The Role of TRAIL-Signalling in Doxorubicin-Induced Cardiotoxicity

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by Michelle Sims

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ABSTRACT

Introduction: Doxorubicin (DOX) is a widely prescribed chemotherapeutic for the treatment of solid and haematologic malignancies. However, its clinical utility is limited by irreversible cardiotoxicity, which can lead to lifelong, sometimes fatal, heart complications. Recent evidence suggests the involvement of TNF-related apoptosis-inducing ligand (TRAIL) which, through binding to its death receptors (DR4 and DR5), initiates a signalling cascade leading to cell death. We hypothesise that DOX sensitises cardiomyocytes to TRAIL-induced death through modulation of this pathway.

Method: Using cultured human cardiomyocytes, we assessed the ability of DOX and TRAIL to cause cardiomyocyte death, and interrogated molecular factors involved in the TRAIL apoptotic signalling pathway using flow cytometry and Western blot. Wild-type and $TRAIL^{-/-}$ mice (n=7 per group) were used to evaluate the effect of TRAIL on DOX-induced cardiotoxicity. Cardiac function was assessed using echocardiography to measure ventricular ejection fraction (LVEF) and fractional shortening (FS).

Results: We showed that (i) TRAIL was significantly cytotoxic to cardiomyocytes in the presence of DOX ($p<0.0001$); (ii) surface expression of DR5 increased ~2-fold following DOX treatment in cardiomyocytes; (iii) expression of X-linked apoptotic inhibitor by cardiomyocytes decreased ~20-fold in the presence of DOX+TRAIL, and (iv) in wild-type mice, DOX caused a 16% ($p<0.001$) and 24% ($p<0.001$) reduction in LVEF and FS respectively, whereas DOX-treated $TRAIL^{-/-}$ mice had no significant reduction in cardiac function.

Discussion: Our data support the hypothesis that DOX sensitises cardiomyocytes to TRAIL-induced death. Collectively, these findings reveal a novel mechanism which could lead to therapeutic intervention to limit or eliminate DOX-induced cardiotoxicity.

1. INTRODUCTION

Doxorubicin (DOX) is an anthracycline chemotherapeutic used widely in the treatment of both adult and paediatric cancers. Its uses range from solid tumours including breast, lung, and testicular malignancies, to haematologic cancers such as leukaemias and lymphomas[1]. While DOX has strong anti-neoplastic potency, it causes many side effects, including nausea, gastrointestinal disturbances, and myelosuppression[2]. Cardiotoxicity is the most severe and dose-limiting effect of DOX treatment[3]. This toxicity has a cumulative, dose-related effect on cardiomyocytes, the heart cells responsible for myocardial contractility. Cardiomyocytes are non-regenerative in the adult heart; therefore loss of cardiomyocytes results in hypertrophic remodelling of the remaining cells and fibrotic scarring, as shown in myocardial infraction[4].

Chronic cardiotoxicity is characterised by permanent morphological and functional changes to the heart following DOX treatment. This manifests as a reduction of LVEF and contractile function, causing diastolic dysfunction[5]. The severity of cardiotoxicity ranges from asymptomatic to life-threatening congestive heart failure (CHF). With the current cumulative dose limit of 550mg/m^2, the incidence of CHF is between 7 and 26%[6, 7]. However, once CHF has developed, the 5-year survival rate is 50%[6]. Currently, there is no approved medicine for the prevention of DOX-induced cardiotoxicity.

The primary chemotherapeutic action of DOX is intercalation between DNA base pairs, stabilising the DNA-topoisomerase-II complex, and inhibiting DNA and RNA synthesis. Topoisomerase-II is responsible for nicking double-stranded DNA during replication, which relaxes the unwinding helix to prevent supercoiling[8]. By stabilising this complex, DOX prevents free nucleotide ligation after double-strand breakage, inhibiting cell replication and transcription[9]. This cytotoxic mechanism

targets highly replicating cells such as cancer cells. However, cardiomyocytes are not highly replicating cells, suggesting another mechanism is responsible for DOX's cardiotoxicity.

Many mechanisms have been proposed to contribute to DOX-induced cardiotoxicity. A by-product of DOX metabolism is the formation of reactive oxygen species (ROS)[10]. ROS can induce oxidative stress, triggering apoptosis in cancer cells and normal cells, and was hypothesised to cause cardiomyocyte damage and apoptosis by DOX[11, 12]. However, recent studies show the formation of ROS may not be the major mechanism of cardiotoxicity[13, 14]. Other mechanisms proposed include disruption of intracellular homeostasis[15], mitochondrial dysfunction[16], and inhibition of DNA and protein synthesis[17, 18]. Despite this research, the pathogenetic pathway of DOX cardiotoxicity remains unclear.

DOX initiates the intrinsic apoptotic signalling pathway in cells[19]. Activation of this pathway is the result of cell injury, from DNA damage or cellular stress, which causes the mitochondria to realise cytochrome c, leading to apoptosis (Fig 1). Activation of the intrinsic apoptotic pathway can also occur through BH3 interacting-domain death-agonist (BID), a protein primarily involved in the extrinsic apoptotic pathway, suggesting an apoptotic "cross-talk" mechanism.

The extrinsic apoptotic pathway is also affected by DOX; this pathway is activated by TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is a naturally occurring cytokine present in the blood and tissue; however, its role under normal and pathological conditions is still somewhat unknown. In the immune system, TRAIL is expressed on T-cells, Natural Killer cells, macrophages and dendritic cells[20]. In this setting, TRAIL plays a crucial role in tumour-surveillance and apoptosis-induction of viral and bacterial infected cells[21, 22]. The extrinsic apoptotic pathway is activated when TRAIL binds to its death receptors, TRAIL-1 (DR4) and TRAIL-2 (DR5)[23]. These receptors contain an intracellular death domain required to initiate the apoptotic pathway. TRAIL binding with DR4 or DR5 results in

receptor aggregation and recruitment of adaptor proteins to form a death-inducing signalling complex (DISC)[24] (Fig 1). DISC formation triggers the activation of initiator caspases-8/10, subsequently activating effector caspases-3/7 and poly ADP-ribose polymerase (PARP-1), resulting in apoptosis. Inhibitors of this pathway include X-linked inhibitor of apoptosis (XIAP), which can inhibit both apoptotic pathways through blocking effector caspase-3, 7, and 9. TRAIL also binds to decoy receptors TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin (OPG). DcR1 and DcR2 both lack a functional death domain, and therefore cannot induce apoptosis[25]. The secreted protein OPG is a soluble receptor with weak binding affinity for TRAIL and lacks both transmembrane and death domain properties required to initiate apoptosis[26].

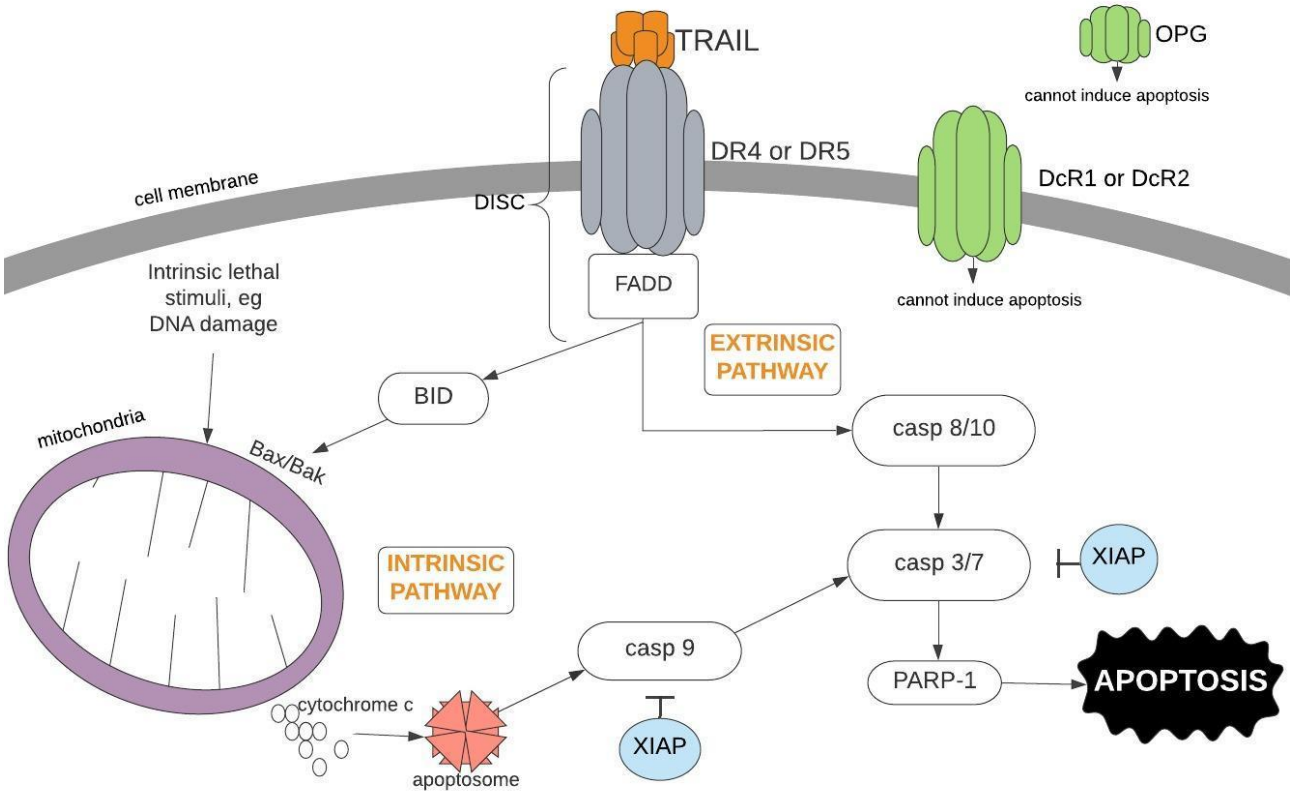


Figure 1. The extrinsic and intrinsic apoptotic pathway.

TRAIL also plays a role in cell proliferation, survival, and migration. Non-apoptotic pathways initiated from TRAIL-binding include nuclear factor-κB, protein kinase C, protein kinase B, and mitogen-activated protein kinase pathways[27]. These non-cytotoxic pathways mediated by TRAIL indicate the dual function of its cognate receptors and caspases, revealing TRAIL's important role in tissue homeostasis.

Many studies have explored TRAIL's chemotherapeutic effects, given its natural tumour-surveillance role. However, issues such as tumour resistance[28], promotion of malignancy via survival pathways[29], and short serum half-life[30] have hindered progress. Further research has revealed the administration of DOX sensitises some cancer cells to TRAIL-induced death[31-33]. This sensitisation may occur through several mechanisms; one mechanism observed is the upregulation of both DR4 and DR5, with modulation of DR5 being more pronounced[34, 35]. DOX can also alter this pathway downstream through downregulating pathway inhibitors, as observed in prostate carcinomas[36, 37]. Because of this synergistic apoptotic mechanism, research has focused on the co-administration of TRAIL and DOX as a potential chemotherapeutic regimen[32]. However, the effect of TRAIL and DOX on cardiomyocytes is currently unknown and previously unexamined.

TRAIL, DR4 and DR5 are all highly expressed in the heart[38]; despite this, cardiomyocytes are seemingly resistant to the apoptotic effects of TRAIL. This is possibly owing to the high expression of both XIAP and DcR1 in cardiomyocytes[39]. At normal physiological conditions, cardiomyocytes have been shown to benefit from TRAIL-mediated growth and survival pathways. Studies have shown the risk of mortality inversely correlates with levels of TRAIL in coronary heart disease[40], acute myocardial infarction[41], and heart failure[42]. However, presence of DOX may alter the TRAIL pathway's natural homeostasis in cardiomyocytes in favour of apoptosis induction.

Sensitisation of cardiomyocytes to TRAIL-induced death may be a key mechanism of DOX-induced cardiotoxicity. A study by Zhao and Zhang. shows DOX upregulates the expression of death receptors

at both protein and mRNA levels in cardiomyocytes, with the most pronounced increase in DR4 (~7 fold)[43]. This change in death receptors was associated with increased cytotoxicity. However, this mechanism was investigated using induced pluripotent stem cells-derived cardiomyocytes (IPS-CMs), which phenotype differs to that of adult cardiomyocytes[44]. For clinical relevance, it is therefore necessary to investigate this mechanism in adult primary cardiomyocytes and *in vivo*.

We hypothesise that the principal mechanism of DOX-induced cardiotoxicity is modulation of the extrinsic apoptotic signalling pathway, resulting in sensitisation of cardiomyocytes to TRAIL-induced death. We propose that DOX modulates both survival and apoptotic molecular factors in favour of apoptosis induction. To test this hypothesis we aim to: (i) determine the ability of DOX with and without the presence of TRAIL to elicit human adult cardiomyocyte death; (ii) compare the effect of DOX and TRAIL treatment on cardiomyocytes with cancer cells and fibroblasts; (iii) investigate the effects of DOX and TRAIL on the expression of key mediators of the extrinsic and intrinsic apoptotic pathways in cardiomyocytes; and (iv) utilise TRAIL-knockout mice to investigate the *in vivo* effect of TRAIL on DOX-induced cardiotoxicity.

2. MATERIALS AND METHOD

2.1. Cell culture reagents

Human adult cardiomyocytes (HCMs), media and supplements were purchased from PromoCell, Germany unless otherwise stated. HCMs were cultured in myocyte growth medium supplemented with 0.5 ng/ml epidermal growth factor, 2 ng/ml fibroblast growth factor, 5 µg/ml insulin, 1% penicillin/streptomycin (ThermoScientific, Australia), and 10% foetal bovine serum (FBS, Life Technologies, Australia).

Human foreskin fibroblasts (HFFs) were purchased from American Type Culture Collection (USA), and breast cancer cells MDA-MB231 derivative MDA-MB231-TXSA were gifted by Dr Toshiyuki Yoneda (University of Texas Health Sciences Centre, USA).

HFFs and TXSAs were cultured in Dulbecco's modified eagle medium (ThermoScientific) supplemented with 1% penicillin/streptomycin, and 10% FBS. All cells were cultured in a humidified incubator with 5% CO_2 atmosphere at 37ºC.

2.2. In vitro effects of DOX and TRAIL on HCM, HFF, and TXSA cell viability

To investigate aims (i) and (ii) cells were seeded in 96-well microtiter plates at a density of $8x10^3$ cells per well and allowed to adhere overnight. HCMs and HFF were treated with vehicle (media, control), DOX hydrochloride (Hospira, Australia) (0.5-100µM), TRAIL (Genentech, USA) (5, 10, 100 ng/ml) or a combination of DOX (1 µM) and TRAIL (5, 10, 100 ng/ml). TXSAs were treated with increasing concentrations of DOX (0.4-50 µM) and increasing concentrations of TRAIL (0.1-100 ng/ml). From the TRAIL concentration-response curve, we determined the EC_{15} (5 ng/ml TRAIL), which was then used in combination with increasing concentrations of DOX (0.2-50 µM) in TXSAs. HCMs and HFFs were incubated with treatments for 48-hours while TXSAs were incubated for 24-hours, with six replicate wells for each concentration. After fixing with formalin and staining

with crystal violet, plates were left to dry for 24-hours. Stained wells were resuspended in 10% acetic acid for 1-hour, and optical density values were read at 595nm using an Optima plate reader.

2.3. In vitro effects of DOX and TRAIL on the expression of death receptors in HCMs

Flow cytometry was used to investigate aim (iii). HCMs were seeded in a 24-well microtiter plate at a density of $3x10^4$ cells per well and left to attach overnight. Cells were treated with vehicle (media, control), 5 µM DOX, or 100 ng/ml TRAIL for 24-hours or 48-hours, in triplicate. Cells were then washed with phosphate-buffered saline (PBS), harvested using 2 mM ethylenediaminetetraacetic acid (EDTA) at 37ºC for 5min, and transferred into one tube per treatment group. Cells were then pelleted and washed twice in PBS, then again with ice-cold PBS. All following steps were conducted at 4ºC or on ice. Cells were resuspended in wash buffer of 2% FBS, 2 mM EDTA in PBS (PFE), centrifugated, then resuspended in blocking buffer (5% normal goat serum in PFE) and the tubes placed on ice for 15min. Primary antibodies (Table 1) were added to each tube at a 1:100 dilution and left for 45min in the dark. Cells were then washed three times with PFE and collected by centrifugation. Cells were resuspended and fixed in fluorescent-activated cell sorting fix (4% paraformaldehyde in PBS) for 10min, centrifuged and resuspended in 200µl of PFE. The tubes were stored in darkness at 4°C until analysis. Antibodies for each receptor had been directly conjugated to fluorophores of differing wavelengths by the manufacturer.

Table 1: Mouse anti-human monoclonal primary antibodies

Target Receptor	**Fluorophore**	**Supplier**	**Product no.**
DR4	BV480	BD Biosciences	746250
DR5	AlexaFluor 647	BD Biosciences	565498
DcR1	BV421	BD Biosciences	744764
DcR2	AlexaFluor 488	R&D Systems	FAB633G

2.4. Effects of DOX and TRAIL on the expression of key mediators of the extrinsic and intrinsic apoptotic pathways

Aim (iii) was further investigated using Western blot. Confluent T25 flasks of HCMs were treated with vehicle (media, control), 2.5 µM DOX, 50 ng/ml TRAIL, or 2.5 µM DOX + 50 ng/ml TRAIL, and incubated for 48-hours. At harvest, flasks were washed in ice-cold PBS and lysed in RIPA buffer containing 1:100 dilution of protease inhibitor cocktail (ThermoScientific) for 10min on ice. Lysates were collected into tubes and stored in -80ºC until required. Following a freeze/thaw cycle, lysates were analysed for protein concentration using a bicinchoninic acid (BCA) protein assay kit (ThermoScientific). Using the BCA standard curve, the lysates were normalised to ensure each sample had equal amounts of protein for loading. Samples were run on an electrophoresis 4-12% precast polyacrylamide gel (BioRad, USA) and transferred to a nitrocellulose membrane.

After washing twice in tris-buffered saline with 0.1% tween-20 (TBST), membranes were blocked with 5% milk overnight in 4ºC, then probed with primary antibody overnight (Table 2). After washing, the corresponding secondary antibody was incubated on the membrane for 1-hour at room temperature. All blots were visualised using enhanced chemiluminescence (GE life sciences, UK),

imaged with ChemiDoc Touch imaging system (BioRad), and analysed through ImageLab (BioRad). Anti-β-actin primary antibody was used for the loading control in semi-quantitative analysis.

Table 2: Antibodies used in Western blot analyses

Primary antibody	**Supplier**	**Product no.**	**Dilution**	**Secondary antibody, 1:5000 dilution (**in 1% milk TBST)
Mouse anti-β-actin	abcam	ab8226	1:5000	Goat anti-mouse IgG-HRP sc2005
Rabbit anti-PARP-1	Merck	11835238001	1:1500	Goat anti-rabbit IgG-HRP sc2004
Mouse anti-caspase-3	Cell Signalling	9668S	1:1500	Goat anti-mouse IgG-HRP sc2005
Rabbit anti-BID	Cell Signalling	2002S	1:1000	Goat anti-rabbit IgG-HRP sc2004
Mouse anti-caspase-9	Cell Signalling	9502S	1:1000	Goat anti-mouse IgG-HRP sc2005
Mouse anti-DR5	Novus Biologicals	NBP2-80066	1:500	Goat anti-mouse IgG-HRP sc2005
Mouse anti-caspase-10	MBL	MO59-3	1:1000	Goat anti-mouse IgG-HRP sc2005
Goat anti-XIAP	R&D Systems	AF8221	1:1000	Donkey anti-goat IgG-HRP sc2020

2.5. DOX-induced cardiotoxicity in TRAIL-knockout mice

To investigate aim (iv), wild-type and TRAIL-knockout[45] 4-6 week old C57BL/6 mice were donated from Dr Mary Kavurma (the Heart Research Institute, NSW). Mice were acclimatised in the Animal Housing facility (The Queen Elizabeth Hospital) for one week before treatment. The weight and physical wellbeing of the animals were monitored continuously throughout the experiment in accordance with the Laboratory Animal Services. All experiments were approved by the University of Adelaide Animal Ethics Committee (34020).

Mice were treated with saline (vehicle) or 4 mg/kg of DOX once every five days for a cumulative dose of 16 mg/kg. Treatment groups were: wild-type vehicle n=8, wild-type DOX-treated n=7, $TRAIL^{-/-}$ vehicle n=7, $TRAIL^{-/-}$DOX-treated n=7. The dose was administered via intraperitoneal injection while under isoflurane gas anaesthetic (Faulding Pharmaceuticals, Australia). Echocardiography (Vivid iq, GE Healthcare, Australia) was used to examine cardiovascular function three days after the last dose was administered. Echocardiography was performed by a blinded clinical echocardiographer, whilst the mice were under continuous isoflurane gas anaesthetic (1-3%). M-mode analysis was used to assess LVEF and FS (n=3). Mice were humanely killed through cardiac puncture whilst under isoflurane gas anaesthetic and their organs harvested.

2.6 Statistical analyses

2-way ANOVA was used to compare between groups in cell viability and mice echocardiography data. Differences between groups were considered significant at $p<0.05$. Data are represented as mean ± standard deviation (SD).

3. RESULTS

3.1. In vitro effects of DOX and TRAIL on HCM, HFF, and TXSA cell viability

After 24-hour incubation with DOX, HFFs showed a decrease in cell viability by 25% ($p<0.0001$) to 51% ($p<0.0001$) in a concentration-dependent manner (Fig 2.A). 48-hour DOX-treatment caused HFF viability to decrease between 44% ($p<0.0001$) and 68% ($p<0.0001$).

Following 24-hours of treatment with DOX, HCMs showed no significant decrease in viability (Fig 2.B). However, following 48-hour DOX treatment, cell viability decreased between 12% ($p<0.001$) to 27% ($p<0.0001$) in a concentration-dependant manner.

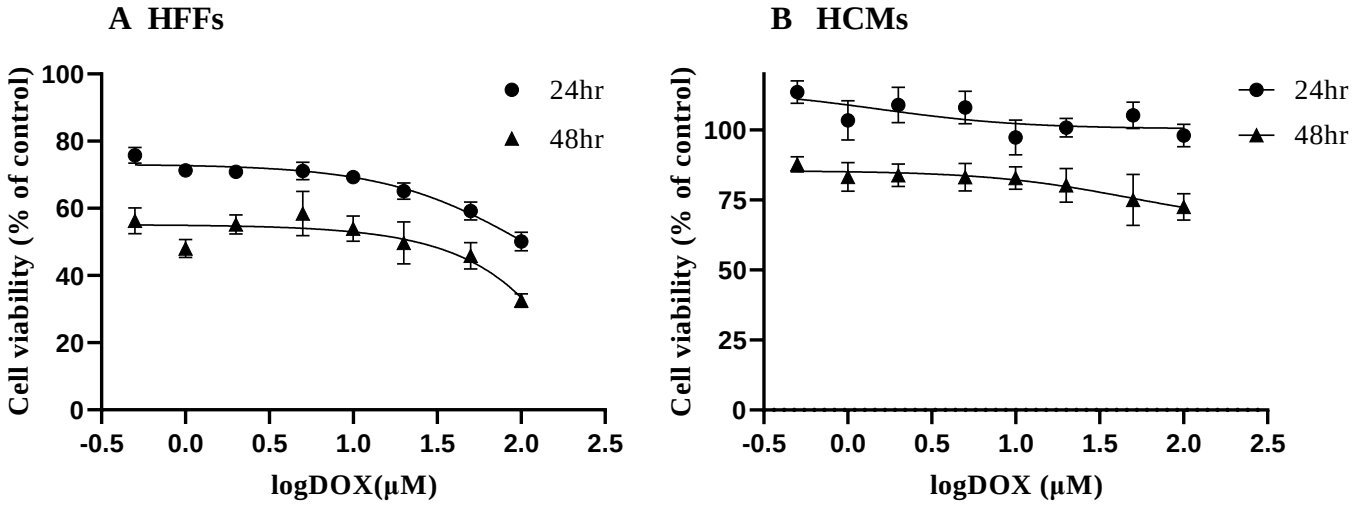


Figure 2. Sigmoidal dose-response of DOX treatment at 24- and 48-hour incubations in (**A**) HFFs (n=6) and **(B)** HCMs (n=6). Statistical analysis through 2-way ANOVA. Data are expressed as mean ±SD.

TXSA breast cancer cells treated with increasing concentrations of DOX for 24-hours had an EC_{50} of 3 μM (95% confidence interval: 2.347- 4.167) (Fig 3). TXSAs co-treated with 5ng/ml of TRAIL and with increasing concentrations of DOX showed an EC_{50} of 0.3 μM (95% confidence interval: 0.2601-

0.3654). The increased apoptotic effect of TRAIL on DOX-treated cells is shown by a shift in the concentration-response curve to the left.

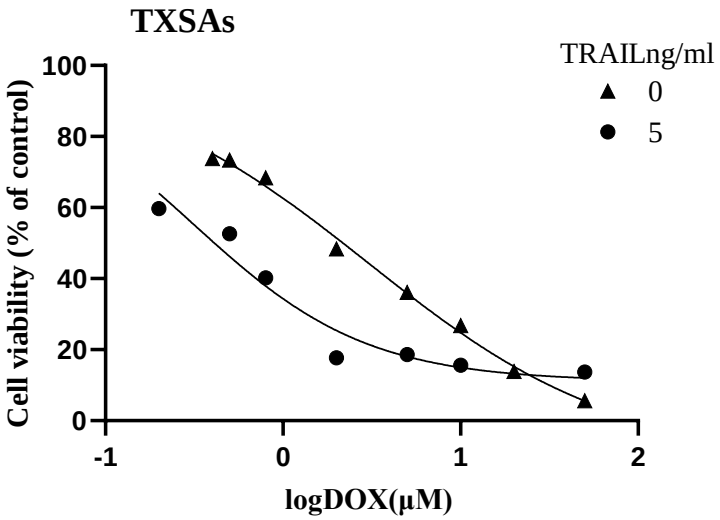


Figure 3. Sigmoidal dose-response of DOX treatment in TXSAs alone and with 5 ng/ml TRAIL (n=6). Data are expressed as mean ±SD.

The effects of combining DOX and TRAIL on HFF viability are shown in Fig 4. TRAIL alone had no effect on viability after 24- or 48-hour incubation. Following 24-hour treatment with DOX, HFF viability decreased 37%. After 48-hour treatment with DOX, viability decreased 42% from vehicle-treated cells. The co-treatment of DOX and TRAIL caused a further decrease in HFF viability compared DOX-only treated cells (up to 19% decrease).

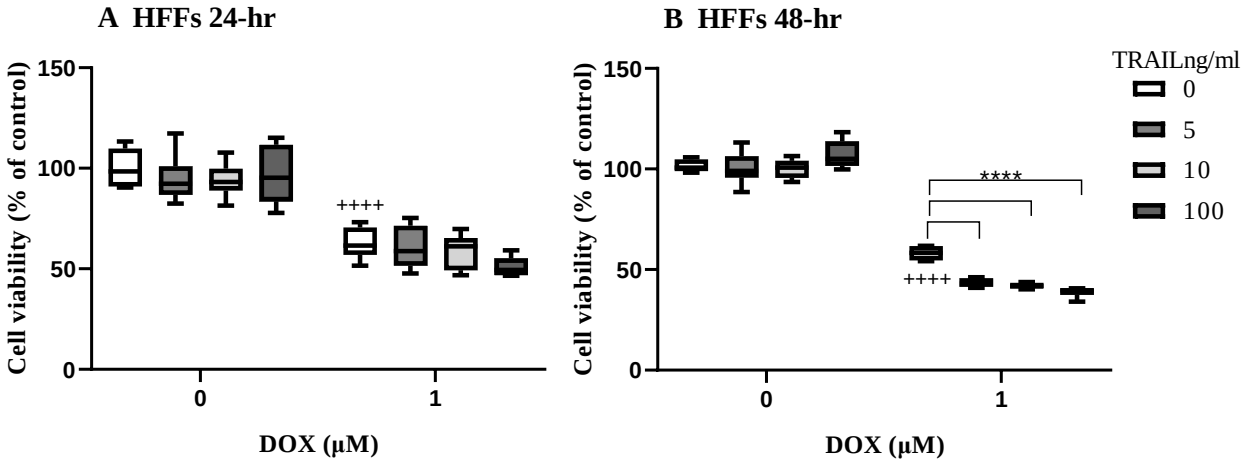


Figure 4. Viability of HFFs after (**A**) 24- and (**B**) 48-hour incubations with vehicle, TRAIL alone and with 1 µM DOX (n=6). ++++ $p<0.0001$ compared to vehicle-only. **** $p<0.0001$ compared to DOX-only. Statistical analysis through 2-way ANOVA. Data are expressed as mean ±SD.

The effects of combining DOX and TRAIL on HCM viability are shown in Fig 5. TRAIL alone did not affect HCM viability, except in the 24-hour incubation where 10 and 100 ng/ml of TRAIL caused a significant increase in viability. DOX alone caused no significant decrease in HCM viability following either 24- or 48-hour incubations. However, after 48-hour incubation, the co-treatment of DOX and TRAIL caused a significant decrease in HCM viability compared to DOX-only treated cells in a TRAIL concentration-dependent manner (up to 43% decrease).

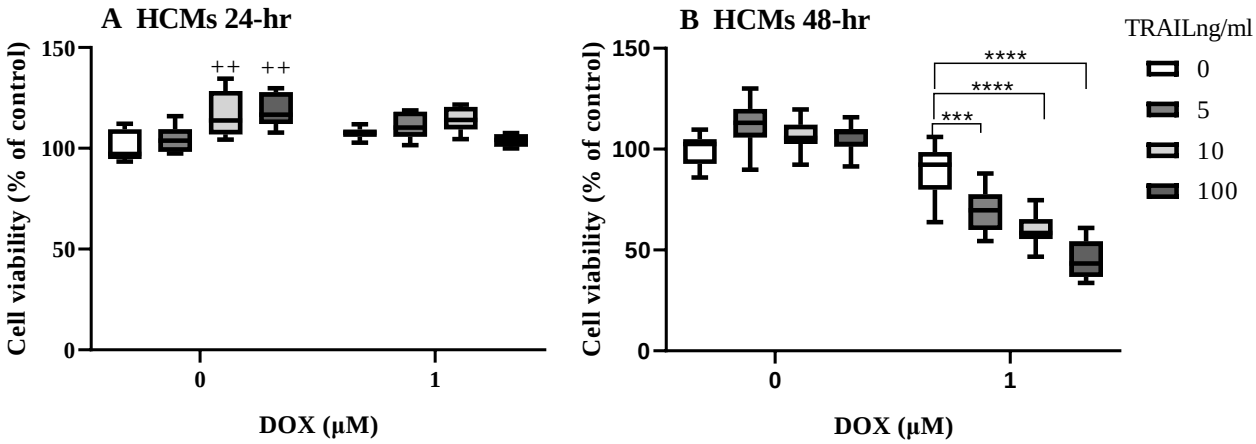


Figure 5. Viability of HCMs after (**A**) 24- and (**B**) 48-hour incubations with vehicle, TRAIL alone and with 1 μM of DOX (n=6). ++ $p<0.01$ compared to vehicle-only. *** $p=0.0001$, **** $p<0.0001$ compared to DOX-only. Statistical analysis through 2-way ANOVA. Data are expressed as mean ±SD.

3.2. Effects of DOX and TRAIL on the expression of death receptors

Treatment of HCMs with DOX or TRAIL for 24-hours did not affect cell surface expression of decoy receptors or DR4 (Fig 6.A). However, DR5 surface expression had a ~1.5-fold increase in the presence of DOX when compared to untreated HCM's.

48-hour incubation of HCMs with TRAIL had no difference in surface expression of the death and decoy receptors, except for a slight decrease (~25%) in DR5 expression compared to untreated cells (Fig 6.B). DOX-treated HCMs had a ~1.8-fold increase in DcR1 and a ~2-fold increase in DR5. No change was observed in the expression of DR4 and DcR1 following DOX-treatment.

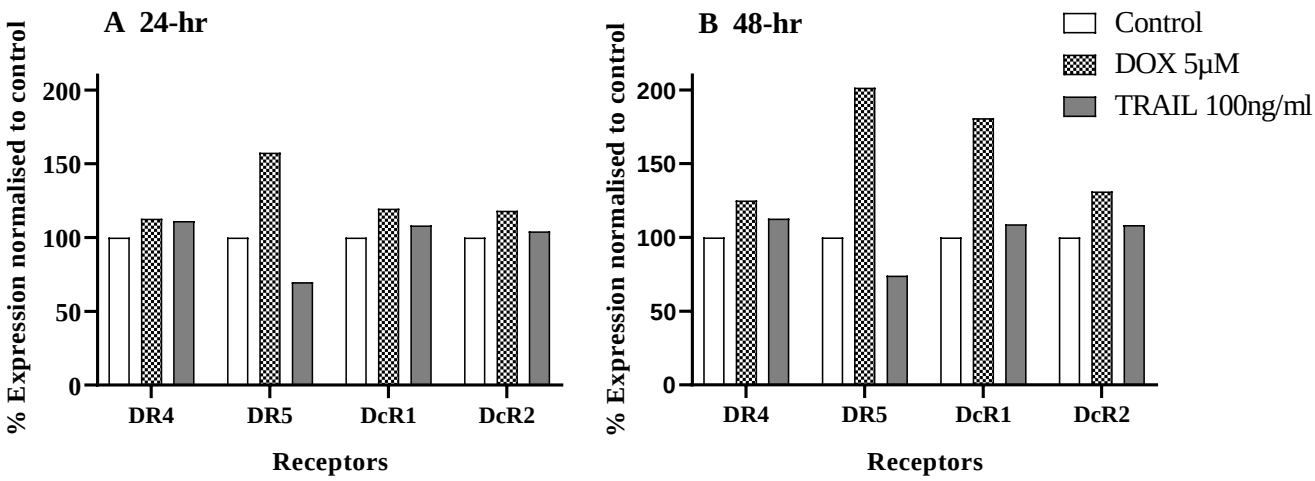


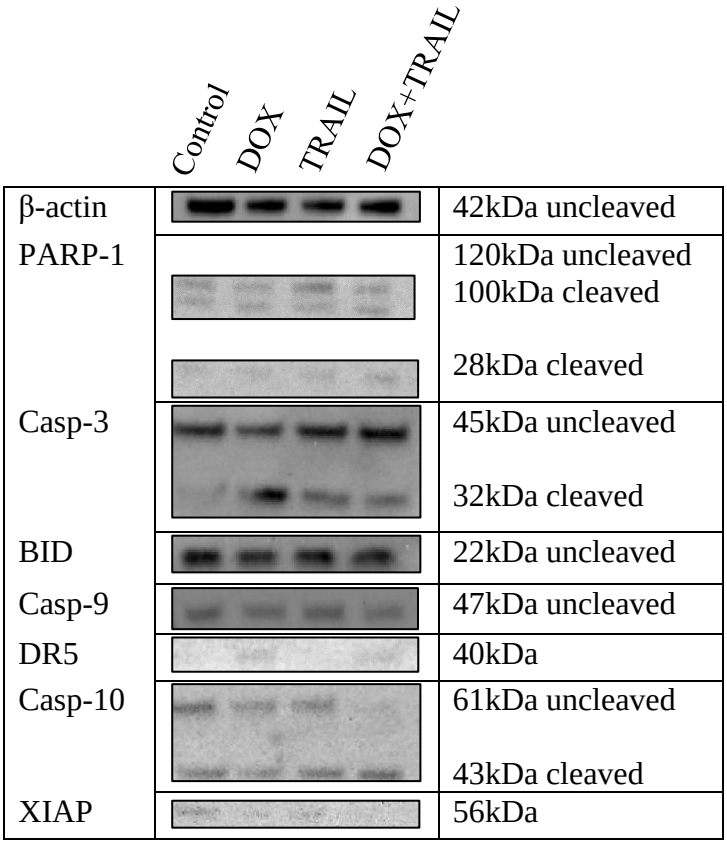
Figure 6. Expression of TRAIL death and decoy receptors at (**A**) 24- and (**B**) 48-hours on the surface of HCMs after incubation with vehicle, 5 μM DOX, or 100 ng/ml TRAIL (n=1).

3.3 Effects of DOX and TRAIL on the expression of key mediators of the extrinsic and intrinsic apoptotic signalling pathways

Protein analysis via Western blot revealed uncleaved and cleaved products, see Table 3. Cleavage of proteins indicates activation has occurred, allowing the protein to carry out its function. Uncleaved and cleaved products of PARP-1 can be observed in HCMs (Fig 7A). DOX and DOX+TRAIL treated HCMs showed higher concentration of total cleaved protein than uncleaved (~1.5 and 1.7-fold, respectively).

Uncleaved and cleaved caspase-3 in HCMs following treatment is shown in Fig 7B. DOX-treated HCMs had the most caspase-3 activation, with the presence of cleaved product ~1.6 fold higher than uncleaved product. While TRAIL and DOX+TRAIL treated HCMs had higher levels of uncleaved protein, activated caspase-3 was still detected.

Table 3: Membranes post-incubation with appropriate primary and secondary antibodies, including the molecular weight of proteins identified.



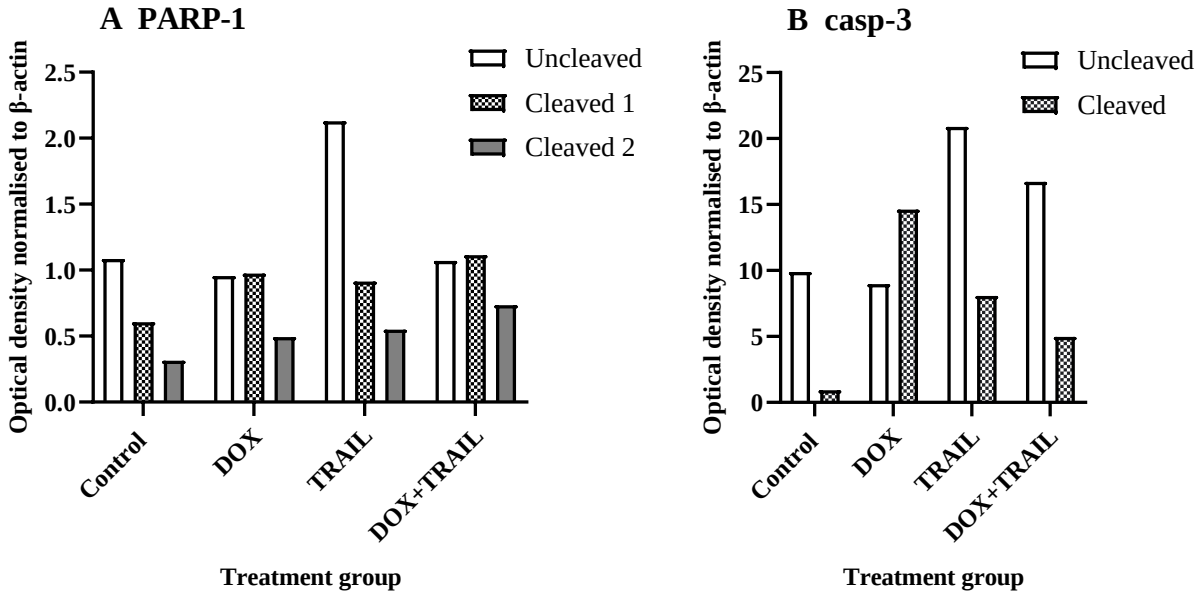


Figure 7. Markers of cellular stress and apoptosis. ***(*A**) Expression of uncleaved and cleaved PARP-1 and, (**B**) uncleaved and cleaved caspase-3 in HCMs incubated for 48-hours with vehicle, 2.5 µM DOX, 50 ng/ml TRAIL, and 2.5µM DOX + 50 ng/ml TRAIL (n=1).

There were no cleaved BID or cleaved caspase-9 proteins observed in any treatment group (Fig 8). However, a decrease in expression of both uncleaved BID and caspase-9 following DOX and DOX+TRAIL treatment was observed, suggesting activation.

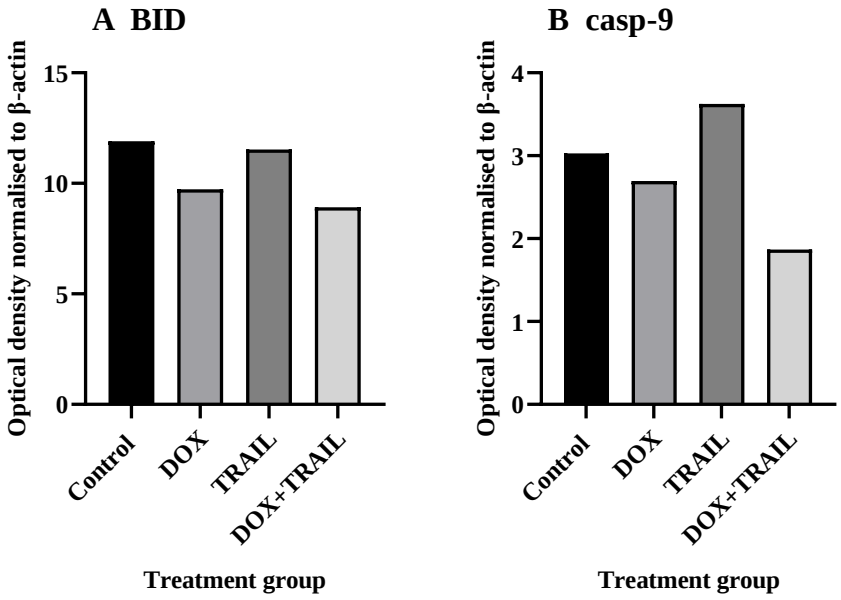


Figure 8. Markers of intrinsic apoptosis. (**A**) Expression of uncleaved BID and, (**B**) expression of pro-caspase-9 in HCMs incubated for 48hours with vehicle, 2.5 µM DOX, 50 ng/ml TRAIL, and 2.5 µM DOX + 50 ng/ml TRAIL (n=1).

Total DR5 expression increased ~4-fold in DOX and ~3.4-fold in DOX+TRAIL treated HCMs (Fig 9.A). Caspase-10 activation was greatest in DOX+TRAIL treated HCMs, with the presence of cleaved product ~4.7-fold higher than uncleaved (Fig 9.B). XIAP expression was decreased ~4.3-fold in DOX, and ~20.4-fold DOX+TRAIL treated HCMs (Fig 9.C).

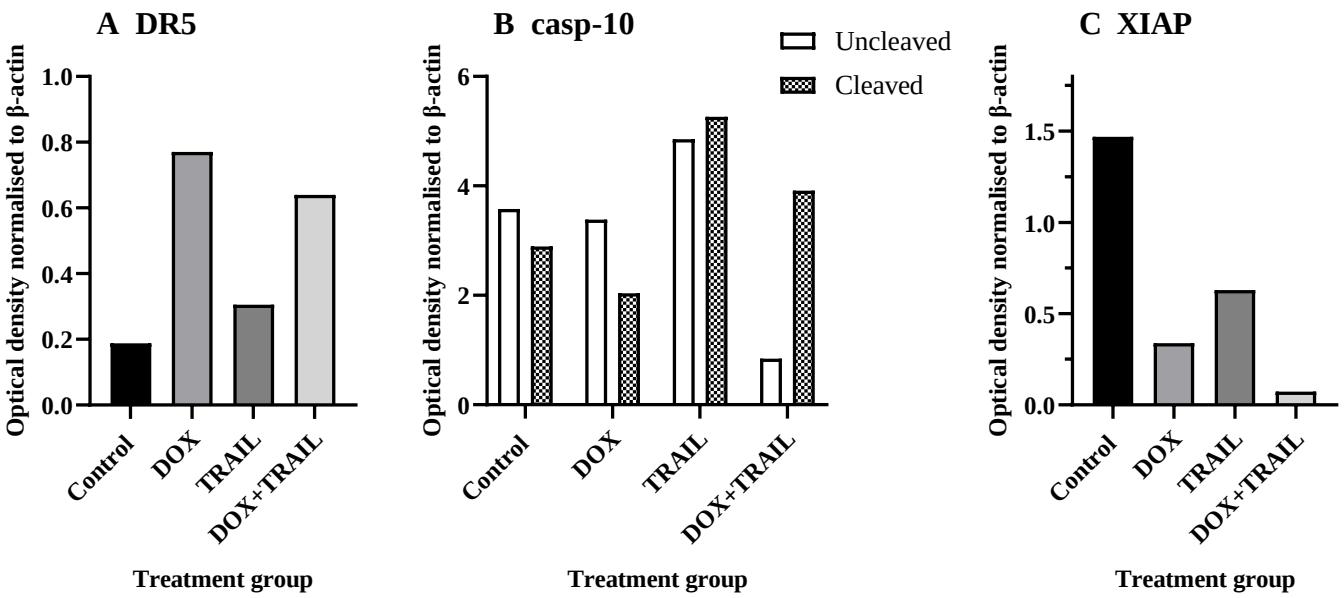


Figure 9. Extrinsic apoptotic pathway. (**A**) Total expression of DR5, (**B**) expression of uncleaved and cleaved caspase-10, and (**C**) inhibitor of both extrinsic and intrinsic apoptosis, XIAP, in HCMs incubated for 48-hours with vehicle, 2.5 µM DOX, 50 ng/ml TRAIL, and 2.5 µM DOX + 50 ng/ml TRAIL (n=1).

3.3 DOX-induced cardiotoxicity in TRAIL-knockout mice

DOX-treated wild-type mice had significant reduction in LVEF as compared to vehicle-treated wild-type (~16%, Fig. 10.A). TRAIL$^{-/-}$ DOX-treated mice had no significant reduction in LVEF as compared to their vehicle-treated counterparts (Fig 10.B).

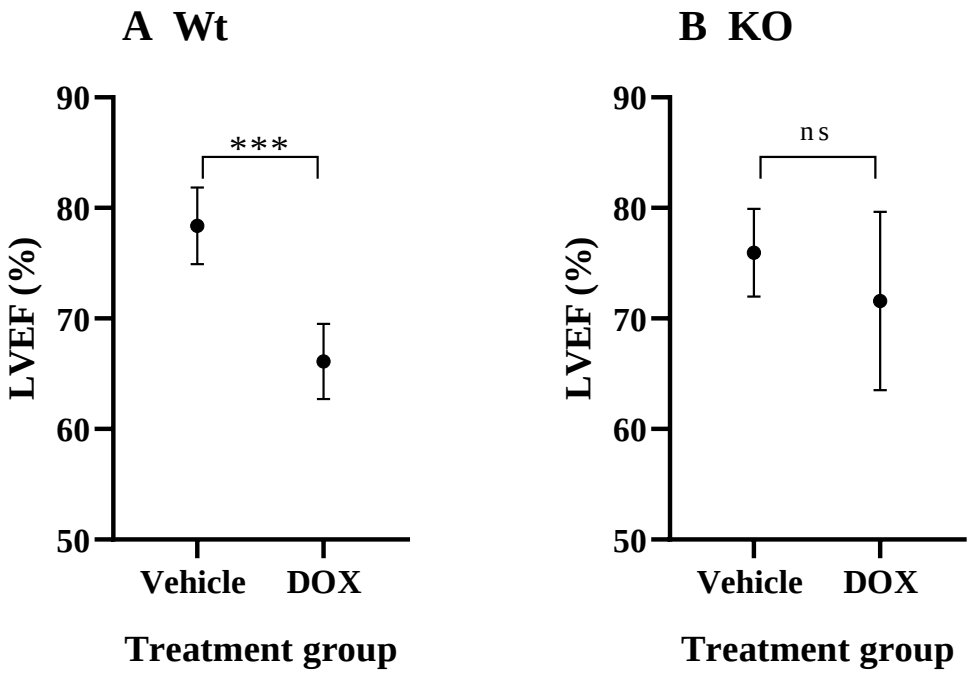


Figure 10. LVEF in (**A**) DOX-treated (n=7) and vehicle-treated wild-type mice (n=8) ($p < 0.001$) and (**B**) DOX-treated (n=7) and vehicle-treated TRAIL$^{-/-}$ mice (n=7). Statistical analysis through 2-way ANOVA. Data are expressed as mean ±SD.

DOX-treated wild-type mice had significantly reduced FS as compared to vehicle-treated wild-type (~24%, Fig 11.A). DOX-treated TRAIL$^{-/-}$ mice had no significant reduction in FS as compared to their vehicle-treated counterparts (Fig 11. B).

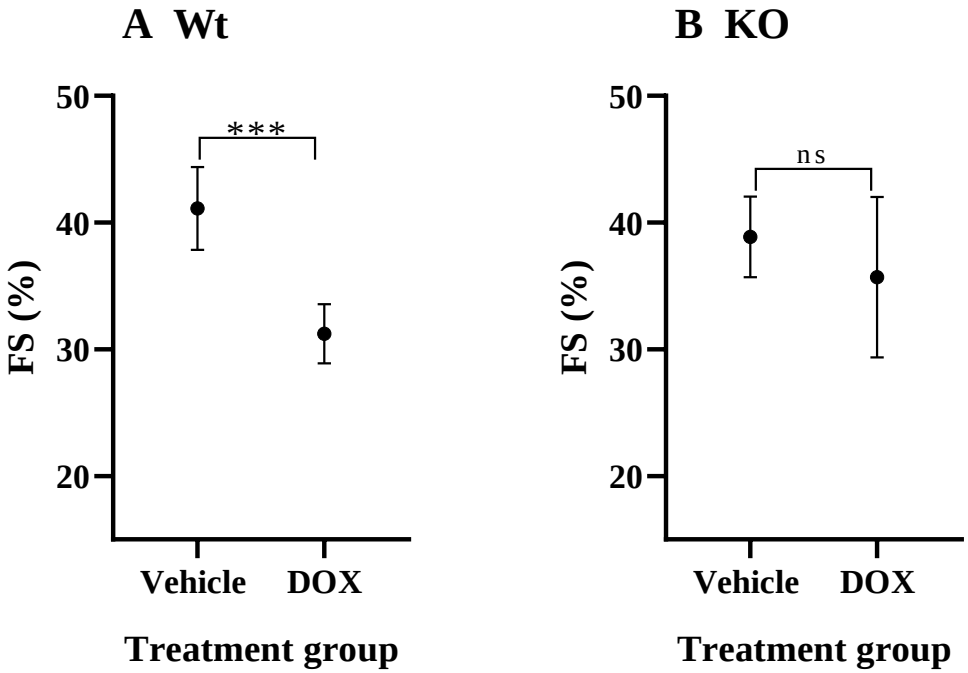


Figure 11. FS in **(A)** DOX-treated (n=7) and vehicle-treated wild-type mice (n=8) ($p<0.001$) and **(B)** DOX-treated (n=7) and vehicle-treated TRAIL$^{-/-}$ mice (n=7). Statistical analysis through 2-way ANOVA. Data are expressed as mean ±SD.

4. DISCUSSION

Studies have shown that treatment of DOX enhances the ability of TRAIL to induce apoptosis in cancer cells[36, 37, 46]. Modulation of the extrinsic and intrinsic apoptotic pathway has been identified as the mechanism in which DOX sensitises cancer cells to TRAIL-induced death[47-49]. Here, we have proposed a similar mechanism is occurring in cardiomyocytes.

When treated with DOX alone, HCMs had no significant decrease in viability. However, DOX caused a significant decrease in viability in both HFFs and TXSAs. An explanation for this decreased viability is that these are highly replicating cells and therefore, sensitivity to DOX is likely due to topoisomerase II inhibition. Interestingly, TXSAs but not HCMs or HFFs were sensitive to the treatment of TRAIL alone, and this may be due to high expression of death receptors on the surface of the TXSAs as reported by Zinonos et al.[50].

As suggested in previous studies[40, 51], TRAIL had pro-survival effects on cardiomyocytes. Following treatment with TRAIL, HCM viability increased, and protein analysis revealed greater presence of apoptotic proteins in inactivated form.

Following treatment with the combination of DOX and TRAIL, all three cell types were affected. This suggests DOX has sensitised HCMs to TRAIL-induced death. The lack of regeneration abilities in cardiomyocytes necessitates long-term survival of these cells in order to prevent irreversible cardiac damage[52]. Therefore, the death of cardiomyocytes, via this DOX and TRAIL apoptotic mechanism, may be deleterious to the heart.

These results are comparable to those reported by Zhao and Zhang. However, they reported higher DOX toxicity at lower concentrations, and this could be because IPS-CMs replicate more rapidly than HCMs[52]. DOX concentrations used in our *in vitro* studies were higher and more relevant to concentrations used clinically[53]. In order to investigate the effects of low TRAIL we also included a 5 ng/ml concentration, similar to what was used in the Zhao and Zhang study.

DOX may have sensitised the cardiomyocytes to TRAIL-induced apoptosis by increasing the expression of DR5. Interestingly, and unlike our findings here, Zhao and Zhang observed the most profound increase in DR4, not DR5[43]. In the same study, a DR5 neutralising antibody was used to prevent apoptosis in IPS-CMs by blocking TRAIL binding, contradicting their observation of DR4 being the primary receptor affected by DOX. Other than replication rates, IPS-CMs differ from adult cardiomyocytes in structural, electrophysiological, and metabiological properties, with similarities to that of embryonic cardiomyocytes[54]. These phenotypic differences between IPS-CMs and adult HCMs may explain the difference in results.

Increased caspase-10 activation following DOX treatment has been observed in cancer cells[55]. In cardiomyocytes, we have observed an increase in caspase-10 activation following co-treatment with DOX and TRAIL. This finding has not been reported previously and may represent an important mechanism by which DOX sensitises cardiomyocytes to TRAIL-induced death.

Decreased expression of XIAP following DOX treatment has been shown in HCMs in one previous study[56]. However, the addition of TRAIL causing a further decrease in XIAP expression in DOX-treated HCMs is a novel finding. Upregulation of DR5 may increase TRAIL-binding, and in addition, decreased inhibition of the pathway downstream, creating a perfect pro-apoptotic environment for the death of cardiomyocytes. Following DOX treatment, changes in proteins which are indicative of intrinsic and extrinsic pathway activation occurred. However, markers of the intrinsic apoptotic

pathway such as BID or caspase-9 activation show negligible change, whereas markers of the extrinsic pathway, such as caspase-10 and DR5, have been upregulated, suggesting cardiomyocyte death is occurring primarily through the extrinsic pathway. Further research into this complex pathway is required to confirm these findings and seek a full understanding of DOX's molecular effects in cardiomyocytes.

In this study pathways other than extrinsic and intrinsic apoptosis were not investigated. Therefore, the effects of DOX treatment on the TRAIL survival pathways in cardiomyocytes remains unexamined. It is also important to consider cardiomyocytes in cell culture do not bear the same strain as cardiomyocytes in the body, which require large amounts of energy to contract continually. Treating cells with DOX and TRAIL cannot replicate the pharmacokinetics in a living body; however, we have supported this data with *in vivo* studies.

Clinically, LVEF and FS values are commonly used to diagnose cardiomyopathies[57]. During chemotherapy, a reduction in LVEF of ≥10% is considered left ventricular dysfunction, and further therapy is discontinued[58]. In DOX-treated wild-type mice, we observed a decrease in LVEF of 24%, suggesting clinically defined DOX-induced cardiotoxicity occurred and was measurable in the wild-type mice. Various studies in wild-type mice have comparable results after treatment with similar concentrations of DOX[59, 60], some of which additionally observed changes in radial strain[61], a more sensitive echocardiographic measurement.

In TRAIL$^{-/-}$ mice, DOX treatment demonstrated no significant reduction in cardiac function, suggesting lack of TRAIL is cardioprotective against DOX. To our knowledge, this is the first *in vivo* report to demonstrate a causative role for TRAIL in DOX-induced cardiotoxicity.

In contrast to previous studies[62, 63], our application of a low dose, long-duration regimen of DOX to induce chronic cardiotoxicity provides a mouse model that is more consistent with morphological

changes observed clinically[64]. A limitation is that mice only have one apoptosis-inducing TRAIL receptor, DR5[65]. This is a homologue of both human DR4 and DR5 (76 and 79% homology, respectively); however, it has similar binding affinity for TRAIL to that of human DR4 and DR5.

Future studies would examine the effect of TRAIL-deficiency on the anticancer efficacy of DOX treatment. Any attempt to block TRAIL-signalling during DOX treatment as a means to protect the heart must not compromise the anticancer efficacy of DOX. To investigate this, a TRAIL-blocking therapeutic would be given in conjunction with DOX treatment in our mouse cancer model, where both cardio-protection and anticancer efficacy is assessed simultaneously.

Potential therapeutics to inhibit the TRAIL-signalling pathway include the use of anti-TRAIL antibodies. These antibodies can bind circulating and membrane-bound TRAIL, preventing it from binding to its death receptors and preventing apoptosis induction. Another potential therapeutic target is using soluble fusion proteins as "decoy receptors" to block TRAIL from binding to DR5. These include soluble receptors DR5 or OPG, which can bind TRAIL with varying degrees of affinity. Some of these proteins have been formulated and tested for their TRAIL-blocking ability *in vitro* and *in vivo*[66, 67], and our preliminary data for blocking the TRAIL-signalling pathway is promising.

5. CONCLUSION

We have shown for the first time that TRAIL is causatively involved in DOX-induced cardiotoxicity, a mechanism previously unexplored in adult primary human cardiomyocytes or *in vivo*. Investigations into blocking TRAIL-signalling could lead to a new class of cardioprotective agents to prevent DOX-induced cardiotoxicity.

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