Biochemical Pharmacology, Vol. 56, pp. 351–359, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00030-6

Cytotoxic and DNA-Damaging Properties of *N*-[2-(Dimethylamino)ethyl]acridine-4-Carboxamide (DACA) and Its Analogues

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ABSTRACT. An antitumor drug *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA) and its three close structural analogs *N*-[2-(hydroxyethylamino)ethyl]acridine-4-carboxamide (DACAH), *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide (amino-DACA), and *N*-[2-(hydroxyethylamino)ethyl]-9-aminoacridine-4-carboxamide (amino-DACAH) were studied for their ability to inhibit RNA synthesis *in vitro* and to form topoisomerase II-mediated DNA lesions in relation to cell-killing activity. All tested compounds induced chromatin lesions characteristic of topoisomerase II-blocking drugs (DNA breaks and DNA-protein cross-links) in treated cells, but were much less active than reference antileukemic acridine *m*-AMSA (4′-(9-acridinylamino)-methanesulfon-*m*-anisidide). The ability to form these lesions was dependent on the structure of the 4-carboxamide side-chain, which seems to be an important factor affecting the drug transport rate through cell membrane. A 4-carboxamide chain with an *N*-2-(dimethylamino)ethyl moiety resulted in more efficient transport through cell membranes, higher cytotoxicity, and DNA-damaging activity. The mode of action of acridine-4-carboxamides was further elucidated by their incubation with cells in the presence of antitopoisomerase II agents of a known mechanism of inhibition. These were: bisdioxopiperazine (ICRF-187), a catalytic inhibitor of topoisomerase II, and etoposide (VP-16), an inducer of a cleavable complex of the enzyme with DNA. The cytotoxicity of DACA and its analogs was not antagonized by preincubating cells with ICRF-187. All tested acridines protected cells against DNA breakage induced by VP-16, but the extent of protection varied significantly. Amino-DACA, which easily penetrates cell membrane, fully inhibited DNA break formation, whereas other analogs exhibited a low degree of protection when used at high concentration. Our results suggest that the acridine-4-carboxamides discussed here are poor topoisomerase II poisons and that this enzyme is not their main target. BIOCHEM PHARMACOL **56**;3:351–359, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. antitumor acridines; ICRF-187; topoisomerase II inhibitors

Acridine derivatives are known to have a broad spectrum of biologic activity which seems to be related to their interaction with DNA. A number of acridines have been tested for their antitumor properties and some have entered clinical trials [1, 2]. Acridine-4-carboxamides are of interest as a new generation of intercalators which are active against solid tumors and leukemias [3, 4]. Some members of this group were studied for their capacity to bind to DNA [5, 6], for their DNA-damaging activity in whole cells and isolated nuclei [7], and for their inhibitory effect on RNA synthesis in a cell-free system [8].

In this study we focused on DACA§ and amino-DACA, as these compounds are most promising among acridine-4-carboxamides, with DACA currently in phase I clinical trials. Despite the fact that these acridines have similar structures, they exhibited a significant difference in the association constants of the DNA-drug complexes [9] and different antitumor specificities, as well as varied structure-activity relationships [4]. The aim of this work was to compare the cytotoxic and DNA-damaging properties of DACA and amino-DACA in cultured L1210 cells. As amsacrine, the most prominent member of the acridine anticancer drugs, is believed to exert its antileukemic effect

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Received 16 July 1997; accepted 2 January 1998.

§ *Abbreviations:* amino-DACA, *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide; amino-DACAH, *N*-[2-(hydroxyethyl]amino)ethyl]-9-aminoacridine-4-carboxamide; *cis*-DDP, *cis*-diamminedichloroplatinum(II); DACA, *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide; DACAH, *N*-[2-(hydroxyethylamino)ethyl]acridine-4-carboxamide; ICRF-187, bisdioxopiperazine; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; and VP-16, etoposide.

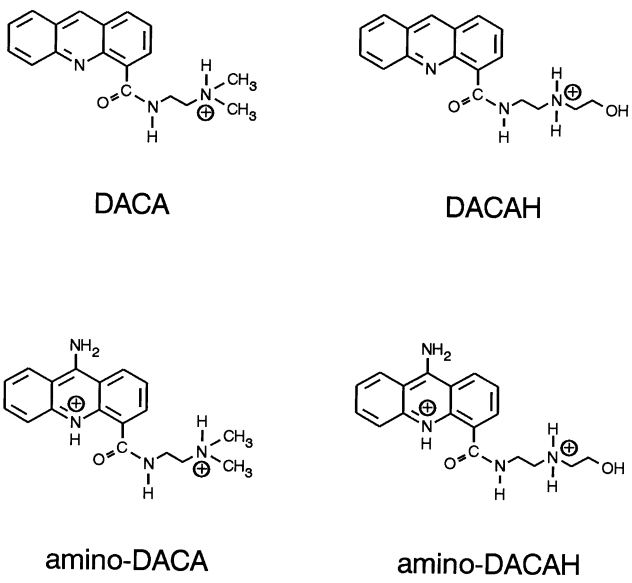

FIG. 1. Structures of acridines tested in this study.

by topoisomerase II blockage [10], we were mainly interested in DNA lesions characteristic of topoisomerase II-trapping compounds. Wakelin and Denny [11] proposed a model of the acridine-4-carboxamide-DNA complexes suggesting intercalation of acridine ring between base pairs with the 4-carboxamide moiety located in the minor groove. The structure of the substituent at position 4 seems to be the important factor in the stabilization of the drug-DNA complex as well as in the GC specificity of these acridines [6]. In order to verify whether changes in the 4-carboxamide residue may influence the cytotoxicity and genotoxic activity of acridine-4-carboxamides, two analogs of DACA and amino-DACA with a modified side-chain were also included in this study (see Fig. 1 for structures).

As shown by Jensen and coworkers [12–14], topoisomerase II targeting drugs can interfere with the enzyme catalytic cycle by inhibiting any one of four different steps of the DNA relaxation reaction. Because the mechanism of action of some topoisomerase inhibitors is relatively well understood (e.g. VP-16, *m*-AMSA, and ICRF-187), it is possible to design experiments in which these drugs, acting on different steps of the topoisomerase II-catalyzed reaction, exhibit antagonistic effects [12–14]. This kind of methodological approach was also used in the current work. By coincubating ICRF-187 or VP-16 with acridine-4-carboxamides, we attempted to explain whether DACA and its analogs trap topoisomerase II at the cleavable complex step or whether they act as catalytic inhibitors.

DNA-interacting compounds affect the function of enzymes which catalyze different DNA-dependent reactions, and studies in cell-free systems are useful to study drug-DNA interactions and/or drug-protein interactions [15]. In some cases, correlation has been shown between inhibition of RNA synthesis *in vitro* and cytotoxicity [5, 16]. Because DACA and amino-DACA differ in their DNA-binding parameters [5, 9], it was of interest to investigate whether these compounds exhibit different effects on DNA-dependent RNA synthesis and whether a correlation exists between the inhibitory effect and cytotoxicity. To answer these questions, we measured the influence of tested acridine-4-carboxamide compounds on overall RNA synthesis in a system containing phage T7 DNA and *Escherichia coli* DNA-dependent RNA polymerase.

MATERIAL AND METHODS

Drugs

The anticancer acridines DACA, DACAH, amino-DACA, amino-DACAH and *m*-AMSA, the latter used as reference compound, were synthesized as described earlier [3, 17]. VP-16 and ICRF-187 were gifts from Dr. A. Składanowski.

Cells and Cytotoxicity Assay

Mouse leukemia L1210 cells were cultured in RPMI 1630 medium (Sigma) supplemented with 10% fetal calf serum (Life Technologies), gentamycin (50 μg/mL) and 0.02 M HEPES buffer (Life Technologies). Cytotoxic effects were assayed by measuring inhibitory effects on L1210 cell proliferation. In this assay, cells were seeded in 3-mL aliquots in 6-mL tissue culture tubes (Corning) at a concentration of 2×10^4 cells/mL and exposed to drugs for 1 hr at 37°. After 72 hr of incubation in fresh growth medium, the cell number relative to control was determined by a tetrazolium dye method [18].

DNA Damage Measurements

The presence of DNA strand breaks and interstrand cross-links was assayed by means of alkaline elution [19]. L1210 cultures at a density of 2×10^5 cells/mL were incubated with 0.02 μCi/mL of [^{14}C]thymidine (Chemapol) for 24 hr and then treated with drugs for 1 hr at 37°. Suspensions were centrifuged, pellets were washed with cold PBS, and alkaline elution was performed essentially as described [19], except that the pumping rate was 0.1 mL/min and tetrapropylammonium hydroxide in the elution buffer was replaced by tetraethylammonium (Sigma). Lysates from cells treated with DACA, DACAH and amino-DACAH were deproteinized by 30-min digestion at 20° with 0.5 mg/mL of proteinase K (Sigma) dissolved in 0.01 M EDTA pH 10. For measurement of DNA strand breaks induced by amino-DACA, the time of digestion was prolonged to 2 hr and the concentration of proteinase K was 2 mg/mL. In the interstrand cross-link assay, the control and drug-treated cells were irradiated on ice with a dose of 3 Gy of γ radiation. To measure DNA-protein cross-links, the procedure of Strniste and Rall [20], slightly modified as described previously [21], was applied.

Uptake of Acridine-4-Carboxamides

To study the transport of amino-DACA and amino-DACAH through cell membranes, the procedure of Sullivan *et al.* was used [22], except that drug concentrations were estimated by fluorometric measurements instead of radioactivity assays. L1210 cells were concentrated to 10^7 cells/mL and treated in growth medium with 10 μM drugs at 37°. The aliquots of cell suspension (0.2 mL) were mixed with 5 mL of ice-cold PBS and centrifuged at 1500 *g* for 5 min, then washed twice with ice-cold PBS. Final cell pellets were resuspended in 2.2 mL of PBS. The fluorescence intensity of these suspensions was measured at 20° by a Perkin-Elmer LS 50 B spectrofluorimeter. Optimal excitation and emission wavelengths were 260 and 454 nm, respectively. To estimate the drug concentrations, calibration curves in the concentration range 0.01–0.4 μM were prepared. Because the fluorescence was slightly quenched in the presence of cells, each point of the calibration curves was measured in the presence of 2×10^6 cells. The low fluorescence of DACA and DACAH over the entire range of excitation and emission wavelengths did not allow transport studies to be performed for these acridines.

Inhibition of RNA Synthesis

The effects of acridine-4-carboxamides on overall RNA synthesis were assayed as described previously [23], with *E. coli* DNA-dependent RNA polymerase except that the incubation time was 10 min. Drug concentrations decreasing RNA synthesis to 50% of control (IC_{50}) were read from the inhibition curves.

RESULTS

Cytotoxicity and Inhibitory Effect on RNA Synthesis

Acridine-4-carboxamides inhibited the proliferation of L1210 cells. Their cytotoxic potencies expressed as ED_{50} values are shown in Table 1. Amino-DACA and amino-DACAH, both possessing an amino group in position 9 of the chromophore ring, were significantly more cytotoxic than their des-amino analogs DACA and DACAH. When the cytotoxic activities of amino-DACA and DACA were compared with those of amino-DACAH and DACAH, it was seen that replacement of the *N*-2-(dimethylamino)ethyl moiety by *N*-2-(hydroxylamino)ethyl decreased the cytotoxic potency. This effect was especially striking in the pair of analogs amino-DACA and amino-DACAH where ED_{50} values differed by a factor of 60. Among tested acridine-4-carboxamides, only amino-DACA was more toxic toward L1210 cells than the reference acridine *m*-AMSA.

Amino-DACA and amino-DACAH were more potent inhibitors of RNA synthesis than their *des*-amino analogs (Table 1). All tested carboxamides inhibited overall RNA synthesis to a greater extent than *m*-AMSA. The comparison of cytotoxicity and inhibitory effects exhibited in the cell-free RNA synthesis system showed that there was no correlation between these properties.

TABLE 1. The cytotoxic activity of acridine-4-carboxamide derivatives and their effect on the overall RNA synthesis *in vitro*

Compound	Cytotoxic activity ED_{50}(μM)	Inhibition of RNA synthesis IC_{50}(μM)
m-AMSA	0.32 ± 0.04	124.0 ± 17.0
DACA	22.1 ± 3.7	35.8 ± 3.2
DACAH	44.0 ± 6.1	20.2 ± 5.8
amino-DACA	0.24 ± 0.05	13.6 ± 1.4
amino-DACAH	14.5 ± 2.7	9.1 ± 0.1

ED_{50}, the acridine concentration effective in inhibiting 50% of the cell growth after 1-hr exposure of L1210 cells to the drug. IC_{50}, drug concentration inhibiting RNA synthesis *in vitro* to 50% of control value. Data are means of 3–4 independent experiments ± SD.

Effect of ICRF-187 on Cytotoxicity of Acridine-4-Carboxamides

To investigate the effect of ICRF-187, a catalytic inhibitor of topoisomerase II on the cytotoxicity of tested compounds, L1210 cells were incubated with 100 μM ICRF-187 for 15 min prior to a 60-min treatment with acridines at concentrations equal to ED_{50}. As seen from Fig. 2, ICRF-187 did not significantly change the cytotoxic properties of DACA and its analogs. The cytotoxic effect of VP-16, a classic topoisomerase II poison, was considerably reduced. The experiments were repeated with 200 μM ICRF-187 and the results were very similar (data not shown).

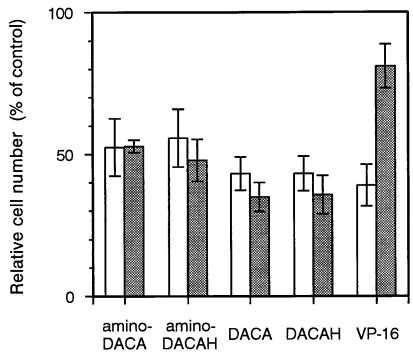

FIG. 2. Effect of ICRF-187 on the cytotoxicity of acridine-4-carboxamides and VP-16. L1210 cells were incubated for 15 min in growth medium at 37° with 100 μM ICRF-187 followed by treatment for 60 min with acridines or VP-16 at doses equal to ED_{50}. The relative cell number was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described in Materials and Methods. In the presence of ICRF-187 alone, the rate of cell proliferation was slightly reduced to 89–92% of the control. (□) cells treated without ICRF-187, (▨) cells treated in the presence of ICRF-187. Data are means ± SD of three experiments.

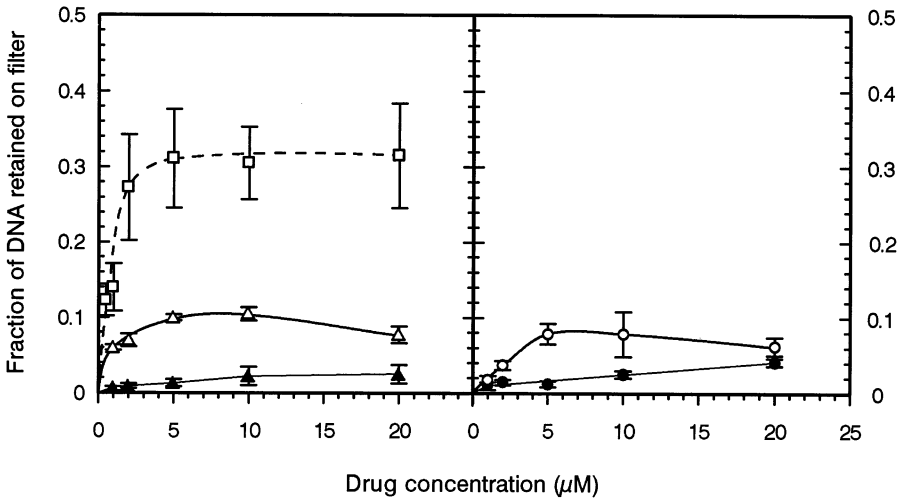

FIG. 3. DNA-protein cross-links induced in L1210 cells by acridine-4-carboxamides. Cells were treated with drugs for 1 hr at 37° and DNA-protein cross-links were measured by the nitrocellulose filter binding assay. (□) *m*-AMSA, (△) DACA, (▲) DACAH, (○) amino-DACA, (●) amino-DACAH. The points are means ± SD of four independent experiments.

DNA-Protein Cross-linking

To assess DNA-protein cross-links caused by acridine-4-carboxamides, we used a filter binding assay based on the adsorption of DNA-protein complexes on nitrocellulose [20]. In this procedure, the fraction of radioactive DNA retained on the filter corresponds to the cross-link frequency. Dependence of DNA-protein cross-link formation on acridine concentration after 1-hr exposure is shown in Fig. 3. A large difference was observed in the ability to induce DNA-protein cross-links between acridine-4-carboxamides and *m*-AMSA. Maximal levels of these lesions, expressed as a fraction of DNA bound to proteins, reached 0.3 for *m*-AMSA and was approximately 4–15 times higher than that measured for other acridines. Frequency of DNA-protein cross-links formed by acridine-4-carboxamides showed different dependence on the drug concentration than *m*-AMSA. Whereas *m*-AMSA induced cross-linking saturated above 2 μM, DNA-protein cross-links produced by DACA and amino-DACA peaked between 5 and 10 μM and then decreased. Analogs with a modified 4-side-chain (DACAH and amino-DACAH) exhibited very low DNA-protein cross-linking activity, inducing barely detectable amounts of DNA-protein cross-links (Fig. 3).

DNA Breakage

DNA topoisomerase II-trapping drugs are known to produce DNA breaks. In this study, the ability of acridine-4-carboxamides to induce DNA strand breaks was assayed by the DNA alkaline elution technique. As shown in Fig. 4, DNA-breaking activities of acridine-4-carboxamides varied significantly. DACA and DACAH produced DNA breaks in a concentration-dependent manner. Contrary to the curve obtained for *m*-AMSA, which reached plateau between 5 and 20 μM, DACA- and DACAH-induced DNA strand breaks tended to increase up to the highest concentration tested. A different DNA breakage dependence on drug concentration was observed in the case of the most cytotoxic acridine, amino-DACA. This drug produced a bell-shaped concentration dependence curve reaching a maximal level of breaks in the range of 2–10 μM. When

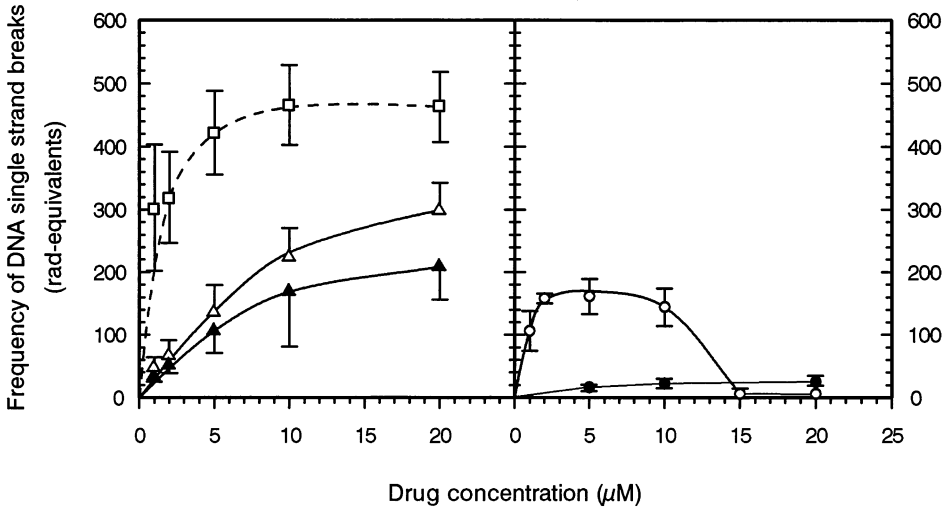

FIG. 4. DNA strand breaks induced in L1210 cells by acridine-4-carboxamides. Cells were exposed to acridines for 1 hr at 37° and subjected to the alkaline elution procedure. (□) *m*-AMSA, (△) DACA, (▲) DACAH, (○) amino-DACA, (●) amino-DACAH. The points are means ± SD of four independent experiments.

concentrations of amino-DACA exceeded 10 μM, DNA breaks were not detected.

During experiments with amino-DACA, it was found that this acridine induced DNA-protein cross-links which exhibited an unusual resistance to proteinase K digestion. Topoisomerase II inhibitors are known to simultaneously produce DNA strand breaks and DNA-protein cross-links having approximately the same frequencies [24, 25]. Therefore, to estimate DNA strand breaks induced by topoisomerase inhibitors, cell lysates are deproteinized to avoid DNA breaks masked by DNA-protein cross-links. The standard procedure used in DNA alkaline elution assays involves the proteinase K digestion of DNA-protein complexes adsorbed on filters [19]. In our experiments, incubation of DNA for 30 min on filters at 20° with 0.5 mg/mL of proteinase K dissolved in lysis solution (2% SDS, 0.01 M EDTA pH 10) was sufficient to detect DNA breaks produced by all tested acridines except amino-DACA. DNA breaks induced by this drug appeared after 2-hr digestion with proteinase K solution at an increased concentration of 2 mg/mL. No change in DNA break frequencies was observed when DNA from cells treated with other tested acridines was subjected to the longer digestion (data not shown).

Antagonistic Effects of Acridine-4-Carboxamides on VP-16-Induced DNA Breakage

The frequency of DNA strand breaks induced in L1210 cells simultaneously treated with VP-16 and acridines was estimated by alkaline elution (Fig. 5). Among tested compounds, only amino-DACA exhibited a strong antagonistic effect on topoisomerase II-mediated DNA cleavage by VP-16. At 10 μM amino-DACA, a slight decrease in break frequency was noted; when concentration of this acridine was brought up to 20 and 50 μM, DNA breakage was completely inhibited. Other acridine-4-carboxamides did not efficiently protect L1210 cells against VP-16-induced DNA breaks. At lower concentrations, an additive effect in the formation of breaks was observed. A low degree of protection was obtained at the highest 50-μM concentration of these acridines (Fig. 5).

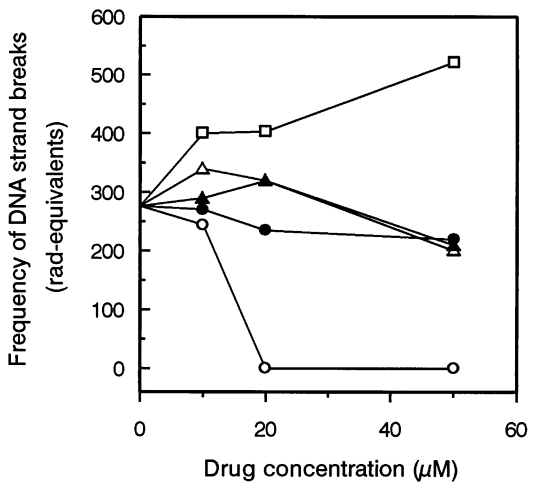

FIG. 5. The effects of acridine-4-carboxamides and *m*-AMSA on the frequency of DNA strand breaks induced in L1210 cells by VP-16. Cells were preincubated with the indicated concentrations of acridines for 15 min at 37° followed by treatment for 60 min with 3 μM VP-16. (□) *m*-AMSA, (△) DACA, (▲) DACAH, (○) amino-DACA, (●) amino-DACAH.

DNA Interstrand Cross-linking

The results of alkaline elution assays which were aimed to detect DNA interstrand cross-links produced by amino-DACA are shown in Fig. 6. In these experiments, the rates of elution of DNA from untreated cells and from those treated with amino-DACA or with the cross-linking agent *cis*-DDP were compared. Prior to lysis, the cells were irradiated with 3 Gy of γ radiation in order to obtain fragmented DNA characterized by fast elution [19]. In this assay, the presence of interstrand cross-links is evidenced by the decrease in the elution rate of DNA from drug-treated and irradiated cells as shown for cells incubated for 1 hr with 10 μM *cis*-DDP (Fig. 6). The elution curve observed in the case of DNA from cells treated for 1 hr with 20 μM amino-DACA and immediately subjected to the alkaline elution procedure suggests the presence of a low level of interstrand cross-links, as DNA from these cells eluted at a rate slightly lower than that of control cells. This effect was not observed for amino-DACA concentrations lower than 20 μM (data not shown). When L1210 cells treated with amino-DACA were incubated in drug-free medium for 1 hr after drug removal, the elution curve was almost identical to the control.

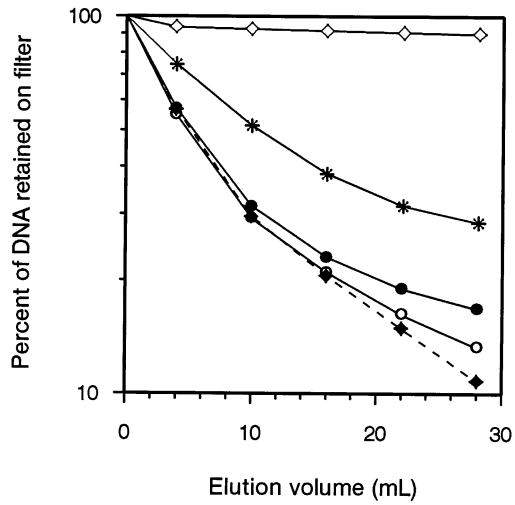

FIG. 6. DNA alkaline elution kinetics of L1210 cells treated with 20 μM amino-DACA for 1 hr at 37°. (●) cells treated with the drug, irradiated with 3 Gy of γ g radiation and immediately lysed, (○) cells treated with the drug and then incubated in drug-free medium for 1 hr prior to irradiation and lysis, (◆) irradiated control cells, (◇) nonirradiated control cells. (*) positive control, e.g. cells treated for 1 hr at 37° with 10 μM *cis*-DDP, irradiated and immediately lysed.

Uptake Studies

A striking difference in the transport rates of amino-DACA and amino-DACAH through cell membrane was found (Fig. 7). Amino-DACA was taken up quickly, reaching plateau within 20 min. When cells were washed and incubated further in drug-free medium, the drug exit was also rapid, but *ca.* 20% of amino-DACA molecules were retained in the cell at 60 min after cell washing. Contrary to amino-DACA, its analogue with the modified side-chain was taken up very slowly. After 60 min of drug treatment, the amount of amino-DACAH accumulated in the cells did not reach plateau and was approximately 10 times lower than that measured for amino-DACA.

DISCUSSION

Numerous studies have indicated that many clinically useful anticancer intercalators act as topoisomerase II poisons [26, 27]. Acridine derivatives, which are well known for their affinity to DNA, seem to be a potential source of drugs which target enzymes involved in DNA functions, e.g. the topoisomerases. In fact, in the last decade a series of highly cytotoxic acridines exhibiting antitumor activity *in vivo* have been developed [3, 4, 28]. The characteristic feature of all of these compounds is a charged carboxamide side-chain in position 4. Perhaps the most extensively studied member of this class of compounds is DACA, given its promising activity against a number of experimental murine tumors [29] and its ability to overcome P-glycoprotein-dependent multidrug resistance as well as resistance resulting from reduced topoisomerase II activity [30]. In this study, we have used close structural analogs of DACA to examine the structure-activity relationships for cytotoxicity and the ability to induce topoisomerase II-mediated chromatin lesions.

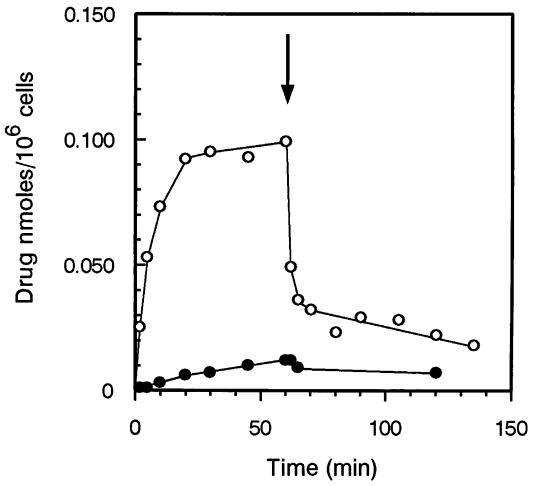

FIG. 7. Kinetics of uptake and efflux of amino-DACA and amino-DACAH. L1210 cells at the density of 10^7/mL were incubated with 10-μM acridines in growth medium at 37°. The arrow shows time of drug removal. The amount of drug accumulated in cells was estimated by fluorometric measurements.

Cytotoxic Activity and Inhibition of RNA Synthesis In Vitro

Both of these properties in this set of acridine-4-carboxamides depend mainly on the presence of a 9-amino group. Analogs with a 9-amino group (amino-DACA and amino-DACAH) are clearly more cytotoxic than DACA and DACAH (Table 1). The same compounds were also more efficient in inhibiting total RNA synthesis *in vitro*. The differences in the effect on RNA synthesis among acridine-4-carboxamides seem to be linked to their capacity to bind DNA. 9-Aminoacridines are known to form strong intercalative complexes with polynucleotides, and DNA-binding properties of DACA and amino-DACA were recently compared in terms of association constants and base specificity. Depending on the type of polynucleotide and assay method, the 9-amino derivative, amino-DACA was found to form complexes characterized by association constants which were *ca.* 5.5–15 times higher than those measured for DACA [9, 31]. *m*-AMSA, an even weaker DNA binder than DACA [31], was also the weakest RNA polymerase inhibitor (Table 1). This observation agrees with the report of Piestrzeniewicz *et al.* [8], who found correlation between the inhibition of RNA synthesis *in vitro* by acridine-4-carboxamides and their DNA-binding constants. Whereas the capabilities of acridine-4-carboxamides and *m*-AMSA to inhibit RNA synthesis *in vitro* can be explained on the basis of their affinities for the DNA template, the cytotoxic activities cannot. The most active pair of acridines, i.e., amino-DACA and *m*-AMSA, which exhibited similar cytotoxicities (Table 1), represent the strongest and the weakest DNA binders, respectively [9, 31]. The present results indicate that a very important factor influencing cytotoxicity of acridine-4-carboxamides is their transport rate through the cell membrane. Amino-DACA and amino-DACAH, which exhibited a substantial difference in cytotoxic potency, also greatly differed in their capacity to cross cell membrane. The amount of the drug accumulated in cells after 60 min of treatment reached a level tenfold lower for the less cytotoxic analogue amino-DACAH than for amino-DACA. A great difference in the uptake of amino-DACA and amino-DACAH is perhaps related to their different lipophilicities [32], as these compounds do not vary significantly in their pK_a values for the side-chain cationic center [9, 32].

The lower cytotoxic activity of DACA when compared to its 9-amino analogue amino-DACA does not seem to be related to the transport. This is suggested by the data of Baguley (manuscript in preparation), who found by a radioactivity assay that the uptake and efflux of DACA was very rapid. The uptake of DACAH is not known, but because the lipophilicity of this drug is decreased by the presence of a hydroxyethylamino moiety at the 4-side-chain, its low uptake, as in the case of amino-DACAH, cannot be excluded.

Earlier studies have shown that topoisomerase II appears to be the main cellular target for acridine-4-carboxamides

[7, 33]. Woynarowski *et al.* described the "non-classic" (when compared with *m*-AMSA) mechanism of topoisomerase II-trapping by acridine-4-carboxamides [7]. These authors suggested that the specific mechanism of topoisomerase II-blocking by acridine-4-carboxamides reflected the lack of an anilino group in position 9 of the acridine ring. This bulky substituent of *m*-AMSA lying in the minor groove of DNA was considered to be the fragment interacting with the topoisomerase II protein [34]. A model of binding of acridine-4-carboxamides with DNA was proposed [11], and recently supported [9], in which the 4-carboxamide chain is placed in the minor groove of B-DNA. The detailed mechanism of of topoisomerase II interaction with DNA as well as molecular structures of ternary complexes of the enzyme with its poisons have not been elucidated as yet. However, it seems reasonable to hypothesize that the 4-carboxamide chain in the minor groove of DNA may interact with topoisomerase. To verify this hypothesis, we have compared DNA lesions produced in L1210 cells by DACA and amino-DACA with those produced by their analogs possessing a modified side-chain (Fig. 1).

Topoisomerase poisons produce characteristic cellular lesions: DNA breaks accompanied by DNA-protein cross-links [24–27]. DACA and its analogs were able to induce these types of lesion in cultured L1210 cells, but they exhibited different activities. (Figs. 3 and 4). The carboxamide chain with a *N*-2-(dimethylamino)ethyl residue seems to be a structure favored for higher activity. Both analogs with this side-chain were more active than their *N*-2-(hydroxyethylamino)ethyl analogs in DNA breaks and DNA-protein cross-link induction. A large difference was noticed between amino-DACA and amino-DACAH. The latter compound was barely active, producing very low DNA break and DNA-protein cross-link frequencies. The cytotoxic activity of acridine-4-carboxamides seems to be linked to the level of chromatin lesions only when very close structural analogs are compared. Amino-DACA produced more DNA breaks and DNA-protein cross-links than amino-DACAH and was also more cytotoxic. The same relationship was observed between DACA and DACAH. On the basis of DNA damage measurements and uptake studies (Figs. 3, 4 and 7), it can be concluded that the differences in the biologic activity of acridine-4-carboxamide derivatives may be attributed to the rate of their transport through the cell membrane. The hypothesis as to the important role the 4-side-chain structure may play in the formation and stabilization of cleavable complex of topoisomerase II with DNA remains valid but requires further investigation.

Possibility of Topoisomerase II as Main Target for Acridine-4-Carboxamides

Earlier studies suggested that topoisomerase II was the main target for acridine-4-carboxamides, while pointing out the nonclassical mechanism of their action [7]. More recently, experiments with purified preparations of topoisomerases I and II have evidenced the capability of amino-DACA and DACA to stimulate DNA cleavage mediated by these enzymes, suggesting that topoisomerase I may be an additional target for DACA and related compounds [31]. It has also been demonstrated that human leukemia cell lines having low topoisomerase II activity and exhibiting resistance to classic topoisomerase II poison, *m*-AMSA, are sensitive to DACA and amino-DACA [27, 35]. Thus, topoisomerase II does not seem to be the only target for acridine-4-carboxamides. To further elucidate this problem, we performed experiments with ICRF-187, a catalytic inhibitor of topoisomerase II, which is known to antagonize the cytotoxicity of topoisomerase II poisons when incubated with cells simultaneously [14]. As demonstrated in Fig. 2, ICRF-187 reduced the cytotoxicity of the reference topoisomerase II poison VP-16, but such an effect was not observed for acridine-4-carboxamides. There are two possible explanations for this result. The first is that the acridine-4-carboxamide mechanism of action resembles that of clerocidine, to date the only known topoisomerase II poison for which cytotoxicity is not antagonized by ICRF-187 [14]. This seems, however, unlikely as clerocidin is not an intercalator but binds covalently to topoisomerase II, producing a very stable cleavable complex [36]. There is no experimental evidence for the covalent binding of acridine-4-carboxamides to topoisomerase II, or even for formation by these drugs of a cleavable complex of the enzyme which cannot be dissociated by heat or high ionic strength. Therefore, the second explanation, that acridine-4-carboxamides in L1210 cells do not stabilize the cleavable complex of topoisomerase II with DNA efficiently, despite their ability to form DNA breaks and DNA-protein cross-links, is favored. The results of experiments on the protective action of DACA analogs against DNA breaks induced by VP-16 confirmed this view (Fig. 6). Amino-DACA, a strong intercalator which penetrates cell membrane easily, completely protected cells against VP-16. The protection occurred at the same concentration when self-inhibition of DNA breakage by amino-DACA was noted (Figs. 4 and 6). This may point to the possible dual mode of action of amino-DACA as postulated for other intercalators [37]. At low concentrations, this drug may trap topoisomerase II at the cleavable complex step, whereas at higher concentrations it inhibits binding of the enzyme to its DNA substrate by untwisting the DNA through the intercalation process. Another strong intercalator, amino-DACAH, exhibited a poor effect, but this is understandable as the uptake of this acridine was very low. The weak protective effect of DACA and DACAH toward VP-16 is probably related to the low binding affinities of these drugs to DNA. Thus, some doubts may be raised if the most important drug of the current study, DACA, interferes with topoisomerase II in cells. Such a notion is inconsistent with the conclusions of previous studies [7] and the ability of this acridine to produce chromatin lesions characteristic of topoisomerase II-blocking drugs (Figs. 3 and 4). A possible explanation for

this disagreement is that DACA-induced DNA strand breaks and DNA-protein cross-links may also reflect, other than topoisomerase II blocking, intracellular reactivities of this drug. In fact, recent data suggest the involvement of topoisomerase I in the cytotoxic action of DACA [31, 35]. As alkaline elution does not reveal whether DNA lesions are produced by topoisomerase I or II blockage, this lack of direct proof permits one to hypothesize that a substantial fraction of DNA breaks and DNA-protein cross-links observed in cells treated with DACA is mediated by topoisomerase I.

Both 9-aminoacridines (amino-DACA and amino-DACAH) appear to be surprisingly cytotoxic in the light of their rather poor DNA-damaging activity. The possibility that amino-DACA is not only a topoisomerase II inhibitor but may also possess DNA cross-linking activity involved in its cytotoxic action was suggested earlier [7, 38]. Using DNA alkaline elution, we investigated this suggestion, seeking evidence for the presence of DNA interstrand cross-links in amino-DACA-treated cells. The elution profiles obtained for cells treated with amino-DACA are similar to the profile produced by the reference cross-linking agent, *cis*-DDP (Fig. 6). These results, however, should be interpreted with caution. A decrease in the DNA elution rate was observed only at a very high amino-DACA concentration which exceeded ED_{50} by factor of 80. This dose of the drug evidently went beyond the pharmacologically reasonable range, making it difficult to deduce the biologic meaning of this effect. Changes in DNA elution patterns were only observed immediately after drug treatment, and disappeared quickly after drug removal, suggesting that the cross-linking effect of acridine-4-carboxamides is distinct from the two-step reaction described for classic cross-linkers [39]. A second possibility, that the decrease in the elution rates is not due to interstrand cross-links induced by amino-DACA but to the incompletely digested DNA-protein complexes, should also be considered. As mentioned in the Results section, the lysates of cells incubated with amino-DACA required more extensive proteinase K digestion in order to remove proteins masking DNA breaks. Because the presence of protein on filters during DNA interstrand cross-link assay affects elution kinetics [19], it seems possible that at a very high amino-DACA concentration some level of DNA-protein cross-links would remain and decrease the DNA elution rate, giving an artificial interstrand cross-linking effect. The reason for the relative resistance of DNA-protein cross-links induced by amino-DACA to proteinase K is not clear, particularly as this acridine produced a much lower frequency of DNA-protein cross-links than did *m*-AMSA (Fig. 3). It can be assumed that DNA-protein complexes occurring in the presence of amino-DACA are not limited to the topoisomerase II-drug-DNA ternary complexes, but that some large particles of condensed chromatin may also be formed. This hypothetical explanation for our results is based on the data of Darzynkiewicz and Kapuściński [40], who found condensation of chromatin by several intercalators, including charged acridine derivatives. Such large DNA-protein particles require more time and a higher proteinase K concentration to be digested, and may be responsible for the relative resistance of amino-DACA-induced DNA-protein cross-links to deproteinization. Thus, the involvement of DNA interstrand cross-linking in the mode of action of amino-DACA was not evidenced in this work.

In conclusion, this work demonstrates that the stabilization of the cleavable complex of topoisomerase II with DNA by acridine-4-carboxamides is not as important for the cytotoxic action of this class of compounds as for the classic poisons *m*-AMSA or VP-16. Derivatives which can penetrate cell membrane easily and are able to form a strong intercalative complex with DNA, for instance amino-DACA, may enhance DNA cleavage by topoisomerase II only when used at low doses. At higher concentrations they act as catalytic inhibitors. The mechanism of action of *des*-amino derivatives possessing lower affinity to DNA (DACA) is less clear. Their ability to poison topoisomerase II is also poor, and these compounds are not efficient catalytic inhibitors of the enzyme. Thus, our work confirmed recent suggestions concerning the existence of an additional cellular target for DACA, e.g. topoisomerase I [31, 35]. In the case of the most cytotoxic compound, amino-DACA, another mechanism is suggested to be involved in its cytotoxic action, presumably resulting from the induction of chromatin condensation.

We thank Anna Koza for her expert technical assistance. This work was supported by Grant No. 4-0238-91-01 of the State Committee for Scientific Research.

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