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Modification of Cellular Efflux and Cytotoxicity of Adriamycin by Biscoclaulin Alkaloid *In Vitro**

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Abstract—*The intracellular uptake, retention and cytotoxicity of adriamycin (ADR) combined with a biscoclaulin alkaloid, cepharanthine, were investigated by flow cytometry in NIH 3T3 cells. Cepharanthine suppressed the efflux of ADR in a similar fashion to verapamil. The intracellular uptake and retention of ADR were increased gradually by* 0.1 *to* 1 μg/ml *of cepharanthine and reached a plateau at* > 1 μg/ml. *Cepharanthine, which had no toxic action on survival, increased intracellular ADR uptake by about* 20% *for* 1 h *incubation at* 37°C, *and increased cellular ADR retention after incubation in an ADR free medium for* 4 h *from* 15% *to* 75%. *The cytotoxicity of ADR was enhanced* 5*-fold in the cells pre- and co-incubated with cepharanthine. When cepharanthine was present in the medium before, during and for colony formation* (10 *days) after incubation with ADR, the cytotoxicity increased to about* 300*-fold. Furthermore, an increase in intracellular uptake of ADR was induced by an elevated temperature of* 43°C, *and the efflux of ADR was inhibited by cepharanthine. A high level of intracellular ADR was maintained during the treatment. These results suggest a possible novel use of cepharanthine to improve the drug sensitivity of tumors resistant to ADR.*

INTRODUCTION

MAJOR causes of failure in cancer therapy are acquired resistance to anticancer drugs and the selection and proliferation of drug-resistant clones during treatment due to the heterogeneity of the tumor during treatment [1, 2]. The extent of accumulation and retention of antineoplastic agents within tumor cells is the most important determinant of cytotoxicity [3]. If acceleration of influx to the cells and inhibition of the efflux from the cells could be modified, the efficacy of the drug may be improved. Recently, it has been demonstrated that hyperthermia can increase uptake of some drugs [4–6]. We reported that hyperthermia increased intracellular adriamycin (ADR) uptake and its increase resulted from an increased influx [7, 8]. Meanwhile, attempts to inhibit efflux of the anticancer agent and to enhance their cytotoxicity by various drugs have also been investigated [9–14]. For clinical use, suitable potentiating agents are required, which have low toxicity.

We reported in a preliminary study that cepharanthine, a biscolaulin alkaloid, suppressed the efflux of ADR [15]. In the present study, we have analysed modification of the intracellular uptake, retention and cytotoxicity of ADR by cepharanthine and also demonstrated that the combination of ADR and cepharanthine is potentially useful in cancer chemotherapy.

MATERIALS AND METHODS

Cells

NIH 3T3 cells were used for the experiments. A detailed description of our standard growth techniques has been published previously [16]. In brief, NIH 3T3 cells (a gift from Prof. Baserga, Temple University, Philadelphia, PA) were grown in monolayer and maintained in plastic dishes (Falcon) in Dulbecco's modified Eagle's medium (DMEM) (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented by penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% calf serum (Gibco). Exponentially growing cells were obtained 2 days after seeding 2×10^5 cells per 25 cm^2 in DMEM

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+ 10% calf serum. The cells were used at a density of about $5 \times 10^4/cm^2$ for experiments.

Drugs

ADR was provided by Farmitalia Carlo Erba Co., Ltd., Italy. Verapamil was obtained from Eisai Co., Tokyo, Japan. Cepharanthine was kindly supplied by Kakken Pharmaceutical Co., Ltd., Tokyo, Japan. In our experiments, ADR was used immediately after dissolving it in DMEM for each experiment and the cells exposed to ADR under yellow light [8]. Verapamil was used at a concentration of 2.3 μg/ml as it was at a non-toxic concentration between 0.3 to 9.1 μg/ml [10]. Verapamil and cepharanthine were dissolved in 99.5% ethanol and diluted to adequate concentration with DMEM. Final concentrations of ethanol were less than 1%. This concentration of ethanol did not affect intracellular uptake, retention and cytotoxicity of ADR (data not shown). The cells were prepared in an adequate medium volume, flushed with a 5% CO_2 + 95% air mixture for a few minutes, and, after addition of drugs, incubated at the required temperature.

Hyperthermia

Heat treatment was conducted by submerging a flask (Falcon) in a circulating water bath. The temperature of the water bath was monitored by thermistor probes with an accuracy of ± 0.05°C. The time required to reach temperature equilibrium was within 3 min after immersion.

Cytotoxic study

Cell viability was assayed only in terms of proliferative ability (colony formation). The plating efficiency of the control under these conditions was about 55%. Each experiment was repeated at least twice. Data were a compilation of two or more experiments.

Intracellular ADR measurement

When ADR is excited with a 488 nm laser beam, it emits a characteristic fluorescent spectrum with peaks at 556 and 582 nm [17, 18]. Therefore, quantitative measurements of intracellular uptake and retention of ADR can be obtained rapidly and easily using flow cytometry [7, 8, 12–15, 18–22]. A detailed description of this method has been published previously [8]. In brief, following exposure to ADR, tripsinized cell suspensions were placed in an ice box and immediately used for analysis with a fluorescence activated cell sorter. All flow cytometric measurements were made with a Becton Dickinson fluorescence-activated cell sorter (FACS III). ADR-treated cells were analysed with an excitation at 488 nm (1 W power), and an emission integrated above 530 nm. We determined the true mean intracellular ADR levels by subtracting the mean spontaneous fluorescence of untreated cells from ADR-treated cells. This procedure was used for the determination of the intracellular ADR content presented in this paper. Each experiment was repeated at least twice. Data were a compilation of two or more experiments.

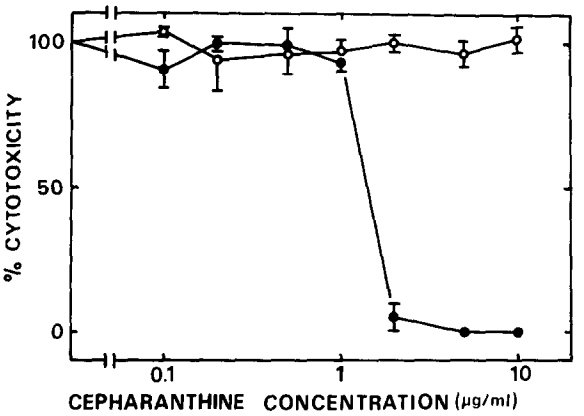

Fig. 1. Toxicity of cepharanthine. ○: 1 h exposure of cepharanthine at 37°C, ●: continuous exposure of cepharanthine for 1 h and during colony formation at 37°C (10 days). Bars = S.E.

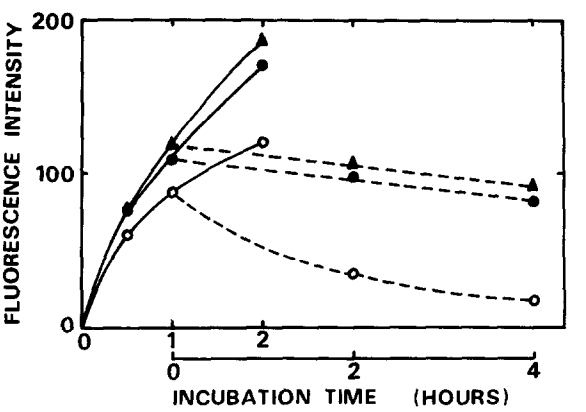

Fig. 2. Comparison of effects of verapamil and cepharanthine on intracellular uptake and retention of ADR at 37°C. The cells were exposed to ADR at 5 μg/ml in combination with verapamil or cepharanthine (solid lines), rinsed three times with DMEM and incubated in ADR-free medium containing the same drugs (dotted lines). ○: No further additions, ▲: medium containing 2.3 μg/ml verapamil, ●: medium containing 1 μg/ml cepharanthine. Both cerapamil and cepharanthine are added 30 min before ADR exposure. Points = mean of two experiments.

RESULTS

Toxicity of cepharanthine

After the cells were incubated in the presence of 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg/ml of cepharanthine for 1 h, the cytotoxicity of cepharanthine was studied by colony formation with or without the same concentrations of cepharanthine in complete medium (Fig. 1). Exposure of cepharanthine for 1 h was non-toxic even at 10 μg/ml. Continuous exposure of cepharanthine for 10 days was non-toxic at 0.1–1 μg/ml and toxic at 2 μg/ml.

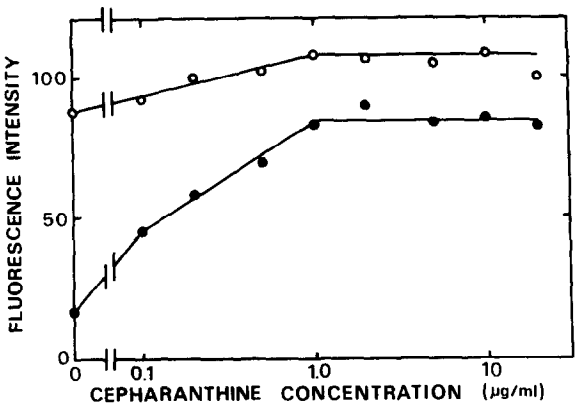

Fig. 3. Effects of cepharanthine concentrations on intracellular uptake and retention of ADR at 37°C. *The cells were exposed to* 5 μg/ml *ADR at* 1 h *in combinations of various concentrations of cepharanthine* (○). *Then, the cells were rinsed three times with DMEM, changed to ADR-free medium containing the same concentrations of cepharanthine and incubated for* 4 h (●). *Cepharanthine was added* 30 min *before ADR exposure. Points = mean of two experiments.*

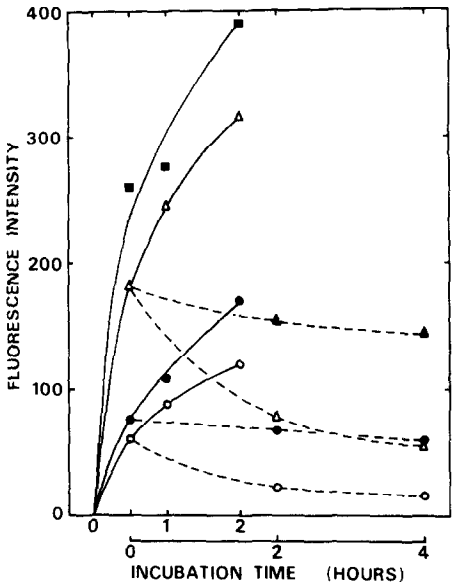

Fig. 4. Intracellular uptake and retention of ADR combined with cepharanthine and hyperthermia. The cells were exposed to ADR of 5 μg/ml *at* 37°C (*solid lines*), *rinsed three times with DMEM and incubated in ADR-free medium at* 37°C (*dotted lines*). ○: *No further additions at* 37°C, ●: *medium containing* 1 μg/ml *cepharanthine at* 37°C. △: *Incubation at* 43°C, ■: *medium containing* 1 μg/ml *cepharanthine at* 43°C, ▲: *post-incubation with* 1 μg/ml *cepharanthine. Cepharanthine was added* 30 min *before ADR exposure. Points = mean of two experiments.*

Effect of verapamil and cepharanthine on intracellular uptake and retention of ADR

Intracellular uptake and retention of ADR were analysed in combinations with or without verapamil or cepharanthine (Fig. 2). The cellular uptake of ADR increased gradually depending on incubation time. Verapamil added to the medium of 2.3 μg/ml increased intracellular ADR uptake by about 30% at 1 h and by about 50% at 2 h. Cepharanthine added to the medium of 1 μg/ml increased it by about 30% at 1 h and about 40% at 2 h. The extent of the effect of verapamil and cepharanthine on the cellular uptake of ADR was similar. When the cells were incubated in the presence of ADR without verapamil or cepharanthine at 1 h and then changed into ADR-free medium without drugs, the cellular retention of ADR was decreased exponentially (retention rate at 4 h was about 15%). The retention of ADR at 4 h was 77% when verapamil had been present in the medium, before, during and after exposure to ADR. For cepharanthine, it was 75%. Verapamil and cepharanthine inhibited ADR efflux. The inhibitory effects of 1 μg/ml cepharanthine were similar to those of 2.3 μg/ml verapamil which was a suppressor of ADR efflux. To determine the optimal dose of cepharanthine, the intracellular uptake and retention of ADR were measured after incubation with various concentrations of cepharanthine (Fig. 3). The intracellular uptake and retention were increaed gradually by 0.1–1 μg/ml of cepharanthine and reached a plateau at more than 1 μg/ml. This indicated that cepharanthine at 1 μg/ml was an optimal and low dose.

The intracellular uptake of ADR was measured after simultaneous exposure to cepharanthine and/or hyperthermia at 43°C (Fig. 4). As reported previously [7, 8], hypethermia markedly increased intracellular ADR uptake. Cepharanthine combined with hyperthermia increased intracellular uptake of ADR by abut 30% as compared to hyperthermia alone. In a study of efflux (Fig. 4), the efflux capability was unaffected by hyperthermia at 43°C [8] and cepharanthine inhibited the efflux of cellular ADR which had been increased by hyperthermia (retention rate; 70% at 4 h).

Potentiation of ADR by cepharanthine

The cytotoxicity of ADR combined with cepharanthine at a non-toxic dose betwen 0.1 and 1 μg/ml was compared with that of ADR alone. The data in Fig. 5 indicated the surviving fraction ratio, calculated by dividing the cytotoxicity of ADR in combination with cepharanthine by the cytotoxicity of ADR alone (surviving fraction for 30 min incubation with 2 μg/ml of ADR alone was about 6×10^{-2}). When the concentrations of cepharanthine increased, the cytotoxicity of ADR was enhanced progressively. Cepharanthine at 1 μg/ml in pre- and co-incubation with ADR enhanced ADR cytotoxicity about 5-fold. Furthermore, when cepharanthine was present in the medium before, during and for colony formation (10 days) after incubation with ADR, the cytotoxicity increased to about 300-fold.

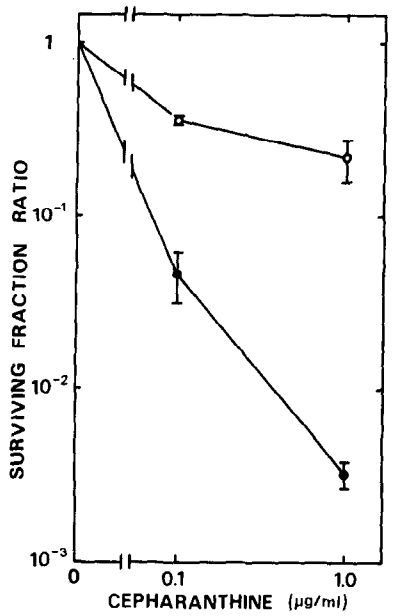

Fig. 5. Effects of cepharanthine on cytotoxicity of ADR. The cells were exposed to 2 μg/ml *ADR for* 30 min *in combinations of different concentrations of cepharanthine. Cepharanthine was added* 30 min *before ADR exposure.* ○: *Pre- and co-incubation of cepharanthine;* ●: *pre-, co- and post-incubation of cepharanthine (during colony formation). Bars = S.E.*

DISCUSSION

ADR is a powerful anthracycline antibiotic with clinical activity against a wide variety of human cancers. It is one of the anticancer agents which is used most frequently alone or combined with other anticancer agents. Theoretically, ADR exerts its cytotoxicity by intercalating with DNA and producing subsequent changes in nucleic acid synthesis [23, 24]. The intracellular uptake and retention of ADR are important factors which influence its effectiveness [3]. It has been reported that a good correlation was found between cell survival and intracellular uptake of ADR [8, 19]. In various experimental systems, an ADR-resistant subline was associated with a reduced cellular accumulation and retention of ADR [9, 25, 26]. The decreased cellular accumulation and cellular retention of ADR have been demonstrated to attribute to enhanced active efflux of intracellular ADR by an energy-dependent mechanism [25].

More recently, calcium antagonists, calmodulin inhibitors and some other drugs have been shown to increase drug uptake and increase sensitivity to ADR treatment in ADR-resistant P388 mouse leukemia by an inhibition of ADR efflux from the cells [9–11, 13, 14]. In our experiment (Fig. 2), verapamil, a calcium antagonist, added to the medium in a concentration of 2.3 μg/ml inhibited ADR efflux from the cells. Cepharanthine (1 μg/ml) added to the medium, a non-toxic dose, also suppressed the efflux of cellular ADR. The extent of efflux inhibition of cepharanthine and verapamil was the same in the concentrations of both drugs used. Cepharanthine at 1 μg/ml was a non-toxic, maximum and optimal dose as an efflux blocker of ADR (Figs. 1, 3). As predicted from these results, cepharanthine potentiated the cytotoxicity of ADR (Fig. 4). Tsuruo et al. [9] speculated that the inhibition of ADR efflux by calcium antagonists and calmodulin inhibitors might be mediated via alterations in calcium fluxes, and intracellular calcium and calmodulin might be related to the drug efflux mechanism in the plasma membrane of the cells. On the other hand, Tsuruo *et al.* [11] demonstrated that quinidine which has no calcium channel blocking action inhibits efflux of cellular ADR and enhances the cytotoxicity of ADR in resistant tumor cells. However, it must be considered that the interaction of calcium antagonists and quinidine with the membrane of tumor cells might lead to an inhibition of drug efflux from resistant tumor cells [27, 28]. Quinidine can induce a decrease in the intracellular level of free calcium to the same extent as a calcium channel blocker, although the processes are different from each other [29].

Cepharanthine inhibits the activity of phospholipase A_2 [30] and phospholipase C [31] which are membrane-bound enzymes. Release of calcium from microsomes and calcium-dependent reactions are depressed indirectly by the latter. Cepharanthine also inhibits release of membrane-bound calcium [30]. Thus, cepharanthine interacts by a decrease in intracellular level of free calcium. However, Kessel *et al.* [32] reported that there was no relationship between calcium fluxes and anthracycline transport. In order to clarify whether cellular calcium is related to the drug efflux mechanism in the plasma membrane of the cells, the localization of cellular calcium would have to be identified.

Cepharanthine also decreases membrane fluidity and acts as a potent membrane stabilizing agent [33]. Cepharanthine incorporated into the plasma membrane and was believed to cause rearrangement of the lipid bilayer [34, 35]. Recently, several laboratories have reported that cells resistant to ADR have an alteration in the lipid fluidity of the cell surface [36–38]. The interaction of cepharanthine with the cell membrane might lead to the inhibition of drug efflux from the cells. The exact mechanism of the inhibition of ADR efflux is unknown.

The influx of ADR is due to passive transport and is influenced by the permeability of the cell membrane [8, 20, 21, 39]. We reported that hyperthermia induced an increase of ADR influx by increasing the permeability of the cell membrane to ADR, and thus enhancing its cytotoxicity [7, 8]. Cepharanthine also inhibited the efflux of the increased intracellular ADR induced by hyperthermia (Fig. 5). We reported that both influx and efflux to ADR were different in various cell lines

[22]. The combinations of ADR, cepharanthine and hyperthermia may be a promising way to overcome the acquired resistant and natural resistant tumor cells to ADR.

The use of verapamil in cancer therapy might be difficult as the drug produces coronary vasodilation. The most exciting feature of cepharanthine is its low toxicity; Makidono *et al.* [40] demonstrated that an i.v. dose of 40–60 mg cepharanthine per day for 2 months in man had no side effects [40]. It might be possible to overcome drug resistance practically by using the approach described in this paper.

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