Cancer Chemother Pharmacol (1994) 34: 159–165

Cancer Chemotherapy and Pharmacology

Novel carbamate analogues of amsacrine with activity against non-cycling murine and human tumour cells

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Received: 24 September 1993/Accepted: 14 December 1993

Abstract. The cytotoxicity of a class of compounds related to the topoisomerase-II poison amsacrine was investigated against plateau-phase murine Lewis lung carcinoma cells (LLTC), HCT-8 human colon carcinoma cells and other cell lines. Methyl *N*-[4-(9-acridinylamino)-2-methoxy-phenyl]carbamate hydrochloride and the corresponding demethoxy compound, which contain a methylcarbamate instead of the methylsulphonylamino group, manifested relatively high cytotoxic activity against plateau-phase cells as measured by clonogenic survival. The concentration of drug required for a given cytotoxic effect on plateau-phase cells was about 2 times higher than that required for an equitoxic effect on actively proliferating cells. In contrast, at least 5 times more amsacrine, doxorubicin or etoposide was needed for an equitoxic effect on plateau-phase cells. Cells taken directly from subcutaneous LLTC tumours and exposed to drugs displayed the same differential drug sensitivity to the carbamate compounds, suggesting that the plateau-phase cells provide an appropriate model for cells growing in vivo. The greater cytotoxicity of the carbamate drugs was shown to depend critically on the provision of an energy source such as glucose, suggesting that nutrient starvation both in plateau-phase cells and in tumours induced a glucose-sensitive resistance mechanism. It is suggested that the carbamate analogues of amsacrine recognize a form of topoisomerase II, possibly topoisomerase IIβ, the activity of which increases relative to that of topoisomerase IIα in non-cycling cells, and might be used to devise new strategies for the treatment of solid tumours.

Introduction

Resistant cell populations within solid tumours are primarily responsible for the ineffectiveness of chemotherapy in many types of cancer. Resistance may be inherent in the phenotype of a cell or induced as a result of exposure to metabolic or other stress within the tumour and is of particular importance to the action of drugs, including the anthracyclines, epipodophyllotoxins, amsacrine and mitoxantrone, which target DNA topoisomerase II [6, 7]. The development of new drugs or treatment strategies that will overcome such resistance [27] is thus critical to progress in clinical treatment.

A major research aim of this laboratory has been the design of new topoisomerase II-directed agents that overcome resistance mechanisms. A major type of resistance to such agents involves enhanced drug efflux associated with the expression of P-glycoprotein [15]. The synthesis of a large series of acridine derivatives resulted in the development of amsacrine [9], the amsacrine analogue 9-[(2-methoxy-4-methylsulphonylamino)phenylamino]-N,5-dimethyl-4-acridinecarboxamide isethionate (CI-921) [3] and *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide dihydrochlori-de (DACA) [1], which are capable of overcoming this type of multidrug resistance [5, 20, 22, 29]. A second type of multidrug resistance, often termed "atypical" multidrug resistance, does not involve altered drug transport or DNA repair but rather is associated with lower amounts of topoisomerase II and, possibly, altered enzyme phosphorylation [6, 8, 33]. Using a series of Jurkat leukaemia cell lines that have been characterized as having the latter type of resistance [32], we have shown that DACA is largely capable of overcoming such resistance as measured by in vitro growth inhibition assays [20].

In the course of screening 9-anilinoacridine derivatives for biological activity, we have found some unusual properties associated with methyl *N*-[4-(9-acridinylamino)-phenyl]carbamate hydrochloride (AMCA) as well as with the corresponding methoxy derivative methyl *N*-[4-(9-acridinylamino)-2-methoxyphenyl]carbamate hydrochloride (mAMCA), which is the carbamate analogue of amsacrine

This research was supported by the Cancer Society of New Zealand, by its Auckland Division, and by the Health Research Council of New Zealand.

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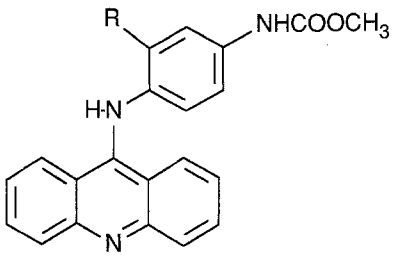

Fig. 1. Structures of AMCA (R = H) and mAMCA (R = OCH_3) as the free bases

(see Fig. 1 for structures). Both AMCA and mAMCA are more potent than amsacrine or AMSA (amsacrine lacking the 3'-methoxy group) as inhibitors of herpesvirus growth in cultured BHK cells [21]. Furthermore, AMCA is active in vitro and in vivo against a P-glycoprotein containing multidrug-resistant P388 line (P/DACT) as well as against an amsacrine-resistant P388 cell line (P/AMSA) that demonstrates atypical multidrug resistance [5]. In this report we describe the unexpectedly high activity of AMCA and mAMCA against Lewis lung carcinoma cells growing either at high density in culture or as subcutaneous tumours in mice. The studies also demonstrate a distinct but concomitant role of cellular energy supply in establishing the resistant state.

Materials and methods

Materials. The drugs used and their sources were as follows: amsacrine and CI-921 isethionate; Parke-Davis Research (Ann Arbor, Mich., USA); doxorubicin, Farmitalia Carlo Erba (Italy); and etoposide, Bristol Arzneimittel (Germany). Other cytotoxic compounds were synthesized in this laboratory by Drs. W. A. Denny, G. J. Atwell, and G.W. Rewcastle. Other sources were as follows: streptomycin sulphate, Serva; propidium iodide and pronase, Calbiochem; penicillin, pepsin, ribonuclease and 2,4-dinitrophenol (DNP), Sigma; 5-bromodeoxyuridine (BUdR)/5-fluorodeoxyuridine, Amersham; rat anti-BUdR antibody, Seralab (Sussex, UK); sheep anti-rat IgG fluorescein-conjugated antibody, Silenus (Victoria, Australia); α-modified minimum essential medium (MEM) and foetal bovine serum (FBS), Gibco; trypsin, Difco; and tissue culture plasticware, Nunc or Falcon.

Clonogenic assays. LLTC (murine Lewis lung carcinoma cells adapted to tissue culture) [36] and HCT-8 human colon carcinoma cells were obtained from Dr R. C. Jackson. Other human colon carcinoma lines were sourced as previously reported [18]. Cells were cultured in α-modified MEM supplemented with FBS (10%, v/v), penicillin (100 U/ml) and streptomycin sulphate (100 μg/ml) [18, 19]. Detachment from plastic was effected by trypsin (0.07%, w/v) in citrate-buffered saline (KCl, 0.134 *M*; trisodium citrate dihydrate: 0.015 *M*, pH 7.6). Cells were seeded into 100-mm dishes at 10^5 cells/ml (15 ml/dish of α-MEM supplemented with FBS and antibiotics) and cultured for 18 h to provide exponentially growing cells and for between 4 and 8 days to provide cultures approaching or at saturation density. Cells were recovered by trypsinization (HCT-8 cells required syringing to produce single-cell suspensions), collected by centrifugation and exposed to cytotoxic drugs in polystyrene tubes in fresh medium (Falcon 2057 tubes; 10^5 cells/ml, 5 ml/tube) for 1 h at 37° C. Control tubes received drug solvent (ethanol) equivalent to the highest concentration present in drug-containing tubes. After incubation, cells were collected by centrifugation, washed twice, counted in a particle counter (Coulter Electronics) and diluted, and varying numbers of cells were plated in 60-mm dishes containing α-MEM supplemented with 5% FBS and antibiotics. After 10 days of incubation, cultures were fixed and stained with methylene blue (5 g/l) in 50% aqueous ethanol. Colonies consisting of at least 50 cells were counted. C_{10} and C_{30} values were defined as the drug concentrations that reduced cell survival to 10% and 30%, respectively, of that of control cultures. The average plating efficiencies for control LLTC cultures after 1, 4 and 6 days in culture were 0.98, 0.96 and 0.71, respectively, whereas those for HCT-8 cultures after 1, 4 and 8 days were 1.07, 0.83 and 0.73, respectively.

One series of clonogenicity assays was performed with LLTC cells recovered from large subcutaneous tumours arising in BDF_1 mice some 3–4 weeks after the inoculation of 10^6 cultured LLTC cells as described elsewhere [19, 24]. Briefly, tumours were minced with crossed scalpels and disaggregated with pronase (1 mg/ml) in full growth medium at a ratio of 60 mg tumour tissue/ml (40 min, 37° C). Cells were collected by centrifugation and washed to remove pronase prior to drug exposure. Recovered cells either were assayed for drug sensitivity immediately or were cultured overnight in an airtight box containing a humidified atmosphere of 5% CO_2 and 5% O_2 in nitrogen to provide cells recruited into cycle. Cells plated for colony formation were co-cultured with ^{60}Co-gamma-irradiated (35 Gy) autologous feeder cells (10^5/dish) in an atmosphere containing a reduced O_2 concentration (5%, v/v). The average plating efficiency of cells recovered from subcutaneous tumours was 0.6.

Flow cytometry. LLTC cell cultures (10^6 cells) were labeled for 60 min with BUdR (10 μ*M*)/5-fluorodeoxyuridine (0.1 μ*M*), fixed in 70% aqueous ethanol at −20° C for at least 16 h, centrifuged (200 *g*, 7 min), washed, resuspended in a minimal amount of phosphate-buffered saline (PBS)/2% FBS and incubated with a solution of pepsin (0.2 mg/ml) in 2 *N* HCl (1 ml) with occasional mixing at 37° for 30 min. The suspension was diluted to 10 ml with PBS/FBS and the pellet was collected by centrifugation (200 *g*, 7 min), resuspended in PBS/FBS and washed twice with PBS/FBS (10 ml) and once with PBS/0.5% Tween 20 (2 ml). Cells were resuspended in PBS/0.5% Tween 20 (50 μl) and stained with a 1: 10 dilution of anti-BUdR antibody for 1 h at room temperature. Cells were then washed and resuspended and incubated with sheep anti-rat IgG fluorescein conjugate (1: 100 dilution). PBS (0.5 ml) was added and the cells were centrifuged for 7 min, washed once with PBS (0.5 ml) and resuspended in PBS (0.5 ml) containing propidium iodide (20 μg/ml) and ribonuclease (1 mg/ml). After standing at room temperature for 15 min the cell suspension was analyzed on an Epics Profile flow cytometer (Coulter Electronics, Florida, USA), correcting for cell doublets [24]. Data were analyzed using MULTIPLUS software (Phoenix Flow Systems, San Diego, USA).

Results

Comparison of the cytotoxicity of AMCA, mAMCA and other topoisomerase-II poisons against cultured cells at high density

LLTC cells were established in culture and grown for 1, 4 or 6 days, providing cell densities of 2.7×10^5, 1.5×10^6 and 1.8×10^6, respectively. The S-phase fraction, determined by BUdR labeling of the DNA and two-dimensional flow cytometry, comprised 50%, 6% and 4%, respectively, of these cell populations, indicating a progressive reduction of cycling cells as the incubation time increased. The proportions of G_2/M-phase cells remained relatively constant at 5.3%, 5.3% and 6.6%, respectively, of the total number of cells. The cytotoxicity of AMCA and mAMCA was compared with that of AMSA, amsacrine, etoposide and doxorubicin against cells that had been either actively proliferating (day 1) or at high density (day 4) before being

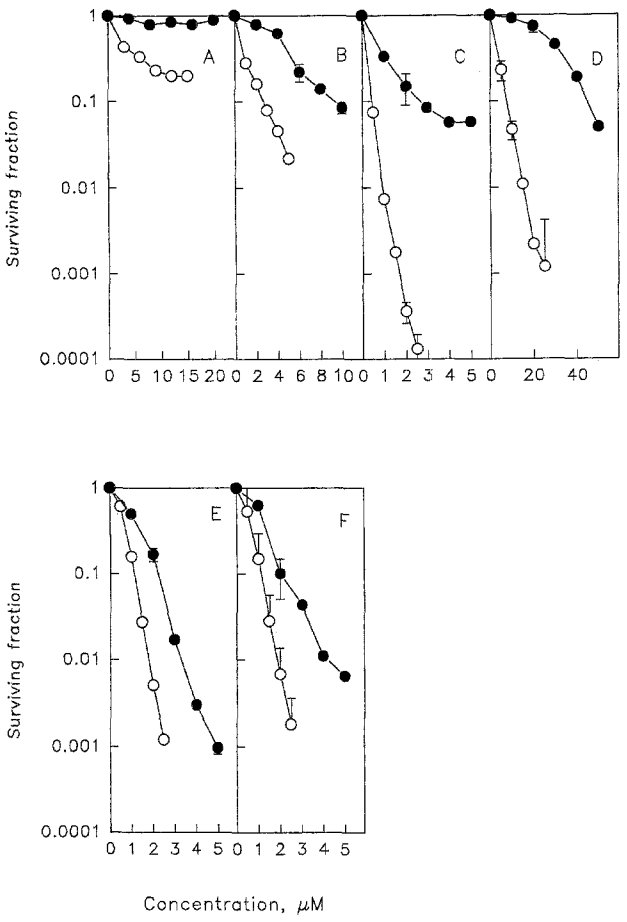

Fig. 2. A–F. Comparison of the cytotoxicity of **A** AMSA, **B** amsacrine, **C** doxorubicin, **D** etoposide, **E** AMCA and **F** mAMCA. LLTC cells were established in culture at 10^5 cells/ml and exposed to cytotoxic drugs in fresh medium after 1 day (○) or 4 days (●)

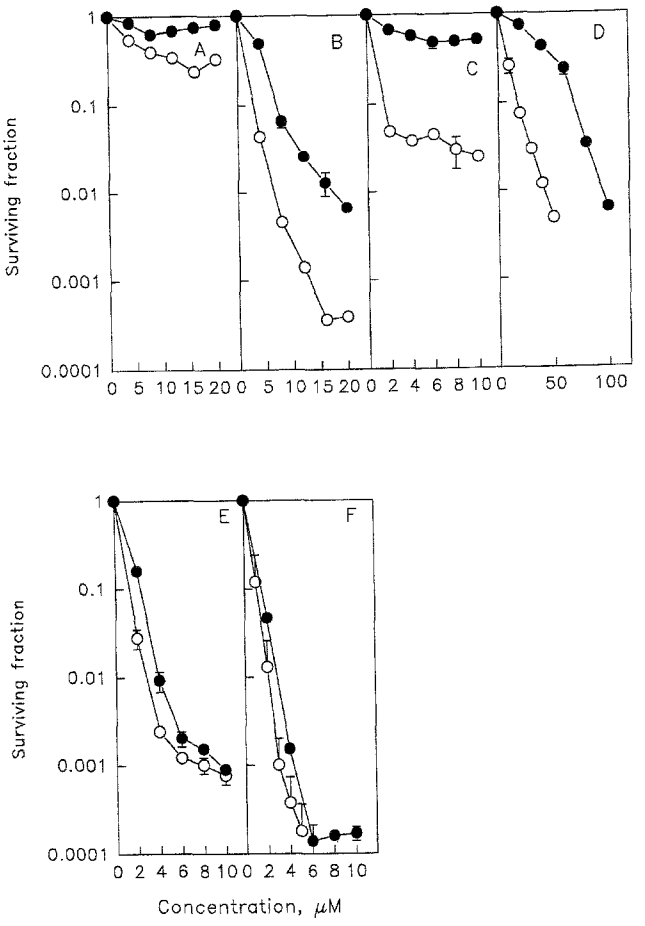

Fig. 3. A–F. Comparison of the cytotoxicity of **A** AMSA, **B** amsacrine, **C** doxorubicin, **D** etoposide, **E** AMCA and **F** mAMCA. HCT-8 cells were established in culture at 10^5 cells/ml and exposed to cytotoxic drugs in fresh medium after 1 day (○) or 4 days (●)

transferred to fresh medium for the drug exposure. AMCA and mAMCA killed plateau-phase cells more efficiently than amsacrine, AMSA, doxorubicin or etoposide (Fig. 2). The C_{10} values obtained for plateau-phase cells relative to rapidly growing cells were also lower for the carbamate compounds than for the other compounds (Table 1). Similar results were obtained for HCT-8 cells (Fig. 3) and for HT-29, LoVo and SW620 human colon carcinoma cells (results not shown).

C_{10} and C_{30} values were compared from the 1st to the 6th (LLTC) or 8th day (HCT-8) of culture to determine whether the higher activity of the latter compound was maintained as the cells entered a more profound quiescent phase of growth (LLTC cells did not survive a 7th day of culture without feeding). The C_{10} value obtained for amsacrine was unattainable after 4 or more days, whereas the C_{30} value increased with time to over 10-fold that of the day-1 value (Fig. 4). In contrast, the C_{10} and C_{30} values obtained for the carbamate analogue remained within 2- to 3-fold those of the day-1 value.

Comparison of the cytotoxicity of carbamates with that of other topoisomerase-II poisons against cells from subcutaneous tumours

Subcutaneous LLTC tumours were removed from mice, disaggregated, resuspended in fresh medium and tested for drug sensitivity either immediately after disaggregation or following overnight culture to allow resumption of proliferation. Previous experiments had established that LLTC cells isolated directly from tumours had a slightly higher G_1-phase fraction than did plateau-phase LLTC cells but that those cultured overnight showed cytokinetics similar to those of asynchronous low-density cultures [19, 24]. The activity of AMCA and mAMCA was compared with that of AMSA, amsacrine, CI-921 and doxorubicin. As shown in Fig. 5 and Table 1, 4 times as much AMCA or mAMCA was required to effect 90% killing of freshly isolated cells relative to cultured, cycling cells. For CI-921 and doxorubicin, the corresponding ratios were 10 and at least 6.6, respectively. Amsacrine did not reduce the cloning efficiency of freshly isolated cells by 90% at any concentration tested.

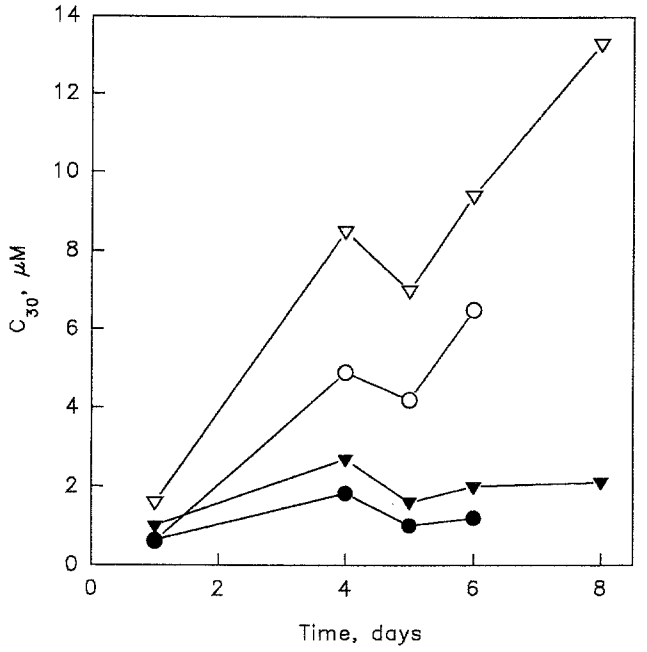

Fig. 4. Time course of drug sensitivity. LLTC cells (*circles*) or HCT-8 cells (*triangles*) were established in culture at 10^5 cells/ml for the indicated number of days and then harvested, resuspended in fresh medium and exposed to amsacrine (*open symbols*) or mAMCA (*filled symbols*)

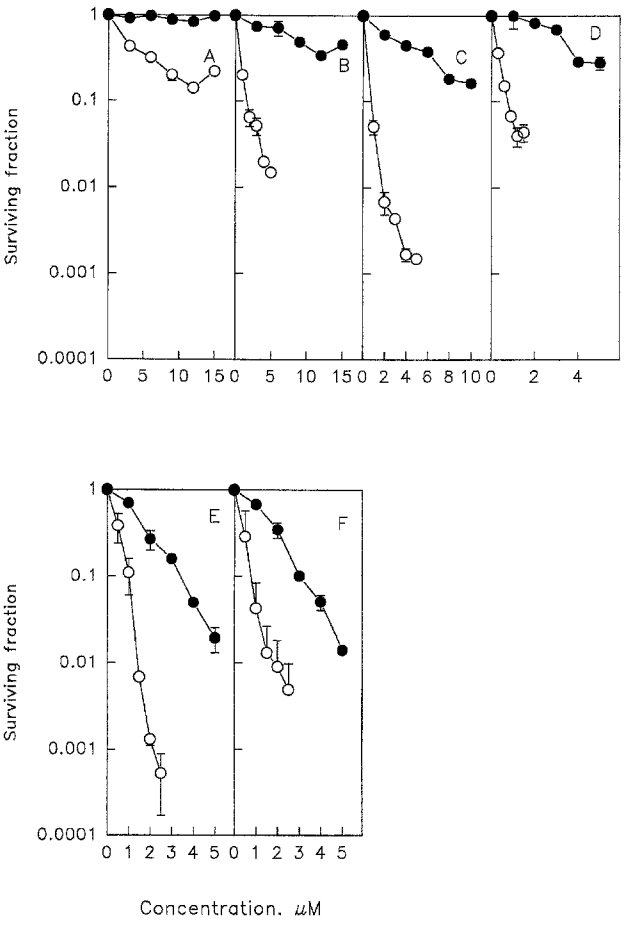

Fig. 5. A–F. Comparison of the cytotoxicity of **A** AMSA, **B** amsacrine, **C** CI-921, **D** doxorubicin, **E** AMCA and **F** mAMCA against LLTC cells recovered from large subcutaneous tumours. LLTC tumours were excised and disaggregated and the cells were exposed to cytotoxic compounds in fresh medium either immediately (●) or after culture for 1 day at 10^5 cells/ml (○)

Effects of medium depletion

To test whether components in the medium would be likely to modulate cellular sensitivity, LLTC cells that had been cultured for 6 days were harvested and resuspended in either fresh medium, spent medium (supernatant from plateau-phase cells), or spent medium supplemented with glucose or pyruvate. It was found that cells in spent medium were highly refractory to mAMCA and that sensitivity was induced to an equivalent degree by resuspending the cells in fresh medium or in spent medium containing added glucose (1 mg/ml; Fig. 6). Sensitivity was partially restored by the presence of pyruvate (2 m*M*). The role of energy availability in drug sensitivity was further shown by using the respiratory uncoupler DNP. Addition of DNP (100 μ*M*) further increased the resistance of cells in spent medium but had no effect on cells saturated with glucose. The stimulatory effect of fresh medium or of glucose on cytotoxicity was pronounced with mAMCA but progressively lower with CI-921, doxorubicin, and etoposide (Fig. 7).

Discussion

Cultured cells that have entered a non-proliferating (plateau) phase have provided one of the earliest in vitro models for the resistance of solid tumours, and decreased proliferation is associated with increased resistance to many cytotoxic drugs [34]. Such cytokinetic resistance is strongly expressed against agents that target the enzyme topoisomerase II, including doxorubicin, etoposide, amsa-

Table 1. C_{10} values obtained for exponential, plateau-phase and ex vivo LLTC cells

Compound	C_{10} (μ*M*)		Ratio	C_{10} (μ*M*)		Ratio
	Exponential (E)	Plateau (P)	(P/E)	Ex vivo (V)	After 24 h (A)	(V/A)
AMCA	1.1 ±0.0	2.5±0.2	2.3	3.5±0.1	0.92±0.11	3.9
mAMCA	1.0 ±0.1	2.6±0.6	2.6	2.5±0.5	0.63±0.13	4.0
AMSA	[a]	[a]	–	[a]	[a]	–
Amsacrine	2.1 ±0.5	10.6±0.6	5.0	[a]	1.6	–
CI-921	0.61±0.09	4.3±0.5	7.4	7.1	0.7	10
Doxorubicin	0.43	2.7	6.3	>5	0.76	>6
Etoposide	7.7	45	5.8			

[a] Minimal attainable survival >10%

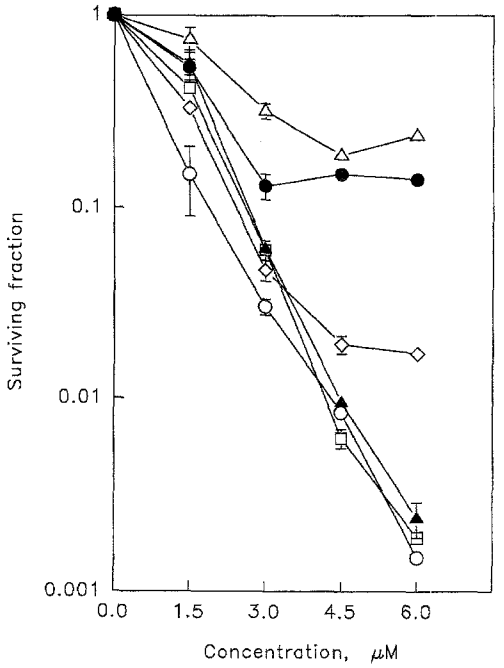

Fig. 6. Effect of modification of energy availability on the cytotoxicity of mAMCA against plateau-phase cells. LLTC cells at high density were recovered by treatment with trypsin and resuspended in fresh medium (○), spent medium (●), spent medium plus pyruvate (◇), spent medium plus glucose (□), spent medium plus 2,4-dinitrophenol (△) or spent medium plus glucose plus 2,4-dinitrophenol (▲). Cells were then exposed to mAMCA for 1 h before being washed and plated

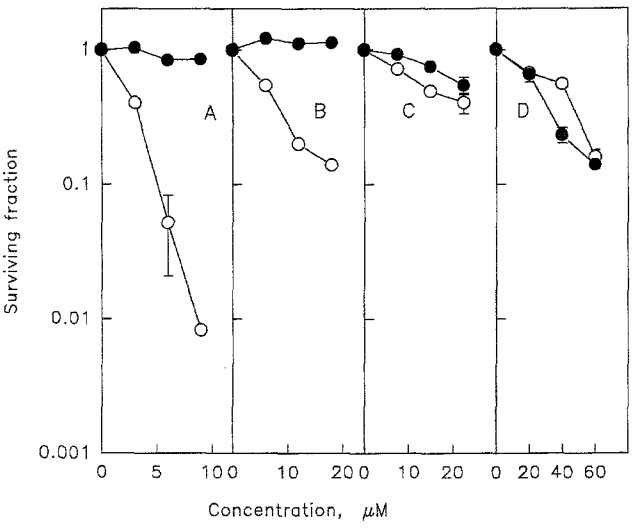

Fig. 7. A–D. Effect of modification of energy availability on the cytotoxicity of **A** mAMCA, **B** CI-921, **C** doxorubicin and **D** etoposide. LLTC cells at high density (6 days of culture) were recovered by treatment with trypsin, resuspended in spent medium (●), or spent medium plus glucose (○) and exposed to drug for 1 h before being washed and plated

crine, CI-921 and DACA [4, 14, 16, 24]. Using a standard method of trypsinization of cultured cells and clonogenic assay, we demonstrated that the amsacrine analogues AMCA and mAMCA are capable of efficient killing of plateau-phase murine LLTC and human HCT-8 cells. These compounds are capable of killing at least 99.9% of such populations with a potency that is approximately half of that required for exponentially growing cells. These results contrast with those obtained using amsacrine, doxorubicin and etoposide, whose potency is considerably reduced following culture for 4 days (Figs. 2, 3; Table 1). Incubation of cells for periods longer than 4 days further reduces drug sensitivity (Figs 4, 7). Similar results were obtained in several other human cell lines, suggesting that the phenomenon is not cell-line- or species-specific. Furthermore, analogous results are obtained when LLTC cells freshly recovered from subcutaneous tumours are exposed to drug in vitro and assayed for survival of clonogenic cells (Fig. 5, Table 1). This result, together with previous data [19, 24], suggests that tumour cell lines in the plateau phase provide informative models for the drug-sensitivity properties of solid tumours.

One possible explanation for the activity of AMCA and mAMCA on plateau-phase cultures is that they do not act on topoisomerase II but rather target DNA directly, for instance by reacting chemically with DNA. It is known that a number of DNA-reactive compounds do not distinguish between logarithmic and plateau-phase populations [14]. However, AMCA and mAMCA are not highly chemically reactive, and AMCA is known to stimulate the formation of DNA-protein cross-links [22], a property characteristic of topoisomerase poisons. Furthermore, the toxicity of AMCA is modulated by Hoechst 33342, i.e. is increased at low Hoechst concentrations and decreased at high concentrations (data not shown) in a manner similar to that reported for amsacrine [17]. It therefore seems likely that AMCA and mAMCA act on a form of topoisomerase II that is in some way altered both in plateau-phase cultured cells and in cells derived from solid tumours. It is noteworthy that doxorubicin and etoposide consistently provide survival curves of different shape with plateau-phase cells (etoposide displays a pronounced shoulder, whereas doxorubicin is less effective at high concentrations), with amsacrine providing one of intermediate shape (Figs 2, 3). This suggests differences in the way in which these drugs interact with topoisomerase enzymes. It is of interest that mAMCA is the most active of a number of amsacrine analogues in preventing the replication of herpesvirus in cultured BHK cells [21], suggesting that a similarly altered topoisomerase II is utilized for virus replication in these cells.

Plateau-phase LLTC cells as well as cells taken from solid LLTC tumours have an increased proportion with a G_1-phase DNA content relative to those that are proliferating rapidly. A high degree of linear correlation exists between the calculated G_1-phase transit time of LLTC cells and the logarithmic C_{10} values obtained for amsacrine, CI-921, doxorubicin and etoposide [24]. Topoisomerase II is degraded during the G_1 phase [23] and topoisomerase IIβ-predominates in non-proliferating cells, suggesting that the IIβ (180 kDa) isoenzyme is degraded at a lower rate than the IIα (170 kDa) isoenzyme [13, 25]. Topoisomerase IIβ is intrinsically less sensitive than topoisomerase IIα to amsacrine, doxorubicin and etoposide [10, 28]. It is known [5] that AMCA is active both in vitro and in vivo against a cell line (P/AMSA) that contains an increased proportion of topoisomerase IIβ [28]. A possible explanation for our re-

sults is therefore that AMCA and mAMCA display increased selectivity for topoisomerase IIβ. The X-ray crystal structures of 9-anilinoacridines, as well as model-building studies, suggest that the anilino substituents lie in the DNA minor groove, pointing away from the DNA, and are thus capable of making contact with a second macromolecule such as topoisomerase II [2, 22, 37]. The carbamate group of AMCA and mAMCA might provide for slightly different molecular contacts, thus allowing for such altered selectivity. Further work is required to determine whether topoisomerase IIβ is the target for AMCA and mAMCA.

AMCA and mAMCA possess in vivo activity against the Lewis lung carcinoma growing as lung nodules but have thus far proved to be inactive against LLTC cells growing as subcutaneous tumours in mice (unpublished results). The studies on nutrient availability provide clues to the cause of this resistance. Plateau-phase LLTC cells require the presence of an added energy source to respond fully to AMCA and mAMCA (Figs. 6, 7). Glucose deprivation or metabolic inhibitors such as 2-deoxyglucose, DNP and novobiocin have been shown to suppress the cytotoxicity of a variety of topoisomerase-II poisons [11, 26, 30]. Both glucose starvation and 2-deoxyglucose induce resistance to doxorubicin and etoposide in Chinese hamster ovary (CHO) cells by a process that takes several hours to develop, involves the loss of topoisomerase II from the cell nucleus and may involve the induction of glucose-regulated proteins. DNP and novobiocin, which can affect mitochondrial energy production [12, 31], have little effect on the production of amsacrine-induced protein-DNA cross-links or DNA breaks and therefore appear to act at a step subsequent to the generation of topoisomerase II-DNA complexes [31, 35]. As can be seen from the results reported herein (Fig. 6), DNP increases the resistance of plateau-phase cells to mAMCA in the absence of glucose but does not affect mAMCA activity in the presence of added glucose. Thus, energy deprivation in plateau-phase LLTC cells results in a rapidly reversible resistance mechanism that is distinct from the adaptive response to glucose deprivation in CHO cells, which takes several hours for complete reversal after glucose re-addition [11, 26, 30]. The reduction of the drug sensitivity of plateau-phase LLTC cells by nutrient deprivation suggests that nutrient deprivation in LLTC tumours growing in vivo may contribute to the lack of drug sensitivity.

In conclusion, the identification of AMCA and mAMCA as having unexpectedly high activity against non-cycling cells suggests a new strategy for drug design: modifying topoisomerase poisons to take advantage of altered expression of topoisomerase in these cells. A number of new compounds with properties similar to those of AMCA and mAMCA have now been produced in this laboratory and are undergoing further testing. The results shown in Figs. 6 and 7 suggest that successful in vivo therapy of non-cycling cell populations may require the combination of an appropriately designed drug with a strategy for counteracting the nutrient deprivation of this tumour cell population.

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