Prodrug of ICRF-193 provides promising protective effects against chronic anthracycline cardiotoxicity on a rabbit model in vivo

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Abstract

The anthracycline (ANT) anticancer drugs such as doxorubicin or daunorubicin (DAU) can cause serious myocardial injury and chronic cardiac dysfunction in cancer survivors. A bisdioxopiperazine agent dexrazoxane has been developed as a cardioprotective drug to prevent these adverse events, but it is uncertain whether it is the best representative of the class. This study used a rabbit model of chronic ANT cardiotoxicity to examine another bisdioxopiperazine compound called GK-667, a water-soluble prodrug of ICRF-193, as a potential cardioprotectant. The cardiotoxicity was induced by DAU (3 mg/kg, i.v. weekly, 10 weeks), and GK-667 (1 or 5 mg/kg, i.v.) was administered before each DAU dose. The treatment with GK-667 was well tolerated and provided full protection against DAU-induced mortality and left ventricular (LV) dysfunction (determined by echocardiography and LV catheterization). Markers of cardiac damage/dysfunction revealed minor cardiac damage in the group co-treated with GK-667 in the lower dose, whereas almost full protection was achieved with the higher dose. This was associated with similar prevention of DAU-induced dysregulation of redox and calcium homeostasis proteins. GK-667 dose-dependently prevented p53-mediated DNA damage response in the LV myocardium not only in the chronic experiment but also after single DAU administration. These effects appear essential for cardioprotection, presumably because of the topoisomerase IIβ inhibition provided by its active metabolite ICRF-193. In addition, GK-667 administration did not alter the plasma pharmacokinetics of DAU and its main metabolite daunorubicinol in rabbits in vivo. Hence, GK-667 merits further investigation as a promising drug candidate for cardioprotection against chronic ANT cardiotoxicity.
Keywords

Anthracycline cardiotoxicity, cardioprotection, dexrazoxane, bisdioxopiperazine, ICRF-193, topoisomerase II beta

Abbreviations

ANP, atrial natriuretic peptide; ANT, anthracycline; AUC, area under the curve; BNP, brain natriuretic peptide; cTnT, cardiac troponin T; CTR, control; DAU, daunorubicin; DAUol, daunorubicinol; DEX, dexrazoxane; GK-627 (rac-ICRF-239), (±)-4,4’-(propane-1,2-diyl)bis(1-methylpiperazine-2,6-dione); GK-667, meso-(butane-2,3-diyl)bis(2,6-dioxopiperazine-4,1-diyl)bis(methylene)-bis(2-aminoacetate) hydrochloride; HO1, heme oxygenase 1; ICRF-193, meso-4,4’-(butan-2,3-diyl)bis(piperazine-2,6-dione); kDNA, kinetoplast DNA; LMV, last measured value; LV, left ventricle/ventricular; LVFS, left ventricular fractional shortening; MDM2, MDM2 proto-oncogene; NOX2, NADPH oxidase 2; NOX4, NADPH oxidase 4; p16 (CDKN2A), cyclin dependent kinase inhibitor 2A; p21 (CDKN1A), cyclin dependent kinase inhibitor 1A; p53, tumor suppressor p53; p53R2, ribonucleotide reductase regulatory TP53 inducible subunit M2B; ROS, reactive oxygen species; RT-qPCR, reverse transcription quantitative real-time PCR; RyR2, ryanodine receptor 2; SERCA2, sarcoplasmic/endoplasmic reticulum calcium ATPase 2; SOD2, mitochondrial superoxide dismutase 2; TOP2B, topoisomerase IIβ.
1. Introduction

Although approved for clinical use almost 50 years ago, anthracycline (ANT) anticancer drugs such as doxorubicin, epirubicin, or daunorubicin (DAU), are still indispensable in many current chemotherapeutic protocols. However, ANTs can also induce a cumulative dose-dependent cardiotoxicity, which is largely irreversible and can present as cardiomyopathy and heart failure several months or years after the chemotherapy [1, 2]. ANT-induced cardiotoxicity has been traditionally associated with oxidative damage to the heart [3, 4]; therefore, different natural and synthetic antioxidants and reactive oxygen species scavengers have been widely studied as potential cardioprotectants. However, no such agent has been successfully translated into clinical settings to protect the hearts of cancer patients against ANT toxicity [5, 6]. More recently, an alternative theory of ANT cardiotoxicity development has been proposed [7, 8]. It postulates that ANT cardiotoxicity is triggered by a specific interaction of ANTs with topoisomerase IIβ (TOP2B) in the heart, which results in DNA damage and a series of other downstream events leading to myocardial injury and heart failure.

The bisdioxopiperazine agent dexrazoxane (DEX, ICRF-187) (Suppl. Fig. 1B) is the only cardioprotective drug approved by regulatory authorities to prevent ANT cardiotoxicity in clinical practice [5, 9]. It has been long perceived as a prodrug that prevents ANT-induced oxidative damage through iron-chelating effects of its active metabolite—ADR-925; however, more recent findings point at the interaction of the parent compound with TOP2B [10-12]. The clinical use of DEX has been limited owing to initial uncertainty about an interference with the anticancer effect of ANTs and/or the increased risk of secondary malignancies. While these concerns were not confirmed by outcomes of the vast majority of randomized clinical trials performed so far and their meta-analyses, a reluctance towards its wider clinical use remains and is only slowly and partially subsiding [5, 9, 13].
Although bisdioxopiperazine agents have been systematically studied for their anticancer effects [14, 15], analyses of their cardioprotective effects were less frequent and comprehensive. Therefore, it has not been clear whether a more potent and suitable derivative could be found in this drug class to serve as a cardioprotectant against ANT cardiotoxicity. Ours and other research groups have previously observed that even very minor modifications to the chemical structure of DEX can markedly diminish or completely abolish its cardioprotective potential against ANT cardiotoxicity and this corresponded well with decreased/abated TOP2(B) interactions [11, 12, 16, 17]. These results have been explained by protein crystallography, docking and molecular modelling studies, which confirmed that the so-called DEX-binding site on the TOP2 enzyme provides only limited scope for structural modifications of bisdioxopiperazines [12, 18, 19]. Conversely, we and others have recently found that ICRF-193 (Suppl. Fig. 1A), which differs from DEX by only one methyl group, shows markedly higher potency than DEX in both TOP2B inhibition and cytoprotection against ANT cardiotoxicity in vitro [12, 19]. ICRF-193 is the only bisdioxopiperazine derivative that has been identified to have superior potency than DEX, but its very poor solubility in aqueous media precluded testing beyond in vitro studies. To overcome this issue, we have recently prepared and characterized its water-soluble prodrugs and identified compound GK-667 (Suppl. Fig. 1A) as a new candidate for in vivo investigations [20]. GK-667 showed rapid release of active ICRF-193 both in vitro (in cell culture media and rabbit plasma) and in vivo (in rabbits). Incubation of isolated neonatal cardiomyocytes with GK-667 induced significant intracellular concentrations of active ICRF-193 and showed high cytoprotective potency against ANT toxicity in this in vitro bioassay. Furthermore, the in vitro toxicity of GK-667 itself was low, and its administration to rabbits resulted in promisingly high plasma concentrations in vivo [20]. However, the translatability of these findings to complex in vivo conditions of chronic experiments was still unclear.

Therefore, we aimed to determine whether the selected prodrug of ICRF-193, i.e., compound GK-667, can provide a well-tolerated, potent, and dose-dependent protection against chronic ANT...
cardiotoxicity in a DEX-validated in vivo rabbit model. Furthermore, molecular aspects of the cardiotoxicity and cardioprotection were addressed with a particular focus on DNA damage response. We also investigated whether the diminished cardiotoxicity is associated with altered plasma concentrations of ANT (or its major metabolite) in vivo.
2. Materials and Methods

2.1 Drugs and chemicals

GK-667 (prodrug of ICRF-193), ICRF-193, and GK-627 (rac-ICRF-239) (Suppl. Fig. 1C) were synthesized and characterized in-house as described previously [11, 20]. GK-667 and GK-627 were used as tetra- and dihydro-chloride salts, respectively. DAU (hydrochloride salt, pharmaceutical grade) was purchased from Euroasia’s (India). The identity and purity of all studied substances (including the purchased DAU) were confirmed in-house by high-performance liquid chromatography mass spectrometry (HPLC–MS). For in vivo experiments, all the solutions were prepared freshly prior to administration by dissolution of the substances in saline (B. Braun, Germany) followed by filtration through an antimicrobial filter (0.22-µm porosity, Carl Roth GmbH + Co. KG, Germany).

2.2 Animal experiments

Adult, male New Zealand White rabbits (n=104, age: 12–16 weeks; Velaz, Czech Republic) were used. The study was approved by the Animal Welfare Committee of Charles University, Faculty of Medicine in Hradec Králové (No. 12981/2019-2) and was carried out in the same institution in accordance with EU Directive 2010/63/EU and ARRIVE Guidelines [21]. Animals were caged individually under standard conditions (temperature 18°C, relative humidity: 40–50%, 12-h–long illumination period) with ad libitum access to a standard rabbit chow diet (KO-16; Velas, a.s., Czech Republic) and tap water. The behavior and mortality of the rabbits were monitored daily, while body weight was recorded weekly. All animals underwent at least 2 weeks of acclimatization in the animal unit before randomization to study groups.

2.2.1 In vivo chronic cardioprotection study

The rabbits (3.4±0.2 kg, n=47) were randomly divided into five groups receiving intravenous (i.v.) treatment to the marginal ear vein once a week for 10 consecutive weeks. Chronic cardiotoxicity was induced with DAU (3 mg/kg, n=10). GK-667 was administered as either a 1 mg/kg (n=10) or 5 mg/kg
(n=10) dose, 30 min before each DAU dose to the contralateral ear. Doses and intravenous route of administration of GK-667 were set based on the previous pharmacokinetic study [20] and pilot experiments probing tolerability and efficacy of its combination with DAU. The timing of administration with respect to DAU was adopted from recommendations for clinical use of DEX [22] and previous experiments with DEX in our rabbit model [11, 23]. The reference group (n=7) received GK-667 alone in the higher studied dose (i.e., 5 mg/kg), while the control group (n=10) received saline (1 ml/kg).

Mild anesthesia comprising ketamine (30 mg/kg; Narkamon 100 mg/mL; Bioveta a.s., Czech Republic) and midazolam (1.25 mg/kg; Midazolam Kalceks; Kalceks AS, Latvia) was administered intramuscularly for non-invasive procedures. Individually titrated pentobarbital anesthesia (approximately 10 mg/kg, i.v.; Sigma-Aldrich, USA) was used for invasive hemodynamic measurement of cardiac function. Animals were then euthanized with pentobarbital overdose.

During the necropsy, peritoneal and pleural effusions were monitored. The heart was rapidly excised, washed, and briefly retrogradely perfused with ice-cold saline. The transverse sections of the heart ventricles were obtained for histological examination. The rest of the left ventricular (LV) myocardium was shock-frozen in liquid nitrogen. Frozen samples were later pulverized in liquid nitrogen and stored at -80°C until further analyses.

**Cardiac function examination**

The LV systolic function was examined noninvasively by echocardiography (Vivid 4, 10 MHz probe; GE Healthcare, USA) at the beginning of the experiment and weekly from the 8th week until the end of the experiment. LV fractional shortening (LVFS) was calculated from LV end-systolic and end-diastolic diameters obtained during left parasternal long- and short-axis M-mode scanning, as described previously [24].
Invasive hemodynamic measurement of LV function was performed at the scheduled end of the study. A Mikro-Tip pressure catheter (2.3F; Millar instruments, USA) connected to a data acquisition system (Powerlab; ADInstruments Pty., New Zealand) was inserted into the LV via the left common carotid artery. Index $dP/dt_{max}$ (first derivative of the LV pressure change in the isovolumic contraction) was calculated using LabChart 6 software (ADInstruments Pty). The stabilization period for each animal was at least 15 min.

**Cardiac troponin T analysis in plasma**

Before the 1st, 5th, 8th, and 10th drug administration and before the invasive hemodynamic measurements at the end of the study, blood samples were taken from the central ear artery. Elecsys Troponin T-high sensitive STAT test (Roche Diagnostics, Switzerland), with a detection limit of 0.003 μg/L, was used for determination of plasma concentrations of cardiac troponin T (cTnT).

**Histological examination of ventricular myocardium**

The transverse sections of the heart ventricles were immersed in 4% neutral formaldehyde and embedded in paraffin. Serial paraffin sections (5-μm thick) were stained with Masson’s blue trichrome. Photomicrographs were taken using an Olympus BX51 microscope equipped with a DP71 digital camera (Olympus, Japan) and QuickPHOTO 3.0 software (PROMICRA, Czech Republic).

**2.2.2 Acute in vivo experiments for analysis of p53-dependent DNA damage response in the LV myocardium after single DAU dose**

The rabbits (3.4±0.1 kg, n=35) were randomized into five groups (n=7/group). The first group received single dose of DAU (3 mg/kg, i.v.), while the control group received saline (1 ml/kg, i.v.). Two other groups received GK-667 before the single DAU dose exactly like in the chronic study (1 or 5 mg/kg, i.v. 30 min before DAU into the contralateral ear). Serving as a negative control, the last group
of animals received compound GK-627, another close DEX derivative, which was unable to inhibit TOP2B and provide any protection against chronic ANT cardiotoxicity in rabbits in our previous study [11]. The dosing schedule for GK-627 was adopted from that previous study (60 mg/kg intraperitoneally 30 min before the DAU dose). The study was completed 6 h after the DAU dose, when the rabbits were euthanized by pentobarbital overdose. This time interval was selected according to pilot experiments monitoring DNA damage response in the LV up to 24 h after single DAU dose.

2.2.3 In vivo study to analyze the potential effects of GK-667 administration on plasma pharmacokinetics of DAU

While the pharmacokinetics of GK-667 in rabbits has been reported previously [20], it remained unknown whether the pharmacokinetics of DAU and its main metabolite daunorubicinol (DAUol) are altered by GK-667 administration.

The rabbits (3.3±0.1 kg, n=22) were treated with single dose of DAU (3 mg/kg, i.v.) either alone (n=11) or in combination with the higher studied dose of GK-667 (i.e., 5 mg/kg, i.v., n=11), and the experiment was completed 12 or 24 h after DAU administration (n=5 and n=6, respectively). During the experiment, blood was sampled at predefined intervals between 3 min and 24 h. Blood sampling was omitted between 8 and 12 h in rabbits sampled for 24-h period. Plasma was immediately separated from whole blood, shock-frozen in liquid nitrogen, and stored at -80°C until further analysis.

Rabbit plasma (200 μL) was spiked with isotopically labelled internal standard solutions (daunorubicin-13C-d3 and daunorubicinol-13C-d3, Toronto Research Chemicals, Canada). Samples were mixed with 1800 μL of a mixture of chloroform:methanol, (4:1, v/v), and DAU and DAUol were extracted by vortexing (5 min, 8–12×100 rpm). The resulting organic layer was taken and evaporated
to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of methanol, vortexed, filtered, and 10 μL of the sample were injected onto the column. The analysis was carried out on a Kinetex C18 column (100x2.1 mm, 1.7 μm) with the same type of guard column (both Phenomenex, USA). The column was maintained at 40°C and the autosampler thermostat at 8°C. The mobile phase A was 0.0025% formic acid in water (v/v), and mobile phase B was acetonitrile. The following gradient was used: 0.0–4.0 (20–60% B) and 4.1–5.5 (20% B). The flow rate was set at 0.35 mL/min. Agilent 1290 Infinity II LC with Triple Quad LC/MS–6400 series (Agilent Technologies, USA) equipped with Agilent Jet Stream–Electrospray Ionization operating in a positive mode was used. Quantitation was performed in the selected reaction monitoring mode. The data were analyzed by Agilent MassHunter Quantitative Analysis software (Agilent Technologies). The method was validated within the concentration range 0.25–1000 ng/mL for both DAU and DAUol in plasma according to the EMA Guideline on bioanalytical method validation [25].

2.3 Molecular analyses in LV myocardium

2.3.1 RNA isolation and quantitative real-time PCR

Total RNA was isolated with TRI Reagent (Sigma-Aldrich), reversely transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit, and qPCR analysis was performed with a QuantStudio 7 Flex Real-Time PCR System using TaqMan Fast Universal PCR Master Mix (all from Applied Biosystems, USA). Assays for gene expression analysis of ANP, FNI, p16, RyR2, and SERCA2 were also purchased from Applied Biosystems. Assays for gene expression analysis of BNP, COL1A1, COL4A2, HO1, HPRT1, MDM2, NOX2, NOX4, p21, p53R2, and SOD2 were purchased from Generi Biotech, Czech Republic. Detailed description of the assays can be found in Supplementary Table 1. The relative gene expression was calculated using the Pfaffl method [26] with hypoxanthine phosphoribosyltransferase 1 (HPRT1) used as a reference gene.

2.3.2 Western blot analysis of p53 protein level
The LV myocardium was homogenized in Cell Lysis Buffer (Cell Signaling Technology, USA) supplemented with protease (cOmplete™, EDTA-free Protease Inhibitor Cocktail; Roche Diagnostics, Switzerland) and phosphatase inhibitors (Halt™ Phosphatase Inhibitor Cocktail; Thermo Fisher Scientific Inc., USA). Proteins were separated using TGX Stain-Free precast gels (Bio-Rad, USA) and transferred onto a PVDF membrane (Immobilon-P; Sigma-Aldrich). Incubation with mouse anti-p53 purified primary antibody (BP53-12; Exbio Praha a.s., Czech Republic; dilution 1:1000) and anti-mouse secondary antibody (P0447, Polyclonal Goat Anti-Mouse Immunoglobulin/HRP; DAKO Denmark A/S, Denmark; dilution 1:1000) followed. BM Chemiluminescence Western Blotting Substrate (Roche) and Fusion Solo S coupled with CCD camera (Vilber Lourmat Sté, France) were used for signal detection. Densitometric quantification of the bands was done in Quantity One software (Bio-Rad). The total protein in each lane on the PVDF membrane was visualized with Stain-Free imaging technology (Bio-Rad).

2.3.3 Caspase activity assay
LV myocardium was homogenized in Cell Lysis Buffer (BioVision, USA) on ice. Activity of executioner caspase 3 was determined using luminescence Caspase-Glo® 3/7 Reagent (Promega Corporation, USA) assay according to the manufacturer’s instructions. The luminescence was measured on microplate reader Tecan M200Pro (Tecan Group, Switzerland) using a kinetic protocol.

2.4 In vitro experiments
2.4.1 In vitro inhibition of TOP2B activity
Recombinant human TOP2B (Inspiralis, UK) was incubated for 30 min at 37°C with kDNA (isolated in-house as described previously [27]) in a reaction buffer (55 mM Tris-HCl, pH 7.5, 135 mM NaCl, 10 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 3 mM ATP, 100 μg/mL bovine serum albumin) with different concentrations (1–1000 µM) of GK-667, ICRF-193, and GK-627 diluted in dimethyl sulfoxide (final concentration: 1%). The reaction was stopped by addition of equal volume of gel loading buffer (pH...
8.0) consisting of 40% (w/v) sucrose, 10 mM EDTA, 0.5 mg/mL bromophenol blue, and 100 mM Tris-HCl. The samples were loaded on 1% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and electrophoresed at 3 V/cm for approximately 1 h. Gels were stained with SYBR™ Safe (Thermo Fisher Scientific Inc.) and visualized using a Gel Doc EZ with ImageLab software (Bio-Rad).

2.4.2 Antiproliferative effects of GK-667 alone and in combination with DAU

Leukemic cell line HL-60 (American Type Culture Collection, USA) was cultured as described previously [11]. For antiproliferative activity assessment, the cells were plated on 96-well plates (10,000 cells/well) and incubated with DAU (15 nM = IC₅₀) and/or GK-667 (0.1–100 μM) dissolved in dimethyl sulfoxide (final concentration in each well: 0.1%) for 72 h at 37°C. The MTT assay described by Vávrová et al. [28] was used to determine the proliferation rate of the cells. For each concentration, 4–5 independent experiments were performed.

2.5 Data analysis

Data were analyzed for statistical significance (p<0.05) by SigmaStat 3.5 software (SPSS, USA) using one-way ANOVA (followed by Holm–Sidak’s post-hoc test), ANOVA on ranks (followed by Dunn’s post-hoc test), t-test or Mann–Whitney U test according to the data characteristics and type of particular comparison. Graphical presentation of the data and the area under the curve (AUC) values were determined using GraphPad Prism 8.3 (GraphPad Software, USA). Data are presented as means±SD or as medians with box and whisker plots representing the interquartile range and 5th–95th percentile, respectively, unless stated otherwise.
3. Results

3.1 In vivo chronic cardioprotective study

3.1.1 Co-treatment of rabbits with GK-667 and DAU was well tolerated and prevented DAU-induced premature deaths, pathological post-mortem findings, and increase in cardiac damage biomarkers

DAU (3 mg/kg) administered weekly for 10 weeks induced premature deaths in 2 out of 10 animals (in the 9th and 10th week of the experiment, i.e., 20% mortality). Necropsy examination of these animals showed marked biventricular dilation and massive hydrothorax and ascites which confirmed that the premature deaths were due to the development of ANT cardiotoxicity. In contrast, all animals in both combination groups of GK-667 with DAU survived until the end of the study, and the same was true for the group with GK-667 alone.

Repeated administration of GK-667 alone was well tolerated by rabbits, as evidenced by body weight gain matching the control group (relative body weight at the end of the experiment vs. at the beginning of the study was 120.1±4.1% and 119.4±12.5% in the GK-667 alone and control group, respectively). The combination of GK-667 with DAU was also well tolerated, as documented by insignificantly higher relative body weight at the end of the study as compared with the DAU group (116.3±7.3%, 115.8±8.9%, and 108.1±9.3% in the GK667(1)+DAU, GK-667(5)+DAU, and DAU group, respectively). No conspicuous changes in animal appearance/behavior and no local damage at the site of administration of GK-667 was noted. In the DAU group, apparent signs of blood congestion were found during the necropsy of animals at the end of the study or after the premature death (total incidence of hydrothorax and ascites in this group was 30% and 40%, respectively). No such signs were found during necropsy of animals from both combination groups, GK-667-alone, and the control group.

Plasma concentrations of cTnT, as a selective and sensitive marker of cardiac damage, were significantly higher in the DAU group than the control group at the end of the experiment (Fig. 1A) and
the same was true for the AUC of cTnT plasma concentrations determined during the whole experiment (Fig. 1B). Co-treatment with GK-667 in the lower dose (i.e., 1 mg/kg) decreased the DAU-induced cTnT levels, but it was still significantly higher than in the control group. Co-treatment with the higher dose of GK-667 (i.e., 5 mg/kg) prevented the significant increase of cTnT, and the results were significantly different from the DAU group. Treatment with GK-667 alone had no significant influence on cTnT levels.

3.1.2 GK-667 co-treatment prevented DAU-induced LV dysfunction

Echocardiographic examination of LV systolic function showed a significant decline in LVFS in the DAU group (Fig. 1C), whereas no change was detectable in both combination groups also receiving GK-667; the LVFS values in the combination groups were also significantly higher than those in the DAU group. In animals surviving until the scheduled end of the experiment, the LV systolic function was examined also by LV catheterization with dP/dt_max as the main parameter of systolic function (Fig. 1D). This approach independently confirmed the echocardiographic findings and yielded the same statistically significant differences. The treatment with GK-667 alone did not have any impact on LV systolic function in both examinations.

Determination of gene expression of biomarkers of wall stress and heart failure (atrial and brain natriuretic peptide – ANP and BNP) in LV myocardium revealed a statistically significant rise in the DAU group with a significant interindividual variability reflecting largely severity of the LV dysfunction (Fig. 1E–F). Co-treatment with GK-667 in the lower dose (1 mg/kg) decreased median values of both biomarkers, but the results were not significantly different to the DAU group and remained significantly higher than in the control group. Co-treatment with GK-667 in the higher dose (5 mg/kg) was more effective and prevented the significant induction of expression of both biomarkers. In the case of ANP, statistically significant improvement as compared with the DAU group was found. The treatment with GK-667 alone had no significant impact on the gene expression of both biomarkers.
3.1.3 GK-667 co-treatment prevented DAU-induced morphological changes in the myocardium and expression of markers of fibrosis

Histological examination of the myocardium in the DAU group confirmed the presence of conspicuous focal degenerative changes particularly in the LV free wall and interventricular septum. This consisted of loss of myofibrils and vacuolization of cardiomyocyte cytoplasm (Fig. 2A). More pronounced damage resulted in cell death of cardiomyocytes that was healed with replacement fibrosis. These changes were less frequent and severe in the group co-treated with the lower dose of GK-667, but the improvement was only partial as some degenerative changes were still recognizable. However, co-treatment with the higher dose of GK-667 yielded almost full protection from these changes as nearly intact myocardium was observed in all animals of this group. The treatment with GK-667 alone had no influence on the myocardial morphology.

The quantitative examination of gene expression of fibrosis markers in the LV myocardium (fibronectin 1, collagen I, and collagen IV) (Fig. 2B–D) confirmed their marked increase in the DAU group. The group that received co-treatment with the higher dose of GK-667 yielded significantly lower values of all three markers than the DAU group. In the case of fibronectin 1 and collagen I, the results matched the value of the control group. The results obtained with the lower dose of GK-667 showed only partial improvements in terms of both quantitative and statistical outcomes.

3.1.4 GK-667 co-treatment prevented DAU-induced impairment of expression of proteins involved in redox and calcium homeostasis in the LV myocardium

The DAU treatment induced a marked and significant increase of gene expression of ROS-generating enzymes in the LV myocardium (NADPH oxidase 2 and 4 – NOX2 and NOX4, Fig. 3A–B), increased expression of antioxidant and cytoprotective heme oxygenase 1 (HO1, Fig. 3C), and decreased expression of mitochondrial antioxidant superoxide dismutase 2 (SOD2, Fig. 3D). These changes were
reduced by GK-667 co-treatment, especially with the higher dose. The results were quite similar to those of the control group and were significantly different to those observed in the DAU group in all parameters. In the group co-treated with the lower dose of GK-667 only moderate quantitative changes were observed, but these were rather rarely statistically significant as compared with the DAU group.

Gene expression of calcium handling proteins (sarcoplasmic/endoplasmic reticulum calcium ATPase 2 – SERCA2 and ryanodine receptor 2 – RyR2) was also considerably lower in the LV myocardium of the animals from the DAU group (Fig. 3E–F). The combination of GK-667 in the lower dose significantly reduced the decline induced by DAU administration whereas almost complete protection was found in the combination group with the higher dose of the studied drug.

3.1.5 GK-667 co-treatment prevented DAU-induced DNA damage response and activation of executioner apoptotic caspases in the LV myocardium

Chronic DAU treatment induced significant accumulation of myocardial p53, which is a key molecule orchestrating DNA damage response (Fig. 4A). In the groups co-treated with GK-667, the dose-dependent reduction of p53 accumulation was observed and the trend generally resembled that seen in other molecular markers discussed above. As p53 is an essential transcriptional activator of a battery of target genes, we sought to determine whether selected target genes show the corresponding response. Analysis of gene expression of p21, MDM2, p53R2, and p16 at the mRNA level (Fig. 4B–E) revealed significant induction of these p53 target molecules in the LV myocardium of animals belonging to the DAU group and dose-dependent reduction in the groups co-treated with GK-667. The combination of DAU with the lower dose of GK-667 induced only partial and often insignificant reduction as compared with the DAU group. The higher dose of GK-667 resulted in more effective reduction of gene expression of these p53 targets with significant difference in all but one (p16) target. Given that the activation of p53-dependent DNA damage response commonly results in
apoptosis via several molecular pathways, we analyzed the activity of executioner myocardial caspase 3. The results confirmed the increased activity of caspase 3/7 in the LV myocardium of the DAU group (Fig. 4F). In groups co-treated with GK-667, the activity was lower, particularly in the group treated with the higher dose of GK-667, where significant and effective prevention of caspase 3/7 activity induction was noted.

3.2 Acute in vivo study showed dose-dependent prevention of DAU-induced DNA damage response by GK-667 in the LV myocardium

In further experiments, we aimed to analyze the DNA damage response following the single DAU dose (the same as in the chronic experiments), where the changes can be directly attributed to DAU exposure without interference of the heart failure phenotype.

In these experiments, we found that single DAU exposure induced a strong (~8-fold) accumulation of p53 protein in the LV myocardium 6 h post-treatment and this alteration was effectively prevented by GK-667 co-treatment (Fig. 5A). The effect of GK-667 was already apparent with the lower dose of GK-667, but there was no statistical difference with respect to either the DAU group or the control group. With the higher dose of GK-667, the suppression of DAU-induced p53 accumulation was more effective and the values were significantly lower than in the DAU group. Similar findings were obtained in the analysis of gene expression of p53-target genes, namely p21 and MDM2 (Fig. 5B–C). The DAU-induced relative up-regulation of p53R2 was also significant, but only moderate in quantity, and it was only partially reduced by treatment with the higher dose of GK-667 (Fig. 5D).

In these experiments, we also studied in parallel a very close DEX derivative (N,N’-dimethyl dexrazoxane, GK-627), which we have previously reported as lacking any cardioprotective potential on our chronic rabbit model [11]. It is noteworthy that this bisdioxopiperazine derivative did not have
any impact on DAU-induced p53-dependent DNA damage response when administered in the same
dose and route as in the previous chronic study. Hence, these results strongly suggest a link between
the ability of GK-667 to prevent DAU-induced myocardial DNA damage response and its
cardioprotective efficacy against chronic DAU cardiotoxicity.

3.3 Active metabolite of GK-667 (ICRF-193) effectively inhibited TOP2B activity in vitro which
contrasted with the activity of the parent prodrug and non-cardioprotective bisdioxopiperazine
derivative GK-627

The decatenation assay was performed to determine whether GK-667 can interact with TOP2B on its
own or whether it is only a prodrug releasing ICRF-193. In this assay, the effects of GK-667, ICRF-193,
and GK-627 on TOP2B activity were studied in concentrations ranging from 1 to 1000 µM (Fig. 5E).
Inhibition of TOP2B activity by GK-667 was observed only in the highest concentration used (1000
µM), which is by far unachievable in vivo [20]. The short incubation time (30 min) and specific serum-
free buffer used in this assay did not allow effective conversion of GK-667 to ICRF-193 and thus
enabled us to study the direct effect of the prodrug. ICRF-193 itself potently and dose-dependently
inhibited TOP2B activity from the lowest concentration used and the effect was well pronounced in
10 µM which is close to the C_max of ICRF-193 in plasma (~11 µM) after administration of higher dose
of GK-667 (5 mg/kg) to rabbits [20]. In contrast, GK-627, which did not affect the DAU-induced DNA
damage response, showed no inhibitory effect under the same conditions in concentrations up to
1000 µM.

3.4 Administration of GK-667 did not interfere with the plasma pharmacokinetics of DAU and its
main metabolite DAUol

Even though GK-667 was shown to be cardioprotective in the chronic study, we could not exclude
that these effects may be (co)-determined by an interference with the pharmacokinetics of DAU in
vivo. Therefore, in another set of animals, a pharmacokinetic experiment with administration of DAU
alone or in combination with the higher dose of GK-667 was performed. GK-667 did not significantly influence plasma concentrations of either DAU or DAUol during the 24 h after DAU administration (Fig. 6A). The concentration profiles of both analytes were almost matching each other in the studied groups. Similarly, AUC<sub>0-24h</sub> was almost the same for both DAU and DAUol in this experiment, suggesting that total body exposure to DAU/DAUol was unaffected by GK-667 co-treatment (Fig. 6B).

3.5 GK-667 did not protect leukemic HL-60 cells from DAU-induced toxicity in a pilot in vitro experiment

Although the primary aim of this study was to assess the cardioprotective potential of GK-667 in vivo, we wanted to exclude the probability that the protection could be also similarly applicable to cancer cells. Thus, we analyzed the in vitro effects of GK-667 alone and in combination with DAU on the proliferation of leukemic cell line HL-60 (a model cancer in which DAU is clinically indicated). The results of this pilot study (Suppl. Fig. 4) showed concentration-dependent inhibition of proliferation of HL-60 cells by GK-667 alone with IC<sub>50</sub> = 0.5 µM. When DAU at its IC<sub>50</sub> (~15 nM, [12]) was combined with increasing concentrations of GK-667, an insignificant further decrease of cellular viability/proliferation of HL-60 cells was observed up to 1 µM of GK-667. In the higher concentrations of GK-667, further dose-dependent significant decrease in viability of HL-60 cells was noted. Hence, GK-667 showed no apparent potential to protect cancer cells against DAU toxicity, as it did not decrease the anticancer effect of DAU in HL-60 cells in this pilot study.
4. Discussion

In this study, we showed that GK-667, a water-soluble prodrug of ICRF-193, effectively and completely prevented DAU-induced premature deaths, signs of blood congestion, and LV dysfunction in vivo in a rabbit model of chronic ANT cardiotoxicity. This effect was seen upon administration of 1 and 5 mg/kg GK-667, corresponding to 0.76 and 3.78 mg/kg, respectively, of a free base of the prodrug (containing 0.47 and 2.34 mg/kg of active ICRF-193). Analysis of biomarkers of cardiac damage and LV dysfunction as well as histological examination of the LV myocardium revealed a subclinical damage in the combination group co-treated with the lower dose of GK-667 (1 mg/kg), whereas almost full protection was found in the group co-treated with the higher dose of GK-667 (5 mg/kg). These findings imply that the cardioprotection with GK-667 is dose-dependent and highly effective, especially when the significant cumulative dose of DAU (30 mg/kg) and the incidence of heart failure-related mortality in the DAU group is taken into account.

Very few other bisdioxopiperazines (namely ICRF-154, sobuzoxane, and bimolane) have been shown to have some cardioprotective potential [28-33], and none of them were more effective than DEX. Dose dependency of the protective effects of DEX against ANT cardiotoxicity has been established in mice, rats, and dogs and expressed as a dose ratio of DEX:ANT [34]. Herein, DEX provided detectable cardioprotection starting from the ratio of 5:1, but it was more significant and consistent in the ratio-range of 10–20:1 that was selected for later clinical trials. Partial cardioprotection of DEX was also previously found in a rabbit model of chronic DAU cardiotoxicity using the dose ratio 7.8:1 to ANT [35] and almost full protection was found at the ratio 20:1 [11, 23, 24, 36]. In the present experiments, we described the cardioprotective effects of GK-667 (as a free-base) in dose ratios of 0.25–1.25:1 to ANT. These comparisons suggest that GK-667 is currently the most potent cardioprotectant of this class. Not only did GK-667 provide almost complete cardioprotection close to 1:1 ratio to ANT, but it was also very well tolerated both alone and in combination with ANT.
Moreover, GK-667 can be easily formulated for i.v. administration and unlike with DEX, there is no need to ensure enantiomeric purity to achieve reasonable water solubility.

Other research groups and ours have previously attempted to modify the DEX molecule to modulate its cardioprotective effects [11, 12, 16, 17, 19, 29]. The vast majority of these attempts, including very minor modifications in the chemical structure such as the addition of one carbon, resulted in diminished or completely lost cardioprotective potential, and these findings correlated with abated potential to inhibit TOP2. Recently, Hasinoff et al. [19] and our group [12] have independently reported that ICRF-193 is a more potent cardioprotectant against ANT toxicity than DEX in vitro in neonatal cardiomyocytes, and this ability corresponded well with its higher potential to inhibit TOP2B. However, ICRF-193 is extremely poorly water-soluble (≤5 µg/mL at room temperature) precluding its use for in vivo studies. Hence, to overcome this issue, we synthesized several prodrugs of ICRF-193 [20] and identified compound GK-667 as the most promising candidate for this chronic in vivo cardioprotective study. Our results have shown that GK-667 is an interesting drug candidate, as it provided effective and dose-dependent cardioprotection against chronic ANT cardiotoxicity in a well-defined non-roden animal model.

In a previous study, we described the release of ICRF-193 from GK-667 in vitro and studied the pharmacokinetics of the prodrug, ICRF-193, and its ring-opened metabolite in rabbits [20]. The prodrug GK-667 was found to be effectively metabolized to its active metabolite ICRF-193 in cell culture media (c_{max} of ICRF-193 was reached after a 1-h incubation when the vast majority of the parent prodrug disappeared). The bioactivation was even faster in rabbit plasma in vitro (with c_{max} of ICRF-193 at 20–30 min of incubation) and especially in rabbits in vivo. C_{max} of ICRF-193 in plasma after administration of GK-667 to rabbits (5 mg/kg, i.v. – the same dose as in this study) was reached in the 5th min (approximately 11 µM). On the contrary, the concentrations of parent GK-667 did not exceed 3 µM in the same time point and dropped below the lower limit of quantification (0.1 µM) in the 30th
min. The concentration of ICRF-193 decreased more slowly and dropped below the lower limit of quantification (0.01 µM) in all animals 6 h after GK-667 administration. The elimination half-life of ICRF-193 was slightly shorter (0.8 vs. 2.0 h), and the AUC correspondingly lower than previously found for DEX in the same model [20]. However, these pharmacokinetic differences relative to DEX did not limit the cardioprotective potential of ICRF-193 in our model, because the effect was comparable to DEX administered in the higher dose ratios to ANT.

The traditional understanding of DEX-induced cardioprotection emphasized metal chelation and corresponding reduction of ANT-induced oxidative damage to the heart. However, emerging concepts suggest that a specific pharmacological mechanism may be essential instead, namely the catalytic inhibition of TOP2B in the cardiomyocytes, which could prevent poisoning of this enzyme by ANT [11, 12]. This idea was reinforced by a seminal study that showed that genetic knockout of this enzyme prevents the development of chronic ANT cardiotoxicity [7]. The latter was associated with the prevention of DNA damage and activation of the DNA damage response, while the vast majority of events were downstream of that.

In our chronic cardioprotective experiments, we found an accumulation of p53 protein in the LV myocardium of DAU-treated animals and this was accompanied by transcriptional activation of p53 target genes. These events were prevented by GK-667 administration in a dose-dependent manner. However, the interpretation of this finding is likely not straightforward, because increased p53 levels and activation of its downstream signaling pathways have been described in failing human and animal hearts of various etiology [37, 38]. Nevertheless, GK-667 was also shown to dose-dependently and effectively prevent the p53-mediated DNA damage response induced in the heart by a single DAU dose, while the non-cardioprotective DEX derivative (GK-627) was completely ineffective under the same conditions. This occurred despite the ability of GK-627 to undergo metabolism to a metal-chelating metabolite and high plasma concentrations of both GK-627 and its metabolite in rabbits.
These results suggest that the mechanisms of cardioprotective effects of GK-667 are attributable to the prevention of DAU-induced DNA damage response involving the p53 signaling pathway. Based on data from the TOP2B activity assay and our previous reports, this seems explainable by the ability of ICRF-193 released from the prodrug to effectively interact with TOP2B and thereby prevent poisoning of the enzyme in cardiomyocytes by ANTs [12, 20]. Co-treatment with GK-667 also uniformly prevented other DAU-induced molecular disturbances in chronic experiments (e.g., impaired expression of calcium and ROS handling proteins) and thus they likely occur downstream of the TOP2B, as has been also suggested by Zhang et al. [7].

Some other pharmacological interventions reported in the literature may also be at least partially associated with the inhibition of ANT-induced and TOP2B-mediated cardiomyocyte damage, especially when the events downstream of TOP2B are incompletely understood. For instance, Amgalan et al. [39] recently reported that pharmacological inhibition of BAX is an effective cardioprotective strategy. Indeed, BAX is also an important part of p53-mediated DNA damage response [40, 41]. However, further studies are needed to fully understand the cardioprotective potential of agents targeting events significantly downstream of the ANT-TOP2B interaction or mechanistic pathways that appear to be completely independent of TOP2B.

Our pharmacokinetic experiments also excluded that the cardioprotection stems from or is co-determined by the decreased exposure of rabbits to ANT, as co-treatment with GK-667 did not affect the plasma concentrations and AUC of either DAU or DAUol. In fact, this is also a pre-requisite for the potential further development of such agents as cardioprotectants, because any distinct alteration in ANT pharmacokinetics would be an issue. These findings are similar to those previously reported for DEX [42].
In contrast to the potent cytoprotective effects of GK-667 against DAU toxicity in isolated neonatal cardiomyocytes *in vitro* [20], the pilot *in vitro* experiments with leukemic cell line HL-60 suggested that co-treatment with GK-667 does not diminish the anticancer effect of DAU. The present findings are very similar to those previously reported for ICRF-193, which also did not diminish the anticancer activity of DAU in HL-60 cells, while effectively protected isolated neonatal cardiomyocytes against DAU-induced toxicity [12]. The similarity between these results can be explained by the effective bioactivation of GK-667 to its active metabolite ICRF-193 in cell cultures *in vitro* [20]. Bisdioxopiperazine agents such as ICRF-193 and DEX are not isoform-specific TOP2 inhibitors; therefore, they also induce potent catalytic inhibition of topoisomerase IIα in cancer cells [12, 43]. While this could implicate concerns about the potential negative impact of DEX on anticancer effects of ANTs, it should be noted that the clinical trials and their meta-analyses have found little evidence to support this view [9, 13, 44]. Nevertheless, more detailed analyses involving multiple cancer cell lines and *in vivo* xenograft models, along with mechanistic investigations, are warranted to properly assess the interaction of GK-667 with ANT in cancer cells.

In conclusion, using a non-rodent animal model, we demonstrated that GK-667 is, to the best of our knowledge, the most effective cardioprotective agent of the bisdioxopiperazine class against chronic ANT cardiotoxicity *in vivo*, while it is very well tolerated both alone and in combination with ANT. Further investigations excluded any significant impact of GK-667 on the plasma pharmacokinetics of ANT and thus, the observed effects should only be determined by the pharmacodynamic effects of the drug. The results of the present study together with our previous findings indicate that mechanisms of the cardioprotective effects of GK-667 are attributable to the prevention of DAU-induced and p53-mediated DNA damage response in the myocardium via inhibition of TOP2B by ICRF-193 released from the prodrug (see Fig. 7 for schematic summary). Therefore, GK-667 is an interesting drug candidate for further advanced study and development.
Clinical perspectives

(i) Dexrazoxane is the only drug approved for prevention of anthracycline cardiotoxicity; however, its clinical use is limited, which may be co-determined by a possibility that it is not the best representative of the bisdioxopiperazine class.

(ii) The present experimental study demonstrates that this premise may be valid because another bisdioxopiperazine agent (GK-667) shows highly effective and well-tolerated cardioprotective effects against chronic anthracycline cardiotoxicity in much lower dose ratios to anthracycline without interfering with the anthracycline pharmacokinetics.

(iii) The cardioprotective effects were found attributable to prevention of anthracycline-induced and p53-mediated DNA damage response in the myocardium via inhibition of topoisomerase IIβ by ICRF-193 released from the prodrug. These results further support a changing view on the primary mechanisms of chronic anthracycline cardiotoxicity development and targets for effective cardioprotection.

Data availability statement

The data related to this article are available in the article itself, its online supplementary material, or upon reasonable request to the corresponding author.

Acknowledgement

The authors thank Mrs. Klára Lindrová, Jitka Pohorská, and Dagmar Ježková for their excellent laboratory assistance. The authors would also like to thank Prof. Julius Lukeš and Eva Kriegová for the culture of *Crithidia fasciculata* used for kDNA isolation.

Funding

This study was supported by the Czech Science Foundation (GAČR) [Grant no.: 21-16195S]; Charles University Research Programme Progres [Grant no.: Q40/5]; Charles University Grant Agency (GAUK)
[Grant no.: 1204120]; and by project INOMED reg. No. CZ.02.1.01/0.0/0.0/18_069/0010046: Pre-application research into innovative medicines and medical technologies project co-funded by the ERDF.

**Declarations of interest**

PK-B, GK, JK, AJ, TŠ, PŠ-K, JR, and MŠ are co-inventors on Czech patent application No. PV 2020-26 and The Patent Cooperation Treaty (PCT) application No. PCT/IB2021/050285. The authors declare no other conflict of interest.
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Figure legends

Figure 1. Co-treatment with GK-667 and daunorubicin prevented daunorubicin-induced increase of biomarkers of cardiac damage and left ventricular dysfunction.

Plasma concentrations of cardiac troponin T (cTnT) as a biomarker of cardiac damage - last measured values (LMV) during the experiment (A) and area under the curve (AUC) of cTnT plasma concentrations determined during the whole experiment (B). Left ventricular (LV) systolic function was examined by echocardiography as LV fractional shortening (LVFS) and displayed as LMV (C) and by LV catheterization at the scheduled end of the study (index \(dP/dt_{\text{max}}\)) (D). Gene expression of biomarkers of wall stress and heart failure in LV myocardium, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) were evaluated by RT-qPCR (E, F). Data are expressed as medians with box and whisker plots representing the interquartile range and 5th–95th percentiles, respectively. Statistical significance was evaluated using one-way ANOVA (followed by Holm–Sidak’s post-hoc test) or ANOVA on ranks (followed by Dunn’s post-hoc test) according to the data characteristics. Symbols indicate statistically significant differences (\(p<0.05\)) in comparison with the following: “*”, the control group (CTR); “#”, the GK_{667}(5) group receiving GK-667 alone (5 mg/kg); and “d”, the daunorubicin group (DAU). In combination groups, the compound GK-667 was administered intravenously 30 min before each DAU administration at doses of 1 and 5 mg/kg [GK_{667}(1)+DAU and GK_{667}(5)+DAU, respectively]. Each group included 10 rabbits with the exception of the group treated with GK-667 alone (n=7). Unscheduled deaths reduced the number of evaluated animals in the DAU group to n=9 (E, F) and n=8 (D).

Figure 2. GK-667 co-treatment prevented daunorubicin-induced morphological changes in the myocardium and expression of markers of fibrosis.

Histopathological evaluation of left ventricular (LV) myocardium (Masson’s blue trichrome staining) revealed normal myocardium in the control group (CTR) and the group treated with GK-667 alone [GK_{667}(5) group]. Focal degenerative changes of cardiomyocytes (with vacuolized cytoplasm) and replacement fibrosis (thick blue collagen fibers) were observed in the LV myocardium and interventricular septum of animals from the daunorubicin (DAU) group. These changes were incompletely reduced in the myocardium of the animals co-treated with 1 mg/kg of GK-667 [GK_{667}(1)+DAU group] as some foci with milder degenerative changes were still recognizable. In the group co-treated with 5 mg/kg of GK-667 [GK_{667}(5)+DAU], the prevention of DAU-induced pathological changes was almost complete and the myocardium resembled the control group. Bar 50 \(\mu\)m (A). Gene expression of markers of fibrosis, fibronectin 1 (FN1), collagen I (COL1A1), and collagen IV (COL4A2) in the LV myocardium examined by RT-qPCR (B, C, and D, respectively). Data are
expressed as medians with box and whisker plots representing the interquartile range and 5th–95th percentiles, respectively. Statistical significance was evaluated using one-way ANOVA (followed by Holm–Sidak’s post-hoc test) or ANOVA on ranks (followed by Dunn’s post-hoc test) according to the data characteristics. Symbols indicate statistically significant differences (p<0.05) in comparison with the following: “∗”, the control group (CTR); “#”, the GK667(S) group receiving GK-667 alone (5 mg/kg); and “d”, the daunorubicin group (DAU). Each group included 10 rabbits with the exception of the group treated with GK-667 alone (n=7). Unscheduled deaths reduced the number of evaluated myocardial samples in the DAU group to n=9 in figures B, C, and D.

Figure 3. GK-667 co-treatment prevented daunorubicin-induced impairment of expression of proteins involved in redox and calcium homeostasis in the left ventricular myocardium.

Gene expression of redox homeostasis enzymes, NADPH oxidase 2 – gp91phox subunit (NOX2), NADPH oxidase 4 (NOX4), heme oxygenase 1 (HO1), and mitochondrial superoxide dismutase 2 (SOD2) in the LV myocardium were examined by RT-qPCR (A, B, C, and D, respectively). Gene expression of calcium handling proteins, sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2), and ryanodine receptor 2 (RyR2) was determined using the same technique (E and F, respectively). Data are expressed as medians with box and whisker plots representing the interquartile range and 5th–95th percentiles, respectively. Statistical significance was evaluated using one-way ANOVA (followed by Holm–Sidak’s post-hoc test) or ANOVA on ranks (followed by Dunn’s post-hoc test) according to the data characteristics. Symbols indicate statistically significant differences (p<0.05) in comparison with the following: “∗”, the control group (CTR); “#”, the GK667(S) group receiving GK-667 alone (5 mg/kg); “d”, the daunorubicin group (DAU); and “†”, the GK667(1)+DAU group. In the combination groups, the compound GK-667 was administered intravenously 30 min before each DAU administration at doses of 1 and 5 mg/kg [GK667(1)+DAU and GK667(5)+DAU, respectively]. Each group contained 10 rabbits with the exception of the group treated with GK-667 alone (n=7). Unscheduled deaths reduced the number of evaluated myocardial samples in the DAU group to n=9 in all figures (A–F).

Figure 4. GK-667 co-treatment prevented daunorubicin-induced DNA damage response and activation of executioner apoptotic caspases in the left ventricular myocardium from the chronic experiment.

Myocardial p53 (molecular weight = 53 kD) was analyzed by western blotting, and total protein on the PVDF membrane was visualized by Stain-Free imaging technology (Bio-Rad, USA) (A). Un-cropped images of the original representative western blot can be seen in Suppl. Fig. 2. Gene expression of p53 target genes in the left ventricular (LV) myocardium examined by RT-qPCR – cyclin dependent
kinase inhibitor 1A (p21) (B), MDM2 proto-oncogene (MDM2) (C), ribonucleotide reductase regulatory TP53 inducible subunit M2B (p53R2) (D), and cyclin-dependent kinase inhibitor 2A (p16) (E). The activity of caspase 3/7 in the LV myocardium (F). Data are expressed as medians with box and whisker plots representing the interquartile range and 5th–95th percentiles, respectively. Statistical significance was evaluated using one-way ANOVA (followed by Holm–Sidak’s post-hoc test) or ANOVA on ranks (followed by Dunn’s post-hoc test) according to the data characteristics. Symbols indicate statistically significant differences (p<0.05) in comparison with the following: “*”, the control group (CTR); “#”, the GK667(5) group receiving GK-667 alone (5 mg/kg); “d”, the daunorubicin group (DAU); and “‡” the GK667(1)+DAU group. In the combination groups, the compound GK-667 was administered intravenously 30 min before each DAU administration at doses of 1 and 5 mg/kg [GK667(1)+DAU and GK667(5)+DAU, respectively]. Each group contained 10 rabbits with the exception of the group treated with GK-667 alone (n=7). Unscheduled deaths reduced the number of evaluated myocardial samples in the DAU group to n=9 in all figures (A–F).

**Figure 5. Prevention of DNA damage response in the left ventricular myocardium after single daunorubicin administration to rabbits in vivo and topoisomerase IIβ activity assay in vitro.**

Selected molecules associated with DNA damage response were analyzed in the left ventricular (LV) myocardium 6 h after single administration of DAU (3 mg/kg, i.v.) to rabbits either alone or in combination with the studied drugs (A–D). Myocardial p53 (molecular weight ≈ 53 kD) was analyzed by western blotting, and total protein on the PVDF membrane was visualized by Stain-Free imaging technology (Bio-Rad, USA) (A). Un-cropped images of the original representative western blot can be seen in Suppl. Fig. 3. Expression of p53 target genes in the LV myocardium was examined by RT-qPCR – cyclin dependent kinase inhibitor 1A (p21) (B), MDM2 proto-oncogene (MDM2) (C), and ribonucleotide reductase regulatory TP53 inducible subunit M2B (p53R2) (D). Data are expressed as medians with box and whisker plots representing the interquartile range and 5th–95th percentiles, respectively. Statistical significance was evaluated using one-way ANOVA on ranks followed by Dunn’s post-hoc test. Symbols indicate statistically significant differences (p<0.05) in comparison with the following: “*”, the control group (CTR); “d”, the daunorubicin group (DAU); and “‡”, the GK627+DAU group. In the combination groups, the compound GK-667 was administered as in the chronic cardioprotective study, i.e., intravenously 30 min before DAU administration at doses of 1 and 5 mg/kg [GK667(1)+DAU and GK667(5)+DAU, respectively]. A close DEX derivative GK-627, which was previously shown to be free of cardioprotective effects in chronic rabbit model [11] was employed as the negative control. The dose and route of GK-627 administration were adopted from the above-mentioned study (60 mg/kg, intraperitoneally 30 min before DAU). Each studied group contained 7 rabbits. In addition, the effects of studied compounds (1–1000 µM) on the activity of
human recombinant topoisomerase IIβ (TOP2B) enzyme were studied in vitro (E). The active TOP2B was characterized by the ability to release minicircles (product) from kinetoplast DNA (kDNA, substrate). TOP2B inhibited by the drug cannot process kDNA to individual minicircles which is detected as a lower signal of the product on gel electrophoresis. (+/-) means presence/absence of the enzyme and it serves as a negative and positive control. The representative picture of the analysis is shown.

**Figure 6. Administration of GK-667 did not interfere with plasma pharmacokinetics of daunorubicin (DAU) and its main metabolite daunorubicinol (DAUol).**

Plasma concentrations of DAU and its main metabolite DAUol were determined after single administration of DAU (3 mg/kg, i.v.) to rabbits either alone (n=11) or in combination with GK-667 (5 mg/kg, i.v. 30 min before DAU, n=11) by using a validated UHPLC-MS/MS assay (A). Data are expressed as mean±SD. No statistically significant differences in concentrations of both DAU and DAUol were found between the GK-667 treated and untreated groups (p>0.05). AUC_{0-24h} for both DAU and DAUol was calculated using GraphPad software for 6 animals in each group undergoing the whole 24-h experiment (B). Medians with boxes and whiskers representing the interquartile range and 5th–95th percentiles are shown. Statistical significance (p<0.05) was evaluated using t-test or Mann-Whitney U test according to the data characteristics. “n.s.” – non-significant difference (p>0.05).

**Figure 7. Mechanisms of cardioprotective effects of bisdioxopiperazine compound GK-667 against chronic anthracycline cardiotoxicity – a schematic overview.**

Abbreviations used: ANT, anthracycline; TOP2B, topoisomerase IIβ.
A  
\( p53 \) (protein)  
6 hours

B  
\( p21 \) (mRNA)  
6 hours

C  
\( MDM2 \) (mRNA)  
6 hours

D  
\( p53R2 \) (mRNA)  
6 hours

E  
substrate  
(kDNA)

product  
(released minicircles)

| TOP2B | + | - |
|-------|---|---|
| \( GK_{667} [\mu M] \) | 1 | 10 | 100 | 1000 |
| \( ICRF-193 [\mu M] \) | 1 | 10 | 100 | 1000 |
| \( GK_{627} [\mu M] \) | 1 | 10 | 100 | 1000 |
A

**DAU/DAUol plasma concentrations**

- **DAU** after DAU
- **DAU** after Gk667(5)+DAU
- **DAUol** after DAU
- **DAUol** after Gk667(5)+DAU

**time (min)**

| Time (min) | DAU | DAU after Gk667(5)+DAU | DAUol | DAUol after Gk667(5)+DAU |
|------------|-----|------------------------|-------|-------------------------|
| 0          |     |                        |       |                         |
| 10         |     |                        |       |                         |
| 20         |     |                        |       |                         |
| 30         |     |                        |       |                         |
| 40         |     |                        |       |                         |
| 50         |     |                        |       |                         |
| 60         |     |                        |       |                         |
| 70         |     |                        |       |                         |
| 80         |     |                        |       |                         |
| 90         |     |                        |       |                         |
| 100        |     |                        |       |                         |
| 120        |     |                        |       |                         |
| 240        |     |                        |       |                         |
| 360        |     |                        |       |                         |
| 480        |     |                        |       |                         |
| 600        |     |                        |       |                         |
| 720        |     |                        |       |                         |
| 1080       |     |                        |       |                         |
| 1440       |     |                        |       |                         |

B

**AUC<sub>0-24h</sub> of plasma concentrations**

- **DAU**
- **DAUol**

**μM x h**

- **DAU**
- **DAUol**

- n.s.
GK-667 \rightarrow \text{ICRF-193} \quad \text{bioactivation}

\text{ANT pharmacokinetics} \rightarrow \text{not altered}

\text{cardioprotection} \rightarrow \text{cardiotoxicity}

\text{anthracyclines} (\text{ANT})

\text{inhibition}

\text{poisoning}

\text{p53 dependent DNA damage response}

\text{heart failure}