Cinchoine, a Potent Efflux Inhibitor to Circumvent Anthracycline Resistance

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ABSTRACT

Circumvention of multidrug resistance is a new field of investigation in cancer chemotherapy, and safe and potent multidrug resistance inhibitors are needed for clinical use. We investigated several analogues of quinoline for their ability to increase anthracycline uptake in resistant cancer cells. Cinchoine was the most potent inhibitor of anthracycline resistance in vitro, and its activity was little altered by serum proteins. Serum from rats treated with i.v. cinchoine produced greater uptake of doxorubicin in cancer cells (DHD/K12/PROb rat colon cells and K562/ADM human leukemic cells) than did serum from quinoline-treated rats (ex vivo assay). Cinchoine was more effective than quinoline in reducing tumor mass and increasing the survival of rats inoculated i.p. with DHD/K12/PROb cells and treated i.p. with deoxydoxorubicin. Moreover, the acute toxicity of cinchoine in rats and mice was lower than that of other quinoline-related compounds. The lower toxicity and greater potentiation of in vivo anthracycline activity produced by cinchoine are favorable characteristics for its use as an anti-multidrug resistance agent in future clinical trials.

INTRODUCTION

Primary or acquired resistance of cancer cells to anthracyclines is part of a larger phenomenon described as MDR (1). MDR is common to anthracyclines, Vinca alkaloids, mitoxantrone, etoposide, and daunomycin. MDR results from the expression of a 180,000 membrane glycoprotein (GP 180), which acts as a drug-efflux pump (2). GP 180 or its encoding gene (mdr1) has been detected in many human cancers, either in untreated tumors or after previous chemotherapy (3). A large variety of chemically unrelated compounds, such as verapamil (4), amiodarone (5), cyclosporins (6), and quinidine (7), inhibit drug efflux, facilitate intracellular accumulation, and restore the cytotoxicity of anthracyclines to resistant cells in vitro.

Several clinical trials aiming to circumvent MDR have been reported recently (8-10). The most well studied MDR inhibitor is verapamil; however, the cardiovascular toxicity of this calcium channel blocker prevents its clinical use in the necessary concentrations (11). We reported previously that quinine reached a sufficiently high level in patient serum to inhibit the anthracycline resistance of rat colon cancer cells (12) or human leukemic cells (13).

In the present work, we investigated several quinidine-derived compounds for their anti-MDR activity. We report that cinchoine is more efficient than quinidine in potentiating anthracycline cytotoxicity both in vitro and in vivo.

MATERIALS AND METHODS

Cancer Cells. The DHD/K12/PROb cancer cell line was derived in our laboratory from a chemically induced colon cancer in syngeneic BDIX rats (14). The resistance of DHD/K12/PROb cells to anthracyclines results from increased drug efflux (15), which is inhibited by amiodarone (5), verapamil, and quinidine in vitro (12). The presence of the mdr1 gene product was established in DHD/K12/PROb cells by Northern blot, using the pCHPl probe (American Type Culture Collection, Rockville, MD) (data not shown). DHD/K12/PROb cells were grown as a monolayer in Ham's F10 medium supplemented with 10% fetal bovine serum. For experiments, the cells were detached from the culture flask by a 10-min treatment with EDTA (0.2 mg/ml) and trypsin (2.5 mg/ml), in Hanks' medium without calcium or magnesium. The human MDR myelogenous leukemia cell line K562/ADM was obtained from Dr. Tsuruo (Cancer Chemotherapy Center, Tokyo, Japan). Cells were grown in suspension in RPMI 1640 medium supplemented with 2 mm l-glutamine, 25 mm HEPES, and 10% fetal bovine serum at 37°C, in a humidified atmosphere of 5% CO2 and 95% air. The K562/ADM cell line was continuously cultured in the presence of DXR (0.2 μM) to maintain a stable MDR phenotype.

Drugs. DXR was obtained from Roger Bellon (Neuilly, France). DeoDXR was purchased from Farmitalia Carlo Erba (Milan, Italy). Amiodarone hydrochloride was obtained from Labaz (Bordeaux, France). Verapamil hydrochloride was purchased from Biosedra (Malakoff, France). Cinchoine hydrochloride, cinchonidine hydrochloride, quinidine, and quinidine hydrochloride were obtained from Sigma (La Verpilliere, France). Hydroquinidine hydrochloride was obtained from Aldrich Chimie (Steinheim, Germany). [14C]-DXR (specific activity, 1.85 GBq/mmol) was obtained from Amersham (Les Ulis, France).

Animals and Treatment. Acute toxicity produced by resistance modifiers was evaluated using 4-5-week-old male BALB/c mice (Iffa Credo, Lyon, France), weighing 25-30 g, and male BDIX rats. Groups of three mice or three rats received a single i.p. injection of drug diluted in 1 ml 0.9% NaCl. The dose was increased in steps of 25 mg/kg until animal death. The maximal tolerated dose was defined as the step immediately below the dose that induced acute death of animals (minimal observation time was 24 h).

BDIX rats were bred in our laboratory. The animals were anesthetized with ether before drug injection or blood collection by cardiac puncture. Serum was separated from blood and stored at -80°C until assays.

Female BDIX rats, 5-7 months old and weighing about 200 g, were used to evaluate the enhancement of deoDXR antitumor activity by resistance modifiers. Rats were inoculated i.p. with 1 × 106 DHD/K12/PROb cells and were then treated 24 h after cell inoculation. Cinchoine and quinidine (100 mg/kg in 0.5 ml 0.9% NaCl) were injected i.m. 1 h before the i.p. treatment (1 ml 0.9% NaCl supplemented or not with 0.5 mg/kg deoDXR). DeoDXR was preferred to DXR, due to its greater activity on DHD/K12/PROb cells in vivo. Animals were killed 6 weeks after the cell injection. Tumor nodules of the peritoneal carcinomatosis were excised and weighed. Student's t test was used to compare tumor weight in different animal groups.

Three- to 4-month-old male BDIX rats, weighing 300-350 g, were used to study the survival induced by the resistance modifiers in deoDXR-treated rats. Rats were inoculated i.p. with 2 × 106 DHD/K12/PROb cells. Animals were treated 24 h after the cancer cell inoculation. The control and the deoDXR-treated groups received an i.v. injection of 1 ml 0.9% NaCl. The quinine and cinchoine groups received drugs at the maximal tolerated i.v. dose for quinine (45 mg/
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kg in 1 ml 0.9% NaCl). Rats received an i.p. injection of 4 ml of 5% glucose, supplemented or not (control group) with deoDXR (0.5 mg/kg), 30 min after the i.v. injection. Log-rank test was used to compare the survival curves.

Cell DXR Uptake. DHD/K12/PROb cells (2 × 10⁶) were seeded in microtiter plates (24 wells/plate) and cultured for 24 h. Cells were incubated for 4 h at 37°C with 20 μM DXR (97% nonradioactive DXR; 3% [¹⁴C]-DXR) diluted in 0.5 ml of serum-free Ham's F-10 medium or rat serum. After incubation, cells were rinsed three times with ice-cold phosphate-buffered saline, trypsinized, and transferred into counting vials with 3 ml scintillant liquid (LKB, Stockholm, Sweden). The radioactivity was measured on a β scintillation counter (LKB 1214; Rackbeta, Stockholm, Sweden). A similar procedure was used to quantify the cellular uptake of DXR in K562/ADM cells; cells (5 × 10⁶) were incubated for 4 h in rat serum in the presence of 10 μM DXR (98% nonradioactive DXR; 2% [¹⁴C]-DXR), rinsed, and centrifuged three times with phosphate-buffered saline before radioactivity counting.

Assay of Quinine and Cinchonine Concentrations in Rat Serum. Rat serum (0.1 or 0.2 ml for quinine and cinchonine assays, respectively) was mixed with 0.1 ml of 5 N NaOH and 0.1 ml of an internal standard (hydroquinidine). Extraction was performed using a 5-ml mixture of dichloromethane and isooamyl alcohol (98:2; v:v). The organic phase was evaporated under a nitrogen stream. The dry residue was dissolved in the mobile phase and injected into a high performance liquid chromatography apparatus (Waters, Millipore, St Quentin, France). The mobile phase was a mixture of acetonitrile and 0.045 M potassium phosphate buffer, pH 3.8 (1:4, v:v). The stationary phase was a Lichrospher RP-Select B 5-μm column (Merck, Darmstadt, Germany). Drugs were detected by fluorimetry, at excitation and emission wave-lengths of 350 and 440 nm for quinine assays and 365 and 480 nm for cinchonine assays.

RESULTS

In vitro Effect of Resistance Modifiers on Cell DXR Uptake. The increase of cell DXR uptake induced by resistance modifiers was evaluated after incubation of DHD/K12/PROb cells in serum-free Ham's F-10 medium or rat serum. Activity of resistance modifiers at 10 and 20 μM in serum-free medium, in decreasing order, was amiodarone > verapamil > cinchonine > quinidine > hydroquinidine > quinine > cinchonidine. Incubation with rat serum modified greatly the in vitro activity of resistance modifiers: cinchonine = verapamil > quinidine > quinine > hydroquinidine > cinchonidine > amiodarone. Decrease of resistance-modifying activity in the presence of serum was evident for amiodarone and, to a lesser degree, for verapamil. However, quinine-derived compound activity on DXR accumulation was modified less by serum. Cell uptake of anthracycline alone was also decreased by the addition of serum.

Acute Toxicity of Resistance Modifiers. Acute toxicity of resistance modifiers was studied in BALB/c mice and in BDIX rats after a single i.p. injection (Table 1). Cinchonine was less toxic in both species.

Cell DXR Uptake in the Presence of Serum from Treated Rats. BDIX rats were treated by i.v. injection of quinine or cinchonine at various doses. Rats were killed at different times after injection (Fig. 2). In this ex vivo assay, cinchonine-con-
Table 1  Acute toxicity of anthracycline resistance modifiers in BALB/c mice and BDIX rats

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<tr>
<th>Resistance-modifying agent</th>
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Fig. 2. DXR uptake in DHD/K12/PROb cells incubated with 20 μM DXR diluted in serum of resistance modifier-treated rats; animals were treated i.v. with quinine or cinchonine at various doses (C, 10 mg/kg; O, 25 mg/kg; ■, 45 mg/kg) and killed at different times after injection. , serum of control rats. Each point is the mean of six measurements (±SD) (two measurements and three rats per point).

Fig. 3. DXR uptake in DHD/K12/PROb cells in the presence of treated rat serum as a function of the serum concentration of quinine (○) and cinchonine (●) (r = 0.83 for quinine; r = 0.94 for cinchonine).

Fig. 4. DXR uptake in K562/ADM human leukemic cells incubated with 10 μM DXR diluted in serum of resistance modifier-treated rats. Animals were treated i.v. with quinine (O) or cinchonine (●), at a dose of 45 mg/kg, and were killed at different times after injection. , serum of control rats. Each point is the mean of six measurements (±SD) (two measurements and three rats per point).

Fig. 5. Tumor weight of BDIX rats with peritoneal carcinomatosis induced by DHD/K12/PROb cells. Animals were treated, 24 h after the cancer cell injection, with i.p. deoDXR (0.5 mg/kg), preceded or not by an i.m. injection of quinine or cinchonine (100 mg/kg). Columns, mean tumor weight of five rats (±SD).
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Fig. 6. Survival of BDIX rats with peritoneal carcinomatosis induced by DHD/K12/PROb cells (six rats/group). D, control group. DeoDXR group (O) received an i.p. injection of 0.5 mg/kg deoDXR, quinine (■) and cinchonine (•) groups received an i.v. injection of quinine or cinchonine (45 mg/kg) 30 min before an i.p. injection of 0.5 mg/kg deoDXR.

other hand, quinine (45 mg/kg) produced no effect on rat survival in this experiment.

Pharmacokinetics of Quinine and Cinchonine in Rat Serum. Cinchonine and quinine, after i.v. injection, showed biexponential curves of elimination in rat serum. The residence time in the blood circulation was longer for cinchonine, compared with quinine (Fig. 7). Half-time distribution and elimination were determined by a graphical method: \( t_{1/2,h} = 1 \) h and \( t_{1/2,e} = 11 \) h for cinchonine; \( t_{1/2,h} = 0.33 \) h and \( t_{1/2,e} = 2 \) h for quinine.

DISCUSSION

In the present work, we investigated five quinine-derived compounds for the ability to increase DXR uptake in resistant cancer cells. This activity was compared with that of known MDR-modifying agents, such as verapamil, amiodarone, and quinine. Several other quinine-related compounds have little or no effect on anthracycline resistance of DHD/K12/PROb cells: chloroquine, quinoline, quinacrine, quinoline yellow, 1,4-benzoquinone, 4-quinoline carboxyaldehyde, and 4-hydroxyquinoline (data not shown). None of the five selected quinine-derived compounds were more active than amiodarone or verapamil when assayed in serum-free medium. However, very different results were obtained when the DXR uptake assay was performed in rat serum. Cinchonine was then a very potent inhibitor of anthracycline efflux, at least as active as verapamil. The resistance-modifying activity of quinine-derived compounds was little altered by the presence of serum, whereas the anti-MDR activity of verapamil and amiodarone was greatly diminished in the presence of serum protein. The deleterious effect of serum on amiodarone activity is probably related to the affinity of amiodarone for binding (95–99%) to serum proteins, as we have previously reported (16).

For potential clinical use of quinine-derived compounds, we investigated their acute toxicity in rats and mice. In both species, cinchonine was the least toxic quinine-derived compound; our results are in agreement with previous literature on quinine-derived compounds (17). Quinidine and hydroquinidine are antiarrhythmic drugs used in clinical practice. However, both of these drugs have toxic effects at concentrations higher than 17 \( \mu M \) in patient serum (blockade of auriculoventricular conduction, bradycardia, and syncope). These cardiovascular side effects could hamper their clinical use as anti-MDR agents. Similar cardiovascular side effects on cardiac conduction associated with hypotension have been observed when high dose verapamil has been used, by i.v. infusion, as an anti-MDR agent (11).

We reported previously (12, 13) that quinine can reach a sufficient concentration in patient serum (24–37 \( \mu M \)) to lower anthracycline resistance of MDR cancer cells. Quinine is less toxic and can be readily administered as a continuous infusion. However, we currently report that quinine is less active than cinchonine in \textit{in vitro} and \textit{in vivo} assays. The greater activity of cinchonine, compared to quinine, for \textit{in vivo} potentiation of anthracyclines results from its greater intrinsic activity on cancer cell drug efflux and from higher serum concentrations and slower elimination following i.v. injection.

Cinchonine, like quinine, is a natural extract of cinchona bark that was used as an antimalaria agent with acceptable toxicity before World War II (18). It was discontinued for clinical use due to the higher costs of extraction from the natural source, compared to quinine. Quinine-cinchonine and quini-
Cinchonine and cinchonidine are two pairs of diastereoisomers (Fig. 8), each differing in the stereochemistry of the carbinal function (C-9). Since cinchonine is more active than its stereoisomer cinchonidine, the configuration of the asymmetric C-9 carbon seems to play a role in the anti-MDR activity of cinchona alkaloid compounds. Such a difference in activity on MDR and tumor cells have been investigated recently. It was demonstrated by which quinidine exerts its action on the drug efflux function and a decrease of in vivo toxicity have also been reported for the two stereoisomers, L- and D-, of verapamil (19). Mechanisms by which quinidine exerts its action on the drug efflux function of tumor cells have been investigated recently. It was demonstrated that quinidine competes with the antineoplastic agents at a drug-binding site of GP 180 (20). The difference in the in vitro activity of the quinine-derived compounds could result from different affinity for this drug-binding site on GP 180. However, a lipophilic agent such as quinidine could interact with membrane lipids of tumor cells and lead to an indirect inhibition of the drug efflux by modification of membrane fluidity (21).

Cinchonine is more active than quinine on anthracycline drug efflux from rat colon cancer cells, as well as resistant human leukemic cells. It is known that MDR is an important cause of chemotherapy failure (13, 22-24). The greater inhibition of efflux from rat colon cancer cells, as well as resistant human leukemic cells, tumour levels can be detected. Proc. Am. Soc. Clin. Oncol., 1991.

**REFERENCES**


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