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A Reassessment of the Mechanism of Action of 4′-[(9-Acridinyl)-amino]methanesulphon-*m*-anisidide*

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Abstract—*The conclusion that treating cells with 4′-[(9-acridinyl)-amino]methanesulphon-*m*-anisidide (m*AMSA*) causes an alkali-dependent decrease in the size of their DNA was reinvestigated. Evidence is presented that alkali is not needed to detect an effect of m*AMSA* on the sedimentation rate of DNA released from m*AMSA*-treated PY815 cells and that m*AMSA* causes double-strand breaks in cellular DNA.*

INTRODUCTION

THE PROMISING anticancer drug *m*AMSA† is currently undergoing clinical trials in several countries [1]. Its mechansim of action is unknown, although various studies have shown that *m*AMSA binds to isolated DNA [2–4]. This, together with the observations that *m*AMSA induces limited DNA damage in treated cells [5] and causes cell-cycle-specific chromosome damage [6], has led to the idea that *m*AMSA affects cycling cells by interacting with their DNA. However, attempts to demonstrate effects of *m*AMSA on DNA or RNA syntheses have not shown any convincing effects at low but physiologically active drug concentrations [7–9], and comparisons of the antitumour activity of *m*AMSA and its analogues with their ability to bind to DNA have shown no clear correlations between these parameters [3, 4]. Because attempts to induce breakage of isolated DNA, DNA in isolated nuclei or DNA in swollen cells with *m*AMSA were unsuccessful [10, 11] and the observed effects of *m*AMSA on DNA have been confined to intact cycling cells [5, 6, 9–11], it has been suggested that the interaction of *m*AMSA with DNA might not fully explain its antitumour activity [7, 12].

Burr-Furlong *et al.* [11] lysed *m*AMSA-treated [^{3}H]-thymidine-labelled L1210 leukemia cells with DOC† in alkali and fractionated the DNA in alkaline sucrose density gradients to demonstrate that *m*AMSA caused a shift in the sedimentation coefficient of the DNA from > 170S to about 30S. Since no changes in the sedimentation coefficient of DNA were observed when cells lysed in neutral solution with or without 70% formamide were fractionated on neutral sucrose gradients, they concluded that *m*AMSA causes alkali-sensitive lesions at a limited number of sites in DNA. The lesions were not identified, but the appearance of 30S DNA in alkali suggested that subsequent DNA breakage had occurred in alkali.

We obtained essentially similar results when [^{3}H]-thymidine-labelled *m*AMSA-treated PY815 mouse mastocytoma cells were lysed with LDS† in alkali and fractionated on alkaline sucrose density gradients [10]. However, in subsequent studies we found that the [^{3}H]-thymidine required to label PY815 cell DNA sufficiently for analysis on sucrose gradients stopped the growth of the cells after 10–15 hr, and 1 g serum gradient fractionation [13] indicated that the cells ceased growing in late G1 or early S phase (A. Forster, unpublished observation). Because it was possible that *m*AMSA might only break the DNA of cells at this particular stage of the cell cycle, we therefore re-examined the effects of *m*AMSA on PY815

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†The abbreviations used are: LDS, lithium dodecylsulphate; DOC, sodium deoxycholate; *m*AMSA, 4′-[(9-acridinyl)-amino]methanesulphon-*m*-anisidide.

cells. The results show that alkali is not necessary to detect effects of *m*AMSA on the sedimentation coefficient of PY815 cell DNA and that *m*AMSA decreases the sedimentation coefficient of DNA in [^{14}C]-thymidine-labelled PY815 cells under conditions where the radioisotope does not affect growth.

MATERIALS AND METHODS

PY815 mouse mastocytoma cells were cultured in suspension in RPMI 1640 medium supplemented with 10% neonatal calf serum at 37°C in a 'National' incubator. The culture doubling-time was 10 hr. DNA was uniformly labelled by seeding cells at a density of 5×10^4 cells/ml into fresh medium containing 0.5 μCi/ml of [^{3}H]-thymidine (sp. act. 47 Ci/mmol) and cultures were grown for 15–20 hr to a density of $7–8 \times 10^4$ cells/ml. There was no further uptake of [^{3}H]-thymidine into acid-precipitable material after 10–12 hr. Growth inhibition that occurred after 15–20 hr was due to the radioisotope, since growth was not affected when cells were grown with the same concentration of non-radioactive thymidine. For some experiments, cells were labelled with [^{14}C]-thymidine (57 mCi/mmol; 0.1 μCi/ml) for 18 hr. This procedure did not affect cell growth.

Cells labelled with [^{3}H]- or [^{14}C]-thymidine were treated with 4 μM *m*AMSA at 37°C for 30 min. Untreated controls received an equivalent volume of water. The cells were recovered by centrifugation at 500 ***g*** for 5 mins at 4°C, washed twice with 5 volumes of ice-cold phosphate-buffered saline and finally resuspended in phosphate-buffered saline to a density of 1×10^5 cells/ml. The cells were then lysed on top of neutral or alkaline sucrose density gradients [10, 11] to avoid shearing DNA by pipetting. To prepare neutral sucrose gradients, an 0.1 ml or 0.2 ml aliquot of the resuspended cells ($1–2 \times 10^4$ cells) was loaded onto a pre-formed 5 ml 5–20% linear neutral sucrose density gradient overlayed with 0.3 ml of neutral lysing solution. The lysing solution consisted of 0.5% LDS, 0.01M Na_2 EDTA, 0.1M Tris–HCl buffer, pH 8.0, and 0.01M NaCl. The sucrose solutions were prepared with the same solution but with only 0.01% LDS. Gradients were 'cushioned' with 0.3 ml of 2.3M sucrose. After lysis of cells in the dark at 20°C for the required time, the gradients were centrifuged in a Spinco SW50.1 rotor at 25,000 revs/min for 1 hr at 18°C in a Spinco L_2A ultracentrifuge. Fractions were recovered by piercing the bottom of the centrifuge tube. Lithium salts were used to avoid precipitating the detergent. The procedure for alkaline gradients was essentially the same as that for neutral gradients. The lysis mixture contained 0.5% LDS, 0.5M LiOH and 0.01M Na_2 EDTA. The gradients contained 0.01% LDS, 0.3M LiOH and 0.01M EDTA, and centrifugation was for 2 hr.

The radioactive DNA in each fraction was measured by precipitating the DNA together with 100 μg bovine serum albumin and 0.4 ml of ice-cold 10% trichloroacetic acid. After 10 min at 0°C the precipitates were collected on Whatman GF/C glass-fibre filters, dried and the radioactivity associated with the filters measured in a liquid scintillation spectrometer. Previous experiments confirmed that more than 95% of the incorporated [^{3}H]-thymidine was present in DNA.

When cells were lysed in neutral and then alkaline solutions, lysis was carried out in microfuge tubes and the alkaline lysate was layered onto gradients using a wide-bore disposable polypropylene pipette tip.

RESULTS

Figure 1a shows the sedimentation profile of [^{3}H]-thymidine-labelled PY815 cell DNA after lysis of *m*AMSA-treated cells for 1.5 hr in alkaline LDS and sedimentation in an alkaline LDS-sucrose density gradient. A substantially reduced sedimentation rate was observed for the DNA from *m*AMSA-treated cells, while the bulk of the DNA from untreated cells sedimented to the bottom of the gradients [cf. 10, 11]. This paralleled the results of Burr-Furlong *et al.* [11], who lysed *m*AMSA-treated L1210 cells with alkaline DOC and sedimented the DNA in alkaline sucrose density gradients. However, when PY815 cells were lysed in neutral LDS for 1.5 hr or 18 hr, then sedimented in neutral LDS-sucrose gradients, the DNA of *m*AMSA-treated cells again sedimented more slowly than that of untreated cells (Fig. 1b). The same effect was obtained when 50 μg/ml proteinase K was included in the neutral lysis mixture to eliminate possible nuclease activity [14]. This result contrasted with Burr-Furlong *et al.* [11], who observed no reduction in the sedimentation rate of DNA from *m*AMSA-treated L1210 cells after lysis in neutral 1% DOC solutions, and it suggested that the detergent action of DOC was too weak to release DNA from chromatin following lysis of the cells. To test this possibility we substituted 1% DOC for LDS in the neutral lysis mixture and the sucrose gradients. Under these conditions there was no slower sedimenting DNA in gradients of *m*AMSA-treated cells, confirming that DOC was less effective than

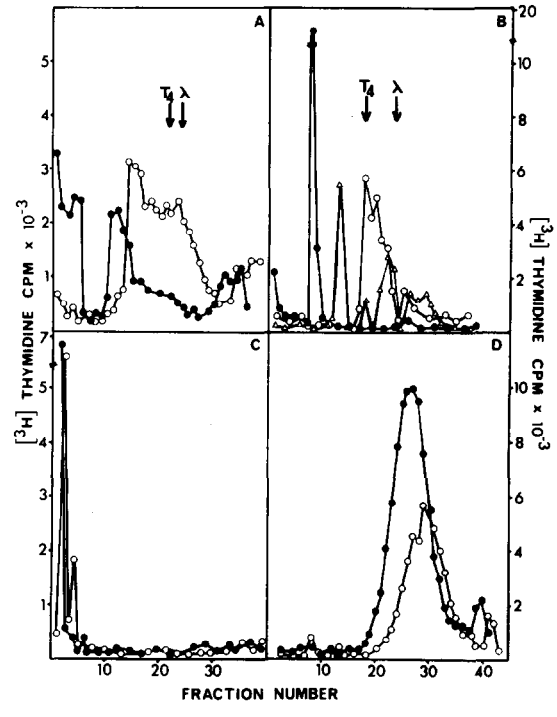

Fig. 1. Effects of various lysis procedures on sedimentation of [^{3}H]*-thymidine-labelled PY*815 *cell DNA following* m*AMSA treatment. (A) Sedimentation of DNA from* m*AMSA-treated PY*815 *cells in alkaline sucrose gradients following lysis in alkaline LDS for* 1.5 hr *at* 20°C. *Sedimentation was right to left.* O——O m*AMSA-treated cells;* ●——● *untreated cells. (B) Sedimentation of DNA from* m*AMSA-treated PY*815 *cells in neutral sucrose gradients following lysis in neutral LDS for* 1.5 *or* 18 hr *at* 20°C. *The untreated cell DNA profile was identical whether lysis was for* 1.5 *or* 18 hr. Δ——Δ m*AMSA-treated cells lysed for* 1.5 hr; O——O m*AMSA-treated cells lysed for* 18 hr; ●——● *untreated cells lysed for* 18 hr. *(C) Sedimentation of DNA from* m*AMSA-treated PY*815 *cells in neutral sucrose gradients following lysis in neutral* 1% *DOC for* 1.5 hr *at* 20°C. O——O m*AMSA-treated cells;* ●——● *untreated cells. (D) Sedimentation of DNA from* m*AMSA-treated PY*815 *cells in alkaline sucrose gradients following lysis for* 18 hr *in neutral LDS then* 1.5 hr *in alkaline LDS.* O——O m*AMSA-treated cells;* ●——● *untreated cells. Arrows indicate positions of sedimentation of* [^{3}H]*-thymidine-labelled phage λ and T4 DNAs.*

LDS in releasing DNA in neutral solution (Fig. 1c). These results demonstrated that alkali was not essential to detect effects on the DNA of *m*AMSA-treated cells when a sufficiently strong detergent was employed.

Prolonged (18 or 48 hr) lysis of untreated PY815 cells with neutral LDS and proteinase K did not decrease the sedimentation rate of DNA in neutral LDS-sucrose gradients, suggesting that enzymic degradation of the DNA during the lysis procedure was not responsible for the decreased sedimentation rate of DNA from *m*AMSA-treated cells. However, when *m*AMSA-treated or untreated cells were lysed in neutral LDS solution for 18 hr at 20°C, and then held in alkaline LDS for 1.5 hr prior to alkaline sucrose gradient fractionation, both treated and untreated cells produced slower sedimenting DNA of identical size (Fig. 1d). Since lysing untreated cells for 1.5 hr with alkaline LDS did not reduce the sedimentation rate of their DNA (Fig. 1a), this experiment suggested that the 18 hr period of neutral rather than alkali lysis was responsible for the lower sedimentation rate of the DNA. Single-strand nicking of DNA during longer neutral lysis did not appear to be involved because the DNA of *m*AMSA-treated cells lysed in neutral solution for 2 or 18 hr, then for the 1.5 hr in alkali, sedimented at the same position in alkaline sucrose gradients. These results raised the question of whether the observed effects were in fact due to DNA breakage, or whether they might result from a slow unfolding or dissociation of chromatin which was assisted by alkali or prior *m*AMSA-treatment of cells. Kohn [15] has criticized the use of sedimentation rates as a criterion for establishing that DNA breakage has occurred, and pointed out the difficulties in distinguishing between DNA breakage and unfolding with large molecules of DNA. The importance of the time factor was emphasized by the fact that alkaline-LDS lysis of untreated cells for 5 hr instead of the usual 1.5 hr at 20°C, followed by alkaline sucrose gradient fractionation, produced DNA with an intermediate sedimentation rate (Fig. 2a). It was unlikely that this effect resulted from damage to the DNA by alkali because there was little if any change in the sedimentation rate of DNA from *m*AMSA-treated cells lysed in alkaline-LDS for 20 min or 5 hr at 20°C (Figs 2a, b). To test the possibility that the slower

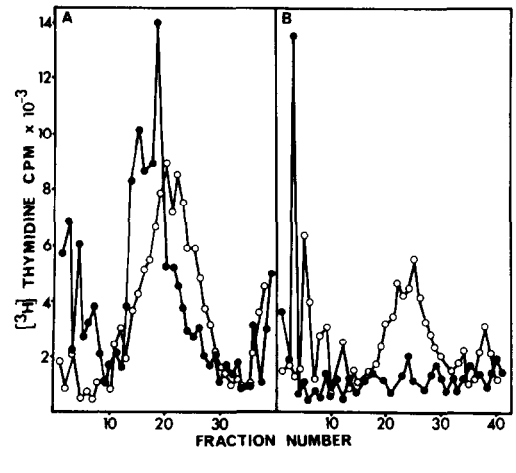

Fig. 2. Effect of variation in alkaline lysis time on sedimentation of DNA from m*AMSA-treated PY*815 *cells in alkaline sucrose gradients. (A) Cells lysed for* 5 hr *(B) cells lysed for* 20 min. O——O m*AMSA-treated cells;* ●——● *untreated cells.*

sedimenting DNA from *m*AMSA-treated cells resulted from unfolding of chromatin structures, the viscosity of PY815 cell lysates was measured with an Ostwald viscometer. Treating cells with *m*AMSA for 30 min substantially reduced the viscosity of both neutral and alkaline lysates, consistent with DNA breakage rather than unfolding of chromatin structures, which might have been expected to increase viscosity.

To eliminate the possibility that the effects of *m*AMSA on [^{3}H]-thymidine-labelled cell DNA were due to inhibition of the cell cycle at a drug-sensitive stage, cells were also labelled with [^{14}C]-thymidine under conditions that did not affect growth, then treated with *m*AMSA for 1.5 hr and fractionated on alkaline sucrose gradients. The DNA of the *m*AMSA-treated cells again sedimented more slowly than that of untreated cells, confirming the absence of cell-cycle-specific effects.

Further doubt was thrown on the conclusion that *m*AMSA must interact with DNA to cause lesions in the DNA of PY815 cells by the fact that treating PY815 cells with the mitochondrial inhibitors 2,4 dinitrophenol or carbonyl cyanide-*m*-chlorophenylhydrazone also caused a reduction in the sedimentation rate of their DNA after lysing the cells in alkaline LDS and sedimenting the DNA in an alkaline sucrose gradient (Fig. 3). Since there is no evidence to suggest that these reagents intercalate into or interact with DNA, this result weakens the argument that *m*AMSA must interact with DNA to cause the observed effects on DNA.

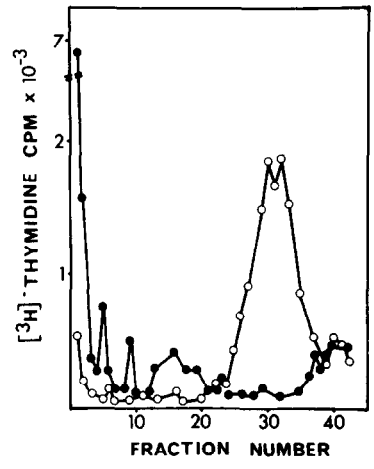

Fig. 3. Effect of 2,4 dinitrophenol on sedimentation of DNA from PY815 cells in alkaline sucrose gradients. Cells were treated with 5 mM 2,4 dinitrophenol at 37°C for 30 min and lysed for 20 min in alkaline LDS as described in Methods. O——O *2,4 Dinitrophenol-treated cells;* ●——● *untreated cells.*

DISCUSSION

The above results suggest that *m*AMSA treatment causes double-strand breaks in PY815 cell DNA which is released as slower sedimenting DNA in neutral or alkaline sucrose gradients when a strong detergent is utilised. No evidence was obtained that the slower sedimenting DNA resulted from unfolding of chromatin structures, a possibility that was only considered briefly by Burr-Furlong *et al.* [11]. A similar conclusion has recently been reported concerning the mechanism of action of other intercalating agents [16].

We have no clear explanation why prolonged neutral lysis of untreated PY815 cells, followed by brief (1.5 hr) alkaline lysis, produced slower sedimenting DNA, whereas brief alkaline lysis alone did not. It is possible that some single-strand nicks were introduced into the DNA during prolonged neutral lysis and that in alkali these decreased the sedimentation rate of DNA from untreated cells more dramatically than that from *m*AMSA-treated cells. Substantial changes in DNA size are necessary to observe significant shifts in sedimentation profiles on sucrose density gradients.

The DNA from *m*AMSA-treated cells sedimented faster than phage T4 DNA, indicating a size greater than 120×10^6, although compared with λ or T4 DNA the sedimentation profile was broader, suggesting greater heterogeneity in size of the cellular DNA. A comparison of the sedimentation rates of T4, λ and *m*AMSA-treated DNA [17] suggests a median size class for the latter DNA of approximately 2×10^8. This might be consistent with *m*AMSA introducing one break per replicon or chromosome loop, raising the possibility that the drug affects topoisomerase action on DNA [15, 16]. We did not detect an effect of inhibiting PY815 cell DNA synthesis with novobiocin on the action of *m*AMSA on cellular DNA. However, the mechanism of action of novobiocin on mammalian cells is not yet resolved.

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