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ORIGINAL ARTICLE

Distamycin A and derivatives as synergic drugs in cisplatin-sensitive and -resistant ovarian cancer cells

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Abstract Acquired resistance to cisplatin (cDDP) is a multifactorial process that represents one of the main problems in ovarian cancer therapy. Distamycin A is a minor groove DNA binder whose toxicity has limited its use and prompted the synthesis of derivatives such as NAX001 and NAX002**,** which have a carbamoyl moiety and different numbers of pyrrolamidine groups. Their interaction with a B-DNA model and with an extended-TATA box model, [Polyd(AT)], was investigated using isothermal titration calorimetry (ITC) to better understand their mechanism of interaction with DNA and therefore better explain their cellular effects. Distamycin A interactions with Dickerson and Poly[d(AT)$_6$] oligonucleotides show a different thermodynamic with respect to NAX002. The bulkier distamycin A analogue shows a non optimal binding to DNA due to its additional pyrrolamidine group. Cellular assays performed on cDDP-sensitive and -resistant cells showed that these compounds, distamycin A in particular, affect the expression of folate cycle enzymes even at cellular level. The optimal interaction of distamycin A with DNA may account for the down-regulation of both dihydrofolate reductase (DHFR) and thymidylate synthase (TS) and the up-regulation of spermidine/spermine N1-acetyltransferase (SSAT) caused by this compound. These effects seem differently modulated by the cDDP-resistance phenotype. NAX002 which presents a lower affinity to DNA and slightly affected these enzymes, showed a synergic inhibition profile in combination with cDDP. In addition, their combination with cDDP or polyamine analogues increased cell sensitivity to the drugs suggesting that these interactions may have potential for development in the treatment of ovarian carcinoma.

Keywords Distamycin A · Polyamines · DNA recognition · Ovarian cancer · Cisplatin-resistance · Thymidylate synthase

Abbreviations

cDDP	Cisplatin or cis-diamminedichloroplatinum
Put	Putrescine
Spd	Spermidine
Spm	Spermine
SSAT	Spermidine/spermine N1-acetyltransferase
BESpm: N1	N12-bisethylspermine
DENSpm	N1,N11-diethylnorspermine
ITC	Isothermal titration calorimetry
DHFR	Dihydrofolate reductase
TS	Thymidylate synthase
5-FU	5-fluorouracil
MTX	Methotrexate
CH_2FH_4	5,10-methylenetetrahydrofolate
FH_4	Tetrahydrofolate

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DAPI	4,6-diamidino-2-phenylindole dihydrochloride
Hoechst 33258	Bisbenzimide H 33258

Introduction

Ovarian cancer is a malignant tumour that is the fifth most common cause of cancer death in women. The standard first-line treatment for ovarian cancer is a combination of paclitaxel and a Pt derivative drug such as cisplatin (cDDP) or carboplatin alone.

Acquired resistance to cDDP and its derivatives is a multifactorial process involving many mechanisms; enhanced DNA synthesis and repair is the most common feature of resistance among almost all resistant cell lines studied. The over-expression of both dihydrofolate reductase (DHFR) and thymidylate synthase (TS) enzymes plays an important role in cDDP resistance in a large survey of human ovarian carcinoma cell lines (Scanlon et al. 1988). In this context, the discovery of drugs aimed at impairing the activity of these enzymes, thus circumventing cDDP-resistance, is of great interest.

Most antitumour drugs act by causing DNA cleavage that leads to apoptosis. Amplification of their cytotoxic effects through drug combinations is expected to treat cancer with reduced doses, resulting in reduced side effects. It has been demonstrated that DNA cleavage induced by antitumour drugs is enhanced by DNA binding ligands. DNA intercalating agents and DNA minor groove-binder also bind to TS mRNA site 1 construct (Cho and Rando 2000). In particular, the DNA minor groove binders DAPI, Hoechst 33258 and distamycin A demonstrated TS RNA binding affinities similar to the classical intercalator drugs quinacrine and proflavine ($K_d = 530$, 730 and 1,120 nM respectively). Distamycin A, an antibiotic produced by *Streptomyces distallicus,* is an oligopeptide containing three pyrrolic rings and has binding affinity at the relatively narrow AT-rich sequences of the minor groove of double-strand DNA (Arcamone et al. 1964; Abu-Daya and Fox 1997). DNA-binding ligands, including minor groove binders such as distamycin A and intercalators, are considered amplifiers of the DNA damaging effects of anticancer drugs such as bleomycin or duocarmycin A (Hiraku et al. 2002).

Binding of distamycin A to DNA has been characterised through different biophysical studies, including X-ray crystallography. The X-ray crystallographic complex of distamycin A with the Dickerson oligonucleotide [d(CGC AAATTTGCG)] (Kawanishi and Hiraku 2004) has shown that distamycin A establishes a hydrogen bond network with the carbonyl groups of the thymine or with the adenine N3 in the minor groove. The three pyrrolic rings of distamycin A presented a 15° torsion between A and B and 11° between B and C that resulted in a non-planar compound (Kawanishi and Hiraku 2004). The only planar portion, i.e. the terminal amidic group, followed the natural trend of the B-DNA minor groove. The compound occupies the DNA minor groove in the AT-rich segment in a full-contact manner, leaving no space for solvent molecules to interfere in the complex formation. The binding specificity of the compound to AT-rich sequences can be explained by the ability of distamycin A to strongly interact with adenines and thymines in the minor groove. Furthermore, the presence of the guanine N2 amino group provides steric hindrance that interferes with binding of distamycin A in GC-rich sequences of the minor groove. Another important feature, part of the network of hydrogen bonds, is Van der Waals interactions between the pyrrolic rings of the compounds and the heteroaromatic ring of the nucleotide bases. In addition, the terminal amidine group of distamycin A interacts electrostatically with the negative charge of the DNA the phosphate group; distamycin A is expected to form 1:1 and 2:1 complexes with B-DNA sequences (Coll and Christin 1987; Uytterhoeven and Sponer 2002).

Distamycin A exhibited anticancer, antiviral and in vitro antimalarial activity (Lombardi and Crisanti 1997). However, the high toxicity associated with distamycin A precludes its development as a drug; thus, some synthetic analogues of distamycin A have been developed, including NAX001, NAX002 and NAX003 (Fig. 1), which are carbamoyl analogues of distamycin A that differ in the number of pyrrolamidine groups. Some of them have proven to be highly active and less toxic in vitro (Lombardi and Crisanti 1997).

The naturally occurring polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are essential for cell growth and differentiation, and their elevated concentrations in cancer cells (Wallace and Fraser 2004), suggest a link between polyamine content regulation and carcinogenesis. The polyamine pathway is therefore an attractive target in the development of anticancer strategies (Wallace and Niiranen 2007). Recently, it has been reported that traditional antifolates alone, such as 5-fluorouracil (5-FU), affected polyamine pathways to reduce the levels of polyamines (Zhang et al. 2003) and that the combined modulation of folate cycle by traditional antifolates and polyamine metabolism by Spm analogues negatively affected colon cancer cell growth (Choi et al. 2005). In addition, the polyamine key catabolic enzyme spermidine/spermine N1-acetyl transferase (SSAT; EC 2.3.1.57) has been confirmed as an important anticancer target, being inducible in response to 5-FU or Pt drugs in combination with Spm analogues in parental and resistant colon cancer cell lines (Allen et al. 2007). In this regard, we have also

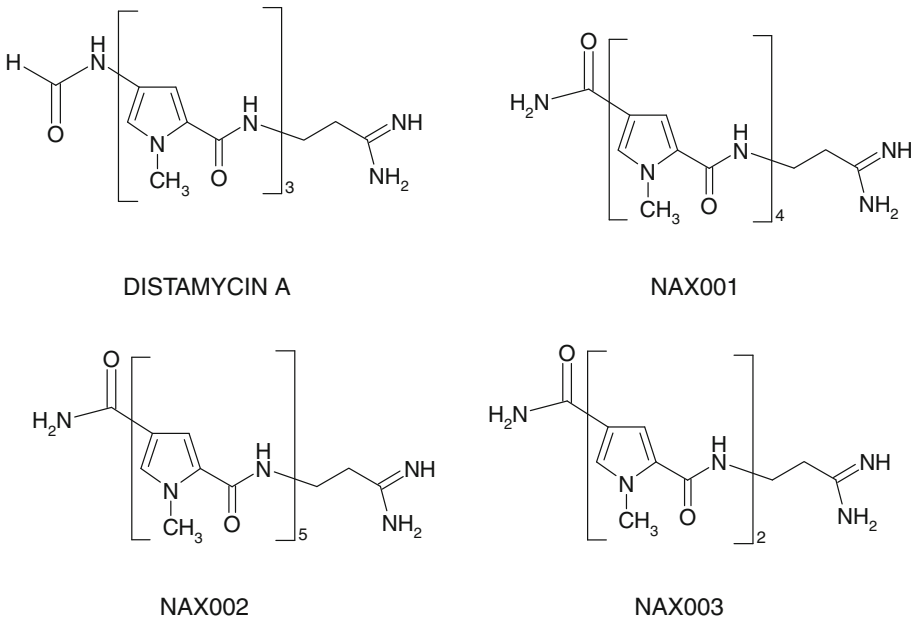

Fig. 1 Structure of distamycin A and its derivatives

recently reported that combinations of novel folate cycle inhibitors with quinoxaline structure and drugs that specifically target polyamine metabolism, such as diethylderivatives of norspermine (DENSPM) or spermine (BESpm), have synergistic effect in killing cisplatin-sensitive and drug-resistant daughter human ovarian cell lines (Marverti et al. 2009). Besides, we have previously indicated the limited induction of SSAT activity as an important determinant of the reduced response to BESpm in the cDDP-resistant human ovarian cancer C13* cells (Marverti et al. 2005).

In the present paper we explored the binding mechanisms to DNA of distamycin A derivatives, using an ITC assay in comparison with distamycin A to better explain the cellular effects. First, we studied distamycin A and its derivatives binding to the Dickerson [d(CGCAAATT TGCG)] and the Poly[d(AT)$_6$] oligonucleotides. The oligonucleotides for binding studies are well known as the B-DNA model (Dickerson) and the extended TATA box region model (Poly[d(AT)$_6$]).

Then, we studied for the first time, the effects of distamycin A and its two synthetic analogues on TS expression in vivo in a cDDP-sensitive human ovarian cancer cell line, 2008, and their resistant counterpart, C13*, which showed cross-resistance to classic TS and DHFR inhibitors, such as 5-fluorouracil (5-FU) and methotrexate (MTX). The cross-resistance phenotype is due to the higher expression of folate cycle enzymes rose during selection of the cDDP-resistance phenotype (Marverti et al. 2009). We have also studied, for the first time, the nature of the interaction between distamycin A and a derivative with either cDDP or a polyamine analogue against these ovarian carcinoma cell models.

Experimental section

Materials

2-(*N*-Morpholino)ethanesulfonic acid monohydrate (MES), ethylenediaminetetra-acetic acid (EDTA), sodium chloride (NaCl), and netropsin were purchased from SIGMA. Distamycin A (3-[1-methyl-4-[1-methyl-4-(1-methyl-4-carboxamidopyrrol-2-carboxamido)pyrrol-2-carboxamido] pyrrol-2-carboxamido]propionamidine Hydrochloride), NAX001 (3-[1-methyl-4-[1-methyl-4-[(1-methyl-4-(1-methyl-4-carboxamidopyrrol-2-carboxamido)pyrrol-2-carboxamido] pyrrol-2-carboxamido]pyrrol-2-carboxamido]propionamidine Hydrochloride), NAX002 (3-[1-methyl-4-[1-methyl-4-[(1-methyl-4-[(1-methyl-4-(1-methyl-4-carboxamidopyrrol-2-carboxamido)pyrrol-2-carboxamido]pyrrol-2-carboxamido] pyrrol-2-carboxamido] pyrrol-2-carboxamido]propionamidine Hydrochloride) and NAX003 (3-[1-methyl-4-[1-methyl-4-carboxamidopyrrol-2-carboxamido)pyrrol-2-carboxamido]propionamidine Hydrochloride) were synthesised by NAXOSPHARMA s.r.l. (Milan, Italy), as reported in (Lombardi and Crisanti 1997). Dickerson oligonucleotides (dCGCAAATTTGCG), poly[d(AT)] (dATATATATATAT), and poly[d(A)d(T)] (dAAAAAATTTTTT) were purchased from MWG Biotech. All chemicals used were of the highest purity available.

[5-^{3}H]dUMP (20 Ci/mmol), was purchased from Moravek Biochemicals (Brea, CA, USA). BESpm was kindly supplied by Hoechst Marion Roussel Inc. (Cincinnati, OH, USA). DENSpm was purchased from Tocris Bioscience (UK). 1-[^{14}C]acetyl coenzyme (1.89 GBq/mmol) was purchased from Perkin Elmer Italia (Milan, Italy). All other

chemicals were purchased from Sigma-Aldrich S.r.L. (Milan, Italy), except otherwise indicated.

Buffers

The MES buffer consisted of 0.01 M MES, 0.001 M EDTA and 0.2 M NaCl at pH 6.95. Titrant solutions were constituted as a 0.2 mM solution of distamycin A, netropsin, NAX001 or NAX02 in MES buffer. The titrated solution consisted of 100 μL of the stock solution of DNA (0.3 mM) added to a 1,400 μL volume of MES buffer.

ITC techniques

DNA annealing

An annealing reaction was performed with each single strand to obtain double-strand DNA following the MWG annealing protocol (http://www.mwg-biotech.com). The degree of purity was checked after each annealing process.

Isothermal titration calorimetry

For the calorimetric experiments, we used a VP-ITC from MicroCal®. Experiments were initiated by filling the reference and sample cells, respectively, with approximately 1.8 mL of MES buffer and oligonucleotide-containing solutions. Both solutions were thoroughly degassed before loading into the calorimeter cells. Cell loading was accomplished using a 2.5 mL Hamilton Gastight® syringe via a 20-μL injection series with an equilibration time of 4 min for each injection. Heat effects arising from dilution of the titrant were measured in a separate experiment where the titration and reference cells were both filled with buffer solution. These contributions to the observed reaction heat measurements were subtracted from the corresponding total. The heat effect of the oligonucleotide dilutions was negligible in all cases and was not reported in the figures. The experiments were conducted at pH 6.95. Before all experiments, the melting temperature of the DNA duplex was checked. Data analysis and curve fitting were performed using the ITC data analysis software supplied by MicroCal (ORIGIN 7.0).

Biological assays

Cell lines

The 2008 cell line was established from a patient with advanced cystadenocarcinoma of the ovary. The cDDP-resistant C13* subline, which is approximately 15-fold resistant to cDDP, was derived from the parent 2008 cell line by monthly exposure to cDDP, followed by chronic exposure to step-wise increases in cDDP concentration (Andrews and Albright 1992). The cell lines were grown in monolayers in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum and 50 μg/ml gentamycin sulphate. All cell media and serum were purchased from Lonza (Verviers, Belgium). Cultures were equilibrated with humidified 5% CO_2 in air at 37°C. All studies were performed in mycoplasma negative cells, as routinely determined using the MycoAlert Mycoplasma detection kit (Lonza, Walkersville, MD, USA). Protein content in the assays was estimated using the Lowry method (Lowry et al. 1951), unless otherwise indicated.

Cell growth assay

Cell growth was determined using a modified crystal violet assay (Kueng et al. 1989). On selected days, the tissue culture medium was removed and the cell monolayer fixed with methanol and stained with 0.2% crystal violet solution in 20% methanol for at least 30 min. After being washed several times with distilled water to remove excess dye, the cells were left to dry. The incorporated dye was solubilised in acidified isopropanol (1 N HCl:2-propanol, 1:10). After appropriate dilution, absorbance was determined spectrophotometrically at 540 nm. The extracted dye was proportional to cell number. The percentage of cytotoxicity was calculated by comparing the absorbance of cultures exposed to the drug to un-exposed (control) cultures.

TS catalytic assay

Cells used for the enzyme assay were harvested by trypsinisation when they were in an exponential growth phase, washed with PBS and used or stored at −20°C. Cell pellets were thawed by the addition of ice-cold lysis buffer (200 mM Tris–HCl, pH 7.4, 20 mM 2-mercaptoethanol, 100 mM NaF and 1% Triton X-100), sonicated (3 × 5 s), and subsequently centrifuged at 14,000×*g* for 15 min at 4°C. The supernatant was used for enzyme assays. The TS catalytic assay was conducted according to a previously reported method (van Triest et al. 1999); the assay determined the catalytic activity of TS by measuring the amounts of ^{3}H released during the TS catalyzed conversion of [5-^{3}H]dUMP to dTMP. Briefly, the assay consisted of the enzymes in assay buffer (lysis buffer without Triton X-100) and 650 μM 5,10-methylenetetrahydrofolate in a final volume of 50 μl. The reaction was started by adding [5-^{3}H]dUMP (1 μM final concentration, specific activity 5 mCi/mol), followed by incubation at 37°C for 60 min and stopped by adding 50 μl of ice-cold 35% trichloroacetic acid. Residual [5-^{3}H]dUMP was removed by adding

250 µl of 10% neutral activated charcoal. The charcoal was removed by centrifugation at 14,000×*g* for 15 min at 4°C, and a 150-µL sample of the supernatant was assayed for tritium radioactivity by liquid scintillation counting in the liquid scintillation analyzer Tri-Carb 2100 (Packard). For each cell line, the linearity of [5-^{3}H]dUMP conversion with respect to amount of protein and time was established.

Western blotting

Cells were harvested, washed twice in ice-cold 1× PBS, and resuspended in a buffer consisting of 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, and 0.1% SDS. Cells were lysed by freeze–thaw three times followed by sonication using three 2–3 s bursts. The insoluble debris was removed by centrifugation at 15,000×*g* for 30 min. Protein concentrations were determined using the Lowry method. Twenty-five micrograms of each protein sample was resolved by SDS-PAGE (12%). The gels were electroblotted onto hydrophobic polyvinylidene difluoride membranes (Hybond^{TM}-P PVDF, GE Healthcare Bio-Science, Uppsala, Sweden). Antibody staining was performed with a chemiluminescence detection system (ECL Plus Western Blotting Detection Reagent, GE Healthcare Bio-Science, Uppsala, Sweden), using a 1:500 dilution of the mouse anti-human TS (TS106) monoclonal primary antibody (Invitrogen S.r.L., Milan, Italy), 1:1,000 dilutions of the mouse anti-human DHFR monoclonal antibody (Tebu-Bio, Milan, Italy) and 1:1,000 of mouse anti-human *β*-tubulin antibody (Sigma-Aldrich S.r.L., Milan, Italy) in conjunction with a 1:3,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (GE Healthcare Bio-Science, Uppsala, Sweden). For western blot analysis of SSAT, electrotransfer to nitrocellulose membrane was followed by incubation with a polyclonal anti-SSAT antibody, prepared as described previously (Allen et al. 2007), and detection using the Lumi-Light Plus detection solution (Roche). Quantification of signal intensity was performed by densitometry on a GS-800 calibrated densitometer (Bio-Rad) and analysed by using Quantity One software (Bio-Rad, CA, USA).

Real-time reverse transcription-PCR analysis

Total RNA was extracted from the cultured cells using TRI reagent (Sigma-Aldrich S.r.L., Milan, Italy). Reverse transcription was performed with 2 µg of total RNA using random primers (Promega, Milan, Italy) and M-MLV reverse transcriptase (Promega, Milan, Italy). Real time RT-PCR was performed with 10 ng of cDNA using Power SYBR® Green PCR Master Mix (Applied Biosystems, Monza (MI), Italy) and an ABI PRISM 7900 HT Sequence Detection System (SDS, Applied Biosystems, Monza (MI), Italy), followed by a dissociation curve analysis and subsequent agarose gel electrophoresis to confirm amplification. The following primer sets were used: TS [PubMed, CoreNucleotide: NM_001071.1], forward: 5′-CAGATTATTCAGGACAGGGAGTT-3′, reverse: 5′-CATCAGAGGAAGATCTCTTGGATT-3′; DHFR [PubMed, CoreNucleotide: NM_000791.3], forward: 5′-TGCACAAATGGGGACGA-3′, reverse: 5′-GGAAATATCTGAATTCATTCCTGAG-3′; and GAPDH [PubMed, CoreNucleotide: NM_002046.3], forward: 5′-CAAGGTCATCCATGACAACTTTG-3′, reverse: 5′-GGGCCATCCACAGTCTTCTG-3′. SSAT primer sequences were, forward: 5′-TTATAGAGGCTTTGGCATAGGA-3′, reverse: 5′-TCATTGCAACCTGGCTTAGA-3′. The amount of target, normalised to an endogenous reference (glyceraldehydes-3-phosphate dehydrogenase, GAPDH) and relative to a calibrator (2008 cell line or untreated sample), was given by $2^{-\Delta\Delta Ct}$ calculation (Arocho et al. 2006). All experiments were carried out three times in triplicate; amplification plots were analysed using the ABI Prism 7900 HT SDS version 2.1 software (Applied Biosystems, Monza (MI), Italy).

Assay of SSAT activity

SSAT activity was measured as previously described (Casero et al. 1994). The cells were harvested, washed twice in PBS, and suspended in a buffer containing 10 mM tris(hydroxymethyl)aminomethane (pH 7.2) and 1 mM dithiothreitol. This suspension was freeze- thawed twice, then cytosolic aliquots were incubated in 100 mM tris(hydroxymethyl)aminomethane (pH 8.0), 3 mM Spd and 0.5 nmol 1-[^{14}C]acetyl coenzyme A in a final volume of 50 µL for 10 min at 30°C. The reaction was stopped by adding of 10 µL 1 M NH_2-OH HCl and boiling in water for 3 min. The resulting samples were spotted onto P-81 phosphocellulose discs and radioactivity measured by scintillation counting. The amount of cytosol added to the final reaction mixture was adjusted to maintain the enzyme/substrate concentrations within the linear range. Enzyme activity is expressed as pmol [^{14}C]acetylspermidine formed/min/mg protein.

Synergy analysis

The nature of the combination between cisplatin and the compounds, combined simultaneously at a fixed ratio, was determined using median-effect analysis with the CalcuSyn ver. 2.0 software (Biosoft, Cambridge, UK), based on a previously described method (Chou and Talalay 1984), which calculates a non-exclusive case combination index (CI) for every fraction affected (FA), a measure of the drug

interaction effects. CI values of less than or greater than 1 indicated synergy and antagonism, respectively, whereas a CI value of 1 indicated additive effects of the drugs. Growth inhibition was assayed by the crystal violet assay and the cytotoxicity of the combination was compared with the cytotoxicity of each drug alone in every experiment, and each experiment was performed at least 3 times.

Statistical analysis

All values report the mean ± SEM, unless otherwise indicated. Statistical significance was estimated by two-tailed Student's *t*-test performed using Microsoft Excel software; a difference was considered significant at $*P < 0.05$ or $**P < 0.01$.

Results and discussion

ITC distamycin A, NAX001 and NAX002 interaction studies with Dickerson and poly[d(AT)] oligonucleotides

The ITC studies of the interaction of distamycin A with the Dickerson oligonucleotide [d(CGCAAATTTGCG)] were performed at four different temperatures (see SI, Figs. 1, 2, 3, 4) while the interaction with poly[d(AT)] oligonucleotide was performed at 293 K (See SI Fig. 5). Thermodynamic parameters for distamycin A interaction with the Dickerson and poly[d(AT)] oligonucleotides are reported in Table 1. The best-fit experimental curve was found to be a "one-site binding model" for the interaction with poly[d(AT)] and a "two-sites sequential binding model" for the Dickerson oligonucleotide interaction at all temperatures. Thermodynamic parameters for distamycin A interaction with the Dickerson oligonucleotide at each temperature are reported in the supplementary data (SI, Table 1) and the K_b (equilibrium constant) temperature-dependence for both binding sites has been determined (see SI, Fig. 6).

The two binding sites presented different thermodynamic parameters. The interaction in the first binding site is enthalpically and entropically favoured, suggesting an important involvement of H-bond formation possibly together with water molecule release. The release of these molecules, together with extended surface contact between the compounds and the double-strand, may contribute to the favourable positive entropic variation. The binding process to the first site exhibits a ΔH value less favourable than the second, but it is entropically more favoured, exhibiting a positive ΔS value (see SI Table 1). The second binding site is enthalpically favoured, showing a ΔH value approximately three times larger than that for the first binding site, leading to the negative and unfavourable ΔS variation. The different thermodynamic behaviour between the two binding sites has been suggested to be correlated with a ligand-induced structural modification by the binding of the first ligand molecule. This conformational change thermodynamically limits the binding of the second molecule (negative cooperativity) (Boots and de Bokx 1989).

The thermodynamic parameters characterising NAX001 and NAX002 interactions with the Dickerson oligonucleotide and the extended TATA box-model oligonucleotide (poly[d(AT)] has been obtained at 293 K but could not be studied at higher temperatures because of the instability of the double strand. The optimally fitting equation for the integrated peak values corresponds to a "one site binding" model for both interactions. Thermodynamic parameters characterising the two interactions are reported in Table 1. The interaction of NAX001 and NAX002 with the B-DNA model oligonucleotide (Dickerson) is endothermic, whereas the interaction with the extended TATA box-model oligonucleotide (poly[d(AT)] is exothermic, (see SI, Fig. 7a–d).

We observed different interaction patterns when comparing the thermodynamic data obtained from the interaction of each of the three ligands (distamycin A, NAX001 and NAX002) with the Dickerson oligonucleotide. Distamycin A exhibited an exothermic binding profile (enthalpy variation, $\Delta H = -18.03$ kJ/mol), whereas NAX001 and

Table 1 Thermodynamic parameters for distamycin A, NAX001 and NAX002 interaction with the Dickerson and poly[d(AT)] oligonucleotides at 293 K

Syringe	Sample cell	Conc. (μM)	K_b (10^6 M^{-1})	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)	K_d (nM)
Distamycin	Dickerson	10	70.1 ± 0.21	−18.03 ± 0.36	26.74 ± 0.51	−44.77	14
	Poly[d(AT)]	10	3.60 ± 0.24	−16.54 ± 0.28	20.24 ± 0.51	−36.77	281
NAX001	Dickerson	10	2.31 ± 0.28	27.47 ± 0.64	63.17 ± 0.94	−35.70	433
	Poly[d(AT)]	10	1.11 ± 0.31	−20.05 ± 0.78	11.3 ± 0.81	−31.35	909
NAX002	Dickerson	10	2.03 ± 0.42	27.22 ± 0.35	62.61 ± 0.47	−35.39	493
	Poly[d(AT)]	10	6.35 ± 0.84	−84.11 ± 0.15	−45.93 ± 0.50	−38.18	157

NAX002 exhibited an endothermic profile ($\Delta H = 27.47$ and 27.22 kJ/mol respectively). On the other side, distamycin A analogues showed a larger favourable entropic contribution ($T\Delta S = 63.17$ and 62.61 kJ/mol respectively, where ΔS is the entropy variation) compared to distamycin A ($T\Delta S = 14.91$). Distamycin A interaction with the Dickerson oligonucleotide is enthalpically and entropically driven whereas for the studied analogues interaction is entirely entropically driven. Thermodynamic parameters obtained from the Dickerson oligonucleotide titrations indicate a high binding-site specificity for distamycin A, involving the presence of H-bond formation, compared to a more important involvement of surface–surface interactions between the analogues and the oligonucleotide (Gómez and Freire 1995; Spolar et al. 1992).

Distamycin A binding to the Dickerson oligonucleotide in the first binding site induces torsion in the oligonucleotide that prevents insertion of water molecules. These water molecules cannot interfere with the binding of distamycin A, leading to a more stable interaction with a greater affinity constant. On the contrary, NAX001 and NAX002, which have one and two pyrrole rings, respectively, more than distamycin A, require a higher energetic contribution for the binding. Our experiments suggested that both ligands do not bind completely with the oligonucleotide, leaving a free mobile fragment that is responsible for the higher entropic contribution compared to distamycin A. This mobile fragment allowed a higher solvent molecules accessibility to the binding sites (in contrast to distamycin A) leading to a higher K_d (therefore lower affinity) compared to that found for distamycin A (14 nM for distamycin A, 433 nM for NAX001 and 493 nM for NAX002).

The interaction with Poly[d(AT)] is enthalpically and entropically favourable for distamycin A and NAX001, whereas it is entropically unfavourable for NAX002. The last showed a larger negative ΔH value (−84.11 kJ/mol) with respect to the other two molecules (−20.05 and −18.03 kJ/mol, NAX001 and distamycin A respectively). Distamycin A binding to the Poly[d(AT)] showed a thermodynamic profile similar to that observed for the high-affinity site (first binding site) found in titrating the Dickerson oligonucleotide, suggesting a similar interaction. But, unlike Dickerson oligonucleotide, only one molecule can bind the double strand. It is possible that a change in torsion induced by the binding of the first molecule induces a conformational change that is not suitable for the binding of a second molecule. This is rational, considering that the Poly[d(AT)] oligonucleotide is not a suitable model for the B-DNA stereochemistry and conformation. NAX001 and NAX002 showed different thermodynamic behaviour. NAX001 with a positive entropic contribution ($T\Delta S = 11.3$ kJoule/mol K) showed a higher affinity for Poly[d(AT)] compared to NAX002, which shows a larger positive enthalpic variation accompanied by an unfavourable entropic contribution ($T\Delta S = -45.93$ kJ/mol K). It is possible that NAX002, due to its additional pyrrolic ring compared to NAX001, is able to form more hydrogen bonds and to block water molecules upon binding. NAX001 may be more complementary with the double strand, thus displacing and substituting solvent molecules (Table 1).

Biological evaluation

Cell growth inhibition and modulation of folate cycle enzymes

It has been shown that DNA intercalating agents and DNA minor groove-binding drugs also bind to the TS mRNA site 1 construct (Cho and Rando 2000). Among these compounds, the DNA minor groove binder distamycin A binds to TS mRNA with a greater affinity than that of the classical DNA intercalator drugs, quinacrine and proflavine. In the present study, we evaluated the effects of distamycin A and some derivatives on cell growth and, for the first time in vivo, on TS expression in a cDDP-sensitive human ovarian cancer cell line, 2008, and its resistant counterpart, C13*. Although the inhibition of cell growth and the reduction of TS expression was almost negligible for NAX001 and NAX003 (data not shown), NAX002 and distamycin A at 40 μM decreased the cell survival of both cell lines by 20-25% (Fig. 2a). TS activity and TS protein level, as well as DHFR protein content, were decreased by both compounds in a dose-dependent manner, in particular in cDDP-sensitive 2008 cells (Fig. 2b). In addition, both the TS expression and the DHFR protein level of the cDDP-sensitive line were more responsive to distamycin A than to NAX002 (Fig. 2c), indicating only a partial correlation with cell growth effects.

Effects of combining the novel folate cycle inhibitors with cDDP on cell growth

The nature of the combination of cisplatin with the studied compounds, combined simultaneously at a fixed ratio, was determined by median-effect analysis using the software CalcuSyn ver. 2.0 (Biosoft, Cambridge, UK). This analysis, based on a method previously described (Chou and Talalay 1984), calculates a non-exclusive case combination index (CI) for every fraction affected (FA), which is a measure of drug interaction. CI values of less than or greater than 1 indicate synergy or antagonism, respectively, whereas a CI value of 1 indicates an additive effect of the drugs. Although all compounds were barely cytotoxic against the

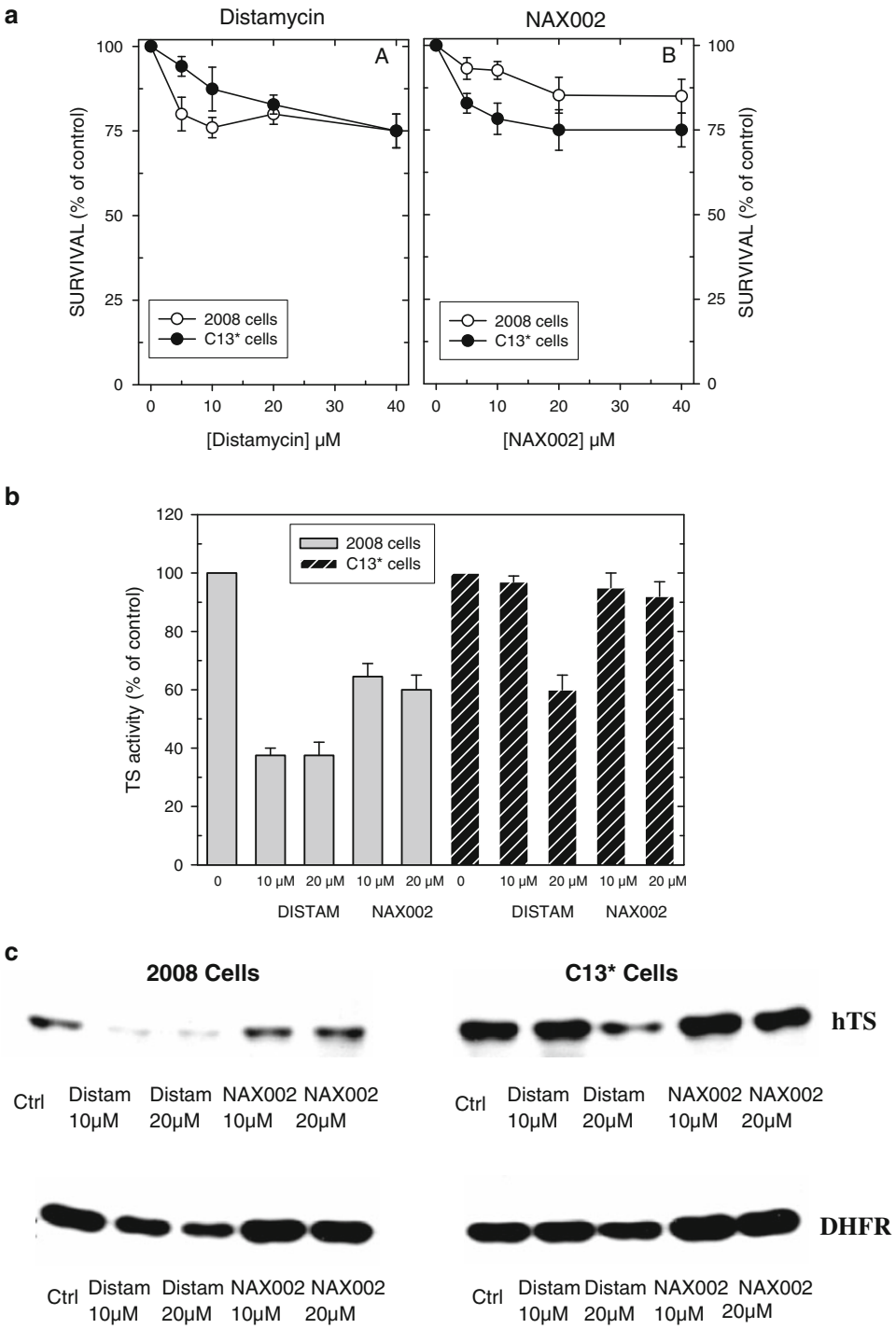

Fig. 2 Effects of distamycin A and NAX002 on cell growth and on TS and DHFR expression in 2008 and C13* cells. **a** The dose-dependent effects of distamycin A and NAX002 on the growth of 2008 cells (*open circles*) and C13* cells (*closed circles*) were determined after a 3-day exposure. Results represent the mean of five separate experiments performed in duplicate.TS activity and Western blot analysis of TS and DHFR protein levels in 2008 and C13* cells (**b**, **c**). Protein levels were determined after a 72-h treatment with the indicated concentrations of the compounds. TS and DHFR protein levels in panels C were assayed in cell line extracts as described under "Materials". hTS monomer and hDHFR monomer, molecular mass 35 and 21 kDa, respectively, are indicated on the right. Equal lane loading was confirmed using a mouse anti-human β-tubulin antibody. Results represent the mean of four separate experiments performed in duplicate. *Error bars* SEM

cell lines when administered alone, when distamycin A was combined with cDDP, the resulting effects were additive in the 2008 cells and mostly synergistic in C13* cells (Fig. 3a). The combination of NAX002 with cDDP was mainly synergistic in both sensitive and resistant cell lines, confirming that these compounds may facilitate cDDP activity (Fig. 3a).

Both cDDP and NAX002 have been reported as telomerase inhibitors (Burger et al. 1997; Zaffaroni et al. 2002; Zhang et al. 2002), thus the synergistic interaction of NAX002 with cDDP both in sensitive and in resistant cells is probably the result, at least in part, of an inhibitory action of the two drugs directed against the same target, such as the activity of telomerase. On the other hand, the positive effects of distamycin A on cDDP may be mainly explained as a consequence of the enhanced impairment of TS expression, caused by the proper combinations of cDDP. It is well-known that cDDP increases the TS

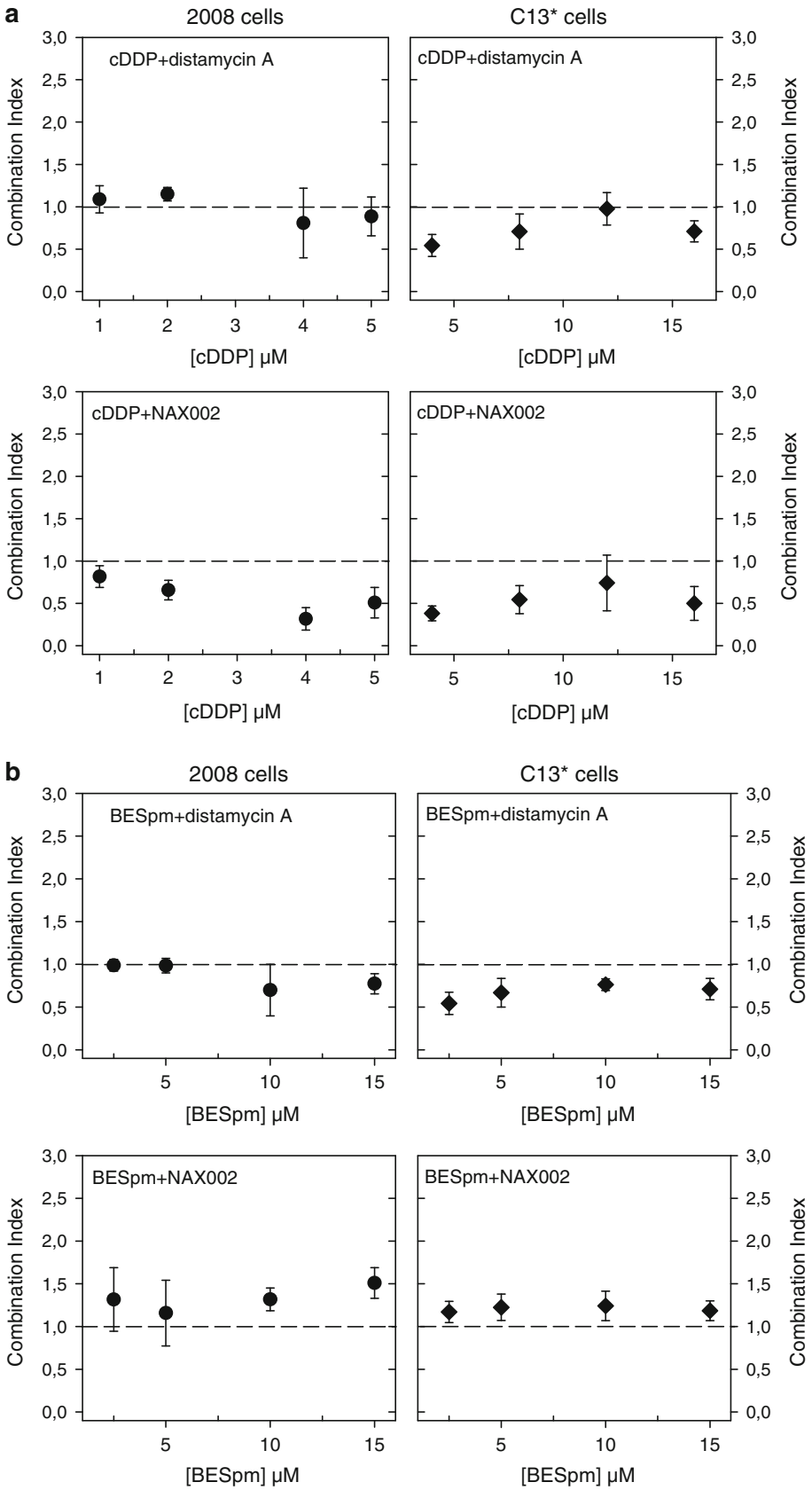

Fig. 3 Median-effect analysis of drug combinations at the proper constant ratios. 2008 cells (*left panels*) and for the C13* cells (*right panels*) were allowed to attach overnight and then simultaneously treated with distamycin A (*upper panels*) or with NAX002 (*lower panels*) and with the indicated concentrations of cDDP (**a**) or BESpm (**b**) in 24-well plates. Following a 3-day exposure, cells were stained with crystal violet. Results are expressed as combination indexes (CIs), where CIs < 1 indicate synergistic effects, CIs > 1 indicate antagonistic effects, CIs $= 1$ indicate additivity. Results represent the mean of three separate experiments performed in duplicate. *Error bars* SEM

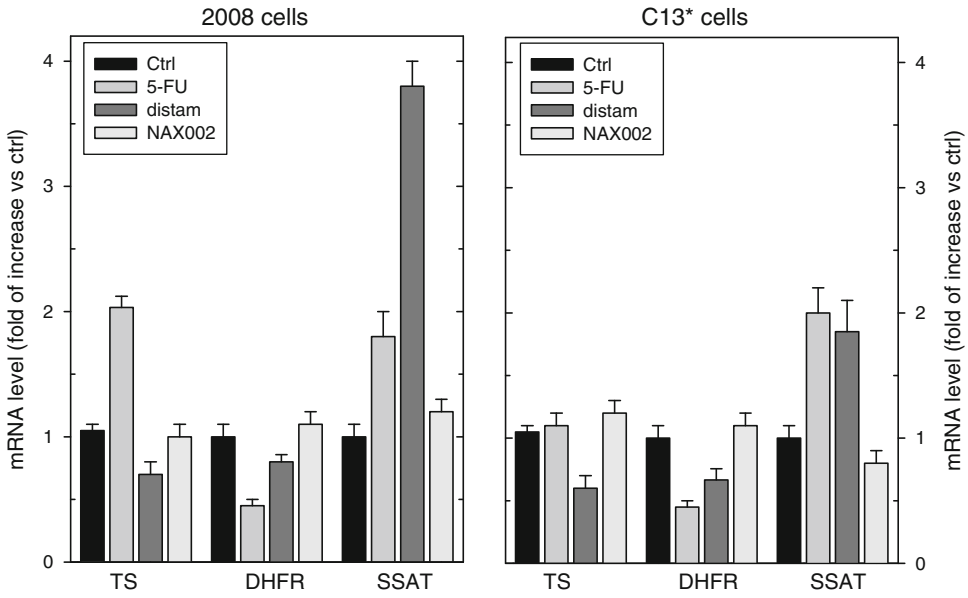

Fig. 4 Comparison of the effects of 5-FU, NAX002 and distamycin A on TS, DHFR and SSAT mRNA expression in 2008 and C13* cells. RT-PCR analysis of TS, DHFR and SSAT mRNA was performed in 2008 and C13* cells treated for 72 h with 20 μM of the indicated compounds. The amount of TS mRNA was normalised to glyceraldehyde-3-phosphate dehydrogenase mRNA. Results represent the mean of three separate experiments performed in duplicate. *Error bars* SEM

inhibition by the classical folate inhibitor 5-FU by inhibiting the transport of L-methionine and indirectly causing the elevation of intracellular levels of CH_2FH_4 and FH_4 in tumour cells in vitro and in vivo (Araki et al. 2000). Therefore, it is likely that even in our cell model, particularly in sensitive cells, cDDP could increase TS down-regulation induced by distamycin A, accounting for the combined effects.

Cellular effects of combining the novel folate cycle inhibitors and Spm analogue in ovarian cell lines

An increasing amount of evidence indicates that the concurrent modulation of both the folate cycle and the polyamine metabolism may represent a valid strategy for treating cancer cell growth from different tumour types. In particular, the combination of classical inhibitors of TS, such as 5-FU with inducing agents of the key catabolic enzyme of polyamine catabolism, spermidine/spermine N1-acetyltransferase (SSAT), such as N1,N11-diethylnorspermine (DENSpm), has proven therapeutic efficacy against tumour cell lines in vitro (Choi et al. 2005). SSAT was also shown to be inducible in response to 5-FU or platinum drugs, both in parental and in cisplatin-resistant cell lines (Allen et al. 2007).

Because over-expression of the folate cycle enzymes TS and DHFR is a significant component of enhanced DNA repair capacity, hence a major mechanism of resistance in cDDP-resistant cell lines, we also evaluated the effects of distamycin A and derivatives combined with polyamine analogues also in the human ovarian cDDP-resistant C13* cells. These resistant cells have a lower sensitivity to both the spermine analogue N1,N12-bisethylspermine (BESpm) (Marverti et al. 1998) and the traditional anti-folates, in comparison with their cDDP-sensitive counterparts, 2008 cells.

As expected, RT-PCR analysis revealed that 5-FU almost doubled TS mRNA level in 2008 cells only, whereas it increased SSAT mRNA level and lowered DHFR mRNA content in both 2008 and C13* cells. Distamycin A decreased both TS and DHFR expression by approximately 50% in both cell lines, whereas it up-regulated SSAT expression by approximately fourfold and twofold in sensitive and resistant cells, respectively. On the contrary, NAX002 did not significantly change the expression of the three enzymes, both in 2008 and in C13* cells (Fig. 4). These data showed, for the first time, that these minor groove binders may modulate TS mRNA in vivo, thus affecting the translation and enzyme activity of sensitive cells and also suggested that differences may exist in the effect on TS mRNA between 2008 and C13* cells.

The up-regulation of SSAT expression was more evident in the presence of BESpm, whose enzyme-induction effects were increased by distamycin A but not by NAX002 in both cell lines (Fig. 5a). We have previously shown that SSAT expression is less induced by the analogue in the resistant cells compared to sensitive cells (Marverti et al. 2010). Of note, in the presence of these TS modulators, 5-FU and distamycin A, but not NAX002, induction of SSAT activity resumed at a level near that of sensitive cells (Fig. 5b). This activity partly correlated with the enhanced SSAT protein level and resulted in cell growth inhibition.

As a result of the induction of SSAT expression alone or in combination with BESpm, only distamycin A synergistically increased cell growth inhibition by BESpm,

especially in the resistant cell line (Fig. 3b). On the other hand, the additive or slight antagonistic effect of NAX002 combined with the spermine analogue could be explained with the poor induction of SSAT expression by this derivative.

It has been reported that bisethylderivatives of polyamines are good inhibitors of cell growth because much weaker DNA aggregators than parent polyamines, leading to relaxation of chromatin structure (Basu et al. 1989) and thus distamycin A may favour, more than NAX002, the interaction and the effects of the analogue with DNA, partly accounting for the enhanced cytotoxicity of the combinations.

Besides, the enhanced induction of SSAT by the combination of 5-FU and DENSpm (Choi et al. 2005), and by some quinoxaline compounds and Spm analogues combination (Marverti et al. 2010), leads to acetylated Spm or Spd and to reactive oxygen species (Agostinelli et al. 2004). Results from our study showed that most of the combinations of novel folate cycle inhibitors with Spm analogues indeed elevated cellular hydrogen peroxide content, which correlating with Spm depletion, may initiate the apoptotic cascade and account for synergistic interactions (Marverti et al. 2010). In fact, these products are oxidized by polyamine oxidase, producing hydrogen peroxide, which at high levels damages mitochondrial membranes resulting in cytochrome *c* release, which in turn, activates members of caspase families and triggers the cascade to apoptosis (reviewed in Marra et al. 2007). Again, in the present study it is likely that the overexpression of SSAT by the combination of the analogue and distamycin A results in elevation of reactive oxygen species which may account for the synergistic effects on cell growth.

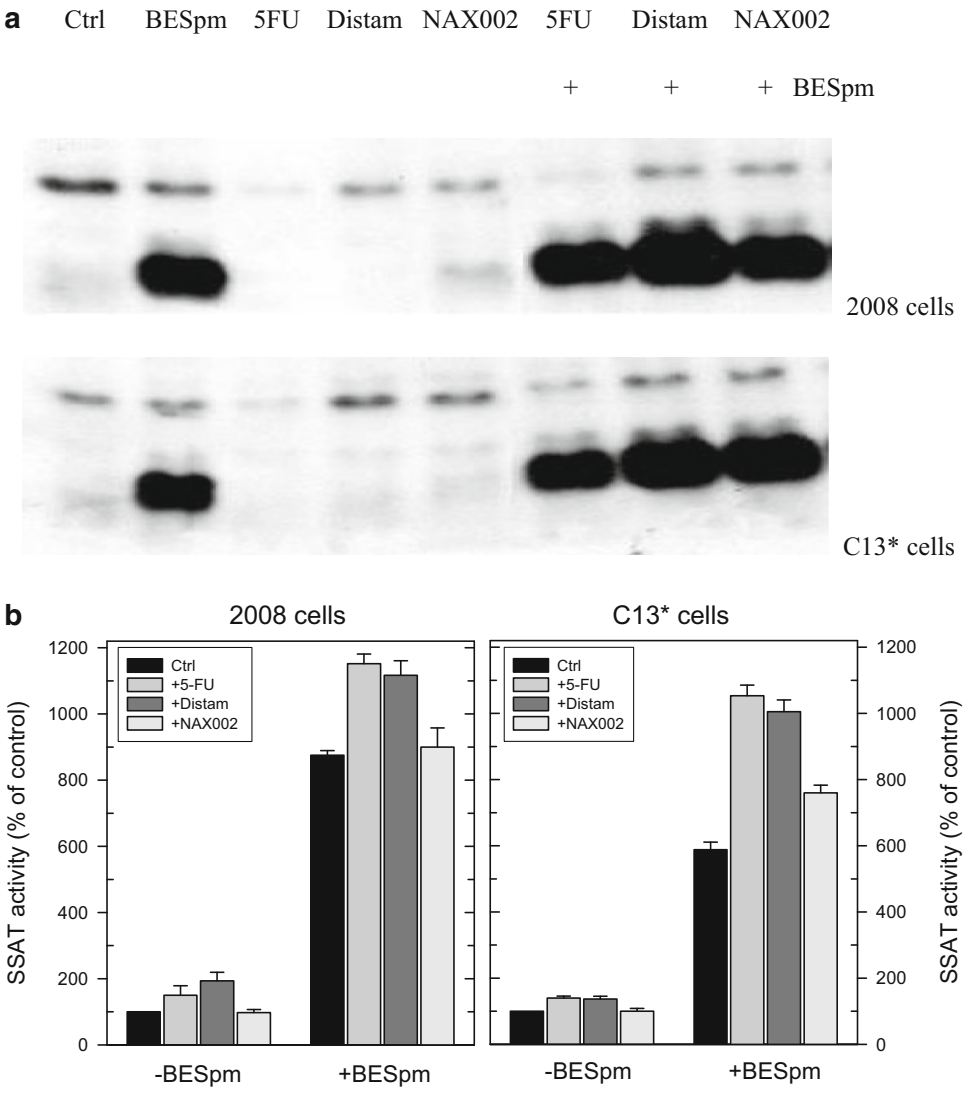

Fig. 5 Effects of 5-FU, NAX002 and distamycin A on SSAT expression in 2008 and C13* cells. **a** Western blot analysis of SSAT protein concentration and **b** SSAT activity of 2008 and C13* cells after incubation with 20 μM of the indicated compounds for 3 days in the presence or absence of BESpm. Results represent the mean of three separate experiments performed in duplicate. *Error bars* SEM

Conclusions

Distamycin A derivatives were studied to identify compounds with improved biological activity in ovarian cancer cells that are sensitive or resistant to cDDP treatment. Distamycin A has been largely studied, and in this work, new structural analogues are presented. To explain the interaction of NAX001 and NAX002 with their DNA target at the cellular level, we studied their interaction with a B-DNA double strand model (Dickerson oligonucleotide) and an extended TATA box double strand model [Polyd(AT)] and characterised their thermodynamic interaction profile in comparison with distamycin A. Through thermodynamic analysis, NAX001 and NAX002 were found to have a different thermodynamic profile compared to distamycin A when interacting with the oligonucleotides. The NAX compounds showed a "one binding site" pattern interaction and higher ΔS contribution because of the presence in their structure of longer polypyrrolamidine residues that cannot completely fit the DNA groove. In particular, NAX002 has a different mechanism of interaction compared to NAX001 and distamycin A that involves an increased number of H-bonds.

Noteworthy, the biochemical studies show, for the first time, that distamycin A and its derivatives affect the expression of folate cycle enzymes even at the cellular level; in particular, these minor groove binders are able to modulate TS mRNA in vivo, thus affecting the translation and enzyme activity. This effect was more evident in sensitive cells and suggest that differences may exist in the TS mRNA of the two cell lines, as a consequence of the cDDP-resistance phenotype.

Our results also show that these compounds, showing different affinity for DNA, modulate the effects on cell growth of cDDP and BESpm. In fact, the data suggest that distamycin A which binds DNA with high affinity may affect the transcription of genes, such as TS and DHFR, causing down-regulation or induce up-regulation of others such as SSAT, reinforcing the induction of this enzyme by the spermine analogue.

On the contrary, the lower affinity binding of NAX002 in comparison with distamycin A may account for the scant effects on the expression of the genes of the enzymes studied, thus differently affecting the action of BESpm in the drug combinations.

Overall, the differential effects of NAX002 seem more dependent on mechanisms only slightly involving TS, DHFR and SSAT. Therefore, to explain the synergistic interaction of NAX002 with cDDP both in sensitive and in resistant cells, the involvement of an inhibitory action against the activity of telomerase, as a common target, has been proposed.

Thus, our results provide further evidence that, in the cDDP-resistant C13* cells, the re-establishment of BESpm sensitivity requires increased expression of SSAT. Moreover, they further confirm that TS inhibitors, either classical or not, target two major cellular processes, i.e. polyamine metabolism as well as DNA synthesis and repair. These two processes may have intimate inter-relationships because polyamines bind DNA and RNA and affect chromosomal structure, RNA stability, and translation. These findings suggest that, to overcome the mechanism of cDDP-resistance based on enhanced DNA synthesis and repair, these polypyrrolamidine derivatives may be combined with cDDP or Spm analogues for more effective cytotoxicity in human ovarian cancer cells also through modulation of the polyamine pathway.

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Conflict of interest The authors declare that they have no conflict of interest.

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