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m-AMSA AS A PROBE FOR TRANSPORT PHENOMENA ASSOCIATED WITH ANTHRACYCLINE RESISTANCE

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Abstract—Kinetics of transport of the acridine derivative 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (m-AMSA) were examined in P388 murine leukemia cells and in P388/ADR, a subline selected for adriamycin resistance and cross-resistant to a variety of drugs including m-AMSA. Compared with the drug-responsive parent cell line, P388/ADR cells showed impaired accumulation of m-AMSA and an enhanced rate of drug exodus. Competition studies demonstrated structural specificity of the outward transport process. There was a low degree of intracellular m-AMSA binding, and steady-state drug levels were reached in <1 min. These results suggest that m-AMSA will be a useful probe for studying transport systems associated with anthracycline resistance.

Impaired drug accumulation is associated with development of resistance to a series of natural products, including the anthracyclines, vinca alkaloids and actinomycin D. This phenomenon is apparently mediated by an energy-dependent outward transport system that has been studied in P388/ADR, an adriamycin-resistant subline of the P388 murine leukemia [1–7], and in other similarly-derived cell lines [8–13]. The structural specificity of this outward transport system has been partially characterized [1, 11]. Skovsgaard [12] demonstrated that the relatively non-toxic substrate *N*-acetyldaunorubicin competed with DNR† exodus and promoted DNR toxicity in a drug-resistant Ehrlich ascites cell line.

Intracellular drug binding can complicate interpretation of transport kinetics associated with this mode of drug resistance [14, 15]. Therefore, it would be helpful to have a substrate that exhibits a low degree of intracellular binding. The P388/ADR cell line is cross-resistant [6] to m-AMSA, a synthetic acridine derivative [16]. The time–course accumulation of m-AMSA by L1210 cells is proportional to the extracellular drug level, if this is kept below 10 μM. Washing in fresh medium resulted in loss of >80% of accumulated drug [17, 18]. These results suggest that m-AMSA might be a useful probe for examination of transport phenomena associated with anthracycline resistance. A preliminary report of these studies has been presented [19].

MATERIALS AND METHODS

Cell lines. The P388 murine leukemia and the adriamycin-resistant subline (P388/ADR) were maintained in suspension culture using Fischer's medium supplemented with 10% horse serum and 1 μM mercaptoethanol.

Drugs. [9-^{14}C]-m-AMSA (20 Ci/mole) was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH. Purity of the compound was >98% by TLC assay. Non-radioactive acridines were provided by Drs. Bruce Cain and Bruce Baguley, University of Auckland, New Zealand.

Growth studies. Cells were treated for 1 hr with m-AMSA and other specified agents. The cells were then washed and diluted appropriately into growth medium + 0.2% agar. Colonies of control vs treated cells were counted after 7 days.

Transport studies. These studies were carried out at cell densities of 2×10^6/ml (7 mg/ml, wet weight) in HEPES-buffered Fischer's medium (GIBCO, Grand Island, NY). The pH was usually adjusted to 7.2, although some experiments were carried out over a pH range of 6 to 8.5. The concentration of labeled m-AMSA was 0.5 μM (3600 counts per min per ml of cell suspension). In no experiment was >20% of total radioactivity accumulated by cells. In some studies, m-AMSA analogs [20] were present during incubations, and their effects on steady-state accumulation of labeled m-AMSA were assessed. To determine the effect of energy depletion, glucose-free medium containing 10 mM sodium azide was used for some transport studies. Incubations were terminated by rapid chilling and centrifugation (30 sec, 200 *g*). Pellets were washed once with 150 mM NaCl at 0°, and drug uptake was assessed by liquid scintillation counting.

For outward transport studies, cells were first loaded with labeled m-AMSA during 10-min incuba-

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† Abbreviations: DNR, daunorubicin; ADR, adriamycin; m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (NSC 249992); LC_{50}, drug concentrations which cause a 50% cell kill; EC_{100}, drug concentrations which increase the steady-state uptake of m-AMSA by 100% in P388/ADR cells; and HEPES, 4-(2-hydropyethyl)-1-piperazine-ethanesulfonic acid.

tions at 37°. So that similar initial intracellular drug levels were obtained, loading concentrations of m-AMSA were 0.5 μM for P388 and 3 μM for P388/ADR cells. Drug-loaded cells were then suspended in growth medium (2×10^6 cells/ml) at 37°. In some experiments, the medium contained non-labeled m-AMSA analogs. At specified intervals, 500 μl of the cell suspension was mixed rapidly with an equal volume of 1.2 mM NaI + 2 mM $HgCl_2$ in 150 mM NaCl at 0° [21]. This inhibited further outward transport of m-AMSA. The cells were collected as before, and intracellular drug levels were estimated by liquid scintillation counting.

Kinetic studies. Kinetics of drug exodus were analyzed using the BMDP computer program designed by the Department of Biomathematics, University of California at Los Angeles. The size and half-life of intracellular drug compartments were thereby derived.

RESULTS

Studies on drug accumulation. The time course of m-AMSA uptake by P388 and P388/ADR cells at pH 7.2 is shown in Fig. 1. A drug distribution ratio of 1 corresponds to accumulation of 20 pmoles of m-AMSA/10^7 cells. Steady-state accumulation was reached within 0.5 min. Impaired drug accumulation by P388/ADR cells is apparent. In glucose-free medium containing 10 mM sodium azide, the steady-state m-AMSA accumulation by P388/ADR cells was increased to approximately 450 pmoles/10^7 cells. Drug accumulation by P388 cells was increased by <10%. In other studies, we examined the effect of pH variation on m-AMSA uptake; the results shown in Fig. 1 were not altered over a pH range of 6 to 8.5.

Structures of m-AMSA analogs are shown in Fig. 2. Drug analog concentrations required to double steady-state m-AMSA accumulation (EC_{100} values) are shown in Table 1. The capacity of these analogs to promote net m-AMSA uptake was not a function of drug toxicity; LC_{50} values for the AMSA analogs [20] are compared with EC_{100} levels in Table 1.

The time course of drug exodus from P388 and P388/ADR cells is shown in Fig. 3. The initial level

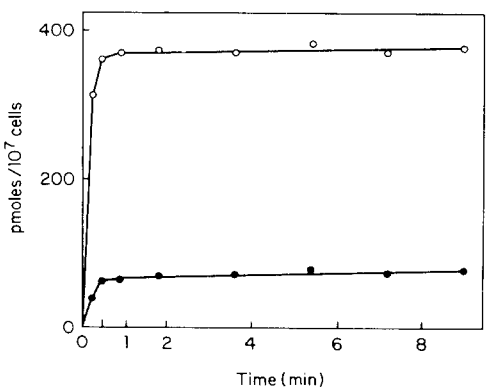

Fig. 1. Time course of [^{14}C]-m-AMSA accumulation by P388 (○) and P388/ADR (●) cells at 37°.

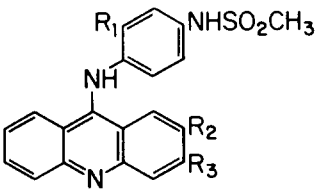

Fig. 2. m-AMSA analogs described in Table 1.

Table 1. Exodus inhibition versus toxicity of AMSA analogs*

Drug†	Substituents	LC_{50}‡	EC_{100}§
SN11841‖	R_1 = OMe	35	4.0
SN10106	R_1 = OMe, R_3 = NH_2	6	60
SN11838		35	50
SN12736	R_2 = Me	680	3.0
SN12723	R_2 = OMe	2,600	5.2
SN20279	R_1 = F	3,000	5.0
SN18738	R_1 = NO_2	17,000	6.6
SN20278	R_1 = I	30,000	5.3

* These data represent means of three experiments with a range of ±15% of the values shown here.

† Drug reference number. University of Auckland. Structures are shown in Fig. 2. R_{1-3} = H unless otherwise specified.

‡ Drug concentration (ng/ml) required for a 50% cell kill (24-hr exposure).

§ Drug concentration (μg/ml) required for 100% promotion of m-AMSA steady-state accumulation in P388/ADR cells.

‖ m-AMSA.

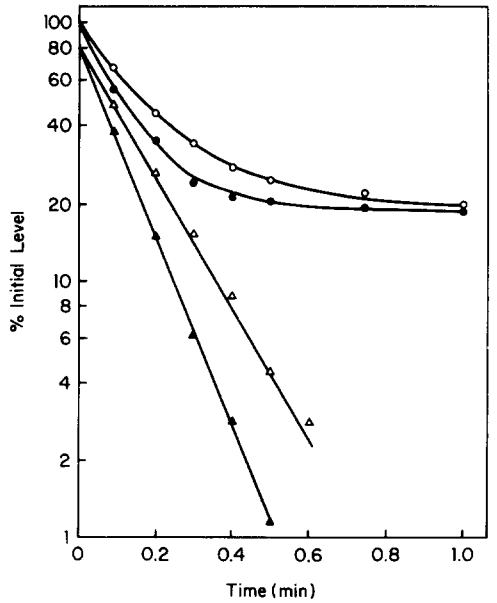

Fig. 3. Time course of m-AMSA exodus from P388 (○) and P388/ADR (●) cells. Computer-generated kinetics of exodus of the loosely-bound pools: P388 (△), P388/ADR (▲).

of intracellular drug was approximately 360 pmoles/10^7 cells for each cell line. Approximately 80% of the initially accumulated drug was rapidly lost, with the remainder more tightly bound. Computer analysis of five replicate experiments yielded control $T_{\frac{1}{2}}$ values of 0.084 ± 0.014 min for the loosely-bound drug pool in P388/ADR cells and 0.123 ± 0.011 min in P388. In both cell lines, the tightly-bound drug pool showed a half-life of >50 min. The fast phase of m-AMSA exodus was substantially slowed at 0° ($T_{\frac{1}{2}}$ = approximately 20 min). In glucose-free medium containing 10 mM sodium azide, the half-life of the loosely-bound drug pool was 0.125 ± 0.010 min in both cell lines.

We examined efflux of labeled m-AMSA in the presence of EC_{100} levels of three of the analogs described in Table 1: SN 10106, 12736 and 20278. In all such experiments, the half-life of the fast phase of exodus was increased to 0.120 ± 0.015 min. In other studies, we found that m-AMSA analogs, at concentrations shown in Table 1, increased m-AMSA uptake by less than 10% in P388 cells and did not detectably affect drug efflux from these cells.

The LC_{50} levels (24-hr exposure) of m-AMSA were 0.02 μg/ml for P388 and 0.5 μg/ml for P388/ADR. Addition of an EC_{100} level of SN 20278 or SN 18738 to P388 cultures did not affect cell growth, nor did this affect the LC_{50} level of m-AMSA. In contrast, such additions to P388/ADR cultures reduced the LC_{50} level of m-AMSA to 0.05 μM.

DISCUSSION

Resistance to anthracyclines and other natural products in several cell lines is associated with an energy-dependent exodus process [1–13] that limits intracellular drug accumulation. To determine whether a similar phenomenon could explain the cross-resistance of the P388/ADR cell line to m-AMSA [6], we examined m-AMSA transport in P388 and P388/ADR cells. The kinetics of m-AMSA transport were very rapid; equilibrium was achieved within 0.5 min. Resistance was associated with both decreased steady-state accumulation of m-AMSA (Fig. 1) and more rapid efflux of drug (Fig. 3). These results are consistent with similar reports concerning the kinetics of anthracycline transport in P388 vs P388/ADR cells [3–5, 10, 13]. DNR accumulation was strongly promoted by increased pH [10]; this effect was not observed with m-AMSA. A low degree of intracellular m-AMSA binding was also demonstrated (Fig. 3).

Data described here indicate that m-AMSA is a useful probe for examination of transport phenomena associated with anthracycline resistance. Minor variations in pH did not alter steady-state drug accumulation, and a substantial intracellular pool of free drug was obtained for exodus measurements. Because of the rapid rate of m-AMSA uptake, we could not determine the initial rates of drug uptake or the zero-time drug-cell association that presumably represents cell-surface binding. More rapid sampling techniques should permit determination of these values, so that a mathematical model can be developed to test the hypothesis that enhanced m-AMSA exodus is the major determinant of the steady-state intracellular drug concentration.

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