Cardioprotective effects of inorganic nitrate/nitrite in chronic anthracycline cardiotoxicity: Comparison with dexrazoxane

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Abstract

Dexrazoxane (DEX) is a clinically available cardioprotectant that reduces the toxicity induced by anthracycline (ANT) anticancer drugs; however, DEX is seldom used and its action is poorly understood. Inorganic nitrate/nitrite has shown promising results in myocardial ischemia–reperfusion injury and recently in acute high-dose ANT cardiotoxicity. However, the utility of this approach for overcoming clinically more relevant chronic forms of cardiotoxicity remains elusive. Hence, in this study, the protective potential of inorganic nitrate and nitrite against chronic ANT cardiotoxicity was investigated, and the results were compared to those using DEX. Chronic cardiotoxicity was induced in rabbits with daunorubicin (DAU). Sodium nitrate (1 g/L) was administered daily in drinking water, while sodium nitrite (0.15 or 5 mg/kg) or DEX (60 mg/kg) was administered parenterally before each DAU dose. Although oral nitrate induced a marked increase in plasma NOx, it showed no improvement in DAU-induced mortality, myocardial damage or heart failure. Instead, the higher nitrite dose reduced the incidence of end-stage cardiotoxicity, prevented related premature deaths and significantly ameliorated several molecular and cellular perturbations induced by DAU, particularly those concerning mitochondria. The latter result was also confirmed in vitro. Nevertheless, inorganic nitrite failed to prevent DAU-induced cardiac dysfunction and molecular remodeling in vivo and failed to overcome the cytotoxicity of DAU to cardiomyocytes in vitro. In contrast, DEX completely prevented all of the investigated molecular, cellular and functional perturbations that were induced by DAU. Our data suggest that the difference in cardioprotective efficacy between DEX and inorganic nitrite may be related to their different abilities to address a recently proposed upstream target for ANT cardiotoxicity.

1. Introduction

Anthracycline antibiotics (ANTs, e.g., doxorubicin or daunorubicin) are important anticancer drugs that are well known for their remarkable clinical efficacy as well as for an unfavorable cardiac safety profile. While a limitation of total cumulative dose certainly decreased the incidence of symptomatic cardiotoxicity in ANT-treated patients, it unfortunately failed to eliminate the cardiotoxicity burden completely, which is important in light of the rapidly growing number of long-term cancer survivors who are exposed to ANTs [1,2]. ANT cardiotoxicity has both acute and chronic forms which differ in on-set, manifestation and clinical impact [3]. Acute forms are mostly represented by subclinical changes in cardiovascular functions occurring soon after drug administration and are only rarely a significant clinical issue [3,4]. In contrast, chronic forms developing months or years after chemotherapy are particularly feared because they are associated with irreversible dilated cardiomyopathy and the resulting heart failure poorly responds to standard treatment [3,5] highlighting the importance of preventive strategies including pharmacological cardioprotection. So far, dexrazoxane (DEX) is the only drug that has been approved for this indication [5,6]. Although its cardioprotective...
Potential has been firmly confirmed in many experimental and clinical studies [2,7], currently, DEX is seldom used due to concerns related to its adverse reactions [6]. The cardioprotective effects of DEX have been traditionally explained by the iron chelating properties of its metabolite [2,7,8], but experiments with stronger and more selective intracellular iron chelators of different chemical structures did not bring any DEX successor [7]. Hence, the search for a safe and effective cardioprotectant against chronic ANT cardiotoxicity continues.

Inorganic nitrite and nitrate have been long perceived as biologically inert products of nitric oxide (NO) metabolism, but in the last two decades it has been revealed that they may also be a reservoir and alternative source of NO in the organism [9]. Dietary inorganic nitrate can be reduced by mammalian gastrointestinal microflora to nitrite which is subsequently absorbed into the bloodstream; the efficacy of this process is enhanced by the entero-salivary circulation of nitrates. Blood nitrites may be reduced to NO in various tissues including the myocardium, particularly under hypoxia and acidosis [9,10]. In light of this finding, multiple experimental studies have demonstrated the significant cardioprotective potential of dietary nitrate and enteral/parenteral nitrite against myocardial ischemia–reperfusion (I/R) injury [11–17] and clinical trials have been subsequently initiated [18,19]. While the mechanisms are not completely understood, it seems that cardioprotective effects involve an activation of the canonical NO/cGMP/PKG pathway resulting in the prevention of mitochondrial depolarization, the production of oxidative stress and apoptotic cell death [9,10]. Nitrite and/or NO can also regulate the function of many important cellular and mitochondrial proteins via S-nitrosylation [9,10,20]. This process involves respiratory chain subunits, particularly complex I, with the subsequent partial inhibition of its enzymatic activity and the reduction of oxidative stress in the mitochondria [16,20]. Inorganic nitrite has also been demonstrated as cardioprotective, even under normoxic conditions via protein kinase A [17]. The inhibition of mitochondrial fission and the activation of AMP kinase are key downstream events of this treatment. Furthermore, nitrite has been suggested to activate mitochondrial biogenesis to increase a number of functionally efficient mitochondria [21]. A recent study has also shown that dietary sodium nitrate (1 g/L in drinking water) can induce significant protection against acute ANT cardiotoxicity induced in mice with a single high dose of doxorubicin [22]. However, the translatability and efficacy of this approach in clinically more relevant chronic ANT cardiotoxicity induced by the repeated exposure of therapeutic doses remains unknown, along with the underlying molecular events.

Because nitrate/nitrite therapy has been previously reported to positively affect numerous targets that are commonly associated with the development of ANT cardiotoxicity [7] and because dietary nitrate showed promise against acute high-dose ANT cardiotoxicity [22], in the present study, we sought to determine whether this cardioprotective intervention is effective against chronic ANT cardiotoxicity. Furthermore, the results were compared with those generated using DEX, the only clinically available cardioprotectant, and the molecular mechanisms were investigated.

2. Materials and methods

Detailed description of the Materials and methods can be found in Supplementary materials and methods.

2.1. Animals and experimental design

Male Chinchilla rabbits (n = 54, VELAZ, Czech Republic) had free access to the standard pellet diet and drinking water. Chronic ANT cardiotoxicity was induced by DAU on a well-established schedule (3 mg/kg i.v., weekly for ten weeks, n = 9) [23,24]. Another group of rabbits (n = 9) received sodium nitrate (1 g/L) in their drinking water one week before and continuously throughout the DAU treatment on the above-described schedule. This supplementation with inorganic nitrate has been previously shown to be effective against acute ANT cardiotoxicity [22]. In addition, other groups of rabbits received sodium nitrite (0.15 and 5 mg/kg, n = 7 in each) in a 30-minute intravenous infusion that was initiated 45 min before each DAU dose. The lower selected dose was within the typical dose range (0.07 to 0.17 mg/kg) showing consistent cardioprotective effects in different animal models of myocardial (I/R) injury [13,15], while the higher dose corresponds to the maximal recommended doses to treat acute cyanide poisoning (5 to 6 mg/kg) [25]. DEX (60 mg/kg i.p.) was administered as a model cardioprotectant 30 min before each DAU dose (n = 7). The control group received saline (1 mL/kg i.v., n = 9) or nitrate-alone (nitrate group, n = 6), as described above.

After animal sacrifice, the heart was rapidly excised and washed, and transverse sections of ventricles were taken for histological examination. The rest of the free LV wall was pulverized in liquid nitrogen and stored at −80 °C.

2.2. Examination of left ventricular function

The LV systolic function was examined using echocardiography (Vivid 4, 10-MHz probe, GE Medical Systems Ultrasound, UK) in light anesthesia (ketamine and midazolam), and the LV fractional shortening (LV FS) was calculated as an index of the systolic function. LV catheterization examination was performed in pentobarbital anesthesia using a Mikro-Tip pressure catheter (2.3F, Millar Instruments, TX USA) and The Chart 5.4.2 software (ADInstruments, Australia) was used for data analysis and for the calculation of the indexes of systolic and diastolic function (dP/dt max and dP/dt min, respectively).

2.3. Determination of cardiac troponin T in plasma

Plasma concentrations of cardiac troponin T were determined using Elecsys Tropo T hs STAT (Roche Diagnostics, Switzerland) according to the manufacturer’s recommendations with a 0.003 μg/L limit of detection.

2.4. Histological processing of left ventricular myocardium

Hearts were transversely cut through both ventricles and fixed in 4% neutral formaldehyde. Serial paraffin sections (6 μm thick) were stained with hematoxylin and eosin and Masson’s blue trichrome.

2.5. Biochemical analyses — NOx in plasma, myocardial lipoperoxidation and citrate synthase activity

Plasma samples for the determination of NOx (nitrate and nitrite) concentrations were obtained at the end of the study and were frozen at −80 °C. The analysis was performed using an HPLC method as described by Woitzik et al. [26]. Only the NOx levels are reported because the pre-analytical conditions of this experiment were not controlled and validated for the precise separate determination of the concentrations of nitrate and nitrite, which are otherwise prone to artifacts [27].

Malondialdehyde (MDA) was determined in the LV myocardial samples as a marker of lipoperoxidation by the thiobarbituric acid-independent HPLC method according to Pilz et al. [28].

The citrate synthase activity was assayed according to De Sousa et al. [29] as an enzyme-coupled formation of citric acid from acetyl coenzyme A and oxaloacetic acid.

2.6. RNA isolation and quantitative real-time PCR

Total RNA was isolated from the LV myocardium using TRIzol reagent (Sigma-Aldrich, MO, USA). After reverse transcription (High Capacity cDNA Reverse Transcription Kit), qPCR was performed with the TaqMan Fast Universal PCR Master Mix (all from Applied Biosystems, CA, USA). The mean threshold cycle values were transformed into
relative expression using the Pfaffl method [30]. Commercial gene expression assays were obtained from Applied Biosystems (CA, USA) and Generi Biotech (Hradec Králové, Czech Republic) (see Supplementary Table 1). The expression data were normalized by Hprt1 expression.

2.7. Cell cultures and isolated neonatal cardiomyocytes

The rat embryonic heart-derived H9c2 myoblast cell line (ATCC, VA, USA) was incubated 24 h before the experiments in serum-free DMEM cell culture (Sigma-Aldrich, MO, USA) to stop cellular proliferation. H9c2 cells were then incubated with a freshly prepared solution of sodium nitrite in a concentration range of 0.01 to 30 mM for 24 h alone or were pre-incubated with the inorganic nitrite for 45 min and then co-incubated with 1.2 μM DAU for 3 h followed by a 20-hour DAU-free period.

Primary rat neonatal ventricular cardiomyocytes (NVCMs) were isolated as described in the Supplementary material and methods. Prior to the experiment, the medium was changed to serum- and pyruvate-free DMEM/F12 with 1% P/S. The myocytes were incubated with freshly prepared sodium nitrite or DEX (both 0.1 mM) for 48 h alone or pre-incubated with inorganic nitrite at a concentration ranging from 0.1 to 30 mM or 0.1 mM DEX for 3 h and then co-incubated with 1.2 μM DAU for 3 h, followed by a 48-hour DAU-free period. The single concentration of DEX that was used in the cell experiments was selected based on previous studies [31].

2.8. Cell viability

The viability of H9c2 cells was measured using an MTT assay (Sigma-Aldrich, MO, USA) according to the manufacturer’s instructions. The cell viability of experimental cells was expressed relative to that of the untreated controls (100%).

Lactate dehydrogenase (LDH) activity in the culture medium was used as a marker of cytotoxicity in neonatal myocytes. The control wells were treated with lysis buffer to determine the total cellular LDH activity and data were expressed as a percent of total LDH activity.

2.9. Fluorescence microscopy and quantification of mitochondrial inner membrane potential

The mitochondrial inner membrane potential (ΔΨm) of H9c2 cells was evaluated using an Eclipse inverted epifluorescence microscope (Nikon, Japan). The JC1 probe (Molecular Probes, OR, USA) was used for the ΔΨm assessments. The cells were loaded with the JC1, washed twice with culture medium and examined using fluorescence microscopy.

To quantify the mitochondrial inner membrane potential, H9c2 cells were incubated with tested compounds and then loaded with JC1 probe (5 μM). The cells were rinsed twice and the fluorescence of the JC1 probe was measured using an Infinite M200 microplate reader (Tecan, Austria).

2.10. Determination of activities of caspase 3/7 and 9

To measure the activities of caspase 3/7 and 9, commercial luminescent kits (Caspase-Glo Assays, Promega, WI, USA) were used following the manufacturer’s instructions. The measurements were performed in homogenates from H9c2 cells or pulverized LV myocardium prepared with Cell Lysis Buffer (BioVision, CA, USA). The luminescent units were normalized to the protein content and the results are shown as a fold-increase in caspase activity compared with that of the control samples.

2.11. Western blotting

In this analysis, H9c2 (3 million cells per dish) and NVCM (4.8 million cells per dish) were incubated with 100 μM DEX or inorganic nitrite for 24 h. The LV myocardia were harvested 12 h after the administration of DEX (60 mg/kg i.p.) or the higher dose of nitrite (5 mg/kg i.v.) to rabbits. The cell pellets and pulverized myocardial samples were homogenized in ice-cold RIPA buffer that was supplemented with protease and phosphatase inhibitors. For immunoblotting, equal amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane by electroblotting. Immunodetection was performed with primary antibody to topoisomerase I/II (Abcam, United Kingdom) and anti-rabbit secondary antibody conjugated with horseradish peroxidase (GE Healthcare, United Kingdom). Densitometric quantitation was performed using Quantity One software (Bio-Rad, CA, USA). GAPDH was used as a loading control.

2.12. Determinations of protein concentrations

The BCA Protein Assay Kit (Sigma-Aldrich, MO, USA) was used for the determination of protein concentrations.

2.13. Statistical analysis

Statistical analyses were performed using SigmaStat 3.5 (SPSS, IL, USA). The data are presented as the mean ± SD or median along with the interquartile range according to the data distribution character, and the statistical significance was determined using either One-Way ANOVA or One-Way ANOVA on Ranks. Differences were considered statistically significant at p < 0.05.

3. Results

3.1. General toxicity

DAU treatment resulted in premature death in three out of nine animals between the 9th and 11th weeks (Fig. 1A) with marked LV dilatation and signs of blood congestion (massive hydrothorax and ascites (35 to 75 mL)). Dietary supplementation with inorganic nitrate did not prevent DAU-induced mortality, and marked pleural and abdominal effusions (7 to 125 mL) together with the dilation of heart chambers were observed. The combination of DAU with a low dose of parenteral nitrite (0.15 mg/kg) showed only moderate mortality (14%), but it was accompanied by pleural and peritoneal effusion (90 and 20 mL, respectively), along with the dilation of cardiac chambers. Interestingly, the combination with a higher dose of inorganic nitrite (5 mg/kg) or with DEX resulted in complete animal survival without external signs of poor health. No signs of blood congestion or LV dilatation were observed in the DEX + DAU group, whereas in the group of 5 mg/kg nitrite with DAU, two animals showed moderate pleural effusion (~5 mL), and a single animal suffered from slight ascites (~5 mL). No abnormalities were found in the control and nitrate-alone groups and the well-being of these animals was evidenced by a significant increase in body weight (Fig. 1B and C). However, all of the animals receiving DAU (alone or in combinations) showed significantly lower body weight gain compared with that of the controls. Of the combination groups, only the DEX + DAU group showed a significantly higher weight gain than that of the DAU-alone group (Fig. 1C).

Determination of the plasma NOx at the end of the study confirmed significant systemic exposure (Fig. 1D) due to the dietary supplementation of inorganic nitrate which corresponded well with the monitored intake of water with the supplement. The NOx concentrations were 20-times higher in both groups receiving oral nitrate compared with those of control group. All of the other groups showed values similar to those of the controls which also concerned both of the nitrite-treated groups because the blood sampling was performed at the end of the study, i.e., a week after the last nitrite dose.
3.2. Cardiac function and biomarkers of cardiac damage

Echocardiographically determined fractional shortening revealed a significant decline in the LV systolic function in the DAU group (Fig. 2A). Impaired LV systolic performance was also found in all of the combination groups with the exception of the DEX + DAU group, in which the functional parameter almost matched the control values. Similar results were also obtained following an invasive examination of the LV systolic and diastolic performance in animals surviving until the end of the study (Fig. 2B and C). The gene expression of ANP in the LV was used to further support these findings (Fig. 2D). Marked induction was found in the DAU group as well as in combination groups receiving dietary nitrate and the lower dose of parenteral nitrite. Although the findings in the group that was co-treated with the higher nitrite dose showed a significant up-regulation compared with that of the controls, the median values were more than 5-fold lower than those in the other nitrate/nitrite combination groups. No significant elevation of ANP was observed in the DEX + DAU group.

The area under the curve of plasma concentrations of cardiac tropo-
in T throughout the entire experiment (Fig. 2E) showed a similar significant increase in all of the combination groups with the exception of the DEX + DAU group. The determination of the plasma concentrations of this marker at the end of the experiment (Fig. 2F) provided data of similar statistical significance. A trend toward the lower median values was again noted in animals receiving a combination with the higher nitrite dose.

3.3. Histological examination of the left ventricular myocardium

Chronic DAU treatment induced typical focal morphological changes in the LV myocardium ranging from fragmentation and loss of myofibrils to cytoplasmic vacuolization and finally to cardiomyocyte necrosis/death with replacement fibrosis (Fig. 2G – DAU). The changes were particularly severe and frequent in prematurely dead animals. DEX co-treatment almost completely prevented all of these changes, and myocyte degeneration was almost completely absent or occurred at a minimum intensity (Fig. 2G – DEX + DAU). On the other hand, the combination of dietary nitrate with DAU showed severe morphological changes (Fig. 2G – nitrate + DAU) that were qualitatively and quantitatively similar to those found in the DAU group. The combination of a low nitrite dose with DAU also did not significantly improve DAU-induced morphological changes (Fig. 2G – nitrite(0.15) + DAU). The situation appeared to be somewhat different in the group that was co-treated with the higher nitrite dose (Fig. 2G – nitrite(5) + DAU a, b). This result particularly concerned the presence of advanced changes representing late stages of DAU-induced cardiomyopathy (severe damage affecting larger parts of the myocardium of the LV and interventricular septum). The extent of the damage (i.e., the size and number of foci of seriously altered myocytes and replacement fibrosis) was rather lower compared with that of the DAU group. The majority of the animals in the combination group with the higher nitrite dose thus showed rather moderate morphological changes in the LV myocardium. However, there was still a clear difference in the overall myocardial morphology compared with the morphologies of the DEX-co-treated and control animals.

To further describe the fibrosis of the LV myocardium, we also analyzed relative changes in collagen I and IV expression (Fig. S1A and B). In agreement with the histological examination, we found a distinct up-regulation of collagen I expression not only in the DAU group but also in the combination groups with nitrate and a low dose of nitrite. No significant change was found in the DEX + DAU group compared with the controls, but despite obvious variability, no significant change was also found in the combination...
with the higher dose of nitrite either. In contrast, collagen IV was significantly up-regulated in all of the groups receiving DAU with the exception of the DEX + DAU group. DAU treatment also induced the up-regulation of vimentin (non-myocyte intermediate filament) at the mRNA level which was significantly prevented only by DEX co-administration (Fig. S1C).

Fig. 2. The effects of inorganic nitrate/nitrite and dexrazoxane treatment on daunorubicin-induced cardiac dysfunction, biomarkers of cardiac damage and morphology of the left ventricular myocardium. A) Echocardiographic examination of left ventricular systolic function (LV fractional shortening, the last measured value). Invasive examination of left ventricular systolic function (index dp/dt<sub>max</sub>, B), and diastolic function (index dp/dt<sub>min</sub>, C) at the end of the study. D) Expression of atrial natriuretic peptide (ANP) in the left ventricle. Plasma concentrations of cardiac troponin T were expressed as areas under curve (E) or as the last measured values (F). The statistical significance between the groups was determined by a 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 character. The letters/symbols above the columns indicate statistically significant differences (p ≤ 0.05) compared with the “c” — control group, “n” — nitrate-alone group and “#” — DEX + DAU group. DAU — daunorubicin and DEX — dexrazoxane. G) Histological examination of the left ventricular myocardium — Masson’s blue trichrome staining. Normal morphology was found in the control and dietary nitrate-alone group. Marked degenerative changes and replacement fibrosis were often present in the myocardium of the daunorubicin (DAU) group, and similar findings were observed in the combination groups with dietary nitrate (nitrate + DAU) and the lower dose of parenteral nitrite — nitrite(0.15) + DAU. Somewhat different findings were noted in the group that was co-treated with the higher nitrite dose — nitrite(5) + DAU. Here, advanced myocardial changes representing late stages of cardiomyopathy (b) were relatively less frequent and the majority of animals in this group showed only moderate morphological changes (a). In contrast, DEX co-treatment (DEX + DAU) almost completely prevented all of the morphological alterations that were induced by DAU in the LV myocardium and the findings were very close to those found in the control group. Collagen fibers are stained in blue. Bar: 50 μm.
3.4. Oxidative stress-related parameters

A gene expression analysis of NOX2 and NOX4 in the LV myocardium revealed a marked induction in the DAU group and combination group with dietary nitrate (Fig. 3A and B). A moderate and insignificant increase of NOX2 expression compared with the controls was found in both of the combination groups with parenteral nitrite. Interestingly, changes in NOX4 expression, in which the activity corresponds well with gene expression, revealed a difference between both nitrite doses — the combination with the lower nitrite dose showed a significant increase compared with the controls whereas this was not the case with the higher dose. On the other hand, the gene expression of heme oxygenase 1 was significantly increased in all of the DAU-treated groups with the only exception of the DEX + DAU group (Fig. 3C). DEX was also the only agent that protected against DAU-induced myocardial lipoperoxidation (Fig. 3D).

3.5. Mitochondrial effects and related analysis

DAU treatment induced a significant decrease in citrate synthase activity (Fig. 4A) in the LV myocardium, indicating decreased mitochondrial content. While nitrate supplementation showed no impact on the development of this pathological change, DEX co-treatment completely prevented this. Interestingly, significant improvement was found with nitrite co-treatment. DAU also induced a distinct decrease in the gene expression of mitochondrial superoxide dismutase (MnSOD, Fig. 4B) that was prevented by DEX and by high-dose nitrite co-treatment. We also investigated the impact of the treatments on mitochondrial biogenesis, the perturbation of which has been previously implicated in the development of chronic ANT cardiotoxicity [32–34]. Accordingly, we observed a severe down-regulation of mitochondrial transcription factor A (TFAM, Fig. 4C) which coordinates expression from the nuclear and mitochondrial genome and suppresses the expression of the complex I subunits that are encoded by both the mitochondrial (Nd4, Fig. 4D) and nuclear (Ndufs2, Fig. 4E) genomes. In contrast to nitrate and the lower dose of nitrite, both DEX and the higher dose of nitrite significantly protected hearts from an impairment of expression of TFAM (Fig. 4C), along with the complex I subunits that were encoded by both genomes (Fig. 4D and E). Similar results were also found in the expression of the ADP/ATP translocator of the inner mitochondrial membrane (Fig. 4F). Moreover, DEX and parenteral nitrite (particularly the higher dose) prevented the DAU-induced increase in caspase 3/7 activity (Fig. 4G), suggesting a reduction in apoptotic cell death. Similar changes were observed in the case of caspase 9 (Fig. 4H), which implicates the link to the mitochondrial apoptotic pathway. The same effects were noted with the lower nitrite dose and dietary nitrate. DAU treatment also decreased the expression of Park2 which is an important regulator of mitophagy. While DEX and nitrite (particularly in the higher dose) were found to prevent this event, dietary nitrate showed virtually no effect (Fig. 4I).

Fig. 3. Left ventricular expression of selected pro- and anti-oxidant proteins and lipoperoxidation. Relative gene expression of A, B) NOX2 and NOX4 (NADPH oxidases), and C) heme oxygenase 1 (HO1) in the left ventricular myocardium. D) HPLC determined the total malondialdehyde (MDA) content in the left ventricle as a marker of lipoperoxidation. The data are presented as the mean ± SD or median with interquartile range. The statistical significance between the groups was determined by a 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 character. The letters/symbols above the columns indicate statistically significant differences (p < 0.05) compared with the “c” — control group, “n” — nitrate-alone group and “#” — DEX + DAU group. DAU — daunorubicin and DEX — dexrazoxane.
3.6. Calcium handling, myofibrillar and related proteins

ANT cardiotoxicity has been associated with the impaired expression of calcium handling and sarcomeric proteins. DAU treatment induced a marked decrease in the expression of calcium handling proteins such as SERCA2a (Fig. 5A) and cardiac ryanodine receptor 2 (Fig. 5B). Co-treatment with dietary nitrate had no impact on these changes, although nitrate supplementation alone induced a significant increase in expression at the mRNA level. On the other hand, these events were effectively prevented by co-treatment with both DEX and parenteral nitrite, especially at the higher employed dose. DAU treatment also suppressed the gene expression of myosin light and heavy chains as well as titin (Fig. 5C–E, respectively), and dietary nitrate again showed little to no effect on these perturbations, while DEX was able to prevent the changes completely. An improvement was found with nitrite co-treatment, where statistical significance was again more often observed with the higher dose. On the other hand, only DEX prevented the DAU-induced tendency toward the up-regulation of CARP (Ankrd1, Fig. 5F) which acts as an important mechanosensor within the myofibrils. Similarly, only DEX effectively preserved the normal gene expression of desmin (Fig. 5G).

3.7. Inorganic nitrite and DAU-induced toxicity in vitro

Using H9c2 cells, we demonstrated that a clinically relevant concentration of DAU may induce the loss of the mitochondrial inner membrane potential, as indicated by changes in the red/green ratio of the JC1 probe (Fig. 6A and B). Inorganic nitrite showed a significant, although only partial protection against this effect at wide range of concentrations. Furthermore, DAU increased the activity of caspase 9...
belonging to the mitochondrial apoptotic pathway (Fig. 6C), and this was significantly, albeit only partially, ameliorated by co-incubation with inorganic nitrite. Accordingly, inorganic nitrite also significantly decreased the DAU-induced activation of executive caspase 3/7 (Fig. 6D). The toxicity of inorganic nitrite-alone to H9c2 cells was negligible across a wide range of concentrations (Fig. 6E). However, this toxicity failed to substantially improve DAU-induced viability loss. The only difference was a very slight but significant improvement in cell viability at a very high nitrite concentration (30 mM). These findings were also confirmed using isolated neonatal cardiomyocytes (Fig. 7A). Here, nitrite co-treatment had no significant effect on DAU-induced loss of cell viability which contrasted with the significant cytoprotective effects of DEX in the same settings. Other schedules of nitrite exposure were tested and, in addition to DAU, doxorubicin was also employed, but without significant improvement in the overall cardioprotective efficacy of the tested agent (data not shown).

The obvious difference in the efficacy of cardioprotection as provided by inorganic nitrite and DEX in vitro and in vivo drove us to examine their impact on the protein level of topoisomerase IIβ (TOP2b) which has been recently suggested to be a primary target for ANT in cardiomyocytes [35]. DEX exposure consistently and significantly depleted the TOP2b protein in both primary neonatal cardiomyocytes and H9c2 cells as well as in rabbit hearts (Fig. 7B–D) under conditions in which DEX was found to be cardioprotective. In contrast, nitrite showed no effect on this protein in either of these biological models.

4. Discussion

ANTs can redox cycle within the heart to induce oxidative stress, and mitochondrial complex I has been implicated as the major catalyst of this process [36]. ANTs have been shown to induce profound mitochondrial damage with the attenuation of anti-oxidative defense, impaired
Oxidative phosphorylation, calcium-overload and depolarization of inner mitochondrial membrane [2,7]. Furthermore, we [37] and others [32–34] have demonstrated that chronic ANT cardiotoxicity is associated with the impairment of mitochondrial biogenesis. Cardioprotection with nitrate/nitrite has been previously reported to positively affect all of the above-mentioned events in I/R injury [9,10].

**Fig. 6.** Impact of inorganic nitrite on daunorubicin-induced mitochondrial depolarization, mitochondrial apoptosis signaling and cytotoxicity in H9c2 cells. A) Changes in the mitochondrial membrane potential as determined with the JC1 probe. B) Epifluorescence images of H9c2 cells after loading with the JC1 probe. Red emission reflects the mitochondrial inner membrane potential-dependent accumulation of probe dimers in actively respiring mitochondria, and green fluorescence indicates monomers of the probe released into the cytoplasm after mitochondrial depolarization. Bars 100 μm. Activity of mitochondrial caspase 9 (C) and executive caspase 3/7 (D) as determined by luminescence assay. E) Effects of inorganic nitrite on the daunorubicin-induced cytotoxicity in H9c2 myoblasts. The toxicity was determined by MTT assay and expressed as a percentage of the untreated control group (100%). H9c2 cells were incubated with inorganic nitrite for 24 h alone or pre-incubated with inorganic nitrite for 45 min and then co-incubated with DAU for 3 h, followed by a 20-hour DAU-free period. The data are presented as the mean ± SD. The statistical significance between the groups was determined by a One-Way ANOVA (followed by Holm-Sidak’s post-hoc test). The letters/symbols above the columns indicate statistically significant differences (p < 0.05) compared with the *— control and nitrite-alone group and “d” — DAU group. DAU — daunorubicin.
Inorganic nitrite is deemed to release NO mostly in the affected tissue (when administered in safe doses) and it does not suffer from rapid tolerance development [10]. In addition, sodium nitrite has been safely used for decades for the intravenous treatment of cyanide poisoning [10]. Our in vitro experiments also revealed that inorganic nitrite does not diminish the anticancer efficacy of ANTs even at high concentrations (Fig. S2), which is important in light of some previously published concerns [38]. For these reasons, nitrate/nitrite therapy appeared to be a promising candidate for cardioprotective assessment using the well-established model of chronic ANT cardiotoxicity and for comparison with DEX, the only approved cardioprotectant.

Oral nitrate treatment with the same dose as in the previous study using the acute cardiotoxicity model [22] was well tolerated in rabbits and was associated with a marked increase (20-fold) in plasma NOx that was much higher than the 2-fold increase that was observed in the latter study (with a short supplementation period). However, our data conclusively demonstrate that this approach is unable to prevent or significantly reduce the development of chronic ANT cardiotoxicity in our model. To overcome possible concerns about the limited bioactivation of nitrate to nitrite, we also used i.v. treatment with inorganic nitrite before each chemotherapy cycle. The lower dose of nitrite (0.15 mg/kg) is within the dose range that was used previously for cardioprotection in myocardial I/R injury [13,15,16]. Considering that the i.v. infusion of 0.035 mg/kg of nitrite to rabbits increased its plasma levels by only ~2.5-fold over the baseline [39], our treatment with ~4-fold higher dose of nitrite is likely to provide only a mild to moderate increase in its plasma concentrations. This treatment resulted in only negligible cardioprotective effects against chronic ANT cardiotoxicity as animal survival, necropsy findings, and the functional and histological examination of the LVs did not show a notable improvement compared to the DAU group. After pilot experiments, the second nitrite dose was set to the maximum recommended i.v. dose that was used in the acute cyanide poisoning settings [25]. We have recognized the potential of the agent to reduce the incidence of end-stage cardiotoxicity induced by DAU and to prevent associated premature deaths. Furthermore, we observed that the higher nitrite dose significantly ameliorated certain DAU-induced changes such as loss of mitochondria, mitochondrial biogenesis impairment, decreased MnSOD expression and decreased activation of mitochondrial and global apoptotic signaling. The potential of this treatment to partially reduce DAU-induced mitochondrial damage and apoptosis was confirmed in vitro. We previously observed that DEX can effectively prevent these ANT-induced alterations in vitro [31,40] and in vivo [23,37]. The protection from DAU-induced perturbation of the gene expression of essential sarcomeric and calcium

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**Fig. 7.** Impact of inorganic nitrite and dexrazoxane on daunorubicin-induced cytotoxicity in isolated neonatal ventricular cardiomyocytes (NVCMs) and effects of these agents on topoisomerase IIβ (TOP2b) protein levels. A) The cytotoxicity of inorganic nitrite or DEX alone (100 μM) as well as in combination with DAU was assessed in NVCMs by lactate dehydrogenase (LDH) release into the culture medium. NVCM cells were incubated with inorganic nitrite for 48 h alone or pre-incubated with inorganic nitrite for 3 h and then co-incubated with DAU for 3 h, followed by a 48-hour DAU-free period. Western blot analysis of the level of TOP2b protein in H9c2 myoblasts (B), NVCM cells (C) and rabbit left ventricle myocardium (D). In this analysis, H9c2 and NVCM cells were incubated with 100 μM dexrazoxane and nitrite for 24 h. The left ventricular myocardium were harvested 12 h after the administration of dexrazoxane (60 mg/kg i.p.) or nitrite (5 mg/kg i.v.) to rabbits. A single concentration of DEX utilized in the cell experiments was selected based on previous experiments. The data are presented as the mean ± SD. The statistical significance between the groups was determined by a One-Way ANOVA (followed by Holm–Sidak’s post-hoc test). The letters/symbols above the columns indicate statistically significant differences (p < 0.05) compared with the *— control and nitrite-alone group and #— DEX + DAU group. DAU — daunorubicin. DEX — dexrazoxane.
handling proteins was also observed. Nevertheless, this treatment evidently failed to prevent or significantly reduce cardiac dysfunction as determined by both echocardiography and LV catheterization as well as the biochemical analysis of ANP. Similarly, this treatment was unable to consistently prevent troponin T increase, myocardial lipoperoxidation, and the cellular and molecular remodeling of the myocardium. Thus, we found nitrite treatment to be unable to protect both H9c2 cells and primary cardiomyocytes from DAU-induced loss of viability.

Our data indicate that high nitrite doses may positively modulate some pathways that are involved in the pathogenesis of chronic ANT cardiotoxicity and may partially affect some clinical cardiotoxicity endpoints. Unfortunately, further dose-escalation above the recommended dose is not possible from a clinical viewpoint because of the well-described safety concerns at higher doses [25]. A requirement of higher nitrite doses for the achievement of at least a partial benefit in our setting is strikingly different from the experience with this agent in I/R injury [9]. This difference may be caused by the markedly enhanced bioactivation of nitrite to NO under I/R conditions involving hypoxia and acidosis. Our data with oral nitrate supplementation differ from those previously published using the model of acute ANT cardiotoxicity [22]. This result was not caused by the lower exposure of our animals to nitrate, as we found much higher systemic NOx levels. While different ANTs (doxorubicin vs. DAU) have been used in these studies, they should not be the main issue because chronic cardiotoxicity is known to be the class effect of all effective ANTs and because DEX protects the heart against this phenomenon irrespectively of ANT derivative employment [7]. We preferred DAU over doxorubicin in the present study to avoid complications related to serious extra-cardiac toxicities of doxorubicin in rabbits [41]. Importantly, DAU reproducibly induces a dose-dependent chronic cardiotoxicity with all of the typical hallmarks under these settings. However, the difference in the efficacy of nitrate supplementation in the present and previous study may be related to significant differences between both models of ANT cardiotoxicity. In fact, many previously tested therapeutic agents with promising outcomes in acute high dose models have failed later when tested in clinically more relevant models of chronic ANT cardiotoxicity [7]. This result also concerned several classical antioxidants (e.g., acetylcysteine or vitamin E) or selective iron chelators (such as deferoxamine or deferiprone) [7]. Furthermore, we did not find any increase in NOx after chronic ANT treatment with clinically relevant doses, while a single supratherapeutic dose of doxorubicin did induce an increase in NOx [22], which further implies possible differences in cardiac damage induced by these two approaches as also previously shown [42].

Indeed, the overall efficacy of cardioprotection with nitrite in our chronic ANT cardiotoxicity settings was evidently limited, which contrasted with the unequivocal cardioprotective efficiency of DEX. Good protection with DEX was achieved not only in all of the molecular parameters that were positively modulated by inorganic nitrite but also in the other parameters that are examined in the present study. Most importantly, in our study, DEX almost completely prevented the development of the functional, morphological and biochemical hallmarks of chronic ANT cardiotoxicity. In sharp contrast to the present findings, we previously reported that DEX had only minor cardioprotective effects in myocardial I/R injury model in vitro and in vivo [43]. To achieve these limited benefits, much higher doses of DEX were necessary. The apparently different efficacy of DEX and nitrite in I/R injury and chronic ANT cardiotoxicity settings suggests only limited overlaps of the mechanisms that are essential for the development of both cardiac pathologies. Altogether, these data may suggest more specific upstream effects of DEX in chronic ANT cardiotoxicity that can effectively prevent all pathophysiological consequences, while nitrite may modulate only several downstream mechanisms in the cascade.

While the confirmation of the upstream target for the cardioprotective action of DEX is beyond the scope of this study, we have addressed this point at least in part to determine potential directions for further investigations. Indeed, a recent seminal study proposed TOP2b as a new upstream target for ANT in the heart. TOP2b is a dominant isofrm in terminally differentiated cells including cardiomyocytes, while TOP2a is the most important isofrm and target for ANTs in rapidly dividing cancer cells. Interestingly, conditional TOP2b knockout in cardiomyocytes rescued the myocardium from the development of chronic ANT cardiotoxicity as well as other events such as oxidative stress, mitochondrial damage and apoptosis. Of note, DEX was originally developed as an anticancer drug and TOP2 was subsequently recognized as its main target in cancer cells [7]. Although the iron chelation hypothesis is still often used to explain the cardioprotective action of DEX, we and others have shown little to no cardioprotective effect of stronger and more selective intracellular iron chelators [7]. In addition, we have recently shown that DEX exposure actually does not elicit distinct intracellular chelation in cardiomyocytes [40]. However, we cannot rule out that intracellular chelation is involved in the protective effects of DEX in other tissues in addition to the heart (e.g., protection against bleomycin-induced lung injury) [44]. Hence, in the present study, we compared the effects of DEX and nitrite on the protein level of TOP2b in H9c2 cells, primary cardiomyocytes and rabbit hearts to determine whether and how they affect this new upstream target. The striking difference between both agents in the ability to manipulate TOP2b protein levels suggests that interaction with this target could be involved in the different cardioprotective efficacies. This effect of DEX has been noted previously in vitro in myoblasts [45], and its importance in cardioprotection has been strengthened by our recent study showing that close DEX derivatives lacking effects on TOP2b depletion were cardioprotective neither in vitro nor in vivo despite their retained chelating properties [40]. Hence, it is possible that DEX can pharmacologically deplete the upstream target of ANTs in the myocardium and thus make the heart resistant to cardiotoxicity, as demonstrated similarly by Zhang et al. [35] using a genetic approach. Nevertheless, it remains to be clarified whether TOP2b depletion at the protein level is required for effective cardioprotection or whether enzyme inhibition is a sufficient step for this purpose. While further research is certainly needed, the inability of inorganic nitrite to interact with specific upstream targets of ANTs may explain the limited efficacy of these cardioprotective interventions compared with DEX.

5. Conclusions

Nitrate/nitrite therapy showed only limited cardioprotective potential in chronic ANT cardiotoxicity settings which is in contrast with previous data from myocardial I/R injury and acute high dose ANT cardiotoxicity. A relatively high nitrite dose was required for the positive modulation of some pathways participating in chronic ANT cardiotoxicity and was found to reduce incidence of end-stage cardiotoxicity and related premature deaths; however, this dose was not sufficient for the prevention of most of the other cardiotoxicity endpoints. This result contrasted with the remarkable efficacy of DEX in all of the studied parameters which suggests its rather upstream target. Although further studies are needed, the ability to affect the proximal target within the cardiomyocytes may be important for cardioprotection and may explain the differences in efficacy of DEX and nitrite in chronic ANT cardiotoxicity. The present data also suggest that effects of the possible cardioprotectants on TOP2b in chronic ANT cardiotoxicity merit further study.

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