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Accumulation of anthracenyl-amino acid topoisomerase I and II inhibitors in drug-sensitive and drug-resistant human ovarian cancer cell lines determined by high-performance liquid chromatography

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Abstract Anthracenyl amino acid/dipeptide conjugates (AADC) represent novel structures rationally designed for their DNA-binding properties. A high-performance liquid chromatography method is described for simultaneous determination of five compounds that exhibit novel mechanisms of action as topoisomerase I and II inhibitors. The method uses an Apex ODS-2 column and a mobile phase of 0.25 *M* ammonium acetate/trifluoroacetic acid (pH 3) in methanol with gradient elution. Selective detection is achieved by monitoring at 545 nm, with limits of detection ranging between 2 and 4 ng on the column. AADC are recovered from cell sonicates by solid-phase extraction using C2 cartridges, with extraction efficiencies ranging from 84% to 95%. Drug uptake studies were performed with three active compounds in the human ovarian cancer cell line A2780 and its multi-drug-resistant counterpart 2780^{AD}. Marked differences were observed in the pattern of cellular accumulation produced by each compound. NU/ICRF 505 (tyrosine derivative) was taken up most avidly, reaching plateau levels of 4000 pmol/10^6 cells after 2 h, with no difference being apparent between A2780 and 2780^{AD}. NU/ICRF 510 (arginine derivative) accumulated slowly in A2780, failing to achieve an equilibrium after 4 h, and appeared to be completely excluded from 2780^{AD}. NU/ICRF 500 (serine derivative) was most rapidly taken up by A2780, producing a plateau of 800 pmol/10^6 cells after only 30 min with approximately 3-fold less accumulation in 2780^{AD}. These results are correlated to the chemosensitivity of the two cell lines to the three compounds.

Keywords Anthracenyl-amino acids · HPLC analysis · Drug uptake Drug resistance · Topoisomerase I and II

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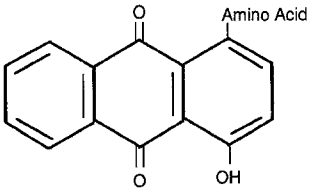
Introduction

The nuclear enzymes DNA topoisomerase (topo) I and II represent two of the most important cellular targets for rational design of new anticancer drugs [7, 9]. Classic inhibitors, which include the clinically useful drugs doxorubicin, mitoxantrone, etoposide, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (mAMSA) and the camptothecins, interfere with the breakage-reunion stage in the catalytic cycle of these enzymes and trap the protein in a covalent complex (termed the cleavable complex) with DNA [17, 26, 31]. Thus, inhibitors poison the endogenous enzyme to damage DNA and ultimately produce cytotoxicity. Existing topo inhibitors suffer from drawbacks, including host toxicity due to other mechanisms of action, such as free radical generation; genotoxicity as a consequence of extensive site-specific DNA cleavage, which has even been linked to secondary malignancies; and the major clinical problem of cancer-cell drug resistance (for review see [10]).

More recently, new classes of topo inhibitors have been described, including agents that induce limited cleavage at specific DNA sequence elements [18] and pure catalytic inhibitors that do not work through the cleavable complex and do not produce DNA cleavage [4, 23, 30]. In addition, catalytic enzyme inhibitors have been shown not to be recognised by certain cancer-cell drug-resistance phenotypes [3]. Anthracenyl amino acid/dipeptide conjugates (AADC) are novel chemical structures rationally designed to bind less avidly to DNA and, therefore, are anticipated to produce less genotoxicity [13]. They exhibit unfavourable one-electron reduction potentials for conversion into toxic free radicals in vivo and are non-cross-resistant against cancer cell lines [13, 14], but they do possess a range of specificities as inhibitors of topo I and II [24, 25] (see Fig. 1). To aid the progress of these interesting new molecules through early development as potential anticancer drugs, a high-performance liquid



Codename	Amino Acid	C-terminus	[a]DNA Binding	[b]Mechanism of topo Inhibition	Molecular Weight	[c]IC50 (μM) A2780	[c]IC50 (μM) 2780^{AD}
NU/ICRF 500	Serine	$-CONHNH_2$	weak intercalation	catalytic inhibitor topo II	341	5.3	8.4
NU/ICRF 505	Tyrosine	$-COC_2H_5$	nil	stabilizes topo I cleavable complexes	431	4.4	7.7
NU/ICRF 510	Arginine	$-COCH_3$	strong intercalation	catalytic inhibitor topo II	396	11.2	>50
NU/ICRF 600	Gly-Phe	-COOH	nil	stabilizes topo I cleavable complexes, catalytic inhibitor topo I	446	ND	ND
NU/ICRF 602	Gly-Gly	-COOH	nil	catalytic inhibitor topo I and II	354	ND	ND

Fig. 1 Chemical structures, biochemical properties and in vitro activity of AADC. A2780 is a human ovarian cancer cell line and 2780^{AD} is its multi-drug-resistant counterpart. NU/ICRF 500 was marginally cross-resistant in 2780^{AD}; NU/ICRF 505 was nominally cross-resistant against 2780^{AD} and NU/ICRF 510 was effectively inactive against 2780^{AD}. [a] Determined by UV-thermal melt analysis and DNA-unwinding assays [24, 25]. [b] Data from Meikle et al. [24, 25]. [c] Data from Cummings et al. ([14]; manuscript in preparation). (*ND*: Not determined)

chromatography (HPLC) method and a solid-phase sample preparation technique were developed for the simultaneous determination of five lead compounds from this series (NU/ICRF 500, 505, 510, 600 and 602; for structures see Fig. 1). The methodology was utilised to perform drug accumulation studies with three of the most active compounds (NU/ICRF 500, 505 and 510) in the human ovarian cancer cell line A2780 and its multi-drug-resistant (MDR) counterpart 2780^{AD}.

Materials and methods

Chemicals and drug standards

All methanol was of HPLC reagent grade and was obtained from Rathburn Chemicals (Walkerburn, UK); ammonium acetate was of HPLC reagent grade and was supplied by FSA Laboratory Supplies (Loughborough, UK); and trifluoroacetic acid was of protein-sequencing grade and was obtained from Sigma Chemical Co. (Poole, UK). Dimethylsulphoxide (DMSO) was of spectroscopic grade and was supplied from NBS Biological (Hatfield, UK). Water was deionised and double-distilled in a quartz glass still. All other general chemicals were of the highest grade available commercially and were used as received.

AADC were synthesised through the reaction of α-amino acid esters with [2H, 3H]-9,10-dihydroxyanthracene-1,4-dione as described in detail (Cummings and Mincher, UK patent GB 9205859.3; International Application Number PCT/GB93/00546, published 30 September 1993 and filed with the American Patent Office, 14 October 1994). The above-mentioned procedure results (after oxidation) in monosubstitution of the anthraquinone nucleus uncontaminated by bis-substitution products. The highly crystalline, optically pure *N*-anthracenyl amino acid derivatives were characterised by electron-impact mass spectrometry, elemental analysis, proton nuclear magnetic resonance (NMR) and infra-red spectroscopy. They were purified by preparative thin-layer chromatography (TLC), column chromatography and recrystallisation. All analytical standards were dissolved and diluted in DMSO and were stored refrigerated at 4°C for no longer than 2 weeks.

High-performance liquid chromatography

The apparatus consisted of a Hewlett-Packard Model 1090 liquid chromatograph with a diode-array detector (set at 545 nm with a reference wavelength of 400 nm) configured as reported previously [12]. The stationary phase was an Apex ODS-2 (25 cm × 4.6 mm inside diameter) stainless-steel analytical column and an Apex ODS-2 (1 cm × 4.6 mm inside diameter) stainless-steel pre-column (supplied by Crawford Scientific, Strathaven, UK). The mobile phase comprised ammonium acetate [0.25 *M*, adjusted to pH 3 with 25% (v/v) trifluoroacetic acid] as buffer A and methanol as solvent B. Gradient elution was employed at a flow rate of 1 ml/min and at 40°C using the following linear programme: t = 0, 40% solvent B; t = 10 min, 100% solvent B; t = 12 min, 100% solvent B; and t = 16 min, 40% solvent B. The total run time was 20 min, which allowed 4 min for complete re-equilibration of the mobile phase to occur (as determined spectrophotometrically using the diode-array detector).

Solid-phase extraction

AADC were extracted from cancer cell sonicates (up to 3 ml/10^6 cells) using Bond Elut C2 mini-columns (100 mg sorbent, 1-ml reservoirs; Phenomenex, Macclesfield, UK) operating under negative pressure. Columns were first activated with 1 ml methanol, then washed with 1 ml of water prior to loading of the sample. After sample loading, columns were washed twice with 1 ml of water and finally eluted with 400 μl of 90% methanol: 10% 1 *M* ammonium acetate (v/v). Final eluents were then filtered and immediately transferred to capped autosampler vials, and 50 μl was analyzed by HPLC as described above.

Drug accumulation studies in cancer cells

The A2780 human ovarian cancer cell line and its MDR counterpart 2780^{AD} were kindly provided by Drs. T.C. Hamilton and R.F. Ozols (Medicine Branch, Division of Cancer Treatment, NCI, Bethesda, Md. USA). In our hands, 2780^{AD} is 1460-fold resistant to doxorubicin and 5.5-fold resistant to camptothecin (Cummings et al., manuscript in preparation). Cells were grown as monolayers in RPMI 1640 medium supplemented with 5% heat-inactivated foetal calf serum containing a 1% antibiotic mixture under standard tissue-culture conditions and were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Drug accumulation studies were performed in 12.5-cm^2 tissue-culture flasks (Falcon Plastics, Becton Dickinson Labware, Franklin Lakes, N.J., USA) containing approximately 10^6 cells in 4 ml of media, with 2–5 replicate flasks being set up for each time point. Cells were incubated with 10 μM of each of the three compounds under investigation (NU/ICRF 500, 505 and 510) for the following time points: 0, 10, 30, 60, 120, and 240 min and, occasionally, 360 and 480 min. At the end of each incubation, drug-containing medium was removed and the monolayer cells were washed twice with ice-cold phosphate-buffered saline. Monolayer cells were then disrupted by addition of 2 ml of cold distilled water, mechanically harvested from flasks by scraping with the addition of a further 1 ml of distilled water and finally subjected to a brief period (10 s) of sonication to complete the disruption process.

Trypsinisation of cells was avoided, as phenol red present in the trypsin/versene mixture interfered with the HPLC method. Cell sonicates were stored on ice for no more than 2 h and then analyzed. For the uptake studies performed at 4°C, flasks were temperature-equilibrated for 1 h prior to the addition of drug-containing medium. In each drug uptake study performed, three separate flasks of cells were set aside for determination of cell numbers, and the results were expressed as picomoles of drug per 10^6 cells.

Results

High-performance liquid chromatography

AADC are amphipathic molecules containing a strongly hydrophobic (non-water-soluble) anthraquinone chromophore and a more water-soluble amino acid side chain that can be neutral (NU/ICRF 500), positively charged (NU/ICRF 510), negatively charged (NU/ICRF 600 and 602) or hydrophobic (NU/ICRF 505). Thus, a unique set of HPLC conditions are necessary to determine all five compounds on the same chromatogram. The characteristics of the method developed are contained in Table 1, and a chromatogram illustrating the separation of a mixture of standards is presented in Fig. 2. In keeping with related anthraquinoid structures such as mitoxantrone [8, 28], ametantrone [22] and C1941 (biantrazole, DUP941) [16], a high-ionic-strength buffer was required together with a reversed-phase (C-18) HPLC column to reduce secondary column interactions and peak tailing. For full resolution of all five compounds a pH adjustment to 3.0 was necessary and trifluoroacetic acid, which is commonly used in the HPLC analysis of peptides, was adopted for this purpose. All five compounds share almost identical visible absorption spectra with a maximum at 545 nm, and this wavelength was chosen for highly selective detection free of interference from endogenous substances co-extracted from cancer cells. However, a monitor wavelength of 545 nm did result in a 2- to 3-fold reduction in the limit of detection as compared with the standard UV monitor wavelength of 254 nm.

Solid-phase extraction sample preparation

Typical recoveries of all five compounds when 1 μg was added to 10^6 A2780 or 2780^{AD} human ovarian cancer cells suspended in 3 ml of distilled water were: NU/ICRF 500, 95.4 ± 4.1%; NU/ICRF 505, 85.5 ± 5.3%; NU/ICRF 510, 91.8 ± 4.4%; NU/ICRF 600, 94.4 ± 3.8%; and NU/ICRF 602, 83.5 ± 2.2%. In each case, high and reproducible extraction efficiencies were recorded. Thus, if required, one compound could act as an internal standard for the others. The sample preparation technique did not extract any endogenous substances that could potentially interfere with the identification of AADC in cancer cells (data not shown).

Solid-phase extraction has been adopted in preference to liquid:liquid extraction techniques or XAD extraction columns, which have been employed with mitoxantrone and ametantrone [15, 22, 27]. These techniques have been criticised as being tedious and time-consuming and as offering poor sensitivity [16].

Table 1 HPLC of AADC topoisomerase I and II inhibitors. For structures and biological properties of AADC see Fig. 1, for experimental details of the HPLC methodology see Materials and methods

Code name	Retention time (min ± CV)[a]	Limit of detection On column (ng)	Limit of detection After extraction (ng/ml)	Calibration curves Range: 5–5000 ng ($y = mx + c$)[b]	Regression correlation r^2
NU/ICRF 500	9.2 ± 0.3	2	16	$y = 1.49x + 0.52$	1.000
NU/ICRF 505	13.0 ± 0.5	2	16	$y = 1.28x + 1.70$	1.000
NU/ICRF 510	11.2 ± 0.4	2	16	$y = 2.12x + 0.84$	0.999
NU/ICRF 600	12.2 ± 1.0	4	32	$y = 3.42x - 2.37$	1.000
NU/ICRF 602	10.0 ± 0.6	4	32	$y = 3.22x - 0.68$	0.999

[a]Within-day coefficient of variation in retention time

[b]y = Concentration, x = integrated peak area, m is the gradient of the calibration curve and c is the intercept with the y-axis

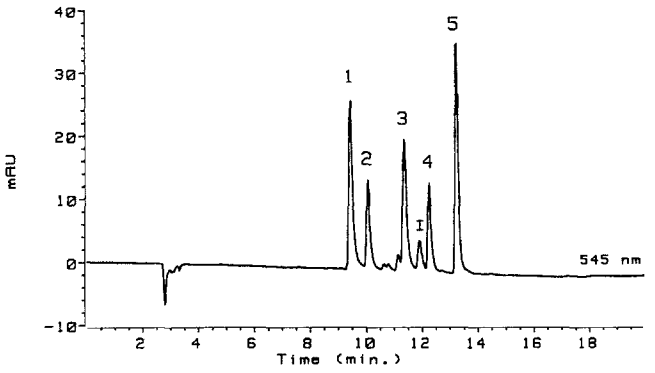


Fig. 2 A typical HPLC chromatogram illustrating the separation of a standard mixture of 5 AADC. Each component represents 400 ng (on the column) and peaks are identified as follows: *1* NU/ICRF 500, *2* NU/ICRF 602, *3* NU/ICRF 510, *4* NU/ICRF 600, *5* NU/ICRF 505. For structures and properties of AADC see Fig. 1. *Peak I* is an impurity

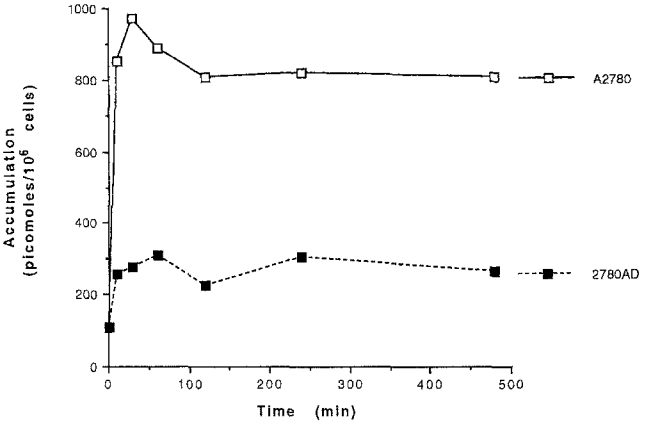


Fig. 3 Concentration-time profiles for uptake/accumulation of NU/ICRF 500 in human ovarian cancer cell line A2780 and its multi-drug-resistant counterpart 2780^{AD} as measured at 37°C. Each point represents the average result of studies performed in duplicate flasks, and cells were exposed to a drug concentration of 10 μ*M*

High-sensitivity extraction of mitoxantrone from plasma has been reported using the negatively charged ion-pairing agent hexane sulphonic acid with dichloromethane [32], but this technique would clearly not be applicable to all the AADC under investigation. Solid-phase extraction has previously been applied to mitoxantrone and biantrazole utilising C2 and C18 sorbents, but the final elution step required methanolic-HCl (0.5 *M* or concentrated HCl) [16, 26], which would almost certainly hydrolyse peptide bonds present in AADC. The technique developed in this present work avoids strong acids or bases in the final elution step to ensure the recovery of all five compounds with equal and high efficiency and prevent sample degradation.

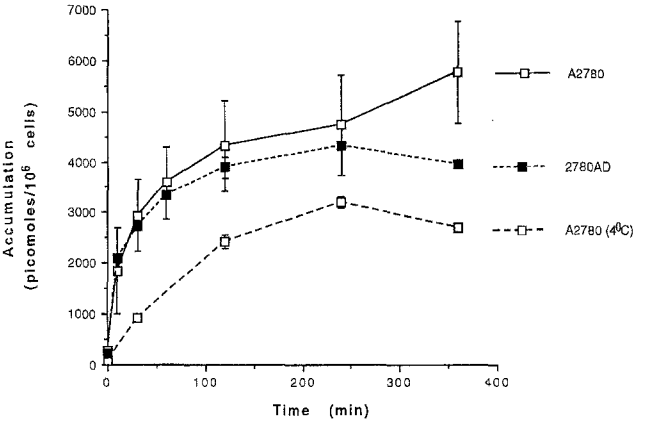


Fig. 4 Concentration-time profiles for uptake/accumulation of NU/ICRF 505 in A2780 and its multi-drug-resistant counterpart 2780^{AD} as measured at 37°C. Each point represents the mean value ± SD for 3–5 replicates, and cells were exposed to a drug concentration of 10 μ*M*. In addition, uptake/accumulation was determined in A2780 at 4°C

Drug accumulation studies

Concentration/time profiles generated for the uptake/accumulation of NU/ICRF 500, 505 and 510 in A2780 and 2780^{AD} are illustrated in Figs. 3, 4 and 5, respectively. Each compound produced its own characteristic and markedly different pattern of uptake. NU/ICRF 500 was the first compound (at 30 min) to reach plateau levels of approximately 800 pmol/10^6 cells for A2780 and 250 pmol/10^6 cells for 2780^{AD} (see Fig. 3). NU/ICRF 510 failed to reach an equilibrium phase of uptake/accumulation in A2780 even after a 4-h incubation period, when levels were approaching 1500 pmol/10^6 cells (Fig. 5). Concentrations determined in 2780^{AD} at 4 h were not significantly different from those measured at time zero, suggesting that this compound is effectively excluded from the resistant cell line. NU/ICRF 505 was most avidly taken up into cells, producing plateau concentrations of around 4000 pmol/10^6 cells after approximately 2 h. No significant difference was observed between A2780 and 2780^{AD} with the exception of the 6-h time point (see Fig. 4). The high avidity of NU/ICRF 505 for cells is further illustrated by the observation that at 4°C, whereas uptake was slowed down, plateau levels were reduced only to around 3000 pmol/10^6 cells (see Fig. 4). These data for AADC are to be compared against the uptake profile of mitoxantrone in L1210 cells, where a plateau of 480 pmol/10^6 cells was recorded after 30 min when cells had been exposed to 4 μ*M* drug [6], and that of daunorubicin, where a plateau of approximately 1000 pmol/10^6 cells was reached after 30 min when P388S cells had been exposed to 9.5 μ*M* drug [21].

No evidence of metabolism of any of the compounds was detected throughout the various different incuba-

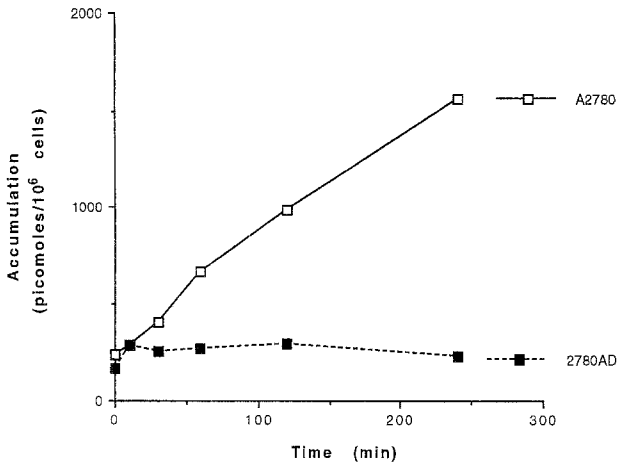


Fig. 5 Concentration-time profiles for uptake/accumulation of NU/ICRF 510 in A2780 and its multi-drug-resistant counterpart 2780^{AD} as measured at 37°C. Each point represents the average result of studies performed in duplicate flasks, and cells were exposed to a drug concentration of 10 μM

tions. Analysis of the tissue-culture medium after 4 h of drug incubation revealed that 92.0% of the NU/ICRF 500 was intact, 75.9% of the NU/ICRF 505 was intact and 93.4% of the NU/ICRF 510 was intact. The lower recovery of NU/ICRF 505 may be due to oxidation of the phenol group in this compound to species that bind covalently to biological molecules or polymerise. Such products are unlikely to be detected by conventional HPLC techniques. This possibility is currently under investigation.

Discussion

AADC are now established as a new chemical class of topoisomerase inhibitors that have novel mechanisms of action and are non-cross-resistant or nominally cross-resistant in cells expressing classic MDR or atypical MDR phenotypes (see Fig. 1; Cummings et al., manuscript in preparation) [14, 24, 25]. To aid rationalization as to which agents should be promoted to the preclinical animal pharmacology phase of anticancer drug development, accumulation studies were carried out with three of the most active compounds in the human ovarian cancer cell line A2780 and its MDR counterpart 2780^{AD}. In addition, these studies were performed to provide insights into potential mechanisms of drug resistance or explain the lack of drug resistance exhibited by these compounds (see Fig. 1).

Perhaps the most striking observations concerned NU/ICRF 510, the arginine derivative that binds to DNA with high affinity and is a catalytic inhibitor of topo II. Two interesting results were obtained. First, in drug-sensitive A2780, uptake of NU/ICRF 510 proceeded very slowly in comparison with that of related drugs such as mitoxantrone [6] and the anthracycline daunorubicin [21]. Second, NU/ICRF 510 was effectively excluded from drug-resistant 2780^{AD}, strongly indicating that this compound is a substrate for the plasma membrane MDR efflux-pump P-170 glycoprotein (P-170), which is highly overexpressed in this cell line [1]. The unique chemical property that distinguishes NU/ICRF 510 from the two other AADC under investigation is the presence of a full positive charge, and this feature is probably responsible for both recognition by P-170 in 2780^{AD} and slower uptake in A2780. The presence of a positively charged amino group in the anthracycline doxorubicin slows down its uptake in HL-60 cells, and replacement of this group with a more hydrophobic *N*-dimethyl residue enhances drug uptake and increases plateau levels of accumulation by 6-fold [5]. Replacement of the positively charged amino function in doxorubicin with a hydroxyl group increases drug uptake 3-fold and overcomes MDR in the human melanoma cell line 8266S [29]. Studies with a series of fluorescent dyes have also shown that the positively charged analogues are actively effluxed in MDR P388/ADR cells (along with neutral species but not anionic derivatives) [20]. NU/ICRF 510 is the least active of the three compounds in A2780 and is without activity in 2780^{AD} (see Fig. 1). These results can therefore probably be explained largely on the basis of cellular drug kinetics, whereby uptake is delayed in A2780 and is effectively non-existent in 2780^{AD}.

The drug uptake/accumulation profiles produced by the serine derivative NU/ICRF 500 were very similar both quantitatively and qualitatively to those of other closely related anticancer drugs such as mitoxantrone and daunorubicin [6, 21]. A 3-fold reduction was recorded in 2780^{AD}, again indicating recognition by P-170. Such a reduction in accumulation can lead to a 38-fold loss in cytotoxicity as in the case of daunorubicin [21], yet NU/ICRF 500 was only marginally less active in 2780^{AD} (see Fig. 1). However, NU/ICRF 500 is a catalytic inhibitor of topo II and does not work through the cleavable complex. From this point of view it is noteworthy that as well as overexpressing P-170, 2780^{AD} underexpresses topo IIα by 4-fold and topo IIβ by 2.5-fold (Cummings et al., manuscript in preparation). Therefore, this cell line also displays the drug-resistance phenotype known as atypical-MDR [2] or altered topo MDR and should be highly resistant to topo inhibitors, regardless of whether they are actively extruded by P-170. This argument tends to hold true for classic topo inhibitors that stabilize the cleavable complex, where relationships between increased enzyme expression and increased cytotoxicity have been established [10]. It is tempting to speculate that as a catalytic inhibitor, where the normal rules of enzyme kinetics will apply, NU/ICRF 500 does not need to accumulate to the same extent in

2780^{AD}, since there is less enzyme present in this cell line for inhibition to occur. A similar argument may be applicable in the case of the human leukaemic cell lines CEM/VM-1 and -1–5, both of which display reduced cellular topo II activities but are non-cross-resistant to the topo II catalytic inhibitors merbarone, aclarubicin and fostriecin [3]. However, it must be stated that at present it is unclear exactly how catalytic inhibitors are non-cross-resistant.

NU/ICRF 505, which works through the topo I cleavable complex, proved to be the only compound that was not recognised by P-170 on the basis of the experimental finding that uptake did not differ in A2780 as compared with 2780^{AD}. This compound was only nominally cross-resistant in 2780^{AD} (1.8-fold), which may be explained by the finding that the resistant cell line has a 1.5-fold reduction in topo I expression (Cummings et al., manuscript in preparation). Although NU/ICRF 505 was most avidly taken up into cells (4- to 5-fold greater accumulation as compared with NU/ICRF 500), it was no more effective than NU/ICRF 500 against A2780. Of the three compounds studied, NU/ICRF 505 is the most lipophilic, a property known to overcome MDR in related compounds such as the anthrapyrazoles [21]. A situation similar to that in NU/ICRF 505 has been reported for the lipophilic anthracycline analogue 4′-deoxydoxorubicin, whereby this derivative achieved intracellular levels 300-fold in excess of those of the parent drug doxorubicin in the L-DAN cell line but was no more cytotoxic [19]. Indeed, increased drug uptake is sometimes associated with decreased drug potency since when the chemical structure of a drug is modified, other parameters are likely to be altered as well as membrane transport [5]. Nevertheless, although increased drug uptake at the in vitro cellular level may not confer an advantage from a mechanistic point of view, it could provide a major therapeutic benefit in vivo. Taking the analogy of 4′ -deoxydoxorubicin further, although this derivative's improved cellular uptake does not increase its cytotoxicity, its increased liphophilicity enhances its penetration into a sub-cutaneously growing MC 40A rat sarcoma by 2.5-fold and circumvents drug resistance [11]. Thus, the drug uptake properties of NU/ICRF 505 would tend to suggest that this would be the most appropriate compound for further progress into the preclinical pharmacology stage of anticancer drug development.

In summary, an HPLC method and a sample preparation technique were developed for the determination of five different AADC in cancer cells. The methodology was applied to study the uptake of three active compounds in the A2780 cell line and its MDR counterpart 2780^{AD}. The results reveal that each compound is taken up in a characteristic and markedly different manner, which is nevertheless consistent with their activity in the two cell lines and their mechanisms of action as topoisomerase I and II inhibitors.

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