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TITLE: Induction of Multidrug Resistance in Human Cells by Transient Exposure to Different Chemotherapeutic Drugs

AUTHOR: Preet M. **Chaudhary,** Igor B. Roninson <1>

ABSTRACT: Background: The MDR1 (multidrug resistance) gene (also known as PGY1), which encodes the transmembrane efflux pump P-glycoprotein (Pgp), confers resistance to Pgp-transported cytotoxic drugs in tissue culture. The increase in MDR1 expression in tumors after chemotherapy is usually attributed to selection of pre-existing multidrug-resistant cells by Pgp-transported drugs. MDR1 expression in tissue culture can be increased by several types of stress-inducing treatment, including agents that activate protein kinase C (PKC). Previous studies, however, failed to demonstrate that short-term exposure to any chemotherapeutic drug can induce the expression of the resident MDR1 gene in drug-sensitive human cells. Purpose: This study was designed to utilize highly sensitive assays to determine if transient exposure to chemotherapeutic drugs would have an effect on MDR1 expression in human cells and to assess if PKC inhibitors would influence such an effect. Methods: We analyzed the MDR1 gene expression in several human cell lines derived from leukemias or solid tumors, after treatment with different cytotoxic drugs, in the presence or absence of PKC inhibitors. Pgp function and expression were studied by flow cytometric assays, and MDR1 messenger RNA (mRNA) was assayed by polymerase chain reaction. Results: Transient exposure to chemotherapeutic drugs, including agents that are not transported by Pgp, induced Pgp and MDR1 mRNA expression in subpopulations of treated cells in most of the tested cell lines. This induction was observed along with microscopically detectable cell damage. The drug-induced MDR1 expression and the associated twofold to threefold increase in resistance to vinblastine were sustained in K562 leukemia cells for at least several weeks after the removal of the drug. Drug-mediated MDR1 induction was blocked by nonspecific protein kinase inhibitors that are active against PKC, but not by a protein kinase inhibitor ineffective against PKC. Conclusions: Expression of the human MDR1 mRNA and Pgp can be induced in response to cellular damage by cytotoxic drugs regardless of whether the drugs are transported by Pgp. This induction can be prevented by protein kinase inhibitors. Implications: Induction of MDR1 expression in response to cellular damage may account for increased MDR1 expression in treated human tumors. Protein kinase inhibitors may be useful in preventing the emergence of multidrug resistance during cancer chemotherapy.

TEXT:
The best known form of multidrug resistance in human cells results from increased expression of the MDR1

gene (also known as PGY1) which encodes P-glycoprotein (Pgp), an energy-dependent efflux pump for various lipophilic compounds [n1-n3]. Cell lines with constitutively increased MDR1 expression, isolated by one or more steps of cytotoxic selection, are cross-resistant to Pgp substrates, such as vinca alkaloids, anthracyclines, or epipodophyllotoxins, but not to the drugs that are not transported by Pgp (e.g., alkylating agents or methotrexate). MDR1 is expressed in a large proportion of human tumors [n3-n5], and its expression in several different forms of cancer was shown to be associated with a lack of response to combination chemotherapy [n6-n9]. The associations with the clinical resistance were observed even when the average levels of MDR1 messenger RNA (mRNA) in the tumor were only slightly higher than those in a Pgp-negative drug-sensitive cell line [n9] or when Pgp expression was confined to a minor subpopulation of cells in a tumor section [n6,n7]. The highest levels of MDR1 mRNA or Pgp are usually found in malignancies arising from MDR1-positive normal tissues (e.g., renal, adrenocortical, or colorectal carcinomas) [n4]. In other types of cancer, MDR1 expression is usually low or undetectable prior to treatment, but it is frequently increased during the progression of the disease and, most noticeably, after chemotherapy [n3-n9]. The increase in MDR1 expression in treated tumors is usually attributed to the selection of pre-existing multidrug-resistant cells by Pgp-transported chemotherapeutic drugs [n3], in accordance with a mutation-selection hypothesis for drug resistance in cancer [n10].

The possibility that MDR1 expression may be directly induced, rather than selected, by Pgp-transported drugs has been previously investigated by several groups (including our laboratory). No evidence for MDR1 induction in human cells by short-term treatment with Pgp substrates or other cytotoxic drugs has been obtained by conventional hybridization techniques [n2,n11], although it was reported that the number of Pgp-positive cells in a human mesothelioma line was increased after prolonged exposure to some Pgp substrates [n12]. In contrast, a rodent homologue of MDR1 was induced in several cell types in response to different forms of stress including short-term treatment with cytotoxic drugs [n11,n13,n14], suggesting an essential difference between human and rodent tissues in their MDR1 regulation [n11]. Several studies have indicated, however, that the human MDR1 gene may also, under certain conditions, be susceptible to stress induction. Thus, MDR1 expression in some human cell lines was increased by treatment with heat shock or arsenite [n15,n16] or with certain differentiating agents [n17,n18]. Some cytotoxic Pgp substrates, as well as cultivation in serum-free medium, were reported to stimulate transcription of a reporter gene from the human MDR1 promoter [n19,n20], although no parallel induction of the endogenous MDR1 gene was observed [n20].

The negative results on human MDR1 induction by cytotoxic drugs had been obtained by use of techniques that were unlikely to detect low-level or heterogeneous expression of MDR1, even though such low-level expression in human tumors was associated with poor prognosis. We have therefore decided to use highly sensitive assays, including flow cytometric analysis of Pgp function and expression and polymerase chain reaction (PCR)-based detection of MDR1 mRNA, to test the possibility that drug treatment may induce MDR1 expression in at least some of the treated human cells. Our interest in re-examining the direct induction hypothesis was also prompted by studies suggesting that treatment of cells with different cytotoxic drugs activates protein kinase C (PKC) [n21-n23] and by our recent finding that PKC-activating agents can induce MDR1 expression in human cells [n24].

In the present study, we have found that MDR1 expression in a subpopulation of cells from several human cell lines can indeed be induced by transient exposure to different cytotoxic drugs, many of which are not transported by Pgp. Drug-mediated MDR1 induction was sensitive to protein kinase inhibitors which are active against PKC, but not to a non-PKC-specific inhibitor. These findings have implications for the origin of multidrug resistance in vitro and in vivo and suggest an approach to preventing the emergence of

multidrug resistance during cancer chemotherapy.

Materials and Methods

Human Cell Lines and Drug Treatment

K562, H9, KG1, and HL60 leukemia cell lines were maintained in RPMI-1640 medium, and EJ and KB-3-1 carcinoma cells were maintained in Dulbecco's modified Eagle medium. The media were supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 100 U/mL penicillin, and 100 mu g/mL streptomycin (GIBCO BRL, Grand Island, N.Y.). All the cytotoxic drugs were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cells (2.5-5 X 10<5>) were plated in each well of a six-well plate in the absence or presence of different drugs. In most experiments, drug-treated cells were collected for flow cytometric assays or RNA extraction only after drug-induced cell damage became detectable microscopically by cell swelling and alterations in cell shape and granularity. For the vinblastine inhibition assay, cells (in duplicate) were plated in a 96-well microtiter plate at 3000 cells per well and were allowed to grow in increasing concentrations of vinblastine. Inhibition of cell growth in drug-treated relative to untreated cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [n25].

Flow Cytometric Assays

Pgp activity was analyzed by rhodamine 123 (Rh123) or 3,3'-diethyloxacarbocyanine iodide [DiOC[2](3)] efflux assays. For these assays, the cells were stained either with 100 ng/mL Rh123 (Kodak Co., Rochester, N.Y.) or with 10 ng/mL DiOC[2](3) (Molecular Probe, Inc., Eugene, Ore.) for 10 minutes. After two washes, the cells were allowed to efflux the dye for 3 hours for Rh123 or for 2 hours for DiOC[2](3) at 37 degrees C in drug-free medium, as previously described [n26]. The assays were carried out in the presence or in the absence of 30 mu *M* verapamil, a Pgp inhibitor. KG1 cells were stained with 100 ng/mL Rh123 for 3 hours at 37 degrees C and were analyzed without efflux. Flow cytometric analysis and fluorescence-activated cell sorting were carried out as described [n26]; nonviable cells were excluded from the analysis on the basis of their abnormal size and granularity or, in experiments not utilizing phycoerythrin, by the accumulation of propidium iodide.

For two-color analysis of Pgp function and expression, 2 X 10<5> cells were first stained by DiOC[2](3) efflux as described above, except that DiOC[2](3) was used at a concentration of 3 ng/mL. After a 2-hour efflux, cells were labeled with phycoerythrin by indirect immunofluorescence with 2 mu g of the primary antibody [Pgp-specific mouse monoclonal antibody UIC2 [n27] or mouse IgG2a isotype control; Sigma Chemical Co.] and 10 mu g of the secondary antibody [phycoerythrin-conjugated F(ab')[2] fragments of sheep anti-mouse IgG; Sigma Chemical Co.]. Indirect immunofluorescence labeling of KG1 cells was carried out with 1 mu g of the secondary antibody per 2 X 10<5> cells. Expression of differentiation-related antigenic markers was analyzed by direct immunofluorescence labeling using phycoerythrin-conjugated mouse monoclonal antibodies against CD33, HLA-DR (Becton Dickinson, San Jose, Calif.), and CD13 (Coulter, Hialeah, Fla.) as well as a fluorescein isothiocyanate-conjugated antibody against CD15 (Dako, Carpinteria, Calif.). Flow cytometric analysis was carried out as previously described [n26].

Complementary DNA-PCR Analysis of MDR1 mRNA

RNA from drug-treated or untreated cells, grown in a single well of a six-well plate, was extracted by a small-scale sodium dodecyl sulfate extraction procedure [n28]. Complementary DNA (cDNA) synthesis and PCR were carried out by a modification of a previously described procedure [n5], with the following changes.

1) To improve the yield of PCR products from small samples, *Taq* DNA polymerase was added to the PCR mixtures after the samples were initially heated to 94 degrees C ("hot start"). This modification has necessitated a re-evaluation of the previously determined kinetics of PCR amplification of the MDR1 and beta[2]-microglobulin (internal control) cDNA sequences in a standard series of cell lines with increasing levels of MDR1 mRNA [n5], to determine the maximum number of PCR cycles providing for exponential amplification of these cDNA sequences. The optimum number of PCR cycles was found to be 29 for MDR1 and 21 for beta[2]-microglobulin. In contrast to the previously described procedure [n5], the hot-start PCR for these cDNAs had to be carried out in separate tubes. <32>P-labeled PCR products were mixed prior to electrophoresis and detected by autoradiography. 2) To account for differential RNA degradation and the associated differences in the efficiency of reverse transcription and PCR between drug-treated and untreated cells, we used the cDNA preparations in preliminary assays to amplify beta[2]-microglobulin cDNA alone. Amounts of cDNA preparations providing the same yield of the beta[2]-microglobulin-specific PCR product were determined by these assays and were then used for MDR1-specific PCR.

Results

We have initially tested whether cytarabine, an effective antileukemic drug which is not transported by Pgp and therefore should not select for MDR1 expression, would have an effect on Pgp function or expression in K562 leukemia cells. K562 cells express a low level of MDR1 mRNA and no detectable Pgp on their surface [n24]. As a primary assay for Pgp function, we have used the efflux of the Pgp-transported fluorescent dyes Rh123 or DiOC[2](3) [n26,n29]. Flow cytometric analysis showed that only a few (0.26% +/- 0.05% [mean +/- SD]) of the untreated K562 cells showed dye efflux sensitive to the Pgp inhibitor verapamil. In contrast, exposure of K562 cells to cytarabine for 12-72 hours led to the emergence of a subpopulation of 3%-17% Rh123 "dull" (i.e., less fluorescent) cells showing verapamil-sensitive dye efflux (Fig. 1, A). Under the most frequently used treatment conditions (10 mu *M* cytarabine for 48 hours), 7% +/- 1.5% (mean +/- SD) of the cells appeared to be dull. The emergence of the dull cells was paralleled by a dose-dependent increase in MDR1 mRNA expression relative to beta[2]-microglobulin (internal control) in cytarabine-treated K562 cells, as detected by PCR amplification of cDNA sequences (cDNA-PCR) (Fig. 2, A). Under conditions of maximal induction, the relative level of MDR1 mRNA in K562 cells approached that of the KB-8-5 carcinoma cell line with a low level of multidrug resistance [n5] (data not shown).

We have also tested other chemotherapeutic drugs for the ability to induce MDR1 expression in K562 cells. Doxorubicin, daunorubicin, vinblastine, etoposide, methotrexate, fluorouracil, chlorambucil, cisplatin, and hydroxyurea were found to induce MDR1 mRNA expression (Fig. 2, B) and the efflux of Rh123 or DiOC[2](3) (Fig. 1, A; data not shown). The last five of these drugs, like cytarabine, are not transported by Pgp [n1], indicating that MDR1 induction was not preferentially associated with Pgp substrates. The subpopulation of dull cells in the treated populations ranged from 3% to 10%, depending on the type and the dose of drug and the length of exposure. In all cases, MDR1 induction became detectable at the same time as visible cell damage, as evidenced by cell swelling, increased granularity, altered cell shape, and growth inhibition (Fig. 2, A; data not shown).

To determine whether drug-induced MDR1 expression was a stable or transient effect, we treated K562 cells with cytotoxic concentrations of cytarabine, doxorubicin, chlorambucil, or methotrexate for 3-5 days and then allowed them to grow in the absence of the drugs. At different time points, MDR1 expression in the surviving cells was analyzed by single-color or two-color flow cytometric assays based on dye efflux and immunofluorescence labeling with the Pgp-specific monoclonal antibody UIC2 [n27] (Fig. 1, B; data not shown) or by cDNA-PCR analysis of MDR1 mRNA (Fig. 2, E). MDR1 expression in a subpopulation of

treated cells was maintained for at least several weeks after the removal of the drugs (up to 11 weeks of observation in the cytarabine-treated cells). Rh123-dull and Rh123-bright subpopulations of the cytarabine-treated K562 cells, separated by fluorescence-activated cell sorting after 6 weeks of growth in the absence of the drug, showed no apparent differences in their size, shape, granularity, and expression of the differentiation-related antigenic markers CD13, CD15, CD33, and HLA-DR (data not shown), indicating that MDR1 expression was not related to the state of differentiation. The presence of multidrug-resistant cells 6 weeks after the removal of cytarabine or doxorubicin was also confirmed by a growth inhibition assay with vinblastine, a Pgp substrate. Vinblastine resistance, characterized by an approximately twofold to threefold increase in the ID[10] value (i.e., drug concentration that decreases cell growth by 90%), was specifically associated with the Rh123-dull subpopulation of K562 cells (Fig. 3). Thus, drug-induced MDR1 expression, with its associated drug resistance, is a stable phenomenon not limited to dying or terminally differentiating cells.

The ability of cytotoxic drugs to induce MDR1 expression was not limited to K562 cells. Thus, the KG1 leukemia cell line, which expresses a moderate amount of Pgp even without drug treatment, increased Pgp expression in 11%-12% of the cells after treatment with cytarabine, as seen by Rh123 accumulation and UIC2 antibody immuno-reactivity (Fig. 1, C). Cytarabine also activated MDR1 mRNA expression in H9 leukemia (Fig. 4), KB-3-1 carcinoma (Fig. 2, C), and EJ carcinoma cells (Fig. 2, D), although the magnitude of induction appeared to be somewhat lower in the carcinoma cell lines. In addition, MDR1 mRNA expression was induced in H9 cells by treatment with doxorubicin, vinblastine, and methotrexate (Fig. 4) and in KB-3-1 cells by treatment with doxorubicin (Fig. 2, C). We have been unable, however, to detect Pgp induction in HL60 leukemia cells treated with the same drugs (data not shown). We have also noticed that continuous passage of some cell lines in the absence of drugs for several months led to a small increase in MDR1 expression. The observed changes, detectable by cDNA-PCR and dye efflux assays, were much weaker than those observed after drug exposure (e.g., Pgp-positive K562 cells increased from 0.26% +/- 0.05% to 0.83% +/- 0.32% [means +/- SD] after prolonged cultivation, as opposed to 3%-17% Pgp-positive cells observed after treatment with different drugs).

We have previously found that MDR1 expression in most of the drug-inducible cell lines tested in the present study could also be induced by treatment with the PKC agonists phorbol ester and diacylglycerol [n24]. To test if PKC could be involved in MDR1 induction by cytotoxic drugs, we have analyzed the ability of the potent, but nonspecific, PKC inhibitors staurosporine and H7 to block MDR1 mRNA induction in H9 cells. These compounds, added at concentrations known to provide efficient inhibition of PKC (0.03 mu *M* staurosporine and 10-100 mu *M* H7), prevented MDR1 induction by cytarabine, doxorubicin, methotrexate, and vinblastine, as determined by cDNA-PCR (Fig. 4) and, in the case of cytarabine, by dye efflux assays (data not shown). No inhibition was observed with 60 mu *M* Iso-H7, a structural analogue of H7 with a 10-fold weaker inhibitory effect [n30]. Staurosporine and H7 also inhibited MDR1 induction by cytarabine in K562 cells (data not shown). Since no well-characterized inhibitors specific for PKC were available, we have compared the effects of increasing doses of H7 (IC[50] [i.e., 50% inhibitory concentration] = 6.0 mu *M* for PKC and 3.0 mu *M* for protein kinase A) and HA1004, a non-PKC-specific protein kinase inhibitor (IC[50] = 40 mu *M* for PKC and 2.3 mu *M* for protein kinase A) [n31]. As shown in Fig. 4, A, H7 inhibited MDR1 induction by cytarabine at 10 mu *M* or higher concentrations, but HA1004 showed no detectable inhibition even at 60 mu *M*. These results are consistent with a role for PKC in MDR1 induction by cytotoxic drugs.

Discussion

We have found that the treatment of several human cell lines with different cytotoxic drugs results in the

induction of MDR1 mRNA and Pgp expression in a subpopulation of the treated cells. MDR1 induction was observed after treatment not only with Pgp substrates, but also with different drugs that are not transported by Pgp and therefore should not select for Pgp-expressing cells. This observation, together with the short times of drug exposure sufficient for MDR1 induction (less than one cell generation in many experiments), indicates that cytotoxic selection for pre-existing MDR1-expressing cells could not be responsible for the emergence of the Pgp-positive subpopulations. MDR1 induction in our study was observed only in the presence of morphologically detectable cell damage. These results suggest that MDR1 induction may represent a general (albeit relatively weak) response to cellular damage. It seems likely that damage-induced MDR1 activation in tumor cells would occur during chemotherapy, and it may account at least in part for the observed increase in the incidence of MDR1 expression in drug-treated human tumors.

In K562 cells, drug-induced MDR1 expression was maintained for a prolonged period after the drugs were removed and resulted in a twofold to threefold increase in vinblastine resistance. Such an increase is likely to decrease the response to chemotherapy under clinical conditions [n32], and it should facilitate the selection of genetic variants with even higher levels of drug resistance. It will be important to determine if the response to drug damage involves the induction of resistance to any drugs other than those transported by Pgp, since clinical correlative studies suggested that MDR1 expression may be associated with tumors possessing multiple mechanisms of drug resistance [n9]. Such an analysis would also point out the drugs that are more likely to maintain their efficacy in subsequent rounds of chemotherapy. Thus, K562 cells that survived the treatment with cytarabine showed increased MDR1 expression and resistance to vinblastine, but they remained as sensitive to cytarabine as the untreated cells (data not shown).

MDR1 induction by cytotoxic drugs was blocked by the potent, though nonspecific, PKC inhibitors staurosporine and H7 but not by a protein kinase inhibitor, HA1004, which is not effective against PKC. This finding, together with the previously observed induction of MDR1 expression by PKC agonists [n24], lends support to the hypothesis [n33] that PKC plays a central role in cellular response to different types of cytotoxic damage. Interestingly, in vitro selected multidrug-resistant cell lines frequently contain elevated levels of PKC [n34-n36]. The relatively nonspecific nature of protein kinase inhibitors used in the present study, however, does not allow one to rule out an involvement of some protein kinase other than PKC. The availability of inhibitors with proven specificity for PKC or its individual subtypes would make it possible to ascertain the role of this enzyme in the induction of multidrug resistance by cytotoxic agents. The results of the present study indicate, however, that protein kinase inhibitors active against PKC provide a potentially useful pharmacologic approach to preventing the emergence of multidrug resistance during cancer chemotherapy.

In a recent study, Kioka et al. [n16] reported that an arsenite-induced increase in MDR1 expression in a hepatocarcinoma cell line could be prevented with the flavonoid quercetin. Although quercetin is a PKC inhibitor [n37], it is unclear whether PKC inhibition was responsible for the observed effect. Arsenite is believed to promote transcription by acting at the heat shock response element, which is found in several RNA polymerase II promoters, including the promoter of the MDR1 gene [n15]. Non-flavonoid PKC inhibitors, however, were reported to be inactive in inhibiting the response to heat shock [n38]. It remains to be determined whether the effect of quercetin observed by Kioka et al. [n16] was due to the inhibition of the heat shock response element-mediated stimulation of MDR1 transcription by arsenite or whether quercetin, like PKC inhibitors tested in the present study, acted to prevent the general response to damage in arsenite-treated cells.

PKC-mediated transcriptional stimulation is believed to be exerted through the interaction of the Jun and Fos

proteins with the AP-1 promoter element [n39,n40]. A non-canonical form of the AP-1 element is found in the major (down-stream) promoter of the human MDR1 gene [n41,n42]. A potential role for the AP-1 sequence in drug-mediated induction of the human MDR1 gene is consistent with the reports that some of the cytotoxic drugs tested in our study were also capable of inducing reporter gene expression from the downstream promoter of MDR1 [n19,n20]. The increase in the MDR1 mRNA levels that we observed after drug treatment, however, could be due to mRNA stabilization rather than to increased transcription. The molecular basis of MDR1 induction in drug-treated cells and the mechanism responsible for the maintenance of MDR1 expression after the removal of the drugs are currently under investigation.
Notes

Affiliation of authors: Department of Genetics, University of Illinois at Chicago, Chicago, Ill.

Present address: P. M. Chaudhary, Department of Medicine, Northwestern University McGaw Medical Center, Chicago, IL 60611.

Correspondence to: Igor B. Roninson, Ph.D., Department of Genetics (M/C 669), University of Illinois at Chicago, 808 South Wood St., Chicago, IL 60612.

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SUPPLEMENTARY INFORMATION: <1> See "Notes" section following "References."

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GRAPHIC: Figure 1, Flow cytometric analysis of drug-induced MDR1 expression. Fluorescence intensity is shown on a logarithmic scale. A) Efflux of Pgp-transported fluorescent dyes from K562 leukemia cells in the absence (left) or in the presence (right) of 30 mu M verapamil (VER). Top panel: Rh123 efflux from untreated cells and from cells treated with 50 mu M cytarabine (ARA) for 12 hours or with 10 mu M cytarabine for 2 or 3 days. Bottom panel: DiOC[2](3) efflux from untreated cells and from cells treated with 1 mu g/mL vinblastine (VBL) for 36 hours. B) Contour density maps of K562 cells maintained in drug-free media after exposure to different drugs and analyzed by two-color flow cytometry using DiOC[2](3) (horizontal axis) and either UIC2 antibody (left) or IgG2a isotype control (right), indirectly labeled with phycoerythrin (PE) (vertical axis). Top to bottom: untreated cells, cells treated with 60 ng/mL doxorubicin (ADR) for 3 days and grown without drug for 5 weeks, cells treated with 30 mu M chlorambucil (CHL) for 5 days and grown without drug for 2 weeks, cells treated with 10 mu M cytarabine (ARA) for 3 days and grown without drug for 5 weeks (this experiment utilized one-half the amount of the secondary antibody used in the other assays), and Rh123-dull population of cells obtained after treatment with cytarabine (ARA) and isolated by fluorescence-activated cell sorting 6 weeks after removal from the drug. C) Increased Pgp expression in cytarabine (ARA-C)-treated KG1 leukemia cells. Left: Rh123 accumulation in untreated cells or cells treated with 10 mu M cytarabine for 1.5 days. Right: indirect immunofluorescence labeling of the same cells with anti-Pgp UIC2 antibody or IgG2a isotype control; Figure 2, cDNA-PCR analysis of MDR1 mRNA expression in drug-treated cells. In each lane, the upper band (167 base pair [bp]) corresponds to MDR1, and the lower band (120 bp) corresponds to beta[2]-microglobulin-specific PCR products [n5], amplified in separate tubes.

A) MDR1 induction in K562 leukemia cells by cytarabine (Ara-C). Cells were exposed to the indicated concentrations of cytarabine for 4.5 days. The growth of these cells relative to that of untreated cells was determined by the MTT assay [n25] in parallel with RNA extraction. B) MDR1 induction in K562 cells treated with different drugs. The times of drug exposure are indicated. The drugs and their concentrations are as follows: -, untreated cells; daunorubicin (DAU), 250 ng/mL; doxorubicin (ADR), 500 ng/mL; vinblastine (VBL), 20 ng/mL; etoposide (VP), 1 mu g/mL; methotrexate (MTX), 200 ng/mL; cisplatin (CDDP), 3 mu g/mL; chlorambucil (CHL), 50 mu M; fluorouracil (5FU), 2 mu g/mL; and hydroxyurea (HU), 30 mu M. C) MDR1 induction in KB-3-1 carcinoma cells, untreated or treated for 2 days with 200 ng/mL doxorubicin (ADR) or 10 mu M cytarabine (ARA). D) MDR1 induction in EJ carcinoma cells, untreated (-) or treated for 4 days with 10 mu M cytarabine (ARA). E) Maintenance of drug-induced MDR1 expression in K562 cells. Cells were treated for 3 days with 10 mu M cytarabine (ARA), 60 ng/mL doxorubicin (ADR), or 200 ng/mL methotrexate (MTX) and were cultured in drug-free medium for the indicated period of time; Figure 3, Vinblastine resistance in cytarabine- or doxorubicin-treated K562 leukemia cells. Each point represents the mean of duplicate measurements; the variance for each point was 20% or less of the mean. A) Growth inhibition by vinblastine in untreated and cytarabine (ARA)- or doxorubicin (ADR)-treated cells. Cells were treated with 10 mu M cytarabine or 60 ng/mL doxorubicin for 3 days and were then grown without drug for 6 weeks. Pgp expression in these cell populations is illustrated in Fig. 1, B. Vinblastine inhibition assay was carried out for 10 days. B) Growth inhibition by vinblastine in untreated cells and Rh123-dull and Rh123-bright populations of cytarabine-treated cells. Treated cells, 6 weeks after removal from the drug, were separated into Rh123-dull and Rh123-bright populations. The Rh123-dull population was more than 60% pure (Fig. 1, B), and the Rh123-bright population was 90%-95% pure. One week after cell sorting, vinblastine inhibition assay was carried out for 7 days; Figure 4, Effect of protein kinase inhibitors on MDR1 mRNA induction by cytotoxic drugs in H9 leukemia cells. In each experiment, the inhibitors staurosporine (ST), H7, Iso-H7 (IH7), or HA1004 were added twice, the first time immediately prior to the addition of the corresponding drug and the second time after the specified period of time. MDR1 mRNA expression was analyzed as in Fig. 2. A) H9 cells, untreated or treated with 50 mu M cytarabine (ARA) for 22 hours. The inhibitors were added at the indicated concentrations at the beginning of the experiment and 16 hours later. B) H9 cells, untreated or treated with 200 ng/mL doxorubicin (ADR) for 22 hours. Equal amounts of inhibitors (0.03 mu M staurosporine or 10 mu M H7, HA1004 [HA], or Iso-H7 [IH7]) were added at the beginning of the experiment and 16 hours later. C) H9 cells, untreated or treated with 40 ng/mL vinblastine (VBL) or 200 ng/mL methotrexate (MTX) for 36 hours. Equal amounts of inhibitors (0.1 mu M staurosporine or 50 mu M H7) were added at the beginning of the experiment and 24 hours later.