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BIOCHEMISTRY OF TOPOISOMERASE I AND II INHIBITION BY ANTHRACENYL-AMINO ACID CONJUGATES

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Abstract—Mono-conjugation of an anthraquinone nucleus with a range of naturally occurring amino acids chemically modified at their C-terminus has been adopted as a synthetic approach in the rational design of novel topoisomerase (topo) inhibitors. The biochemistry of topo I and II inhibition has been investigated for a series of 16 new compounds (NU/ICRF 500–515) from which structure–activity relationships have been investigated. Only three compounds could be demonstrated to bind to DNA: two serine derivatives (NU/ICRFs 500 and 506) and an arginine derivative (NU/ICRF 510). In decatenation and relaxation assays with purified enzyme, several compounds were shown to be potent catalytic inhibitors of topo II (100% inhibition at 5 μg/mL (10–15 μM) or less) without stabilizing cleavable complex formation. These included the three DNA binding species (of which NU/ICRF 506 was the most active) and a dihydroxyphenylalanine analogue (NU/ICRF 513). Both NU/ICRFs 500 and 506 were further shown to antagonize DNA cleavage induced by amsacrine. Only NU/ICRF 506 unequivocally inhibited the catalytic activity of topo I without induction of DNA cleavage, and was the only combined topo I and II catalytic inhibitor. One compound, NU/ICRF 505 (tyrosine conjugate), stabilized topo I cleavable complexes without inhibiting the catalytic activity of topo I and II. Modifications to the structure of NU/ICRF 505 revealed that the presence of an unhindered hydroxyl on the tyrosine ring and a more hydrophobic ethyl ester at the amino acid C-terminal were both essential, suggesting a highly specific interaction between drug, enzyme and DNA in the ternary complex. Molecular modelling studies suggested that the observed differences in topo inhibition are a consequence of major conformational alterations brought about by small changes in the amino acid substituent, and confirmed a rigid structural requirement for the induction of topo I cleavage, in addition to a less rigid structural requirement for topo II inhibition. A strong correlation was observed between topo inhibition and *in vitro* cytotoxicity against the human ovarian cancer cell line A2780 (IC_{50} range 3.4–11.6 μM), suggesting a mechanism of cell kill, at least in part, involving topo inhibition.

Key words: topoisomerases I and II; anthracenyl-amino acids; catalytic inhibition; DNA cleavage; antagonism; cytotoxicity

DNA topo I and II§ are nuclear enzymes which alter the topology of DNA through strand breakage, strand passage and religation [1, 2]. They have been demonstrated to be involved in most aspects of DNA metabolism including replication, transcription, recombination, chromosome condensation and segregation, and have been shown in yeast models to be essential for cell survival [3–5]. In addition to studies of cellular function, topoisomerases have been identified as the primary target for a number of clinically important antitumour agents [6, 7]. These include inhibitors of topo I, such as the plant alkaloid, camptothecin, and its semisynthetic derivatives [8, 9], and the topo II inhibitors such as anthracyclines [10], acridines [11], ellipticines [12], epipodophyllotoxins [13] and anthracenediones [14]. All of these drugs, although structurally diverse, have been shown to poison topoisomerases by stabilizing a putative reaction intermediate, termed the cleavable complex, in which the enzyme remains covalently attached to either one strand (topo I) or both strands (topo II) of DNA following topo-mediated strand breakage [11, 15–17].

However, many of the recent topo inhibitors do not stabilize topo-mediated cleavable complexes and have, in some instances, been shown to antagonize DNA cleavage induced by existing anticancer drugs such as etoposide and amsacrine [18, 19]. These drugs have been reviewed elsewhere [20], although examples include the topo II catalytic inhibitors fostriecin [21], ICRF-154 and ICRF-193 [19], suramin [22], merbarone [23], the topo I catalytic inhibitor

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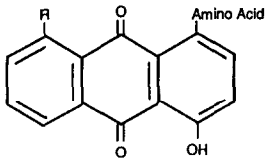
§ Abbreviations: amsacrine, 4′-(9-acridinylamino)-methanesulphon-*m*-anisidide; kDNA, DNA purified from the kinetoplast of *Crithidia fasciculata*; T_m, DNA melting temperature; topo, DNA topoisomerase; TLC, thin-layer chromatography.



Codename	Amino Acid	Modified C-terminus
NU/ICRF 500	Serine	$CONHNH_2$
NU/ICRF 501	Tyrosine	$CONHNH_2$
NU/ICRF 502	Alanine	$CONHNH_2$
NU/ICRF 503	Serine	$COOC_2H_5$
NU/ICRF 504	Alanine	$COOCH_3$
NU/ICRF 505	Tyrosine	$COOC_2H_5$
NU/ICRF 506	Serine	$CONHNH_2$
NU/ICRF 507	Phenylalanine	$COOC_2H_5$
NU/ICRF 508	Phenylalanine	$COOCH_3$
NU/ICRF 509	Threonine	$COOCH_3$
NU/ICRF 510	Arginine	$COOCH_3$
NU/ICRF 511	Cysteine	$COOC_2H_5$
NU/ICRF 512	Methionine	$COOCH_3$
NU/ICRF 513	Dihydroxyphenylalanine	$COOCH_3$
NU/ICRF 514	Tyrosine	$COOCH_3$
NU/ICRF 515	Serine	$CONHNHCH_2CH_2OH$

'R' is normally an 'H' with the exception of NU/ICRF 506 where it is an 'OH'

Fig. 1. Structures of anthracenyl-amino acids. [2*H*,3*H*]-9,10-dihydroxyanthracene-1,4-dione was reacted with a number of different α-amino acids to give a range of monosubstituted anthraquinone-amino acid conjugates, designated NU/ICRF 500-515. Further variation has been obtained by modifying the C-terminus of each amino acid prior to linking onto the anthracenyl ring.

β-lapachone [24] and the dual topo I and II catalytic inhibitor Sanguiin H-6 [25]. They have further been shown to be actively cytotoxic [21, 26] and non-cross-resistant in cell lines exhibiting resistance mechanisms to topo inhibitors which work through cleavable complex formation [27], revealing their potential as anticancer drugs.

Previous studies have investigated the potential use of peptide conjugates of anthraquinones as redox active drugs through the metal-chelating properties of the peptide [28]. In this study we report for the first time on the biochemistry of topo inhibition by 16 compounds from a novel series of anthraquinones mono-conjugated to a range of naturally occurring L-amino acids chemically modified at their C-terminus (designated NU/ICRF 500–NU/ICRF 515; illustrated in Fig. 1). Within this series a spectrum of topo I and II inhibitory activities were identified, allowing for the evaluation of structure–activity studies with the aid of molecular modelling. Finally, *in vitro* cytotoxicity was determined against the human ovarian cancer cell line A2780.

MATERIALS AND METHODS

Drugs and chemical reagents. Anthracenedione-amino acid conjugates were synthesized through the reaction of α-amino acid esters with [2*H*,3*H*]-9,10-dihydroxy-anthracene-1,4-dione (for full details of chemical synthesis and characterization procedures see Cummings and Mincher, UK patent GB 9205859.3; International Application Number PCT/GB93/00546, 30 September, 1993). Structures were characterized by electron impact mass spectrometry, proton NMR and infrared spectroscopy. Reaction mixtures were purified by preparative TLC, silica gel column chromatography and finally recrystallization. Purity of the crystalline *N*-anthracenyl amino acid derivatives was confirmed by TLC. Stock solutions of drugs were made up fresh for each assay in DMSO at a top concentration of 50 μg/mL and diluted down appropriately. Drug solutions were added so that the final concentration of DMSO did not exceed 1%; at this concentration topo I and II activities were unaffected.

Other drugs which were used as positive controls in appropriate assays included camptothecin (Sigma Chemical Co, Poole, U.K.), doxorubicin (Farmitalia Carloerba Ltd, St Albans, U.K.) and amsacrine (a gift from Dr A. Sumner, MRC Human Genetics Unit, Western General Hospital, Edinburgh, Scotland). Stock solutions of these drugs were made up at the appropriate concentration in DMSO as for anthracenyl-amino acids.

All other chemicals and reagents used were of analytical grade and readily available commercially unless stated otherwise.

Enzymes. Topo II was isolated and purified from cultured HeLa cells growing in exponential growth phase (ICRF cell production services, Clare Hall Labs, South Mimms, Potters Bar, U.K.) using a protocol based on the methods of Miller *et al.* [29] and Drake *et al.* [30]. Using this approach, topo II was recovered from HeLa cell nuclear extracts by sequential application to hydroxylapatite, phosphocellulose P11, Sepharose Q and Sepharose S, all of which was carried out on a Gradifrac™ compact chromatography system (Pharmacia LKB Biotechnology, Milton Keynes, U.K.). Topo II activity of recovered fractions at each stage was determined using the decatenation assay in the presence of ATP (see below). The final fraction was assayed for topo I and endonuclease activity using the relaxation assay described below in which a lack of relaxed topomers and nicked DNA formation ruled out topo I and endonuclease contamination respectively. Immediately following purification, a decatenation assay was performed to characterize the activity of the enzyme pool. One unit of enzyme activity was defined as the amount of topo II required to fully decatenate 0.3 μg kDNA in the standard reaction conditions described below. The purified enzyme was stored at $-80°$ in 50% glycerol until further use.

Human topo I was purchased from TopoGEN Inc. (OH, U.S.A.).

Oligonucleotides and DNA substrates. The oligonucleotides for use in UV melting studies (CGTACG and CGCGAATTCGCG) were prepared on an Applied Biosystems AB1 380B DNA synthesizer and purified by Professor T. Brown (Department of Chemistry, University of Edinburgh, Scotland). Supercoiled plasmid pBR322 was obtained from Boehringer Mannheim (Lewes, Sussex, U.K.);

linearized plasmid for use as a standard in topo II cleavage assays was obtained by incubation of pBR322 with the restriction endonuclease Hind III at 37° overnight. Kinetoplast DNA was obtained from TopoGEN Inc. (OH, U.S.A.).

DNA binding studies (UV-thermal melt analysis). T_m of oligonucleotide duplexes and oligonucleotide–drug complexes were determined on a Perkin-Elmer λ 15 UV–Vis spectrometer equipped with a Peltier block, and controlled by an IBM P52 microcomputer. Samples were heated at a rate of 1°/min and the DNA melting process monitored at 264 nm. Data were collected and processed using the PECSS 2 software package provided by Perkin-Elmer. Reactions conditions were the same as those described by Leonard *et al.* [31].

DNA binding studies (unwinding assay). DNA unwinding effects caused by intercalation were assayed according to the method of Pommier *et al.* [32]. For the preparation of relaxed DNA, supercoiled pBR322 DNA was incubated with excess topo I in a reaction buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$ and 15 μg/mL BSA (buffer A) at 37° for 30 min. The relaxed DNA was retrieved using the nucleon II DNA extraction kit manufactured by Scotlab (Coatbridge, U.K.) and extracted samples concentrated using a centricon-50 microconcentrator (Amicon Ltd, Stonehouse, U.K.). Unwinding reactions were performed in 45 μL reaction volumes containing 1 μg relaxed DNA and excess topo I in buffer A. Reactions were stopped by the addition of 5 μL of a solution containing 5% SDS of 5 mg/mL proteinase K (prewarmed at 37°) for 30 min. After this time, 5 μL stop buffer was added (50% sucrose, 50 mM EDTA, 0.1% bromophenol blue, 0.01% SDS). Samples were analysed by agarose gel electrophoresis in 1% gels made with 40 mM Tris–acetate, 1 mM EDTA buffer. Following electrophoresis, gels were stained with 1 μg/mL ethidium bromide for 30 min, destained with several washings of distilled water, and photographed on a UV light box.

Decatenation assay. Topo II activity was measured by the ATP-dependent decatenation of kDNA in which an interlocking kDNA network is decatenated to individual DNA minicircles and small catenanes [33]. Reaction buffer, consisting of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.5 mM EDTA, 30 μg/mL BSA, 1 mM ATP, 0.5 mM dithiothreitol and 100 μg/mL kDNA was mixed with drug and enzyme (in this order) in a total volume of 45 μL and reaction mixtures incubated for 30 min at 37°. The reaction was stopped and reaction products analysed by agarose gel electrophoresis as for the unwinding assay except using 89 mM Tris-borate (pH 8.3), 2 mM EDTA buffer. Assays were repeated at least three times and inhibition of enzyme activity by each drug then given a rating depending on whether greater than 50% inhibition was observed at 1, 5 or 50 μg/mL final drug concentration.

Relaxation assay. Both topo I and II activities were assayed by the relaxation of supercoiled plasmid pBR322 DNA. For topo I assays, reaction conditions were the same as the unwinding assay in which reaction mixtures of buffer, plasmid (0.5 μg), drug and enzyme were added in this order and incubated for 30 min at 37°. Each assay was carried out at least three times and inhibition by each compound given a rating as described for the decatenation assay. The same procedure was followed for topo II relaxation except using the reaction buffer described for the decatenation assay above.

DNA cleavage reactions. Agarose gel electrophoresis assays for topo-mediated DNA cleavage have been well characterized (topo I [34]; topo II [35]). DNA cleavage reaction conditions were the same as for the topo I or II relaxation assays except that the concentration of enzyme was approximately 20 times higher than that required for full relaxation of pBR322. After 30 min, however, reactions were terminated by the addition of 5 μL of a solution containing 5% SDS and 5 mg/mL proteinase K (prewarmed to 37°). Following an additional incubation at 37° for 30 min, the reaction was stopped and the samples electrophoresed as described for the unwinding assay, with the exception that the agarose gels contained 1 μg/mL ethidium bromide. Gels were visualized as above and each negative scanned using a densitometric scanner. The area of each peak was measured and the percentage of linear DNA (topo II) or nicked DNA (topo I) calculated.

Cell culture and in vitro *cytotoxicity.* The A2780 human ovarian cancer cell line was obtained from the Medicine Branch of the Division of Cancer Treatment (NCI, Bethesda, U.S.A.). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum containing a 1% antibiotic mixture under standard tissue culture conditions, and were maintained at 37° in a humidified atmosphere of 5% CO_2 in air.

The cytotoxicity of drugs was measured using cells in exponential growth phase and, in the majority of cases, employed a slightly modified version of the MTT assay described by Plumb *et al.* [36]. Cells were plated at a density of 500–750 per well in 96-well flat-bottomed plates and allowed to attach for 2–3 days. Medium was replaced with either 200 μL fresh medium (control) or medium containing drug at a range of concentrations. Following 24 hr exposure to drug, cells were incubated with fresh drug-free medium for 48–72 hr prior to MTT addition. Medium and MTT were removed after 4 hr, and 200 μL DMSO added. Each plate was read at 540 nm using a Bio-Rad model 255 EIA microplate reader (Bio-Rad Laboratories, Hemel Hempstead, U.K.). A linear, or near linear, relationship between absorbance and cell number was obtained in preliminary experiments.

In experiments with compounds analysed in the later stages of this work, cell number was initially estimated using a Coulter Counter ZM (Coulter Electronics Ltd, Luton, U.K.). The procedure was exactly the same except that $3–5 \times 10^4$ cells were plated in 12.5 cm^2 flasks, and that after the final incubation in drug-free medium for 48–72 hr, cells were trypsinized prior to counting. Results from Coulter counting experiments were used to determine the range of concentrations to be applied in subsequent experiments with the MTT assay, from which all IC_{50} values were determined.

Table 1. DNA binding studies with selected anthracenyl-amino acids using UV-melt analysis

Drug	ΔT_m(K) CGTACG	CGCGAATTCGCG
Doxorubicin	19.5 ± 1.7	10.7 ± 1.5
NU/ICRF 500	1.2 ± 0.5	1.3 ± 0.4
NU/ICRF 502	0	0
NU/ICRF 505	0	0
NU/ICRF 506	4–9	ND
NU/ICRF 509	0	0
NU/ICRF 510	4.2 ± 1.1	2.1 ± 1.2
NU/ICRF 513	0	0

DNA binding effects were investigated by looking for an increase in the thermal denaturation temperature (ΔT_m) of known DNA sequences following incubation with drug (see the Materials and Methods section for incubation conditions). Results shown were from at least three separate melt cycles. ND, not determined.

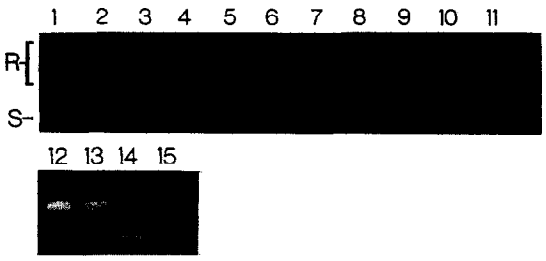


Fig. 2. DNA unwinding by anthracenyl-amino acids. Assay conditions were as described in the Materials and Methods section. Unwinding assay with increasing concentrations of NU/ICRFs 500, 506 and 510. Lane 1, supercoiled pBR322 DNA; lane 2, relaxed DNA control; lane 3, relaxed DNA plus enzyme; lane 4, 100 μM amsacrine; lanes 5, 9 and 12, NU/ICRFs 500, 510 and 506, respectively, at 1 μg/mL; lanes 6, 10 and 13, as for 5, 9 and 12, except at 5 μg/mL; lanes 7 and 14, NU/ICRFs 500 and 506 at 25 μg/mL; lanes 8, 11 and 15, as for 5, 9 and 12, except at 50 μg/mL.

Drugs were dissolved in DMSO immediately prior to use as for studies with the purified enzyme (except using a final DMSO concentration of 0.3–0.5%), and made up to the required concentration with medium.

Molecular modelling studies. The molecular modelling was performed using the Quanta and Charmm programs (Molecular Simulations, Burlington, MA, U.S.A.). All the compounds were minimized using the Steepest Descents program (50 iterations) followed by minimization using the Adopted Basis Newton–Raphson procedure (800 iterations). A conformational search of the anthraquinone-NH and the NH-C torsions was then performed using the conformational search program from Quanta. For each structure the torsion angles of the two selected bonds were altered stepwise by 30° over 360° and minimized at these fixed positions by the Adopted Basis Newton–Raphson procedure following each step. This process afforded a two-dimensional contour plot which was analysed to discover the minimum energy conformations associated with the selected torsion angles of the structures. A further conformational search on the minimum energy conformation derived above was then carried out on the next bond (C–CH_2). This process afforded a plot of torsion angle versus potential energy. In general, the CH–CH_2 bond is conformationally flexible, although the difference in energy between the lowest and highest energy conformations is a few kcal/mol. In these studies, the structures corresponding to their minimum potential energies were chosen, saved, and later used in drawing and overlapping the compounds. Eight structures (NU/ICRFs 500, 504, 505, 506, 510, 512, 513 and 514) were overlaid using the Molecular Similarity program from Quanta. Eight equivalent atoms from the anthraquinone ring system were overlaid, leaving the amino acid substituent in its preferred minimum energy conformation for each structure.

RESULTS

Only NU/ICRFs 500, 506 and 510 bind to DNA

The ability of an agent to alter the thermal denaturation profile of DNA can be used to indicate the ability of the compound to bind to DNA [37]. The hexamer and dodecamer oligonucleotide sequences used in this study are well established as either preferred sequences for DNA intercalators (hexamer) or minor groove binders (dodecamer) [38]. Both NU/ICRFs 500 and 510 gave a small but significant rise in T_m (Table 1), an effect observed for both sequences. NU/ICRF 506 consistently gave a large ΔT_m with the hexamer, ranging from 4 to 9 K. Such a large variation in melting temperature is indicative of a more complex, but less stable, binding mechanism. However, all three compounds showed relatively low ΔT_m values compared to doxorubicin, and a topo I/DNA unwinding assay was subsequently used as a more sensitive alternative method for the determination of DNA binding (Fig. 2). Again, out of the 16 compounds in the series, only NU/ICRFs 500, 506, and 510 produced DNA unwinding effects, as determined by reformation of supercoiled DNA from relaxed starting material. The least marked of these three compounds was NU/ICRF 500, which produced partial unwinding at 25 and 50 μg/mL (with no difference in the extent of unwinding at both these concentrations). NU/ICRF 506 produced full unwinding at 25 μg/mL whereas NU/ICRF 510 produced this effect at 5 μg/mL. These results are supported by observations made during topo-catalysed relaxation assays in which both these compounds caused marked retardation of the supercoiled DNA bands during electrophoresis of the reaction mixtures (see Fig. 3B, lane 15 for an example).

Different anthracenyl-amino acids show varying levels of inhibition of topo I and II catalytic activity

The ability of each anthracenyl-amino acid to

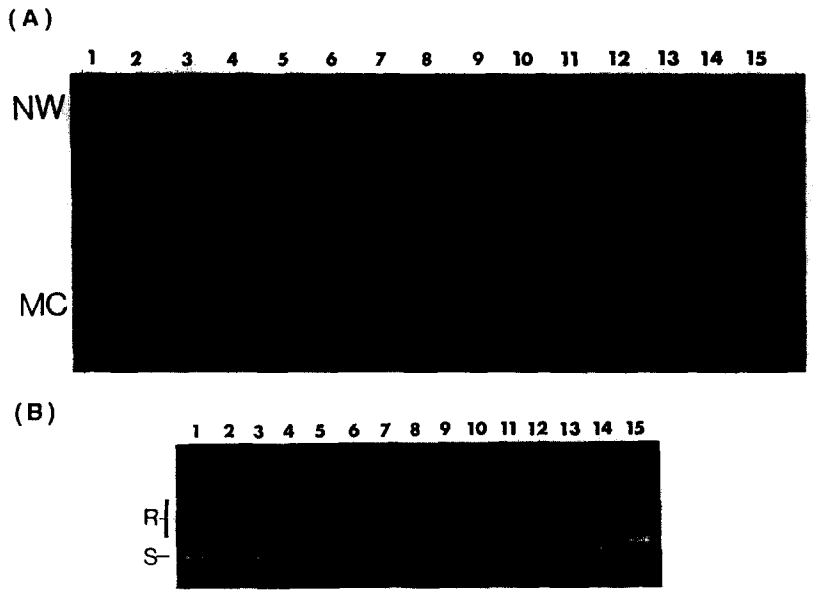


Fig. 3. Inhibition of topo II catalytic activity by anthracenyl-amino acids. Assay methods were described in the Materials and Methods section. (A) Inhibition of decatenation activity of topo II. Lane 1, no enzyme; lane 2, decatenated standard (TopoGEN Inc., OH, U.S.A.); lane 3, enzyme control; lanes 4, 7, 10 and 13, NU/ICRFs 505, 512, 500 and 509 respectively at 1 μg/mL; lanes 5, 8, 11 and 14, as for 4, 7, 10 and 13 except at 5 μg/mL; lanes 6, 9, 12 and 15 as for 4, 7, 10 and 13 except at 50 μg/mL. NW, kinetoplast DNA; MC, minicircular monomeric DNA. (B) Inhibition of topo II-catalysed relaxation of supercoiled DNA. Lane 1, no enzyme; lane 2, enzyme control; lane 3, 50 μM amsacrine; lanes 4, 7, 10 and 13, NU/ICRFs 500, 509, 505 and 506 respectively at 1 μg/mL; lanes 5, 8, 11 and 14 as for 4, 7, 10 and 13 except at 5 μg/mL; lanes 6, 9, 12 and 15 as for 4, 7, 10 and 13 except at 50 μg/mL. R, relaxed closed circular DNA; S, supercoiled closed circular DNA.

Table 2. Summary of topoisomerase inhibition by anthracenyl-amino acids and corresponding *in vitro* cytotoxicity

	Topo I			Topo II						
	Relaxation			Decatenation			Relaxation			*In vitro* cytotoxicity (IC_{50}, μM)
	50	5	1	50	5	1	50	5	1	
NU/ICRF 500	++	—	—	++	++	—	++	—	—	5.4
NU/ICRF 501	+	—	—	—	—	—	—	—	—	Not active
NU/ICRF 502	+	—	—	++	—	—	—	—	—	Not active
NU/ICRF 503	—	—	—	++	+	—	—	—	—	Not active
NU/ICRF 504	—	—	—	—	—	—	—	—	—	Not active
NU/ICRF 505	—	—	—	—	—	—	—	—	—	7.4
NU/ICRF 506	++	++	—	++	++	+	++	+	—	3.4
NU/ICRF 507	—	—	—	—	—	—	—	—	—	Not active
NU/ICRF 508	—	—	—	—	—	—	—	—	—	Not active
NU/ICRF 509	+	—	—	+	—	—	+	—	—	Not active
NU/ICRF 510	++	++	—	++	++	—	++	++	—	11.2
NU/ICRF 511	—	—	—	++	—	—	+	—	—	ND
NU/ICRF 512	—	—	—	++	—	—	+	—	—	Not active
NU/ICRF 513	+	—	—	++	++	—	++	—	—	10.6
NU/ICRF 514	—	—	—	+	—	—	+	—	—	ND
NU/ICRF 515	—	—	—	—	—	—	—	—	—	11.6
							Doxorubicin			1.5×10^{-3}
							Camptothecin			7.9×10^{-3}

Topoisomerase inhibition by each of the 16 anthracenyl-amino acids studied (NU/ICRF 500–515) was determined using the assays described in the Materials and Methods section and illustrated in Fig. 3. Greater than 50% inhibition of enzyme activity at 50, 5 or 1 μg/mL was indicated by ++ (evidence of inhibition below 50% was denoted by a single +). The concentrations of each drug giving 50% inhibition of cell survival (IC_{50}) were determined using A2780 cells as described in the Materials and Methods section. IC_{50} values greater than 100 μM were denoted 'not active'. ND, not determined.

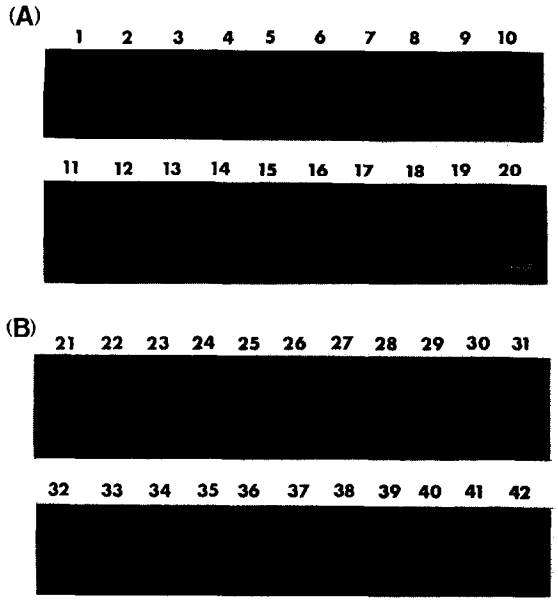
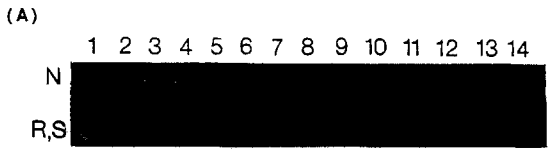


Fig. 4. Only NU/ICRF 506 inhibits topo I-mediated relaxation. Topo I relaxation assays were carried out under the conditions described in the Materials and Methods section. (A) Decreasing concentrations of topo I were reacted with pBR322 in the absence (upper panel; lanes 1–10) or presence of 10 μg/mL NU/ICRF 506 (lower panel; lanes 11–20). Topoisomerase I levels per reaction in lanes 1–10 and 11–20 were 6, 4, 3, 2.5, 2, 1.6, 1.2, 0.8, 0.4, and 0 units, respectively. (B) Decreasing concentrations of topo I were reacted with pBR322 in the absence (upper panel; lanes 21–31) or presence of 2.5 μg/mL NU/ICRF 510 (lower panel; lanes 32–42). Topoisomerase I levels per reaction in lanes 21–31 and 32–42 were 10, 8, 6, 5, 4, 3, 2, 1.5, 1, 0.5 and 0 units, respectively.



(B)

Compound	% Nicked DNA
Camptothecin (0.1 μM)	24
Camptothecin (50 μM)	54
NU/ICRF 505	38
NU/ICRF 507	6
NU/ICRF 508	4
NU/ICRF 513	0
NU/ICRF 514	0

Fig. 5. Induction of topo I-mediated DNA cleavage by NU/ICRF 505. In panel (A) topo I cleavage reactions were carried out using the agarose gel electrophoresis assay described in the Materials and Methods section. Lane 1, substrate pBR322 DNA; lane 2, no drug; lane 3, 0.1 μM camptothecin; lane 4, 50 μM camptothecin; lanes 5, 7, 9, 11 and 13, DNA plus NU/ICRFs 505, 507, 508, 513 and 514, respectively, at 50 μg/mL without enzyme; lanes 6, 8, 10, 12 and 14 as for 5, 7, 9, 11 and 13 except with excess enzyme. Values for percentage nicked DNA formation (as determined by densitometric scanning of each gel negative) are shown in panel (B) for those compounds shown in (A). N, nicked relaxed DNA; R, relaxed DNA; S, supercoiled DNA.

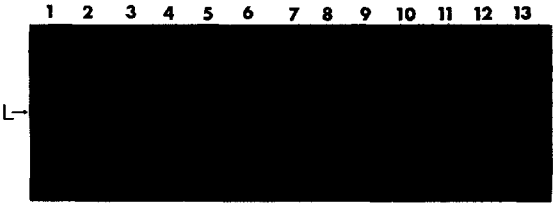


Fig. 6. Anthracenyl-amino acids do not induce topo II-mediated cleavage. The ability of each drug to induce topo II-mediated cleavage was determined using the assay conditions described in the Materials and Methods section in which double-stranded DNA cleavage and the corresponding formation of linear DNA molecules (L) can be detected by agarose gel electrophoresis. Lane 1, substrate DNA control; lane 2, no drug; lane 3, linearized DNA standard; lane 4, 10 μM amsacrine; lanes 5, 8 and 11, NU/ICRFs 500, 505 and 509 respectively at 1 μg/mL; lanes 6, 9 and 12 as for 5, 8 and 11 except at 5 μg/mL; lanes 7, 10 and 13 as for 5, 8 and 11 except at 50 μg/mL.

inhibit topo I (relaxation) and topo II (decatenation; relaxation) catalysed reactions was investigated (see Fig. 3 for examples). Inhibition of topo II catalytic activity was observed for a number of compounds, of which NU/ICRFs 506, 510, 500 and 513 were the most active in this order (Table 2). Apparent inhibition of topo I relaxation by NU/ICRFs 500 and 510 (Table 2) was shown to be due to DNA intercalation rather than inhibition of topo I activity using the topo I/DNA unwinding assay (Fig. 2). NU/ICRF 506, on the other hand, was shown to be a genuine inhibitor of topo I-mediated relaxation in an enzyme titration experiment which can differentiate between DNA unwinding and enzyme inhibition (Fig. 4A). NU/ICRFs 500 and 510 did not inhibit topo I activity using the same experimental approach (see Fig. 4B for assay with NU/ICRF 510). NU/ICRF 506 was also the most potent inhibitor of topo II activity, producing 100% inhibition between 1 and 5 μg/mL. NU/ICRFs 500, 510 and 513 also produced 100% inhibition of topo II activity at 5 μg/mL although these compounds, unlike NU/ICRF 506, did not show any evidence of inhibition at 1 μg/mL.

NU/ICRF 505 stimulates topo I-mediated DNA cleavage

An agarose gel electrophoresis assay was employed to determine the amount of single-stranded DNA breakage resulting from potential stimulation of topo I-mediated cleavable complex formation by each of the 16 compounds. Of all the compounds, only NU/ICRF 505 produced significant cleavage effects (38% nicked DNA formation, Fig. 5). These results contradict the findings of catalytic inhibition assays in which NU/ICRF 505 did not inhibit the catalytic activity of topo I or II (Table 2). However, the extent of DNA cleavage with this compound was lower than that for camptothecin (54% nicked DNA formation for 50 μM camptothecin; 38% for 50 μg/mL (approx. 150 μM) NU/ICRF 505).

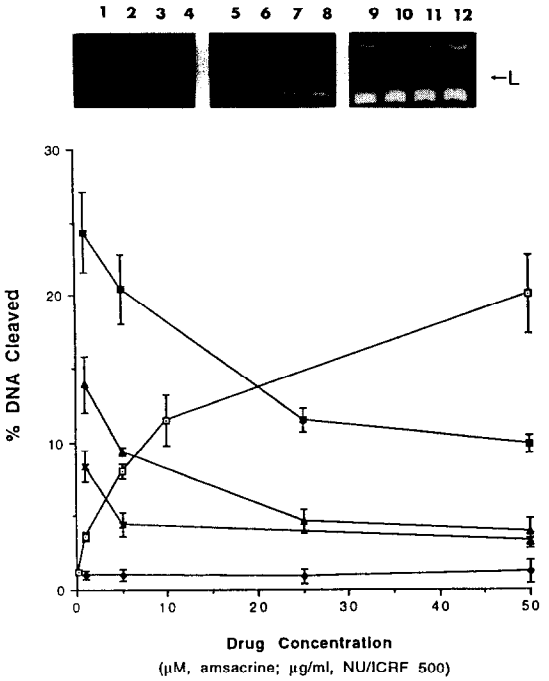


Fig. 7. NU/ICRF 500 partially inhibits amsacrine-induced topo II-mediated cleavage. Drug-induced double-stranded DNA cleavage of DNA was detected by the formation of linear molecules (L) as described in the Materials and Methods section. The effect of NU/ICRF 500 at final concentrations of 1, 5, 25 and 50 μg/mL on topo II cleavage induced by 5, 10 and 50 μM amsacrine is shown in the gel inserts. Lanes 1, 5 and 9, NU/ICRF 500 at 1 μg/mL; lanes 2, 6 and 10, NU/ICRF 500 at 5 μg/mL; lanes 3, 7 and 11, NU/ICRF 500 at 25 μg/mL; lanes 4, 8 and 12, NU/ICRF 500 at 50 μg/mL. Lanes 1–4 also contained 5 μM amsacrine; lanes 5–8, 10 μM amsacrine; lanes 9–12, 50 μM amsacrine. The results shown in the gel inserts were quantified by densitometric scanning of negatives from at least three separate experiments. The percentage of linear DNA formed in the total amount of DNA for each reaction mixture was calculated and plotted as the percentage of DNA cleaved. —⊡—, amsacrine; —◆—, NU/ICRF 500; —×—, NU/ICRF 500 + 5 μM amsacrine; —▲—, NU/ICRF 500 + 10 μM amsacrine; —■—, NU/ICRF 500 + 50 μM amsacrine.

The topo I cleavage assay shown in Fig. 5 is of particular interest since the five compounds included (NU/ICRFs 505, 507, 508, 513 and 514) are all structurally very similar. Since NU/ICRF 505 was the only compound inducing substantial topo I-mediated DNA cleavage, this has important implications from a structure–activity perspective and will be discussed later.

Anthracenyl-amino acids do not induce topo II-mediated cleavage but NU/ICRF 500 inhibits cleavable complex formation induced by amsacrine

In an attempt to determine whether anthracenyl-amino acids stimulate topo II-mediated DNA cleavage, supercoiled pBR322 DNA was incubated with topo II in the presence of increasing amounts of each compound (see Fig. 6 for examples with NU/ICRFs 500, 505 and 509). No increase in linear DNA was detected by the addition of any of the 16 compounds in the series under the same conditions which produced noticeable cleavage using 10 μM amsacrine. Because of the apparent similarity in the mechanism of anthracenyl-amino acids to a number of other non-cleavage inducing topo inhibitors, experiments were performed to determine whether the topo II inhibitor NU/ICRF 500 could reduce the amount of cleavage induced by amsacrine (Fig. 7). At both 5 and 10 μM amsacrine, NU/ICRF 500 reduced the amount of linear DNA formation down to almost insignificant levels at 25 and 50 μg/mL, although at 50 μM this effect was only partial (i.e. about 50% reduction in the double-stranded cleavage induced by amsacrine).

Essentially the same effect was observed for NU/ICRF 506 (results not shown).

Energy minimized conformations of various anthracenyl-amino acids reveal important differences between structurally similar compounds

Molecular modelling was carried out to derive predicted energy minimized conformations of NU/ICRFs 500, 504, 505, 506, 510, 512, 513 and 514. These compounds were selected for analysis due to their range in ability to inhibit topo I and II, even though several are structurally very similar. Overlay of each energy minimized conformation revealed that each amino acid substituent projects out from the planar anthraquinone ring system in a different conformation (Fig. 8). The implications for this observation regarding the various levels of topo inhibition observed for these compounds will be discussed later.

Some anthracenyl-amino acids are actively cytotoxic, with a level of cytotoxicity which correlates with inhibition of purified enzyme

A preliminary screening programme was established to determine whether anthracenyl-amino acids could be cytotoxic to cells. In these experiments, a range of drug concentrations were used to determine the IC_{50} value for each of the 16 compounds against the human ovarian cancer cell line A2780. Results from these experiments corresponded very closely with the pattern of inhibition observed with purified enzyme (Table 2). For example, the most potent inhibitor of enzyme activity, NU/ICRF 506, also had the lowest IC_{50} value of 3.4 μM. Similarly, NU/ICRFs 500, 510 and 513 were also actively cytotoxic with IC_{50} values in the low μM range (5.4, 11.2 and 10.6, respectively). These values are again in general agreement with their ability to inhibit enzyme activity *in vitro*. NU/ICRF 515, which was inactive in assays with purified enzyme, showed a level of cytotoxicity (IC_{50}, 11.6 μM) not consistent with this compound acting through topo inhibition.

NU/ICRF 505, the apparent inducer of topo I-mediated DNA cleavage, was also actively cytotoxic in the low μM range (IC_{50} of 7.1 μM) which corresponds well with its postulated mechanism of action.

DISCUSSION

Mono-conjugation of an anthraquinone nucleus

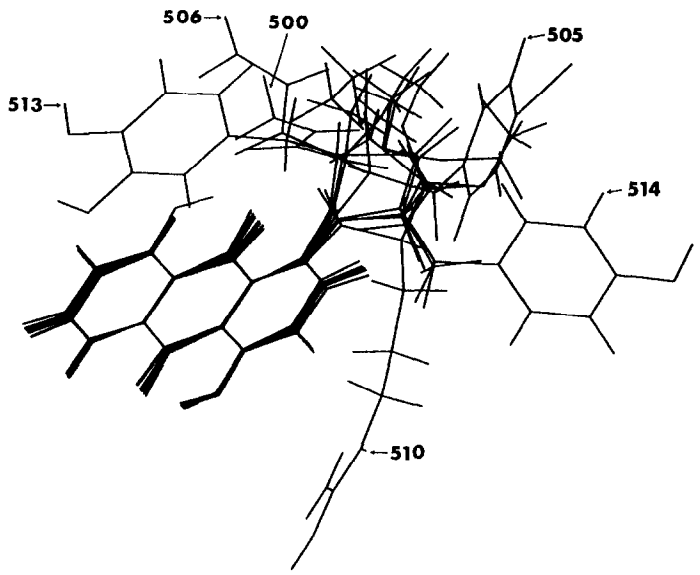


Fig. 8. Molecular modelling of anthracenyl-amino acids. Energy minimized conformations were derived for NU/ICRFs 500, 504, 505, 506, 510, 512, 513 and 514 using the Quanta and Charmm programs detailed in the Materials and Methods section. Each conformation was overlaid around the anthraquinone ring, leaving each amino side-chain substituent in its preferred minimum energy conformation. Those compounds relevant to the discussion are indicated by an arrow and appropriately labelled (i.e. NU/ICRFs 500, 505, 506, 510, 513 and 514).

with a range of naturally occurring amino acids has been adopted for the first time as a synthetic approach to rationally design novel topoisomerase inhibitors. By further chemically modifying the C-terminus of the amino acids and by substitution of the anthraquinone ring system with additional hydroxyl groups, structure–activity relationships for topo inhibition were explored. The working hypothesis was that the anthraquinone moiety would intercalate DNA and the amino acid substituent may interact with the enzyme [39]. This follows from structural studies with intercalators which revealed that most of these drugs contain a side-chain which projects from one of the grooves of DNA, allowing for direct contact with the topo protein [40]. The importance of side-chain substituents in anthracenedione derivatives and their role in modulating drug activity has been well documented (for a review see Ref. 41). While the majority of topo II active anthracenediones are bis-substituted, recent work has implied that just one of the hydroxyethylaminoethylamino side-chains in mitoxantrone is sufficient for activity [14]. Therefore, it is not surprising that a number of monosubstituted anthracenedione-amino acid derivatives described in this paper are effective inhibitors of topo II.

The most active topo II inhibitors, NU/ICRFs 500 and 506 (Fig. 3; Table 2), are serine conjugates with the same C-terminus and differ only by way of the additional hydroxyl group on the 'C' ring of NU/ICRF 506 (Fig. 1). Elevated topo II inhibition by compounds containing additional hydroxyl groups in key positions has been observed for mitoxantrone and its less active analogue ametantrone [14]. The molecular basis for this observation has been explained as an increased stability of DNA binding through the formation of intermolecular hydrogen bonds [42] and, indeed, in this study NU/ICRF 506 has been shown to exhibit greater binding to DNA than NU/ICRF 500. In addition, comparison of the minimum energy conformations of NU/ICRFs 500 and 506 revealed that the presence of the additional hydroxyl group alters the orientation of the serine residue dramatically such that it extends away from the ring system. This may, therefore, provide a rationale for the elevated topo II inhibitory activity of NU/ICRF 506 and its selective inhibition of topo I catalytic activity, despite almost identical empirical formulae.

The importance of hydroxyl groups in the amino acid substituent and hence potential hydrogen bonding interactions with the enzyme is shown with another of the active compounds, NU/ICRF 513, which has a dihydroxyphenylalanine substitution (Fig. 1) and was a potent topo II inhibitor (Table 2). Very similar structural analogues of this compound, which have the same modified C-terminus but differ only by way of the number of phenolic hydroxyl groups in the amino acid (phenylalanine and tyrosine for NU/ICRFs 508 and 514, respectively), are not nearly as active as topo II catalytic inhibitors. Molecular modelling studies have been employed here in an attempt to explain these observations, as was similarly carried out recently by Capranico *et al.* [43] to identify drug conformations determining sequence-selectivity of drugs inducing topo II-mediated cleavage. Derivation of energy minimized conformations for these anthracenyl-

amino acids revealed that substitution in the amino acid phenyl ring with extra hydroxyl groups dramatically altered the three-dimensional structure of the compound (Fig. 8). For example, the dihydroxy phenyl ring in NU/ICRF 513 is located in parallel with the anthraquinone ring, whereas the phenyl ring in NU/ICRFs 508 and 514 projects out from the ring system in different directions. It is possible, therefore, to conclude from the topo II inhibition data with NU/ICRFs 500, 506, 510 and 513, that topo II inhibition by such structurally diverse compounds implies a less rigid structural consensus for such inhibitory activity.

NU/ICRF 510, the arginine derivative, was shown to be a potent inhibitor of topo II catalytic activity. This compound also displayed a high affinity for DNA, and topo II inhibition may, therefore, be due to prevention of the enzyme interacting with the DNA–drug complex, as has been shown for several minor groove binding agents including distamycin [44] and netropsin [45]. This is supported by observations made during topo II assays in which an excess amount of enzyme could not overcome inhibition by this compound (results not shown).

NU/ICRF 505 was the only compound able to induce topo I-mediated DNA cleavage (Fig. 5), at a level of cleavage below that of camptothecin. Importantly, a number of very similar structural analogues of this compound (NU/ICRFs 507, 508, 513 and 514) were not able to produce this effect. The reason for this is not clear but again molecular modelling highlighted the fact that in this group of compounds the phenyl ring of the amino acid was in each case in a different orientation (see Fig. 8). These data can be interpreted as indicating a more rigid structural requirement for stabilization of topo I cleavable complexes and suggest a highly specific interaction between drug, DNA and enzyme in the ternary complex. A number of structure–activity studies have emphasized the importance of such interactions, including hydroxy analogues of podophyllin congeners [46], the 9-hydroxy eternal group in ellipticines [47], the 9-hydroxy position in the anthracycline ring system [48], and the 9-anilino ring system of amsacrines [49], all of which markedly affect cleavable complex formation by these drugs.

In addition to the amino acid substituent, the nature of the C-terminal modification also appears to be critical for stabilization of topo I cleavable complexes. Modification of the ethyl ester group of NU/ICRF 505 to a less hydrophobic hydrazide in NU/ICRF 501, for example, completely abolished the DNA cleavage activity. These observations are consistent with the model of the topo II ternary complex recently proposed by Morjani *et al.* [39] using a series of intoplicine derivatives, where the drug binds by hydrogen bonding deep into a hydrophobic pocket of the enzyme.

The most active compounds inhibiting topo II (NU/ICRFs 500, 506 and 510) also appeared to inhibit topo I relaxation (Table 2; Fig. 3B), although a topo I relaxation assay in which decreasing enzyme levels are employed together with a constant level of DNA in the presence or absence of drug to separate DNA unwinding from catalytic inhibition [32] indicated that NU/ICRF 506 was the only compound able to inhibit topo I-mediated relaxation (Fig. 4). Further evidence for topo I inhibition by NU/ICRF 506 is with the original topo I relaxation assay (Fig. 3B; lanes 13–15) in which inhibition is apparent at 5 μg/mL. This cannot be due to DNA intercalation since unwinding effects with this compound were not apparent at this drug concentration (Fig. 2, lane 13). These results, therefore, indicate that NU/ICRF 506 is the only anthracenyl-amino acid in the series of 16 compounds which inhibits the catalytic activity of topo I, in addition to being the most potent inhibitor of topo II activity. Such dual inhibition of topo I and II is now recognized as a desirable property by potentially overcoming resistance mechanisms associated with changes in topo levels [50, 51].

The most active anthracenyl-amino acids, NU/ICRFs 506 and 500, appear to fall into the expanding category of topo targeting drugs that apparently do not stabilize covalent DNA-enzyme intermediates. The most recent compounds in this category [18, 19, 22] were also shown to inhibit DNA cleavage induced by classic topo poisons. In this study NU/ICRFs 500 and 506 markedly reduced amsacrine-induced topo II-mediated DNA cleavage at 5 and 10 μM, with a partial reduction at 50 μM (Fig. 7). As for other catalytic inhibitors [19], inhibition by NU/ICRF 500 could be overcome by addition of excess enzyme (data not shown), suggesting direct interaction between the drug and enzyme. A protein clamp model has been proposed recently to explain the mechanism of topo II catalytic inhibition by bisdioxopiperazines [52] which may be relevant to inhibition by NU/ICRFs 500 and 506. However, the cytotoxicity of amsacrine has also been shown to be reduced by novobiocin, a coumarin-based drug which inhibits topo II-catalysed ATP hydrolysis [53], and this is clearly an important possible alternative mechanism for the reduction in amsacrine-induced DNA cleavage by NU/ICRFs 500 and 506.

The most active anthracenyl-amino acids in topo-catalysed reactions with purified enzyme, with the exception of NU/ICRF 515, were also those compounds with highest levels of *in vitro* cytotoxicity against A2780 human ovarian cancer cells (Table 2). Therefore, as with other catalytic inhibitors [21, 22, 26], anthracenyl-amino acids appear to be actively cytotoxic to cells. Furthermore, cytotoxic doses of the most active compounds in cell culture appear similar to those inhibiting the activity of the purified enzyme. Clearly this preliminary screen with A2780 cells will have to be extended to a number of different cell lines in order to further characterize the cytotoxicity of these compounds and deduce potential mechanisms of drug resistance [50, 51, 54]. In addition to the determination of topo I and II expression levels in these cell lines, additional studies should investigate cellular uptake since the rate of uptake (in addition to possible intracellular metabolism) will have an important bearing on the levels of cytotoxicity observed for the topo active compounds in the series.

In summary, we have shown that several compounds from a new series of 16 anthracenedione-amino acid conjugates are potent inhibitors of DNA topoisomerases, and that the most active compounds

are actively cytotoxic *in vitro*. Future studies will aim to deduce the actual cytotoxic mechanisms of existing compounds, in addition to the rational design of structural analogues of these compounds, in an attempt to produce more effective topo inhibitors and achieve the ultimate goal of application in the clinic.

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