Reversal of Multidrug Resistance by Synthetic Isoprenoids in the KB Human Cancer Cell Line

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ABSTRACT

A colchicine resistant clone, CH'-24, derived from the human carcinoma KB cell line is extensively resistant to multiple drugs including vinblastine, vincristine, Adriamycin, actinomycin D, and daunomycin. In comparison with KB cells, very low accumulation of daunomycin or vincristine is observed in multidrug-resistant cells. Two isoprenoids with 9 to 10 isoprene chains (polyisoprenoids), N-(p-methylbenzyl)decaprenylamine and N-solanesyl-N,N'-bis(3,4-dimethoxybenzyl)ethylenediamine overcome the multidrug resistance almost completely in cultured CH'-24, whereas they only slightly sensitized the parental KB cells to antitumor agents. Both isoprenoids enhance the accumulation of vincristine or daunomycin in CH'-24, possibly by inhibiting efflux and also by enhancing influx of antitumor agents. A verapamil-like structure of N-solanesyl-N,N'-bis(3,4-dimethoxybenzyl)ethylenediamine is discussed in relation to its ability to overcome drug resistance.

INTRODUCTION

Resistance to drugs whether natural or acquired is the major obstacle in clinical cancer therapy to be solved. Methods to assay drug resistance and new more effective agents to overcome drug resistance are therefore of great importance. Multidrug-resistant cell lines for test systems have been developed either by selecting drug-resistant clones spontaneously or from mutagenized cultured mammalian cells in vitro (1-6) or by selecting drug resistance are therefore of great importance. Multidrug resistance almost completely in cultured CH'-24, whereas they only slightly sensitized the parental KB cells to anticancer agents. Both isoprenoids enhance the accumulation of vincristine or daunomycin in CH'-24, possibly by inhibiting efflux and also by enhancing influx of antitumor agents. A verapamil-like structure of N-solanesyl-N,N'-bis(3,4-dimethoxybenzyl)ethylenediamine is discussed in relation to its ability to overcome drug resistance.

MATERIALS AND METHODS

Cell Lines and Culture. A human KB epidermal carcinomacell line (KB-3-1) and a multidrug-resistant clone, KB-CH'-24 (CH'-24) derived from the KB cell line were used. Properties of CH'-24 and parental KB clone have been described previously (1, 12, 13). Cells were grown in monolayer in MEM4 (Nissui Seiyaku Co., Tokyo, Japan) containing 10% NCS (Microbiological Associates, Bethesda, MD), Bactopeptone (1 mg/ml; Difco Laboratories, Detroit, MI), glutamine (0.292 mg/ml), 10% NCS (Microbiological Associates, Bethesda, MD), Bactopeptone (1 mg/ml; Difco Laboratories, Detroit, MI), glutamine (0.292 mg/ml), and penicillin (100 units/ml) (14).

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1 This work was supported by a grant-in-aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan.2 Present address: Central Research Laboratory, Nisshin Flour Milling Co., Saitama 354, Japan.3 To whom requests for reprints should be addressed.

1 The abbreviations used are: MEM, minimal essential medium; NCS, newborn calf serum; SDB, N-solanesyl-N,N'-bis(3,4-dimethoxybenzyl)ethylenediamine; PMB, N-(p-methylbenzyl)decaprenylamine; HCl, ADAM, Adriamycin; VCR, vincristine; VBL, vinblastine; ACD, actinomycin D; DAU, daunomycin; DIO, that concentration of drug that reduces the plating efficiency of a cell line to 10% of its original value; PBS, phosphate-buffered saline (in g/liter: NaCl, 8.9; NaHPO4, 12H20, 2.9; KCl, 0.2; KH2PO4, 0.2); CHO, Chinese hamster ovary.

Chemicals. Radioisotopic compounds and chemicals were obtained from the following sources. [3H]VCR (specific activity, 5.9 Ci/mmol) and [3H]DAU (specific activity, 3.8 Ci/mmol) were obtained from Amersham and New England Nuclear, respectively. Radiochemical purity of [3H]VCR and [3H]DAU was greater than 98% based on high pressure liquid chromatography and thin layer chromatography. [3H]-Thymidine (specific activity, 20 Ci/mmol) was obtained from New England Nuclear; colchicine, ADM, VCR, VBL, ACD, DAU were from Sigma Chemical Co., St. Louis, MO; verapamil was a kind gift from Eisai Chemical Co., Tokyo, Japan; PMB and SDB were synthesized and used in this study (10).

Colony Formation Assay to Test Circumvention of Drug Resistance. Cell survival was measured by colony formation as described previously (10, 14). To assay colony formation, we first plated 300 cells of KB or CH'-24 in a 60-mm dish in the absence of drugs at 37 °C for 18 h, after which they were exposed to drugs and incubated for an additional 10 days. The number of colonies was counted after Giemsa staining. All drugs were freshly prepared in physiological saline or dimethyl sulfoxide and isoprenoids were dissolved in absolute ethanol. All control experiments were done by adding the same amount of saline and/or dimethyl sulfoxide and/or absolute ethanol. Each trial was done with triplicate dishes.

D10s were determined from dose response curves of the parental KB cell line and CH'-24 subline. Relative resistance to drugs was determined by dividing the D10 of KB with isoprenoids or D10 of CH'-24 with or without isoprenoids by the D10 of KB without isoprenoids as described previously (13).

Drug Accumulation. Cells (4 x 10^5/dish) were incubated and treated with isoprenoids at the indicated concentrations for 20 h at 37°C. Blank plates were also prepared simultaneously using 2 ml of MEM with 10% NCS, the pH of which was adjusted to 7.3. Then, medium was replaced with serum-free MEM and the cells were incubated with 34 nM [3H]-DAU or 22 nM [3H]VCR for 4 h in the presence or absence of isoprenoids. Cells were then washed once with ice-cold PBS (g/liter: NaCl, 8.0; NaHPO4, 12H20, 2.9; KCl, 0.2; KH2PO4, 0.2) at 4°C and harvested with a rubber scraper. This procedure with a scraper caused cell damage of only less than 10% of the total cell population when observed by electron microscope. Harvesting with the rubber scraper showed almost similar data of accumulation as with trypsin treatment. The cells were then washed three times with ice-cold PBS at 4°C and cellular pellets were suspended in 0.9 ml of H2O and mixed thoroughly with 10 ml of Scintisol EX-H (Wako Chemical Co., Osaka, Japan); then, radioactivity was counted.

Drug Efflux. Exponentially growing cells (4 x 10^5/dish) and "no cell" plates (blank) were incubated in the presence or absence of isoprenoids for 20 h and medium was then changed to serum-free MEM with or without isoprenoids. KB cells in the absence or presence of isoprenoids or CH'-24 cells in the absence of isoprenoids were incubated for 60 min at 37°C with 34 nM [3H]DAU or 22 nM [3H]VCR. To achieve equivalent levels of radioactive DAU and VCR accumulation prior to the efflux study, CH'-24 cells in the absence of isoprenoids were incubated with 203 nM [3H]DAU or 130 nM [3H]VCR for 60 min at 37°C. After 60 min of incubation, each dish was washed once with ice-cold PBS at 4°C and efflux was followed over 90 min at 37°C in serum-free medium with or without isoprenoids. At indicated times, cells were harvested and the radioactivity was counted.

Drug Influx. Exponentially growing cells (1 x 10^5/dish) and blank plates were incubated in MEM containing 10% NCS without or with various doses of isoprenoids for 20 h at 37°C. Medium was changed to glucose- and serum-free Hanks' balanced salt solution containing 1 mM...
2,4-dinitrophenol and various doses of isoprenoids. The cells were further incubated with 34 nm [3H]DAU or 22 nm [3H]VCR for 10 min at 37°C. Each dish was then washed three times with ice-cold PBS at 4°C. The cells were harvested and radioactivity associated with the cells was counted.

DNA Synthesis. Exponentially growing cells (4 x 10⁵/dish) and blank plates were incubated in the presence or absence of SDB for 20 h; then, medium was replaced with serum-free MEM and the cells were incubated with various doses of DAU or 22 nM [3H]VCR for 10 min at 37°C. Each dish was then washed three times with ice-cold PBS at 4°C. The cells were harvested and radioactivity associated with the cells was counted.

RESULTS

Effect of Isoprenoids on Survival of KB and Chr-24 in the Absence or Presence of Anticancer Agents. A multidrug-resistant clone, Chr-24, is resistant to much higher concentrations of ADM, VCR, VBL, ACD, and DAU than is the parental KB cell (1). We examined whether 2 isoprenoids with 9 to 10 isoprene units, SDB and PMB, could overcome the multidrug resistance in Chr-24. Chemical structures of the two isoprenoids are presented in Fig. 1. Dose response curves of VCR with or without the isoprenoids were assayed by colony formation. Figs. 2 and 3 show the effect of SDB and PMB on the sensitivity to VCR of the parent and the multidrug-resistant clone. Chr-24 had about 90-fold greater resistance to VCR than did the parental KB cell. The sensitivity of KB to VCR was slightly enhanced in the presence of SDB, 17 or 34 µg/ml (Fig. 2), and also in the presence of PMB, 108 or 216 µg/ml (Fig. 3). Combining VCR with SDB or PMB overcame drug resistance to VCR in Chr-24 cells. SDB at 34 µg/ml (Fig. 2) or PMB at 216 µg/ml (Fig. 3) almost completely reversed drug resistance to the level of that in KB cells. The cell survival fraction of both KB and Chr-24 cells was decreased by less than 10% compared to controls in the presence of SDB alone, 17 or 34 µg/ml and PMB alone, 108 or 216 µg/ml.

Table 1 summarizes the data on relative resistance in KB and Chr-24 which are derived from dose response curves as seen in Figs. 2 and 3. Three different concentrations of isoprenoids were used with various doses of ADM, VCR, VBL, ACD, and DAU. Chr-24 was resistant to about 30- to 90-fold higher doses of these anticancer agents (Table 1). The highest doses of the isoprenoids (SDB, 68 µg/ml and PMB, 432 µg/ml) were found to block the cell survival by about 50% of control. The sensitivity of Chr-24 cells to ADM was increased 8, 35, 4, and 6 times by SDB, 17 and 34 µg/ml and PMB, 108 and 216 µg/ml, respectively, but that of the parental KB was increased only 1- to 2-fold by these isoprenoids. Sensitivity to other anticancer agents such as VCR, VBL, ACD, and DAU in Chr-24 cells was almost completely restored to the level in KB cells when 2
isoprenoids were combined (Table 1); but once again the 2 isoprenoids affected sensitivity to various anticancer agents in the sensitive parental clone little if at all.

**Effect of Isoprenoids on Drug Accumulation in Chr'-24 Cells.** To explore how 2 isoprenoids overcome multidrug resistance, we examined their effects on the accumulation of DAU or VCR in KB and Chr'-24 cells. Time kinetics for DAU accumulation was followed over 6 h when KB and Chr'-24 were incubated with [\(^3\)H]DAU in the absence or presence of SDB, 17 and 34 \(\mu\)g/ml (Fig. 4). Both cell lines were first exposed to SDB for 20 h and then incubated with [\(^3\)H]DAU for the indicated times. The cellular accumulation of [\(^3\)H]DAU in Chr'-24 was about one-third of that in KB. Saturation time for the accumulation was, respectively, observed when KB cells were incubated for more than 2.5 h and when Chr'-24 cells were incubated for more than 4 h. Addition of SDB, 17 or 34 \(\mu\)g/ml enhanced the DAU accumulation in Chr'-24 cells, but it only slightly enhanced the accumulation in KB cells.

Then we compared dose-response effects of SDB and PMB on the cellular accumulation of [\(^3\)H]DAU or [\(^3\)H]VCR in KB and Chr'-24. Both cell lines were first incubated with various doses of SDB or PMB, then [\(^3\)H]DAU or [\(^3\)H]VCR was added to the medium and followed by further incubation for 4 h. The intracellular level of DAU and VCR in Chr'-24 cells was one-third and one-fourth of that in KB cells, respectively (Fig. 5). Addition of SDB at 17 to 34 \(\mu\)g/ml or PMB at 108 to 216 \(\mu\)g/ml enhanced accumulation of radioactive DAU in Chr'-24 cells to the level of that of KB cells without the isoprenoids (Fig. 5). In the sensitive KB cells, accumulation of DAU is enhanced up to 1.8-fold by the isoprenoids. Very similar data were observed with VCR: SDB enhanced the cellular accumulation of VCR in Chr'-24 5-fold at 34 \(\mu\)g/ml and 8-fold at 68 \(\mu\)g/ml, respectively, in comparison to the level in the absence of the isoprenoid. Cell survival was not affected by various doses of two isoprenoids under the accumulation assay when the cells were exposed to the isoprenoids for 1 day. The effect of PMB on accumulation of VCR was somewhat weak in comparison with SDB (Fig. 5); however, the level of intracellular VCR in Chr'-24 cells was significantly increased as a function of PMB doses.

To study whether the intracellular drug level is correlated with overcoming effects of drug resistance by SDB, we assayed DNA synthesis of KB and Chr'-24 cells treated with SDB and DAU under conditions similar to the drug accumulation study in Fig. 5. Table 2 shows relatively higher resistance of Chr'-24 cells to various doses of DAU than do KB cells when assayed by [\(^3\)H]thymidine incorporation. SDB at 17 and 34 \(\mu\)g/ml could not further enhance the inhibitory effect of DAU alone on DNA synthesis in KB cells whereas the same doses of SDB could synergistically enhance the effect of DAU in Chr'-24 cells. The inhibitory levels in Chr'-24 cells under the combination of DAU and SDB appear to be comparable to the level in KB cells treated with DAU alone (Table 2).

**Effect of Isoprenoids on Efflux and Influx of Drugs in Chr'-24 Cells.** Enhanced efflux has been previously reported to be involved in the acquisition of drug resistance in mouse P388 leukemia (15, 16). Fojo et al. (12) have recently suggested that drug uptake as well as efflux is altered in multidrug-resistant Chr'-24 cells. We examined whether increased accumulation of anticancer agents in Chr'-24 by isoprenoids is due to altered permeability efflux and/or influx.

To test the effect of isoprenoids on drug efflux activity, the cells were exposed to [\(^3\)H]DAU or [\(^3\)H]VCR for 60 min followed by incubation with or without isoprenoids in medium containing no serum (Fig. 6). To equate the cellular level of VCR or DAU in Chr'-24 with that of KB, a 6-fold higher amount of radioactive DAU or VCR was used for the assay of Chr'-24 in the absence of isoprenoids. More than 60% of cell associated radioactive DAU or VCR in Chr'-24 was released into medium after 30 min of incubation in the absence of drug at 37°C, and there was a slight subsequent release of radioactive drugs. In contrast, in the parent KB cells, the release of DAU or VCR into the medium proceeded much more slowly in comparison to KB cells without (O) or with SDB, 17 (\(\bullet\)) or 34 \(\mu\)g/ml (A). Values, mean ± SD (bars) of triplicate trials.
with Chr-24, and more than 60% of the initial activity was still retained after 90 min of incubation (Fig. 6). Efflux of DAU from Chr-24 cells was significantly blocked by SDB, 34 \mu g/ml or PMB, 216 \mu g/ml. More than 50% of initial DAU or VCR activity remained in SDB- or PMB-treated Chr-24 cells. Inhibition comparable to that with DAU by 2 isoprenoids of drug efflux from Chr-24 cells was also observed when the efflux was followed with VCR (Fig. 6). Almost similar curves of efflux as in Fig. 6 were observed when cells preexposed to [\textsuperscript{3}H]DAU or [\textsuperscript{3}H]VCR for 4 h were then followed by efflux assay (data not shown). SDB at 34 \mu g/ml or PMB at 216 \mu g/ml had no cytotoxic activity against KB and Chr-24 under conditions for efflux study. The efflux of DAU or VCR from Chr-24 cells was significantly more rapid in comparison with the parental clone, and isoprenoids blocked the efflux. In the sensitive KB cells, treatment with isoprenoids also retarded efflux, but much less than in Chr-24 cells.

The effect of isoprenoids on uptake or influx of drug was also examined. 2,4-Dinitrophenol is an inhibitor of the release of DAU or VCR from ADM-resistant P388 leukemia cells (15). Both KB and Chr-24 cells were treated with 2,4-dinitrophenol, resulting in almost complete blockage of the release activity, and the effect of isoprenoids on influx activity was then tested. Treatment of Chr-24 cells with 2,4-dinitrophenol increased the accumulation of DAU or VCR nearly to the level of dinitrophenol-treated KB cells (Fig. 7). As a function of dose of 2 isoprenoids, accumulation of DAU into Chr-24 cells increased up to 3-fold more than in the absence of isoprenoids whereas the accumulation into KB cells increased about 1.2-fold. Fig. 7 also shows increasing accumulation activity of VCR when both 2,4-dinitrophenol and SDB or PMB were present. The stimulatory effect of the two isoprenoids on accumulation of VCR in KB cells was only slight if any. Accumulation of VCR in Chr-24 was enhanced about 2-fold when SDB, 68 \mu g/ml or PMB, 432 \mu g/ml was present (Fig. 7). PMB at 432 \mu g/ml or SDB at 68 \mu g/ml was found to show no cytotoxic effects under treatment conditions as seen in Fig. 7.

Fig. 7. Effect of isoprenoids on drug influx. KB (D) and Chr-24 (L) cells preexposed to various doses of SDB or PMB were incubated with [\textsuperscript{3}H]DAU or [\textsuperscript{3}H]VCR in the presence of 1 \textsuperscript{mm} 2,4-dinitrophenol and various doses of isoprenoids for 10 min, and cell-associated radioactivity was measured. Values, mean ± SD (bars) of triplicate dishes.

**DISCUSSION**

Anthracycline resistance in P388 leukemia (8, 9), in Ehrlich carcinoma (17), and in CHO cells (2, 6) had been earlier supposed to be due to reduced permeability of DAU and ADM. Further study has suggested that active outward transport (efflux) is involved in the anthracycline resistance in Ehrlich carcinoma cells (17, 18) and in P388 leukemia (15); however, the existence of such active efflux in the cellular transport of anthracyclines has been debated (19). In colchicine-resistant cell lines with multidrug resistance derived from CHO, reduced permeability of drugs was originally suggested to be a plausible mechanism for acquired resistance (4). In the colchicine-resistant clone, Chr-24, with multidrug resistance derived from the KB cell line, reduced drug accumulation might be caused by activating efflux and/or decreasing uptake of DAU or VCR (12). Concerning the molecular mechanism for multiple drug resistance, increased expression of a cell surface glycoprotein has been reported in the multidrug-resistant clones derived from CHO cells (20), Chinese hamster lung cells (5), and a human lymphoblastoid cell line (3). Specific DNA sequences correlated with multidrug resistance in Chinese hamster cells are amplified (21). Amplification of the surface glycoprotein genes was observed in the multidrug-resistant CHO clone (22) and monoclonal antibody against the surface glycoprotein in the resistant clone has been recently prepared (23). By contrast, in Chr-24 derived from KB cells, decreased expression of the surface glycoprotein was recently reported (24). At present one cannot still correlate precisely the altered expression of the membranous proteins with the multiple drug resistance.

In our present study, DAU and SDB show a synergistic effect on cellular DNA synthesis under conditions similar to the drug-accumulation study (Table 2). This result suggests that overcoming the effect of drug resistance by SDB might be due to enhanced intracellular levels of drug. Concerning any plausible correlation of intracellular levels of anticancer agents with levels of drug sensitivity, either the influx or efflux pathway appears to be altered in the acquired drug-resistant phenotype. One can thus expect to overcome multidrug resistance by changing either
one or both pathways. So far drugs which overcome drug resistance affect the efflux pathway of anticancer agents in mouse leukemia cells or human ovarian cancer cells. Such drugs include verapamil or other calcium influx blockers (16, 25, 26) and trifluoperazine or other calmodulin inhibitors (25, 27). Siegfried et al. (28) compared anthracycline concentration in the sarcoma 180 cell line of varying sensitivity, and they suggested that reduced uptake or increased efflux of the anthracycline are not the sole determinants of resistance in all of the sublines. By contrast, during the sequential development of the multidrug-resistant CHO-24 cells, each step for acquisition of drug resistance appears to be related to decreasing drug accumulation (1, 12). In CHO-24 cells, verapamil may reverse VBL resistance by changes in VBL influx (12). Phenothiazine calmodulin inhibitors like thioridazine or trifluoperazine are also found to reverse the resistance in CHO-24, but W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide], a well known inhibitor of calmodulin, cannot (13). Thioridazine, but not W-7, affects accumulation of anticancer agents (13). These data may suggest that the development of multidrug resistance or the reversal of drug resistance is partly due to altered membrane permeability. Of course other possibilities besides enhanced efflux or decreased influx (19) should be considered.

We have previously shown that several polypropenoids could potentiate anticancer agents against cultured mammalian cells as well as transplantable murine tumors (10, 11); PMB is a potentiatior in vitro as well as in vivo. We also found that SDB potentiated various anticancer agents.5 Our previous study has also shown that PMB enhanced accumulation of ADM possibly through interfering with the efflux pathway in cultured Chinese hamster V79 cells (10). The cellular accumulation in CHO-24 cells could be remedied almost completely to the level of the parental KB cells when polypropenoids were added. In the presence of 2,4-dinitrophenol, polypropenoids further enhanced the cellular uptake of DAU or VCR. These data lead us to suggest that enhanced uptake and/or reduction of efflux might be involved in the overcoming of multiple drug resistance in CHO-24 cells by the isoprenoids. Unlike verapamil or thioridazine, the isopropenoids appear to have dual activities: potentiation of anticancer agents and circumvention of drug resistance. A combination of anticancer agents with these isopropenoids might give a favorable approach to anticancer chemotherapy.

It is very interesting that part of the chemical structure in one of two polypropenoids, SDB, is very similar to that of verapamil; however, another polypropenoid, PMB, does not carry the verapamil-like structure, but still reverses drug resistance in a way similar to SDB. Isopropen structure rather than verapamil-like structure in SDB appears to affect closely the circumventing action; nevertheless, there appears to be no chemical calion blocking action in SDB (29). These polypropenoids are found to overcome drug resistance in mice bearing drug-resistant P388 leukemia in vivo (29). Further study is necessary to understand how cellular accumulation is controlled at the molecular level in the presence of polypropenoids.

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REFERENCES

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