Amelioration of doxorubicin-induced cardiotoxicity by an anticancer-antioxidant
dual-function compound, HO-3867

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Running Title

Amelioration of DOX-mediated cardiotoxicity by HO-3867

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List of Abbreviations

FAK - focal adhesion kinase
FAS - fatty acid synthase
LVID - left-ventricular internal diameter
PTEN - phosphatase and tensin homolog on chromosome 10
ROS - reactive oxygen species
STAT3 - signal transducer and activator of transcription-3
Abstract

Doxorubicin (DOX) is a commonly-used drug for the treatment of cancer. The development of resistance to DOX is common, and high cumulative doses cause potentially lethal cardiac side-effects. HO-3867, a synthetic curcumin analog, has been shown to exhibit both anticancer and cardioprotective effects. However, its cardioprotection in the setting of a conventional cancer therapy has not been established. This work investigated the use of HO-3867 and DOX to achieve a complementary outcome, i.e. increased toxicity towards cancer cells, and reduced cardiac toxicity. Combination treatment was investigated using DOX-resistant MCF-7 breast cancer cells (MCF-7 MDR) and Balb/c mice. Lower doses of HO-3867 and DOX (5 and 2.5 μM, respectively), reduce viability of MCF-7 MDR cells to an extent significantly greater than that when either drug was used alone, an effect equivalent to that induced by exposure to 50 μM DOX. In normal cardiac cells, the loss of viability from combination treatment was significantly lower than that induced by 50 μM of DOX. Increases in apoptotic markers, e.g. cleaved-caspase-3, and decreases in FAS and pAkt expressions were observed by western blotting. Mice treated with both HO-3867 and DOX showed significant improvement in cardiac functional parameters when compared to mice treated with DOX alone. Reduced expression of Bcl-2 and pAkt was observed in mice treated with DOX alone, while mice given combination treatment showed levels similar to control. The study indicates that combination treatment of HO-3867 and DOX is a viable option for treatment of cancer with reduced cardiotoxic side effects.
Introduction

Doxorubicin (DOX) is commonly used in the treatment of several malignancies, including breast cancer. Although effective in the initial stages, repeated administration of DOX may lead to the development of drug resistance, which necessitates the use of increased doses to achieve sufficient therapeutic effect. Since increasing the dose increases the rate at which high cumulative doses are reached, DOX-resistance is a serious issue to overcome (Szakacs et al., 2006). In addition, at high cumulative doses of DOX, cardiotoxic side-effects, such as congestive heart failure, dilated cardiomyopathy and death, become increasingly more probable (Unverferth et al., 1982; Dunn, 1994; Jain, 2000; Tokarska-Schlattner et al., 2006; Takemura and Fujiwara, 2007; Ewer and Ewer, 2010). The pathogenesis of DOX-induced cardiotoxicity and heart failure is complex and may involve various signaling mechanisms including oxidative stress, mitochondrial dysfunction/damage, and activation of mitogen-activated protein kinases (MAPK) (Kalyanaraman et al., 2002; Jin et al., 2003; Small et al., 2007; Takemura and Fujiwara, 2007; Vibet et al., 2008). There is increasing identification that activation of MAPK, p38 and JNK contribute to DOX-induced cell death and cardiotoxicity (Kim and Freeman, 2003; Timolati et al., 2006). Although the exact mechanism of DOX cardiotoxicity is not currently known, DOX-mediated generation of reactive oxygen species (ROS) has been implicated as at least partially responsible (Zhou et al., 2001; Kalyanaraman et al., 2002; Kim et al., 2006). To ameliorate ROS-induced toxicity, there is a need to use detoxicants, such as antioxidants, that can protect cardiac cells by scavenging the oxidants (Cao and Li, 2004; Khan et al., 2006; Danz et al., 2009; Li et al., 2010).

HO-3867 is a synthetic curcumin analog that we previously have shown to have antioxidant as well as anticancer properties (Selvendiran et al., 2010a; Selvendiran et al., 2010b; Selvendiran et al., 2010c).
al., 2010c). The compound targets STAT3 pathways in various cancer cell lines and inhibits xenograft ovarian tumor growth, without causing any significant side effects to healthy cells (Selvendiran et al.). In addition, we showed that HO-3867 inhibits Akt and MAPK expression by activation of PTEN (phosphatase and tensin homolog on chromosome 10) in serum-stimulated (proliferating) human smooth muscle cells (Selvendiran et al., 2009). Further, HO-3867 has been shown to have protective effects on healthy cells, at least partially due to its antioxidant properties (Selvendiran et al., 2010a). As such, it is an obvious complement to DOX for cancer therapeutics, as both of these drugs exhibit anti-tumor properties, and HO-3867 is additionally effective in scavenging ROS.

In this work, we examined the effects of DOX and HO-3867 combination treatment in vitro on breast cancer cells, including a drug-resistant strain. Normal cardiac and aortic endothelial cells were used as controls. We then examined the effect of combination treatment on the development of cardiotoxic side-effects in vivo using a mouse model of breast tumor. The results showed that combination treatment allowed for equivalent loss of cell viability in cancerous cells when compared to a high dose of DOX, while reducing the toxic effects in healthy cells. Further, it was observed that combination treatment eliminated the cardiotoxic side-effects of DOX.

Materials and Methods

Materials

HO-3867 was synthesized as reported (Selvendiran et al., 2010a; Kalai et al., 2011 ). 20-mM stock solutions of the compound were freshly prepared in dimethylsulfoxide (DMSO). Cell-culture medium (DMEM), fetal-bovine serum (FBS), antibiotics, sodium pyruvate, trypsin, and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY). Other cell
culture medium was purchased from ATCC (DMEM: Manassas, Virginia) and Lonza (SmBM: Basel, Switzerland). Polyvinylidene fluoride (PVDF) membranes and molecular-weight markers were obtained from Bio-Rad (Hercules, CA). Antibodies against, pAkt, Akt, pERK1/2, ERK1/2, FAS, Bcl-2, Bcl-xL, Caspase-3, -7, PARP and actin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies specific for α-tubulin, cyclin D1, cyclin D2, p53, p21, and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotech (GE Healthcare, Piscataway, NJ). DOX and all other reagents, of analytical grade or higher, were purchased from Sigma-Aldrich.

**Cell lines and culture**

MCF-7 human breast and MCF-7 MDR multi-drug resistant breast cancer cell lines were used. The MCF-7 MDR cells are partially resistant to anthracyclines, including DOX. Human aortic endothelial cells (HAEC) and cardiomyocytes (H9C2) were used as noncancerous (healthy) controls. The MCF-7 and MCF-7 MDR cells were grown in DMEM, H9C2 cells were grown in ATCC DMEM, and HAEC were grown in SmBM. All cell media were supplemented with 10% FBS, 2% sodium pyruvate, 1% penicillin and 1% streptomycin. Cells were grown in a 75-mm flask to 70% confluence at 37° C in an atmosphere of 5% CO$_2$ and 95% air. Cells were routinely trypsinized (0.05% trypsin/EDTA) and counted using an automated counter (NucleoCounter, New Brunswick Scientific, Edison, NJ).

**Cell-viability assay**

Cell survival was assessed by MTT assay. Cells, grown to ~80% confluence in 75-mm flasks, were trypsinized, counted, seeded in 96-well plates with an average population of 7,000
cells/well, incubated overnight, and then treated for 24 hours with DOX alone (2.5 μM, 25 μM, or 50 μM), HO-3867 alone (5 μM) or in combination (2.5 μM DOX and 5 μM HO-3867). All experiments were done using 6 replicates and repeated at least three times. Untreated cells were used as controls.

**Annexin-V assay**

Cells were treated with the drugs for a period of 24 h, trypsinized, washed in PBS, and treated with Annexin-V and PI staining. Cells were sorted and analyzed by flow cytometry. Untreated cells were used as controls.

**Immunoblot analysis**

Cells in their respective media were treated with DOX alone (2.5 μM), HO-3867 alone (5 μM), or in combination using these concentrations for 24 h. Equal volumes of DMSO (0.1% v/v) were present in each treatment. Following treatment, the cell lysates were prepared in nondenaturing lysis buffer containing 10-mM Tris-HCl (pH 7.4), 150-mM NaCl, 1% Triton X-100, 1-mM EDTA, 1-mM EGTA, 0.3-mM phenylmethylsulfonyl fluoride, 0.2-mM sodium orthovanadate, 0.5% NP40, 1-μg/ml aprotinin, and 1-μg/ml leupetin. The lysates were centrifuged at 10,000× g for 20 min at 4°C, and the supernatant was separated. The protein concentration in the lysates was determined using a Pierce detergent-compatible protein assay kit. For Western blotting, 25 to 50 μg of protein lysate per sample was denatured in 2× SDS-PAGE sample buffer and subjected to SDS-PAGE on a 10% tris-glycine gel. The separated proteins were transferred to a PVDF membrane and blocked with 5% nonfat milk powder (w/v) in TBST (10-mM Tris, 10-mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. The membranes were then incubated with the primary antibodies. The bound antibodies were detected with horseradish
peroxidase (HRP)-labeled sheep anti-mouse IgG or HRP-labeled donkey anti-rabbit IgG using an enhanced chemiluminescence detection system (ECL Advanced kit). Protein expression levels were quantified using Image Gauge version 3.45.

**Balb/c mouse handling and treatment**

Male Balb/c mice of approximately 20 grams weight were split into four groups: control, DOX-treated, HO-3867-treated or combination-treated. DOX was administered as an IP injection of 2.5 mg/kg weekly for four weeks. HO-3867 was given continuously as 100 ppm in the feed. Combination-treated animals received both DOX injections and HO-3867 feed. After 5 weeks, the animals were euthanized. Tissue samples were collected at this time. Ventricular sections from euthanized mouse hearts were homogenized and treated using the same lysis buffer described above, after which the procedure for immunoblot analysis was the same as that described above for cells.

**Echocardiography for cardiac functional analysis**

Cardiac function was analyzed using echocardiography at baseline prior to the beginning of DOX treatment and at 4 weeks after DOX treatment. Mice were anesthetized using 1.5-2% isofluorane and M-mode ultrasound images were acquired using a Vevo-2100 (Visualsonics; Toronto, Canada) high-resolution ultrasound rodent imaging system.

**Histopathology**

Slices of recovered heart tissues were fixed in 10% formalin for 48 hours at room temperature, dehydrated by graded ethanol, and embedded in paraffin. Tissue sections (5-μm thick) were deparaffinized with xylene, stained with hematoxylin and eosin (H&E) and representative
photomicrographs were taken using an inverted fluorescence microscope (Nikon TE 2000, Japan).

Data analysis

The statistical significance of the results was evaluated using Student’s t-test, or ANOVA, as appropriate. A p value of less than 0.05 was considered significant.

Results

Combination treatment with HO-3867 and DOX reduces cell viability in MCF-7 and MCF-7 MDR breast cancer cell lines, while having less effect on heart and aortic cells

Using MTT assay, we examined the viability of several cell lines after treatment with low doses of DOX and HO-3867 alone (2.5 μM and 5 μM, respectively) or a combination of both of these concentrations. We compared these with effects observed using higher doses of DOX alone (25 and 50 μM). The results showed that combination treatment reduced the viability of MCF-7 and MCF-7 MDR breast cancer cells to an extent significantly greater than that seen by treatment with either drug alone (Fig. 1). Treatment with the higher doses of DOX had a similar effect on the viability of the cells. MCF-7 cells had slightly lower viability after treatment with DOX than MCF-7 MDR cells. In the non-cancerous cell lines, H9C2 and HAEC, combination-treated cells had a significantly higher viability than those cells treated with higher doses of DOX. The results suggested that the combination treatment was more cytotoxic to cancer cells than DOX or HO-3867 alone, at the doses used. Further, the combination treatment induced much less cytotoxicity to normal cells.
Combination treatment with lowered doses of HO-3867 and DOX induces more apoptosis in MCF-7 MDR cells than treatment with either drug alone

The inhibition of cell growth in tumor cells is usually associated with an increase in apoptosis. In order to analyze the effect of HO-3867 with DOX on apoptosis, we performed Annexin-V flow cytometry. In both MCF-7 MDR and HAEC cells, the higher dose of DOX induced a greater percentage of apoptotic cells (Fig. 2A). The apoptotic fraction was significantly increased in cells given combination treatment, when compared to those given DOX or HO-3867 alone in MCF-7 (Fig. 2B) and MCF-7 MDR (Fig. 2B) cells. The results indicated that the combination treatment induced substantially higher apoptosis in cancer cells than DOX or HO-3867 alone, at the doses used.

Combination treatment of MCF-7 MDR cells with HO-3867 and DOX reduces expression of cell-cycle-related proteins and increases expression of apoptotic markers

Western blotting was used to examine the effect of combination treatment on cell-cycle and apoptotic protein expressions in MCF-7 MDR cells. Cells treated with both DOX and HO-3867 had reduced levels of cyclin-D1 and cyclin-D2 when compared to those treated with either drug alone, or control (Fig. 3A). The expressions of caspases 3, 7 and 9, as well as cleaved PARP, were increased in combination-treatment cells when compared to treatment with either drugs used alone, or control (Fig. 3B). The results revealed that the combination treatment caused greater inhibition of cell cycle-promoting proteins and promotion of apoptosis in cancer cells than DOX or HO-3867 alone, at the doses used.
Combination treatment of MCF-7 MDR cells with HO-3867 and DOX decreases expression of FAS and pAkt (Ser 473)

Fatty acid synthase (FAS) is overexpressed in a variety of tumors and play a key role in the migration and invasion of cancer cells. A number of reports have shown that anticancer agents induce apoptosis, in part, by blocking the activation of FAS and Akt pathways (Wrede et al., 2002; Coticchia et al., 2009; Zecchin et al., 2011). Akt prevents cells from undergoing apoptosis by inhibiting pro-apoptotic factors (Coticchia et al., 2009; Srinivasan et al., 2009). Recently, we have shown that HO-3867 suppressed the migration and invasion of the ovarian cancer cells by inhibiting the expression/activity of FAS (Selvendiran et al., 2010b). Hence, we determined the expression levels of FAS and Akt in cells treated with DOX and HO-3867 by Western-blot analysis. The results showed that FAS, Akt, and pAkt (Ser 473) were significantly decreased with combination treatment (Fig. 4).

Combination treatment of Balb/c mice with HO-3867 and DOX decreases the incidence of cardiotoxic side-effects, compared to DOX treatment alone

We examined the effect of combination treatment using an in vivo mice model of DOX-induced cardiotoxicity. Mice were treated with DOX alone or a combination of DOX and HO-3867 for 4 weeks and examined using M-mode echocardiography (Fig. 5A). Ejection fraction was significantly decreased in mice given DOX treatment alone, and mice given combination treatment had significantly greater ejection fraction by comparison (Fig. 5B). Similarly, fractional shortening was significantly less in the DOX-treated group, but not in the combination-treated group when compared to control. However, there were no significant differences in left ventricular dimensions at systole and diastole between the groups. Histopathological analysis on animals treated with DOX alone showed characteristic cardiotoxic
lesions, including mild to moderate multifocal cardiomyocyte degeneration, vacuolation, interstitial edema and mild inflammatory cell infiltrates. Hearts from combination-treatment mice were devoid of such lesions, and appeared histologically similar to that of untreated control animals (Fig. 5C). The results established that the combination treatment induced less cardiac dysfunction than DOX alone.

Combination treatment of Balb/c mice with HO-3867 and DOX increases the expression of pro-survival proteins in heart tissue compared to treatment with DOX alone

After 5 weeks of DOX treatment, the animals were euthanized and the ventricular sections of the heart were harvested. Protein levels in these sections were analyzed by Western blotting. Levels of pAkt (Ser 473) and Bcl-2 were decreased in DOX-treated mice, but not in the combination-treated mice (Fig. 6). Conversely, p21 levels were increased in DOX-treated mice. This increase was not seen in combination-treated mice, in which p21 levels were similar to those seen in control mice. Our results indicate that HO-3867 induced upregulation of Akt and Bcl-2 proteins may account, at least in part, for the cardioprotective effects observed in the combination-treated animals. The results established that the combination treatment upregulated the levels of prosurvival proteins in the heart.

Discussion

Resistance to chemotherapy is a commonly-encountered impediment in the treatment of breast cancer (Pivot et al., 2000; Rossi et al., 2005). Such resistance can prove to be a major limitation to the effectiveness of the treatment, necessitating the use of increased doses which can lead to debilitating consequences that substantially reduce the quality of life of patients. In the case of DOX, increasing doses can lead to severe cardiac complications that may eventually promote an
early death in patients (Unverferth et al., 1982; Jain, 2000; Takemura and Fujiwara, 2007; Ewer and Ewer, 2010). As such, development of strategies to counter drug resistance, while reducing both short-term and long-term impediments is necessary. This study indicates that a low-dosage combination treatment using both HO-3867 and DOX can be more effective at inhibiting breast cancer cell growth than either drug working alone. Moreover, this combination therapy alleviates many of the side effects of high-dose DOX treatment.

This study shows that combination treatment of HO-3867 with a low dose of DOX has a similar effect on the viability of both wild-type and drug-resistant breast cancer cells when compared to a much greater dose of DOX alone. The effect upon normal cardiomyocytes and aortic endothelial cells is markedly in contrast to that in cancer cells. The viability of the noncancerous (healthy) cells treated with the combination therapy is over three times greater than that of cells treated with DOX. A similar trend is seen in the percentage of apoptotic cells in combination-treated cultures. It is clear that combination treatment with low-dose DOX and HO-3867 would reduce the amount of DOX exposure necessary to treat breast cancer. Since side effects of DOX are known to be related to the total cumulative dose given, this is an important factor in assessing the clinical utility of such treatment.

The p53 tumor suppressor protein is to promote the induction of p21. The induction of p21 causes subsequent cell-cycle arrest by binding of the cyclin-cdk complex. In this study, we have shown that combination treatment of MDR cancer cells with HO-3867 and DOX resulted in the accumulation of p53, p21 and p27. The arrest of cell-cycle progression in tumor cells is usually associated with induction of apoptosis. Many chemotherapeutic agents induce apoptosis by activation of the p53-dependent pathway. The effect of the combination treatment is further seen
in cell-cycle protein expression and expression of apoptotic markers. The expressions of cyclins D1 and D2 are reduced by combination treatment, while no effect is seen with treatment by either HO-3867 or DOX alone. Similarly, expression of cleaved caspases 3 and 7 is greatly increased by combination treatment, which is an indication of increased apoptosis-promoting signaling. These results indicate that the loss of viability seen in combination treatment may be caused by the complementary effects of both cell-cycle arrest and the induction of apoptosis.

Combination treatment significantly reduced the expression of FAS and both pAkt (Ser 473) and total Akt. This implies that the mechanism of action for the combination treatment may involve one or both of these pathways. Our results indicate that FAS inhibition was solely caused by HO-3867 alone, as the combination treatment had no further inhibition (Figure 4). We have recently shown that HO-3867 is capable of downregulating FAS and FAK (focal adhesion kinase) thereby inhibiting the migration and invasion of human ovarian cancer cells (Selvendiran et al., 2010b). As both FAS and Akt are often overexpressed in breast cancers (Esslimani-Sahla et al., 2007; Vazquez-Martin et al., 2008; Wu et al., 2008), this indicates that this combination may have clinical utility in these cases. It has previously been shown that inhibition of FAS and Akt can lead to apoptosis in breast cancer, as well as other types of cancer (Coticchia et al., 2009; Zecchin et al., 2011). Conversely, cells overexpressing FAS and Akt exhibit resistance to apoptosis (Liu et al., 2008). By reducing the expression of FAS and Akt, the combination treatment may induce apoptosis through the mitochondrial pathway.

Side effects from DOX treatment commonly result in a loss of cardiac function. In the present study, the combination treatment permitted the use of reduced doses of DOX while maintaining the same level of cancer inhibition. Mice given combination treatment had significantly better
cardiac function after 4 weeks than their counterparts given DOX alone. Both ejection fraction and fractional shortening were significantly reduced by DOX treatment, whereas mice given combination treatment experienced no such reduction. Further, microscopic analysis of ventricular slices confirms that DOX-treated hearts are clearly physiologically different from control animals, while combination-treated animals have hearts that are physiologically similar to the control group. This similarity of the combination-treated animals to their control counterparts is also seen in the protein expression in the hearts. Both pAkt (Ser 473) and Bcl-2 are significantly reduced in the DOX-treated animals, but the expression of both proteins is recovered in the combination-treated group. This is especially interesting given the effect of combination treatment seen in cancer cells. In cancer cells the combination-treatment reduces the expression of pAkt (Ser 473) compared to DOX treatment alone. In normal heart tissue, however, the effect of the combination is precisely the opposite: combination treatment results in a greater expression of pAkt (Ser 473) than that seen in DOX-treated animals.

The design of HO-3867 was intended to provide both anticancer and antioxidant properties with cell-type dependent cytotoxicity or protection (Kalai et al., 2011). Many chemotherapeutic agents such DOX act by producing free radicals, which may increase oxidative stress in normal cells (Sinha and Mimnaugh, 1990; Minotti et al., 2004). HO-3867 has been shown to differentiate between healthy and cancerous cells and selectively protect healthy cells by scavenging free radicals (Selvendiran et al., 2010a). The protective (antioxidant) function stems from the N-hydroxypyrroline moiety of HO-3867, which is capable of scavenging oxygen radicals in cells that have normal oxygenation or redox status (Samuni et al., 2004). Most tumors are hypoxic in nature, and their cellular environment is more reducing (for example, thiol-rich) when compared to healthy cells (Kuppusamy et al., 1998; Ilangovan et al., 2002; Kuppusamy et
al., 2002; Yamada et al., 2002). Under the reductive conditions in cancer cells, the antioxidant efficacy of HO-3867 is inhibited (Mitchell et al., 2001). We have previously shown that HO-3867 exhibited significant growth arrest and apoptosis in a number of human cancer cell lines including breast, colon, head and neck, liver, lung, ovarian, and prostate cancer with no apparent toxicity to noncancerous cells (Selvendiran et al., 2010a). We observed that the anticancer activity HO-3867 was mediated by inhibition of STAT3 phosphorylation at Tyr705 and Ser727 residues and induction of apoptotic markers cleaved caspase-3 and PARP (Selvendiran et al., 2010c). The protective activity of HO-3867 towards noncancerous cells was shown to be mediated by the ability of the compound to confer selective anti-oxidant protection to the healthy cells. We further demonstrated that HO-3867 significantly inhibited the growth of the ovarian xenograft tumors (A2780) in a dosage-dependent manner (Selvendiran et al., 2010c). Western-blot analyses of the xenograft tumor tissues confirmed that HO-3867 inhibited pSTAT3 (Tyr705 and Ser727) and pJAK1 and increased apoptotic markers cleaved caspase-3 and PARP. In addition, it was also observed that HO-3867 suppressed the migration and invasion of the ovarian cancer cells by inhibiting the expression/activity of FAS and FAK proteins (Selvendiran et al., 2010b). While our previous studies suggested the potential of HO-3867 as a safe and effective anticancer agent for cancer therapy, the present study, for the first time, provides confirmatory evidence that HO-3867 protects healthy tissue in an in vivo model of cardiotoxicity.

This study clearly demonstrated that combination treatment with DOX and HO-3867 can allow for greater toxicity to breast cancer cells while reducing cardiac side-effects. Such reduction of side-effects is seen in two ways: first, by reducing the dose of DOX necessary to inhibit drug-resistant cancer cell growth, and second, by directly reducing the cardiac side-effects of DOX.
even while maintaining the same dose. As such, combination treatment with both DOX and H0-3867 has clear potential as a therapy for the management of drug-resistant breast cancer.

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**Authorship Contributions**

Participated in research design: Dayton, Selvendiran, P. Kuppusamy

Conducted experiments: Dayton, M.L. Kuppusamy, Meduru, Naidu, Khan

Contributed new reagents or analytic tools: Kalai, Hideg

Performed data analysis: Dayton, Selvendiran, Meduru, Khan

Wrote or contributed to the writing of the manuscript: Dayton, Selvendiran, P. Kuppusamy

**References**


Footnotes

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Legends for Figures

Figure 1. DOX+HO-3867 combination treatment decreases viability in cancer cells, has less toxic effect upon normal cells. MTT assay of breast cancer cells (MCF-7, MCF-7 MDR) and normal cells (HAEC, H9C2) was performed using various concentrations of DOX and HO-3867 (structure shown) for 24 hours. Combination treatment shows a greater loss of viability in cancer cells than treatment with either drug alone, at the same dose. Loss of viability is comparable to that seen in cells treated with a greater dose of DOX. Conversely, loss of viability in normal cells is significantly less than that in cells given a higher dose of DOX. *p<0.05 versus DOX 2.5 μM and HO-3867 5 μM, **p<0.05 versus DOX 50 μM. Values are mean±SD, n=6.

Figure 2. DOX+HO-3867 combination treatment induces a greater percentage of Annexin-V positive cancer cells than treatment with either drug alone. (A) Annexin-V flow-cytometry data from MCF-7 MDR and H9C2 cells treated with either 5- or 50-μM DOX for 24 hours. Annexin-V flow-cytometry data from MCF-7 (B) and MCF-7 MDR (C) cells treated with either DOX, HO-3867 or both (DOX: 2.5 μM, HO-3867 5 μM) for 24 hours. Values in the bar graph are mean±SD, n=3. *p<0.05 versus all other groups.

Figure 3: DOX+HO-3867 combination treatment decreases expression of cyclins D1 and D2, and induces expression of apoptotic markers. Western blotting was performed on MCF-7 MDR cells treated with DOX, HO-3867 or both (DOX: 2.5 μM, HO-3867 5 μM) for 24 hours. (A) The effect of treatment on cell cycle-related proteins and densitometric analysis of visualized bands for p53 and Cyclin D1 expressions. (B) The effect of treatment on apoptotic marker proteins and quantified the cleaved caspase-3 and PARP expressions.
Figure 4: DOX+HO-3867 combination treatment decreases expression of FAS and pAkt.

Western blotting was performed on MCF-7 MDR cells treated with DOX, HO-3867 or both (DOX: 2.5 μM, HO-3867 5 μM) for 24 hours. (A) Representative blots showing the effect of treatment on proteins in the FAS and Akt signaling pathways. (B) Quantitative representation of FAS and pAkt $^{\text{Ser}473}$. Values are mean±SD, n=3. *p<0.05 versus control and DOX treatment. **p<0.05 versus all other groups.

Figure 5. DOX+HO-3867 combination treatment protects cardiac tissue in vivo. Mice were treated with DOX or DOX+HO-3867. Echocardiography was performed after 4 weeks, and tissues were harvested after 5 weeks. (A) Representative M-mode echocardiograms of control, DOX-treated and combination-treated groups showing wall motion. (B) Changes in ejection fraction (EF), fractional shortening (FS), left-ventricular internal diameter at systole (LVIDs), and left-ventricular internal diameter at diastole (LVIDd) in the three groups. *p<0.05 versus control, **p<0.05 versus DOX-treated. Values are mean±SD, n=6.

DOX+HO-3867 treatment ameliorated the significant decrease in the ejection fraction and fractional shortening induced by DOX. (C) Representative images (200x magnification) of H&E staining performed on cross-sections harvested after 5 weeks treatment with DOX or DOX+HO-3867. Cross-sections from animals treated with DOX alone exhibited lesions characteristic of DOX toxicity, such as cardiomyocyte degeneration and cytoplasmic vacuolation, while the cross-sections from the animals given combination treatment appeared normal, comparable to control.

Figure 6: DOX+HO-3867 combination treatment induces protective proteins in heart tissue in vivo. Western blotting was performed on harvested ventricular tissue from mice after 5

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weeks treatment with DOX, HO-3867 or both. DOX significantly suppress the pAkt and Bcl-2 proteins, HO-3867 combination with DOX reversed these proteins in cardiac tissues.
Figure 2
Figure 3

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Protein Expressions (a.u.)

- p53
- Cyclin D1
- Cle. Cas-3
- Cle. PARP

Control DOX HO-3867 DOX+ HO-3867
Figure 4
Figure 5

A

Control  DOX  DOX+HO-3867

B

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C

Control  DOX  DOX+HO-3867

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Figure 5
Figure 6