J Drug Interact Res
一 般 論 文
26(3) 01-08 (2003)

Reversal of Doxorubicin Resistance in K562 Human Leukemia Cells by Oxatomide

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(Received November 21, 2002
Accepted December 12, 2002)

Abstract

Antiallergic drug, oxatomide, was examined for its reversing effect on multidrug-resistant tumor cells. Oxatomide synergistically potentiated the cytotoxicity of doxorubicin in doxorubicin-resistant K562 cells (K562/DXR) at concentrations of 1-10 μM, but had hardly any synergistic effect on the parental cell line (K562), which does not express the P-glycoprotein, at the same concentration. Furthermore, oxatomide increased the intracellular accumulation of rhodamine-123, a substrate for the multidrug-resistance pump, in the K562/DXR cells, and inhibits the P-glycoprotein activity (restoration of calcein retention) 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

Introduction

The anthracycline antibiotic doxorubicin is one of the most active drugs in the treatment of acute lymphatic leukemia. However, one of the serious obstacles to optimal cancer chemotherapy is the development of drug-resistant tumor cells during treatment. Multidrug resistance (MDR) is frequently characterized by cross-resistance to functionally and structurally unrelated drugs and by decrease of drug accumulation in resistant cells compared with their parental cell lines[1,2]. The MDR phenotype includes cross-resistance to anthracyclines, Vinca alkaloids, podophyllotoxins, taxanes, and other cytotoxic compounds, with increased expression of a membrane protein termed P-glycoprotein. P-glycoprotein functions as an efflux pump, which is found on the surface

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membranes of resistant cells, and serves to transport antitumor drugs. Reversal or modulation of MDR has been achieved using a wide variety of pharmacological agents including calcium channel blockers (e.g., verapamil, nifedipine, and diltiazem), cyclosporins (e.g., cyclosporin-A and PSC-833), cardiovascular drugs (e.g., dipyridamole and quinidine), steroid analogs (e.g., tamoxifen and progesterone), antibiotics (e.g., cefoperazone and erythromycin), calmodulin inhibitors (e.g., fluphenazine and trifluoperazine), and antimalarials (e.g., quinacrine and quinine). The mechanism by which most of these agents reverse MDR is caused by competitively inhibiting the binding of the chemotherapeutic agents to P-glycoprotein and is unrelated to their primary pharmacological mechanisms[2]. However, to date, no effective resistance modifier has been useful in the clinic; and generally the use of such an agent is dose-limited due to toxicity or unacceptable side effects[1,2]. Therefore, a multidrug-resistance blocker with fewer side effects was needed.

Detailed examination of the structural features of drugs that sensitize multidrug-resistance cells to chemotherapy has led to a number of important conclusions[3,4]. For example, Zamora et al.[3] demonstrated that a planar hydrophobic ring and a positively charged amino group are present in a series of active indole alkaloids. And, Hait et al.[4] reported 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[5,6]. We previously reported that astemizole, which is an antihistamine and substrate for CYP3A4, is also an inhibitor of P-glycoprotein[7]. In addition, we found that oxatomide, which has a similar structure but has not been reported to be a substrate for CYP3A4, is also an inhibitor of P-glycoprotein in a preliminary study. Based on the data, we examined whether or not oxatomide could potentiate doxorubicin (DXR) and overcome MDR *in vitro*, and compared those 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

A Human leukemic cell line (K562) and a multidrug-resistant clone (K562/DXR), derived from it, were used. The properties of K562/DXR and the parental K562 cells have been described previously[8]. 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 ℃ in a humidified 5% CO_2-95% air incubator under standard conditions. The K562/DXR cell line was maintained in medium containing 1 μM doxorubicin. Doxorubicin was washed out at least 3 days before the experiments.

Chemicals

Doxorubicin hydrochloride (Adriamycin, Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan) was obtained commercially. Calcein-AM was

obtained from Molecular Probes Europe (Leiden, The Netherlands). Rhodamine-123, propidium iodide, verapamil hydrochloride and oxatomide were obtained from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved in dimethylsulfoxide (at 10 nmol) and stored at -20 ℃. Light exposure was kept to a minimum for all drugs used.

Cytotoxicity assay

Cells were inoculated into each well of 96-well plates with 50 μL of the culture medium at 4 x 10^4 cells. After an overnight incubation, 50 μL of antitumor drug solution or 25 μL of doxorubicn and 25 μL of multidrug-resistant blocker solution, at final concentration of indicated dose were added in triplicate and the plates incubated for 48 hr. After treatment, cytotoxicity was assessed by the trypan blue 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 Rhodamine-123

P-glycoprotein function was measured by the ability of cells to accumulate rhodamine-123 in the presence or absence of oxatomide.

Rhodamine-123 was dissolved in ethanol and stored as a stock solution (5 mg/dL) at 4 ℃. Cells (2x10^6) were incubated at 37℃ for 1 hr with rhodamine-123 (10 μM). For treatment with oxatomide, oxatomide was added 10 min prior to the addition of rhodamine-123. Cells were then washed once with ice-cold PBS, resuspended in ice-cold PBS containing 50 μg/mL propidium iodide and fluorescence analysed immediately using a Becton Dickinson FACScan flow cytometer as previously described [9]. Ten thousand cells were counted. Dead cells which took up propidium iodide were excluded from the analysis by gating. All determinations were in duplicate and experiments were repeated at least three times. Mean fluorescence is recorded from the histogram and data are expressed as mean fluorescence channel numbers.

Assay for P-glycoprotein inhibitory activity

The assays for p-glycoprotein activity and inhibition were performed by use of the standard calcein-AM efflux method reported in detailed earlier [10]. Briefly, K562/DXR cells were exposed to the oxatomide or verapamil for 15 min at 37℃; calcein-AM (0.25 μM) was then added and the cells were kept at 37℃ for further 15 min. After three washes by centrifugation, flicking and cell resuspension in the medium, inhibition of P-glycoprotein function in K562/DXR cells was measured as calcein specific fluorescence and expressed as percentage of the calcein retention in K562 cells. The P-glycoprotein-modulator IC_{50} values were defined as the concentrations (μM) which , in K562/DXR cells, restored of the calcein retention shown by similarly treated parental K562 cells. For the compounds tested in the present paper, calcein retention by the K562 cells was not affected even by the highest tested compound concentration.

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 Cytotoxicity of Doxorubicin

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

But in K562 cells, oxatomide at 10 μM did not synergize the growth-inhibitory action of doxorubicin; at the concentrations examined and in the absence of doxorubicin, no cytotoxicity toward any K564 cell was detected by the trypan blue exclusion test. Oxatomide circumvented resistance to vinblastine (VBL) in K562/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[9,11]. These results suggest that oxatomide is effective against P-glycoprotein-positive tumors.

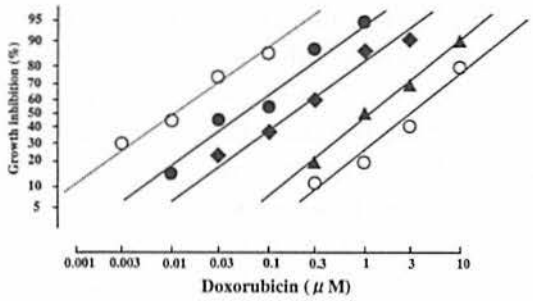

Fig1 Effect of Oxatomide on the Growth Inhibitory Action of Doxorubicin in K562 and K562/DXR Cells

K562 (dotted line) or K562/DXR (linked line) cells (4 x 10^4 cells/well) were incubated with various concentrations of doxorubicin in the absence or presence of three different concentrations of oxatomide for 48 hr. Drug concentrations of oxatomide were 0 (○), 1 (▲), 3 (◆) and 10 (●) μ M. Cell viability was estimated by trypan blue dye exclusion. Each value represents the mean value for three different experiments performed in triplicate; the SDs of the means were less than 15 % and omitted.

Cellular uptake of rhodamine-123

To explore how oxatomide potentiate the cytotoxicity of doxorubicin, the effects of the oxatomide on the accumulation of rhodamine-123 in K562/DXR was investigated. Rhodamine-123 is a fluorescence dye that is a substrate for

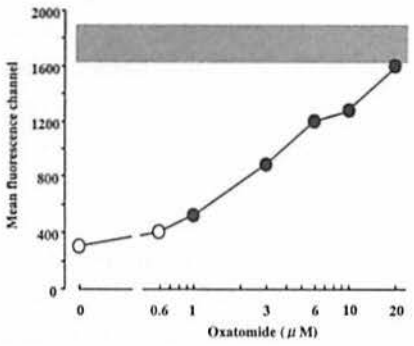

Fig2 Effect of Oxatomide on Rhodamine-123 Accumulation in K562 and K562/DXR cells

Cells (2 x 10^6 cells) were loaded with 10 μM rhodamine-123 in the presence or absence of various concentrations of oxatomide for 60 min and rhodamine-123 uptake measured, using FACScan in K562 and in K562/DXR (linked line). Data are expressed as mean fluorescence channel numbers; the SDs of the means were less than 10 % and are omitted from the figure. Dotted bars express the mean ±S.D. of fluorescence channel numbers of K562 cells. Closed circles mean significance of differences of values with oxatomide from the corresponding control.

P-glycoprotein and rapidly transported out of multidrug-resistance cells[12,13]. Thus, rhodamine-123 uptake can be used as a simple and convenient way of assessing the impact of various treatments on the multidrug-resistance phenotype.

Cells were incubated with rhodamine-123 in the presence or absence of various concentrations of oxatomide and the cell suspension was centrifuged (2 min at 200 g) at 4℃. Cells were suspended in an equal volume of rhodamine-123free medium at 37℃, and intracellular accumulation was measured using FACScan. Rhodamine-123 efficiently accumulated in K562 cells at 37℃, and verapamil at 10 μM did not affect this accumulation; 0.9, 1.2 and 2.4-fold retention was observed at 1, 10 and 20 μM verapamil, respectively. In K562/DXR cells, the accumulation of rhodamine-123 was extremely reduced as compared to in K562-sensitive cells at 37 ℃ (Fig. 2). Oxatomide, in a dose-dependent manner, restored the rhodamine-123 accumulation in K562/DXR to a level comparable to that in K562 cells. Indeed, oxatomide at 20 μM enhanced the accumulation of rhodamine- 123 in K562/DXR cells to an extent almost comparable with that observed in parental K562 cells incubated without oxatomide. When verapamil was added at a final concentration of 10-30 μM to the K562/DXR cells, verapamil potentiated the accumulation of rhodamine-123 in a dose-related manner; 1.6, 3.3 and 24-fold retention was observed at 10, 20 and 30 μM verapamil, respectively. Oxatomide at 1, 3, 6, 10 and 30 μM enhanced the accumulation of rhodamine-123 more strongly than did verapamil. Furthermore, oxatomide augmented the intracellular accumulation of rhodamine-123 in K562/VBL cells (data not shown). When the cells were incubated at 0℃ to suppress the intracellular energy system, no potentiation of doxorubicin accumulation caused by either oxatomide or verapamil was observed (data not shown). Consequently, the overcoming effect on drug resistance seems to be closely

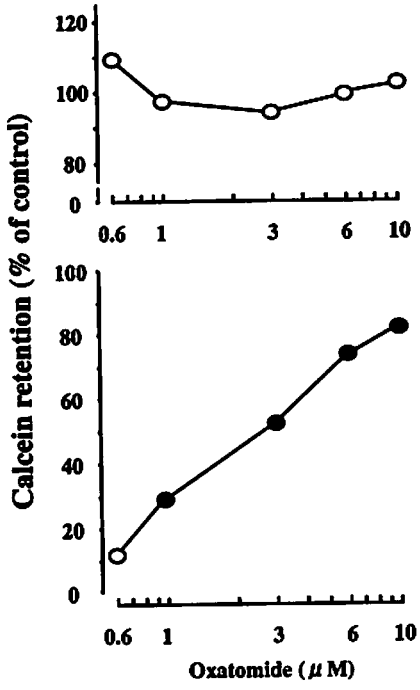

Fig3 Effect of Oxatomide on Calcein-AM Retention in K562 and K562/DXR Cells

Cells (2 x 10^6 cells) were loaded to the oxatomide or verapamil for 15 min, and calcein-AM was then added and the cells were kept for 15 min and calcein-AM uptake was determined by fluorimetrically measuring in K562 (top panel) and in K562/DXR (bottom panel). The retention of calcein-AM shows the level of calcein-AM-specific fluorescence which is expressed, in the case of K562 cells (top panel), as percentage of the untreated K562 cell one, and in the case of K562/DXR cells (bottom panel), as percentage of the similarly treated K562 cell one. Data are expressed as mean fluorescence channel numbers; the SD of the means were less than 10 % and were omitted. Closed circles indicate values in the presence of oxatomide that significant different from the corresponding values in the control.

related to the potency of oxatomide for doxorubicin accu-mulation in K562/DXR cells.

Inhibition of P-glycoprotein function by oxatomide

The inhibition of P-glycoprotein function is measured by the oxatomide dose-dependent inhibition of cellular uptake of the P-glycoprotein probe calcein-AM by K562/DXR, using K562 cells as controls. The interference with the efflux of calcein-AM which is entering the plasma membrane, reduces the amount of free calcein in the cytosol, the cellular fluorescence being the quantitative read-out[10]. Tiberghien and Loor (1996) have reported that MDR-reversing agents inhibites uptake of the P-glycoprotein probe calcein-AM in the cytosol of P-glycoprotein-expressing tumor cells[10]. Using this system, to study the interactions of P-glycoprotein with oxatomide, the effect of cellular uptake of the calcein-AM by K562/DXR cells was examined. Oxatomide and verapamil were thus compared for their capacity to inhibit the P-glycoprotein func-tion of K562/DXR cells, using K562 cells as control, with the resulting mean (n=3) dose-response curves.

Oxatomide and verapamil did not affect unspecifically the cellular uptake of the non-fluorescent P-glycoprotein-substrate calcein-AM and the level of the specific fluorescence signal of free calcein in the cytosol of K562 cells. Oxatomide potently increased the retention of free calcein in a dose-dependent manner, and verapamil had a calcein retention of 15.5% at 6 μM, 29.3% at 10 μM, 53.5% at 20 μM and 63.4% at 30 μM in the K562/DXR (Fig. 3).

With the P-glycoprotein-expressing 562/DXR cells, oxatomide (IC_{50}= 3.2±0.9 μM) was 7.2-fold more potent than verapamil (IC_{50}= 23.2± 6.5 μM) for P-glycoprotein inhibition. Oxatomide is endowed with a substantial P-glycoprotein inhibitory capacity. This result suggests that oxatomide directly interacts with P-glycoprotein and inhibits the transport of doxorubicin.

In comparison to oxatomide, using the same assay system to measure P-glycoprotein inhibition, early characterized MDR-reversing agents such as mefloquine, quinidine and the other show IC_{50} of 20~30 μM and higher[2,9,11].

Discussion

We have shown that in an MDR-positive human chronic myelogenous leukemia cell line K562/DXR, oxatomide can function as an MDR modulator, reversing resistance to doxorubicin. In contrast, oxatomide does not potentiate the growth-inhibitory activity of doxorubicin 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 doxorubicin. P-glycoprotein has an important role in the expression of the doxorubicin-resistant phenotype[2] and K562/DXR cells express high levels of P-glycoprotein[8]. Since the primary difference between the K562/DXR and K562 cell lines is the expression of P-glycoprotein, we evaluated the effect of oxatomide on P-glycoprotein activity in efflux experiments with rhodamine-123 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. Oxatomide was able to reverse the cellular accumulation defect of rhodamine-123, a substrate for the multidrug-resistance pump[12,13], in the K562/DXR cells, increasing the cellular levels of these compounds to those observed in the parental cell line, K562. This result supports the concept that oxatomide is interacting in some way to inhibit the function of P-glycoprotein.

Although the mechanism by which oxatomide reverses doxorubicin 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 fluorimetric results showed that oxatomide reduced the efflux of rhodamine-123 from K562/DXR cells in a dose-dependent manner. Furthermore, oxatomide, which was ineffective in synergizing the inhibitory activity of rhodamine-123 on cell growth, did not prevent the effect of doxorubicin in K562 cells.

Treatment strategies to overcome drug resistance have included various chemosensitizers[2,12-14]. Several *in vitro* studies have suggested that the mechanism of reversing MDR with these agents involves competition with cytotoxic drug-binding sites on P-glycoprotein[2,7,9,11,15]; uptake of calcein-AM is a valuable technique for the evaluation of drug binding sites and the elucidation of the mechanism of action of multidrug-resistance modifiers. Calcein-AM was an often used efflux agent for P-glycoprotein[11,16]. Oxatomide inhibited the calcein-AM efflux of P-glycoprotein efficiently and more strongly than verapamil.

Furthermore, the accumulation of rhodamine-123 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[17].

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

In conclusion, as a new multidrug-resistance modifier, oxatomide showed strong blocker of multidrug-resistant 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 maybe make oxatomide a candidate multidrug-resistance modifier.

Acknowledgement

This research was supported in part by a Grant-in-Aid for Science Research Promotion Fund (Japan Private School Promotion Fundation).

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