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Apoptosis induced by doxorubicin and cinchonine in P388 multidrug-resistant cells

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Abstract

Acquired drug resistance is a major factor in the failure of doxorubicin-based cancer chemotherapy. We determined the ability of cinchonine to reverse doxorubicin drug resistance in a doxorubicin-resistant leukaemia cell line (mouse P388/DOX). A non-cytotoxic concentration of cinchonine (10 μM) increased the sensitivity to doxorubicin of multidrug-resistant P388/DOX cells and significantly enhanced the doxorubicin-induced apoptosis and DNA fragmentation in resistant cells, but had no effect in parent cells. Time-course studies demonstrated that DNA fragmentation was present 24 h after incubation with doxorubicin and cinchonine, indicating that DNA degradation was a preceding event. In cultured cells, cinchonine increased the intracellular accumulation of doxorubicin in the resistant cells in a dose-dependent manner. Using flow cytometry to measure the inhibition of the P-glycoprotein (P-gp) dependent efflux of rhodamine 123, cinchonine was found to be considerably more effective than quinine. The results with cinchonine suggest that there may be quinine derivatives with a similar capacity to inhibit drug transport by P-gp. Additionally, the G_2/M phase cell population in resistant cells is increased by doxorubicin/cinchonine treatment. Exposure of resistant cells to 1 μM doxorubicin and 10 μM cinchonine resulted in the expression of Fas (APO-1/CD95) in cells after 6 h. These studies demonstrate that the cell killing effects of doxorubicin and cinchonine in resistant cells are mediated, at least in part, by the induction of apoptosis.

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Introduction

Multidrug resistance (MDR) of human tumours is one of the major reasons for the failure of chemotherapy in refractory cancer patients (Lehnert 1998). Exposure to agents such as anthracyclines, epipodophyllotoxins or vinca alkaloids confers resistance to a wide range of compounds with no apparent structural or functional similarities to the selective agents (Naito & Tsuruo 1998). Thus, MDR can be intrinsic or acquired, depending on the time of its occurrence, either at diagnosis or during chemotherapy. Furthermore, the mechanisms underlying MDR have also been extensively studied.

One of the major mechanisms in MDR cells is initiated by the P-glycoprotein (P-gp), encoded by the MDR gene (MDR1) in human cancer cells (Zubercova & Babusikova 1998). Also, the presence of mouse and hamster MDR genes has been confirmed in MDR cells (Bosch & Croop 1996). The P-gp has been sequenced and shown to encode a membrane P-gp with 12 transmembrane domains and two nucleotide-binding sites (Hafkemeyer et al 1998). It functions as an ATP-dependent active efflux pump of anticancer agents in human cancer cells. The P-gp expressing

resistant cells are frequently characterized by diminished drug accumulation, such as of the anthracycline drug doxorubicin in resistant cells compared with drug-sensitive cells (Bradshaw & Arceci 1998). Recently, other drug resistance proteins, including MDR-associated protein (MRP1) and cMOAT (MRP2), were also identified in MDR cells (Borst et al 1999; Hinoshita et al 2000). A functional study of MRP1 has shown that it has the potential to act as a transporter of glutathione conjugates, which has been shown to be a central detoxification pathway in chemotherapeutic agents (Borst et al 2000). Furthermore, several other resistance proteins (glutathione S-transferase, metallothionein, thioredoxin, topoisomerase I, II) have been shown to be regulated in resistant cells and their molecular mechanisms are believed to contribute to the MDR phenotype (Volm 1998).

Reversal of MDR has been accomplished by a number of agents including verapamil, quinidine, calmodulin antagonists, and ciclosporin A (Bosch & Croop 1996; Petriz et al 1997). Possible circumvention mechanisms of MDR include cell membrane alterations and decreased efflux via competition for binding sites on the transport protein P-gp. Unfortunately, the concentration of many of these agents necessary to reverse drug resistance is difficult to achieve in-vivo.

Quinine has been shown to bind to P-gp and partially reverse drug resistance in-vitro (Solary et al 1991). Indeed, quinine has been used in preliminary trials in combination with mitoxantrone and aracytine for the treatment of acute leukaemias (Solary et al 1992). More recently, another quinine analogue, cinchonine, has also been shown to be an efficient agent both in-vitro and in-vivo (Genne et al 1992, 1994, 1995). These studies have suggested that cinchonine of low toxicity may be useful in the clinical treatment of drug resistant tumours, although the mechanism by which it achieves this is not fully understood.

The purpose of the present study was to analyse the effects of cinchonine on the cytotoxic activity of doxorubicin in murine P388 leukaemia P-gp expressing cells, to investigate the effects of cinchonine on intracellular drug accumulation, the cell cycle distribution, and further show how Fas is expressed in relation to doxorubicin-induced apoptosis.

Materials and Methods

Drugs and chemicals

Cinchonine hydrochloride, melphalan, cytarabine, propidium iodide (PI) and RNase A were purchased from Sigma Chemical (St Louis, MO); doxorubicin was from Kyowa Hakko Kogyo Chemical (Tokyo, Japan); vincristine sulfate was from Shionogi (Osaka, Japan); etoposide was from Nihon Kayaku (Tokyo, Japan); quinine hydrochloride was from Nakalai Tesque (Kyoto, Japan); cisplatin (CDDP) was from Bristol-Myers Squibb (Tokyo, Japan); pirarubicin, penicillin G potassium and streptomycin sulfate were from Meiji Seika (Tokyo, Japan); RPMI 1640 medium was from Nipro Co., Ltd (Osaka, Japan); fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS). All other chemicals were of reagent grade and are available commercially. Each drug was dissolved in phosphate-buffered saline (PBS) and filtered through a 0.20-μm syringe filter (Coring, NY).

Cells and cultures

The drug-sensitive mouse leukaemia cell line P388 (P388/S) used in this study was kindly supplied by the Japanese Cancer Research Resource Bank, Tokyo, Japan. The resistant sublines were selected for resistance to doxorubicin or CDDP by gradually increasing doxorubicin or CDDP exposure over a period of 6 months. The P388/DOX subline was selected for resistance by gradually exposing the cells to 0.5 μM doxorubicin, whereas the P388/CDDP cells were selected by gradually increasing the drug concentration to 3 μM CDDP. Cells were grown in RPMI 1640 medium supplemented with 10% FBS in the presence of 5×10^{-5} M 2-mercaptoethanol, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37°C in a 5% CO_2–95% air atmosphere.

Cytotoxicity assay

Cell concentrations and viability were determined by hemocytometer counts of cells excluding 0.1% trypan blue dye. Cytotoxicity was also assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma Chemical, MO) based colorimetric assay (Heo et al 1990). Cells (1×10^4/well) were plated in 96-well round-based plates (Corning, NY). To study the cytotoxic effects of doxorubicin alone and the combined effects of cinchonine and doxorubicin, cells were exposed to different concentrations of the drugs for 48 h at 37°C in an atmosphere of 5% CO_2 in air. After completion of the drug treatment, 10 μL MTT (5 mg mL^{-1}) solution was added to each well followed by 100 μL complete medium. After incubation for 4 h at 37°C, the MTT solution was removed. The incorporated formazan crys-

tals in viable cells were solubilized with 100 μL dimethylsulfoxide (Nacalai Tesque, Inc., Kyoto, Japan). Cells for each experimental data point were plated into at least eight wells, and the absorbance of each well was then read at 540 nm, using an ELISA analyser (Model ETY-96; Toyo Sokki, Kanagawa, Japan). The surviving fraction was determined by dividing the absorbance of treated wells by that of the control wells.

Apoptosis assay

Quantification of apoptotic cell death was done by measuring the hypodiploid peak in DNA multiploid histograms obtained by flow cytometry (Díez-Fernández et al 1998). For the analysis of DNA content, cells were stained with PI (10 μg mL^{-1}), and the emitted fluorescence of the DNA–PI complex was analysed in a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA) in the FL2-A channel.

DNA fragmentation assay

DNA fragmentation was measured by quantification of BrdU-labelled DNA fragments using the Cellular DNA Fragmentation ELISA (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Briefly, a cytosolic fraction ($500 \times g$ supernatant) of approximately 10^4 cells was used as the antigen source in a sandwich ELISA with a primary anti-DNA antibody coated to the microtiter plate and a secondary anti-BrdU antibody coupled to peroxidase.

Doxorubicin accumulation

Cells (1×10^6 cells mL^{-1}) were pre-incubated with cinchonine (1–100 μM) for 15 min at 37°C in an atmosphere of 5% CO_2 in air. Subsequently, doxorubicin was added to obtain a final concentration of 2 μg mL^{-1}. After a 60-min incubation period, treated cells were immediately centrifuged at $600 \times g$ and washed twice with ice-cold PBS. The cell pellet was then resuspended in 0.3 M HCl in 50% ethanol, sonicated and then centrifuged at $3000 \times g$. The doxorubicin concentration in the supernatant was determined spectrofluorimetrically (Bachur et al 1970) using a Hitachi spectrofluorimeter (650-10S type). Excitation and emission wavelengths of 470 and 585 nm, respectively, were used for the fluorimetric analysis of doxorubicin. The cellular doxorubicin concentration was derived from standard curves prepared using known amounts of drug.

Rhodamine 123 retention assay

For efflux experiments, fresh medium containing 2 μM rhodamine 123 was added to the cells (1×10^6 cells mL^{-1}) and incubated at 37°C for 1 h. They were then rinsed once with medium and incubated again in fresh medium without rhodamine 123, but with cinchonine at 37°C for 1 h. Intracellular rhodamine 123 fluorescence was measured (Buckingham et al 1996) on FACScan. The median fluorescence of rhodamine 123 was used as a quantitative measure of intracellular fluorochrome accumulation and used as a parameter for P-gp inhibition. Data are expressed as the percentage of cell-associated fluorescence intensity measured in P388/S control cells.

Cell cycle analysis

The frequency of cells in the various cell cycle phases was determined by enumerating the distribution of nuclei containing double-stranded DNA, using flow cytometry. Samples of 1×10^6 cells were fixed in 70% ethanol on ice for at least 2 h and centrifuged. The pellets were treated with RNase (200 μg mL^{-1}) at room temperature for 30 min and then incubated with PI (10 μg mL^{-1}) for at least 10 min. The stained cells were analysed with FACScan using CellQuest and ModiFit LT software.

Fas (APO-1/CD95) analysis by flow cytometry

P388/S or P388/DOX cell numbers were adjusted to 5×10^5 cells mL^{-1}. The cells were exposed to 1 μM doxorubicin in the presence or absence of cinchonine (1–10 μM) at 37°C for 6 h, and then washed three times with ice-cold PBS, after which 10 μg mL^{-1} anti-mouse Fas monoclonal antibody (RFM6, Medical & Biological Laboratories Co., Nagoya, Japan) was added. The samples were shaken for 10 min and allowed to stand for 20 min at room temperature. Subsequently, the samples were washed twice with PBS (2% FBS, 0.1% NaN_3), FITC-labelled goat F(ab′)2 anti-rat IgG (Biosource International Co., Camarillo, CA) was added followed by incubation at room temperature for 30 min. The cells were again washed twice with PBS (2% FBS, 0.1% NaN_3). The samples were resuspended in PBS (2% FBS, 0.1% NaN_3). Fluorescence intensity was analysed by flow cytometry.

Statistical analysis

All results are expressed as the mean or the mean $\pm$ s.d. of three experiments. One-way analysis of variance was

used to evaluate possible differences between treatments and controls. $P < 0.05$ was considered significant.

Results

Cytotoxic activity of doxorubicin and other anticancer drugs in P388/S and P388/DOX cells

To determine the cytotoxic activity of anticancer drugs including doxorubicin, the MTT assay was performed (Table 1). The IC50 of doxorubicin was 0.02 μM in P388/S and 0.81 μM in P388/DOX cells. The P388/DOX cells were 40.5-fold more resistant to doxorubicin than the parent cell line. Compared with the parent P388/S cells, P388/DOX cells also were resistant to pirarubicin (4.5-fold greater degree of resistance), vincristine (17-fold), etoposide (20-fold), melphalan (1.9-fold), and cytarabine (3-fold). P388/DOX cells were considered to be of a multidrug-resistant phenotype, although the cells did not show cross-resistance to CDDP.

Modulatory effect of cinchonine or quinine on doxorubicin cytotoxicity

The ability of cinchonine or quinine to modulate doxorubicin cytotoxicity was studied. Various doses of doxorubicin were added to growth medium and incubated for 48 h, with or without cinchonine or quinine. Non-cytotoxic concentrations of cinchonine and quinine (2 and 10 μM) slightly increased the sensitivity to doxorubicin by 1.8- and 9-fold in P388/S cells, respectively (data not shown). Moreover, cinchonine (2 and 10 μM) markedly enhanced doxorubicin cytotoxicity by 4.2- and 26.7-fold for P388/DOX cells. The effect of cinchonine on CDDP cytotoxicity was also examined in P388/S and P388/CDDP cells. The combination of 10 μM cinchonine and CDDP resulted in a slight increase in the cytotoxicity of P388/CDDP cells (data not shown). No significant increase in the cytotoxicity by cinchonine was observed in P388/S cells treated with CDDP.

Table 1 Cytotoxic effect of antitumour drugs on doxorubicin-sensitive (P388/S) and doxorubicin-resistant (P388/DOX) cells.

Drugs	IC50 (μM)[a]		Relative resistance[b]
	P388/S	P388/DOX	
Doxorubicin	0.020	0.810	40.5
Pirarubicin	0.022	0.100	4.5
Vincristine	0.003	0.051	17.0
Etoposide	0.072	1.440	20.0
Melphalan	3.200	5.943	1.9
Cytarabine	0.027	0.082	3.0
CDDP	0.500	0.520	1.0

Cells (2×10^4 cells mL^{-1}) were exposed to antitumour drugs for 48 h. Data are the means of three independent experiments done in triplicate. [a]IC50 (μM) concentrations were determined by the MTT assay. [b]Relative resistance is the ratio of the IC50 for P388/DOX cells to the IC50 for P388/S cells.

Enhancement of doxorubicin-induced apoptosis in resistant cells by cinchonine

The effect of cinchonine on doxorubicin-induced apoptosis was also analysed by flow cytometry. Various doses (0.01–0.1 μM) of doxorubicin were added to a growth medium, which was incubated for 24 h with or without cinchonine (2–10 μM). The lowest concentration of doxorubicin (0.01 μM) produced 37% apoptosis of P388/S cells in 24 h-cultures (Figure 1), whereas no such doxorubicin-induced apoptosis was observed in P388/DOX cells (data not shown). In the case of resistant cells, a combination of 10 μM cinchonine with 0.1 μM doxorubicin resulted in a marked increase in apoptotic cells (41%) compared with 0.1 μM doxorubicin alone (apoptotic cells; 9%). No significant increase in apoptotic cells by cinchonine was observed in P388/S cells treated with doxorubicin. Thus, non-cytotoxic concentrations of 10 μM cinchonine increased sensitivity to doxorubicin 4.6-fold in P388/DOX cells.

Doxorubicin-induced DNA fragmentation and enhancing effect by cinchonine

To explore whether drug treatment resulted in DNA fragmentation, we used an ELISA to determine the amount of fragmented BrdU-labelled DNA. Drugs were added to the growth medium and incubated for 6–48 h. A significant time-dependent increase in the DNA fragmentation by doxorubicin (0.1 μM) was observed in P388/S cells, whereas no such enhancement of doxorubicin-induced DNA fragmentation was detected in P388/DOX cells (Figure 2). In addition, cinchonine (10 μM) reversed the decreased DNA fragmentation induced by doxorubicin (0.1 μM) in resistant cells and resulted in a marked increase in cellular DNA fragmentation compared with that of resistant cells treated with doxorubicin alone. This demonstrated that doxo-

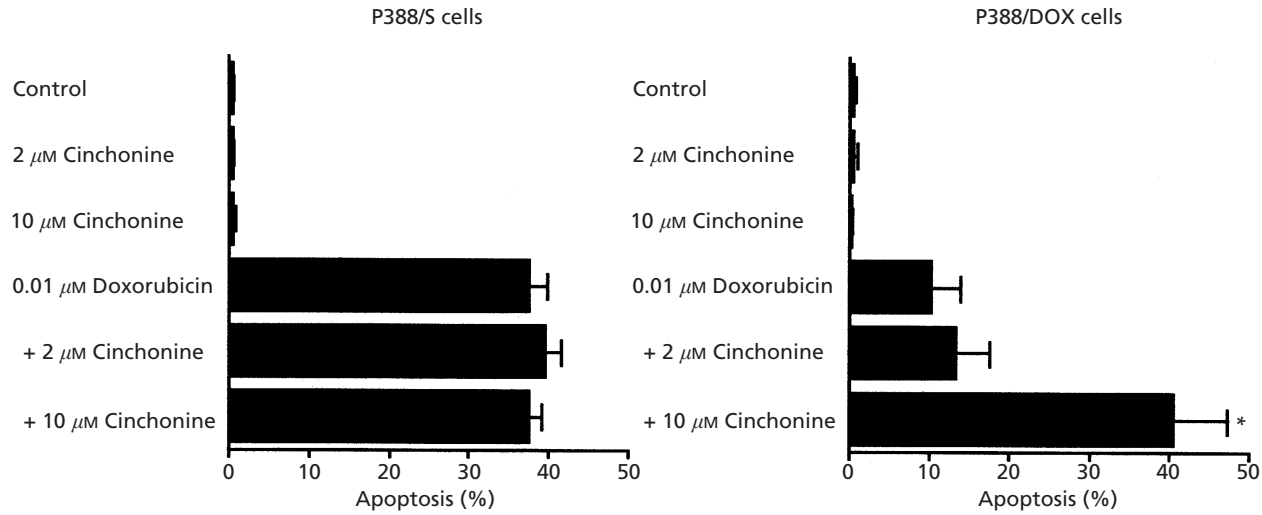

Figure 1 Apoptosis induced by doxorubicin in the presence or absence of cinchonine in P388/S and P388/DOX cells. Cells were cultured with doxorubicin (0.01 and 0.1 μM) for 24 h in the presence or absence of cinchonine (2 and 10 μM) and then stained with the DNA-intercalating dye PI before flow cytometric analysis. Data are expressed as the mean $\pm$ s.d. of three experiments. $*P < 0.05$, significantly different compared with the 0.1 μM doxorubicin-treated group.

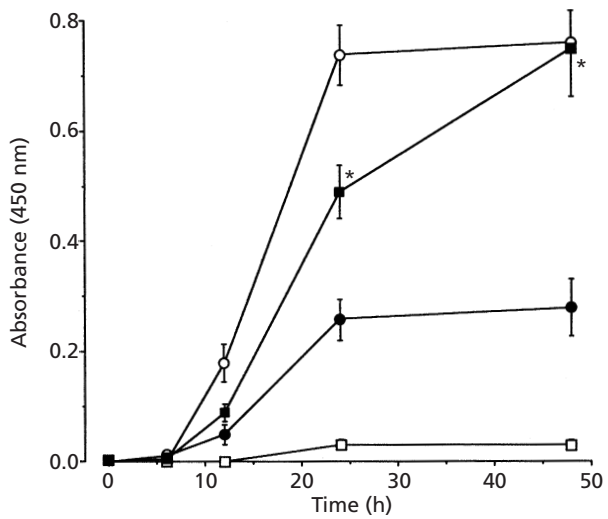

Figure 2 Cellular DNA fragmentation induced by doxorubicin in the presence or absence of cinchonine in P388/S and P388/DOX cells. Cells were exposed to doxorubicin (0.1 μM) in the presence or absence of cinchonine (10 μM) for 6–48 h at 37°C, and then DNA fragmentation was measured by Cellular DNA Fragmentation ELISA. ○, doxorubicin-treated P388/S cells; ●, doxorubicin-treated P388/DOX cells; □, cinchonine-treated P388/DOX cells; ■, doxorubicin plus cinchonine-treated P388/DOX cells. Data are expressed as the mean $\pm$ s.d. of three experiments. $*P < 0.05$, significantly different compared with the 0.1 μM doxorubicin-treated P388/DOX cells.

rubicin/cinchonine-treated resistant cells exhibit a marked increase in DNA fragmentation proportional to the number of cell cultures compared with control.

Effect of cinchonine on drug accumulation of doxorubicin

To determine if an alteration of the intracellular drug concentration is involved in the mechanism of drug resistance in P388 cells, we compared the uptake of doxorubicin by P388/S and P388/DOX cells. The intracellular concentration of doxorubicin was 0.247 ± 0.008 $\mu g/10^6$ cells in P388/S and 0.102 ± 0.002 $\mu g/10^6$ cells in P388/DOX cells. There was a 2.4-fold reduction in the accumulation of doxorubicin in P388/DOX cells relative to those in P388/S cells after 1 h of exposure to drug (Figure 3). The cellular accumulation of doxorubicin was also measured after exposure to doxorubicin with cinchonine. In P388/DOX cells, the presence of 1–100 μM cinchonine produced a large increase in the amount of intracellular doxorubicin, whereas no such increase in intracellular accumulation of doxorubicin was observed in P388/S cells.

Effect of cinchonine on rhodamine 123 retention

The effect of cinchonine on P-gp function was examined in P388/S and P388/DOX cells using rhodamine 123. Cells were exposed to 2 μM rhodamine 123 and rinsed with medium and then incubated with or without cinchonine. Decreased cellular retention of rhodamine 123 was observed in P388/DOX cells compared with sensitive cells (Figure 4). Cinchonine (10 and 50 μM) increased cellular retention of rhodamine 123 in P388/

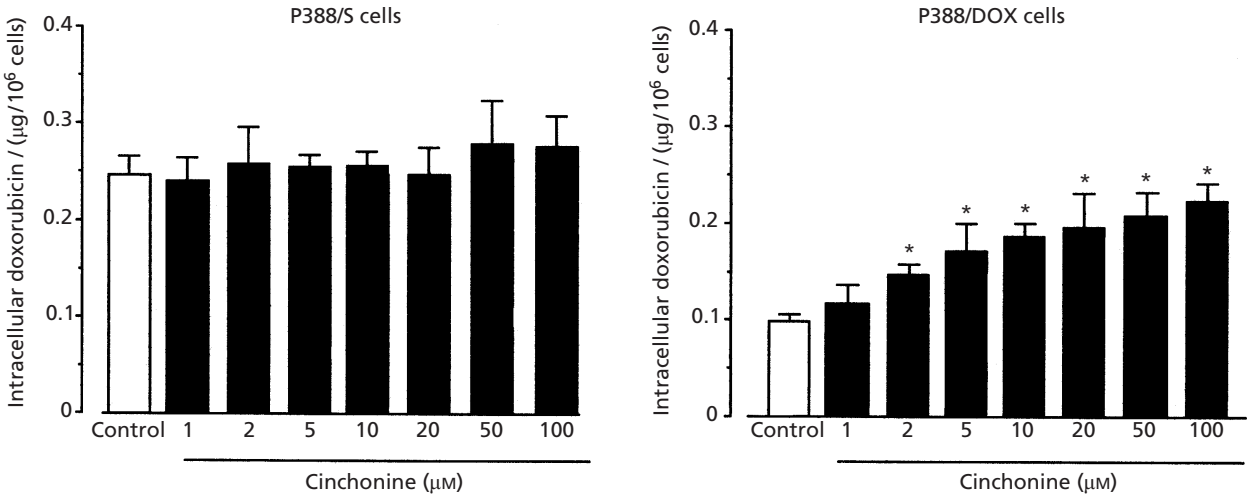

Figure 3 Effects of various concentrations of cinchonine on doxorubicin accumulation in P388 leukaemia cells. P388/S and P388/DOX cells were incubated with doxorubicin (2 μM) for 1 h in the absence or presence of cinchonine (1–100 μM). Data are expressed as the mean $\pm$ s.d. of three independent experiments performed in triplicate. *$P < 0.05$, significantly different compared with the control group.

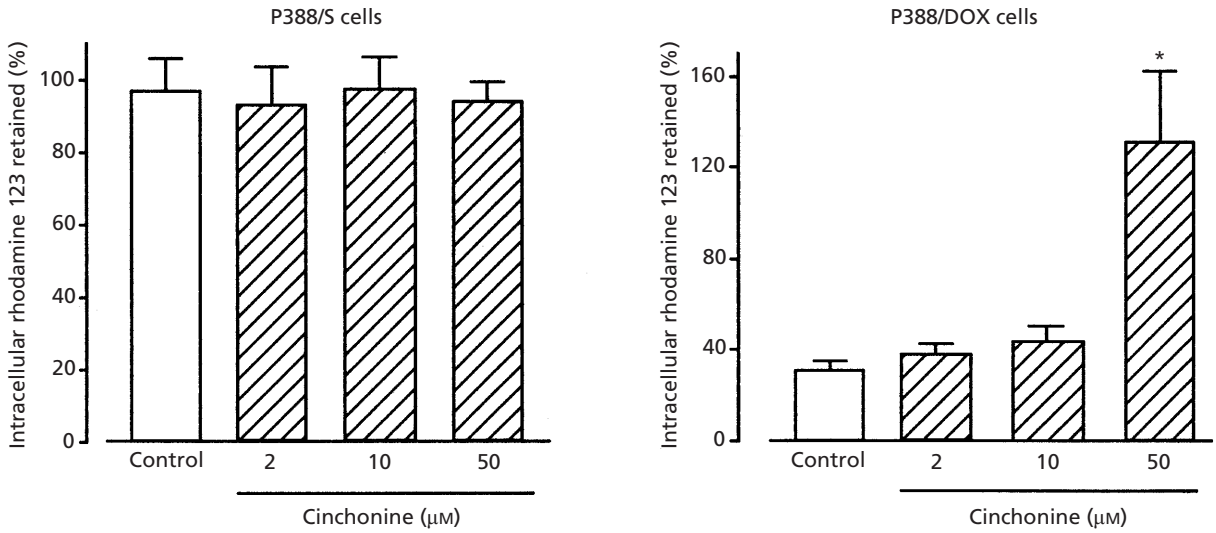

Figure 4 Effect of cinchonine on retention of rhodamine 123 fluorescence in P388/S and P388/DOX cells. Cells were incubated with the indicated concentration of cinchonine for 1 h before measuring rhodamine 123 retention by flow cytometry. Data are expressed as the mean $\pm$ s.d. of three independent experiments performed in triplicate. *$P < 0.05$, significantly different compared with the control group.

DOX cells, whereas no such increase of rhodamine 123 was observed in the sensitive cells.

Effects of cinchonine on cell cycle kinetics

Attached cells were analysed by flow cytometry after exposure to doxorubicin (0.01–0.1 μM) for 24 h (Figure 5). In the control cultures, or those treated with 0.01 μM doxorubicin, 10–12% of cells were in G_2/M phase. Cinchonine was also found to be less effective on cell cycle progression. The significant percentage of cells remaining in the G_2/M population indicated the presence of a G_2 block. As shown in Figure 6, doxorubicin (0.1 μM) treatment resulted in a marked G_2/M blocking effect compared with the control P388/S cells, whereas no such increase in the G_2/M phase produced by doxo-

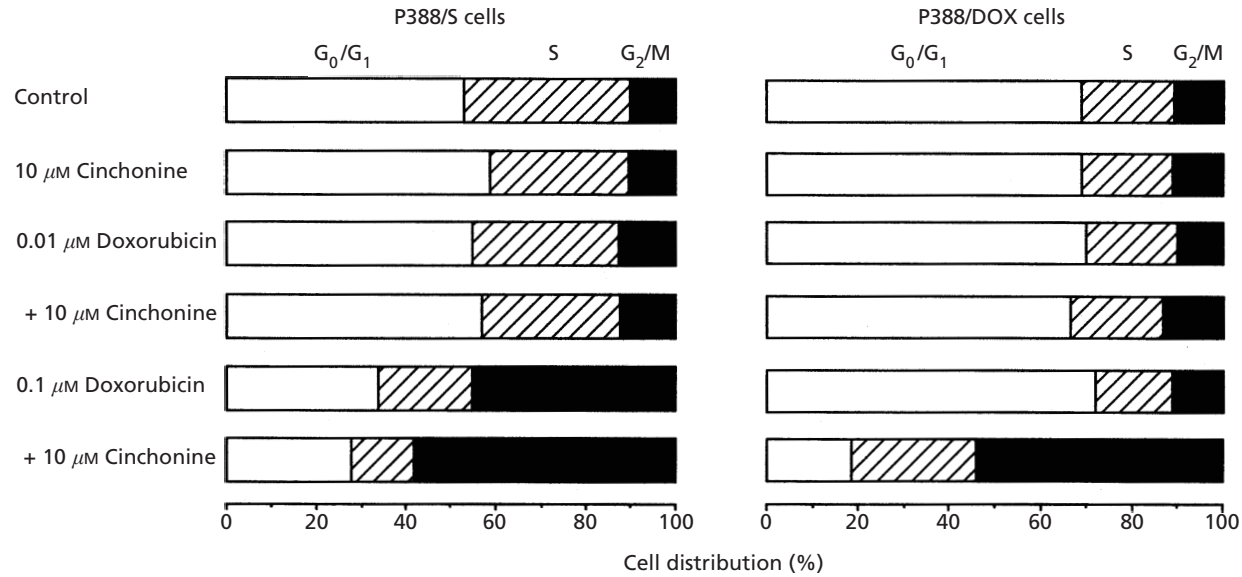

Figure 5 Effect of cinchonine on cell cycle arrest induced by doxorubicin. P388/S and P388/DOX cells were incubated with or without cinchonine (10 μM) for 10 min. The cells were then exposed to doxorubicin for 48 h. The cell cycle was measured by flow cytometry. Data are expressed as the means of three experiments.

rubicin was observed in P388/DOX cells. However, after treatment for 24 h with 0.1 μM doxorubicin and 10 μM cinchonine, this distribution shifted to 54% in G_2/M, 28% in S phase, and 18% in G_0/G_1. Therefore, cinchonine (10 μM) had a marked effect on doxorubicin (0.1 μM) induced G_2/M blockade in resistant cells.

Fas expression by cinchonine

To determine any correlation between the sensitivity to doxorubicin/cinchonine observed in the cell line studied and levels of Fas expression, we measured the Fas expression of P388/S and P388/DOX cells. The cells were exposed to doxorubicin (1 μM) in the presence or absence of cinchonine (2 and 10 μM) at 37°C under 5% CO_2 for 6 h. Doxorubicin (1 μM) induced Fas expression in P388/S cells after 6 h, whereas no such Fas expression was observed in P388/DOX cells (Figure 6). In addition, cinchonine (2 and 10 μM) intensified Fas expression in P388/DOX treated with doxorubicin (1 μM).

Discussion

The cinchona alkaloids, such as quinine, quinidine, cinchonine and cinchonidine, have been used as effective antimalarial drugs (Barennes et al 1995). They are structurally similar but quantitatively show different effects on various physiological actions, for example effects on neutrophil function (el Benna & Labro 1990), MDR (Genne et al 1992, 1994, 1995), phospholipid synthesis and K^+-channels (Pelassy & Aussel 1993), as well as cardiac arrhythmias. It was reported that cinchonine inhibits calcium-signalling cascade on human platelet aggregation (Shah et al 1998) and quinine specifically interferes with the synthesis of phospholipids in Jurkat T-cells (Pelassy & Aussel 1993). Here we showed that cinchonine induced apoptosis of the topoisomerase II inhibitor doxorubicin through interference with Fas signalling by affecting the function of P-gp in MDR cells.

The development of drugs that can reverse resistance by blocking the function or synthesis of P-gp may be a key step in improving treatment modalities. We have previously shown that cepharanthine (Kisara et al 1995), tacrolimus hydrate (Wu et al 1996) and rifampicin (Furusawa et al 1997) modulate anthracycline cytotoxicity and retention in MDR cells. The reversing or chemosensitizing agents represent an interesting way to circumvent drug resistance in cancer cells although, for most, the high doses required to inhibit P-gp function lead to toxic effects as demonstrated in clinical trials (Damiani et al 1998). Therefore, reversal of MDR via selective inhibition of P-gp function is of great theoretical interest and practical importance. In addition,

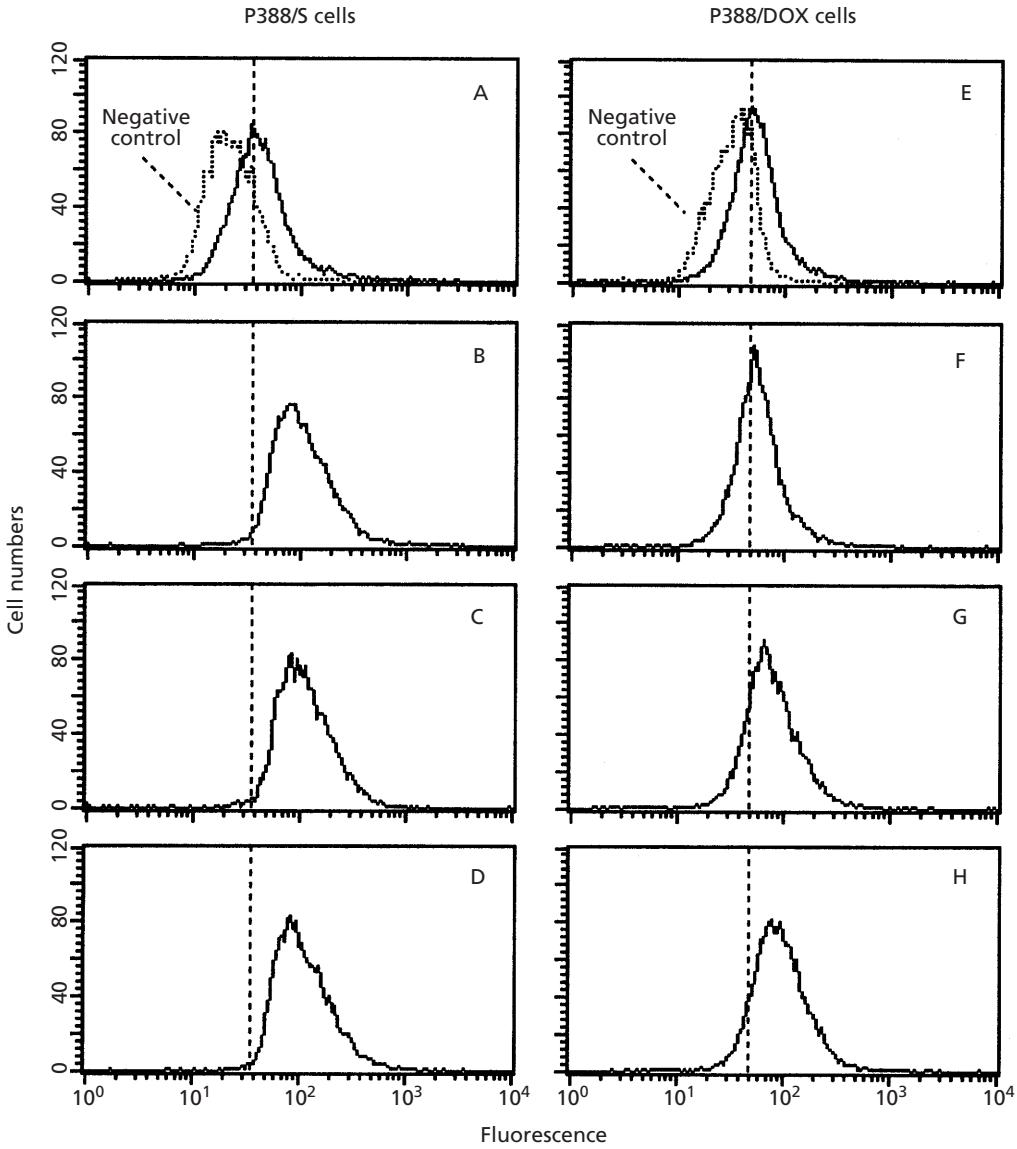

Figure 6 Flow cytometric frequency distribution of Fas (APO-1/CD95) expression in P388/S and P388/DOX cells. Cells were exposed to doxorubicin (1 μM) for 6 h in the presence or absence of cinchonine (2–10 μM): control (A, E); 1 μM doxorubicin (B, F); 1μM doxorubicin plus 2 μM cinchonine (C, G); 1 μM doxorubicin plus 10 μM cinchonine (D, H).

cinchonine has much lower toxicity compared with other quinine-related compounds (Genne et al 1992).

In the MTT assay, doxorubicin resistant P388/DOX cells showed a strong cross-resistance to vincristine, etoposide and a slight cross-resistance to pirarubicin, melphalan, and ara-C. Consequently, P388/DOX cells were considered to be of a MDR phenotype, although the cells did not show cross-resistance to cisplatin. Further MDR1 protein in P388/DOX was identified using its amino acid composition combined with protein database searches (ExPASy and EMBL PROP-SEARCH) (Shindo et al 1998).

In the drug combination study using the MTT assay and flow cytometric assays, we demonstrated that treatment of P388 cells with 10 μM cinchonine enhances doxorubicin-induced cytotoxicity and apoptosis towards the resistant P388/DOX line but not towards the sensitive P388/S line. Using the Cellular DNA Fragmentation ELISA assay to quantify normal and apoptotic (DNA fragmented) cells, doxorubicin-induced

DNA fragmentation towards P388/DOX cells was found to be potentiated by cinchonine. These results suggest that cinchonine induces apoptosis of doxorubicin through a marked cleavage of DNA into nucleosomal length fragments in MDR cells. The importance of apoptosis has been increasingly appreciated during the last decade. Apoptosis and cell proliferation are two opposing yet tightly controlled events (Wang & Wang 1999). Apoptosis has been accepted as the predominant mechanism of drug-induced cell death in pre-clinical experimental models and in clinically sensitive tumours. However, drug-induced cell death can include acute or delayed apoptosis, necrosis, or a delayed mitotic death, and requires further delineation for their relative contribution to tumour responses in-vivo (Houghton 1999). Apoptosis can be described by multiple pathways converging from numerous different initiating events and insults, such as anticancer agents (Konopleva et al 1999). In these pathways, caspases are the key effector molecules of the physiological process known as apoptosis (Habibovic et al 2000). The processing and activation of a caspase can be regulated by molecules such as FADD, APAF-1, Bcl-2 family members, FLIP and IAPs (Ekert et al 1999). However, the change of caspase activity and its molecules in mechanism by which cinchonine modulates drug resistance is as yet unclear.

Recently, cinchonine has been shown to increase the drug sensitivity of doxorubicin in P-gp positive cancer cells (Genne et al 1997). Although the mechanism whereby cinchonine modulates drug resistance remains unclear, studies suggest that cinchonine modulation is due to inhibition of drug transport. In our study, cinchonine increased intracellular doxorubicin, but had no significant effect on doxorubicin accumulation in sensitive cells. This is similar to the results of a previous study showing that some reversing agents had no effect on drug-sensitive cells (Wu et al 1996; Furusawa et al 1997). The increase in doxorubicin accumulation in P388/DOX cells with the use of a modulator was associated with an increase in doxorubicin cytotoxicity and apoptosis in resistant cells. It seems that mechanisms which regulate cellular drug concentrations are responsible for the cytotoxic potential of the anthracycline. It is well known that rhodamine 123 provides a good substrate for MDR-associated P-gp (Muller et al 2000). Cellular retention of this dye can therefore be used to reflect the activity of P-gp in MDR cancer cells. In our experiments cinchonine increased the retention of rhodamine 123 in resistant cells. These observations suggest that cinchonine inhibits the P-gp function as drug transporter in resistant cells.

The P-gp, which is functionally active at the beginning of the cell cycle, is synthesized late in G_2 phase (Tarasiuk et al 1993). Because the modulatory effect of cinchonine requires at least one cell cycle, it is possible that changes in cell-cycle kinetics are necessary for modulation of MDR. Despite the effect of the combination of doxorubicin and cinchonine on the cell cycle kinetics of P388/DOX cells, the modulators had no effect on the doxorubicin sensitivity of P388/S cells. Modulation occurred only in MDR cells, and cinchonine caused a block in G_2/M in MDR cells, hence cell kinetic changes may be sufficient for modulation to occur. Thus, these data showed that cinchonine modified the doxorubicin effect during the cell cycle progression.

Aborted apoptosis in neoplastic cells could be a critical factor in resistance to both natural defences and to clinical therapy (Schmitt & Lowe 1999). Acquired resistance of tumour cells to apoptosis is of major concern in cancer therapy, particularly in cases of recurrent disease, and the mechanisms underlying this phenomenon have yet to be fully elucidated. Fas is a type I transmembrane glycoprotein, the cytoplasmic domain of which encodes a conserved death domain homologous to that encoded by other members of the tumour necrosis factor receptor supergene family (Sheikh & Fornace 2000). Binding of Fas ligand or anti-Fas antibodies induces Fas receptor trimerization and leads to the assembly of a signal transduction complex that results in activation of the caspase proteolytic cascade (Villunger et al 1997). Recently, doxorubicin was reported to induce Fas expression to mediate apoptosis through the Fas–Fas ligand system (Tolomeo et al 1998), suggesting the involvement of alternative p53-independent pathways at least in this system. In the present study, doxorubicin also induce Fas expression in sensitive cells, whereas no Fas expression was observed in resistant cells. Additionally, cinchonine enhanced doxorubicin-induced Fas expression in sensitive cells. These finding suggest that Fas-mediated doxorubicin-induced apoptosis of resistant cells is modulated by cinchonine. Further, Friesen et al (1999) reported that induction of Fas ligand expression by cytotoxic drugs is modulated by the cellular redox state and mitochondria-derived reactive oxygen species in drug-resistant tumour cells.

The results of the present study indicate that the antimalarial drug cinchonine potentiates the sensitivity of resistant cells to doxorubicin. Cinchonine induces Fas-mediated apoptosis by doxorubicin and enhances doxorubicin accumulation in MDR cells, at least partly via inhibition of P-gp function. This study demonstrates that cinchonine can modulate doxorubicin-induced apoptosis by enhancing Fas expression in MDR cells.

Further investigation is required to clarify the reversing mechanism of cinchonine to acquired resistant cells.

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