

Purine analogs sensitize the multidrug resistant cell line (NCI-H460/R) to doxorubicin and stimulate the cell growth inhibitory effect of verapamil

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Summary The resistant cell line NCI-H460/R and its counterpart NCI-H460 were used to investigate the ability of purine analogs to overcome multidrug resistance (MDR) that seriously limit the efficacy of lung cancer regimens with chemotherapeutic agents. Two purine analogs, sulfinosine (SF) and 8-Cl-cAMP, exerted dose-dependent effects on cell growth in both parental and resistant cell lines. They significantly decreased *mdr1* expression in NCI-H460/R cells. Low concentrations (1 μ M) of SF and 8-Cl-cAMP in combination with doxorubicin (DOX) exerted synergistic growth inhibition in both cell lines. Pretreatment with SF and 8-Cl-cAMP improved the sensitivity to DOX more than verapamil (VER), the standard modulator of MDR. The increased accumulation of DOX observed after the treatment with SF and 8-Cl-cAMP was consistent with the results obtained with VER. VER stimulated the effect of 8-Cl-cAMP on DOX cytotoxicity and *mdr1* expression. Combinations of either SF or 8-Cl-cAMP with VER at clinically acceptable concentrations exhibited synergistic effects on cell growth inhibition in the resistant cell line. SF and 8-Cl-cAMP modulated MDR in NCI-H460/R cells, especially when applied before DOX administration. This feature, together with their ability to reverse MDR, renders the purine analogs (in combination with VER) as potential candidates for improving the clinical activity of existing lung cancer therapeutics.

Keywords Drug combination · Multidrug resistance · Sulfinosine · 8-Cl-cAMP · Doxorubicin · Verapamil

Introduction

MDR is the main cause of lung cancer treatment failure. The predictors of a poor outcome are increased expression of drug export proteins and drug inactivation as a result of increased enzyme activities [1]. MDR frequently correlates with overexpression of the ABC transporters in cell membranes that actively pump anticancer drugs out of cells, i.e. P-glycoprotein (P-gp) and/or MDR-associated protein 1 (MRP1) [2].

To obtain a better understanding of the mechanisms of drug resistance, many *in vitro*-selected cell lines have been produced in the presence of continuous or pulsed exposure to drugs [3–5]. A DOX-resistant cell line NCI-H460/R characterized previously [6] was employed in the present study. This cell line was established from large cell neuroendocrine carcinoma (LCNEC), the most aggressive form of non-small cell lung carcinoma (NSCLC).

Acquisition of the MDR phenotype includes additional mechanisms aside from the emergence of increased P-gp expression. The inhibition of transport proteins are prerequisites for the effective reversion of the MDR phenotype. The potential of synthetic compounds and compounds originating from plants for MDR reversion are under scrutiny [7–10].

Combinations of two or more drugs and/or adjustments of the dosage and administration schedule can improve the efficacy of cancer therapy. Combined therapy with anti-metabolites, including purine analogs, and DNA-damaging agents and topoisomerase II inhibitors has had good results in generally incurable malignancies such as lung and colon

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cancers and are opening new possibilities in treatments of solid tumors [11].

In the present work, we examined the effect of combinations of DOX with two purine analogs, SF and 8-Cl-cAMP, on human LCNEC cell lines: sensitive NCI-H460 and MDR-resistant NCI-H460/R lines. SF reverses DOX resistance in NCI-H460/R cells when applied alone [8] and in combination with curcumin [12]. 8-Cl-cAMP was shown to synergistically increase the growth-inhibitory effects of paclitaxel or cisplatin in a number of cell lines derived from human breast, lung, ovarian, colon, head carcinoma and melanoma [13, 14]. Also, 8-Cl-cAMP acts synergistically with 9-*cis*-RA, 13-*cis*-RA and all-*trans*-RA and inhibits the viability of Ewing's sarcoma CHP-100 cells [15]. A synergistic effect of SF and 8-Cl-cAMP was observed in the human neuroblastoma cell line [16].

SF (2-amino-9-beta-D-ribofuranosylpurine-6-sulfonamide) is a comparatively new anti-neoplastic agent. SF displays considerable anticancer activity against non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) cell lines. It inhibits cell growth and induces apoptosis *in vitro* [17]. Derivatives of SF are metabolized by the cell's glutathione system. The ready formation of adducts between SF and sulfhydryl compounds (glutathione and cysteine) is probably responsible for the observed lowering of glutathione levels by SF and subsequent induction of cell death. These findings have stimulated investigations of MDR reversal by SF in the LCNEC cell line [8, 12].

8-Cl-cAMP is a site-specific cAMP analog that selectively down-regulates the RI α subunit of PKA I, a signaling protein directly involved in various cellular functions, including cell proliferation, differentiation and neoplastic transformation and mediation of the mitogenic effects of different oncogenes and growth factors [18]. 8-Cl-cAMP is the first cAMP analog to have entered clinical trials in over 30 years of research [19]. The effect of 8-Cl-cAMP on the reduction of P-gp synthesis and *mdr1* mRNA levels was revealed in the MCF-7 breast cancer resistant line [20]. The authors proposed that activated PKA affected the promoter region of *mdr1*.

Little information is available concerning the sensitizing effects of SF and 8-Cl-cAMP and their interaction with DOX. In order to elucidate the potential of SF and 8-Cl-cAMP for MDR reversal, we examined the anti-proliferative effects of these two agents on sensitive NCI-H460 and resistant NCI-H460/R cells. We investigated whether SF and 8-Cl-cAMP inhibit *mdr1* mRNA synthesis and studied the interactions between SF and 8-Cl-cAMP with DOX. Different schedules of administration were also examined. Pretreatment with low concentrations of SF increased the sensitivity of NCI-H460/R cells to DOX to a greater degree than the conventional chemo-sensitizing agent VER. We describe for the first time the synergistic effects of the combinations of SF and VER and of 8-Cl-cAMP and VER. Our results are consistent with

the finding that the Ca²⁺ channel blocker VER significantly stimulates the actions of several anti-cancer drugs that are not directly affected by P-gp [21]. The obtained results suggest that SF and 8-Cl-cAMP have a potential for MDR reversal in the resistant NCI-H460/R cell line. This study provides a new approach for a combined treatment with SF and 8-Cl-cAMP based on the modulation of responsiveness to DOX.

Materials and methods

Drugs

SF ([R,S]-2-amino-9- β -D-ribofuranosylpurine-6-sulfonamide) was synthesized from 6-thioguanosine according to the published procedure [22] and kept at -20°C . DOX solution was obtained from EBEWE Arzneimittel GmbH, Vienna, Austria and kept at -20°C . 8-Cl-cAMP (8-chloroadenosine 3'5'-cyclic monophosphate) was purchased from the BIOLOG Life Science Institute, Bremen, Germany. VER was purchased from Sigma-Aldrich Chemie GmbH, Germany and kept at room temperature. SF and VER were diluted in water before use.

Chemicals

RPMI 1640 medium, the antibiotic-antimycotic solution, L-glutamine and trypsin/EDTA were purchased from PAA, Vienna, Austria; fetal bovine serum (FBS) and sulforhodamine B (SRB) from Sigma-Aldrich Chemie GmbH, Germany and BrdU from Roche Applied Science.

Cells and cell culture

The NCI-H460 cell line was purchased from the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 4.5 g/l glucose, 10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 $\mu\text{g}/\text{ml}$ amphotericin B solution at 37°C in a humidified 5% CO₂ atmosphere. NCI-H460/R cells were originally selected from NCI-H460 cells and cultured in a medium containing 100 nM DOX [6]. Both cell lines were sub cultured at 72 h intervals using 0.25% trypsin/EDTA and seeded into a fresh medium at the following densities: 8,000 cells/cm² for NCI-H460 and 16,000 cells/cm² for NCI-H460/R.

BrdU labeling

Cells grown in 75 cm² tissue flasks were trypsinised, seeded into flat-bottom 96-well tissue culture plates (2,000 cells/well for NCI-H460 and 4,000 cells/well for NCI-H460/R) and

incubated overnight. The cells were then treated with SF (1–50 μM), 8-Cl-cAMP (1–50 μM) and DOX (5–100 nM for NCI-H460 and 100–1500 nM for NCI-H460/R) for 72 h. Untreated control and treated cells were incubated with the thymidine analog bromodeoxyuridine (BrdU) 4 h prior to the end of the incubation period. After fixation, the cells were processed according to the manufacturer's protocol (Roche Applied Science). The relative incorporation of BrdU was determined by absorbance reading at 450 nm, with correction at 670 nm (LKB 5060–006 Micro Plate Reader, Vienna, Austria).

Sulforhodamine B chemosensitivity assay

Cells grown in 25 cm^2 tissue flasks were trypsinized, seeded into flat-bottom, 96-well tissue culture plates and incubated overnight. The 72 h treatments were performed on NCI-H460 and NCI-H460/R cells that were seeded at densities of 2,000 cells/well and 4,000 cells/well, respectively. The treatments with SF, 8-Cl-cAMP and VER that lasted two weeks were performed in 75 cm^2 flasks on resistant cells that were seeded at a lower density (4,000 cells/ cm^2). The additional DOX treatment included a 24 h period of growth in fresh medium in flat-bottom 96-well tissue culture plates and a 72 h treatment with different concentrations of DOX ranging from 100–5,000 nM. This was followed by a slightly modified sulforhodamine B (SRB) chemosensitivity assay. The cells in 96-well plates were fixed in 50% trichloroacetic acid (50 μl /well) for 1 h at 4°C, rinsed in tap water and stained with 0.4% (w/v) sulforhodamine B in 1% acetic acid (50 μl /well) for 30 min at room temperature. The cells were then rinsed three times in 1% acetic acid to remove the unbound stain. The protein-bound stain was extracted with 200 μl 10 mM Tris base (pH 10.5) per well. The optical density was read at 540 nm, with correction at 670 nm (LKB 5060–006 Micro Plate Reader, Vienna, Austria). Growth inhibition (I) was determined according to the following equation:

$$I(\%) = (1 - (A \text{ treated sample}/A \text{ untreated control}) \times 100$$

The IC₅₀ value is defined as the concentration of the drug that inhibits cell growth by 50%. The IC₅₀ for the drug was calculated by linear regression analysis using Excel software.

Median effect analysis

The nature of the interaction between SF and DOX was analyzed with Calcsyn software that uses the combination index method of Chou and Talalay [23] and is based on the multiple drug effect equation. The analysis required that at least three or more data points for each single drug were

available in each experiment. The non-constant ratio combination design was chosen to assess the effect of both drugs in combination. The incorporated software generates a fraction-affected CI table, graph and a classic isobologram. Values of CI < 1 point to a pronounced additive effect or synergism i.e. the smaller value, the greater the degree of synergy. A value of CI = 1 indicates an additive effect, and values of CI > 1 point to an antagonistic effect. Each CI ratio shown here represents the mean value derived from two separate experiments.

DOX accumulation and efflux analysis

DOX accumulation was analyzed by flow cytometry utilizing the ability of DOX to emit fluorescence. The intensity of the fluorescence is proportional to DOX accumulation [24]. Studies were carried out with VER, a Pgp/MRP inhibitor, and the purine nucleoside analogs SF and 8-Cl-cAMP. Pretreated and untreated NCI-H460/R cells were grown to 80% confluence in 75 cm^2 flasks, trypsinized and resuspended in 10 ml centrifuge tubes in a DOX-containing medium. The DOX preloaded cells were incubated at 37°C in 5% CO₂ for 1 h. The cells were then pelleted by centrifugation, resuspended in growth medium and maintained at 4°C for 1 h (accumulation), or in growth medium at 37°C in 5% CO₂ for 1 h (efflux). At the end of the accumulation and efflux periods, the cells were washed. Cold PBS and 10% FBS were added and the cells were kept on ice in the dark until flow cytometric analysis. The samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson, Oxford, United Kingdom). The orange fluorescence of DOX was assessed on fluorescence channel 2 at 570 nm. A minimum of 10,000 events were assayed for each sample. Differences in the shape of the curves were quantified using a Komogorov-Smirnov nonparametric statistic. P values were calculated (available on request) in CellQuest Pro and run on a Macintosh computer.

RNA extraction and RT-PCR

Total RNA was extracted with TRIzol from NCI-H460 and NCI-H460/R cells that were either untreated or treated with IC₅₀ doses of SF and 8-Cl-cAMP. Samples of NCI-H460/R untreated cells and cells treated four times during 2 weeks with the same concentration (1 μM) of SF, 8-Cl-cAMP and VER, as well as with the combinations 1 μM SF and 1 μM VER, 1 μM 8-Cl-cAMP and 1 μM VER were also analyzed. The quality of the RNA was determined by agarose gel electrophoresis/ethidium bromide staining. RNA was quantified by spectrophotometry. About 5 μg of total RNA was reverse-transcribed using M-MLV reverse transcriptase. The gene coding for P-gp (*mdr1*) [25] was investigated. *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) [26] served as

an internal control and was co-amplified with the gene of interest in all of the PCR experiments. The primer sequences and product sizes for each gene were described earlier [6]. The PCR reactions were performed on the GeneAmp® PCR System 9700 (Applied Bioscience) under the following conditions: one cycle at 94°C for 5 min, 25 for NCI-H460/R cells and 33 cycles for NCI-H460 at 94°C for 15 s, at 56°C for 30 s, at 72°C for 30 s, and at 4°C indefinitely. The optimal ratio of *gapdh* to *mdr1* primers for linear amplification conditions was 1:3. For each RT-PCR, a negative control without template was performed (not shown). All PCR reactions were performed at least five times. The PCR products were loaded onto 2% agarose gels, stained with ethidium bromide and photographed under UV light. Multi-Analyst/PC Software Image Analysis System (Bio-Rad Gel Doc 1000) was used for densitometric analysis.

Statistical analysis

Statistical analysis was tested by one-way analysis of variance (ANOVA). When statistical significance was

observed, the Tukey honest significant difference (HSD) test was used. Statistical significance was accepted when $p < 0.05$. Groups of data that did not have a normal distribution were analyzed by the non-parametric U-test. The observed differences were considered statistically significant if the probability level was $p < 0.05$.

Results

The effects of SF and 8-Cl-cAMP on cell proliferation and *mdr1* mRNA expression

The BrdU assay which detects the amount of BrdU incorporated into DNA was used to test the anti-proliferative effect of SF and 8-Cl-cAMP in NCI-H460 and NCI-H460/R cell lines. SF and 8-Cl-cAMP caused dose-dependent inhibition of cell growth in both cell lines (Fig. 1a, b). SF and 8-Cl-cAMP brought about statistically significant ($p < 0.05$) cell growth inhibition at 2.5 μM in the parental line (Fig. 1a, b). The IC₅₀ value in NCI-H460 cells for SF was 4.8 μM and

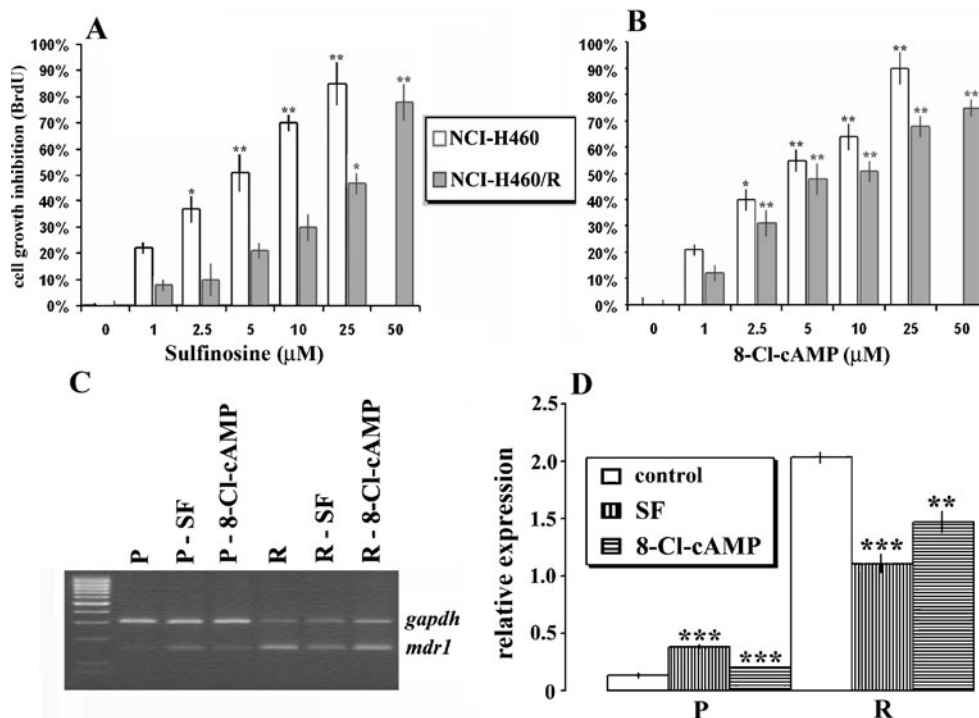


Fig. 1 SF and 8-Cl-cAMP induce dose-dependent inhibition of cell proliferation in parental and resistant cell lines and SF and 8-Cl-cAMP decrease the level of *mdr1* mRNA expression in the resistant cell line. **a**—the effects of different concentrations of SF on cell proliferation in NCI-H460 and NCI-H460/R cells. **b**—the effects of different concentrations of 8-Cl-cAMP on cell proliferation in NCI-H460 and NCI-H460/R cells. The statistical significance in all of the treatments was compared to the untreated control and is presented as $p < 0.05$ (*) and $p < 0.01$ (**). Mean values were obtained from five independent experiments ($n=5$). **c**—the amplified specimens of the *mdr1* gene

were visualized with ethidium-bromide in agarose gel next to a DNA ladder (100 bp) in cell lines NCI-H460, untreated (P) and treated (P-SF), (P-8-Cl-cAMP) and NCI-H460/R cells, untreated (R) and treated (R-SF), (R-8-Cl-cAMP). The PCR product of *gapdh* was co-amplified with *mdr1*. **d**—the relative expression of *mdr1* gene was calculated relative to *gapdh* expression. The statistical significance between the controls and treatments is presented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Mean values were obtained from five or more independent experiments ($n \geq 5$)

4.2 μM for 8-Cl-cAMP. Treatment of the resistant line with SF was statistically significant ($p < 0.05$) at 25 μM (Fig. 1a) and with 8-Cl-cAMP ($p < 0.01$) at 2.5 μM (Fig. 1b). The IC₅₀ values in the resistant line were 27.4 μM for SF and 8.3 μM for 8-Cl-cAMP.

The levels of *mdr1* mRNA expression were analyzed in RNA samples isolated from NCI-H460 and NCI-H460/R untreated cells and cells that were treated with SF and 8-Cl-cAMP. Parental cells were treated with 5 μM SF and with 5 μM 8-Cl-cAMP, while the resistant cells were treated with 25 μM SF and 10 μM 8-Cl-cAMP (Fig. 1c). Statistically significant changes in *mdr1* mRNA expression levels were observed in NCI-H460 and NCI-H460/R cells after the treatments. SF and 8-Cl-cAMP increased *mdr1* mRNA expression in the parental line (Fig. 1d). The level of *mdr1* mRNA expression after the treatment with SF exceeded the levels of expression observed in untreated cells (2.8-fold; $p < 0.001$) and after the 8-Cl-cAMP treatment (1.5-fold; $p < 0.001$). In contrast, SF and 8-Cl-cAMP decreased *mdr1* mRNA expression in the resistant line (Fig. 1d). SF exhibited a stronger effect. It led to a 45% decrease in the level of *mdr1* mRNA expression ($p < 0.001$). 8-Cl-cAMP reduced the level of expression by 28% ($p < 0.01$).

The effects of combined application of SF and 8-Cl-cAMP with DOX

The effects of the application of either SF or 8-Cl-cAMP and DOX on NCI-H460 and NCI-H460/R cell lines were examined by the BrdU assay. The same concentration of SF and 8-Cl-cAMP (1 μM) was used in both lines. The range of DOX concentrations (5–100 nM) applied to the parental line was lower than the concentrations (100–1500 nM) applied to the resistant line (Table 1). The nature of the mutual effects of SF and DOX and of 8-Cl-cAMP and DOX was established from the combination index (CI). The obtained results revealed strong synergistic effects of SF and DOX and of 8-Cl-cAMP and DOX ($\text{CI} < 1$) in parental cells at all of the tested concentrations (Table 1). The results obtained with the combination of SF and DOX point to a

synergistic effect in resistant cells (Table 1). The greatest effect was obtained by applying 1 μM SF with 500 nM DOX. The combination of 1 μM SF and 200 nM DOX exhibited an additive effect ($\text{CI} = 1$). In resistant cells, lower DOX concentrations (100 nM and 200 nM) applied with 1 μM 8-Cl-cAMP were antagonistic ($\text{CI} > 1$), whereas other combinations of DOX and 8-Cl-cAMP were synergistic (Table 1). The greatest effect was obtained with 1 μM 8-Cl-cAMP and 500 nM DOX. These results revealed synergism between SF and DOX in NCI-H460 and NCI-H460/R cells and 8-Cl-cAMP and DOX in NCI-H460 cells. Lower DOX concentrations in combination with 8-Cl-cAMP were antagonistic in NCI-H460/R cells. Higher DOX concentrations in combination with 8-Cl-cAMP were synergistic.

Enhancement of DOX chemosensitivity in resistant cells treated with SF, 8-Cl-cAMP and VER

The individual effects of VER, SF and 8-Cl-cAMP on cell growth in the NCI-H460/R line were studied by the SRB assay which detects the binding of sulforhodamine B dye to proteins in living cells (Fig. 2a). Statistically significant ($p < 0.05$) inhibition of cell growth inhibition was obtained with 10 μM VER (Fig. 2a). The highest concentration of VER caused 40% inhibition. Statistically significant inhibition of cell growth ($p < 0.05$) was achieved with 10 μM SF and 5 μM 8-Cl-cAMP (Fig. 2a). Nearly 50% inhibition was obtained with 21.4 μM SF and 13 μM 8-Cl-cAMP, whereas VER at concentrations greater than 100 μM caused 50% inhibition.

The efficacy of the different simultaneous treatments of DOX with either SF, 8-Cl-cAMP or VER was obtained from the coefficient of “relative reversion” – the relation between the IC₅₀ value for DOX and the IC₅₀ value for the combined treatment with DOX and each of the above agents. In these experiments, lower concentrations of SF, 8-Cl-cAMP and VER which do not lead to significant inhibition of cell growth (Table 2) were used. The combination of VER with DOX was the most effective whereas SF with DOX and 8-Cl-cAMP with DOX were less so. The relative reversions achieved with 1 μM and 2 μM VER were 10.38 and 24.9,

Table 1 Combination of low concentration (1 μM) of SF and 8-Cl-cAMP with DOX in parental and resistant cell lines: effect on cell proliferation

NCI-H460			NCI-H460/R		
DOX (nM)	CI + SF (1 μM)	CI + 8-Cl-cAMP (1 μM)	DOX (nM)	CI + SF(1 μM)	CI + 8-Cl-cAMP (1 μM)
5	0.247 (S)	0.317 (S)	100	0.523 (S)	2.180 (AN)
10	0.330 (S)	0.384 (S)	200	0.902 (AD)	1.741 (AN)
20	0.458 (S)	0.603 (S)	500	0.425 (S)	0.557 (S)
50	0.199 (S)	0.366 (S)	1000	0.766 (S)	0.779 (S)
100	0.295 (S)	0.184 (S)	1500	0.828 (S)	0.792 (S)

AD—additive effect ($C = 0.9$ – 1.1); AN—antagonism ($C > 1$); S—synergism ($C < 1$)

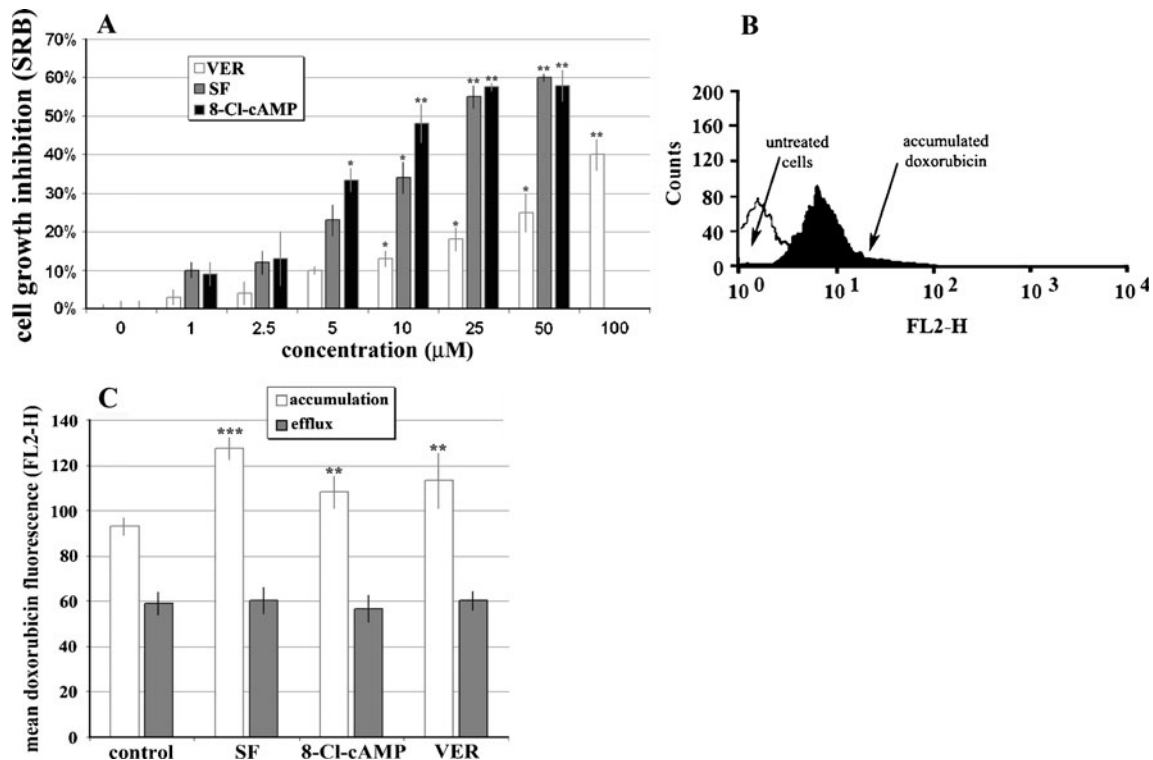


Fig. 2 VER, SF and 8-Cl-cAMP inhibit cell growth and increase DOX accumulation in the resistant cell line. **a**—the effects of different concentrations of VER, SF, and 8-Cl-cAMP on cell growth in NCI-H460/R cells. The statistical significance in all treatments compared to the untreated control is shown as $p < 0.05$ (*) and $p < 0.01$ (**). Mean values were obtained from five independent experiments ($n = 5$). **b**—the accumulation of DOX in NCI-H460/R untreated cells (flow

cytometric plot). **c**—the calculated accumulation and DOX efflux in untreated NCI-H460/R cells (R) and in NCI-H460/R cells that were treated for two-weeks with 1 μM of SF, 8-Cl-cAMP and VER. The statistical significance between the control and treatments is presented as $p < 0.01$ (**) and $p < 0.001$ (***)). Mean values were obtained from three independent experiments ($n = 3$)

respectively (Table 2). The relative reversion coefficients for 1 μM and 2 μM VER were 10.38 and 24.9, respectively (Table 2). The coefficients of reversion obtained with the same concentrations of SF were 1.68 and 1.98, respectively. The relative coefficients of reversion that were brought about by 1 μM and 2 μM 8-Cl-cAMP were 1.25 and 2.04, respectively (Table 2).

To further analyze the potential for reversal of SF and 8-Cl-cAMP, NCI-H460/R cells were pretreated for two weeks either with SF, 8-Cl-cAMP or with VER during two cell passage cycles that were carried out in one week. The first

cycle lasted 72 h and the second lasted 96 h. Thus, the two-week pretreatments included four treatments with the same concentrations (either 1 μM or 2 μM). The lower concentrations led to 20–40% ($p < 0.05$) inhibition of cell growth. After the pretreatments, the cells were seeded in fresh medium and treated with DOX (100–5000 nM) for the next 72 h. The most pronounced reversal was obtained when NCI-H460/R cells were subjected to a pretreatment with SF. The coefficients of relative reversion obtained with 1 μM and 2 μM SF were 4.12 and 6.87, respectively (Table 3). The coefficients of relative reversion obtained with the same

Table 2 Relative reversion of resistance to DOX in simultaneous treatment with SF, 8-Cl-cAMP and VER

Drugs	Concentration (μM)	IC ₅₀ (μM)	Relative reversion
DOX		2.49 \pm 0.16	
SF	1	1.48 \pm 0.07	1.68
(+ DOX)	2	1.26 \pm 0.08	1.98
8-Cl-cAMP	1	2.00 \pm 0.10	1.25
(+ DOX)	2	1.22 \pm 0.07	2.04
VER	1	0.24 \pm 0.05	10.38
(+ DOX)	2	0.10 \pm 0.03	24.90

Table 3 Relative reversion of resistance to DOX in subsequent treatment with SF, 8-Cl-cAMP and VER

Drugs	Concentration(μ M)	IC ₅₀ (μ M)	Relative reversion
DOX		2.06 \pm 0.12	
SF	1	0.50 \pm 0.01	4.12
(+ DOX)	2	0.30 \pm 0.03	6.87
8-Cl-cAMP	1	1.80 \pm 0.07	1.14
(+ DOX)	2	0.50 \pm 0.05	4.12
VER	1	0.77 \pm 0.05	2.68
(+ DOX)	2	0.79 \pm 0.09	2.61

concentrations of 8-Cl-cAMP were 1.14 and 4.12. After the pretreatment with VER the coefficients of relative reversion were 2.68 and 2.61 (Table 3).

DOX accumulation and efflux were examined in untreated NCI-H460/R cells (Fig. 2b) and in cells that were treated four times during two weeks with either 1 μ M SF, 8-Cl-cAMP or VER. The two-week pretreatment with SF led to a statistically significant 37% ($p < 0.001$) increase in DOX accumulation in comparison with the control (Fig. 2c). 8-Cl-cAMP and VER also increased the accumulation of DOX for about 20% ($p < 0.01$). DOX efflux remained unchanged with respect to the control in all of the treatments.

Interactions between SF and VER and 8-Cl-cAMP and VER

The effects of SF and 8-Cl-cAMP on chemosensitivity to DOX in the NCI-H460/R line were tested after the cells were pretreated with 1 μ M SF and 1 μ M VER, and with 1 μ M 8-Cl-cAMP and 1 μ M VER. The effects of the combinations were compared with the control, i.e. cells that were not pretreated, and to cells pretreated with either 1 μ M SF or 1 μ M 8-Cl-cAMP. The profiles of cell growth inhibition were similar in cells pretreated with SF and cells pretreated with the combination of SF and VER. High statistical significance ($p < 0.001$; in comparison with the control) was observed in both SF- and SF+VER-pretreated cells that were subsequently treated with 500 nM DOX (Fig. 3a). Both pretreatments decreased the IC₅₀ value for DOX from 2,000 nM to 500 nM. The combination was still efficient, promoting about 58% growth inhibition in cells treated with 500 nM DOX, whereas the treatment with only SF led to 50% inhibition (Fig. 3a).

The pretreatment with 1 μ M 8-Cl-cAMP did not significantly reduce the IC₅₀ value for DOX. In cells that were treated with 2,000 nM DOX, individually applied 8-Cl-cAMP induced 53% inhibition of cell growth while the combination of 8-Cl-cAMP and VER caused 86% inhibition (Fig. 3b). The combination of 8-Cl-cAMP and VER decreased the IC₅₀ value for DOX almost ten times ($p < 0.01$). Thus, in cells

treated with 200 nM DOX, the combination achieved 48% inhibition of cell growth (Fig. 3b).

The level of expression of *mdr1* mRNA was analyzed in untreated NCI-H460/R cells and in cells treated four times during two weeks with the same concentration (1 μ M) of SF, 8-Cl-cAMP and VER, as well as with the following combinations: 1 μ M SF and 1 μ M VER, 1 μ M 8-Cl-cAMP and 1 μ M VER (Fig. 3c). Statistically significant inhibition ($p < 0.01$) of *mdr1* mRNA expression was observed only after the combined treatment with 1 μ M 8-Cl-cAMP and 1 μ M VER. This combination decreased the level of *mdr1* mRNA expression by 38.5% with respect to the control (Fig. 3d).

We next assessed the nature of the combined application of VER with SF and 8-Cl-cAMP with the combination index (CI). The calculated combined effects of the tested substances are shown in Table 4. Lower concentrations of SF (1 and 2.5 μ M) and VER (2.5 μ M) and of 8-Cl-cAMP (1 and 2.5 μ M) and VER (1 and 2.5 μ M) exhibited significant synergism ($CI \leq 0.5$) (Table 4). Combined treatments with higher concentrations (5 μ M VER with 5 μ M and 10 μ M SF or 8-Cl-cAMP, respectively) elicited a weaker synergistic effect ($0.5 < CI < 1$). A clear dose-dependent increase in CI values was not observed.

Discussion

MDR continues to present a major challenge to chemotherapy of lung carcinoma. In the present study we examined the effects of combinations of DOX and two purine analogs, SF and 8-Cl-cAMP on human sensitive (NCI-H460) and MDR resistant (NCI-H460/R) LCNEC cell lines. SF promotes the reversion of DOX resistance in NCI-H460/R both alone [8] and in combination with curcumin [12]. Synergistically enhanced effects of SF and 8-Cl-cAMP were observed in the human neuroblastoma cell line [16]. 8-Cl-cAMP also synergistically increases the growth-inhibitory effects of either paclitaxel or cisplatin in a number of cell lines [13, 14].

Using the BrdU proliferation assay, we showed that SF and 8-Cl-cAMP promoted dose-dependent inhibition of cell growth in both parental and resistant cell lines. The

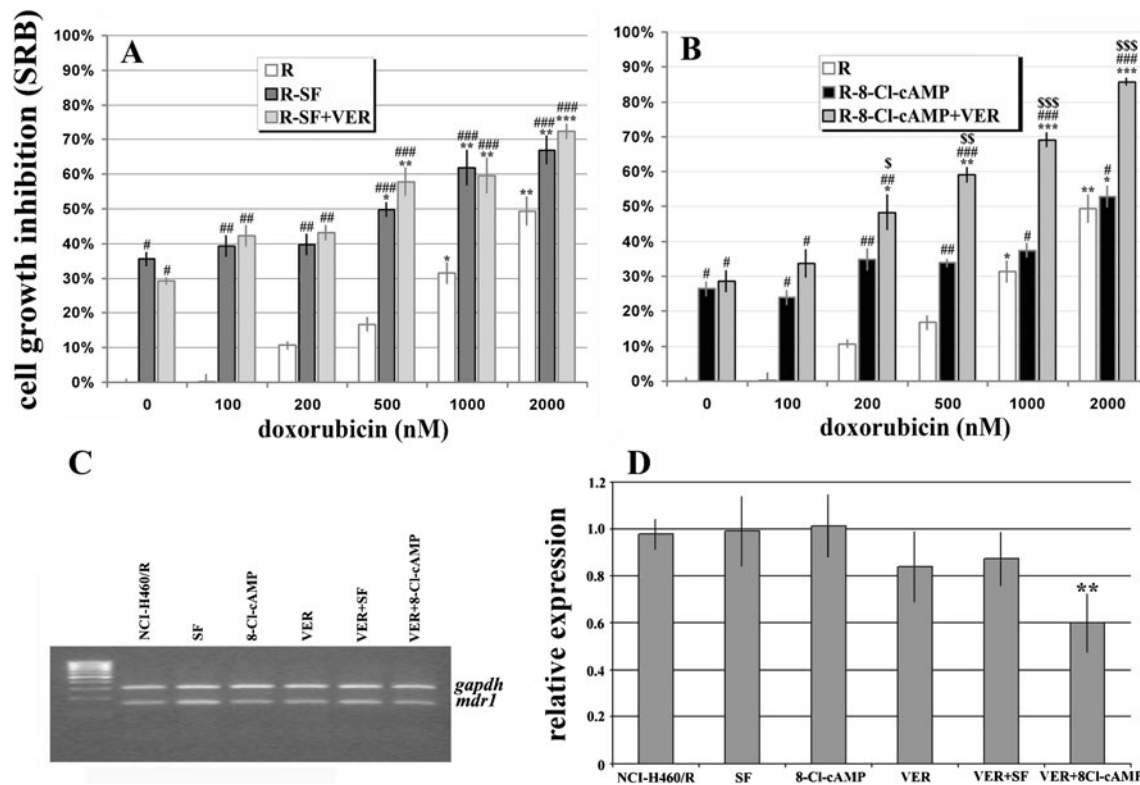


Fig. 3 VER stimulates the effect of 8-Cl-cAMP on DOX cytotoxicity and *mdr1* expression in the resistant cell line. **a**—inhibition of cell growth in NCI-H460/R cells treated with DOX (R); inhibition of cell growth in NCI-H460/R cells that were pretreated for two-weeks with SF either alone (R-SF) or in combination (R-SF + VER). **b**—inhibition of cell growth in NCI-H460/R cells treated with DOX (R); inhibition of cell growth in NCI-H460/R cells that were pretreated for two-weeks with 8-Cl-cAMP either alone (R-8-Cl-cAMP) or in combination (R-8-Cl-cAMP + VER). The statistical significance between the individual pretreatments and combined pretreatments is shown as: $p < 0.05$ (\$), $p < 0.01$ (\$\$) and $p < 0.001$ (\$\$\$). The statistical significance between R and individual pretreatments and the combined pretreatments is shown as: $p < 0.05$ (#), $p < 0.01$ (##) and $p < 0.001$ (###). The statistical significance in all of the treatments

compared to the untreated control is shown as: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Mean values were obtained from five independent experiments ($n = 5$). **c**—the amplified specimens of *mdr1* gene were visualized with ethidium-bromide in agarose gel next to a DNA ladder (100 bp) and examined in the following cells: NCI-H460/R, untreated (R) and treated for two-weeks with individual one agent, i.e. (R-SF), (R-8-Cl-cAMP), (R-VER), and with the following combinations of agents: (R-VER + SF), (R-VER + 8-Cl-cAMP). The PCR product of *gapdh* was co-amplified with *mdr1*. **d**—the relative expression of *mdr1* gene was calculated relative to *gapdh* expression. The statistical significance between the control and treatments is presented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Mean values were obtained from five or more independent experiments ($n \geq 5$)

sensitivity of parental NCI-H460 cells was similar for both compounds. The obtained IC₅₀ values (4.8 μ M for SF and 4.2 μ M for 8-Cl-cAMP) match the micro molar range of the clinically effective concentrations of the purine analogs [16, 17, 19]. Higher concentrations were applied to resistant NCI-H460/R cells. Results of the BrdU and SRB assays showed that the IC₅₀ for the resistant line was 5-fold higher

for SF and 2.5-fold higher for 8-Cl-cAMP than in the parental line. Comparing the obtained results, we found that 8-Cl-cAMP inhibited the proliferation of resistant cells with lower concentrations compared to the concentrations that reduced cell viability. The inhibition of RI α (PKA subunit) by 8-Cl-cAMP can affect the activity of DNA polymerases and significantly slow down cell proliferation [27].

Table 4 Combined effect of VER with SF and 8-Cl-cAMP: effect on cell growth in resistant cell line

SF (μ M)	8-Cl-cAMP (μ M)	VER (μ M)	CI SF + VER	CI 8-Cl-cAMP + VER
1	1	1	0.427 (S)	0.415 (S)
2.5	2.5	1	0.482 (S)	0.237 (S)
1	1	2.5	0.399 (S)	0.186 (S)
2.5	2.5	2.5	0.505 (S)	0.348 (S)
5	5	5	0.667 (S)	0.598 (S)
10	10	5	0.800 (S)	0.719 (S)

AD—additive effect (C = 0.9–1.1); AN—antagonism (C > 1); S—synergism (C < 1)

Analysis of *mdr1* mRNA expression in parental and resistant cells treated with inhibitory concentrations of SF and 8-Cl-cAMP showed that increased IC₅₀ values in resistant cells did not result from “cross-resistance” to these two substances. In NCI-H460/R cells, both SF and 8-Cl-cAMP significantly reduced the level of *mdr1* expression. This observation encouraged us to examine the potential of these substances for MDR reversion. The response of parental cells treated with SF and 8-Cl-cAMP differs from the response of resistant cells. Treatments with SF and 8-Cl-cAMP induced a significant increase of *mdr1* expression. Although they are not substrates for P-gp, increased *mdr1* expression in NCI-H460 cells suggests that SF and 8-Cl-cAMP after long-term administration could lead to the development of resistance.

Combined therapy with several chemotherapeutic agents aims to increase the efficacy of the applied drugs, overcome the problem of resistance and diminish side effects [28]. The combined effects of SF and DOX and of 8-Cl-cAMP and DOX have not been investigated. To test their effects on the cell proliferation, we combined a broad range of DOX concentrations with comparatively low concentration of SF and 8-Cl-cAMP (1 μ M). A strong synergistic anti-proliferative effect of SF and 8-Cl-cAMP in combination with DOX was observed in the parental line. In resistant NCI-H460/R cells, the combination of DOX with lower concentrations than the respective IC₅₀ values for SF and 8-Cl-cAMP produced a synergistic effect. This result is very important because the concentrations of SF and 8-Cl-cAMP were 25 and 10 times lower, respectively, than the IC₅₀ value that achieved significant reduction of *mdr1* expression in NCI-H460/R cells.

We compared the results obtained after administration of SF, 8-Cl-cAMP and VER in combination with DOX to the effect of DOX only in the resistant line. After calculating the coefficient of “relative reversion”, we established that the combined treatment with VER was ten times more efficient in decreasing the IC₅₀ value for DOX than SF and 8-Cl-cAMP. Although there is synergism between SF and DOX and 8-Cl-cAMP and DOX in the resistant cells, lower concentrations of SF and 8-Cl-cAMP (1 and 2 μ M, respectively) could not reverse the resistance.

The efficacy of the combination in some cases depended on the sequence the agents were applied. The effect of the nucleoside analogs in combination with classical chemotherapeutic agents in several lung cancer cell lines depends on the order of treatment [29]. In addition, the synergistic effect increases when 8-Cl-cAMP is administrated after paclitaxel or cisplatin [13]. Considering that SF and 8-Cl-cAMP decrease the expression level of *mdr1* mRNA in the NCI-H460/R line, we applied both agents prior to the DOX treatment. We wanted to determine the effect of long-term

exposure of resistant cells to the non-inhibiting concentrations of SF and 8-Cl-cAMP. For two weeks NCI-H460/R cells were treated with lower concentrations (1 and 2 μ M) of SF and either 8-Cl-cAMP or VER, respectively, followed by the examination of the sensitivity to DOX. After calculating the coefficient of “relative reversion”, we found that the 2 μ M SF and 2 μ M 8-Cl-cAMP were 3.5- and 2-fold, respectively, more efficient in reverting resistance after the pretreatment than the simultaneous treatment. In addition, the efficiency of the VER pretreatment was 9.5-fold lower in comparison with the simultaneous treatment. The greater potential for reversal of resistance after the pretreatments with SF and 8-Cl-cAMP was probably due to their effect on gene expression whereas the higher resistance reversal potential of VER observed after the simultaneous treatment was probably the consequence of its binding to P-gp. After the two-week treatment with SF, 8-Cl-cAMP and VER, we observed a significant increase of DOX accumulation in NCI-H460/R cells, with the biggest increase observed after the treatment with SF.

The possibility that VER can improve the effect of SF and 8-Cl-cAMP by reversing the resistance of NCI-H460/R cells was examined. To that end, 1 μ M VER was combined with 1 μ M SF and 1 μ M 8-Cl-cAMP, and applied as a two-week pretreatment. VER did not affect the reversion of resistance caused by SF, whereas in the case of 8-Cl-cAMP, VER brought about a 10-fold improvement of reversion compared to the treatment with 8-Cl-cAMP alone. The reversion that was achieved by the combination of VER and 8-Cl-cAMP was four times higher than the reversion attained after the treatment with VER alone. The inhibition of *mdr1* gene expression in NCI-H460/R cells after the two-week treatment suggests that the combined effect of VER and 8-Cl-cAMP resulted from their effect on *mdr1* mRNA levels.

The effects of SF and 8-Cl-cAMP in combination with VER, on cell growth were examined in the NCI-H460/R line. Concentrations from 1–10 μ M were used. A strong synergistic effect was observed with lower concentrations (1 and 2.5 μ M). At these concentrations VER is acceptable for clinical application. The combination of 8-Cl-cAMP and VER reversed resistance by affecting the synthesis of P-gp. However, the synergistic inhibition of cell growth was probably achieved through two different pathways: inhibition of cell proliferation and disturbance of the Ca²⁺ equilibrium [27, 30]. Synergism between SF and VER probably includes two convergent pathways that lead to glutathione loss [31, 32].

The effects of purine analogs during cancer treatment include the reversion of resistance. In view of their considerable efficacy and moderate toxicity, the purine analogs SF and 8-Cl-cAMP are suitable for combining with other chemotherapeutic agents [11]. They act synergistical-

ly with DOX. We tested the effects of their concentrations and the sequence at which they were applied. In light of their inhibitory effects on cell growth, their impact on *mdr1* expression and the accumulation of DOX, we conclude that purine analogs represent useful agents for MDR reversion. Further studies of the effects of these agents need to be extended to *in vivo* experimental systems such as the orthotopic model of NCI-H460/R cells, before firm conclusions can be drawn as to their capability of reversing the MDR phenotype in NSCLC carcinoma and their possible therapeutic potential.

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