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4-Demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyl-daunorubicin (PNU-159548): A promising new candidate for chemotherapeutic treatment of osteosarcoma patients

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Abstract

The effectiveness of the alkycycline 4-demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyl-daunorubicin (PNU-159548, ladirubicin), a new drug with high antitumour activity against a broad range of neoplasms, was evaluated by using a panel of 32 human osteosarcoma cell lines, including cell lines resistant to doxorubicin, methotrexate, or cisplatin. PNU-159548 resulted to be highly active in all cell lines. No cross-resistance was found with conventional drugs, being PNU-159548 active also in cells resistant to doxorubicin and with a multidrug resistance phenotype (associated with *MDR1* gene/P-glycoprotein overexpression), as well as in cells resistant to methotrexate or to cisplatin. Analysis of drug–drug interactions showed that PNU-159548 could be successfully used in combination with all the most important drugs currently used in OS chemotherapy. In fact, the simultaneous administration of PNU-159548 and doxorubicin, methotrexate, or cisplatin produced mostly additive or synergistic effects. Sequential exposure to PNU-159548 followed immediately by doxorubicin, methotrexate, or cisplatin was the most effective sequence of administration, invariably resulting in additive or synergistic effects in both drug-sensitive and drug-resistant osteosarcoma cell lines. In conclusion, the high *in vitro* effectiveness, the absence of cross-resistance with doxorubicin, methotrexate, or cisplatin and the possibility to be successfully used in combination with these drugs indicate PNU-159548 as a promising candidate to be considered for planning new therapeutic regimens for osteosarcoma patients, who show a decreased response to conventional chemotherapy.

Keywords: Osteosarcoma; Drug resistance; P-glycoprotein; Alkycyclines; Anthracyclines; Chemotherapy

1. Introduction

Osteosarcoma (OS), the most common primary malignant tumour of bone, is characterised by an extremely aggressive clinical course with rapid development of metastases in 40–50% of patients [1–4]. Although the adoption of adjuvant and neoadjuvant chemotherapy has significantly improved the prognosis of patients with this tumour, a considerable number of OS patients develop drug resistance and die because of the disease progression [4–6].

The current neoadjuvant chemotherapy protocols for high-grade OS are based on doxorubicin (DX), high-dose methotrexate (MTX), and *cis*-dichloro-diammine-platinum (CDDP), with the addition of ifosfamide in the post-operative phase [4–7]. Several clinical studies

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have demonstrated that inherent or acquired resistance to chemotherapeutic agents is the major obstacle to successful treatment of OS [4–6,8–10]. Therefore, treatments with agents that are able to circumvent the mechanisms responsible for drug resistance could become the basis for innovative therapeutic regimens aimed to increase the drug response rate and to improve the clinical outcome of OS patients.

Clinical evidence has shown that DX is the most important drug for high-grade OS chemotherapy [11–13]. Clinical unresponsiveness to DX is mostly determined by the overexpression of P-glycoprotein (a membrane protein encoded by the *MDR1* gene), which has been demonstrated to be one of the most important adverse prognostic factors for OS patients [14–17]. Preliminary trials with first- and second-generation resistance modifiers, aimed to overcome the P-glycoprotein-mediated drug resistance, have only been partially successful, revealing the need for an improvement in the ability of these drugs to completely revert the multidrug resistance (MDR) phenotype at non-toxic conditions [18].

A possible alternative to resistance modifiers is represented by novel antitumour compounds, like a new class of alkycyclines, which was shown to be active in several drug resistant tumours [19]. The lead compound of this new class of drugs is the 4-demethoxy-3′-deamino-3′aziridinyl-4′methylsulphonyl-daunorubicin (PNU-159548, ladirubicin), which has been demonstrated to be effective against tumour cells overexpressing P-glycoprotein or resistant to alkylating agents (including cisplatin and cyclophosphamide) or topoisomerase I and II inhibitors [20]. Moreover, *in vivo* studies have shown that PNU-159548 has a significantly lower cardiotoxicity than DX, further proposing this drug as a possible candidate for overcoming drug resistance and collateral toxicity of DX [19–21]. On the basis of this pre-clinical evidence, PNU-159548 has been included in Phase I and II clinical studies.

So far, no data have been reported about PNU-159548 effectiveness in OS cells. Therefore, aim of this study was the pre-clinical assessment of the *in vitro* efficacy of this new agent on drug-sensitive and drug-resistant human OS cell lines, together with the evaluation of its interactions with the drugs that are most commonly used in OS chemotherapy.

2. Materials and methods

2.1. Drugs

DX, MTX and CDDP were purchased from Sigma–Aldrich (Milan, Italy). PNU-159548 was synthesised by Pharmacia Corporation (Milan, Italy). Stock solutions of DX (2 mg/ml), MTX (25 mg/ml), and CDDP (500 μg/ml) were stored at 4 °C. PNU-159548 was dissolved in absolute ethanol and stock solution aliquots (1 mg/ml) were stored at −80 °C. For all the drugs, working concentrations were prepared by diluting stock solutions in culture medium immediately before use.

2.2. Cell lines

The *in vitro* effectiveness of PNU-159548 was assessed on a panel of 10 human OS cell lines and 22 drug-resistant variants (Table 1). The human OS cell lines U-2OS, Saos-2 and MG-63 were obtained from the ATCC (Rockville, MD), whereas the other seven human OS cell lines were established from clinical specimens obtained from untreated OS patients at the Laboratorio di Ricerca Oncologica of the Istituti Ortopedici Rizzoli (Bologna, Italy) [22]. Drug-resistant variants were obtained by exposing the drug-sensitive U-2OS and Saos-2 cell lines to *in vitro* stepwise increased concentrations of DX [23,24], MTX [25,26], or CDDP. *In vitro* continuous exposure to each drug resulted in the selection of a series of drug-resistant variants that were identified with the name of the parental cell line, followed by the name and concentration of the drug that was used for the selection. Drug concentrations refer to ng/ml or, when clearly specified, to μg/ml (Table 1). All cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM), supplemented with penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen Italia, Milan, Italy) and 10% heat-inactivated fetal bovine serum (FBS; Biowhittaker Europe, Cambrex-Verviers, Belgium). Each drug resistant variant was continuously cultured in presence of the drug concentration used for the selection. All cell lines were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. In vitro cytotoxicity

In the first set of experiments, the *in vitro* effectiveness of PNU-159548 was determined on the 10 drug-sensitive cell lines by assessment of growth inhibition after 96 h of drug exposure at dosages (0.01–0.03 μM) that were significantly lower than the PNU-159548 plasma levels that had been reached in Phase I clinical studies [27,28], and compared with those of equimolar concentrations of DX.

In addition, for each cell line, the sensitivity to the different drugs was calculated from dose response curves and expressed as IC50 (drug concentration resulting in 50% inhibition of cell growth after 96 h of drug treatment). To determine the IC50 values, 20,000 cells/cm^2 were seeded in IMDM 10% FBS and after 24 h medium was changed with IMDM with 10% FBS without (control) or with different drug concentrations. After 96 h, cells were harvested and counted by Trypan blue dye exclusion to estimate the

Table 1
Panel of human osteosarcoma cell lines considered for the assessment of *in vitro* effectiveness of PNU-159548

Cell line	Origin[a]	Gender/age of patient (years)	IC50 ng/ml (μM) relative to selection drug	Fold-increase in drug resistance compared to parental cell line
U-2OS	OS			
Saos-2	OS			
MG-63	OS			
IOR/OS9	BM obl. OS	Male/15		
IOR/OS10	PRI fbl. OS	Female/10		
IOR/OS14	PRI obl. OS	Male/14		
IOR/OS15	PRI obl. OS	Female/12		
IOR/OS18	LM obl. OS	Male/33		
IOR/MOS	PRI obl. OS	Female/13		
SARG	PRI OS	Male/25		
DX-resistant variants				
U-2OS/DX30	U-2OS		79.9 (0.14)	14
U-2OS/DX100	U-2OS		319.5 (0.55)	55
U-2OS/DX580	U-2OS		1827.0 (3.15)	315
Saos-2/DX30	Saos-2		535.9 (0.92)	70
Saos-2/DX100	Saos-2		788.9 (1.36)	102
Saos-2/DX580	Saos-2		2529.4 (4.36)	328
MTX-resistant variants				
U-2OS/MTX3	U-2OS		11.9 (0.03)	3
U-2OS/MTX30	U-2OS		72.3 (0.16)	21
U-2OS/MTX100	U-2OS		240.9 (0.53)	69
U-2OS/MTX300	U-2OS		471.0 (1.04)	135
Saos-2/MTX30	Saos-2		198.1 (0.44)	15
Saos-2/MTX100	Saos-2		316.1 (0.70)	24
Saos-2/MTX300	Saos-2		1454.2 (3.20)	109
Saos-2/MTX1 μg	Saos-2		3740.0 (8.23)	281
CDDP-resistant variants				
U-2OS/CDDP300	U-2OS		415.3 (1.38)	4
U-2OS/CDDP600	U-2OS		706.1 (2.35)	6
U-2OS/CDDP1 μg	U-2OS		1116.0 (3.72)	10
U-2OS/CDDP2 μg	U-2OS		2754.0 (9.18)	24
Saos-2/CDDP300	Saos-2		382.3 (1.27)	3
Saos-2/CDDP600	Saos-2		935.2 (3.12)	8
Saos-2/CDDP1 μg	Saos-2		1907.1 (6.35)	17
Saos-2/CDDP10 μg	Saos-2		21400.0 (71.30)	190

[a] OS, high-grade osteosarcoma; PRI, primary tumor; BM, bone metastasis; LM, lung metastasis; obl., osteoblastic; fbl., fibroblastic.

percentages of growth inhibition compared to the appropriate control. The IC50 values were calculated by taking into consideration at least three independent experiments.

2.4. Drug–drug interactions

To investigate the *in vitro* interactions between PNU-159548 and the conventional drugs used in OS chemotherapy, human OS cell lines were treated with different regimens of drug combinations.

In the experiments of simultaneous drug exposure, cell lines were treated with different two-drugs combinations, consisting in the simultaneous administration of PNU-159548 together with DX, MTX or CDDP. In these experiments all drugs were used at equitoxic concentrations, corresponding to dosages resulting in 30% growth inhibition (IC30) after 96 h of single drug treatment.

In the experiments of drug sequence evaluation, OS cells were sequentially exposed for a total of 96 h to equitoxic concentrations of PNU-159548 and then, after its removal, to DX, MTX or CDDP. The same schedule was repeated by exposing cells to one of the conventional drugs followed by PNU-159548. In these experiments all drugs were used at equitoxic concentrations, corresponding to dosages resulting in 30% growth inhibition (IC30) after 48 h of single drug treatment.

To define how PNU-159548 interacted with the other drugs (in terms of synergism, antagonism or additivity), the resulting inhibition of cell growth was compared with that obtained in single-drug administration experiments. The combined effects of

each two-drugs combination was analysed by using the median effect plot method [29], and by calculating the coefficient of drug interaction (CI) with the following equation:

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2},$$

where $(Dx)_1$ and $(Dx)_2$ are, respectively, the doses of drug 1 and drug 2, which are required to produce an x% effect alone; $(D)_1$ and $(D)_2$ are, respectively, the doses of drug 1 and drug 2, which are required to produce the same x% effect in combination. When $CI = 1$ the interaction was considered additive; when $CI < 1$ the interaction was considered synergistic; when $CI > 1$ the interaction was considered antagonistic.

2.5. Cell cycle analysis

Assessment of the PNU-159548 effects on cell cycle was performed by seeding 20,000 cells/cm^2 in IMDM 10% FBS. After 24 h, medium was changed with IMDM 10% FBS without (control) or with the PNU-159548-IC50 concentration of each cell line. After 24, 48 and 72 h of drug treatment, cells were incubated with 10 μM bromodeoxyuridine (Sigma–Aldrich) for 1 h in a humidified 5% CO_2 atmosphere at 37 °C, harvested and finally fixed in 70% ethanol for 30 min. After DNA denaturation with 2 N HCl, cells were processed for indirect immunofluorescence staining with the B44 anti-bromodeoxyuridine monoclonal antibody (Becton Dickinson, San Jose, CA) diluted 1:4, followed by an anti-mouse FITC antibody (Sigma–Aldrich) diluted 1:20 in phosphate buffered saline solution. For the simultaneous determination of DNA content, cell suspensions were stained with 20 μg/ml propidium iodide (Sigma–Aldrich). All samples were analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

2.6. Apoptosis assays

For the detection and quantification of apoptosis, 10,000 cells/cm^2 cells were seeded in IMDM 10% FBS in two series of 60 mm Petri dishes. After 24 h, medium was changed with IMDM 10% FBS without (control) or with the PNU-159548-IC50 concentration of each cell line. For the morphological assessment of apoptotic nuclei after 24, 48, and 72 h of drug treatment, cells of the first series of dishes were fixed in methanol:acetic acid (3:1 vol:vol) for 15 min and then stained with 50 ng/ml Hoechst 33258 (Sigma–Aldrich). Cell nuclei showing three or more bodies of chromatin addensation were considered as apoptotic. For each sample, the percentage of apoptotic nuclei was determined by counting 1000 cells.

In addition to the morphological assessment of apoptotic nuclei, detection and quantification of apoptotic cells was performed by the annexin-V assay on the second series of dishes. This assay, which is based on the flow cytometric analysis of annexin-V-FITC labelling on harvested cell suspensions, was performed by using the MBL MEBCYTO Apoptosis kit (Medical and Biological Laboratories, Naka-ku Nagoya, Japan) by following the manufacturer's instructions. Staining with 20 μg/ml propidium iodide (Sigma–Aldrich) was used to discriminate necrotic cells (which showed simultaneous annexin-V-FITC and DNA staining) from apoptotic cells (which showed only annexin-V-FITC staining).

2.7. Intracellular uptake of DX and PNU-159548

By taking advantage from the natural red fluorescence of DX and PNU-159548, the intracellular drug uptake was evaluated and quantified on all the drug-sensitive cell lines, as well as on the U-2OS or Saos-2 variants with the highest level of resistance to DX, MTX or CDDP. To define the optimal conditions for PNU-159548 incorporation, suspensions of 200,000 cells/ml IMDM 10% FBS from U-2OS and Saos-2 cell lines were incubated with concentrations of PNU-159548 ranging from 0.01 to 0.20 μM at 37 °C for different times (from 15 min to 4 h). After drug incorporation, cell suspensions were incubated with 1 mg/ml fluorescein diacetate (Sigma–Aldrich) for 10 min at 4 °C, washed with phosphate-buffered saline solution (PBS) and immediately analysed with a fluorescence microscope. Drug incorporation was evaluated only in living cells, which were identified by the fluorescein diacetate green fluorescence, as previously described for DX uptake and efflux analyses [30,31]. Optimal conditions to estimate PNU-159548 incorporation were fixed at 0.08 μM PNU-159548 for 30 min at 37 °C, which invariably resulted in less than 3% cell death. Therefore, for each cell line, the amount of intracellular drug incorporation was assessed and quantified by flow cytometry (FACSCalibur; Becton Dickinson) after incubation of cell suspensions with 0.08 μM PNU-159548 or DX at 37 °C for 30 min.

To analyse the pattern of intracellular distribution of DX and PNU-159548, drug incorporation was performed on both cell suspensions and adherent cells. In the latter case, 10,000 cells/cm^2 cells were seeded in 60-mm Petri dishes with IMDM 10% FBS. After 24 h, medium was changed with 0.08 μM PNU-159548 or DX and cells were incubated for 30 min at 37 °C. After drug removal, cell layers were briefly washed with PBS and then immediately analysed with a fluorescence microscope equipped with a Photometrics Sensys charge-coupled device (CCD) camera (QUIPS XL Genetic Workstations; Abbott-Vysis Inc., Downers Grove, IL). The same fluorescence microscopy facilities were employed to analyse the pattern of intracellular drugs distribution in cell suspensions.

2.8. Statistical analyses

Differences among averages were analysed using a two-sided Student's *t*-test.

The IC50 value for each drug was calculated from the linear transformation of its dose–response curve. For each drug, the relative resistance index of each drug-resistant variant was expressed as the ratio of its IC50 value to the IC50 value of the corresponding parental cell line.

3. Results

3.1. In vitro cytotoxicity

A first comparison between the *in vitro* efficacy of PNU-159548 and DX was performed by using drug concentrations of 0.01 and 0.03 μM, which were at least ten times lower than the PNU-159548 plasma levels that had been reached in Phase I clinical studies at the Phase II selected dose [27,28]. All cell lines resulted to be highly sensitive to both PNU-159548 and DX, without any statistically significant difference between these two agents, despite the low drug concentrations used in these experiments (data not shown).

To better compare the efficacy of these two drugs, IC50 for both PNU-159548 and DX were determined for each cell line (Table 2). The comparison between PNU-159548- and DX-IC50 values confirmed that, in drug-sensitive OS cell lines, the two drugs exhibit a very similar *in vitro* activity. In fact, no statistically significant difference was found between the IC50 values for PNU-159548 (which ranged from 0.002 to 0.050 μM, with an average value of 0.022 μM) and those for DX (which ranged from 0.003 to 0.033 μM, with an average of 0.017 μM) (Table 2).

The determination of PNU-159548-IC50 values of drug-resistant variants revealed a remarkable sensitivity to PNU-159548 in all cell lines, with IC50 values ranging from 0.002 to 0.044 μM (average of 0.015 μM) (Table 3). In particular, PNU-159548 showed a similar activity in parental U-2OS or Saos-2 cell lines and in their DX- and MTX-resistant variants, without any statistically significant difference in their respective IC50 values (Table 3). CDDP-resistant variants resulted to be slightly less sensitive to PNU-159548 than parental cell lines and all the other drug-resistant variants, but still showed PNU-159548-IC50 values in the range of those of the other cell lines considered in this study (Tables 2 and 3). The difference between PNU-159548-IC50 values of CDDP-resistant variants and of the other cell lines never resulted to be statistically significant.

By considering together all these results, it appears that the average PNU-159548-IC50 value of all the cell lines tested in this study was pretty low (0.016 μM) and that there was any significant difference between the average PNU-159548-IC50 value of drug-sensitive (0.017 μM) and drug-resistant (0.015 μM) cell lines. This evidence indicates a general high effectiveness of PNU-159548 on OS cells and the lack of cross-resistance with conventional drugs.

Table 2
Comparison between sensitivity to PNU-159548 and DX in 10 drug-sensitive, human osteosarcoma cell lines

Cell line	IC50[a] ng/ml (μM)	
	PNU-159548	DX
U-2OS	1.4 (0.002)	5.8 (0.010)
Saos-2	4.1 (0.007)	7.7 (0.013)
MG-63	4.2 (0.007)	12.7 (0.022)
IOR/OS9	27.5 (0.046)	4.6 (0.008)
IOR/OS10	20.4 (0.034)	1.8 (0.003)
IOR/OS14	15.1 (0.025)	14.5 (0.025)
IOR/OS15	30.2 (0.050)	4.3 (0.017)
IOR/OS18	12.6 (0.021)	19.3 (0.033)
IOR/MOS	10.2 (0.017)	17.4 (0.030)
SARG	3.6 (0.006)	7.4 (0.013)

Data show the mean value of at least three different experiments.
[a] IC50, inhibitory concentration of 50% cell growth after 96 h of *in vitro* drug treatment.

Table 3
In vitro sensitivity to PNU-159548 of the 22 drug-resistant variants derived from U-2OS and Saos-2 human osteosarcoma cell lines

Cell line	IC50[a] ng/ml (μM)	Ratio to IC50 for PNU-159548 of correspondent parental cell line
DX-resistant variants		
U-2OS/DX30	2.0 (0.003)	1
U-2OS/DX100	2.5 (0.004)	2
U-2OS/DX580	5.6 (0.009)	4
Saos-2/DX30	5.4 (0.009)	1
Saos-2/DX100	5.5 (0.009)	1
Saos-2/DX580	8.2 (0.014)	2
MTX-resistant variants		
U-2OS/MTX3	1.3 (0.002)	1
U-2OS/MTX30	2.0 (0.003)	1
U-2OS/MTX100	2.6 (0.004)	2
U-2OS/MTX300	3.3 (0.005)	2
Saos-2/MTX30	4.0 (0.007)	1
Saos-2/MTX100	6.7 (0.011)	2
Saos-2/MTX300	8.3 (0.014)	2
Saos-2/MTX1 μg	9.3 (0.015)	2
CDDP-resistant variants		
U-2OS/CDDP300	10.2 (0.017)	7
U-2OS/CDDP600	11.3 (0.019)	8
U-2OS/CDDP1 μg	11.4 (0.019)	8
U-2OS/CDDP2 μg	12.1 (0.020)	9
Saos-2/CDDP300	14.6 (0.024)	4
Saos-2/CDDP600	21.5 (0.036)	5
Saos-2/CDDP1 μg	22.3 (0.037)	5
Saos-2/CDDP10 μg	26.3 (0.044)	6

Data show the mean value of at least two different experiments.
[a] IC50, inhibitory concentration of 50% cell growth after 96 h of *in vitro* drug treatment.

3.2. Drug combination analyses

The *in vitro* efficacy of PNU-159548 in combination with DX, MTX or CDDP was evaluated after either simultaneous or sequential two-drugs exposure. As shown in Table 4, simultaneous exposure of drug-sensitive cell lines to PNU-159548 and to one of the conventional drugs produced mostly additive or synergistic interactions. Only few antagonistic interactions were observed between PNU-159548 and DX (IOR/OS14 cell line), PNU-159548 and MTX (U-2OS, Saos-2 and SARG cell lines), or PNU-159548 and CDDP (IOR/OS18 cell line).

In the group of drug-resistant variants, simultaneous two-drugs exposure was performed using PNU-159548 together with the drug of selection. In DX-resistant variants, simultaneous exposure to PNU-159548 and DX resulted in a synergistic (U-2OS variants) or additive (Saos-2 variants) effect (Table 4). An additive effect was also found when PNU-159548 was used in association with CDDP in CDDP-resistant variants, whereas in MTX-resistant variants only antagonistic effects were observed after simultaneous treatment with PNU-159548 and MTX (Table 4).

Table 4
Effects of the simultaneous administration of equitoxic concentrations (corresponding to the IC30 for each cell line) of PNU-159548 and DX, MTX or CDDP evaluated by growth inhibition analyses and the median effect plot method

Cell line	Combined effects of PNU-159548 with:		
	DX	MTX	CDDP
U-2OS	ADD	*ant*	ADD
Saos-2	ADD	*ant*	ADD
MG-63	ADD	ADD	ADD
IOR/OS9	ADD	ADD	ADD
IOR/OS10	ADD	SYN	ADD
IOR/OS14	*ant*	SYN	ADD
IOR/OS15	ADD	ADD	ADD
IOR/OS18	SYN	ADD	*ant*
IOR/MOS	SYN	SYN	ADD
SARG	ADD	*ant*	ADD
DX-resistant variants			
U-2OS/DX30	SYN		
U-2OS/DX100	SYN		
U-2OS/DX580	SYN		
Saos-2/DX30	ADD		
Saos-2/DX100	ADD		
Saos-2/DX580	ADD		
MTX-resistant variants			
U-2OS/MTX3		*ant*	
U-2OS/MTX30		*ant*	
U-2OS/MTX100		*ant*	
U-2OS/MTX300		*ant*	
Saos-2/MTX30		*ant*	
Saos-2/MTX100		*ant*	
Saos-2/MTX300		*ant*	
Saos-2/MTX1 μg		*ant*	
CDDP-resistant variants			
U-2OS/CDDP300			ADD
U-2OS/CDDP600			ADD
U-2OS/CDDP1 μg			ADD
U-2OS/CDDP2 μg			ADD
Saos-2/CDDP300			ADD
Saos-2/CDDP600			SYN
Saos-2/CDDP1 μg			ADD
Saos-2/CDDP10 μg			ADD

ADD, additive; SYN, synergistic; *ant*, antagonistic.

Sequential exposure experiments were done by using as first drug the PNU-159548 followed immediately by DX, MTX, or CDDP, as well as by using the reversed order of drug exposure (DX, MTX, or CDDP as first drug followed by PNU-159548). Analyses of sequential drug exposure were performed on parental cell lines (U-2OS and Saos-2) and on their drug-resistant variants with the highest level of resistance to DX, MTX or CDDP. In drug-resistant variants, the determination of CI was performed by treating the cells with PNU-159548 and the drug of selection. As shown in Table 5, in both drug-sensitive and drug-resistant cell lines, additive or synergistic effects were observed when cells were treated with PNU-159548 as first drug followed immediately by DX, MTX or CDDP. The reversed order of drug exposure produced an entirely different picture, always resulting in an antagonistic interaction, with the only exception of an additive effect with DX in drug-sensitive cell lines (Table 5).

3.3. Cell cycle perturbations and apoptosis

The analysis of cell cycle phase perturbations induced by PNU-159548 after different exposure times were examined in all drug-sensitive OS cell lines, as well as in the U-2OS and Saos-2 variants with the highest level of resistance to DX, MTX or CDDP. Treatment of cell lines with their respective IC50 dose of PNU-159548 induced an accumulation of cells in the G2/M phases, with a parallel decrease in the percentage of cells in G0/G1 phases (Fig. 1). The peak of G2/M accumulation was reached after 48–72 h of drug treatment, whereas shorter incubation times did not produce any evident effect on cell cycle phase distribution. These data suggest that, in OS cells, PNU-159548 induces both a partial blockage of cells in G2/M phase and a delay of cell progression from G1 to G2-M rather than an accumulation in the S phase, differently from findings reported in other experimental models [19].

Like described for cell cycle analysis, also the effects of PNU-159548 on apoptosis was evaluated after treatment with dosages corresponding to the IC50 of each cell line. Morphological evaluation of apoptosis after nuclear staining with Hoechst 33258 did not show any statistically significant increase in the percentage of apoptotic cells compared to the appropriate controls, neither in drug-sensitive nor in drug-resistant cell lines

Table 5
Effects of the sequential administration of equitoxic concentrations (corresponding to the IC30 after 48 h of drug treatment for each cell line) of PNU-159548 and DX, MTX or CDDP

Cell line	Drug sequence					
	PNU-159548 ⇓ DX	PNU-159548 ⇓ MTX	PNU-159548 ⇓ CDDP	DX ⇓ PNU-159548	MTX ⇓ PNU-159548	CDDP ⇓ PNU-159548
U-2OS	ADD	ADD	ADD	ADD	*ant*	*ant*
Saos-2	ADD	ADD	ADD	ADD	*ant*	*ant*
DX-resistant variants						
U-2OS/DX100	SYN			*ant*		
U-2OS/DX580	SYN			*ant*		
Saos-2/DX100	ADD			*ant*		
Saos-2/DX580	ADD			*ant*		
MTX-resistant variants						
U-2OS/MTX100		ADD			*ant*	
U-2OS/MTX300		ADD			*ant*	
Saos-2/MTX300		ADD			*ant*	
Saos-2/MTX1 μg		ADD			*ant*	
CDDP-resistant variants						
U-2OS/CDDP300			ADD			*ant*
U-2OS/CDDP1 μg			ADD			*ant*
U-2OS/CDDP300			ADD			*ant*
Saos-2/CDDP1 μg			ADD			*ant*

Drug treatment was performed for a total of 96 h and drug interactions were evaluated by growth inhibition analyses and the median effect plot method.
ADD, additive; SYN, synergistic; *ant*, antagonistic.

(data not shown). In agreement with that, also the annexin-V assay did not detect any induction of cell apotosis or necrosis (data not shown).

Taken together, cell cycle and apoptosis data indicate that, under these experimental conditions, PNU-159548 exerts a cytostatic rather than a cytotoxic effect in both drug-sensitive and drug-resistant OS cells.

3.4. Intracellular drug uptake

Analysis of intracellular drug uptake by flow cytometry showed that all cell lines incorporated PNU-159548 in the vast majority of cells (Fig. 2A). Evaluation of DX incorporation showed similar results, with the only exception of DX-resistant variants, in which the percentage of DX-incorporating cells was significantly lower than in all the other cell lines ($P < 0.05$; Fig. 2A). Flow cytometric measurement of drug incorporation showed similar intracellular amounts of PNU-159548 in all cell lines (Fig. 2B). Quantification of DX incorporation showed similar results, with only a slightly, statistically not significant decreased intracellular drug accumulation in DX-resistant cell lines. However, it has to be considered that, in DX-resistant variants, data concerning the intracellular levels of DX derived from the few DX-incorporating cells.

Analysis of drugs intracellular distribution by fluorescence microscopy showed that all cell lines presented both cytoplasmic and nuclear uptake of PNU-159548, whereas DX intracellular distribution appeared to be mostly nuclear in all the drug-sensitive and drug-resistant cell lines, with the exception of the P-glycoprotein-over-expressing DX-resistant variants, which accumulated the drug in the cytoplasm and not in the nucleus. To better describe these results, representative patterns of intracellular DX- and PNU-159548 incorporation are shown in Fig. 3. In particular, Fig. 3A shows the nuclear accumulation of DX in the U-2OS parental cell line, which is not present in its P-glycoprotein-over-expressing DX-resistant U-2OS/DX580 variant (Fig. 3B). Differently from DX, the intracellular uptake of PNU-159548 did not differ between the U-2OS (Fig. 3C) and the U-2OS/DX580 (Fig. 3D) cell lines, being the drug accumulated in both nucleus and cytoplasm.

Taken together, these data indicate that, differently from DX, PNU-159548 intracellular accumulation and distribution is not influenced or reduced by the presence of high levels of P-glycoprotein.

4. Discussion

Clinical evidence has shown that anthracyclines are the most important drugs for high-grade OS chemotherapy. In particular, DX has gained a major place in OS treatment, although toxic side effects (mainly a dose-related cardiomyopathy) and inherent or acquired drug

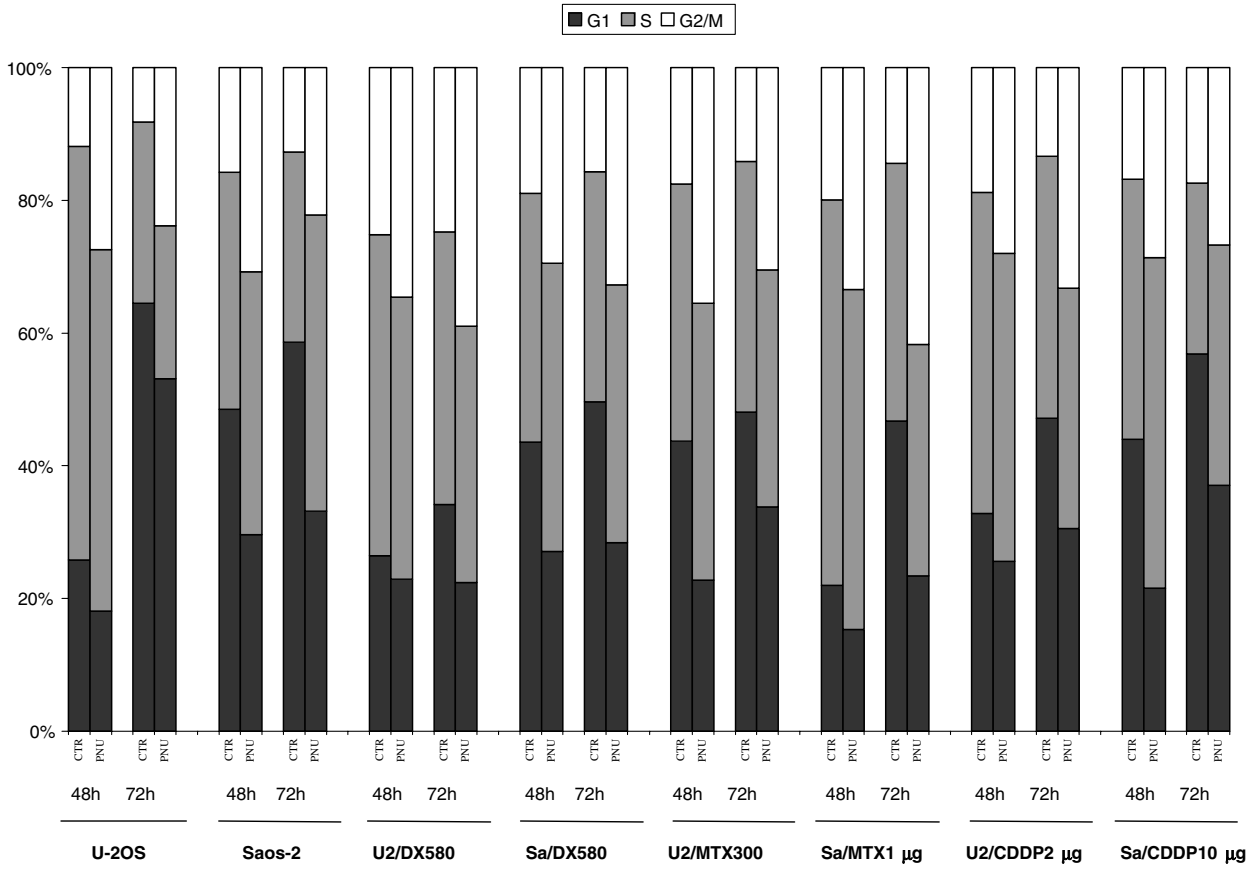

Fig. 1. Cell cycle phase perturbations induced by equitoxic doses of PNU-159548 (corresponding to the IC50 concentration of each cell line) in U-2OS and Saos-2 human osteosarcoma cell lines, and in their variants with the highest level of resistance to DX, MTX or CDDP. All the other drug-sensitive human osteosarcoma cell lines showed cell cycle phase distributions very similar to those of U-2OS and Saos-2 cell lines (data not shown in this figure). Data refer to the percentage of cells for each cell cycle phase and are representative of at least two different experiments.

resistance (mostly due to increased levels of the *MDR-1* gene-product P-glycoprotein) limit its clinical efficacy [11–13,32]. Since anthracyclines are widely used, not only for the treatment of OS but also of many other human tumours, in the past ten years extensive research has been directed toward means of reducing toxicity and increasing activity of this class of powerful drugs. One of the strategies that has been used is the synthesis of new anticancer agents through the modification of the anthracycline chemical structure.

PNU-159548 (ladirubicin) is the prototype of a novel class of anthracycline derivatives with a dual mode of action: it intercalates into DNA and alkylates guanine at N^7 position and adenine in N^3 position in the DNA major groove [33]. The pattern of *in vitro* and *in vivo* activity of PNU-159548 has been studied in murine and human tumours, together with its pre-clinical pharmacokinetic and toxicological profile [19,20]. So far, *in vitro* studies have shown that PNU-159548 is active against a variety of murine and human tumour cell lines, including rapidly proliferating murine leukaemias and human colon, ovarian, and prostatic carcinoma cell lines [19,20]. *In vivo*, PNU-159548 has been resulted to be highly active against human ovarian, breast, pancreatic, colon, epidermoid, gliobastoma and small lung cancer xenografts [19].

The toxicological profile of PNU-159548 has been pre-clinically defined in mice, rats, and dogs, and target organs were identified after single and repeated-cyclic-dose administrations [19]. The collateral toxic effects mainly consisted in myelosuppression, lymphoid organ cell depletion, and intestinal toxicity and resulted to be dose-related and reversible [19]. Moreover, it is interesting to note that in animals, at equimyelotoxic doses, PNU-159548 was remarkably less cardiotoxic than DX [19]. In agreement with that, Phase I studies on patients with a variety of solid tumours did not reveal any significant cardiotoxicity of PNU-159548 at doses ranging from 1 to 16 mg/m^2, resulting in a peak plasma concentration (C_{max}) ranging from 0.5 to 1.5 µM [27,28]. The combination of intercalating and alkylating activities within the same molecule, without the cardiotoxic side effects of anthracyclines, has therefore indicated PNU-159548 as excellent candidate for clinical development in oncology, with particular regard to those neoplasms in which, like in OS, anthracyclines

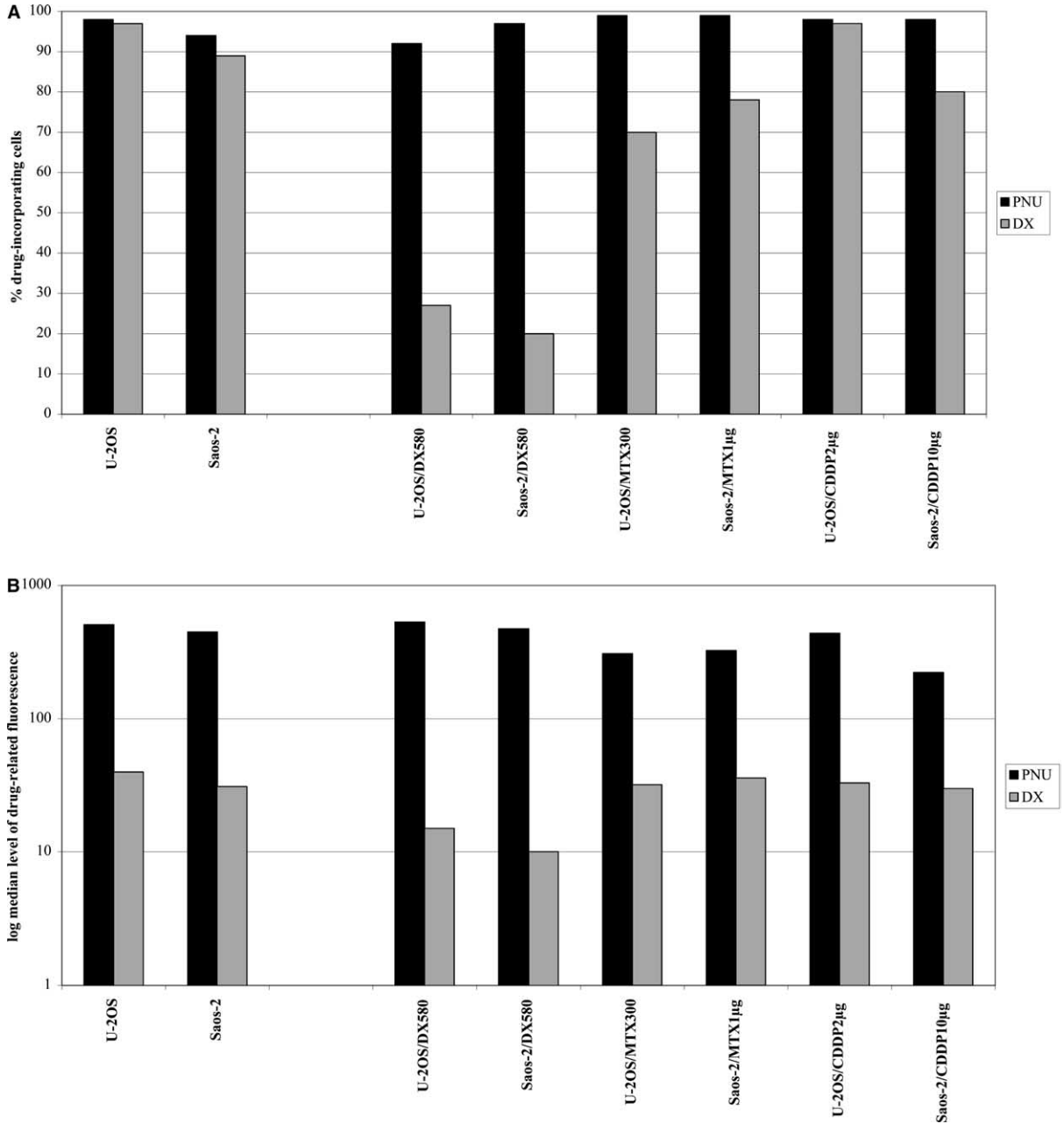

Fig. 2. Incorporation of doxorubicin and PNU-159548 in U-2OS and Saos-2 human osteosarcoma cell lines and in their variants with the highest level of resistance to DX, MTX or CDDP. (A) The percentage of incorporating cells; (B) the intracellular drug content in drug-incorporating cells after incubation with 0.08 μM PNU-159548 or DX. All the other drug-sensitive human osteosarcoma cell lines showed results which were very similar to those of U-2OS and Saos-2 cell lines (data not shown in this figure). Data are representative of at least three different experiments.

play a key role in the chemotherapeutic treatment of the disease.

So far, no data have been reported concerning the PNU-159548 activity in OS. To get some insight into the efficacy of PNU-159548 in OS cells, we have investigated its cytotoxicity and effectiveness in a large panel of drug-sensitive and drug-resistant human OS cell lines. In our experimental models, PNU-159548 resulted to be very effective in both drug-sensitive and drug-resistant cell lines. Of particular interest was the finding that this drug retained its efficacy also in DX-resistant OS cell lines, which present a MDR phenotype as a consequence of *MDR-1* gene amplification/over-expression and increased levels of P-glycoprotein [23,24]. According to these results, the intracellular uptake of PNU-159548 was not influenced by the presence of high levels of P-glycoprotein, being the PNU-159548 able to accumulate at the same extent in both drug-sensitive and DX-resistant cell lines. These data indicate that the simultaneous presence of an anthracycline backbone and an

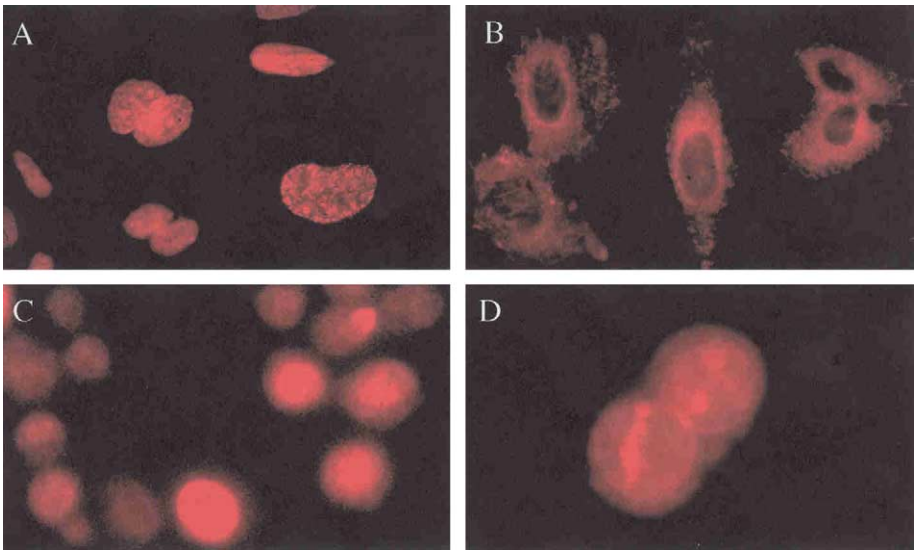

Fig. 3. Pattern of intracellular incorporation of DX and PNU-159548 in drug-sensitive and DX-resistant osteosarcoma cells. (A) Incorporation of DX and (C) PNU-159548 in U-2OS cell line. (B) Incorporation of DX and (D) PNU-159548 in U-2OS/DX580 cell line. Original magnification: A–C, 400×; D, 1000×.

alkylating moiety inside the PNU-159548 molecule may lead to mechanisms of action and resistance different from those of anthracyclines.

In our experimental models, PNU-159548 resulted to be active also against OS cell lines resistant to MTX or CDDP, with an efficacy that was similar to that found in drug-sensitive cell lines. These data indicate the lack of cross-resistance mechanisms between PNU-159548 and MTX or CDDP, which are, together with DX, the most widely used anticancer agents for OS chemotherapy. Of particular interest was also the evidence that all the OS cell lines exhibited PNU-159548 IC50 values that were at least ten times lower than the PNU-159548 C_{max}, which has been reached in Phase I clinical trials [27,28], suggesting that the treatment of OS patients with PNU-159548 may be feasible and effective at clinically usable dosages.

To verify the possibility of effectively combining PNU-159548 with conventional anticancer drugs, we investigated the interactions between PNU-159548 and conventional drugs (DX, MTX and CDDP) on both drug-sensitive and drug-resistant cell lines. The reason for assessing drug combination efficacy also on drug-resistant variants was that they may more closely resemble the clinical situation of OS patients who are refractory to conventional treatments, and therefore give useful information concerning the best *in vitro* scheme of drug administration, which should be taken into consideration for planning innovative treatment schedules for drug-unresponsive OS patients.

Simultaneous exposure to PNU-159548 and DX, MTX or CDDP of drug-sensitive OS cell lines mostly resulted in additive or synergistic interactions. Data obtained in drug-resistant variants showed that PNU-159548 could be efficiently used in combination with DX or CDDP, but not with MTX. In fact, simultaneous administration of PNU-159548 and DX or CDDP resulted in additive or synergistic effects in cells resistant to these two drugs, whereas in MTX-resistant variants the combination of PNU-159548 and MTX led invariably to antagonistic effects.

Only few data are available so far to explain the possible reasons for these different drug combination effects. The ability of PNU-159548 to intercalate and alkylate DNA may explain its positive interaction with other DNA-targeting agents with different mechanisms of action like DX or CDDP, also in cells that are resistant to these drugs.

The antagonistic interaction of PNU-159548 with MTX, which was observed in three drug-sensitive cell lines and in all MTX-resistant variants, may be partly explained by the effects of PNU-159548 on cell cycle. In fact, MTX efficacy is strictly related to the presence of actively growing cells [34]. Therefore, the partial blockage of cell cycle induced by PNU-159548, which was more pronounced in the three drug-sensitive cell lines in which the interaction with MTX was antagonistic compared to the others, may be partly responsible for the simultaneous reduction of MTX effectiveness. Since the same effects of PNU-159548 on cell cycle were also present in MTX-resistant variants, the simultaneous administration of both drugs may have completely abrogated the low effectiveness that MTX still maintains in these cells, resulting in the observed antagonistic effect.

Additional interesting findings derived from the experiments of sequential two-drugs exposure. The highest *in vitro* efficacy was reached when PNU-159548 was

used as first drug followed immediately by DX, MTX or CDDP. These administration sequences invariably led to additive or synergistic effects in both drug-sensitive and drug-resistant cell lines. These results may be explained by the high sensitivity to PNU-159548 of both drug-sensitive and drug-resistant cell lines: the relevant drug response observed in all cell lines after exposure to PNU-159548 may also be responsible for a subsequent enhanced sensitivity or reduced resistance of the same cells to conventional drugs. In other words, it is possible that effectiveness of conventional drugs is potentiated on cells that have been previously damaged by the DNA alkylation activity of PNU-159548.

On the other hand, it has to be taken into account that, in drug resistant variants, all the other drug administration sequences resulted to have antagonistic effects. This evidence suggests that a first exposure to the drug against which these cells are resistant negatively affects or compromises their subsequent sensitivity to PNU-159548 through mechanisms that still remain to be determined.

In conclusion, the new anticancer agent PNU-159548 has shown strong activity in a large panel of drug-sensitive and drug-resistant human OS cell lines. These findings, together with the observed absence of cross-resistance with DX, MTX or CDDP and the possibility to be successfully used in combination with these drugs, indicate that PNU-159548 may be considered as a promising candidate for planning new therapeutic regimens for OS patients, although the treatment protocols should be carefully designed on the basis of our preclinical evidence. Moreover, data obtained from drug interaction analyses support the clinical use of PNU-159548 together with conventional drugs also in OS patients who are unresponsive to standard chemotherapy, and may therefore be considered of potential clinical utility for new recovery treatments.

Conflict of interest

Authors disclose any financial and personal relationships with other people or organisations that could have inappropriately influenced or biased their work.

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