Effect of Bisbenzylisoquinoline (Biscolaurine) Alkaloids on Multidrug Resistance in KB Human Cancer Cells

Norio Shiraishi, Shin-ichi Akiyama, Masayuki Nakagawa, Michio Kobayashi, and Michihiko Kuwano

Department of Biochemistry and Surgery, Oita Medical School, Hachinohe, Oita 879-56, Japan

ABSTRACT

Cepharanthine, a bisbenzylisoquinoline (biscolaurine) alkaloid, completely overcomes resistance of a multidrug-resistant subline, ChR-24, derived from human KB carcinoma cells, to vincristine, actinomycin D, and daunomycin, and partially overcomes resistance to Adriamycin. Another biscolaurine alkaloid, berberine, partially overcomes resistance to these anticancer agents. Accumulation of [3H]daunomycin in ChR-24 cells is about 10% of that in both the parental KB and revertant cell line (Rev-2) which is derived from ChR-24. Cepharanthine prominently increases the accumulation of daunomycin in resistant ChR-24 cells, but not in parental KB and Rev-2 cells. Enhanced efflux of daunomycin from the resistant cells is completely inhibited by cepharanthine. Cellular uptake of [3H]daunomycin is not significantly affected in the resistant cells by cepharanthine. Accumulation of [3H]cepharanthine is observed at similar levels in both KB and ChR-24. Phosphatidylserine specifically inhibited the accumulation of [3H]cepharanthine in KB and ChR-24 cells when tested by adding various phospholipids such as phosphatidylycerine, phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin to culture medium. The enhanced accumulation of [3H]daunomycin in cepharanthine-treated ChR-24 cells is inhibited in the presence of 20 μg/ml phosphatidylserine. Cepharanthine may overcome multidrug resistance by binding to phosphatidylycerine in the plasma membrane and perturbing membrane function.

INTRODUCTION

Multidrug resistance can be overcome by verapamil, a calcium channel blocker (1, 2). The effect of verapamil seems not to be directly related to its calcium channel blocking activity (3). Our recent study indicates that three phenothiazine calmodulin inhibitors, thioridazine, trifluoperazine, and chlorpromazine, can also overcome multidrug resistance in a cultured human cancer cell line. Circumvention of multidrug resistance by these inhibitors seems not to be mediated through their inhibitory action on calmodulin (4). We have recently reported that isopenoids can also overcome multidrug resistance (5-7). The detailed mechanism of circumvention of multidrug resistance by these agents is not known. However, most of the hydrophobic agents which overcome drug resistance are amphiphatic and cationic, and they can presumably interact with certain polar lipids.

Cepharanthine (see Fig. 1), a bisbenzylisoquinoline (biscolaurine) alkaloid, is extracted from a menispermaceous plant, Stephania cepharantha Hayata. The alkaloid is cationic and amphiphatic and has been reported to decrease fluidity of various biological membranes (8). Cepharanthine inhibits the release of histamine from rat peritoneal mast cells (9), and it induces morphological changes of human erythrocytes (10). Because of the known capacity of biscolaurine alkaloids to affect cell membranes, we have tested the ability of cepharanthine and other biscolaurine alkaloids to overcome multidrug resistance. We find that these agents also overcome multidrug resistance in human KB cells.

Received 9/2/86; revised 1/6/87; accepted 1/29/87.

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.

1This work was supported by a grant-in-aid for cancer research from the Ministry of Education, Science, and Culture of Japan.

2To whom requests for reprints should be addressed.

MATERIALS AND METHODS

Cell Lines and Cell Culture. KB-3-1 (KB) was derived from a single clone of human KB epidermoid carcinoma cells after two subclonings (11). A multidrug-resistant mutant, KB-ChR-24 (ChR-24), was selected with increasing concentrations of colchicine (11). Rev-2 is a cloned spontaneous revertant of ChR-24, isolated from a population of ChR-24 cells grown in the absence of colchicine for 3 mo. Cells were grown in monolayer in MEM1 (Nissui Seiyaku Co., Tokyo, Japan) containing 10% newborn calf serum (Microbiological Associates, Bethesda, MD), 1 mg/ml Bactopeptone (Difco Laboratories, Detroit, MI), 0.292 mg/ml glutamine, 100 μg/ml kanamycin, and 100 units/ml penicillin.

Drugs and Chemicals. Adriamycin, vincristine, actinomycin D, and daunomycin were obtained from Sigma Chemical Co., St. Louis, MO, and cepharanthine and all other biscolaurine alkaloids were kindly donated to us by Kakén Pharmaceutical Co., Ltd., Osaka, Japan. [3H]Daunomycin (3.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and [3H]cepharanthine was presented to us from Dai-ichi Pharmaceutical Co., Ltd., Tokyo, Japan. Phospholipids such as phosphatidylcholine, phosphatidylycerine, phosphatidylethanolamine, and sphingomyelin were obtained from Sigma Chemical Co., St. Louis, MO.

Cell Survival by Colony Formation. Cell survival was determined by plating 300 cells in 60-mm dishes in the absence of any drug. The various drugs were added 16 h later. After incubation for 10 days at 37°C, the colonies were stained with 0.5% methylene blue in 50% ethanol and counted. Solutions of all the drugs were freshly prepared before use in dimethyl sulfoxide. Relative resistance was determined by dividing the D0 of KB with biscolaurine alkaloids or D0 of ChR-24 with or without the alkaloids by the D0 of KB without the alkaloids.

Drug Accumulation. Cells (4 × 10^6/dish) were plated and incubated overnight at 37°C. Then medium was replaced with serum-free MEM, and the cells were incubated with 0.25 μCi/ml [3H]daunomycin for 60 min with or without cepharanthine. Cells were washed once with cold PBS and then exposed to 0.5 μCi/ml [3H]daunomycin for 10 days at 37°C, isolated from a population of ChR-24 cells grown in the absence of colchicine for 3 mo. Cells were grown in monolayer in MEM containing 0.05 μCi/ml [3H]cepharanthine. After incubation for 60 min, the cells were washed 3 times with cold PBS, and the cellular pellets were suspended in 0.7 ml of H2O and mixed thoroughly with 7 ml of Scintisol EX-H (Wako Chemical Co., Osaka, Japan). Then radioactivities were determined.

Drug Efflux and Influx. For the study of drug efflux, cells (4 × 10^3/ dish) were incubated overnight, and then medium was changed to fresh medium. KB cells and ChR-24 cells were incubated with 0.25 μCi/ml and 1 μCi/ml [3H]daunomycin, respectively, at 37°C for 60 min to obtain the same accumulation of [3H]daunomycin in both cell lines. Each dish was washed 3 times with PBS and added to fresh serum-free medium with or without cepharanthine, incubated for the indicated times at 37°C, harvested, and then counted.

For the study of drug influx, cells were plated and incubated overnight, and then the medium was changed to glucose-free, serum-free Hank's balanced salt solution. Cells were incubated for 15 min at 37°C and then exposed to 0.5 μCi/ml [3H]daunomycin for 1 min. Each dish was washed 3 times with ice-cold PBS, harvested, and then counted.

Effect of Phospholipids on the Accumulation of [3H]Cepharanthine. Exponentially growing cells (8 × 10^5/dish) were incubated for 60 min at 37°C in fresh serum-free MEM with or without 10 or 20 μg/ml liposomes prepared from each phospholipid, containing 0.05 μCi/ml [3H]cepharanthine. After incubation for 60 min, the cells were washed 3 times with cold PBS and harvested for counting their radioactivities. To prepare the liposomes, phospholipids were added to 5 ml serum-free MEM and shaken by hand for 2 min, and the suspension was then...
CEPHARANTHINE AND MULTIDRUG RESISTANCE

Fig. 1. Structure of cepharanthine.

Table 1 Effect of cepharanthine on relative resistance of ChR-24 cells to agents

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>ADR*</th>
<th>VCR</th>
<th>ACT-D</th>
<th>DAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>ChR-24</td>
<td>147.3 ± 4.1</td>
<td>104.5 ± 3.0</td>
<td>43.2 ± 1.6</td>
<td>17.7 ± 1.1</td>
</tr>
<tr>
<td>1.0</td>
<td>7.2 ± 1.0</td>
<td>1.8 ± 0.6</td>
<td>1.7 ± 0.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>2.0</td>
<td>5.9 ± 0.8</td>
<td>1.2 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

* ADR, Adriamycin; VCR, vincristine; ACT-D, actinomycin D; DAU, daunomycin.

RESULTS

Circumvention of the Multidrug Resistance in Human Cancer Cells by Biscoclaurine Alkaloids. Multidrug-resistant ChR-24 cells were isolated from the human carcinoma KB cell line after stepwise selection by colchicine resistance (11). ChR-24 cells are resistant to from 18- to 147-fold higher concentrations of Adriamycin, daunomycin, vincristine, and actinomycin D (Table 1). We determined whether cepharanthine could overcome multidrug resistance in ChR-24 cells. ChR-24 cells were slightly more sensitive to the alkaloids than KB cells. We used two concentrations of the alkaloid. The lower concentration of the alkaloid did not block colony formation, whereas the higher one reduced the colony formation of both cell lines to 80 to 90% of initial value. Fig. 2 shows an example of our colony formation assays to test overcoming effects of a combination of alkaloids and anticancer agents. ChR-24 showed about 18-fold higher resistance to daunomycin than KB. Combination of daunomycin with 1.0 or 2.0 μg/ml cepharanthine overcame the drug resistance in ChR-24, and the dose-response curves under the combinations were very similar to those of KB in the presence of daunomycin with or without cepharanthine. Cepharanthine did not significantly enhance the cellular sensitivity of KB to daunomycin (Fig. 2a). Table 1 summarizes data from dose-response curves of various combinations of cepharanthine and anticancer agents. Other biscoclaurine alkaloids, isotetradrine, tetradrine, and fangchinoline, showed the similar effects on the sensitivity of the resistant cells to anticancer agents. They almost completely overcome resistance in ChR-24 cells to vincristine, actinomycin D, and daunomycin, and partially overcome resistance to Adriamycin (data not shown). Another biscoclaurine alkaloid, berbamine, has a weaker affinity for membranes compared with other alkaloids. This alkaloid showed a similar but weaker effect than other alkaloids on multidrug resistance. Fig. 2b shows that berbamine partially overcomes the resistance of ChR-24 cells to daunomycin. These alkaloids, however, only slightly enhanced the sensitivity of the parental cells to many anticancer drugs.

Effect of Cepharanthine on Cellular Accumulation of [3H]-Daunomycin. The amount of daunomycin accumulated for 1 h, and the effect of cepharanthine on this accumulation was examined (Fig. 3). The intracellular levels of daunomycin in the presence of various concentrations of cepharanthine in KB (○), △, □) and ChR-24 (▲, ●) were about one-tenth that in KB cells, whereas that in the revertant cell line, Rev-2, was about the same as that in KB cells. The accumulation of daunomycin in both KB cells and Rev-2 cells treated with cepharanthine was not significantly affected. In contrast, cepharanthine at 0.5 or 2.0 μg/ml increased about 7-fold the accumulation of daunomycin in ChR-24 cells.

Effect of Cepharanthine on Efflux and Influx of [3H]-Daunomycin in KB and ChR-24 Cells. We have observed enhanced influx of daunomycin and vincristine from resistant cells and consequently examined whether increased accumulation of anticancer agents in ChR-24 cells by cepharanthine was due to inhibition of drug efflux. After incubation of the cells for 1 h

Fig. 2. Effect of cepharanthine and berbamine on drug resistance in KB cells. The effect of cepharanthine (a) and berbamine (b) on drug resistance in KB (○, △, □) and ChR-24 (▲, ●) in the presence of 0 μg (○, △), 1.0 μg (○, △), and 2.0 μg (▲, ●) cepharanthine (a) and berbamine (b)/ml was examined by the colony formation assay. Points, mean of triplicate experiments; bars, SE.

Fig. 3. Effect of cepharanthine on the accumulation of daunomycin in KB, ChR-24, and Rev-2 cells. The intracellular levels of daunomycin in the presence of various concentrations of cepharanthine in KB (○), ChR-24 (▲), and Rev-2 (●) cells were determined as described in "Materials and Methods." Columns, mean from triplicate experiments; bars, SE.
in the absence of cepharanthine, about 60% of daunomycin was lost from ChR-24 cells, whereas more than 90% of daunomycin was retained in KB cells. Addition of 2 µg/ml cepharanthine to the culture medium almost completely inhibited this efflux of daunomycin from ChR-24 cells. In contrast, cepharanthine did not affect the efflux activity of daunomycin from the sensitive KB cells (Fig. 4).

The effect of cepharanthine on uptake or influx of daunomycin was also examined. According to the assay reported by Fojo et al. (2), we assayed cellular uptake of [³H]daunomycin for 1 min (Fig. 5). Fig. 5 shows that there is not a significant difference in the cellular uptake activities between KB and ChR-24 cells in the absence or presence of cepharanthine. From data in Fig. 5, cepharanthine appeared not to enhance the cellular uptake or influx activity of anticancer agents in the multidrug-resistant cells.

Effect of Phospholipids on the Uptake of [³H]Cepharanthine into Cells. Cepharanthine is cationic and amphipathic. Lüllman et al. (12) reported that cationic and amphipathic drugs bind to certain polar lipids, resulting in complex formation. To determine which phospholipids strongly interact with cepharanthine, we assayed the effect of various phospholipids on cellular accumulation of [³H]cepharanthine. As shown in Fig. 6, the accumulation of [³H]cepharanthine was similarly observed in both KB and ChR-24 cells. When 10 or 20 µg/ml of phosphatidylinerine were added to the medium, accumulation of cepharanthine decreased to 20% of its initial value in both cell lines. Other phospholipids, phosphatidycholine, phosphatidylethanolamine, and sphingomyelin, had no such effect on the accumulation of cepharanthine at the same concentration as that of phosphatidylinerine.

The effect of phosphatidylinerine was examined if it blocked enhanced cellular accumulation by cepharanthine of daunomycin in both KB and ChR-24 cells. In accordance with Fig. 3, accumulation of [³H]daunomycin in ChR-24 is about 20% of the parental cell line (Fig. 7). Treatment with 100 ng/ml cepharanthine increased the cellular level of [³H]daunomycin.

![Image](image_url)
about 3-fold higher than in the absence of the alkaloid, but addition of 20 μg/ml phosphatidylserine blocked about 30% of the enhancement in Chβ-24 cells by cepharanthine (Fig. 7a). By contrast, addition of phosphatidylserine did not affect the cellular accumulation of daunomycin in KB cells. As seen in Fig. 7b, 1 μg/ml of cepharanthine enhanced the accumulation of [3H]daunomycin about 3-fold in Chβ-24, and phosphatidylserine again blocked the enhancement by about 30%. Phosphatidylserine alone at 20 μg/ml did not affect the cellular accumulation of daunomycin in Chβ-24 cells.

**DISCUSSION**

In our previous study (4), we suggested a role for increased drug efflux in the development of the multidrug-resistant phenotype in the KB human carcinoma cells. Drug resistance in Chβ-24 cells can be overcome by inhibitory drugs of efflux including calcium channel blockers like verapamil (1, 2); phe-nothiazine calmodulin inhibitors like thiordiazine, trifluoperazine, and chlorpromazine (4); lysosomotropic amines like chloroquine and propranolol (13); and some isopenoids like SDB-ethylidenediamine and N-(P-methylbenzyl)decaprenylamine (5-7). One common feature of the agents which overcome multidrug resistance is that all of them are cationic and amphipathic. In the present study, we examined another cationic amphipathic agent, cepharanthine, and its analogues. They are all found to overcome multidrug resistance in human KB cells. Cepharanthine increases drug accumulation through its inhibition of drug efflux (Fig. 4).

Cationic amphipathic drugs are reported to interact with polar lipids, especially with phosphatidylserine (12). They induce lipidosis with a myelin-like structure in the lysosome (14). We observed myelin-like lamellated structures in lysosomes of KB and HeLa cells treated with thiordiazine (15) and SDB-ethylidenediamine (16), respectively. Our recent study also shows the appearance of similar structures in cepharanthine-treated KB or Chβ-24 cells.4 As relevant data to correlate membranous lipids with the overcoming effect by amphipathic agents of drug resistance, our present study presents the specific interaction of cepharanthine with phosphatidylserine. Cellular accumulation of [3H]cepharanthine in KB or Chβ-24 cells is almost completely blocked only by phosphatidylserine (Fig. 6). Cepharanthine-induced enhancement of [3H]daunomycin accumulation in Chβ-24 is also blocked by phosphatidylserine, but not by other phospholipids, although the inhibitory effect is partial (Fig. 7). One way in which cepharanthine and other cationic amphipathic agents overcome multidrug resistance would be by binding to phosphatidylserine in the plasma membrane and perturbing membrane function.

Cornwell et al. (17) have investigated the biochemical basis of reduced drug accumulation using membrane vesicles from KB, KB-C4, a highly multidrug-resistant cell line, and KB-R1, drug-sensitive revertant. The appearance of M, 170,000 mem-branous glycoproteins is proposed to be closely correlated with multidrug resistance (18). Cornwell et al. (17, 19) show the specific labeling of a M, 150,000 to 170,000 glycoprotein with two analogues of vinblastine that could be photoactivated in membrane vesicles from the multidrug-resistant KB cells, but not in those parental KB cells or revertant KB-R1 cells. Verapamil blocks the specific labeling of a M, 170,000 protein with a photoaffinity analogue of vinblastine in membrane vesicles from the multidrug-resistant cells (17). Cepharanthine also blocks this photoaffinity labeling by vinblastine analogues.5 Although the molecular mechanism of drug efflux is not known, a M, 150,000 to 170,000 membrane protein that is labeled with a photoaffinity analogue of vinblastine (17, 19) may be correlated with the drug efflux; and the function of this protein may be influenced by the membrane lipids, especially phosphatidylserine. Our present study suggests a plausible involvement of membrane lipids and proteins in the development of the multidrug resistance. Further study to clarify roles of membrane lipids in the acquisition of multidrug resistance is now in progress in our laboratory.

**ACKNOWLEDGMENTS**

We thank Dr. Michael M. Gottesman (National Cancer Institute, NIH) for critical reading of this manuscript and for critical comment. We also thank Junko Kikuchi for technical assistance.

**REFERENCES**


* Akiyama, S., unpublished data.
Effect of Bisbenzylisoquinoline (Biscoclaurine) Alkaloids on Multidrug Resistance in KB Human Cancer Cells

Norio Shiraishi, Shin-ichi Akiyama, Masayuki Nakagawa, et al.