Evidence that mAMSA induces topoisomerase action

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Evidence is presented that the topoisomerase inhibitors novobiocin and coumermycin inhibit the production of double-strand breaks in mouse mastocytoma cell nuclear DNA by the anticancer drug 4'[(9-acridinyl)amino]-methanesulphon-*m*-anisidide (mAMSA). Novobiocin did not inhibit resealing of DNA breaks induced by mAMSA. It is suggested that mAMSA intercalation into DNA induces the action of a type II topoisomerase. mAMSA and oAMSA were equally effective in breaking the DNA in isolated nuclei.

mAMSA *oAMSA* *DNA fragmentation* *Topoisomerase*

1. INTRODUCTION

The anticancer drug 4'[(9-acridinyl)amino]-methanesulphon-*m*-anisidide (mAMSA) induces breaks in the nuclear DNA of cells or isolated nuclei, probably after intercalation of the drug into DNA [1]. In either case the broken DNA becomes linked to protein [2,3]. Because the protein-DNA cross-links produced in isolated nuclei by mAMSA are rapidly resealed upon dilution of the drug it has been suggested that they are intermediates in the response to mAMSA that are exposed when DNA is prepared from the cells [2,3]. These and other observations [1] have led to the suggestion that a topoisomerase breaks, and becomes linked to, DNA in mAMSA-treated cells or nuclei in response to the altered DNA structure caused by drug intercalation [2,3]. In support of this possibility we present evidence that mAMSA induces double-strand breaks in the DNA of isolated PY815 mouse mastocytoma cell nuclei and that known topoisomerase inhibitors negate the action of mAMSA. These observations suggest that mAMSA induces the action of a type II topoisomerase.

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2. MATERIALS AND METHODS

Novobiocin and coumermycin were products of Sigma. mAMSA and oAMSA were kind gifts from Dr B.C. Baguley. Log-phase PY815 mouse mastocytoma cells were grown as in [4]. Nuclei were prepared from the cells as in [3] but with 5 mM EGTA and resuspended in their nuclei buffer with 5 mM EGTA prior to drug treatment or lysis. To measure the effects of drugs on DNA, nuclei from $1-3 \times 10^6$ cells in 0.1–0.2 ml buffer were treated with drugs for 10 min as described in the text then diluted to 3 ml with nuclei buffer, lysed by adding 2 ml of neutral lysis buffer (Li dodecylsulphate (2% w/v) 20 mM Tris–HCl buffer (pH 8.0), 20 mM NaCl, 20 mM Na + EDTA) and rocked gently for 2 h at 20°C in darkness. Lysis in neutral solutions was used to reveal double-strand breaks in DNA. DNA breakage was measured with a simple viscometer as previously described [5]. The validity of measuring viscosity to detect DNA breakage has since been confirmed [6].

3. RESULTS

We previously used an alkaline lysis procedure and viscometry to detect single-strand breaks pro-

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duced in intact cellular DNA by mAMSA treatment [5]. At that time attempts to use neutral lysis to detect the production of double-strand breaks in the DNA of mAMSA-treated cells was thwarted by gelatinous lumps that failed to disperse in the lysates [5]. A similar problem was encountered when isolated nuclei in small volumes (0.1–0.2 ml) were treated with mAMSA and subsequently lysed in 5 ml neutral lysis solution. However the problem was overcome by diluting and dispersing the mAMSA-treated or untreated nuclei with 3 ml of nuclei buffer immediately prior to addition of the neutral lysis mixture, when a uniformly viscous neutral nuclei lysate suitable for viscosity measurement was obtained (see section 2).

Fig.1 shows that the viscosity of neutral lysates of isolated PY815 mouse mastocytoma cell nuclei increased with increasing concentrations of nuclei. Fig.1 also illustrates the effect of pretreating the isolated nuclei with mAMSA for 10 min on the viscosity of subsequent neutral lysates. It is clear that mAMSA treatment caused a substantial

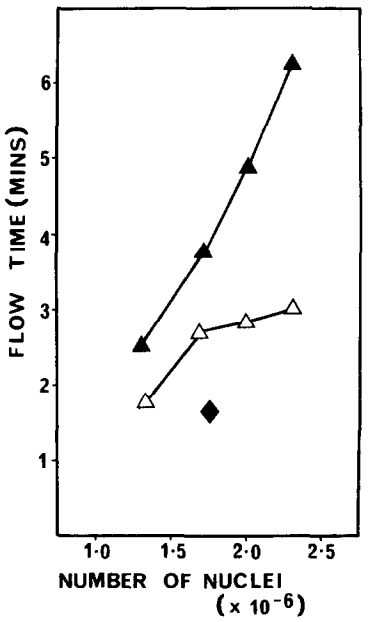

Fig.1. Effect of mAMSA on the viscosity of neutral nuclei lysates. Nuclei for this experiment were isolated with 5 mM ATP. Incubation was at 30°C. (▲) Nuclei incubated 10 min; (△) nuclei treated with mAMSA for 10 min; (◆) nuclei incubated with pancreatic deoxyribonuclease I (30 μg/ml) for 10 min.

Table 1

Resealing of mAMSA-induced breaks in the DNA of isolated nuclei

Treatment[a]	Flow rate (min)[b]
Untreated nuclei	4.42
+ 4 μM mAMSA	2.14
+ 4 μM mAMSA, then 30-fold diluted	4.43

[a] 3×10^6 nuclei per assay
[b] Mean of 3 measurements

reduction in the viscosity of the nuclei lysates consistent with the introduction of double-strand breaks in the nuclear DNA, a result that paralleled earlier demonstrations that mAMSA produces double-strand breaks in the DNA of intact cells [2,4,5]. Preincubating the nuclei for 10 min with 30 μg/ml pancreatic deoxyribonuclease I (EC 3.1–3.5) prior to lysis dramatically reduced viscosity confirming that it was due to DNA.

When PY815 cell nuclei pretreated with 4 μM mAMSA for 10 min in 0.1 ml buffer were diluted to 3 ml, reincubated at 30°C for 5 min, then lysed with 2 ml neutral lysis solution, there was a recovery in the viscosity of the lysates relative to that of mAMSA-treated nuclei indicating the rejoining of breaks in the nuclear DNA (table 1). A similar observation was made with L1210 cell nuclei in [5].

Because the production of rejoinable double-strand breaks in nuclear DNA could be explained by the action of a topoisomerase [7], the ability of the topoisomerase inhibitors novobiocin and coumermycin to negate the effects of mAMSA on nuclear DNA was examined. Preincubation of isolated nuclei for 1 min with 100 μg/ml of either novobiocin or coumermycin prior to the addition of 4 μM mAMSA substantially prevented the reduction in viscosity normally seen when nuclei treated with mAMSA alone for 10 min were lysed in neutral solution (table 2A,B). Concentrations of the inhibitors below 20 μg/ml were ineffective. Novobiocin or coumermycin (100 μg/ml) did not prevent the rejoining of DNA when the drugs were added to mAMSA-treated nuclei just prior to dilution and reincubation to allow DNA rejoining (table 2C).

Table 2

Inhibition of nuclear DNA breakage by novobiocin or coumermycin

Treatment[a]		Flow rate (min)[2]
(A)	Control (no drugs)	5.55
	+ Coumermycin (100 μg/ml)	6.18
	+ mAMSA (4 μM)	2.02
	+ Coumermycin + mAMSA	4.72
(B)	Control (no drugs)	5.16
	+ Novobiocin (100 μg/ml)	5.41
	+ mAMSA (4 μM)	2.51
	+ Novobiocin + mAMSA	4.23
(C)	Control (no drugs)	4.05
	+ mAMSA (4 μM)	2.44
	+ Novobiocin (100 μg/ml)	4.12
	+ mAMSA then novobiocin (100 μg/ml) during rejoining	4.08

[a] A and B = 3×10^6 nuclei per assay. Incubation with drugs for 10 min at 30°C. C = 2.5×10^6 nuclei per assay

[b] Mean of 3 measurements

The effect of oAMSA on DNA breakage in isolated nuclei was also measured. This analogue of mAMSA is dramatically less cytotoxic than mAMSA and it has much less effect on DNA in intact cells [2,8]. Treatment of isolated PY815 cell nuclei with o- or mAMSA was almost equally effective in reducing the viscosity of subsequent neutral lysates over a range of drug concentrations (fig.2).

4. DISCUSSION

In an earlier publication we reported unsuccessful attempts to develop an isolated nuclei system sensitive to the drug mAMSA [8]. Subsequently, we inadvertently found that mAMSA can be demonstrated to break the DNA in isolated PY815 mouse mastocytoma cell nuclei if the calcium chelating agent EGTA is included in the medium used to isolate nuclei. While our research was in progress authors in [3] reported that mAMSA breaks the DNA in isolated L1210 cell nuclei and these authors also included EGTA in their nuclei medium. It appears likely that EGTA is essential to inhibit calcium-dependent DNA endonucleases in nuclei preparations in order to detect breakage of DNA induced by mAMSA [9]. However, despite the inclusion of EGTA, isolated nuclei still appeared to contain some broken DNA since the viscosity of neutral PY815 cell nuclei lysates was lower than that of an equivalent number of whole cells suggesting that some DNA breakage occurred during the isolation of nuclei. Furthermore, the reduction in viscosity of lysates of intact cells after treating cells with mAMSA was greater than that obtained when isolated nuclei were treated with mAMSA. We believe that these effects are probably a consequence of unavoidable breaking of some of the supercoiled loops in nuclear DNA during the preparation of nuclei so that DNA in the loops is no longer distorted by mAMSA intercalation and therefore not attacked by topoisomerase. In some experiments, in an attempt to induce repair of nicks in DNA, 5 mM ATP was included in all of the nuclei isolation and incubation buffers. This produced nuclei lysates with higher viscosities and a greater effect of mAMSA-treatment on viscosity was observed. However it did not restore the viscosity to that produced by an equivalent number of whole cells. Both authors in [3] and [10] were unable to avoid some breakage of nuclear DNA during preparation of L1210 cell nuclei.

Our results with novobiocin and coumermycin together with earlier demonstrations that mAMSA causes double-strand breaks in nuclear DNA [2,4]

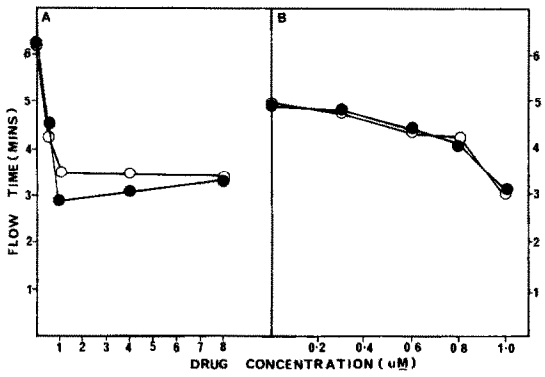

Fig.2. Comparison of the effects of o- and mAMSA on the viscosity of nuclei lysates: (A) 2.2×10^6 nuclei per assay; (B) 2×10^6 nuclei per assay; (○) oAMSA-treated nuclei; (●) mAMSA-treated nuclei.

and that protein is linked to the DNA fragments from mAMSA-treated cells or nuclei [2,3] suggest that a type II topoisomerase may be responsible for breaking the DNA in mAMSA-treated cells or nuclei because eukaryotype I topoisomerases cause only single-strand discontinuities in DNA [7]. However, whether the cytotoxic action of mAMSA actually results from topoisomerase action and DNA breakage in intact cells remains to be established. It is also possible that intercalated mAMSA prevents the resealing of topoisomerase II cross-linked DNA in cells leading to overt chromosome damage and rearrangement which is ultimately responsible for cell death [11,12]. The greater susceptibility of S phase cells to low concentrations of mAMSA [13,14] is consistent with the idea that interference with topoisomerase action might explain the action of mAMSA since topoisomerases are known to be required during DNA synthesis [7] and interference with their function at that time would probably have dramatic consequences on DNA replication, chromosome assembly and cell survival.

The fact that oAMSA breaks the DNA of isolated nuclei as effectively as mAMSA, although it is substantially less cytotoxic to intact cells than mAMSA, suggests that the cytotoxicity of these drugs may not be related to DNA breakage. However, oAMSA may penetrate cells poorly, or be more rapidly inactivated in the cytoplasm and hence fail to enter the nucleus, therefore no definitive statement regarding the actual mechanism of cytotoxicity of the drugs can yet be made. It would be worthwhile to identify the exact reason for the different activities of o- and mAMSA since it might also limit the action of mAMSA to some extent.

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