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Original Paper

Cellular Responses to Methyl-*N*-[4-(9-Acridinylamino)-2-Methoxyphenyl]Carbamate Hydrochloride, an Analogue of Amsacrine Active Against Non-proliferating Cells

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The acridine derivative m-AMCA (methyl-*N*-[4-(9-acridinylamino)-2-methoxyphenyl]carbamate hydrochloride), a carbamate analogue of the toposiomerase II poison amsacrine, is distinguished by its high cytotoxicity against non-cycling tumour cells. We compared the response of cultured Lewis lung carcinoma cells to m-AMCA, amsacrine and the topoisomerase I poison camptothecin. The DNA polymerase inhibitor aphidicolin reversed the cytotoxicity of camptothecin fully, that of amsacrine partially, and that of m-AMCA minimally. The ability of m-AMCA to induce the enzyme poly(ADP-ribose)polymerase (PARP) was markedly lower than that of camptothecin or amsacrine. Cell cycle responses to m-AMCA and amsacrine were similar, with slowing of progress through S-phase and arrest in G_2-phase. These cell cycle changes were also observed when plateau phase cultures were exposed to drug for 1 h, washed free of drug and cultured in fresh medium, with m-AMCA having a more pronounced effect than amsacrine and camptothecin having no effect. We also examined the role of p53 protein in the response using cultured human H460 cells. Both m-AMCA and amsacrine induced p53 protein expression in proliferating but not in non-proliferating H460 cells, and induced $p21^{WAF1}$ regardless of proliferation status. Both induced G_1-phase cell cycle arrest. It is suggested that two cytotoxicity mechanisms can be distinguished using these drugs. The first is specific for S-phase cells, is reversed by aphidicolin and induces PARP activity. The second is cell cycle non-specific, does not induce PARP and is unaffected by aphidicolin. Camptothecin activates only the first, m-AMCA primarily the second and amsacrine activates both. © **1997 Elsevier Science Ltd.**

Key words: topoisomerase, p53, poly(ADP)ribose polymerase, amsacrine and cell cycle
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INTRODUCTION

DRUG-RESISTANT CELL populations within solid tumours are primarily responsible for the ineffectiveness of cancer chemotherapy and are of particular importance to the action of drugs which target the enzyme DNA topoisomerase II [1, 2]. As part of an approach to design new topoisomerase II-directed agents that overcome resistance mechanisms, three drugs developed in this laboratory have progressed to clinical trial. These drugs, amsacrine [3], the amsacrine analogue asulacrine (9-[2-methoxy-4-methylsulphonylamino)phenylamino]-*N*,5-dimethyl-4-acridinecarboxamide) [4] and *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide [5], are all able to overcome one or more resistance mechanisms [6–9]. However, each of these, like currently available topoisomerase II-directed clinical anticancer drugs, have greatly reduced activity against non-cycling cells [10], highlighting the importance of cytokinetic resistance in limiting cancer therapy.

We have previously reported that the drug methyl *N*-[4-(9-acridinylamino)-2-methoxyphenyl]-carbamate hydro-

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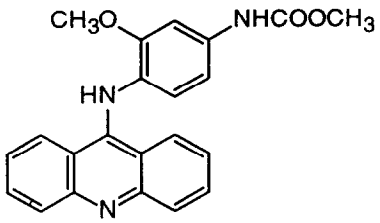
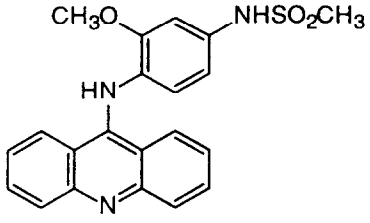


m-AMCA



Amsacrine

Figure 1. Structure of m-AMCA (a) and amsacrine (b) in the free base forms.

chloride (m-AMCA), the carbamate analogue of amsacrine (see Figure 1 for structures) is active *in vitro* and *in vivo* not only against cell lines exhibiting some types of multidrug resistance [6], but also against plateau phase cultured carcinoma cells [11]. Cells growing at a high density in culture provide a good model for the cytokinetics of the same cells growing as subcutaneous tumours in mice [10–12]. m-AMCA is therefore of particular interest in the development of new drugs for slowly growing tumours. It appears to target topoisomerase II since resistant lines with reduced topoisomerase II exhibit cross-resistance to m-AMCA [13, 14]. Studies with purified topoisomerase II indicate that although small differences are evident in the sequence preferences for DNA cleavage in response to m-AMCA and amsacrine, both drugs target topoisomerase IIα and IIβ [14].

In this paper, we have attempted to distinguish the cellular response to m-AMCA from that to the classical topoisomerase II poison amsacrine, comparing it also with the topoisomerase I poison camptothecin. Firstly, we have compared the effect of the drugs on the cytokinetics of cultured cell lines, one of which expresses wild-type p53 protein and one of which has a mutant phenotype [15, 16]. Secondly, we have measured the induction of the enzyme poly(ADP-ribose)polymerase (PARP) and the expression of p53 and $p21^{WAF1}$ proteins as internal indicators of DNA damage. PARP is a chromatin bound enzyme that catalyses the attachment of poly(ADP-ribose) to nuclear proteins, using NAD^+ as a substrate, and its activity is stimulated by the presence of single- or double-strand DNA breaks [17]. The p53 protein activates the transcription of a number of genes in response to DNA damage [18]. The p21 protein, the synthesis of which is stimulated by activated p53 protein, has a variety of actions, particularly in relation to regulation of the cell division cycle [19].

METHODS AND MATERIALS

Materials

Anilinoacridine derivatives were synthesised in this laboratory by W. A. Denny, G. J. Atwell and G. W. Rewcastle. PD 128763 (3,4-dihydro-5-methyl-1(2*H*)-isoquinoline; PD128) was kindly supplied by the Parke-Davis Division of the Warner-Lambert Co., Ann Arbor, Michigan, U.S.A. Aphidicolin was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Cell lines

The LLTC cell line was developed from the Lewis lung carcinoma [20] at the Southern Research Institute, Birmingham, Alabama, U.S.A. and was originally provided by the Parke-Davis Division of the Warner-Lambert Co. The H460 cell line was originally derived from the pleural fluid of a male with a large cell lung carcinoma in 1982 [21]. Cells were taken from frozen stock (approximately 10^6 cells/tube) and passaged weekly for approximately 4 months. Cells were cultured in α-modified minimal essential medium (α-MEM) supplemented with fetal bovine serum (FBS), penicillin and streptomycin as previously described [8, 11].

Poly(ADP-ribose) synthesis assay

LLTC cells were plated at 10^5 cells/ml in 100 mm dishes containing 15 ml α-MEM supplemented with FBS (5%) per plate as previously described [22, 23]. Cells were exposed to the topoisomerase poison for 1 h at 37°C, and cells were then detached from the plates by treatment with 0.07% trypsin in citrate saline and collected by centrifugation. Poly(ADP-ribose) synthesis was determined according to the procedure of Benjamin and Gill [24]. Cells were resuspended in a reaction buffer (2.5 ml) containing 5% dextran T-150, 10 mM $MgCl_2$, 0.05% Triton X-100, 30 μM non-radioactive NAD^+, [^{3}H]-NAD^+ (0.3 μCi/ml) and 40 mM Tris-HCl, pH 8.0. After the pellet was dispersed, the reaction mixture was incubated at 35°C. Aliquots (1 ml) were withdrawn after 10 min and added to 2 ml of ice cold 10% trichloroacetic acid (TCA). The precipitates were collected on Whatman GF/C filters (Whatman, Maidstone, U.K.), washed three times with 10% TCA, dried and their radioactivity measured by liquid scintillation counting. [^{3}H]-ADP ribose incorporation in treated cells was expressed as a ratio to that in untreated cells.

Cell proliferation assays

LLTC cells were incubated at 37°C in 96-well plates at a density of 1000 cells per well (150 μl total volume) with appropriate inhibitors for 4 days. Growth inhibition was determined after fixing the cultures by staining with 0.4% (w/v) sulphorhodamine B in 1% acetic acid [25]. Unbound dye was removed by four washes with 1% acetic acid and protein-bound dye was extracted with 10 mM unbuffered Tris. A 96-well microplate photometer (MR 600, Dynatech Instruments Inc., California, U.S.A.) was used for determination of absorbance with 570 nm as the sample wavelength and 410 nm as the reference wavelength. The IC_{50} was defined as the drug concentration required to reduce the number of cells in a culture by 50% with respect to control cultures.

Clonogenic assays

LLTC cells were plated at 10^5 cells/ml and grown for 17–18 h to produce exponential phase cells and 4 days to produce plateau phase cells. The proliferation status of the cultures was assessed by flow cytometry using propidium staining of DNA [12]. Exponential phase cells contained

32% G_1-phase and 62% S-phase cells, while plateau phase cells contained 91% G_1-phase and 2% S-phase cells. Cells were recovered from culture plates by treatment with 0.07% trypsin in citrate saline, collected by centrifugation and resuspended in tubes containing growth medium/10% FBS (5 ml) at 10^5 cells/ml. Aphidicolin was added to tubes at a concentration (3 μM) that was previously determined to be optimal for reversal of amsacrine toxicity [26]. After 15 min, camptothecin, m-AMCA or amsacrine was added for a further 1 h incubation. The cells were then collected by centrifugation to remove drug-containing medium, washed twice. Cells were resuspended in growth medium/5% FBS, counted in a particle counter (Coulter Electronics, Australia), diluted serially and plated in 60 mm dishes containing 5 ml growth medium/5% FBS. After 10 days the cultures were fixed and stained with methylene blue (5 g/l) in 50% aqueous ethanol. Colonies comprising over 50 cells were counted.

Flow cytometry

Methods for the analysis of DNA content were similar to those used previously [12]. Cells were collected by centrifugation, washed in phosphate-buffered saline (PBS; 2 ml), fixed in methanol (1 ml) and stored at −20°C until flow cytometric analysis. Samples were resuspended in PBS/0.5% Tween 20 (2 ml) together with ribonuclease A (50 μl, 10 mg/ml) and incubated at 37°C for 20 min. After centrifugation, the pellet was resuspended in PBS (0.5 ml) with propidium iodide (25 μg/ml) and kept at room temperature for 15 min. The cell suspension was analysed on an FACScan flow cytometer (Beckton Dickinson, San Jose, California, U.S.A.) with correction for cell doublets. Data were processed using MULTIPLUS software (Phoenix Flow Systems, San Diego, California, U.S.A.).

In order to measure p53 content by immunofluorescence, H460 cells were plated at 10^5 cells/ml in 60 mm dishes containing 5 ml of α-MEM supplemented with FBS (5%) per plate. After overnight culture, cells were treated for 4–24 h with drug at a concentration which provided a surviving fraction of approximately 10%. Cells were detached from plates by treatment with 0.07% trypsin in citrate saline, collected by centrifugation and fixed in 1.6 ml of 0.5% paraformaldehyde in PBS for 5 min. Fixed cells were divided into duplicate samples, centrifuged and permeabilised in 1 ml of ice-cold 0.1% TritonX-100 in PBS for 3 min. After further centrifugation, samples were washed in 2 ml of ice-cold PBS supplemented with FBS (2%) and resuspended in 100 μl of PBS/FBS (2%). Human specific fluoresceinated anti-p53 mouse monoclonal antibody (5 μl; DAKO-p53/DO-7, supplied by DAKO Corporation, Carpinteria, California, U.S.A.) was added to one sample at a concentration of 1 mg antibody/10^6 cells while fluoresceinated isotype mouse IgG antibody (DAKO) was added at the same concentration to the other. Cells were incubated for 1 h at 4°C, washed, and resuspended in ice-cold PBS/FBS (2%) for analysis by flow cytometry.

Immunohistochemistry

Exponential phase H460 cells were exposed to amsacrine (1.25 μM) or m-AMCA (0.75 μM) for 4 h. Plateau phase H460 cells were detached, resuspended to 10^5 cells/ml and exposed to amsacrine (2.5 μM) or m-AMCA (1.5 μM) for 4 h. Cells were washed with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. Fixed cells were stored in PBS until immunohistochemical staining was performed. Cells were incubated overnight with a specific polyclonal antiserum (200 μl/well) diluted in immunobuffer (PBS plus methiolate). The anti-

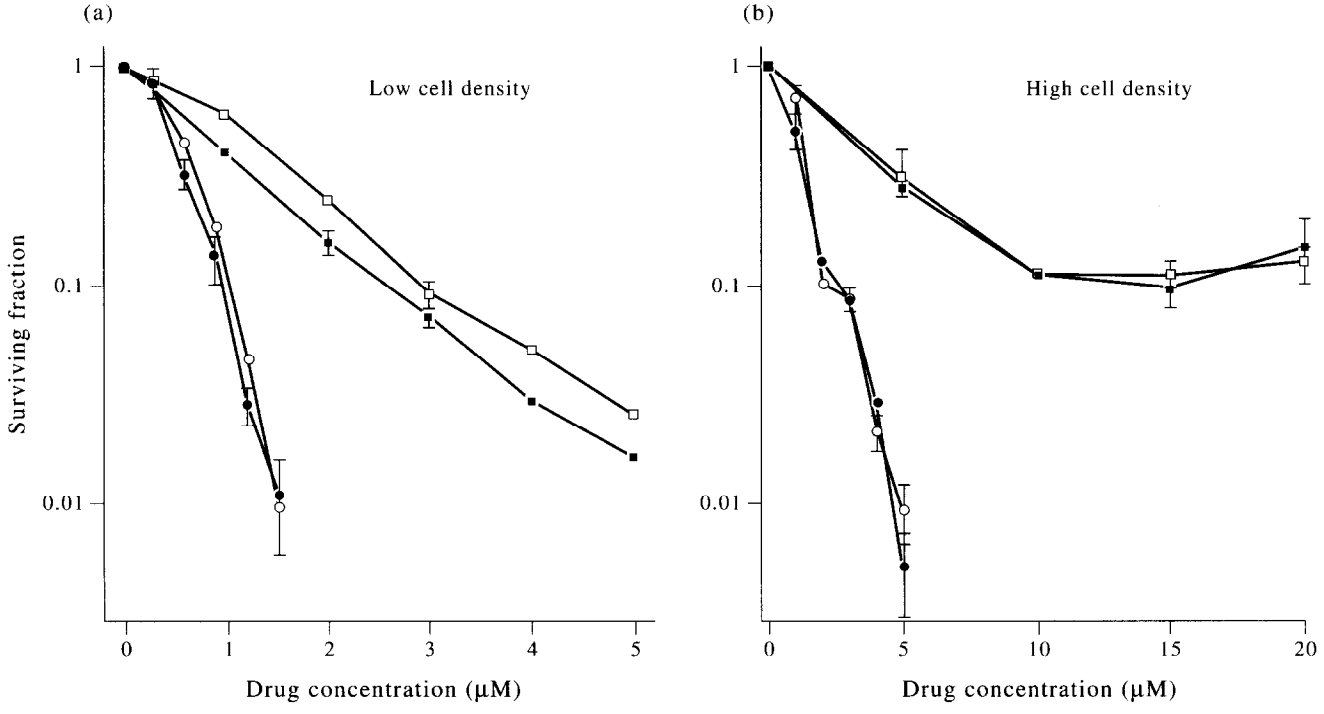


Figure 2. Comparison of the cytotoxicity of amsacrine ■, and m-AMCA ●, against LLTC cells in exponential phase (a) or in plateau phase (b) incubated with drug for 1 h in the presence (□, ○) or absence (■, ●) of aphidicolin (3 μM). Cells were then assayed for survival.

bodies were as follows: p53 (DAKO DO-7, 1:250 dilution, mouse), p21 (designated p21A, Santa Cruz SC397, 1:500 dilution, rabbit) and WAF1 (designiated p21B, Oncogene Science #OP64,1:250 dilution, mouse). After the primary antibody solution was removed, cells were washed three times in PBS (5 min per wash) and incubated overnight at 4°C with biotinylated goat anti-rabbit (or mouse) serum, diluted in immunobuffer. This was followed by three washes (PBS) and incubation with peroxidase (ExtrAvidin, 250 µl, 1:250 dilution) for 2 h at room temperature. After removal of the peroxidase and 3 washes, the reaction was visualised by the addition of diaminobenzidine (DAB; 0.5 mg/ml in 0.1 M phosphate buffer) with H_2O_2 (0.01%) for 10 min. DAB was removed, cells were washed twice in PBS and then left in PBS for viewing. Cells were viewed and photographed using an inverted microscope and camera. Five fields of view, corresponding to between 200 and 500 cells, were counted in each well to estimate the percentage of positively stained cells. Examination of plates for all treatments was conducted in a single session to ensure uniformity of scoring.

RESULTS

Effect of aphidicolin on m-AMCA and amsacrine activity

In order to determine whether the cytotoxicity of m-AMCA was dependent on DNA replication, experiments incorporating the DNA synthesis inhibitor aphidicolin were performed. This is known to reduce the cytotoxicity of amsacrine, etoposide and camptothecin [26–29]. LLTC cells growing both in exponential phase (50% cells in S-phase) and in plateau phase (minimal cells in S-phase) were tested. In exponential phase cell cultures, aphidicolin (3 µM) reversed the cytotoxicity of camptothecin (2 µM; data not shown), but had no effect on that of m-AMCA. The reduction by aphidicolin of the cytotoxicity of amsacrine (Figure 2) was reproducible, significant ($P < 0.05$) and of a magnitude which was consistent with other reports [26, 30]. Aphidicolin did not affect the cytotoxicity of amsacrine or m-AMCA in plateau phase cells (Figure 2(b)).

Induction of PARP by topoisomerase poisons

LLTC cells were treated with different DNA damaging agents for 1 h at cytotoxic concentrations then analysed for PARP activity (Table 1). As expected [31], camptothecin and amsacrine induced increases in NAD incorporation indicative of PARP induction. In contrast, cells exposed to m-AMCA showed a barely detectable elevation in PARP activity. In a converse experiment, the effect of the potent PARP inhibitor PD128 [32] was tested using cultures of LLTC cells exposed for 4 days to drugs either alone or in the presence of PD128 (250 µM). Addition of PD128 caused decreases in the IC_{50} values from 8.5 to 3.7 nM for amsacrine, from 46 to 7.8 nM for camptothecin, but only from 37 to 26 nM for m-AMCA.

Table 1. Induction of PARP by topoisomerase poisons. LLTC cells were exposed to topoisomerase poisons at equitoxic concentrations for 1 h. After this cells were permeabilised and [^{3}H]-NAD incorporation was measured. The control incorporation was 370 ± 39 cpm (mean ± SEM)

Treatment	Concentration (µM)	Ratio to control
Camptothecin	2	2.0
Amsacrine	10	2.2 ± 0.3
m-AMCA	6	1.3 ± 0.2

Effects of topoisomerase poisons on the cell cycle of exponential phase LLTC cells

In order to examine the effects of m-AMCA on the cell cycle, LLTC cells were exposed to m-AMCA (3 µM) or amsacrine (5 µM) for 1 h, sufficient to provide approximately 99% reduction in cell viability (Figure 2). Cells were also exposed to camptothecin (1 µM) for 1 h, sufficient to kill the entire S-phase fraction. Cells were washed to remove drug and incubated in fresh growth medium for various times up to 23 h, then analysed by flow cytometry. Results from multiple profiles were used to calculate the proportions of cells in each phase of the cell cycle, and typical profiles are shown in Figure 3. After a 1 h exposure to m-AMCA or amsacrine followed by incubation in fresh growth medium for 7 h, the G_1-phase fraction had virtually all entered S-phase, while pre-existing S-phase cells had been slowed or arrested in the cell cycle. The DNA content of the resulting S-phase component increased with time and had moved to the G_2/M-phase position by 24 h. In contrast, cells exposed to camptothecin showed a progressive decrease in G_1-phase and a corresponding increase of cells in G_2/M-phase with time, with little increase in S-phase content.

Effects of topoisomerase poisons on the cell cycle of plateau phase LLTC cells

We wished to determine whether cells exposed to drugs in the plateau phase exhibited subsequent changes in cytokinetics upon re-entry into cycle. LLTC cells were exposed for 1 h to m-AMCA (6 µM), amsacrine (20 µM) or camptothecin (10 µM). For m-AMCA and amsacrine, this corresponded to a similar degree of cytotoxicity to that used for exponential phase cells. Cells were washed following drug exposure and allowed to grow for various times in drug-free medium before analysis by flow cytometry. Typical profiles, representative of many experiments, are shown in Figure 4. The proportion of cells in G_1-phase immediately after drug exposure was 91%, consistent with cells being out of cycle. For both control cells and cells treated with each of the three drugs, the proportion of cells in G_1-phase remained constant up to 8 h after drug treatment, then fell progressively to less than 30% by 18 h after drug treatment, indicating that entry into S-phase was not affected by the presence of drug. By 24 h after drug treatment there was a divergence in the response of the cells to different drugs. For both control and camptothecin treated cells, the percentage of G_1-phase cells increased as cells re-entered G_1. In contrast, for cells treated with m-AMCA and amsacrine, a progressive accumulation in late S- and G_2-phase occurred, accompanied by a further decrease in the proportion of G_1-phase cells. The effect was greater for m-AMCA than for amsacrine.

Effects of topoisomerase poisons on the cell cycle of exponential phase H460 cells

In order to compare the effect of the drugs on a cell line expressing a functional p53 pathway, H460 cells were exposed to the three drugs under similar conditions to those used for LLTC cells. The time of culture after a 1 h drug exposure was increased from 7 h to 17 h, in keeping with the longer cycle time of this cell line. Typical profiles for

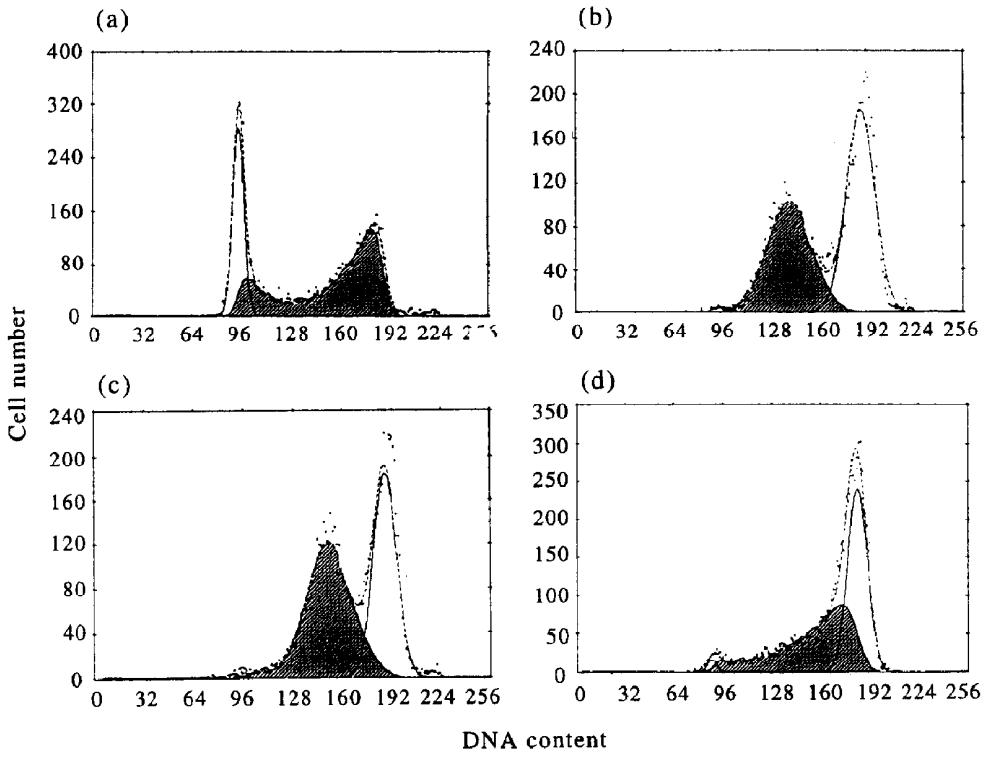


Figure 3. Flow cytometric analysis of propidium-stained exponential phase LLTC cells 7 h after an incubation for 1 h with (a) no drug, (b) m-AMCA (3 μM), (c) amsacrine (5 μM) or (d) camptothecin (1 μM). Fitted G_1-phase, S-phase and G_2/M-phase components are shown for each profile, with the S-phase component shaded for clarity.

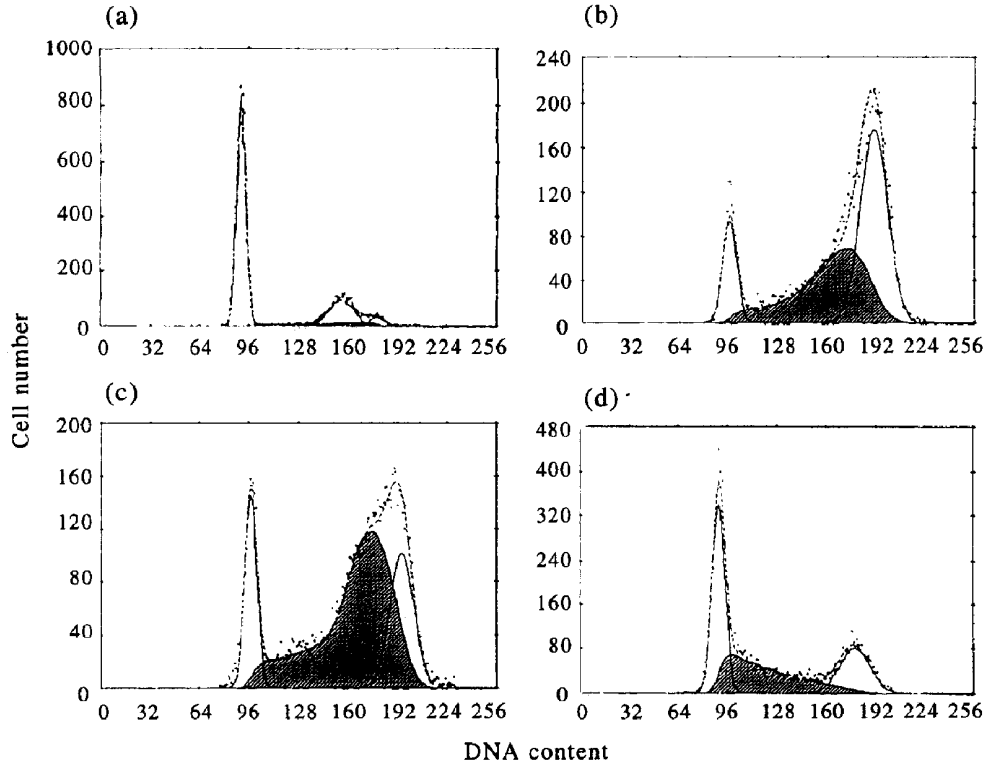


Figure 4. Flow cytometric analysis of propidium-stained plateau-phase LLTC cells at the beginning of the incubation (a) and then 23 h after a 1 h incubation with (b) m-AMCA (6 μM), (c) amsacrine (20 μM) or (d) camptothecin (10 μM). Fitted G_1-phase, S-phase and G_2/M-phase components are shown for each profile, with the S-phase component shaded for clarity.

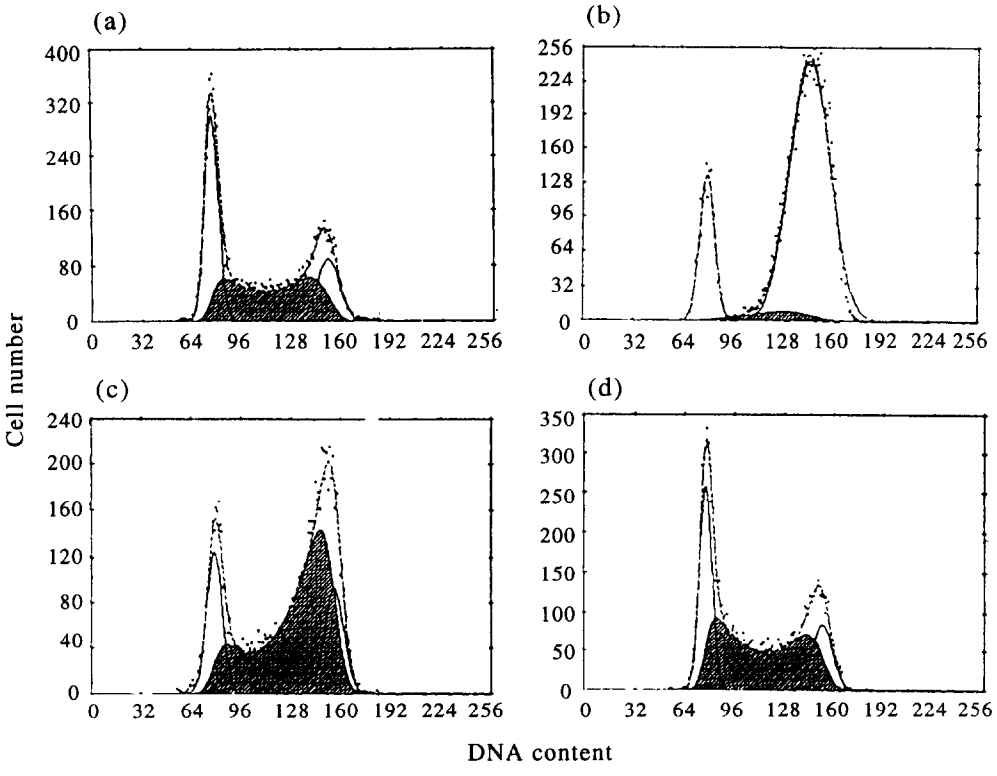


Figure 5. Flow cytometric analysis of propidium-stained exponential phase H460 cells at the beginning of the incubation (a) and then 17 h after a 1 h incubation with (b) m-AMCA (3 μM), (c) amsacrine (5 μM) or (d) camptothecin (1 μM). Fitted G_1-phase, S-phase and G_2/M-phase components are shown for each profile with the S-phase component shaded for clarity.

control, m-AMCA, amsacrine and camptothecin treated cells are shown in Figure 5. S-phase slowing was observable 6 h after a 1 h exposure to m-AMCA, consistent with that seen with LLTC cells. However, in contrast to LLTC behaviour, a proportion of cells was arrested in G_1-phase. After 18 h, virtually all S-phase cells had moved through to G_2/M-phase. In the case of amsacrine, S-phase cells accumulated at 6 h but were still present after 18 h, indicating an apparently increased effect on S-phase transit relative to that of m-AMCA. Camptothecin treated cells did exhibit some S-phase slowing at 6 h but this was not followed by a significant G_2/M-phase block at 18 h.

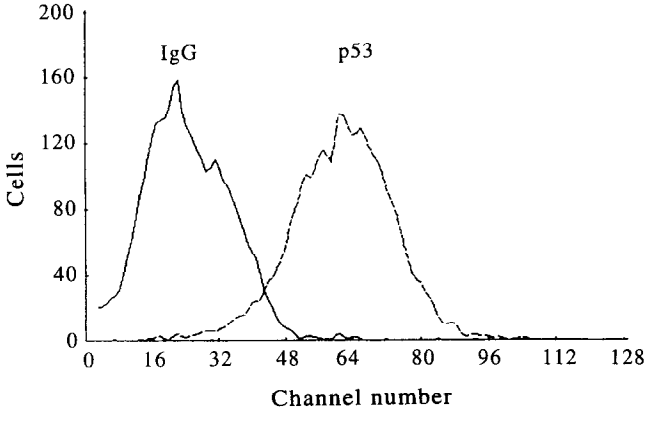


Figure 6. Fluorescence intensity of antibody staining in exponential-phase H460 cells exposed to m-AMCA (0.75 μM) for 16 h. Cells stained with antibody to p53 are shown by the broken line and cells stained with isotype antibody by the solid line.

Induction of p53 and p21 proteins

The above results suggested an induction of the p53 pathway was occurring in H460 cells, leading to expression of

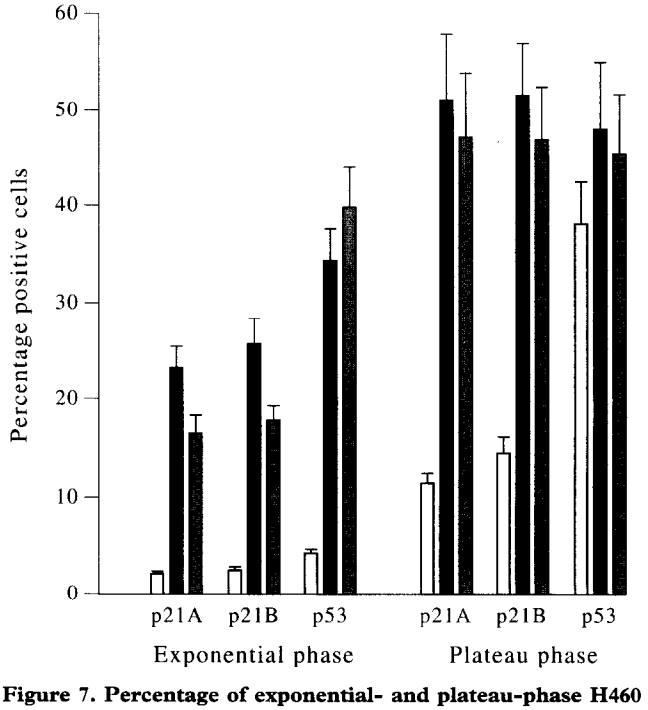


Figure 7. Percentage of exponential- and plateau-phase H460 cells staining positively to antibodies to p53 or p21 proteins (p21A and p21B indicate two different antibody preparations) in the absence of drug (unshaded bars), following treatment with amsacrine (black) and following treatment with m-AMCA (shaded).

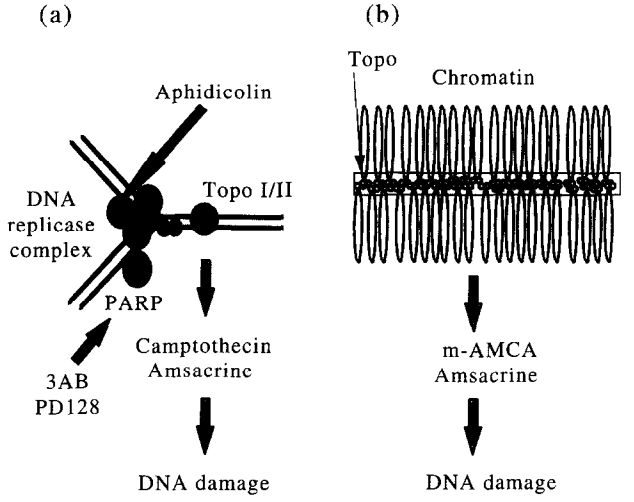


Figure 8. A model for two pathways of topoisomerase induced DNA damage. The first (a) is specifically stimulated by camptothecin. It occurs only in S-phase and is modulated by the DNA replication inhibitor aphidicolin, by prior inhibition of PARP activity, or by elimination of S-phase cells at high culture density. The second pathway (b) is specifically stimulated by m-AMCA. It is cell cycle phase non-specific and relatively insensitive to aphidicolin, PARP inhibition and proliferation status. Amsacrine stimulates both pathways.

$p21^{WAF1}$ and consequent G_1-phase arrest. The induction of p53 protein was measured in exponential phase H460 cells by flow cytometry in response to m-AMCA and amsacrine. Induction was observed both 4 h and 16 h after exposure to either drug; results for m-AMCA are shown in Figure 6. When plateau-phase cells were similarly exposed to m-AMCA or amsacrine, the intensity of p53 staining was not above that of the isotype antibody control.

To investigate whether $p21^{WAF1}$ was induced in H460 cells, an immunohistochemical detection method was used to score individual cells for the induction of both p53 and p21 proteins following exposure to m-AMCA and amsacrine. Increases in the proportion of cells staining for both p53 and p21 proteins were observed in exponential-phase cells, while in plateau-phase cells increased staining was observed only for p21 protein (Figure 7). Untreated plateau-phase cells appeared to stain more frequently for p53 protein than exponential-phase cells. It was difficult to relate this to the flow cytometric studies, since in the latter a single broad distribution was observed with a maximum at similar staining intensity to that of exponential-phase cells.

DISCUSSION

Our results demonstrate both similarities and differences in the actions of m-AMCA and amsacrine. m-AMCA is similar to amsacrine in its induction of p53 and its perturbation of the cell cycle of both exponential- and plateau-phase cells. However, it contrasts with amsacrine and camptothecin [28] in that its activity is unaffected by the DNA polymerase inhibitor aphidicolin (Figure 2). Its activity is slightly affected by the PARP inhibitor PD128, and it is clear from Table 1 that m-AMCA is a poor inducer of PARP. PARP copurifies with DNA replication forms [33], suggesting that along with DNA polymerase it is part of the DNA replicative apparatus. From these two observations, m-AMCA appears to exert little of its cytotoxic action through disruption of the replicative apparatus.

Studies with purified topoisomerase II firmly establish the ability of m-AMCA to promote enzyme-mediated DNA cleavage [14]. Western blotting of exponential- and plateau-phase Lewis lung carcinoma cells indicates that a 2-fold decrease in cellular sensitivity to m-AMCA is associated with a 10-fold decrease in topoisomerase II (G.J. Finlay, unpublished results), suggesting that only a fraction of cellular topoisomerase II is a target for m-AMCA. It is known that topoisomerase II exists in two forms of differing extractability. A proportion is bound tightly to the chromosome matrix, associated with the bases of chromatin loops, while the remainder is in a more easily extractable form, probably associated with transcription and replication complexes [34–36]. It has been suggested that exchange between subunits of adjacent topoisomerase II homodimers at the bases of chromatin loops, which would result in a non-homologous recombination and DNA deletions, is stimulated by topoisomerase II poisons such as amsacrine and etoposide [37]. The lack of dependence of m-AMCA on DNA replication suggests that topoisomerase II on chromatin loops forms the target enzyme for m-AMCA. Our preliminary results indicate that m-AMCA also induces large DNA deletions, consistent with the proposed mechanism.

m-AMCA and amsacrine cause DNA damage as measured by the formation of protein-linked DNA breaks [9]. We have determined the cellular response to DNA damage induced by these drugs, asking in particular whether the response of non-proliferating cells is similar to that of proliferating cells. Treatment of exponential phase LLTC induces slowing of progress in S-phase and arrest in G_2-phase (Figure 3), a typical response to DNA damage. Notably, when plateau-phase LLTC cells are exposed to drugs, washed to remove drug and allowed to grow, the progress of cells through the cycle is slowed in S-phase and subsequently arrested in G_2-phase (Figure 4). The similarity of the cellular responses under two different proliferative conditions argues strongly that both m-AMCA and amsacrine induce similar DNA lesions and that the cell reacts to this damage by cell cycle changes which, in the case of plateau-phase cells, occur many hours after drug exposure. The results contrast with those with camptothecin, where exposure in plateau phase has no effect on the subsequent cell cycle behaviour.

It is clear that m-AMCA does not cause G_1-phase arrest of LLTC cells (Figures 3 and 4). LLTC cells have a mutant p53 pathway, raising the question of whether p53 induction in a wild-type cell line could be used as an internal measure of DNA damage. Experiments carried out with H460 cells, which have an intact p53 pathway, confirm that m-AMCA and amsacrine induce not only S-phase slowing and G_2-arrest, but also G_1-phase arrest (Figure 5). This suggests in turn that m-AMCA and amsacrine activate a DNA damage-sensitive pathway of p53 activation with consequent increases in the amounts of $p21^{WAF1}$ and G_1-phase arrest [19]. The H460 cell line is highly sensitive to topoisomerase II-directed agents [8], possibly implicating the p53 pathway as contributing to chemosensitivity.

Both camptothecin and amsacrine are known to induce p53 protein production, with that by camptothecin being dependent on DNA replication [38]. In comparing cellular responses of H460 cells to m-AMCA and amsacrine, both flow cytometric (Figure 6) and immunohistochemical (Figure 7) assays have been used to identify cells with

increased p53 protein content. The assay systems confirm the induction of p53 protein following exposure of exponential phase H460 cells to m-AMCA and amsacrine, consistent with the observed cell cycle effects. The level of $p21^{WAF1}$ protein becomes elevated in exponential-phase H460 cells in response to m-AMCA and amsacrine (Figure 7). Surprisingly, in plateau-phase cells drug exposure leads to an increase in the amounts of p21 protein staining but without a corresponding increase in p53 protein staining. It is possible that the p53 protein in plateau-phase cells becomes activiated in response to DNA damage and increases the synthesis of p21 but not of itself.

In conclusion, the results are consistent with a model incorporating two topoisomerase-mediated cytotoxicity mechanisms, as shown in Figure 8. We hypothesise that in both proliferating and non-proliferating cells, topoisomerase II, probably associated with chromatin, forms the target for the action of m-AMCA and amsacrine. It is evident from Figure 2 that m-AMCA is approximately 5-fold more potent than amsacrine in this activity. The other (S-phase specific) cytotoxicity mechanism involves the DNA replication complex, is activated selectively by camptothecin, and is an important mechanism for amsacrine cytotoxicity. The reason that m-AMCA is ineffective against this mechanism is as yet unclear. While further work is in progress to resolve this issue, it is clear that the unique action of m-AMCA on topoisomerase II will provide a useful means of further understanding this enzyme, as well as better exploiting it as a means of cancer therapy.

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