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# A novel compound DT-010 protects against doxorubicin-induced cardiotoxicity in zebrafish and H9c2 cells by inhibiting reactive oxygen species-mediated apoptotic and autophagic pathways

Fan Tang[a,1], Xinhua Zhou[a,1], Liang Wang[b], Luchen Shan[c], Chuwen Li[a], Hefeng Zhou[a], Simon Ming-Yuen Lee[a,*], Maggie Pui-Man Hoi[a,**]

[a] State Key Laboratory of Quality Research in Chinese Medicine and Institute of Chinese Medical Sciences, University of Macau, Macau
[b] Institute of Biomedical and Pharmaceutical Sciences, Guangdong University of Technology, Guangzhou, China
[c] Institute of New Drug Research, College of Pharmacy, Jinan University, Guangzhou, China

## ARTICLE INFO

*Chemical compounds studied in this article:*
Danshensu (PubChem CID: 11600642) , (R)-3-(3,4-Dihydroxyphenyl)-2-hydroxypropanoic acid
Doxorubicin (PubChem CID: 443939) , (7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl] oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione
DT-010, 4-(3,5,6-trimethylpyrazin-2-yl) hepta-1,6-dien-4-yl (R)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoate
Tetramethylpyrazine (PubChem CID: 14296) , 2,3,5,6-tetramethylpyrazine

*Keywords:*
Cardioprotection
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H9c2 cardiomyocytes

## ABSTRACT

Doxorubicin (Dox) is an effective anti-cancer agent but limited by its cardiotoxicity, thus the search for pharmacological agents for enhancing anti-cancer activities and protecting against cardiotoxicity has been a subject of great interest. We have previously reported the synergistic anti-cancer effects of a novel compound DT-010. In the present study, we further investigated the cardioprotective effects of DT-010 in zebrafish embryos in vivo and the molecular underlying mechanisms in H9c2 cardiomyocytes in vitro. We showed that DT-010 prevented the Dox-induced morphological distortions in the zebrafish heart and the associated cardiac impairments, and especially improved ventricular functions. By using H9c2 cells model, we showed that DT-010 directly inhibited the generation of reactive oxygen species by Dox and protected cell death and cellular damage. We further observed that DT-010 protected against Dox-induced myocardiopathy via inhibiting downstream molecular pathways in response to oxidative stress, including reactive oxygen species-mediated MAPK signaling pathways ERK and JNK, and apoptotic pathways involving the activation of caspase 3, caspase 7, and PARP signaling. Recent studies also suggest the importance of alterations in cardiac autophagy in Dox cardiotoxicity. We further showed that DT-010 could inhibit the induction of autophagosomes formation by Dox via regulating the upstream Akt/AMPK/mTOR signaling. Since Dox-induced cardiotoxicity is multifactorial, our results suggest that multi-functional agent such as DT-010 might be an effective therapeutic agent for combating cardiotoxicity associated with chemotherapeutic agents such as Dox.

## 1. Introduction

The cardiotoxicity induced by broad-spectrum anti-cancer anthracyclines such as doxorubicin (Dox) may manifest as an initial acute effect with transient tachycardia, or a delayed cardiomyopathy with cumulative dose-dependent congestive heart failure that persists long after drug treatment discontinued (Swain et al., 2003; Volkova and Russell, 2011). Therefore, combination therapies with cardioprotective agents to improve cancer treatment regimens have been a subject of great interest (Liu et al., 2017; Ojha et al., 2016). Previous studies demonstrated DT-010, a derivative of danshensu (DSS) and tetramethylpyrazine (TMP) (Fig. 1), was an cardioprotective agent against ischemic myocardial injury (Zhang et al., 2017), and in MCF-7 breast cancer cells enhanced Dox chemotherapeutic efficacy (Wang et al., 2016a, 2016b). In the present study, we aimed to further investigate the cardioprotective potential and the underlying mechanisms of DT-010 against Dox-induced cardiotoxicity.

Dox-induced cardiotoxicity is multifactorial and not yet fully understood (Cappetta et al., 2017). A number of molecular mechanisms were implicated in the pathogenesis of Dox-induced cardiotoxicity,

* Correspondence to: Institute of Chinese Medical Sciences, University of Macau, Avenide da Universidade, Room 7003, N22 Building, Taipa, Macau.
** Correspondence to: Institute of Chinese Medical Sciences, University of Macau, Avenide da Universidade, Room 7012, N22 Building, Taipa, Macau.
*E-mail addresses:* simonlee@umac.mo (S.M.-Y. Lee), maghoi@umac.mo (M.P.-M. Hoi).
[1] These authors contributed equally to this work.

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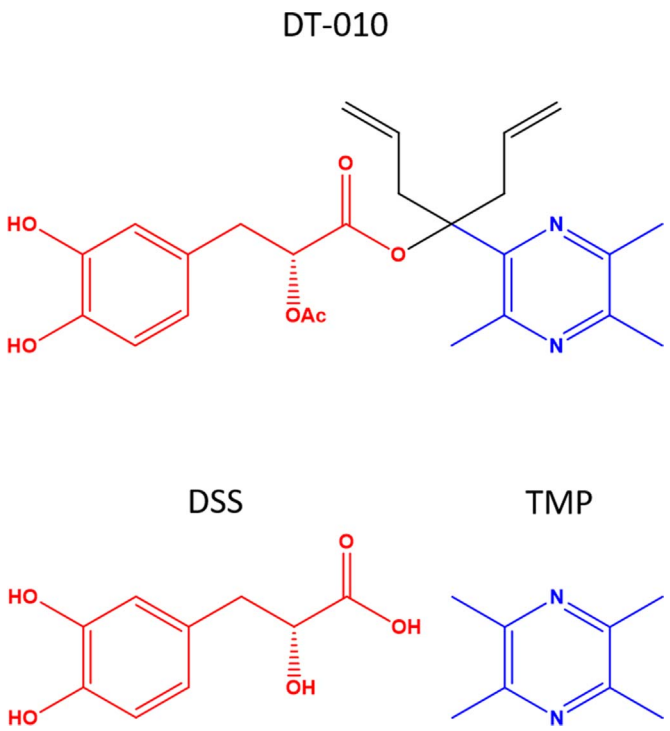


**Fig. 1.** Chemical structures of DT-010 and its parent compounds danshensu (DSS) and tetramethylprazine (TMP).

including reactive oxygen species generation, mitochondrial dysfunction, cardiomyocytes apoptosis, and autophagy dysregulation (Arola et al., 2000; Dirks-Naylor, 2013; Octavia et al., 2012). It is known that Dox produces reactive oxygen species through multiple pathways at intracellular locations such as mitochondria, sarcoplasmic reticulum and cytoplasmic compartments (Sterba et al., 2013). Besides, persistently high levels of intracellular reactive oxygen species trigger mitochondrial dysfunction and apoptotic signaling pathways in cardiomyocytes (Minotti et al., 2004; Octavia et al., 2012). Furthermore, damaged mitochondria become a major source of reactive oxygen species promoting pathological production and accumulation of oxidative stress in cardiomyocytes (Chen and Zweier, 2014).

More recently, the dysregulation of autophagy in the myocardium was shown to play a contributing role in Dox-induced cardiomyopathy (Bartlett et al., 2017). Autophagy is a cellular homeostasis process that removes and/or recycles damaged and unwanted proteins for normal function and new cell formation (Mizushima and Komatsu, 2011). Therefore, autophagy is likely to be stimulated in response to stress stimuli such as redox injury and mitochondrial damage triggered by Dox. Dox-induced alterations in cardiac autophagy was reported in various in vitro and in vivo models, although whether Dox upregulates or downregulates autophagy is not yet clear (Dirks-Naylor, 2013). Moreover, it is now recognized that the interplay between autophagy and apoptosis plays very important roles in heart diseases (Marino et al., 2014; Nishida et al., 2008). Several stress-responsive signaling pathways, such as the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), regulate both autophagy and apoptosis (Marino et al., 2014; Xu et al., 2015; Zhou et al., 2015).

In the present study, we showed that DT-010 co-treatment significantly prevented cardiac impairments induced by Dox in *Tg (cmlc2:GFP)* zebrafish in vivo. We further elucidated potential molecular mechanisms underlying the cardioprotective effects of DT-010 in H9c2 cardiomyocytes. We showed that DT-010 reduced the generation of reactive oxygen species, inhibited reactive oxygen species-mediated ERK and JNK activation and caspase-dependent apoptosis induced by Dox. In addition, DT-010 inhibited Dox-induced autophagy activity by inhibiting autophagosome formation via upstream Akt/AMPK/mTOR signaling.

## 2. Materials and methods

### 2.1. Materials

DT-010 (purity > 98%, Fig. 1) was synthesized at Jinan University, China. Danshensu (DSS) was purchased from Xian Honson Biotechnology company (Xian, Shanxi, China). Tetramethylpiperidine (TMP) was purchased from Shanghai Banghai Chemical cooperation (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), Penicillin/Streptomycin (Pen/Strep) were purchased from Thermo Fisher Scientific company (Waltham, MA, USA). P-AKT/AKT, P-AMPK/AMPK, P-mTOR/mTOR, LC3A/B, Cl-Caspase-7/Caspase7, Cl-Caspase3/Caspase3, Cl-PARP/PARP, P-ERK/ERK, P-JNK/JNK, β-actin, HRP-linked antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was purchased from Invitrogen (Carlsbad, CA, USA). Rapamycin, 3-Methyladenine (3-MA) and N-Acetyl-L-cysteine (NAC) were from Sigma (St Louis, MO, USA). Amersham ECL Prime Western Blotting Detection Reagent was purchased from GE Healthcare Life Sciences (Chicago, IN, USA).

### 2.2. Maintenance of zebrafish

Transgenic zebrafish *Tg(cmlc2:GFP)* with GFP (green fluorescent protein) specifically expressed in the myocardial cells were used following instructions as previously described (Huang et al., 2003). The maintenance of zebrafish was performed according to the Zebrafish Handbook (Westerfield, 1995). Briefly, adult zebrafish were kept at aquaculture system with the light cycles and fed with brine shrimp twice a day. Zebrafish experiments were conducted under the standard regulations (Ethical guidelines of the ICMS, University of Macau).

### 2.3. Zebrafish embryo collection and drug treatment

The zebrafish embryos were prepared as previously reported (Chen et al., 2015; Tang et al., 2010). Briefly, pair-wise mating (3–12 months old) was used to generate zebrafish embryos. Fertilized embryos were selected by microscopic examination for further experiments. Embryos were then raised in embryo medium containing 1-phenyl-2-thiourea (200 mM) at 27 ± 1 °C for 2 days. Zebrafish embryos of 2 days post-fertilization (dpf) were distributed into a 12-well microplate (12–15 fish per well), and were treated with Dox (35 μM) in the presence or absence of DT-010 (20, 30, or 40 μM) for another 48 h. Four parameters (heartbeat, stroke volume, cardiac output, fractional shortening) were calculated to evaluate the cardiac functions. Movie sequences of zebrafish heart were recorded by the Cell^R imaging system comprising IX71 microscope. Measurements of cardiac functions were performed as previously described (Wang et al., 2016c). Briefly, the lengths of longitudinal axis (a) and lateral axis (b) were measured and ventricular volume was calculated with the formula $V = 4/3\pi ab^2$. Stroke volume was the difference between end-diastolic volume and end-systolic volume. Cardiac output was the product of stroke volume and heartbeats (1 min). Fractional shortening (%) was calculated by formula: (diastolic diameter – systolic diameter)/(systolic diameter) × 100%.

### 2.4. Cell culture

H9c2 cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100IU ml-1 penicillin-streptomycin (Pen/Strep) in a humidified incubator with 5% $CO_2$ at 37 °C. The cells were used until they reached 80% confluence, and were differentiated with 10 nM retinoic acid for 7 days in DMEM medium