Dr. Péter Deres

Prevention of doxorubicin-induced acute cardiotoxicity by experimental antioxidant compounds and PARP inhibitors

PhD thesis

Program leader: Prof. Dr. Kálmán Tóth

University of Pécs
Medical School
1st Department of Medicine
Pécs

2008.

INTRODUCTION

Doxorubicin

The anthracycline antibiotic doxorubicin or adriamycin is a chemotherapeutic drug from intercalating agents. Its molecule contains an aminosugar (daunosamine) linked through a glycosidic bond to adriamycinone, a red-pigmented naphtacenequinine nucleus. The exact mechanism of action of doxorubicin is unclear, though it is thought to interact with DNA by intercalation. The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA, while the aminosugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site. Thus topoisomerase II is inhibited.

This very lipophylic agent with its long half-life is widely used against a variety of cancers, but clinical use of doxorubicin is largely limited by its cardiotoxic side-effects. As it is described, 30% of patients treated for advanced carcinoma by repeated infusions of doxorubicin showed heart failure with marked hypotension, tachycardia, necroenzyme elevation (CK, GOT, LDH), R wave reduction. These patients were found to be refractory to inotropic drugs as well as mechanical circulatory assist devices. The incidence of dilatative cariomyopathy and heart failure increases sharply above a cumulative dose of 550 mg/m^2 body surface area. In some patients, the first signs of damage appeared 20 years after discontinuation of the treatment.

Great deals of efforts have been expended in preventing the cardiotoxic side-effects of doxorubicin mainly by scavenging free radicals or chelating iron.

Experimental drugs

Antioxidant molecules and enzymes can potentially limit oxidative injury, but most of them are not readily internalized within myocardial cells, or they cannot reach the right cell compartment to exert their protective effect.

H-2545, a compound with 3-carboxamido-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole moiety, and its metabolite (H-2954) can scavenge free radicals. The molecular structure of H-2545 and H-2954, a stable nitroxide free radical gives these compounds the ability to accumulate in cell membranes and as so exert their antioxidant effect at the primary site of radical formation preventing further cell damage.

In the case of H-2641 the well-known Vaughan-Williams Class I/B mexiletine (which can accumulate into membranes) was substituted with 3-carbonyl-2,2,5,5-tetrametil-2,5-dihydro-1*H*-pyrrolle. Modified mexiletine derivatives were proved earlier to have antioxidant effects; inorganic phosphates, which had been depleted during ischemia, were regenerated; the extent of lipid peroxidation, protein oxidation and ssDNA break formation could also be decreased more favourable than by mexiletine.

For this consideration we selected H-2641 and H-2693 and examined whether they could protect against doxorubicin-induced cell damage. During our experiments we calculated intracellular pH and myocardial energetic parameters.

We previously proved in our ischemic-reperfusion experiments, that PARP-inhibitors (PARPI) could moderate free radical-mediated damages. As we supposed the mechanism of doxorubicin-induced cardiotoxicity acted mainly through free radicals, observing the possible protective effect of PARPI against doxorubicin-induced acute cardiotoxicity seemed to be evident. Quinazoline is a compound made up of two fused six-membered simple aromatic rings, a benzene ring and a pyrimidine ring. It has PARP inhibitor activity, as it's well-known. We investigated the effect of a widely used 4-hydroxyquinazoline (4OHQ) on doxorubicin-induced myocardial damage.

Aims

In our Langendorff-perfusion model monitorized by ^{31}P NMR spectroscopy, we investigated the effect of the H-2545 - that can accumulate in cell membranes -, on doxorubicin-induced acute cardiotoxicity by observing the parameters of energy metabolism, contractile function, myocardial oxidative damage, as well as Akt phosphorylation.

By using H-2641 and H-2693 we wanted to prove the “Hideg-paradigm”, which states that the quick reacting oxidative stress can only be moderated/prevented by molecules capable of connecting site-specifically to the site of damage and having the ability to modify ROS to non-oxidant molecules.

We studied whether the PARP inhibitor, similarly to ischemia-reperfusion experiments, could express its favourable effect on doxorubicin-induced damage. We also investigated if PARPI activated protective PI3-kinase-Akt-GSK signal transduction pathway (which was observed earlier) took a part in presumably protective effect of PARPI on doxorubicin-induced injuries.

..

Methods

Chemicals

The synthesis of H-2545, H-2954, H-2641 and H-2954 has already been published. Malondialdehyde-bis (diethylacetal) was obtained from Merck (Darmstadt, Germany). All other reagents were of the highest purity commercially available.

Animals

The hearts of adult male CFY rats weighing 250-300 g were used for the Langendorff heart perfusion experiments. Investigations meet the guidelines of European Communities Council Directive of 24 November 1986 (86/609/EEC).

Heart perfusion experiments

Rats were anesthetized with ketamine, 200 mg/kg intraperitoneally and heparinized with sodium heparin (100 IU/rat i.p.). Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 mmHg, at 37^{o}C. The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, 0.6 mM octanoic acid, and additionally doxorubicin (100 μM), and/or H-2545 or H-2954 (5, 10, and 20 μM), H-2641, H-2693 (20 μM), and 4-hydroxyquinazoline (100 μM). The perfusate was adjusted to pH 7.40 and bubbled with 95% O_2/ 5% CO_2 through a glass oxygenator. At the end of 60-min perfusion hearts were freeze clamped.

NMR spectroscopy

NMR spectra were recorded with a Varian ^{UNITY}INOVA 400 WB (Varian Inc., Palo Alto, CA, USA) instrument. ^{31}P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z•SPEC® 20 mm broadband probe (Nalorac Co., Martinez, CA, USA), applying WALTZ-16 proton decoupling (γB2=1.2 kHz) during acquisition. Field homogeneity was adjusted by following the ^{1}H signal (w1/2=10-15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each FID (Free Induction Decay: the primary transient signal generated by the sample after an excitation by a radiofrequency pulse, which provides the spectrum after Fourier transformation). 45° flip angle pulses were employed after a 1.25 s recycle delay, and transients were acquired over a 10 kHz spectral width in 0.25 s. Under the above circumstances the relative concentrations of the species can be taken to be proportional to

the peak areas, since interpulse delays exceeded 4-5×T_1 values of the metabolites to be analyzed in ^{31}P experiments. Amounts of each phosphate molecule (creatine phosphate, ATP, and inorganic phosphate) were calculated on the ^{31}P NMR spectroscopic recordings of each heart as the area under the curve of the phosphate group is specific for the given phosphate. Once the amounts were measured, we assigned 100% to the initial values and expressed the values of the subsequent time points as the percentage of the starting value of each individual heart (except for Pi, where the amounts are shown in arbitrary units). Myocardial pH can be calculated using chemical shift (δ) of inorganic phosphate from creatine phosphate: $pH= 6.77 + \log [(\delta-3.23)/(5.70-\delta)$.

Determination of heart function

A latex balloon was inserted into the left ventricle through the mitral valve and filled to achieve an end-diastolic pressure of 8-12 mmHg. All measurements were performed at the same balloon volume. Hearts were selected on the basis of the stability of high-energy phosphates (assessed by NMR) during a control period of 15 min before the experiment. Experimental drugs were added after the 15-min control period. Functional data of rat hearts (LVDP – left ventricle developed pressure, RPP – rate-pressure product, HR – heart rate, +dP/dt, and -dP/dt) were monitored during the perfusion.

Lipid peroxidation and protein oxidation

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined using a modification of a method described by Tzeng et al. Using malondialdehyde standard TBARS were calculated as nmol/g wet tissue. On the other hand, the freeze-clamped perfused heart tissues were homogenized with 1 ml 4% perchloric acid and the protein content was collected by centrifugation.

To determine the protein carbonyl content fifty mg of freeze-clamped perfused heart tissue were homogenized with 1 ml 4% perchloric acid and the protein content was collected by centrifugation. The protein carbonyl content was determined by the 2,4-dinitrophenylhydrazine-method.

Western blot analysis

Fifty mg of heart samples were homogenized in ice-cold Tris buffer (50mM, pH=8.0) and harvested in 2x concentrated SDS-polyacrylamide gel electrophoretic sample buffer. Proteins were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose

membranes. After blocking (2 hours with 3% non-fat milk in Tris buffered saline) membranes were probed overnight at 4˚C with antibodies recognizing the following antigens: phospho-specific Akt-1/protein kinase B-α Ser^{473} (1:1000 dilution) and non-phosphorylated Akt/PKB (1:1000; Cell Signaling Technology, Beverly, MA, USA). Membranes were washed six times for 5 minutes in Tris buffered saline (pH 7.5) containing 0.2% Tween (TBST) prior to addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution; BioRad, Budapest, Hungary). Membranes were washed six times for 5 minutes in TBST and the antibody-antigen complexes were visualized on conventional films by means of enhanced chemiluminescence. Optical densities were determined by the commercially available ImageJ 1.31v (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) software.

Cell viability assay

Cell lines from human cervix epitheloid carcinoma (HeLa), human pancreatic carcinoma (PANC-1), and human hepatocellular carcinoma (HEPG-2) were seeded into 96-well plates at a starting density of $2.5x10^4$ cell/well and cultured overnight. The following day, doxorubicin and/or H-2545, H-2954 or 4-hydroxyquinazoline was added to the medium at the indicated concentrations. Twenty-four hours later, 0.5% of the water-soluble mitochondrial dye (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide, MTT^+) was added. Incubation was continued for 3 more hours, the medium was removed and the water insoluble blue formasan dye formed stoichiometrically from MTT^+ was dissolved by acidic isopropanol. Optical densities were determined by an ELISA reader (Anthos Labtech 2010) at 550 nm wavelength. All experiments were run in at least four parallels and repeated three times.

Statistical Analysis

After F probe significant differences were evaluated by use of paired Student`s t test and *p* values below 0.05 were considered to be significant.

RESULTS

H-2545, H-2954

Protective effect of H-2545 and its metabolite against doxorubicin-induced myocardial energy impairment

Evaluation of the time-course of creatine phosphate (PCr) revealed that at the end of one-hour perfusion the level of PCr was markedly reduced in doxorubicin-treated hearts. The depletion of high-energy phosphates was prevented by both H-2545 and H-2954 administered at 10 and 20 μM. Although the well-known antioxidant dihydrolipoamide (DHLA) was inefficient in preventing the fall in PCr level in 20 μM, it proved to be protective against doxorubicin-induced energy depletion in higher doses (200 μM). Similar changes were observed in the level of myocardial ATP with the aforementioned treatments. Inorganic phosphate accumulated in doxorubicin-treated hearts, which represents poor energetic state.

Preservation of cardiac contractile function by the antioxidant treatment

At the end of the adaptation period LVDP was 78.9±5.1 mmHg, RPP was $22.3\pm1.44\times10^3$ mmHg/min, dP/dt_{max} was 2479±207 mmHg/s, dP/dt_{min} was 1756±71 mmHg/s, and the average heart rate was 197±19 beats/min. During one-hour perfusion with doxorubicin left ventricular developed pressure decreased compared to the initial value, but addition of H-2545 and its metabolite at 20 μM to the perfusate could prevent the functional deterioration. Doxorubicin, as opposed to the values measured in control group, caused significant depression in rate-pressure product, which was ameliorated ($p<0.05$) by our experimental drugs (H-2545 and H-2954). Two other functional parameters were also lowered by doxorubicin in our experimental protocol. +dP/dt and -dP/dt showed decreased values on doxorubicin administration, which was partly reversed by both H-2545 and H-2954.

Doxorubicin-induced lipid peroxidation and protein oxidation were prevented by H-2545 and H-2954

Harmful effect of doxorubicin on several cell organelles has been previously described. In our present experiment indicators of the level of myocardial oxidative damage such as lipid peroxidation and protein oxidation proved to be markedly elevated upon doxorubicin administration. Lipid peroxidation caused by doxorubicin was characterized by the formation of thiobarbituric acid reactive substances (TBARS). When doxorubicin was

administered alone, the level of TBARS increased almost three-fold, which was prevented by the co-administration of H-2545 and H-2954. DHLA in the same order of concentration (20 μM) could not lower lipid peroxidation.

Free radicals formed on doxorubicin administration can also induce the oxidation of proteins in the cardiomyocytes, which can be characterized by the quantity of protein-bound aldehyde groups. Doxorubicin significantly increased the quantity of protein-bound aldehyde groups. However, the presence of H-2545 or H-2954 during doxorubicin administration markedly diminished that.

Akt phosphorylation

Doxorubicin treatment brought about an intensive Akt phosphorylation compared to a basal level of phosphorylation in untreated hearts, eventually indicating a strong effect of free radicals on tyrosine-kinase/Akt signaling pathway. This activation was reduced if H-2545/H-2954 were added to doxorubicin-treated hearts, presumably because the antioxidant agents prevented the activation of Akt kinase cascade. DHLA did not mitigate Akt phosphorylation at 20 μM, however, 200 μM DHLA was sufficient to do so.

Unaltered anticancer effect of doxorubicin in the presence of H-2545

Because doxorubicin is given to eliminate malignant cells, it is of relevance that any other drug co-administered with doxorubicin does not affect its anticancer properties. To evaluate whether H-2545 and its metabolite could modify the chemotherapeutic effect of doxorubicin, we applied doxorubicin in varying concentrations together with H-2545 and H-2954 to malignant cell cultures (HeLa, PANC-1, HEPG-2). Co-administration of the antioxidant agents had no significant impact on doxorubicin-triggered cell death.

H-2641, H-2693

Protective effect of H-2641 and H-2693 against doxorubicin-induced myocardial energy impairment

At the end of one-hour perfusion the level of PCr was markedly reduced in doxorubicin-treated hearts. The depletion of high-energy phosphates was prevented by both

H-2641 and H-2693. Similar changes were observed in the level of myocardial ATP with the aforementioned treatments. Inorganic phosphate accumulated in doxorubicin-treated hearts, which was prevented by administration of H-2641 and H-2693.

Protective effect of H-2641 and H-2693 against doxorubicin-induced myocardial pH decrease

Intracellular pH significantly decreased by the end of one-hour doxorubicin perfusion. H-2641 and H-2693 could prevent unfavorable changes of pH.

4-hydroxyquinazoline

Protective effect of 4-hydroxyquinazoline against doxorubicin-induced myocardial energy impairment

At the end of one-hour perfusion levels of high-energy phospates were markedly reduced in doxorubicin-treated hearts. The depletion of high-energy phosphates and creatine-phosphates was significantly moderated by 4-hydroxyquinazoline. This favourable effect on high-energy phosphates was decreased by the inhibition of protective PI3-kinase in wortmannin treated groups. In the groups treated with 4OHQ, wortmannin or both, we could not observe significant changes from control values. Inorganic phosphate was accumulated in doxorubicin treated hearts, which could be moderated by 4OHQ.

Protective effect of 4OHQ against doxorubicin-induced myocardial pH decrease

The intracellular pH significantly fell by the end of one-hour doxorubicin perfusion. It was prevented by co-administration of 4OHQ, but inhibiting of protective signal transduction pathway, the protective effect of 4OHQ on doxorubicin-induced pH fall was not occurred.

Akt phosphorylation

Similar to our earlier experiments, doxorubicin treatment brought Akt and GSK phosphorylation compared to a basal level of phosphorylation in untreated hearts. This activation could not be reduced by wortmannin which lead us to conclude that doxorubicin-

induced phosphorylation is independent from PI3-kinase pathway. PARPI markedly increased the phosporylation of Akt and GSK which was moderated by PI3-inhibitor.

Unaltered anticancer effect of doxorubicin in the presence of PARPI

Co-administration of PARPI 4OHQ – even if using 100 μM - had no significant impact on antineoplastic effect of doxorubicin on malignant HeLa, PANC-1, HEPG-2 cell lines.

New results, observations

1. Co-administration of antioxidant H-2545 moderated doxorubicin-induced acute cardiotoxicity without affecting the antineoplastic effect of doxorubicin.
2. In every respect of cardiovascular protection, H-2545 and its metabolite H-2954 were more advantageous than the well-known antioxidant dihydrolipoamide.
3. The quick reacting oxidative stress can only be moderated/prevented by molecules capable of connecting site-specifically to the site of damage and having the ability to modify ROS to non-oxidant molecules. This “Hideg-paradigm” was strengthened by our experiments with cardioprotective H-2545, its metabolite, H-2641 and H-2693.
4. It was proved that (similarly to ischemia-reperfusion experiments) the PARP inhibitor 4-hydroxyquinazoline decreased the doxorubicin-induced myocardial damage and activated (partially through PI3-kinase pathway) the protective Akt-GSK signal transduction pathway without affecting the antineoplastic effect of doxorubicin.
5. Our results underline that free radical mechanisms play key role in doxorubicin-induced acute cardiotoxicity.

ACKNOWLEDGEMENT

First I would like to express my gratitude to Prof. Dr. Kálmán Tóth, my supervisor, for showing me the commitment towards the subject, for the given support, and patience. I would like to thank to Prof. Dr. Kálmán Hideg, for having the opportunity to work together with his research group, and for providing the study materials. For the advices given, the research instruments and materials provided, and collectively for the institution as a whole I would like to thank to Prof. Dr. Balázs Sümegi.

I'm further grateful to my co-workers, Dr. Róbert Halmosi, Dr. Ambrus Tóth, Dr. Krisztina Kovács, Dr. Anita Pálfi, Dr. Katalin Hantó, Dr. Gyöngyi Kiss, who provided direct and indispensable aid in my research.

Related to my work as well, I gratefully and fondly think about Dr. Erzsébet Ősz, the NMR operator. I would like to thank both the practical and theoretical work of Dr. Zoltán Berente NMR operator.

I would like to express my gratitude here for the numerous aid given by the Department of Biochemistry and Medical Chemistry employees, Gyöngyi, Heni, Irma, Girán Laci, Berci bátyó.

I would separately like to express my gratitude in these lines to my family, who without consideration to their enduring fatigue and financial sacrifices supported my research, as well as for the multitude of patience and commitment which this paper required for it to be born.