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Unusual dynamics of killing of cultured Lewis lung cells by the DNA-intercalating antitumour agent *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide*

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Summary. The cytotoxicity of *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide (AC; NSC 601316), a new experimental DNA-intercalating antitumour drug, against a cultured Lewis lung adenocarcinoma cell line was compared with that of the DNA-intercalating antitumour drug amsacrine. In contrast to amsacrine, AC demonstrated self-inhibition of cytotoxicity following short (3–9 h) incubation periods and exponential killing (with a shoulder) after long (24–72 h) periods of incubation. The difference between these drugs was best demonstrated using a constant concentration × time (C × T) exposure (AC, 12 μmol h l^{-1}; amsacrine, 3 μmol h l^{-1}). In contrast to amsacrine, AC was minimally effective over exposure periods of ≤1 h and maximally effective over intermediate periods (4–6 h). The results suggest the possibility of designing AC administration protocols that maximise the drug's cytotoxicity towards solid tumours, which, because of diffusion barriers, are subjected to longer drug exposures than are well-vascularised tumours.

Introduction

Acridine carboxamide (AC) was developed in the course of a programme conducted in this laboratory to design and synthesise acridine derivatives with antitumour activity. Following the development of amsacrine [5], an extensive analogue-development programme led to the discovery that a positively charged side chain at position 4 of 9-aminoacridine conferred good activity against experimental leukaemia [2]. In the course of analogue development to optimise activity against solid tumours, it was found that removal of the 9-amino group provided decreased acridine base strength, increased lipophilicity, and cure rates of 90% in advanced Lewis lung tumours in mice [3]. One feature of the action of AC is that when it is injected i.p., it shows more activity against remotely sited (i.v. inoculated) Lewis lung tumour than against proximally sited (i.p. inoculated) P388 or L1210 leukaemia [3, 7]. Although AC is as cytotoxic towards cultured Lewis lung cells as it is against L1210 cells in culture, its solid tumour selectivity in vivo is unexpected. Because of its unique properties, AC is now undergoing preclinical toxicology and pharmacology testing under the auspices of the Cancer Research Campaign, United Kingdom, in preparation for clinical trial.

AC appears to target the enzyme topoisomerase II, since it induces the formation of protein-DNA cross-links in cultured L1210 cells and is 13-fold less active in vitro against a P388 subline that displays altered topoisomerase II isozyme expression [13]. However, AC differs from many other topoisomerase II poisons such as amsacrine, doxorubicin, ellipticine, etoposide, and mitoxantrone in that it does not have an easily oxidisable or reducible quinone or quinoneimine group. It has been suggested that the potential of the latter compounds for redox cycling contributes to their cytotoxicity [13], and it is noteworthy that although AC is more potent than amsacrine in inducing DNA-protein cross-links, it is less potent than amsacrine as a cytotoxic agent [13]. Studies on the action of AC may therefore contribute to our knowledge of the multiple modes of action of cytotoxic agents.

One feature of the in vitro toxicity of AC is the self-inhibition of cytotoxicity at high drug concentrations [7]. Thus, as measured by cell survival, optimal killing of cultured Lewis lung cells exposed to the drug for 1 h is achieved at a concentration of 3 μM, whereas drug concentrations above 15 μM are almost non-cytotoxic. In the present study, we extended this finding by measuring cell killing by AC over a range of exposure periods and concentrations and comparing the results to those obtained using another antitumour acridine, the clinical agent amsacrine. We show that the self-inhibition phenomenon

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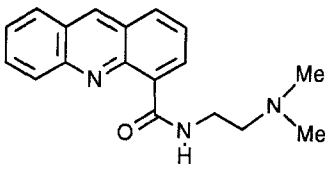

Fig. 1. Structure of AC

gives rise to unique pharmacodynamic properties that help explain the high experimental activity of AC against solid tumours. The phenomenon may also have implications for its clinical use against solid tumours.

Materials and methods

Chemicals. AC hydrochloride (Fig. 1) was synthesised in this laboratory [3]. Amsacrine isethionate was obtained from the Parke-Davis Division of the Warner-Lambert Company (Ann Arbor, Mich., USA). The compounds were dissolved in 50% (v/v) aqueous ethanol to obtain stock solutions of 2–5 mmol/l and were stored at –20° C.

Clonogenicity assays. The LLTC cell line was developed from the Lewis lung carcinoma [15] at the Southern Research Institute (Birmingham, Ala., USA) and was originally obtained from Dr R. C. Jackson, Parke-Davis Division of Warner-Lambert. LLTC cells were plated at 10^5 cells/ml in 100-mm dishes containing 15 ml growth medium [αMEM supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU penicillin/ml, and 100 μg streptomycin/ml] per plate as previously described [7, 8]. After 18 h, cells were detached from the plates by treatment with 0.07% trypsin (Difco) in citrate-saline, collected by centrifugation, and resuspended to a concentration of 10^5 cells/ml in growth medium in plastic tubes (5 ml/tube). After incubation at 37° C for various periods of up to 3 h in the presence of a range of concentrations of cytotoxic agents, cells were washed twice, counted, diluted serially, and plated in 60-mm dishes at 10^2, 10^3, and 10^4 cells/plate. Cultures were fixed and stained after 10 days using methylene blue (5 g/l) in 50% (v/v) aqueous ethanol. Colonies comprising over 50 cells were counted. Plates containing no colonies at the lowest dilution were arbitrarily scored at a percentage of survival of 0.001%.

Alternatively, LLTC cells were plated at 10^5 cells/ml in 96-well plates (150 μl growth medium/well). Cells were incubated overnight (17–18 h) to allow attachment to plates. After incubation, growth medium was removed, drug diluted in medium was added (eight wells for each drug concentration), and cells were incubated at 37° C for various intervals. Cells were harvested by removing the media and then trypsinising and transferring the cells to test tubes containing fresh medium (2 ml). Cells were counted and clonogenic cells were determined by plating as described above. After incubation periods of longer than 18 h, culture supernatants (which contained a small number of cells) were retained and added to the trypsinised cell suspension prior to washing and plating.

Results

Concentration dependence of drug cytotoxicity

Following short drug-exposure periods, self-inhibition of cytotoxicity was observed at high concentrations of AC, as previously found after 1-h exposures [7]. This behaviour contrasted with that of another antitumour agent, amsacrine (Fig. 2). As the duration of exposure was increased, the maximal degree of cell killing increased (Fig. 3). Following exposure periods of 24 h and greater, the concentration dependence of killing by AC became exponential (with a small shoulder), with high degrees of cell killing

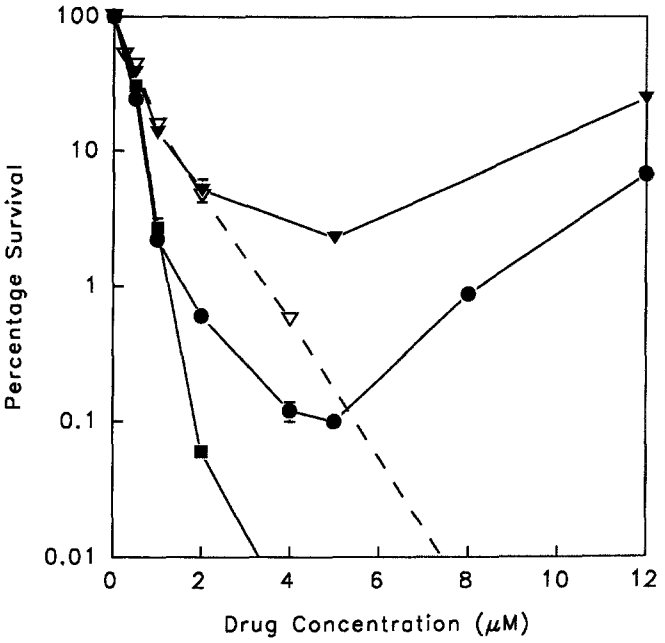

Fig. 2. Relationship between the cytotoxicity of AC towards LLTC cells and the concentration over short-term exposures of 1 (▼), 3 (●), and 6 h (■). For purposes of comparison, the plot for amsacrine at 1 h (▽) is shown. The off-scale value (5 μM) is zero

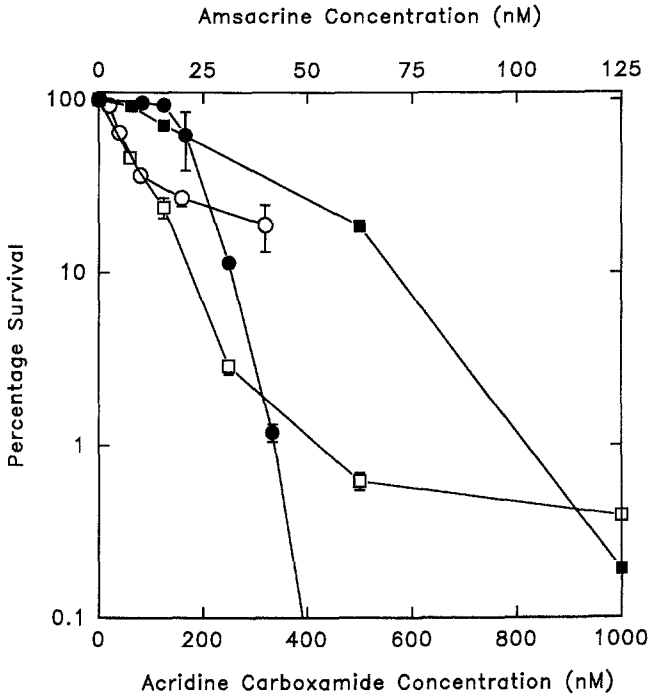

Fig. 3. Relationship between the cytotoxicity of AC towards LLTC cells and the concentration over long-term exposures of 24 (■) and 72 h (●). For purposes of comparison, plots for amsacrine at 24 (□) and 72 h (○) are shown. The off-scale value (500 nM) is zero

occurring at high drug concentrations (Fig. 3). This behaviour contrasted with that of amsacrine, which showed a survival curve indicative of a resistant sub-population. Amsacrine was apparently less cytotoxic after exposure for 3 days than it was after exposure for 1 day. This was probably a consequence of the regrowth of surviving cells,

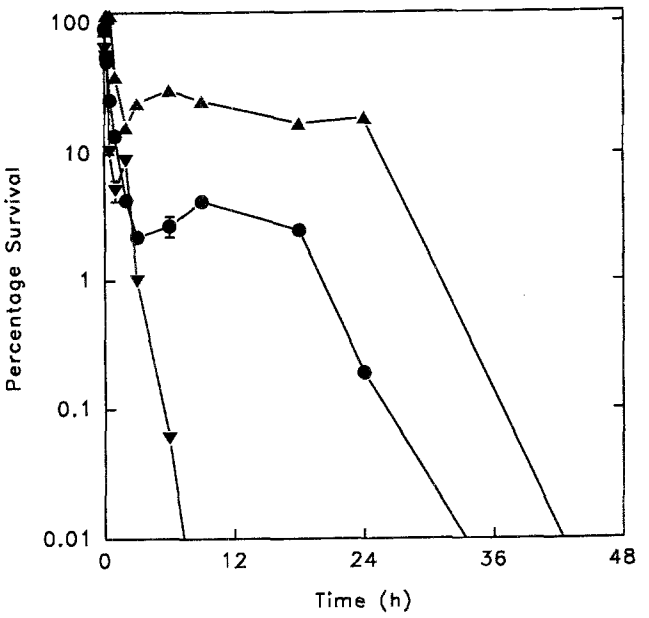

Fig. 4. Relationship between the cytotoxicity of AC towards LLTC cells and the duration of exposure at constant concentrations of 0.5 (▲), 1.0 (●), and 2.0 μM (▼). The off-scale values are zero

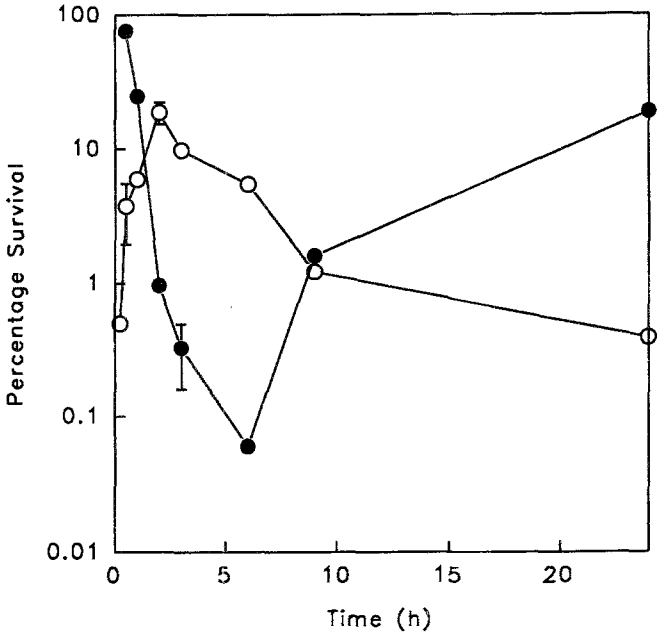

Fig. 5. Relationship between the cytotoxicity of AC towards LLTC cells and the duration of exposure to AC (●) at a constant C×T value of 12 μmol h l^{-1} and to amsacrine (○) at a constant $c \times T$ value of 3 μmol h l^{-1}

since amsacrine is known to be degraded in tissue-culture medium [4].

Time dependence of drug cytotoxicity

When cell survival at a constant drug concentration was plotted for certain concentrations of AC, a biphasic pattern pattern of cell killing was observed (Fig. 4). This was not found for amsacrine (not shown). Drug cytotoxicity at constant C × T was also plotted as a function of time. Short exposures to AC were relatively ineffective, and optimal killing of LLTC cells was found following exposure periods of 4–6 h (Fig. 5). In contrast, amsacrine was least effective after an intermediate (2 h) exposure period.

Discussion

As shown in Figs. 2–5, the cytotoxic properties of AC differed in two important respects from those of another acridine-based antitumour agent, amsacrine. First, following short exposure periods (≤3 h), AC displayed optimal activity at 5 μM and demonstrated self-inhibition at higher concentrations (Fig. 2). Second, AC exhibited an unusual time dependence in its killing of LLTC cells (Fig. 4), in which at certain concentrations, an early phase of killing was followed by a drug-resistant period and then by a further period of killing. The self-inhibition effect is probably related to the inhibition of AC and other topoisomerase II-directed agents by the simple intercalator 9-aminoacridine [8]. In the latter case, efficient reversal of cytotoxicity was observed at a 9-aminoacridine concentration of 5 μM, corresponding to an uptake ratio (cell-associated: extracellular drug concentrations) of approx. 930 and a maximal theoretical DNA-binding ratio of 0.28 drug molecules/bp, although the latter figure would be expected to be lower because of drug binding at other cellular sites [8].

Our experiments using AC indicate that cellular uptake is rapid and proportional to external drug concentration, with an uptake ratio of approx. 450 being observed (manuscript in preparation). It is thus probable that the self-inhibition of AC at concentrations above 5 μM reflects the same phenomenon observed with 9-aminoacridine. Self-inhibition of cytotoxicity parallels the effect of AC on the production of DNA-protein cross-links in L1210 cells, whereby stimulation is maximal at a drug concentration of between 1 and 5 μM but decreases sharply at concentrations of 10 μM or higher [13]. Therefore, the most likely explanation of this effect is that drug intercalation induces a conformational change in the DNA, hindering the formation of the cleavable complex of topoisomerase II with DNA. Work is in progress to determine the basis for the unusual time dependence of cell killing by AC.

Early preclinical pharmacological investigations of anticancer agents have focused mainly on the pharmacokinetic properties of drugs. More recently, it has been acknowledged that the pharmacodynamic properties of these drugs, encompassing the relationship between therapeutic response and drug concentration, are important considerations [1, 12]. Emphasis has been placed on the C × T product, which provides the best prediction of response to an anticancer drug. The present results demonstrate the unusual dependence of the cytotoxicity of AC on its C × T product.

C × T relationship can be visualised conveniently when cytotoxicity at constant C × T values is plotted as a function of time as shown in Fig. 6. Eichholtz-Wirth [6] has shown that there is a direct relationship between the C × T product of doxorubicin and the biological effect of the drug

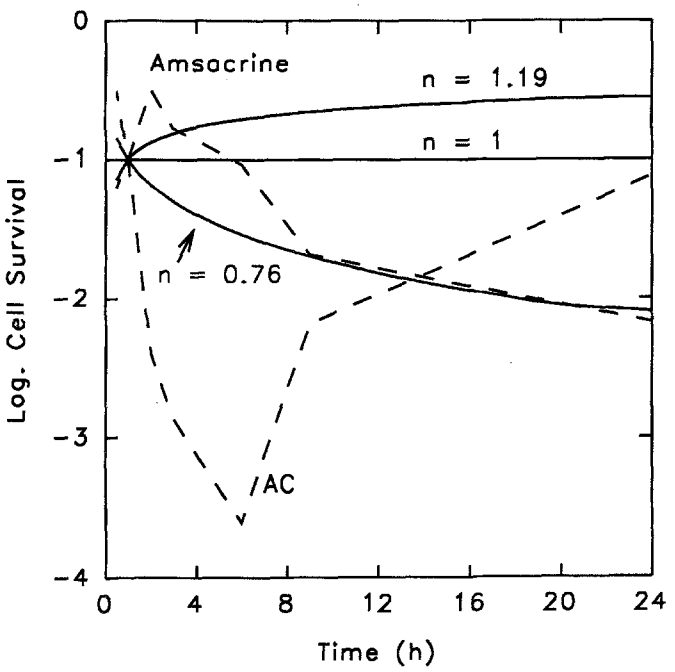

Fig. 6. Comparison of theoretical relationships obtained between cytotoxicity at constant $C \times T$ and time of exposure. Examples are shown for $C^n \times T$ $n = 0.76$ (5-fluorouracil) and $n = 1.19$ (doxorubicin) [1]. Dashed lines show data from Fig. 5 for AC and amsacrine. All curves are normalised for 90% killing at a time of 1 h

as measured by clonogenic survival tests. Ozawa et al. [11] have demonstrated that the cytotoxicity of cycle phase-non-specific drugs, including Adriamycin, cisplatin, and neocarzinostatin, are $C \times T$-dependent if a correction for drug decomposition in media is employed. Such relationships would correspond to horizontal lines when drawn on Fig. 6. Reports of deviations from this relationship have been described [1, 9, 11]. On the basis of bacterial models, Skipper [14] has suggested, that the correlation between cytotoxicity and drug exposure often takes the form of $C^n \times T$. The $C^n \times T$ relationship has been used to investigate pharmacodynamic properties of a selection of anticancer agents in vitro [1]. When $n < 1$, as for 5-fluorouracil, cell killing is optimal following long exposure periods, producing a descending curve when plotted as in Fig. 6. When $n > 1$, as for doxorubicin and etoposide, killing is optimal after shorter exposures, producing an ascending curve when plotted as in Fig. 6. The present results obtained for amsacrine correspond to a curve with $n > 1$, except that since amsacrine is degraded with time in culture medium [4], the curve is distorted towards lower cytotoxicity following longer drug-exposure periods [11]. In contrast, AC, which is stable under culture conditions (unpublished data) shows a completely different dependence on exposure time. Killing is minimal at high drug concentrations following short exposures (a consequence of self-inhibition of toxicity) and maximal following intermediate exposure periods (Fig. 6).

The importance of this finding is best understood by considering that the administration of a drug as a pulse dose results in a high peak plasma concentration and, provided that the drug is rapidly degraded, a short exposure period. As the drug passes through successive diffusion barriers provided by the multicellular structure of a tumour, the peak drug exposure decreases but the duration of exposure increases. This behaviour is well demonstrated by amsacrine and its analogue CI-921 in mice, in which the half-life of unmetabolised drug in a subcutaneous solid tumour is 4- to 8-fold longer than that in plasma [10]. Similar results have been obtained for AC (Paxton, personal communication). A single i.p. injection of AC would thus lead to a high "pulse" concentration in the peritoneum but to an exposure period of several hours in a remotely sited tumour. This may explain why an i.p. injection of AC is less effective against i.p. implanted leukaemia than it is against Lewis lung carcinoma growing as lung nodules [2, 7]. This result may also have application in the design of clinical protocols, whereby a short infusion would provide a relatively short exposure to drug and therefore result in minimal cytotoxicity towards well-vascularised tissues such as bone marrow while providing longer retention and, consequently, greater cytotoxicity towards solid tumours. Thus, the unusual pharmacodynamics of AC could provide the basis for a new principle in the treatment of solid tumours.

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