Circumvention of Acquired Resistance to Doxorubicin in K562 Human Leukemia Cells by Oxatomide

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We studied the effect of oxatomide, an antiallergic drug, on the resistance of K562 cells to doxorubicin. Oxatomide synergistically potentiated the cytotoxicity of doxorubicin in doxorubicin-resistant K562 cells (K562/DXR) at a concentration of 1—10 μM, but had hardly any synergistic effect on the parental cell line (K562) at the same concentration. Oxatomide inhibit P-glycoprotein pump-efflux activity and the binding of [3H]-azidopine to the cell-surface protein P-glycoprotein, in a dose-related manner. These results indicate that oxatomide reverses the multidrug-resistance phenotype through direct interaction with P-glycoprotein.

Key words K562 cell; oxatomide; verapamil; doxorubicin; multidrug resistance

A serious barrier to the treatment of patients with any form of cancer remains the emergence of cancer cells that are refractory to chemotherapy. The resistance is frequently mediated by overexpression of P-glycoprotein, which functions as an ATP-dependent drug-efflux pump. A number of agents, including verapamil and cyclosporin A, bind to and inhibit P-glycoprotein to reverse multidrug resistance (MDR) in vitro. However, to date no effective resistance modifier has been useful in the clinic; generally the use of such agents is dose limited due to toxicity or unacceptable side effects. Therefore a MDR blocker with fewer side effects is needed.

Detailed examination of the structural features of drugs that sensitize MDR cells to chemotherapy has led to a number of important conclusions. For example, Zamora et al. demonstrated that a planar hydrophobic ring and a positively charged amino group are present in a series of active indole alkaloids. And, Ford et al. studied a series of substituted phenothiazines and found that the hydrophobicity of the ring, the length of the methylene bridge, and the charge on the terminal amino group are directly related to activity. Furthermore, many researchers have reported that all substrates for P-glycoprotein are those (or inhibitors) for CYP3A4, a drug-metabolizing enzyme. We previously reported that astemizole, which is an antihistaminic substance for CYP3A4, was also found to be an inhibitor of P-glycoprotein in a preliminary study. Based on the data, we examined whether oxatomide could potentiate doxorubicin (DXR) and overcome MDR in vitro, and compared the effects with those of verapamil, a known resistance modifier. We used the human leukemic cell line K562, which does not express P-glycoprotein, and its DXR-resistant variant K562/DXR, which expresses the MDR phenotype.

MATERIALS AND METHODS

Cell Lines and Culture The human chronic myelogenous leukemia cell line K562 was obtained from the Cancer Cell Repository (Tohoku University). K562 and an MDR clone, K562/DXR, derived from the K562 cell line were used. The properties of K562/DXR and parental K562 cells have been described previously. Cells were routinely kept in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin G (100 U/ml)/streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO2–95% air incubator under standard conditions. The K562/DXR cell line was maintained in medium containing DXR 1 μM. DXR was washed out at least 3 d before the experiments.

Chemicals Drugs used in the present experiments were as follows: DXR (Sigma), vinblastine (VBL) (Exal, Shionogi Pharmaceutical Co.), verapamil (Sigma), oxatomide (Celtect, Kyowa Hakko Pharmaceutical Co.), and [3H]-azidopine (Amersham International). All cell culture reagents were obtained from GibcoBRL (Life Technologies Ltd.). All other reagents were of the highest available grade and were supplied by either Sigma or Nacalai Tesque. Oxatomide was dissolved in dimethylsulfoxide (DMSO). DMSO at concentrations lower than 0.5% had no effect on cell growth. Light exposure was kept to a minimum for all drugs used.

Cytotoxicity Assay After treatment, cytotoxicity was determined by the trypan blue dye-exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue and the cells were counted with a hemocytometer. Results are expressed as the percentage of dead cells (ratio of stained cells vs. the control number of cells).

Cellular Uptake of DXR To estimate the concentration of DXR in vitro, 1×10^6 cells/ml were resuspended in culture tubes in the presence or absence of various concentrations of oxatomide. DXR (4 μM/ml) was added to the cells, gently mixed, and incubated at 37 °C. Tubes were removed at 45 min, and cells for intracellular DXR accumulation were analyzed by FACScan (Becton-Dickinson Co., San Jose, CA, U.S.A.). Some 10000 cells were counted. All experiments were done in triplicate. Mean fluorescence was recorded.

Fig. 1. Chemical Structure of Oxatomide

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from the histogram, and data are expressed in the text as mean fluorescence channel numbers.

Binding of [3H]-Azidopine to Plasma Membrane of K562/DXR Cells Membrane vesicles from K562/DXR cells were prepared as described\(^7\) from cells grown in 24 \times 24 mm dishes under standard growth conditions. Protein concentrations were determined by the method of Bradford.\(^9\) Membrane vesicles (50 μg of protein) were photolabeled in 40 mM Tris–HCl buffer (pH 7.2) containing 4% DMSO and 100 nm [3H]-azidopine in a final volume of 25 μl in the presence or absence of various drugs. Photolabeled membranes were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using gradient gels (4—20%). A total of 20 μg of protein was loaded onto each lane. The gel was fixed, treated with the fluorographic reagent Amplify (Amersham Japan), dried, and then exposed to Kodak XAR-5 films at −70 °C for 12—14 d.

Statistical Analysis Statistical comparisons were carried out using Student’s t-test for unpaired two-tailed comparisons. A p-value of less than 0.05 was considered significant.

RESULTS

Effect of Oxatomide on DXR Cytotoxicity Drug sensitivity was measured using the trypan blue dye-exclusion test as an indicator. Cells were incubated in the presence or absence of various concentrations of oxatomide and DXR. As shown in Fig. 2, K562/DXR cells showed a 416 fold greater resistance to DXR than parental K562 cells. When added at a final concentration of 1, 3, and 10 μM to the cells, oxatomide potentiated the cytotoxicity of DXR in a dose-related manner, but complete reversal was not attained at oxatomide 10 μM in K562/DXR cells. Oxatomide itself (10 μM) was not cytotoxic to the K562/DXR cells.

However, in K562 cells, oxatomide 10 μM did not synergize the growth-inhibitory action of DXR: at the concentrations examined and in the absence of DXR, no cytotoxicity toward K562 cells was detected by the trypan blue dye-exclusion test. Oxatomide circumvented resistance to VBL in K562/DXR/VBL as well as K562/DXR cells (data not shown). K562/DXR and K562/VBL cells are known to express P-glycoprotein, but K562 cells do not.\(^8\) These results suggest that oxatomide is effective against P-glycoprotein-positive tumors.

Effect of Oxatomide on Intracellular DXR Accumulation To explore how oxatomide potentiates the cytotoxicity of DXR, the effects of oxatomide on the accumulation of DXR in K562/DXR was investigated. Cells were incubated with doxorubicin in the presence or absence of various concentrations of oxatomide and intracellular accumulation was measured using FACSscan. DXR efficiently accumulated in K562 cells at 37 °C, and oxatomide and verapamil at 3 μM did not affect this accumulation. In K562/DXR cells, the accumulation of DXR was significantly reduced as compared to that in K562-sensitive cells at 37 °C (Fig. 3). Oxatomide dose-dependently restored DXR accumulation in K562/DXR to a level comparable to that in K562 cells. Oxatomide 3 μM enhanced the accumulation of DXR in K562/DXR cells to an extent almost comparable with that observed in parental K562 cells incubated without oxatomide. Verapamil 3 μM caused moderate enhancement of DXR accumulation in K562/DXR cells. When the cells were incubated at 0 °C to suppress the intracellular energy system, no potentiation of DXR accumulation by either oxatomide or verapamil was observed (data not shown). Consequently, the overcoming of drug resistance appears to be closely related to the potency of oxatomide against DXR accumulation in K562/DXR cells.

Inhibition of [3H]-Azidopine Photolabeling of P-Glycoprotein by Oxatomide Azidopine, a photoactive analogue of dihydropyridine, photolabels P-glycoprotein in plasma membranes of MDR cells; this labeling is inhibited by VBL and some calcium channel blockers.\(^2\) Yusa and Tsuruo have reported that a photoactive analogue of verapamil photolabels P-glycoprotein in the plasma membranes of K562/DXR cells.\(^16\) Using this photolabeling system, we investigated whether oxatomide inhibited the [3H]-azidopine photolabeling of P-glycoprotein. As shown in Fig. 3, [3H]-azidopine specifically labeled a 170000- to 180000-Da protein in K562/DXR cells but not in drug-sensitive K562 cells. The photolabeling of P-glycoprotein in K562/DXR membranes by [3H]-azidopine was inhibited by oxatomide in a concen-
Fig. 4. The Inhibitory Effects of Oxatomide on [3H]-Azidopine Photolabeling of P-Glycoprotein

Membrane vesicles (50 μg of protein) from K562/DXR cells were incubated with 100 nM [3H]-azidopine in the presence of oxatomide and verapamil at 10 or 100 μM. After solubilization, photolabeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Typical photo-labeling patterns are presented. Another experiment produced the same pattern.

tration-dependent manner, and oxatomide inhibited P-glycoprotein labeling at 10 μM partially and at 100 μM strongly. Verapamil 100 μM also partially inhibited P-glycoprotein labeling (Fig. 4), but at 10 μM had no effect. This result suggests that oxatomide interacts directly with P-glycoprotein and inhibits the transport of DXR.

DISCUSSION

Our results show for the first time that oxatomide synergizes the inhibitory effect of DXR on the growth of K562/DXR human leukemic cells. In these cells, at concentrations of oxatomide ranging from 1 to 10 μM, there is a dose–response relationship in reversing DXR resistance. In contrast, oxatomide does not potentiate the growth-inhibitory activity of DXR in the parental cell line, which does not express P-glycoprotein. These findings suggest a role for P-glycoprotein in determining the capacity of oxatomide to synergize the growth-inhibitory effect of DXR. P-glycoprotein has an important role in the expression of the DXR-resistant phenotype,2,11 and K562/DXR cells express high levels of P-glycoprotein.8 Thus we evaluated the effect of oxatomide on P-glycoprotein activity in efflux experiments with DXR which has been found to be transported by P-glycoprotein and is now recognized as a useful tool for studying P-glycoprotein efflux activity by flow cytometry. This technique, unlike other biochemical methods, provides information concerning the activity of P-glycoprotein.

Although the mechanism by which oxatomide reverses DXR resistance in vitro remains to be fully clarified, our results indicate that this antiallergic drug can act by modulating the activity of P-glycoprotein in K562/DXR cells. Our cytofluorimetric results showed that oxatomide reduced the efflux of DXR from K562/DXR cells in a dose-dependent manner. Furthermore, oxatomide, which was ineffective in synergizing the inhibitory effect of DXR on cell growth, did not prevent the effect in K562 cells.

Treatment strategies to overcome drug resistance have included various chemosensitizers.1,2,12,13 Several in vitro studies have suggested that the mechanism of reversing MDR with these agents involves competition with cytotoxic drug-binding sites on P-glycoprotein14; photoaffinity labeling of P-glycoprotein is a valuable technique for the evaluation of drug binding sites and the elucidation of the mechanism of action of MDR modifiers. [3H]-Azidopine is often used as a photoaffinity labeling agent for P-glycoprotein.1,2 Oxatomide inhibited the [3H]-azidopine photolabeling of P-glycoprotein efficiently and more strongly than verapamil. Furthermore, the accumulation of DXR in K562/DXR cells was increased more efficiently by oxatomide than by verapamil. These results suggest that the mechanism of action of oxatomide for reversing MDR is similar to that of verapamil. It has been reported that verapamil binds competitively to the drug-binding site on P-glycoprotein and is transported from resistant cells by a mechanism similar to that of antitumor agents.15

To overcome MDR, the use of oxatomide in combination with anticancer drugs may be clinically important because the cytotoxicity of anticancer drugs is increased, and as result their dose can be reduced. However, to use oxatomide as a drug to overcome MDR, detailed studies are also necessary on the development of its side effects at effective blood concentrations.

In conclusion, as a new MDR modifier, oxatomide blocked the MDR function by a mechanism similar to that of verapamil. Oxatomide increases intracellular concentrations of chemotherapeutic agents by inhibiting the function of P-glycoprotein. These properties make oxatomide a candidate MDR modifier.

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REFERENCES