Biochemical Pharmacology, Vol. 45, No. 2, pp. 401–406, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

TERFENADINE (SELDANE®): A NEW DRUG FOR RESTORING SENSITIVITY TO MULTIDRUG RESISTANT CANCER CELLS*

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(*Received* 1 *June* 1992; *accepted* 23 *September* 1992)

Abstract—In our efforts to identify clinically effective drugs for reversing multidrug resistance (MDR) mediated by P-glycoprotein, we tested terfenadine for anti-MDR activity because it appeared to sensitize a patient to doxorubicin and because it met structural requirements defined for this activity. Terfenadine sensitized MCF-7/ADR human breast cancer cells and L1210/VMDRC.06 murine leukemia cells to doxorubicin. At concentrations $\leq 10\ \mu M$, terfenadine decreased the IC_{50} to doxorubicin by up to 25-fold against MCF-7/ADR cells and completely restored sensitivity to L1210/VMDRC.06 cells. The drug had no effect on the sensitive, parental cell lines and enhanced activity of other drugs affected by the MDR phenotype. Terfenadine was as potent as *trans*-flupenthixol, one of the most active modulators of MDR. The mechanism of action of terfenadine appeared to be due to inhibition of the function of P-glycoprotein since it augmented the accumulation of doxorubicin and inhibited the efflux of rhodamine 123 from MDR lines but had no effect on drug accumulation or efflux in sensitive cells. Terfenadine displaced azidopine from P-glycoprotein, but at concentrations higher than expected based on its overall potency. Since terfenadine is clinically available, has numerous structural derivatives available for study, and has a relatively low toxicity profile, this drug and drugs of its class should be evaluated for future clinical trials.

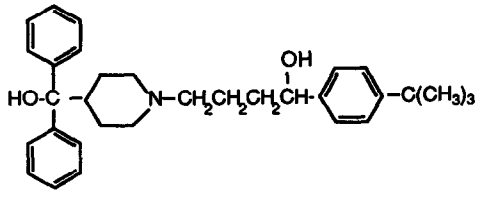

Fig. 1. Structure of terfenadine.

Detailed examination of the structural features of drugs that sensitize multidrug resistance (MDR)§ cells to chemotherapy has led to a number of important conclusions [1–4]. For example, Zamora *et al.* [3] demonstrated that a planar hydrophobic ring and a positively charged amino group are present in a series of active indole alkaloids. Our laboratory studied a series of substituted phenothiazines and found that the hydrophobicity of the ring, the length of the methylene bridge and the charge on the terminal amino group are directly related to activity [1]. Based on these data, we identified *trans*-flupenthixol, a thioxathene with greater activity as a chemosensitizer than verapamil or the phenothiazines [2]. Furthermore, this stereoisomer lacks undesirable clinical side effects [1, 2], presumably due to its weak affinity for dopamine receptors [5].

Terfenadine is a non-sedating antihistamine which conforms to the structural features for reversal of MDR predicted by our previous analyses (Fig. 1). We became interested in this compound when a patient treated at the Yale Comprehensive Cancer Center developed unexpectedly severe myelosuppression while receiving a chemotherapy regimen which included doxorubicin [6]. Review of the medication record showed that the only other drug this patient received was terfenadine. Based on the observation of Chaudhary and Roninson that CD34 positive hematopoietic stem cells express P-glycoprotein [7], we postulated that the myelosuppression in this patient might have been due to an interaction between doxorubicin and terfenadine. In fact, this patient developed identical myelosuppression when terfenadine was discontinued, but the initial clinical observation was the impetus for the studies we now report.

MATERIALS AND METHODS

Cell lines. MCF-7/ADR cells were a gift from Dr. Kenneth Cowan, National Cancer Institute. These cells were grown as monolayers in RPMI 1640

* This work was presented in part at the annual meeting of the American Association of Cancer Research, 1992.

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§ Abbreviations: MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; and PBS, phosphate-buffered saline.

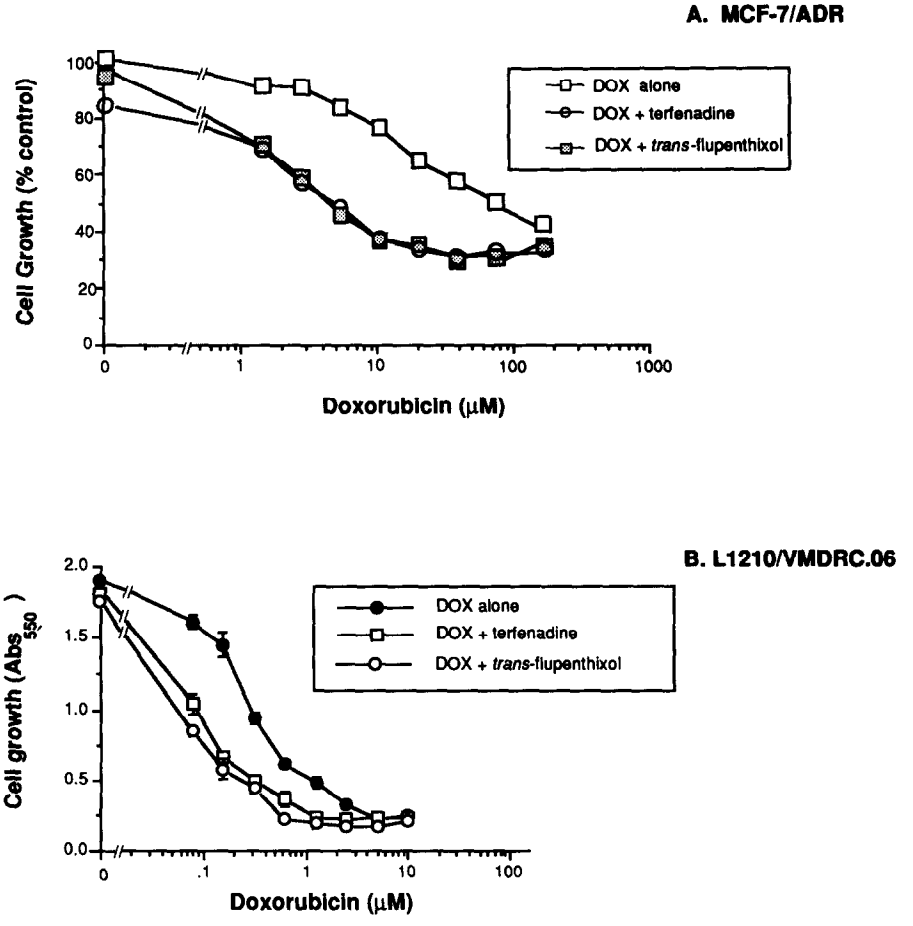

Fig. 2. Effects of terfenadine and *trans*-flupenthixol on the sensitivity of MDR cells to doxorubicin. (A) MCF-7/ADR cells were exposed to 0–200 μM doxorubicin for 48 hr in the absence or presence of a 5 μM concentration of the modulator. Cell number was evaluated by methylene blue staining and spectrophotometric analysis [11]. Each point is the mean of quadruplicate determinations from a representative of three experiments which differed by less than 5%. (B) L1210/VMDRC.06 cells were exposed to 0–10 μM doxorubicin for 48 hr in the absence or presence of a 3 μM concentration of the modulator. Cell viability was determined by the MTT assay as previously described [10]. Each point is the mean ± SEM of quadruplicate determinations from a representative of three experiments.

medium supplemented with 5% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air as previously described [1, 2]. L1210/VMDRC.06 cells are a subclone of L1210 leukemia that were infected with a retroviral expression vector containing a full length cDNA for the human *MDR1* gene [8] and grown in suspension as previously described [9]. Parental lines were grown identically to the resistant clones. KB/V20C cells, a subclone of the KB/V human carcinoma line, were grown in RPMI 1640 medium supplemented with 5% FBS and 2 nm vincristine. All cells were checked routinely and found to be free of contamination with Mycoplasma and fungi.

In vitro sensitivity to drugs. The effects of drugs on cell viability were studied for each sensitive and resistant cell line during exponential growth using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) spectrophotometric assay [10]. The effects of chemosensitizers on drug resistance were determined by exposing cells to a broad range of concentrations of cytotoxic drugs for 3 or 48 hr in the presence of several concentrations of chemosensitizers (0–10 μM) or their vehicles. Cell number was determined by methylene blue staining and spectrophotometric analysis [11] or by electronic counting using a Coulter model ZB (Coulter Inc., Hialeah, FL).

Cellular accumulation of doxorubicin. MCF-7/ADR cells were incubated with 0–20 μM doxorubicin at 37° for 3 hr, by which time doxorubicin accumulation had reached steady state. Aliquots of cells were removed, immediately centrifuged for 60 sec at 11,000 *g* and washed three times with ice-cold phosphate-buffered saline (PBS). Cell pellets were resuspended in 0.3 N HCl in 50% ethanol and sonicated for 10 pulses at 200 Ws with a Tekmar cell sonicator (Tekmar, Cincinnati, OH). The

concentration of doxorubicin in the supernatants was determined spectrofluorometrically [12]. The presence of chemosensitizers was shown to have a minimal effect on the emission or absorbance spectra of doxorubicin which was accounted for in all experiments.

Rhodamine 123 *accumulation and efflux.* Sensitive and resistant L1210 cells were incubated with 1 μM rhodamine 123 in the presence or absence of 5 μM terfenadine at 37° for 60 min. Following this period of drug accumulation, cells were washed and resuspended in medium containing terfenadine or vehicle. During the accumulation phase and efflux phase of the experiment, 150-μL aliquots were removed at 15-min intervals and diluted in ice-cold PBS containing 1% bovine serum albumin (BSA) and 0.05% azide. Samples were analyzed immediately by flow cytometry as previously described [13]. Fluorescence was measured as the mean channel value using arbitrary units.

Binding of [^{3}H]*azidopine to P-glycoprotein.* The ability of drugs to displace labeled azidopine from P-glycoprotein was assayed by a modification of the method of Safa [14]. Plasma membranes (50 μg protein) prepared from parental KB cells or KB/V20C cells, a subclone of a vincristine-resistant line, were photoaffinity labeled with 200 nm [^{3}H]-azidopine (sp. act. 56 Ci/mmol) in 40 mM Tris, pH 7.4, containing 4% dimethyl sulfoxide in a final volume of 50 μL in the absence or presence of 0–100 μM terfenadine, verapamil or *trans*-flupenthixol. Photoaffinity-labeled membranes were solubilized in sodium dodecyl sulfate (SDS) and separated by electrophoresis in a 7.5% SDS/polyacrylamide gel. The labeled bands were visualized by autoradiography.

Drugs and reagents. Terfenadine was obtained from Dr. Albert Carr of Marion Merrell Dow, Inc. (Cincinnati, OH), and *trans*-flupenthixol from Dr. John Hyttel of H. Lundbeck Pharmaceuticals (Copenhagen, Denmark). Doxorubicin, colchicine and verapamil were obtained from Sigma (St. Louis, MO). Etoposide was purchased from Bristol Laboratories (Evanston, IN), vincristine sulfate from LyphoMed, Inc. (Rosemont, IL), [^{3}H]azidopine from Amersham (Arlington Heights, IL) and rhodamine 123 from Eastman Kodak (Rochester, NY).

RESULTS

Effect of terfenadine on cellular proliferation and multidrug resistance. Terfenadine sensitized MDR cells to doxorubicin. Following a 48-hr exposure, the IC_{50} of doxorubicin against MCF-7/ADR cells decreased from 150 to 7 μM in the presence of 5 μM terfenadine (Fig. 2A). Against L1210/VMDRC.06 cells, terfenadine decreased the IC_{50} of doxorubicin from 400 to 90 nm (Fig. 2B). Terfenadine had no effect on the sensitivity of either parental cell line to doxorubicin (data not shown). Following a 3-hr exposure, terfenadine also increased the sensitivity of L1210/VMDRC.06 cells to doxorubicin alone (1.3 μM) compared to that of the combination (0.4 μM).

Terfenadine sensitized L1210/VMDRC.06 cells to other drugs affected by the MDR phenotype, including vincristine, colchicine and etoposide (Table 1). It was most effective when combined with doxorubicin or vincristine (4-fold) and less effective with colchicine or etoposide (1.5- to 2-fold).

Table 1. Effect of terfenadine (2 μM) on the viability of L1210/VMDRC.06 cells exposed to chemotherapeutic drugs affected by P-glycoprotein

	IC_{50} (μM)	
	Drug	+ Terfenadine
Doxorubicin	0.4	0.1
Vincristine	0.04	0.01
Colchicine	0.4	0.2
Etoposide	0.7	0.4

Each value is the mean of quadruplicate determinations.

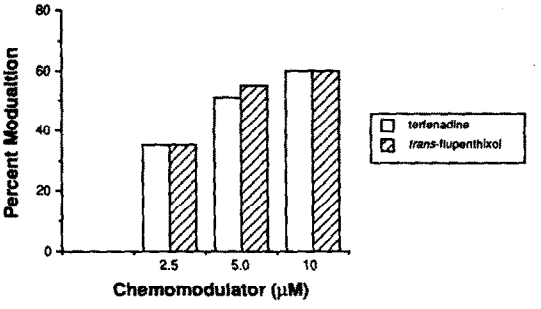

Fig. 3. Concentration–response relationship of terfenadine and *trans*-flupenthixol in sensitizing MCF-7/ADR cells to doxorubicin. Cells were exposed to 12.5 μM doxorubicin for 48 hr in the presence or absence of 0–10 μM chemosensitizer. Percent modulation was derived by dividing the viability of cells treated with doxorubicin plus chemosensitizer by the viability of cells treated with doxorubicin alone (×100). Each bar is the mean of quadruplicate determinations from a representative experiment.

Comparison of terfenadine to trans-*flupenthixol.* Terfenadine was as effective (Fig. 2) and as potent (Fig. 3) as *trans*-flupenthixol in sensitizing MCF-7/ADR cells to doxorubicin. Terfenadine alone was somewhat more potent than *trans*-flupenthixol in inhibiting cell growth (Fig. 2) producing 15% inhibition at 5 μM compared to 5% for *trans*-flupenthixol at the identical concentration. It was almost twice as potent against L1210 cells.

Terfenadine and *trans*-flupenthixol both increased the accumulation of doxorubicin in MCF-7/ADR cells by 2- to 3-fold at 5 μM (Fig. 4).

Effect of terfenadine on rhodamine 123 *accumulation and efflux.* Terfenadine increased the accumulation of rhodamine 123 in L1210/VMDRC.06 cells by 8- to 10-fold but had no effect on drug accumulation in sensitive cells (Fig. 5). Following a 60-min period of rhodamine 123 accumulation, cells were washed and resuspended in medium containing

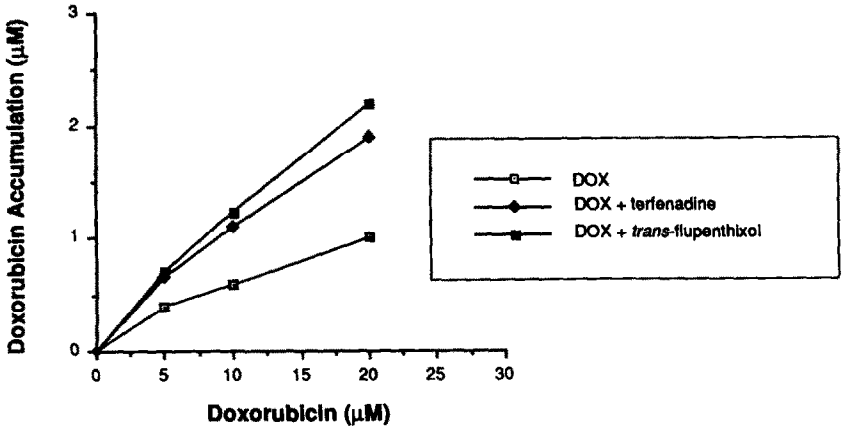

Fig. 4. Effects of terfenadine and *trans*-flupenthixol on the accumulation of doxorubicin in MCF-7/ADR cells. Concentration–response curves were run for doxorubicin alone or in the presence of a 5 μM concentration of the modulator. Doxorubicin was measured in the acid-soluble supernatant after 3 hr of incubation. Each point is the mean of duplicate determinations.

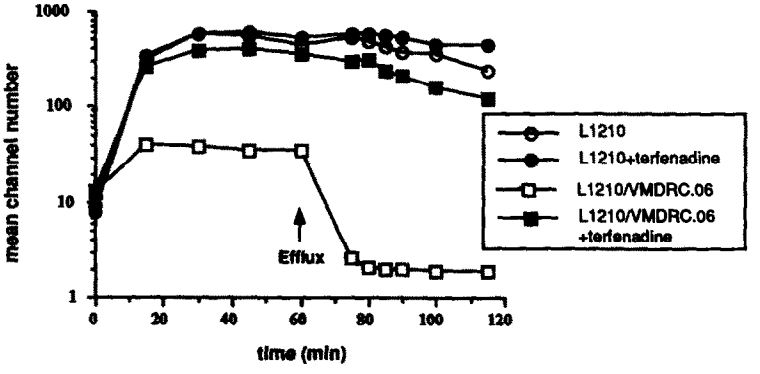

Fig. 5. Effect of terfenadine on the accumulation and efflux of rhodamine 123. Sensitive and resistant L1210 cells were incubated with 1 μM rhodamine in the presence or absence of 5 μM terfenadine at 37° for 60 min. Cells were then washed and resuspended in medium containing terfenadine or vehicle. During the accumulation and efflux phases of the experiment, aliquots were removed at 15-min intervals and diluted in ice-cold PBS containing 1% BSA and 0.05% azide. Samples were analyzed immediately by flow cytometry. Fluorescence was measured as the mean channel value using arbitrary units. Each point is the mean channel value obtained from analysis of 5,000 cells.

terfenadine or vehicle. Figure 5 also demonstrates that terfenadine markedly inhibited the efflux of rhodamine 123 from the resistant cells but had no effect on the sensitive line.

Effect of terfenadine on azidopine binding to P-glycoprotein. Terfenadine inhibited the binding of azidopine to P-glycoprotein (Fig. 6). However, it was far less potent than *trans*-flupenthixol or verapamil.

DISCUSSION

These studies show that terfenadine sensitizes MDR MCF-7 and L1210 cells to doxorubicin and other drugs affected by the MDR phenotype. These results support our earlier work, which suggested that drugs containing a hydrophobic ring separated by a 4 carbon methylene bridge from a positively charged amino group, possess structural features that predict activity for chemomodulation [1, 2].

Terfenadine was as active as *trans*-flupenthixol in restoring sensitivity to doxorubicin (Figs 2 and 3) and in increasing accumulation of doxorubicin in MDR cells (Fig. 4). *Trans*-Flupenthixol was discovered by screening compounds conforming to the structural motif described above [1, 2]. It was directly compared to verapamil and numerous phenothiazines and was found to be several fold more potent [1, 2]. Terfenadine and *trans*-flupenthixol produced maximum modulation without displaying intrinsic toxicity at concentrations of 2–5 μM. We and others have shown that cyclosporin A produces similar effects at concentrations of 0.8–1.2 μM (1–2 μg/mL) [15].

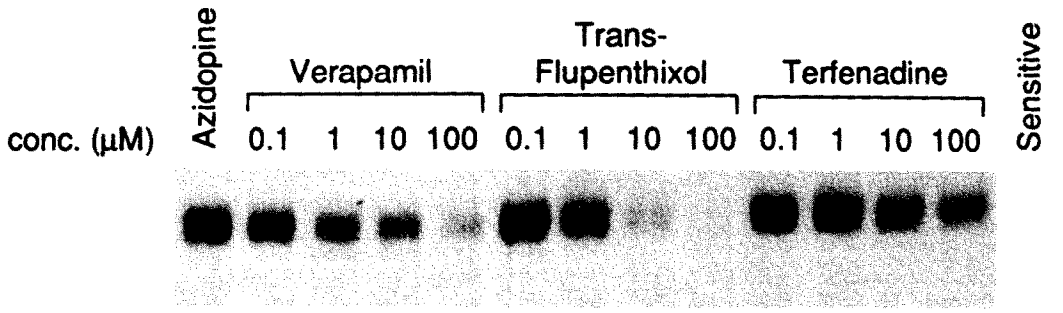

Fig. 6. Effects of terfenadine, *trans*-flupenthixol and verapamil on the displacement of [^{3}H]azidopine from P-glycoprotein. Plasma membrane protein (50 μg) prepared from KB/V20C cells was photoaffinity labeled with 200 nM [^{3}H]azidopine in the presence or absence of 0–100 μM terfenadine, *trans*-flupenthixol, or verapamil as described in Materials amd Methods.

The mechanism of action of terfenadine appears to be mediated through inhibition of P-glycoprotein. Accordingly, it had no effect on the sensitivity of parental cell lines, while it completely restored the sensitivity to L1210/VMDRC.06, a line which expresses the human *MDR1* gene [8]. The mechanism of resistance in the L1210/VMDRC.06 line is predominantly mediated by P-glycoprotein since it does not appear to have alterations in topoisomerase II or changes in glutathione content [8]. The ability of terfenadine to increase the accumulation of doxorubicin in MCF-7/ADR cells (Fig. 4) and rhodamine 123 in L1210/VMDRC.06 cells (Fig. 5) and to block the efflux of rhodamine 123, a compound known to be a substrate for the putative drug-transporter [13], strongly supports the conclusion that terfenadine acts by interfering with a process associated with the expression of P-glycoprotein. Although terfenadine increased doxorubicin accumulation by 2- to 3-fold, it sensitized MCF-7/ADR cells by 10- to 15-fold. This disproportion between drug accumulation and cytotoxicity is frequently observed [15] but remains incompletely understood.

Because of compelling evidence in support of a specific action of terfenadine on P-glycoprotein, the results of displacement of [^{3}H]azidopine from P-glycoprotein were somewhat unexpected but not without precedent. For example, the concentration of drugs such as vinblastine or doxorubicin required to displace azidopine often requires a 2000-fold excess over the photoaffinity probe [16], and certain drugs known to be affected by P-glycoprotein, such as colchicine, do not alter the photoaffinity labeling of the protein [17]. There are several explanations for these results. For example, terfenadine may act at a site near but not identical to that of azidopine on P-glycoprotein, or it may produce its effect by several mechanisms including inhibiting enzymes having a putative role in modulating the function of P-glycoprotein such as protein kinase C [18–20]. These alternatives are currently under investigation.

Although many drugs have been shown to sensitize MDR cells, most have proven to be unacceptably toxic when moved to the clinic [21]. Terfenadine, at usual doses, is relatively non-toxic. Not only does it lack the usual sedating effects of other antihistamines, but it has been safely administered to animals in doses of over 100 mg/kg [22]. However, case reports have appeared in which patients overdosing on terfenadine demonstrated prolongation of the QTc interval during cardiac monitoring [23], indicating that changes in cardiac conduction could become problematic when the drug is used at higher doses. *In vivo*, terfenadine is rapidly converted to a carboxylic acid metabolite so that the concentration of the parent drug is virtually undetectable in the serum [22]. The activity of the metabolites against MDR cells should also be investigated since if they lose activity its usefulness *in vivo* could be limited.

The identification of terfenadine as a potent modulator of MDR is important for several reasons. First, these results confirm the structure–activity relationships previously reported by our group and by others for identifying chemosensitizers [1–4]. Second, terfenadine represents a new class of clinically available drugs that could be exploited for use in patients harboring malignancies that express P-glycoprotein and display the MDR phenotype. Third, it will be possible to search for more potent and effective derivatives for future evaluation in the clinic since many cogeners of terfenadine have been synthesized and are available for study.

Acknowledgements—This work was supported by grants from the Public Health Service (CA 04388, CA 44358 and CA 08341).

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