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RESEARCH ARTICLE

The novel butyrate derivative phenylalanine-butyramide protects from doxorubicin-induced cardiotoxicity

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Aims Butyric acid (BUT), a short chain fatty acid produced daily by the gut microbiota, has proven beneficial in models of cardiovascular diseases. With advancements in cancer survival, an increasing number of patients are at risk of anticancer drug cardiotoxicity. Here we assess whether the novel BUT derivative phenylalanine-butyramide (FBA) protects from doxorubicin (DOXO) cardiotoxicity, by decreasing oxidative stress and improving mitochondrial function.

Methods and results In C57BL6 mice, DOXO produced left ventricular dilatation assessed by echocardiography. FBA prevented left ventricular dilatation, fibrosis and cardiomyocyte apoptosis when co-administered with DOXO. DOXO increased atrial natriuretic peptide, brain natriuretic peptide, connective tissue growth factor, and matrix metalloproteinase-2 mRNAs, which were not elevated on co-treatment with FBA. DOXO, but not FBA + DOXO mice, also showed higher nitrotyrosine levels, and increased inducible nitric oxide synthase expression. Accordingly, DOXO hearts showed lower levels of intracellular catalase vs. sham, while pre-treatment with FBA prevented this decrease. We then assessed for reactive oxygen species (ROS) emission: DOXO induced increased activity of mitochondrial superoxide dismutase and higher production of H_2O_2, which were blunted by FBA pre-treatment. FBA also ameliorated mitochondrial state 3 and state 4 respiration rates that were compromised by DOXO. Furthermore, in DOXO animals, the mitochondrial degree of coupling was significantly increased vs. sham, while FBA was able to prevent such increase, contributing to limit ROS production, Finally, FBA reduced DOXO damage in human cellular models, and increased the tumour-killing action of DOXO.

Conclusions Phenylalanine-butyramide protects against experimental doxorubicin cardiotoxicity. Such protection is accompanied by reduction in oxidative stress and amelioration of mitochondrial function.

Keywords Phenylalanine-butyramide • Doxorubicin cardiotoxicity • Heart failure • Oxidative and nitrosative stress

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Introduction

Cardiovascular complications of cancer therapies are important concerns that limit treatment of oncologic patients,[1−3] emerging as a relevant clinical problem during and after oncologic treatments. Anthracyclines (ANT) are well known for their cardiotoxicity,[4] as their successful use can be hampered by progressive cardiac dilatation, contractile dysfunction, and ultimately heart failure (HF). A remarkable number of strategies has been studied to reduce, prevent, or attenuate ANT-induced cardiotoxicity. Besides limiting cumulative ANT doses and using common HF drugs,[1,3] the interest of the scientific community has also been focusing on antioxidant drugs. The use of dexrazoxane[5] has been clinically evaluated in doxorubicin-treated children with acute lymphoblastic leukaemia, resulting in reduced myocardial injury, as indicated by decreased serum troponin T.[6] However, none of these strategies is unanimously recommended, emphasizing the need for further studies.[7]

Growing evidence shows that the gut microbiota may be an attractive target for the development of novel therapeutic approaches for the treatment of chronic diseases, such as cardiovascular diseases.[8] Butyric acid (BUT), a short chain fatty acid (SCFA) produced daily by the gut microbiota, has already proven beneficial when administered exogenously in several experimental models of cardiovascular diseases,[9−12] but its translation into clinical practice is difficult because of its unpleasant organoleptic properties (e.g. its intense smell of stale cheese) and its rapid degradation that alters its stability, with a despicable pharmacokinetic profile. On the other hand, phenylalanine-butyramide (FBA), a novel synthetic derivative of BUT,[13] has a toxicological profile comparable to that of BUT, but is tasteless and odourless and can be envisioned as a promising novel compound that can be used for a wide range of conditions, including treatment of cardiovascular diseases.[14] Interestingly, FBA has been shown to modulate mitochondrial function and has been proposed as a novel therapeutic approach against obesity and insulin resistance.[15] Therefore, in this work, we aim at assessing whether ANT-induced cardiotoxicity can be prevented by exogenous administration of FBA by decreasing oxidative stress and improving mitochondrial function.

Methods

Doxorubicin treatment protocol *in vivo*

C57BL/6 mice (2−4-month-old, Envigo Srl Italy, San Pietro al Natisone, Udine, Italy) were injected with a cumulative dose of 7 mg/kg doxorubicin (DOXO) via seven daily intraperitoneal injections (1 mg/kg/die i.p., DOXO group). No mortality was associated with this dosing regimen.

Another group of mice was treated with oral gavage with FBA (30 mg/kg/day),[16,17] provided by Prof. Calignano (US patent US 2011 00983.19A1; 28 April 2011), daily for 21 days (FBA group). A third group of mice, after 14 days of FBA, received co-administration of FBA (1 h before i.p. administration of DOXO) and DOXO, at the same doses used in the DOXO group (FBA + DOXO group). Sham animals were used as controls (*Figure 1A*).

For *ex vivo* analyses, animals were sacrificed by cervical dislocation after anaesthesia with tilotamine (0.09 mg/g), zolazepam (0.09 mg/g), and 0.01% xylazine (0.04 mL/g); hearts were then excised and processed for further studies. Eight to 10 animals per group were studied for all protocols.

Transthoracic echocardiography

In vivo cardiac function was assessed by transthoracic echocardiography in sedated wild-type C57Bl/6 mice using a Vevo 770 High-Resolution In Vivo Micro-Imaging System (VisualSonics, Toronto, ON, Canada, 40 MHz transducer). Studies and analysis were performed blinded to heart condition.

More details are available in the online supplementary *Methods S1*.

The experiments described herein were conducted in accordance with the Italian regulations for experimentation on animals. All *in vivo* experiments were carried out with ethics committee approval and met the standards required by Directive 2010/63/EU of the European Parliament.

Immunohistological analysis

Hearts from each group were rapidly excised, washed in cold phosphate-buffered saline (Sigma-Aldrich, D8537) and fixed in 4% formalin, and embedded in paraffin. Cardiac fibrosis was evaluated by staining 5 μm-thick tissue sections. Heart slices were dewaxed, rehydrated and subjected to haematoxylin-eosin staining. Cross-section cardiomyocyte area was evaluated using ImageJ software.

Interstitial fibrosis analysis

Interstitial fibrosis was analysed by PicroSirius Red staining. Briefly, heart slices were dewaxed, rehydrated and incubated with PicroSirius Red 0.1% (Fluka, Buchs, Switzerland) in saturated aqueous solution of picric acid (Fluka, Buchs, Switzerland) for 1 h. Slices were then washed 2 s with acidified water (0.5% acetic acid glacial solution), dehydrated and mounted. The positively stained (red) fibrotic area was measured using ImageJ software. The average percentage of fibrosis to total area was calculated in five images for each heart.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to evaluate cardiac apoptosis, according to the manufacturer's protocol (*in situ* cell death detection Kit, Roche Applied Science, Penzberg, Germany). Apoptosis was indicated as the ratio of TUNEL-positive nuclei/DAPI-stained nuclei in five images per heart.

Gene expression analysis by quantitative real-time polymerase chain reaction

Total RNA was isolated from mice hearts using the Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA) and quantified using the Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). For cDNA synthesis, 1 μg total RNA was transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

More details are available in the online supplementary *Methods S1*.

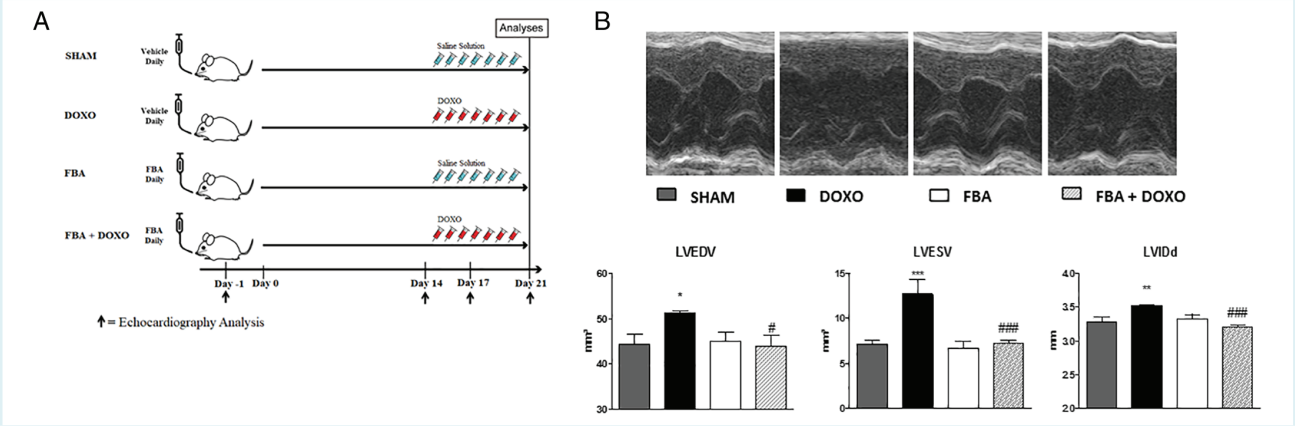

Figure 1 (*A*) Schematic protocol for mice treatments and echocardiography. C57/BL6 mice were randomly divided into four groups. At day 0, mice in the phenylalanine-butyramide (FBA) and FBA + doxorubicin (DOXO) groups were pre-treated with FBA daily for 21 days by oral gavage, while sham and DOXO mice received vehicle as control. At day 15, DOXO and FBA + DOXO mice were injected with DOXO 1 h after daily pre-treatment with vehicle or FBA for the next 7 days, while sham and FBA mice were treated with saline solution. At day 21, mice were sacrificed for *ex vivo* analysis. Heart function was monitored by echocardiography analysis at days −1, 14, 17 and 21. (*B*) FBA prevents left ventricular dilatation induced by DOXO. Top: sample M-mode short-axis echocardiographic images showing left ventricular dilatation induced by DOXO, and the protective effects of FBA in the FBA + DOXO group. Bottom: in mice treated with FBA + DOXO, left ventricular end-diastolic volume, left ventricular end-systolic volume and left ventricular internal dimension in diastole are significantly smaller compared to DOXO. $*P < 0.05$ vs. sham; $**P < 0.005$ vs. sham; $***P < 0.0005$ vs. sham; $\#P < 0.05$ vs. DOXO; $\#\#\#P < 0.0005$ vs. DOXO.

Western blotting

Heart tissues were homogenized and protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Total protein lysates (50 μg) were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (240 mA for 40 min at room temperature).

For further details, see the online supplementary *Methods S1*.

Mitochondrial isolation procedure

Hearts were finely minced and washed in a medium containing 100 mmol/LKCl, 50 mmol/L Tris, pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 5 mmol/L EGTA, 1 g/L fatty acid-free bovine serum albumin (BSA). Tissue fragments were treated with protease nagarse (E.C. 3.4.21.62; 1 mg/g tissue) for 5 min, washed, homogenized for 1 min with the above medium (1:8, wt/v) in a Potter Elvehjem homogenizer (Heidolph, Kelheim, Germany) set at 500 r.p.m. (4 strokes/min) and filtered. The homogenate was centrifuged at 3000 × *g* for 10 min, the mitochondrial pellet was washed twice, re-suspended in a medium containing 250 mmol/L sucrose, 50 mmol/L Tris, pH 7.5, 1 g/L fatty acid-free BSA and centrifuged at 700 × *g* for 10 min.

For further details, see the online supplementary *Methods S1*.

Assessment of markers for oxidative damage and antioxidant enzymes

The rate of mitochondrial H_2O_2 release was assayed by following the linear increase in fluorescence (excitation 312 nm, emission 420 nm) due to the oxidation of homovanillic acid in the presence of horseradish peroxidase. Superoxide dismutase specific activity was measured in a medium containing 0.1 mm EDTA, 2 mm KCN, 50 mm KH_2PO_4, pH 7.8, 20 mm cytochrome c, 5 mm xanthyne, and 0.01 U of xanthyne oxidase. Determinations were carried out spectrophotometrically (550 nm) at 25°C.

For further details, see the online supplementary *Methods S1*.

Measurement of mitochondrial functional parameters

Mitochondrial oxygen consumption was estimated by a Clark type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) maintained in a water-jacketed chamber at 30°C.

For further details, see the online supplementary *Methods S1*.

Cell culture

Human-induced pluripotent stem cell (hiPSC)-derived cardiomyocytes obtained from Ncardia (Cor.4U cardiomyocytes, Ax-B-HC02-MPC, Cologne, Germany) were stored as a frozen stock with at least 10^6 cells/1 mL vial in liquid nitrogen.

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC) and grown in EGM medium.

The human breast cancer cell line MCF-7 was obtained from the ATCC. Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) release was measured with the Pierce™ LDH Cytotoxicity Assay Kit (Termofisher™ #88954). Cells were thawed according to the manufacturer's standard protocol and seeded at a density of 35×10^3 cells/well in a 96-well coated with 10 μg/mL

bovine fibronectin (Sigma Aldrich, St. Louis, MO) dissolved in PBS with Calcium and Magnesium (DPBS Sigma-Aldrich, D8662) overnight at 4°C. Cells were grown in Cor.4U Culture Media (Ncardia) and the medium was changed daily for 2 days before performing the experiment.

For further details, see the online supplementary *Methods S1*.

MTT assay

The viability of cells after concentration- and time-dependent treatments was determined using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. All treatments were done using 3×10^4 cells/well in 24 wells plate and allowed to grow for 24 h. The medium was replaced and different doses of DOXO and FBA, alone or in combination, were added to the culture medium for 3 days.

For further details, see the online supplementary *Methods S1*.

Statistical analysis

Echocardiographic analyses were performed blind to treatment group. Data are presented as mean ± standard deviation. Statistical analyses were undertaken using Prism 5 (GraphPad Software, San Diego, CA, USA).

For most studies, between-group differences were assessed by Student's *t*-test or one-way ANOVA or two-way ANOVA as appropriate, followed by Bonferroni post-hoc test. Statistical significance was defined as $P < 0.05$, unless differently specified.

Results

Phenylalanine-butyramide prevents doxorubicin-induced left ventricular dysfunction

First, we tested the protective effects of FBA on heart damage induced by DOXO. To this end, groups of 8–10 mice received 7 i.p. injections of DOXO (DOXO group) or, after 14 days of pre-treatment with FBA alone, DOXO and FBA were administered together for 7 days (FBA + DOXO group). Another group of animals was treated with FBA alone for 21 days (FBA group) (*Figure 1A*).

After 7 days of treatment with DOXO, transthoracic echocardiography *in vivo* showed left ventricular (LV) dilatation: LV end-diastolic volume (LVEDV) was $51.31 \pm 0.32\,mm^3$ vs. $44.40 \pm 2.19\,mm^3$ in sham ($P < 0.05$); LV end-systolic volume (LVESV) was $12.70 \pm 1.62\,mm^3$ in DOXO vs. $7.11 \pm 0.51\,mm^3$ in sham ($P < 0.0005$); LV end-diastolic internal dimension (LVIDd) was 3.28 ± 0.07 mm vs. 3.52 ± 0.01 mm in sham ($P < 0.005$) (*Figure 1B*).

Intriguingly, treatment with FBA resulted in a significant inhibition of all DOXO-induced effects (*Figure 1*). Indeed, mice from the FBA + DOXO group had significantly smaller left ventricles compared to DOXO: LVEDV was $43.84 \pm 2.61\,mm^3$, LVESV was $7.24 \pm 0.35\,mm^3$, and LVIDd was 3.20 ± 0.03 mm.

Mice administered with FBA alone did not show significant differences compared to sham (*Figure 1B*).

Phenylalanine-butyramide prevents doxorubicin-induced cardiac remodelling and injury

Next, cardiac fibrosis, and cardiomyocyte size and apoptosis, three key hallmarks of DOXO cardiotoxicity, were analysed in explanted hearts. PicroSirius red staining showed a significant increase of interstitial fibrosis in DOXO hearts, which was counteracted by FBA co-administration (*Figure 2A*). Similarly, FBA prevented the reduction in cardiomyocyte size induced by DOXO (*Figure 2B*). In line with these results, DOXO induced apoptosis, prevented by FBA administration (*Figure 2C*).

Phenylalanine-butyramide blunts the increase in ANP, BNP, CTGF and MMP-2 induced by doxorubicin

Hearts were also processed for mRNA expression and detection of myocardial metabolic alterations. A significant increase of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) gene expression, markers of heart dysfunction, was found in the DOXO group compared to sham. Oral treatment with FBA prevented such increase, compared to the DOXO group (*Figure 3A*). In DOXO animals, the increase in the expression of ANP and BNP was paralleled also by a significant increase of connective tissue growth factor (CTGF) and matrix metalloproteinase-2 (MMP-2) gene expression compared to sham, confirming activation of heart remodelling. Co-treatment with FBA significantly reduced these mRNAs compared to DOXO (*Figure 3B*).

Phenylalanine-butyramide prevents the increase of oxidative and nitrosative stress induced by doxorubicin

One of the main mechanisms of DOXO-induced cardiotoxicity involves an increase in oxidative and nitrosative stress.[4,5,7,18] Hence, we tested whether the protection conferred by FBA was achieved by re-establishing the nitroso–redox balance. The evaluation of nitrosative stress was performed by analysing S-nitrosylation of tyrosine residues in intracellular proteins and through the quantification of inducible nitric oxide synthase (iNOS). As shown in *Figure 3B*, compared to sham, DOXO animals showed significantly higher nitrotyrosine levels, in agreement with significantly increased iNOS expression. On the contrary, mice receiving FBA + DOXO showed both significantly lower nitrotyrosine levels and iNOS expression.

We then evaluated the expression of the antioxidant enzyme catalase. DOXO hearts showed significantly lower levels of intracellular catalase vs. sham, while pre-treatment with FBA prevented this decrease ($P < 0.05$ vs. DOXO) (*Figure 3B*).

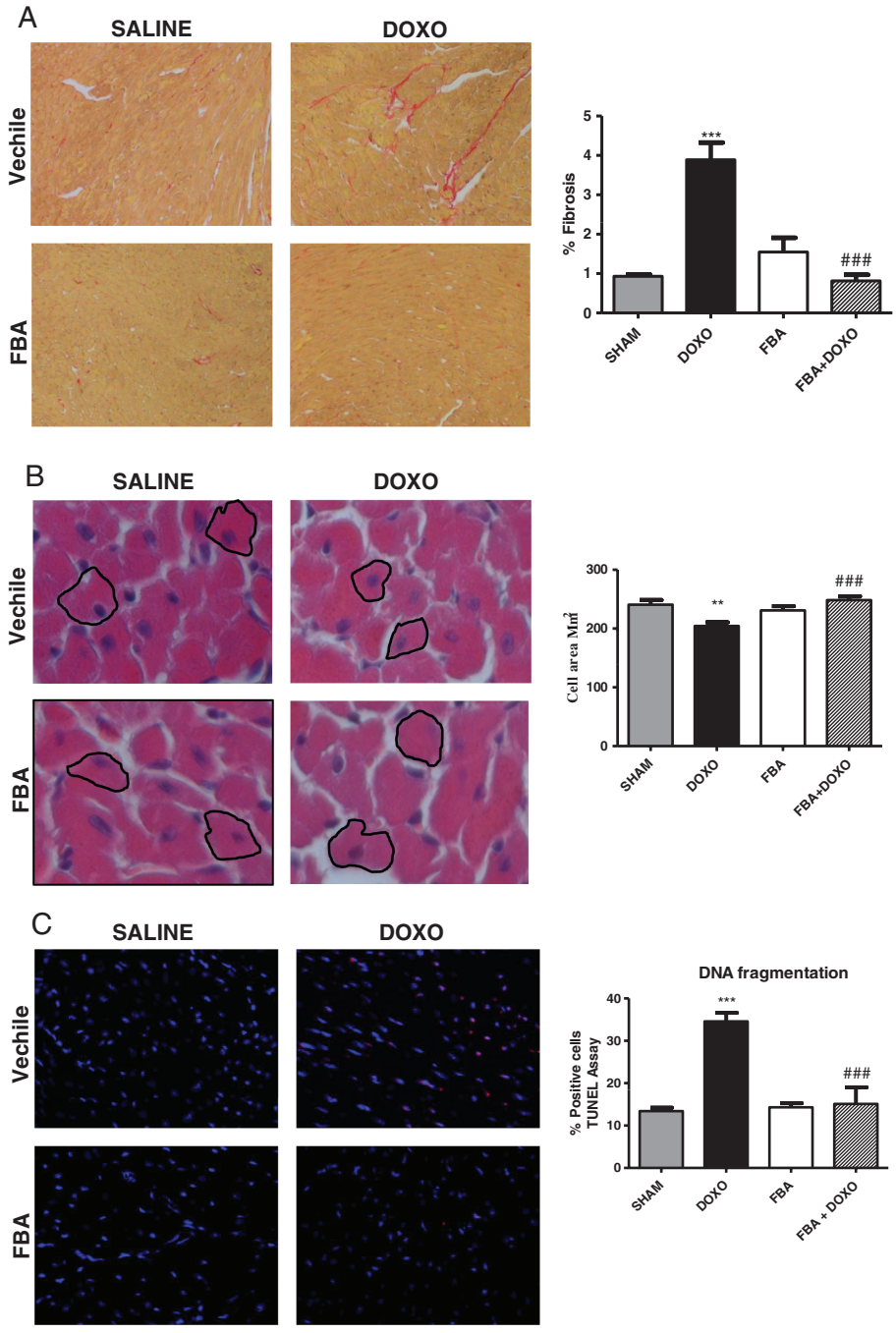

Figure 2 Phenylalanine-butyramide (FBA) protects heart from remodelling induced by doxorubicin (DOXO). (*A*) FBA reduces interstitial fibrosis provoked by DOXO in the heart. (*B*) FBA protects the heart from reduction of cardiomyocyte size induced by DOXO. (*C*) Cell damage and DNA fragmentation is prevented by FBA administration in comparison with DOXO. $^{**}P < 0.005$ vs. sham; $^{***}P < 0.0005$ vs. sham; $^{\#\#\#}P < 0.0005$ vs. DOXO by one-way ANOVA analysis followed by Bonferroni post-hoc test.

Phenylalanine-butyramide counteracts mitochondrial dysfunction induced by doxorubicin

When we assessed for mitochondrial reactive oxygen species (ROS) release, we found that DOXO induced higher production of H_2O_2 and significantly increased activity of mitochondrial superoxide dismutase (*Figure 4A* and *4B*), which were significantly blunted by pre-treatment with FBA.

Mitochondrial state 3 respiration rate, evaluated after ADP addition, using succinate as substrate, decreased in the DOXO group, but FBA pre-treatment determined a significant improvement.

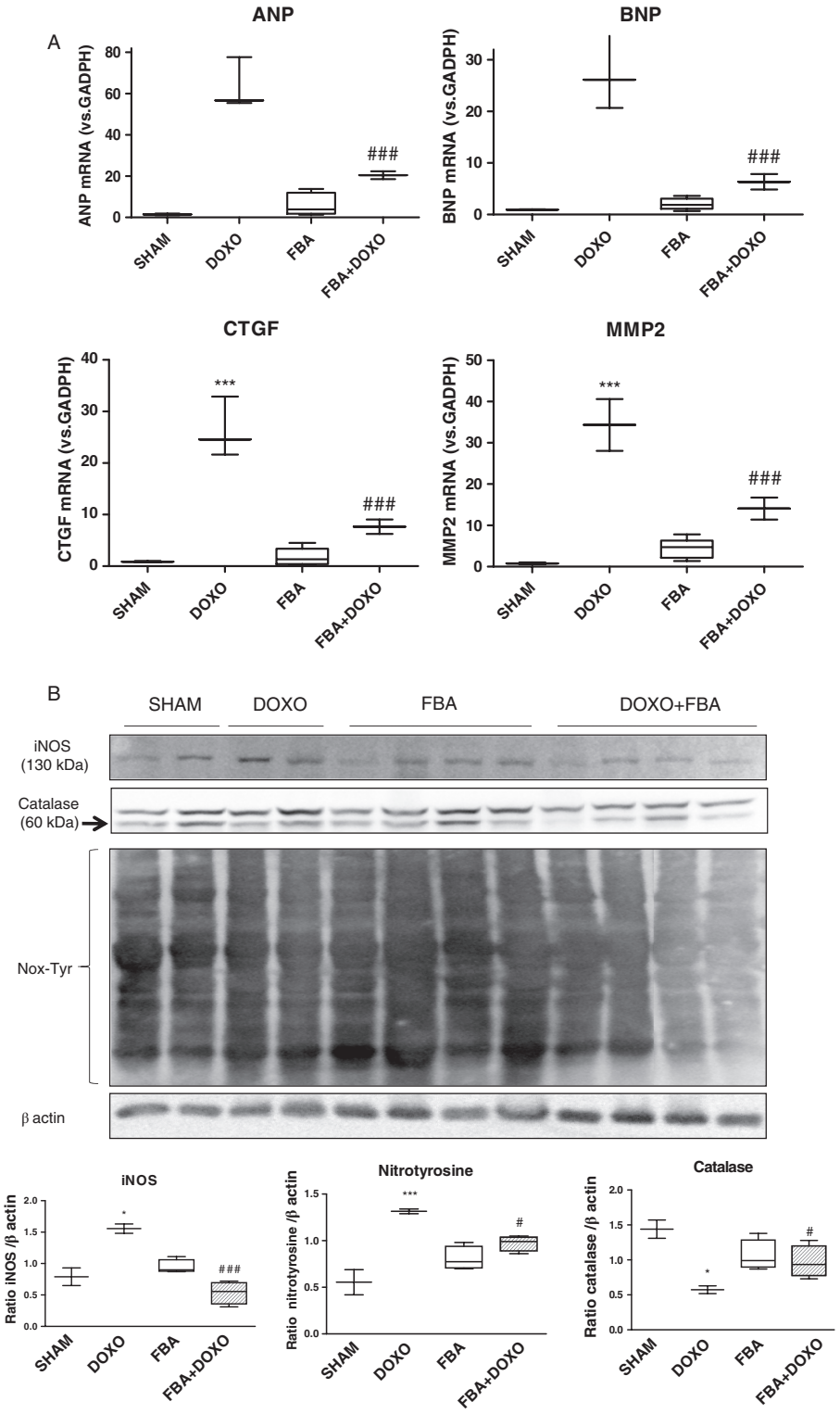

Figure 3 Phenylalanine-butyramide (FBA) reduces mRNA levels of genes elevated during cardiac stress and remodelling and counteracts the increase in nitrosative stress caused by doxorubicin (DOXO). (*A*) Expression levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), connective tissue growth factor (CTGF), matrix metalloproteinae-2 (MMP-2) are increased in mice treated with DOXO, while they are lower in the FBA + DOXO group vs. DOXO. ***$P < 0.05$ vs. sham; ###$P < 0.05$ vs. DOXO. (*B*) FBA decreases protein nitrosylation, blunts the increase of inducible nitric oxide synthase (iNOS) and the reduction of catalase induced by DOXO. *$P < 0.05$ vs. sham; ***$P < 0.0005$ vs. sham; #$P < 0.05$ vs. DOXO; ###$P < 0.0005$ vs. DOXO.

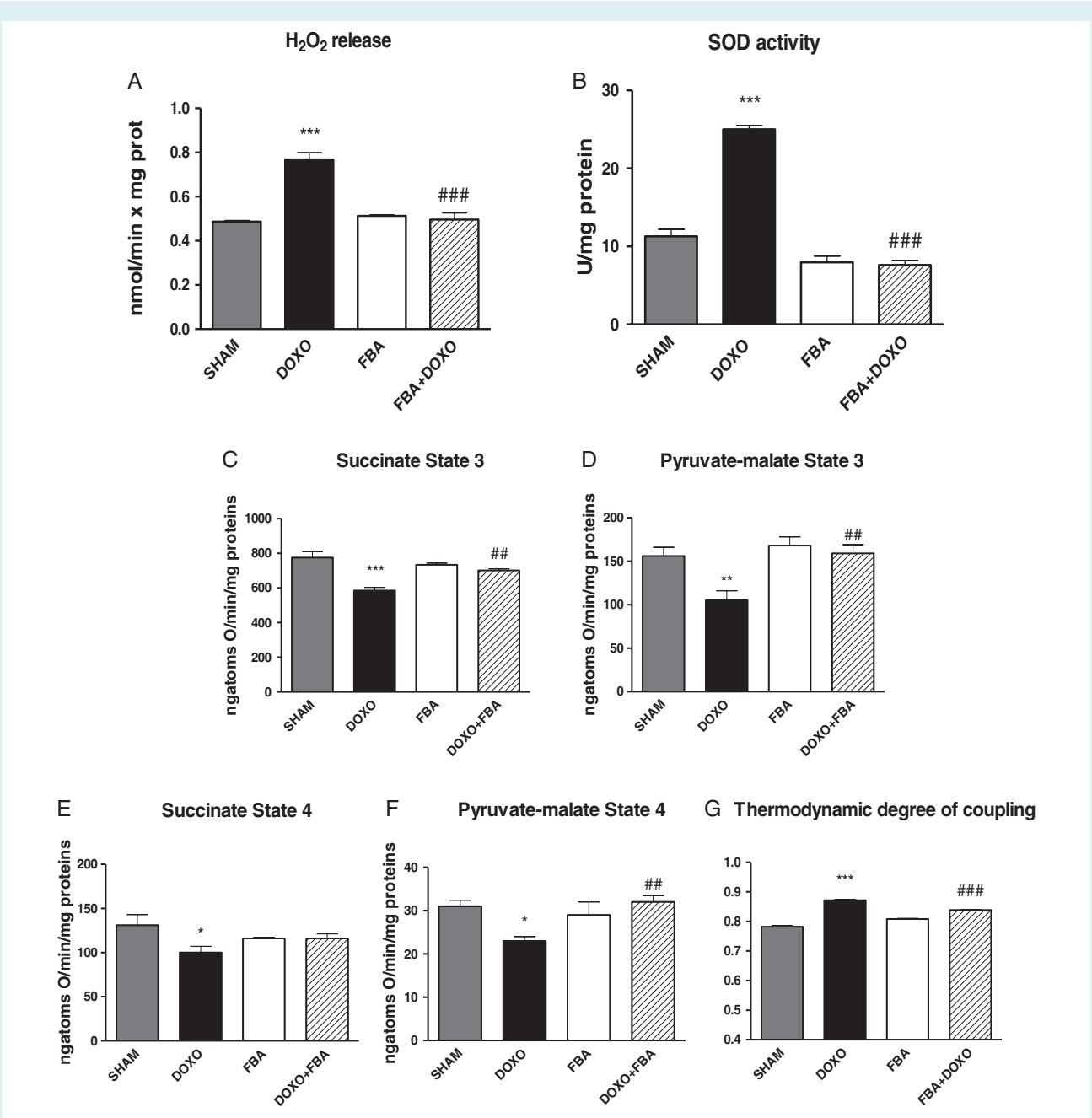

Figure 4 Phenylalanine-butyramide (FBA) prevents mitochondrial reactive oxygen species release and counteracts mitochondrial dysfunction induced by doxorubicin (DOXO). DOXO-triggered H_2O_2 production (*A*) and superoxide dismutase (SOD) activity (*B*) are blunted after pre-treatment with FBA compared to DOXO alone ***$P < 0.0005$ vs. sham; ###$P < 0.0005$ vs. DOXO. Oxygen consumption rates decrease in states 3 and 4 of mitochondrial respiration in the DOXO group (*C*–*F*), while they are preserved after treatment with FBA in the presence of succinate (*C*, *E*) or pyruvate-malate (*D*, *F*). Thermodynamic degree of coupling (*G*) is significantly preserved in the FBA + DOXO group vs. DOXO. *$P < 0.05$ vs. sham; **$P < 0.005$ vs. sham; ***$P < 0.0005$ vs. sham; #$P < 0.05$ vs. DOXO; ##$P < 0.005$ vs. DOXO; ###$P < 0.0005$ vs. DOXO.

The same was true when state 3 was evaluated using pyruvate/malate as substrate (*Figure 4C* and *4D*). State 4 respiration rates, using succinate or pyruvate, were also decreased in DOXO hearts compared to sham, but were increased when mice were pre-treated with FBA (*Figure 4E* and *4F*).

To test for mitochondrial efficiency, we measured oxygen consumption in the presence of oligomycin and FCCP. In the DOXO group, the mitochondrial degree of coupling was significantly increased compared to sham, while FBA was able to prevent such increase, thus contributing to limit ROS production (*Figure 4G*).

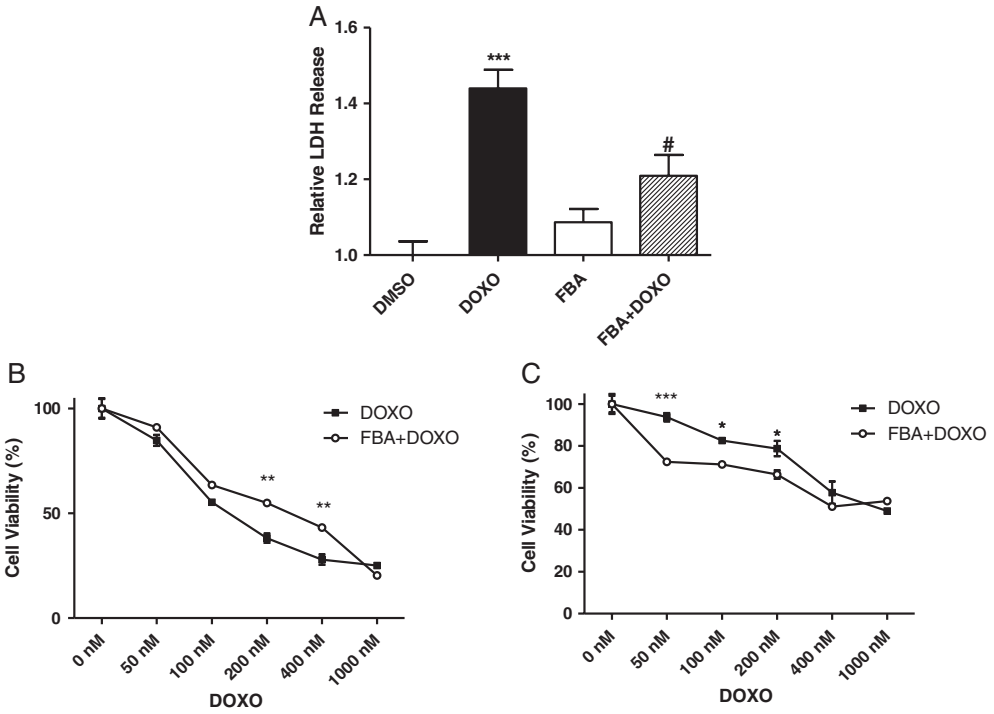

Figure 5 Phenylalanine-butyramide (FBA) reduces the toxicity of doxorubicin (DOXO) in human cardiac cellular models while increasing its tumour-killing action in cancer cells. (*A*) FBA reduces the toxicity of DOXO in human-induced pluripotent stem cell cardiomyocytes as measured by relative lactate dehydrogenase (LDH) release. ***$P < 0.0005$ vs. dimethylsulfoxide (DMSO); #$P < 0.05$ vs. DOXO by one-way ANOVA followed by Bonferroni post-hoc test. (*B*) FBA protects human umbilical vein endothelial cells against DOXO-induced mortality and (*C*) increases the tumour-killing action of DOXO in MCF-7 cells after exposure to increasing concentrations of DOXO. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ by two-way ANOVA followed by Bonferroni post-hoc test.

Respiratory control ratio values were indicative of high quality of mitochondrial preparations indicated by high in all groups (data not shown).

Phenylalanine-butyramide reduces doxorubicin damage in human cellular models and increases the tumour-killing action of doxorubicin

The protective effect of FBA was eventually tested in various normal and transformed human cell types relevant to cardioncology. First, FBA was administered to hiPSC-derived cardiomyocytes and its cardioprotective potential analysed after treatment with DOXO. In line with our findings, FBA significantly protected from cell damage and LDH release induced by DOXO (*Figure 5A*).

We then evaluated the effect of FBA on endothelial cells. As shown in *Figure 5B*, pre-treatment with FBA reduced the mortality of HUVEC induced by different doses of DOXO.

Finally, the effect of FBA in combination with different doses of DOXO was investigated in MCF-7 breast cancer cells. FBA pre-treatment did not interfere, but instead synergized with DOXO to block tumour cell growth (*Figure 5C*).

Discussion

Butyric acid, a short chain fatty acid produced daily in nanomole concentrations by the gut microbiota, has proven beneficial when administered to models of cardiovascular diseases. Cardiotoxicity from ANTs represents a great limitation for the clinical use of these drugs. Here we show for the first time that exogenous administration of FBA (a synthetic derivative of BUT) can prevent DOXO-induced cardiotoxicity in a murine model, by counteracting oxidative stress and improving mitochondrial function. In addition, FBA reduces DOXO damage in human cardiovascular cellular models and increases the tumour-killing action of DOXO.

Among the widely studied mechanisms underlying ANT cardiotoxicity, the increase in oxidative and nitrosative stress plays a major role.[4,5,18,19] It is well established that at physiologic levels ROS are key signalling molecules in normal cardiac function, growth adaptations and matrix changes but higher levels are linked to several heart diseases playing a central role in the pathophysiology of atherosclerosis, ischaemia/reperfusion injury.[20–24] DOXO destabilizes the delicate balance between ROS and reactive nitrogen species production and antioxidant enzyme levels, thus directly and indirectly inducing apoptosis, disarraying extracellular matrix, dysregulating Ca^{2+} homeostasis, and severely damaging mitochondrial function. Furthermore, DOXO cardiotoxicity is mediated, at least

in part, by changes in the high-energy phosphate pool, endothelin-1 levels, disturbances of myocardial adrenergic signalling, nuclear factor-kappa B and Toll-like receptor-2 pathway[25] and the selective inhibition of topoisomerase IIβ.[26] Another key factor in the genesis of myocardial dysfunction induced by DOXO is represented by mitochondrial impairment. Indeed, it is known that ANTs induce an impairment of the ATP pool, subverting the intracellular energetic homeostasis and destabilizing the oxidative phosphorylation through a direct damage to the mitochondrial function with subsequent activation of apoptotic mechanisms.[4,19]

Several strategies have been developed in order to protect the heart from DOXO cardiotoxicity, including limitation of cumulative dose and molecules aimed at lowering oxidative stress. However, the use of these agents is somehow still controversial.[5–7] Great interest has been paid to the potentiality of prebiotics and probiotics in reducing cardiovascular risk, sustaining the key role of gut microbiota in preventing cardiovascular diseases such as hypertension, metabolic syndrome and hypercholesterolaemia.[8,27] Among the production of intestinal fermentation mediated by gut microbiota, BUT is one of the most attractive, guaranteeing about 60–70% of the energy consumed by the epithelial cells of the colon. Mechanisms of action of BUT are multiple, but many of these are related to its epigenetic ability to regulate gene expressions, exerting anti-inflammatory effects with a remarkable translational value for prevention of atherogenesis, diet-induced obesity[13] and insulin resistance,[15] hypercholesterolaemia, inflammatory bowel diseases, as reviewed by our group.[14]

McKay and Mathers[28] demonstrated that BUT can exert histone deacetylase (HDAC) inhibition at supra-physiological concentrations (5 mM), thus modifying the expression of critical genes associated with physiologic and pathologic processes. In the heart, HDAC inhibition *in vivo* and *in vitro* antagonizes hypertrophic stimulation of myocytes, supporting the notion of histone hyperacetylation-dependent signalling of cardiac hypertrophy[29] and seems to attenuate ischaemic injury in the heart as well as in other tissues.[30] Also, HDAC inhibitors are successfully used polychemotherapeutic agents for several tumours.[30]

Among HDAC inhibitors, BUT has already been proven to have anti-neoplastic properties *in vitro*.[14,31] In addition, Tarasenko and co-workers[10] demonstrated *in vivo* and *ex vivo* that butyroyl-oxymethyldiethyl phosphate (AN-7, a BUT-derivative) can augment the cytotoxic effects of DOXO while protecting cardiomyocytes against cardiotoxicity.[11,31] Interestingly, a BUT analogue, sodium-4-phenylbutyrate (4-PBA), has been approved for the treatment of urea cycle disorders[32] and has been also shown to alleviate cardiac apoptosis and myocardial dysfunction induced by DOXO, mainly through reduction in endoplasmic reticulum stress-initiated apoptotic signalling and HDAC-inhibition mechanisms.[32,33] As for these evidences, BUT could represent an optimal strategy to simultaneously synergize anti-neoplastic effects of DOXO and overcome its cardiotoxicity.

The greatest limitation to the clinical use of BUT (and its analogues) is represented by its unpleasant organoleptic and unsuitable pharmacokinetic properties, thus directly limiting its compliance. On the other hand, FBA presents as a solid, poorly hygroscopic, easy to weight molecule, stable to acids and alkalis and able to continuously release BUT into the small and large bowel over time. Moreover, FBA has a toxicological profile comparable to that of BUT, and, importantly, does not carry the typical stench of BUT and its analogues (among which PBA) and it is practically tasteless.[15] Our experimental study shows that pre-treatment with FBA prevents LV dysfunction induced by DOXO lowering DOXO-induced oxidative and nitrosative stress, and improving mitochondrial function compared to hearts treated with DOXO alone. We believe that thanks to its organoleptic and pharmacokinetic features, oral assumption of FBA could be easily tolerated by oncological adult patients and successfully administered even to the paediatric population. The data obtained on hiPSCs cardiomyocytes and HUVEC, together with the ability of FBA to synergize with the tumour-killing effects of DOXO constitute promising grounds for clinical translation of our findings.

Study limitations

Anthracyclines are administered to cancer patients, while in our study we used cancer-free wild-type mice. Nevertheless, C57BL6 mice, that are usually employed in murine HF models, also have a compromised immune system that in part may mimic cancer. Further studies involving cancer-bearing mice will be necessary to better understand the complex interactions between FBA, ANTs, heart and cancer, and to eventually characterize FBA anti-tumour potential.

Conclusions

Besides average HF drugs, there are no specific treatments for DOXO-induced cardiotoxicity. The perspective of using a diet-derived molecule, with great compliance and an optimal cost:yield ratio is very attractive. Thanks to its odourless and tasteless properties, FBA could be safely administered to oncological patients. Our study shows for the first time that FBA, a novel synthetic derivative of BUT, is able to blunt DOXO experimental cardiotoxicity *in vivo*, via a mechanism that involves lowering of oxidative and nitrosative stress and improvement of mitochondrial function. Further investigations are necessary to better establish the potentiality of FBA in cardioncology, also to further explore the mechanisms of action described for other BUT-like molecules.[11,32]

Supplementary Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Methods S1. Supplementary methods.

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