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On the Mechanism of Action of 4′-[(9-Acridinyl)-Amino] Methanesulphon-*m*-Anisidide*

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Abstract—*When cultures of PY*815 *mouse mastocytoma cells were treated with the antitumour agent* 4′-[(9-*acridinyl*)-*amino*]*methanesulphon-m-anisidine* (m-*AMSA*) *for* 15 min *or* 2 hr *at* 37°C *then applied to alkaline sucrose density gradients the DNA released was smaller than the DNA of untreated cells. Under similar conditions* m-*AMSA failed to affect the size of DNA in swollen cells, or in isolated nuclei incubated with or without added cytoplasmic extracts. The tumour inactive* m-*AMSA analogue* 4′[(9-*acridinyl*)-*amino*] *methanesulphon-*o-*anisidide did not affect the size of DNA in cells treated with the drug for* 15 min *at* 37°C *while after* 2 hr *a small part of the DNA was cleaved to material of size intermediate between that of untreated and* m-*AMSA-treated cells.* In toto *the results suggest that additional factors are involved in the action of* m-*AMSA on cellular DNA.*

INTRODUCTION

THE COMPOUND 4′-[9(-acridinyl)-amino] methanesulphon-*m*-anisidide (*m*-AMSA) is a promising synthetic anticancer agent which has undergone numerous preclinical trials [1–3] and is now being evaluated in phase II clinical trials under the auspices of the Drug Research and Development Programme, Division of Cancer Treatment, National Cancer Institute, U.S.A. *m*-AMSA binds strongly to isolated DNA where it is thought to intercalate between adjacent base pairs in a manner similar to the parent molecule 9-aminoacridine [4, 5]. At low drug concentrations *m*-AMSA is particularly toxic to cycling cells [6, and personal observations, R.K.R.] and its addition to growing L1210 leukaemia cells [7] or PY815 mouse mastocytoma cells [8] stops growth and causes breaks in DNA that are readily detectable on alkaline sucrose density gradients. There was no evidence that the drug directly breaks isolated DNA [7]. When added to Chinese hamster ovary cells *m*-AMSA caused cell-cycle-specific chromosome damage. Cells at the S–G_2 boundary were most sensitive to its action, while cells at the G_1–S boundary were less affected and cells in S or G_1 were least affected [6, 9]. These effects on chromosome structure led Deaven *et al.* [9] to suggest that *m*-AMSA is most effective at times when chromatin is undergoing structural reorganization. Despite these observations it has not been possible to demonstrate a direct degrading of *m*-AMSA on DNA *in vitro* and the exact mechanism of action of the drug is unknown.

While investigating the possibility that intercalation of *m*-AMSA into DNA *in vivo* might induce the unscheduled action of an untwistase [10] thereby producing the breaks in DNA detectable in alkaline sucrose density gradients [11, 12] we prepared nuclei from PY815 mastocytoma cells and examined the action of *m*-AMSA on their DNA *in vitro*. We report the results of these studies which suggest that in addition to *m*-AMSA other factors in cells are necessary for *m*-AMSA-induced breakage of DNA. From the data it seems unlikely that DNA-breakage results from the action of an untwistase.

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MATERIALS AND METHODS

PY815 mouse mastocytoma cells were grown in Dulbecco's modified Eagles medium with 10% calf serum in a National -CO_2 incubator. Mid-log-phase cells were used for all experiments. To radioactively label DNA, cells were usually grown for 15–20 hr in medium containing 0.5 μCi/ml [^{3}H] thymidine (56.9 Ci/mmole). Nuclei were isolated from cells previously washed with 0.17M NaCl, 3.4mM KCl, 0.01M $Na_2HPO_4.12\ H_2O$, 0.018M KH_2PO_4 (PBS) and swollen in 0.01M KCl, 0.01M Tris–HCl buffer, pH 7.4, 1.5mM $MgCl_2$ (TKM buffer) for 15 min at 0°C. The swollen cells were broken with a tight-fitting Dounce homogeniser and the nuclei recovered by centrifugation of the homogenate through a layer of 0.4M sucrose in TKM buffer for 5 min at 500 ***g***. The resulting pellet of nuclei with some associated cytoplasmic membranes was resuspended either in TKM buffer or in PBS and used immediately. To prepare a cytoplasmic fraction the supernatant was re-centrifuged at 900 ***g*** for 10 min. Aliquots (0.1 ml) of the resulting supernatant fraction were added to nuclei in PBS (0.5 ml) as required. All operations were performed at 0–4°C. Cells or nuclei were always incubated with drugs in darkness to avoid possible effects of light on intercalated drug molecules.

To fractionate DNA alkaline 5–20% linear sucrose density gradients were employed at 20°C essentially as described by Walker and Ewart [13] but with lithium dodecylsulphate and LiOH instead of sodium dodecylsulphate and NaOH. Gradients (4.4 ml) containing 0.01% Li dodecylsulphate, 0.3M LiOH and 1 mM Na_2 EDTA were prepared over a 0.3 ml cushion of 2.3 M sucrose. Lysis solution (0.3 ml) containing 0.2% Li dodecylsulphate, 0.5 M LiOH and 10 mM Na_2 EDTA was carefully layered onto the gradients and the cell or nuclei suspension (0.1 ml) was layered onto the lysis solution. The gradients were left for 90 min at 20°C in darkness before centrifugation at 25,000 rev/min for 2 hr at 20°C in a Spinco SW50L rotor. Fractions were collected from the bottom of the gradients and the DNA in each fraction was precipitated together with 50 μg bovine serum albumin carrier by the addition of 1 ml 5% trichloroacetic acid at 0–4°C. The resulting precipitates were collected on Whatman GF/C glass fiber filters and the associated radioactivity measured using a Beckman liquid scintillation spectrometer.

Some variation in the exact position of sedimentation of the DNA of untreated control cells was observed during our experiments. This first became apparent when [^{3}H] thymidine of lower specific activity (40.1 Ci/mmole) was used to pre-label cells before treatment with *o*- and *m*-AMSA. The DNA of control cells then sedimented further into the gradients than the DNA of previously studied control cells grown with 56.9 Ci/mmole [^{3}H] thymidine. Since the reduction in DNA size after treatment with *m*-AMSA still occurred no attempt was made to resolve whether the altered sedimentation position of control cell DNA resulted from increased radiation damage due to the higher specific activity thymidine, or whether it was due to an inherent variability in the layering and lysis procedure when cells were applied to the gradients.

RESULTS

Initial experiments confirmed that incubating PY815 cells with 4 μM *m*-AMSA for 2 hr at 37°C in culture medium caused DNA breakage (cf. [7, 8]) as evidenced by the reduced size of DNA in alkaline gradients (Fig. 1a). The effect on DNA was rapid since it was also observed after only 15 min treatment of cells with *m*-AMSA. Cells washed with PBS and subsequently incubated with *m*-AMSA in PBS for 15 min also produced smaller DNA on gradients than untreated cells suggesting that the effect of the drug was not directly growth related. Furthermore the bulk of the DNA was reduced in size suggesting that the effect of *m*-AMSA on DNA was not cell-cycle-stage dependent. These conclusions were reinforced by the fact that inhibiting respiration of cells with 40 mM NaN_3 10 min prior to addition of *m*-AMSA for 15 min did not stop the action of the drug on DNA although in separate experiments this azide concentration stopped growth and reduced [^{3}H] thymidine incorporation into DNA by more than 95%. Thus the effect of *m*-AMSA did not appear to require continuous respiration or growth.

When nuclei were incubated with or without *m*-AMSA in TKM buffer for 15 min at 37°C their DNA was reduced in size after either treatment (Fig. 1b). However the size of DNA was not affected when the nuclei were incubated in PBS with or without *m*-AMSA (Fig. 1b) suggesting that in TKM buffer the Mg^{2+} ions were responsible for

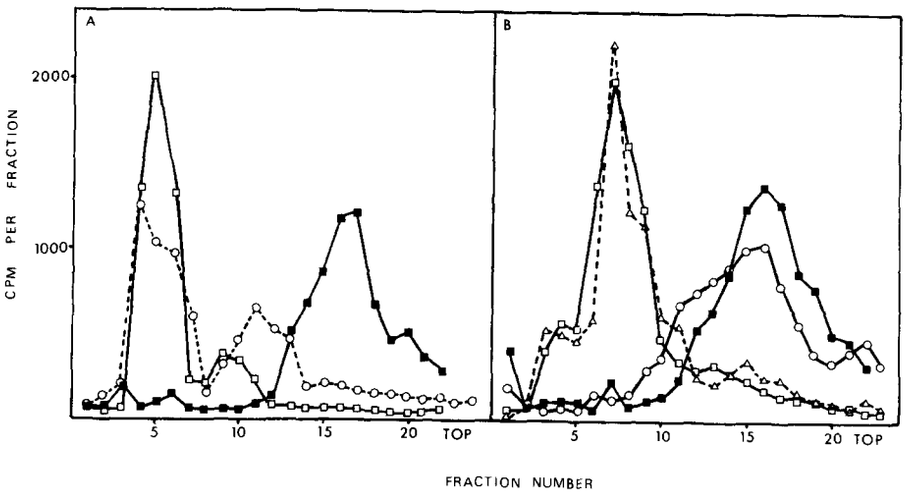

Fig. 1. (*A*) *Alkaline sucrose density gradients of cells treated for* 2 hr *with* o-*AMSA or* m-*AMSA.* □—□, *no drugs*; ○- - - - ○, m-*AMSA* ■—■ m-*AMSA.* (*B*) *Alkaline sucrose density gradients of nuclei treated for* 15 min *with* m-*AMSA.* □—□, *no drugs in PBS;* △- - - -△, m-*AMSA in PBS;* ○—○, *no drug in TKM buffer;* ■—■, m-*AMSA in TKM buffer.*

DNA breakage, perhaps by activating nuclear endonucleases [14–16]. Adding cytoplasmic extracts to nuclei incubated with *m*-AMSA in PBS did not assist DNA breakage, while addition of 10 mM mercaptoethanol did not affect the size of DNA produced in the presence of *m*-AMSA. Thus, *m*-AMSA did not appear to act like Bleomycin [17]. *In toto* these experiments suggested either that some component necessary for *m*-AMSA action on DNA was inactivated or lost during the preparation of nuclei, or that modification of *m*-AMSA was necessary in intact cells before it could induce breaks in DNA.

Because the size of nuclear DNA was not affected by incubating nuclei with *m*-AMSA in PBS and since during the preparation of nuclei cells were swollen in TKM buffer, the effect of swelling cells on the subsequent action of *m*-AMSA was examined. *m*-AMSA failed to induce breaks in DNA when cells previously swollen in TKM buffer for 15 min at 0°C were recovered and treated with the drug in PBS. This result also suggested that some necessary factor required for DNA cleavage was inactivated or lost from the cells during the swelling process. It was not possible to incubate the swollen cells in TKM buffer with *m*-AMSA and apply the mixture directly to the alkaline gradients because the DNA then sedimented to the bottom of the gradients. Since this did not occur with isolated nuclei in TKM buffer it is thought to have resulted from formation of a Mg^{2+}-dodecylsulphate–membrane–DNA complex [18].

The effect on PY815 cell DNA of 4′-[(9-acridinyl)-amino] methanesulphon-*o*-anisidide (*o*-AMSA) was also examined. The latter drug is a very close analogue of *m*-AMSA yet it is inactive against L1210 cells *in vivo* [19] and some 50-fold less potent than *m*-AMSA in its effect on growth of PY815 cells in culture [8]. The DNA in *o*-AMSA-treated cells was not reduced in size when cultures were treated with drug for 15 min at 37°C and subsequently fractionated on alkaline sucrose density gradients. Treatment with *o*-AMSA for 2 hr did appear to cause breaks in a part of the DNA giving some product of intermediate size, but the bulk of the DNA was not affected by *o*-AMSA (Fig. 1a). Thus at equal concentrations *o*-AMSA was significantly less effective than *m*-AMSA in its effects upon DNA.

DISCUSSION

In eukaryotes untwistase is present in nuclei [20] and *in vitro* the enzyme has been shown to exhibit maximum activity between 0.15–0.2 M Na^+ or K^+ in the absence of Mg^{2+} or at much lower salt concentrations in the presence of Mg^{2+} [10]. The failure of *m*-AMSA to induce breaks in the DNA of

isolated nuclei or of swollen but otherwise whole cells incubated in PBS or TKM buffer under conditions suitable for intercalation and untwistase action suggests therefore that the scission of DNA in *m*-AMSA-treated cells does not result from the action of untwistase following intercalation of *m*-AMSA into DNA. Nevertheless some factor in addition to *m*-AMSA did appear to be required to induce breaks in DNA and the relative effects of *m*-AMSA and *o*-AMSA on DNA size did correspond with their effects on growth and did not exclude the possibility that the antitumour action of *m*-AMSA might arise either directly or indirectly from effects on DNA.

Waring [4] did not find a correlation between DNA binding to PM2 DNA and biological action on L1210 cells *in vivo* of a series of analogues of *m*-AMSA, suggesting that if DNA binding is involved in the antitumour action of the drug it must be of a rather subtle nature. In particular there was no difference detected in the binding of *o*- and *m*-AMSA to DNA or in the effects of the drugs on DNA unwinding [4]. In view of the almost identical structures of the two drugs these results are not surprising and they suggest that if the drugs do act directly upon DNA then there must be a more subtle component to their action than mere intercalation to explain the different effects of *o*- and *m*-AMSA observed in our experiments. The possible existence of a very specific type of DNA binding to a unique and rare type of site is suggested by the fact that the DNA in *m*-AMSA-treated cells fragments to material of large and uniform size on alkaline sucrose gradients [7]. However this could reflect the structure of chromatin or other factors rather than any particular binding specificity of the drugs to DNA.

Mülbacher and Ralph [21] were unable to detect an effect of *m*-AMSA on DNA synthesis in isolated L1210 cell nuclei while Gormley *et al.* [22] could find no marked base-pair or sequence specificity in the binding of *m*-AMSA to DNA, nor any convincing effect on cellular nucleic acid polymerases at the low drug concentrations active *in vivo*. Furthermore, although Tobey *et al.* [6] have proposed that *m*-AMSA belongs to a general class of intercalating agents along with adriamycin, it has recently been reported that adriamycin affects the gel–liquid–crystal transition temperature of membranes and that a congener of adriamycin that does not enter the cell nucleus or bind to DNA is as active as the parent drug [23]. These facts suggest that this general class of compounds might not in fact act directly upon nuclear DNA and that other possible modes of *m*-AMSA action should be considered (cf. [22]).

For example, the observation that incubating nuclei in TKM buffer in the absence of *m*-AMSA produced breaks in DNA and a product of similar size to that obtained following *m*-AMSA treatment of intact cells suggests that *m*-AMSA might affect the compartmentalisation of divalent cations in cells, thereby inducing specific endonuclease action in the nucleus [16]. Such an effect could explain the failure of *m*-AMSA to affect DNA in swollen cells or isolated nuclei. It has already been demonstrated that the DNA intercalating drug ethidium affects organelles such as mitochondria and membranes [24, 25] while the antitumour effectiveness of several bifunctional diacridines showed a highly significant inverse correlation with their effect on phenomena associated with plasma membranes, and all of the diacridines bound to membranes [26]. However at present the possibility cannot be excluded that *m*-AMSA is modified in intact cells to produce an active derivative as is the case with many aromatic hydrocarbons that react with DNA (cf. [27]). Wilson *et al.* [28] have already demonstrated that both *m*-AMSA and *o*-AMSA can be modified *in vivo*. Failure to modify the drug would then explain its lack of effect with swollen cells or nuclei preparations. In the absence of readily available, highly radioactive *m*-AMSA derivatives, it is at present extremely difficult to distinguish between the various alternatives.

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