0.7	1.5	8.5 ± 1.1	0.2 ± 0.2	7.3 ± 0.4	21 ±0.5	68
PTX-PEG-NP	p.o.	55.9 ± 3.1	4.0 ± 0.9	1.5	10.3 ± 0.5	0.5 ± 0.2	8.5 ± 0.1	26 ± 0.5	79

AUC: area under the concentration-time curve from time 0 to ∞; C_{max}: peak plasma concentration; T_{max}: time to peak plasma concentration; $t_{1/2\,z}$: half-life of the terminal phase; Cl: clearance; V: volume of distribution; MRT: mean residence time; Fr: relative oral bioavailability.

In addition, the mean residence time (MRT) of the drug in plasma and $t_{1/2}$ were found between 20 and 26-times higher when paclitaxel was administered in the nanoparticle formulations by the oral route than when administered as Taxol® by the i.v. route. When Taxol® was i.v. administered the clearance of paclitaxel was 2.7 l/h and the volume of distribution of the drug was 7l, approximately. Interestingly, the clearance values of the anticancer drug when loaded in nanoparticles were always relatively lower than for the commercial Taxol®. On the contrary, regarding the volume of distribution for paclitaxel, all treatments demonstrated a similar value.

Finally, the relative oral bioavailability of paclitaxel when incorporated to poly(anhydride) nanoparticles varied from 60 to 80% depending on the formulation. The highest relative oral bioavailability of the drug was obtained for the pegylated nanoparticles PTX-PEG-NP (79%), followed by the PTX-HPCD-NP (72%), followed by PTX-CD-PEG-NP (68%) and finally the lowest achieved by PTX-CD-NP (61%).

As observed from the results, there is a good relationship between P_{app}/R data (*ex vivo*) and AUC calculations (*in vivo*). **Figure 7** shows the correlation between the permeability parameters obtained in the Ussing chambers and the AUC obtained from the plasmatic curves. As observed, for the formulation with the lowest permeability (PTX-CD-NP), the AUC was the smallest and consequently, the relative oral bioavailability Fr (61%). In contrast, when the permeability of the drug through the intestinal membrane of rat increased, such rise was also obtained for the AUC and

subsequently, for the Fr. So herein, we could establish a correlation between these two parameters: absorption ratio as permeability rate and the AUC of the plasmatic curve.

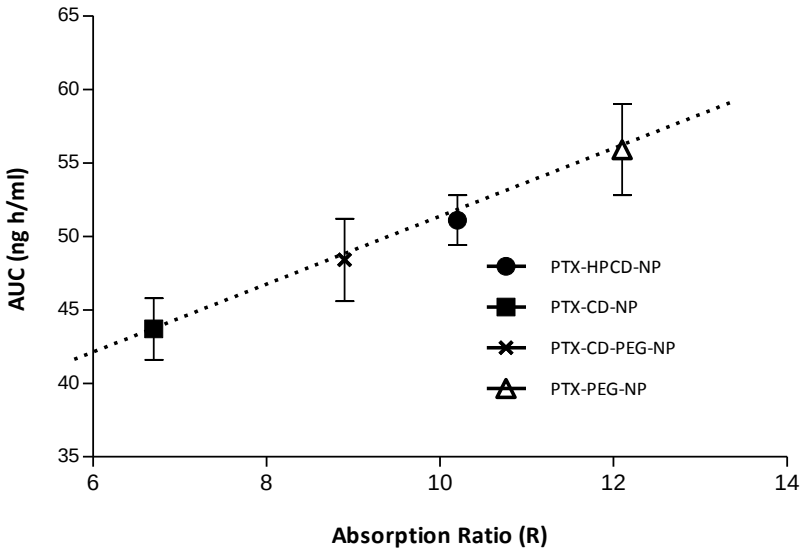

Figure 7. Correlation between the absorption ratio (R) from the Ussing chambers and the AUC from the pharmacokinetic study for the different poly(anhydride) nanoparticles studied.

Discussion

In the recent past, previous works have already described the capabilities of poly(anhydride) nanoparticles in combination with either cyclodextrins or PEG to promote both the intestinal permeability and oral bioavailability of paclitaxel in rats [21, 25, 27]. Both types of compounds, cyclodextrins and PEG, permit to increase the drug loading and encapsulation efficiency of the anticancer molecule in the poly(anhydride) nanoparticles. When these carriers are orally administered, their hydrophilic surface would facilitate (at least in part) the diffusion across the mucus layer and promote the development of adhesive interactions in close contact with the absorptive layer of the enterocytes [25, 34, 35]. This fate of crossing the mucus layer would be higher for pegylated nanoparticles than for nanoparticles combined with cyclodextrins [27]. Once the nanoparticles would be anchored in the mucosa, they would release their content and the “in situ” presence of either PEG or cyclodextrins would facilitate their inhibitory task on the Pgp and CYP3A4 [28-30].

In this context, the aim of this work was to explore the potential of combining both strategies with poly(anhydride) nanoparticles and, thus, to determine if the incorporation of paclitaxel as inclusion complex with CD in pegylated nanoparticles would also modify the intestinal permeability and oral

bioavailability of this anticancer drug in C57BL/6J female mice. In addition, this work would allow to gain insight about the fate of the poly(anhydride) nanoparticles *in vivo* and to identify certain correlation between their behavior *in vitro* or *ex vivo* and *in vivo*.

Overall, when paclitaxel was encapsulated in the form of inclusion complexes with cyclodextrins, the resulting nanoparticles displayed a mean size higher than those in which poly(ethylene glycol) was used **(table 1)**. In this way, it was also clear that the presence of PEG reduced the mean size of the resulting carriers. Concerning the drug loading, the nature of the cyclodextrin selected to prepare the inclusion complex with paclitaxel clearly influenced this parameter. Thus, the use of HPCD allowed us to increase 3-times the paclitaxel loading compared to CD. In both cases, the amount of the drug associated with the resulting nanoparticles was found similar to values previously reported [21]. For pegylated nanoparticles, the paclitaxel loading was high (about 11% by weight) but lower than the data previously reported [27]. This difference could be related to a lower incubation time between the drug, PEG and the poly(anhydride) before the formation of nanoparticles (30 min *vs*. 1h).

The release profile of paclitaxel from the different types of poly(anhydride) nanoparticles was clearly dependent on the pH conditions. Under acid pH conditions, no release of paclitaxel was observed. This fact can be explained by the low propensity of the poly(anhydride) polymer to undergo hydrolysis in an acid medium (SGF) [36] preventing the erosion of the nanoparticles. On the contrary, when the experiment was carried out in simulated intestinal fluid, the release of the drug occurred and, interestingly, it was found to be dependent of the composition of the nanoparticles. In fact, under these conditions, the hydrolysis of anhydride groups would yield two carboxylic acids groups, which would cause the swelling of the nanoparticles. Interestingly, pegylation of nanoparticles induced a much more rapid release of paclitaxel from nanoparticles in simulated intestinal fluid than non-pegylated ones. These results agree well with the observation reported by Yu and coworkers who demonstrated that pegylation of poly(anhydride) microparticles (around 1 mm) accelerated the release rate of bovine serum albumin [37].

Concerning the permeability studies of paclitaxel in Ussing chambers, the asymmetric permeation found for free paclitaxel (Taxol®) when both absorptive and excretory directions were assessed with an efflux ratio (S-M) (calculated by equation 2) of about 20, confirmed the presence of an active mechanism for which paclitaxel is a good substrate [38, 39]. Similarly, when verapamil, a selective Pgp inhibitor, was added to the donor chamber, the amount of paclitaxel absorbed through the intestinal membrane increased in all cases, with no differences between the commercial Taxol® and the poly(anyhydride) nanoparticles (P_{app} values around $32x10^{-6}$ cm/s). These

results are in agreement with previous studies with pegylated nanoparticles [27] or nanoparticles loaded with PTX-HPCD inclusion complex [25].

In our work, when PTX was included in the different nanoparticles, the permeability and the absorption ratios of the anticancer agent increased significantly compared to the control (Taxol®), confirming a significant decrease in the efflux transport of paclitaxel which can be considered as a clear evidence of the inhibition of the active pump by both cyclodextrins and PEG. However, this effect seemed to be more intense when nanoparticle formulations were prepared with PEG. This fact again can be directly related to the ability of poly(ethylene glycol) to yield nanoparticles with a more slippery surface that could permit an easier penetration through the mucus layer of the gut than nanoparticles containing cyclodextrins. Another interesting thing to highlight would be the good correlation between the *in vitro* release profiles from nanoparticles in SIF and the P_{app} obtained by the Ussing chamber technique of the anticancer drug, confirming the inhibitory effect on Pgp and cythocrome P450 for all the nanoparticle formulations tested.

For the *in vivo* studies, a single dose of 10 mg/kg was selected. When Taxol® was administered by the intravenous route, the profile of the curve was biphasic **(figure 5)** and similar to that published previously [40]. In contrast, after the oral administration of Taxol®, no paclitaxel plasma levels could be estimated since they were not detectable. So, the oral bioavailability of Taxol® could not be estimated. In any case, these values have been previously reported to be between 2 and 10.5 % [10, 41].

When paclitaxel was loaded in nanoparticles and orally administered to mice, the plasma levels of paclitaxel were high and prolonged in time **(figure 6)**. Thus, pegylated nanoparticles demonstrated the longest plasma levels in mice as previously described in rat [27], reaching 72 hours post-administration with the highest C_{max}. Nanoparticles loaded with either PTX-HPCD or PTX-CD complexes induced paclitaxel plasma levels for at least 24 h, whereas when the PTX-CD inclusion complex was loaded in nanoparticles in the presence of PEG, therapeutic plasma levels were maintained for at least 2 days **(figure 6)** and evidenced by the high MRT (around 22 hours) and low clearance values (0.2-0.7 l/h) **(table 2)**.

In any case, these prolonged plasma levels can also be explained by an increased residence time of the pharmaceutical dosage form (nanoparticles) in close contact with the absorption site. As a consequence, the calculated relative oral bioavailability of paclitaxel when formulated in the nanoparticles was high and around 60%. Again, pegylation of nanoparticles presented the highest increase in the relative oral bioavailability of the anticancer agent. Interestingly, for all the formulations tested (Taxol® and nanoparticles) the volume of distribution was calculated to be

around 7-8 liters. These values would be an indirect probe that paclitaxel (and not the nanoparticles) is absorbed and, then, distributed in the body of the animal. In addition, these values are much higher than the total body water in mice (around 14 g or 69% of body weight [42]) suggesting that the drug is extensively distributed to tissues.

In summary, the incorporation of cyclodextrins and/or PEG to poly(anhydride) nanoparticles appeared to be an adequate strategy to promote the oral bioavailability of paclitaxel and maintain plasma levels of paclitaxel for at least 24 hours in C57BL/6J female mice. The addition of PEG to the poly(anhydride) nanoparticles containing the PTX-CD complexes presented a more slippery surface that would allow a deeper penetration of the nanocarriers in the intestinal mucosa where contact with the absorptive membrane would be established and finally, promoting the absorption of paclitaxel and inhibiting the Pgp efflux pump. These results were interesting in the cases of PTX-PEG-NP and PTX-CD-PEG-NP which provided a relative oral bioavailability of paclitaxel in mice of about 68% and 79%, respectively.

Acknowledgements

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References

[1] M. Borner, W. Scheithauer, C. Twelves, J. Maroun, H. Wilke, Answering patients' needs: Oral alternatives to intravenous therapy, Oncologist, 6 (2001) 12-16.
[2] V.J. O'Neill, C.J. Twelves, Oral cancer treatment: developments in chemotherapy and beyond, British Journal of Cancer, 87 (2002) 933-937.
[3] J. Batlle, E. Arranz, J. de Castro Carpeño, E. Sáez, P. Auñón, A. Sánchez, M. Barón, Oral chemotherapy: potential benefits and limitations, Clinical and Translational Oncology, 6 (2004) 335-340.
[4] T.R. Halfdanarson, A. Jatoi, Oral Cancer Chemotherapy: The Critical Interplay between Patient Education and Patient Safety, Current Oncology Reports, 12 (2010) 247-252.
[5] S.N. Weingart, E. Brown, P.B. Bach, K. Eng, S.A. Johnson, T.M. Kuzel, T.S. Langbaum, R.D. Leedy, R.J. Muller, L.N. Newcomer, S. O'Brien, D. Reinke, M. Rubino, L. Saltz, R.S. Walters, NCCN Task Force Report: Oral chemotherapy, Journal of the National Comprehensive Cancer Network : JNCCN, 6 Suppl 3 (2008) S1-14.
[6] E.K. Rowinsky, R.C. Donehower, Drug-Therapy - Paclitaxel (Taxol), New England Journal of Medicine, 332 (1995) 1004-1014.
[7] D.G.I. Kingston, Tubulin-Interactive Natural Products as Anticancer Agents, Journal of Natural Products, 72 (2009) 507-515.
[8] R.A. Holton, H.B. Kim, C. Somoza, F. Liang, R.J. Biediger, P.D. Boatman, M. Shindo, C.C. Smith, S.C. Kim, H. Nadizadeh, Y. Suzuki, C.L. Tao, P. Vu, S.H. Tang, P.S. Zhang, K.K. Murthi, L.N. Gentile, J.H. Liu, First total

synthesis of taxol .2. Completion of the c-ring and d-ring, Journal of the American Chemical Society, 116 (1994) 1599-1600.
[9] R.A. Holton, C. Somoza, H.B. Kim, F. Liang, R.J. Biediger, P.D. Boatman, M. Shindo, C.C. Smith, S.C. Kim, H. Nadizadeh, Y. Suzuki, C.L. Tao, P. Vu, S.H. Tang, P.S. Zhang, K.K. Murthi, L.N. Gentile, J.H. Liu, First total synthesis of taxol .1. Functionalization of the b-ring, Journal of the American Chemical Society, 116 (1994) 1597-1598.
[10] S. Peltier, J.M. Oger, F. Lagarce, W. Couet, J.P. Benoit, Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules, Pharmaceutical Research, 23 (2006) 1243-1250.
[11] T. Brooks, H. Minderman, K.L. O'Loughlin, P. Pera, I. Ojima, M.R. Baer, R.J. Bernacki, Taxane-based reversal agents modulate drug resistance mediated by P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein, Molecular Cancer Therapeutics, 2 (2003) 1195-1205.
[12] J. van Asperen, O. van Tellingen, M.A. van der Valk, M. Rozenhart, J.H. Beijnen, Enhanced oral absorption and decreased elimination of paclitaxel in mice cotreated with cyclosporin A, Clinical Cancer Research, 4 (1998) 2293-2297.
[13] J.S. Woo, C.H. Lee, C.K. Shim, S.J. Hwang, Enhanced oral bioavailability of paclitaxel by coadministration of the P-glycoprotein inhibitor KR30031, Pharmaceutical Research, 20 (2003) 24-30.
[14] E. Lee, J. Lee, I.-H. Lee, M. Yu, H. Kim, S.Y. Chae, S. Jon, Conjugated Chitosan as a Novel Platform for Oral Delivery of Paclitaxel, Journal of Medicinal Chemistry, 51 (2008) 6442-6449.
[15] F. Fenyvesi, T. Kiss, E. Fenyvesi, L. Szente, S. Veszelka, M.A. Deli, J. Varadi, P. Feher, Z. Ujhelyi, A. Tosaki, M. Vecsernyes, I. Bacskay, Randomly Methylated beta-Cyclodextrin Derivatives Enhance Taxol Permeability Through Human Intestinal Epithelial Caco-2 Cell Monolayer, Journal of Pharmaceutical Sciences, 100 (2011) 4734-4744.
[16] P. Gao, B.D. Rush, W.P. Pfund, T.H. Huang, J.M. Bauer, W. Morozowich, M.S. Kuo, M.J. Hageman, Development of a supersaturable SEDDS (S-SEDDS) formulation of paclitaxel with improved oral bioavailability, Journal of Pharmaceutical Sciences, 92 (2003) 2386-2398.
[17] S. Khandavilli, R. Panchagnula, Nanoemulsions as versatile formulations for paclitaxel delivery: Peroral and dermal delivery studies in rats, Journal of Investigative Dermatology, 127 (2007) 154-162.
[18] R. Mo, X. Jin, N. Li, C. Ju, M. Sun, C. Zhang, Q. Ping, The mechanism of enhancement on oral absorption of paclitaxel by N-octyl-O-sulfate chitosan micelles, Biomaterials, 32 (2011) 4609-4620.
[19] L. Ye, K. Letchford, M. Heller, R. Liggins, D. Guan, J.N. Kizhakkedathu, D.E. Brooks, J.K. Jackson, H.M. Burt, Synthesis and Characterization of Carboxylic Acid Conjugated, Hydrophobically Derivatized, Hyperbranched Polyglycerols as Nanoparticulate Drug Carriers for Cisplatin, Biomacromolecules, 12 (2011) 145-155.
[20] K. Yoncheva, P. Calleja, M. Agüeros, P. Petrov, I. Miladinova, C. Tsvetanov, J.M. Irache, Stabilized micelles as delivery vehicles for paclitaxel, International Journal of Pharmaceutics, 436 (2012) 258-264.
[21] M. Agueros, V. Zabaleta, S. Espuelas, M.A. Campanero, J.M. Irache, Increased oral bioavailability of paclitaxel by its encapsulation through complex formation with cyclodextrins in poly(anhydride) nanoparticles, Journal of Controlled Release, 145 (2010) 2-8.
[22] C. Fonseca, S. Simoes, R. Gaspar, Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity, Journal of Controlled Release, 83 (2002) 273-286.
[23] E. Roger, F. Lagarce, E. Garcion, J.P. Benoit, Lipid nanocarriers improve paclitaxel transport throughout human intestinal epithelial cells by using vesicle-mediated transcytosis, Journal of Controlled Release, 140 (2009) 174-181.
[24] M. Agueros, S. Espuelas, I. Esparza, P. Calleja, I. Penuelas, G. Ponchel, J.M. Irache, Cyclodextrin-poly(anhydride) nanoparticles as new vehicles for oral drug delivery, Expert Opinion on Drug Delivery, 8 (2011) 721-734.
[25] M. Agueros, L. Ruiz-Gaton, C. Vauthier, K. Bouchemal, S. Espuelas, G. Ponchel, J.M. Irache, Combined hydroxypropyl-beta-cyclodextrin and poly(anhydride) nanoparticles improve the oral permeability of paclitaxel, European Journal of Pharmaceutical Sciences, 38 (2009) 405-413.

[26] P. Arbos, M.A. Campanero, M.A. Arnangoa, M.J. Renedo, J.M. Irache, Influence of the surface characteristics of PVM/MA nanoparticles on their bioadhesive properties, Journal of Controlled Release, 89 (2003) 19-30.
[27] V. Zabaleta, G. Ponchel, H. Salman, M. Agueros, C. Vauthier, J.M. Irache, Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: Permeability and pharmacokinetic study, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 81 (2012) 514-523.
[28] F. Fenyvesi, E. Fenyvesi, L. Szente, K. Goda, Z. Bacso, I. Bacskay, J. Varadi, T. Kiss, E. Molnar, T. Janaky, G. Szabo, Jr., M. Vecsernyes, P-glycoprotein inhibition by membrane cholesterol modulation, European Journal of Pharmaceutical Sciences, 34 (2008) 236-242.
[29] M. Ishikawa, H. Yoshi, T. Furuta, Interaction of modified cyclodextrins with cytochrome P-450, Bioscience Biotechnology and Biochemistry, 69 (2005) 246-248.
[30] S.W. Wang, J. Monagle, C. McNulty, D. Putnam, H.M. Chen, Determination of P-glycoprotein inhibition by excipients and their combinations using an integrated high-throughput process, Journal of Pharmaceutical Sciences, 93 (2004) 2755-2767.
[31] I. Bravo-Osuna, C. Vauthier, H. Chacun, G. Ponchel, Specific permeability modulation of intestinal paracellular pathway by chitosan-poly(isobutylcyanoacrylate) core-shell nanoparticles, European Journal of Pharmaceutics and Biopharmaceutics, 69 (2008) 436-444.
[32] C. Wadell, E. Bjork, O. Camber, Nasal drug delivery - evaluation of an in vitro model using porcine nasal mucosa, European Journal of Pharmaceutical Sciences, 7 (1999) 197-206.
[33] M.G. Lionetto, M.E. Giordano, F. De Nuccio, G. Nicolardi, E.K. Hoffmann, T. Schettino, Hypotonicity induced K+ and anion conductive pathways activation in eel intestinal epithelium, Journal of Experimental Biology, 208 (2005) 749-760.
[34] K. Yoncheva, L. Guembe, M.A. Campanero, J.M. Irache, Evaluation of bioadhesive potential and intestinal transport of pegylated poly(anhydride) nanoparticles, International Journal of Pharmaceutics, 334 (2007) 156-165.
[35] K. Yoncheva, E. Lizarraga, J.M. Irache, Pegylated nanoparticles based on poly(methyl vinyl ether-co-maleic anhydride): preparation and evaluation of their bioadhesive properties, European Journal of Pharmaceutical Sciences, 24 (2005) 411-419.
[36] Q.X. Cai, K.J. Zhu, D. Chen, L.P. Gao, Synthesis, characterization and in vitro release of 5-aminosalicylic acid and 5-acetyl aminosalicylic acid of polyanhydride - P(CBFAS), European Journal of Pharmaceutics and Biopharmaceutics, 55 (2003) 203-208.
[37] Y. Yu, T. Lu, W. Zhao, W. Sun, T. Chen, Preparation and Characterization of BSA-Loaded Microspheres Based on Polyanhydrides, Journal of Applied Polymer Science, 121 (2011) 352-358.
[38] M.V.S. Varma, R. Panchagnula, Enhanced oral paclitaxel absorption with vitamin E-TPGS: Effect on solubility and permeability in vitro, in situ and in vivo, European Journal of Pharmaceutical Sciences, 25 (2005) 445-453.
[39] M.V.S. Varma, R. Panchagnula, Prediction of in vivo intestinal absorption enhancement on P-glycoprotein inhibition, from rat in situ permeability, Journal of Pharmaceutical Sciences, 94 (2005) 1694-1704.
[40] A. Sparreboom, O. vanTellingen, W.J. Nooijen, J.H. Beijnen, Nonlinear pharmacokinetics of paclitaxel in mice results from the pharmaceutical vehicle Cremophor EL, Cancer Research, 56 (1996) 2112-2115.
[41] S.C. Yang, R.N. Gursoy, G. Lambert, S. Benita, Enhanced oral absorption of paclitaxel in a novel self-microemulsifying drug delivery system with or without concomitant use of P-glycoprotein inhibitors, Pharmaceutical Research, 21 (2004) 261-270.
[42] T.P. Faulkner, S.B. Cantleberry, V.J. Watts, A.S. Hussain, Comparative Pharmacokinetics Of Ethanol In Inbred Strains Of Mice Using Doses Based On Total-Body Water, Alcoholism-Clinical and Experimental Research, 14 (1990) 82-86.

CHAPTER 4

Pharmacokinetics and antitumor efficacy of paclitaxel-cyclodextrin complexes loaded in mucus-penetrating nanoparticles when administered by oral route to mice

Abstract

Paclitaxel, a class IV drug by the Biopharmaceutical Classification System, is widely used against several types of cancers such as refractory ovarian or non-small-cell lung cancer in clinics. It has very low water solubility and low intestinal permeability which limit its administration by the oral route. In addition, paclitaxel is highly metabolized by cytochrome P450 and it is substrate of the P-glycoprotein present in the gut. Therefore, to overcome these drawbacks and assay the oral administration of the cytotoxic agent, an interesting strategy is the encapsulation of the drug in polymeric nanosystems which enable the oral administration. So, paclitaxel was encapsulated in poly(anhydride) nanoparticles combined with cyclodextrins and/or poly(ethylene glycol) 2000 and the obtained nanoparticle formulations were administered orally to mice. In the pharmacokinetic studies performed at 25 mg/kg dose, the plasma levels of the anticancer agents were maintained for at least 24 hours, with the longest times for the pegylated nanoparticles (72h). On the other hand, the organ distribution studies carried out revealed that paclitaxel underwent a similar distribution when orally administered in nanoparticles as when intravenously injected implying that the drug is absorbed at the gastrointestinal level and widely distributed mainly to liver, kidney, spleen, ovaries and intestine. Finally, when evaluating the efficacy of the different poly(anhydride) nanoparticles loading paclitaxel, a significant tumor inhibition was observed for the pegylated nanoparticles orally administered compared to Taxol®.

Introduction

Paclitaxel (PTX) is a widely used anticancer agent in patients with advanced breast and resistant ovarian cancers as well as non-small cell lung cancer [1], among other clinical uses. It was first described in the 1970s but it was not until the early 1990s that it was approved by the FDA and consequently used in clinical practice. It works by promoting the stabilization of the microtubules during cell replication and therefore, stopping the cell cycle inducing apoptosis [2]. However, paclitaxel presents a major limitation to its clinical use: it has low water solubility (<0.3 mg/mL) and therefore, it has to be solubilized using a mixture of ethanol and Cremophor EL® (1:1, v/v). Cremophor EL® is a polyoxyethylene castor oil derivative that has been proven to be responsible for the severe hypersensitivity reactions observed in patients after the drug administration. Cremophor EL® exhibits a high risk in inducing allergic reactions and neurotoxicity, mainly peripheral neuropathy [3, 4]. To minimize or avoid these reactions, patients have to be pretreated with antihistamines and corticosteroids. In addition, the route of administration of the anticancer agent is generally by means of the intravenous route. This route presents certain risks for the patient and it requires specially qualified staff. In intravenous treatments, patients have to undergo long infusion times (at least 3 hours for paclitaxel), hospitalization (in case of severe side effects) and repeated cycles every 3 weeks until disease regression or no response to treatment. In the cases of no response to treatment, patients have to suffer the inconvenience of having a new treatment prescribed and administered again intravenously.

In this context, the oral alternative to the traditional intravenous chemotherapy is a new trend in research that is gaining interest. For a given drug, the oral administration offers more advantages over the injected ones such as a better patient compliance, less frequency of clinic visits, ease of therapeutic regimes and a possible lower cost [5]. The oral administration of anticancer medication would imply an increase in the patient's quality of life since patients would feel freer and less dependent on hospital care, gaining in autonomy [6].

Nevertheless, paclitaxel has a low oral bioavailability since it is rapidly metabolized by the cytochrome P450 and it is substrate of the P-glycoprotein (Pgp) efflux pump. Both mechanisms are highly expressed in the gastrointestinal (g.i.) tract limiting the permeation of the drugs through the intestinal membrane [7]. For PTX, plenty of work has been developed in order to overcome these drawbacks and several alternatives have arisen lately to attempt the oral administration of the cytotoxic agent. Such alternatives vary from the co-administration of Taxol® with selective Pgp inhibitors such as cyclosporin A or verapamil [8-10], to the encapsulation of the anticancer drug in

drug delivery systems, i.e. micelles [11, 12], self-microemulsifying formulations [13], lipid nanoparticles [14-16] or biodegradable polymeric nanoparticle [17-20].

Biodegradable nanoparticles are promising carriers to improve the oral administration of drugs (namely anticancer agents), vaccines and therapeutic proteins [21]. Such drug delivery systems help to enhance the oral bioavailability of poorly water soluble drugs and furthermore, protect from degradation and promote the absorption of the drugs at the gastrointestinal tract. In such a way, poly(anhydride) nanoparticles prepared from Gantrez AN® polymer have been described as interesting carriers for oral delivery [22]. Gantrez AN® has been reported to present bioadhesive properties within the gastrointestinal mucosa and a low oral toxicity [23]. Moreover, previous works combined the poly(anhydride) nanoparticles with the use of excipients, such as cyclodextrins and poly(ethylene glycol), which have been identified as Pgp inhibitors [24-26], in order to enhance the oral bioavailability of PTX in rats and increase the residence time of the drug delivery systems in the g.i. tract.

Therefore, the major objective of this work was to assess the *in vivo* efficacy of orally administered poly(anhydride) nanoparticles loaded with paclitaxel combined with cyclodextrins or poly(ethylene glycol) in C57BL/6J female mice. For this purpose, pharmacokinetic and tissue distribution studies were developed. Finally, the antitumor activity in tumor-bearing mice against Lewis lung carcinoma cell line(3LL) was also evaluated.

Materials and methods

1. Materials

Paclitaxel (USP XXVI, grade>99.5%) and docetaxel (grade>99.0%) were purchased from 21CECpharm (London, UK). Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) (PMV/MA) [Gantrez® AN 119; MW 200,000] was purchased from ISP (Barcelona, Spain). Taxol® was provided by Bristol-Myers-Squibb (NY, USA). Phosphate buffered saline (PBS), glycine, β-cyclodextrin (CD) and 2-hydroxylpropyl-β-cyclodextrin (HPCD) were obtained from Sigma Aldrich (Germany) and disodium edetate (EDTA) and poly(ethylene glycol) 2000 (PEG2000) were provided by Fluka (Switzerland). Acetone, ethanol, t-buthylmethylether and acetonitrile were obtained from Merck (Darmstadt, Germany). Deionised reagent water (18.2 MΩ resistivity) was prepared by a water purification system (Wasserlab, Spain). All reagents and chemicals used were of analytical grade.

Lewis Lung Carcinoma cell line was obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were cultured in RPMI 1640 medium (GibCo, Life Technologies, UK) supplemented with L-glutamine, 10% fetal calf serum, streptomycin and penicillin (10%) (Invitrogen, Carlsbad, California, USA) and passaged by trypsinization.

2. Preparation of paclitaxel-cyclodextrin complexes

The preparation of inclusion complexes of paclitaxel and cyclodextrins (HPCD or CD) was performed, by the evaporation method, in the molar ratio 1:1 as described previously [27]. Briefly, 10 mg of PTX were dissolved in 2 ml of ethanol. Once dissolved completely, this solution was then added to 8 ml water, containing the oligosaccharide. After agitation of the mixture for 72 h, ethanol was evaporated under reduced pressure (Büchi R-144, Switzerland) and the resulting suspensions filtered through a 0.45 μm membrane filter. Finally, the obtained clear solution was evaporated under vacuum at a temperature of 50°C in the rotary evaporator in order to obtain a solid dry residue.

3. Preparation of poly(anhydride) nanoparticles

Paclitaxel-cyclodextrin inclusion complexes (PTX-HPCD or PTX-CD) were encapsulated in poly(anhydride) nanoparticles by a solvent displacement method as described previously [19]. Pegylated nanoparticles with PEG2000 were prepared following the method published by Zabaleta et al. [20] with minor modifications.

3.1. Preparation of poly(anhydride) nanoparticles loaded with paclitaxel-cyclodextrin complexes: PTX-HPCD-NP and PTX-CD-NP

Paclitaxel, 8.8 mg as inclusion complex with either HPCD or CD, was dispersed in 5 ml of an acetone solution containing 100 mg of the poly(anhydride) polymer previously dissolved. The mixture was magnetically stirred for 30 minutes at room temperature. After, the nanoparticles were formed by the addition of an ethanol/water mixture (1:1, v/v). After elimination of the organic solvents, the resulting suspensions were purified by centrifugation 27,000x*g*, for 20 min. The supernatants were removed and the pellets resuspended in water. Finally, the formulations were frozen and lyophilized (Genesis 12EL, Virtis, USA) using sucrose (5% w/v) as cryoprotector.

For the identification of the different formulations the following abbreviations were used: PTX-HPCD-NP (nanoparticles containing the PTX-HPCD inclusion complex) and PTX-CD-NP (nanoparticles containing the PTX-CD inclusion complex).

3.2. Preparation of pegylated nanoparticles loaded with PTX-CD (PTX-CD-PEG-NP)

In this case, paclitaxel (8.6 mg as inclusion complex with CD) was dispersed in 5 ml of acetone containing 100 mg poly(anhydride) and 12.5 mg PEG2000 previously dissolved. The mixture was magnetically stirred for 30 min at room temperature. Afterwards, the nanoparticles were formed by the addition of a mixture of ethanol and water (1:1 by vol.). The resulting suspension was purified by centrifugation and, finally, freeze-dried using sucrose (5%) as cryoprotector.

3.3. Preparation of pegylated nanoparticles loaded with PTX (PTX-PEG-NP)

For this purpose the PEG with a molecular weight of 2000 was selected. The formulations were obtained following the procedure reported by Zabaleta et al. [20] with minor modifications. Ten mg PTX were incubated with 100 mg Gantrez® AN and 12.5 mg PEG2000 under magnetic stirring for 30 minutes in 5 ml acetone. Then, nanoparticles were formed by the addition of 10 ml of ethanol followed by the addition of 10 ml of an aqueous solution containing glycine (50 mg) and disodium edetate (20 mg). The organic solvents were eliminated by evaporation under reduced pressure and the nanoparticle suspensions purified in Vivaspin tubes (300,000 MWCO, Sartorius Group, Germany) at 3,000x*g* for 20 min. The pellets were resuspended in water and the purification step was repeated twice. Finally, the formulations were frozen and then freeze-dried using sucrose (5%) as cryoprotector.

4. Characterization of poly(anhydride) nanoparticles

4.1. Physicochemical characterization

The mean hydrodynamic diameter of the nanoparticles and the zeta potential were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments Ltd., Worcestershire, UK). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1:10) and measured at 25 C by dynamic light scattering angle of 90°C. The zeta potential was measured in 0.1 mM KCl solution. The yield of the process was calculated by gravimetry as described previously [22]. Concisely, the yield was calculated as the difference between the initial amount of polymer used and the weight of the freeze-dried samples.

4.2. Quantification of the amounts of PEG and CD associated with the nanoparticles

The amounts of poly(ethylene glycol) and cyclodextrins (either HPCD or CD) bound to the nanoparticles were estimated by HPLC as published elsewhere [28, 29]. The excipients, PEG2000,

HPCD or CD, associated were calculated by quantification in the supernatants collected from the purification steps when preparing the nanoparticles. The technique assessed was HPLC (Agilent model 1100 series LC, Germany) attached to an Evaporative Light Scattering Detector (ELSD) (Alltech, Illinois, USA).

Each sample was assayed in triplicate and results were expressed as the amount of poly(ethylene glycol) or cyclodextrin per mg of nanoparticle.

4.3. Paclitaxel content in nanoparticles

The amount of paclitaxel loaded in the nanoparticles was quantified by HPLC-UV following [19]. Briefly, the equipment was an Agilent model 1100 series LC and a diode-array detector set at 228 nm. The chromatographic system was equipped with a reversed-phase 150 mm x 3 mm C18 Phenomenex Gemini column (particle size 5 µm). The mobile phase, pumped at 0.5 ml/min, was a mixture of phosphate buffer (0.01 M, pH 2) and acetonitrile (50:50, v/v). The column was placed at 30°C and the injection volume was 100 µl. Docetaxel (DCX) was used as internal standard. Calibration curves were designed over the range of 80–7000 ng/ml (r^2>0.999). For analysis, nanoparticles were solubilized with acetonitrile (1:5 v/v). Each sample was assayed in triplicate and results were expressed as the amount of PTX (µg) per mg nanoparticles.

5. Pharmacokinetic studies

For the pharmacokinetic study, C57BL/6J female mice (average weight 20-22 g) were obtained from Harlan (Barcelona, Spain). Animals were kept in standard animal facilities with 6 animals per cage and given free access to food and drinking water. They were housed in a temperature and humidity controlled room with 12-hour on-off light cycles. All the animal experiments were approved by the Ethical Committee for Animal Experimentation at the University of Navarra (protocol number: 147-11) in agreement with the European guidelines on animal experiments (86/609/EU).

For this purpose, mice were randomly divided into 6 groups. The experimental groups were: (a) PTX-HPCD-NP, (b) PTX-CD-NP, (c) PTX-PEG-NP and (d) PTX-CD-PEG-NP. Each animal received PTX loaded in poly(anhydride) nanoparticles at a dose of 25 mg/kg body weight orally with a blunt needle via the esophagus into the stomach. The nanoparticles formulations were administered dispersed in water in a final volume of 300 µl. As controls, one group of animals received Taxol® intravenously via tail vein and another group was treated with the commercial formulation orally; in both cases at a dose of 25 mg/kg body weight. Previous to the oral administration of the

formulations, animals were fasted overnight to avoid interference with the absorption, allowing free access to water.

Blood samples (300 µl) were obtained from 3 animals per time point at 0 min, 10 min, 30 min, 1, 3, 6, 8, 24, 48 and 72 hours after administration. EDTA was used as anticoagulant agent. Blood volume was recovered intraperitoneally with an equal volume of normal saline solution pre-heated at body temperature. Samples were immediately placed on ice and centrifuged at 2500*xg* for 10 minutes. Plasma was separated into clean tubes and kept frozen at -80°C until HPLC-UV analysis.

5.1. Determination of PTX plasma concentration by HPLC-UV

The amount of paclitaxel was determined in plasma by HPLC-UV [27]. Calibration curves were used for the conversion of the PTX/DCX chromatographic area to the concentration. Calibrator and quality control samples were prepared by adding appropriate volumes of standard PTX in ethanol solution to drug free plasma. Calibration curves were designed over the range 80–3200 ng/ml (r^2>0.999). An aliquot (100 µl) of plasma was mixed with 25 µl of internal standard solution (docetaxel, 4 µg/ml in ethanol, previously evaporated). After vortex mixing, liquid–liquid extraction was accomplished by adding 4 ml of tert-buthylmethylether following vortex gentle agitation (1 min). The mixture was centrifuged for 10 min at 5000 rpm, and then, the organic layer was transferred to a clean tube and evaporated until dry (Savant, Barcelona, Spain). Finally, the residue was dissolved in 125 µl of reconstitution solution (acetonitrile–phosphate buffer 0.01 M pH=2; 50:50 v/v) and transferred to auto-sampler vials, capped and placed in the HPLC auto-sampler. A hundred microlitre-aliquot of each sample was injected onto the HPLC column. The limit of quantification was calculated to be 80 ng/ml with a relative standard deviation of 5.2%. Accuracy values during the same day (intraday assay) at low, medium and high concentrations of PTX were always within the acceptable limits (less than 5%) at all concentrations tested.

5.2. Calculation of pharmacokinetic parameters

The pharmacokinetic analysis of concentration-time data, obtained from the administration of the different formulations, was performed based on a non-compartmental model using WinNonlin 5.2 software (Pharsight Corporation, Mountain View, USA). With this purpose, the following parameters were estimated: total area under the curve (AUC), half-life of the terminal phase ($t_{1/2}$), mean residence time (MRT), peak plasma concentration (C_{max}) and the time to reach the peak plasma concentration (T_{max}). In addition, the relative oral bioavailability (F %) of paclitaxel was calculated using the ratio of dose-normalized AUC values following oral and i.v. administrations:

$$F = \frac{AUC\ oral}{AUC\ i.v.}\ x\ 100\ [eq.1]$$

where AUC_{oral} and $AUC_{i.v.}$ correspond to the areas under the plasmatic curve for the oral and intravenous administrations, respectively.

6. Organ distribution of paclitaxel

For these *in vivo* studies C57BL/6J female mice (average weight 20-22 g) were obtained from Harlan (Barcelona, Spain). Animals were kept in standard animal facilities with 6 animals per cage and given free access to food and drinking water. They were housed in a temperature and humidity controlled room with 12-hour on-off light cycles. Experiments were approved by the Ethical Committee for Animal Experimentation at the University of Navarra (protocol number: 129-11) in agreement with the European guidelines on animal experiments (86/609/EU).

To study the amount of anticancer drug in different organs after administration, C57BL/6J mice received paclitaxel loaded in the poly(anhydride) nanoparticles orally at a dose of 25 mg/kg. The treatment groups were: PTX-HPCD-NP, PTX-CD-NP, PTX-CD-PEG-NP and PTX-PEG-NP. In addition, a group of mice was treated intravenously with the commercial formulation, Taxol®, at the same dose (25 mg/kg) as control.

After administration, mice were sacrificed at different time points by cervical dislocation under isoflurane anesthesia and the following organs were harvested: liver, lung, spleen, kidneys, ovaries, stomach and intestine. In the group receiving the commercial formulation, animals were sacrificed at 30 min, 3h, 8h and 24 hours post-administration. On the other hand, the animals treated with the pegylated nanoparticles (PTX-PEG-NP) were sacrificed at 3h, 8h, 24h and 72 hours. Finally, for the rest of the treatment groups (PTX-HPCD-NP, PTX-CD-NP and PTX-CD-PEG-NP), the animals were killed at 8 hours exclusively.

Before quantifying the amount of paclitaxel in the different tissues, organs were individually weighed and homogenized in 1 ml of PBS pH 7.4 using a Mini-bead Beater (BioSpect Products Inc, Oklahoma, USA). Later, the homogenized organs were centrifuged at 10,000*xg* for 10 minutes. The supernatants were then collected and stored at -80°C until analysis.

6.1. Measurement of PTX levels in tissue samples by HPLC

For the determination of paclitaxel content in the different tissue samples, a liquid-liquid extraction method followed by reverse-phase HPLC analysis was performed. The extraction method was adapted from Agüeros et al [19]. Standardized calibration curves were used for each organ. As

internal standard, docetaxel was used and the conversion of the PTX/DCX chromatographic areas to concentration was performed.

Aliquots of 200 µl of the selected tissues were mixed with 25 µl of the DCX solution (5µg/ml in ethanol, previously evaporated). After mixing, a liquid-liquid extraction was accomplished by adding 4 ml of t-buthylmethylether followed by vortex gentle mixing. Next, the mixture was centrifuged at 5000 rpm for 5 min and then, the clear organic layer was transferred to clean tubes and evaporated until complete dryness. Finally, the residue was dissolved in 125 µl of acetonitrile-phosphate buffer 0.01 M pH2, 50:50 v/v, transferred to cap vials and placed in the HPLC autosampler and the amount of PTX quantified by UV detection (228 nm.).

A new calibration was done for every set of sample measurements for each of the tissues studied. The interday variability of the calibration coefficients was less than 15%. The detection limit was 250 ng/ml for the organs.

7. Antitumor efficacy studies

The experimental protocol (number: 129-11) involving use of animals for this research was approved by Ethical Committee for Animal Experimentation at the University of Navarra in agreement with European guidelines on animal experiments (86/609/EU).

C57BL/6J female mice, 4 to 6-week old, weighing 20-22 g, were purchased from Harlan (Barcelona, Spain) and housed under standard animal facilities with 8 animals per cage and given free access to food and drinking water. Housing conditions were maintained by control temperature and humidity and with 12-hour on-off light cycles. The animals were allowed to acclimate for at least 1 week before any experiments.

7.1. Animal model

In vivo antitumor efficacy of the poly(anhydride) nanoparticles was evaluated with a tumor model set up by inoculation of Lewis Lung Carcinoma cell line (3LL) to mice. Before the implantation in animals, 3LL cell line was maintained at 37°C and 5% CO_2 in RPMI supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Prior to the inoculation of cells to the animals, a mycoplasma assay was performed to ensure the absence of contaminants in the cell culture samples.

On the day of the experiments, 3LL cells (1×10^5) were mixed with Growth Factor Reduced Matrigel Matrix (BD Biosciences, California, USA) (1:1, v/v), and injected subcutaneously on the right back flank of C57BL/6J female mice under light isoflurane anesthesia.

7.2. Antitumor study

Treatments were started on day 8 after inoculation when tumor volumes were palpable and reached approximately 100 mm^3. On that day (considered day 1 of treatment), mice were randomly distributed into the following groups: tumor growth control group, PTX injection treatment group (commercial Taxol® administered intravenously) and the nanoparticle treatment groups (PTX-HPCD-NP, PTX-CD-NP, PTX- CD-PEG-NP and PTX- PEG-NP) administered orally. Each group consisted of 8 tumor-bearing animals. On that same day, animals were marked for further identification.

In the antitumor activity experiment, Taxol® was diluted with sterile normal saline (0.9%) to facilitate the administration. The selected dose for the commercial formulation was 10 mg/kg body weight (around 0.2 g of anticancer drug) and administered on a daily regimen via the tail vein to mice. The lyophilized nanoparticle formulations were resuspended in water prior to the oral administration. An equivalent dose to 25 mg/kg of paclitaxel formulated in poly(anhydride) nanoparticles was orally administered to the animals. The treatment schedule for the nanoparticles was as follows. For the pegylated nanoparticles, PTX-PEG-NP, mice received the PTX dose in nanoparticles every 3 days. The cyclodextrin containing formulations, PTX-HPCD-NP, PTX-CD-NP and PTX-CD-PEG-NP were administered on a daily basis. As established in the approved protocol, when tumor volumes reached 1500 mm^3, animals were sacrificed considering the volume of the tumor excessive and life-threatening.

Throughout the study, mice were weighed and tumors were measured with a caliper every two days. Tumor volumes were calculated according to the following formula:

$$Tumor\ Volume\ (mm^3) = \frac{L\ x\ W^2}{2}\ [eq.2]$$

In which the *L* corresponded to the largest diameter and *W* to the shortest diameter of the tumor, perpendicular to length.

In addition, tumor growth delay and tumor doubling time (DT) were determined as described elsewhere as pharmacodynamic parameters [30, 31]. Tumor growth delay (TGD) is calculated as the time in days required for tumors to reach a mean volume of 500 mm^3.

The tumor doubling time (*DT*) was calculated using the following equation:

$$DT = \left(t_f - t_0\right) x \frac{\log 2}{\log V_f - \log V_0} \; [eq.3]$$

In which $t_f - t_0$ corresponds to the length between two measurements and V_f and V_0 indicate the tumor volumes at two points of measurements.

7.3. Measurement of vascular endothelial growth factor (VEGF)

In addition, blood samples were obtained in order to evaluate the vascular endothelial growth factor (VEGF). For this purpose, a commercial kit (Mouse VEGF Immunoassay Quantikine® ELISA kit, R&D Systems, Minneapolis, Minnesota, USA) was used. Blood samples were obtained at the beginning of the study (basal levels of VEGF in plasma). Throughout the experiment, blood was extracted every 2 days to 3 animals in each group randomly. For this purpose, 300 µl of blood were extracted from the mice in the different treatment groups and plasma was recovered by centrifugation at 2500x*g* for 10 min and frozen at -80^{o}C until analysis. The samples were processed as specified in the commercial kit and the plate was read at 450 and 540 nm using a microtite plate reader (iEMS Reader MF, Labsystems, Helsinki, Finland). A standard curve constructed with mouse VEGF was carried out to extrapolate the experimental data.

8. Statistical analysis

Data are expressed as the mean ± S.D. of at least three experiments. One-way ANOVA with Bonferroni post-test, Mann-Whitney U-test or Kruskall-Wallis tests were used to investigate statistical differences. In all cases, $p<0.05$ was considered to be statistically significant. All data processing was performed using GraphPad Prism 4.0 statistical software (GraphPad Software, USA).

Results

1. Preparation and characterization of poly(anhydride) nanoparticles

Poly(anhydride) nanoparticles were successfully prepared by the solvent displacement method. The main physicochemical characteristics of the different poly(anhydride) nanoparticle formulations loaded with PTX are summarized in **table 1**. In the first place, nanoparticles containing the drug-cyclodextrin complexes displayed bigger sizes than the nanoparticles including the poly(ethylene glycol). For the PTX-HPCD-NP, the sizes were close to 300 nm while for the other formulation containing cyclodextrin, PTX-CD-NP; sizes were slightly smaller, around 265 nm. However, when the poly(anhydride) nanoparticles were prepared with PEG (PTX-PEG-NP), sizes

turned out to be below 200 nm. It is interesting to note that the addition of PEG to the formulation containing PTX-CD complexes, PTX-CD-PEG-NP, rendered nanoparticles of smaller sizes (17% reduction).

Table 1. Physicochemical characterization of the poly(anhydride) nanoparticles obtained.

Formulation	Size (nm)	Zeta Potential (mV)	Yield (%)	Poly(ethylene glycol) content (μg PEG/ mg NP)	Cyclodextrin content (μg CD/mg NP)	Paclitaxel Loading (μg PTX/mg NP)
PTX-HPCD-NP	295 ±	-45 ± 3	70 ± 5	-	75.3 ± 3.5	149 ± 3.1
PTX-CD-NP	255 ±	-46 ± 6	73 ± 4	-	88.4 ± 6.9	49 ± 3.8
PTX-CD-PEG-NP	220 ±	-55 ± 3	65 ± 3	42.2 ± 2.4	98.3 ± 5.8	69 ± 3.1
PTX-PEG-NP	193 ±	- 53 ± 2	62 ± 4	55.6 ± 2.2	-	112 ± 4.2

Data are expressed as mean ± S.D. (n=3). PTX-HPCD-NP: paclitaxel complexed with 2-hydroxylpropyl-β-cyclodextrin and loaded in poly(anhydride) nanoparticles; PTX-CD-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles; PTX-PEG-NP: paclitaxel loaded in poly(anhydride) nanoparticles combined with PEG2000; PTX-CD-PEG-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles combined with PEG2000.

Regarding the zeta potential, it is interesting to highlight that the nanoparticles containing PEG (PTX-CD-PEG-NP and PTX-PEG-NP) presented a slightly more negative surface charge, around -55 mV. The nanoparticles formulated with just the CDs displayed a surface charge around -48 mV. Furthermore, the yield of the process was calculated to be between 60 and 76% for the PEG and CD containing formulations, respectively.

The amounts of excipients (cyclodextrins and PEG) associated with the different formulations were calculated. On one hand, for the cyclodextrin containing nanoparticles, the amount of oligosaccharide was dependent on the type of cyclodextrin used. Thus, under the experimental conditions here described, HPCD showed a higher ability to associate with the poly(anhydride) polymer than β-cyclodextrin. In all, the amount of cyclodextrin combined to the nanoparticles was similar for the 2 formulations containing β-CD (PTX-CD-NP and PTX-CD-PEG-NP: 80-88 μg/mg NP approximately) and relatively higher for PTX-HPCD-NP (95 μg/mg NP). On the other hand, for PEG, the amount of excipient associated with the formulations was different. When PEG was added to the formulation containing paclitaxel as a complex with CD, the amount of PEG was decreased (42 μg/mg NP) compared to the nanoparticles with no cyclodextrin at all, PTX-PEG-NP (55 μg/mg NP).

Focusing on the amount of PTX loaded in the nanoparticles, differences were obtained between the formulations. Depending on the cyclodextrin used, the amount of PTX varied

significantly. For the nanoparticles formulated with HPCD, the PTX content was estimated in 150 µg PTX/mg NP. However, for the nanoparticles containing β-cyclodextrin (PTX-CD-NP), the amount of PTX was fairly lower (3 times lower), around 50 µg/mg NP. In addition, when poly(ethylene glycol) was added to the formulation containing PTX-CD complexes, almost a 40% increase in the drug loading was observed, from 50 to 70 µg/mg NP. Finally, for PTX-PEG-NP, the amount of PTX was calculated to be around 110 µg/mg NP.

2. Pharmacokinetic study

The plasma concentration-time profile of PTX after a single i.v. administration of Taxol® at 25 mg/kg is shown in **figure 1**. The plasma profile after the i.v. injection of the commercial formulation diluted in normal saline (0.9%) presented a nonlinear profile. Levels of paclitaxel in plasma were quantifiable till 12 hours post-administration.

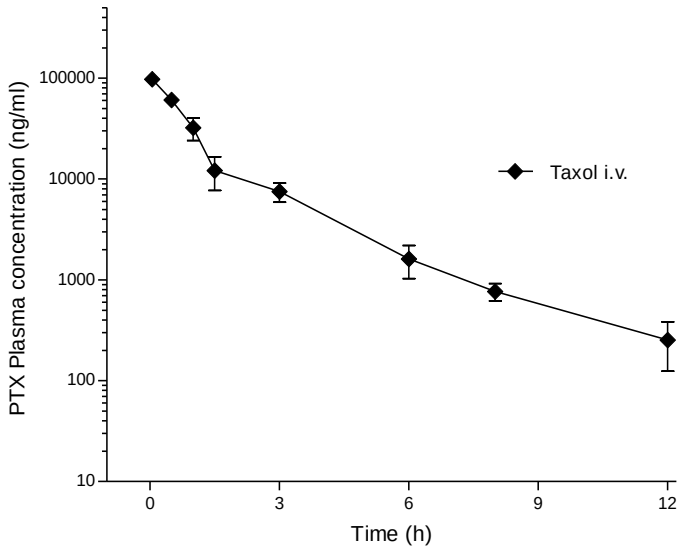

Figure 1. Plasma paclitaxel concentration-time profile after the intravenous administration of a 25 mg/kg dose of Taxol®. Data are expressed as mean ± S.D., n=3 per time point.

Figure 2 shows the plasma concentration profiles of paclitaxel after a single oral dose of 25 mg/kg to laboratory animals when administered as commercial Taxol® or encapsulated in poly(anhydride) nanoparticles. When commercial Taxol® was administered to mice by the oral route, PTX plasma levels were detected at low concentrations. The drug plasma levels for Taxol® orally administered were sort of maintained up to 6 hours but decreased rapidly, displaying no detectable levels after 8 hours. Thus, these plasma levels for the commercial formulation were in all cases, close to the quantification limit in plasma of the HPLC technique (80 ng/ml).

On the contrary, the paclitaxel plasma levels found after the oral administration of the loaded poly(anhydride) nanoparticles were significantly higher than for the oral Taxol®. The observed increase in plasma was between 10-15-fold higher. In all cases, there was an initial rapid rise in the anticancer plasma levels for the first 2 hours reaching the maximum concentration (C_{max}) followed by slow decline which was prolonged for at least 24 hours for PTX-HPCD-NP and PTX-CD-NP and for about 72 hours for the PEG containing formulations (PTX-PEG-NP and PTX-CD-PEG-NP).

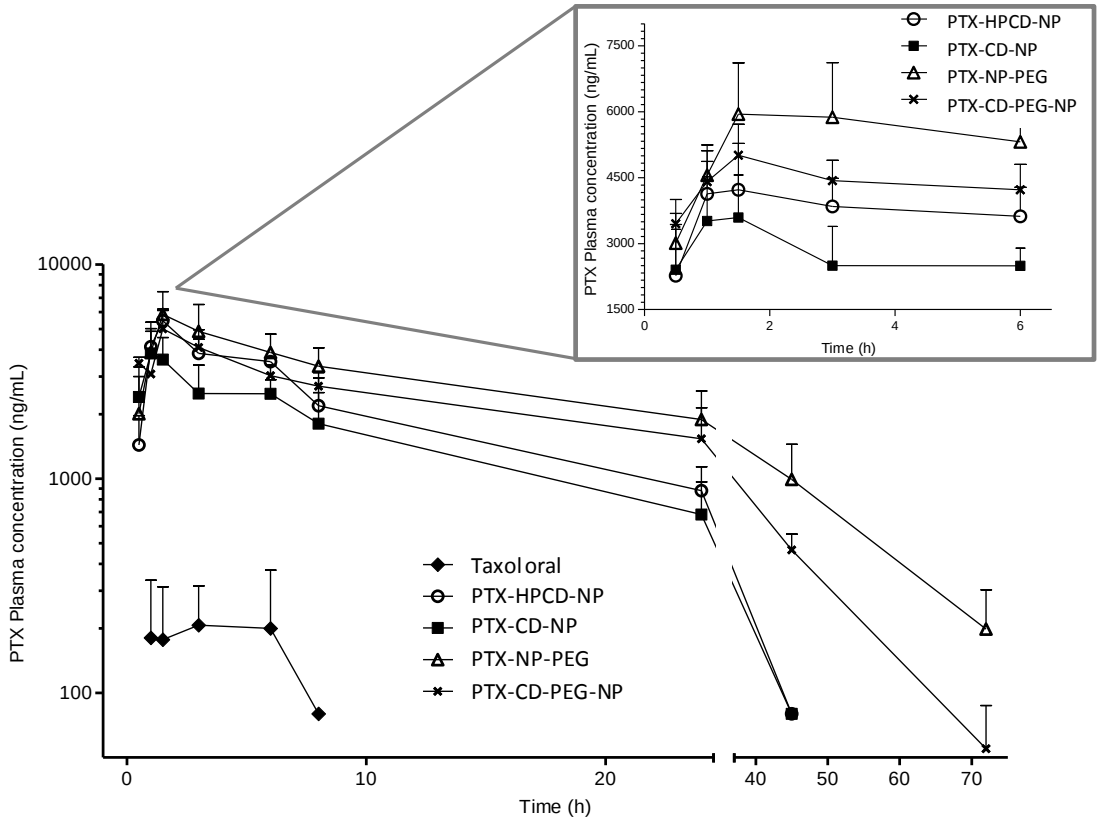

Figure 2. Paclitaxel plasma levels after the administration of a single oral dose of 25 mg/kg. Animals received Taxol® or paclitaxel-loaded nanoparticles: PTX-HPCD-NP, PTX-CD-NP, PTX-CD-PEG-NP and PTX-PEG-NP. Data are expressed as mean ± S.D. (n=3). PTX-HPCD-NP: paclitaxel complexed with 2-hydroxyl-propyl-β-cyclodextrin and loaded in poly(anhydride) nanoparticles; PTX-CD-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles; PTX-PEG-NP: paclitaxel loaded in poly(anhydride) nanoparticles combined with PEG2000; PTX-CD-PEG-NP: paclitaxel complexed with β-cyclodextrin and loaded in poly(anhydride)nanoparticles combined with PEG2000.

Table 2 summarizes the main pharmacokinetic parameters derived from the oral and intravenous plasma curves estimated by a non-compartmental analysis. Firstly, for the commercial formulation administered by means of the i.v. route, the mean value of AUC was 100 µg h/ml. The maximum concentration (C_{max}) was 113 µg/ml. The MRT was 2.25h and the half-life of the terminal phase ($t_{1/2z}$) of the curve was estimated to be 2.5 h.

As seen in **table 2**, the paclitaxel C_{max} for the nanoparticle formulations was found to be about 17-20 times higher than for the orally administered commercial formulation. Within the different poly(anhydride) nanoparticle formulations, the rank order of C_{max} values obtained was as follows: PTX-PEG-NP> PTX-CD-PEG-NP> PTX-HPCD-NP> PTX-CD-NP. Similarly as for the maximum concentration values, the highest AUC value was obtained for the PEG containing nanoparticles (PTX-PEG-NP). In addition, this AUC values for PTX-PEG-NP were 38%, 48% and 21.5% higher than for PTX-HPCD-NP, PTX-CD-NP and PTX-CD-PEG-NP, respectively. However, in all cases the T_{max} appeared to be similar, independent of formulation (T_{max}= 1-1.5 h)

Table 2. Pharmacokinetic parameters of paclitaxel obtained after the intravenous and oral administration of the commercial Taxol® and poly(anhydride) nanoparticles at a single dose of 25 mg/kg to C57BL/6J female mice. Data expressed as mean ± S.D. (n= 3)

Formulation	Route	AUC (µg h/mL)	C_{max} (µg/mL)	T_{max} (h)	MRT (h)	$t_{½ z}$ (h)	Fr (%)
Taxol®	i.v.	101.2 ± 5.7	112.9 ± 6.7	0.05	3 ± 0.7	2.5 ± 0.3	100
Taxol®	p.o.	2.3 ± 0.9	0.2 ± 0.1	1	1 ±0.8	2.20 ± 0.6	2.3
PTX-HPCD-NP	p.o.	59.4 ± 5.7	5.2 ± 2.8	1.5	23 ± 2.3	15.3 ± 1.4	58.6
PTX-CD-NP	p.o.	55.3 ± 5.2	3.6 ± 1.3	1.5	18 ± 1.6	13.6 ± 1.5	54.6
PTX-CD-PEG-NP	p.o.	67.4 ± 3.8	5.0 ± 2.8	1.5	31 ± 2.8	17.1 ± 1.6	66.6
PTX-PEG-NP	p.o.	81.9 ± 3.0	5.7 ± 2.6	1.5	29 ± 3.1	18.3 ± 1.2	81.1

AUC: Area under the concentration-time curve; C_{max}: peak plasma concentration; T_{max}: time to reach peak plasma concentration; MRT: mean residence time; $t_{1/2z}$: half-life of the terminal phase, Fr: relative oral bioavailability.

Additionally, the mean residence time of the drug in plasma when administered encapsulated in poly(anhydride) nanoparticles was between 18 to 30 hours. In all cases, again, these values were significantly higher than those obtained for Taxol® (1.3h). In this way, the values of $t_{1/2z}$ for the nanoparticles formulations were similar, between 14 to 18h for all the nanoparticle treatments.

Finally, the relative oral bioavailability of PTX delivered in nanoparticles was calculated to be around 55% and 59% for PTX-CD-NP and PTX-HPCD-NP, and 67% and 81% for PTX-CD-PEG-NP and PTX-PEG-NP, respectively. In all cases, these values were 33-fold higher (on average) than the bioavailability estimated for the commercial formulation of Taxol® (Fr= 2.3%).

3. Tissue distribution of paclitaxel

Tissue distribution of paclitaxel after intravenous administration of Taxol® and oral administration of poly(anhydride) nanoparticles were compared in C57BL/6J female mice. **Figures 3A** and **3B** represent the amount of PTX found in the studied organs (liver, kidneys, spleen, ovaries, lung, stomach and intestine) at different times after the intravenous administration of Taxol® or the oral administration of PTX-PEG-NP, respectively. As it can be seen in **figures 3A** and **3B**, paclitaxel underwent a rapid and wide distribution in the evaluated organs. Following the intravenous administration, at the shortest time evaluated (30 minutes), the highest concentration of PTX was found in liver (around 30 µg PTX/mg tissue), followed by kidney (25 µg PTX/mg tissue), spleen (15 µg/mg tissue) and lung (10 µg/mg tissue). However, after 24 hours, the drug concentration in the mentioned organs was remarkably decreased, as expected, and a higher concentration was found in the intestine (PTX amount at 24 hours post-administration=20 µg/mg tissue).

On the other hand, when paclitaxel was administered loaded in poly(anhydride) nanoparticles formulated with PEG, the amounts of the anticancer agent found in the selected organs followed a similar trend to that obtained for Taxol®. The highest drug amount was found once again in liver (with a similar value to that of the intravenous formulation=30µg PTX/mg tissue), although with a certain delay, at 3 hours post-administration. The main distribution at 3 hours after administration of PTX-PEG-NP was in liver (33 µg/mg tissue), followed by kidneys (around 30 µg/mg tissue), lung (35 µg/mg tissue) and ovaries (15 µg/mg tissue). As time increased, the drug amounts in tissues decreased except in the case of the intestine, which displayed higher levels (25 µg PTX/mg tissue approximately) at the longest times evaluated, 72 hours. This again was the same trend as observed for the commercial formulation.

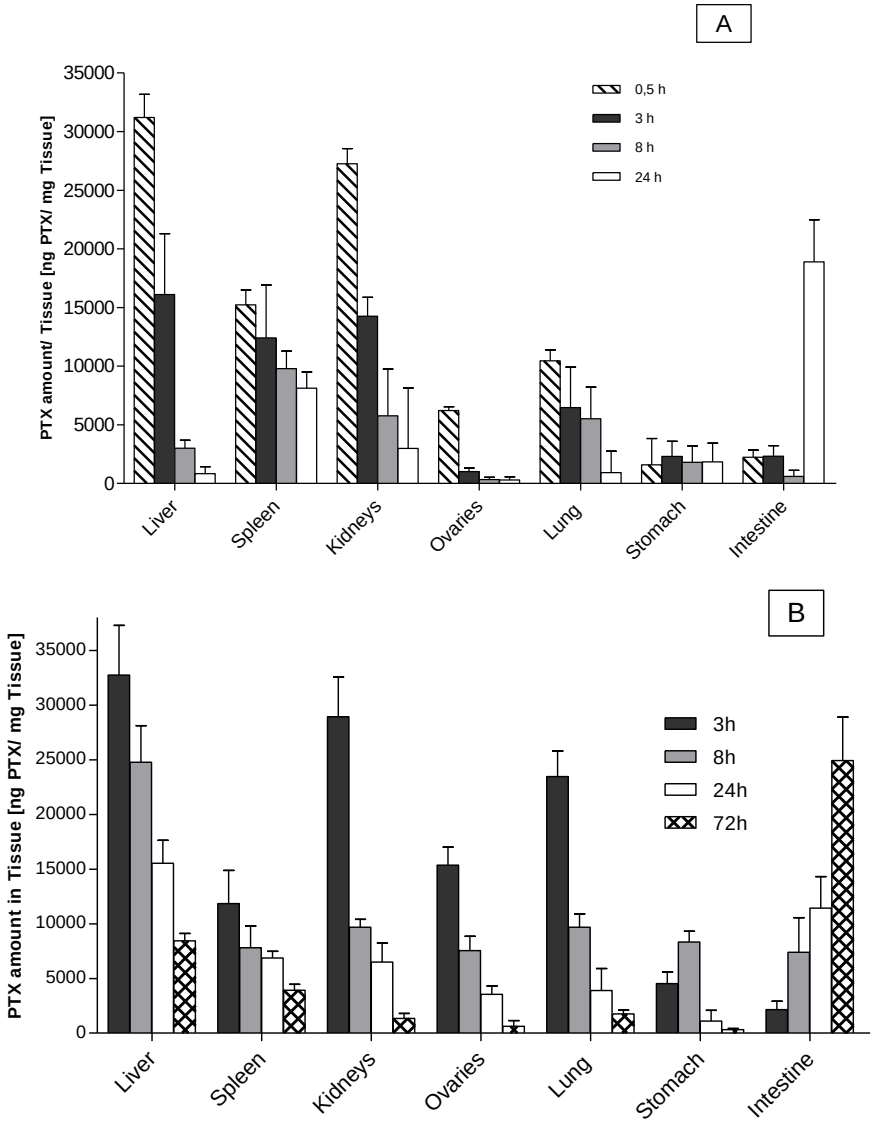

Figure 3b. Organ distribution time profiles of paclitaxel in C57BL/6J female mice after intravenous administration of Taxol® (A) or the oral administration of paclitaxel-loaded in pegylated nanoparticles (PTX-PEG-NP) (B). All mice received a single dose of 25mg/kg. Data expressed as mean ± S.D. (n=4)

Figure 4 shows the comparative amount of anticancer drug in various organs of mice after oral administration of the nanoparticles and intravenous Taxol®, respectively, at a dose of 25 mg/kg 8 hours after administration. Focusing on the nanoparticle formulations by the oral route, in general, the amounts of anticancer drug found in the extracted organs at 8 hours after administration were higher than for Taxol®. Statistical analysis using one-way ANOVA test ($p<0.05$) indicated that the differences among the different nanoparticle groups and the commercial Taxol® were significant. In addition, the largest amounts of drug were found in all cases in liver, kidneys, ovaries and intestine. In these organs (liver, kidneys, ovaries and intestine), the amount of paclitaxel found was at least 7-fold, 11.5-fold, 4.5-fold and 5.5-fold higher than for the commercial

formulation, respectively. However, slight differences were observed between nanoparticle formulations, mainly in liver and intestine. In the liver, pegylated nanoparticles (PTX-PEG-NP and PTX-CD-PEG-NP) appeared to display certain higher drug levels than PTX-HPCD-NP and PTX-CD-NP (1.5 times). In the intestine, at 8 hours post administration the highest drug amounts were obtained for PTX-HPCD-NP. These paclitaxel levels were 1.5 times higher for PTX-HPCD-NP than for the rest of the poly(anhydride) nanoparticle formulations. In contrast, the levels of paclitaxel in ovaries for the poly(anhydride) nanoparticles studied were alike (around 5 µg/mg tissue).

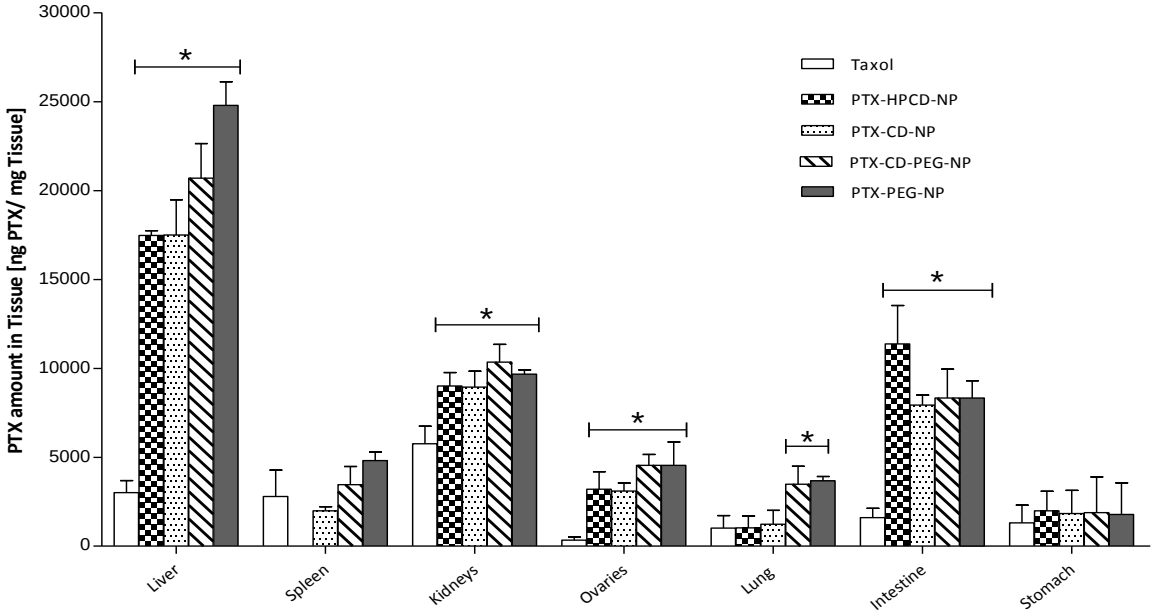

Figure 4. Comparative organ distribution of paclitaxel following the oral administration of the different poly(anhydride) nanoparticles loaded with paclitaxel and the intravenous administration of Taxol® (dose=25 mg/kg) at 8 hours after administration in C57BL/6J mice. Data expressed as mean ± S.D. (n=4). *p<0.05 Taxol® vs. nanoparticle formulations: PTX-HPCD-NP, PTX-CD-NP, PTX-CD-PEG-NP and PTX-PEG-NP.

Regarding the levels of drug in lung, only the poly(ethylene glycol) containing formulations (PTX-CD-PEG-NP and PTX-PEG-NP), presented statistically significant different values when compared to the commercial formulation of the anticancer drug. Furthermore, in the lung, the amounts of drug found for the PEG containing formulations (PTX-PEG-NP and PTX-CD-PEG-NP) were almost 3 times higher than for PTX-HPCD-NP and PTX-CD-NP (for these 2 formulations the paclitaxel amount was rather similar). In the other studied organs (spleen and stomach), the drug amounts were similar to those observed for the Taxol® group with no statistical differences.

4. Antitumor activity

Antitumor efficacy of PTX encapsulated in poly(anhydride) nanoparticles was evaluated and compared to the commercial formulation, Taxol®, in a subcutaneous tumor model set up by inoculation of non-small cell lung cancer cell line (3LL) in C57BL/6J female mice. After implantation of tumor cells, mice were observed daily until the tumor mass was palpable and reached a measurable volume of around 100 mm^3, approximately 8 days after implantation, considering this as day 1 of treatment.

Figure 5 represents the mean tumor volumes (in mm^3) throughout the days of treatment. As it can be observed**,** on day 1 (beginning of treatment) tumor volumes were similar in all groups with a mean volume of 100 mm^3, approximately. Focusing on tumor size, no size regression was observed in the growth control group, as expected. In this group, tumors grew from the beginning of the experiment reaching larger volumes than 2000 mm^3 at the end of the study (day 11). On the other hand, the daily intravenous administration of the commercial formulation, Taxol®, was capable of maintaining the tumor volume around 100 mm^3 up to day 5. After the fifth day, the tumor volume increased rapidly presenting on day 10 the biggest volumes of all the treatment groups (volume>1000 mm^3).Yet, for the poly(anhydride) nanoparticles groups, tumor volumes grew at a slower rate. Nonetheless, differences were observed between formulations, more specifically after 7 days of treatment. From day 7 onwards, in the group treated with the β-cyclodextrin containing formulation (PTX-CD-NP), tumors grew at a faster rate than for the rest of the nanoparticles and at the end of the study, mice presented tumor volumes of 700-800 mm^3, approximately. The evolution of the tumor volume in PTX-CD-PEG-NP and PTX-HPCD-NP was similar, with a more sustained growth and a final tumor volume of around 575 and 650 mm^3, respectively. In contrast, at the endpoint of the treatment, the treatment based on PTX-PEG-NP displayed the smallest tumor sizes (volume<500 mm^3) of all the poly(anhydride) groups.

So, the pegylated nanoparticles (PTX-PEG-NP) administered every 3 days as well as the strategy combining PEG and β-cyclodextrin (PTX-CD-PEG-NP) administered daily were capable of reducing significantly ($p<0.01$) the tumor sizes in mice compared to intravenous Taxol®, administered every day. In addition, PTX-HPCD-NP also reduced significantly ($p<0.05$) tumor volumes when compared to Taxol®. However, when the poly(anhydride) nanoparticle treatments were compared amongst them, differences were not statistically significant, even though a reduction in the tumor was evidenced throughout the efficacy study performed.

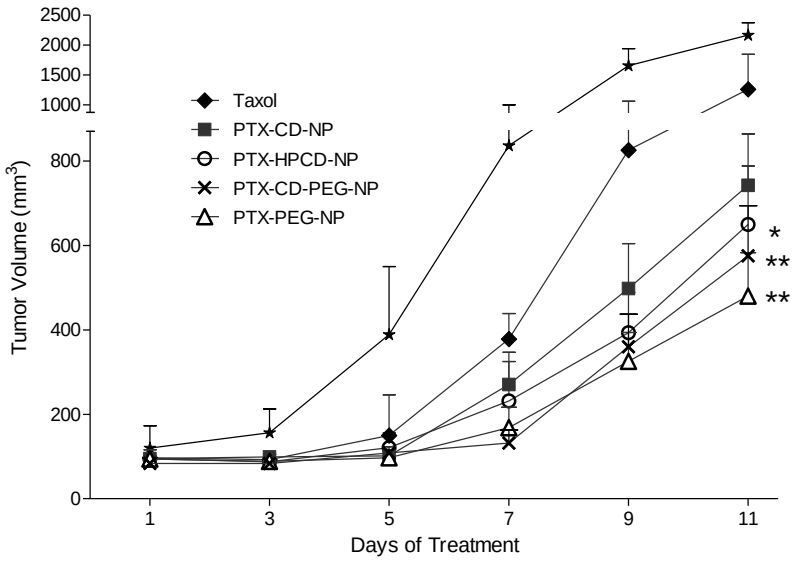

Figure 5. Comparative tumor growth inhibition by intravenous Taxol® (dose 10 mg/kg) or oral PTX loaded in poly(anhydride) nanoparticles (dose 25 mg/kg) in 3LL tumor-bearing C57BL/6J female mice. Results expressed as mean ± S.D. (n=6). *p<0.05 ANOVA + Bonferroni post-test (PTX-HPCD-NP vs. Taxol® i.v.); **p<0.01 ANOVA + Bonferroni post-test (PTX-PEG-NP and PTX-CD-PEG-NP vs. Taxol® i.v.)

The pharmacodynamic variables of antitumor response to the different treatments were determined by measuring the growth delay and the volume doubling times (DT). As shown in **table 3**, the untreated group, the Taxol® treated group and the group receiving PTX-CD-NP presented doubling times of 1.91, 1.62 and 1.74 days, respectively. The animals treated with the poly(anhydride) nanoparticles containing HPCD had an increase in the doubling times as compared to Taxol® (2.34 days vs. 1.62 days). In addition, the treatment groups administered the nanoparticles containing PEG (PTX-CD-PEG-NP and PTX-PEG-NP) had similar DT values (2.5 and 2.6 days, respectively).

Table 3. Pharmacodynamic parameters after paclitaxel treatment either as commercial Taxol® formulation or loaded in poly(anhydride) nanoparticles

Treatment Groups	Tumor Volume Doubling Time (DT) (days)	Tumor Growth Delay (days)
Growth Control (untreated)	1.9 ± 0.7	5
Taxol® i.v.	1.6 ± 0.5	7.5
PTX-HPCD-NP	2.3 ± 0.3	9.5
PTX-CD-NP	1.8 ± 0.4	9
PTX-CD-PEG-NP	2.5 ± 0.3	10.2
PTX-PEG-NP	2.6 ± 0.3	11

Values shown as mean ± S.D. n=8.

On the other hand, comparing the growth delay values (time to reach a mean tumor volume of 500 mm^3) when animals were treated with the intravenous Taxol® the delay in growth was around 7.5 days (2.5 days higher than for the untreated animals). Besides, the formulation of paclitaxel in nanoparticles clearly inhibited the growth of the tumor achieving values from 9 to 11 days in growth delay with no substantial differences between treatments. When considering the nanoparticle formulations against the commercial intravenous formulation, the tumor growth delay was at least 2 days higher, meaning that mice receiving the poly(anhydride) nanoparticles orally presented slower tumor growth rates than Taxol®.

Moreover, VEGF was evaluated as an angiogenesis marker in plasma since it is directly related to tumor growth. The VEGF profiles on different days throughout the study for the different groups are plotted in **figure 6**. The amounts of VEGF at the beginning of the experiment were the plasma levels obtained prior to starting the experiment, namely the basal VEGF levels in mice on the day of the implantation of the tumors. These basal levels were found to be around 15 pg/ml, on average, in all mice. As observed in **figure 6**, in general, despite the administration of the different treatments, there was an increase in the VEGF concentration in plasma for all the groups. On the initial days of treatment, levels of the angiogenesis marker were maintained similar. However, at the end of the study levels rose and differences were then observed between the treatments. At this final moment, on day 11, the concentrations of VEGF in plasma were around 5 to 10 times higher than the basal levels for all the treatments evaluated. It is interesting to point out that while for the Taxol® receiving group, the increase was of 10-fold compared to the basal levels, for the nanoparticle groups this rise was not as pronounced (5-7-fold) except for PTX-CD-NP (in this case, the VEGF concentration rose 8-fold).

In addition, statistical differences ($p<0.05$) were observed when the values of VEGF for the nanoparticle formulations (except for PTX-CD-NP) were compared to the commercial Taxol® on the last day of treatment. In addition, interestingly, statistically significant differences were also obtained when PTX-CD-NP were compared to the rest of the nanoparticle formulations. The VEGF values for PTX-CD-NP were around 1.5-fold higher than for the other poly(anhydride) nanoparticle treatment groups.

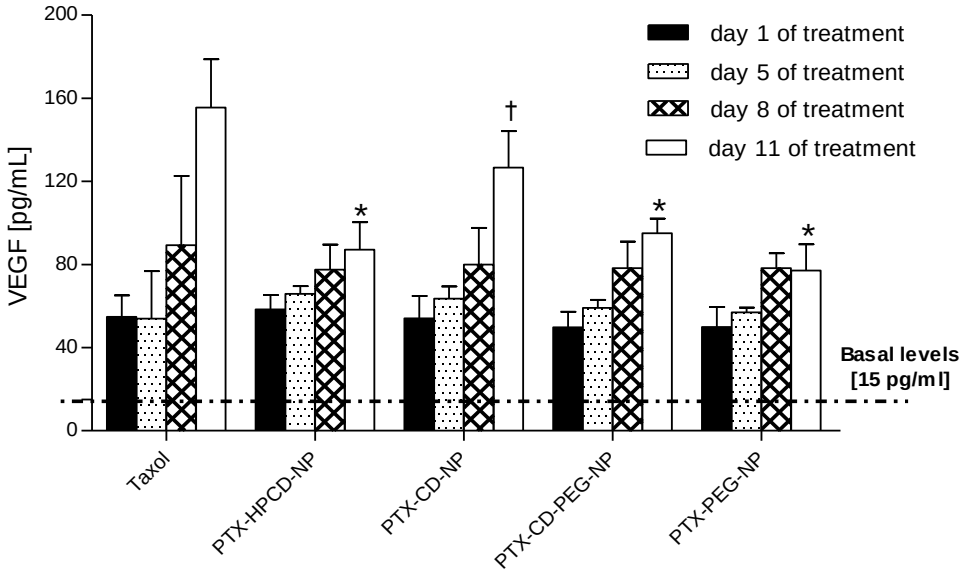

Figure 6. Vascular endothelial growth factor (VEGF) concentration in plasma for the different treatment groups: Taxol® and PTX loaded poly(anhydride) nanoparticles, i.v. and orally administered respectively, on different days throughout the treatment. Data expressed as mean ± S.D. (n=3) *p<0.05 Mann-Whitney U-test PTX-HPCD-NP vs. Taxol®, PTX-CD-PEG-NP vs. Taxol®, PTX-PEG-NP vs. Taxol®.† p<0.05 Mann Whitney U-test PTX-CD-NP vs. PTX-HPCD-NP, PTX-CD-PEG-NP and PTX-PEG-NP.

Discussion

As for numerous anticancer agents, paclitaxel, classified as class IV by the Biopharmaceutical Classification System, is highly lipophilic implying it cannot be easily solubilized in water and it presents a low permeability through intestinal membrane. Therefore, its oral bioavailability is very low. The marketed and commonly used formulation of paclitaxel, Taxol®, contains a mixture of ethanol and Cremophor EL® as excipients for its intravenous administration. The latter, Cremophor EL®, has been described as the responsible agent for the severe hypersensitivity reactions that may occur after administration, despite the premedication [3]. In addition, patients have to suffer the inconvenience of the hospital treatment associated with the intravenous route. For these reasons, the oral administration of paclitaxel represents a major breakthrough and is currently being investigated. From a clinical point of view, the oral route would facilitate the treatment in the chronic regimes such as cancer may be. In these circumstances, a prolonged exposure to the anticancer drug may have certain pharmacodynamic advantages over sporadic intravenous regimes [32].

Thus, different alternatives have been recently published to increase the oral bioavailability of the anticancer agent. In line with this, the poly(anhydride) nanoparticles and their combination with cyclodextrins and poly(ethylene glycol) have been reported as an interesting approach for the oral administration of paclitaxel [19, 20, 27]. On one hand, poly(anhydride) nanoparticles may increase the residence time of the loaded drug in contact with the mucosa [22, 23]. And on the other hand, the use of cyclodextrins and PEG has been reported to increase the drug loading in these nanoparticles [19, 20, 27]. In these previous studies, poly(anhydride) nanoparticles loaded with PTX combined with either oligosaccharides or poly(ethylene glycol) were able to increase the relative oral bioavailability of the drug in rat up to 70-80%, approximately. Cyclodextrins permitted the formation of complexes with the drug prior to its encapsulation leading to an increase in the stability of the drug in the gastrointestinal fluids and promoted the absorption of the drug by interacting with the mucosa and disrupting the activity of Pgp [33]. On the other hand, it has been demonstrated that the presence of PEG in the nanocarriers facilitates the interaction with the mucosa due to the hydrophilic nature of PEG and therefore, increase the residence time of the delivery systems in the g.i. tract and subsequently, enhance the absorption of paclitaxel [23]. In addition, both cyclodextrins and PEG have been described as moderate inhibitors of the intestinal efflux pump Pgp and cytochrome P450 [24-26].

Following the line of these previous works, the strategy here described combined the use of the two excipients, poly(ethylene glycol) and cyclodextrins, to perform *in vivo* studies in C57BL/6J female mice. The poly(anhydride) nanoparticles were obtained by a simple desolvation of the poly(anhydride) polymer in ethanol as described previously by Arbos et al [22]. The results here obtained were similar to those reported by previous authors [19, 20]. However, due to the minor modifications carried out for the pegylated nanoparticles the amount of anticancer drug loaded into poly(anhydride) nanoparticles varied slightly; from 150 µg /mg NP reported by Zabaleta et al [20] to 110 µg /mg NP. The drug content was found to be dependent on the excipients used in the formulations. Thus, there were differences in the drug loading according to the cyclodextrin selected. When HPCD was incorporated to the formulations, the amount of drug increased compared to β-cyclodextrin. Besides, when PEG was added to the formulation containing PTX-CD complexes, an increase in the drug loading was evidenced.

For the pharmacokinetic study, a single dose of 25 mg/kg was selected. When Taxol® was administered intravenously to mice; a peak of paclitaxel concentration in plasma appeared at the shortest sampling time, presenting a nonlinear pharmacokinetic profile. This profile is characteristic of the anticancer drug as described previously and associated with the presence of Cremophor EL® in the formula [34]. At the administered dose, the PTX plasma levels diminished rapidly and 12

hours later, no drug levels could be quantified. From the plasma curve, the PK parameters were estimated. The pharmacokinetic values obtained are comparable to the earlier reported by other authors [34, 35]. However, when the commercial Taxol® was administered by the oral route, the plasma concentrations of paclitaxel were rather low. The calculated oral bioavailability for Taxol® was around 2.5%, in agreement with previously reported values varying from 2 to 10.5% [16, 36]. On the other hand, when paclitaxel loaded in poly(anhydride) nanoparticles was administered orally to mice, the plasma levels of the anticancer drug were higher and more sustained in time. Thus, paclitaxel was found in plasma for at least 24 hours after administration for the case of PTX-HPCD-NP and PTX-CD-NP and for around 72 hours for the formulations containing PEG, PTX-PEG-NP and PTX-CD-PEG-NP. These plasma levels in all cases could be considered pharmacologically active since they were above the clinical therapeutic threshold of 0.01mM (equivalent to 85 ng/ml approximately) [37]. Besides, when PTX was encapsulated in poly(anhydride) nanoparticles, the MRT increased compared to Taxol® relating with the sustained plasma levels and besides, there was an increase in the MRT of 20-30 times when orally administered meaning that the drug is available in plasma for a longer time.

Overall, all the relative bioavailability data obtained for the different poly(anhydride) nanoparticles were high, varying from 55-60% for PTX-CD-NP and PTX-HPCD-NP and from 67 to 81% for the PEG containing nanoparticles, PTX-CD-PEG-NP and PTX-PEG-NP. Other published works that enhanced the oral bioavailability of the drug for instance, Oostendorp et al demonstrated that the administration of PTX in self-emulsifying formulations increased the oral bioavailability of the anticancer drug in mice up to 20% [13]. Similarly, when PTX was incorporated to polymeric micelles [11] and administered orally, the oral bioavailability of the drug rose up to 40%.

The results of tissue distribution showed the presence of paclitaxel mainly in liver, kidney and lung at the initial hours after the intravenous administration of Taxol®. At 24 hours post-administration, the highest levels were obtained in intestine, as expected since paclitaxel has been described as to suffer elimination by feces in mice [10]. Similarly, the evaluation of the drug levels in organs after the oral administration of the drug loaded in the different poly(anhydride) nanoparticles assessed the highest amounts of anticancer agent in liver, kidneys, intestine, lung and ovaries, such as mentioned for the Taxol®. In fact, the wide distribution of the drug in liver indicated the possibility of first-pass metabolism [10]. Previous published results on organ distribution already stated the wide distribution of the anticancer drug, decreasing with time [14, 38]. The presence of the drug in organs clearly correlates with a systemic effect related to the drug since the drug is first absorbed at the gastrointestinal level and it is rapidly distributed in plasma reaching the different tissues where it would finally act as anticancer agent.

For the tumor model in mice, Lewis Lung Carcinoma (3LL) cells were used. The 3LL cell line was selected considering that PTX is active and widely used against non-small cell lung cancer in clinics. The antitumor efficacy was studied by measuring the tumor volume after implantation. Using this model, the poly(anhydride) nanoparticles loaded with paclitaxel and administered by means of the oral route were able to diminish the tumor growth compared to the Taxol® administered intravenously on a daily basis. It is interesting to highlight that the lowest tumor volume was obtained for the pegylated nanoparticles, PTX-PEG-NP, which were administered every 3 days and the effect on tumor growth of PTX-HPCD-NP and PTX-CD-PEG-NP (administered once a day) was similar since both formulations achieved similar results throughout the study. These results appear to indicate that the presence of sustained and not very high levels of paclitaxel in plasma could be efficient to reduce tumor mass in tumor bearing mice. Furthermore, no signs of toxicity were observed in the nanoparticle receiving animals, indicative of the biocompatibility of the poly(anhydride) nanoparticles in this study.

In addition, VEGF was measured as angiogenesis factor related to tumor growth. All these antitumor effects were in agreement with the plasmatic levels of VEGF**.** Vascular endothelial growth factor (VEGF) is a chemical signal produced by cells that stimulates the growth of new blood vessels. This is especially relevant in tumors since it is known that tumor cells induce the formation of new blood vessels by secreting growth factors. These new vessels are weaker and leaky in order to facilitate the arrival of blood to all the cells in the tumor [39]. Taking into account that tumor cells produce VEGF and that PTX induces tumor cell death, VEGF plasmatic levels in the treatment groups would be expected to decrease. However, this effect was not clearly observed in the treatment groups in which paclitaxel was acting against tumor cells. Indeed, VEGF did not diminish in blood but there was slower increase, especially in those animals receiving the nanoparticles treatments. This was directly correlated with the tumor growth. In both cases, animals administered the poly(anhydride) nanoparticle formulations displayed tumor growth and VEGF values reduced if compared to commercial Taxol® receiving animals.

Comparing the results obtained *in vivo* for the different nanoparticle formulations, it appears clear that the presence of PEG provided a rather higher ability than cyclodextrins to promote the absorption of the drug at the intestinal mucosa. In fact, regarding the poly(anhydride) nanoparticles using CD and PEG, the combination of the 2 excipients apparently favored the interaction of the nanoparticles with the mucosa at the g.i. level and promoted the absorption of the anticancer drug once released displaying higher drug levels in plasma and tissues than for the nanoparticles formulated with just CD. Herein, the presence of PEG would permit a deeper penetration of the drug delivery system into the mucosa and there, establish stronger interactions with the mucosa

thanks to the hydrophilic nature of the PEG molecule and finally, enhance the absorption of the encapsulated drug at the surface of the enterocytes. Since PTX-HPCD-NP and PTX-CD-NP formulations presented bigger sizes and no PEG was attached to them, the capacity of the carriers to penetrate in the mucus could be more limited remaining instead in more superficial layers. As a result, the plasma and tissue concentrations appeared to be slightly reduced (1.5-3 times lower levels) for poly(anhydride) nanoparticles containing cyclodextrins: HPCD or CD than for the pegylated ones: PTX-CD-PEG-NP and PTX-PEG-NP). In this study, poly(anhydride) nanoparticles provided the drug with certain protection at the gastrointestinal tract level and prolonged the transit time of the drug thanks to the bioadhesive properties of the polymer [23]. In this way, the plasma and tissue distribution levels reached were sufficient to inhibit or slow down growth in a tumor model in C57BL/6J female mice. On the other hand, the formulations containing PEG demonstrated a more sustained profile in plasma and tissue implying that the presence of PEG, mainly on the surface of the nanoparticles, helps to increase the residence time of the nanocarriers at the intestinal level.

In conclusion, the work here presented demonstrated that the combination of poly(anhydride) polymeric nanoparticles with cyclodextrins and poly(ethylene glycol) increased the loading of paclitaxel conferring the drug the possibility to interact with the gastrointestinal mucosa and enhancing the absorption at the intestinal surface. This is reflected in the maintained plasma levels of the drug and the organ distribution achieved after the oral administration of the paclitaxel encapsulated in the poly(anhydride) nanocarriers. In addition, these nanoparticles were able to slow down the tumor growth in a murine model using 3LL tumor cell line.

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References

[1] E.K. Rowinsky, R.C. Donehower, Drug-Therapy - Paclitaxel (Taxol), New England Journal of Medicine, 332 (1995) 1004-1014.
[2] S.B. Horwitz, Taxol (paclitaxel): mechanisms of action, Annals of oncology : official journal of the European Society for Medical Oncology / ESMO, 5 Suppl 6 (1994) S3-6.
[3] H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom, Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation, European Journal of Cancer, 37 (2001) 1590-1598.
[4] J.S. Kloover, M.A. den Bakker, H. Gelderblom, J.P. van Meerbeeck, Fatal outcome of a hypersensitivity reaction to paclitaxel: a critical review of premedication regimens, British Journal of Cancer, 90 (2004) 304-305.
[5] S. Irshad, N. Maisey, Considerations when choosing oral chemotherapy: identifying and responding to patient need, European Journal of Cancer Care, 19 (2010) 5-11.
[6] I. Kuppens, P. Breedveld, J.H. Beijnen, J.H.M. Schellens, Modulation of oral drug bioavailability: From preclinical mechanism to therapeutic application, Cancer Investigation, 23 (2005) 443-464.
[7] H.-J. Yao, R.-J. Ju, X.-X. Wang, Y. Zhang, R.-J. Li, Y. Yu, L. Zhang, W.-L. Lu, The antitumor efficacy of functional paclitaxel nanomicelles in treating resistant breast cancers by oral delivery, Biomaterials, 32 (2011) 3285-3302.
[8] M.M. Malingre, W.W.T. Huinink, K. Duchin, J.H.M. Schellens, J.H. Beijnen, Pharmacokinetics of oral cyclosporin A when co-administered to enhance the oral absorption of paclitaxel, Anti-Cancer Drugs, 12 (2001) 591-593.
[9] M.M. Malingre, W.W.T. Huinink, M. Mackay, J.H.M. Schellens, J.H. Beijnen, Pharmacokinetics of oral cyclosporin A when co-administered to enhance the absorption of orally administered docetaxel, European Journal of Clinical Pharmacology, 57 (2001) 305-307.
[10] A. Sparreboom, J. vanAsperen, U. Mayer, A.H. Schinkel, J.W. Smit, D.K.F. Meijer, P. Borst, W.J. Nooijen, J.H. Beijnen, O. vanTellingen, Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine, Proceedings of the National Academy of Sciences of the United States of America, 94 (1997) 2031-2035.
[11] R. Mo, X. Jin, N. Li, C. Ju, M. Sun, C. Zhang, Q. Ping, The mechanism of enhancement on oral absorption of paclitaxel by N-octyl-O-sulfate chitosan micelles, Biomaterials, 32 (2011) 4609-4620.
[12] K. Yoncheva, P. Calleja, M. Agüeros, P. Petrov, I. Miladinova, C. Tsvetanov, J.M. Irache, Stabilized micelles as delivery vehicles for paclitaxel, International Journal of Pharmaceutics, 436 (2012) 258-264.
[13] R.L. Oostendorp, T. Buckle, G. Lambert, J.S. Garrigue, J.H. Beijnen, J.H.M. Schellens, O. van Tellingen, Paclitaxel in self-micro emulsifying formulations: oral bioavailability study in mice, Investigational New Drugs, 29 (2011) 768-776.
[14] D. Pandita, A. Ahuja, V. Lather, B. Benjamin, T. Dutta, T. Velpandian, R.K. Khar, Development of Lipid-Based Nanoparticles for Enhancing the Oral Bioavailability of Paclitaxel, Aaps Pharmscitech, 12 (2011) 712-722.
[15] D. Pandita, A. Ahuja, T. Velpandian, V. Lather, T. Dutta, R.K. Khar, Characterization and in vitro assessment of paclitaxel loaded lipid nanoparticles formulated using modified solvent injection technique, Pharmazie, 64 (2009) 301-310.
[16] S. Peltier, J.M. Oger, F. Lagarce, W. Couet, J.P. Benoit, Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules, Pharmaceutical Research, 23 (2006) 1243-1250.
[17] C. Fonseca, S. Simoes, R. Gaspar, Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity, Journal of Controlled Release, 83 (2002) 273-286.
[18] L. Zhao, S.-S. Feng, Enhanced Oral Bioavailability of Paclitaxel Formulated in Vitamin E-TPGS Emulsified Nanoparticles of Biodegradable Polymers: In Vitro and In Vivo Studies, Journal of Pharmaceutical Sciences, 99 (2010) 3552-3560.

[19] M. Agueros, V. Zabaleta, S. Espuelas, M.A. Campanero, J.M. Irache, Increased oral bioavailability of paclitaxel by its encapsulation through complex formation with cyclodextrins in poly(anhydride) nanoparticles, Journal of Controlled Release, 145 (2010) 2-8.
[20] V. Zabaleta, G. Ponchel, H. Salman, M. Agueros, C. Vauthier, J.M. Irache, Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: Permeability and pharmacokinetic study, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 81 (2012) 514-523.
[21] A. des Rieux, V. Fievez, M. Garinot, Y.-J. Schneider, V. Preat, Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach, Journal of Controlled Release, 116 (2006) 1-27.
[22] P. Arbos, M.A. Campanero, M.A. Arnangoa, M.J. Renedo, J.M. Irache, Influence of the surface characteristics of PVM/MA nanoparticles on their bioadhesive properties, Journal of Controlled Release, 89 (2003) 19-30.
[23] K. Yoncheva, L. Guembe, M.A. Campanero, J.M. Irache, Evaluation of bioadhesive potential and intestinal transport of pegylated poly(anhydride) nanoparticles, International Journal of Pharmaceutics, 334 (2007) 156-165.
[24] F. Fenyvesi, E. Fenyvesi, L. Szente, K. Goda, Z. Bacso, I. Bacskay, J. Varadi, T. Kiss, E. Molnar, T. Janaky, G. Szabo, Jr., M. Vecsernyes, P-glycoprotein inhibition by membrane cholesterol modulation, European Journal of Pharmaceutical Sciences, 34 (2008) 236-242.
[25] E.D. Hugger, B.L. Novak, P.S. Burton, K.L. Audus, R.T. Borchardt, A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity in vitro, Journal of Pharmaceutical Sciences, 91 (2002) 1991-2002.
[26] M. Ishikawa, H. Yoshi, T. Furuta, Interaction of modified cyclodextrins with cytochrome P-450, Bioscience Biotechnology and Biochemistry, 69 (2005) 246-248.
[27] M. Agueros, L. Ruiz-Gaton, C. Vauthier, K. Bouchemal, S. Espuelas, G. Ponchel, J.M. Irache, Combined hydroxypropyl-beta-cyclodextrin and poly(anhydride) nanoparticles improve the oral permeability of paclitaxel, European Journal of Pharmaceutical Sciences, 38 (2009) 405-413.
[28] M. Agueros, M.A. Campanero, J.M. Irache, Simultaneous quantification of different cyclodextrins and Gantrez by HPLC with evaporative light scattering detection, Journal of Pharmaceutical and Biomedical Analysis, 39 (2005) 495-502.
[29] V. Zabaleta, M.A. Campanero, J.M. Irache, An HPLC with evaporative light scattering detection method for the quantification of PEGs and Gantrez in PEGylated nanoparticles, Journal of Pharmaceutical and Biomedical Analysis, 44 (2007) 1072-1078.
[30] S. Ozono, N. Miyao, T. Igarashi, K. Marumo, H. Nakazawa, M. Fukuda, T. Tsushima, N. Tokuda, J. Kawamura, M. Murai, R. Collaboration Grp Japanese Soc, Tumor doubling time of renal cell carcinoma measured by CT: Collaboration of Japanese Society of Renal Cancer, Japanese Journal of Clinical Oncology, 34 (2004) 82-85.
[31] H. Devalapally, Z. Duan, M.V. Seiden, M.M. Amiji, Modulation of drug resistance in ovarian adenocarcinoma by enhancing intracellular ceramide using tamoxifen-loaded biodegradable polymeric nanoparticles, Clinical Cancer Research, 14 (2008) 3193-3203.
[32] S. Kakolyris, G. Samonis, M. Koukourakis, I. Vlachonicolis, G. Chalkiadakis, K. Kalbakis, I. Souglakos, S. Agelaki, P. Toloudis, V. Georgoulias, Treatment of non-small-cell lung cancer with prolonged oral etoposide, American Journal of Clinical Oncology-Cancer Clinical Trials, 21 (1998) 505-508.
[33] T. Loftsson, D. Hreinsdottir, M. Masson, Evaluation of cyclodextrin solubilization of drugs, International Journal of Pharmaceutics, 302 (2005) 18-28.
[34] A. Sparreboom, O. vanTellingen, W.J. Nooijen, J.H. Beijnen, Nonlinear pharmacokinetics of paclitaxel in mice results from the pharmaceutical vehicle Cremophor EL, Cancer Research, 56 (1996) 2112-2115.
[35] S.H. Lee, S.D. Yoo, K.H. Lee, Rapid and sensitive determination of paclitaxel in mouse plasma by high-performance liquid chromatography, Journal of Chromatography B, 724 (1999) 357-363.
[36] T.K. Yeh, Z. Lu, M.G. Wientjes, J.L.S. Au, Formulating paclitaxel in nanoparticles alters its disposition, Pharmaceutical Research, 22 (2005) 867-874.

[37] S.C. Yang, R.N. Gursoy, G. Lambert, S. Benita, Enhanced oral absorption of paclitaxel in a novel self-microemulsifying drug delivery system with or without concomitant use of P-glycoprotein inhibitors, Pharmaceutical Research, 21 (2004) 261-270.
[38] A. Sparreboom, O. vanTellingen, W.J. Nooijen, J.H. Beijnen, Tissue distribution, metabolism and excretion of paclitaxel in mice, Anti-Cancer Drugs, 7 (1996) 78-86.
[39] D.O. Holtz, R.T. Krafty, A. Mohamed-Hadley, L. Zhang, I. Alagkiozidis, B. Leiby, W. Guo, P.A. Gimotty, G. Coukos, Should tumor VEGF expression influence decisions on combining low-dose chemotherapy with antiangiogenic therapy? Preclinical modeling in ovarian cancer, Journal of Translational Medicine, 6 (2008).

CHAPTER 5

1-Methyl-tryptophan improves the antitumor efficacy of paclitaxel loaded in poly(anhydride) nanoparticles

Abstract

Indoleamine-2,3-dioxygenase (IDO) is an immunosuppressive enzyme responsible for the immune tolerance characteristics of certain biological settings, such as cancer inducing tolerance to tumor antigens. It is widely expressed by cancer cells as well as by dendritic cells surrounding the tumor. Recently, 1-methyl-tryptophan (1-MT) has been described as a specific inhibitor of IDO enzyme and investigation is being carried out to combine 1-MT with traditional chemotherapeutic agents. However, 1-MT has a very low water solubility hampering its administration *in vivo*. Therefore, the nanocrystal strategy presented in this work would enhance its dissolution rate and facilitate its administration. Nanocrystals were formulated using two different surfactants, Pluronic F68 and vitamin E-TPGS. *In vitro* release studies demonstrated that a more extensive release of 1-MT was achieved from the formulation containing vitamin E-TPGS. In addition, for the *in vivo* studies nanocrystals of 1-MT were co-administered with paclitaxel encapsulated in poly(anhydride) nanoparticles, a well known cytotoxic agent broadly used in clinics. Orally administered paclitaxel encapsulated in poly(anhydride) nanoparticles and subcutaneous nanocrystals of 1-MT were able to reduce tumor growth in C57BL/6J female mice. Taken together, our results would facilitate cancer treatment by combining two different strategies: oral chemotherapy and immunotherapy to enhance antitumor immunity.

Introduction

Indoleamine-2,3-dioxygenase (IDO) is a tryptophan catabolizing enzyme with a crucial activity: it plays an important role in immune control [1]. Thus, cells expressing IDO are capable of suppressing local T cell responses promoting immune tolerance under various physiological and pathological conditions, i.e. infectious diseases, fetal rejection, organ transplantation, inflammation, auto-immune disorders and cancer. Recently, IDO has been related to tumor immune-resistance since it appears to be chronically activated in patients with cancer [2-4]. Moreover, IDO is over-expressed in a wide variety of tumor types as well as by the dendritic cells (DC) that are located in the tumor-draining lymph nodes [3, 5]. In all, IDO represents an important immune regulatory enzyme *in vivo* and it is considered of medical relevance. As such, it represents an ideal target for therapeutic intervention. Based on the mechanisms of IDO-induced tumor tolerance, a pharmacologic blockade of the IDO enzyme could be clinically useful in reversing tumor immune-resistance and in enhancing chemotherapy treatments. So, a new challenge in immunotherapy is to develop strategies that can effectively overcome tumor-induced immunosuppression and evasion, while safely augmenting antitumor responses [6].

In this context, recent developments have selected 1-methyl-tryptophan (1-MT) as an interesting candidate for immunotherapy and for its combination with classical chemotherapy. 1-MT is a methylated tryptophan with anti-immunosuppressive activity. It inhibits the indoleamine-2,3-dioxygenase enzyme which may increase or maintain tryptophan levels important for T cell function. Tryptophan depletion is correlated with immune-suppression involving T cell arrest. The problem associated with this molecule is its low water solubility which limits its administration. Nevertheless, 1-MT is currently used in clinical trials combined with either vaccines or chemotherapy in several kinds of solid tumors such as refractory metastatic prostate cancer or invasive metastatic breast cancer. Recently published works have already assayed the combination of the two mentioned strategies, immunotherapy and chemotherapy. In their work, Ou et al. [7] combined the use of a dendritic cell/Lewis lung carcinoma fusion vaccine and the IDO inhibitor 1-MT. Other authors, such as Hou et al. [8], evaluated the capacity of the 2 stereoisomers of 1-MT to inhibit IDO in dendritic cells in different tumor models in mice.

Paclitaxel (PTX) is an anticancer agent widely used against different types of tumors such as metastatic breast and refractory ovarian cancers or non-small cell lung cancer. It is classified as class IV by the Biopharmaceutical Classification System (BCS) implying it has low water solubility and a very low permeability through biological membranes. Currently, in clinic, PTX is administered by means of the intravenous (i.v.) route and the vehicles that the commercialized formulations

contain are a mixture (1:1, v/v) of ethanol and Cremophor EL®. The latter is a polyethoxylated castor oil which has been described as the responsible agent of the severe hypersensitivity reactions that may occur to patients when receiving treatment [9]. In these cases, patients have to be pre-medicated with antihistamines, H_1 and H_2, and corticosteroids to prevent severe side effects [9]. In addition, paclitaxel presents a very low oral bioavailability (<3%) [10] which it is mainly due to the effect of P-glycoprotein (Pgp), an efflux pump expressed mostly in the intestinal epithelium, and the cythocrome P450, hampering the oral administration of the anticancer agent. Recently, some works have demonstrated that the encapsulation of paclitaxel in poly(anhydride) nanoparticles increases its oral bioavailability up to 70% in rat [11, 12]. In these works, cyclodextrins and poly(ethylene glycol) were used as excipients to both increase the drug loading in the resulting nanoparticles and to disturb the activity of intestinal Pgp and the drug metabolizing enzyme *cytochrome P450* [13, 14]. Many other works are being developed in order to attempt the oral administration of PTX. Examples include its co-administration with Pgp and/or CYP3A4 inhibitors (i.e. verapamil or cyclosporin A) [15] or its encapsulation in drug delivery systems (micelles, lipid nanoparticles) [16, 17].

Following the mentioned previous works and the increasing interest in complementing chemotherapy with certain immunotherapy strategies, we assayed *in vivo* the efficacy of combining a selective IDO inhibitor, 1-MT, with an oral formulation of paclitaxel based on poly(anhydride) nanoparticles. So, in the present study, we sought to evaluate the anti-tumor efficacy of nanocrystals of 1-methyl-tryptophan in a Lewis lung carcinoma tumor (3LL) model in C57BL/6J female mice, as a single agent (orally or subcutaneously administered) or in combination with paclitaxel-β-cyclodextrin complexes encapsulated in poly(anhydride) nanoparticles administered by means of the oral route.

Materials and methods

1. Materials

Paclitaxel (USP XXVI, grade>99.5%) was purchased from 21CECpharm (London, UK). Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) (PMV/MA) [Gantrez® AN 119; MW 200,000] was purchased from ISP (Barcelona, Spain). Taxol® was provided by Bristol-Myers-Squibb (NY, USA). β-cyclodextrin, phosphate buffered saline (PBS) and 1-methyl-tryptophan were obtained from Sigma Aldrich (Germany). Acetone, ethanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Reagents used for fluorimetric assays were o-phtalaldehyde from Merck (Darmstad, Germany), 2-mercaptoethanol from Sigma (St. Louis, MO, USA) and boric acid and

diethyl ether from Panreac Química S.A. (Barcelona, Spain). All other reagents and chemicals used were of analytical grade.

Lewis Lung Carcinoma (3LL) cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). Cells were cultured in RPMI 1640 medium (GibCo, Life Technologies, UK) supplemented with L-glutamine, 10% fetal calf serum, streptomycin and penicillin (10%) (Invitrogen, Carlsbad, California, USA) and passaged by trypsinization.

2. Preparation of microcrystals of 1-Methyl-tryptophan

Microcrystals were prepared by a single emulsion technique previously described. Briefly, 200 mg of 1-methyl-tryptophan were dispersed in 2 ml dichloromethane and on the other hand, a solution of polyvynil alcohol (PVA) 2% (w/v) was prepared. The solution containing 1-MT was added to the PVA solution and the emulsion was obtained under sonication for 30 seconds. Once obtained, the organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland). Finally, the obtained formulation was purified by centrifugation at 21,000 x*g* for 20 minutes at 4°C and lyophilized (Genesis 12EL, Virtis, USA).

3. Preparation of nanocrystals of 1-Methyl-tryptophan

Nanocrystals of 1-methyl-tryptophan were prepared by nanoprecipitation method previously described. For this purpose, 30 mg of 1-methyl-tryptophan were dissolved in 1 ml of ethanol by adding 20 µl of HCl 37%. The solution was stirred in an ultrasound bath for 5 minutes until the complete dissolution of 1-MT (solution A). On the other hand, 100 mg of either Vitamin E-TPGS or Pluronic F68 (as surfactant) were dissolved in 5 ml PBS (solution B) at room temperature. Once dissolved, solution A containing 1-MT was added onto B, containing the surfactant, with the help of a syringe. Then, the mixture was stirred for 15 minutes and the organic solvents were eliminated under reduced pressure at a temperature below 40°C. Finally, the formation of the nanocrystals was achieved by the addition of NaOH (1 N) to a pH of 7. The obtained nanocrystals were lyophilized.

4. Preparation of PTX-β-cyclodextrin complex (PTX-CD)

The preparation of inclusion complexes of paclitaxel and β-cyclodextrin (PTX-CD) was performed, by the evaporation method, in the molar ratio 1:1 as described previously in literature [11]. On one hand, 10 mg paclitaxel were dissolved in 2 ml ethanol. This solution was then added to 8 ml water, containing the oligosaccharide. After agitation of the mixture for 72 h, ethanol was

evaporated under reduced pressure and the resulting suspensions filtered through a 0.45 µm membrane filter. Finally, the obtained clear solution was evaporated under vacuum at a temperature of 50°C in the rotary evaporator in order to obtain a solid dry residue.

5. Preparation of poly(anhydride) nanoparticles loaded with PTX-β-cyclodextrin complexes (PTX-CD-NP)

Paclitaxel-cyclodextrin inclusion complexes (PTX-CD) were encapsulated in poly(anhydride) nanoparticles by a solvent displacement method as described previously [11]. Briefly, paclitaxel (8.8 mg as inclusion complex with β-cyclodextrin) was dispersed in 5 ml of acetone containing 100 mg poly(anhydride) previously dissolved. The mixture was magnetically stirred for 30 min at room temperature. Then, nanoparticles were formed by the addition of an ethanol/water mixture (1:1, v/v). After elimination of the organic solvents, the resulting suspensions were purified by centrifugation 27,000x*g* for 20 min. The supernatants were removed and the pellets resuspended in water. Finally, the formulations were frozen and freeze-dried using sucrose (5% w/w) as cryoprotector.

6. Characterization of the formulations

6.1. Physicochemical characterization

The mean hydrodynamic diameter and the zeta potential of the crystals and nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments Ltd., Worcestershire, UK). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1:10) and measured at 25°C by dynamic light scattering angle of 90°C. The zeta potential was determined as follows: 200 µL of the samples were diluted in 2 ml of a 0.1 mM KCl solution.

6.2. X-ray diffractometry (XRD) of 1-Methyl-tryptophan samples

Powder X-ray diffraction patterns were collected on a X Bruker axs D8 Advance diffractometer (Karlsruhe, Germany) using a Ni filter, CuK_{α} radiation , a voltage of 40kV and a current of 20 mA. The scanning rate was 1º/min, the time constant 3s/step over a 2θ interval of 2-40°.

6.3. Differential thermal analysis (DTA) of 1-Methyl-tryptophan samples

Thermal analysis was performed on a simultaneous TGA/sDTA 851 Mettler Toledo thermoanalyzer. The thermal behaviour was studied by heating about 10-20 mg of sample in a pierced aluminium crucible with a scan rate of 5°C/min from 25 to 300°C in static air atmosphere and N_2 (20 mL min^{-1}) as purge gas.

6.4. Paclitaxel content in poly(anhydride) nanoparticles

The amount of paclitaxel loaded into nanoparticles was quantified by HPLC [11]. Briefly, the equipment was an Agilent model 1100 series LC and a diode-array detector set at 228 nm. The chromatographic system was equipped with a reversed-phase 150 mm x3 mm C18 Phenomenex Gemini column (particle size 5 μm) and a precolumn filled with the same material. The mobile phase, pumped at 0.5 ml/min, was a mixture of phosphate buffer (0.01 M, pH 2) and acetonitrile (50:50, v/v). The column was placed at 30°C and the injection volume was 100 μL. Docetaxel (DCX) was used as internal standard. Calibration curves were designed over the range of 80–7000 ng/ml (r^2>0.999). The limit of quantification was calculated to be 80 ng/ml. Nanoparticles were solubilized with acetonitrile (1:5, v/v). Samples were assayed in triplicate and results were expressed as the amount of PTX in μg per mg nanoparticles.

7. *In vitro* release studies of 1-Methyl-tryptophan

Release experiments were conducted under sink conditions in 1 ml of phosphate buffered saline pH 7.4. Samples were incubated at 37°C. The study was performed under orbital shaking in a rotatory plate (FALC F200, Falc intruments, Treviglio, Italy) for 1 week. For each time point, 333 μg of 1-MT formulated in nanocrystals were dispersed in 1 ml of PBS medium. At appropriate intervals, sample tubes were collected and centrifuged at 27,000 x*g* for 20 minutes. Supernatants were collected and frozen until analysis.

7.1. 1-Methyl-tryptophan quantification

The amount of 1-MT in the supernatants was determined by fluorimetry after the derivation of the amino acid with o-phthalaldehyde. To quantify 1-MT, supernatants were diluted in 0.4 M boric acid pH 9.7 and 50 μl were placed in a 96-well microplate (TPP, Trasadingen, Switzerland). Then, 50 μl of fluorimetric assay solution (0.04% (w/v) o-phthalaldehyde, 0.1% (v/v) diethyl ether, 0.2% (v/v) β-mercaptoethanol in boric acid) were added to the samples and fluorescence was measured in a Tecan GENios fluorimeter (Tecan Group Ltd, Maennedorf, Switzerland) (excitation wavelength 340 nm, emission wavelength 450 nm). 1-MT content was determined by extrapolating

the fluorescence values from a calibration curve prepared with standard 1-MT solutions in boric acid 0.4 M pH 9.7.

8. Antitumor efficacy studies

The experimental protocol (number: 129-11) involving the use of animals was approved by the Ethical Committee for Animal Experimentation at the University of Navarra in agreement with European guidelines on animal experiments (86/609/EU).

8.1. Animal model

C57BL/6J female mice, 4-6-week old (20-22 g) were purchased from Harlan (Barcelona, Spain) and were housed under standard animal facilities with 8 animals per cage and given free access to food and drinking water. Housing conditions were maintained by controlled temperature and humidity and with 12-hour on-off light cycles. Animals were allowed to acclimate for 1 week before any experiments.

In vivo antitumor efficacy was evaluated in a tumor model set up by inoculation of Lewis Lung Carcinoma cell line (3LL) in mice. Before the implantation in animals, 3LL cells were maintained at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Before inoculating the cells, a mycoplasma assay was performed to ensure the absence of contaminants in the cell culture samples.

On the day of the experiment, 3LL cells (1×10^5) were mixed with Growth Factor Reduced Matrigel® Matrix (BD Biosciences, California, USA) (1:1, v/v), and injected subcutaneously on the right back flank of mice under light anesthesia.

8.2. Antitumor study

Once the tumors were established subcutaneously and began to grow, animals were distributed into treatment groups. The groups were the following: (i) PTX injection (commercial Taxol® administered intravenously); (ii) oral (p.o.) nanocrystals of 1-MT formulated with vitamin E-TPGS; (iii) subcutaneous (s.c.) nanocrystals of 1-MT with vitamin E-TPGS; (iv) oral nanoparticle treatment group (PTX-CD-NP) and two groups that received the combination of nanocrystals of 1-methyl-tryptophan and nanoparticles: (v) oral 1-MT plus PTX-CD-NP and (vi) s.c. 1-MT plus oral PTX-CD-NP. Besides, a tumor growth control group was also included. Each group consisted of 8 tumor-bearing animals.

Treatments were started on day 10 after inoculation when tumor volumes were palpable and reached sizes of approximately 100 mm^3. However, one day prior to the initiation of the selected treatments, a first administration of 1-methyl-tryptophan (either orally or subcutaneously at a dose of 100 mg/kg) was carried out for the animals in the groups that included 1-MT as treatment.

For a more convenient administration, Taxol® was diluted with sterile normal saline (0.9% w/v). The selected dose for the commercial formulation was 10 mg/kg (around 0.2 g of anticancer drug) and administered on a daily regimen via the tail vein. The lyophilized nanoparticle formulation was resuspended in water each day prior to the oral administration. An equivalent dose to 25 mg/kg of paclitaxel formulated in poly(anhydride) nanoparticles was administered to the animals orally every day. However, different administration schedules were followed for the nanocrystals although in both routes the selected dose was the same, 100 mg/kg. Thus, the oral treatment was administered every day by dispersion of the nanocrystals in water while animals received the subcutaneous treatment every two days resuspended in PBS, injected close to the location of the tumor.

Throughout the study, tumors were measured with a caliper every two days. Tumor volumes were calculated according to the following formula:

$$Tumor\ Volume\ (mm^3) = \frac{L\ x\ W^2}{2}\ [eq.1]$$

In which *L* corresponded to the largest diameter and *W* to the shortest diameter of the tumor, perpendicular to length.

As established in the approved animal protocol, when tumor volumes reached 2000 mm^3 approximately, animals were sacrificed considering the volume of the tumor excessive and life-threatening.

In addition, tumor growth delay (TGD) was determined as pharmacodynamic parameter [18]. Tumor growth delay was calculated as the time, in days, required for tumors to reach a mean volume of 500 mm^3.

8.3. Measurement of vascular endothelial growth factor (VEGF)

In addition, blood samples were obtained throughout the study in order to evaluate the vascular endothelial growth factor (VEGF). For this purpose, a commercial kit (Mouse VEGF Immunoassay Quantikine® ELISA kit, R&D Systems, Minneapolis, Minnesota, USA) was used. Initially, blood samples were obtained at the beginning of the study setting these as basal levels of

VEGF in plasma. Throughout the experiment, blood was extracted every 2 days to 3 animals in each group randomly. For this purpose, 300 µl of blood were extracted from the mice in the different treatment groups and plasma was recovered by centrifugation at 2500 x*g* for 10 min and frozen at -80°C until analysis. The samples were processed as specified in the commercial kit and the plate was read at 450 and 540 nm using a microtite plate reader (iEMS Reader MF, Labsystems, Helsinki, Finland). A standard curve constructed with mouse VEGF was carried out to extrapolate the experimental data.

9. Statistical analysis

Data are expressed as the mean ± S.D. of at least three experiments. One-way ANOVA with Bonferroni post-test or Mann-Whitney U tests were used to investigate statistical differences. In all cases, $p<0.05$ was considered to be statistically significant. All data processing was performed using GraphPad Prism 4.0 statistical software (GraphPad Software, USA).

Results

1. Preparation and characterization of nanoparticles

Poly(anhydride) nanoparticles were successfully prepared by the solvent displacement method. The main physicochemical characteristics of the poly(anhydride) nanoparticle formulation loaded with PTX (PTX-CD-NP) are summarized in **table 1**. Nanoparticles displayed sizes around 265 nm. In addition, the surface charge of the poly(anhydride) nanoparticles was around -50 mV. Regarding the amount of paclitaxel encapsulated in the nanoparticles, it was estimated to be around 50 µg/mg NP. Finally, the yield of the process was calculated to be around 75%.

Table 1. Physicochemical characterization of poly(anhydride) nanoparticles loaded with paclitaxel-cyclodextrin complexes (PTX-CD-NP). Data are expressed as mean ± S.D. (n=4).

Formulation	Size (nm)	Zeta Potential (mV)	Yield (%)	Paclitaxel Loading (µg PTX/mg NP)
PTX-CD-NP	263 ± 7	-48 ± 6	71 ± 4	53 ± 2.5

2. Preparation and characterization of 1-MT crystals

On the other hand, the results obtained for the formulations containing 1-methyl-tryptophan are shown in **table 2**. For the microcrystal formulation, the size was around 15-20 µm. The nanocrystals displayed different sizes depending on the surfactant used. In this way, the

formulation containing Pluronic F68 presented bigger sizes (5 times bigger approximately) than the nanocrystals obtained with vitamin E-TPGS. In both cases, the polydispersity index was similar, around 0.3-0.4 implying a relative heterogeneity of these samples. Regarding the zeta potential of the 1-MT containing samples, the values ranged from -4 mV to -35 mV, in all cases negative values.

Table 2. Physicochemical characterization of microcrystals and nanocrystals of 1-methyl-tryptophan (1-MT). Data are expressed as mean ± S.D. (n=4).

Formulation	Size	PDI	Zeta Potential (mV)
Microcrystals	14 ± 3 μm	0.2 ± 0.1	-30 ± 2
Nanocrystals Pluronic F68	150 ± 27 nm	0.4 ± 0.2	-4 ± 2
Nanocrystals Vit E-TPGS	24 ± 21 nm	0.4 ± 0.1	-27 ± 5

Peaks in the X-ray diffractograms indicated the presence of crystal structure while no peaks were observed in the presence of amorphous structure. X-ray diffraction analysis of the nanocrystal formulations was performed to determine the crystalline form of the active drug substance and reduction in the crystalline form of the active substance. The X-ray diffraction patterns of the nanocrystal formulations, Pluronic F68, vitamin E-TPGS and 1-MT, as well as the physical mixture of 1-MT with each surfactant are shown in **figure 1**. **Figure 1a** corresponds to the diffractograms of the Pluronic samples and **figure 1b** to the vit E-TPGS.

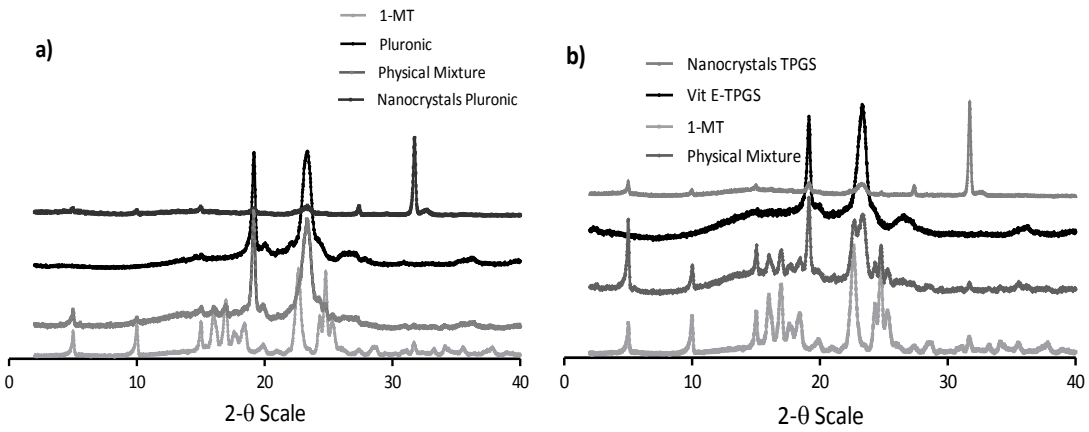

Figure 1. X-ray diffractograms of a) samples containing Pluronic F68 and b) samples containing vitamin E-TPGS.

The crystalline structure of 1-MT was clearly seen in the X-ray diffractograms due to the presence of numerous reflections with high intensity. On the other hand, the diffractograms of Pluronic F68 and vit E-TPGS showed two characteristic peaks at 19.1° and 23.2 2θ; while the physical mixtures presented the superposition of both components.

The nanocrystal formulations obtained with Pluronic F68 and vit E-TPGS displayed some reflections of 1-MT and certain belonging to the surfactants, in all cases, with rather low intensity. However, a high intensity reflection at 31.6° 2θ, corresponding to 1-MT, was observed. Thus, the presence of the surfactant favors the preparation of small crystals of 1-MT.

Differential thermal analyses were carried out to examine the thermal behavior of the components used in the nanocrystal formulations. The DTA thermograms of 1-MT showed a sharp endothermic peak corresponding to the melting point of the drug with a high crystallinity **(Figure 2)**. In contrast, Pluronic F68 and vitamin E-TPGS exhibited a glass transition at 50° and 30°C, respectively.

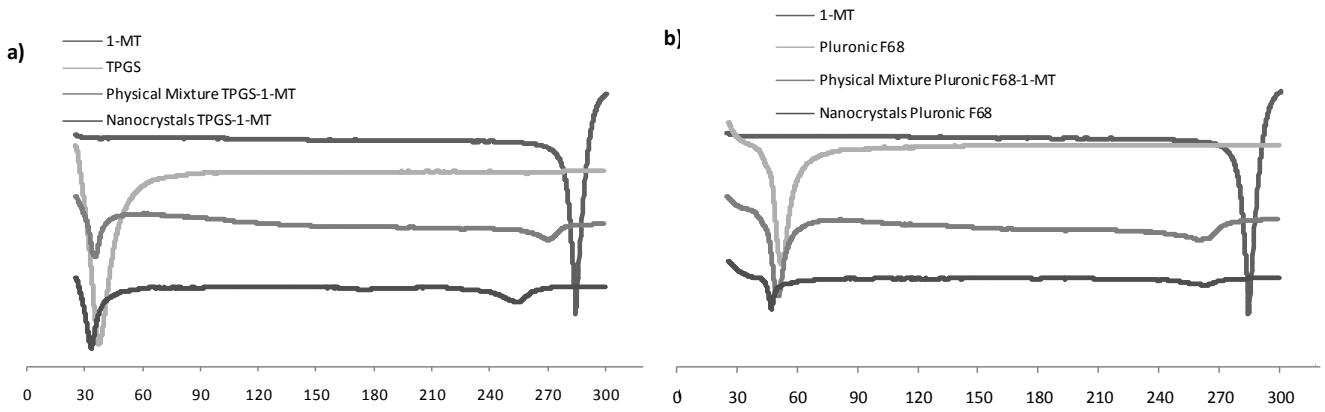

Figure 2. Differential thermal analysis of a)the nanocrystal formulation and physical mixture of the samples containing Pluronic F68 and b)the nanocrystal formulation and physical mixture of the samples containing vitamin E-TPGS, as well as 1-Methyl-tryptophan.

Additionally, in the thermograms of the nanocrystals and physical mixtures, the endothermic peaks corresponding to the surfactants and 1-MT could be detected. However, in the formulations with Pluronic F68 and vitamin E-TPGS, the drug, 1-MT, melted at a slightly lower temperature (>260°C) than in the physical mixture (270°C). These data would indicate the formation of small crystals of 1-MT in the presence of the surfactants confirming the results obtained by X-ray diffractometry.

2.1 *In vitro* drug release studies

1-MT release profile was evaluated by incubating the formulations in PBS (pH 7.4) at 37°C. **Figure 2** represents the release profiles of 1-MT from the different assayed formulations as

cumulative percentage of drug released as a function of time. From the release profiles, it can be highlighted that the release behavior of 1-methyl-tryptophan to the PBS medium displayed a rapid initial release of the drug (burst effect) observed at the shortest times followed by a more sustained second phase, reaching a plateau. However, this plateau was achieved at different release percentage for the different studied formulations.

For the microcrystals, the release of 1-methyl-tryptophan at the initial studied times (30 minutes) was around 15%, while for the nanocrystal formulations the initial burst effect was higher. In fact, for the vitamin E-TPGS containing formulation the largest amount of drug was released at 30 minutes (40% approximately). In contrast, for the Pluronic F68 containing formulation this initial release was lower (around 10%) and the release rate was more sustained if compared to the other nanocrystal formulation. It can be said that the Pluronic F68 nanocrystal formulation did not suffer from a marked burst effect compared to the other 1-MT formulations.

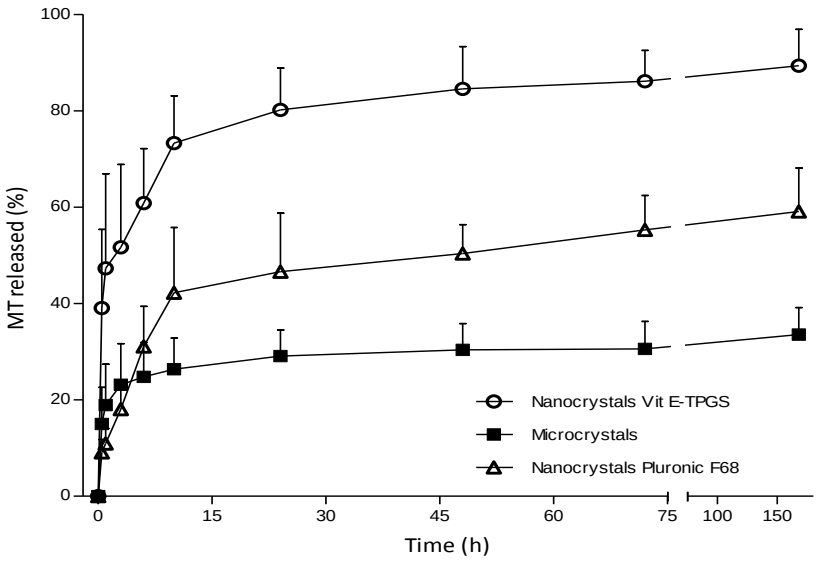

Figure 2. *In vitro* release profiles of the 1-methyl-tryptophan containing formulations after incubation in PBS medium (pH 7.4) at 37°C. Data expressed as mean ± S.D. (n=3)

On the other hand, looking at the plateau phase of the release curve, the amount of drug released from the different formulations in this phase was rather low compared to the initial phase. Herein, the plateau levels were achieved at 25%, 42% and 75-80% for the microcrystals, nanocrystals formulated with Pluronic F68 and vitamin E-TPGS, respectively. Additionally, the times to reach the plateau were around 10 hours in all cases. These release levels of drug achieved in the plateau for the 3 studied formulations were maintained until the end of the study.

3. Antitumor activity

Antitumor efficacy of 1-methyl-tryptophan alone or in combination with PTX-CD complexes encapsulated in poly(anhydride) nanoparticles was evaluated and compared to the commercial formulation, Taxol®, in a subcutaneous tumor model set up by inoculation of non-small cell lung cancer cell line in C57BL/6J female mice. After implantation of tumor cells, mice were observed daily until the tumor mass was palpable and reached a measurable volume of around 100 mm^3, approximately 10 days after inoculation, considering this day 1 of treatment.

Figure 3 represents the mean tumor volumes (in mm^3) throughout the days of treatment of the different animal groups. Once tumors were established subcutaneously, they developed for 10 days and grew to 100 mm^3 at a constant rate. At this point, the different treatment regimens started. On day 1 of treatment, all mice presented similar tumor volumes and the first dose of paclitaxel loaded in nanoparticles was administered orally. One day before the first dose of the anticancer agent, the animals in the 1-MT groups were administered a first dose of the immunoregulatory agent, either orally or subcutaneously close to the location of the tumor at a dose of 100 mg/kg in both cases.

As it can be observed, tumor volumes in all treatment groups were kept similar for the first 5 days of treatment. However, after the fifth day, differences began to be noticeable **(figure 3)**.From this point onwards; the tumor growth control group presented an exponential growth tendency reaching a final volume of 2000 mm^3 at the end of the study. On the other hand, the daily intravenous administration of the commercial formulation, Taxol®, was capable of maintaining the tumor volume around 100 mm^3 up to day 8. After, the tumor volume increased rapidly presenting on day 13 a final volume of 1100 mm^3, approximately.

Looking at the initial volumes displayed by the treatments based on 1-MT as single therapy, tumors were maintained around 100-150 mm^3 but from day 7 approximately, the growth was increased remarkably. In fact, in the terminal phase of the tumor growth, the volumes reached in these groups of single therapy were similar to that of the control group receiving the commercial Taxol®. At the endpoint, mice in the treatment groups with 1-MT as monotherapy presented tumor sizes of around 1000-1200 mm^3. In contrast, the monotherapy regimen based on the poly(anhydride) nanoparticles loaded with PTX was able to control the tumor growth in a more sustained manner but at the end of the study a relative increase in tumor volume was observed. The mean final volume reached in this group was 680 mm^3, approximately.

Finally, the groups receiving the combined regimens consisting of 1-MT and poly(anhydride) nanoparticles loaded with the anticancer agent presented a slower tumor growth rate. Interestingly, the orally administered combined therapy was capable of maintaining similar tumor sizes than the PTX-CD-NP as single treatment while the s.c. 1-MT plus PTX-CD-NP presented a more sustained volume with a very low growth rate. For PTX-CD-NP and 1-MT oral plus PTX-CD-NP, tumor volumes on day 13 (end of study) were similar, around 600 mm^3 and 500 mm^3, respectively. On the other hand, the other combined therapy consisting of s.c. 1-MT and oral PTX-CD-NP, maintained a slower growth rate throughout the whole study. On day 13, endpoint of the study, the tumor volumes presented by the animals in this group were the smallest, with a mean volume of 300 mm^3, approximately.

Comparing the tumor volumes of the groups receiving monotherapy and the combined strategies, it is interesting to point out that the trend in tumor growth observed in the animals receiving monotherapy, either 1-MT or PTX-CD-NP on their own, was characterized by a higher growth rate from day 8 onwards and tumor volumes increased between 1.5 to 3 times at the end of the study compared to the combined strategies, PTX-CD-NP plus nanocrystals of 1-MT p.o. or s.c..

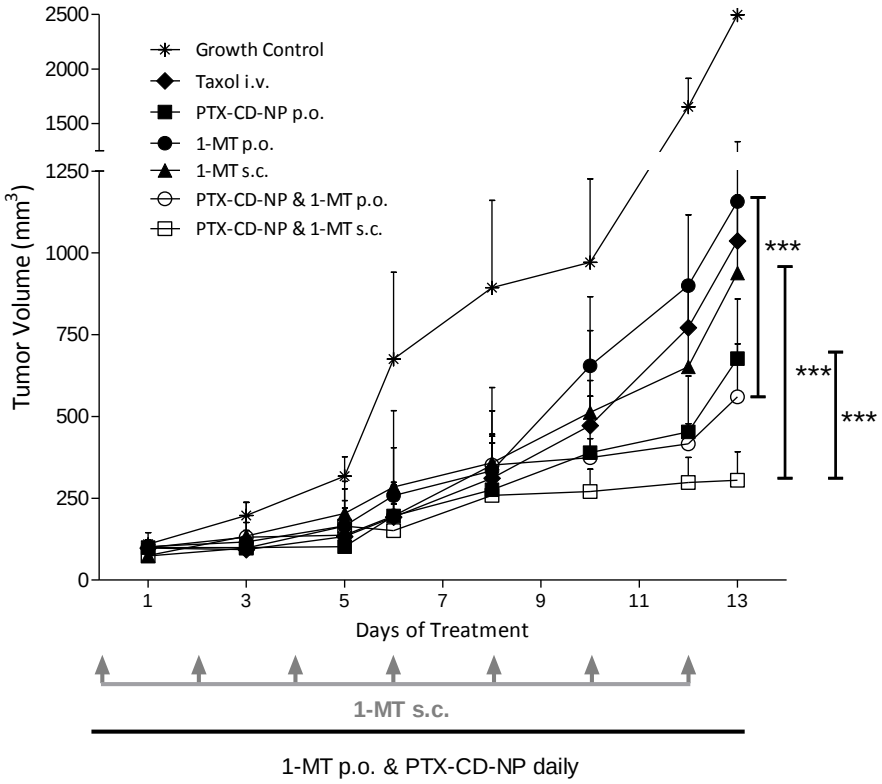

Figure 3. Comparative tumor growth inhibition by i.v. Taxol® (dose 10 mg/kg), oral PTX-CD loaded in poly(anhydride) nanoparticles (PTX-CD-NP, dose 25 mg/kg), oral (p.o.) and subcutaneously (s.c.) 1-methyl-tryptophan (1-MT) (dose 100 mg/kg) and the combined administration of 1-methyl-tryptophan (p.o. or s.c.; dose 100 mg/kg) and PTX-CD loaded in poly(anhydride) nanoparticles (p.o.) (dose 25 mg/kg) in Lewis-lung (3LL) tumor-bearing C57BL/6J female mice. Data expressed as mean ± S.D. (n=8). The criterion for statistics was to compare monotherapies of 1-MT vs. combined strategies of nanoparticles and 1-MT, monotherapies and combination therapies between themselves and finally, Taxol® *vs.* the other treatments. ***p<0.001 PTX-CD-NP *vs*. PTX-CD-NP & 1-MT s.c.; 1-MT s.c. *vs* PTX-CD-NP & 1-MT s.c.; 1-MT p.o. *vs*. PTX-CD-NP & 1-MT p.o. Additionally, statistical differences (data not shown) were obtained between Taxol® and PTX-CD-NP (p<0.01) and Taxol® *vs*. PTX-CD-NP & MT p.o. and Taxol® *vs*.PTX-CD-NP & MT s.c. (p<0.001). No statistical differences were obtained for the comparison between MT s.c. *vs*. MT p.o. as monotherapies.

From a pharmacodynamic point of view, tumor growth delay was evaluated as a parameter of efficacy for the different treatment groups, summarized in **Table 3**. As seen in **table 3**, while Taxol® and 1-MT as monotherapy (1-MT p.o. and 1-MT s.c.) managed to delay tumor growth for 10 days and PTX-CD-NP as single treatment strategy delayed tumor growth for 12 days, the combination between nanoparticles and 1-MT (s.c. or p.o.) presented the highest growth delays.

Interestingly, at the end of the study for the combined treatment based on PTX-CD-NP & 1-MT s.c., tumor volumes were lower than 500 mm^3.

Table 3. Tumor growth delay as pharmacodynamic parameter to evaluate the efficacy of the different treatments. It represents the time, in days, to reach a mean tumor volume of 500 mm^3.

Treatment Groups	Tumor growth delay (days)
Growth Control	5.5 days
i.v. Taxol®	10 days
1-MT p.o.	9 days
1-MT s.c.	10 days
PTX-CD-NP	12 days
PTX-CD-NP & 1-MT p.o.	≈13 days
PTX-CD-NP & 1-MT s.c.	>13 days

Besides, VEGF was evaluated as an angiogenesis marker in plasma since it could be related to tumor growth. The VEGF profiles on different days throughout the study for the different groups are plotted in **figure 4**. The basal levels of VEGF for the different treatment groups were found to be lower than 20 pg/ml. These were the plasmatic levels of VEGF before implantation of tumor cells in mice. As observed in **figure 4**, in general, in spite of the fact that animals were treated against the tumor, the VEGF concentration in plasma increased. At the beginning of the treatments, for the first 7 days, levels were more sustained and no significant increases were observed, except for the animals administered i.v. Taxol®. However, from day 7 to the end of the study, bigger differences were obtained amongst the treatment groups. The rise in the VEGF plasmatic levels in the different treatment groups varied from 1.5-fold to 3-fold for PTX-CD-NP and 1-MT as monotherapy, respectively. In these cases, on day 13, end of treatment, the concentrations of VEGF in plasma were significantly higher than the initial concentrations. For the combined treatment consisting of oral PTX nanoparticles and 1-MT s.c., the final concentrations of VEGF were the lowest (75 pg/ml approximately), around 2 times lower than the monotherapies based on 1-MT.

If comparing VEGF levels of the 2 combined treatments, the animals receiving the subcutaneous 1-MT presented a 1.6-fold lower plasma concentration than the ones receiving the oral 1-MT (77 pg/ml for the s.c. 1-MT and PTX-CD-NP *vs.* 126 pg/ml for the p.o. 1-MT and PTX-CD-NP). These amounts of VEGF for the s.c. 1-MT and PTX-CD-NP were maintained 1.6-fold lower compared to the single therapy consisting of PTX-CD-NP. Interestingly, looking at the VEGF plasma

concentrations of the PTX-CD-NP and the combination of the nanoparticles with oral 1-MT similar levels were found throughout the study and especially at the end.

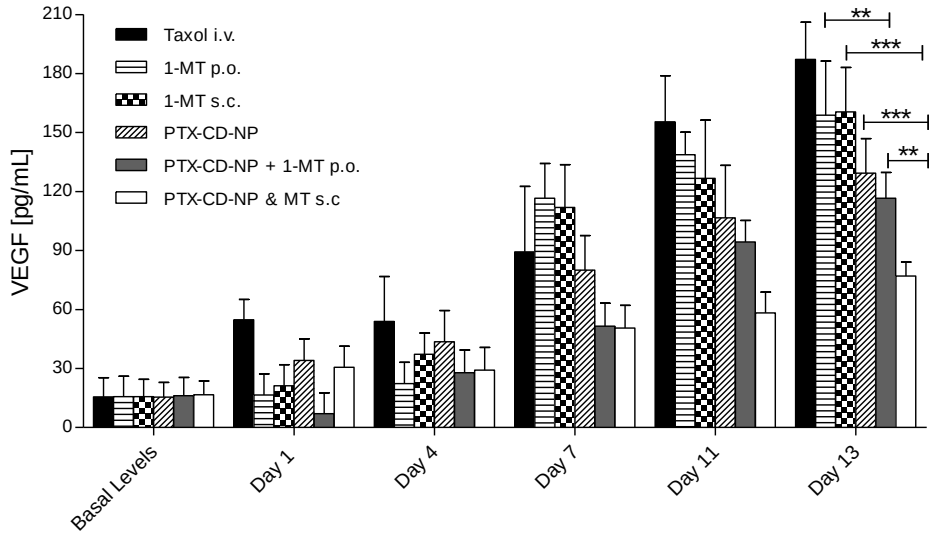

Figure 4. Vascular endothelial growth factor (VEGF) concentrations in plasma for the different treatment groups: intravenous Taxol®, 1-methyl-tryptophan formulation as single treatment (oral or subcutaneously) or as combined treatment with paclitaxel complexed with β-cyclodextrin loaded in poly(anhydride) nanoparticles. Data expressed as mean ± S.D. (n=3). The criterion for statistics was to compare monotherapies of 1-MT vs. combined strategies of nanoparticles and 1-MT, monotherapies and combination therapies between themselves and finally, Taxol® *vs.* the other treatments. ** p<0.01 1-MT p.o. vs PTX-CD-NP & 1-MT p.o.; PTX-CD-NP & 1-MT p.o. vs. PTX-CD-NP & 1-MT s.c.; ***p<0.001 PTX-CD-NP vs. PTX-CD-NP & 1-MT s.c.; 1-MT s.c. vs. PTX-CD-NP & 1-MT s.c. Besides, statistical differences (data not shown) were obtained between Taxol® and PTX-CD-NP; Taxol® vs. PTX-CD-NP & MT p.o. and Taxol® vs. PTX-CD-NP & MT s.c. (p<0.001). No statistical differences were obtained for the comparison between MT s.c. vs MT p.o. as monotherapies.

In addition, statistical differences (p<0.05) were observed. The main differences were observed from day 7 to day 13. On day 7 the main statistical differences (p<0.05) were obtained amongst the intravenous Taxol® and the groups administered 1-MT and amongst the single therapies of 1-MT and the combined therapies of PTX-CD-NP and 1-MT. These differences were maintained similar on day 11. On day 13, the plasma levels of VEGF in the Taxol® group were statistically higher than in the groups receiving the poly(anhydride) nanoparticles either as monotherapy or combined with 1-MT. Interestingly, on day 13 differences statistically significant were observed between the two combined (oral and subcutaneous) therapies of poly(anhydride) nanoparticles encapsulating paclitaxel and 1-MT.

Discussion

Immune escape is one of the main characteristics of cancer although little is known in regards to the genetics and the mechanism of this evasion. This immune escape seems to be suppressed by several mechanisms [7]. Among them, IDO has recently been identified as an impeding enzyme to a successful cancer immunotherapy. Previous works have demonstrated that IDO can be expressed by not only tumor cells themselves but by other non-malignant surrounding cells in response to the presence of the tumor [3, 19]. In addition, more recent findings have suggested the expression of IDO within tumor draining lymph nodes [7] in a murine tumor model of Lewis Lung carcinoma. Tumor cells expressing IDO become rapidly resistant to immunologic rejection. This finding was rapidly related to the capacity that tumor cells present at evading the natural immune response and subsequently, limit the efficacy of the anticancer treatment. Thus, to improve the antitumor efficacy of tumor immunotherapy, it could be helpful to add an IDO selective inhibitor to the conventional chemotherapy schedule to overcome this immune tolerance [20]. The most common IDO inhibitor under investigation is 1-methyl-tryptophan although recently other inhibitors have demonstrated certain activity, such as brassinins [21]. Since 1-MT has selectivity for IDO, especially for IDO2 isoform, it is already in clinical trials in combination with chemotherapy treatments in order to achieve a synergistic effect of both strategies to increase the effect of antitumor drugs. From a therapeutic view, drugs that inhibit IDO represent a new class of immunomodulatory agents. The initial application of these immunomodulatory drugs is likely to be as adjuvants used in combination with other anticancer therapies [20].

Herein, following this trend, nowadays under investigation which is gaining great interest, we combined the use of 1-MT as inhibitor of IDO with paclitaxel loaded in poly(anhydride) nanoparticles, administered orally, as chemotherapy treatment against a non small-cell lung cancer cell line set up in C57BL/6J female mice. The use of paclitaxel loaded in poly(anhydride) nanoparticles is an interesting approach to avoid the intravenous route since the oral route has been described as the most convenient route of administration for drugs and as the preferred route mentioned by patients receiving chronic treatment schedules [22, 23]. An important advantage of the oral chemotherapy is related to the reduction of the side effects which appear in patients undergoing treatment. In this work, for the oral administration of paclitaxel, a class IV drug by the Biopharmaceutical Classification System (BCS), poly(anhydride) nanoparticles were used as drug delivery systems. Previous works have already obtained promising results loading paclitaxel in these nanoparticles combining with cyclodextrins or poly(ethylene glycol) in order to increase the drug loading [11, 12]. The obtained nanoparticles in this work displayed sizes similar to those

reported by Agüeros previously (250 nm approximately) with a drug loading of 50 µg/mg of nanoparticle [11].

For the administration of 1-methyl-tryptophan, due to the low water solubility of the molecule, the nanocrystal strategy was selected. Microcrystals were prepared by a single emulsion technique while nanocrystals were prepared by a simple precipitation method in the presence of stabilizing agents such as Pluronic F68 and vitamin E-TPGS. The preparation of microcrystals/nanocrystals of poorly water-soluble drugs by these techniques in the presence of hydrophilic surfactants followed by lyophilization is suitable for the enhancement of drug dissolution [24]. Later, the *in vitro* drug release to a PBS medium was carried out. The release profiles obtained for the different nanocrystals formulations were able to release the drug to the medium at a higher rate than the microcrystals. When formulating the drug in nanocrystals, the surface is smaller facilitating the release of the drug. The nanocrystals containing vitamin E-TPGS managed to release a larger amount of 1-MT to the medium than the ones containing Pluronic F68. So, the nanocrystal formulation obtained with vitamin E-TPGS was selected for *in vivo* evaluation.

For the *in vivo* efficacy studies, a subcutaneous tumor was set up in C57BL/6J mice using a Lewis Lung carcinoma cell line. Once tumors were palpable, treatment regimens began. The intravenous commercial formulation of paclitaxel, Taxol®, maintained constant tumor volumes for the initial days but later, volume increased in an exponential trend. Similarly, 1-MT as single therapeutic agent was not able to control tumor growth by itself, displaying a comparable trend than Taxol®. Interestingly, 1-MT administered subcutaneously either alone or in combination was capable of displaying tumor volumes lower than the oral 1-MT. An explanation for this could be related to the route of administration. Oral administration tends to potentiate the immune system as a whole while the s.c. administration is more located and would be more specific at the site of the desired action. In this context, recent *in vivo* studies showed that blocking IDO with 1-MT can limit tumor growth in rodents [3, 25, 26]. However, because 1-MT on its own only retarded growth and did not prevent progression, concerns were raised about its use as a monotherapeutic drug. In all, the combined strategies based on 1-MT and PTX-CD-NP, either p.o. or s.c., reduced tumor growth and at the end of the study mice presented the smallest tumors in all treatment groups but better efficacy profile was obtained for the combination based on PTX-CD-NP and s.c. 1-MT.

Previously published works have already attempted the use of an IDO inhibitor, such as 1-MT, to potentiate cancer chemotherapy. However, these works which used several anticancer agents combined 1-MT with the traditional intravenous chemotherapy used in clinics. Muller et al. studied the inhibitory effect of 1-methyl-tryptophan on IDO enzyme and the expression of the *Bin1,*

the gene that controls the expression of the *Indo* gene which encodes the IDO enzyme in cancer suppression [3, 25]. This work combined the IDO inhibition with commonly used chemotherapeutic agents such as cisplatin, 5-fluorouracil, or paclitaxel. In their studies, the 1-MT was administered in pellets implanted subcutaneously with a time-release speed of 20 mg/day and the paclitaxel was administered by means of the intravenous route at a dose of 13.3 mg/kg 3 times per week for a period of 2 weeks. They evaluated the effect of the use of the IDO inhibitor on a transgenic mouse model of breast cancer and reported that 1-MT on its own retarded but did not arrest outgrowth of tumors. However, when combined with cytotoxic agents, tumor regressions were observed even under conditions where single agents were ineffective. They found that IDO inhibitors potentiated the efficacy of certain cytotoxic drugs without increasing their side effects, although the mechanism by which the cooperation is established is not yet clear [3, 25]. Additionally, Hou and coworkers worked on the inhibition of IDO by the stereoisomers of 1-MT and correlating this inhibitory effect with antitumor therapies [8]. For this purpose, they administered 1-methyl-tryptophan subcutaneously in pellets or orally in different tumor models (i.e. melanoma, breast carcinoma) and evaluated the efficacy of the 2 isomers in inhibiting IDO and promoting the anticancer therapy (chemotherapy or radiation). In all these mentioned works, the combination of 1-MT is mainly based on the already existing traditional chemotherapy, this is, mainly intravenous treatments. In fact, activating the immune system for therapeutic benefit in cancer has long been a goal in immunology and oncology [27].

These studies propose IDO as an attractive target for the development of small-molecule immunomodulatory drugs to safely modulate the efficacy of standard chemotherapeutic agents [25]. The increasing works related to the combination of different immune strategies and cancer therapies (both chemotherapy and radiotherapies) have established a significant breakthrough in cancer treatment and several of these strategies are already under clinical trials in humans. In fact, regimens combining chemotherapy and immunotherapy are gaining interest in clinics [28] since they are easily applicable and from a mechanistic point of view, chemotherapy causes cell death which releases tumor antigens [28].

In our study, 1-MT enhanced reduction of tumor growth since 1-MT could inhibit IDO-mediated immune suppressive microenvironment in tumor tissue and in combination with chemotherapy, the effect turned out to be significantly higher. So, it can be said that the combination of 1-methyl-tryptophan and paclitaxel loaded in poly(anhydride) nanoparticles had a synergistic effect on delaying tumor growth. In addition, the use of the oral route seems rather attractive for the treatment of chronic-like diseases, especially cancer. In summary, the combination of poly(anhydride) nanoparticles for the oral delivery of paclitaxel and the

nanocrystals of 1-methyl-tryptophan as inhibitor enzyme of IDO appeared to be an adequate strategy to promote the efficacy of the chemotherapeutic treatment.

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References

[1] N.J.C. King, S.R. Thomas, Molecules in focus: Indoleamine 2,3-dioxygenase, International Journal of Biochemistry & Cell Biology, 39 (2007) 2167-2172.
[2] A. Huang, D. Fuchs, B. Widner, C. Glover, D.C. Henderson, T.G. Allen-Mersh, Serum tryptophan decrease correlates with immune activation and impaired quality of life in colorectal cancer, British Journal of Cancer, 86 (2002) 1691-1696.
[3] C. Uyttenhove, L. Pilotte, I. Theate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, B.J. Van den Eynde, Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase, Nature Medicine, 9 (2003) 1269-1274.
[4] G. Weinlich, C. Murr, L. Richardsen, C. Winkler, D. Fuchs, Decreased serum tryptophan concentration predicts poor prognosis in malignant melanoma patients, Dermatology, 214 (2007) 8-14.
[5] D.H. Munn, E. Shafizadeh, J.T. Attwood, I. Bondarev, A. Pashine, A.L. Mellor, Inhibition of T cell proliferation by macrophage tryptophan catabolism, Journal of Experimental Medicine, 189 (1999) 1363-1372.
[6] L. Jia, K. Schweikart, J. Tomaszewski, J.G. Page, P.E. Noker, S.A. Buhrow, J.M. Reid, M.M. Ames, D.H. Munn, Toxicology and pharmacokinetics of 1-methyl-D-tryptophan: Absence of toxicity due to saturating absorption, Food and Chemical Toxicology, 46 (2008) 203-211.
[7] X. Ou, S. Cai, P. Liu, J. Zeng, Y. He, X. Wu, J. Du, Enhancement of dendritic cell-tumor fusion vaccine potency by indoleamine-pyrrole 2,3-dioxygenase inhibitor, 1-MT, Journal of Cancer Research and Clinical Oncology, 134 (2008) 525-533.
[8] D.-Y. Hou, A.J. Muller, M.D. Sharma, J. DuHadaway, T. Banerjee, M. Johnson, A.L. Mellor, G.C. Prendergasts, D.H. Munn, Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses, Cancer Research, 67 (2007) 792-801.
[9] H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom, Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation, European Journal of Cancer, 37 (2001) 1590-1598.
[10] A. Sparreboom, J. vanAsperen, U. Mayer, A.H. Schinkel, J.W. Smit, D.K.F. Meijer, P. Borst, W.J. Nooijen, J.H. Beijnen, O. vanTellingen, Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine, Proceedings of the National Academy of Sciences of the United States of America, 94 (1997) 2031-2035.
[11] M. Agueros, V. Zabaleta, S. Espuelas, M.A. Campanero, J.M. Irache, Increased oral bioavailability of paclitaxel by its encapsulation through complex formation with cyclodextrins in poly(anhydride) nanoparticles, Journal of Controlled Release, 145 (2010) 2-8.
[12] V. Zabaleta, G. Ponchel, H. Salman, M. Agueros, C. Vauthier, J.M. Irache, Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: Permeability and pharmacokinetic study, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 81 (2012) 514-523.

[13] F. Fenyvesi, E. Fenyvesi, L. Szente, K. Goda, Z. Bacso, I. Bacskay, J. Varadi, T. Kiss, E. Molnar, T. Janaky, G. Szabo, Jr., M. Vecsernyes, P-glycoprotein inhibition by membrane cholesterol modulation, European Journal of Pharmaceutical Sciences, 34 (2008) 236-242.
[14] S.W. Wang, J. Monagle, C. McNulty, D. Putnam, H.M. Chen, Determination of P-glycoprotein inhibition by excipients and their combinations using an integrated high-throughput process, Journal of Pharmaceutical Sciences, 93 (2004) 2755-2767.
[15] M.M. Malingre, W.W.T. Huinink, K. Duchin, J.H.M. Schellens, J.H. Beijnen, Pharmacokinetics of oral cyclosporin A when co-administered to enhance the oral absorption of paclitaxel, Anti-Cancer Drugs, 12 (2001) 591-593.
[16] S. Peltier, J.M. Oger, F. Lagarce, W. Couet, J.P. Benoit, Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules, Pharmaceutical Research, 23 (2006) 1243-1250.
[17] K. Yoncheva, P. Calleja, M. Agueros, P. Petrov, I. Miladinova, C. Tsvetanov, J.M. Irache, Stabilized micelles as delivery vehicles for paclitaxel, International journal of pharmaceutics, 436 (2012) 258-264.
[18] H. Devalapally, Z. Duan, M.V. Seiden, M.M. Amiji, Modulation of drug resistance in ovarian adenocarcinoma by enhancing intracellular ceramide using tamoxifen-loaded biodegradable polymeric nanoparticles, Clinical Cancer Research, 14 (2008) 3193-3203.
[19] M. Friberg, R. Jennings, M. Alsarraj, S. Dessureault, A. Cantor, M. Extermann, A.L. Mellor, D.H. Munn, S.J. Antonia, Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection, International Journal of Cancer, 101 (2002) 151-155.
[20] D.H. Munn, A.L. Mellor, IDO and tolerance to tumors, Trends in Molecular Medicine, 10 (2004) 15-18.
[21] T. Banerjee, J.B. DuHadaway, P. Gaspari, E. Sutanto-Ward, D.H. Munn, A.L. Mellor, W.P. Malachowski, G.C. Prendergast, A.J. Muller, A key in vivo antitumor mechanism of action of natural product-based brassinins is inhibition of indoleamine 2,3-dioxygenase, Oncogene, 27 (2008) 2851-2857.
[22] T.R. Halfdanarson, A. Jatoi, Oral Cancer Chemotherapy: The Critical Interplay Between Patient Education and Patient Safety, Current Oncology Reports, 12 (2010) 247-252.
[23] V.J. O'Neill, C.J. Twelves, Oral cancer treatment: developments in chemotherapy and beyond, British Journal of Cancer, 87 (2002) 933-937.
[24] N. Rasenack, H. Hartenhauer, B.W. Muller, Microcrystals for dissolution rate enhancement of poorly water-soluble drugs, International Journal of Pharmaceutics, 254 (2003) 137-145.
[25] A.J. Muller, J.B. DuHadaway, P.S. Donover, E. Sutanto-Ward, G.C. Prendergast, Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy, Nature Medicine, 11 (2005) 312-319.
[26] B.K. Choi, T. Asai, D.S. Vinay, Y.H. Kim, B.S. Kwon, 4-1BB-mediated amelioration of experimental autoimmune uveoretinitis is caused by indoleamine 2,3-dioxygenase-dependent mechanisms, Cytokine, 34 (2006) 233-242.
[27] I. Mellman, G. Coukos, G. Dranoff, Cancer immunotherapy comes of age, Nature, 480 (2011) 480-489.
[28] R.A. Lake, B.W.S. Robinson, Opinion - Immunotherapy and chemotherapy - a practical partnership, Nature Reviews Cancer, 5 (2005) 397-405.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

Cancer is one of the most important diseases in the world with a high incidence, prevalence and mortality. In the year 2010, in Europe almost 2,500,000 new cases of cancer were diagnosed with a mortality of around 50% [1]. Future predictions in oncology are not encouraging, with an estimated 3.2 million new cases and 1.7 million of deaths yearly worldwide. However, thanks to research in oncology, new findings each day are making it possible to know a little more about mechanisms, treatments and prevention. In this regards, we now know enough to prevent at least 30% of all cancers and how to treat many others.

Cancer treatment is based on surgical intervention, radiotherapy, immunotherapy and chemotherapy. The required treatment depends mainly on the tumor type and the stage of the disease as well as the patient's physical condition. Focusing on chemotherapy, the traditional standard regimen has been by means of the intravenous route. Nevertheless, currently, there is growing consensus that the oral route is the most convenient route of administration for drugs and it could be desirable for chronic-like treatments, such as cancer [2].

- **Limitations to the oral administration of anticancer drugs**

Many anticancer agents, including paclitaxel, are classified as class IV by the Biopharmaceutical Classification System, implying these drugs present very low water solubility and low permeability. In addition, many anticancer drugs are substrates of the P-glycoprotein and cytochrome P450, both highly expressed in the gastrointestinal tract. As a consequence, the oral administration of these drugs is rather hampered.

- **Why choose the oral route for chemotherapy?**

Oral chemotherapy consisting of drug delivery systems could be advantageous for many cancer patients who have to suffer the inconvenience of hospital life with continuous clinic visits and intravenous injections with the increasing risk of associated infections. It would facilitate treatments since the patients could be at home with no need of special equipment and no specialized staff. In addition, the patient by receiving an oral treatment feels greater autonomy since he is more responsible for the treatment too [3].

Therefore, many different attempts have been going on for the past years to develop new dosing regimens, new drugs or improved delivery systems (prodrugs, polymer-drug conjugates, self-microemulsifying drug delivery systems (SMEDDS), micelles or nanoparticles) with enhanced oral properties. In this context, it is where our works could be placed. Polymeric nanoparticles have

demonstrated bioadhesive properties within the intestinal mucosa [4] and ability to control the release of the loaded drug, appearing as potential benefits for the oral administration of paclitaxel.

- **What results did we obtain?**

The first step, once the nanoparticles were formulated **(Chapter 3)**, was the evaluation of the intestinal permeability *ex vivo* in rat. Thus, the encapsulation of paclitaxel in poly(anhydride) nanoparticles increased the intestinal permeability of the drug; a rise in the apparent permeability and efflux ratio was observed. Interestingly, the pegylated nanoparticles (PTX-PEG-NP and PTX-CD-PEG-NP) presented the highest values of P_{app} and efflux ratios while the formulation containing PTX-CD complexes alone had lower values.

In addition, *in vitro* release studies **(Chapter 3)** were performed in simulated gastric and intestinal fluids since the formulations are intended for the oral route. It is noteworthy that under the acidic conditions of the gastric fluid no drug was released for any of the evaluated formulation. Interestingly, the release profile in the intestinal fluid for PTX-CD-NP followed a biphasic pattern.

Once the *in vitro* studies were completed, we proposed the *in vivo* studies in C57BL/6J female mice. For the pharmacokinetic studies (doses 10 and 25 mg/kg body weight), the oral bioavailability (F) of the drug encapsulated in poly(anhydride) nanoparticles increased up to 80% for the pegylated nanoparticles (PTX-PEG-NP) while the cyclodextrin containing formulations reached a F value between 60 and 70%, β-cyclodextrin and HPCD respectively. Interestingly, the plasma profile was characterized by relatively stable drug blood levels, providing more consistent therapeutic effect than frequent doses of short acting medications. **(Chapters 3 and 4)**

Then, organ distribution was studied in mice. The main organs where paclitaxel is distributed were: liver, kidney, ovaries, intestine and lung. No different distribution to organs was obtained for the oral paclitaxel encapsulated in poly(anhydride) nanoparticles compared to the intravenous commercial Taxol®. The drug appeared in the same organs with a delay in time. The delay in the apparition of the cytotoxic drug in the different tissues could be associated with the fact that the drug prior to being distributed must be released from the delivery system and subsequently, absorbed at the enterocytes' surface and then, passed to the bloodstream. **(Chapter 4)**

Finally, paclitaxel encapsulated in poly(anhydride) nanoparticles, combined with cyclodextrin and/or poly(ethylene glycol) was evaluated in a murine tumor model using a non-small cell lung cancer cell line (3LL). Mice received paclitaxel poly(anhydride) nanoparticles orally on a daily schedule for the cyclodextrin containing formulations and every 3 days for the pegylated nanoparticles. As a result, the pegylated nanoparticles were able to inhibit tumor growth and mice

displayed the lowest tumor volumes. On the other hand, the tumor growth inhibition effect of the cyclodextrin nanoparticles varied depending on the cyclodextrin. **(Chapter 4)**

- **Why combine chemotherapy with immunotherapy?**

Cancer cells are able to elude immune response and as a result, grow in an uncontrolled way. In fact, tumors can establish immunoregulatory networks that can lead to tolerance. Immunotherapy is a promising alternative treatment that can be combined with chemotherapy [5]. The immunotherapy treatment is aimed at directing the host immune response against tumor cells (without harming healthy cells). In addition, by its combination with chemotherapy regimens the effect of both mechanisms could be amplified achieving a more successful treatment. Many targets have been proposed and different combinations have already been proved in clinics (i.e. monoclonal antibodies combined with chemotherapeutic agents). In this work **(Chapter 5)**, we have focused on IDO, indoleamine-2,3-dioxygenase, which is an enzyme that is over-expressed in tumors leading to immunosuppression and no recognition of cancer antigens by the host immune system. So, to inhibit IDO, 1-methyltryptophan (1-MT) is the most common inhibitor [6].

- **What did we obtain by combining paclitaxel loaded poly(anhydride) nanoparticles with 1-MT as inhibitor of IDO?**

Antitumor assays were performed to evaluate the capacity of 1-MT as monotherapy or combined with paclitaxel-cyclodextrin poly(anhydride) nanoparticles (PTX-CD-NP) to inhibit tumor growth. The immunotherapy with 1-MT was initiated one day before the administration of the poly(anhydride) nanoparticles in order to stimulate the immune system. Two routes of administration were evaluated for 1-MT, oral and subcutaneous (s.c.) near the tumor site, whereas paclitaxel loaded in poly(anhydride) nanoparticles was administered by means of the oral route. Subcutaneous 1-MT in combination with nanoparticles was capable of maintaining the smallest tumor volumes compared to 1-MT s.c. as monotherapy and compared to the oral combination of poly(anhydride) nanoparticles and 1-MT. **(Chapter 5)**

In all, the work here proposed could set an example for the development of further oral chemotherapy as interesting strategy to treat tumors in order to facilitate drug administration for patients and clinical staff.

FUTURE PERSPECTIVES

The following possible steps could be proposed in line with the results obtained in this work.

- ✓ **Evaluation of the antitumor activity of paclitaxel encapsulated in poly(anhydride) nanoparticles against different human tumor cell lines.**

Up to now, the efficacy studies performed in mice have been done using a mouse Lewis lung carcinoma cell line (3LL). Further studies, *in vitro* and *in vivo,* could be developed using other type of cell lines of different tumors: breast, ovarian,…

- ✓ **Toxicity studies.**

Paclitaxel has been described to present toxic side effects. The main toxicological effects related to the administration of Taxol® in clinics are myelosuppression, nausea and vomiting, hair loss and peripheral neuropathy. Therefore, toxicological studies would be of interest for the paclitaxel-cyclodextrin pegylated poly(anhydride) nanoparticles in order to evaluate the presence or reduction of the side effects described for the anticancer drug.

- ✓ **Study of optimization of paclitaxel dose aiming at metronomic schedules.**

A new study could be aimed at reducing the dose of the anticancer agent, as stated in the metronomic therapies. Metronomic therapies have been proposed as alternatives to the traditional anticancer regimens where the maximum tolerated dose is administered followed by several drug-free weeks in order to let bone marrow recovery [7]. In fact, the idea of the metronomic therapies is to combine the cytotoxic effect of anticancer drugs with antiangiogenic properties.

Thus, due to the controlled and prolonged drug release profile that poly(anhydride) nanoparticles display, the attempt for metronomic scheduling could be of interest; allowing the reduction of the dose with a more frequent drug administration and assay the combination with other interesting molecules that could enhance the anticancer efficacy.

REFERENCES

[1] R. Siegel, D. Naishadham, A. Jemal, Cancer Statistics, 2012, CA-Cancer J. Clin., 62 (2012) 10-29.
[2] V.J. O'Neill, C.J. Twelves, Oral cancer treatment: developments in chemotherapy and beyond, British Journal of Cancer, 87 (2002) 933-937.
[3] S.N. Weingart, P.B. Bach, K. Eng, S.A. Johnson, T.M. Kuzel, T.S. Langbaum, R.D. Leedy, R.J. Muller, L.N. Newcomer, S. O'Brien, D. Reinke, M. Rubino, R.S. Walters, NCCN Task Force Report: Oral chemotherapy, SO - J Natl Compr Canc Netw. 2008 Mar;6 Suppl 3:S1-14., (2008).
[4] M. Agueros, P. Areses, M. Angel Campanero, H. Salman, G. Quincoces, I. Penuelas, J. Manuel Irache, Bioadhesive properties and biodistribution of cyclodextrin-poly(anhydride) nanoparticles, European Journal of Pharmaceutical Sciences, 37 (2009) 231-240.
[5] I. Mellman, G. Coukos, G. Dranoff, Cancer immunotherapy comes of age, Nature, 480 (2011) 480-489.
[6] C. Uyttenhove, L. Pilotte, I. Theate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, B.J. Van den Eynde, Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase, Nature Medicine, 9 (2003) 1269-1274.
[7] R.S. Kerbel, B.A. Kamen, The anti-angiogenic basis of metronomic chemotherapy, Nature Reviews Cancer, 4 (2004) 423-436.

CHAPTER 7

CONCLUSIONS

Conclusions

The experimental work compiled in this work has been focused on the attempt to increase the oral bioavailability of the antitumor drug paclitaxel. All the results obtained for this work have led us to conclude the following:

1. The formulation of paclitaxel in poly(anhydride) nanoparticles combined with either β-cyclodextrin and/or poly(ethylene glycol) increased the apparent permeability of the drug through rat intestinal sections in an *ex vivo* model in Ussing chambers. The absorption ratio (R) increased 10-12-fold approximately for the nanoparticle formulations compared to commercial Taxol®.

2. Oral pharmacokinetic studies of commercial Taxol® revealed that no plasma levels could be detected since no absorption of the drug occurred due to the presence of P-glycoprotein and cytochrome P450 at the intestinal surface. However, when paclitaxel was encapsulated in poly(anhydride) nanoparticles, the drug could be absorbed through the intestinal membrane and sustained plasma levels of the drug in plasma were observed at the two doses evaluated: 10 and 25 mg/kg body weight. In addition, the cyclodextrins and poly(ethylene glycol) could facilitate the absorption of the anticancer drug by inhibiting P-glycoprotein and cytochrome P450.

3. After the oral administration of poly(anhydride) nanoparticles loaded with paclitaxel-cyclodextrin complexes or combined with poly(ethylene glycol) 2000 to C57BL/6J mice, drug levels in plasma were maintained for around 25-30 hours for the 2 studied doses (10 and 25 mg/kg).

4. The encapsulation of paclitaxel in poly(anhydride) nanoparticles combined with cyclodextrins and poly(ethylene glycol) increased the oral bioavailability of the cytotoxic drug up to 80% for pegylated formulations and between 60% and 70% for cyclodextrin nanoparticles in C57BL/6J female mice.

5. The organ distribution studies carried out in mice revealed that the encapsulation of paclitaxel in poly(anhydride) nanoparticles did not alter its distribution. The main organs where paclitaxel could be found after its administration, were liver, kidneys, ovaries and lung, both for the intravenous and oral formulations. Besides, at the longest studies times, paclitaxel was mainly found in intestine since it is known that it is excreted by feces.

6. In experiments performed in a murine tumor model using Lewis lung carcinoma cell line (3LL), paclitaxel encapsulated in poly(anhydride) nanoparticles slowed down significantly the growth of the implanted tumor cells, compared to the commercial Taxol® formulation.

7. The oral administration of paclitaxel encapsulated in poly(anhydride) nanoparticles presented a slight antiangiogenic effect since the levels of vascular endothelial growth factor (VEGF) were maintained lower than in the Taxol® group. These lower levels could be related to the capacity of the orally administered cytotoxic drug to restrain tumor growth.

8. Nanocrystal formulations of 1-methyltryptophan were prepared by a simple precipitation method using Pluronic F68 and vitamin E-TPGS as surfactants. In addition, microcrystals were obtained by a simple emulsion technique using poly(vinyl alcohol) 2% as aqueous phase.

9. 1-Methyl-tryptophan formulated with vitamin E-TPGS, especially when administered subcutaneously, reduced significantly tumor volumes when combined with orally administered paclitaxel encapsulated in cyclodextrin poly(anhydride) nanoparticles in a Lewis lung carcinoma tumor cell line model implanted in C57BL/6J female mice. The combination of 1-methyl-tryptophan and paclitaxel loaded poly(anhydride) nanoparticles was a successful strategy to enhance the antitumor efficacy of the anticancer drug.

CONCLUSIONES

Conclusiones generales

El trabajo experimental recogido en esta memoria se ha enfocado hacia la mejora de la biodisponibilidad oral del fármaco anticanceroso, paclitaxel. Los resultados obtenidos en esta tesis doctoral nos han permitido concluir lo siguiente:

1. La formulación de paclitaxel en nanopartículas de poli(anhídrido) combinadas con β-ciclodextrina y/o con poli etilenglicol 2000 aumentó la permeabilidad aparente del fármaco a través de segmentos de intestino de rata en un modelo *ex vivo* en las cámaras de Ussing. El ratio de absorción (R) aumentó en torno a 10-12 veces aproximadamente para las nanopartículas comparado con el medicamento comercial Taxol®.

2. Los estudios farmacocinéticos por vía oral del Taxol® comercial revelaron que no se pudieron detectar niveles plasmáticos de fármaco ya que la absorción del fármaco está impedida por la presencia de la P-glicoproteína y el citocromo P450 a nivel intestinal. Sin embargo, cuando el paclitaxel se administró encapsulado en nanopartículas de poli(anhídrido), el fármaco pudo ser absorbido a través de la mucosa intestinal y se observaron niveles plasmáticos sostenidos del fármaco a las dos dosis estudiadas (10 y 25 mg/kg). Además, las ciclodextrinas y el poli etilenglicol podrían facilitar la absorción del anticanceroso gracias a la inhibición de la P-glicoproteína y del citocromo P450 en la superficie intestinal.

3. Tras la administración oral de las nanopartículas de poli(anhídrido) con paclitaxel complejado con ciclodextrinas o combinado con poli etilenglicol 2000 a ratones C57BL/6J, los niveles plasmáticos obtenidos se mantuvieron en el tiempo entre 25-30 horas para las dos dosis estudias (10 and 25 mg/kg).

4. La encapsulación de paclitaxel en nanopartículas de poli(anhídrido) combinadas con ciclodextrinas y poli etilenglicol aumentó la biodisponibilidad oral del citotóxico hasta un 80% para las formulaciones pegiladas y entre un 60 y un 70% para las nanopartículas con ciclodextrinas en ratones hembra C57BL/6J.

5. Los estudios de distribución llevados a cabo en ratones revelaron que la encapsulación de paclitaxel en nanopartículas de poli(anhídrido) no alteraron su distribución. Su principal acumulación fue en el hígado, riñones, ovarios y pulmón, tanto para su administración

intravenosa como oral. Además, a los tiempos más largos evaluados, paclitaxel se acumuló principalmente en el intestino ya que es conocida su excreción por heces.

6. En los experimentos llevados a cabo en un modelo tumoral con células tumorales de pulmón de Lewis (3LL), el paclitaxel encapsulado en nanopartículas de poli(anhídrido) ralentizó el crecimiento de las células tumorales implantadas, comparado con el medicamento comercial Taxol®.

7. La administración de paclitaxel encapsulado en nanopartículas de poli(anhídrido) presentó un ligero efecto antiangiogénico puesto que los niveles plasmáticos del factor de crecimiento del endotelio vascular (VEGF) se mantuvieron más bajos que en el grupo recibiendo Taxol®. Estos niveles bajos podrían estar relacionados con la capacidad del fármaco administrado por vía oral de limitar el crecimiento tumoral.

8. Los nanocristales de 1-metil-triptófano se prepararon por un método de precipitación usando Pluronic F68 y vitamin E-TPGS como tensioactivos. Asimismo, también se prepararon microcristales por el método de emulsión simple con poli(vinil alcohol) al 2% como fase acuosa.

9. El 1-metil-triptófano formulado con vitamina E-TPGS redujo significativamente el volumen tumoral al combinarlo con paclitaxel complejado con ciclodextrina encapsulado en nanopartículas de poli(anhídrido) administrado por vía oral en un modelo tumoral de cáncer de pulmón de Lewis implantado en ratones hembra C57BL/6J, especialmente al administrar el 1-metil-triptófano por vía subcutánea. La combinación de 1-metil-triptófano y nanopartículas de poli(anhídrido) con paclitaxel mejoró la eficacia antitumoral del fármaco anticanceroso.

CHAPTER 8

ANNEXES

ANNEXE 1

Cyclodextrin-poly(anhydride)nanoparticles as new vehicles for oral drug delivery

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ANNEXE 2

Molecular buckets: cyclodextrins for oral cancer therapy

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ANEXO 3

Stabilized micelles as delivery vehicles for paclitaxel

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