Table 4. The distribution of total glutathione S-transferase activity among various organs of the rat

Organ	Total activity (μmoles/organ/100 g rat/min) Unactivated microsomal	Cytosolic
Liver	9.100 (100.0)	466 (100.0)
Kidney	0.097 (1.0)	18.8 (4.0)
Lung	0.036 (0.4)	5.61 (1.0)
Adrenal	0.006 (0.0)	0.177 (0.0)
Testicle	1.060 (12.0)	132 (28.0)
Spleen	0.041 (0.5)	1.27 (0.3)
Brain	0.012 (0.1)	2.50 (0.5)
Heart	0.006 (0.0)	1.78 (0.4)
Thymus	0.002 (0.0)	0.78 (0.2)

The figures in parentheses are percentages of the liver value.

The total activated liver microsomal glutathione S-transferase activity can be calculated as 167 μmoles/liver/100 g rat/min [700 nmoles/mg microsomal protein × 17.7 mg microsomal protein/g liver × 4.08 g liver/100 g rat × 3 (to correct for the incomplete recovery of microsomes [12])]. Thus, if we assume that the microsomal glutathione S-transferase can be activated *in vivo* as it can *in vitro* (an assumption for which we as yet only have indirect evidence [14]), the largest capacity for conjugation of 1-chloro-2,4-dinitrobenzene and, presumably, of similar substrates with glutathione is found in the liver cytosol, the liver endoplasmic reticulum, and the testicle cytosol.

The most important observation presented here is that the N-ethylmaleimide-activatable microsomal glutathione S-transferase seems to be found only in the liver and is absent from the other organs examined. This is rather unusual, since most other drug-metabolizing enzymes are found in a variety of different tissues (see, for example, Table 2). Thus, if the N-ethylmaleimide-activatable microsomal glutathione S-transferase has some function other than conjugation of xenobiotics and/or their metabolites with glutathione, then this function must be localized mainly, if not exclusively, in the liver.

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Impairment by ruthenium red of anticancer drug cytotoxicity in CCRF-CEM cells

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It was shown recently that ruthenium red (RR), an inorganic dye, reduced both the cellular accumulation and the cytotoxicity of vinblastine (VLB) toward murine cells in culture [1]. It has been known for some time that RR binds to plasma membrane mucopolysaccharides and glycoproteins, and this property has been exploited in the staining of mucopolysaccharides for electron microscopy [2]. The dye is also known to inhibit both Ca^{2+} transport in mitochondria [3] and Ca^{2+}-ATPase activity in sarcoplasmic reticulum [4].

To account for the reversal of VLB cytotoxicity by RR in KB cells, it was proposed [1] that, besides the reduction in drug accumulation, the dye-induced inhibition of Ca^{2+}-ATPase could lead to an increase in intracellular Ca^{2+}, which in turn might eventually protect microtubules from binding by VLB.

The purpose of our present study was to analyze further this phenomenon of impaired drug toxicity by RR. Specifically, we wished to investigate the implied role of microtubules in this phenomenon and did so by examining

the effect of RR on the cytotoxicity of several classes of anticancer drugs, including some that are not thought to interact with microtubules.

Materials and methods

Cells and culture conditions. CCRF-CEM (CEM) cells were grown in minimal essential medium (Hanks' salts) and 10% fetal bovine serum as described elsewhere [5]. For growth experiments, cells were incubated in multiwell plates at a concentration of 2×10^5/ml with or without drugs; after 48 hr, the cell concentrations were determined by the use of a Coulter counter (model ZBI). The IC_{50} value is defined as the concentration of drug required to inhibit the growth of cells by 50% after 48 hr. Accordingly, the fold-increase in cytotoxic drug concentration was determined by dividing the IC_{50} of the RR-treated cells by the IC_{50} of the controls.

Assay of ATPase activity. Cells in the logarithmic phase of growth ($5–10 \times 10^5$/ml) were washed (twice) in phosphate-buffered saline (PBS, as described in Ref. 5) without Ca^{2+} or Mg^{2+} (pH 7.4). The cells were suspended at a concentration of 1.5×10^6/ml in 30 mM Tris-acetate buffer (pH 8), containing the following chemicals (in $M \times 10^{-3}$): ouabain, 0.2; NaCl, 90; KCl, 50; $CaCl_2$, 2.0; $MgCl_2$, 1.0; and RR at various concentrations. The assay, which was a modification of that of Karasaki and Okigaki [6], was started by preincubating 3×10^5 cells in 200 μl of buffer in tubes for 5 min at 37°. The ATPase reaction was initiated by adding 50 μl of 1 mM [γ-^{32}P]ATP (sp. act. 60 mCi/mmole) to the tubes; it was stopped after 20 min, as described [6]. Experiments were also performed in Ca^{2+}-free buffer containing ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) (2 mM) and $MgCl_2$ (1 mM).

Chemicals and supplies. RR, colchicine (CLC), podophyllotoxin (POD), molybdic acid, ouabain, EGTA, Trizma base, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) and ATP were purchased from the Sigma Chemical Co. (St. Louis, MO); VLB and vincristine (VCR) were supplied by Eli Lilly & Co (Indianapolis, IN), through the courtesy of Dr. Gerald L. Thompson. Adriamycin (ADR) was obtained from the Adria Laboratories (Wilmington, DE), and cytosine arabinoside (ara-C) was from Upjohn (Kalamazoo, MI); all these compounds were dissolved in 0.9% NaCl solution. VM-26 was a gift of the Bristol Laboratories (Syracuse, NY) through the courtesy of Dr. William T. Bradner. VM-26 was first dissolved in a solution of 0.9% NaCl, containing 35% dimethyl sulfoxide (DMSO; Fisher Scientific Co., Fairlawn, NJ), and then brought to volume with 0.9% NaCl. The highest final concentration of DMSO in culture was 0.69% and inhibited the growth of cells by < 5%, when compared to non-DMSO-treated controls. [γ-^{32}P]ATP (sp. act. 9.7 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). All other supplies were obtained from commercial sources.

Results

Effect of RR on the cytotoxity of VLB. The growth of CEM cells in different concentrations of VLB in the absence or presence of RR is shown in Fig. 1. VLB and RR were added to the cultures at the same time on day 0. RR (1 μg/ml) had no discernible independent effect on cell growth (left panel, [VLB] = 0 nM). VLB was progressively toxic to the cells, the greatest effect occurring between 5 and 10 nM. RR almost completely reversed the VLB-induced cytotoxicity when the alkaloid was present at concentrations between 10 and 100 nM; additionally, the dye partially prevented the cytotoxicity of 500 nM VLB (far right panel).

Effect of RR on the cytotoxicity of other oncolytic drugs. We next wished to determine if the effect of RR was specific for VLB and other Vinca alkaloids, or if its effect was more general. To this end, we examined the effect of RR on the cytotoxicity of different classes of oncolytic agents represented by the following drugs: VLB and VCR (Vinca alkaloids); VM-26 (epipodophyllotoxin) and ADR (anthracycline), the latter two being compounds which apparently do not interact [7] or interact weakly [8] with tubulin,

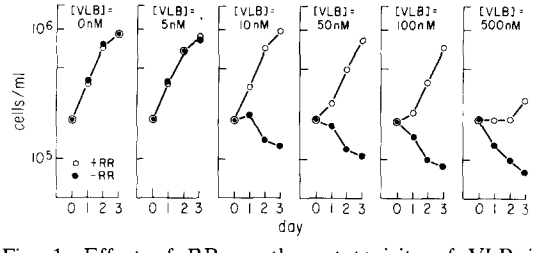

Fig. 1. Effect of RR on the cytotoxicity of VLB in CCRF-CEM cells. Cultures were seeded in multiwell dishes at 2×10^5 cells/ml in the abence (●) or presence (○) of 1 μg RR/ml and concentrations of VLB ranging from 0 to 500 nM (see the top of each panel). Cells were taken for counting with a Coulter counter (model ZBI) at 24, 48 and 72 hr.

Table 1. Effect of ruthenium red on drug cytotoxicity in CCRF-CEM cells*

Drug	Molecular wt	IC_{50}† [$M \times 10^{-9}$] Without RR	With RR	Fold-increase in cytotoxic concentration‡	P§
VLB	912	9.4 ± 9.6‖	75.5 ± 64.9	8.0	<0.01
VCR	923	0.39 ± 0.30	3.38 ± 1.56	8.7	<0.001
VM-26	657	89.9 ± 78.7	904 ± 1190	10.1	<0.05
ADR	556	67.2 ± 17.2	285 ± 146	4.2	<0.001
POD	414	20.3 ± 3.1	21.0 ± 2.6	1.03	NS
CLC	399	11.0 ± 2.3	9.9 ± 1.2	0.92	NS
Ara-C	243	18.7 ± 2.3	19.3 ± 3.1	1.03	NS

* Cells, diluted to a density of 2×10^5/ml, were exposed to the drug in the absence and presence of 1 μg RR/ml for 48 hr, at which time they were counted.

† The IC_{50} value is defined as the drug concentration that inhibits the growth of the cells by 50% after 48 hr.

‡ The fold-increase in cytotoxic concentration was determined by dividing the IC_{50} of the RR-treated cells by the IC_{50} of the controls.

§ P values were determined by Student's *t*-test; NS = not significant. The number of separate experiments for each drug ranged from three to nine.

‖ Mean ± S.D.

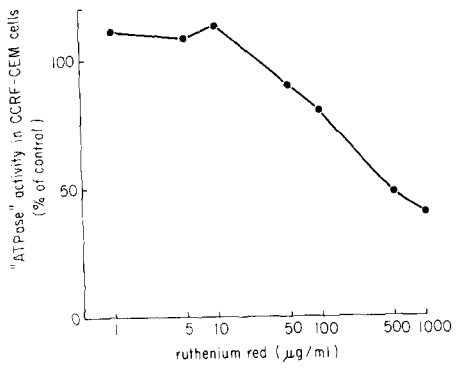

Fig. 2. Effect of RR on the ATPase activity of CCRF-CEM cells. Cells were incubated for 20 min in a ouabain-containing buffer, as described in Materials and Methods. ATPase activity was determined according to a modification of the method of Karasaki and Okigaki [6]. Since no specific Ca^{2+}-stimulated ATPase activity could be observed in these cells, the total ouabain-insensitive activity measured has been designated "ATPase". See text for further details. Control (100%) activity was 7.18 nmoles P_i liberated per 10^6 cells per 20 min.

respectively; ara-C (nucleoside antimetabolite); and CLC and POD, antimitotic compounds whose tubulin binding site is different from that of VLB and VCR [9, 10]. As can be seen in Table 1, RR (1 μg/ml) not only diminished the cytotoxicity of VLB by 8-fold, but it also diminished the cytotoxic effects of VCR, VM-26 and ADR, increasing the IC_{50} of these compounds by 9-, 10- and 4-fold respectively. In contrast to these observations, RR had no effect on the cytotoxic actions of CLC, POD or ara-C.

Reversal of the effect of RR on drug cytotoxicity. CEM cells were incubated in the presence or absence of 1 μg RR/ml for 48 hr, washed (twice), resuspended in fresh medium, and then tested for their sensitivity to VM-26 as before. It was found that the drug sensitivity of the cells preincubated with RR was the same as that of the controls, the IC_{50} values for VM-26 being 5.9×10^{-8} M and 6.1×10^{-8} M, respectively, indicating that the effect of RR was reversible.

Effect of RR on ouabain-insensitive ATPase activity of whole CEM cells. In our whole CEM cells, we found ouabain-insensitive ATPase activity to be stimulated to approximately the same extent in the presence of either Ca^{2+} (2 mM) or Mg^{2+} (1 mM) or both. Interestingly, the maximal activation of ATPase activity in this system was obtained in Ca^{2+}-free buffer containing Mg^{2+} (1 mM) and EGTA (2 mM) (data not shown); because this whole cell activity was not stimulated solely by Ca^{2+}, we designate this total ouabain-insensitive activity "ATPase". Similar results showing no stimulation by Ca^{2+} have been demonstrated in rat brain synaptosomal vesicles [11]. RR had no effect on whole CEM cell "ATPase" activity when the dye was present up to a concentration of 10 μg/ml (Fig. 2); however, under our assay conditions, 50 to 1000 μg of RR/ml caused a progressive inhibition of this ATPase activity by 16 to 62% respectively.

Discussion

RR has been shown to inhibit both Ca^{2+} transport in isolated mitochondria [3] and Ca^{2+}-stimulated ATPase activity in erythrocyte membranes [12]; inhibition of this enzyme by RR has also been seen in whole (KB) cells [1]. The effect of RR on plasma membrane Ca^{2+}-ATPase, however, appears to be rather controversial: for example, inhibition was not observed in lymphocytes [13]; moreover, in red cell membranes, inhibition was seen by some investigators [12] but not by others [14]. Additionally, with regard to the study by Tsuruo *et al.* [1], it should be noted that complete reversal of VLB cytotoxicity was obtained with concentrations of RR that inhibited the Ca^{2+}-ATPase activity of KB cells by only ≈12%, indicating that inhibition of this enzyme activity is not the primary mechanism responsible for the reversal of VLB cytotoxicity. Indeed, we observed no inhibition of "ATPase" activity by ≤ 10 μg RR/ml (Fig. 2), dye concentrations which are > 10 times that which caused diminished drug toxicity (Fig. 1 and Table 1).

Our results further demonstrate that the effect of RR on drug cytotoxicity is most likely unrelated to any direct effect on tubulin or microtubules as RR had no effect on the cytotoxicity of either CLC or POD, which are tubulin-binding agents. Morever, we could find no effect of the dye on either the 24-hr steady-state accumulation of $^{45}Ca^{2+}$, or its retention by the CEM cells (unpublished results). Accordingly, our experiments examining drug cytotoxicity, whole cell ouabain-insensitive ATPase activity, and $^{45}Ca^{2+}$ uptake tend to contradict the notion of Tsuruo *et al.* [1] that the effect of RR is specific for tubulin-binding agents and may be mediated through an action on microtubules, possibly by inhibition of Ca^{2+}-ATPase activity.

If then, RR is most likely not acting on microtubules or Ca^{2+}-ATPase, how might the reversal of drug cytotoxicity be mediated? The major features that Vinca alkaloids have in common with CLC, POD, ADR and VM-26 are that they are (a) "natural" products, or semi-synthetic derivatives of natural products, and (b) large heterocyclic molecules (their molecular weights range from 399 for CLC to 923 for VCR). Moreover, cells selected for (high) resistance to some of these agents have been shown to express cross-resistance to others [15, 16], and this has been attributed to alterations of the plasma membrane [16, 17 and *]. That CLC and POD, whose cytotoxic actions are not reversed by RR, have the lowest molecular weights of all these natural product compounds indicates that the size of the molecule may be important in the phenomenon of dye reversal of toxicity. Accordingly, we suggest that RR might act at the level of the plasma membrane, perhaps by reversibly perturbing local domains through interactions with mucopolysaccharides. In this regard, it would be of much interest to study the effects of RR on the relative drug sensitivities of cells having different proportions of mucopolysaccharides.

In summary, using cultured human leukemic lymphoblasts (CCRF-CEM), we have studied the effect of ruthenium red (RR) on the cytotoxicity of various oncolytic agents. RR is an inorganic dye which is known to bind to plasma membrane mucopolysaccharides and to inhibit Ca^{2+}–Mg^{2+} ATPase activity of the membrane. We have found that RR reduces the cytotoxicity of the tubulin-binding Vinca alkaloids VLB and VCR, as well as that of the anthracycline, ADR, and the epipodophyllotoxin, VM-26. In contrast, RR does not affect the cytotoxicity of other tubulin-binding agents, CLC and POD. Finally, we were unable to observe any effect of RR on total ouabain-insensitive, Ca^{2+}–Mg^{2+} ATPase activity at dye concentrations that were effective in reducing drug cytotoxicity. These data indicate that the reversal of drug cytotoxicity by RR is not mediated by inhibition of Ca^{2+}–Mg^{2+} ATPase, nor is it specific for antimicrotubular agents.

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Cytotoxicity of 2′-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine and its relationship to deoxycytidine deaminase

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2′-Fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC) and several structural analogs were synthesized recently by Fox and coworkers [1]. Some of them were found to have good activity against herpes simplex virus (HSV) [2, 3]. The selectivity of these compounds against HSV is related to their behavior as substrates of HSV-induced thymidine kinase [4] versus cellular thymidine kinase. The mechanism of their antiviral action may be related to the inhibition of DNA polymerases by the triphosphate derivatives of those pyrimidine nucleoside analogs [2, 5]. The cytotoxicity of FIAC was found to vary with the cell lines used for examination [2, 6]. When 2′-fluoro-5-iodo-1β-arabinofuranosyluracil (FIAU), the deaminated analog of FIAC, was evaluated for its cytotoxic effects, FIAU was found to be equal to, or more toxic than, FIAC in most of the cell lines tested [2, 6]. This suggested the possibility that the FIAC cytotoxicity could, to a certain extent, be dependent on its transformation to FIAU through the action of dCyd deaminase. The deamination of FIAC to FIAU by mouse and human leukemic cell dCyd deaminase was demonstrated previously although no detailed kinetic behaviors were given [7, 8]. In view of the apparent kinetic difference between the dCyd deaminases of humans and mice [9, 10], we examined how FIAC and its analogs behaved as substrates of human dCyd deminase and whether dCyd deminase played an important role in the action of FIAC. The results are discussed in this paper.

Materials and methods

Chemicals. dCyd and most of the other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO. The [2-^{14}C]dCyd was purchased from Moravek Biochemicals, Brea, CA. The 5-ethynyl araCyd and 5-ethynyl, 2′-F-araCyd were sent by Dr. Sharma and Dr. Bobek of Roswell Park Memorial Institute, Buffalo, NY. All the other analogs were synthesized in the laboratory of Dr. J. J. Fox at Memorial Sloan Kettering Cancer Center, New York, NY.

Enzyme assay. The procedure was essentially the same as that published by Chabner *et al.* [9] for routine dCyd deaminase assay except that the components of the assay were modified. The reaction mixture contained 0.6 mM [2-^{14}C]dCyd, 45 μCi/mmole, 0.6 mg/ml bovine serum albumin, 5 mM dithiothreitol (DTT), and 2.5 mM Tris–HCl, pH 8.0, and enzyme in a total volume of 100 μl. After incubation at 37° for 30 min, 50 μl of 1.2 N trichloroacetic acid (TCA) was added. After centrifugation, 100 μl of supernatant fluid was loaded on a 0.5 × 2.5 cm Dowex 50 H^+ column. Then 1.5 ml of H_2O was used to elute the [2-^{14}C]dUrd which was mixed with aqueous counting fluid and counted in a Beckman LC 100 liquid scintillation counter. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 nmole dUrd/min at 37°.

For those analogs which were not available in a radiolabeled form, the reaction conditions were essentially the same as above except that 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 8.0 (0.01 M), was used instead of Tris–HCl buffer. These assays were terminated by addition of 4 vol. of cold methanol. The supernatant fraction was removed and mixed with equal volumes of solution containing internal standard (usually