APR-246 combined with 3-deazaneplanocin A, panobinostat or temozolomide reduces clonogenicity and induces apoptosis in glioblastoma cells

JAVIER DE LA ROSA[1], ALEJANDRO URDICIAIN[1], MARÍA V. ZELAYA[2], IDOYA ZAZPE[3], BÁRBARA MELÉNDEZ[4], JUAN A. REY[5], MIGUEL A. IDOATE[6] and JAVIER S. CASTRESANA[1]

[1]Department of Biochemistry and Genetics, University of Navarra School of Sciences; Departments of [2]Pathology and [3]Neurosurgery, Hospital Complex of Navarra, 31008 Pamplona; [4]Molecular Pathology Research Unit, Virgen de la Salud Hospital, 45005 Toledo; [5]IdiPaz Research Unit, La Paz University Hospital, 28046 Madrid; [6]Department of Pathology, University of Navarra Clinic, 31008 Pamplona, Spain

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Abstract. Glioblastoma is the most malignant brain tumor and presents high resistance to chemotherapy and radiotherapy. Surgery, radiotherapy and chemotherapy with temozolomide are the only treatments against this tumor. New targeted therapies, including epigenetic modulators such as 3-deazaneplanocin A (DZ-Nep; an EZH2 inhibitor) and panobinostat (a histone deacetylase inhibitor) are being tested *in vitro*, together with temozolomide. The present study combined APR-246 with DZ-Nep, panobinostat and teomozolomide in order to explore the possibility of restoring p53 function in mutated cases of glioblastoma. Following the Chou-Talalay method it was demonstrated that APR-246 acts in an additive manner together with the other compounds, reducing clonogenicity and inducing apoptosis in glioblastoma cells independently of p53 status.

Introduction

Glioblastoma is the most malignant type of brain tumor and it exhibits a high degree of resistance to chemotherapy and radiotherapy (1,2). Novel targeted therapies are required to treat patients with this tumor. In our previous studies, the effects of 3-deazaneplanocin A (DZ-Nep; an EZH2 inhibitor) and panobinostat [a histone deacetylase (HDAC) inhibitor] were assessed on regular and temozolomide-resistant glioblastoma cells, and showed that DZ-Nep combined with panobinostat exerted the most notable synergistic effect (3,4) However, additional variants of drug combinations are required, particularly as a subset of gliomas harbor TP53 mutations (5-7). The agents PRIMA-1 and PRIMA-1MET (also called APR-246) can alter the three-dimensional structure of mutated p53 (8-10).

Previous studies have highlighted the apparently contradictory results of the initial p53 studies, in which p53 was considered as an oncogene (11-13). In other tumor suppressor genes, the appearance of mutations results in almost completely, or completely abrogated expression (11); however, the mutated variant of p53 appears to possess a very long half-life, leading to its accumulation in high amounts (12). Some variants of this monoallelically mutated and stable p53 protein can exert a dominant-negative effect on the normal p53 protein encoded by the remaining normal allele (14,15). This may be due to the formation of tetramers composed of mutant and normal monomers, which together do not make up a protein with tumor suppressor functions (16,17).

It has been further shown that the appearance of mutations of TP53 in human tumors is usually followed by loss of heterozygosity at the corresponding locus, which suggests that there is a selective advantage in the expression of mutant p53 with respect to the expression of normal p53, leading to the disappearance of normal p53 expression even after mutation of only one of its two alleles (14). These and other findings have resulted in the development of the 'gain-of-function' hypothesis of mutated p53, which postulates that TP53 mutations do not only cause the loss of the tumor suppressor function of the natural protein, but also bestow p53 novel capabilities that promote progression of tumorigenesis (18).

In vivo experiments have shown that tumors expressing mutant p53 are more aggressive or metastatic compared with those expressing normal p53 or not expressing it at all (19), and they may even show greater resistance to temozolomide (20,21). Furthermore, several studies have demonstrated that the TP53 mutation status is a powerful prognostic marker in certain types of tumors, particularly breast cancer (22,23).

All these functions possessed/acquired by p53 represent possible therapeutic targets, although there is an added

Correspondence to: Professor Javier S. Castresana, Department of Biochemistry and Genetics, University of Navarra School of Sciences, Irunlarrea 1, 31008 Pamplona, Spain
E-mail: jscastresana@unav.es

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difficulty in the treatment of mutated p53, as not all tumors will harbor the same mutation of TP53, which may vary by tumor type or ethnicity of the patient (24,25). Each mutation acts differently in p53, altering its ability to bind DNA, its three-dimensional conformation, or both, leading to the loss of the physiological function of p53, to a dominant-negative inhibition on the normal allele of TP53, or to the gain of acquired tumor functions (13,18). These variants increase the difficulty of understanding how mutations in TP53 affect the progression of the disease or treatment outcome.

Reversal of the mutant conformation of p53 to the normal functional conformation, if successful, is noteworth, as i) mutant p53 gain-of-function activity will be limited and ii) p53 tumor suppressor function will be recovered. In addition, as mutant p53 is normally expressed at high levels, the impact of this therapeutic strategy on the tumor cell is considerable. PRIMA-1, or its analogs, have been shown to be safe in a phase I clinical study (26). PRIMA-1 can be metabolized into other compounds, such as methylene quinuclidinone (MQ), which covalently binds to the central domain of mutated p53 and restructures it back to its physiological conformation (9). In addition, MQ can induce apoptosis independently of p53 by inducing oxidative stress; the sum of these two mechanisms leads to cell death (27).

To date, studies have assessed the use of PRIMA-1 or APR-246 for the treatment of glioma cells. Notably, some studies suggested that the cytotoxic effects caused by PRIMA-1 or APR-246 in glioma cell lines is independent on the molecular status of p53 (28,29). However, these studies are very recent; thus, the study of the synergy of APR-246 with other drugs is a niche which is yet to be explored.

Temozolomide is used clinically against glioblastoma, and any other compound under investigation should be checked versus temozolomide. Therefore, the present study used DZ-Nep (30) and panobinostat (31) because they are inhibitors of epigenetic regulators (DZ-Nep inhibits the EZH2 histone methyl transferase, and panobinostat inhibits HDAC). After demonstrating some synergies in our previously published study (3), APR-246 was used, in order to possibly restore p53 function in mutated glioblastoma. In this sense, and following on from our previous study (3), the synergistic effects of DZ-Nep, panobinostat and temozolomide against glioblastoma cells were assessed *in vitro*. Cells were treated with APR-246 and one of each of the aforementioned chemical compounds in order to determine whether APR-246 exhibited a synergistic, additive or antagonistic activity.

Materials and methods

Cell lines and primary tumours. A172, U87MG, T98G, MOG-C-CCM, LN405 and GOS-3 human glioblastoma cell lines were used in the present study. All cell lines were obtained from the American Type Culture Collection (ATCC). The conditions for cell culture and molecular profiling of the cell lines used are described in detail in our previous study (3). Of note, A172, U87MG and GOS-3 are wild-type p53 cell lines, whereas T98G, MOG-C-CCM and LN405 are mutated p53 cell lines (3).

The U87MG cell line, which was obtained from ATCC, was found to be misidentified, as it is not the original glioblastoma cell line established in 1968 at the University of Uppsala (web.expasy.org/cellosaurus/CVCL_0022), but rather a glioblastoma cell line of unknown origin. Additionally, the GOS-3 cell line has been shown to be contaminated, and is a U-343MGa derivative (web.expasy.org/cellosaurus/CVCL_2050).

The cell lines established with increased resistance to temozolomide, were termed A172-TMZR and LN405-TMZR (3). The present study was authorized by The Ethics Committee of the University of Navarra (approval no. CEI0502012). Cell lines derived from primary tumors were termed PE8 and PE9 (3). Short tandem repeat (STR) profiles of PE8 and PE9 (Figs. S1-S5) were analyzed and the resulting alleles of the 16 STR loci were listed for further analysis (Tables SI and SII).

Pharmacological treatments. APR-246 (Cayman Chemical Company), and DZ-Nep, panobinostat and temozolomide (Sigma-Aldrich; Merck KGaA), alone or as combined treatments (APR-246 combined with any one of the other three treatments; DZ-Nep, panobinostat or temozolomide), were used to treat the cell lines and primary cultures. A stock solution of the drugs was prepared by diluting them in DMSO at a concentration of 100 mM (APR-246), 33.45 mM (DZ-Nep), 500 μM (panobinostat) and 51.5 mM (temozolomide). The drugs were stored in aliquots at -20˚C (APR-246, DZ-Nep and temozolomide) or at -80˚C (panobinostat). Each drug was diluted in medium to the corresponding concentration of each experiment. All experimental treatments were performed with the same quantity of DMSO diluted in medium, which was always <0.1%.

Cell proliferation was measured using a colorimetric MTT assay (Sigma Aldrich; Merck KGaA), as described previously (3). The concentration range used for APR-246, DZ-Nep and temozolomide was 1-200 μM. The concentration range for treatment with panobinostat was 1-200 nM.

Drug combination studies and synergy quantification. Drug combinations were used to assess the synergistic potential of the drugs used, using the Chou-Talalay method as described in our previous study (3,32). This method, based on the combination index theorem, allows for quantitative determination of the interactions of the effects produced by drugs, defining a combinatorial index (CI); where, CI <1 defines a synergistic effect, CI >1 defines an antagonistic effect and CI =1 is equivalent to an additive effect (32). To obtain the combinatorial indices, Compusyn was used (33) (version 1.0, from https://www.combosyn.com) which is capable of calculating the CI indices from the cellular lethality observed when cells are treated with a combination of two to three drugs (33). Combinations of three drugs were analyzed using a constant ratio (DZ-Nep, 5 μM; panobinostat, 0.01 μM; temozolomide, 12.5 μM). APR-246 was included using a non-constant ratio. The drug concentrations used in the present study are shown in Table I.

Cellular and molecular studies. The study was authorized by the Ethics Committee of the University of Navarra (approval no. CEI0502012) for conducting research on the genetics of tumors of the nervous system. All samples were fully anonymized prior to accessing; therefore, patient details were not known. Patients provided written informed consent for the

Table I. Pharmacological compounds used in the present study.

A, Single studies

Cell line	APR-246, μM	DZ-Nep, μM	Panobinostat, μM	Temozolomide, μM
A172	35	5	0.02	0.2
A172-TMZR	35	5	0.02	0.2
U87MG	35	5	0.02	0.2
LN405	65	5	0.02	0.2
LN405-TMZR	65	5	0.02	0.2
T98G	65	5	0.02	0.2
GOS-3	65	5	0.02	0.2
MOG-G-CCM	35	5	0.02	0.2
PE8	35	5	0.02	0.2
PE9	35	5	0.02	0.2

B, Double combination studies

Cell line	APR-246, μM	DZ-Nep, μM	Panobinostat, μM	Temozolomide, μM
U87MG	33-41	5-80	0.01-0.16	12.5-200
LN405	70-86	5-80	0.01-0.16	12.5-200
LN405-TMZR	62-78	5-80	0.01-0.16	12.5-200
GOS-3	62-78	5-80	0.01-0.16	12.5-200
MOG-G-CCM	33-41	5-80	0.01-0.16	12.5-200

C, Triple combination studies

Cell line	APR-246, μM	DZ-Nep, μM	Panobinostat, μM	Temozolomide, μM
U87MG	11-13.67	1.66-26.67	0.003-0.053	4.167-66.67
LN405	23.33-28.67	1.66-26.68	0.003-0.054	4.167-66.68
LN405-TMZR	21-26	1.66-26.69	0.003-0.055	4.167-66.69
GOS-3	21-26	1.66-26.70	0.003-0.056	4.167-66.70
MOG-G-CCM	11-13.67	1.66-26.71	0.003-0.057	4.167-66.71

DZ-Nep, 3-deazaneplanocin A.

use of their samples for research. Sample collection took place between January 2012 and December 2016. The only inclusion criterion was diagnosis of glioblastoma and there were no exclusion criteria. Primary tumors obtained from the Hospital Complex of Navarra were cultured and frozen in vials at -80˚C and in liquid nitrogen. The remainder of the tumor tissue was cut with scalpels into ~22-mm thick fragments, which were treated with 0.1% trypsin and later mechanically processed until tumor tissue was disintegrated into individual cells. The trypsinized fragments were pipetted up and down using a 5-ml pipette one to 20 times, and the liquid was passed through a Falcon® 40 μm Cell Strainer filter (Corning, Inc.). The filtrate was finally centrifuged at 700 x g for 5 min at room temperature to obtain the cell pellet. The cells were cultured with neurosphere medium in flasks and pretreated overnight at 4˚C with laminin (Sigma-Aldrich; Merck KGaA) at 10 μg/ml final concentration. The neurosphere medium used was DMEM + F12 (Gibco; Thermo Fisher Scientific, Inc.) in the absence of serum and supplemented with 1X B27 (Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (Sigma-Aldrich; Merck KGaA) and 20 ng/ml basic fibroblast growth factor (Sigma-Aldrich; Thermo Fisher Scientific, Inc.). The cell lines derived from primary tumors used in this study were termed PE8 and PE9.

Cell lines and primary tumors were incubated for 72 h with APR-246, DZ-Nep, panobinostat or temozolomide using the concentrations/combinations shown in Table I, and as described previously (3). The effects of drugs on the activation of apoptosis were studied using a Caspase-Glo 3/7 Assay kit (Promega Corporation) according to the manufacturers' instructions in A172, A172-TMZR and LN405 cells (3). Soft agar colony formation assays, colony formation assays in adherent conditions, quantitative PCR following mRNA extraction and reverse transcription were performed as

Table II. Primary and secondary antibodies used in the study.

Antibody	Molecular weight, kDa	Company	Cat. no.	Specie	Dilution	Type
Anti p53 (DO-1)	53	Santa Cruz Biotechnology, Inc.	sc-126	Mouse (mAb)	1:2,000	Primary
Anti p21 (F-5)	21	Santa Cruz Biotechnology, Inc.	sc-6246	Mouse (mAb)	1:2,000	Primary
Anti-Bax	20	Cell Signaling Technology, Inc.	#2772	Rabbit (pAb)	1:2,000	Primary
Anti-β-actin	42	Sigma-Aldrich; Merck KGaA	A5441	Mouse (mAb)	1:20,000	Primary
Anti-rabbit igg-HRP	-	Santa Cruz Biotechnology, Inc.	sc-2004	Goat (pAb)	1:5,000[a] 1:500[b] 1:2,000[c]	Secondary
Anti-mouse igg-HRP	-	Santa Cruz Biotechnology, Inc.	sc-2005	Goat (pAb)	1:20000[d]	Secondary

Dilution of secondary antibody for [a]p53, [b]p21, [c]Bax and [d]β-actin. mAb, monoclonal antibody; pAb, polyclonal.

described in our previous study (3). IC_{50} of APR-246 was used for soft agar, colony formation assays and RT-qPCR experiments, while concentrations of panobinostat, DZ-Nep and temozolomide were used as published elsewhere (3).

Western blots. Cell precipitates were extracted using RIPA buffer (described below). The suspension obtained was incubated at 4˚C for 10 min with shaking, and subsequently centrifuged at 10,000 x g for 20 min at 4˚C. Protein supernatants were quantified using the BCA Protein Assay kit (Promega Corporation).

The composition of the RIPA buffer consisted of: 50 mM TRIS (Bio-Rad Laboratories), 150 mM NaCl (Panreac Química SLU), 0.5% Triton® X-100 and 0.5% sodium deoxycholate (both from Sigma-Aldrich; Merck KGaA). TRIS and NaCl solutions were adjusted to pH 8.0 with 1 M HCl (PANREAC), before adding other compounds. The buffer was supplemented with 1 mM PMSF (Sigma-Aldrich; Merck KGaA), DTT at final concentration 10 mM (Invitrogen; Thermo Fisher Scientific, Inc.) and 1x Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.). PMSF was previously dissolved in isopropanol (Panreac Química SLU) until obtaining a stock solution of 100 mM.

In total, 30 μg of total proteins from each sample were loaded per well and electrophoresed on SDS-PAGE and 12% polyacrylamide gels were run for 1 h at 120 V. Proteins were transferred to a nitrocellulose membrane (Amersham; Cyvita). The membranes were blocked for 30 min at room temperature, in TBS or 5% skimmed milk, depending on the protein to be studied. The membranes were incubated for 5 min with TBS-Tween 0.1% three times and were incubated overnight at 4˚C with the primary antibodies (Table II). Incubation with the secondary antibodies (Table II) was carried out for 1 h at room temperature after three washes with TBS-Tween 0.1%. All washing steps with TBS-Tween were done at room temperature. Proteins were visualized using LI-COR (LI-COR Biosciences) or enhanced chemiluminescence detection (Chemi-IR Detection kit; cat. no. 926-32234; LI-COR Biosciences) after incubation with applicable secondary antibodies. All antibody incubations were done in PBS blocking buffer (LI-COR Biosciences). The LI-COR detection was performed using an ODDISSEY CLx system (LI-COR Biosciences). The protein expression levels of p53 and p21 in A172, A172-TMZR and U87MG cells (expressing wild-type p53), and in LN405, LN405-TMZR and T98G cells (expressing mutant p53) were assessed after 24 h of treatment with DZ-Nep, APR-246, or a combination of the two.

Apoptotic index. To establish a qualitative apoptotic index, the ratio (Bax+Noxa/Bcl-2+BCL-XL) was created by adding the fold changes of mRNA expression after treatments in the different glioblastoma cell lines to build up a simplified model of apoptosis. Ratios <1 were considered as antiapoptotic, while ratios >1 were considered as proapoptotic.

Statistical analysis. Three independent experiments were performed. All data are presented as the mean ± standard deviation. All data are normalized with respect to controls. All control values were normalized to 1. To analyze the differences observed between the different pharmacological conditions, ANOVA was performed followed by Tukey's post hoc test using GraphPad Prism 7 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

APR-246 and DZ-Nep combined treatment

APR-246 and DZ-Nep combined treatment exerts an additive effect. The effects of the aforementioned drugs, either combined or alone, were assessed using U87MG, LN405, LN405-TMZR, MOG-G-CCM and GOS3 cell lines. The interactions between APR-246 and any of the other drugs were studied following a non-constant ratio, since, due to the shape of the dose/effect curve of APR-246, the range of APR-246 concentrations with which an effect of partial lethality was observed was very narrow and, thus, does not permit working at concentrations far from its IC_{50}. Therefore, for each cell line, a range of different concentrations of APR-246 close to the corresponding IC_{50} of the cell line were used (Table SIII).

APR-246 and DZ-Nep together exerted an additive effect (Table SIII) in all cell lines when they were administered in simultaneous 72-h double treatments (A72D72), in all drug combinations assessed (CI, 0.840-1.205). The additive effects were also maintained when cells were pretreated with

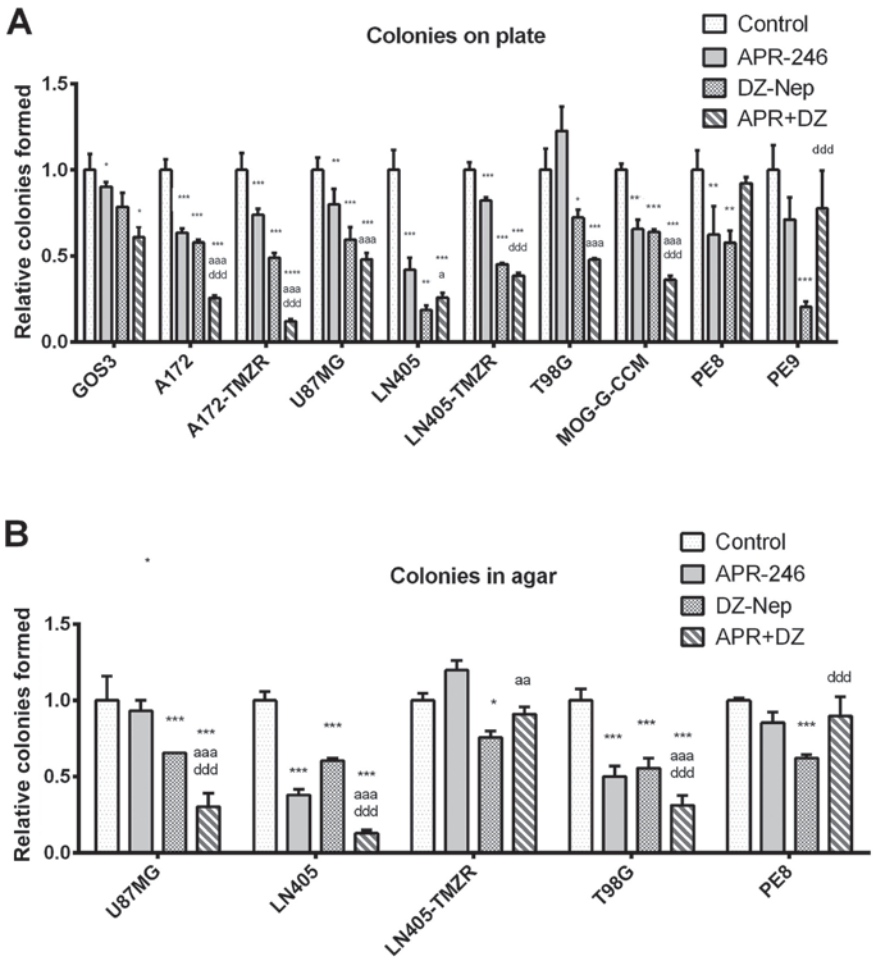

Figure 1. Colony formation capacity in (A) adherent conditions or (B) in soft agar after APR-246, DZ-Nep, or combined treatment (APR+DZ). Data show the mean ± standard deviation of three independent repeats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control; ^{a}P<0.05, ^{aa}P<0.01 and ^{aaa}P<0.001 double treatment vs. individual treatment of APR-246. ^{ddd}P<0.001 double treatment vs. individual treatment of DZ-Nep (Tukey's test). DZ-Nep or DZ, 3-deazaneplanocin A; CI, Combinational Index; Fa, affected cellular fraction.

APR-246 and subsequently treated with DZ-Nep (A24D48; CI, 0.931-1.009), or pretreated with DZ-Nep and subsequently treated with APR-246 (D24A48; CI, 1.056-1.132) in the GOS-3 cells. The A24D48 pretreatment generated additive CI values in the U87MG cells (CI values 1.175, 1.050 and 1.090), and synergistic values in drug combinations of higher concentrations in the MOG-G-CCM cells (CI values 0.589 and 0.335). By contrast, D24A48 generated highly antagonistic CI values in these two cell lines (CI values higher than 10.525 in U87MG, and higher than 23.153 in MOG-G-CCM). LN405 and LN405-TMZR cell lines (Table SIII) exerted additive or slightly antagonistic values in the D24A48 treatments (CI, 1.080-1.231 for LN405; CI, 1.124-2.092 for LN405-TMZR), and additive values, slightly antagonistic, or very antagonistic values in the A24D48 treatments (CI, 1.122-12.49 for LN405; 1.293-7.041 for LN405-TMZR). The biggest affected fractions were obtained in the simultaneous combined treatment. The CI values were collected and the logarithms of the CIs are shown in Fig. S6.

APR-246 and DZ-Nep combined treatment reduces clonogenicity of glioblastoma cells. GOS-3 cells, when treated with a combination of DZ-Nep and APR-246, resulted in a significant reduction in its ability to form colonies in adherent conditions when compared with control cells (P<0.05; Fig. 1A). PE8 and PE9 primary tumor cells did not show significant differences in clonogenicity following the double treatment. LN405 cells were more sensitive compared with LN405-TMZR cells to the individual treatments of APR-246 or DZ-Nep, and to the APR-246 and DZ-Nep combined treatment. Similarly, A172-TMZR cells were more sensitive, compared with A172 cells, to the double treatment, since with the combined treatment A172-TMZR formed a smaller number of relative colonies compared with A172 (P<0.001). The remaining cell lines (U87MG, T98G and MOG-G-CCM) exhibited a significant reduction in the number of colonies formed when double simultaneous treatment was compared to control (P<0.001; Fig. 1A).

These results were confirmed by the soft agar colony formation assays. There was a significant decrease in the number of colonies formed in T98G, U87MG and LN405 cells, when treated simultaneously with APR-246 and DZ-Nep, but not in the LN405-TMZR or PE8 cells (all P<0.001). LN405-TMZR cells also exhibited an increase when treated with APR-246 (Fig. 1B).

APR-246 and DZ-Nep combined treatment increases apoptosis of glioblastoma cells. The caspase activation levels of A172, A172-TMZR and LN405 cells were assessed after 2 h of DZ-Nep or APR-246 treatment alone, and after APR-246 and DZ-Nep double treatment (Fig. 2). Individual

Table III. Antiapoptotic and proapoptotic ratios after treatments of the glioblastoma cell lines.

	Drug combinations						
Cell line	A	D	P	T	A+D	A+P	A+T
A172	1.08	1.32	1.33	1.15	2.06	2.31	1.44
LN405	2.52	2.19	2.14	2.39	2.88	3.61	2.44
LN405-TMZR	0.56	0.92	1.49	0.50	0.49	1.01	0.40
MOG-G-CCM	0.48	1.39	0.86	1.05	0.49	0.37	0.44
T98G	2.38	1.57	1.96	2.05	1.61	2.95	1.52
PE8	2.28	1.87	8.20	1.56	3.37	1.07	7.76

Ratio (Bax + Noxa/Bcl-2 + BCL-XL), created by the addition of the fold changes of mRNA expression after treatments in the different glioblastoma cell lines, to build up a simplified model of apoptosis. A, APR-246; D, 3-deazaneplanocin A, P, panobinostat, T, temozolomide. Ratios <1 might be considered as antiapoptotic, while ratios >1 might be considered as proapoptotic.

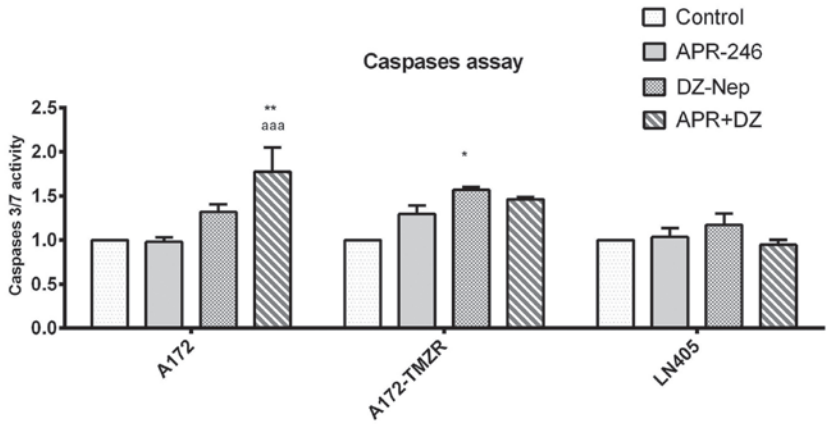

Figure 2. Activation of caspases-3/7 following treatment with APR-246, DZ-Nep or combined treatment (APR+DZ). Data are presented as the mean ± standard deviation of three independent repeats. *P<0.05 and **P<0.01 vs. control; ^{aaa}P<0.001 double treatment vs. individual treatment of APR-246. DZ-Nep or DZ, 3-deazaneplanocin A.

treatment with APR-246 did not significantly increase the level of caspase activation compared with the control treatment in the three lines studied. DZ-Nep resulted in a slight increase in caspase activation in the three cell lines, and the difference was significant in A172-TMZR cells. However, the double treatment of APR-246 with DZ-Nep only resulted in an increase in activated caspases with respect to the individual treatments in A172 cells (P<0.01)

The combination of APR-246 and DZ-Nep significantly increased the expression of BAX mRNA in T98G cells (P<0.001), and particularly in LN405 cells, where the highest increase in BAX expression was observed (P<0.0001). No significant increases in BAX expression were detected in the A172, LN405-TMZR, MOG-G-CCM and PE8 cells (Fig. 3A). There was a significant decrease observed in MOC-G-CCM cells treated with APR-246 alone (P<0.05). The APR-246 and DZ-Nep combination significantly increased NOXA expression in LN405, PE8 and A172 (all P<0.0001) cells. The double treatment increased NOXA expression in the A172 cells, but with no differences when compared with the individual treatment of APR-246 (P<0.01). The double treatment did not result in significant alterations in NOXA expression in the T98G or MOG-G-CCM cells. Individual treatment with DZ-Nep increased NOXA expression significantly in MOG-G-CCM cells (P<0.0001; Fig. 3B).

The combination of APR-246 and DZ-Nep significantly decreased BCL-2 mRNA expression levels in A172 (P<0.0001), MOG-G-CCM (P<0.001), T98G (P<0.05) and PE8 cells (P<0.001), whereas LN405 and LN405-TMZR cells exhibited increased BCL-2 expression levels following combined treatment (P<0.0001; Fig. 4).

Double treatment with APR-246 and DZ-Nep significantly increased BCL-XL expression in A172 (P<0.01), LN504 (P<0.001) and MOG-G-CCM (P<0.0001). LN405-TMZR and T98G cells also increased BCL-XL expression, but differences were not statistically significant (Fig. 4 and Table III).

APR-246 and DZ-Nep combined treatment activates the p53 pathway in cell lines with wild-type but not mutant p53. Both DZ-Nep and APR-246 increased the p53 expression levels in A172 ells (Fig. 5). Basal p53 expression in p53 mutant cell lines was higher, and the treatments did not significantly alter p53 expression. Combined treatment of APR-246 and DZ-Nep further increased the expression levels of p53 in A172, A172-TMZR, U87MG, LN405 and LN405-TMZR cells. No increase of p53 was observed in T98G cells, a cell line with a very high basal expression level of mutant

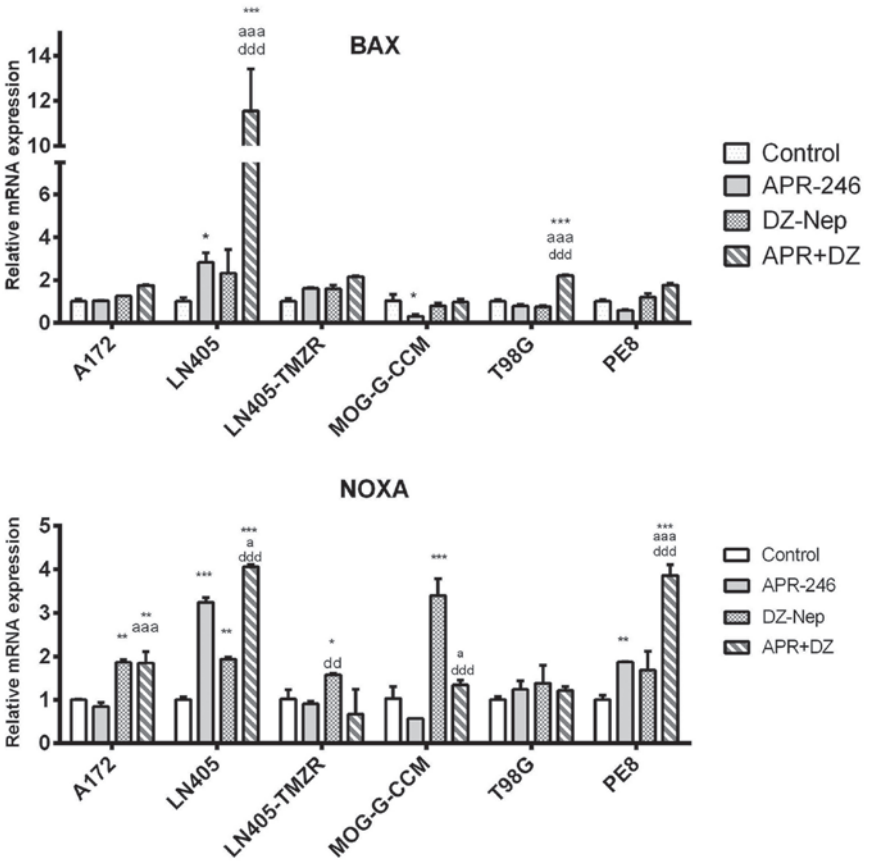

Figure 3. BAX and NOXA mRNA expression in cell lines treated for 72 h with APR-246, DZ-Nep or a combination of both (APR+DZ). Data are presented as the mean ± standard deviation of three independent repeats. *P<0.05, **P<0.01 and ***P<0.001 vs. control; ^{a}P<0.05 and ^{aaa}P<0.001 double treatment vs. individual treatment of APR-246; ^{dd}P<0.01 and ^{ddd}P<0.001 double treatment vs. individual treatment of DZ-Nep. DZ-Nep or DZ, 3-deazaneplanocin A.

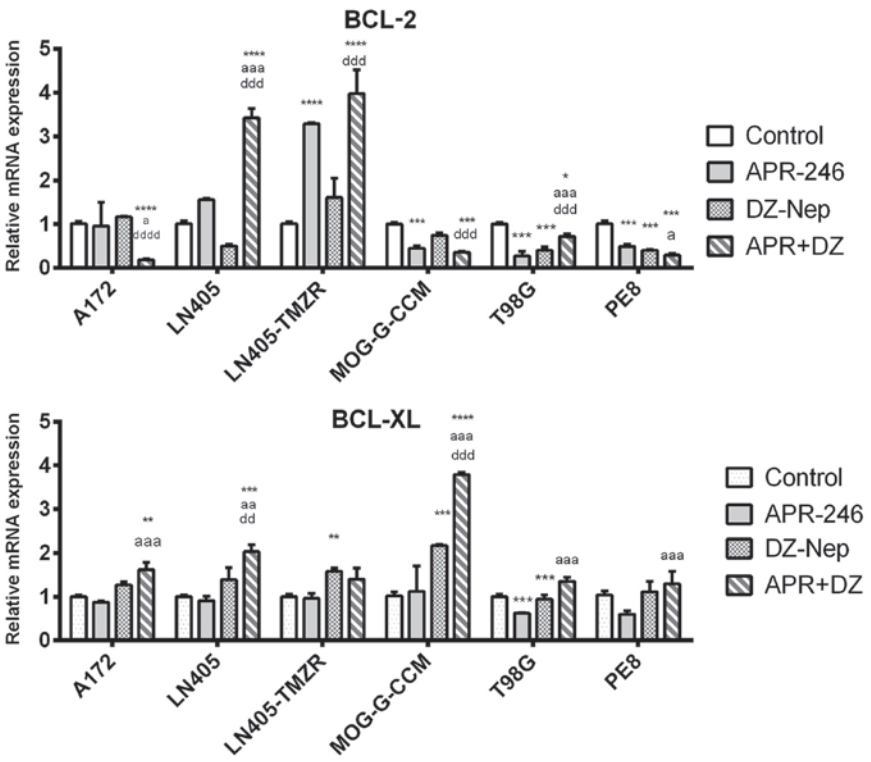

Figure 4. BCL-2 and BCL-XL mRNA expression in cell lines treated for 72 h with APR-246, DZ-Nep or a combination of both (APR+DZ). Data are presented as the mean ± standard deviation of three independent repeats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control; ^{a}P<0.05, ^{aa}P<0.01 and ^{aaa}P<0.001 double treatment vs. individual treatment of APR-246; ^{dd}P<0.01, ^{ddd}P<0.001 and ^{dddd}P<0.0001, double treatment vs. individual treatment of DZ-Nep. DZ-Nep or DZ, 3-deazaneplanocin A.

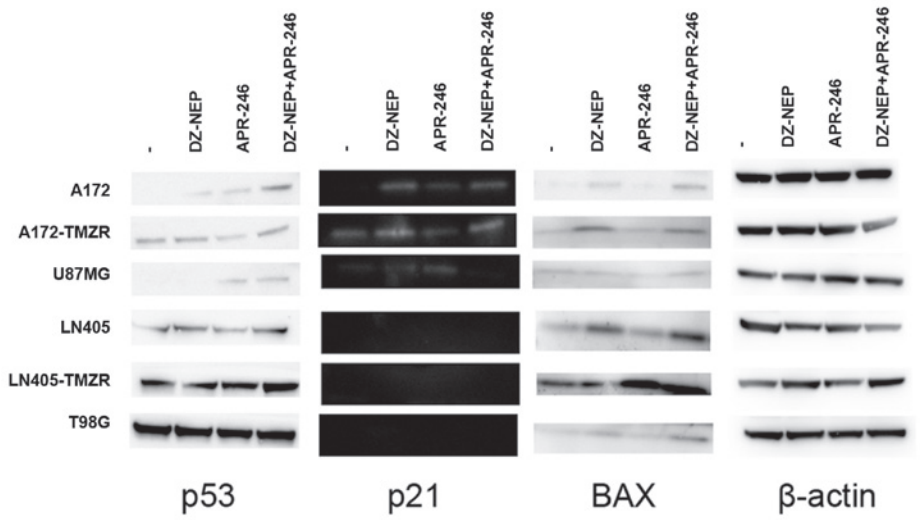

Figure 5. Expression of p53, p21 and Bax proteins in A172, A172-TMZR, U87MG, LN405, LN405-TMZR and T98G cell lines treated for 24 h with APR-246, DZ-Nep or both. p53: 53 kDa, p21: 21 kDa, bax: 17 kDa, β-actin: 42 kDa. β-act, β-actin; DZ-Nep, 3-deazaneplanocin A.

p53. Notably, the basal expression levels of p53 were higher in the temozolomide-resistant cells compared with their respective parental cell lines (A172-TMZR vs. A172 and LN405-TMZR vs. LN405).

These treatments produced similar changes in the expression of p21 (Fig. 5), the effector protein of the p53 pathway (34), but only in cells expressing native p53. DZ-Nep, APR-246 and its combination increased p21 expression in A172 cells, with the highest expression levels observed with DZ-Nep, alone or in the combination treatment. A172-TMZR basal expression of p21 was higher compared with that of A172 cells, which only increased with DZ-Nep alone and with the combined treatment. Although p21 expression was detected in U87MG cells after the individual treatments with DZ-Nep and APR-246, no increase in p21 was detected following double treatment.

In the mutant p53 cells (LN405, LN405-TMZR and T98G), no expression of p21 was detected following these treatments, which suggests that p53 was not reactivated due to APR-246.

Finally, BAX expression was detected in both the wild-type and mutant p53 cells. In A172, A172-TMZR and U87MG cells, the highest expression of BAX was observed following individual treatment with DZ-Nep, or with the combined treatment, whereas in the LN405 and T98G cells the highest expression of BAX was observed in the cells subjected to combined treatment. In the LN405-TMZR cells, the greatest increases in expression were detected in cells treated with APR-246, alone or in the combined treatment. It should also be noted that the temozolomide-resistant cells exhibited higher expression of BAX compared with their respective parental cells, and this was particularly true for LN405-TMZR.

APR-246 and panobinostat combined treatment

APR-246 and panobinostat combined treatment exerts an additive effect. The simultaneous combination treatment with APR-246 and panobinostat for 72 h (A72P72) predominantly showed additive CI values, 0.957-1.010, in the GOS-3 and MOG-G-CCM cells (Table SIV and Fig. S7). U87MG cells showed synergistic values (CI, 0.693-0.748). For LN405 cells, additive CI values were shown in the treatments with lower concentrations of drugs (CI, 0.969-1.087), whereas at higher drug concentrations, CI values were antagonistic (CI values of 1.279, 1.498 and 1.851). LN405-TMZR cells exhibited additive values in only two of the combination studies (CI values of 1.076 and 1.127).

When pretreated with APR-246 followed by treatment with panobinostat (A24P48) and vice versa (P24A48), additive CI values were observed in all the treatments in GOS-3 cells (CI, 0.927-1.044). MOG-G-CCM cells showed synergistic values in the P24A48 (CI, 0.421) and A24P48 (CI, 0.794 and 0.8084) treatments at the highest concentrations; additive values appeared in most combinations; and even antagonistic values (CI, 58.722 for A24P48; 2.659 and 2.170 for P24A48) were observed at the lowest concentrations of APR-246 and panobinostat. Similarly, in the U87MG cell line, additive values were found at the highest concentration of drugs in the A24P48 therapy (CI, 0.897-1.135), whereas synergistic values were observed at the highest drug concentration combinations of P24A48 (CI values of 0.216 and 0.202).

Despite working with lower concentrations of APR-246 in LN405-TMZR cells compared with in the parental LN405 cells, higher affected cellular fractions (Fa) were obtained in the LN405-TMZR cell line in the higher concentrations of the double treatment (Table SIV and Fig. S7).

No synergistic CI values were found in the A24P48 pretreatment therapy for LN405 cells; however, the highest concentrations of drugs were found to act synergistically in LN405-TMZR cells (CI, 0.765 and 0.602). The P24A48 pretreatment therapy showed additive CI values at low concentrations in LN405 cells (CI, 0.993 and 0.985), and synergistic values at the highest concentrations (CI, 0.794, 0724 and 0.611). Although these additive values were not observed in LN405-TMZR cells at low concentrations (CI, 1.521 and 1.563), synergy was higher in the LN405-TMZR cells compared with the LN405 cells at the highest concentrations, especially in the treatment of higher concentrations (CI, 0.794, 0.640 and 0.343). The logarithms of the CIs are presented in Fig. S7.

APR-246 and panobinostat combination reduces colony formation. The GOS-3 cell line showed no changes in colony

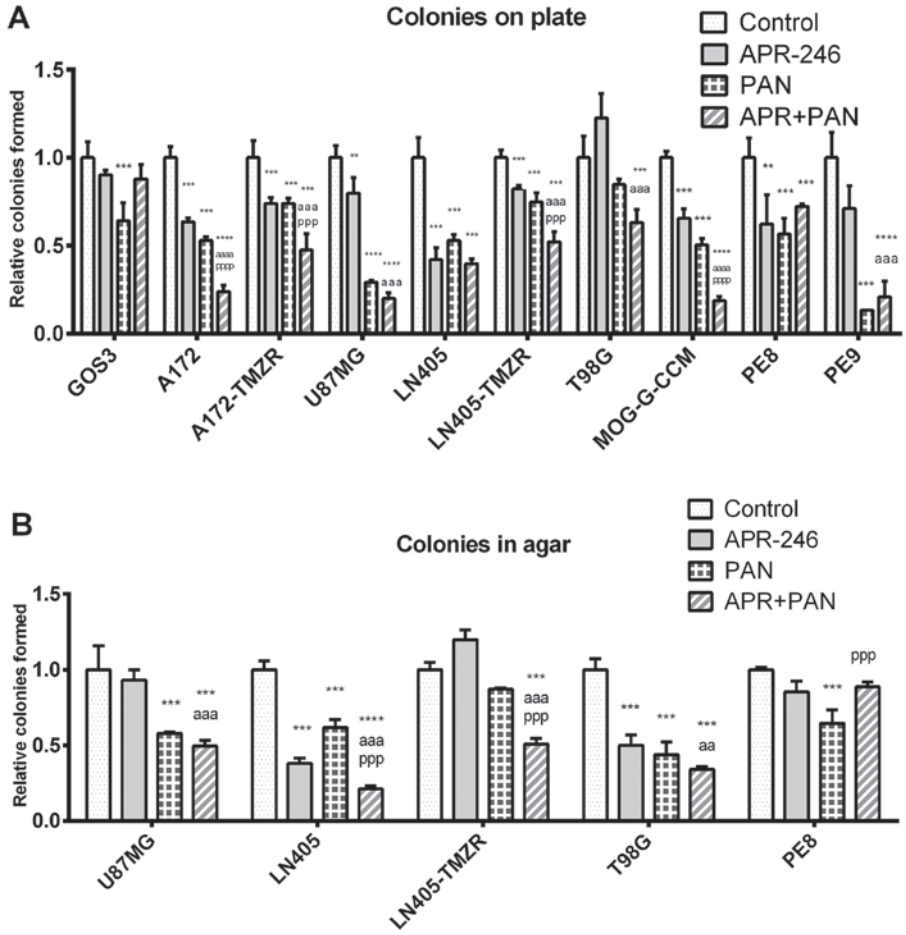

Figure 6. Colony formation capacity in (A) adherent conditions (B) or in soft agar after APR-246, PAN, or combined treatment (APR+PAN). Data show the mean ± standard deviation of three independent repeats. **P<0.01, ***P<0.001 and ****P<0.0001 vs. control; ^{aa}P<0.01, ^{aaa}P<0.001 and ^{aaaa}P<0.0001, double treatment vs. individual treatment of APR-246; ^{ppp}P<0.001 and ^{pppp}P<0.0001, double treatment vs. individual treatment of PAN. PAN, panobinostat.

formation potential in the plate when treated with a combination of APR-246 and panobinostat compared with the control treatment. Several cell lines exhibited a significant decreased in their colony formation capacity, both in the plate or in agar, when compared with the control treatment. A172, A172-TMZR, LN405-TMZR, and MOG-G-CCM exhibited a significant decrease in their colony formation capacity in plate when treated with the double combination and compared with the other treatments (P<0.001 for A172-TMZR and LN405-TMZR; P<0.0001 for A172 and MOG-G-CCM). The double treatment in U87MG, T98G and PE9 was also significant when compared with the control treatment (P<0.001), but not with the panobinostat treatment. LN405 and PE8 showed a significant decrease in the double treatment when compared to the control (P<0.001), but not with the other treatments. Lastly, GOS3 showed only a significant decrease in the individual treatment of panobinostat (P<0.001).

Regarding the colony formation in agar, the double treatment showed a significant decrease in the colonies formed by LN405 and LN405-TMZR cell lines when compared to the control and the individual treatments (P<0.001). U87MG and T98G cell lines showed a significant decrease in the double treatment when compared to the control (P<0.001) and APR-246 (P<0.001) for U87MG and P<0.01 for T98G), but not when compared to panobinostat. In the PE8 line, the higher decrease was observed in the individual treatment of panobinostat.

Several cell lines exhibited significant decreases in their colony formation capacity, both in the plate or in agar, when compared with the control treatment. Additionally, the combined treatment of APR-246 with panobinostat significantly reduced colony formation compared with either drug alone in A172, A172-TMZR, LN405-TMZR and MOG-G-CCM cells in the plates (P<0.001 for A172-TMZR, LN405-TMZR; P<0.0001 for A172 and MOG-G-CCM; Fig. 6A). LN405-TMZR showed reduced colony formation in both the plates and the agar when treated with the combination of drugs (P<0.001; Fig. 6B). LN405 cells showed no differences in colony formation capacity in plates when treated with APR-246 alone or combined with panobinostat, although significant differences were observed in the colony formation ability of LN405 cells in the soft agar (P<0.0001). Similarly, colony formation was not altered by APR-246 combined with panobinostat compared with panobinostat alone in U87MG and T98G cells, both in plates and in soft agar. GOS3, PE8 and PE9 cells did not display significant differences in colony formation potential on plates following combination treatment.

APR-246 and panobinostat combined treatment increases apoptosis. The level of caspase activation in the A172, A172-TMZR and LN405 cell lines was assessed after 2 h of treatment with APR-246, panobinostat, and the simultaneous double treatment of APR-246 and panobinostat (Fig. 7).

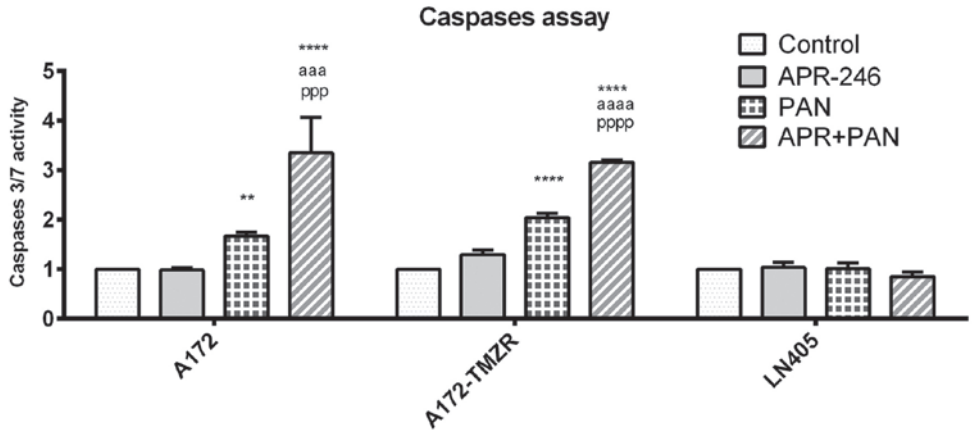

Figure 7. Activation of caspases-3/7 following treatment with APR-246, PAN or combined treatment (APR+PAN). Data are presented as the mean ± standard deviation of three independent repeats. **P<0.01 and ****P<0.0001 vs. control; ^{aaa}P<0.001 and ^{aaaa}P<0.0001 double treatment vs. individual treatment of APR-246; ^{ppp}P<0.001 and ^{pppp}P<0.0001 double treatment vs. individual treatment of PAN. PAN, panobinostat.

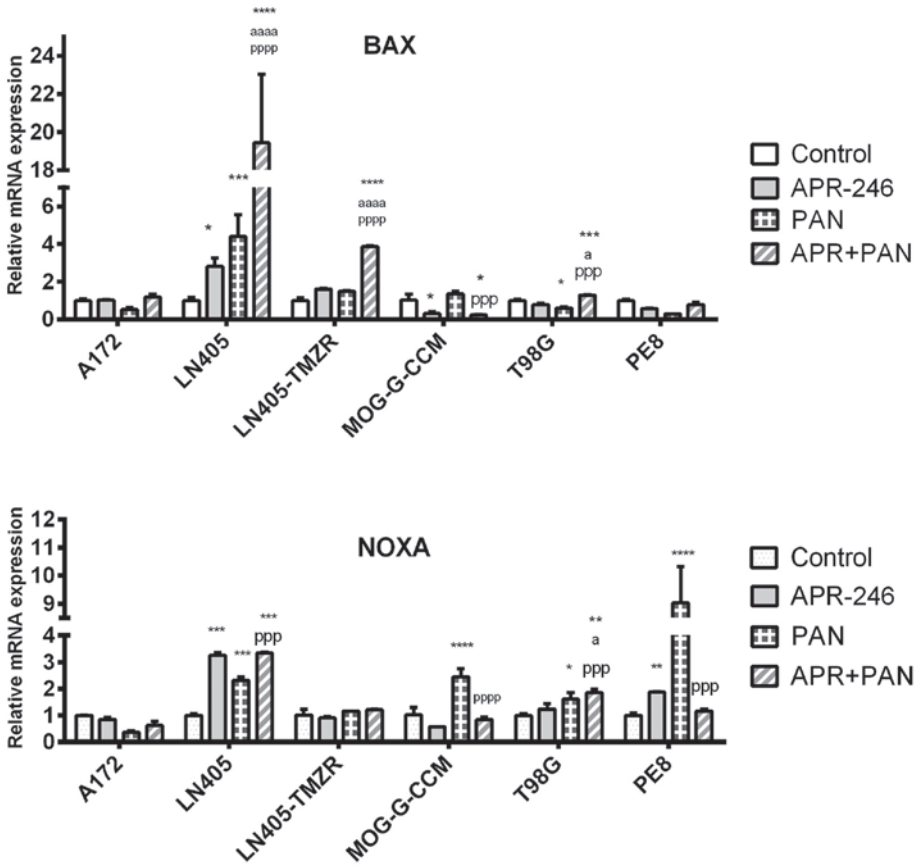

Figure 8. BAX and NOXA mRNA expression in cell lines treated for 72 h with APR-246, PAN or a combination of both (APR+PAN). Data are presented as the mean ± standard deviation of three independent repeats. *P<0.05, **P<0.01 and ***P<0.001 vs. control; ^{a}P<0.05 and ^{aaa}P<0.001 double treatment vs. individual treatment of APR-246; ^{ppp}P<0.001, double treatment vs. individual treatment of PAN. PAN, panobinostat.

Treatment with panobinostat resulted in a significant increase in the activation of caspases in A172 (P<0.01) and A172-TMZR (P<0.0001) cells. The combination treatment of APR-246 with panobinostat resulted in a significant increase in activated caspase levels in the A172 and A172-TMZR cells (P<0.0001), but not in the LN405 cells.

The combination of APR-246 and panobinostat significantly increased the expression of BAX mRNA in T98G, LN405 and LN405-TMZR cells (P<0.001 for T98G; P<0.0001 for LN405 and LN405-TMZR), particularly in the LN405 cell line. The remaining cell lines assessed did not exhibit a notable increase in BAX expression; MOG-G-CCM cells exhibited a significant decrease in BAX expression (P<0.05; Fig. 8).

The double treatment of APR-246 and panobinostat significantly increased NOXA expression only in T98G

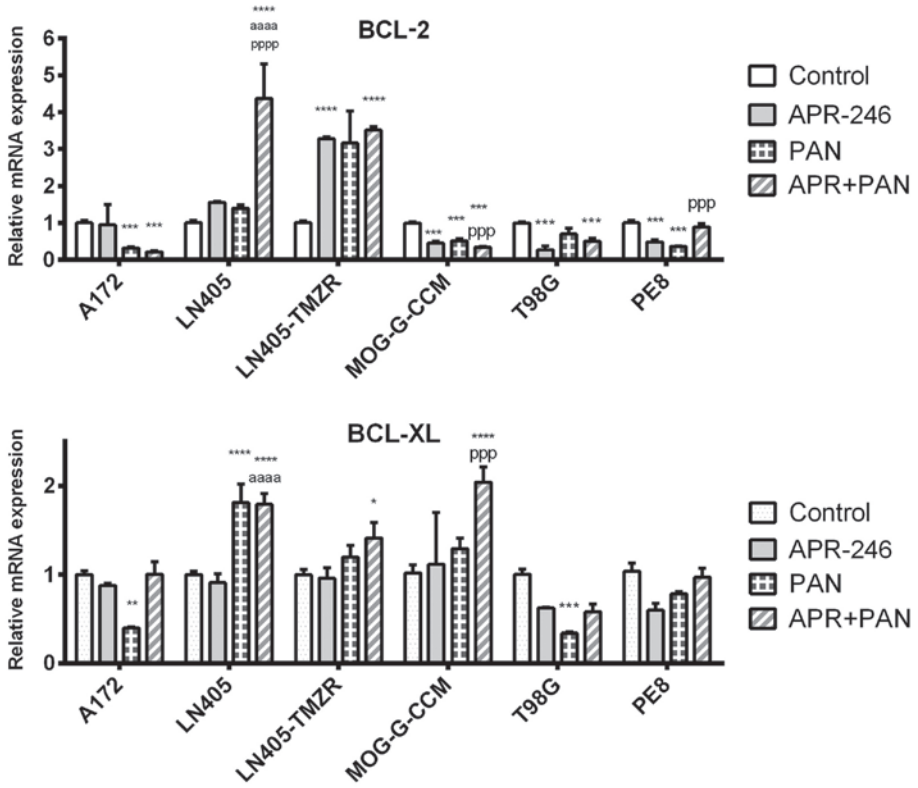

Figure 9. BCL-2 and BCL-XL mRNA expression in cell lines treated for 72 h with APR-246, PAN or a combination of both (APR+PAN). Data are presented as the mean ± standard deviation of three independent repeats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control; ^{aaaa}P<0.001, double treatment vs. individual treatment of APR-246; ^{ppp}P<0.001 and ^{pppp}P<0.001 double treatment vs. individual treatment of PAN. PAN, panobinostat.

cells (P<0.01). Although a significant increase in NOXA was detected by the combination treatment of APR-246 and panobinostat in LN405 cells (P<0.001), it was not significantly different from the NOXA increase produced by treatment with APR-246 alone in LN405 cells (Fig. 8).

The combination of APR-246 and panobinostat significantly decreased the expression of BCL-2 mRNA in A172, MOG-G-CCM and T98G (P<0.001). An increase in the expression of BCL-2 was detected in LN405 and LN405-TMZR cells (P<0.0001; Fig. 9).

In PE8 cells, although the individual treatments of APR-246 or panobinostat showed a significant decrease in BCL-2 expression when compared to the control (P<0.001) the double treatment did not show significant differences in BCL-2 expression.

The combined treatment with APR-246 and panobinostat significantly increased the expression of BCL-XL in LN405, LN405-TMZR and MOG-G-CCM cells (P<0.0001 for LN405 and MOG-G-CCM, P<0.05 for LN405-TMZR) (Fig. 9), but the double treatment did not show differences with the individual treatment of APR-246 in LN405 cells (P<0.001) (Table III).

APR-246 and temozolomide combined treatment

APR-246 and temozolomide combined treatment exerts an additive effect. Combined treatment of APR-246 and temozolomide (A72T72) exhibited additive CI values in all cell lines except U87MG cells, where only one additive value (CI, 1.092) was found in the combination of drugs at the highest concentrations. LN405 cells exhibited slightly higher affected fractions compared with LN405-TMZR cells. In particular, LN405-TMZR showed an slightly antagonistic CI value in the combination at the highest concentrations (CI, 1.201) (Table SV).

Pretreatments with APR-246 followed by post-treatment with temozolomide (A24T48) resulted in additive CI values in U87MG (CI, 0.899-0.949). This same pretreatment showed additive CI values in GOS-3 cells (CI, 0.944-1.005), and it also exhibited a synergistic CI value at the highest concentration (CI, 0.529) (Fig. S8 and Table SV).

When comparing LN405 with LN405-TMZR cells, higher affected fraction was observed in LN405-TMZR cells (between 0.259-0.439 in LN405-TMZR; and between 0.127-0.408 in LN405 cells). The CIs showed an additive effect of the two drugs in LN405 cells, whereas the LN405-TMZR CIs were found to be antagonistic at the two lowest drug concentrations (CI, 1.237 and 1.544), additive at average concentrations (CI, 0.960 and 0.954) and synergistic at the highest drug concentrations (CI, 0.510) (Fig. S8 and Table SV). This may be due to pre-sensitization of the cells with temozolomide resulting in breaks in the DNA (35), thus increasing the apoptotic effect of APR-246.

When pretreated with temozolomide followed by a post-treatment with APR-246, additive CI values were observed in the GOS-3 cells (CI, 1.008-1.165) and LN405 cells (CI, 0.952-1.015), and antagonistic values in the remaining cell lines. In addition, the affected fraction of LN405-TMZR (from 0.199 to 0.334) was smaller compared with that of LN405 (from 0.288 to 0.456), probably due to the fact that pretreatment with temozolomide was ineffective in a cell line already resistant to temozolomide (Fig. S8 and Table SV). The logarithms of the CIs are plotted in Fig. S8.

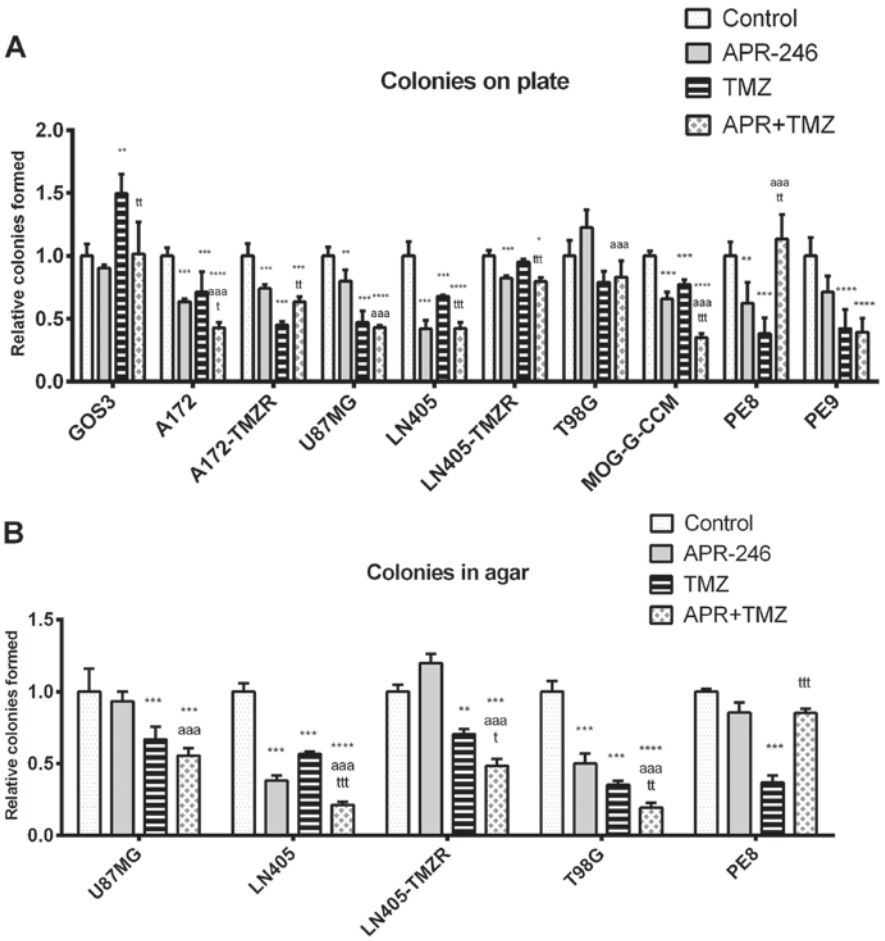

Figure 10. Colony formation capacity in (A) adherent conditions or (B) or in soft agar after APR-246, TMZ or combined treatment (APR+TMZ). Data show the mean ± standard deviation of three independent repeats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control; ^{aaa}P<0.001 double treatment vs. individual treatment of APR-246; ^{t}P<0.05, ^{tt}P<0.01 and ^{ttt}P<0.001 double treatment vs. individual treatment of TMZ. TMZ, temozolomide.

APR-246 and temozolomide combined treatment reduces cell clonogenicity. The double treatment of APR-246 with temozolomide was able to significantly decrease the formation of colonies in the plates in A172, A172-TMZR, U87MG, LN405, and MOG-G-CCM cells, and in the PE9 primary tumor cells, but not in the PE8 primary tumor cells, when compared with the control treatment (all P<0.001; Fig. 10A). However, the combined treatment was only more effective compared with the two individual treatments alone (APR-246 and temozolomide) in A172 and MOG-G-CCM cells.

In GOS3, A172-TMZR, LN405 and LN405-TMZR cells, no significant differences were observed between the combined treatment with APR-246 and temozolomide compared with treatment with APR-246 alone, whereas in the U87MG and PE9 cells, no differences were found with the combined treatment when compared with temozolomide alone (Fig. 10A).

In the colony formation assays under anchorage independent conditions, significant differences were observed in the colonies formed when treated with both APR-246 and temozolomide (fewer colonies formed) compared with either drug alone in the LN405, LN405-TMZR and T98G cells, all of which possess a mutant p53 subunit (P<0.0001 for LN405 and T98G; P<0.001 for LN405-TMZR) (Fig. 10B). Finally, although the combined treatment resulted in the greatest inhibition of colony formation in agar in U87MG cells, the difference between the combined treatment and temozolomide alone was not significant. The most effective treatment against PE8 primary tumor cells was temozolomide alone.

APR-246 and temozolomide combined treatment increases apoptosis. The levels of caspase-3/7 activation in the A172, A172-TMZR and LN405 cells after 2 h of treatment were assessed following treatment with APR-246, temozolomide or their combination (Fig. 11). In A172 cells, both the temozolomide and the combined treatments achieved a significant increase of caspases (P<0.01), but there were no differences between the individual treatment of temozolomide and the combined treatment. The simultaneous double treatment did not result in changes in caspase activation in any of the cell lines studied.

The combination of APR-246 and temozolomide only significantly increased BAX expression in the LN405 cells (P<0.0001; Fig. 12). A significant increase of NOXA was observed in the LN405 and PE8 cells (P<0.0001; Fig. 12). In the remaining cell lines, the combined treatment did not increase the expression levels of these proapoptotic genes.

The double treatment with APR-246 and temozolomide for 72 h significantly decreased the expression of BCL-2 in MOG-G-CCM, T98G and PE8 cells (P<0.001). BCL-2 expression was increased in LN405 and LN405-TMZR cells (P<0.0001; Fig. 13).

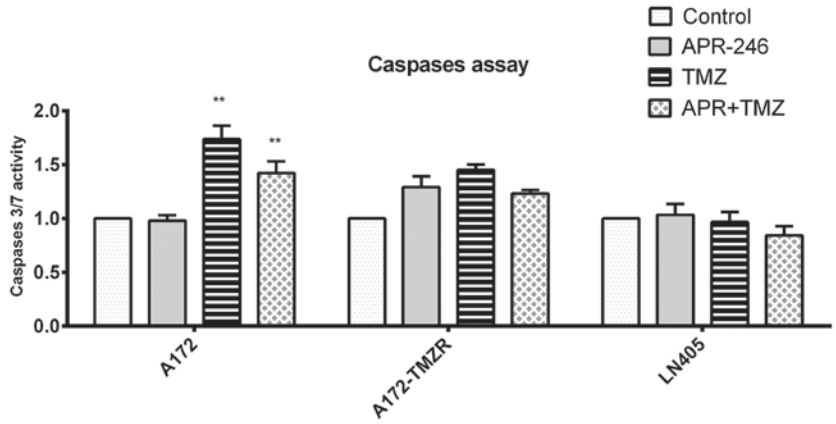

Figure 11. Activation of caspases-3/7 following treatment with APR-246, TMZ or combined treatment (APR+TMZ). Data are presented as the mean ± standard deviation of three independent repeats. **P<0.01 vs. control. TMZ, temozolomide.

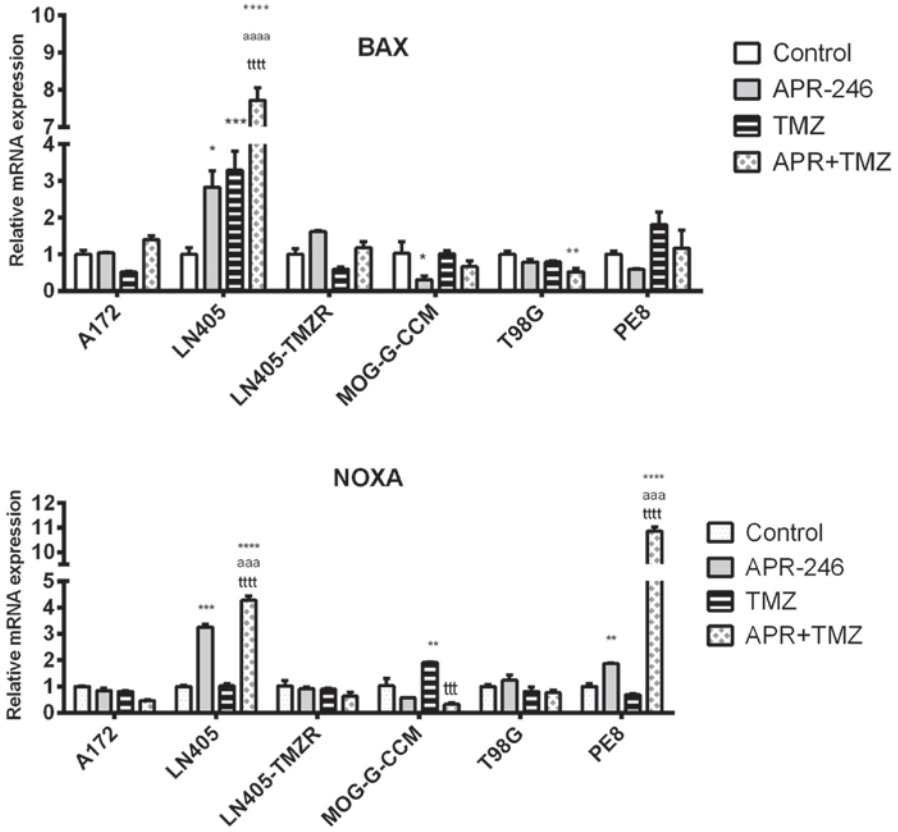

Figure 12. BAX and NOXA mRNA expression in cell lines treated for 72 h with APR-246, TMZ or a combination of both (APR+TMZ). Data are presented as the mean ± standard deviation of three independent repeats. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control; aaaP<0.001 and aaaaP<0.0001, double treatment vs. individual treatment of APR-246; tttP<0.001 and ttttP<0.0001, double treatment vs. individual treatment of TMZ. TMZ, temozolomide.

APR-246 and temozolomide in combination significantly increased the expression of BCL-XL in LN405 and MOG-G-CCM cells (P<0.0001), and decreased BCL-XL expression in T98G cells (P<0.001), although this inhibition did not differ significantly from the inhibition caused by treatment with temozolomide alone (Fig. 13) (Table III).

APR-246 and temozolomide combined treatment activates the p53 pathway in cell lines containing the wild-type but not mutant p53. The effect of a 24-h treatment with APR-246, temozolomide, or their combination, on the expression of p53, p21 and BAX protein levels were assessed in all the cell lines (Fig. 14). A172, A172-TMZR, LN405 and LN405-TMZR cells exhibited increased p53 expression after the individual treatments of temozolomide or APR-246; the increase in p53 expression was greater with the combined treatment of APR-246 and temozolomide. The increase in p53 produced by the different treatments in temozolomide-resistant cells was greater compared with that produced in the control lines (A172-TMZR vs. A172 and LN405-TMZR vs. LN405). In U87MG cells, the greatest

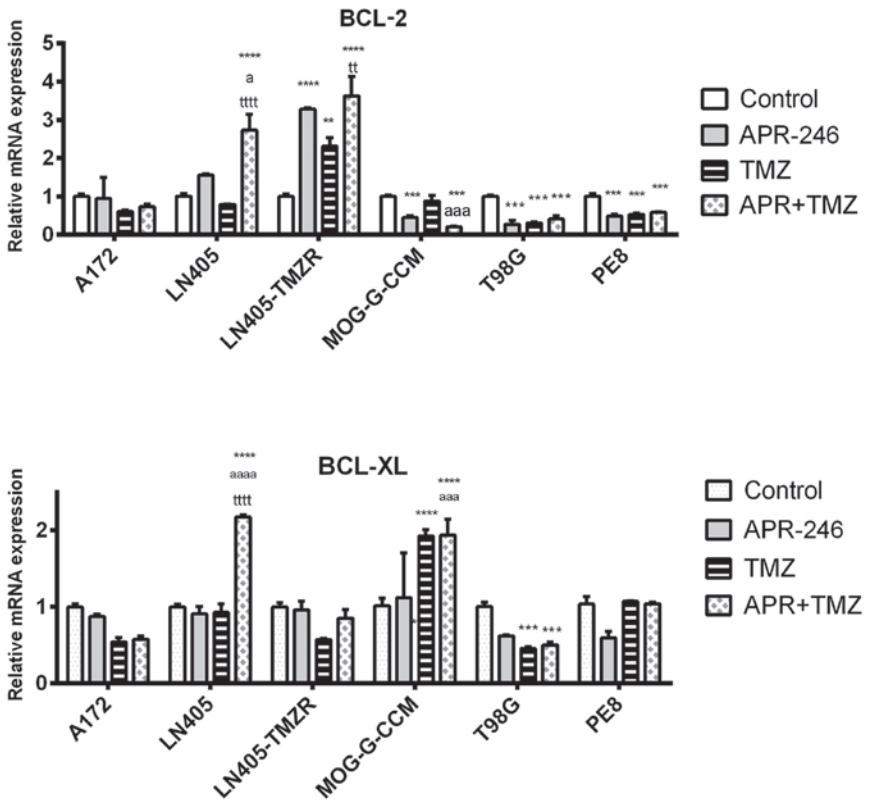

Figure 13. BCL-2 and BCL-XL mRNA expression in cell lines treated for 72 h with APR-246, TMZ or a combination of both (APR+TMZ). Data are presented as the mean ± standard deviation of three independent repeats. **P<0.01, ***P<0.001 and ****P<0.0001 vs. control; ^{a}P<0.05, ^{aaa}P<0.001 and ^{aaaa}P<0.0001 double treatment vs. individual treatment of APR-246; ^{tt}P<0.01 and ^{tttt}P<0.0001, double treatment vs. individual treatment of TMZ. TMZ, temozolomide.

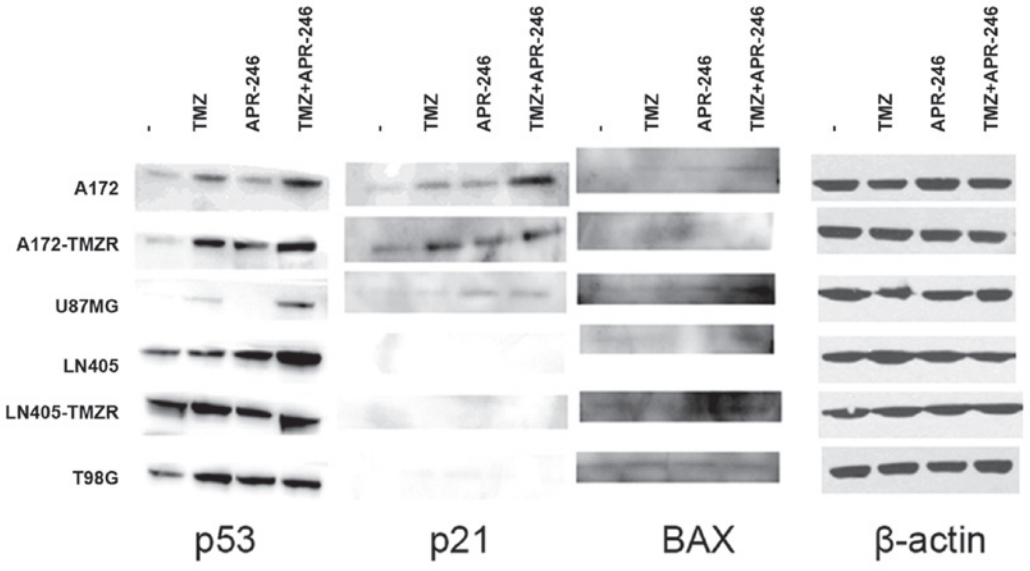

Figure 14. Expression of p53, p21 and Bax proteins in A172, A172-TMZR, U87MG, LN405, LN405-TMZR and T98G cell lines treated for 24 h with APR-246, TMZ or both (TMZ + APR-246). p53: 53 kDa, p21: 21 kDa, bax: 17 kDa, β-act: 42 kDa. TMZ, temozolamide; β-act, β-actin.

increase was observed in cells treated with the combination of drugs.

Similar effects on p21 expression were observed in the cells expressing wild-type p53 following treatment. APR-246, temozolomide and their combined treatment increased the expression of p21 in A172 and A172-TMZR cells, and the highest increase in expression was observed in cells treated with APR-246 and temozolomide combined. A172-TMZR cells expressed higher levels of p21 compared with A172 cells under basal conditions and after treatments. p21 expression in the U87MG cells was detected after APR-246 treatment and after the APR-246 and temozolomide combined treatment.

No p21 expression was detected before the treatments in the mutant p53 cells (LN405, LN405-TMZR and T98G).

Additionally, the highest expression of BAX was observed in all cell lines following combined treatment with APR-246 and temozolomide, except in A172-TMZR and T98G cells, which did not show changes in any of the treatments.

Synergistic effect of the triple drug combination. Cells were treated for 72 h with the triple drug combinations. APR-246 was always included in the treatments, together with two more drugs among DZ-Nep, panobinostat or temozolomide. To avoid excessive cellular lethality, lower concentrations of each drug were used, equivalent to one-third of the concentrations used in combinatorial studies of drug pairs (Table I). All triple combinations included APR-246 and were analyzed with Compusyn software following a non-constant quotient. The triple treatment with APR-246, panobinostat and temozolomide for 72 h showed CI values of considerable synergy in all cell lines (CI values smaller than 0.668) (Table SVI; Fig. S9). CI values obtained for the LN405 cell line (CI, 0.210-0.484) were again indicative of considerable synergy compared with the LN405-TMZR line (CI, 0.427-0.668).

The triple combination of APR-246, DZ-Nep and panobinostat for 72 h (Table SVII and Fig. S10) showed CI indices of considerable synergy in all treatments and in all cell lines (CI, 0.216-0.513), except for the LN405-TMZR, where combinations at lower concentrations resulted in CI values suggestive of an additive effect (CI values of 0.829 and 0.964).

APR-246, DZ-Nep and temozolomide combined in a triple treatment for 72 h resulted in highly synergistic CI values in all cell lines, (CI, 0.161-0.792) (Table SVIII and Fig. S11). CI values for LN405 cells (CI, 0.174-0.183) revealed a greater synergy compared with those of LN405-TMZR cells (CI, 0.467-0.792).

Discussion

The most frequent treatment used for glioblastoma consists of the most extensive possible surgical resection of the tumor, followed by temozolomide, with or without radiotherapy. However, the mean survival time for patients who receive this treatment is 14.7 months (36,37). This problem underscores the importance of effective therapies against glioblastoma. APR-246 may serve as a suitable candidate for treatment, particularly when combined with other established drugs (38,39). Thus, the synergistic effects of APR-246 combined with temozolomide, DZ-Nep and panobinostat were assessed in a continuation to our previous study on the epigenetic modulation of glioblastoma (3).

In the present study, it was investigated whether double or triple drug combinations of APR-246 with DZ-Nep, panobinostat and temozolomide could inhibit tumor growth efficiently. The Chou-Talalay method was used to determine whether the different combinations of drugs produced an additive, synergistic or antagonistic effects. Cells were treated with drugs in pairs and their effects on clonogenicity, apoptosis and on the p53 pathway were assayed. Triple treatment combinations were also tested.

APR-246 treatment of the mutated p53 glioblastoma cell lines (T98G, LN405, LN405-TMZR and MOG-G-CCM) did not result in reactivation of the p53 pathway, as p21 expression was observed. An increase in expression of genes associated with apoptosis was observed, although this may have been independent of p53, and may rather be attributed to induction of oxidative stress produced by APR-246. When APR-246 is metabolized to its MQ metabolite, the binding of MQ to the thiol group of proline-rich protein 13 inhibits the reducing activity of TxR1, and converts it into an enzyme with NADPH oxidase activity. This change causes an increase in cellular oxidative stress, which triggers apoptosis (40). The present study demonstrated that APR-246 increased p53 expression in both mutant and wild-type p53 cells. It may be possible that the increase in oxidative stress produced by MQ induced an increase in p53 expression, activating its pathway in cells expressing wild-type p53.

However, the increase of p53 expression in the mutant p53 cells did not lead to activation of the p53 pathway in the aforementioned mutant cell lines. Previous studies reported similar results, in which APR-246 does not reactivate the p53 pathway in T98G tumor cell line (29,41). Patyka *et al* (29) showed the inability of APR-246 to restore p53 activity in mutant p53 glioblastoma cells, such as T98G. It is possible that this lack of reactivation of mutated p53 in the glioblastoma cells may be due to specific mutations of p53 not recognized by APR-246, or to the presence of isoforms of p53 that exhibit fewer binding sites for the drug (42-44). In any case, understanding the lack of activity of APR-246 may allow for improvement of reactivation therapies against mutated p53 tumors.

To date, although relatively little is known regarding the possible interactions between MGMT and p53 proteins, a certain link between these proteins has been demonstrated. For example, there is a correlation between the presence of mutant p53 and low expression of MGMT (45), although it would be useful to deepen our understanding of this correlation. Patyka *et al* (29) suggested that the sensitivity of cells to APR-246 is due to a decrease in the expression of MGMT, and not to the molecular status of p53. This may explain the acquired sensitivity to APR-246 in cell lines with increased resistance to temozolomide, such as A172-TMZR and LN405-TMZR. Perhaps one of the mechanisms through which the cells develop increased resistance to temozolomide is the increase in the expression of MGMT, which leads to more efficient repair of the damage in cellular DNA produced by temozolomide and ensures successful DNA replication during mitosis (46,47). The cells would become somewhat 'addicted' to this increase in MGMT, such that treatment with APR-246 capable of decreasing MGMT levels would lead to a mitotic disruption in the cell, which together with the oxidative damage caused by APR-246 may initiate apoptosis.

Other studies have suggested that temozolomide increases long-term oxidative stress in the cells (48). Oxidative stress, if not produced at lethal levels, may favor carcinogenesis and tumor progression, including the acquisition of resistance to temozolomide (49). Further treatment with APR-246 increases the levels of oxidative stress above the threshold tolerated by cells, triggering cell death.

Conversely, temozolomide-resistant cell lines (A172-TMZR and LN405-TMZR) exhibited greater basal expression of p53 compared with their respective parental lines (A172 and LN405). This may be due to the fact that A172-TMZR and LN405-TMZR cells, after surviving prolonged treatment with temozolomide at high concentrations by increasing

their resistance to the drug, likely possess DNA damage that activates p53 expression, although resistance to temozolomide may slow the cellular apoptotic response (50) .

Treatment with APR-246, through increasing oxidative stress, may initiate the activation of accumulated p53 in A172-TMZR cells, producing apoptosis at lower doses compared with in the parental line. However, this hypothesis is not applicable to LN405-TMZR cells, as they express mutated p53, therefore apoptosis would occur independently of p53.

In the present study, different paired combinations of APR-246 with DZ-Nep, panobinostat and temozolomide were tested, and their cellular effect was measured based on short-term caspase activation, the expression of proapoptotic and antiapoptotic genes, changes in expression of p53 and p21 proteins, as well as clonogenicity. Western blot analysis demonstrated that the individual treatment with DZ-Nep increased p53 expression. Several studies have reported an interaction between DZ-Nep and the p53 pathway. For example, it has been shown that the sensitivity to DZ-Nep in gastric (51) or thyroid (52) tumor cells is dependent on the molecular status of p53, making mutant p53-expressing cells more resistant to DZ-Nep (51,52). DZ-Nep inhibited the conjugation of ubiquitin to native p53, thus increasing the stability of p53 and activating its target genes, such as MDM2, p21 and GADD45 (51). This was not observed in the mutant p53-expressing cells, which were found to be more resistant to DZ-Nep. Furthermore, PRIMA-1 reactivates p53 in mutant p53 expressing cells, restoring their sensitivity to DZ-Nep (52).

However, APR-246 did not reactivate p53 in the mutant p53-expressing cell lines (T98G, LN405 and LN405-TMZR) in the present study. This may explain the absence of a synergistic effect in the combinatorial experiments with APR-246 and DZ-Nep in the p53 mutant cell lines. However, the possibility that simultaneous double treatments exerted an additive effect in all the cell lines studied, regardless of the molecular status of p53, should also be considered. This additive effect may be explained by two possible mechanisms: First, the accumulation of activated p53 induced by DZ-Nep treatment of the native p53 cell lines would be added to the activation of p53 caused by APR-246. However, this would not explain the additive effect observed in the mutant p53 cell lines, where p53 has no activity. The other possible explanation would be based on the accumulation of oxidative stress. As aforementioned, APR-246 can increase oxidative stress within tumor cells. DZ-Nep, on the other hand, can also increase apoptosis through the dysregulation of the cellular redox balance, as previously described reported (53). The oxidative effects induced by APR-246 and DZ-Nep may explain why these drugs produced an additive effect.

Pretreatment with APR-246 followed by post-treatment with DZ-Nep, and vice versa, did not produce Fa or CI as effectively as the double simultaneous treatment. It was hypothesized that cells may have adapted to drug-induced changes without exceeding the threshold of damage to allow an apoptotic response. APR-246 and DZ-Nep combined treatment increased BAX and NOXA expression in almost all cell lines studied. The most notably affected cell line was LN405 and not LN405-TMZR, which exhibited the lowest expression of NOXA. LN405-TMZR was the least affected cell line by this double treatment in the colony formation assay. Thus, it is possible that the acquisition of temozolomide resistance may have enabled these cells to resist DZ-Nep, by preventing the cooperation of DZ-Nep with APR-246 that would instead produce a greater effect. On the contrary, a greater inhibitory effect on clonogenicity was observed in A172-TMZR cells when compared with the A172 cells. It may be the case that A172-TMZR cells, when expressing native p53, display an increased pharmacological response when APR-246 and DZ-Nep are used concurrently. Although no synergistic effect was observed in the joint action of these two drugs, the presence of an additive effect is a notable factor for the improvement of existing brain tumor therapies. Finally, the combination of these two drugs may be more promising in other types of tumors where APR-246 can reactivate mutated p53, and a synergistic effect may be observed.

The combinatorial studies performed with APR-246 and panobinostat also demonstrated an additive effect in most of the cell lines studied, apart from U87MG cells, where a slightly synergistic effect was observed. By contrast, a slightly antagonistic effect was observed in LN405 and LN405-TMZR cells. It is noteworthy that, when pretreated with APR-246 followed by panobinostat (and vice versa) the Fa and CI were similar to those obtained by the simultaneous double treatment in the majority of the cell lines. Several studies have shown multiple links among HDACs, the action of panobinostat and the p53 pathway. For example, it has been demonstrated that HDACs can inhibit the expression of p53-dependent genes, such as BAX (54). Other studies have shown that the molecular status of p53 may affect the cellular response to panobinostat (55), whereas treatment with panobinostat increases the expression of p53 and p21 in lung tumor (56) and thyroid cancer (57) cell lines. These studies may explain the additive effect of the combination of APR-246 and panobinostat, through the proposed mechanism for the combination of APR-246 and DZ-Nep. On the one hand, panobinostat or APR-246 individually increased the expression of p53. The accumulation of p53 activated by the combined drug treatment would lead to the additive behavior of the double treatment, although this would only activate apoptosis in the cell lines expressing native p53. However, the ability of panobinostat to induce oxidative stress and DNA damage in the cells has also been described (58-60). The mechanism of cellular oxidative stress accumulation exceeding a tolerable pre-apoptotic threshold for the cell may also take place, regardless of the molecular status of p53. A conjunction of the two mechanisms is also possible, and would explain the synergy observed in U87MG cells, which express wild-type p53.

As aforementioned, pretreatment with APR-246 for 24 h followed by a 48-h post-treatment with panobinostat (and vice versa) resulted in Fa similar to those obtained by the simultaneous double treatment of APR-246 and panobinostat for 72 h. This was not observed in pretreatment tests performed with the combination of APR-246 and DZ-Nep, or APR-246 and temozolomide. This might be explained, in our experimental conditions, by assuming that panobinostat, unlike DZ-Nep or temozolomide, might behave as a particularly fast-acting drug, such that administration of panobinostat in the form of pre- or post-treatment, combined with a post- or pre-treatment of APR-246, could be sufficient to have a significant impact on cell viability. The combination of APR-246 and panobinostat

also exerted an effect on the expression levels of the pro-apoptotic BAX and NOXA genes, particularly in the LN405 line. LN405-TMZR cells, although they exhibited an increase in the expression of these genes, did so to a lesser degree compared with LN405 cells. Although MOG-G-CCM cells did not exhibit such an increase, they did exhibit significantly reduced colony formation in agar, similar to most other cell lines.

In summary, the combination of APR-246 and panobinostat produced a stronger effect compared with that produced by either compound alone. Although this effect displayed an additive pattern, further research on this combination should not be ruled out in order to obtain more effective therapies for glioblastoma. In other types of tumors where APR-246 reactivates mutated p53, it is hypothesized that a synergistic response between the two drugs may be observed. Therefore, future studies examining the effects of APR-246 and panobinostat or other types of cancer may yield promising results.

Temozolomide is an alkylating agent that causes DNA damage, which, if not repaired, may lead to programmed cell death or senescence (61). Thus, molecular alterations preventing the function of the genes that underlie activation of senescence or apoptosis, or alterations enhancing the activity of DNA repair genes, may facilitate resistance to temozolomide (20,21,62). Temozolomide was shown to increase the expression of p53 in the present cells studied, and the expression of p21 in cells with wild-type p53. Temozolomide also promoted the expression of BAX, a proapoptotic gene, regardless of the molecular status of p53.

The combinatorial experiments with simultaneous treatment with APR-246 and temozolomide for 72 h again showed a CI corresponding to an additive effect, except in U87MG cells, where the effect was only additive when treated with the highest concentrations. LN405-TMZR cells also showed a slightly lower effect compared with LN405 cells, particularly when treated with higher concentrations, where the additive effect became antagonistic. The additive effect observed when cells were treated with a combination of APR-246 and temozolomide may be explained by the accumulation of p53 caused by APR-246 or temozolomide, by the accumulation of DNA damage caused by temozolomide or the oxidative stress caused by APR-246. Pretreatment with APR-246 followed by the post-treatment with temozolomide not only resulted in CI values closer to synergistic values compared with in the other treatment modalities, but an additive response was also observed with this treatment modality in U87MG cells. It is possible that the oxidative stress produced by APR-246 resulted in sensitization of cells to temozolomide. In this regard, there are several publications stating that the increase in oxidative stress produced pharmacologically may constitute a potential strategy of sensitization to temozolomide (63-65).

No marked increase in mRNA expression of the BAX and NOXA genes was produced by the combination of APR-246 and temozolomide, except in LN405 cells. This combination led to a greater decrease in colony formation compared with that produced by the individual treatments in A172 and MOG-G-CCM cells.

LN405, LN405-TMZR and T98G cells, on the other hand, were more affected in the colony formation assays in soft agar when treated with the double treatment compared with the individual treatments.

All triple drug combinations (APR-246/DZ-Nep/panobinostat, APR-246/DZ-Nep/temozolomide and APR-246/panobinostat/temozolomide), were found to be highly synergistic. The combination including the DZ-Nep/temozolomide pair, yielded high affected fractions and low CI indices. It is possible that in all drug combinations, each individual compound favored the induction of DNA damage (due to oxidative stress or methylation), which, together with joint overexpression of p53, resulted in apoptosis. Oxidative stress would be more relevant in mutant p53 cell lines, where the mutated p53 would not allow for activation of apoptosis.

In the present study, treatment of glioblastoma cells using a combination of APR-246 with DZ-Nep, panobinostat and/or temozolomide was evaluated. Combinations of the three drugs with APR-246 exerted an additive effect. APR-246, which did not possess p53 restorative capacity in the cell lines studied, was shown to exhibit effective antitumor activity, reducing clonogenicity and inducing apoptosis in glioblastoma cells, independently of the p53 status.

The majority of the cell lines showed a proapoptotic result, as desired, after treatments. But two of them, LN405-TMZR and MOG-G-CCM, provoked an antiapoptotic response after most treatments, and just had a proapoptotic result after a few treatments. Therefore, the proapoptotic ratio may cell line-dependent rather than treatment-dependent. LN405-TMZR and MOG-G-CCM are the cell lines that most increased the expression of the antiapoptotic genes, finally inducing an antiapoptotic ratio. LN405-TMZR is more resistant to temozolomide compared with MOG-G-CCM, and this might help induce the antiapoptotic response.

Some cell lines did not show the expression results that might be expected, as happened with Bcl-xL antiapoptotic marker in some combinations. This was also reported in our previous study (3). Certainly, a higher number of cell lines tested, including more cell lines derived from patients' tumors, would be highly recommended to further explore the antiapoptotic value of these compounds against glioblastoma. Toxicity and side effects of the drugs have not been studied in our *in vitro* experiments, therefore further *in vivo* studies are required.

Unlike our recently published study (3), the present study also tested APR-246, a drug that might revert the mutated p53 function in glioblastoma and that increases ROS (66). The changes induced by APR-246 in the cell lines might potentiate the effects of some other drugs in combination with APR-246.

The present results, together with those of our previous study on the synergistic effects of DZ-Nep, panobinostat and temozolomide (3), demonstrated that DZ-Nep combined with panobinostat produced the greatest synergistic effect in glioblastoma cells, followed by panobinostat and temozolomide (less synergistic) and DZ-Nep and temozolomide (slightly synergistic). The combinations of APR-246 with the three other compounds exerted an additive effect. However, these results are not conclusive. The compounds used have produced a positive response in glioblastoma cells by increasing apoptosis and reducing clonogenicity *in vitro*. The present study, as well as our previous study (3), only demonstrated that these combinations may achieve potential epigenetic regulation when combined with temozolomide and APR-246. Thus, corroborating *in vitro* and *in vivo* assays are required to

further confirm the potential applicability of these combinations. Additionally, the molecular mechanisms regulated by these combinations should be determined in order to further improve our understanding of the pathological processes which take place in glioblastoma cell lines.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JDLR, AU, BM, JAR, MAI and JSC conceived and designed the experiments. JdlR and AU performed the experiments. JdlR performed the statistical analysis and interpreted the data. IZ and MVZ contributed to the acquisition of clinical samples. JDLR, AU and JSC analyzed the data and drafted the manuscript. All authors contributed to revising the manuscript, and all authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by The Ethics Committee of the University of Navarra (approval no. CEI0502012). All samples were fully anonymized prior to accessing and patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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