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Original Paper

From Amsacrine to DACA (N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide): Selectivity for Topoisomerases I and II Among Acridine Derivatives

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A number of acridine derivatives, including the clinical antileukaemia agent amsacrine and the experimental agent DACA (N-[2-(dimethylamino)ethyl]acridine-4-carboxamide), target the enzyme topoisomerase II. We demonstrate here that DACA induces DNA cleavage in the presence of topoisomerase I as well as of topoisomerase II. We also investigate a series of acridine derivatives which link amsacrine to DACA in terms of DNA binding, topoisomerase poisoning and biological activity. The presence of an acridine 4-linked N-2-(dimethylamino)ethyl group provides both a pronounced G-C preference for DNA binding and activity towards topoisomerase I. The removal of the anilino side chain of amsacrine, in combination with the presence of the N-2-(dimethylamino)ethyl group, provides *in vitro* biological activity against "atypical" multidrug resistant leukaemia lines with low topoisomerase II activity. Among these compounds, suppression of the ionisation of the acridine nitrogen to produce the compound DACA is associated with experimental activity against solid tumours. The addition of an acridine 2-chloro substituent to DACA suppresses the stimulation of topoisomerase II-dependent DNA cleavage but increases stimulation of topoisomerase I cleavage. 2-Substitution also increases activity against the "atypical" multidrug resistant cell lines. Overall, the results suggest that augmentation of topoisomerase I-dependent activity in this series by appropriate chemical substitution in this series leads to circumvention of topoisomerase II-mediated multidrug resistance.

Key words: acridine, multidrug resistance, DNA intercalation, topoisomerase
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INTRODUCTION

DNA TOPOISOMERASES are important targets for both antibacterial and anticancer drugs. Compounds such as doxorubicin and etoposide, which target the enzyme topoisomerase II, have an established role in the treatment of a variety of tumours, while derivatives of the plant product camptothecin, which target topoisomerase I, are currently undergoing clinical trial [1]. Some agents, such as actinomycin D [2], saintopine [3], intoplicine [4], indoloquinolinedione derivatives [5], fagaronine derivatives [6] and anthracycline derivatives [7, 8] appear to stimulate DNA cleavage by both enzymes. The development of new topoisomerase poisons with dual activity is topical and interesting.

Acridine derivatives, long known for their antimicrobial properties, have formed the basis for the development of agents which target topoisomerase II [9, 10]. Amsacrine has proven clinical efficacy in the treatment of acute leukaemia [11], and its disubstituted derivative CI-921 has clinical activity against solid tumours [12]. A series of acridine derivatives lacking the anilino side-chain of amsacrine but containing a charged side-chain at the 4-position (Figure 1) display excellent activity against the Lewis lung carcinoma [13]. One of these compounds, N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA) is active against a number of murine tumours [14] and is currently undergoing clinical trial under the auspices of the Cancer Research Campaign, U.K.

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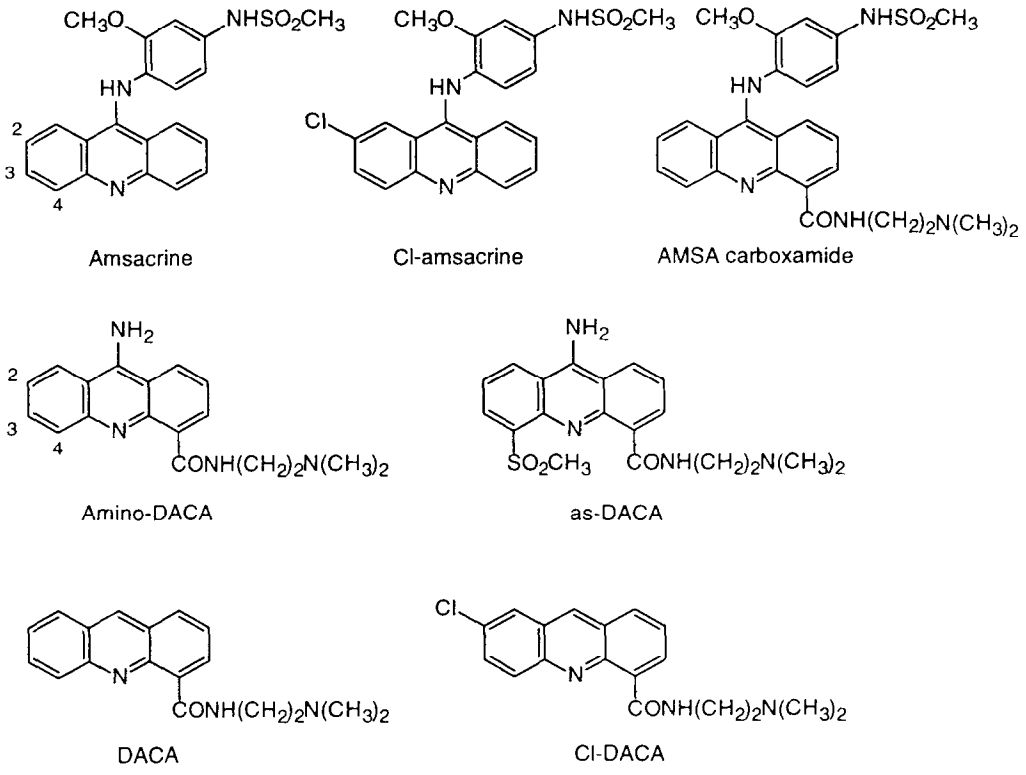

Figure 1. Structures of compounds examined in this study.

Several features of the activity of DACA suggest a unique mode of action. The relationship between cytotoxicity, concentration and drug exposure time is complex, involves self-inhibition at high drug concentrations, and is unlike those of other topoisomerase II poisons [15, 16]. DACA is able to overcome not only P-glycoprotein-mediated multidrug resistance, but also "atypical" resistance involving reduced topoisomerase II activity [17]. In this study, we have traced the structural links between amsacrine and DACA, examining the effect of changing features, one at a time, on the base preference of DNA binding, the ability to overcome multidrug resistance and the ability to stimulate DNA cleavage in the presence of topoisomerase I and topoisomerase II. We discuss these properties in relation to experimental solid tumour activity.

MATERIALS AND METHODS

Materials

Acridine derivatives [13, 17–21] were available from the Cancer Research Laboratory, Auckland, New Zealand. Intoplicine [4] and saintopine [3] were available from the Rhône-Poulenc Rorer Laboratory, Vitry sur Seine, France. Fagaronine [22] was a gift of Dr J.C. Jardillier (Institut Jean Godinot, Reims, France) and the ellipticine derivative, 2-methyl-9-hydroxyellipticinium, was obtained from Sanofi Laboratory (Toulouse, France).

Studies on drug binding to DNA

Drug binding was determined in sodium chloride (sodium acetate buffer, pH 5.0; ionic strength 0.01) by competition with ethidium as previously described [23, 24]. Poly [dA–dT]·poly [dA–dT] and poly [dG–dC]·poly [dG–dC] (Sigma Chemical Co.) were used at concentrations of 20 μM and ethidium bromide (Sigma) at 1.15 μM. The association constants of ethidium for poly [dA–dT]·poly [dA–dT] and poly [dG–dC]·poly [dG–dC] were 9.5×10^6 and 9.9×10^6, respectively [25]. Reduction in ethidium fluorescence was measured in a fluorometer and the drug concentration required to halve the initial fluorescence was determined. Because amsacrine derivatives quench the fluorescence of DNA-bound ethidium, a separate assay employing an excess of DNA was used to correct for fluorescence quenching [26]. Assays were either single determinations or the means of multiple determinations.

Topoisomerase studies

Purification of calf thymus topoisomerase I and II was performed using a nuclear extract preparation, with 200 g of frozen tissue as starting material, by two successive columns of phosphocellulose and hydroxylapatite, as previously described [27]. One unit of topoisomerase I (or II) was defined as the amount of enzyme necessary to relax (or decatenate) 50% of 0.5 μg pBR322 DNA (or kinetoplast DNA) when incubated for 30 min at 37°C under the assay conditions. ^{32}P-end labelled pBR322 DNA was prepared by linearising the DNA with EcoRI restriction endonuclease and labelling its termini with [α-^{32}P]dATP and Klenow polymerase (Boehringer). The 3′-end-labelled DNA was then cut with HindIII restriction endonuclease, generating 4333-base pair and 33-base pair fragments of ^{32}P-3′-end-labelled DNA. Cleavage reactions were performed with topoisomerase I or II using the same reaction mixture, containing 20 mM Tris–HCl, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 30 μg/ml bovine serum albumin, 0.5 mM EDTA, 0.5 mM dithiothreitol and 20000 dpm of 3′-end-labelled pBR322 DNA. Enzyme preparations (10–20 U) in a final volume of 20 μl ATP (1 mM) were added to all except the topoisomerase I reactions. Drugs were added to the reaction mixtures on ice just before the addition of enzyme

preparation. Reactions were incubated at 37°C for 10 min and terminated by the addition of 2 μl of 2.5% SDS, 2.5 mg/ml proteinase K. Samples were further incubated for a period of 30 min at 50°C and were loaded onto a 1% agarose gel in Tris–borate–EDTA (30 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0) buffer with 0.1% SDS. In the topoisomerase I assays, DNA samples were denatured by the addition of 10 μl of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green just before gel loading, whereas in the topoisomerase II assays, samples were not denatured. Gels were dried and autoradiographed for 1 or 2 days (Hyperfilms MP; Amersham, Buckinghamshire, U.K.). Some of the results were from single determinations, while the results for amsacrine and DACA were representative of multiple experiments.

In vitro *cytotoxicity determinations*

Cloned human Jurkat leukaemia cells (designated L_C) were used to derive amsacrine- and doxorubicin-resistant sublines (L_A and L_D, respectively), which were crossresistant to other topo-II poisons [28]. These cells were cultured as described [29] in 96-well plates. Growth of the leukaemia lines was assessed by IC_{50} values (defined where growth corresponded to 50% of that of the control cultures) using the sulphorhodamine B staining method [30]. Assays were carried out in triplicate, and the average coefficient of variation was ±9%.

RESULTS

DNA binding properties

The series of compounds examined is shown in Figure 1. DNA binding preferences were compared (Table 1) by measuring the efficacy of displacing ethidium from the synthetic polymers poly[dA–dT]·poly[dA–dT] and poly[dG–dC]·poly[dG–dC] at low ionic strength, together with correction for fluorescence quenching which is significant for these compounds [21]. Ethidium has slight G–C preference in DNA binding in this assay [25] while amsacrine binds weakly in comparison to ethidium and shows no significant preference [31]. Cl-amsacrine is a derivative of amsacrine with considerably reduced biological activity, indicative of an effect of the 2-chloro group on binding to the target site of the enzyme [24]. Its A–T binding was slightly reduced but its G–C binding was comparable with that of amsacrine. AMSA-carboxamide, which has a charged side-chain at the acridine 4-position, had marked (14-fold) preference for G–C binding and also bound slightly more strongly to the A–T polymer than did amsacrine, while amino-DACA and as-DACA, which lack the anilino side-chain of AMSA-carboxamide, retained G–C preference. DACA, lacking the acridine 9-amino group of amino-DACA, showed reduced DNA binding in comparison to amino-DACA, although binding was G–C selective and was still higher than that of amsacrine. The 7-chloro derivative, Cl-DACA, was tested for comparison with the correspondingly substituted Cl-amsacrine; it showed high DNA binding and showed G–C preference.

Studies with topoisomerase I

The compounds in Table 1 were compared in their ability to stimulate cleavage of DNA at defined sites in the presence of purified topoisomerase I, using camptothecin as a standard. The results, together with comparataive data for camptothecin, fagaronine, saintopine and actinomycin D, are shown in Figures 2 and 3. Amsacrine (not shown) and Cl-amsacrine (Figure 3) were inactive in this assay while faint new cleavage bands were observed with 1 μM of AMSA-carboxamide, and

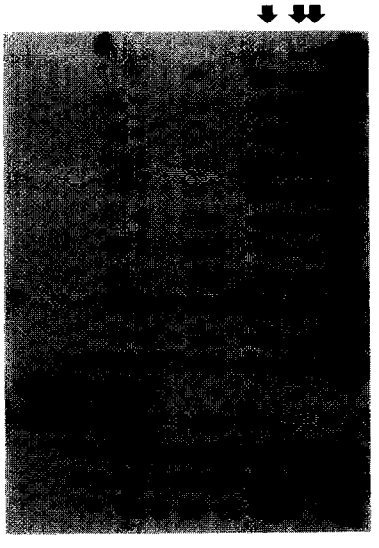

Figure 2. Stimulation by DACA and Cl-DACA of cleavage of topoisomerase I-induced DNA cleavage of the EcoRI/Hind III restriction fragment of pBR322. Lane 1: control DNA. Lane 2: topoisomerase I in the absence of drugs. Lanes 3–7: topoisomerase I plus Cl-DACA. Lanes 8–12: topoisomerase I plus DACA. Lanes 13–17: topoisomerase I plus camptothecin, fagaronine, saintopine, intoplicine and actinomycin D, respectively. Drug concentrations (μM units) are indicated on the figure. Arrows indicate the most prominent cleavage sites for DACA and Cl-DACA.

Table 1. DNA binding and cytotoxicity data for the compounds in the study

No.	Compound*	DNA association constant × 10^{-6}/M [dA–dT]†	[dG–dC]	IC_{50} values for Jurkat lines (nM) Resistance factors shown in brackets L_C	L_A	L_D
1	Amsacrine	0.37	0.45	27	3300 (120)	3000 (110)
2	Cl-amsacrine	0.26	0.48	4800	9300 (1.9)	12200 (2.5)
3	AMSA-carboxamide	0.70	9.6	6.6	36 (54)	290 (45)
4	Amino-DACA	22	50	27	120 (4.5)	170 (6.3)
5	DACA	1.3	3.5	640	1110 (1.7)	1450 (2.3)
6	Cl-DACA	7.4	14	870	630 (0.7)	810 (0.9)
7	as-DACA	21	100	24	350 (15)	2000 (83)

*See Figure 1 for structures. †[dA–dT] = poly [dA–dT]·poly [dA–dT] and [dG–dC] = poly [dG–dC]·poly [dG–dC].

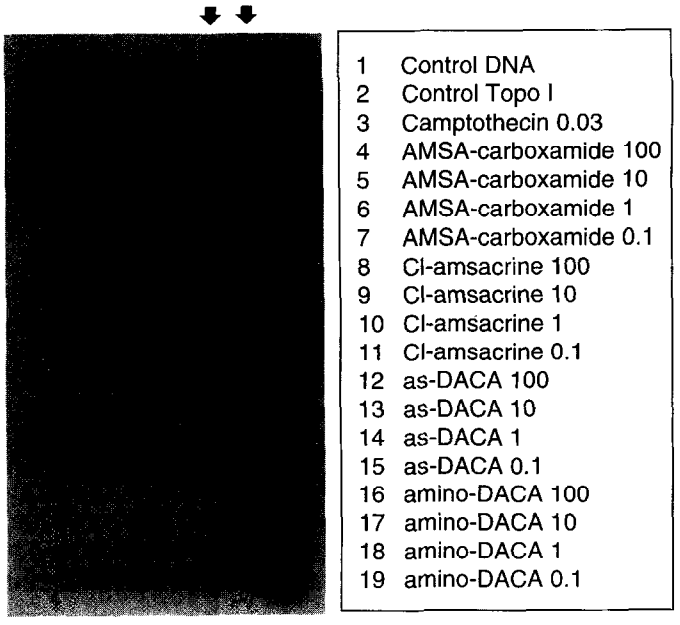

Figure 3. Stimulation by camptothecin, AMSA-carboxamide, Cl-amsacrine, as-DACA and amino-DACA of cleavage of topoisomerase I-induced DNA cleavage of the EcoRI/HindIII restriction fragment of pBR322. Conditions as for Figure 2. Drugs and drug concentrations (μM units) are indicated on the figure. Arrows indicate the most prominent cleavage sites.

cleavage bands were stronger with DACA, amino-DACA, Cl-DACA, and as-DACA. As indicated by the relative positions of the bands, the frequency of DNA sites cleaved was much lower than that for camptothecin or saintopine. Cl-DACA was approximately 4-fold more potent than DACA, while the cleavage sites of amino-DACA and as-DACA were less pronounced than those for DACA. High concentrations of these compounds inhibited cleavage (Figure 3).

Studies with purified topoisomerase II

The compounds in Table 1 were compared with in their ability to stimulate cleavage of DNA at defined sites in the presence of purified topoisomerase II (Figures 4–6). Amsacrine was highly active, as expected, while Cl-amsacrine was considerably less active and slight differences of cleavage sites were detectable (Figure 4). The cleavage patterns of DACA and its derivatives were quite different to those of amsacrine and its derivatives, and also those of other topoisomerase

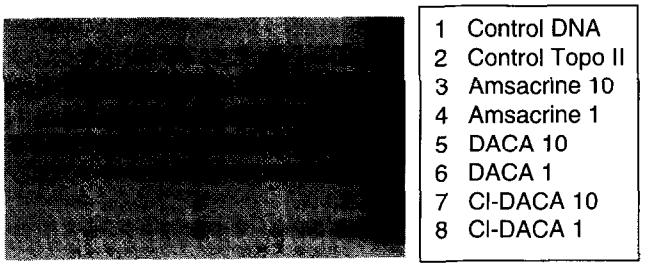

Figure 5. Stimulation by amsacrine, DACA and Cl-DACA of the topoisomerase II-induced DNA cleavage of the EcoRI/HindIII restriction fragment of pBR322. Conditions as described for Figure 4. Drugs and drug concentrations (μM units) are indicated on the figure.

poisons (Figure 5). Among DACA derivatives, some minor differences of cleavage sites, as well as differences in potency, were detectable (Figures 5 and 6). AMSA-carboxamide showed similar activity and a similar cleavage pattern to amsacrine, but in contrast to amsacrine, inhibited topoisomerase II cleavage at high concentration (Figure 5).

Growth inhibition assays

The Jurkat human leukaemia cell line (L_C), together with two independently isolated lines (L_A and L_D), selected for resistance to amsacrine and doxorubicin, respectively, were tested for sensitivity to the compounds in Table 1 by measuring IC_{50} values. L_A and L_D have been previously shown to be highly resistant to amsacrine but sensitive to camptothecin [17], consistent with their reported selective decrease in topoisomerase II activity [27]. Cl-amsacrine was much less cytotoxic than amsacrine and the L_A and L_D lines showed limited cross-resistance. AMSA-carboxamide was highly cytotoxic for L_C cells, with 4-fold higher potency than amsacrine and with greatly attenuated activity against the resistant lines. Amino-DACA was less potent than AMSA-carboxamide but the degree of crossresistance of the resistant lines was reduced from approximately 50-fold to 5-fold. as-DACA showed similar activity to amino-DACA against the L_C line but the degree of crossresistance of the resistant lines rose again to approximately 50-fold. DACA showed a dramatic lowering in the degree of crossresistance to approximately 2-fold while with Cl-DACA, crossresistance had disappeared altogether.

DISCUSSION

The main conclusion of this study is that DACA stimulates DNA cleavage by both topoisomerases I and II. DACA thus

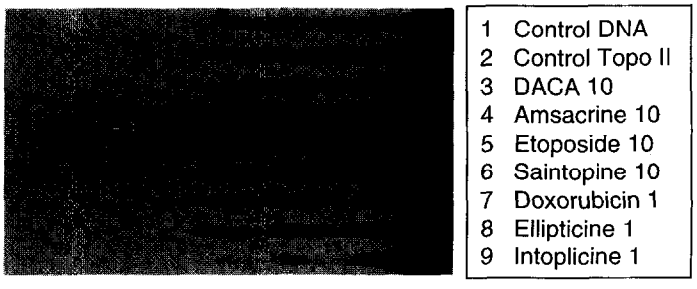

Figure 4. Stimulation by DACA and other drugs of the topoisomerase II-induced DNA cleavage of the EcoRI/HindIII restriction fragment of pBR322. Results are compared with those of other topoisomerase I-directed agents. Lane 1: control DNA. Lane 2: topoisomerase II in the absence of drugs. Lanes 3–9: topoisomerase II plus DACA, amsacrine, etoposide, saintopine, doxorubicin, ellipticinium and intoplicine, respectively. Drug concentrations (μM units) are indicated on the figure.

falls into the same class of DNA intercalators as actinomycin D [2], saintopine [3], intoplicine [4], indoloquinolinedione derivatives [5], fagaronine derivatives [6] and anthracycline derivatives [7, 8]. DACA does not stimulate DNA cleavage by topoisomerase I at as many sites as does camptothecin (Figure 2). Furthermore, two cell lines which have been selected for resistance to camptothecin (P388/CPT$_5$ and DC3/C-10) are sensitive to DACA (data not shown). This situation is similar to that observed with intoplicine [4] and suggests that the amino acid sequence changes in the mutant topoisomerase I in these lines affect the camptothecin binding site on the protein, but that the DNA intercalators either have no protein binding site or interact with topoisomerase I at a different site.

The existence of a series of compounds extending from amsacrine, which poisons only topoisomerase II, to the DACA derivative Cl-DACA, which has a pronounced effect on topoisomerase I, permits an examination of molecular features which are important for interaction with topoisomerases I and II. Although the addition of the charged side-chain has minimal effect on topoisomerase specificity, the concomitant removal of the anilino moiety to provide AMSA-carboxamide causes a pronounced change in topoisomerase II induced cleavage and also promotes topoisomerase I cleavage. It is clear from the above discussion that the presence of the 9-anilino side-chain suppresses the ability of these acridine derivatives to stimulate topoisomerase I cleavage, while the presence of the 2-chloro group decreases the ability to stimulate topoisomerase II. Molecular modelling of amsacrine intercalated into DNA places the 2-position of the acridine close to the phosphodiester backbone. Therefore, 2-substitution may force the molecule into another conformation reducing activity against topoisomerase II and in the case of Cl-DACA, increasing activity against topoisomerase I. A dual DNA binding site model has also been proposed for inhibition of topoisomerases I and II in the intoplicine series; external DNA binding was hypothesised to be associated with topoisomerase II inhibition, while internal binding was associated with topoisomerase I inhibition [32].

The series of acridine derivatives investigated here allows consideration of the basis for G–C preference in DNA binding. Substitution of amsacrine to produce AMSA-carboxamide leads to pronounced G–C binding specificity (Table 1) and this is maintained, although to a reduced degree, in DACA and its derivatives. Models of the intercalative binding of amsacrine have placed the anilino moiety in the minor groove of the DNA double helix [9, 33]. The carboxamide side-chain would therefore be expected to lie in the major groove, which is deeper in G–C rich regions because of the absence of thymine methyl groups. Studies with amsacrine and DACA derivatives have indicated that whereas amsacrine has a rapid dissociation rate from DNA, DACA derivatives and in particular AMSA-carboxamide have much slower dissociation rates [21, 34]. This suggests that the slow dissociation rate is imposed by tight binding of the charged side-chain in the major groove. We suggest that the G–C binding preference may result from interference of side-chain binding in the major groove of these alternating DNA polymers by thymine methyl groups. The slow dissociation rates of DACA and its derivatives from DNA may also be important for the stimulation of topoisomerase I.

An interesting correlation has been obtained between topoisomerase specificity and activity against "atypical" multidrug resistance. The resistant Jurkat leukaemia cells have low topoisomerase II activity and are highly resistant to amsacrine, etoposide and doxorubicin [28, 29]. They appear not to express P-glycoprotein [28], but have not been tested for expression of multiresistance protein (MRP). They have normal sensitivity to camptothecin, suggesting unchanged topoisomerase I activity. As measured by ratios of IC_{50} values, "atypical" multidrug resistance is overcome partially by DACA and completely by Cl-DACA. However, as-DACA is the most selective for topoisomerase II among the DACA derivatives, and is also the least active against the resistant Jurkat lines. One possible conclusion from this result is that topoisomerase I-mediated mechanisms contribute to the circumvention of "atypical" multidrug resistance by DACA and Cl-DACA.

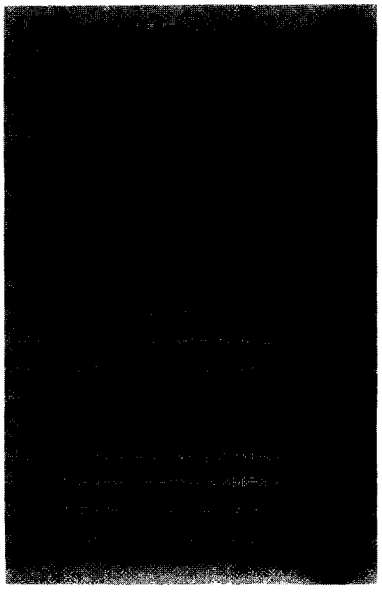

Figure 6. Stimulation by amsacrine, AMSA-carboxamide, Cl-amsacrine, as-DACA and amino-DACA of the topoisomerase II-induced DNA cleavage of the EcoRI/HindIII restriction fragment of pBR322. Conditions as described for Figure 4. Drugs and drug concentrations (μM units) are indicated on the figure.

It is interesting to compare the solid tumour activity of this drug series, as measured against the murine Lewis lung carcinoma. Amsacrine itself has only marginal activity against this tumour [35], while some amsacrine derivatives with suppressed ionisation of the acridine nitrogen have very high activity [36]. Cl-amsacrine has not been tested against the Lewis lung tumour but is inactive against P388 leukaemia. Amino-DACA has no activity against the Lewis lung tumour, but suppression of the ionisation of the acridine nitrogen, as is the case for DACA, Cl-DACA and as-DACA, leads to high activity [13, 20]. It can be concluded that for both the amsacrine and the DACA series, solid tumour activity is enhanced when acridine ionisation is suppressed. Such suppression may improve the ability of the drug to distribute in the poorly vascularised tissue of solid tumours. Activity against the Lewis lung tumour (as measured by percentage increase in life span) is not significantly correlated with either activity against "atypical" multidrug resistant Jurkat cells (as measured by ratios of IC_{50} values) or with topoisomerase I stimulatory activity. Topoisomerase I and II activity has not yet been measured in Lewis lung cells, and although topoisomerase II activity is decreased in the resistant Jurkat cells, the contributions of topoisomerase IIα and β have not been distinguished, and topoisomerase I activity has not been quantitated. Further work is required to characterise these relationships.

In conclusion, it has been demonstrated here that appropriate substitution of the acridine chromophore can control the ability of a drug to stimulate DNA cleavage with either topoisomerase I or II, to bind selectivity to G–C rich DNA sequences, to overcome both "classical" [37] and "atypical" multidrug resistance, and to inhibit the growth of experimental solid tumours *in vivo*. Whereas there is no clear relationship between base preference of DNA binding and biological activity, the ability to stimulate topoisomerase I-dependent DNA cleavage appears to be related to overcoming drug resistance. Experiments are now being carried out to determine whether this result reflects an important role for topoisomerase I as an antitumour target of DACA and its derivatives.

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