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Overexpression of the Multidrug Resistance Genes *mdr1*, *mdr3*, and *mrp* in L1210 Leukemia Cells Resistant to Inhibitors of Ribonucleotide Reductase

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ABSTRACT. L1210 MQ-580 is a murine leukemia cell line resistant to the cytotoxic activity of the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone class of inhibitors of ribonucleotide reductase. The line is cross-resistant to etoposide, daunomycin, and vinblastine. L1210 MQ-580 cells expressed 8-fold resistance to 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP), a relatively newly developed inhibitor of ribonucleotide reductase. The accumulation of [^{14}C]3-AP by L1210 MQ-580 cells was 5- to 6-fold less than by parental L1210 cells. An increased rate of efflux of 3-AP was responsible for the lower steady-state concentration of 3-AP in resistant cells. In reverse transcription–polymerase chain reaction assays, L1210 MQ-580 cells were found to overexpress the multidrug resistance genes *mdr1*, *mdr3*, and *mrp*, but not the *mdr2* gene, compared with parental L1210 cells. Measurement of the steady-state concentration of doxorubicin, a potential substrate for both the *mdr* and *mrp* gene products, demonstrated that L1210 MQ-580 cells accumulated 4-fold less anthracycline than parental cells. These findings indicate that drug efflux is a major determinant of the pattern of cross-resistance of L1210 MQ-580 cells. To extrapolate these observations to the human homologues of the *mdr1*, *mdr3*, and *mrp* murine genes, the effects of 3-AP were measured in L1210/VMDRC0.06 and NIH3T3 36-8-32 cells transfected with human MDR1 and MRP cDNAs, respectively. The transfectants were 2- to 3-fold resistant to the cytotoxic effects of 3-AP and accumulated less [^{14}C]3-AP than their parental mock-transfected counterparts. Moreover, the cytotoxic activity of 3-AP was significantly greater in two double *mrp* gene knockout cell lines than in parental W 9.5 embryonic stem cells. Thus, the results suggest that 3-AP is a substrate for both the P-glycoprotein and MRP and that baseline MRP expression has the capacity to exert a protective role against the toxicity of this agent. BIOCHEM PHARMACOL 54;6:649–655, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. multidrug resistance; ribonucleotide reductase; 3-AP; gene knockout; L1210 leukemia

Ribonucleotide reductase catalyzes a rate-limiting reaction in the synthesis of the 2′-deoxyribonucleoside 5′-triphosphates required for DNA synthesis. Because of the critical role that ribonucleotide reductase plays in the biosynthesis of deoxyribonucleotides, inhibitors directed at this metabolic target should have significant potential as antineoplastic agents [1]. Currently, the only known inhibitor of this enzyme in clinical use is hydroxyurea, which not only is a relatively poor inhibitor of ribonucleotide reductase, but also has a short serum half-life [2]. Nonetheless, the clinical utility of hydroxyurea suggests that other more potent inhibitors of ribonucleotide reductase might well be useful antineoplastic agents, particularly against rapidly proliferating cancers. α-(*N*)-Heterocyclic carboxaldehyde thiosemicarbazones represent a class of agents that are among the most effective inhibitors of ribonucleotide reductase activity identified to date [3–5]. A member of this class of agents, 5-HP[‖], underwent a phase I clinical trial in the early 1970s. Although 5-HP exhibited broad spectrum activity against transplanted animal tumors and spontaneous lymphomas in dogs [6], this agent did not exhibit antineoplastic activity in the phase I trial, presumably due to the rapid formation and elimination of an inactive O-glucuronide conjugate [7]. Recently, a new series of amino-substituted pyridine-2-carboxaldehyde thiosemicarbazones has been synthesized in one of our laboratories;

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‖ *Abbreviations:* 5-HP, 5-hydroxy-2-formylpyridine thiosemicarbazone; 3-AP, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 3-AMP, 3-amino-4-methylpyridine-2-carboxaldehyde thiosemicarbazone; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; VP-16, etoposide; ES, embryonic stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and RT–PCR, reverse transcription–polymerase chain reaction.

3-AP and 3-AMP were found to be the most active members of this series with respect to antileukemic activity *in vivo*, inhibition of cell growth *in vitro*, and inhibition of ribonucleotide reductase activity [8, 9]. 3-AP, which appears to be somewhat more active *in vivo*, against the L1210 leukemia, has been selected for clinical development.

Resistance to multiple chemotherapeutic agents (MDR) is a major obstacle to the successful treatment of most human cancers. The phenomenon of MDR confers upon malignant cells the ability to withstand exposure to lethal doses of many structurally unrelated antineoplastic agents, including the epipodophyllotoxins, the anthracyclines, and the *Vinca* alkaloids [10]. Antimetabolites are generally not included in the spectrum of cross-resistance. MDR has been characterized by the overexpression of a membrane-associated glycoprotein, the P-glycoprotein, which plays a role in drug efflux [10, 11].

With respect to the *mdr* genes, two genes in humans and three genes in mice have been identified, which have a high degree of homology, but different functions. The human *mdr1* gene and the murine *mdr1* and *mdr3* genes can confer multidrug resistance, while the closely related human *mdr3* and murine *mdr2* genes are not involved in MDR, but rather in the transport of phospholipids into the bile. Consequently, mice homozygous for a disruption of the *mdr2* gene present with a severe liver disease associated with the absence of phospholipids in the bile [12].

The recent identification of a 190 kDa membrane transport protein termed MRP in multidrug-resistant small cell lung cancer cells which do not express the P-glycoprotein is consistent with the existence of other non-P-glycoprotein-mediated forms of MDR [13]. In 1992, the MRP gene was cloned and the nucleotide sequence determined [14]. Since it shares sequence homology with the ATP-binding cassette transmembrane transporters, it has been included in this superfamily, which also includes the *mdr1* gene. The amino acid identity between the MRP and the P-glycoprotein, however, is only 15% [14]. Transfection of cells with MRP expression vectors has demonstrated that the protein confers resistance to natural products, such as VP-16, doxorubicin, *Vinca* alkaloids, arsenite, and cadmium [15, 16]. We recently found that in two cell lines in which the expression of the *mrp* gene was abrogated by homologous recombination, the cytotoxic activities of VP-16, teniposide, vincristine, doxorubicin, and sodium arsenite were significantly greater than in the parental wild-type cells [17].

L1210 MQ-580 is a subline of murine leukemia L1210 cells resistant to the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone class of ribonucleotide reductase inhibitors, and exhibits cross-resistance to VP-16, daunomycin, and vinblastine [18]. The ribonucleotide reductase activity in extracts prepared from these resistant cells is elevated only slightly, and the enzyme in cell-free extracts from the L1210 MQ-580 cells exhibits the same sensitivity to inhibitors of ribonucleotide reductase as that from wild-type cells, presumably indicating that a change in ribonucleotide reductase is not the basis for the resistant phenotype [18].

To gain information on the mechanism of resistance of L1210 MQ-580 cells, we have determined whether: (a) the *mdr* and/or *mrp* genes were overexpressed in L1210 MQ-580 cells; (b) the rates of drug transport between L1210 MQ-580 and parental L1210 cells were different; and (c) 3-AP, a ribonucleotide reductase inhibitor was a substrate for the P-glycoprotein and MRP.

MATERIALS AND METHODS

Cell Lines and Growth Inhibition Assay

Wild-type L1210 leukemia cells (originally obtained from the American Type Culture Collection, Rockville, MD), mock-transfected L1210 cells, and sublines L1210 MQ-580 and L1210/VMDRC0.06 were maintained in suspension culture in RPMI 1640 medium supplemented with 10% horse serum. The generation of L1210 MQ-580 cells has been described previously [18]. The sensitivity to 3-AP of L1210 and L1210 MQ-580 cells was determined using the MTS/PMS microtiter plate assay as previously detailed [19]. 3-AP was prepared as a stock solution in DMSO, and the maximum concentration of solvent to which cells were exposed was 0.05%. L1210/VMDRC0.06 is a subclone of the L1210 leukemia transfected with a retroviral expression vector containing the full-length cDNA for the human *mdr1* gene [20, 21]. Mock-transfected NIH3T3 and NIH3T3 36-8-32 cells, transfected with a retroviral expression vector containing the full-length cDNA for the human *mrp* gene [16, 22], were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. All cell lines were grown at 37° in an atmosphere of 5% CO_2 in air and periodically checked for the presence of mycoplasma. Cells were maintained in logarithmic growth by serial subculture at fixed intervals of time. Cell numbers were determined with a Coulter model ZBI particle counter, and short-term viability was ascertained by the exclusion of trypan blue. For assays of growth inhibition, cells were plated at a concentration of 5×10^4 cells/mL and 24 hr later were exposed to 3-AP for 72 hr. Then cells were washed twice with PBS, and the number of viable cells was determined with a Coulter model ZBI particle counter and Channelyzer. Undifferentiated W 9.5 ES cells and MRP double knockout ES MRP-/-1 and ES MRP-/-2 clones [17] were maintained in gelatinized tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, and 1000 units/mL of recombinant leukemia inhibitory factor (Gibco BRL, Grand Island, NY) at 37° in an atmosphere of 10% CO_2 in air. The sensitivity to 3-AP of the knockout cell lines was determined using the MTS/PMS microtiter plate assay as previously detailed [19].

Flow Cytometric Analysis of Doxorubicin

Flow cytometric analysis was performed with a FACS Vantage Flow cytometer (Becton-Dickinson, San Jose,

CA). The cells were excited at 488 nm and emission was collected above 580 nm. A minimum of 20,000 cells was analyzed for each sample. Relative intensity was calculated according to the method of Schmid *et al.* [23].

Intracellular Transport of [^{14}C]3-AP

[^{14}C]3-AP (specific activity 3.6 mCi/mmol) was chemically synthesized in our laboratory as previously described [5]. The cellular flux of radiolabeled 3-AP was measured according to methodology described earlier [24]. The viability of cells after exposure to investigational drugs was always assessed by the exclusion of trypan blue. At various times after the onset of drug treatment, 100 μL of cell suspension was added to a tube containing 10 mL of ice-cold PBS, and cells were collected by centrifugation. An aliquot of the supernatant was saved for measurement of extracellular radioactivity, and the pellet was resuspended in 60 μL of ice-cold PBS and placed in an "oil stop" tube consisting of a 400-μL Eppendorf microfuge tube containing 125 μL of oil (16% Fisher 0121 light paraffin oil and 84% Dow Corning 550 silicon fluid, with a final specific gravity of 1.04 g/mL) layered over 30 μL of 15% trichloroacetic acid and immediately centrifuged for 20 sec at 12,000 *g* using a Beckman model E microfuge. Microfuge tubes were cut through the oil layer and the lower parts placed in glass mini-scintillation vials. After the addition of 200 μL of distilled water, the vials were vortexed vigorously, 5 mL of Ultima Gold scintillation fluid (Packard Co., Meriden, CT) was added, and the radioactivity therein was measured by scintillation spectrometry. Zero-time binding, measured by adding radiolabeled 3-AP to cells prechilled on ice, was always less than 1%. The intracellular volume of L1210 and L1210 MQ-580 cells was calculated for untreated cells and for cells exposed to different concentrations of drugs using $^{3}H_2O$ (18 Ci/mmol) to determine the total water space and [^{14}C]inulin (10 mCi/mmol) to calculate the extracellular space [25].

RT–PCR Assay

Total cellular RNA was isolated from L1210 and L1210 MQ-580 cells by the method of Chomczynski and Sacchi [26]. A single large scale cDNA reaction for use in the different gene-specific amplification reactions was prepared. A 200-μL RT reaction mixture, prepared on ice, contained 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 5 mM $MgCl_2$; 1 mM each of dATP, dCTP, dGTP, and dTTP; 2 units/μL of RNase inhibitor (Promega, Madison, WI); 0.003 A_{260} units of random hexanucleotides (Boehringer Mannheim, Indianapolis, IN); and 0.4 units/μL of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT). Following incubation for 10 min at 25° and for 30 min at 42°, the reaction was terminated by incubation for 5 min at 99°, followed by a 4° quick chill. cDNA amplifications were performed by adding 80 μL of a standard PCR reaction mixture to 20 μL of the large scale

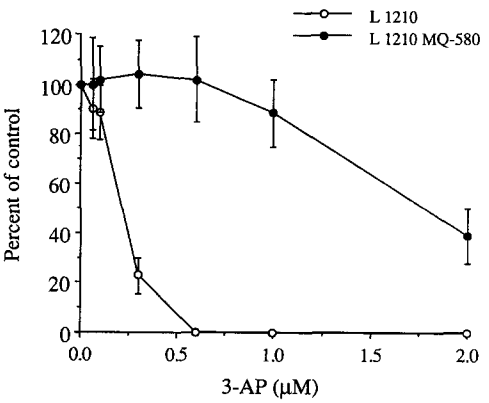

FIG. 1. Effects of 3-AP on the growth of L1210 and L1210 MQ-580 cells. After 3 days of continuous exposure, the sensitivity of cells to 3-AP was measured by the MTS/PMS assay as previously detailed [19] and expressed as a percent of the absorbance values of cells treated with the solvent alone (ranging from 1.0 to 1.5 $\Delta A_{490\ nm}$). Data are the means ± SD of 3–4 separate experiments.

cDNA reaction in the presence of gene-specific primers, followed by incubation at 94° for 4 min and then 28 or 32 cycles of 94° for 30 sec, 55° for 1 min, 72° for 1 min, a final extension at 72° for 10 min, and a quick chill to 4°. The amplimer sequences used were: mrp upstream: 5′-TG CAGTCAGTGTCCTGATGA-3′ and mrp downstream: 5′-TTCTCCTTTGTCCAGGAC-3′, yielding a 341-bp DNA product; mdr1 upstream: 5′-CAGTGTTTGCCAT AGTATTTTCAAGGATTG-3′ and mdr1 downstream: 5′-CCCTTTAACACTAGAAGCATCAC-3′, yielding a 390-bp DNA product; mdr2 upstream: 5′-TATCCGC TATGGCCGTGGGAA-3′ and mdr2 downstream: 5′-ATCGGTGAGCTATCACAATGG-3′, yielding a 400-bp DNA product; mdr3 upstream: 5′-AACAGCG-GTTTCCAGGAGCTGCTGG-3′ and mdr3 downstream: 5′-CATTGCCTGGAAGAACATTCCGATT-3′, yielding a 708-bp DNA product; GAPDH upstream: 5′-CGG GAAGCTTGTGATCAATGG-3′ and GAPDH downstream: 5′-GGCAGTGATGGCATGGACTG-3′, yielding a 358-bp DNA product. Twenty microliters of each amplified reaction mixture was separated on a 2% agarose gel containing 1 μg/mL of ethidium bromide.

RESULTS AND DISCUSSION

We have reported previously that L1210 MQ-580 cells exhibit an 8.7-fold level of resistance to the ribonucleotide reductase inhibitor 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone [18]. We have now ascertained whether L1210 MQ-580 cells exhibit cross-resistance to 3-AP. A comparison of the cytotoxic activities of 3-AP in parental L1210 and L1210 MQ-580 is shown in Fig. 1. The IC_{50} values were 0.21 ± 0.02 μM for L1210 and 1.78 ± 0.3 μM for L1210 MQ-580 cells, resulting in an 8.5-fold level of resistance. To ascertain whether a difference in drug transport might be at least partially responsible for the resistant

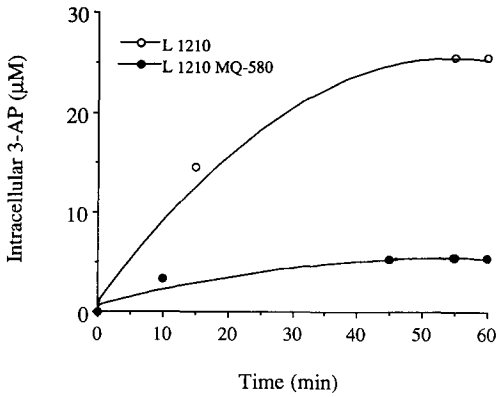

FIG. 2. Time–course of the influx of [^{14}C]3-AP in L1210 and L1210 MQ-580 cells. Cells were incubated with 5 μM [^{14}C]3-AP as described in Materials and Methods, and intracellular radioactivity was measured at different times over a 60-min period. Points represent the means of 2–3 separate experiments in which the variability was less than 10%.

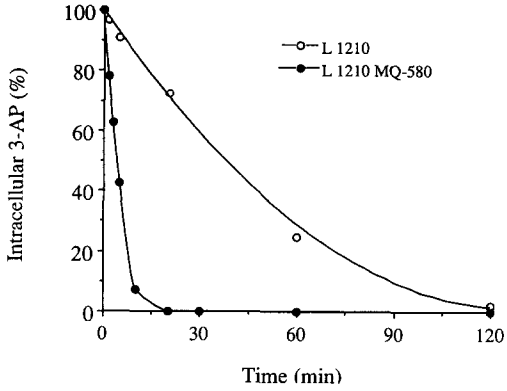

FIG. 3. Efflux of [^{14}C]3-AP in L1210 and L1210 MQ-580 cells. Cells were incubated with 5 μM (L1210) or 28 μM (L1210 MQ-580) [^{14}C]3-AP for 60 min to achieve the same intracellular level of 3-AP, extracellular drug was removed by two washings at 4°, and cells were resuspended in 3-AP-free medium at 37°. Over the next 120 min, the levels of intracellular radioactivity were measured. Points represent the means of 2–4 independent experiments, in which the variability was less than 10%.

phenotype of L1210 MQ-580 cells, the cellular uptake of 5 μM [^{14}C]3-AP by L1210 and L1210 MQ-580 cells was measured after different times of exposure (Fig. 2). 3-AP progressively accumulated in both cell lines. At 45 min, the intracellular levels of [^{14}C]3-AP were five to six times lower in L1210 MQ-580 cells than in parental L1210 cells. To determine whether the rate of efflux of 3-AP was responsible for the marked decrease in the accumulation of this agent in L1210 MQ-580 cells, parental L1210 and L1210 MQ-580 cells were loaded to the same intracellular concentration of [^{14}C]3-AP (about 25 μM) by incubation for 45 min with 5 and 28 μM 3-AP, respectively. Then cells were washed free of excess drug at 0° and resuspended in 3-AP-free medium at 37°. The rate of efflux of 3-AP from L1210 MQ-580 cells was considerably faster than from L1210 cells (Fig. 3), indicating that in L1210 MQ-580 cells one or more membrane proteins responsible for the export of 3-AP were operative. We have reported previously [18] that L1210 MQ-580 cells exhibit a 20- and 16-fold level of resistance to VP-16 and daunomycin, respectively. To determine whether drug transport was at least partially responsible for the pattern of cross-resistance, we measured the cellular uptake of doxorubicin by flow cytometry, demonstrating that the steady-state level of doxorubicin in L1210 MQ-580 cells was three to four times lower than in parental L1210 cells (Fig. 4).

The expression of the *mdr1*, *mdr2*, *mdr3*, and *mrp* genes was analyzed in L1210 and L1210 MQ-580 cells to determine whether one or more of these genes were overexpressed in the resistant variant. The results of RT–PCR assays shown in Fig. 5 revealed an increase in *mdr1*, *mdr3*, and *mrp* expression in L1210 MQ-580 cells compared with parental L1210 cells, while the expression of *mdr2* was unchanged. This finding is in agreement with the current knowledge that *mdr2* is not involved in the phenomenon of drug resistance, its main function being to transport phospholipids into the bile [12]. GAPDH, a ubiquitously ex-

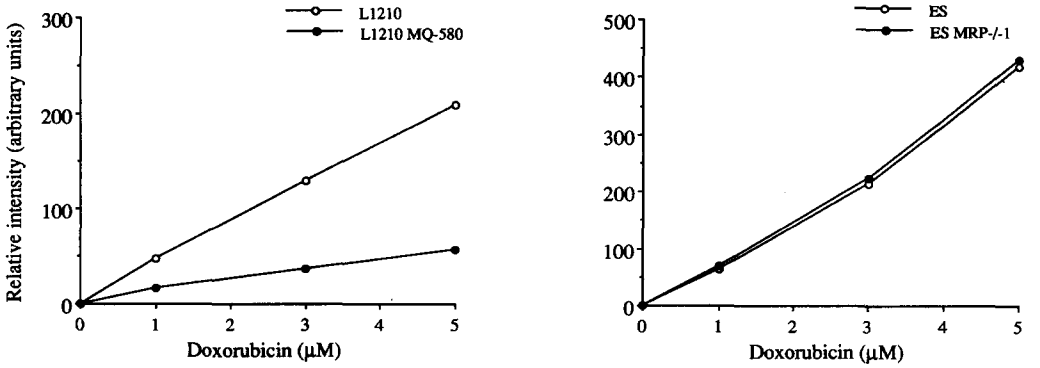

FIG. 4. Steady-state cellular levels of doxorubicin in L1210, L1210 MQ-580, ES, and ES MRP-/-1 cells exposed to various extracellular concentrations of drug. Exposure to doxorubicin was for 2 hr, which resulted in the attainment of steady-state levels of the drug. Doxorubicin fluorescence was measured by flow cytometry, and the results expressed in arbitrary units of fluorescence. Points represent the mean of 2 separate experiments in which the variability was less than 10%.

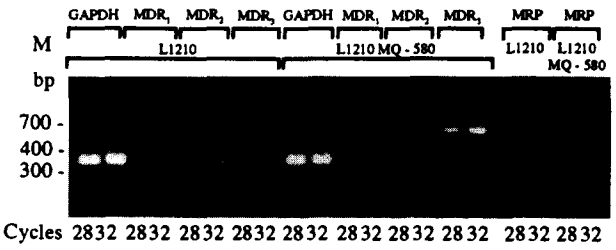

FIG. 5. Relative expression of mdr1, mdr2, mdr3, mrp, and GAPDH mRNAs in L1210 and L1210 MQ-580 cells. cDNAs derived from 100 ng of total RNA were amplified for 28 or 32 cycles of PCR. Twenty percent of the respective PCR reaction products was separated on a 2% agarose gel containing 1 μg/mL of ethidium bromide. The number of PCR amplification cycles is indicated below each lane. M = DNA markers (100-bp DNA ladder).

pressed cell cycle independent gene, was used as a control for RNA integrity and as a gene expression reference point. Clones isolated from L1210 MQ-580 cells also exhibited an increase in *mdr1*, *mdr3*, and *mrp* expression (data not shown), and exhibited a pattern of cross-resistance similar to that of the L1210 MQ-580 cell line [18]. These observations strongly suggest that the L1210 MQ-580 cell line does not derive from the selection of two or more independent clones, each of them overexpressing one resistance gene, but rather from the co-expression of *mdr1*, *mdr3* and *mrp* in the same cells. Furthermore, co-expression of MRP and MDR in the same cell is possible, as found by Brock *et al.* [27] in VP-16 selected H69 small cell lung cancer by double immunocytochemical staining.

To extrapolate these observations to the human homologues of the *mdr1*, *mdr3*, and *mrp* genes, the cytotoxic activity and the intracellular accumulation of 3-AP were measured in MDR1 overexpressing L1210/VMDRC0.06 cells [20, 21] and in MRP overexpressing NIH3T3 36-8-32 cells [22], and the results compared with those of the respective parental cell lines. To compare the sensitivity of parental and resistant cell lines to the cytotoxicity of 3-AP, cell lines were exposed continuously for 3 days to various concentrations of this agent, and the number of viable cells was measured as described in Materials and Methods. The growth curves presented in Fig. 6 show that L1210/VMDRC0.06 and NIH3T3 36-8-32 cells were two to three times more resistant to the cytotoxic activity of 3-AP than their parental counterparts over the range of concentrations employed. The cellular accumulation of ^{14}C-labeled 3-AP by the two parental and the two resistant cell lines was measured after exposure to 5 μM radiolabeled drug for 45 min at 37°, when a steady-state concentration of 3-AP was achieved (Fig. 7). A 2-fold decrease in the accumulation of [^{14}C]3-AP was found in resistant cell lines compared to their respective parental cells. The level of reduction in the accumulation of 3-AP was consistent with the level of resistance to 3-AP exhibited by these cell lines, implying that both MDR1 and MRP are able to export 3-AP from the intracellular compartment.

To ascertain whether MRP protects cells from the toxicity of 3-AP under normal physiological conditions, we compared the sensitivity of MRP double knockout cell lines to 3-AP with that of wild-type ES cells. In the two MRP double knockout ES cell lines, ES MRP-/-1 and ES MRP-/-2, the abrogation of MRP expression results in hypersensitivity to VP-16, teniposide, sodium arsenite, doxorubicin, and vincristine, but not to colchicine and cytosine arabinoside [17]. As shown in Fig. 8, the MRP double knockout clones were also hypersensitive to 3-AP. The IC_{50} values for 3-AP were 0.52 ± 0.09 μM for parental ES cells, 0.31 ± 0.07 μM for clone ES MRP-/-1, and 0.35 ± 0.03 μM for clone ES MRP-/-2, resulting in a 1.68-fold level of hypersensitivity for clone ES MRP-/-1 and a 1.49-fold level of hypersensitivity for clone ES MRP-/-2. Therefore, the abrogation of MRP expression resulted in a moderate level of hypersensitivity to 3-AP, suggesting that physiologically MRP is capable of playing a role in the detoxification of 3-AP. To distinguish between the relative contribution of the overexpression of *mrp* and *mdr* genes to the reduced intracellular levels of doxorubicin present in L1210 MQ-580 cells, flow cytometric analyses of the cellular uptake of doxorubicin were performed in the ES cell lines. The results shown in Fig. 4 demonstrate that the steady-state levels of

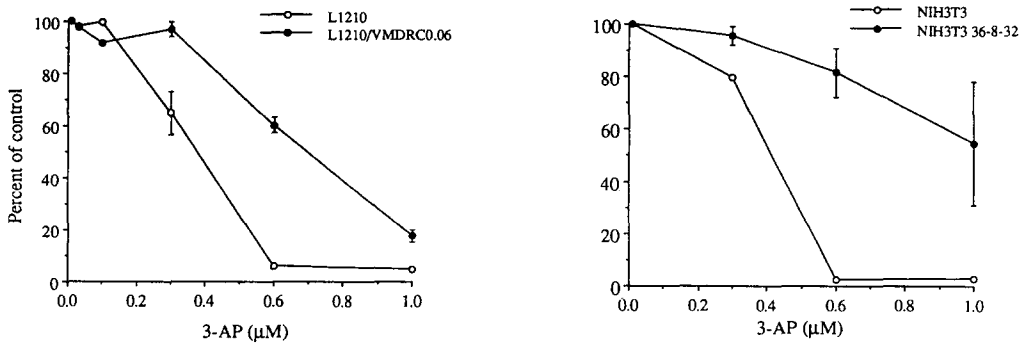

FIG. 6. Effects of 3-AP on the growth of mock-transfected L1210, MDR1-transfected L1210/VMDRC0.06, mock-transfected NIH3T3, and MRP-transfected NIH3T3 36-8-32 cells. After 3 days of continuous exposure to 3-AP, cell viability was determined, and expressed as a percent of cells treated with the solvent alone (ranging from 1.5 to 2 × 10^6 cells/mL). Data are the means ± SD of 3–4 separate experiments.

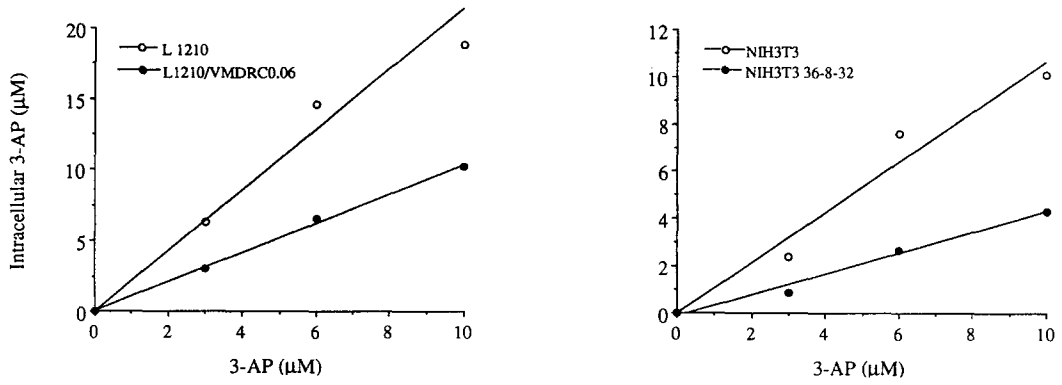

FIG. 7. Steady-state cellular levels of [^{14}C]3-AP in mock-transfected L1210, MDR1-transfected L1210/VMDRC0.06, mock-transfected NIH3T3, and MRP-transfected NIH3T3 36-8-32 cells exposed to various extracellular concentrations of drug. Exposure to [^{14}C]3-AP was for 45 min, which resulted in the attainment of steady-state levels of 3-AP, and intracellular radioactivity was measured as described in Materials and Methods. Points represent the means of 2 separate experiments in which the variability was less than 10%.

doxorubicin in clone ES MRP-/-1 were similar to those found in parental ES cells, suggesting that the *mdr* genes, rather than *mrp*, are responsible for the reduction in intracellular doxorubicin levels observed in L1210 MQ-580 cells.

The α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones represent a new structural class of anticancer agents extruded by an active efflux process. Furthermore, our findings demonstrate that MRP has the potential, under normal conditions, to partially protect normal tissue and presumably neoplastic cells from the toxicity of 3-AP by an active efflux mechanism, and that 3-AP is also a potential substrate for the P-glycoprotein. The non-availability of cell lines in which the *mdr* genes have been knocked out does not allow an analysis of whether the products of the *mdr* genes protect cells from the toxicity of 3-AP under normal conditions. Therefore, we could not assess whether and at what level *mdr1*, *mdr3*, and *mrp* contribute to the resistance pattern of L1210 MQ-580 cells. The great difference in doxorubicin accumulation between L1210 MQ-580 and parental L1210 cells, compared with the equal steady-state concentration of doxorubicin between parental and MRP knockout ES cells, suggests that MDR, rather than MRP expression, is responsible for the increase in doxorubicin accumulation in L1210 MQ-580 cells. However, the relatively low difference in the steady-state intracellular concentration of 3-AP between MRP and MDR1 transfected cell lines and their parental counterparts (about 2-fold) contrasts with the much greater difference (5- to 6-fold) in the intracellular concentration of 3-AP between L1210 MQ-580 and parental L1210 cells. This observation suggests that the effects of the products of the *mdr* and *mrp* genes may be additive or synergistic in their contribution to the effective elimination of a single drug from the intracellular compartment. This concept warrants further investigation, especially in light of the possibility of co-introducing more than one multidrug resistance gene into the bone marrow to protect hematopoietic stem cells from the high dose chemotherapy employed for the treatment of human malignancies.

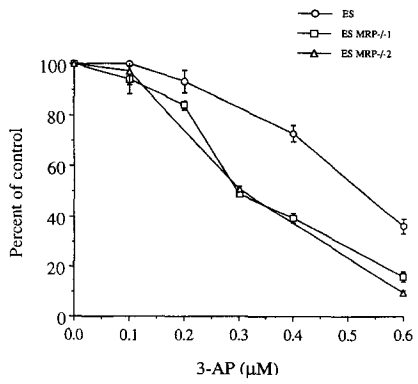

FIG. 8. Effects of 3-AP on the growth of parental ES, and MRP double knockout ES MRP-/-1 and ES MRP-/-2 clones. After 3 days of continuous exposure, the sensitivity of cells to 3-AP was measured by the MTS/PMS assay as previously described [19] and expressed as a percent of the absorbance values of cells treated with the solvent alone (ranging from 1.0 to 1.5 $\Delta A_{490\ nm}$). Data are the means ± SD of 3–4 separate experiments.

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