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Development of Anthracenyl-Amino Acid Conjugates as Topoisomerase I and II Inhibitors that Circumvent Drug Resistance

Jeffrey Cummings, Janet S. Macpherson, Ian Meikle and John F. Smyth*
IMPERIAL CANCER RESEARCH FUND, MEDICAL ONCOLOGY UNIT, WESTERN GENERAL HOSPITAL, EDINBURGH, EH4 2XU, U.K.

ABSTRACT. Anthracenyl-amino acid conjugates (AAC) represent a novel class of topoisomerase (topo) inhibitor. The relationship between mechanism of enzyme inhibition and *in vitro* cytotoxicity has been investigated in a panel of 5 Chinese hamster ovary (CHO) and 2 human ovarian cancer cell lines (A2780) shown to possess different drug resistance phenotypes associated with altered expression of topo I and topo II. From a total of 13 compounds, 4 displayed broad-spectrum activity (IC_{50} ranging from 3.5–29.7 μM). NU/ICRF 500 (topo II catalytic inhibitor) was 1.4-fold more active against CHO ADR-1, which overexpresses topo II and was essentially noncross-resistant in CHO ADR-r (13.9-fold resistant to doxorubicin (DOX)) and 2780^{AD} (1,460-fold resistant to DOX). NU/ICRF 505, which stabilises topo I cleavable complexes, was noncross-resistant in CHO ADR-3 (3.4-fold resistant to camptothecin) and only 1.8-fold cross-resistant in 2780^{AD}. Hypersensitivity was recorded in ADR-r that overexpresses topo I. The most active compound was NU/ICRF 506, a dual catalytic inhibitor of topo I and II. Hypersensitivity was observed in ADR-r (1.4-fold) but not ADR-1, indicating that topo I is the likely nuclear target, and a low level of resistance was seen in the CHO ADR-6 drug transport mutant and 2780^{AD}. The topo II catalytic inhibitor NU/ICRF 513 only produced hypersensitivity in ADR-r. These data suggest that NU/ICRF 500, 505, and 506 induce cell death, at least partly, through topo inhibition. NU/ICRF 513 appears to be cytotoxic *via* a nontopo mechanism of action. In addition, NU/ICRF 505 significantly inhibited the growth of two human xenografts (HT-29 colon cancer and NX002 nonsmall-cell lung cancer) in nude mice after i.p. administration at a dose of 25 mg/kg. The important properties of noncross-resistance and *in vivo* antitumour activity merit further development of AAC as potential new anticancer drugs. BIOCHEM PHARMACOL 52;7:979–990, 1996.

KEY WORDS. anthracenyl-amino acids; topoisomerase I and II; chemosensitivity; antitumour activity; multidrug resistance; atypical-MDR

DNA topoisomerases I and II (topo I and II) continue to be important cellular targets for rational design of new anticancer drugs [1, 2]. Classic inhibitors interfere with the breakage-reunion step in the catalytic cycle of these enzymes, trapping the protein in a putative reaction intermediate (termed the cleavable complex), where the topoisomerase molecule remains covalently attached to either one strand (topo I) or both strands (topo II) of DNA after cleavage [3, 4]. Thus, drugs actually poison the endogenous enzyme to damage DNA. On this basis, classic inhibitors have been shown to be more effective against cell lines with higher topo levels [5–7] and less effective against cell lines with lower topo levels [8, 9]. However, recent clinical studies failed to observe a correlation between topo expression in biopsy material and clinical efficacy of chemotherapy [10, 11], and it has now emerged that several additional factors are required downstream of cleavable complex formation for cell death to occur [12–14].

The majority of clinically effective topo poisons work through the cleavable complex, bind to DNA with high affinity (usually by intercalation), and induce extensive site-specific damage [15]. Such a high degree of genotoxicity is being increasingly linked to the induction of chromosomal abnormalities and the etiology of therapy-related secondary malignancies in the clinic [16, 17], and is probably unnecessary, because in many model systems no clear relationship is observed between bulk DNA damage and cytotoxicity [18]. In addition, most of these drugs are not selective enzyme inhibitors, but exhibit secondary mechanisms of action that contribute to their host tissue toxicity [19]. The major limitation of existing topo inhibitor-based chemotherapy is the problem of drug resistance. In laboratory models, that has taken the form of the MDR† phe-

* Corresponding author. Dr. J. Cummings, Imperial Cancer Research Fund, Medical Oncology Unit, Western General Hospital, Edinburgh, EH4 2XU, U.K. Tel. 0131 332 2471 Ext 2416; FAX 0131 332 8494.

† *Abbreviations:* MDR, multidrug resistance; at-MDR, atypical multidrug resistance; AAC, anthracenyl-amino acid conjugates; CHO, Chinese hamster ovary; FCS, foetal calf serum; pgp, P-170 glycoprotein; DOX, doxorubicin; CPT, camptothecin; TBS, tris buffered saline; TBST, TBS containing tween 20; E^1_7, one electron reduction potential determined at pH 7; RTV, relative tumour volume; T/C, antitumour activity defined as mean volume of drug treated tumours over control tumours × 100; MRP, multidrug-resistance protein; GST, glutathione S-transferase.

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notype and a number of altered topo at-MDR phenotypes [20, 21].

Recently, new compounds have been described that do not stabilize the cleavable complex, but act as pure inhibitors of enzyme activity [22, 23]. One of the significant features of this group is that they antagonize DNA cleavage and the *in vitro* cytotoxicity of conventional poisons [24, 25]. Various unique mechanisms have been proposed to explain catalytic enzyme inhibition [19, 26].

Structure-activity studies and computer modelling are beginning to identify the key molecular features necessary for formation of the ternary complex between drug, DNA, and protein [27, 28]. A polycyclic ring system is required for DNA binding by intercalation and a further aromatic ring substitution, with a sterically unhindered hydroxy or amino function, is necessary to extend out of the plane of DNA and interact with a hydrophobic pocket of the enzyme. This is the basic model employed in the present work to rationally design novel topo inhibitors. An anthraquinone ring was chosen as the potential DNA intercalating motif, and monoconjugation of this nucleus with naturally occurring amino acids modified at their free C-terminal was adopted, to provide chemical diversity to probe different interactions with the topo protein. Emphasis was placed on the more hydrophobic, neutral amino acids containing a side chain hydroxyl group (i.e. tyrosine and serine), as well as more hydrophobic C-terminal modifications (methyl and ethyl esters), for two reasons: first, to decrease DNA binding affinity and reduce the overall level of DNA damage, and second, in an attempt to overcome multidrug resistance [2]. In an initial study, it was confirmed that anthracenyl-amino acid conjugates (AAC) bind only weakly, if at all, to DNA but that they possess a range of specificities as inhibitors of topo I and II (see Fig. 1) [29]. In this report, the relationship between topo inhibition and *in vitro* cytotoxicity has been studied, along with cross-resistance profiles, by determination of activity in a panel of cells expressing varying levels of topo I and II and possessing a number of drug-resistance phenotypes.

MATERIALS AND METHODS

Experimental Compounds

AAC 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 30th September 1993). Camptothecin (CPT) was from the Sigma Chemical Co. (Poole, U.K.) and doxorubicin (DOX) was from Farmitalia Carlo Erba Ltd. (St. Albans, U.K.).

Cell Lines

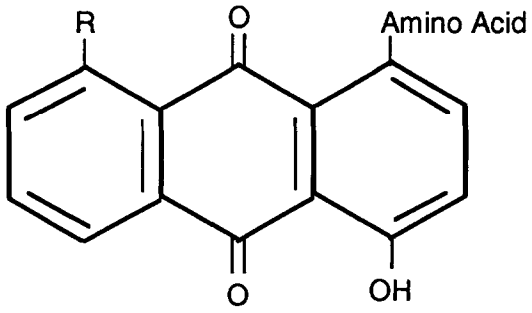
The A2780 human ovarian cancer cell line and its MDR counterpart 2780^{AD} were kindly provided by Drs. T. C. Hamilton and R. F. Ozols, Medicine Branch, Division of Cancer Treatment, NCI, Bethesda, NY, U.S.A. Resistance was derived in 2780^{AD} by stepwise exposure of A2780 to increasing concentrations of DOX. Mutant hypersensitive CHO cell lines ADR-1, ADR-3, and ADR-6 were isolated from CHO-K1 cells in a single selection protocol after 24-hr treatment with the point mutagen ethyl methanesulfonate (300 μg/mL) as described previously [5, 30]. The resistant CHO cell line ADR-r was derived by stepwise exposure of CHO-K1 to increasing concentrations of DOX over a 3-month selection period [31]. A2780 and 2780^{AD} cells were grown as monolayers in RPMI 1640 medium supplemented with 5% heat-inactivated FCS 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. CHO cell lines were maintained under the same conditions except that they were grown in Ham's F-10 nutrient mixture supplemented with 5% FCS and 5% heat-inactivated newborn calf serum. All experiments were performed on cells within 10 passages of each other, during which period phenotypes (see Table 1) remained stable.

P-170 Glycoprotein (Pgp) Expression Determined by Flow Cytometry

Pgp expression in each cell line was measured by a standard immunofluorescence procedure using a FACScan flow cytometer (Becton-Dickson, High Wycombe, U.K.). A population of 50,000 cells were analyzed after treatment with one of three antibodies: MRK 16 (a gift from Dr. Takashi Tsuruo, Tokyo, Japan), human C219, and mouse JSB-1 (a gift from Dr. Henk Broxtermann, Amsterdam, The Netherlands).

Pgp 1 mRNA Levels in CHO Cell Lines Determined by RNase Protection Assay

Total RNA was prepared from CHO cell lines using standard methods. After extraction, and before use in RNase protection assays, the integrity of each RNA preparation was assessed by running samples on a 1% agarose gel. The pgp 1 probe was prepared from a CHO cDNA library (constructed by Dr. D. Simmons, Institute of Molecular Medicine, Oxford, U.K.) using PCR to generate a 354-base pair fragment corresponding to the sequence 1358-1712 of the hamster pgp 1 gene [32]. The fragment generated was subcloned into a pBluescript II vector. To generate sense and antisense transcripts, the T3 or T7 promoters of pBluescript were utilized, after linearization of plasmid with *Eco*R I or *Hind* III, respectively. All radiolabeled transcripts were synthesised *in vitro* using T3 or T7 RNA polymerase and [α-^{32}P]-CTP by the method outlined in [33]. In each RNase protection reaction, an internal loading control of an antisense transcript to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. This probe was digested with *Hind* III and produced a 120-bp protected fragment. Each gel lane was loaded with 50 μg of total RNA. The condi-



Codename	Amino Acid	Modified C-terminus	[a]DNA Binding	[a]Mechanism of topo Inhibition
NU/ICRF 500	Serine	$-NHNH_2$	weak intercalation	catalytic inhibitor topo II
NU/ICRF 501	Tyrosine	$-NHNH_2$	nil	nil
NU/ICRF 502	Alanine	$-NHNH_2$	nil	nil
NU/ICRF 503	Serine	$-OC_2H_5$	nil	weak catalytic inhibitor of topo II
NU/ICRF 504	Alanine	$-OCH_3$	nil	nil
NU/ICRF 505	Tyrosine	$-OC_2H_5$	nil	stabilizes topo I cleavable complexes
NU/ICRF 506	Serine	$-NHNH_2$	intercalation	dual catalytic inhibitor topo I and II
NU/ICRF 507	Phenylalanine	$-OC_2H_5$	nil	nil
NU/ICRF 508	Phenylalanine	$-OCH_3$	nil	nil
NU/ICRF 509	Threonine	$-OCH_3$	nil	nil
NU/ICRF 510	Arginine	$-OCH_3$	strong intercalation	catalytic inhibitor topo II
NU/ICRF 512	Methionine	$-OCH_3$	nil	nil
NU/ICRF 513	Dihydroxyphenyl alanine	$-OCH_3$	nil	catalytic inhibitor topo II

FIG. 1. Chemical structures and biological properties of anthracenyl amino acid conjugates (AAC). R is normally an H with the exception of NU/ICRF 506 where it is an OH. [a]Data taken from [29].

TABLE 1. Characterisation of cell lines used for *in vitro* testing of anthracenyl-amino acid conjugate topoisomerase inhibitors

	pgp immunostaining* MRK-16 (%)	pgp1 mRNA levels†	Dox uptake‡ (1μM)	Topoisomerase expression§			Chemosensitivity: Doxorubicin			Chemosensitivity: Camptothecin		
				IIα	IIβ	I	IC_{50} (10^{-8} M)‖	Fold Res¶	Fold Hyper#	IC_{50} (10^{-8} M)	Fold Res	Fold Hyper
CHO-K1	21.0	1	1	1	1	1	10.2	–	–	21.6	–	–
ADR-1	38.0		1	5↑	2↑	1	4.3	–	2.4	35.2	1.6	–
ADR-3				3↓	2.5↑	1	6.2	–	1.6	73.4	3.4	–
ADR-6	27.9	3↓	2↑	2↓	2↑	1.5↑	5.7	–	1.8	39.9	1.8	–
ADR-r	91.4	14↑	11↓	2↓	2.5↑	2↑	142	13.9	–	15.9	–	1.4
A2780	13.0		1	1	1	1	0.15	–	–	0.79	–	–
2780^{AD}	96.2		23↓	4↓	1	1.5↓	220	1460	–	4.3	5.5	–

* P-170 glycoprotein (pgp) expression was determined by flow cytometry as the % of cells staining positive after treatment with the MRK-16 antibody. † Pgp 1 mRNA levels were measured by an RNase protection assay. ‡ Dox uptake was measured by flow cytometry. § Topoisomerase expression was measured by immunoblot analysis. The blots were scanned and expression in variant cell lines represented as a ratio to the expression in the parental cell line. ‖ IC_{50} values were determined by a standard MTT assay after a 24-hr drug exposure. ¶ Fold resistance and # fold hypersensitivity compared to parental cell lines CHO-K1 and A2780.

tions for annealing and digesting of the RNA-RNA hybrids were as described previously [34].

Doxorubicin Accumulation in Cell Lines Determined by Flow Cytometry

DOX, at a concentration of 1 μM, was added to approximately 10^6 log phase cells growing in 25 cm^2 tissue culture flasks in otherwise normal medium (see above). At the end of incubations, which were performed at 37°C, drug-containing medium was removed quickly and cell monolayers were washed 10 times with ice-cold PBS. Cells were then trypsinised, and analyzed by flow cytometry using the FACScan for DOX content by measuring the drug's fluorescence at 560 nm (emission). No correction factors were applied to account for the quenching in fluorescence that occurs when DOX binds to DNA by intercalation. Incubations were performed in triplicate at the following time points after drug addition; 0, 15, 45, 120, and 240 min. Control drug accumulation studies were essentially as above except that incubations were carried out at 4°C; these control experiments were performed with CHO-K1, ADR-r, A2780, and $A2780^{AD}$ cells.

Topoisomerase I, IIα, and IIβ Protein Expression in Cell Lines

ANTIBODIES. The rabbit polyclonal antibody to human topo I was purchased from TopoGEN Inc. (Columbus, OH, U.S.A.) and was used at a working dilution of 1:1000. The rabbit polyclonal antibody to human topo IIα (working dilution 1:500) was purchased from Cambridge Research Biochemicals Ltd. (Northwich, England). The rabbit polyclonal antibody IHIC2 to topo IIβ (working dilution 1:100) was raised to a recombinant fragment of the C-terminal of the human protein, and was produced at the ICRF Laboratories at Clare Hall (Potters Bar, South Mimms, U.K.) [35]. The protein A horseradish peroxidase conjugate was obtained from Bio-Rad Laboratories (Hemel Hempstead, England) and was used at a working dilution of 1:1000.

PREPARATION OF NUCLEAR EXTRACTS. Nuclear extracts from each of the CHO cell lines and human cancer cell lines were prepared using a slightly modified version of the methods described by Glisson *et al.* [36] and Sullivan *et al.* [37]. Nuclear extracts were also prepared from either HeLa cells (ICRF Cell Production Services, Clare Hall Laboratories) or HL60 promyelocytic leukaemia cells, and were included as standards. All buffers used in this procedure contained a standard cocktail of protease inhibitors, and were added immediately prior to each extraction at appropriate concentrations [38]. Protein concentrations were determined on freshly prepared extracts using an assay (Bio-Rad) based on the method of Bradford [39].

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOT ANALYSIS. Freshly prepared nuclear extracts were diluted so as to enable appropriate volume loadings of 100 μg protein per lane, and proteins were separated by electrophoresis according to the discontinuous gel method of Laemmli [40].

The resolved proteins were transferred to nitrocellulose paper overnight (50 volts; 4°C) using a Bio-Rad transblot cell. Transferred proteins were detected using a modified version of the method described by Towbin *et al.* [41]. Blots were blocked with 3% marvel dried-milk powder in TBS and then probed at room temperature with the appropriate antibody for 2 hr. Following 2 × 10 min washes in TBST, the blots were incubated for 1 hr at room temperature with the protein A horseradish peroxidase conjugate. The blots were then washed sequentially in water, TBST, and TBS before developing, using 4-chloro-1-naphthol. Blots were scanned to quantitate the intensity of the topo bands using a GS 300 Scanning Densitometer operating in the reflectance mode (Hoefer Scientific Instruments, Newcastle-under-Lyme, Staffs, U.K.). Immunoblot analyses were repeated in all cell lines at least 3 times.

Determination of One Electron Reduction Potentials

E^1_7 of 6 representative structures from the whole series were derived by pulse radiolysis using the linear accelerator based at the Paterson Institute for Cancer Research (Manchester, U.K.). Pulses of electrons (50 ns) were used to irradiate drug solutions containing 40–100 μM AAC in an argon-saturated buffer mixture of 0.1 M sodium formate and 0.001 M sodium phosphate, pH 7.0. Light transmitted from drug solutions was passed through a Kratos monochromator into an EMI 9558Q photomultiplier using bandwidths of 20 nm. Changes in optical transmission with time were then recorded, using a Hewlett Packard HP9836s computer fitted to a Tektronix 7612D analogue-to-digital converter.

Drug Sensitivity of Cell Lines

All AAC were initially dissolved in pure DMSO immediately prior to use and diluted to the required concentration with medium, producing a final DMSO concentration of 0.3–0.5%. Final drug concentrations added to cells never exceeded 100 μM due to their limited aqueous solubility. Activity of AAC were determined by an MTT assay after 24-hr drug exposure using a slight modification of the method of Plumb *et al.* [42]. After drug exposure, cells were maintained in culture for a further 48–72 hr, prior to determination of cell number. A linear, or near linear, relationship between optical density and cell number was established in preliminary experiments and this absorbance range was not exceeded in test experiments. MTT assays were repeated 3–8 times. DOX and CPT were evaluated as above: DOX was made up fresh in water and CPT was made up fresh in DMSO.

Studies with Human Tumour Xenografts

The antitumour activity of NU/ICRF 505 was evaluated against two human xenografts: HT-29 colon cancer and NX002 nonsmall-cell lung cancer. Female *nu/nu* ('nude') mice (OLAC Ltd., Oxford, U.K.) were implanted with 2–3 mm^3 fragments of viable tumour and left for approximately 1 month. Animals were randomised into control and drug-treated groups, each of which contained 7 mice. NU/ICRF 505 was given by i.p. administration once a day for 5 consecutive days and was made up fresh in 10% DMSO in sterile water each day prior to injection (the first day of treatment was designated 'Day 0'). Tumour volumes were determined by caliper measurement and calculated using the formula: 0.5 × length × $width^2$. An RTV was then calculated for each individual tumour by dividing the tumour volume on day t by the tumour volume on day 0 multiplied by 100%. Statistical significance between the control and drug-treated groups was determined on separate days of the experiment using Students t-test, and a P value of less than or equal to 0.05 was adopted as the criterion for a significant antitumour effect.

Statistics

The statistical significance of differences in IC_{50} values between parent cell lines CHO-K1 and A2780 and their derivatives (ADR-1, ADR-3, ADR-6, ADR-r, and 2780^{AD}) were determined by the paired Students t-test.

Results

Expression of Pgp and Drug Transport Studies in Human and CHO Cell Lines

The drug-resistant cell line 2780^{AD} was confirmed to be strongly positive for pgp (96.2% of cells) but its drug-sensitive counterpart, A2780, stained only poorly (13.0%) using the MRK-16 antibody (see Table 1). Wild-type CHO-K1 expressed a significant level of pgp (21%). As anticipated, the *in vitro*-derived resistant derivative ADR-r was strongly positive for pgp (91.4%), and the mutant ADR-1 line was similar to (although higher than) the parental line (Table 1). No differences in daunorubicin cellular accumulation have been reported between CHO-K1 and ADR-1 [30]. The ADR-6 mutant also appeared to express the same level of pgp as the wild-type line. However, the drug transport properties of ADR-6 have not been previously characterised, and were further investigated. Figure 2 shows the levels of pgp 1 mRNA in selected CHO cell lines as determined by an RNase protection assay. These results confirm that CHO-K1 contains a significant pgp 1 signal and that ADR-r has elevated levels of pgp 1 mRNA.

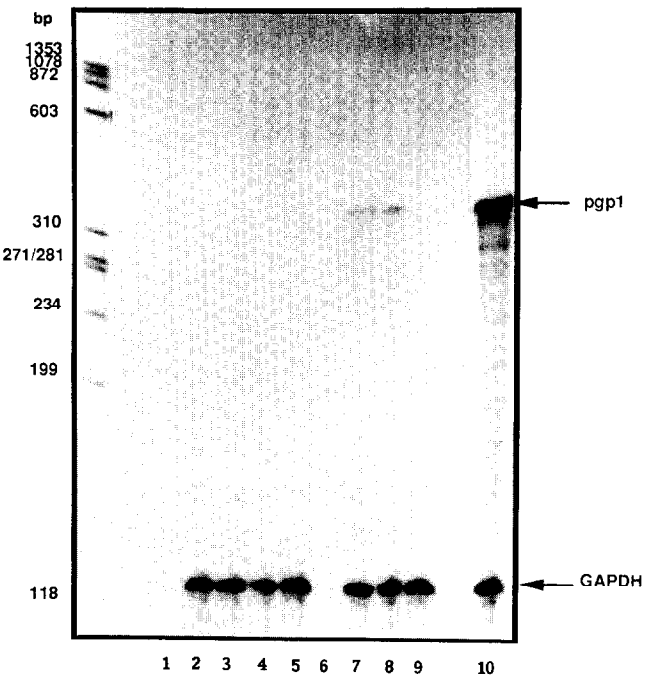


FIG. 2. Pgp 1 mRNA levels in CHO cell lines (see Table 1) were determined by RNase protection assays using a pgp 1 fragment and normalised for equal RNA loading with glyceraldehyde 6-phosphate dehydrogenase. Lanes 1–5 utilized sense probes and lanes 6–10 are antisense. Lanes are identified as follows: 1 and 6, tRNA; 2 and 7, CHO-K1; 3 and 8, ADR-2; 4 and 9, ADR-6; 5 and 10, ADR-r.

Interestingly, ADR-6 appeared to completely lack a pgp 1 mRNA signal, despite the fact that significant pgp positivity was recorded with the panel of three antibodies. This result may be explained by the fact that hamster tumour cells contain three pgp genes (pgp 1–3) [43] and that the antibodies used in the present studies are known to cross-react with at least pgp 3 [44]. Therefore, it is possible that the fluorescence recorded in ADR-6 may be due to cross-reactivity with the pgp 3 gene product that is not actively involved in MDR [43]. Transport studies with 1 μM DOX confirmed that ADR-6 significantly overaccumulated drug, compared to CHO-K1 (Fig. 3). Indeed, DOX accumulation in the whole panel of cells followed the trends expected, based on their pgp expression (Table 1).

Topoisomerase I, IIα, and IIβ Protein Expression in Human and CHO Cell Lines

Topo I, IIα, and IIβ protein levels in the panel of cell lines are shown in Fig. 4 and the fold differences in expression between related cell lines are summarised in Table 1. ADR-1 is a mutant that was isolated due to its hypersensitivity to a range of drugs believed to work through topo II [30]. Subsequently, it was reported to overexpress topo II by 3-fold [5]. These studies have been extended using antibodies that are specific for the 170 kD (α isoenzyme) and the 180 kD (β isoenzyme) forms of the protein. It is now shown that ADR-1 overexpresses the α isoenzyme by 5-fold and the β isoenzyme by 2-fold, but contains comparable levels of topo I. The ADR-3 cell line is also a mutant that was selected for hypersensitivity to topo inhibitors, including marked collateral sensitivity to mitoxantrone (12-fold), but appears to represent a different genetic complementation group from ADR-1 [45]. ADR-3 underexpressed topo IIα by 3-fold and overexpressed topo IIβ by 2.5-fold, but had normal levels of topo I. The transport mutant ADR-6 (see above) had slightly elevated levels of topo I (1.5-fold increase), but a 2-fold reduction in topo IIα and a 2-fold increase in topo IIβ. ADR-r displays multiple mechanisms of resistance including high pgp expression, altered topo, and elevated α-class glutathione S-transferase [31]. ADR-r only had a 2-fold decrease in topo IIα, but a 2.5-fold increase in topo IIβ (see Table 1), suggesting that its high degree of resistance may be predominately due to pgp (see above). Interestingly, ADR-r overexpressed topo I by 2-fold.

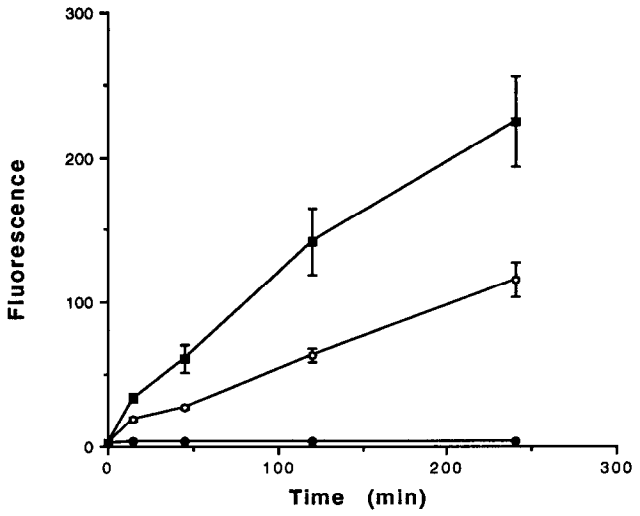


FIG. 3. Drug accumulation of 1 μM doxorubicin in CHO cell lines (see Table 1) determined at 37°C by flow cytometry. ■——■, ADR-6; ○——○, CHO-K1; and ●——●, CHO-K1 incubated at 4°C.

The human ovarian cancer cell line 2780^{AD} is generally considered to possess the classic MDR phenotype [46, 47]. In addition, it is shown that 2780^{AD} has a 4-fold reduction in topo IIα, normal topo IIβ, and a 1.5-fold reduction in topo I (see Table 1). Thus, 2780^{AD} also displays features of the multidrug-resistance phenotype known as at-MDR (atypical or altered topo II) [48]. It is interesting to note that, in a study where 2780^{AD} was reported to be 150-fold resistant to DOX, verapamil was only able to reverse this by 3-fold [47]. Therefore, it is possible that in this cell line, unlike ADR-r, the majority of resistance may be due to the at-MDR phenotype.

Chemosensitivity of Cell Lines to Doxorubicin and Camptothecin

The chemosensitivity of the panel of cell lines to the topo II inhibitor DOX and topo I inhibitor CPT are shown in Table 1. Both these compounds stabilize the cleavable complex [3, 4]. DOX is a good substrate for the multidrug-resistance transporter, whereas CPT does not appear to be recognised by the pump [49]. The results obtained from these studies followed closely the pattern expected. 2780^{AD} and ADR-r were both highly resistant to DOX, whereas 2780^{AD} was marginally resistant to CPT (probably due to reduced topo I expression), and ADR-r was collaterally sensitive to CPT (probably due to increased topo I expression). The transport mutant ADR-6 was cross-sensitive to DOX, but nominally resistant to CPT. ADR-1, which highly overexpresses topo IIα, was cross-sensitive to DOX but 1.6-fold resistant to CPT. Finally, ADR-3 was weakly cross-sensitive to DOX, but significantly resistant (by 3.4-fold) to CPT, and topo I expression was unaltered.

Free Radical Properties of Anthracenyl-Amino Acids

The one-electron reduction potential of a quinone-containing compound is a very good indicator of its likelihood to undergo enzyme catalyzed bioreduction *in vivo* [50]. The one-electron reduction potentials of 6 representative AAC were determined (NU/ICRF 500–505), including compounds with both aliphatic and aromatic substitutions. These studies produced similar E^1_7 for the six compounds ranging from −457 ± 15 mV for NU/ICRF 500 to −496 ± 15 mV for NU/ICRF 501. Compounds with strongly negative values below −250 mV fall outside the range to act as

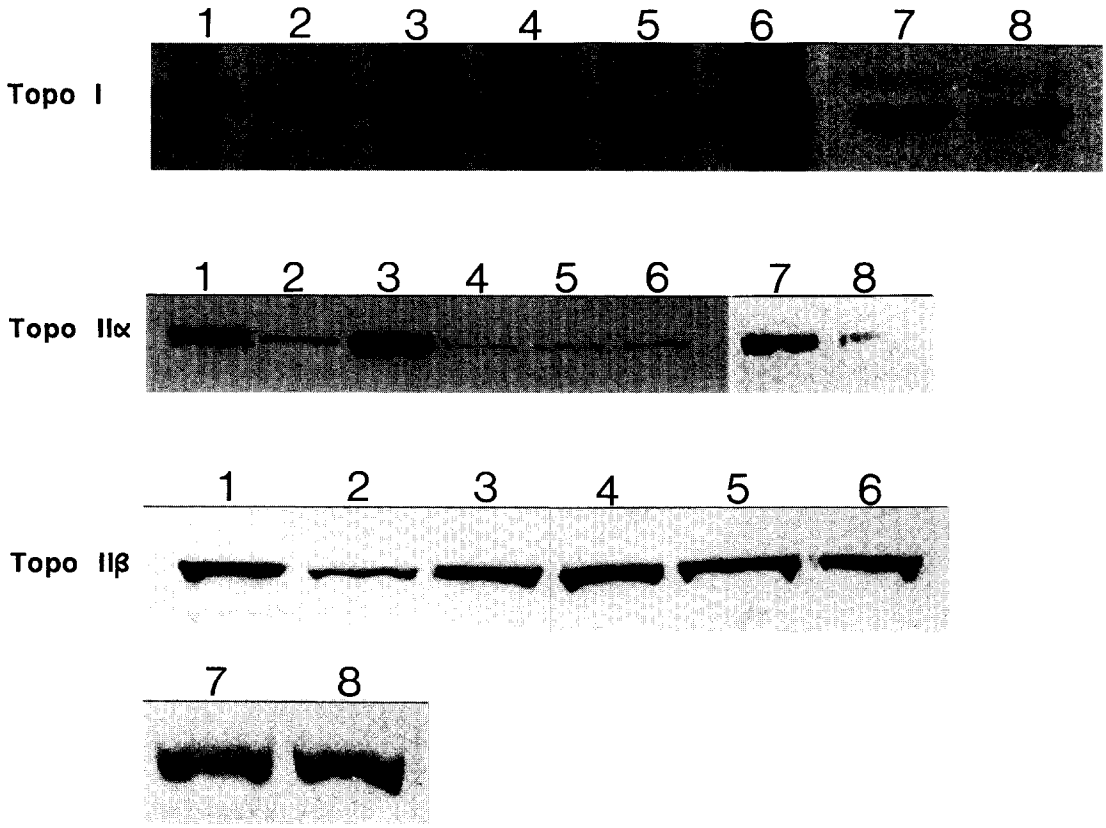


FIG. 4. Topoisomerase expression in CHO and human ovarian cancer (2780) cell lines (see Table 1) measured by immunoblot analysis. Equal loadings were determined by running a separate protein gel stained with Coomassie Blue. Top panel is topoisomerase I; middle panel is topoisomerase IIα, and bottom panel is topoisomerase IIβ. In all three blots, lanes are identified as follows: 1, HeLa cells; 2, CHO-K1; 3, ADR-1; 4, ADR-3; 5, ADR-6; 6, ADR-r; 7, A2780; and 8, 2780^{AD}. In the topo IIβ blot lane 1 is the HL60 cell line.

substrates for most bioreductive enzymes, such as NADPH cytochrome P-450 reductase and NADH cytochrome b_5 reductase [50]. Thus, AAC are most unlikely to undergo enzyme catalyzed bioreduction and induce toxicity through free radical-mediated processes *in vivo*. In fact, related compounds with strongly negative values, such as the anthrapyrazole CI941 (E^1_7 −538 mV) [51], can antagonize free radical generation and lipid peroxidation induced by DOX. Therefore, AAC may actually have a protective role against tissue damage if given in combination with drugs such as DOX.

Chemosensitivity of Cell Lines to Anthracenyl-Amino Acids

A total of 13 new compounds (see Fig. 1) from a closely related structural series were screened for activity. Of the 13 compounds, only 5 are potent topo inhibitors (see Fig. 1), and these are NU/ICRF 500, 505, 506, 510, and 513 [29]. With one exception (NU/ICRF 501), these were the *only* compounds that were active in the panel of cell lines (see Table 2), with IC_{50} values in the μM range (3.5–43.1 μM). The potent DNA binder and topo II catalytic inhibitor NU/ICRF 510 (arginine analogue and only compound to

TABLE 2. *In vitro* activity (IC_{50} values in μM) of anthracenyl-amino acid conjugates against a panel of 5 Chinese hamster ovary (CHO) cell lines and 2 human ovarian cancer cell lines*

	CHO-K1	ADR-1	ADR-3	ADR-6	ADR-r	A2780	A2780^{AD}
NU/ICRF 500	14.7 ± 1.7	10.7 ± 2.5§	10.1 ± 1.6	17.3 ± 2.3§	15.7 ± 1.3	5.3 ± 2.3	8.4 ± 2.3§
NU/ICRF 501	17.4 ± 11.6	20.5 ± 9.7	29.4	16.7 ± 7.5	20.5 ± 0.1	NA‡	33.1 ± 19.5
NU/ICRF 505	19.9 ± 2.4	23.9 ± 5.4	21.2 ± 2.5	22.7 ± 5.6	14.9 ± 0.9‖	4.4 ± 2.7	7.7 ± 1.9‖
NU/ICRF 506	10.7 ± 1.8	9.2 ± 0.9	ND†	13.7 ± 1.0§	7.9 ± 1.9‖	3.5 ± 1.9	5.2 ± 0.9§
NU/ICRF 513	29.7 ± 4.7	43.1 ± 11.0	27.9 ± 1.0	27.7 ± 6.6	15.3 ± 5.1§	21.8 ± 3.2	17.7 ± 5.7

* IC_{50} values were determined by a standard MTT assay after a 24-hr drug exposure as described in detail in Materials and Methods. Each IC_{50} value represents the mean ± SD of N = 3–8 separate MTT assays. †ND, not determined; ‡NA, not active at 100 μM; §$P < 0.05$, ‖$P < 0.01$ compared to parent cell line (Students paired t-test).

carry a full positive charge) was only active against A2780 (IC_{50}, 11.2 μM) and is not included in Table 2. Subsequently, in drug uptake studies, this compound has been shown to be a good substrate for pgp and to be effectively excluded from 2780^{AD} [52].

The degree of resistance/hypersensitivity of the panel of cell lines to the 4 most active compounds is summarised in Table 3. These factors, although small, are derived from the data of Table 2 only where statistical significance was firmly established upon repeated MTT assays up to 8 times over a period of 1–2 years. Results from individual MTT assays are presented in Fig. 5 for NU/ICRF 500 and Fig. 6 for NU/ICRF 513 to illustrate more clearly the magnitude of the effects under investigation.

No evidence of resistance or hypersensitivity in any cell line was seen with NU/ICRF 501, the non-topo inhibitor, consistent with nonspecific cytotoxicity. NU/ICRF 500, the topo II catalytic inhibitor, was more active against ADR-1, which overexpresses topo IIα and β, but less active against transport mutant ADR-6, although this effect was small (see Tables 2 and 3). No cross-resistance was apparent in ADR-r and a small factor of resistance was recorded in 2780^{AD} (1.6-fold, Tables 2 and 3). NU/ICRF 505, which stabilises topo I cleavable complexes, was more effective against drug-resistant ADR-r (which overexpresses topo I) by 1.3-fold, and only 1.9-fold cross-resistant in 2780^{AD} (1.5-fold reduction in topo I). This compound was also noncross-resistant in the CPT-resistant cell line ADR-3. NU/ICRF 506, which is a combined topo I and II catalytic inhibitor and was the most active compound (see Table 2), was also more effective against ADR-r by 1.6-fold but was significantly less active against ADR-6 and 2780^{AD}. Finally, NU/ICRF 513, which is a topo II catalytic inhibitor and was the least active of the 4 compound (Table 2), was completely noncross-resistant against 2780^{AD} but, curiously, was more effective against ADR-r by the largest hypersensitivity factor recorded (1.9-fold).

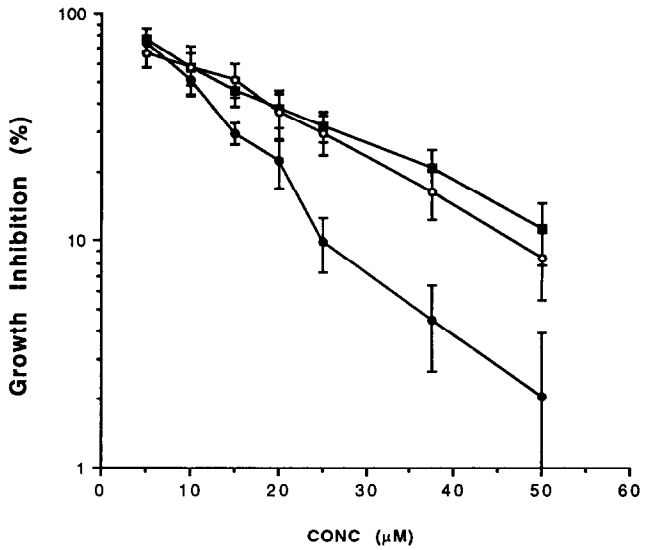


FIG. 5. Chemosensitivity of CHO cell lines (see Table 1) to the anthracenyl amino acid conjugate NU/ICRF 500 (see Fig. 1) determined by a standard MTT assay after a 24-hr drug exposure. The figure shows the results of a typical assay from which the IC_{50} values contained in Table 2 were derived. Each point represents the mean ± SD from N = 8 replicates. ■—■, CHO-K1; ○———○, ADR-6; and ●———●, ADR-1.

Antitumour Activity of NU/ICRF 505

After initial dose-ranging experiments, antitumour activity was established with NU/ICRF 505 at a dose of 25 mg/kg given i.p. on 5 consecutive days. Over a series of 5 antitumour studies in total, this dose produced no toxic deaths or evidence of weight loss in any mouse. The effect of NU/ICRF 505 against HT-29 human colon cancer is shown in Fig. 7 and against NX002 human nonsmall-cell lung cancer is shown in Fig. 8. In both xenografts, a significant reduction in growth was observed at several time points throughout the study. The maximum growth inhibition (mean tumor volume after drug treatment/mean tumour volume after no drug treatment × 100, T/C) was 52% in HT-29 on day 10 and 59% in NX002 on day 14. These effects were reproducible in repeat studies. HT-29 is a well-established human xenograft and has been reported to be resistant to DOX and nitrosoureas, but very sensitive to the topo I inhibitor 9-amino camptothecin (T/C < 10%) [53]. NX002 was established in Edinburgh and is part of the EORTC human tumour xenograft panel: it is resistant to amsacrine and ellipticines, but sensitive to cisplatin and AZQ (T/C < 40%) [54]. Its sensitivity to topo I inhibitors is unknown.

TABLE 3. Fold resistance (r) or hypersensitivity (s) of variant cell lines to anthracenyl-amino acid conjugates*

	NU/ICRF 500	NU/ICRF 505	NU/ICRF 506	NU/ICRF513
ADR-1	1.4-s	NSD†	NSD	NSD
ADR-3	NSD	NSD	ND‡	NSD
ADR-6	1.2-r	NSD	1.3-r	NSD
ADR-r	NSD	1.3-s	1.6-s	1.9-s
2780^{AD}	1.6-r	1.8-r	1.5-r	NSD

* Cell lines are described in Table 1 and anthracenyl-amino acid conjugates in Fig. 1. Fold differences are derived from the IC_{50} data presented in Table 2 only when statistical significance was established, and were calculated as ratios of the mean values between the parent cell lines and their derivatives. †No statistical difference; ‡not determined.

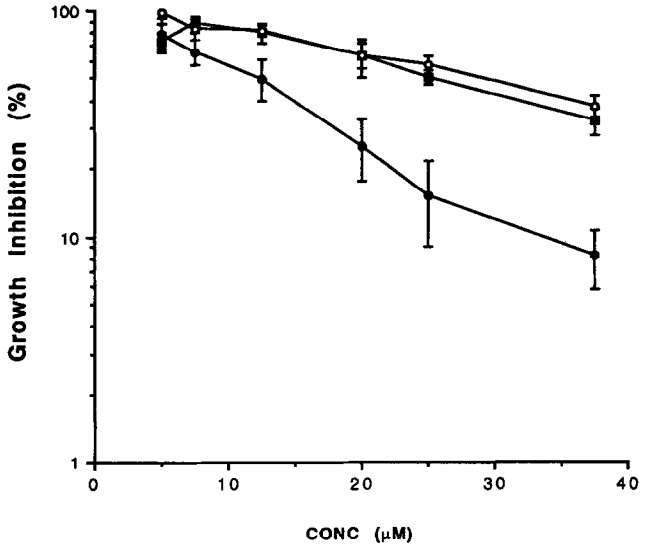


FIG. 6. **Chemosensitivity of CHO cell lines (see Table 1) to the anthracenyl-amino acid conjugate NU/ICRF 513 (see Fig. 1) determined by a standard MTT assay after a 24-hr drug exposure. The figure shows the results of a typical assay, from which the IC_{50} values contained in Table 2 were derived. Each point represents the mean ± SD from N = 8 replicates. ■——■, CHO-K1; ○———○, ADR-3; and ●———●, ADR-r.**

Discussion

The aim of the present study has been to identify potential new candidate anticancer drugs from a novel series of rationally designed anthracenyl-amino acid conjugates (see Fig. 1). Selection criteria adopted were: (a) that a compound should kill cells through topo inhibition but not exhibit secondary mechanisms of action, such as free radical generation, and (b) that the compound should be noncross-resistant in MDR and at-MDR cell lines. The approach employed to achieve the above aims was to evaluate the cytotoxicity of the compounds against a panel of 7 cell lines possessing different levels of topo I and II, and then correlate activity to enzyme inhibitory properties. This approach is based on the assumption that topo protein levels will dictate the response of a cell line to a topo inhibitor.

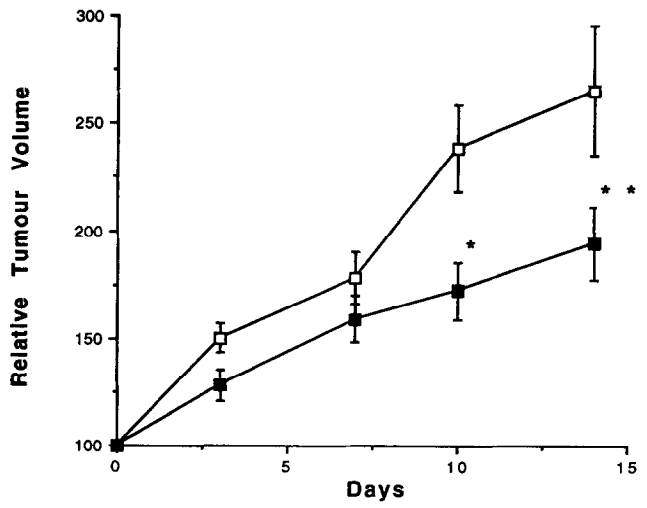


FIG. 7. **Antitumour activity of NU/ICRF 505 against the HT-29 human colon cancer xenograft. The drug was administered i.p. as a 10% DMSO solution in water at a dose of 25 mg/kg on days 0–4. For definition of relative tumour volume (RTV) see Materials and Methods. □——□, control group (N = 7, mean RTV ± SEM); ■——■, drug-treated group (N = 7, mean RTV ± SEM). *P = 0.05 and **P < 0.05 (Students *t* test).**

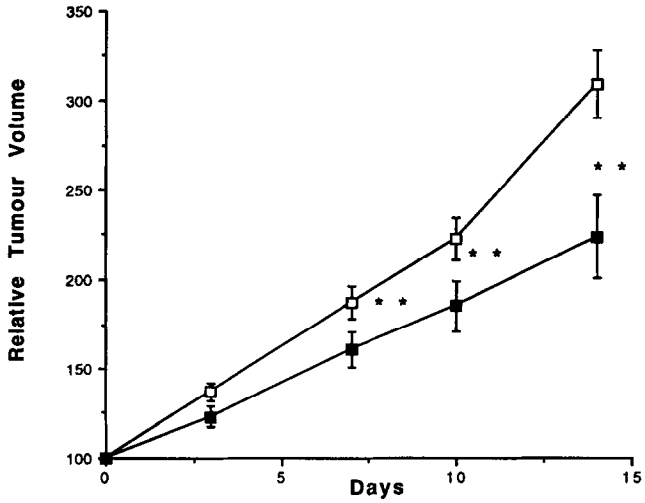


FIG. 8. **Antitumour activity of NU/ICRF 505 against the NX002 human nonsmall-cell lung cancer xenograft. □——□, control group (N = 7, mean RTV ± SEM); ■——■, drug-treated group (N = 7, mean RTV ± SEM). **P < 0.05 (Students *t* test).**

There have been numerous reports indicating a clear relationship between protein levels and chemosensitivity to topo inhibitors, but these tend to involve only 2 or 3 related cell lines where resistance was selected *in vitro* by continuous drug exposure [5, 6, 9, 55, 56]. Other non-topo mediated mechanisms of drug resistance can be activated under such selection pressures including: increased expression of pgp [57]; increased MRP expression [58] and altered GSTs [9]. In the two highly resistant *in vitro* derived cell lines used in the present work, it is clear that multiple mechanisms of resistance are operative [31]. When larger panels have been evaluated, much poorer correlations were apparent [59, 60] and, in a recent study, a negative correlation was observed between topo IIα expression and chemosensitivity to DOX in 6 human leukaemia cell lines [61]. In the present work, a major emphasis has been placed on a series of CHO mutants. Rather than continuous exposure to a selecting drug over months, parental CHO cells were briefly treated with the point mutagen ethyl methanesulfonate in a single-stage selection protocol [5, 30, 45]. The chemosensitivity of the whole panel to the established topo I inhibitor CPT and topo II inhibitor DOX has been demonstrated to correlate well with their biochemical properties. Therefore, it is believed that the comparative activity of AAC in the panel may reflect their underlying mechanism of action.

Five compounds from the series produced broad-spectrum activity, allowing comparisons to be made. One was a non-topo inhibitor (NU/ICRF 501) and it exhibited, as expected, a nonspecific pattern of cytotoxicity. With NU/ICRF 513 (selective topo II catalytic inhibitor) there was no correspondence between cytotoxicity and mechanism of enzyme inhibition, suggesting a nontopo-associated pathway of cell death. In the case of the topo II catalytic inhibitor NU/ICRF 500, there was an apparent positive correlation between topo II expression and levels of cytotoxicity, indicating a mechanism of cell death operating through topo inhibition. Weight has been added to this conclusion from the results of a recent study using cytogenetic tests to characterize the effects of AAC on cells [62]. In that study, treatment of CHO cells with NU/ICRF 500 resulted in a complete inhibition of chromosome segregation at mitosis, a biological function normally attributed to topo II [63]. Furthermore, human lymphocytes exposed to NU/ICRF 500 at its IC_{50} concentration accumulated in the G2/M phases of the cell cycle, consistent with the action of many other non-cleavable complex-forming topo II catalytic inhibitors [64–66].

Perhaps the most significant results were obtained with NU/ICRF 505, which stabilises topo I cleavable complexes. A good correlation was observed between topo I expression and chemosensitivity and no correspondence observed between pgp expression and resistance. These results support a mechanism of action operating exclusively through topo I, and a lack of recognition by pgp, which distinguishes this compound from many CPT analogues [67]. In addition, NU/ICRF 505 was noncross-resistant in the CPT-resistant ADR-3 cell line. NU/ICRF 506, which was the most active compound and is a combined catalytic inhibitor of topo I and topo II, yielded a spectrum of activity that corresponded with topo I expression only and with a lack of recognition by pgp. Studies with yeast would suggest that catalytic inhibition of topo I is not necessarily a cytotoxic event [15]. Nevertheless, in mammalian cells, the topo I catalytic inhibitors xestoquinone, halenaquinone, and TAN-1518A have all been shown to be cytotoxic and to produce cellular abberations consistent with a critical role for the enzyme in DNA replication and gene transcription [23, 68].

It is striking that the topo II catalytic inhibitor NU/ICRF 500 was either noncross-resistant or only nominally cross-resistant (maximum factor 1.6-fold) in the two cell lines that were highly resistant to DOX. The phenomenon of noncross-resistance in at-MDR cell lines has been reported previously for a number of other topo II catalytic inhibitors, such as fostriecin [69], aclarubicin, and merbarone [65]. The surprising feature about this catalytic inhibitor is that there was a positive correlation between topo II expression and *in vitro* cytotoxicity (i.e. the higher the level of expression of enzyme, the greater the activity), paralleling the case of topo II inhibitors that work through the cleavable complex [5, 6, 8, 9]. One might expect to observe, instead, an inverse relationship where increased topo expression resulted in resistance, in keeping with the normal rules of enzyme kinetics for catalytic inhibitors. Such a relationship has been reported with fostriecin, where a cell line (GLC_4/cDDP) with higher levels of topo II activity was chemoresistant and a cell line (GLC_4/ADR) with reduced topo II activity was hypersensitive [69]. Moreover, recent studies with top2-1 mutant yeast cells have also demonstrated a negative correlation between topo II expression and chemosensitivity to the topo II catalytic inhibitors ICRF-159 and -193 [70].

To date, it is unclear exactly how NU/ICRF 500 and 506 function as topo II catalytic inhibitors. NU/ICRF 500 binds weakly to DNA but is effective in antagonising DNA cleavage induced by m-AMSA [29]. Inhibition of enzyme activity can be reversed by the addition of excess protein, rather than DNA substrate, indicative of binding to the enzyme. NU/ICRF 506 interacts more strongly with DNA, and enzyme inhibition cannot be overcome by excess protein [29]; therefore, it is possible that combined catalytic inhibition by this compound is more a general consequence of binding to the double helix, with topo I being more sensitive [71].

In summary, a novel chemical class of topo inhibitor comprising 13 members has been screened against a panel of 7 cell lines with a view to identifying potential new anticancer drugs. Toxicology was, in part, investigated by studying ability to generate free radicals, and the results contained herein indicate that this is unlikely to occur *in vivo*. Three candidate drugs have been identified: NU/ICRF 500, which appeared to be cytotoxic through inhibition of the catalytic activity of topo II; NU/ICRF 505, which appeared to be cytotoxic through stabilisation of topo I cleavable complexes; and, finally, NU/ICRF 506, which appeared to be cytotoxic through catalytic inhibition of topo I. All three compounds were either noncross-resistant or nominally cross-resistant in MDR and at-MDR cell lines. One of the candidate drugs (NU/ICRF 505) was shown to exhibit significant *in vivo* antitumour activity against two human xenografts. The combined properties of reduced toxicity, selective inhibition of DNA topoisomerases, and noncross-resistance merit further development of anthracenyl-amino acid conjugates as a promising new class of anticancer drugs.

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