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Original Paper

A Carbamate Analogue of Amsacrine with Activity Against Non-cycling Cells Stimulates Topoisomerase II Cleavage at DNA Sites Distinct From Those of Amsacrine

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AMCA (methyl *N*-[4-(9-acridinylamino)-2-methoxyphenyl]carbamate hydrochloride), an amsacrine analogue containing a methylcarbamate rather than a methylsulphonamide side chain, contrasts with amsacrine, doxorubicin and etoposide in its relatively high cytotoxicity against non-cycling tumour cells. AMCA bound DNA more tightly than amsacrine, but the DNA base selectivity of binding, as measured by ethidium displacement from poly[dA-dT].[dA-dT] and poly[dG-dC].[dG-dC], was unchanged. AMCA-induced topoisomerase cleavage sites on pBR322, *C-MYC* and SV40 DNA were investigated using agarose or sequencing gels. DNA fragments were end-labelled, incubated with purified topoisomerase II from different mammalian sources and analysed after treatment with sodium dodecylsulphate/proteinase K. AMCA stimulated the cleavage activity of topoisomerase II, but the DNA sequence selectivity of cleavage was different from that of amsacrine and other topoisomerase inhibitors. It was similar to that of the methoxy derivative of AMCA, indicating that the changed specificity resulted from the carbamate group rather than from the methoxy group. The pattern of DNA cleavage induced by AMCA was similar for topoisomerase IIα and IIβ. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: topoisomerase, acridine, intercalation, Lewis lung

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INTRODUCTION

A WIDE variety of antitumour agents, including the anthracyclines, epipodophyllotoxins, amsacrine and mitoxantrone, target DNA topoisomerase II [1, 2]. Despite the success of these agents in the treatment of some types of human cancer, the occurrence of resistance in solid tumours severely limits their effectiveness. Resistance may be either inherent in the phenotype of a cell or induced as a result of treatment, and the development of new drugs or treatment strategies which will overcome such resistance is critical to improved clinical treatment.

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A major type of resistance to such agents involves enhanced drug efflux associated with the expression of either P-glycoprotein [3] or the multiresistance protein MRP [4]. A second type of resistance, often termed "atypical" multidrug resistance, is associated with lower amounts of topoisomerase II and sometimes altered enzyme structure [1, 5, 6]. A variant of the latter type of resistance, which occurs phenotypically in tumour cells growing both *in vitro* and *in vivo*, arises from the depletion of topoisomerase IIα, which occurs as the cell cycle time lengthens [7]. For example, the G_1-phase transit time of Lewis lung carcinoma cells growing in plateau phase cultures or as subcutaneous tumours is increased with respect to that in exponentially growing cells, and is associated with a decrease in sensitivity to a variety of topoisomerase II-directed agents [8].

One class of topoisomerase II-directed agents has been found to differ from all the current clinical agents in its effects on plateau phase cultures. AMCA (methyl *N*-[4-

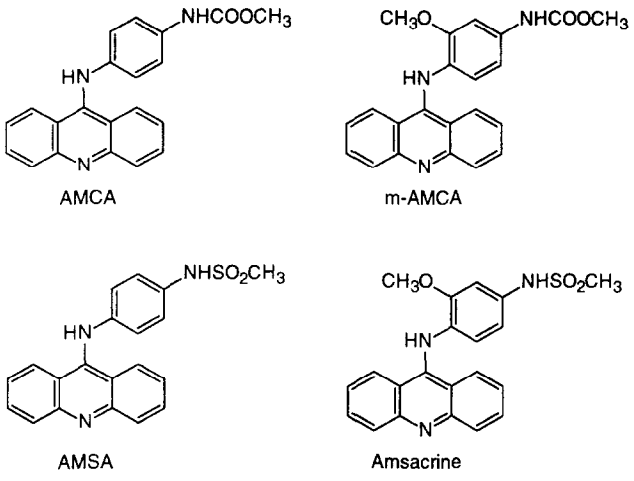

Figure 1. Structures of AMCA, *m*-AMCA, AMSA and amsacrine as the free bases.

(9-acridinylamino)phenyl]carbamate hydrochloride; see Figure 1 for structures), as well as its corresponding methoxy derivative *m*-AMCA (methyl *N*-[4-(9-acridinylamino)-2-methoxyphenyl]carbamate hydrochloride; the carbamate analogue of amsacrine) are remarkably effective in killing non-cycling cells [9]. AMCA is also active *in vitro* and *in vivo* against a P-glycoprotein-containing multidrug-resistant P388 line (P/DACT) and partially overcomes "atypical" multidrug resistance in resistant leukaemia cell lines [10, 11]. Both AMCA and *m*-AMCA are also effective inhibitors of herpes virus growth in cultured BHK cells [12].

In this report, we have investigated possible mechanisms by which AMCA and *m*-AMCA exert their effects against non-cycling cells by examining whether they target the enzyme topoisomerase II. We have also asked whether AMCA distinguishes between the topoisomerase IIα and IIβ enzymes. These isoenzymes have differential degradation rates during the G_1-phase of the cell division cycle [13–15] with the result that topoisomerase IIβ usually predominates in non-proliferating cells and may form a target for selective interaction.

MATERIALS AND METHODS

Materials

Anilinoacridine derivatives were synthesised in this laboratory. Amsacrine was also obtained from the Drug Synthesis and Chemistry Branch, NCI, Bethesda, Maryland, U.S.A. Etoposide was obtained from Bristol-Myers Co. (Wallingford, Connecticut, U.S.A.). Azatoxin was synthesised at the Department of Chemistry, University of Virginia (Charlottesville, Virginia, U.S.A.). Drug stock solutions were made in dimethylsulphoxide (DMSO) at 10 mM and further dilutions were made in distilled water immediately before use. The final concentration of DMSO in the enzymatic reaction did not exceed 1% (v/v), a concentration without detectable effect on *in vitro* topoisomerase II reactions.

Simian virus 40 (SV40), human *C-MYC* DNA, restriction enzymes, T4 polynucleotide kinase, Taq DNA polymerase, and polyacrylamide/bis(acrylamide) were purchased from Life Technologies Inc. (Gaithersburg, Maryland, U.S.A.), from the American Type Culture Collection (Rockville, Maryland, U.S.A.), from Perkin–Elmer (Norwalk, Connecticut, U.S.A.), or from New England Biolabs (Beverley, Massachusetts, U.S.A.). [γ-^{32}P]ATP and [α-^{32}P]dATP were purchased from DuPont NEN (Boston, Massachusetts, U.S.A.). DNA topoisomerase IIα was purified from mouse leukaemia L1210 cell nuclei as described previously, and was stored at −70°C in 40% (v/v) glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 0.2 mM dithiothreitol and 0.1 mM phenylmethanesulphonyl fluoride, pH 6.4. The purified enzyme yielded a single 170 kDa band after silver staining of SDS-polyacrylamide gels [16]. Topoisomerase IIβ was kindly provided by Dr Michael R. Mattern at SmithKline Beecham (King of Prussia, Pennsylvania, U.S.A.). Calf thymus topoisomerase II was purified using an adaptation of the procedures described previously [17]. Primer oligonucleotides for the polymerase chain reaction (PCR) were prepared using a 392 DNA synthesiser from Applied Biosystems (ABi, Foster City, California, U.S.A.) and purified using Abi oligonucleotide purification cartridges.

Preparation of end-labelled DNA fragments

SV40 DNA was labelled at both termini of BclI restriction sites using 1 unit of Taq DNA polymerase and [α-^{32}P]-dATP. ^{32}P-end labelled pBR DNA was prepared by linearising the DNA with EcoRI and labelling its termini with [α-^{32}P]-dATP and Klenow polymerase (Boehringer). The 3′-end labelled DNA was then cut with HindIII. Human *C-MYC* DNA fragments were prepared by PCR. A 254 pair DNA fragment from the first intron was prepared between positions 3035 and 3288, with numbers referring to GenBank genomic positions, as previously described [18, 19]. A 456 pair base DNA fragment containing the nuclear matrix associated region (MAR) was prepared between positions 7303 and 7758 using oligonucleotides: 5′-CCTCACAACCTTGGCTGAGTTCTTG-3′ as sense primer and 5′-TGATGAAAACAAACAGGGATGGTG-3′ as antisense primer. Single end-labelling of the DNA fragment was obtained by 5′-end labelling of one primer oligonucleotide [18, 19]. Approximately 0.1 μg of the *C-MYC* DNA that had been restricted by XhoI and XbaI (junction first exon–first intron) [19] or BclI (MAR region) was used as a template for the PCR.

Topoisomerase II-induced DNA cleavage reactions

DNA cleavage reaction using calf thymus topoisomerase II (20 units) and pBR322 DNA was carried out as described previously [20]. DNA fragments were equilibrated with or without drug in 10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP and 15 μg/ml bovine serum albumin for 5 min before addition of purified topoisomerase II (40–70 ng) or HL-60 nuclear extracts (2 μl, corresponding to the extract from 10^6 nuclei) in 20 μl final reaction volume [21]. Reactions were performed at 37°C for 30 min then stopped by adding sodium dodecyl sulphate (SDS) to a final concentration of 1% and proteinase K to 0.4 mg/ml, followed by incubation for 1 h at 42°C.

Electrophoresis and data analysis

For agarose gel analysis, 3 μl (10×) loading buffer (0.3% bromophenol blue, 16% Ficoll, 10 mM Na_2HPO_4) was added to each sample which was then heated at 65°C for 1–2 min before loading into agarose gels made in 0.1%

SDS and (1×) TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). Agarose gel electrophoresis was at 2 V/cm overnight. Gels were dried and autoradiographed for 1 day (Hyperfilms MP, Amsterdam, The Netherlands). The quantification of drug-induced DNA double-strand breaks in the presence of HL-60 nuclear extracts was carried out as follows. Radioactive gels were scanned using a PhosphorImager (Molecular Dynamic, Sunnyvale, California, U.S.A.). For each lane, the radioactivity was measured in the DNA cleavage products (C), and in the total DNA present in the lane (T). Drug-induced cleavage was expressed as:

$$\text{Per cent DNA cleaved} = 100 \times (C/T - C_0/T_0)/(1 - C_0/T_0)$$

where C_0 and T_0 are the counts for the cleaved and total DNA, in the presence of nuclear extracts without drug, respectively.

For DNA sequence analysis, samples were precipitated with ethanol and resuspended in 2.5 µl loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). Samples were heated to 90°C and immediately loaded into DNA sequencing gels (7% polyacrylamide; 19:1, acrylamide:bis) containing 7M urea in (1×) TBE buffer. Electrophoresis was at 2500 V (60 W) for 4 h. Gels were dried on 3MM paper sheets and autoradiographed with Kodak XAR-2 film.

DNA binding studies

These were determined in sodium acetate/sodium chloride buffer (pH 5.0) at an ionic strength of 0.01 by competition with ethidium as previously described [22, 23]. Poly[dA-dT].poly[dA-dT] and poly[dG-dC].poly[dG-dC] (Sigma Chemical Co.) were used at a concentration of 20 µM and ethidium bromide (Sigma Chemical Co.) at 1.15 µM. The association constants of ethidium for poly[dA-dT].poly[dA-dT] and poly[dG-dC].poly[dG-dC] were 9.5×10^6 and 9.9×10^6, respectively [24]. Reduction in ethidium fluorescence was measured in a fluorometer and the drug concentration required to halve the initial fluorescence was determined. Because amsacrine derivatives quench the fluorescence of DNA-bound ethidium [25], a separate assay employing an excess of DNA was used to correct for fluorescence quenching.

RESULTS

DNA binding properties of amsacrine and its analogues

The binding constants of amsacrine, AMSA (amsacrine lacking the methoxy group), AMCA and *m*-AMCA (AMCA containing the methoxy group) were compared using an ethidium displacement assay method which corrects for the quenching of DNA-bound ethidium [22]. Binding constants of AMSA and AMCA for poly[dA-dT].[dA-dT] and poly[dG-dC].[dG-dC] were very similar to each other with no selectivity between the two copolymers. The addition of a methoxy group (giving amsacrine and *m*-AMCA) decreased the DNA binding affinity in each case by a factor of approximately 4-fold (Table 1). The presence of the carbamate side-chain provided sightly higher binding constants than the methanesulphonamide side-chain.

Table 1. Drug association constants for poly[dA-dT].poly[dA-dT] and poly[dG-dC].poly[dG-dC]

Drug	Poly[dA-dT].poly[dA-dT] K ($\times 10^6$ M^{-1})	Poly[dG-dC].poly[dG-dC] K ($\times 10^6$ M^{-1})
AMCA	1.7	1.3
m-AMCA	0.38	0.39
AMSA	1.1	1.1
Amsacrine	0.37	0.45

Effect of drugs on plateau phase cells

LLTC (murine Lewis lung carcinoma cells adapted to tissue culture) [26] were grown to plateau phase and exposed to each of the drugs for 1 h before clonogenic assay of survival, as previously described [9]. The ranking of drugs for activity was quite different to that for DNA binding, and in particular AMCA and AMSA had different cytotoxicity despite similar DNA binding (Figure 2). The addition of a methoxy group had a much greater effect on the cytotoxicity of AMSA than on that of AMCA.

Induction of topoisomerase II-dependent double-stranded DNA breaks and comparison of cleavage with AMSA, amsacrine, AMCA and m-*AMCA*

The ability of drugs to induce cleavage complexes was tested using pBR322 DNA (^{32}P-labelled at the EcoRI site) and topoisomerase II purified from calf thymus by agarose electrophoresis. Cleavage was observed with all drugs, indicative of trapping of cleavable complexes (Figure 3). The pattern of cleavage formed by AMCA and *m*-AMCA (lanes G–J) was related to, but distinguishable from, that of AMSA and amsacrine (lanes C–F). Despite the low resolution of agarose gel electrophoresis, at least four cleavage sites were found highly stimulated or newly stimulated for AMCA as compared to amsacrine (indicated by arrows). The difference in specificity for cleavage sites was thus

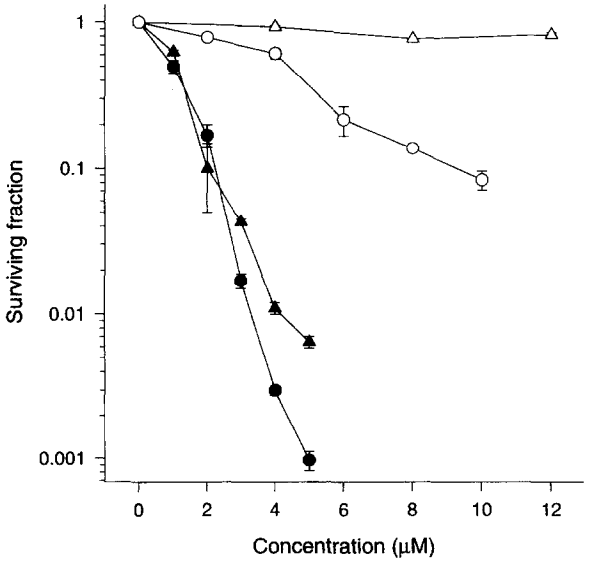

Figure 2. Activity of AMCA (●), *m*-AMCA (▲), AMSA (△), and amsacrine (○) against non-cycling LLTC cells. Data extracted from a previous study [9].

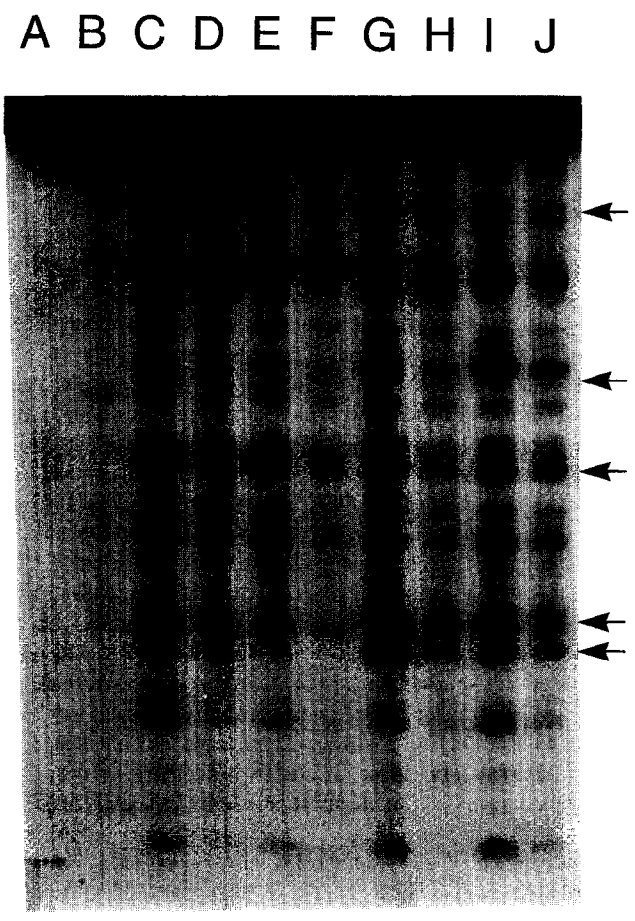

Figure 3. Comparison of topoisomerase II cleavage sites using AMSA, amsacrine, AMCA and *m*-AMCA. The EcoRI/HindIII restriction fragments of pBR322 (^{32}P-labelled at the EcoRI site) was incubated with calf thymus topoisomerase II in the presence of drugs and then analysed by agarose gel electrophoresis. Lane A, DNA control; lane B, topoisomerase II alone; lanes C, D, 10 μM and 1 μM amsacrine; lanes E, F, 10 μM and 1 μM AMSA; lanes G, H, 10 μM and 1 μM *m*-AMCA; lanes I, J, 10 μM and 1 μM AMCA. Arrowheads indicate differences in cleavage sites between amsacrine and AMCA.

found to reside with the 1′-substituent rather than with the methoxy substituent, and the cleavage patterns were similar despite a 4-fold difference in DNA binding affinity. Quantitatively, AMSA induced less cleavage than amsacrine (compare lanes C, D with lanes E, F), but differences between *m*-AMCA, AMCA and amsacrine were not obvious in this experiment. Two additional independent experiments were performed using SV40 DNA and nuclear extracts from cultured HL-60 cells. DNA cleavage induced by AMCA and amsacrine was quantified as a function of the drug concentration (Figure 4). Less than 10% difference in DNA cleavage between the two drugs was observed, indicating that they had similar effectiveness. At the highest concentrations of AMCA, cleavage bands became blurred, possibly due to drug intercalation and consistent with the stronger DNA binding constant of AMCA. In two independent experiments, AMCA induced fewer cleavages than amsacrine.

Sequencing of topoisomerase II cleavage sites in the MAR region of the C-MYC *proto-oncogene*

More precise determination of the differences in cleavage pattern between AMCA and amsacrine was carried out

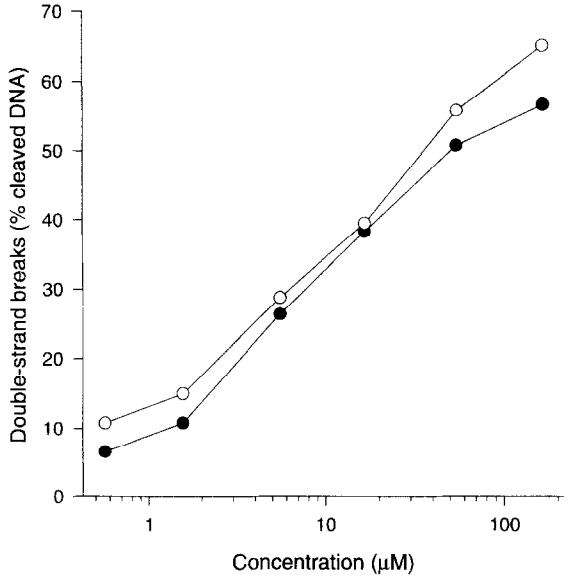

Figure 4. Relative activity of AMCA and amsacrine in inducing DNA cleavage by topoisomerase II. ^{32}P-end-labelled SV 40 DNA was reacted with increasing drug concentrations and HL-60 nuclear extracts for 30 min at 37°C. Reactions were stopped by adding SDS and proteinase K, and DNA fragments were separated on 1% agarose gels containing 0.1% SDS and scored using a PhosphorImager for AMCA (●) or amsacrine (○).

using sequencing gel electrophoresis. The ability of AMCA and amsacrine to stimulate cleavage of the 5′-labelled sense strand of the MAR region of the *C-MYC* proto-oncogene was compared (Figure 5). The pattern of DNA cleavage induced by AMCA was compared with those induced by amsacrine, azatoxin and etoposide. While the pattern induced by AMCA was more similar to that of amsacrine than to those of azatoxin and etoposide, there were also clear differences. For instance, similar bands were found at sites 7482, 7495 and 7566, while differences were found at sites 7374, 7381, 7400 and 7438 (lanes F and G).

To determine whether cleavage specificity varied with drug concentration, a dose response was performed using DNA labelling on the antisense strand. Again, the sequence specificity of AMCA was different from that of amsacrine (Figure 6). With increasing concentrations of AMCA, the intensity of cleavage decreased at some sites with drug concentration (for example, in the upper p25 binding sequence).

Topoisomerase IIα and IIβ cleavage sites

The same strand of the first intron of the *C-MYC* proto-oncogene was used to examine the effect of AMCA on cleavage by purified topoisomerase IIα and IIβ (Figure 7). A comparison was made with etoposide, which distinguishes clearly between these isoenzymes. In contrast, AMCA induced almost the same cleavage patterns with the two enzymes, and only minor differences between the patterns of cleavage sites were observed (as indicated by the arrowheads).

DISCUSSION

The results demonstrate that AMCA and *m*-AMCA are topoisomerase II poisons, trapping cleavable complexes and inducing DNA double strand breaks (Figures 3–7).

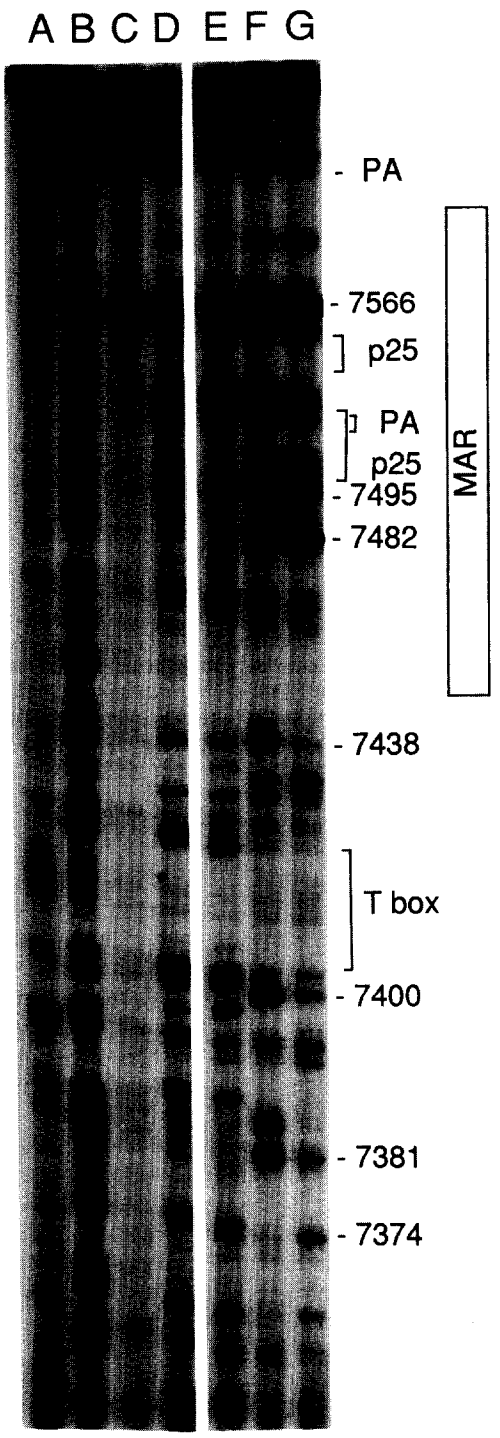

Figure 5. Sequencing of topoisomerase IIα cleavage sites in the MAR region of the *C-MYC* proto-oncogene. 5′-labelled sense strand was prepared by PCR between positions 7303 and 7757 (GenBank numbers). Cleavage reactions were carried out at 37°C for 30 min using purified topoisomerase II and stopped by adding SDS and proteinase K. DNA electrophoresis was in 7% denaturing acrylamide gel. Treatments were: lane A, DNA control; lane B, purine ladder; lane C, topoisomerase II; lane D, 100 μM azatoxin; lane E, 100 μM etoposide; lane F, 10 μM amsacrine; lane G, 10 μM AMCA. Numbers on the right correspond to the genomic position of the nucleotide covalently linked to topoisomerase II. The major component of *C-MYC* MAR (nuclear-matrix-associated region) has been located between positions 7447–7619 [39, 40]. PA, polyadenylation site; p25, binding sequence for a protein involved in HL-60 differentiation; T box, row of 10 thymidylate residues.

However, the pattern of AMCA and *m*-AMCA-induced DNA cleavage by topoisomerase II from four different mammalian sources is different from that induced by amsacrine or AMSA. The data in Figure 3 indicate that this difference is related to the presence of the carbamate side-chain rather than to the presence of the methoxy group. This result is consistent with the findings of Fossé and associates [27] who showed that alteration of the 1′-substituent of amsacrine analogues can change the sequence specificity of topoisomerase II-dependent cleavage. In contrast, substitution of the acridine moiety of amsacrine does not appear to change the specificity of cleavage [28].

DNA binding is well established as an important requirement for biological activity in the amsacrine series, and among a series of acridine-substituted derivatives of amsacrine, both *in vitro* and *in vivo* potency correlates with the DNA association constant [23]. The X-ray crystal structures of DNA–acridine complexes, 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 able to make contacts with a second macromolecule such as topoisomerase II [29–31]. Substitution of AMSA with the methoxy group to give amsacrine causes a 4-fold decrease in DNA binding constant, but a concomitant increase in cytotoxicity (Table 1). A possible explanation of this result is that the buttressing effect of the methoxy group prevents full intercalation of the acridine moiety, thereby reducing DNA binding, but allows better presentation of the methanesulphonamide group to the topoisomerase, thereby increasing biological activity. It is remarkable that in the corresponding pair of carbamate analogues, AMCA and *m*-AMCA, the addition of the methoxy group reduces DNA binding to a similar extent (Table 1), but has essentially no effect on *in vitro* biological potency (Figure 2). A possible hypothesis to explain this result is that AMCA and *m*-AMCA bind as ternary complexes with topoisomerase and DNA with slightly different geometry, so that the buttressing of the methoxy group has less effect on binding of *m*-AMCA to the topoisomerase. This hypothesis is consistent with the altered DNA cleavage patterns observed in Figure 3.

A major aim of this study was to explain the basis for the high activity of AMCA and *m*-AMCA against non-cycling cells. One hypothesis suggested previously [9] was that AMCA and *m*-AMCA might display increased selectivity for topoisomerase IIβ, which is often elevated with respect to the IIα isoenzyme in non-cycling cells [13–15]. Topoisomerase IIβ is predominantly localised in the nucleolus, where along with topoisomerase I, it presumably provides swivel points for RNA synthesis [32]. Resistance of HL-60 cells to the topoisomerase poison mitroxantrone appears to be related to the absence of topoisomerase IIβ [33] and the cytotoxicity of etoposide and doxorubicin may also be related to the expression of topoisomerase IIβ [34]. AMCA is active both *in vitro* and *in vivo* against a cell line (P/AMSA) [10] which contains an increased proportion of topoisomerase IIβ [35], consistent with this hypothesis. However, at least with purified enzyme preparations, AMCA induces almost the same DNA cleavage patterns using purified topoisomerase IIα or IIβ, with only minor differences in intensity (Figure 7). In contrast, etoposide shows distinct differences in cleavage patterns for the two

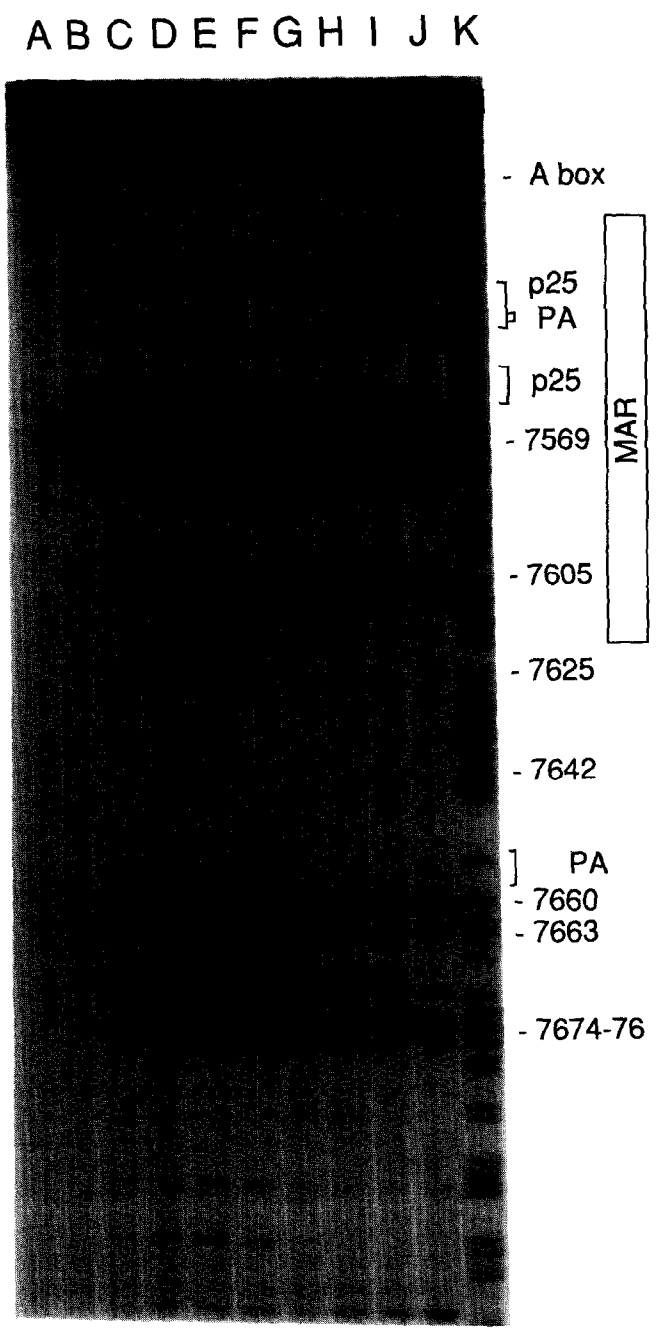

Figure 6. Dose-response of topoisomerase IIα-induced cleavage. Reactions were performed as for Figure 5 except that the antisense strand of the *C-MYC* gene was labelled. Lane A, DNA control; lane B, topoisomerase II; lanes C–F, amsacrine; lanes G–J, AMCA; drug concentrations were 3 μM (lanes C, G) 10 μM (lanes D, H), 30 μM (lanes E, I), and 100 μM (lanes F, J). Lane K, purine ladder. Numbers on the right correspond to the genomic position of the nucleotide covalently linked to topoisomerase II. *C-MYC* MAR has been located between positions 7447–7619. PA, polyadenylation site; p25, binding sequence for a protein involved in HL-60 differentiation; A box, row of 10 adenylate residues.

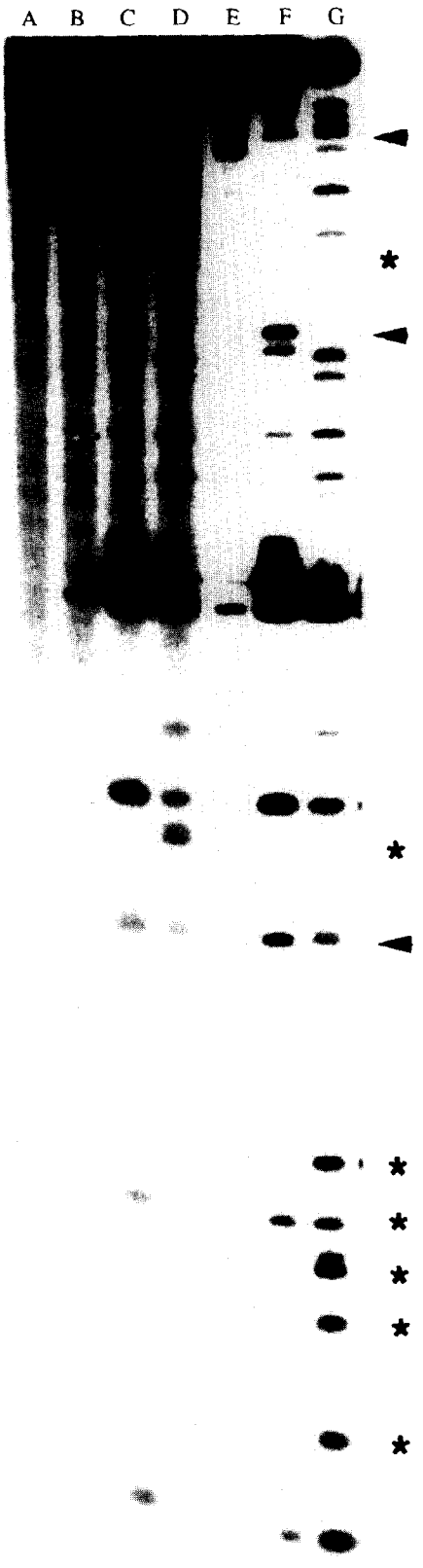

Figure 7. Sequencing of topoisomerase IIα and IIβ cleavage sites in the first intron of the *C-MYC* proto-oncogene. Reactions were performed as above. Lane A, DNA control; lanes B–D, topoisomerase IIβ; lanes E–G, topoisomerase IIα; lanes C, F, 10 μM AMCA; lanes D, G, 100 μM etoposide. Darker exposure of the autoradiogram is shown for reactions with topoisomerase IIβ to compensate for different specific activity of the two enzyme preparations. Differences between topoisomerase IIα and IIβ drug-induced cleavages are indicated with arrowheads for AMCA and with stars for etoposide.

isoenzymes (Figure 7). An argument against the hypothesis that topoisomerase IIβ is the target of *m*-AMCA is provided by the observation that herpes simplex virus replication, which is highly sensitive to *m*-AMCA [12], selectively utilises topoisomerase IIα [36]. A further argument is provided by the striking observation that *o*-AMSA, a biologically inactive analogue of amsacrine with the methoxy group *ortho*

to the methanesulphonamide, simulates DNA cleavage by topoisomerase IIβ rather than by topoisomerase IIα [37].

In conclusion, the results suggest that AMCA and *m*-AMCA act on topoisomerase II, but show little distinction between the IIα and IIβ isoenzymes. While their interaction with the topoisomerase IIα is distinguishable from that of amsacrine in terms of the specificity of induced DNA cleavage, the relationship of these differences to the high activity of AMCA in non-cycling cells remains to be elucidated. We have recently shown that while the DNA polymerase inhibitor aphidicolin reverses part of the cytotoxicity of amsacrine [38], it does not reverse the cytotoxicity of *m*-AMCA (N. Moreland, University of Auckland School of Medicine, New Zealand). Our current studies are aimed at determining whether a lack of interaction with a topoisomerase II target in S-phase is responsible for the activity of AMCA and its analogues against non-cycling cells.

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