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ORIGINAL ARTICLE

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Ketotifen reverses MDR1-mediated multidrug resistance in human breast cancer cells in vitro and alleviates cardiotoxicity induced by doxorubicin in vivo

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Abstract *Purpose*: To investigate the effect of the antihistamine ketotifen on multidrug resistance in human breast cancer cells and doxorubicin toxicity in mice. *Methods*: Clonogenicity assays were used to test the effect of ketotifen on human multidrug resistant breast cancer cell lines exposed to chemotherapeutic agents. Flow cytometry was used to measure accumulation of doxorubicin in cells. Fluorimetry was used to measure accumulation of doxorubicin in cardiac tissues. Histological analysis and toxicity studies in mice were used to test the effect of ketotifen on doxorubicin-induced toxicity. *Results*: Ketotifen was found to restore the sensitivity of P-glycoprotein-overexpressing multidrug-resistant MCF-7/adr cells to doxorubicin, mitoxantrone, VP-16 and vinblastine, but not to methotrexate or camptothecin. Ketotifen, however, was unable to restore sensitivity of BCRP-overexpressing MCF-7/mx cells or MRP-overexpressing MCF-7/vp cells to mitoxantrone or VP-16, respectively. In vivo, pretreatment of mice with ketotifen caused an increased accumulation of doxorubicin in cardiac tissue, consistent with a block in drug clearance. However, unlike verapamil, ketotifen pretreatment did not enhance doxorubicin toxicity but in fact provided protection, both at the level of cardiac tissue damage and in terms of survival. *Conclusions*: Taken together, these observations show that ketotifen is unique in its ability both to reverse multidrug resistance due to P-glycoprotein overexpression and to provide cardioprotection to doxorubicin.

Keywords Multidrug resistance (MDR) · Ketotifen · Doxorubicin · Cardiotoxicity

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Introduction

Intrinsic or acquired resistance to chemotherapeutic agents is a major contributing factor to failure in cancer treatment. Clinical drug resistance often presents as a multidrug resistance (MDR) phenotype, characterized as de novo resistance to a variety of structurally diverse cytotoxic drugs or as developed cross-resistance to chemotherapeutic agents that have never been used in previous chemotherapy [18]. Although the cellular basis underlying drug resistance is not fully understood, several factors have been identified that contribute to its development. These include drug efflux mechanisms, increased drug inactivation (e.g. glutathione-S-transferase and resistance to alkylating agents), drug target mutation (topoisomerase mutation), altered DNA repair and resistance to apoptosis (p53 mutation, bcl-2 overexpression etc.) [1]. Clinical drug resistance may be caused by any one or a combination of these mechanisms.

Increased transmembrane efflux of xenobiotics is one of the best-characterized mechanisms of MDR and is known to be mediated through over-expression of ATP-binding cassette (ABC) transporter superfamily members such as P-glycoprotein (P-gp/MDR1), MDR-associated protein (MRP1), or breast cancer resistance protein (BCRP) [6, 15, 20, 21]. P-gp, the most extensively studied of these transporters, is encoded by the mdr1 gene and has been found to be overexpressed in many tumor cells that are resistant to anthracyclines or vinca alkaloids. Transfection of the mdr1 gene to drug-sensitive cell lines can transfer the MDR phenotype [30]. In about 30–40% of primary and more than 50% of

metastatic breast cancer patient samples, P-gp has been found to be overexpressed [17, 29]. Current data suggest that increased expression of P-gp correlates with adverse prognosis and is associated with poor chemotherapy response and overall survival [29].

The prognostic importance of P-gp overexpression suggests that the ability to prevent or reverse MDR would be clinically valuable. This has led to the identification of a wide variety of compounds that are capable of reversing MDR through the inhibition of P-gp. Preclinical in vitro and in vivo studies in mice using MDR-reversing agents such as verapamil, quinidine and cyclosporin A have demonstrated enhanced anti-MDR tumor activity [10]. Clinical trials have been conducted to evaluate the efficacy of MDR-reversing agents with mixed results. In some cases, serum levels of reversing agents needed to block P-gp could not be achieved. In other cases, Pgp could be blocked but the levels of chemotherapeutic drugs had to be reduced in order to prevent excessive toxicity. However, some small-scale studies in P-gp-positive AML and VAD-refractory multiple myeloma showed that incorporation of verapamil or cyclosporin in chemotherapy significantly improved overall survival [7, 19, 28]. New, more-potent P-gp inhibitors such as PSC388, GF120918, dexverapamil and XR9576 are also currently being evaluated in clinical trials, and preliminary results to date indicate that at minimum it is possible to obtain serum levels of reversing agents sufficient to block P-gp [23, 26]. As mdr1/P-gp is also expressed in certain normal tissues, blockade of P-gp in vivo by reversal drugs inevitably changes drug distribution and metabolism, thus altering the pharmacokinetics of chemotherapeutic agents. As a result, increased accumulation of the drugs in plasma or tissue can cause increased toxicity.

Doxorubicin, one of the most potent chemotherapeutic agents for treating hematological malignancies and solid tumors, has dose-limiting cardiotoxicity both in animal models and in cancer patients. In one study, coadministration of cyclosporin and doxorubicin resulted in 55% and 350% increase in area-under-the-curve (AUC) of doxorubicin and its metabolite doxorubicinol, respectively [2] . PSC388, when used in combination with doxorubicin, increases the AUC of doxorubicin tenfold [13]. Using a murine model, Sridhar et al. have shown that the combination of verapamil and doxorubicin increases peak doxorubicin concentrations in heart tissue by about 40% compared to doxorubicin alone. This increased tissue doxorubicin levels led to severe heart damage and a significantly lower survival rate [27].

Ketotifen is a first-generation antihistamine with store-operated Ca^{2+} channel antagonist properties [11]. As a calcium influx blocker, we have previously demonstrated that ketotifen can induce cell death in an activation-enhanced manner in leukemia cells [14, 25], mast cells [24], and breast cancer cells [31]. In the course of evaluating the ability of ketotifen to induce cell death in breast cancer cells, we observed that ketotifen could sensitize multidrug-resistant human breast cancer cells to doxorubicin. In the study reported here, we found that ketotifen could reverse MDR through inhibition of P-gp. More importantly, we also found that ketotifen reduced cardiotoxicity caused by high-dose doxorubicin in vivo, thus uniquely identifying ketotifen as both a MDR-reversing and a cardioprotective agent.

Materials and methods

Human breast cancer cell lines and culture conditions

MCF-7 (MCF-7/wt) and its multidrug-resistant variant MCF-7/adr cells were obtained from Dr. Ian Tannock (Toronto, Canada). MCF-7/mx and MCF-7/vp cell lines were kind gifts from Dr. Erasmus Schneider (New York, N.Y.). The MCF-7/mx cell line was generated through selection in vitro with mitoxantrone and overexpresses BCRP [20]. The MCF-7/vp cell line was selected with etoposide (VP-16) and overexpresses the MRP gene [22]. All the cell lines were grown routinely as monolayer culture in Dulbecco's minimal essential medium (DMEM) supplemented with L-glutamine (2 m*M*), penicillin, streptomycin and 10% heat-inactivated fetal bovine serum (FBS; GIBCO) in an atmosphere containing 5% CO_2 at 37°C. The cell lines were passaged weekly.

Chemicals

Ketotifen, verapamil and all the chemotherapeutic agents (doxorubicin, VP-16, vinblastine and mitoxantrone) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ketotifen was freshly dissolved in DMSO before use, diluted with culture medium and added to the plate at the indicated concentrations. The final concentration of DMSO was always less than 0.1%. All the other drugs were dissolved either in DMSO (VP-16 and vinblastine) or in saline and stored at −20°C as stock solutions.

Drug treatments and breast cancer clonogenic assay

Exponentially growing MCF-7/wt and its three mutants MCF-7/adr, MCF-7/mx and MCF-7/vp were trypsinized, washed with fresh medium and plated in six-well plates at a density of 1×10^5 cells/ml. Cytotoxic drugs of different concentrations were applied to the cells in the presence or absence of ketotifen or verapamil for 24 h. Both adherent and nonadherent cells were collected and washed with fresh medium. Cell aliquots (5×10^3 cells) were plated in 1 ml 0.3% agar over 1 ml of a 0.5% agar underlayer prepared in Iscove's modified Dulbecco's medium containing 10% horse serum (GIBCO). The upper layer consisted of 20% FBS, 10 μg/ml bovine insulin, 2.5 μg/ml hydrocortisone, 5×10^{-7} *M* 17-β-estradiol (Sigma) and 50 ng/ml epidermal growth factor (R&D Systems, Minneapolis, Minn.). Colonies larger than 50 μm in size were scored after 14 days of incubation at 37°C in a humidified atmosphere of air containing 5% CO_2.

Flow cytometry

As doxorubicin itself is a fluorescent substance, the doxorubicin content in MCF-7/adr cells can be measured with flow cytometry. Briefly, cells (5×10^5 cells/ml) were incubated with 2 μg/ml doxorubicin at 37°C for 2.5 h with or without reversal agents, washed and resuspended in ice-cold PBS. Doxorubicin fluorescence was measured by flow cytometry using a FACStar Plus flow cytometer (FL2, emission at >570 nm, Becton Dickinson); 10^4 cells were analyzed for each sample.

Animals and in vivo treatment

Female Balb/c mice (8–10 weeks of age, 20–22 g body weight) were purchased from Jackson Laboratories (Bar Harbor, Me.). Protocols were approved by the Animal Care Committee of the University Health Network. Animals were divided into six groups of 15 to 20 mice each and received drug treatments as follows: saline, ketotifen 25 mg/kg, verapamil 25 mg/kg, doxorubicin 15 mg/kg plus saline, doxorubicin 15 mg/kg plus ketotifen 25 mg/kg, doxorubicin 15 mg/kg plus verapamil 25 mg/kg. All the treatments were administrated i.p. After treatment, mice were kept in a sterile environment for 6 to 8 weeks. Acute toxicities and survival were observed for the different treatment groups. Mice were killed when they displayed lethargic behavior or any signs of distress. Three mice from each group were killed on day 4 after treatment. Hearts were removed immediately and fixed in 10% neutral buffered formalin. Tissue sections were made from heart tissue and stained with hematoxylin and eosin or with 1% toluidine blue to identify mast cells. All the slides were evaluated by light microscopy for cardiac damage, mast cell density and degranulation.

Doxorubicin concentrations in heart tissues

Three to five mice in each group were treated with the same drug combinations used for survival. Mice were killed 3 h following injection of doxorubicin. Doxorubicin concentrations in heart tissues were determined by fluorometric detection of doxorubicin using the method of Sridhar et al. [27]. Briefly, hearts were excised immediately, rinsed with ice-cold normal saline, minced with scissors, and homogenized in ice-cold ethanol/acid solution (0.3 *N* HCl in 50% ethanol) using a Polytron homogenizer. The homogenates were centrifuged at 20,000 g for 20 min at 4°C. Fluorescence of the supernatants was measured using a Tecan Spectrafluor (excitation wavelength of 468 nm, emission wavelength of 590 nm; Hewlett Packard). The doxorubicin standard curve was made by mixing known amounts of doxorubicin with heart tissue and processing the mixture using an identical protocol. The fluorescence of the supernatant from cardiac tissue without doxorubicin served as background. The concentrations of doxorubicin were normalized to the total protein content of the same tissue.

Statistical analysis

All the colony data were analyzed by two-way analysis of variance (ANOVA), with differences between individual means determined by Bonferroni's post-tests. Data are expressed as means ± SEM. The Kaplan-Meier estimate was used to determine differences in the survival periods for mice following different drug combination treatments.

Results

Ketotifen specifically reverses MDR mediated by P-gp transporter

The toxicity of the cytotoxic drugs was measured by clonogenicity assay. As shown in Fig. 1A, significant dose-dependent reversal of doxorubicin resistance was observed with ketotifen. Beginning at 1 μM, ketotifen restored doxorubicin toxicity while at 10 μM, the MDR phenotype of MCF-7/adr cells was completely reversed. Over this concentration range, ketotifen itself was non-toxic to MCF-7/adr cells. The ability of

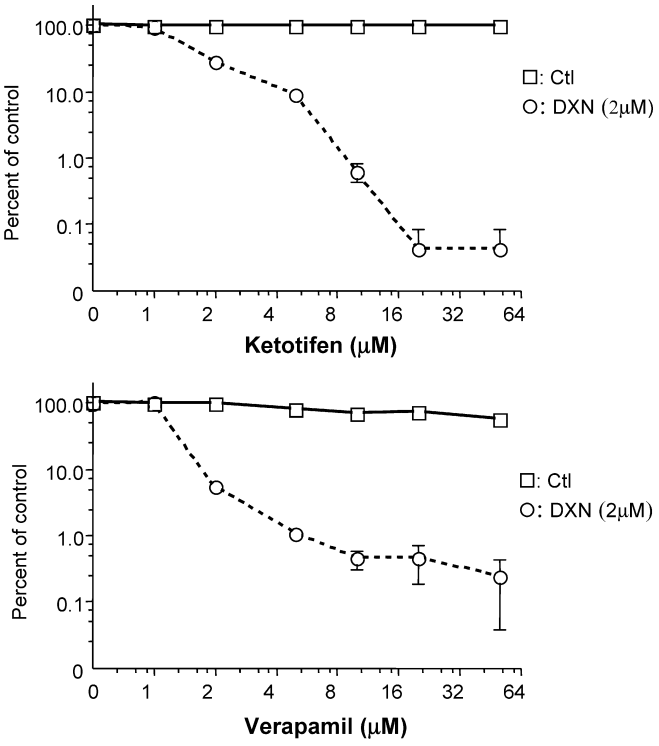

Fig. 1A, B Dose-response curve of ketotifen (**A**) and verapamil (**B**) as MDR reversal compounds. MCF-7/adr cells were treated with different concentrations of reversal compounds with or without 2 μM doxorubicin (*DXN*) for 24 h. Cells were harvested, and plated for growth of breast cancer colonies in triplicate. The data are presented as percent of control clonogenicity in the absence of drug ($P < 0.0001$)

ketotifen to restore sensitivity of MCF-7/adr cells to doxorubicin was compared with that of verapamil. As shown in Fig. 1, both ketotifen and verapamil reversed resistance at similar concentrations. MCF-7/adr cells were also relatively resistant to mitoxantrone, VP-16 and vinblastine. As shown in Fig. 2, sensitivity to these drugs was also restored by 10 μM ketotifen.

The IC_{90} values of different cytotoxic drugs were calculated from dose-response curves for MCF-7/adr or MCF-7/wt cells in the presence or absence of 10 μM of ketotifen. As summarized in Table 1, the IC_{90} levels in MCF-7/adr cells in the presence of ketotifen were almost identical to those for parental MCF-7 cells. In contrast to its reversing activity on MCF-7/adr cells, ketotifen influenced neither the toxicity of mitoxantrone in MCF-7/mx nor the toxicity of VP-16 in MCF-7/vp cells (Fig. 3). These two cell lines exhibit the MDR phenotype by overexpressing BCRP [20] and MRP transporters [22], respectively. Furthermore, in MCF-7/adr cells, ketotifen failed to alter the toxicity of methotrexate or camptothecin (Fig. 3), two drugs that are not subject to P-gp-mediated MDR. Thus, we conclude that ketotifen is a specific reversing agent for MDR associated with P-gp overexpression.

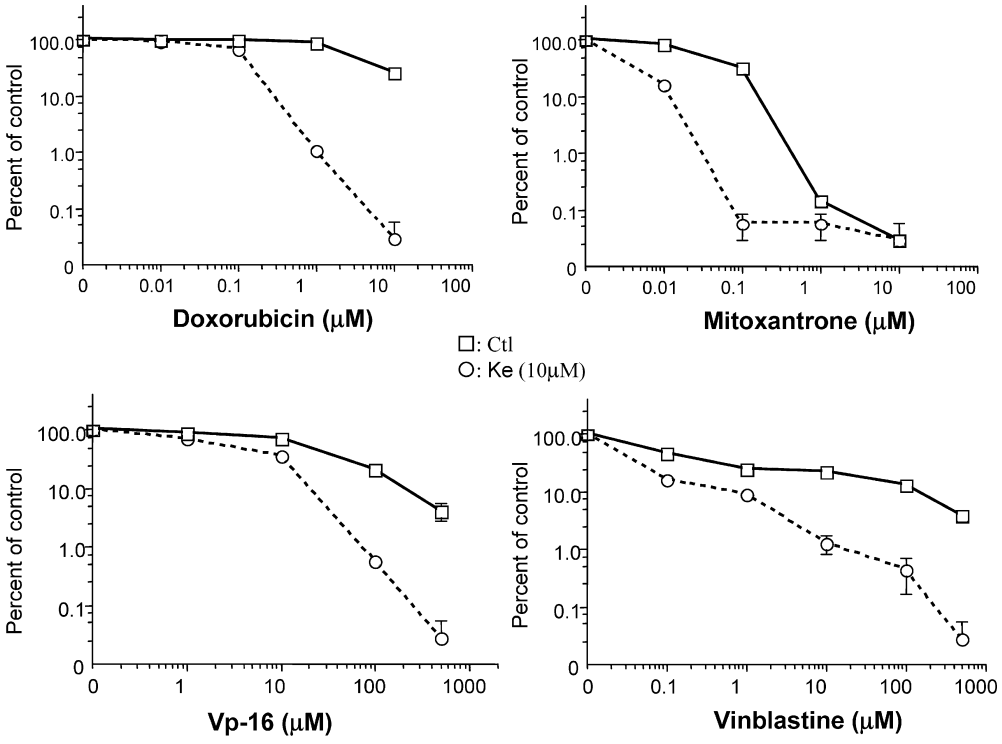

Fig. 2 Influence of 10 μM of ketotifen on the toxicities of four cytotoxic drugs. MCF-7/adr cells were treated with different concentrations of chemotherapeutic agents in the presence or absence of 10 μM ketotifen (*Ke*) for 24 h. Cells were harvested, washed and plated for growth of breast cancer colonies as described in the Materials and methods. The data are presented as percent of control clonogenicity in the absence of drug ($P < 0.0001$)

Table 1 IC_{90} (μM) of cytotoxic drugs in MCF-7/wt and MCF-7/adr cells. The cells were exposed to the indicated drugs for 24 h. The cells were collected and then plated in soft agar as described in Materials and methods. Colonies were enumerated after 2 weeks and IC_{90} values were estimated from the survival curves. All measurements were performed in triplicate

	MCF-7/wt	MCF-7/adr	
		Without ketotifen	With ketotifen
Doxorubicin	0.12	< 10	0.18
Mitroxantrone	0.01	0.22	0.02
VP-16	17	220	20
Vinblastine	0.8	410	1.2

Increased intracellular retention of doxorubicin in ketotifen-treated MCF-7/adr cells

Most MDR-reversing agents act by inhibiting the transporting activity of P-gp. In order to determine if ketotifen inhibited P-gp activity, we used the intrinsic fluorescence of doxorubicin as a marker and measured drug accumulation by flow cytometry. MCF-7/adr cells pretreated with ketotifen or verapamil were exposed to doxorubicin and fluorescence was measured. As shown in Fig. 4, in the presence of either verapamil or ketotifen, fluorescence from doxorubicin increased in the pretreated cells. Ketotifen at 2 μM increased relative fluorescence by 50%, while at 10 μM ketotifen nearly doubled the fluorescence intensity. This result shows that ketotifen caused an accumulation of doxorubicin in MCF-7/adr cells and suggests that ketotifen mediates its reversal ability through the inhibition of drug efflux.

Tissue doxorubicin concentrations in the heart

To explore the possible interactions of ketotifen with cytotoxic drugs in vivo, mice were given i.p. injections of reversal agent, followed by 15 mg/kg doxorubicin. Tissue concentrations of doxorubicin were determined by measuring doxorubicin fluorescence in heart tissue following different time periods after injection and maximal levels of drug were found 3 h after treatment (data not shown). We compared the 3-h time-point values in different groups. As observed with verapamil, pretreatment of mice with ketotifen significantly increased doxorubicin accumulation in the heart in comparison with accumulation in controls (72 ± 5 vs 36 ± 3 ng/mg protein, $P < 0.01$, Fig. 5). This result indicates that, like verapamil, ketotifen causes a buildup of doxorubicin in tissue, likely due to inhibition of normal drug clearance mechanisms [27].

Ketotifen alters cardiac tissue damage

Cardiac tissue damage caused by anthracyclines is well known and characterized by cardiac hypertrophy, vacuolization, disruption of myofibrils and cell loss [4]. In order to characterize the effect of combined MDR-reversing agent plus doxorubicin treatment on heart tissue, mice were treated with ketotifen or verapamil followed by doxorubicin. Heart tissue was fixed 4 days later, sectioned and stained with hematoxylin and eosin. As shown in Fig. 6, mice treated with doxorubicin alone demonstrated well-known pathological changes including dilation of capillaries, myocyte degeneration and vacuolization in left ventricular tissue (Fig. 6B). Addition of verapamil

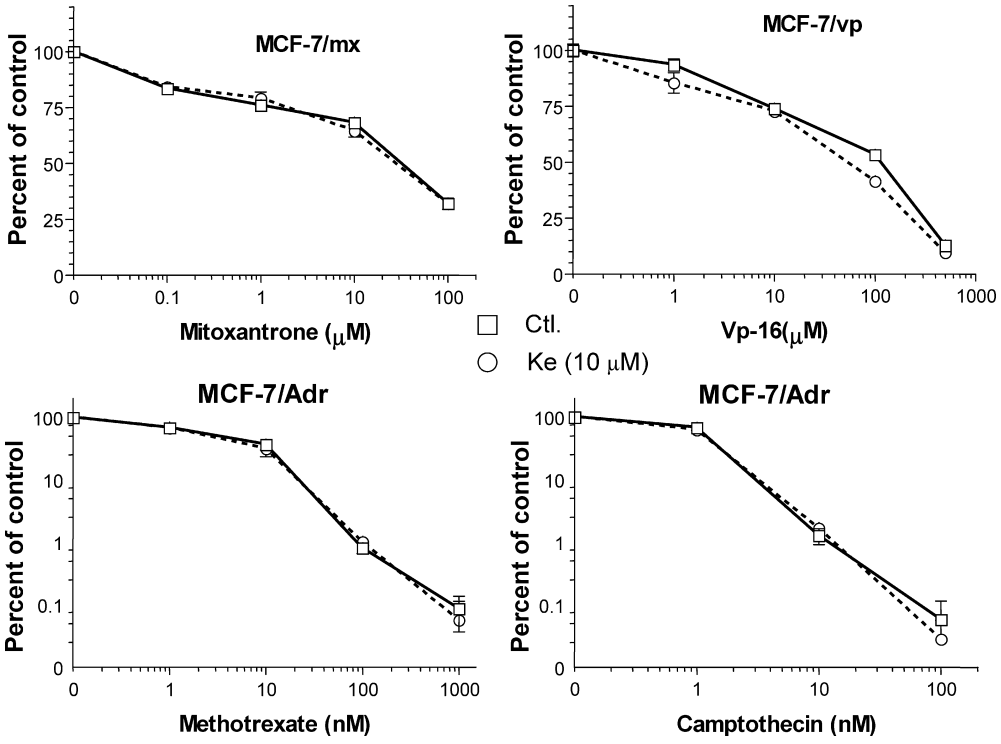

Fig. 3 Ketotifen (*Ke*) failed to reverse MDR of MCF-7/mx and MCF-7/vp cell lines. MCF-7/mx and MCF-7/vp cell lines were treated and assayed as described in Figs. 1 and 2. The toxicities of mitoxantrone and VP-16 were evaluated in MCF-7/mx and MCF-7/vp cells, respectively

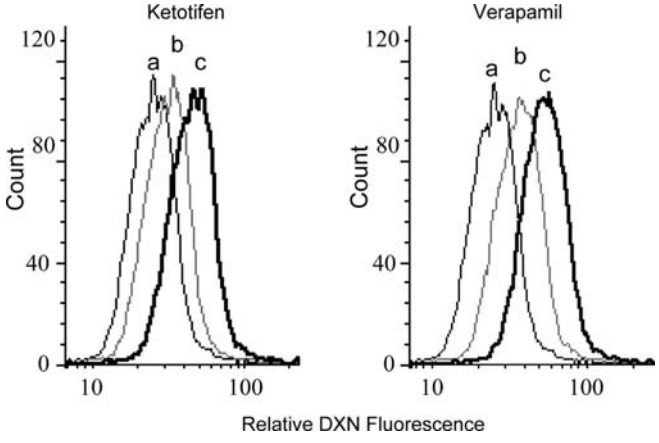

Fig. 4 Flow cytometric analysis of intracellular doxorubicin (*DXN*) retention. MCF-7/adr cells (5×10^5/ml) were incubated with 2 μg/ml doxorubicin at 37°C for 2.5 h in the presence of different concentrations of ketotifen or verapamil. Cells were washed and resuspended in ice-cold PBS. The relative fluorescence of doxorubicin was measured by flow cytometry (*a* 0 μ*M*, *b* 2 μ*M*, *c* 10 μ*M* ketotifen or verapamil)

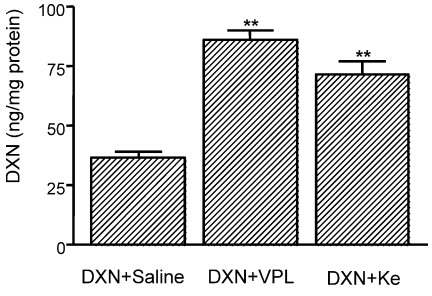

Fig. 5 Doxorubicin accumulation in heart tissue. Mice were treated with i.p. injections of reversal agents ketotifen (*Ke*) or verapamil (*VPL*), followed 30 min later by 15 mg/kg doxorubicin (*DXN*) or saline (as control). Three mice in each group were killed 3 h following injection of doxorubicin, and the hearts were excised, rinsed, minced and homogenized. Tissue doxorubicin was extracted with ice-cold acid ethanol solution (0.3 *N* HCl in 50% ethanol). The doxorubicin in the supernatants was measured by fluorescence spectrometry. All measurements were in triplicate. $^{**}P<0.01$

enhanced cardiac damage caused by doxorubicin (Fig. 6C). In contrast, heart tissue from mice pretreated with ketotifen (25 mg/kg) 30 min before doxorubicin demonstrated observable decreases in the extent of cardiac damage (Fig. 6D) with less cell drop-out, maintenance of myofibril structure and less vacuolization.

Cardiotoxicity and survival

The observation that cardiac damage was reduced in mice receiving ketotifen plus doxorubicin suggests that the addition of ketotifen might also enhance mouse survival. We therefore pretreated mice with ketotifen or verapamil, followed by a single treatment with doxorubicin and followed the animals over 5 weeks. Animals were killed when they showed signs of lethargy or distress. As shown in Fig. 7, the survival rate of mice receiving doxorubicin plus verapamil was significantly lower than among those treated with doxorubicin alone. For the doxorubicin plus verapamil group, the survival rate at day 30 was 0%, with a median survival time of 12.3 days, while 42% of the doxorubicin-alone group survived 30 days after treatment with a median survival of 19.3 days ($P<0.001$). In contrast, pretreatment of mice with ketotifen led to extended survival compared to

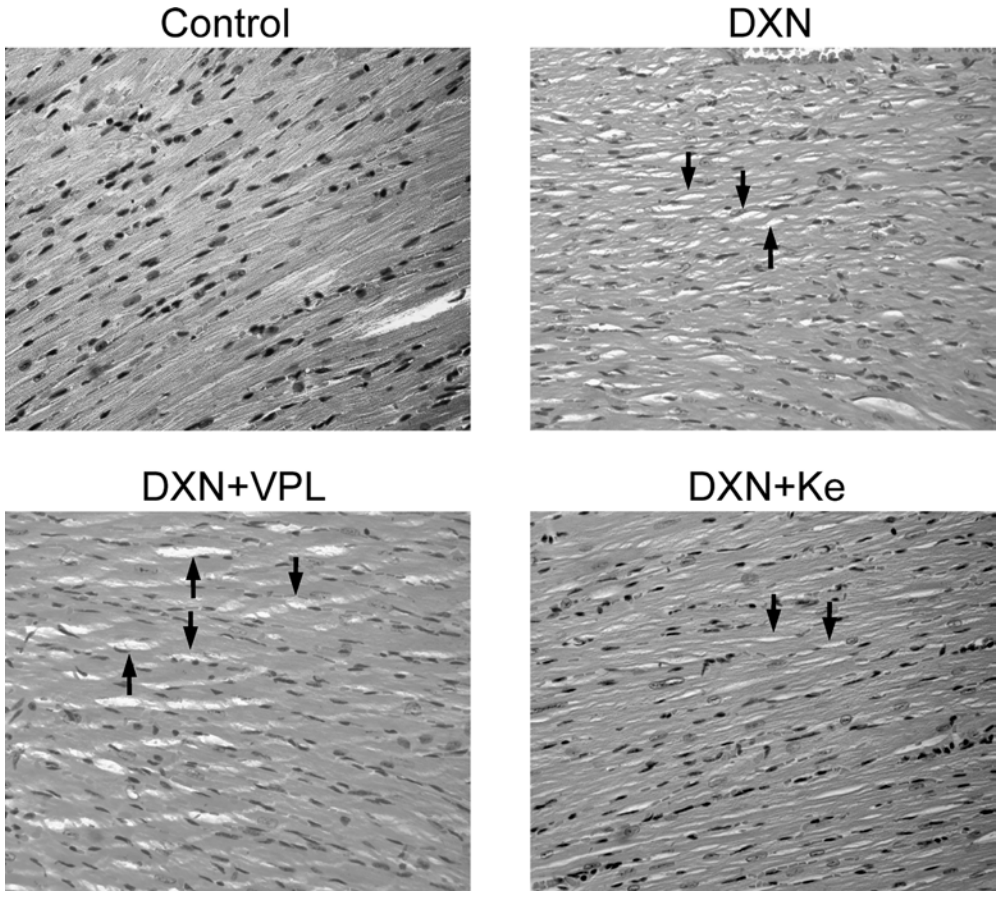

Fig. 6A–D Histological evaluation of cardiotoxicity. Mice were injected with reversal compounds ketotifen (25 mg/kg) or verapamil (25 mg/kg) followed by doxorubicin (15 mg/kg). Three mice in each group were killed 4 days after treatment. The hearts were removed and fixed in 10% formalin. At least three sections were made and stained with H&E. Slides were evaluated by light microscopy (original magnification ×400). In mice receiving doxorubicin alone (**B**, *DXN*), some capillary dilation, degeneration and vacuolization can be readily observed. Combining verapamil with doxorubicin (**C**, *DXN + VPL*) aggravated these manifestations of cardiotoxicity, especially the cytoplasmic vacuolization. Combining ketotifen with doxorubicin (**D**, *DXN + Ke*), on the other hand, alleviated the pathological changes induced by doxorubicin (*arrows* regions with cell drop-out)

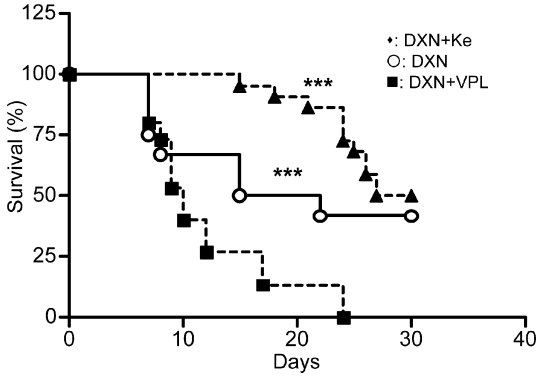

Fig. 7 Modulation of doxorubicin toxicity by verapamil or ketotifen. Mice were injected with reversal compounds ketotifen (*Ke*, 25 mg/kg) or verapamil (*VPL*, 25 mg/kg) followed by doxorubicin (*DXN*, 15 mg/kg). Mice were observed for survival for 30 days following treatment. For the DXN + Ke and DXN + VPL groups, 15 mice per group were used, and for the DXN only group, 10 mice were used. $***P < 0.001$

doxorubicin alone, with a 57% survival rate at day 30 and a median survival time of 23.2 days ($P < 0.001$ vs doxorubicin plus verapamil). Since prolongation of survival in mice treated with ketotifen plus doxorubicin correlated with the protection of ketotifen against the cardiotoxicity induced by doxorubicin, these results suggest that ketotifen enhances survival due to cardioprotection.

Discussion

In this study we showed that the antihistamine ketotifen can reverse MDR in MCF-7/adr cells through inhibition of P-gp. This effect was specific in that cells overexpressing BCRP or MRP were not affected by ketotifen. Furthermore, ketotifen did not modulate the response of MCF-7/adr cells to drugs such as methotrexate or camptothecin that are not subject to efflux by P-gp. At high concentrations, ketotifen also blocks store-operated Ca^{2+} influx and induces activation-enhanced cell death [14, 25, 31]. However, ketotifen's P-gp-inhibitory activity appears to be unrelated to its Ca^{2+} channel-blocking activity since the concentrations required for P-gp inhibition are much lower. Furthermore, we have observed that Ca^{2+} ionophores have no effect on the ability of ketotifen to reverse MDR (Zhang and Berger; unpublished observation), providing additional evidence that ketotifen's MDR-reversing activity is unrelated to its Ca^{2+} channel antagonism. We further demonstrated that pretreatment with ketotifen caused an increased accumulation of doxorubicin in mouse cardiac tissue, consistent with a block in drug clearance. However, we also observed that, unlike verapamil, ketotifen did not enhance doxorubicin toxicity but in fact provided protection, both at the level of cardiac tissue damage and in terms of survival. These observations therefore suggest

that ketotifen is unique in its ability to both reverse MDR due to P-gp overexpression and provide cardioprotection against doxorubicin.

Although the mechanism of cardiotoxicity caused by anthracyclines is not fully understood, it is generally believed that highly active reactive oxygen species (ROS) triggered by anthracycline metabolites may play a central role in the initiation of a series of reactions leading to myocyte damage [12, 16]. While antioxidants have shown some promise as cardioprotective agents in vitro and in animal models, clinical trials have not yet provided consistent benefit [5, 9]. Furthermore, the concern arises that the systemic application of antioxidants may also limit the antitumor efficacy of doxorubicin. Previous studies in which the role of mast cell activation products in anthracycline-mediated cardiotoxicity were investigated have shown that ketotifen can reduce doxorubicin cardiotoxicity and improve overall survival in a murine model [3]. In another study, doxorubicin has been shown to induce mast cell degranulation and histamine release, consistent with a role for mast cell activation in enhancing cardiac damage [8]. In the present study, we confirmed the protective effect of ketotifen. Ketotifen's beneficial effect on survival can be partly attributed to cardiac protection based on the observed decrease in severity of cardiac damage in mice pretreated with ketotifen. It is possible that ketotifen is acting directly on mast cells as has been proposed by Bartoli Klugman et al. [3]. However, we have observed that very high relative concentrations of ketotifen (about 100 μM) are required to block calcium influx and stabilize mast cells in vitro (Soboloff and Berger, unpublished data). Alternatively, it is possible that ketotifen's antihistamine activity is responsible for its cardioprotective action. Further work is required to better define the role of mast cell activation products in anthracycline-induced cardiotoxicity.

The clinical use of anthracyclines is limited by its cardiotoxicity. Furthermore, schemes employing multidrug-reversing agents typically require reductions in chemotherapeutic dose due to inhibition of drug clearance mechanisms. Our observations identifying ketotifen as a multidrug-reversing agent with cardioprotective activity suggest that this unique combination of properties may be clinically useful in the control of multidrug-resistant tumors.

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