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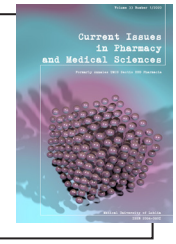

New tirapazamine derivatives protect cardiomyocytes from doxorubicin toxicity

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ABSTRACT

Doxorubicin cardiotoxicity is caused by various mechanisms, most importantly by oxidative stress originating in the mitochondria. Tirapazamine is a hypoxia-activated anticancer experimental drug. Both drugs in normoxia conditions undergo univalent reduction, thus tirapazamine may compete with doxorubicin in univalent reduction enzyme uptake. Herein, tirapazamine derivatives consisted of drug molecules and alkyl chain-connected triphenylphosphine cations that bring about an accumulation in mitochondria. The aim of this study was to evaluate the interaction of newly synthesized tirapazamine derivatives with doxorubicin in rat cardiomyocytes via an vitro model. In the work, H9C2 cells were incubated with combinations of doxorubicin, tirapazamine and seven variants of tirapazamine derivatives. After 24 hours, cell viability was assessed using MTT assay and the results were confirmed by microscopic observation. Tirapazamine in all tested concentrations did not revealed significant protective activity to cardiomyocytes treated with doxorubicine. However, tirapazamine derivatives diminished the cytotoxic effect of doxorubicin regardless of concentration and alkyl chain length. Tirapazamine derivatives have shown protective effects in relation to cardiomyocytes treated with doxorubicin and the mechanism of this phenomenon must be confirmed.

INTRODUCTION

Doxorubicin (DOX) is an anthracycline that in cancer cells acts as a DNA-intercalating agent and also exhibits topoisomerase II inhibitory activity [1]. Its biochemical properties and antitumor activity make doxorubicin one of the most commonly used drugs in treating various types of cancer [2]. Unfortunately, the use of doxorubicin in cancer therapy is limited by a dose-dependent cardiotoxicity which can be observed even years after the last treatment [3]. The toxic effect of doxorubicin is mostly associated with increasing number of reactive oxygen species (ROS) and secondary oxidative stress, which eventually lead to adverse morphologic changes in cells, initially affecting mitochondria [4,5]. Other pathways resulting in doxorubicin-induced cardiotoxicity involve changes in cellular iron homeostasis and reduction in sarcoplasmic Ca^{2+} – ATPase activity [5]. Many antioxidating agents were tested in order to reduce the cardiotoxic effects of doxorubicin, but so far none prove to be clinically useful [6].

In this study, we continue a new approach to protecting cardiomyocytes against doxorubicin via competition. The main concept relies on giving other agents that undergo univalent enzymatic reaction (tirapazamine and its derivates) as competitors of doxorubicin, similarly as ethanol treatment is applied in methanol poisoning. Tirapazamine (TP) is a hypoxia-activated prodrug used in tumor treatment, especially when the tumor cells are anoxic – which may cause resistance to either radio- or chemotherapy [7,8]. The drug alone has moderate antitumor activity, but proved to be useful in combination with other chemotherapeutical agents [9]. Previous study showed that in rat model, tirapazamine reduced heart lipid peroxidation and normalised RyR2 protein level altered by doxorubicin, but there were no significant changes in other parameters of cardiotoxicity

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[10]. The aim of this study was to assess the in vitro effect of combined administration of doxorubicin and newly synthesized tirapazamine derivatives with alkyl chain-connected triphenylphosphine cations that should intensify infiltration of cell membrane and multiply its penetration of the mitochondria.

METHODS

Chemicals

Doxorubicin was purchased from Ebewe Pharma (Unterach, Austria), tirapazamine from Advanced Tech. & Ind. Co. LTD. (China), tirapazamine-derivatives were obtained from the Department of Synthesis and Chemical Technology of Pharmaceutical Substances (Medical University of Lublin, Poland). The derivatives of tirapazamine (4-hydroxy-1-oxido-1,2,4-benzotriazin-1-ium-3-imine) were as follows (Fig. 1a, 1b):

- N2 3-(2-triphenylphosphinyl-ethylamine)-1,2,4-benzotriazine 1-oxide bromide
- N4 3-(2-triphenylphosphinyl-butylamine)-1,2,4-benzotriazine 1-oxide bromide
- N6 3-(2-triphenylphosphinyl-hexylamine)-1,2,4-benzotriazine 1-oxide bromide
- N8 3-(2-triphenylphosphinyl-octylamine)-1,2,4-benzotriazine 1-oxide bromide
- N10 3-(2-triphenylphosphinyl-dodecylamine)-1,2,4-benzotriazine 1-oxide bromide
- N02 3-(2-triphenylphosphinyl-ethylamine)-1,2,4-benzotriazine dioxide bromide
- N04 3-(2-triphenylphosphinyl-butylamine)-1,2,4-benzotriazine dioxide bromide

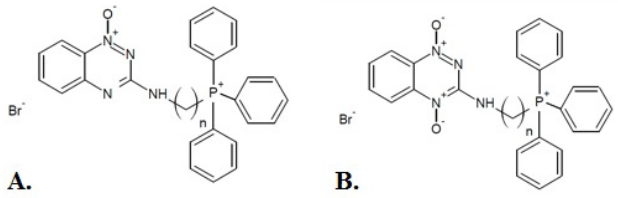

Figure 1. Tirapazamine oxide (A) and dioxide (B) derivatives structure

DMEM (Dublecco's Modified Eagle's Medium), FBS (Fetal Bovine Serum), MTT (thiazolyl blue tetrazolium bromide), tripsin, penicillin-streptomycin solution and amphotericin B were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), DMSO (dimethyl sulfoxide) from POCH S.A. (Gliwice, Poland), PBS from Biomed-Lublin (Lublin, Poland).

Cell Culture

Rat H9C2 cardiac myoblasts (obtained from ATCC – American Tissue Culture Collection, Manassas, VA, USA) were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution in 25 ml flasks. Incubation was carried out at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Upon reaching 80% confluency, the cells were detached via trypsinisation and subcultured to new 25 ml flasks. Cells were seeded in 200µl DMEM supplemented with 10% FBS and plated into 96-well plate approximately 48 h prior to co-treatment with doxorubicin, tirapazamine and its derivates.

Cell cytotoxicity assessment

The cytotoxic effects of tirapazamine derivatives and/or doxorubicin *in vitro* on H9c2 cells were tested by means of rapid colorimetric assay, using MTT, and compared with the untreated controls. The reduction of yellow 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple formazan crystals in living cells was later assessed spectrophotometrically. Cells suspension (2×10^4cell/ml) was dispensed into 96-well microplates and incubated for 48h at 37°C in a fully humidified atmosphere of 5% CO_2. Then, in plate serial dilutions of tirapazamine and its derivatives (chosen concentrations were: 1 µM, 5 µM and 15 µM) and doxorubicin (5 µM) were added to a final volume of 200 µl and incubated for another 24 hrs. Doxorubicin and tirapazamine were used individually as positive controls and the cells incubated in full medium without any drugs were considered as negative controls. After 24 hrs of incubation, all groups underwent MTT testing to evaluate their viability. The incubation lasted for 4 h and was conducted under optimal temperature/humidity conditions. Afterwards, the media was replaced with 200 µl of DMSO in order to release the purple formazan crystals from viable cells, followed by 20 min of shaking. The value of absorbance was read at 540 nm on a Powerwave Microplate Reader (BIO-TEK Instruments INC, USA).

Evaluation of cells morphology

The influence of different concentrations of tirapazamine derivatives with or without doxorubicin on cells morphology was observed by means of a phase contrast microscope (Nikon Eclipse Ti, USA).

Statistics

The results obtained by way of MTT testing were analysed statistically in the STATISTICA v. 10.0 application (StaftSoft, Cracow,Poland), using mean and standard deviation values. To compare more than two groups, one-way analysis of variance ANOVA and post hoc multiple comparisons on the basis of Tukey's HSD test were used.

RESULTS

Doxorubicin (dox) in concentration of 5uM caused decrease of cell viability to 47±7%. Tirapazamine in all tested concentrations did not reveal significant protective activity to cardiomyocytes treated with dox (Fig. 2a). However, tirapazamine derivatives diminished the cytotoxic effect of doxorubicin regardless of concentration and alkyl chain length. What is more, in case of the highest concentration of N6, all concentration of N8 and highest concentration of N10 and NO4, there were not differences in cell viability when compared to control (Fig. 2b-g).

Derivatives N2, N4, N6 15, N8, N10 15, NO4 15 used separately, revealed a slight cytotoxic effect. There were no significant differences in viability of cells treated with lower concentrations of N6, N10 and NO4, in comparison with the control group.

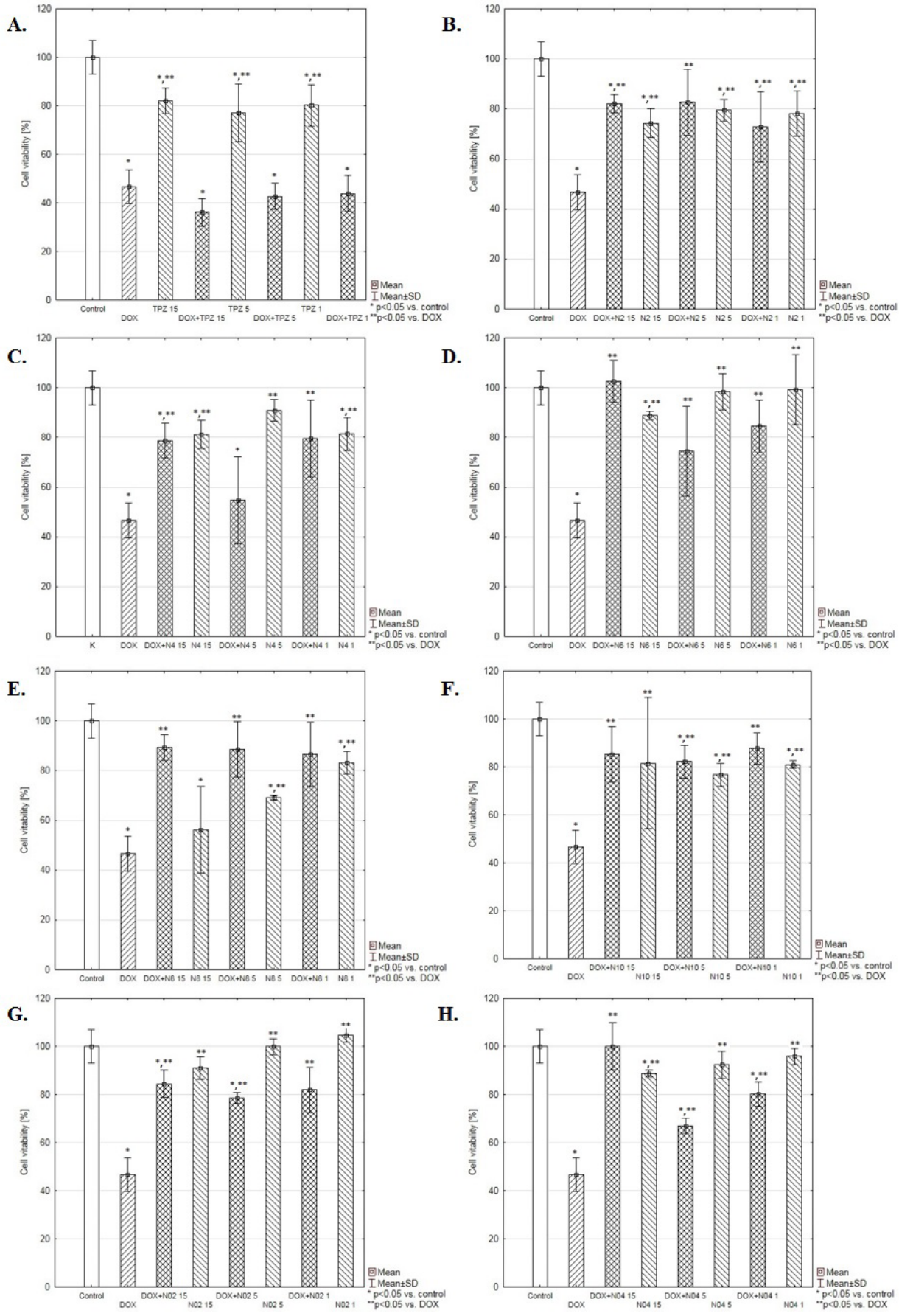

Figure 2a. MTT test results for H9C2 cells treated with doxorubicin (5μM) and tirapazamine (1, 10, 15 μM), expressed as % of values obtained for control cells

Figure 2b-h. MTT test results for H9C2 cells treated with doxorubicin (5μM) and tirapazamine derivative (1, 10, 15 μM), expressed as % of values obtained for control cells (b-N2, c-N4, d-N6, e-N8, f-N10, g-NO2, h-NO4)

Microscopic observations confirmed the results of the MTT assay. Untreated cells show a spindle-like shape with an oval nucleus, localized in the central part of the cell (Fig. 3a). After doxorubicin treatment, a great number of cells appear roundish instead of spindle-shaped, with irregular outline and a wider intercellular gap (Fig. 3b). Incubation with doxorubicin and tirapazamine derivatives resulted in small changes in cell morphology (Fig. 3c). There were much lower numbers of dead cells and cells with shrunken, round and distorted morphology.

DISCUSSION

Cardiotoxicity of doxorubicin is associated with the reactive oxygen species (ROS) formation that occurs simultaneously with redox doxorubicin transformation. The process is related to the activity of various mitochondrial enzymes, including NADPH-oxidoreductases, NADH dehydrogenase, xanthine dehydrogenase and nitric oxide synthase [11]. Doxorubicin reduction conducted by NADH (dehydrogenase) leads to the formation of reactive semiquinone radicals. These react with molecular oxygen, creating superoxide radicals. Simultaneously, this mitochondrial redox cycle produces hydrogen peroxide, as well as hydroxyl radicals. Hydroxyl radicals are the most reactive product of this redox cycle and target the fatty acids present in the lipid bilayer. This leads to irreversible membrane dysfunction, irregular ion transportation and eventually cell death [12]. The aforementioned metabolic pathways combined with moderate antioxidative defense and large density of mitochondria result in far greater susceptibility to ROS-related damage to cardiomyocytes, than to other cells [13]. Due to various pathways leading to ROS formation, ion distribution or membrane potential disruption occurring in mitochondria, finding proper protection against DOX-related cardiomyopathy requires a versatile approach involving many aspects of oxidative stress mechanisms.

Current knowledge of doxorubicin-related cardiomyopathy allows making an argument that tirapazamine might be an appropriate drug to prevent the heart failure that often occurs simultaneously with chemotherapy. This particular benzotriazine derivative runs the same metabolic pathway as doxorubicin, being transformed in cardiomyocytes [9]. In normal conditions, both drugs undergo the same one-electron reduction that leads to free radical formation. Hypoxia, however, leads to tirapazamine exhibiting selective toxicity in targeting DNA [14]. These metabolic similarities result in an assumption that co-administration of both doxorubicin and tirapazamine could provide a unique cardiomyocytes response. Our previous study showed that in a rat model, tirapazamine reduced heart lipid peroxidation and normalised RyR2 protein levels altered by doxorubicin, but there were no significant changes in other parameters of cardiotoxicity [10]. What is interesting, in the skeletal muscle of rats treated with both drugs, concentration of lipid peroxidation product was 5-fold lower than in the muscle of animals treated with DOX only [15]. The promising results of such experiments directed the efforts to finding new tirapazamine derivatives that could prove more efficient in reducing the toxic effects of doxorubicin on cardiomyocytes [7].

RESULTS

The results of this experiment revealed that all examined derivatives protect cardiomyocytes against the cytotoxicity caused by doxorubicin. However, it is not clear if the mechanism of this phenomenon is connected with the suspected competition of the two agents. Still, some kind of interaction definitely occurs. It is generally accepted that mitochondria play a significant role in DOX triggering cardiac specific injury. Heart mitochondria shuttle single electrons to DOX, giving rise to oxygen radical formation via autoxidation of DOX semiquinones. Lipophilic phosphonium cations are able to cross the mitochondrial inner membrane without the assistance of ionophores, and accumulate within the mitochondrial matrix [16]. Directing tirapazamine molecules to the mitochondria could, in our opinion, slow down DOX metabolism and favor the metabolism of the tirapazamine-generated slighter oxidative stress. The hypothesis seems to be confirmed by the results of drug cytotoxicity study – DOX revealed much more toxicity for cardiomyocytes than did tirapazamine. What is more, tirapazamine derivatives also were not toxic to cardiac cells. More intense research, involving mechanism of the phenomenon occurrence is, therefore, necessary to provide a more precise assessment of its therapeutical potential.

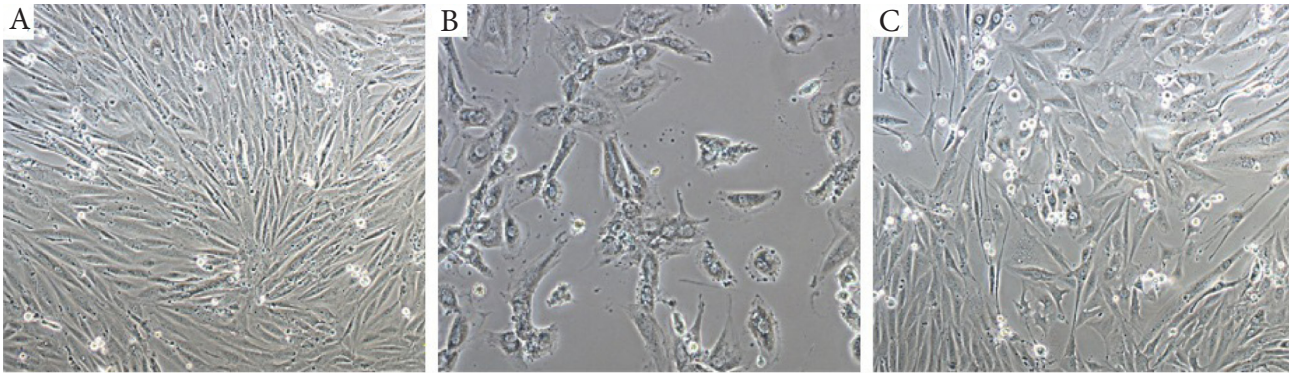

Figure 3. H9C2 ***A.*** control cells, ***B.*** cells after 24 hours post-treatment with doxorubicin (5μM), ***C.*** cells after 24 hours post-treatment with doxorubicin (5 μM) and N8 tirapazamine derivative (1 μM)

CONCLUSIONS

In conclusion, tirapazamine triphenylphosphine derivatives diminished the cytotoxic effect of doxorubicin in H9C2 cells.

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