Transport of AMSA drugs into cells

Sandra Darkin and Raymond K. Ralph+

Cell Biology Department, University of Auckland, Auckland, New Zealand

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The uptake and efflux of radioactive 4′-(9-acridinylamino)methanesulphon-*m*-anisidide (mAMSA) and its inactive congener 4′-(9-acridinylamino)methanesulphon-*o*-anisidide (oAMSA) by PY815 mastocytoma cells were investigated. Both drugs were readily taken up by intact cells although only mAMSA caused DNA scission and is actively cytotoxic to PY815 cells. The microsomal enzyme inhibitors cimetidine or SKF525A increased drug uptake and decreased drug efflux suggesting that drug metabolism could explain the different activities of oAMSA and mAMSA.

mAMSA *oAMSA* *Transport* *PY815 cell*

1. INTRODUCTION

The drug 4′-(9-acridinylamino)methanesulphon-*m*-anisidine (mAMSA) is an active anticancer agent currently in clinical use. It is believed to cause scissions in nuclear DNA by affecting the function of type II topoisomerases [1,2]. While examining the action of various analogues of mAMSA Denny et al. [3] found that the close analogue, 4′-(9-acridinylamino)methanesulphon-*o*-anisidide (oAMSA), which differs in structure only in the position of the $-OCH_3$ moiety was virtually inactive in vivo. Subsequently Ralph [4] and Zwelling et al. [5] reported that oAMSA was substantially less potent than mAMSA in breaking the DNA in cultured cells, despite the fact that the 2 drugs bind to isolated DNA with similar characteristics and intercalate into DNA producing similar unwinding angles [6,7]. Examination of the effect of *o*- and mAMSA on isolated nuclei demonstrated that either drug caused DNA breakage at comparable concentrations [8], suggesting the inactivity of oAMSA in vivo and the failure of oAMSA to break DNA in intact cells might result from inability of the *ortho* analogue to enter cells. Alternatively, it was possible that oAMSA was rapidly inactivated or degraded by cells. In either case it is clear that considerable specificity would be needed at the plasma membrane or in the cell to distinguish the 2 compounds, suggesting either that the AMSA drugs enter cells via a very selective route or that they are very specifically recognized once inside cells. These possibilities are of considerable interest since specific transporters or inactivators of mAMSA or its analogues could be a major impediment to the design and function of more active derivatives. Therefore we examined the question of the inactivity of oAMSA in greater detail. The results showed that oAMSA enters PY815 cells as readily as mAMSA, and they suggest that oAMSA is rapidly inactivated in cells, possibly by microsomal enzymes.

+ To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Drugs*

mAMSA and oAMSA were kindly provided by the Oncology Department of Auckland University. Cimetidine and SKF525A were gifts from Smith, Kline and French.

2.2. *Cells*

PY815 mouse mastocytoma cells were grown in

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RPMI 1640 medium within 10% neonatal calf serum as described [9]. Permeable cells were prepared essentially by the ATP-fluoride method of Makan [10]. Resealing of permeable cells was achieved by centrifuging the cells and resuspending them in buffer A (170 mM NaCl, 3.4 mM KCl, 10 mM $Na_2HPO_4 \cdot 12H_2O$, 18 mM KH_2PO_4) containing 3 mM Mg^{2+} for 15 min at 37°C [11]. Scission of DNA in cells by oAMSA or mAMSA was detected by measuring the viscosity of cell lysates [12].

2.3. *DNA scission*

To compare the effects of oAMSA and mAMSA on DNA in permeable and non-permeable cells 2×10^7 log phase PY815 cells were recovered by centrifugation at $900 \times g$ for 5 min without chilling and washed once at 37°C with buffer A. The cells were resuspended in 9 parts of 0.1 mM Tris-HCl (pH 7.4), 0.05 M NaCl, 50 μM $CaCl_2$, 5 mg/ml Dextran 500 and 1 part 5 mM ATP (buffer B) to 3×10^6 cells/ml then divided into 4×1-ml portions. Samples 1 and 2 served as untreated controls while samples 3 and 4 received 4 μM oAMSA or mAMSA, respectively, for 10 min at 37°C. Parallel control samples of non-permeable cells were resuspended in buffer A instead of buffer B then treated with drugs identically to the permeabilized cells. Scission of DNA in the cells was measured by the viscosity method after recovery of the cells by centrifugation, resuspension in buffer A and cell lysis [12]. After permeabilisation, in excess of 90% of the cells were permeable to trypan blue (0.16%, w/v, in 0.15 M NaCl) whereas 100% of the non-permeable cells excluded trypan blue.

To assess the effect of cimetidine or SKF525A on DNA scission by mAMSA or oAMSA, 3×10^6 PY815 cells in 0.1 ml PBS were pre-treated with 1 mM cimetidine or 1 mM SKF525A for 30 min prior to adding 0.1 or 0.5 μM mAMSA or 4 μM oAMSA for 10 min at 37°C. The cells were then dispersed, lysed in neutral conditions and the viscosity of the lysates was measured [8].

2.4. *Drug uptake and efflux*

To measure mAMSA uptake log phase cells were recovered by centrifugation at $900 \times g$ for 5 min at 37°C, resuspended to 2×10^7 cells/ml in fresh medium and incubated with 0.25 or 0.5 μM [^{14}C]mAMSA (1800 or 3600 cpm or 0.5 ml cell suspension) with or without non-radioactive mAMSA. In some experiments metabolic inhibitors were present to assess their effects on the steady-state accumulation of labelled mAMSA. At intervals, duplicate 0.5 ml aliquots were removed, chilled rapidly, layered over an ice-cold silicon oil-paraffin oil mixture (0.6 ml; 9:1, v/v) in microcentrifuge tubes and centrifuged at $12000 \times g$ for 1 min. The supernatants were discarded and the drained tubes were wiped dry before the bases of the tubes containing the cell pellets were cut off and placed in scintillation vials. The cells were dispersed in 0.5 ml of 0.4 M NaOH by brief sonication then dissolved by incubation at 37°C overnight. Finally radioactivity in the vials was measured in a liquid scintillation spectrometer after adding 3 ml ACS II Amersham scintillant. [^{3}H]oAMSA uptake by cells was measured with 2200 cpm of 0.5 μM [^{3}H]oAMSA per 0.5 ml cell suspension in an identical manner.

To measure drug efflux, cells were loaded with [^{14}C]mAMSA or [^{3}H]oAMSA for 10 min at 37°C as described above, recovered by brief centrifugation at $900 \times g$ and resuspended in fresh growth medium to 2×10^7 cells per ml at 37°C. In some experiments, the fresh medium contained as metabolic inhibitor either cimetidine or SKF525A. At intervals duplicate 0.5 ml aliquots were removed and drug efflux was measured after rapidly chilling the cell suspension and centrifuging the cells through ice-cold silicone-paraffin oil (see above). The cell pellets were recovered and residual cell-associated radioactivity was measured as previously described.

3. RESULTS AND DISCUSSION

Table 1 summarizes initial experiments using the viscosity technique [12] to compare the effect of 4 μM mAMSA or 4 μM oAMSA on DNA scission in permeabilized vs non-permeabilized PY815 cells which suggested that the lower activity of oAMSA might be due to failure of oAMSA to enter intact cells. oAMSA only reduced the viscosity of DNA from permeabilized cells. Fig.1 shows that 0.5 μM [^{14}C]mAMSA was rapidly taken up by PY815 cells, reaching a steady state of 400 pmol per 2×10^7 cells after 5 min and slowly declining after 20 min. Drug bound or taken up at zero time was

Table 1

The effect on DNA of treating permeable or non-permeable PY815 cells with oAMSA or mAMSA

Treatment	Flow time (min:s)	
	Non-permeable cells	Permeable cells
Untreated cells	6:46 ± 0:30	6:09 ± 0:27
+ mAMSA (4 μM)	3:46 ± 0:06	3:31 ± 0:06
+ oAMSA (4 μM)	6:00 ± 0:030	3:39 ± 0:20

determined at 0°C because mAMSA uptake is dramatically slowed below 4°C [13].

Because of the unavailability of radioactive oAMSA, an indirect method was used first to assess whether oAMSA was taken up by cells. When cells were treated simultaneously with 0.5 μM [^{14}C]mAMSA and 0.5 μM oAMSA uptake of [^{14}C]mAMSA was decreased to half that with [^{14}C]mAMSA alone. Furthermore, when cells were incubated with 0.25 μM [^{14}C]mAMSA and 0.25 μM oAMSA or 0.25 μM mAMSA the [^{14}C]-mAMSA taken up was identical and amounted to 400 pmol drug per 2 × 10^7 cells at maximum uptake (not shown). These results confirmed that the cells did not discriminate between oAMSA and mAMSA when taking up the drugs and that oAMSA did not prevent mAMSA uptake.

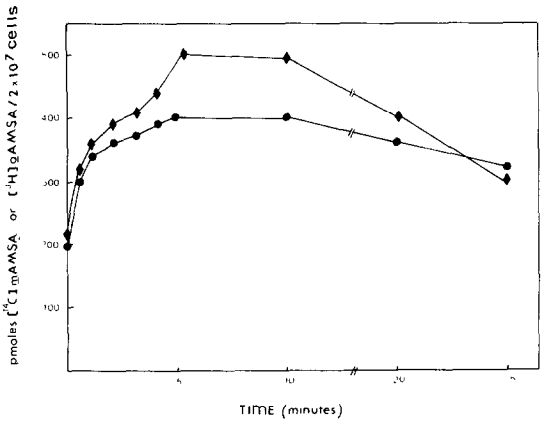

Fig.1. Uptake of radioactive mAMSA and oAMSA. (●—●) 0.5 μM [^{14}C]mAMSA, (◆—◆) 0.5 μM [^{3}H]oAMSA.

When cells were treated with 0.5 μM [^{14}C]-mAMSA plus 2 μM oAMSA, i.e. 2.5 μM total drug, uptake increased to a maximum of 900 pmol total drug per 2 × 10^7 cells after 3 min. Increased uptake at very high mAMSA concentrations was also observed with L1210 cells by Zwelling et al. [13] who conjectured that above a critical concentration mAMSA initiates irreversible sequestration into a new phase that does not contribute to cytotoxicity.

To confirm unequivocally that oAMSA entered cells [^{3}H]oAMSA (spec. act. 500 mCi/mmol) kindly synthesized by Dr W. Denny was used to compare oAMSA and mAMSA uptake. [^{3}H]oAMSA was taken up by PY815 cells as readily as mAMSA, reaching a maximum of approx. 500 pmol per 2 × 10^7 cells in 5 min after which the drug was slowly released from the cells (fig.1).

Because PY815 cells were equally accessible to mAMSA and oAMSA and either drug caused DNA scission in isolated nuclei [8] it was possible that oAMSA was more rapidly degraded than mAMSA in PY815 cells. Therefore, cells were pretreated for 30 min with 1 mM SKF525A or 1 mM cimetidine to inhibit microsomal drug metabolism prior to adding 0.1 μM mAMSA or 4 μM oAMSA to the cultures. At these low drug concentrations mAMSA or oAMSA did not cause DNA scission in the absence of the microsomal enzyme inhibitors and the microsomal enzyme inhibitors strongly potentiated drug action (table 2). SKF525A or cimetidine also substantially increased the cytostatic and cytotoxic effects of 0.5 or 0.1 μM mAMSA or 4 μM oAMSA (not shown). Because oAMSA and mAMSA were equally effective in breaking the DNA in isolated nuclei [8] these results suggested that oAMSA was more rapidly inactivated than mAMSA by PY815 cells. They also suggested that metabolites required for microsomal enzyme action were lost from permeabilized PY815 cells, since oAMSA induced DNA scission in permeabilized, but not in intact cells (table 1).

When the effects of 1 mM SKF525A on uptake of [^{14}C]mAMSA or [^{3}H]oAMSA were examined, maximum uptake of either drug was increased by approx. 60% and net drug efflux did not occur for at least 30 min. Similar results were obtained with 1 mM cimetidine indicating greater retention of oAMSA or mAMSA in the presence of

Table 2

Potentiation of AMSA drug action on DNA by microsomal enzyme inhibitors

Treatment	Flow time (min:s)
(A) Untreated cells	6:30 ± 0:15
(B) + AMSA drugs only	
+ 0.5 μM mAMSA	4:01 ± 0:01
+ 0.1 μM mAMSA	6.37 ± 0:03
+ 4 μM oAMSA	6.38 ± 0:12
(C) + Microsomal inhibitors only	
+ 1 mM SKF525A	6:58 ± 0:03
+ 1 mM cimetidine	6:21 ± 0:11
(D) mAMSA + microsomal inhibitors	
(i) 0.5 μM mAMSA + 1 mM SKF525A	2:35 ± 0:02
(ii) 0.1 μM mAMSA + 1 mM SKF525A	3:00 ± 0:02
(iii) 0.5 μM mAMSA + 1 mM cimetidine	2:59 ± 0:02
(iv) 0.1 μM mAMSA + 1 mM cimetidine	3:00 ± 0:00
(E) oAMSA + microsomal inhibitors	
(i) 4 μM oAMSA + 1 mM SKF525A	3:18 ± 0:15
(ii) 4 μM oAMSA + 1 mM cimetidine	3:58 ± 0:01

Cell density was 3×10^6 per lysate in all cases. Viscosity expressed as mean flow rate of 3 measurements

microsomal inhibitors. Furthermore, when cells preloaded with [^{14}C]mAMSA or [^{3}H]oAMSA for 10 min at 37°C were recovered, resuspended in fresh medium with 1 mM SKF525A or 1 mM cimetidine, and efflux of the radioactive mAMSA was measured over 10 min the microsomal enzyme inhibitors substantially reduced drug efflux compared to that observed with radioactive drugs alone (fig.2). Cimetidine appeared to inhibit oAMSA efflux more than mAMSA efflux. Therefore it was concluded that the microsomal enzyme inhibitors increased the retention of AMSA derivatives by PY815 cells, possibly by decreasing their metabolism.

In view of our results, susceptibility to inactivation by microsomal enzymes may be the major factor influencing activity of different AMSA derivatives. Therefore devising means to inhibit drug metabolism, or possibly chemically substituting existing drugs to increase their resistance to inactivation should be rewarding. A

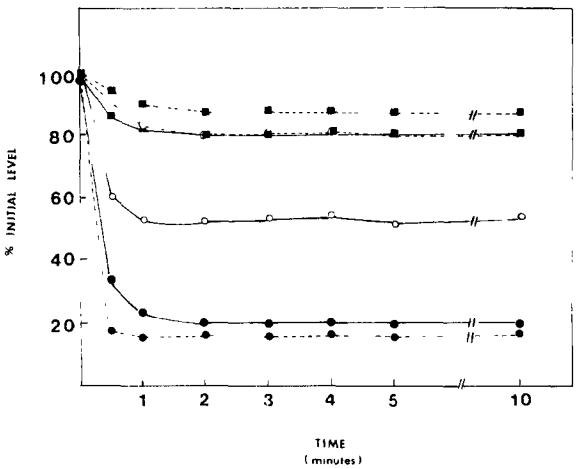

Fig.2. Inhibition of efflux of AMSA drugs by microsomal inhibitors. (●—●) 0.5 μM mAMSA, (■—■) 0.5 μM mAMSA + 1 mM SKF525A, (○—○), 0.5 μM mAMSA + 1 mM cimetidine, (●---●), 0.5 μM oAMSA, (■---■), 0.5 μM oAMSA + 1 mM SKF525A, (○---○), 0.5 μM oAMSA + 1 mM cimetidine.

greater understanding of the mechanism of action of microsomal enzymes on individual drugs would assist these objectives.

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REFERENCES

[1] Ralph, R.K., Marshall, B. and Darkin, S. (1983) Trends Biochem. Sci. 8, 212-214.
[2] Marshall, B. and Ralph, R.K. (1985) Adv. Cancer Res. 44, in press.
[3] Denny, W.A., Atwell, G.J. and Cain, B.F. (1979) J. Med. Chem. 22, 1453-1460.
[4] Ralph, R.K. (1980) Eur. J. Cancer 16, 595-600.
[5] Zwelling, L.A., Michaels, S., Erickson, L.G., Ungerleider, R.S., Nichols, M. and Kohn, K.W. (1981) Biochemistry 20, 6553-6563.
[6] Waring, M.J. (1976) Eur. J. Cancer 12, 995-1001.
[7] Wilson, W.R., Baguley, B.C., Wakelin, L.P.G. and Waring, M.J. (1981) Mol. Pharmacol. 20, 404-414.
[8] Marshall, B., Darkin, S. and Ralph, R.K. (1983) FEBS Lett. 161, 75-78.
[9] Marshall, B. and Ralph, R.K. (1982) Eur. J. Cancer 18, 553-557.
[10] Makan, N.R. (1978) Exp. Cell Res. 114, 417-427.
[11] Gomperts, B.D. (1983) Nature 306, 64-66.
[12] Marshall, B. and Ralph, R.K. (1982) Anal. Biochem. 125, 91-95.
[13] Zwelling, L.A., Kerrigan, D., Michaels, S. and Kohn, K.W. (1982) Biochem. Pharmacol. 42, 2687-2691.