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ORIGINAL ARTICLE

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Mechanism of cytotoxicity of *N*-[2-(dimethylamino)ethyl] acridine-4-carboxamide and of its 7-chloro derivative: the roles of topoisomerases I and II

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Abstract DACA {*N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide}, an acridine derivative that is highly active against solid tumours in mice, is currently in clinical trial. The ability of DACA to overcome "atypical" (topoisomerase II-mediated) multidrug resistance has been hypothesised to stem from its dual topoisomerase I/II specificity. We investigated the topoisomerase specificity of DACA and its 7-chloro derivative (Cl-DACA) using camptothecin and amsacrine as control compounds. In cell-free assays employing supercoiled plasmid DNA, Cl-DACA at 5 μ*M* induced topoisomerase I-mediated DNA breakage, indicating cleavable complex formation (poisoning), and at 10 μ*M* it inhibited relaxation of DNA, consistent with suppression (self-inhibition) of poisoning. In this assay, DACA provided no evidence of poisoning of this enzyme but inhibited its function at concentrations above 10 μ*M*. In DNA cleavage assays utilising purified topoisomerase II, DACA induced breakage of supercoiled plasmid DNA at 5 μ*M* whereas Cl-DACA showed very weak poisoning at 1 μ*M* and inhibition at 5 μ*M*. Under conditions required for the assay of DNA relaxation, Cl-DACA, but not DACA, inhibited topoisomerase II action at 5 μ*M*. The actions of DACA and Cl-DACA could also be distinguished by their ability to form DNA-protein cross-links in H460 human lung carcinoma cells as measured by precipitation of DNA-protein complexes with sodium dodecyl sulfate and potassium chloride. Both drugs stimulated the formation of complexes at low concentrations but inhibited formation at high concentrations. In survival assays with H460 cells, both drugs demonstrated biphasic responses with self-inhibition of cytotoxicity at intermediate drug concentrations. It was concluded that although both drugs have dual topoisomerase I/II specificity, DACA preferentially poisons topoisomerase II and Cl-DACA preferentially poisons topoisomerase I. In addition, drug-induced inhibition of topoisomerase action at higher drug concentrations may mask poisoning in the cell-free assays as well as masking cytotoxicity in cultured cells. A model in which drug binding occludes topoisomerase-binding sites on the DNA can explain this self-inhibition of cytotoxic action.

Key words Dual topoisomerase inhibitors · Acridine · Amsacrine · Camptothecin · Cell cycle

Introduction

DACA {*N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide; see Fig. 1 for structure}, a lipophilic DNA-intercalating acridine derivative developed in this laboratory [1], is notable for its ability to overcome in vitro resistance of several multidrug-resistant lines, including those that express low levels of the enzyme DNA topoisomerase II [6, 8, 16]. Experiments with purified enzymes and defined-sequence DNA have shown that DACA induces DNA breakage by both topoisomerase I and topoisomerase II, suggesting that it has a dual target of cytotoxic action in cells [9]. On the basis of these observations and of its high level of activity against experimental solid tumours, DACA has been tested clinically and has now completed phase I clinical trial.

An important question regarding DACA is whether topoisomerase I might be a significant target for its cytotoxicity. We took advantage of the observation that within the DACA series, topoisomerase specificity might be controlled by appropriate chemical modification. We used the 7-chloro derivative of DACA (Cl-DACA; Fig. 1), which binds DNA approximately 4 times more

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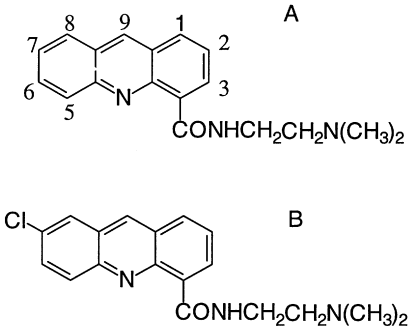

Fig. 1 Structures of DACA (**A**) and Cl-DACA (**B**)

strongly than does DACA [1] and appears in experiments with purified enzymes to be more active than DACA against topoisomerase I [9]. Cl-DACA also has the property, not shown by DACA, of exhibiting stronger activity against a cell line exhibiting topoisomerase II-mediated atypical multidrug resistance as compared with the parental line [9]. This property raises the question of whether it is possible to design drugs that are selectively toxic to multidrug-resistant cells.

In this study we utilised three methods, the formation of topoisomerase cleavable complexes in cell-free systems, the induction of protein-DNA cross-links and the shape of the cytotoxicity curves, for detection of topoisomerase I activity. In each case we compared Cl-DACA with DACA, camptothecin and amsacrine and found that the properties of Cl-DACA and DACA differed from those of camptothecin and amsacrine because of the presence of suppression of topoisomerase poisoning at higher drug concentrations (self-inhibition). We ascribe this to DNA binding and consider the theoretical basis and its implications for mode-of-action studies.

Materials and methods

Materials

DACA, Cl-DACA and amsacrine were synthesised in this laboratory. Camptothecin, penicillin and streptomycin were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). [^{3}H]-Thymidine (78μCi/mmol) was supplied by ICN Pharmaceuticals Inc., (Costa Mesa, USA), and topoisomerase I and II drug screening kits and purified human topoisomerase II were obtained from TopoGEN Inc. (Columbus, Ohio). Purified calf-thymus topoisomerase I was provided by Life Technologies (Gaithersburg, Md.) and biodegradable counting scintillant was obtained from Amersham Corp. (Arlington Heights, Ill., USA). Drugs were prepared as 2-m*M* stock solutions, with acridine derivatives being prepared in 50% (v/v) aqueous ethanol and camptothecin, in dimethyl sulfoxide. Drug solutions were stored at −20 °C.

Topoisomerase I and II relaxation and cleavable-complex formation assays

Topoisomerase I assays were carried out in a reaction (30 μl) containing 50 m*M* TRIS-HCl (pH 7.5), 50 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.1 m*M* ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA) at 30 μg/ml and 0.125 μg pBR322 supercoiled plasmid DNA together with calf-thymus topoisomerase I at 5 units for relaxation assays and at 15 units for cleavable-complex formation assays. Topoisomerase II assays were carried out in a reaction (20 μl) in relaxation and cleavage buffers supplied by the manufacturer (TopoGEN, Inc), pRYG supercoiled plasmid DNA (0.125 μg) and purified human topoisomerase II (10 units for cleavage assays and 4 units for relaxation assays). Reactions were assembled on ice, with drug and topoisomerase being added last, incubated at 37 °C for 30 min and terminated by the addition of pre-warmed sodium dodecyl sulfate (SDS; final concentration 1%) followed by proteinase K treatment (final concentration 50 μg/ml) for an additional 30 min. Relaxation assay samples were loaded onto 1% agarose gels in TAE buffer [40 m*M* TRIS acetate buffer (pH 8.0) containing 1 m*M* EDTA] and electrophoresed at 1.4 V/cm for 14–16 h. Samples from cleavable-complex formation assays were loaded onto 1% agarose gels. For resolution of nicked open circles and linear forms from closed circles, samples were electrophoresed in TAE buffer containing ethidium bromide (05 μg/ml) for topoisomerase II and in 4 × TAE buffer containing ethidium for topoisomerase I experiments. Samples were electrophoresed at 1.5 V/cm for 14–16 h. Amsacrine, DACA and Cl-DACA were diluted in sterile water before their addition to each reaction, whereas camptothecin was diluted in 10% (v/v) aqueous dimethyl sulfoxide.

Cells and culture medium

The H460 non-small-cell lung-carcinoma cell line was obtained from the National Cancer Institute (USA). The cells were cultured in α-modified minimal essential medium supplemented with 5% fetal calf serum (Gibco BRL-Life Technologies Ltd., New Zealand), penicillin (100 units/ml) and streptomycin (100 μg/ml) and were passaged weekly using trypsin (0.07%, w/v) in citrate-buffered saline.

Clonogenicity assays

H460 cells in the exponential phase of growth were exposed to increasing concentrations of cytotoxic agent for 1 h at 37 °C, washed free of drug and plated in triplicate (10^2 or 10^3 cells) in 60-mm dishes. After 10 days of incubation at 37 °C (in an atmosphere of 5% CO_2 in air), cultures were fixed and stained using methylene blue in 50%(v/v) aqueous ethanol. Colonies comprising 50 or more cells were counted [10]. To obtain cells in the exponential phase of growth, H460 cells were plated at 10^5 cells/ml in 100-mm dishes containing 15 ml of culture medium and were incubated for 20 h.

K^+-SDS precipitation assay for protein-DNA complexes

We used a modification of described methods [4, 23]. Cells from confluent cultures of H460 cells were plated into 100-mm dishes containing 15 ml of culture medium at 2×10^5 cells/ml. [^{3}H]-Thymidine (1 μCi/ml) was added and the cultures were incubated for 24 h for labeling of the DNA. Growth medium was removed, cells were washed in phosphate-buffered saline (PBS) and pre-warmed (37 °C) unlabeled medium was added. After 2 h of further incubation, cells were detached with trypsin and resuspended in culture medium at a concentration of 10^6 cells/ml, and 1 ml was added to sterile 1.5-ml microcentrifuge tubes. Cytotoxic drug was added and cells were incubated at 37 °C for 30 min. Following the indicated incubation periods, cells were centrifuged at 200 *g* for 5 min and placed in a heating block at 37 °C. Culture medium was removed by aspiration and the cells were lysed immediately by the addition of 0.5 ml of pre-warmed (37 °C) 2% SDS, 5 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 20 m*M* TRIS-HCl (pH 7.5) followed by vigorous vortexing for 10 s. Lysed samples were then frozen at −20 °C for up to 5 days before KCl precipitation.

After thawing, samples were vortexed at the highest setting for 10 s and then heated for 10 min at 65 °C, and 0.5 ml of a solution of 200 m*M* KCl containing 20 m*M* Tris-HCl (pH 7.5) was added. DNA was sheared by passage of the mixture through a disposable pipette tip (1 ml) five times. Samples (1 ml) were cooled in ice to promote the formation of K^+/SDS precipitates, which were then collected by centrifugation at 3000 *g* for 5 min at 4 °C. The pellet was resuspended in 1 ml of 100 m*M* KCl/20m*M* TRIS-HCl (pH 7.5) by passage five times through a pipette tip. The samples were again heated for 10 min at 65 °C, cooled on ice and centrifuged at 3000 *g* for 5 min at 4 °C. The washing step was repeated once, and the final pellet was solubilised, resuspended in 0.5 ml of water at 65 °C, transferred to scintillation vials containing aqueous accepting scintillant (3 ml) and counted in a liquid scintillation counter. Total cell labeling was ascertained by the resuspension of 10^6 labeled non-drug-treated cells in water (65 °C) and the addition of 3 ml of scintillation fluid.

DNA-binding model

We assume that the topoisomerase (*T*) binds to free DNA sites (*N*) to form complexes (*TN*). A proportion of these can, in turn, form cleavable complexes (*TN**) in which the DNA is cleaved. The relative concentrations of *T*, *TN* and *TN** are provided by the binding constants K_1 and K_2, respectively:

$$K_1 = \frac{[TN]}{[N][T]} \ ,$$

$$K_2 = \frac{[TN^*]}{[TN]} \ .$$

The concentration of cleavable complexes, [*TN**], is then given by the equation:

$$[TN^*] = K_1 K_2 [N][T] \ .$$

Following drug addition, the fraction of DNA-binding sites available to the topoisomerase (*F*) remaining can be estimated by the "nearest neighbour exclusion" equation [13], where *r* is the drug-binding ratio and *n* is the size of the topoisomerase-binding site in nucleotide pairs:

$$F = \frac{(1 - nr)^n}{1 - (n-1)r^{(n-1)}} \ .$$

The addition of drug, by stabilising cleavable complexes, will increase the ratio of [TN*] to [TN] as a function of the drug-binding ratio (*r*). However, it will also decrease the fraction of free topoisomerase-binding sites as a function of the ratio F.

Results

Poisoning and inhibition of topoisomerases I and II in cell-free systems

We first compared the ability of Cl-DACA, DACA and comptothecin to poison topoisomerase I. Ethidium bromide was added to the running buffer and gel for separation of nicked open circles from closed circles. As shown in Fig. 2, camptothecin (100 μ*M*) strongly induced the formation of the nicked open circular form, indicating the formation of cleavable complexes. Cl-DACA also strongly induced formation of the nicked open circular form at 5 μ*M* but did not do so at 10 μ*M*, indicative of inhibition of enzyme activity at the higher concentration. Control experiments showed that drug in the absence of topoisomerase I did not induce formation of the nicked open circular form or otherwise change the electrophoretic mobility of the plasmid DNA. Despite repeated attempts, DACA showed no sign of inducing the formation of the open circular form at the concentrations tested.

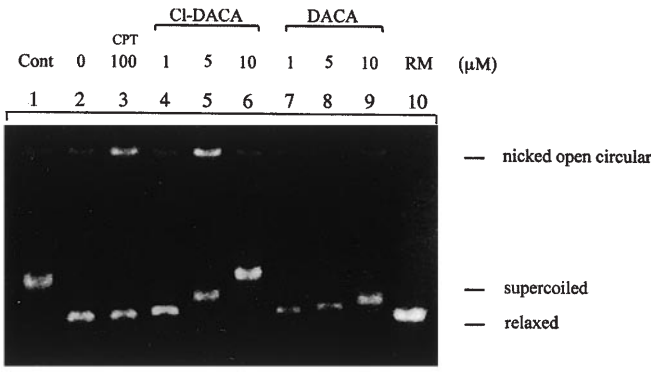

Fig. 2 Topoisomerase I cleavable-complex formation assays performed under conditions favouring DNA cleavage. Gel electrophoresis of supercoiled plasmid pBR322 DNA with no addition (*lane 1*); reacted with topoisomerase I (15 units) in the absence of drugs (*lane 2*); topoisomerase I plus camptothecin at 100 μ*M* (*lane 3*); topoisomerase I plus Cl-DACA at 1 μ*M* (*lane 4*), 5 μ*M* (*lane 5*), and 10 μ*M* (*lane 6*); topoisomerase I plus DACA at 1 μ*M* (*lane 7*), 5 μ*M* ((*lane 8*), and 10 μ*M* (*lane 9*); relaxed marker pBR322 DNA (*lane 10*). Ethidium bromide (0.5 μg/ml) was added to the running buffer and gel for resolution of nicked open circles from closed circles. Supercoiled and relaxed DNA run at the *bottom* of the gel, whereas nicked open circles, which represent the formation of topoisomerase I cleavable complexes, run at the *top*

We next investigated drug-induced inhibition of relaxation of closed circular duplex DNA by purified topoisomerase I. Camptothecin (20 μ*M*) prevented full plasmid relaxation and Cl-DACA at 5 and 10 μ*M* caused a dose-dependent inhibition of topoisomerase I-mediated relaxation (Fig. 3). DACA at concentrations of 5 and 10 μ*M* caused weak inhibition of relaxation as indicated by the absence of a band corresponding to supercoiled DNA. At 10 μ*M* a change in the distribution of the relaxed topoisomers was evident, probably as a result of DNA intercalation by DACA and of a consequent change in the linking number of the topoisomers. Amsacrine (10 μ*M*) had no effect.

We also measured the ability of the drugs to poison topoisomerase II. Amsacrine was a strong inducer of linear DNA formation at 10 μ*M*, indicating induction of cleavable complex formation (Fig. 4). DACA induced the formation of linear DNA at concentrations of 5 μ*M* and above, whereas Cl-DACA did so weakly at 1 μ*M* and even more weakly at 5 μ*M*, consistent with inhibition of topoisomerase II action at this concentration (Fig. 4). Under assay conditions for DNA relaxation, Cl-DACA inhibited topoisomerase II action at 5 μ*M* (data not shown). Thus, DACA was a more potent poison of topoisomerase II than was Cl-DACA, although it was less potent than amsacrine.

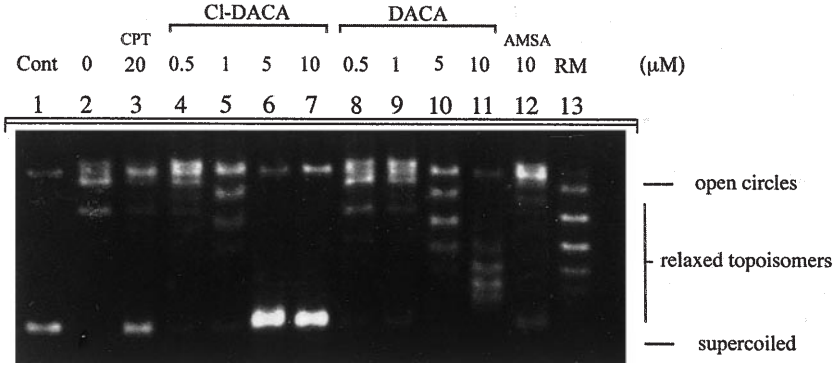

Fig. 3 topoisomerase I cleavable-complex formation assays performed under conditions favouring DNA relaxation. Gel electrophoresis of supercoiled plasmid pBR322 DNA with no addition (*lane 1*); reacted with topoisomerase I (5 units) in the absence of drugs (*lane 2*); topoisomerase I plus camptothecin at 20 μ*M* (*lane 3*); topoisomerase I plus Cl-DACA at 0.5 μ*M* (*lane 4*), 1 μ*M* (*lane 5*), 5 μ*M*) (*lane 6*), and 10 μ*M* (*lane 7*); topoisomerase I plus DACA at 0.5 μ*M* (*lane 8*), 1 μ*M* (*lane 9*), 5 μ*M* (*lane 10*), and 10 μ*M* (*lane 11*); relaxed marker pBR322 DNA (*lane 12*). supercoiled DNA runs at the *bottom* of the gel, relaxed circles run at the *top*, and relaxed topoisomers run at *intermediate positions*

Determination of protein-DNA cross-linking in response to drug treatment

We next examined possible changes in protein-DNA cross-linking following drug exposure. Exponential-phase H460 cells were pre-labeled with [^{3}H]-thymidine, exposed to drug for 30 min and then lysed in SDS. Protein-DNA complexes were precipitated by the addition of KCl and their radioactivity was quantitated. As expected, amsacrine and camptothecin stimulated the formation of protein-DNA complexes in a concentration-dependent manner (Fig. 5). In contrast, DACA exhibited a biphasic effect. At low drug concentrations the formation of protein-DNA complexes was stimulated, whereas at intermediate drug concentrations it was inhibited, consistent with the findings of other investigators [16]. Cl-DACA produced a similar effect, although the amount of complexes formed was slightly lower. Surprisingly, both DACA and Cl-DACA continued to reverse the formation of protein-DNA complexes at higher concentrations, reproducibly reducing the radioactivity associated with the precipitated protein relative to control cells at concentrations of 10 μ*M* and above (Fig. 5). Cl-DACA was the more potent inhibitor of protein-DNA complexes, with 50% inhibition occurring at 20 μ*M* versus 25 μ*M* for DACA.

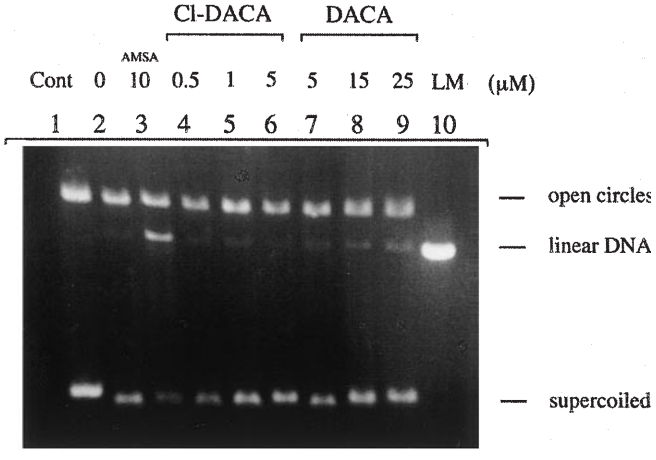

Fig. 4 Topoisomerase II cleavable-complex formation assays. Gel electrophoresis of supercoiled plasmid pRYG DNA with no addition (*lane 1*); reacted with topoisomerase II (10 units) in the absence of drugs (*lane 2*); topoisomerase II plus amsacrine at 10 μ*M* (*lane 3*); topoisomerase II plus Cl-DACA at 0.5 μ*M* (*lane 4*), 1 μ*M* (*lane 5*), and 5 μ*M* (*lane 6*); topoisomerase II plus DACA at 5 μ*M* (*lane 7*), 15 μ*M* (*lane 8*), and 25 μ*M* (*lane 9*); linear marker pRYG DNA (*lane 10*). Ethidium bromide (0.5 μg/ml) was added to the running buffer and gel for resolution of nicked open circles from closed circles. Supercoiled DNA runs at the *bottom* of the gel, open circles run at the *top*, and linear DNA, representing the formation of topoisomerase II cleavable complexes, runs just *below the open circles*

Clonogenic survival curves of Cl-DACA and DACA

One feature of camptothecin is its S-phase selectivity, meaning that survival curves generated for cells exposed for a short period have a highly resistant fraction [5]. We compared the survival curves created for H460 cells exposed for 1 h to Cl-DACA, camptothecin, DACA and amsacrine as shown in Fig. 6. Both Cl-DACA and DACA showed biphasic shapes, with self-inhibition of cytotoxicity occurring at intermediate drug concentrations. The result recorded for DACA was similar to that reported for the murine Lewis lung carcinoma line [10].

An interesting distinction between DACA and Cl-DACA was the inverse order and magnitude of the two phases of the biphasic response. At a low concentration (2 μ*M*), DACA was more effective, causing a maximal cytotoxicity of 90% in comparison with 40% for Cl-DACA. At a higher concentration, Cl-DACA was more effective, causing 99% cell death at 16 μ*M* in comparison with 24 μ*M* for DACA. As both DACA and Cl-DACA are strongly lipophilic drugs, we examined the

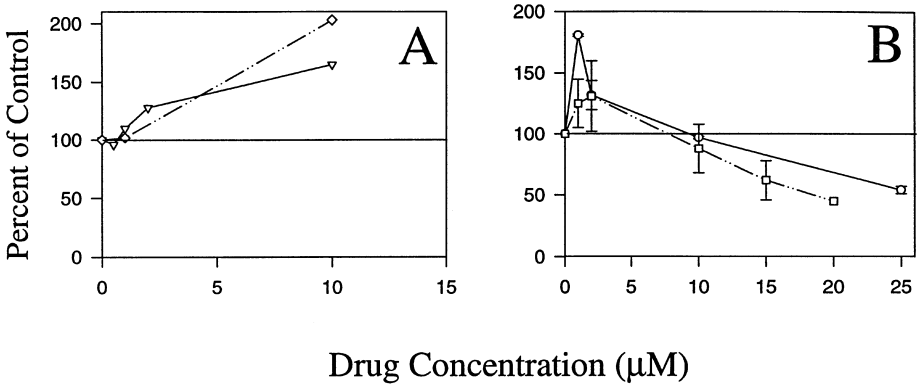

Fig. 5A,B K^+-SDS determination of DNA-protein complexes formed in H460 cells following a 30-min period of exposure to drug. **A** Complexes formed following exposure to amsacrine (▽) and camptothecin (◇). The graph has been redrawn from previously published data [3] to provide comparison. **B** Complexes formed following exposure to Cl-DACA (□) and DACA (○). Data represent mean values (±SEM) plotted for combined duplicate experiments for DACA and combined triplicate experiments for Cl-DACA

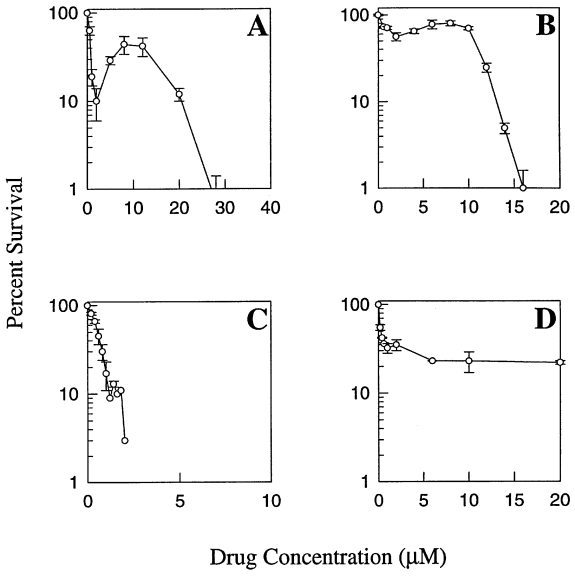

Fig. 6A–D Survival curves generated for H460 cells following a 1-h period of exposure to **A** DACA, **B** Cl-DACA, **C** amsacrine and **D** camptothecin. To facilitate potting, plates that did not contain colonies were arbitrarily scored at 0.001% survival

possibility that killing at high drug concentrations might be due to detergent-like effects acting upon cell membranes, causing membrane collapse and cell death. We measured the ability of drug-treated cells to exclude trypan blue following a 1-h period of exposure to these high concentrations and found that at concentrations of up to 30 μ*M*, >95% of drug-treated cells continued to exclude dye.

Discussion

Topoisomerase-directed DNA-binding agents are termed poisons of topoisomerase enzymes when they stabilise cleavable complexes in which the enzyme is covalently joined to the DNA [20]. DNA-binding agents can also inhibit DNA cleavage by topoisomerases, for instance by preventing binding of the enzyme to DNA. DNA binding can reverse the cytotoxic activity of a poison, as has been shown for topoisomerase II with ethidium [15], 9-aminoacridine [7] and aclarubicin [12] as well as for topoisomerase I with aclarubicin [17]. Analysis of the cytotoxic properties of topoisomerase-directed DNA-binding drugs therefore requires investigation of activity both as a poison and as an inhibitor. This analysis is complex in the case of DACA, which is thought to exhibit dual topoisomerase I/II activity. We used a variety of techniques to dissect this complex action and separated the actions on topoisomerases I and II by supplementary studies with Cl-DACA, a DACA derivative with reduced topoisomerase II activity.

The question of the involvement of topoisomerase I in the cytotoxicity of Cl-DACA and DACA might be resolved with the use of a mutant lacking topoisomerase I activity. However, two camptothecin-resistant mutants, P388/CPT_5 and DC3/C-10, are sensitive to DACA [9]. Most camptothecin-resistant mammalian cell lines contain mutations that change the camptothecin-binding site but preserve topoisomerase activity [19], and a line with reduced topoisomerase I activity has compensatorily higher topoisomerase II activity [22], making the analysis of drugs with dual topoisomerase I/II activity difficult. To our knowledge there is no report of a mammalian cell line completely lacking the topoisomerase I gene.

In experiments with closed circular duplex DNA and purified enzymes, Cl-DACA (0.5–5 μ*M*) is more effective than DACA as a poison of topoisomerase I (Fig. 3), whereas DACA is more effective than Cl-DACA as a poison of topoisomerase II (Fig. 4). These results are consistent with previous studies measuring the topoisomerase-mediated induction of DNA breaks in defined-sequence DNA [9]. However, in contrast to results obtained with camptothecin and amsacrine, we show herein that Cl-DACA and DACA also inhibit the catalytic action of topoisomerases, with Cl-DACA being a more potent inhibitor than DACA. Thus, cleavable complexes, in which the topoisomerase is covalently linked to the DNA, are not seen at high drug concentrations because of this inhibition.

The results of K^+/SDS precipitation assays for drug-induced protein-DNA cross-links in intact cells agree with the results of the cell-free assays. Although both Cl-DACA and DACA increase the formation of

DNA-protein cross-links at low drug concentrations as compared with that occurring in control cells, they decrease the formation of cross-links at higher concentrations (Fig. 5). This behaviour contrasts strongly with that of topoisomerase poisons such as camptothecin and amsacrine. Inhibition of DNA-protein cross-links has previously been observed for the anthracycline drug aclarubicin, and it has been suggested that a proportion of cellular topoisomerase(s) exists natural in a DNA cross-linked form and that addition of the appropriate drug displaces topoisomerase from this form [3].

The biphasic shapes of the survival curves generated for cells exposed for a short period (1 h) to Cl-DACA and DACA (Fig. 6) are unusual and differ from those arising from most other topoisomerase poisons [11]. DACA is strongly cytotoxic at low concentrations (1–5 μM), probably due to topoisomerase II poisoning, since it is reduced in cells with low levels of topoisomerase II activity (unpublished results). The self-inhibition portion of the survival curves resembles the inhibition of DACA cytotoxicity by 9-aminoacridine [7]. Cl-DACA shows low-level but reproducible cytotoxicity at low concentrations (1–5 μM), possibly because of poisoning of topoisomerase I, together with evidence of self-inhibition at higher concentration. The self-inhibition of cytotoxicity of DACA and Cl-DACA may reflect the inhibition of cleavable complex formation observed at higher drug concentrations in the cell-free assays. The cytotoxicity seen for both drugs at even higher drug concentrations (15 and 25 μM) is similar to that observed for aclarubicin [3] and may be a consequence of simultaneous inhibition of topoisomerases I and II. However, it could also involve inhibition of the function of other DNA-associated proteins. Such cytotoxicity is not related directly to topoisomerase II content since it is observed in plateau-phase Lewis lung carcinoma cells that express low levels of topoisomerase IIα as well as in multidrug-resistant cells with reduced topoisomerase II expression (data not shown). Suppression of topoisomerase activity might itself lead to cell death, and further studies are in progress to elucidate the contribution of this process to cytotoxicity.

Self-inhibition of cytotoxicity can be modeled by simple binding equations (see Materials and methods) in which DACA or Cl-DACA bind either to the DNA (thereby decreasing the concentration of available topoisomerase-binding sites) or to the DNA-topoisomerase complex (thereby stabilising cleavable complexes and increasing their concentration). The binding sites for topoisomerase I and II are likely to involve 15–19 contiguous nucleotide pairs [18] and 30 contiguous nucleotide pairs [2], respectively. However, the binding of the enzyme to DNA probably involves the initial recognition of a small segment of DNA (e.g. 5 bp) followed by conformational changes to allow binding to the full site. Assuming a site size of 2 bp for acridine binding [21] and a topoisomerase-initial-binding site size of 5 bp, the concentration of cleavable complexes can be calculated in terms of the added drug concentration. This is shown in Fig. 7, and curves of similar shape can be calculated for other, larger topoisomerase-binding site sizes. If the formation of cleavable complexes is associated with killing, the form of the expression in Fig. 7 can be inverted and used to describe the self-inhibition of cytotoxicity seen in cells exposed to DACA and Cl-DACA in 1-h cytotoxicity assays (Fig. 6).

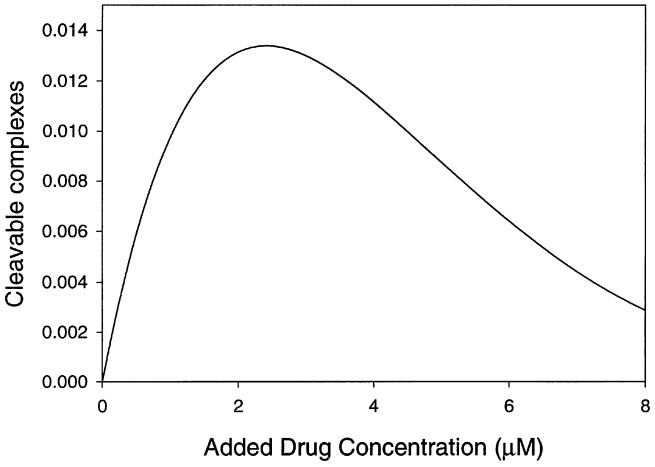

Fig. 7 Relationship between the concentration of DNA-associated topoisomerase in the cleavable complex form (TN*) and the added drug concentration as based on the binding model described in Materials and methods. The values for the drug association constant and the intracellular concentration of nucleotides were set at $5 \times 10^4\ M^{-1}$ and 50 μM, respectively. The binding-site sizes for initial topoisomerase binding and for acridine binding were assumed to be 5 and 2 bp, respectively

In conclusion, the results obtained with Cl-DACA indicate that small changes in structure in the DACA series can alter the balance of activity not only towards topoisomerase I and II but also between enzyme poisoning and enzyme inhibition. They also show that topoisomerase-poisoning activity can be masked by inhibition of cleavable complex formation (Fig. 7). The anthracycline derivative aclarubicin, which has recently been shown to act on topoisomerase I as a poison [14] and as an inhibitor [3], may provide another example of this phenomenon. Thus, a drug concentration that induces only a small proportion of cleavable complexes (e.g. 5%) may be difficult to detect using cell-free topoisomerase analysis, which measures steady-state concentrations. However, the continuous induction of such a small proportion of cleavable complexes over an extended period might enable the induction of a large number of DNA lesions and a correspondingly large cytotoxic effect.

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