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# EFFECTS OF RUTHENIUM RED ON ACCUMULATION AND CYTOTOXICITY OF m-AMSA, VINBLASTINE AND DAUNORUBICIN IN LEUKEMIA CELLS

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**Abstract**—Effects of ruthenium red on accumulation and cytotoxicity of m-AMSA, vinblastine and daunorubicin were examined in the P388 murine leukemia cell line and in an adriamycin-resistant subline, P388/ADR, which is cross-resistant to all three agents. Ruthenium red increased m-AMSA accumulation by both P388 and P388/ADR cells; the extent of this effect was a function of the concentration of both agents. Uptake enhancement occurred within 5 min of exposure of cells to ruthenium red and was readily reversed when cells were suspended in fresh medium. A 24-hr exposure to ruthenium red was needed to affect vinblastine or daunorubicin accumulation, and the effect was substantially less than that observed with m-AMSA. Ruthenium red protected P388 cells from m-AMSA toxicity. These data, together with reports indicating a protective effect of ruthenium red against vinblastine and anthracycline toxicity, suggest that the dye promotes tight binding of m-AMSA and other agents to cellular loci on which toxic effects are not exerted.

Ruthenium red (RR†) is an inorganic dye commonly used as a histochemical stain for cell membrane mucopolysaccharides [1, 2]. In low concentrations, RR interferes with mitochondrial respiration and inhibits calcium uptake [3], exodus [4] and binding [3] by mitochondrial membranes. RR also inhibits $Ca^{2+}$-ATPase activity in sarcoplasmic reticulum [5] and in erythrocyte membranes [6].

Recent reports indicate that RR antagonizes VLB accumulation by tumor cells *in vitro* [7] and protects these cells from the cytotoxic effects of several natural products including VLB, ADR and epipodophyllotoxin [7, 8]. The effect of RR on VLB accumulation was unusual. RR did not affect the initial drug influx, but caused a loss of accumulated drug after 2 hr. RR inhibition of $Ca^{2+}$-ATPase activity suggested that the dye might act by enhancing retention of intracellular calcium, thereby antagonizing VLB binding to microtubules [7]. But, in a later study [8], no correlation was found between the protective effect of RR and inhibition of the $Ca^{2+}$-ATPase activity. Since RR also antagonized effects of VLB and non-tubulin binding agents, it was suggested that the dye might act by altering drug binding to membrane mucopolysaccharides or glycoproteins or both.

The present study was designed to examine the effects of RR on uptake and cytoxicity of m-AMSA, DNR and VLB in the P388 murine leukemia and in an adriamycin-resistant subline (P388/ADR) which is cross-resistant to all three of these agents [9–11]. m-AMSA is a useful substrate for such transport studies because of its low degree of intracellular binding [12, 13].

## MATERIALS AND METHODS

*Cell lines.* Properties of the P388 murine leukemia cell line and its adriamycin-resistant subline, P388/ADR, have been described [11, 14]. Exponentially growing cultures of these cells were maintained in Fischer's medium (GIBCO, Grand Island, NY) containing 10% horse serum, 1 μM mercaptoethanol, and antibiotics.

*Drugs.* [$^{14}$C]-m-AMSA (labeled in the 9 position, 20 Ci/mole) and [$^{14}$C]DNR (labeled in position 14, 30 Ci/mole) were obtained from the Division of Cancer Treatment, NCI. Analyses of DNR [15] and m-AMSA [16] were carried out using TLC procedures. [$^{3}$H]Vinblastine (11.3 Ci/mmole) was purchased from Amersham, Arlington Heights, IL; RR was from the Sigma Chemical Co., St Louis, MO, and from Alfa Products, Danvers, MA. This material was purified as described by Luft [1].

*Transport studies.* Cells were suspended in HEPES-buffered Fischer's medium (pH 7.2) at a density of $2 \times 10^6$/ml (7 mg/ml wet wt). Where specified, RR was added 5 min before addition of labeled drugs. To measure long-term effects of RR, the dye was added to cell cultures 24 hr before transport studies. Approximately steady-state accumulations were reached after incubating cells with 0.5 μM levels of m-AMSA (5 min), DNR or VLB (15 min) at 37°. Incubations were terminated by dilution with cold isotonic NaCl and centrifugation (500 *g*, 30 sec). The cell pellets were washed once

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† Abbreviations: RR, ruthenium red; ADR, adriamycin; m-AMSA, acridinylaminomethanesulfon-*m*-anisidide; DNR, daunorubicin; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; and VLB, vinblastine.

with isotonic NaCl at 0°, and intracellular radioactivity was measured by liquid scintillation counting.

Exodus studies were carried out using cells loaded with labeled drugs as specified above, but in glucose-free medium containing 10 mM $NaN_3$. This medium inhibits the energy-dependent drug exodus system found in the P388/ADR cell line [9, 17–20], so that both P388 and P388/ADR cells attain essentially equal intracellular drug concentrations. In some experiments, loading incubations were carried out in the presence of 0.3 μg/ml RR (0.35 μM). Drug-treated cells were collected by centrifugation and were suspended in fresh medium (containing glucose) at 37°. Aliquots of the suspension were diluted with chilled isotonic NaCl, and the cells were collected by centrifugation, as described above, at 0.2, 0.4, 0.6, 0.8, and 1.0-min intervals following resuspension. Results from exodus experiments were used to calculate the half-life of the loosely-bound drug pool, using a computer program for calculation of non-linear functions. A more thorough description of these procedures is given in Ref. 12.

We also examined the effects of RR on uptake of the non-metabolized amino acid cyclocleucine, uptake of the nucleoside uridine, and incorporation of thymidine into DNA, as described in Ref. 21.

*Cytotoxicity studies.* P388 and P388/ADR cells were incubated for 24 hr in growth medium containing RR, cytotoxic agents, or both. Growth curves of treated versus control cultures were compared. Counting and sizing were carried out using the Coulter Counter/Channelyzer system as described in Ref. 22.

*Drug analyses.* Transformations of DNR and m-AMSA were measured via TLC studies as described in Refs. 15 and 16. The initial purity of these radioactive compounds was >97%. After 15-min incubations, intracellular DNR and m-AMSA pools were also assayed, and effects of addition of 10 μM RR were assessed. In another study, we incubated labeled DNR or m-AMSA (1 μg/ml) together with 10 μg/ml RR at 37° for 30 min. TLC analysis was then used to seek evidence for drug transformations.

## RESULTS

*Drug accumulation studies.* In these experiments, cells were treated with RR for 5 min and then incubated with labeled m-AMSA for an additional 5 min. Figure 1 shows the effect of RR on accumulation of m-AMSA by P388 and P388/ADR cells, at two different m-AMSA levels. Impaired accumulation of m-AMSA by P388/ADR cells [10, 23, 24] is also shown (left side, RR concentration = 0). When the extracellular m-AMSA level was 0.2 μg/ml (approx. 0.5 μM), a RR concentration of 0.3 μg/ml (0.35 μM) caused a doubling of steady-state m-AMSA accumulation in P388 and a substantially greater increase in drug accumulation in P388/ADR. A drug distribution ratio of 1 corresponds to accumulation of 20 pmoles of m-AMSA/$10^7$ cells. As the extracellular RR level was increased, a corresponding increase in m-AMSA accumulation was observed; a plateau was reached when the drug distribution-ratio reached 45 (900 pmoles m-AMSA/$10^7$ cells).

When the m-AMSA level was increased to 3 μg/ml (7.5 μM), a RR concentration of 2 μg/ml (2.5 μM) was required to double the steady-state m-AMSA accumulation in P388 cells (Fig. 1, right). As the extracellular RR level was increased, there was a corresponding increase in steady-state m-AMSA accumulation until a drug distribution-ratio of 55 was reached.

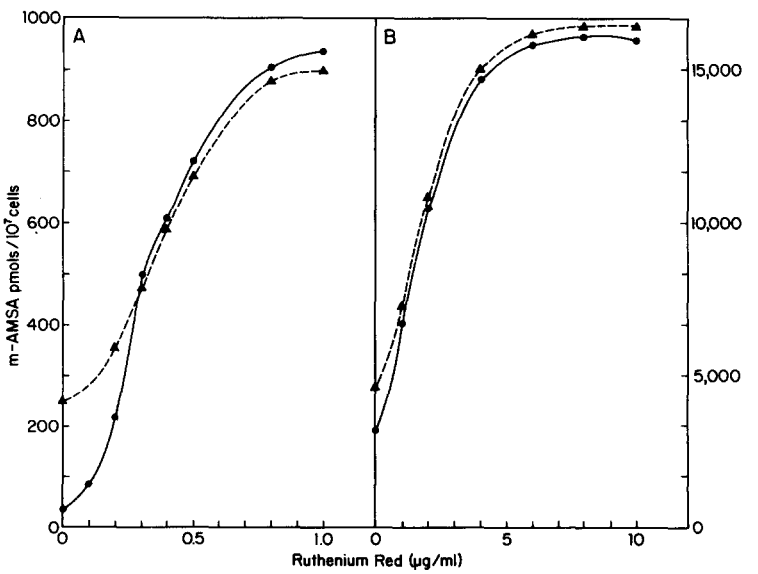


Fig. 1. Enhancement of m-AMSA uptake by RR as a function of the concentration of both drugs. Extracellular m-AMSA concentration, left: 0.2 μg/ml (0.5 μM); right: 3 μg/ml (7.5 μM). Key: P388 (▲), and P388/ADR (●). Cells were first treated with RR for 5 min and then incubated with labeled m-AMSA for an additional 5 min. Data shown represent the mean of three determinations; the range of values was ±10% of those shown.

Table 1. Effects of RR on drug accumulation by P388/ADR cells*

| Exposure to RR | Drug accumulation (%) DNR | VLB | m-AMSA |
|---|---|---|---|
| 5 min | 100 | 100 | 600 |
| 24 hr† | 145 | 135 | 590 |
| 24 hr‡ | 135 | 120 | 170 |

*Cells were treated with 0.3 μg/ml RR for 5 min or 24 hr and then incubated for 5 min with m-AMSA or 15 min with DNR or VLB (drug levels = 0.5 μM). These values represent the mean of five separate experiments with a range of ± 10% of the numbers shown here. The drug accumulation by control cultures (pmoles/$10^7$ cells) was 95 ± 5 (DNR), 200 ± 10 (VLB), and 55 ± 5 (m-AMSA).

† RR was present for the preceding 24 hr, and during the accumulation study.

‡ RR was present for the preceding 24 hr, and then cells were suspended in fresh medium without RR for the drug accumulation study.

In other studies, we found that RR affected neither incorporation of thymidine into DNA nor transport systems responsible for amino acid or nucleoside uptake.

A 15-min exposure of P388 or P388/ADR cells to RR level as high as 10 μg/ml (12 μM) did not affect steady-state VLB or DNR accumulation by either cell line. But VLB and DNR uptakes were increased when cells were grown in medium containing 0.3 μg/ml RR for 24 hr (data for P388/ADR shown in Table 1). Removal of RR from the medium, after the 24 hr incubation, did not change the modest effect on VLB and DNR uptake but markedly reduced the promotion of m-AMSA accumulation.

Results of m-AMSA exodus studies are summarized in Table 2. Cells were loaded with labeled m-AMSA in glucose-free medium containing sodium azide to antagonize an outward transport process [10, 22, 23] which limits drug uptake in P388/ADR cells. A very rapid loss of >80% of intracellular drug was observed when m-AMSA-loaded cells were suspended in fresh medium. The half-life of the loosely-bound drug pool was approximately 0.12 min for P388 cells and 0.08 min for P388/ADR. Drug exodus was markedly prolonged when cells were loaded with [$^{14}$C]-m-AMSA in the presence of RR.

Table 2. Half-time of m-AMSA exodus*

| Conditions | Half-time (min) cell line P388 | P388/ADR |
|---|---|---|
| Azide-loaded | 0.12 ± 0.01 | 0.08 ± 0.01 |
| RR-loaded | 85 ± 6 | 25 ± 3 |

* Kinetics of the initial phase of m-AMSA exodus from cells loaded with radioactive drug in glucose-free medium containing 10 mM $NaN_3$ or in medium (1 mg/ml glucose) containing 0.3 μg/ml RR. Cells were then suspended in fresh medium containing glucose for the determination of m-AMSA efflux. The averages of three determinations each are shown; these varied by less than ± 10% of values shown.

*Cytotoxicity studies.* At an 0.3 μg/ml (0.35 μM) concentration, RR did not affect proliferation of either cell line over a 24-hr interval but did protect P388 cells from the cytotoxic effects of m-AMSA (Table 3).

*TLC analyses.* Intracellular pools of P388 or P388/ADR cells incubated for 15 min in medium containing 0.5 μM labeled DNR or m-AMSA were analyzed by TLC. We found that >80% of recovered radioactivity co-chromatographed with authentic drug samples. This result was not altered by addition of 10 μg/ml RR. Moreover, incubation of labeled DNR or m-AMSA with a 10-fold excess of RR for 30 min, at 37°, caused no detectable drug transformations.

## DISCUSSION

RR protects tumor cells from the cytotoxic effects of several anti-tumor agents *in vitro*, including vincristine, vinblastine and adriamycin [7, 8]. It was suggested [7] that this phenomenon might be mediated via antagonism of drug uptake. In the present study, we first examined interactions between m-AMSA and RR. RR antagonized the cytotoxic effects of m-AMSA on the drug-sensitive P388 cells (Table 3). We found that a 0.3 μg/ml (0.35 μM) extracellular level of RR reversed the lethal effect of a 24-hr exposure to 0.03 μg/ml (0.075 μM) m-AMSA. But this drug combination *promoted* m-AMSA uptake by a factor of 2 (Fig. 1). The P388/ADR cell line was resistant to the toxic effects of 0.1 μg/ml (0.25 μM) m-AMSA (Table 3). m-AMSA toxicity was not affected by the presence of 0.3 μg/ml RR (0.35 μM), although this addition increased m-AMSA uptake by a factor of at least 6 (Table 1).

As is evident from data shown in Fig. 1, promotion of m-AMSA accumulation by RR was a function of the concentration of both drugs. When the concentration of m-AMSA in the medium was increased, a corresponding increase in RR concentration was required to enhance m-AMSA accumulation.

Data shown here suggest that RR promotes m-AMSA uptake into a non-cytotoxic cell compartment where the drug is tightly bound. Substantial retention of accumulated m-AMSA (Table 2) was

Table 3. Effects of RR on m-AMSA toxicity*

| Cell line | m-AMSA (μg/ml) | Ruthenium red (μg/ml) 0.00 | 0.03 | 0.30 |
|---|---|---|---|---|
| P388 | 0 | 80 | | 64 |
| | 0.01 | 76 | 85 | 97 |
| | 0.03 | 9 | 54 | 100 |
| | 0.1 | 7 | 37 | 102 |
| P388/ADR | 0 | 114 | | 114 |
| | 0.01 | 106 | 119 | 103 |
| | 0.03 | 115 | 105 | 108 |
| | 0.1 | 82 | 93 | 100 |

* Percent increase in cell density over 24 hr in medium containing specified levels of m-AMSA and RR. These results represent average values of an experiment carried out in triplicate; the range of numbers was ± 10% of values shown.

observed in both P388 and P388/ADR cells. RR only affected uptake of DNR or VLB by P388 or P388/ADR cells when the time of exposure to RR was 24 hr (Table 1). We conclude that the protection of cells in culture by RR from cytotoxic effects of m-AMSA, DNR and VLB cannot be ascribed to decreased accumulation of these agents. RR instead promotes drug accumulation, but to non-cytotoxic sites. Zwelling [25] also observed a substantial "sequestering" of m-AMSA at non-toxic intracellular loci, when the extracellular m-AMSA levels exceed 10$\mu$M. A similar effect by acridine orange on actinomycin D accumulation, without increased toxicity, has also been reported [10].

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