Potentiation of Pirarubicin Activity in Multidrug Resistant Cells by Rifampicin

Shinobu Furusawa, Shinya Nakano, Jianghong Wu, Ken-ichi Sasaki, Motoaki Takayanagi, and Yoshio Takayanagi

Department of Pharmacology and Toxicology, Cancer Research Institute, Tohoku College of Pharmacy, 4-4-1, Komatsushima, Aoba-ku, Sendai 981, Japan. Received May 16, 1997; accepted August 29, 1997

The effect of the anti-tuberculosis drug rifampicin on pirarubicin activity was investigated in multidrug-resistant cells overexpressing P-glycoprotein. Rifampicin increased the sensitivity of pirarubicin to anthracycline-resistant mouse leukemic P388 cells and significantly enhanced the cytotoxicity and intracellular accumulation of pirarubicin in resistant cells, but had no effect in parent cells. By contrast, two other rifamycins, rifamycin B and SV, had no effect on pirarubicin accumulation in resistant cells. Rifampicin also enhanced pirarubicin-induced apoptosis and G2/M blockade on the cell cycle in resistant cells. These results show that rifampicin enhances the cytotoxic action of pirarubicin in resistant cells, at least partly via the inhibition of cellular pirarubicin efflux.

Key words rifampicin; pirarubicin; multidrug-resistant cell; cytotoxicity; apoptosis; cell cycle

Tumor cells can develop resistance during chemotherapy or can be in intrinsically resistant to a wide variety of diverse chemotherapeutic agents, even in the absence of any previous exposure to these drugs.1) This emergence of multidrug resistance (MDR) is well-recognized and is major obstacle to the successful chemotherapy of cancer.2) A clinically significant class of drug resistance correlates with the expression of a 170 kDa membrane protein, glycoprotein (Pgp), encoded by the MDR1 gene.3) Based on sequence homologies and functional analogies, Pgp belongs to a group of transport proteins that actively transfer hydrophobic molecules, peptides and various drugs across the cell membrane.4) Several types of MDR phenotypes can be functionally reversed by a wide range of chemicals, including calcium-channel blockers,5) calmodulin inhibitors,6) steroids7) and immunosuppressants8,9) that appear to compete with the transporters examined or to modulate active pumping allosterically. However, the clinical utility of MDR modulators may be restricted by dose-limiting toxicity.

Rifampicin, a semi-synthetic antibiotic, is used in the treatment of tuberculosis and has recently been shown to inhibit Pgp function3) and restore sensitivity to cytotoxic drugs. In our experiments we have examined the reversing effect of rifampicin on the activity of an anthracycline derivative, pirarubicin (THP), on Pgp-overexpressing drug-resistant mouse P388 leukemia cells.

MATERIALS AND METHODS

Drugs and Chemicals Rifampicin, rifamycin SV, rifamycin B and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Chemical Co. (U.S.A.). THP was obtained from Meiji Seika Kaisha (Japan). Doxorubicin (DOX) was purchased from Kyowa Hakko Kogyo Chemical Co. (Japan).

Cell Lines and Culture Mouse leukemic P388 (P388/S) cells were kindly supplied by the Japanese Cancer Research Resource Bank. The DOX-resistant P388 cell line (P388/R) was developed from parent cells by growing them in progressively increasing concentrations of a drug. The P388/R cells were approximately 110-fold resistant to DOX, compared with sensitive cells. Cells were maintained in RPMI 1640 medium (Nipro Co., Japan) containing 10% heat-inactivated fetal bovine serum (FBS, JRH Bioscience, Inc., U.S.A.), 5 × 10^-5 m 2-mercaptoethanol, 100 units/ml penicillin and 100 µg/ml streptomycin and were incubated at 37°C in an atmosphere of 5% CO2 in air.

MTT Assay Cells were seeded at a density of 2 × 10^4/ml in 96-well plates and incubated with RPMI 1640 containing 5% FBS and antibiotics in the presence of various concentrations of drugs at 37°C, then cell viability was examined by colorimetric assay with MTT. Absorbance was determined at 540 nm, using an ELISA analyzer.

Detection of Apoptotic Cells To detect apoptotic nuclei, cells were suspended in phosphate-buffered saline (PBS), stained with 5 µg/ml propidium iodide (Sigma Chemical Co., U.S.A.) and analyzed by FACScan (Becton Dickinson Immunocytometry Systems, U.S.A.) using Cell Quest software.

THP Accumulation and Efflux To determine the intracellular THP concentration, cells were treated with 0.5 µM THP in the presence or absence of rifampicin at 37°C for 1–3 h. Intracellular THP fluorescence was measured using a spectrofluorometer with excitation at 470 nm and emission at 585 nm.

Cell Cycle Distributions Single-cell preparations were obtained from control and drug-treated cultures and fixed in ice-cold methanol for 15 min. The cells were removed from the fixative by centrifugation at 900 × g for 5 min and stained for DNA flow cytometry with 50 µg/ml propidium iodide. Thirty minutes before analysis, RNase (Sigma Chemical Co., U.S.A.) was added directly to the stained cell preparations to yield a final concentration of 100 µg/ml. Analysis was performed using FACScan. Single-parameter DNA histograms were collected for 10000 viable cells, and the cell cycle kinetic parameters were calculated from the histograms using the manufacturer’s software (Cell Quest).

© 1997 Pharmaceutical Society of Japan
RESULTS

The effects of rifampicin on THP and DOX cytotoxicity in MDR cells were studied using an MTT assay. Various doses of THP or DOX were added to the growth medium and incubated for 48 h, with or without rifampicin. When the cytotoxicity of the drugs was estimated by comparing the IC₅₀ (50% growth inhibition), the relative resistances to THP and DOX of P388/R cells were 7.7- and 110-fold, respectively (Fig. 1). Non-cytotoxic concentrations of 10 μM rifampicin increased the sensitivity to THP by 2.5-fold in P388/R cells, while no such enhancement of

Fig. 1. Cytotoxic Effect of THP in the Presence or Absence of Rifampicin in P388/S and P388/R Cells

Cells were exposed to THP for 48 h in the presence or absence of 10 μM rifampicin. Drug effects on cell proliferation were then determined using the MTT assay. The number of cells surviving is expressed as a percentage of the number of cells.

Fig. 2. Effects of Rifampicin on Cellular Accumulation and Cellular Retention of THP in P388/S and P388/R Cells

A: Cells were incubated for 1 h with 0.5 μM THP alone or in the presence of rifampicin at various concentrations. Intracellular THP concentrations were then determined by spectrofluorometric assay. B: Cells were incubated for 1 h with 0.5 μM THP alone or in the presence of rifampicin at various concentrations. Cells were then washed twice with ice-cold PBS and reincubated for 3 h in THP-free medium in the presence or absence of rifampicin at various concentrations. Intracellular THP concentrations were then determined by spectrofluorometric assay and expressed relative to initial drug accumulation values. Values are means ± S.E.
THP cytotoxicity was observed in parent cells. Rifampicin (10 μM) also increased the sensitivity to DOX by 3.1-fold in P388/R cells. In addition, the effect of rifampicin on THP-induced apoptosis was also examined by flow cytometry. Cells were exposed to THP for 48 h in the presence or absence of 10 μM rifampicin. In the case of resistant cells, the combination of 10 μM rifampicin with 0.1 μM THP resulted in a marked increase in apoptotic cells (48%) compared with 0.1 μM THP alone (apoptotic cells: 17%). No significant increase in apoptotic cells by rifampicin was observed in P388/S cells treated with THP.

The accumulation of THP in P388/R cells was 1.5 times less than in P388/S cells. Rifampicin (5—100 μM) increased THP accumulation in P388/R in a dose-dependent manner, while no such increase in THP accumulation was observed in P388/S cells (Fig. 2A). By contrast, two other rifamycins, rifamycin B and SV (5—100 μM), had no effect on THP accumulation in P388/R cells (data not shown). THP retention was also increased by rifampicin (5—100 μM) treatment (Fig. 2B). In contrast to P388/R cells, P388/S cells did not exhibit any change in THP retention by rifampicin, regardless of the concentration used.

The cell-cycle changes induced in P388/S and P388/R cells were identical. As illustrated in Fig. 3, 0.1 μM THP treatment results in a marked G2/M blocking effect compared with the control P388/S cells, while no such increase in the G2/M phase due to THP was observed in P388/R cells. However, THP at a higher concentration (0.3 μM) caused an increase in the G2/M phase of P388/R cells. In resistant cells, 10 μM rifampicin reversed the cell-cycle effect of 0.1 μM THP and caused marked G2/M blockade in the cell cycle.

DISCUSSION

Rifampicin is usually administered for a long period with other antituberculosis agents or other classes of drugs. Of clinical importance is the fact that rifampicin accelerates the biotransformation of some other compounds and decreases their plasma concentration and consequent efficacy by the induction of microsomal enzymes.16,17 In addition, Huang et al.18 recently reported that rifampicin suppressed the lipid peroxidation generated by carbon tetrachloride-derived free radicals. Thus, the potential for drug interaction often exists. The present study demonstrates an in vitro reversing effect of rifampicin on the activity of an anthracycline drug in MDR cells.

Apoptosis is a form of active cellular self-destruction or cell suicide.19,20 Biochemically, it has been characterized by chromatin condensation and a requirement for energy as well as RNA and protein synthesis.21,22 THP, a DNA topoisomerase II inhibitor, causes a concentration-dependent induction of apoptosis in cancer cells. Rifampicin enhances THP cytotoxicity towards the resistant P388/R line but not towards the sensitive P388/S line. Rifampicin also enhances DOX cytotoxicity in P388/R cells. Using a flow cytometric method to separate and quantify normal and apoptotic cells, THP-induced apoptosis towards MDR cells was found to be potentiated by rifampicin. These results suggest that rifampicin induces a marked cleavage of DNA into nucleosomal-length fragments in MDR cells. Recently, rifampicin has been shown to enhance anticancer drug activity in resistant cells.13 Although the mechanism by which rifampicin modulates drug resistance is still poorly understood, studies indicate that the rifampicin effect may involve the inhibition of drug transport. In this study, rifampicin increased intracellular THP accumulation in resistant cells, but had no effect on drug 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.23–27 Rifampicin belongs to the group of compounds called rifamycins. However, no effects of rifamycin B or rifamycin SV on intracellular THP accumulation were
observed in MDR cells. These data suggest that some structural features restricted to rifampicin account for its reversing activity on MDR cells. Rifampicin differs from rifamycin B and rifamycin SV in the presence of a piperazinyl amino side group at position 3 of the naphthoquinone ring, which accordingly could play an essential role in the anti-MDR activity of rifampicin. Furthermore, efflux experiments demonstrated that rifampicin decreased THP export from cells, accordingly inhibiting Pgp-mediated drug transport in MDR cells.

It has been shown that the Pgp, which is functionally active at the beginning of the cell cycle, is synthesized late in G2. In our study, 10 μM rifampicin has no effect on the cell-cycle in sensitive and resistant cells. Although THP produced a change in cell distribution in P388/S cells, the anticancer drug had no effect in MDR cells. Rifampicin markedly induced the THP cell-cycle effect and caused an increase in the G2/M phase of MDR cells. These data show that rifampicin modified the THP effect during the cell cycle. Moreover, it is noteworthy that the non-toxic concentration of rifampicin (10 μM) used in the reversing experiment is within the range of plasma concentrations (about 7–9 μg/ml) usually observed 1 h after a normal patient dose (450 mg, p.o.). This last point is likely to be significant since many chemosensitizing agents only act at elevated concentrations.

In summary, we have shown that the antituberculosis drug rifampicin markedly increases THP-induced apoptosis in MDR cells, but has no effect in drug-sensitive cells. Rifampicin also enhances THP influx and inhibits THP efflux in Pgp-overexpressing MDR cells. These results consequently demonstrate that the rifampicin effect is restricted to MDR cells and may reflect a specific interaction between rifampicin and Pgp. Further in vivo experiments are required to establish the reversal mechanism of rifampicin in MDR cells.

Acknowledgements We thank Mr. Yoshinori Inano, Ms. Emi Kitayama and Ms. Yuki Narasaka for their technical assistance.

REFERENCES