CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Organic and Bioorganic Chemistry

Synthesis of cardioprotective ion chelators derived from diethylenetriaminepentaacetic acid

RIGOROSUM THESIS

Supervisor:

Assoc. Prof. PharmDr. Jaroslav Roh, Ph.D.

Hradec Králové **Jan Šůs**

DECLARATION

Hereby I declare that this thesis is my original work. All used literature and sources are listed in the list of used literature at the end of the thesis and are properly cited. This work has not been used to gain equal or different degree.

Hradec Králové 2020 Jan Šůs

Acknowledgement

This thesis was supported by the Centre for the Study of Toxic and Protective Effects of Drugs on Cardiovascular System (UNCE 204019/304019/2012).

In the first place, I would like to thank to my supervisor Assoc. Prof. PharmDr. Jaroslav Roh, Ph.D. for his professional leadership, valuable advices and consultations and always positive and friendly attitude. Thanks to him, I really enjoyed discovering the field of organic chemistry, as well as the time spent in the laboratory.

I would like to thank to leaders of UNCE project Assoc. Prof. PharmDr. Jaroslav Roh, Ph.D.; Prof. PharmDr. Kateřina Vávrová, Ph.D.; Prof. PharmDr. Tomáš Šimůnek, Ph.D. and Assoc. Prof. PharmDr. Martin Štěrba, Ph.D. for the opportunity to participate on this project and elaborate my work under their professional supervision.

I would like to thank Ing. Galina Karabanovich, PhD. for her support and patience during my practical work in the laboratory. I would like to also appreciate all the support I received from my co-workers and friends PharmDr. Tomáš Eisner and Mgr. Marek Kratochvíl.

Last but not least, I would like to thank my parents for their support during my studies.

ABSTRAKT

Univerzita Karlova

Farmaceutická fakulta v Hradci Králové

Katedra organické a bioorganické chemie

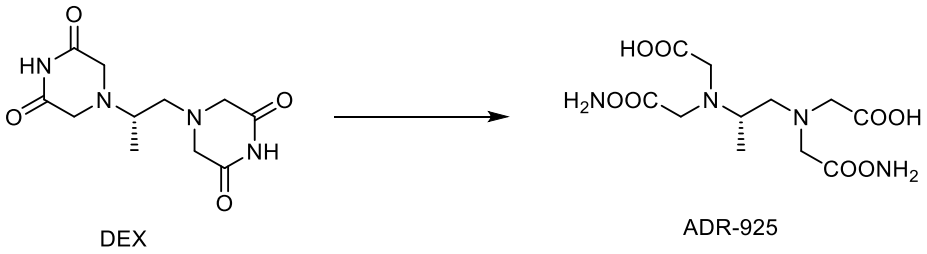
Student: Mgr. Jan Šůs

Školitel: Doc. PharmDr. Jaroslav Roh, Ph.D.

Název rigorózní práce: Syntéza kardioprotektivních chelátorů odvozených od diethylentriaminopentaoctové kyseliny

Anthracykliny (ANTs) jako doxorubicin nebo daunorubicin patří mezi široce rozšířená antineoplastika. Jejich podávání je nicméně spojeno s vysokým rizikem kardiotoxicity. Chronická anthracyklinová kardiotoxicity je charakterizována dilatační kardiomyopatií, s následným rozvojem dysfunkce kontraktility levé srdeční komory a městnavého srdečního selhání. Je předpokládáno, že tvořící se komplexy ANTs s intracelulárními ionty vedou ke vzniku reaktivních forem kyslíku, které způsobují závažné poškození myokardu. Nedávné studie nicméně ukázaly, že mechanismus účinku ANTs je komplexnější a že hlavní úlohu může hrát inhibice topoisomerázy IIβ (TOP2β).

Jedinou látkou s prokázanou klinickou účinností na kardiotoxicitu ANTs je dexrazoxan (DEX). Mechanismu účinku DEX není zcela objasněn, zahrnuje pravděpodobně buď chelataci intracelulárních iontů jejím metabolitem ADR-925 (Obr.1) nebo inhibici TOP2β parentní látkou.

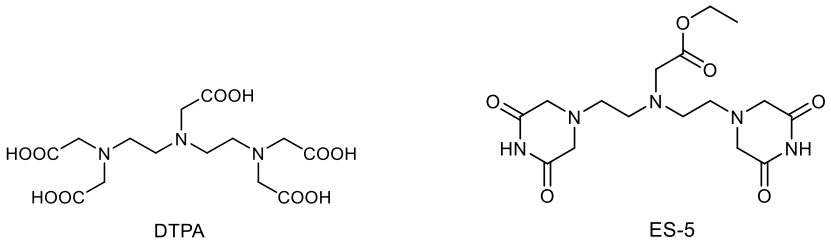


Obr. 1. Dexrazoxan a jeho metabolit ADR-925

Tuto práci lze rozdělit na tři části. V té první jsme připravili metabolit dexrazoxanu ADR-925 v množství dostatečném pro následné *in vitro* a *in vivo* studie s cílem objasnit mechanismus kardioprotekce DEX.

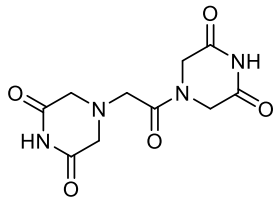
Ve druhé části práce jsme připravili několik potenciálních kardioprotektivních chelátorů železa odvozených od diethylentriaminopentaoctové kyseliny (DTPA, Obr. 2). Hlavní molekula ES-

5 (Obr. 2) byla úspěšně syntetizována a zhodnocena *in vitro* na izolovaných neonatálních potkaních kardiomyocytech a *in vivo* na králičím modelu chronické ANT kardiotoxicity.



Obr. 2. Struktury diethylentriaminopentaoctové kyseliny (DTPA) a ES-5

Ve třetí části jsme se zaměřili na syntézu analogů DEX s modifikovaným spojovacím řetězcem. Byla úspěšně připravena látka JS-X (Obr. 3), která byla určena pro následné *in vitro* a *in vivo* studie.



Obr. 3. Struktura JS-X

ABSTRACT

Charles University

Faculty of Pharmacy in Hradec Králové

Department of Organic and Bioorganic Chemistry

Student: Mgr. Jan Šůs

Supervisors: Assoc. Prof. PharmDr. Jaroslav Roh, Ph. D.

Title of rigorosum thesis: Synthesis of cardioprotective ion chelators derived from diethylenetriaminepentaacetic acid

Anthracyclines (ANTs) such as doxorubicin or daunorubicin are widely used anticancer drugs. However, their administration is associated with high risk of cardiotoxicity. Chronic ANT cardiotoxicity is characterized by dilated cardiomyopathy, with subsequent development of left ventricular contractile dysfunction and congestive heart failure. It is supposed that the complexation of ANTs with intracellular iron ions leads to the formation of reactive oxygen species, which causes serious tissue damage especially in myocardium. However, recent studies showed that the mechanism of action is more complex and the inhibition of topoisomerase IIβ (TOP2β) may play a crucial role.

The only drug preventing cardiotoxicity of ANTs with established clinical efficacy is dexrazoxane (DEX). The mechanism of action of DEX is not fully elucidated, it probably involves either chelation of intracellular ions by its metabolite ADR-925 (Fig. 1) or the inhibition of TOP2β by the parent compound.

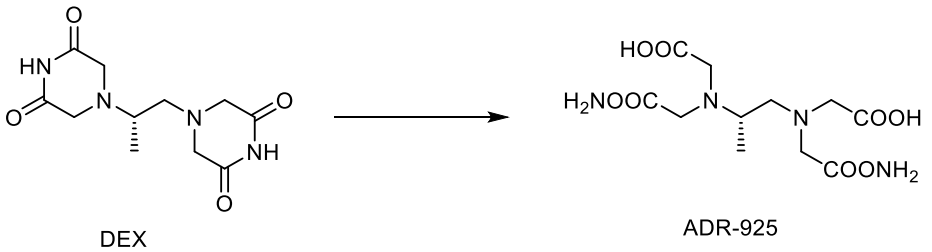


Fig. 1. Dexrazoxane and its metabolite ADR-925

This work may be divided into three parts. In the first part, we synthetized dexrazoxane´s metabolite ADR-925 in the amounts necessary for subsequent *in vitro* and *in vivo* testing in order to study mechanisms of cardioprotection of DEX.

In the second part, we designed several potential cardioprotective iron chelators derived from diethylenetriaminepentaacetic acid (DTPA, Fig. 2). The lead compound ES-5 (Fig. 2) was synthetized and evaluated *in vitro* in isolated neonatal rat cardiomyocytes and *in vivo* in rabbit model of chronic ANTs cardiotoxicity.

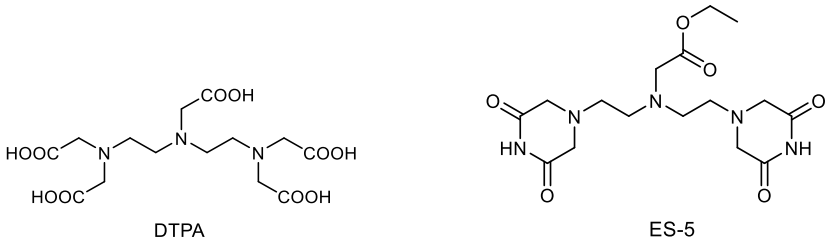


Fig. 2. Structures of diethylenetriaminepentaacetic acid (DTPA) and ES-5

In the third part, we focused on the synthesis of analogues of DEX with modified linker. The compound JS-X (Fig. 3) was synthetized and intended for subsequent *in vitro* and *in vivo* testing.

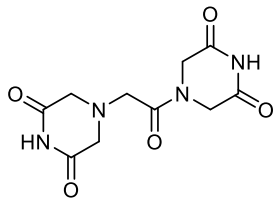


Fig. 3. Structure of JS-X

Contents

1. List of abbreviations

ANT(s) - anthracycline(s)

AUC - area under the curve

C_{max} - maximum plasma concentrations

DAU - daunorubicin

DEX - dexrazoxane

DNA - deoxyribonucleic acid

DOX - doxorubicin

DTPA - diethylenetriaminepentaacetic acid

ECG - electrocardiography

EDTA - ethylenediaminetetraacetic acid

EMA - European Medicines Agency

EU - European Union

FDA - Food and Drug Administration

GTP - guanosine triphosphate

CHF - congestive heart failure

MS - mass spectrometry

NADPH - nicotinamide adenine dinucleotide phosphate

NMR - nuclear magnetic resonance

PPAR - peroxisome proliferator-activated receptor

RAZ - razoxane

Rf - retention factor

ROS - reactive oxygen species

rt – room temperature

SAR - structure-activity relationships

SmPC - summary of product characteristics

TLC - thin layer chromatography

TOP2 - topoisomerase II

US - United States of America

WHO - World Health Organization

2. Theoretical part

2.1. Anthracyclines (ANTs)

2.1.1. Introduction

Cancer is together with cardiovascular diseases leading cause of death in developed countries[1]. Anthracyclines (ANTs) are cytostatic antibiotics belonging to widely used chemotherapeutics and rank among the most effective anticancer drugs ever developed[2,3].

2.1.2. Clinical use, indications

ANTs play an important role in the treatment of various types of neoplastic conditions, including solid tumours such as a small-cell lung cancer, breast cancer, ovarian carcinoma, bladder carcinoma, gastric carcinoma, oesophagus carcinoma, liver carcinoma, colorectal carcinoma, osteosarcoma, adult soft-tissue sarcoma, osteogenic sarcomas, Ewing's sarcoma, Kaposi´s sarcoma, endometrial carcinoma, Wilms' tumour, thyroid cancer and neuroblastoma, and also haematological malignancies such as acute lymphatic leukaemia, acute myeloblastic leukaemia, Hodgkin's and Non-Hodgkin´s lymphomas and multiple myeloma[4,5,6,7,8].

ANTs are often chosen as a frontline treatment in both adult and children patients[3]. There are currently four leading ANTs registered and used in the Czech Republic – doxorubicin (DOX), daunorubicin (DAU), epirubicin and idarubicin. ANTs are predominantly intended for intravenous administration, idarubicin is available also in peroral form.

Although an antibacterial effect of ANT antibiotics was discovered already in 1939, the first ANT called **DAU** was isolated from the bacterium *Streptomyces peucetius, (Actinobacteria* species*)* in early 1960s[9]. **DOX** (also known as adriamycin) was soon isolated from *Streptomyces peucetius, var. caesius* mutated by using *N*-nitroso-*N*-methyl urethane.[10] Its chemical structure is very similar to DAU. Both compound have identical aglycone (a tetracyclic ring with adjacent quinone-hydroquinone groups in rings C-B, a methoxy substituent at C-4 in ring D, and a short side chain at C-9 with a carbonyl at C-13) and sugar (daunosamine, attached by a glycosidic bond to the C-7 of ring A and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl moiety)[11]. The only difference is the introduction of the hydroxyl group to the carbon C14. This slight modification of structure significantly increased the activity of the compound and broadened the range of malignancies, which respond to the treatment. While DAU is effective mainly in the treatment of acute lymphoblastic or myeloblastic leukemias[11,12], DOX was found effective also in a wide range of solid tumours [11,13]. DOX is considered as one of the most effective anticancer drugs, which was ever developed[14].

Soon after the introduction of ANTs into clinical practice, a resistance in tumour cells and ANT-induced cardiomyopathy became a serious complication of the treatment. These facts led to the attempts to further modify the structure and to seek for the analogues with more favourable safety profile, optimized method of administration, as well as for analogues with broader spectrum of clinical efficacy[2]. As a result, epirubicin and idarubicin were introduced to the market.

Epirubicin is a 4-epimer of DOX obtained by an axial-to-equatorial epimerization of the hydroxyl group at C-4 in daunosamine[11]. This modification led to slightly modified pharmacokinetic properties, slightly lower potency, but more favourable safety profile[1,11,15]. Epirubicin is indicated for similar neoplastic conditions as DOX[6]. **Idarubicin** is a semisynthetic 4-demethoxy derivative of DAU. Due to the absence of a methoxy group, its lipophilicity is increased, which particularly leads to higher cellular uptake[16]. Compared to other ANTs, idarubicin is available also in oral dosage form.

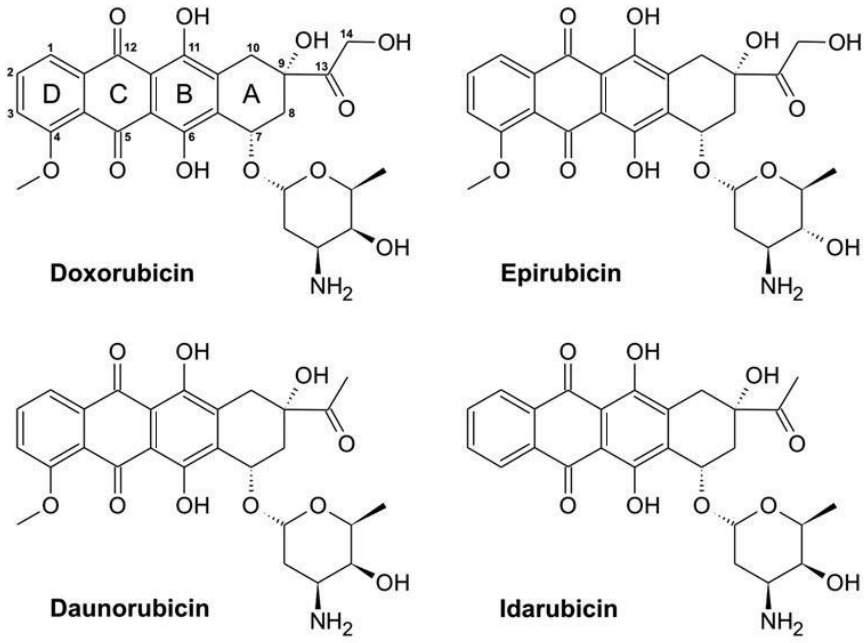


Fig. 4. Chemical structures of the most important ANTs – doxorubicin, daunorubicin, epirubicin and idarubicin[17]

Since the discovery of ANTs in 1960s and their implementation into the treatment regimens, a dramatic enhancement in cancer survival was observed. The increase of 5-year survival from 30% up to 80% was reported in children with leukaemia[18,19]. Although ANTs were discovered more than 50 years ago, they remain crucial in the treatment of many types of malignancies. It was reported than 32% of breast cancer patients[20] and 57-70% of elderly lymphoma patients[21] are treated with ANTs, usually in combination with other

chemotherapeutics. More than 50% available cancer treatment regimens in children are based on ANTs, which results in overall survival rates exceeding 75%[22]. DOX and DAU are included in the WHO Model List Of Essential Medicines[8].

2.1.3. Mechanism of action

The mechanism of action of ANTs is complex and is not fully elucidated. ANTs exert their antineoplastic effect via intercalation into DNA. Nowadays, the antineoplastic activity of ANTs is mainly associated with the inhibition of topoisomerase II (TOP2) - the enzyme which is crucial for control and alteration of the topologic states of DNA during transcription. The inhibition of TOP2 by ANTs results in lethal single and double strand breaks of the DNA helix. Apart from the inhibition of TOP2, also intercalation into nuclear DNA and production of highly reactive oxygen species (ROS) resulting into chromosomal aberrations and subsequent apoptosis probably contribute to the pharmacological activity of ANTs[15,5].

2.1.4. Anthracycline-induced cardiotoxicity

Soon after introduction of ANTs into clinical practice, the cardiomyopathy in the patients treated with them was observed[23,24]. This serious adverse event comprises a spectrum of clinical manifestations, which may result in morbidity, poor quality of life and premature mortality[25].

2.1.4.1. Classification of cardiotoxicity

ANT-induced cardiotoxicity may be classified by the time of onset as acute, subacute or chronic[26].

2.1.4.1.1. Acute cardiotoxicity

Acute forms of cardiotoxicity are relatively rare. The incidence of acute toxicity was reported from 3.2%[27] to 11%[28] of patients treated with ANTs. Acute cardiotoxicity may be manifested as asymptomatic ECG changes or transient decline in left ventricular ejection fraction, but also as severe and potentially lethal arrhythmias, pericarditis, myocarditis or left-ventricular dysfunctions[29,30,31,32,33,34]. Acute cardiotoxicities occur during or immediately after infusion of ANTs, are usually reversible and independent on the ANT dose[29,26]. Mechanism of action of acute cardiotoxicity is not well defined[35].

2.1.4.1.2. Subchronic and chronic cardiotoxicity

Chronic and subacute forms of cardiotoxicity represent the most important adverse events associated with the use of ANTs. This type of cardiotoxicity typically occurs months or years after the completion of chemotherapy with ANTs[36]. Most of the cases are reported within 2-5 years since the therapy commencement[37], nevertheless they may manifest even after 20

years[26]. It is most typically manifested by dilated cardiomyopathy characterized by systolic dysfunction and left-sided congestive heart failure (CHF)[7,38]. The long-term cardiotoxicity caused by the ANTs includes cardiomyocyte death either through necrosis or apoptosis, resulting to irreversible damage of the tissue. It is therefore classified as a type I toxicity[39]. On the contrary, the type II cardiotoxicity may be observed after a treatment with trastuzumab, where a reversible cardiomyocyte dysfunction is present instead of cell death[40]. This severe adverse event usually leads to reduced quality of life or death[29]. The average median survival of 1 year was reported[37].

2.1.4.2. Incidence of cardiotoxicity

There is a comprehensive number of sources on the incidence of ANT-induced cardiotoxicity. It was first reported by van Hoff et al. in 1977, when the retrospective analysis of patients treated with DAU revealed 0.7% incidence of cardiomyopathy in adults and 1.6% in children[41]. Two years later, 2.2% overall incidence of CHF was reported in patients treated by DOX[23]. These retrospective studies also revealed ANT dose-dependent incidence of CHF. Cardiotoxicity was observed in 11% of patients treated with DAU at the dose of 1000 mg/m^2, while the incidence at the dose of 600 mg/m^2 was shown to be 1.5%[41]. Incidence of chronic heart failure was 3%, 7% and 18% at 400, 550 and 700 mg/m^2 doses of DOX, respectively. The overall 2.2% incidence was observed with a median DOX dose of 390 mg/m^2. [23]

In another study conducted by Swain et al., CHF or a significant decline in left ventricular function were observed in 5.1% of patients treated by DOX[42]. Very similar incidence of cardiotoxicity was reported by Abu-Khalaf et al., who observed changes in left ventricular ejection fraction in 5.5% of patients treated with ANTs seven years after the end of chemotherapy[43]. Moreover, the dose dependency of cardiotoxicity was confirmed, as the incidence of CHF was 0.2%, 1.6%, 3.3% and 8.7% at doses 150, 300, 450 and 600 mg/m^2, respectively[42,39]. The results surprisingly showed that also lower doses of DOX may lead to cardiomyopathy. This is supported by the fact, that histopathologic changes on cardiomyocytes were already observed in patients who underwent a chemotherapy with a small dose of 240 mg/m^2 of DOX[32].

In the meta-analysis evaluated by Cardinale et al., cardiotoxicity was observed in approximately 9% out of totally 2625 ANT-treated patients over a median follow-up of 5.2 years[44]. The summary elaborated by McGowan et al. mentions that a clinically symptomatic heart failure develops in 2-4% of patients, 11% of patients suffer from arrhythmias, asymptomatic changes in left ventricular ejection fraction are present in 9-11% of patients and elevated cardiac biomarkers are observed in 30-35% of patients[1].

As the ANTs are widely used for treating pediatric malignancies, the chronic cardiotoxicity is often observed in adult survivals of childhood malignancies[1,45,46]. 23% incidence of late cardiac abnormalities was observed by Steinherz et al. in paediatric patients treated with ANTs after a

follow-up of 10-20 years[47]. Another study reported that up to 65% of the patients treated with DOX in their childhood may have left ventricular abnormalities[48]. A follow-up 1-15 years after DOX treatment revealed myocardium contractility disorders and changes in ventricular wall thickness in 57% in leukaemia paediatric patients[49]. Since the survivals of childhood cancers receive in more than 50% of cases at least one therapy including ANT, the incidence of cardiotoxicity is supposed to be increasing in cancer long-term survivals in the future[18].

To sum up, the risk of cardiotoxicity exists in all patients treated with ANTs including children[50,42,38].

2.1.4.3. Risk factors

As already discussed, the dose of ANTs plays an important role in the development of cardiotoxicity. Apart from ANT cumulative dose, the risk of cardiotoxicity occurrence is increasing with older age, higher body weight and pre-existing co-morbidities such as diabetes or hypertension[3,7,1]. There is also evidence that females are more likely to develop late cardiotoxic events[49] and patients over 60 years have been found to have a four-fold higher risk of developing CHF than patients of 39 years[23]. Additionally, patients who underwent radiation therapy to the mediastinal region, and combination chemotherapy with anticancer agents such as cyclophosphamide, trastuzumab, and taxanes belong to risk groups of patients[15].

2.1.4.4. Mechanism of cardiotoxicity

As the pharmacological mechanism of action of ANTs is not fully elucidated, also the exact pathophysiological mechanism of cardiac toxicity is not known, but it is likely to be multifactorial. It is supposed that ANTs exert their antineoplastic effect via intercalation into DNA, inhibition of TOP2 and formation of ROS[5]. Nowadays, TOP2 is considered as the main mediator of the cardiotoxicity, while ROS generation and other mechanisms contribute. The main hypotheses are described in detail in the following sections.

2.1.4.4.1. Topoisomerase 2β inhibition

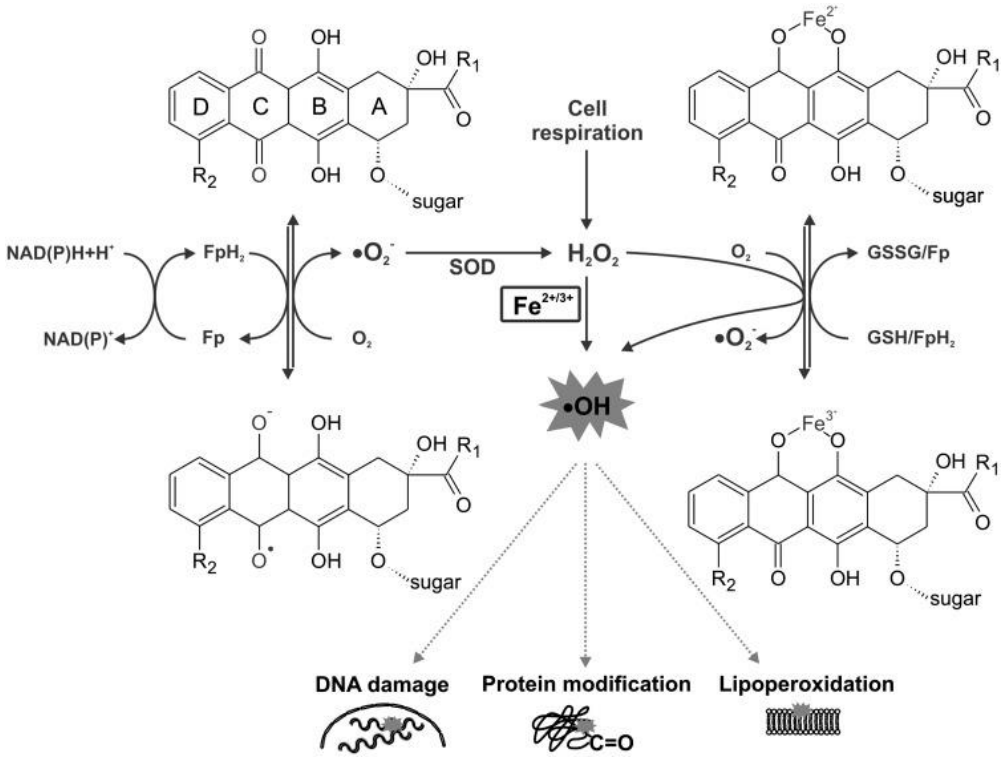
The inhibition of TOP2β is currently believed to be the main mechanism of ANT cardiotoxicity. DNA topoisomerases are the enzymes responsible for temporary single or double-stranded breaks to regulate the topological changes during DNA replication, transcription, recombination and chromatin remodelling[1,51]. Two isoenzymes of TOP2 are present in humans: TOP2α and TOP2β. TOP2α is more prevalent in human body compared to TOP2β and it is highly expressed in proliferating cells. It is necessary for chromosomal segregation and its expression varies during the cell cycle. On the contrary, TOP2β is prevalent mainly in quiescent cells such as cardiomyocytes and its expression remains constant throughout the cell cycle.

After intercalation into DNA, ANTs bind to DNA and TOP2 isoenzymes and form a Top2-anthracycline-DNA complex, which causes single and double strand breaks of the DNA helix. The inhibition of TOP2α leads to the inhibition of DNA replication in the cell cycle G1/G2 and subsequent apoptosis. This mechanism is desirable in the rapidly proliferating malignant cells and is responsible for a high antineoplastic activity of ANTs. On the contrary, when ANTs are bound to the isoenzyme TOP2β, a regulator of oxidative metabolism peroxisome proliferator-activated receptor (PPAR) is inhibited, which leads to the activation of altered P53 tumour suppressor pathway, β-adrenergic signalling, impaired calcium handling, mitochondrial dysfunction and increased apoptosis[1]. As TOP2β is abundantly prevalent in cardiomyocytes, a clinical manifestation of cardiomyopathy may be a consequence.

2.1.4.4.2. Formation of free radicals and oxidative stress

Prior to discovery of the role of TOP2β, the most acknowledged theory elucidating the cardiotoxicity mechanism of action was based on iron ions (Fe)-mediated generation of ROS.

All ANTs share a tetracyclic quinone-hydroquinone moiety. The quinone group may undergo a 1-electron reduction by numerous cellular oxido-reductases, resulting into a formation of a semiquinone radical[52]. In myocardial cells, this is predominantly achieved via an enzymatic pathway involving NADH dehydrogenase (complex I) of the mitochondrial electron transport chain[53]. The parent quinone is regenerated by reducing molecular oxygen to superoxide anion, which dismutates into hydrogen peroxide. It may enter the iron catalyzed Haber–Weiss reaction, resulting into formation of hydroxyl radicals. ANTs are also able to form complexes with free iron ions and undergo a cascade of reactions resulting in further hydroxyl radical production[54]. The hydroxyl radicals are extremely reactive and toxic agents which are causing oxidative stress in the exposed cardiomyocytes by their interference with the mitochondrial electron transport chain. ROS levels may also be increased by free cellular iron and potentiating ferrous-ferric cycling of molecular iron[1]. They impair DNA, modify protein structure and cause lipoperoxidation, which results in cardiomyocyte damage and subsequent death[55]. As the prevalence of oxyradical-scavenging enzymes such as superoxide dismutase, superoxide catalase or glutathione peroxidase in cardiomyocytes is very low and cardiomyocytes are generally rich in mitochondria, these facts make heart very vulnerable to the oxidative stress[56]. The mechanism of main pathways of ANT-induced and iron-catalyzed oxidative stress production is displayed in Fig. 5.



Fe, iron; Fp, flavoprotein; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; NAD(P), nicotinamide adenine dinucleotide (phosphate); $\bullet O_2^-$, superoxide radical; •OH, hydroxyl radical; SOD, superoxide dismutase

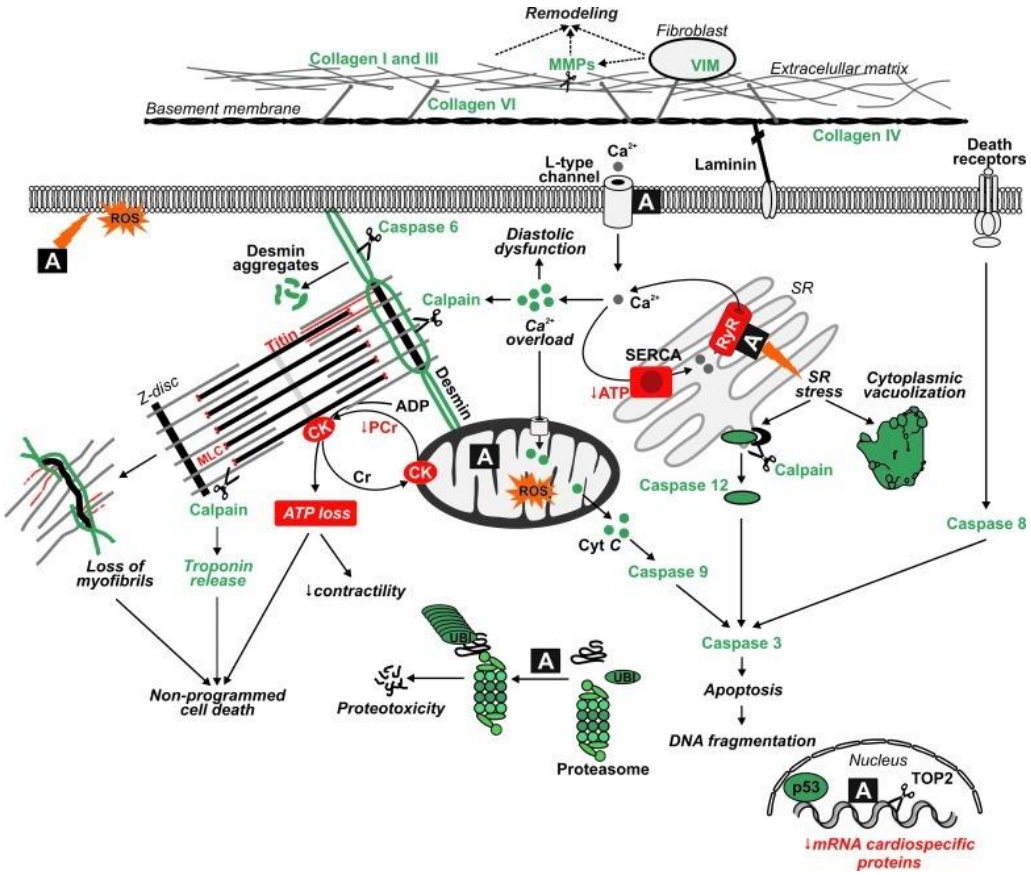
Fig.5. Main pathways of ANT-induced and iron-catalyzed generation of ROS[17]

2.1.4.4.3. Other mechanisms

The administration of ANTs can have also a negative impact on neuregulin-ErbB (NRG) receptor signalling, which is a modulator responsible for controlling of the cell growth in cardiomyocytes, but also in glial cells or skeletal myocytes. A chronic treatment with ANTs probably leads to overexpression of NRG-1 receptor subunit ErbB2, which plays an important role in some malignancies, such as HER2+ breast cancer[1]. By binding of ErbB4 to NRG-1, cardiomyocytes are stimulated and proliferated, which results in heterodimerization of ErbB2 and ErbB4[57]. This is subsequently compensated by cellular hypertrophy, leading to dilated cardiomyopathy and systolic dysfunction of the myocardium[58].

The subunit of GTP enzyme called Ras-related C3 botulinum toxin substrate 1 most probably also contribute to cardiotoxicity of ANTs. They disrupt and fragment a histone associated DNA, which leads to activation of NADPH oxidase and generation of ROS. This is followed by the activation of the programmed cell death caspase-3 enzyme path[59].

Following administration of ANTs to children, c-Kit and cardiac progenitor cells may be decreased, which may result in disruption of repairing mechanisms and instability of myofibrils[60]. This is caused by proteolysis of titin, an important protein responsible for the passive elasticity of muscle, as well as enhanced sequestration of calcium. These both mechanisms can result in diastolic dysfunction[58].



Green color indicates increase, whereas *red color* illustrates decrease due to the treatment. A, anthracycline; CK, creatine kinase; Cr, creatine; Cyt c, cytochrome c; MLC, myosin light chains 1 and 2; MMPs, metalloproteinases; PCr, phosphocreatine; RyR, ryanodine receptor; SERCA, sarco-/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarco-/endoplasmic reticulum; ROS, reactive oxygen species; TOP2, topoisomerase II; VIM, vimentin; UBI, ubiquitin.

Fig.6. Overview of molecular changes associated with development of ANTs cardiotoxicity[17]

2.1.4.5. Prophylaxis and prevention of cardiomyopathy

Range of cardioprotective techniques and therapies have been explored and are described in the following sections.

2.1.4.5.1. **Alteration of pharmacokinetics of anthracyclines**

2.1.4.5.1.1. **Duration and frequency of administration**

Even it is still discussible, the cardiotoxicity of ANTs is believed to be associated with their maximum plasma concentrations (C_{max}) and related penetration of the drug into cardiomyocytes. This is in contrast to their antineoplastic activity, which is correlated mainly with their extent of exposure, represented by the area under the curve (AUC)[11,61]. The administration by a slow infusion instead of intravenous bolus dose may reduce the C_{max} and thus mitigate the exposure of the cardiac tissue[52]. Cochrane review mentions five randomised controlled trials, which concluded the that continuous intravenous infusion, administered for 6 h or longer significantly reduced the risk of CHF (correlated most probably with subclinical cardiac damage) when compared with infusions administered for 1 h or even faster[30,62]. Unfortunately, the pharmacokinetic advantages of slow infusion are counterweighted by another side effects such as myelotoxicity, mucositis, and alopecia, which are resulting from the prolonged exposure to the infusion[63]. Also, DNA-oxidized bases accumulate in normal cells[52]. Additionally, a long hospitalization for several days also poses a significant burden to the patients.

2.1.4.5.1.2. Liposomal formulations

Another strategy how to reduce a risk of cardiotoxicity is the replacement of conventional forms of ANTs by their pegylated liposome encapsulated formulations, which modify pharmacokinetic, as well as pharmacodynamic properties of the drug. Liposomes are composed from bilayers of phospholipids, which are surrounding the active substance. DOX is the compound which has been studied most extensively over this formulation. DOX in its liposomal form occurs mostly encapsulated in liposomes and releases very slowly (over several days) to the plasma, therefore the plasmatic concentration of free DOX is very low. The biodistribution of the active substance to the tissues is changed, the concentration of DOX in tumour is several times higher compared to healthy tissues due to selective uptake and reduced clearance, which is caused mainly by insufficient lymphatic drainage and increased interstitial pressure[64,65]. Moreover, liposomal formulation is not able to penetrate via tight junctions of endothelial lining into the cardiomyocytes and other healthy tissue due its large size[66]. However, they are small enough to cross the very irregular and leaky microvasculature that characterizes solid tumours[11,64]. This is in contrast to conventional formulations of ANT, which are freely diffusible into most of the tissues. As the concentration of free DOX is clearly associated with its toxicity to the issues, especially heart, the cardiotoxicity following administration of liposomal form is significantly decreased compared to the conventional form[67,68]. At the same time, the efficacy of pegylated liposomal DOX has been found to be equal to the conventional form[69].

Currently, there are liposomal formulations of ANTs available in the EU. The available pegylated liposomes of ANTs differ in the ratios between active substance and lipids, as well as in the composition of the lipids. The amount of polyethylene glycol in the liposome affects the pharmacodynamic properties of the drug. As the efficacy and safety of liposomal formulations of ANTs have not been investigated for all indications the conventional forms are approved for, the indications of liposomal formulations are limited. However, they shall be considered for off-label treatment of cancer patients in which cardiovascular risk factors are present.

2.1.4.5.2. Pharmacogenetic prevention

In some patients predispositions for development of heart failure may be observed. The cardiomyopathy may be present even after a treatment with low doses of ANTs, which are normally considered safe[52,64]. There are genetic modifiers such as ANT secondary alcohol metabolites, which may intensify and accelerate the development of cardiotoxicity[52]. Aldehyde reductases are also known to mediate the conversion of ANTs into their secondary alcohol metabolites[70]. In some patients, TOP2β is expressed in higher amounts[71]. Also, coregulating factors of TOP2β such as retinoic acid receptor-γ[72] and ANT transporters polymorphisms[73] may lead in higher predisposition to cardiotoxicity. Also, prooxidant enzymes such as NADPH oxidase[74] and matrix-remodeling enzymes such as type 3 hyaluronan synthase[75] may play a role.

The clinical strategy to prevent administration to the patients with genetic burden comprises the identification of patients at risk[52]. As the nature of cardiotoxicity is multifactorial and there are numerous potential genetic predisposition factors, it is complicated to test all the possible factors and achieve an effective primary prevention. Sometimes, negative screenings for several risk factors may lead to a false-negative conclusion, as there may be some other positive unrevealed factors. If genetic predisposition is identified, the dose of ANTs may be reduced or ANTs can be substituted by another antineoplastic drug. However, this approach is not fully supported by clinical studies.

2.1.4.5.3. Treatment of comorbidities and lifestyle adjustment

As comorbidities such as arterial hypertension or diabetes increase a risk of cardiotoxicity after administration of ANTs, their effective management is therefore needed. Patients may also benefit from a lifestyle adjustment, e.g. weight reduction, smoking cessation and appropriate physical activity[11,76].

2.1.4.5.4. Pharmacological approaches

2.1.4.5.4.1. Co-administration of cardiovascular drugs

The development of cardiotoxicity may be further reduced by the administration of cardiovascular drugs. Significant cardioprotection, measured as left-ventricle ejection fraction, was shown after co-administration of ANTs together with betablockers such as carvedilol[77] or nebivolol[78]. However, another betablocker metoprolol was tested in two studies comprising patients undergoing adjuvant breast cancer therapy and patients treated from lymphoma and it has shown only limited cardiac protection[79,80]. On the other hand, angiotensin II receptor blocker candesartan which was also tested in the study performed by Gulati et al., prevented the decline of left-ventricle ejection fraction[80]. Carvedilol was investigated also in combination with enalapril, a representative of angiotensin converting enzyme inhibitors (ACEi). This clinical trial revealed also a significant level of heart protection. However, enalapril administered alone was found less efficient[79]. It is important to note that the mentioned cardiovascular drugs were examined in studies including small number of patients and only limited long-term follow-up was applied[29]. It is summarized that cardiac protection after cardiovascular drugs administration is achieved mainly through the improvement of compensatory mechanisms of the heart rather than by the protection of the myocytes themselves[29].

2.1.4.5.4.2. Co-administration of statins

Promising cardioprotective effect of statins in breast cancer patients treated by ANTs was shown in the study conducted by Seicean et al.[81]. The study outcome seems to be in accordance with the previously described mechanisms of cardioprotective effects of statins, which are achieved by the activation of nitric oxide synthase[82] and alteration of mitochondrial ATP-sensitive potassium channels opening[83]. However, many patients who were administered statins were in parallel using concomitantly other cardiovascular drugs such as betablockers or ACEi, which puts the direct contribution of statins to the protection of the hearth into question[52].

2.1.4.5.4.3. Treatment with less toxic anthracyclines

As already indicated, epirubicin is a 4-epimer of DOX obtained by an axial-to-equatorial epimerization of the hydroxyl group at C-4 in daunosamine[11]. This modification led to slightly modified pharmacokinetic properties, slightly lower potency, but more favourable safety profile. This was confirmed in controlled clinical trials, where a lower rate of chronic heart failure was observed for epirubicin compared to DOX, while keeping comparable efficacy and survival rate [84]. However, the reduced cardiotoxicity of epirubucin versus DOX is observed only when both compounds are administered in equal amounts. Under these conditions, epirubicin is less efficient due its lower potency and increased clearance. This is compensated by the increase of epirubucin dose, which is unfortunately associated with the increase of risk of cardiotoxicity[85]. The advantage of epirubicin leading from its better safety profiles compared to DOX is therefore counterbalanced by its lower efficacy after the administration of the same dose.

Amrubicin is a representative of the third generation of ANTs, characterized by a lower occurrence of cardiotoxicity. However, its indication is currently limited for the treatment of lung cancer and it is available only in Japan[86]. The clinical trials for bladder carcinoma and gastric cancer are currently ongoing. Chemical structure of amrubicin is depicted in Fig. 7.

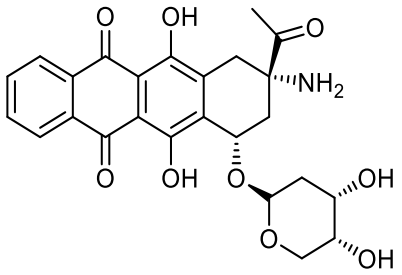


Fig.7. Chemical structure of amrubicin

2.1.4.5.4.4. Antioxidants

Antioxidants, such as vitamin A or E, may decrease the cardiotoxicity of ANTs by behaving as free radical scavengers. Acute cardiotoxicity was mitigated by the administration of vitamin E in mice, however the long-term mortality was not decreased[87,88]. Similarly, the lives of animals were significantly prolonged after administration of vitamin C in mice treated with DOX. There is probably synergistic effect when both vitamins are administered together[56]. Various antioxidants have shown promising results in *in vitro* and *in vivo* studies, however none of them has confirmed an efficacy in clinically relevant chronic models[17].

2.1.4.5.4.5. Dexrazoxane

Although the methods mentioned in the previous chapters may mitigate the risk of ANT-induced cardiotoxicity, the only cardioprotective agent with established preclinical and clinical efficacy is dexrazoxane (DEX). It is also the only medicine approved by EMA and FDA for cardioprotective treatment of ANT-induced cardiotoxicity[89,90].

2.1.4.5.4.5.1. Chemistry of dexrazoxane

DEX belongs to the bisdioxopiperazines and its full chemical name is (*S*)-4-[2-(3,5-dioxopiperazin-1-yl)-propyl]piperazine-2,6-dione. The molecular formula of DEX is $C_{11}H_{16}N_4O_4$, the molecular weight is 268.28 g/mol. The structural formula of DEX is depicted in Fig 8.

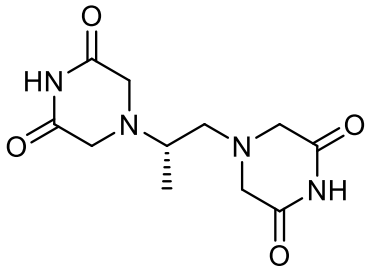


Fig. 8. Structural formula of dexrazoxane

DEX is dextrorotatory isomer of razoxane (a racemic mixture of (+)-(*S*)-dexrazoxane and (-)-(*R*)-levrazoxane). It is a whitish crystalline powder, with the melting point at 191° to 197°C. It is sparingly soluble in water and 0.1 N HCl, slightly soluble in ethanol and methanol, and practically insoluble in nonpolar organic solvents. The pKa is 2.1, an octanol/water partition coefficient (logP) is 0.025.

2.1.4.5.4.5.2. Clinical evidence and use

The bisdioxopiperazine compounds became largely studied for their cardioprotective effects after promising outcomes from several preclinical studies[91]. The study in dogs revealed the cardioprotective effect of razoxane, when it was administered prior to ANTs[91]. Another preclinical study has shown the reduction of the cardiotoxicity and lethality after administration of DAU to non-cancer-bearing Syrian golden hamsters, when pre-treated by DEX[92]. Herman et al. performed another preclinical studies in animals and the cardiac protection ensured by DEX was observed also in beagle dogs, rabbits, and miniature swine, when treated by DOX or DAU[93,94,95]. Positive results from preclinical studies led to examination of DEX in humans, where the cardiac protection was confirmed.

The clinical efficacy of DEX was confirmed in three prospectively randomized placebo-controlled studies in humans. In these studies, either DEX or placebo were administered to patients treated with DOX within the first course of chemotherapy. The cumulative dose of DOX was above 300 mg/m^2, but the upper limit was not specified. Most of the clinical studies were performed in patients with advanced breast cancer, studies comprised also patients with sarcoma and lung cancer[15]. The cardiac function was assessed by measurement of the left ventricular ejection fraction and clinical evaluations. Patients receiving DEX had significantly smaller mean decreases from baseline in left ventricular ejection fraction and lower incidences of CHF compared to placebo group. However, patients with advanced breast cancer receiving treatment consisting of fluorouracil, DOX and cyclophosphamide with DEX had a lower response rate and a shorter time to progression compared to the patients who received the treatment with placebo[96]. A correlation between the cardioprotective benefit of DEX and the cumulative ANT dose was

observed[97]. The pre-treatment with DEX therefore allows the use of higher cumulative doses of ANTs[98,99]. The efficacy of DEX was confirmed both in childhood and adult cancer patients.

DEX is available as sterile, pyrogen-free lyophilizate intended for intravenous administration[96]. DEX is the only drug approved by the US FDA for use as a cardiac protectant in patients exposed to ANTs[52]. The indications of DEX slightly differs between the EU and the US. In the EU, DEX is indicated in adults for the prevention of chronic cumulative cardiotoxicity caused by ANTs use in advanced and/or metastatic breast cancer patients who have received a prior cumulative dose of 300 mg/m^2 of DOX or a prior cumulative dose of 540 mg/m^2 of epirubicin when further ANT treatment is required[100]. In the US, the indication of DEX is slightly narrower, as it is determined for reducing the incidence and severity of cardiomyopathy associated with DOX administration in women with metastatic breast cancer who have received a cumulative DOX dose of 300 mg/m^2 and who will continue to receive DOX therapy to maintain tumour control[96]. The exact indications of DEX are listed in the Table 1.

DEX was designated the orphan drug status in the EU for the treatment of ANT extravasation, which can be associated with intravenous administration of chemotherapeutics. In some cases, the dose may be mistakenly injected or leaked out outside the vein and may be spread to the surrounding tissues. Irritation of the tissues, which may lead also to their necrosis, are the consequences of extravasation. The chemotherapeutic agent forms a depo, which may be responsible for ulcerations over several weeks[101].

DEX has an orphan status also in the US, but for different designation - prevention of cardiomyopathy for children and adolescents up to 16 years of age treated with ANTs. However, its use in children was contraindicated by the EMA over concerns of increased risk of infection, myelosuppression and secondary malignancies, and because its efficacy in children has not been established[89].

Table 1 – Summary of DEX products marketed in the EU and in the US

	EU	**US**
Strengths	500 mg per single dose vial	250 mg or 500 mg per single dose vial
Dosage form	powder for solution for infusion; sterile, pyrogen free, white to off-white, lyophilised powder	sterile, pyrogen-free lyophilizate
Contains	500 mg of DEX as its hydrochloride salt per vial	DEX hydrochloride equivalent to 250/500 mg DEX.
Indications	the prevention of chronic cumulative cardiotoxicity caused by ANT use in	reducing the incidence and severity of cardiomyopathy

	advanced and/or metastatic breast cancer patients who have received a prior cumulative dose of 300 mg/m^2 of DOX or a prior cumulative dose of 540 mg/m^2 of epirubicin when further ANT treatment is required in adults	associated with DOX administration in women with metastatic breast cancer who have received a cumulative DOX dose of 300 mg/m^2 and who will continue to receive DOX therapy to maintain tumour control
Orphan designation	Treatment of ANT extravasations	Prevention of cardiomyopathy for children and adolescents 0 through 16 years of age treated with ANTs
Source	SmPC Cardioxane[100]	Labeling-Package Insert - Zinecard [96]

2.1.4.5.4.5.3. Mechanisms of action

The exact mechanism by which DEX exerts its cardioprotective effect has not been fully elucidated, similarly to the mechanism of ANT cardiotoxicity. There are two main hypotheses for DEX mechanism of action – chelation of iron ions and the inhibition of TOP2.

2.1.4.5.4.5.3.1. Chelation of Fe ions

DEX is a bisdioxopiperazine compound, a ring-closed analogue of ethylenediaminetetraacetic acid (EDTA) (Fig. 9).

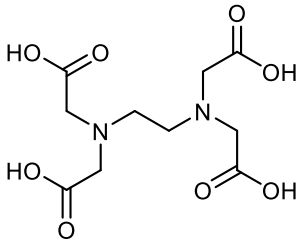


Fig. 9. Ethylenediaminetetraacetic acid (EDTA)

DEX easily penetrates into cardiac cells, where its piperazinedione rings are open and hydrolysed in two steps into diacid diamide compound called ADR-925 (also known as ICRF-198), very similar to EDTA by its structure. The two-step hydrolysis of DEX into ADR-925 and the comparison of its structure with EDTA is displayed in Fig. 10. According to this hypothesis, DEX acts like a prodrug, which easily reaches its site of action due to its enhanced lipophilicity. ADR-925 acts as a strong iron chelator and is able to displace iron from the ANT before it converts O_2^- and H_2O_2 into more toxic highly reactive hydroxyl radicals or equally reactive iron-oxygen

complexes[102]. Cardiac protection is mainly attributed to iron chelating properties of the open ring metabolite (ADR-925), which may inhibit the oxidative injury[103]. However, also DEX itself is capable of chelating iron ions[100].

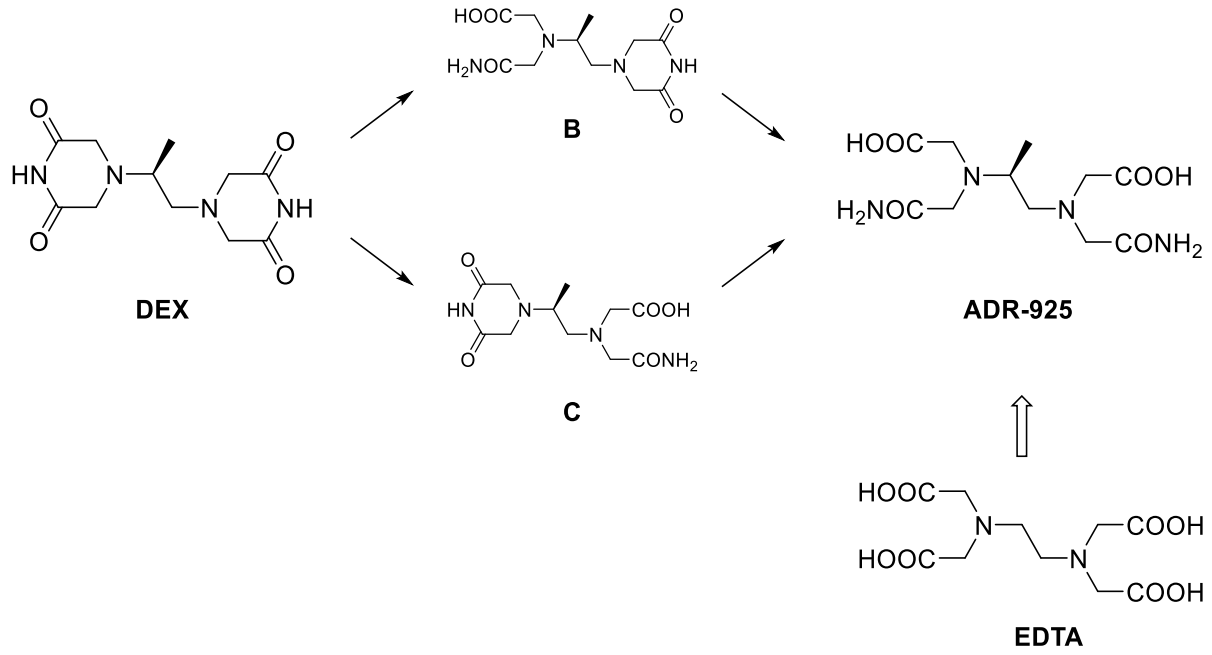


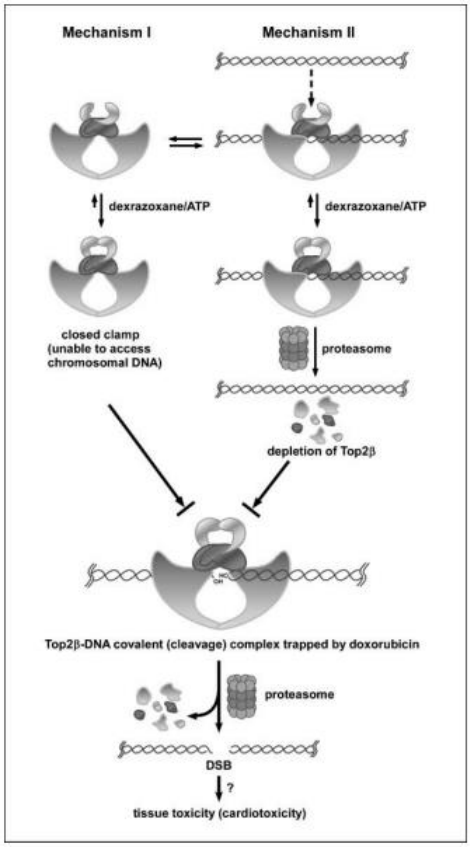
Fig.10. Two-step hydrolysis of DEX to ADR-925 and the structural relationship of ADR-925 and EDTA

2.1.4.5.4.5.3.2. Inhibition of topoisomerase IIβ

Beside the chelating properties, DEX is also a catalytic inhibitor of DNA TOP2β, which is the same target as the ANTs have[15]. However, in contrast to TOP2 poisons such as ANTs, DEX does not induce lethal DNA double-strand breaks[104]. DEX can compete for the ATP-binding site of TOP2β, preventing the formation of ANT-DNA-TOP2β complex and therefore precluding DNA double-strand breaks and subsequent cardiomyocyte death[76,105,106]. While many antioxidants have failed to show a sufficient cardiac protection, the inhibition of TOP2 as the mechanism of action may explain the clinical efficacy of DEX [76,107,108].

The mechanism of cardioprotection of DEX via its inhibition of TOP2β is shown in the Fig.11. TOP2β is shown to exist in two states, TOP2β (Mechanism I) and DNA-bound TOP2β (Mechanism II), at equilibrium. DEX can bind to TOP2β in either state. According to Mechanism I, DEX binds to free TOP2β and stabilizes the closed-clamp conformation of ATP-bound TOP2β. Therefore, it prevents from binding of TOP2β (closed clamp) to chromosomal DNA and thus DOX is unable to trap TOP2β into cleavage complexes. For mechanism II, DEX binds to DNA-bound

TOP2β and stabilizes the closed-clamp conformation of ATP-bound TOPβ. That may trigger proteasomal degradation of TOP2β, i.e. down-regulation of TOP2β. It results into depletion of TOP2β and therefore fewer DOX-trapped TOP2β cleavage complexes. The formation of these complexes leads to DNA double-strand breaks through proteasome-mediated processing, which, could contribute to cell death and possible cardiotoxicity, if not repaired[105].



ATP – Adenosine triphosphate, Top 2β – topoisomerase IIβ, DSB – double-strand break

Fig. 11. Two proposed mechanisms for the antagonistic effect of DEX on DOX-induced DNA damage[105]

Apart from its cardioprotective effect, DEX has shown to be effective also against other types of toxicities associated with oxidative stress[109].

2.1.4.5.4.5.4. Risks associated with dexrazoxane, unmet clinical need

Unfortunately, the prophylaxis with DEX is associated with certain risks. A higher incidence of secondary malignancies has been observed in patients who survived paediatric Hodgkin lymphoma and who were treated with chemotherapeutics together with DEX compared to

chemotherapeutics administered alone[110]. Even though the outcomes of this study are considered controversial, it had a significant impact on the clinical use of DEX. In the EU, DEX is currently contraindicated in children aged 0 to 18 years who are planned to receive a cumulative dose of less than 300 mg/m^2 of DOX or the equivalent cumulative dose of another ANT[100]. In contrary, the use of DEX in children is not limited in the US[96].

The anti-tumour efficacy of ANTs when co-administered with DEX has been questioned in patients suffering from metastatic breast cancer[111], which is probably caused by binding of DEX to TOP2α[112]. Adverse events associated with DEX belong among other complications of the therapy. It can amplify some adverse events of ANTs, such as hematotoxicity and leucopenia[11,113].

Despite indisputable benefit of DEX in the prevention of ANT-induced cardiotoxicity, there are serious risks and complications associated with its clinical use. Some of them have already led to restrictions in certain populations of patient. This fact led to efforts to describe more deeply the structure-activity relationships (SAR) of DEX and to develop its structural analogues with more favourable safety and efficacy profiles. Also, there is a high unmet need for a cardioprotective agent, which could be used in paediatric patients. Surprisingly, only a limited information about SAR of DEX has been available, mainly due to limited amount of its analogues, which has been synthetized and further studied *in vitro* and *in vivo*[17].

3. Aims of the work

The aims of the work may be divided into three sections:

1. **Synthesis of ADR-925**

The aim of the first part was to synthesise a large amount of DEX metabolite ADR-925, which would enable its *in vitro* and *in vivo* evaluation. This would contribute to the study of mechanisms of cardioprotective action of DEX. Currently, the exact mechanism of action of DEX is not fully elucidated and it is not clear, whether it exerts its cardioprotective effect by chelation of metal ions predominantly by its metabolite ADR-925 , or whether the inhibition of TOP2β by the parent compound plays a crucial role. Isolated ADR-925 shall be then tested for its cardioprotective potential *in vitro* and *in vivo* using an appropriate preclinical model of ANT-induced cardiotoxicity. The results could bring a new light on the exact mechanism of action of DEX.

2. **Synthesis of analogues of dexrazoxane derived from diethylenetriaminepentaacetic acid (DTPA)**

In the second part of the work, we synthetized the structural analogues of DEX derived from DTPA. The isolated compounds were intended for the evaluation of their cardioprotective potential *in vitro* on primary cultures of rat isolated neonatal ventricular cardiomyocytes, as well as *in vivo* in a model of chronic ANT-induced heart failure in rabbits.

3. **Synthesis of analogues of dexrazoxane with modified linker**

The third aim of the work was to synthetize and isolate the structural analogues of DEX with modified linker. Similarly to analogues of DEX derived from DTPA, also the analogues with modified linker were intended for the evaluation of their cardioprotective potential *in vitro* on primary cultures of rat isolated neonatal ventricular cardiomyocytes, as well as *in vivo* in a model of chronic ANT-induced heart failure in rabbits.

4. Experimental part

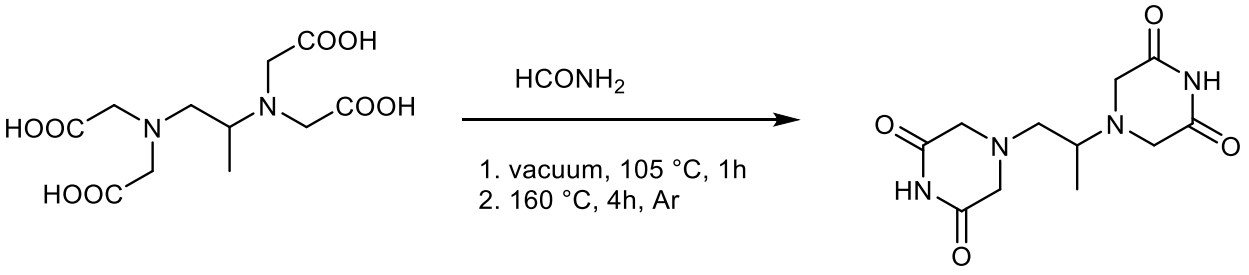
4.1. Synthesis of ADR-925

The aim of the first part was to synthesise ADR-925, main metabolite of razoxane, for *in vitro* and *in vivo* testing. This would help to study the mechanisms of cardioprotective protection of DEX. The synthesis of ADR-925 consists of two subsequent chemical reactions.

4.1.1. Synthesis of razoxane

1,2-Diaminopropane-*N*,*N*,*N*',*N*'-tetraacetic acid (**1**) was used as the starting compound for the preparation of ADR-925. In this first step, **1** was heated in formamide resulting in the cyclization of free carboxylic acids into two imine hetorocycles. The product of this reaction – razoxane (**RAZ**), is the racemic mixture of DEX and levrazoxane.

Reaction:



1 **RAZ**

Reactants:

- 1,2-Diaminopropane-*N*,*N*,*N*',*N*'-tetraacetic acid (**1**)
  - ME = 1
  - M_r = 306.27 g/mol
- $HCONH_2$; formamide
  - solvent
  - M_r = 45.04 g/mol

Approach:

A suspension of **1** (20.146 g, 65.8 mmol) in formamide (approximately 80 mL) was stirred and subsequently heated up to 105 °C under vacuum (30 mbar) for 1 hour. Then, argon was implemented into the reaction apparatus and approximately half amount (40 mL) of formamide

was evaporated under atmospheric pressure at 160 °C within 4 hours. Additional 20 mL the solvent was further evaporated under reduced pressure at the same temperature. The oily mixture containing the rest of the solvent (approximately 20 mL of formamide) was cooled down to the room temperature (rt) and approximately 20 mL of methanol was added into the flask. The arising precipitate was filtered and washed three times with cold methanol (3 × 20 mL). The flask containing filtrate was sealed and put into the refrigerator overnight. In the morning, the same filtering and washing procedure was repeated for the residue present in the flask. The yield of the reaction was approximately 53%. **RAZ** was present in the form of white crystals.

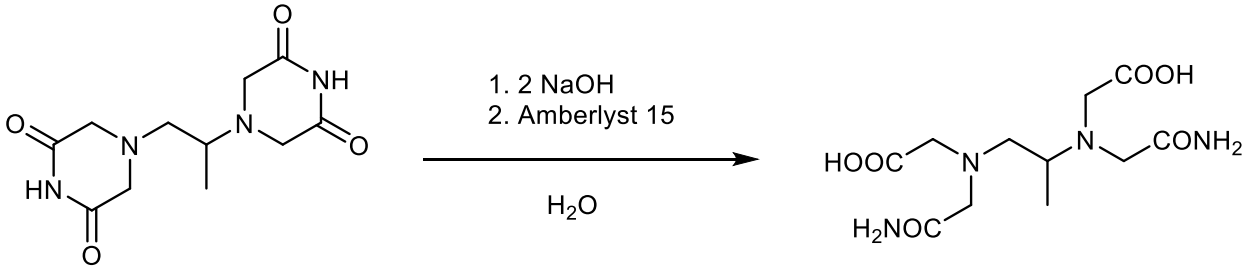
	Reactants		**Product**
Entry	1,2-Diaminopropane-*N*,*N*,*N*',*N*'-tetraacetic acid (**1**)	$HCONH_2$; formamide	4,4'-(Propane-1,2-diyl)bis(piperazine-2,6-dione); razoxane (**RAZ**)
1	m = 20.146 g M_r =306.27 g/mol n = 0.0658 mol	M_r = 45.04 g/mol V = 80 mL solvent	m = 9.353 g M_r = 268.27 g/mol n = 0.0349 mol **Yield**: 53%

^{1}H NMR (300 MHz, DMSO-d_6) δ 11.07 (s, 1H), 11.00 (s, 1H), 3.37 – 3.30 (m, 8H), 3.09 – 2.98 (m, 1H), 2.58 (dd, J = 13.0, 7.8 Hz, 1H), 2.31 (dd, J = 13.0, 6.1 Hz, 1H), 0.88 (d, J = 6.5 Hz, 3H).
^{13}C NMR (75 MHz, DMSO-d_6) δ 172.22, 171.69, 57.71, 55.38, 53.64, 51.54, 12.77.

4.1.2. Basic hydrolysis of razoxane into ADR-925

In this step, **RAZ** underwent a basic hydrolysis using sodium hydroxide (NaOH), resulting to opening of both imine cycles and formation of 2-[(2-Amino-2-oxoethyl)-[2-[(2-amino-2-oxoethyl)-(carboxymethyl)amino]propyl]amino]acetic acid (**ADR-925**).

Reaction:



RAZ **ADR-925**

Reactants:

- 4,4'-(Propane-1,2-diyl)bis(piperazine-2,6-dione) (**RAZ**)
    - ME = 1
    - M_r = 268.27 g/mol
- NaOH; sodium hydroxide
    - ME = 2
    - M_r = 40.00 g/mol

Approach:

RAZ (5 g, 18.6 mmol) was suspended in the water solution of NaOH (2 equiv., 1.47 g; 37.3 mL of 1M solution). The reaction mixture was stirred for 24 hours at laboratory temperature. The TLC (eluent: $CHCl_3$: MeOH = 2:1; detection: UV) was evaluated one hour after the reaction commencement, R_f (product) = 0.05, R_f (reactant) = 0.97). Potential product, as well as the reactant were both observed on the TLC plate. The same TLC was repeated after 24 hours and revealed no reactant left.

Amberlyst 15 - a macro reticular ion exchange resin containing sulfonic group, which acts strongly acidic, was added into the mixture and the pH was adjusted to neutral level (pH = 7). After 1 h, Amberlyst 15 was removed by a simple filtration. The water was evaporated, and the product was put into desiccator, where it was dried under reduced pressure over 7 days.

The yield of the reaction was very high (97%). ADR-925 was present in the form of white crystals. The structure of ADR-925 was confirmed by NMR. Elemental analysis indicated that the ADR-925 was isolated as monohydrate.

	Reactants		**Product**
Entry	4,4'-(Propane-1,2-diyl)bis(piperazine-2,6-dione); razoxane (**RAZ**)	NaOH; sodium hydroxide	2-[(2-Amino-2-oxoethyl)-[-2-[(2-amino-2-oxoethyl)-carboxymethyl)amino]propyl] amino]acetic acid (**ADR-925**)
1	m = 5 g M_r = 268.27 g/mol n = 0.0186 mol	m = 1.47 g M_r = 40.00 g/mol n = 0.0368 mol V = 37.3 mL of 1M solution	m = 5.50 g M_r = 304.30 g/mol n = 0.0181 mol **Yield**: 97%

^{1}H NMR (500 MHz, D_2O) δ 3.90 – 3.73 (m, 4H), 3.68 – 3.49 (m, 5H), 3.23 (dd, *J* = 14.7, 3.9 Hz, 1H), 3.00 (dd, *J* = 14.6, 11.7 Hz, 1H), 1.19 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, D_2O) δ 174.59, 173.84, 173.51, 171.59, 57.10, 56.76, 56.62, 56.42, 53.68, 53.59, 10.60. Anal. Calcd for $C_{11}H_{22}N_4O_7$: C, 40.99; H, 6.88; N, 17.38. Found: C, 40.52; H, 6.62; N, 17.1.

4.2. Synthesis of analogues of dexrazoxane derived from DTPA

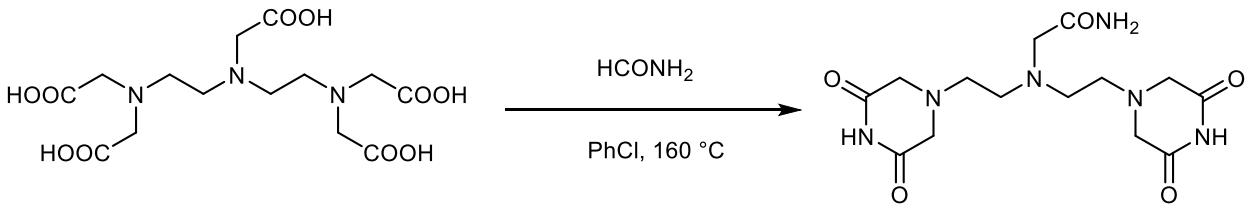
Within this part of the work, structural analogues of DEX derived from DTPA were synthetized. These compounds were intended for the evaluation of their cardioprotective potential *in vitro* on primary cultures of rat isolated neonatal ventricular cardiomyocytes, as well as *in vivo* in a model of chronic ANT-induced heart failure in rabbits.

4.2.1. Synthesis of 2-(bis(2-(3,5-dioxopiperazin-1-yl)ethyl)amino)acetamide (2)

The aim of the following reactions was to synthetize 2-(bis(2-(3,5-dioxopiperazin-1-yl)ethyl)amino)acetamide (**2**) by closing free carboxylic groups of DTPA into two piperazine-2,6-dione cycles and amidation of the fifth carboxyl.

4.2.1.1. Reaction of DTPA with formamide at 160 °C

Reaction:



DTPA **2**

Reactants:

- Diethylenetriaminepentaacetic acid (**DTPA**)
  - ME = 1
  - M_r = 393.35 g/mol
- $HCONH_2$; formamide
  - ME = 5
  - M_r = 45.04 g/mol
- Chlorobenzene
  - M_r = 112.56 g/mol
  - solvent

Approach:

DTPA (1 g, 2.5 mmol) was dissolved in approximately 40 mL of chlorobenzene and formamide was added to the suspension. The molar DTPA: formamide ratio was 1:5. The mixture was heated to

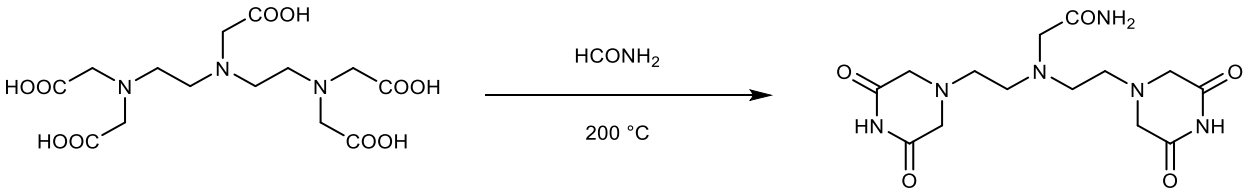
reflux over 4 hours at 160 °C, using Dean-Stark trap. Then, chlorobenzene was evaporated under reduced pressure using vacuum rotary evaporator. Approximately 5 mL of a saturated solution of Na_2CO_3 was added to the oily residue and the resulting mixture was extracted with ethyl acetate (EtOAc). However, no product was detected in the organic extract.

	Reactants		Product
Entry	Diethylenetriaminepentaacetic acid; (**DTPA**)	$HCONH_2$; formamide	2-(bis(2-(3,5-dioxopiperazin-1-yl)ethyl)amino)acetamide (**2**)
1	m = 1 g M_r = 393.35 g/mol n = 0.0025 mol	m = 0.5840 g M_r = 45.04 g/mol n = 0.0130 mol ρ = 1.133 g/cm^3 V = 0.515 mL	m = 0 g M_r =354.37 g/mol n = 0 mol **Yield**: 0 %

4.2.1.2. Reaction of DTPA with formamide at 200 °C

A low temperature was identified as a potential reason of failure of the previous reaction; therefore it was repeated at 200 °C in the absence of chlorobenzene.

Reaction:



DTPA **2**

Reactants:

- Diethylenetriaminepentaacetic acid (**DTPA**)
  - ME = 1
  - M_r = 393.35 g/mol
- $HCONH_2$; formamide
  - M_r = 45.04 g/mol
  - solvent

Approach:

The approach was kept the same as in the previous reaction, however the amounts of reagents differed. **DTPA** (0.5 g; 1.3 mmol) was mixed with approximately 30 mL of formamide. The mixture was heated to 200 °C for 4 hours. Then, formamide was evaporated under reduced pressure using

vacuum rotary evaporator. Approximately 2.5 mL of a saturated solution of Na_2CO_3 was added to the oily residue and the resulting mixture was extracted with ethyl acetate (EtOAc). However, no product was detected in the organic extract.

Methanol (20 mL) was added to the aqueous residue and it was kept in the refrigerator for several days. A small amount of crystals emerged in the flask, but their characterization upon filtration gave no meaningful data.

	Reactants		Product
Entry	Diethylenetriaminepentaacetic acid; (**DTPA**)	$HCONH_2$; formamide	2-(bis(2-(3,5-dioxopiperazin-1-yl)ethyl)amino)acetamide (**2**)
1	m = 0.5 g M_r = 393.35 g/mol n = 0.0013 mol	m = 0.2913 mg M_r = 45.04 g/mol n = 0.0065 mol ρ = 1.133 g/cm^3 V = 0.2571 mL	m = 0 g M_r =354.37 g/mol n = 0 mol **Yield**: 0 %

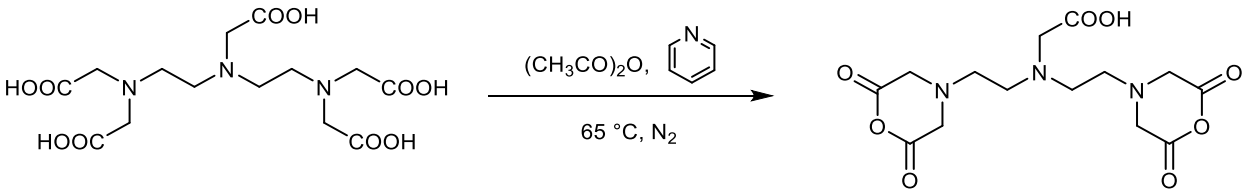
4.2.1.3. Reaction of DTPA with acetic anhydride and subsequent reaction with formamide

The direct reaction between DTPA and formamide in chlorobenzene did not lead to the desired product. Thus, a two-step reaction with acetic anhydrate and formamide was chosen as the alternative pathway.

4.2.1.3.1. Reaction of DTPA with acetic anhydride

In this first step, DTPA shall react with acetic anhydrate to form an DTPA anhydride - an intermediate containing two morpholine-2,6-dione cycles. Reaction was performed according known method[114].

Reaction:



DTPA **AnhDTPA**

Reactants:

- Diethylenetriaminepentaacetic acid (**DTPA**)
  - ME = 1

- M_r = 393.35 g/mol

- $(CH_3CO)_2O$; acetic anhydride
  - ME = 1
  - M_r = 102.09 g/mol
- Pyridine
  - ME = 1
  - M_r = 79.10 g/mol

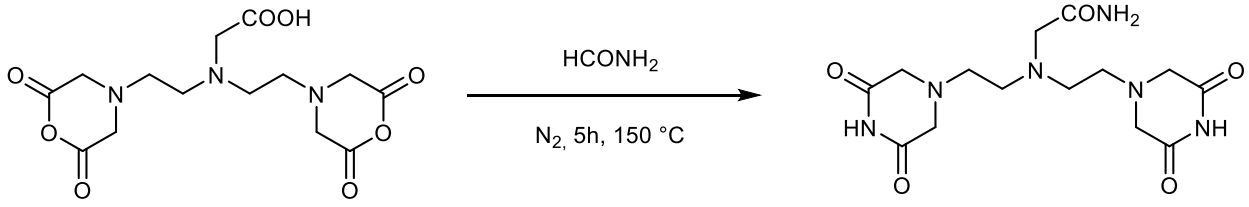
DTPA (3.93 g; 1 mmol) was dissolved in dry pyridine (5 mL) and acetic anhydride (4 mL; 42.4 mmol) was slowly added. The mixture was stirred for 2 hours at 65 °C. Due to the high susceptibility of the acetic anhydride and the product to hydrolysis, the reaction was performed strictly without a presence of water. A nitrogen was applied in order to avoid any influence of humidity. After 2 hours, the mixture was filtered under reduced pressure and the precipitate on the filter was further washed with dry acetonitrile and dry diethyl ether. Then the product was put into desiccator and dried and stored over P_2O_5. The reaction led to the yield of 68% of anhDTPA.

	Reactants			**Product**
Entry	Diethylenetriamine pentaacetic acid; (**DTPA**)	$(CH_3CO)_2O$; acetic anhydrate	pyridine	2-(bis(2-(2,6-dioxomorpholino)ethyl) amino)acetamide; **anhDTPA**
1	m = 3.93 g M_r = 393.35 g/mol n = 0.001 mol	m = 4.328 g M_r = 102.09 g/mol n = 0.0424 mol ρ = 1.082 g/cm^3 V = 4 mL	m = 4.9085 g M_r = 79.10 g/mol n = 0.0621 mol ρ = 0.9819 g/cm^3 V = 5 mL	m = 2.43 g M_r = 357.32 g/mol n = 0.0068 mol **Yield**: 68 %

4.2.1.3.2. Reaction of DTPA anhydride with formamide

In this second step, the anhydride of DTPA shall undergo a conversion into piperazine-2,6-dion cycles when reacting with formamide.

Reaction:



AnhDTPA **2**

Reactants:

- 2-(bis(2-(2,6-dioxomorpholino)ethyl)amino)acetamide (**AnhDTPA**)
  - ME = 1
  - M_r = 357.32 g/mol
- $HCONH_2$ (formamide)
  - solvent
  - M_r = 45.04 g/mol

Approach:

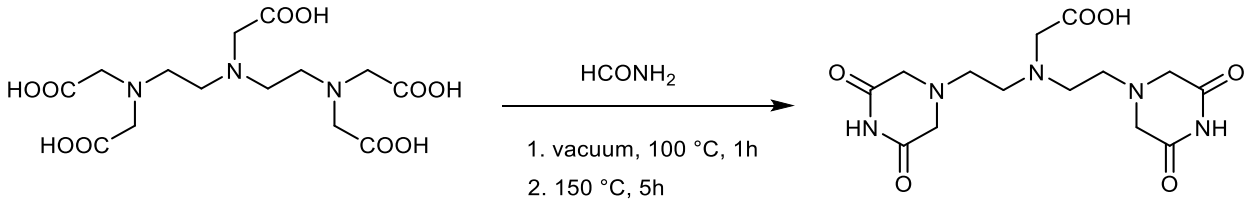
AnhDTPA (0.5 g; 1.4 mmol) was dissolved in dry formamide, the precautions have been applied to prevent occurrence of water in the apparatus. A nitrogen was applied in order to avoid any influence of air humidity. The mixture was heated to 150 °C for 5 hours. After that, the TCL(s) in several eluents ($CHCl_3$: MeOH = 2:1; $CHCl_3$; MeOH: H_2O = 1:1, detection: I_2) were evaluated, however the product was not detected.

	Reactants		**Product**
Entry	2-(bis(2-(2,6-dioxomorpholino)ethyl) amino) acetamide (**AnhDTPA**)	$HCONH_2$; formamide	2-(bis(2-(3,5-dioxopiperazin-1-yl)ethyl)amino)acetamide (**2**)
1	m = 0.5 g M_r =357.32 g/mol n = 0.0014 mol	m = 1.6995 g M_r = 40.00 g/mol n = 0.1416 mol ρ = 1.133 g/cm^3 V = 1.5 mL	m = 0 g M_r = 354.37 g/mol n = 0 mol **Yield**: 0 %

4.2.2. Synthesis of bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (ES-1)

The reaction of DTPA with formamide was repeated under adjusted conditions. The aim of this reaction was to cyclize terminal carboxylates into two piperazine cycles. In contrast to patent literature, the central carboxyl group stayed intact.

Reaction:



DTPA **ES-1**

Reactants:

- Diethylenetriaminepentaacetic acid (**DTPA**)
  - ME = 1
  - M_r = 393.35 g/mol
- $HCONH_2$; formamide
  - M_r = 45.04 g/mol
  - solvent

Approach:

DTPA was dissolved in formamide and the flask was put into the oil bath. The mixture was heated under vacuum (30 mbar) for 1 hour under 100°C. Then, then temperature was increased up to 150 °C and the mixture was heated over 5 hours. The reaction was conducted in the presence of argon. After 5 hours, the product was put on the rotary evaporator and most of formamide was evaporated under reduced pressure. Methanol (30 mL) was added to the residue and it was stored in the freezer overnight, resulting into formation of crystals. The next day, the product was filtered under reduced pressure using Büchner funnel. Crystals were washed with methanol in order to get rid of remaining formamide and dried over P_2O_5. The structure of **ES-1** was confirmed by NMR.

^{1}HNMR (500 MHz, DMSO-d_6) δ 11.06 (s, 2H), 3.34 (s, 8H), 3.31 (s, 2H), 2.78 (t, J = 6.4 Hz, 4H), 2.55 (t, J = 6.4 Hz, 4H);
^{1}H NMR (300 MHz, D_2O) δ 3.82 (s, 2H), 3.56 (s, 8H), 3.50–3.41 (m, 4H), 3.04–2.94 (m, 4H).
^{13}C NMR (126 MHz, DMSO-d_6) δ 171.76, 171.55, 55.31, 55.24, 52.56, 50.90
^{13}C NMR (75 MHz, D_2O) δ 173.45, 170.56, 57.20, 54.83, 51.80, 49.30.

LRMS m/z (APCI) 356.6 (100, M + H+), 357.6 (16%).

	Reactants		**Product**
Entry	Diethylenetriamine pentaacetic acid; (**DTPA**)	$HCONH_2$; formamide	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)
1	m = 10 g M_r = 393.35 g/mol n = 0.0254 mol	m = 45.32 g M_r = 45.04 g/mol n = 0.0062 mol ρ = 1.133 g/cm^3 V = 40 mL	m = 6.11 g M_r = 355.35 g/mol n = 0.0172 mol **Yield**: 68 %
2	m = 50 g M_r = 393.35 g/mol n = 0.1271 mol	m = 226.6 g M_r = 45.04 g/mol n = 5.031 mol ρ = 1.133 g/cm^3	m = 27.66 g M_r = 355.35 g/mol n = 0.0780 mol **Yield**: 61 %

		V = 200 mL	
3	m = 100 g M_r = 393.35 g/mol n = 0.254 mol	m = 453.2 g M_r = 45.04 g/mol n = 0.062 mol ρ = 1.133 g/cm^3 V = 400 mL	m = 57.64 g M_r = 355.35 g/mol n = 0.1622 mol **Yield**: 64 %
4	m = 5 g M_r = 393.35 g/mol n = 0.01271 mol	m = 22.66 g M_r = 45.04 g/mol n = 0.5031 mol ρ = 1.133 g/cm^3 V = 20 mL	m = 2.97 g M_r = 355.35 g/mol n = 0.0084 mol **Yield**: 66 %

ES-1 was the main product of this reaction. In addition, also a small portion of bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycinamide was detected by MS. However, its yield was below 1% and it was not isolated.

4.2.3. Functionalization of free carboxyl group of ES-1

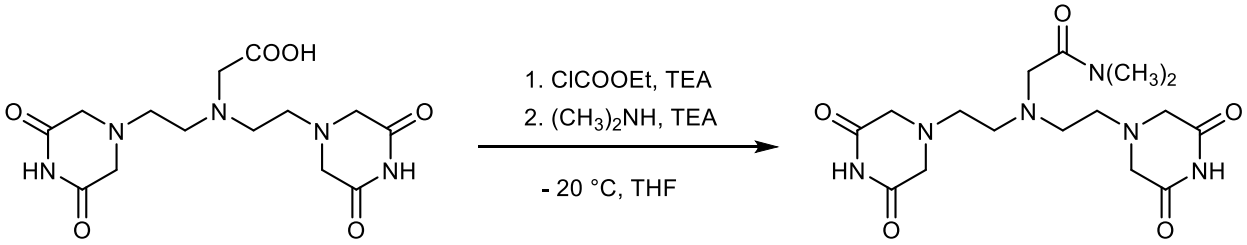
The following reactions were focused on the functionalization of free carboxyl of ES-1 by an appropriate group in order to increase lipophilicity of the final product, which would also increase the permeation via biological membranes.

4.2.3.1. Amidation

4.2.3.1.1. Amidation by dimethylamine

The amidation of free carboxyl group of ES-1 was consecutively conducted using mixed anhydride approach in two different solvents.

4.2.3.1.1.1. Amidation by dimethylamine in tetrahydrofuran



ES-1 **3**

Reactants:

- bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)

- ME = 1
- M_r = 355.35 g/mol

- ClCOOEt; ethyl chloroformate
  - ME = 1
  - M_r = 108.52 g/mol
- TEA; triethylamine
  - ME = 1
  - M_r = 101.193 g/mol
- $(CH_3)_2NH$; dimethyl amine - 2M solution in THF (tetrahydrofurane)
  - ME = 1
  - Mr = 45.08 g/mol
- THF; tetrahydrofuran
  - Mr = 72.107 g/mol
  - solvent

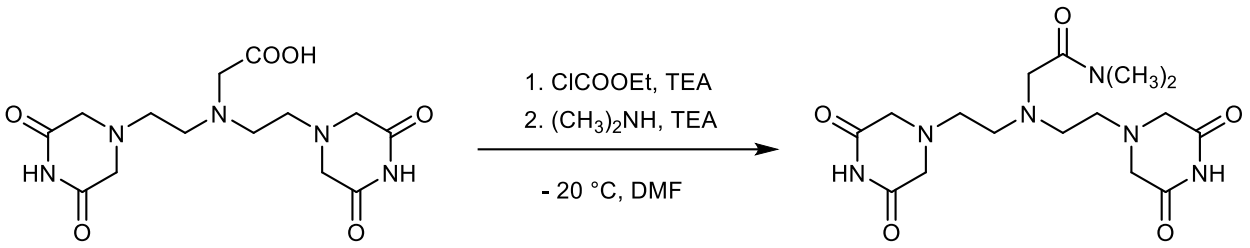
Approach:

ES-1 (0.2 g; 0.56 mmol) was dissolved in THF (5 mL) and TEA (0.078 mL; 0.56 mmol) was slowly added using the injection at the temperature of approximately -20°C and under argon atmosphere. After a while, ClCOOEt (0.053 mL, 0.56 mmol) was added by the injection over the septum and the mixture was kept reacting. After 15 minutes, 2M solution of dimethyl amine in THF (0.28 mL; 5.3 mmol) was added and the mixture was kept stirring for 1 hour, while the flask content slowly reached a laboratory temperature. Then, the reaction mixture was filtered and the precipitate on the filter was washed by THF twice. The samples were taken from the filter and filtrate and they were examined by mass spectrometry. Unfortunately, the crystals from the filter were identified as a parent compound ES-1. A small amount of the desired product was identified in the filtrate, where also ES-1 and its ethyl ester were detected, however, only in trace amounts. Further isolation of the products was not applied due to a very low amount of the substance.

	Reactants				**Product**
Entry	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (ES-1)	ClCOOEt; ethyl carbonochloridate	TEA; triethyl amine	$(CH_3)_2NH$; dimethyl amine – 2M solution in THF	2-((2-(3,5-dioxopiperazin-1-yl)ethyl)(2-(3,5-dioxopiperazin-1-yl)ethyl)amino)-N,N-dimethylacetamide (**3**)
1	m = 0.2 g M_r = 355.35 g/mol n = 0.00056 mol	m = 0.0608 g M_r = 108.52 g/mol n = 0.00056 mol ρ = 1.139 g/cm3 V = 0.053 mL	m = 0.057 g M_r = 101.19 g/mol n = 0.00056 mol ρ = 0.7255 g/cm^3 V = 0.078 mL	m = 0.238 g M_r = 45.08 g/mol n = 0.0053 mol ρ = 0.85 g/cm3 V = 0.28 mL	m = 2.4 mg M_r =382.42 g/mol n = 0.0063 mol **Yield**: 0 %

4.2.3.1.1.2. Amidation by dimethylamine in *N*,*N*-dimethylformamide

Reaction:



ES-1 **3**

Reactants:

- bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)
  - ME = 1
  - M_r = 355.35 g/mol
- ClCOOEt; ethyl chloroformate
  - ME = 1
  - M_r = 108.52 g/mol
- TEA; triethylamine
  - ME = 1
  - M_r = 101.193 g/mol
- $(CH_3)_2NH$; dimethyl amine - 2M solution in DMF (dimethylformamide)
  - ME = 1
  - Mr = 45.08 g/mol
- DMF; dimethylformamide
  - Mr = 73.095 g/mol
  - solvent

Approach:

ES-1 (0.2 g; 0.56 mmol) was dissolved in DMF (5 mL) and TEA (0.078 mL; 0.56 mmol) was slowly added using the injection at the temperature of approximately -20°C and under argon atmosphere. After a while, ClCOOEt (0.053 mL, 0.56 mmol) was inserted by the injection over the septum and the mixture was kept reacting in cold ethanol bath. After 15 minutes, 2M solution of dimethyl amine in DMF (0.28 mL; 5.3 mmol) was added and the mixture was kept stirring for 1.5 hours, while the flask content slowly reached laboratory temperature. Then, the reaction mixture was filtered and the precipitate on the filter was washed with DMF three times. The samples were

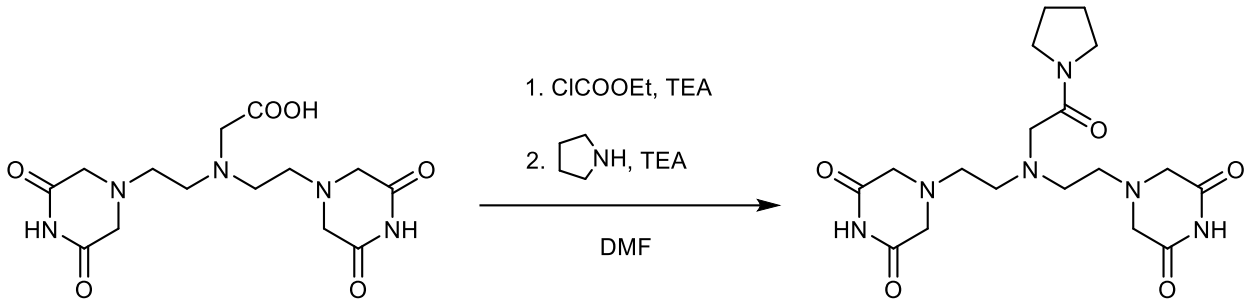
taken from the filter and filtrate and they were examined by mass spectrometry. Unfortunately, as in the case of the previous reaction in THF, the crystals from the filtration paper were mostly identified as a parent compound ES-1. Additionally, the reverse phase TLC from the crystals dissolved in H_2O was applied (eluent: MeOH: H_2O = 1:1, detection: Dragendorff's reagent), with R_f (reactant) = 0.95 and R_f (product) = 0.55. The amount of the detected product was very low. A small amount of the desired product was identified in the filtrate, where also a parent compound ES-1 and its ethyl ester were present. The solvent was evaporated from the filtrate using vacuum rotary evaporator and the residue was allocated into the fridge overnight. The crystals emerged in the flask the next day. They were washed with 2 mL of $NaHCO_3$ and extracted into chloroform. The solution was dried over Na_2SO_4. The reverse phase TLC was applied (eluent: MeOH: H_2O = 1:1, detection: Dragendorff's reagent), with similar results as for the previous TLC: R_f (reactant) = 0.95 and R_f (product) = 0.55. Chloroform was evaporated on rotary evaporator under vacuum. Unfortunately, only a very small amount of crystals was gained. The desired product **3** was detected by mass spectrometry, together with ethyl ester of ES-1. However, the amount of crystals was negligible. Further isolation of ethyl ester of ES-1 and the product was not applied due to a very low amount of the substance.

	Reactants				**Product**
Entry	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)	ClCOOEt; ethyl carbonochloridate	TEA; triethyl amine	$(CH_3)_2NH$; Dimethyl amine – 2M solution in DMF	2-((2-(3,5-dioxopiperazin-1-yl)ethyl)(3-(3,5-dioxopiperazin-1-yl)propyl)amino)-N,N-dimethylacetamide (**3**)
1	m = 0.2 g M_r = 355.35 g/mol n = 0.00056 mol	m = 0.0608 g M_r = 108.52 g/mol n = 0.00056 mol ρ = 1.139 g/cm^3 V = 0.053 mL	m = 0.057 g M_r = 101.19 g/mol n = 0.00056 mol ρ = 0.7255 g/cm^3 V = 0.078 mL	m = 0.238 g M_r = 45.08 g/mol n = 0.0053 mol ρ = 0.85 g/cm^3 V = 0.28 mL	m = 0.0017 g M_r =382.42 g/mol n = 0.0044 mol **Yield**: 0%

The same reaction was also performed in acetonitrile, but with the same results. No product was isolated.

4.2.3.1.2. Amidation by pyrrolidine using mixed anhydride approach

Reaction:



ES-1 **4**

Reactants:

- bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)
  - ME = 1
  - M_r = 355.35 g/mol
- ClCOOEt; ethyl chloroformate
  - ME = 1
  - M_r = 108.52 g/mol
- TEA; triethylamine
  - ME = 1
  - M_r = 101.193 g/mol
- Pyrrolidine
  - ME = 1
  - M_r = 71.12 g/mol
- DMF; dimethylformamide
  - Mr = 73.095 g/mol
  - solvent

Approach:

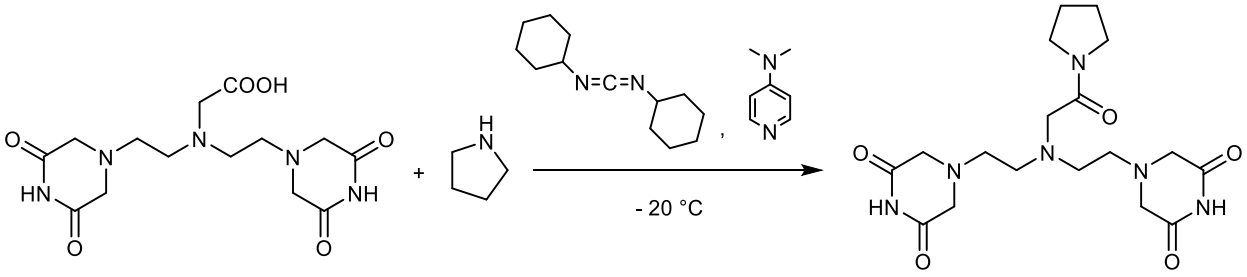
ES-1 (0.2 g; 0.56 mmol) was dissolved in DMF (5 mL) and 0.078 mL of TEA (0.078 mL; 0.56 mmol) was slowly added using the injection. The reaction was conducted under laboratory temperature. After a while, ClCOOEt (0.053 mL; 0.56 mmol) was inserted by the injection over the gum septum. After 15 minutes, 0.2 mL of pyrrolidine (0.2 mL; 2.4 mmol) was added and the mixture was kept stirring for 1 hour. Unfortunately, the subsequent TLC (eluent: CH_3Cl: MeOH = 1:1, detection: UV) did not reveal any product, the reaction was therefore interrupted and stopped.

	Reactants				Product
Entry	bis(2-(3,5-dioxopiperazin-1-	ClCOOEt; ethyl carbonochloridate	TEA; triethyl amine	pyrrolidine	4,4'-(((2-oxo-2-(pyrrolidin-1-yl)ethyl)azanediyl) bis(ethane-2,1-

	yl)ethyl)glycine (**ES-1**)				diyl))bis(piperazine-2,6-dione) (**4**)
1	m = 0.2 g M_r = 355.35 g/mol n = 0.00056 mol	m = 0.0608 g M_r = 108.52 g/mol n = 0.00056 mol ρ = 1.139 g/cm^3 V = 0.053 mL	m = 0.057 g M_r = 101.19 g/mol n = 0.00056 mol ρ = 0.7255 g/cm^3 V = 0.078 mL	m = 0.21732 g M_r = 71.12 g/mol n = 0.0024 mol ρ = 0.866 g/mL V = 0.2 mL	m = 0 g M_r =408.46 g/mol n = 0 mol **Yield**: 0 %

4.2.3.1.3. Amidation by pyrrolidine using *N*,*N*'-dicyclohexylcarbodiimide (DCC)

Reaction:



ES-1 **4**

Reactants:

- bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)
  - ME = 1
  - M_r = 355.35 g/mol
- Pyrrolidine
  - ME = 1
  - M_r = 71.12 g/mol
- N,N'-Dicyclohexylcarbodiimide (DCC)
  - ME = 1
  - M_r = 206.333 g/mol
- 4-Dimethylaminopyridine
  - M_r = 122.17 g/mol
  - catalyzator
- CH_3CN; acetonitril
  - M_r = 41.053 g/mol
  - solvent

Approach:

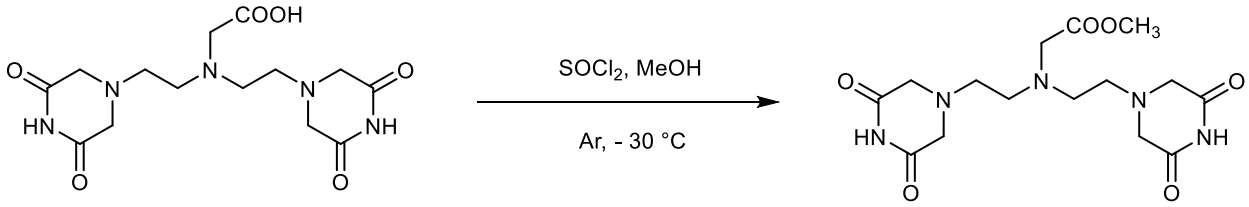
ES-1 (0.5 g; 1.4 mmol) was dissolved in CH_3CN (5 mL) and pyrrolidine (0.12 mL; 1.4 mmol) and a very small amount of 4-dimethylaminopyridine, which acts like a catalyst in this reaction, was added. At this stage, the reaction was conducted under the temperature of approximately -20 °C using ethanol bath. Then, DCC (0.29 g; 1.4 mmol) was added to the reaction mixture and it was slowly brought to laboratory temperature. The content of the flask was stirred for 4 hours and several TLCs in various mobile phases were evaluated. The spots were detected in iodine. In all cases, the TLC revealed several different spots on the TLC plate. The sample was measured also by mass spectrometry. The mixture of various compounds was observed, which made an isolation of the individual compounds impossible. Different adducts of ES-1 with DCC were identified as the major undesired products of this reaction.

	Reactants				**Product**
Entry	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)	pyrrolidine	N,N'-Dicyclohexylcarbodiimide (DCC)	4-Dimethylaminopyridine	4,4'-(((2-oxo-2-(pyrrolidin-1-yl)ethyl)azanediyl)bis(ethane-2,1-diyl))bis(piperazine-2,6-dione) (**4**)
1	m = 0.5 g M_r = 355.35 g/mol n = 0.0014 mol	m = 0.104 g M_r = 71.12 g/mol n = 0.0014 mol ρ = 0.866 g/mL V = 0.12 mL	m = 0.29 g M_r = 206.333 g/mol n = 0.0014 mol	m = 0.01 g M_r = 122.17 g/mol n = 0.0001 mol	m = 0 g M_r =408.46 g/mol n = 0 mol **Yield**: 0 %

4.2.3.2. Esterification

4.2.3.2.1. Esterification using thionyl chloride in methanol

Reaction:



ES-1 **5**

Reactants:

- bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)

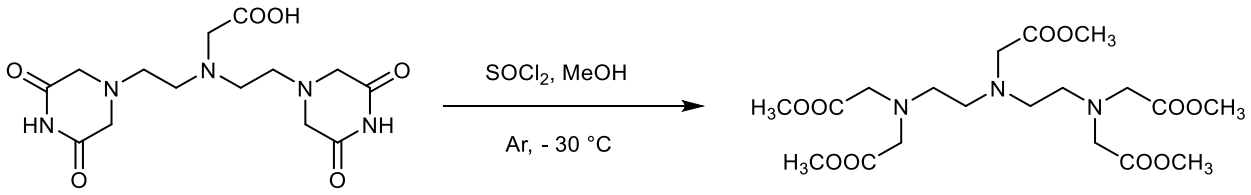
- ME = 1
- M_r = 355.35 g/mol

- $SOCl_2$; thionyl chloride
  - ME = 1
  - M_r = 118.97 g/mol
- MeOH; methanol
  - ME = 1
  - M_r = 79.10 g/mol

Approach:

Methanol (5 mL) was put into cooling bath. The temperature was brought to -30 °C. Thionyl chloride (0.5 mL; 6.9 mmol) was very slowly added to methanol by the injection, which was applied over the septum. The reaction was conducted under argon atmosphere. Then, **ES-1** (0.2 g; 0.6 mmol) was added and the reaction was brought to rt. Then, the volatiles were evaporated using vacuum rotary evaporator. The residue was examined by mass spectrometry. Unfortunately, the desired product was not detected. According to MS, both piperazine cycles of ES-1 were disintegrated and all five carboxyl groups were esterified, leading to the pentamethylester of DTPA (**6**) (Fig. 12).

	Reactants			Product
Entry	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)	$SOCl_2$; thionyl chloride	MeOH; methanol	methyl bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycinate (**5**)
1	m = 0.2 g M_r = 355.35 g/mol n = 0.0006 mol	m = 0.819 g M_r = 118.97 g/mol n = 0.0069 mol ρ = 1.638 g/cm^3 V = 0.5 mL	m = 3.96 g M_r = 34.04 g/mol n = 0.1163 mol ρ = 0.792 g/cm^3 V = 5 mL	m = 0 g M_r = 369.38 g/mol n = 0 mol **Yield**: 0 %



ES-1 **6**

Fig. 12. Esterification of all carboxyl groups, leading to pentamethylester of DTPA (**6**)

	Reactants			Product
Entry	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)	$SOCl_2$; thionyl chloride	MeOH; methanol	Pentamethyl DTPA (**6**)
1	m = 0.2 g M_r = 355.35 g/mol n = 0.0006 mol	m = 0.819 g M_r = 118.97 g/mol n = 0.0069 mol ρ = 1.638 g/cm^3 V = 0.5 mL	m = 3.96 g M_r = 34.04 g/mol n = 0.1163 mol ρ = 0.792 g/cm^3 V = 5 mL	m = 0.143 g M_r = 463.48 g/mol n = 0.0003 mol **Yield**: 55 %

4.2.3.2.2. Esterifications via alkylation of carboxylate under basic conditions

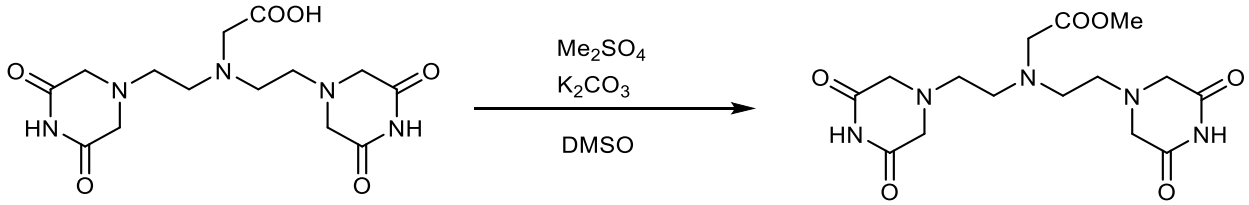
Several attempts to alkylate free carboxylic group of ES-1 under basic conditions were made. The main problem was a low solubility of starting ES-1 in organic solvents. Several attempts resulted in no products (e.g. reaction with benzyl bromide in DMF, DMSO, reaction with bromoethanol in DMF, acetonitrile, DMSO). Finally, DMSO was found to be the only solvent that allowed us to prepare target esters of ES-1.

4.2.3.2.3. Esterification by dimethyl sulphate

The synthesis of methyl ester of ES-1 using dimethyl sulphate was selected as a model reaction and as a precedent for the synthesis of ethyl ester of ES-1, which is believed to be significantly less toxic compared to methyl ester. After cleavage *in vivo*, methyl ester would lead to formation of toxic methanol, which does not make it an optional candidate for the use *in vivo*.

(This reaction was performed in the cooperation with Tomáš Eisner)

Reaction:



ES-1 **7**

Reactants:

- bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)
  - ME = 1

- M_r = 355.35 g/mol

- K_2CO_3; potassium carbonate
  - ME = 0.5
  - M_r = 138.205 g/mol
- Dimethyl sulphate
  - ME = 1
  - M_r = 126.13 g/mol
- DMSO; dimethyl sulfoxide
  - M_r = 78.13 g/mol
  - solvent

Approach:

ES-1 (0.5 g; 1.4 mmol) was dissolved in DMSO (approximately 5 mL) and potassium carbonate (0.097 g; 0.7 mmol) was added into the flask. The reaction was stirred at rt for 15 minutes. Then, dimethyl sulphate (0.13 mL; 1.4 mmol) was slowly inserted and the mixture was kept stirring overnight at rt. The TLC (eluent: acetone; detection: iodine) was evaluated after 2 hours, with the potential product detected with R_f = 0.75. The next day, the content of the flask was diluted with approximately 50 mL of water and the potential product was extracted into ethyl acetate (3 × 50 mL). The TLC (eluent: acetone; detection: iodine) was repeated, with the potential product spotted at the same retention factor (R_f = 0.75). However, apart from the potential product, also several impurities were observed. The organic phase was dried over sodium sulphate and evaporated. The oily residue was purified using a column chromatography on silica gel, with ethyl acetate: acetone = 1:1 as a mobile phase. This procedure led to isolation of the product **7** with the yield of 31%.

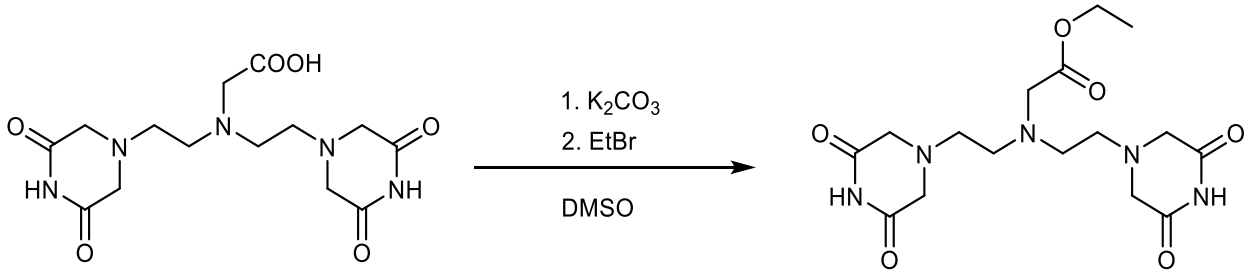
	Reactants			**Product**
Entry	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)	K_2CO_3; Potassium carbonate	Dimethyl sulphate	methyl bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycinate (**7**)
1	m = 0.5 g M_r = 355.35 g/mol n = 0.0014 mol	m = 0.097 g M_r = 138.205 g/mol n = 0.0007 mol	m = 0.173 g M_r = 126.13 g/mol n = 0.0014 mol ρ = 1.33 g/mL V = 0.13 mL	m = 0.1611 g M_r = 369.35 g/mol n = 0.00044 mol **Yield**: 31 %

LRMS m/z (APCI) 370.6 (100, M + H+), 371.5 (17%).

4.2.3.2.4. Esterification by bromoethane

ES-5 was yielded in sufficient amount to allow *in vitro* and *in vivo* studies. (This reaction was performed in the cooperation with Tomáš Eisner, MSc.)

Reaction:



ES-1 **ES-5**

Reactants:

- bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)
  - ME = 1
  - M_r = 355.35 g/mol
- Potassium carbonate
  - ME = 0.5
  - M_r = 138.205 g/mol
- EtBr; bromoethane
  - ME = 0.95
  - M_r = 108.966 g/mol
- DMSO; dimethyl sulfoxide
  - M_r = 78.13 g/mol
  - solvent

Approach:

The previous reaction was repeated, with different alkylating agent. Bromoethane was used instead of 2-bromoethanol. **ES-1** (2 g; 5.6 mmol) was dissolved in DMSO (approximately 8 mL), followed by the half equimolar dose of potassium carbonate (0.4 g; 2.9 mmol). This step led to creation of potassium salt on the carboxyl group of ES-1. After approximately 15 min, alkylating agent bromoethane (0.4 mL; 5.4 mmol) was added. The content of the flask was kept stirring overnight at rt. The next day, the emerged crystals were filtrated using Büchner funnel and DMSO was evaporated from the filtrate under reduced pressure. The residue was subjected to column chromatography, with ethyl acetate as a mobile phase. The product had an appearance of yellow powder and was stored over P_2O_5. The yield of the reaction was 56%.

	Reactants			Product
Entry	bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycine (**ES-1**)	EtBr; bromoethane	K_2CO_3; potassium carbonate	ethyl bis(2-(3,5-dioxopiperazin-1-yl)ethyl)glycinate (**ES-5**)
1	m = 2 g M_r = 355.35 g/mol n = 0.0056 mol	m = 0.584 g M_r = 108.97 g/mol n = 0.0054 mol ρ = 1.46 g/mL V = 0.4 mL	m = 0.4 g M_r = 138.205 g/mol n = 0.0029 mol	m = 1.208 g M_r = 383.40 g/mol n = 0.0032 mol **Yield**: 56 %

Mp 110 °C

^{1}H NMR (300 MHz, DMSO-*d6*) δ 11.06 (s, 2H), 4.02 (q, *J* = 7.1 Hz, 2H), 3.38 (s, 2H), 3.32 (s, 8H), 2.69 (t, *J* = 6.3 Hz, 4H), 2.56–2.46 (m, 4H), 1.16 (t, *J* = 7.1 Hz, 3H)

^{13}C NMR (75 MHz, DMSO-*d6*) δ 171.66, 171.27, 59.85, 55.41, 54.60, 53.27, 50.83, 14.31.

LRMS m/z (APCI) 384.3 (100, M + H+), 385.1 (18%)

4.3. Synthesis of analogues of dexrazoxane with modified linker

In this part we focused on the synthesis and subsequent isolation of the structural analogues of DEX with modified linker. Similarly to analogues of DEX derived from DTPA, also the analogues with modified linker were intended for the evaluation of their cardioprotective potential *in vitro* on primary cultures of rat isolated neonatal ventricular cardiomyocytes, as well as *in vivo* in a model of chronic ANT-induced heart failure in rabbits.

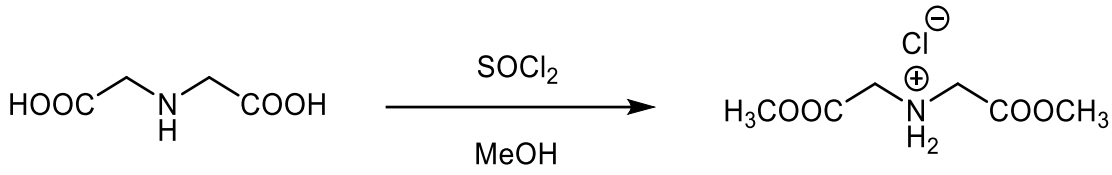
The aim of this part of work was to synthetize 4,4'-(1-oxoethane-1,2-diyl)bis(piperazine-2,6-dione) (**JS-X**). Two approaches were applied and are described in the following sections.

4.3.1. Synthesis of JS-X - Approach 1

The approach 1 consisted of three steps:
1. synthesis of dimethyl ester of iminodiacetic acid
2. connection of two dimethyl esters of iminodiacetic acid using bromoacetyl bromide
3. cyclization using formamide

4.3.1.1. Synthesis of dimethyl iminodiacetate hydrochloride

Reaction:



8 **9**

Reactants:

- Iminodiacetic acid (**8**)
  - ME = 1
  - M_r = 133.10 g/mol
- $SOCl_2$; thionyl chloride
  - ME = 5
  - M_r = 118.97 g/mol
- MeOH; methanol
  - M_r = 32.04 g/mol
  - solvent

Approach:

Methanol (500 mL) was cooled down to -20 °C and $SOCl_2$ (36 mL, 0.5 mol) was added dropwise under vigorous stirring while maintaining the temperature of the reaction mixture below -10 °C. Then, iminodiacetic acid (**8**) (13.3 g, 0.1 mol) was added in one portion. The reaction was kept stirring for one week under laboratory temperature. Then, methanol was evaporated using vacuum rotary evaporator. Re-crystallization from methanol gave dimethyl iminodiacetate hydrochloride (**9**) in 79 or 83% yield (the reaction was performed twice).

	Reactants		**Product**
Entry	Iminodiacetic acid (**8**)	$SOCl_2$; thionyl chloride	dimethyl iminodiacetate hydrochloride (**9**)
1	m = 13.3 g M_r = 133.10 g/mol n = 0.1 mol	m = 58.97 g M_r = 118.97 g/mol n = 0.5 mol ρ = 1.638 g/mL V = 36 mL	m = 15.600 g M_r = 383.40 g/mol n = 0.0407 mol **Yield**: 79 %
2	m = 13.3 g M_r = 133.10 g/mol n = 0.1 mol	m = 58.97 g M_r = 118.97 g/mol n = 0.5 mol ρ = 1.638 g/mL V = 36 mL	m = 16.396 g M_r = 197.62 g/mol n = 0.0830 mol **Yield**: 83 %

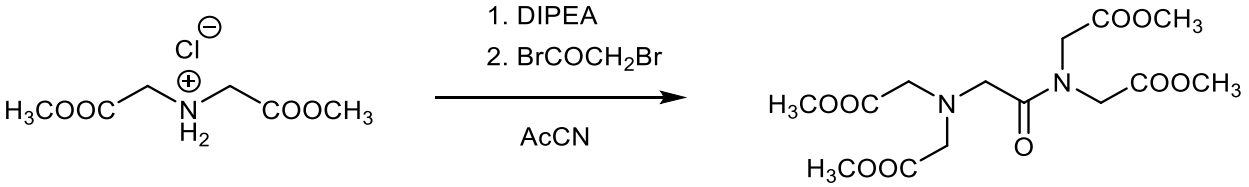
^{1}H NMR (D_2O, 300 MHz): δ = 4.13 (s, 4H), 3.85 (s, 6H).

^{13}C NMR (D_2O, 75 MHz): δ = 168.08, 54.12, 47.66.

Anal. Calcd. for $C_6H_{12}ClNO_4$: C, 36.47; H, 6.12; N, 7.09. Found: C, 36.52; H, 6.01; N, 6.98.

4.3.1.2. Connection of two dimethyl esters of iminodiacetic acid using bromoacetyl bromide

Reaction:



9 **10**

Reactants:

- dimethyl iminodiacetate hydrochloride (**9**)
  - ME = 2
  - M_r = 197.62 g/mol
- *N*,*N*-Diisopropylethylamine; DIPEA
  - ME = 4
  - M_r = 129.247 g/mol
- $BrCOCH_2Br$; bromoacetyl bromide
  - ME = 1
  - M_r = 201.84 g/mol
- CH_3CN; acetonitrile
  - M_r = 41.053 g/mol
  - solvent

Approach:

Dimethyl iminodiacetate hydrochloride (**9**) was dissolved in acetonitrile. Then, DIPEA acting as a base in the reaction, was slowly added using a syringe. After 2 minutes, bromoacetyl bromide was inserted using a syringe. After 1 hour, the TLC (eluent: hexane: ethyl acetate = 1:1; detection: iodine) was evaluated, with R_f (potential product) = 0.05. The TLC was repeated several times

using different mobile phases, in order to select an appropriate mobile phase for subsequent column chromatography. Apart from the product, all TLCs revealed also numerous impurities.

The reaction mixture was stirred for 48 h at rt. Acetonitrile was evaporated, and the residue was dissolved in EtOAc (200 mL). The organic solution was washed with water (3 × 150 mL), dried over Na_2SO_4 and evaporated. The product was purified using column chromatography, chloroform: ethyl acetate = 5:1 was finally selected as a mobile phase. This procedure led to isolation of the product **10**, its structure was confirmed on NMR. The yield of the reaction was 77 - 83% (reaction was performed four times).

	Reactants			**Product**
Entry	bis(2-methoxy-2-oxoethyl)ammonium; dimethyl iminodiacetate hydrochloride (**9**)	N,N-Diisopropylethylamine; DIPEA	$BrCOCH_2Br$; bromoacetyl bromide	Tetramethyl glycinamide-*N*,*N*,*N*',*N*'-tetraacetate (**10**)
1	m = 1 g M_r = 197.62 g/mol n = 0.0051 mol	m = 0.746 g M_r = 129.25 g/mol n = 0.0058 mol ρ = 0.755 g/mL V = 0.988 mL	m = 0.51 g M_r = 201.84 g/mol n = 0.0025 mol ρ = 2.317 g/mL V = 0.22 mL	m = 0.7047 g M_r = 362.33 g/mol n = 0.0019 mol **Yield**: 77 %
2	m = 13.574 g M_r = 197.6 g/mol n = 0.0687 mol	m = 17.756 g M_r = 129.25 g/mol n = 0.1374 mol ρ = 0.755 g/mL V = 23.518 mL	m = 6.933 g M_r = 201.84 g/mol n = 0.0343 mol ρ = 2.317 g/mL V = 3.001 mL	m = 10.072 g M_r = 362.33 g/mol n = 0.028 mol **Yield**: 81 %
3	m = 10 g M_r = 197.6 g/mol n = 0.051 mol	m = 13.08 g M_r = 129.25 g/mol n = 0.101 mol ρ = 0.755 g/mL V =17.326 mL	m = 5.146 g M_r = 201.84 g/mol n = 0.025 mol ρ = 2.317 g/mL V = 2.221 mL	m = 7.275 g M_r = 362.33 g/mol n = 0.0201 mol **Yield**: 79 %
4	m = 16.241 g M_r = 197.6 g/mol n = 0.0822 mol	m = 21.245 g M_r = 129.25 g/mol n = 0.1644 mol ρ = 0.755 g/mL V = 28.139 mL	m = 8.318 g M_r = 201.84 g/mol n = 0.0412 mol ρ = 2.317 g/mL V = 3.59 mL	m = 12.2799 g M_r = 362.33 g/mol n = 0.0339 mol **Yield**: 83 %

M.p.: 57-59 °C

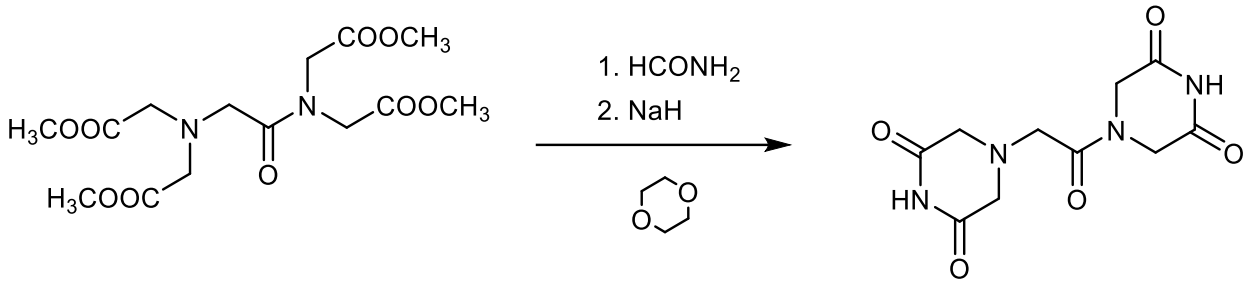
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 4.60 (s, 2H), 4.13 (s, 2H), 3.74 (s, 3H), 3.70 (s, 3H), 3.68 (s, 6H), 3.65 (s, 2H), 3.53 (s, 4H).

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 170.86, 170.04, 169.97, 169.40, 56.52, 54.41, 52.26, 52.12, 51.59, 49.83, 48.19.

MS (APCI+): *m*/z (%) = 363.3 (100) $[M+H]^+$.

4.3.1.3. Synthesis of JS-X by cyclization using formamide and sodium hydride[115]

Reaction:



10 **JS-X**

Reactants:

- Tetramethyl glycinamide-*N*,*N*,*N*',*N*'-tetraacetate (**10**)
  - ME = 1
  - M_r = 362.33 g/mol
- $HCONH_2$; formamide
  - ME=1
  - M_r = 45.04 g/mol
- NaH; sodium hydride
  - ME = 1
  - 60% dispersion in mineral oil
  - M_r = 23.99 g/mol
- 1,4-dioxane
  - Mr = 88.11 g/mol
  - solvent

Approach:

Two mixtures were prepared prior to the reaction. The mixture 1 was prepared by dissolving of **10** (0.905 g; 2.5 mmol) and formamide (0.99 mL; 2.5 mmol) in 12 mL of 1,4-dioxane. The mixture 2 was created by the addition of sodium hydride (1.16 g; 60% dispersion in mineral oil; 29 mmol) into 22 mL of 1,4-dioxane. When both mixtures were ready, the mixture 1 was slowly added into the mixture 2. This step was done under rt and argon atmosphere. When mixed, the content of the flask was left stirring for 36 hours. After approximately 24 hours, the TLC (eluent: chloroform: ethyl acetate = 2:1; detection: iodine) was evaluated, with R_f (potential product) = 0.95 and R_f

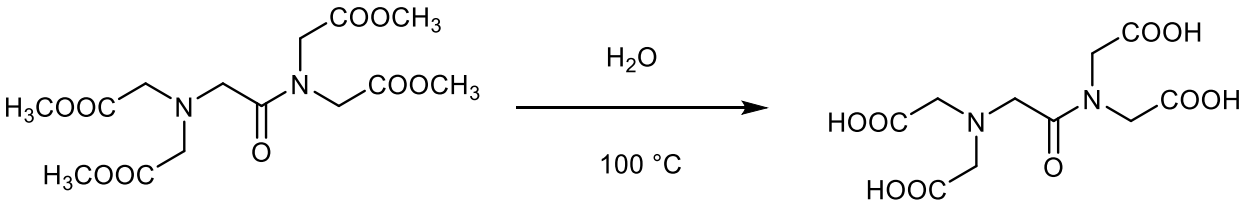
(parent compound) = 0.45. Then, dioxane was evaporated using vacuum rotary evaporator. The oily residue was mixed with approximately 10 mL of diethyl ether and 2.5 g of ice, leading to formation of a suspension. Additionally, approximately 25 mL of water was inserted. Diluted hydrochloric acid was used to neutralize the suspension to pH 7. Unfortunately, the desired product **JS-X** was detected neither in aqueous nor in organic phase.

	Reactants			**Product**
Entry	Tetramethyl glycinamide-*N*,*N*,*N*',*N*'-tetraacetate (**10**)	$HCONH_2$; formamide	NaH; sodium hydride - 60 % dispersion in mineral oil	4,4'-(1-oxoethane-1,2-diyl)bis(piperazine-2,6-dione) (**JS-X**)
1	m = 0.905 g M_r = 362.33 g/mol n = 0.0025 mol	m = 0.132 g M_r = 45.04 g/mol n = 0.0025 mol ρ = 0.133 g/mL V = 0.99 mL	m = 1.16 g M_r = 24.00 g/mol n = 0.029 mol ρ = 1.2 g/mL V = 0.967 mL	m = 0 g M_r = 268.23 g/mol n = 0 mol **Yield**: 0 %

4.3.1.4. Hydrolysis of compound 10

As the cyclization of **10** using formamide was not successful, we tried to hydrolyze methyl esters of **10** in order to get the compound **11**, which would be subsequently used for the next steps to synthetize the desired compound JS-X.

Reaction:



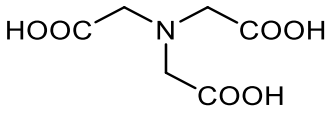
10 **11**

Reactants:

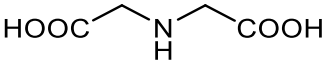
- Tetramethyl glycinamide-*N*,*N*,*N*',*N*'-tetraacetate (**10**)
  - ME = 1
  - M_r = 362.33 g/mol
- H_2O; water
  - M_r = 18.015 g/mol
  - solvent

Approach:

10 (1 g; 2.8 mmol) was placed into the flask and dissolved in water. The solution was then refluxed using a glass condenser. The TLC (eluent: chloroform: ethyl acetate = 1:1; detection: iodine) was evaluated after approximately 1 hour, with R_f (potential product) = 0.6 and R_f (parent compound) = 0.45. The mixture was kept refluxing for 2 hours and then, water was evaporated using vacuum rotary evaporator. The potential product had an appearance of colorless crystals. They were filtrated using Büchner funnel and their structure was determined by the NMR. Unfortunately, the desired product **11** was not confirmed, whereas the crystals were identified as nitrilotriacetic and iminodiacetic acids. (Fig. 13)



12



13

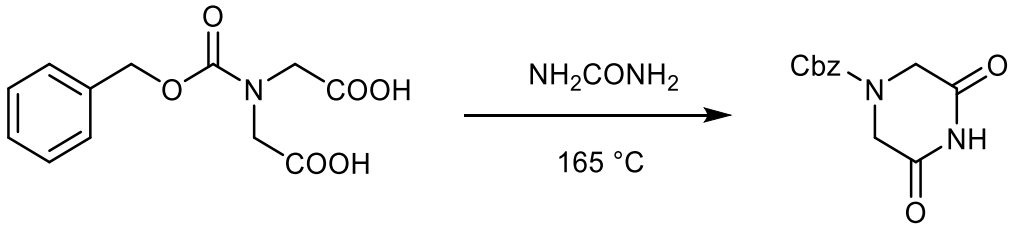
Fig.13. Undesired products of the reactions: nitrilotriacetic acid (**12**) and iminodiaceticacid (**13**)

	Reactants		Product
Entry	Tetramethyl glycinamide-*N*,*N*,*N*',*N*'-tetraacetate (**10**)	H_2O; water	Glycinamide-*N*,*N*,*N*',*N*'-tetraacetic acid (**11**)
1	m = 1 g M_r = 362.33 g/mol n = 0.0028 mol	m = 10 g M_r = 18.015 g/mol n = 0.555 mol ρ = 1 g/mL V = 10 mL	m = 0 g M_r = 306.23 g/mol n = 0 mol **Yield**: 0 %

4.3.2. Synthesis of JS-X - Approach 2

4.3.2.1. Preparation of Cbz-protected piperazine-2,6-dione

Reaction:



14 **15**

Reactants:

- *N*-(Benzyloxycarbonyl)iminodiacetic acid (**14**)

- ME = 1
- M_r = XX g/mol

- Urea (NH_2CONH_2)
  - ME = 1
  - Mr = 60.056 g/mol

Approach:

Cbz-protected iminodiacetic acid (**14**) (20 g; 75.8 mmol) and urea (13.5 g; 224.8 mmol) were put into a round bottomed flask and the mixture of two solid compounds was melted and stirred at 165 °C under reduced pressure for 2 hours. Then, the mixture was slowly cooled down to the rt and approximately 15 mL of methanol was added. The mixture was left stirring for 2 days. Methanol was subsequently evaporated using vacuum rotary evaporator and the oily residue was then partitioned between organic (chloroform) and aqueous phase ($NaHCO_3$ saturated solution). The water residues were dried using sodium sulphate from chloroform fraction. After filtration of sodium sulphate, chloroform was evaporated using vacuum rotary evaporator. Cbz-protected piperazine-2,6-dione (**15**) was isolated, with the yield of 52%.

	Reactants		**Product**
Entry	*N*-(Benzyloxycarbonyl)iminodiacetic acid (**14**)	NH_2CONH_2; urea	4-(Benzyloxycarbonyl)piperazine-2,6-dione (**15**)
1	m = 20 g M_r = 263.85 g/mol n = 0.0758 mol	m = 13.5 g M_r = 60.056 g/mol n = 0.2248 mol	m = 9.785 g M_r = 248.24 g/mol n = 0.0394 mol **Yield**: 52 %

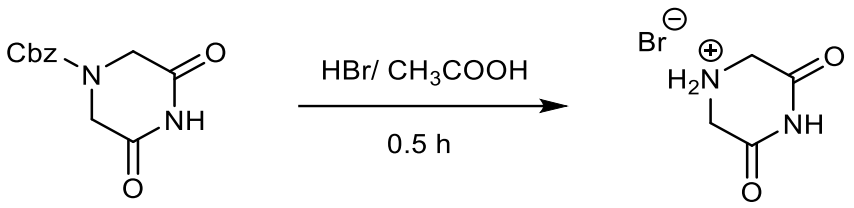
M.p.: 170-172 °C.

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 11.41 (s, 1H), 7.47 – 7.27 (m, 5H), 5.11 (s, 2H), 4.22 (s, 4H).

^{13}C NMR (DMSO-d_6, 75 MHz): δ = 169.58, 154.10, 136.41, 128.65, 128.27, 127.95, 67.32, 46.58.

4.3.2.2. Deprotection of Cbz-protected piperazine-2,6-dione

Reaction:



15 **16**

Reactants:

- 4-(Benzyloxycarbonyl)piperazine-2,6-dione (**15**)
  - ME = 1
  - M_r = 248.24 g/mol
- HBr (33% solution in CH_3COOH)
  - solvent
  - M_r = 80.91 g/mol

Approach:

Cbz-protected piperazine-2,6-dione (**15**) (10 g; 40 mmol) was slowly added into 33% solution of HBr in acetic acid (30 mL) and the emerged suspension was kept stirring for 2 hours at rt. Then, approximately 50 mL of diethyl ether was added. The crystals were filtered and subsequently washed with another volume of diethyl ether. The crystals were put into desiccator over NaOH and were left drying for 2 days. The reaction led to the yield of 98%.

	Reactants		**Product**
Entry	4-(Benzyloxycarbonyl)piperazine-2,6-dione (**15**)	HBr (33% solution in acetic acid)	Piperazine-2,6-dione hydrobromide (**16**)
1	m = 10 g **M_r = 248.24 g/mol** n = 0.04 mol	m = 40.62 g M_r = 80.91g/mol n = 0.5020 mol ro = 1.354 g/mL V = 30 mL	m = 7.699 g M_r = 195.02 g/mol n = 0.0395 mol **Yield**: 98 %

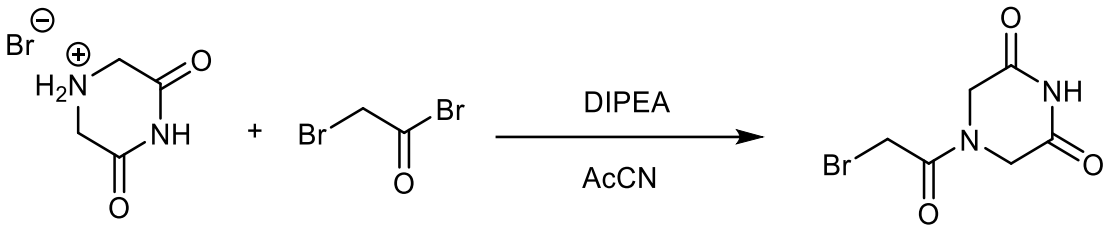
M.p.: decomp. at 290-300 °C.

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 11.83 (s, 1H), 9.84 (br s, 2H), 4.01 (s, 4H).

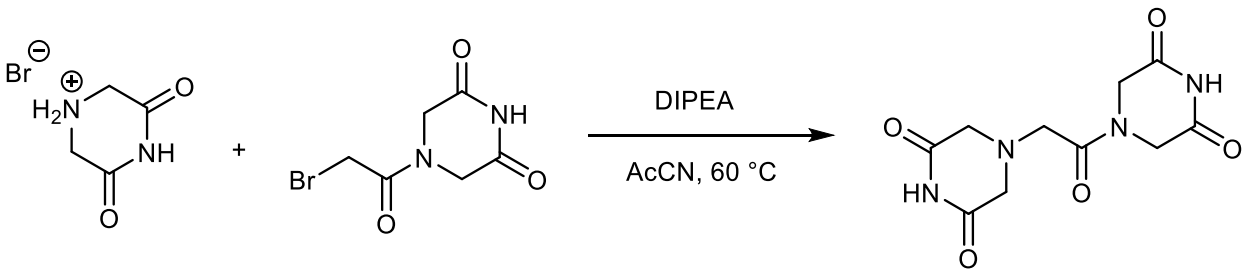
^{13}C NMR (DMSO-d_6, 75 MHz): δ = 166.49, 44.21.

4.3.2.3. Synthesis of JS-X using 2-bromoacetyl bromide

Reaction:



16



16 **JS-X**

Reactants:

- 3,5-dioxopiperazin-1-ium bromide (**16**)
  - ME = 2
  - M_r = 195.02 g/mol
- N,N-Diisopropylethylamine; DIPEA
  - ME = 2
  - M_r = 129.247 g/mol
- 2-bromoacetyl bromide
  - ME = 1
  - M_r = 201.84 g/mol
- CH_3CN; acetonitrile
  - M_r = 41.053 g/mol
  - solvent

Approach:

Piperazine-2,6-dione hydrobromide (5 g; 5.1 mmol) (**16**) was dissolved in acetonitrile and DIPEA (0.988 mL; 5.8 mmol), which plays a role of the base in the reaction, was slowly added. After 2

minutes, bromoacetyl bromide (0.22 mL; 2.5 mmol) was slowly inserted using a syringe. The reaction led to the formation of intermediate 4-(2-bromoacetyl)piperazine-2,6-dione, which subsequently reacted with another molecule of piperazine-2,6-dione and formed the desired product **JS-X**. The mixture was kept stirring for 4 hours at 60 °C. Then we got rid of the solvent using vacuum rotary evaporator and the oily residue was purified using a column chromatography, with chloroform: ethyl acetate = 3:1 as a mobile phase. The product JS-X was isolated, however the yield of the reaction was low (10 %).

	Reactants			Product
Entry	3,5-dioxopiperazin-1-ium bromide (**16**)	N,N-Diisopropylethylamine; DIPEA	$BrCOCH_2Br$; bromoacetyl bromide	4,4'-(1-oxoethane-1,2-diyl)bis(piperazine-2,6-dione) (**JS-X**)
1	m = 5 g M_r = 195.02 g/mol n = 0.0256 mol	m = 3.27 g M_r = 129.25 g/mol n = 0.0253 mol ρ = 0.755 g/mL V = 4.33 mL	m = 0.517 g M_r = 201.84 g/mol n = 0.00256 mol ρ = 2.317 g/mL V = 0.22 mL	m = 1.582 g M_r = 268.23 g/mol n = 0.0059 mol **Yield:** 10 %

M.p.: 247-250 °C.

^{1}H NMR (DMSO-d_6, 500 MHz): δ = 11.36 (s, 1H), 11.10 (s, 1H), 4.30 (s, 2H), 4.24 (s, 2H), 3.50 (s, 2H), 3.42 (s, 4H).

^{13}C NMR (DMSO-d_6, 126 MHz): δ = 171.24, 169.56, 169.35, 167.80, 55.94, 54.58, 47.67, 44.40.

MS (APCI+): *m*/z (%) = 269.0 (100) $[M+H]^+$.

5. Results and discussion

The first aim of this work was successfully fulfilled, a sufficient amount of ADR-925 was synthetized. The reaction consisted of two steps – synthesis of razoxane and subsequent basic hydrolysis resulting in ADR-925. Both reactions were successful, and favourable yields were gained. **ADR-925** was produced in sufficient amount to allow further *in vitro* and mainly *in vivo* testing in order to study the mechanism of cardioprotection exerted by DEX.

The second part of this work was focused on the synthesis of analogues of DEX derived from DTPA. In the beginning, there were several unsuccessful attempts to close free carboxyl groups of DTPA into two imide cycles and substitute the last carboxyl at the same time. Heating of DTPA in formamide at 160 °C and 200 °C, respectively, and two-step approach via anhydride of DTPA were consecutively tried. Therefore, the conditions of the reaction were adjusted, and only four terminal carboxyl groups of DTPA into two imide cycles were cyclized while the last central carboxyl stayed intact. This finding was in contrast to patent literature, where conversion of all five carboxylic groups were described[116]. This reaction led to successful isolation of the product **ES-1**, which was used as a starting compound for further reactions. The synthesis of ES-1 was repeated four times in order to optimize the process parameters such as temperature and allow production of the product in a large scale. The yields of these reactions were approximately 61 – 68%.

In the next step, we focused on functionalization of free carboxylic group. It was presupposed that the substitution of free carboxyl of ES-1 may lead to increased lipophilicity and thus also increased permeability of the compound through biological membranes of cardiomyocytes. Amidation and esterification were tried.

Amidation by dimethyl amine was tried using mixed anhydride approach[117] in three different solvents (THF, DMF, CH_3CN) and none of the reactions led to isolation of the desired dimethylamide of ES-1. Then, pyrrolidine was selected as a promising substituent and two reactions were conducted: amidation by pyrrolidine using ethyl chloroformate and using *N*,*N*'-dicyclohexylcarbodiimide (DCC). Unfortunately, both reactions were unsuccessful and pyrrolidine amide of ES-1 was not isolated. After these failures, amidation was abandoned and we commenced to examine esterification.

Even more reactions based on the esterification of free carboxyl were conducted. These reactions comprised esterification by methanol in thionyl chloride, alkylation with benzyl chloride, 2-bromoethanol, dimethyl sulphate and bromoethane using different combinations of base and solvent (K_2CO_3 and DMSO; DIPEA and THF; DIPEA and CH_3CN; K_2CO_3 and THF; K_2CO_3 and CH_3CN; DIPEA and DMSO). From this set of reactions, esterification by dimethyl sulphate and bromoethane were the most successful ones. The synthesis of methyl ester of ES-1 using dimethyl

sulphate was selected as a model reaction and as a precedent for the synthesis of ethyl ester of ES-1, which was believed to be significantly less toxic compared to methyl ester. After a cleavage in *in vivo* conditions, methyl ester would lead to the formation of toxic methanol, which does not make methyl ester an optional candidate for the use *in vivo*. Even the yield of the reaction was sufficient (31%) and methylester of ES-1 (**11**) was isolated, it was not proceeded to *in vitro* and *in vivo* testing due to reasons stated above.

Preparation of ester using bromoethane led to the isolation of ethylester of ES-1 (**ES-5**). As DMSO was used as a solvent in this reaction, we faced certain issues to get rid of it from the suspension. A portion was evaporated under reduced pressure using vacuum rotary evaporator and the rest was removed during column chromatography. The yield of the reaction was 56% and ES-5 was isolated in sufficient amount for subsequent *in vitro* testing on isolated neonatal rat cardiomyocytes and also *in vivo* preclinical testing on rabbit model of chronic ANTs cardiotoxicity. During this study, ES-5 was administered together with daunorubucin and its cardoprotective effect was examined.

The synthesis of ES-5 and the results of its *in vitro* and *in vivo* studies has been published in the journal with impact factor (see Attachment 1).

A. Jirkovska, J. Roh, O. Lenčová-Popelová, E. Jirkovsky, K. Hruskova, E. Potuckova, H. Jansova, P. Haskova, P. Martinkova, T. Eisner, M. Kratochvil, **J. Sus**, M. Machacek, L. Tichotova, V. Gersl, D. S. Kalinowski, M. Muller, D. Richardson, K. Vavrova, M. Sterba, T. Simunek. Synthesis and analysis of novel analogues of dexrazoxane and its open-ring hydrolysis product for protection against anthracycline cardiotoxicity in vitro and in vivo. *Toxicol. Res.* **2015**, *4*, 1098–1114. (IF_{2015} = 2.161)

Last part of this work was focused on the synthesis of analogues of DEX with modified linker, with the aim to isolate compound **JS-X**, which would be then also tested *in vitro* and *in vivo*. We tried two different approaches. The first approach consisted of three steps: synthesis of dimethyl ester of iminodiacetic acid, connection of two dimethyl esters of iminodiacetic acid using bromoacetyl bromide and finally the cyclization using formamide. First two steps were successful with relatively high yield, however the final cyclization using formamide did not lead to the desired product. Therefore, an alternative approach using piperazine-2,6-dione hydrobromide was tried. This approach also comprised three steps: preparation of Cbz-protected piperazine-2,6-dione, its subsequent deprotection and synthesis of JS-X using 2-bromoacetyl bromide. The yields of the first and second steps were good, the final synthesis of JS-X led to the yield of only 10%. However, the compound JS-X was isolated and used for *in vitro* studies.

6. References

1. McGowan, J. V. *et al.* Anthracycline Chemotherapy and Cardiotoxicity. *Cardiovasc. Drugs Ther.* **31**, 63–75 (2017).

2. Weiss, R.B. (1992) The Anthracyclines Will We Ever Find a Better Doxorubicin Seminars in Oncology, 19, 670-686. - References - Scientific Research Publishing. https://www.scirp.org/(S(vtj3fa45qm1ean45vvffcz55))/reference/ReferencesPapers.aspx?ReferenceID=1843305.

3. Valcovici, M., Andrica, F., Serban, C. & Dragan, S. Cardiotoxicity of anthracycline therapy: Current perspectives. *Arch. Med. Sci.* **12**, 428–435 (2016).

4. Doxorubicin Solution for Injection - Summary of Product Characteristics (SmPC) - (emc). https://www.medicines.org.uk/emc/product/6184/smpc.

5. doxorubicin hydrochloride 2mg/ml solution for infusion - Summary of Product Characteristics (SmPC) - (emc). https://www.medicines.org.uk/emc/product/4613/smpc.

6. PHARMORUBICIN - Summary of Product Characteristics (SmPC) - (emc). https://www.medicines.org.uk/emc/product/5530/smpc.

7. Henriksen, P. A. Anthracycline cardiotoxicity: An update on mechanisms, monitoring and prevention. *Heart* vol. 104 971–977 (2018).

8. World Health Organization. WHO Model List of Essential Medicines: 20th list. *WHO Med. web http//www. who. int/medicines/publications/EML* 62 p. (2017) doi:10.1016/S1473-3099(14)70780-7.

9. Di Marco, A., Gaetani, M., Dorigotti, L., Soldati, M. & Bellini, O. Daunomycin: A New Antibiotic with Antitumor Activity. *Tumori J.* **49**, 203–217 (1963).

10. Arcamone, F. *et al.* Adriamycin, 14-Hydroxydaunomycin, a new antitumor antibiotic from S. peucetius var. caesius. *Biotechnol. Bioeng.* **67**, 704–713 (2000).

11. Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. & Gianni, L. Anthracyclines: Molecular advances and pharmacologie developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* **56**, 185–229 (2004).

12. Creutzig, U. *et al.* Diagnosis and management of acute myeloid leukemia in children and adolescents: Recommendations from an international expert panel. *Blood* **120**, 3167–3205 (2012).

13. Blum, R. H. & Carter, S. K. A new anticancer drug with significant clinical activity. *Ann. Intern. Med.* **80**, 249–259 (1974).

14. Takemura, G. & Fujiwara, H. Doxorubicin-Induced Cardiomyopathy. From the Cardiotoxic Mechanisms to Management. *Prog. Cardiovasc. Dis.* **49**, 330–352 (2007).

15. Langer, S. W. Dexrazoxane for the treatment of chemotherapy-related side effects. *Cancer Manag. Res.* **6**, 357–363 (2014).

16. Zavedos 10 mg Capsules - Summary of Product Characteristics (SmPC) - (emc). https://www.medicines.org.uk/emc/product/2847/smpc.

17. Štěrba, M. *et al.* Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. *Antioxidants Redox Signal.* **18**, 899–929 (2013).

18. Vander Heide, R. S. & L'Ecuyer, T. J. Molecular basis of anthracycline-induced cardiotoxicity. *Hear. Metab.* 1–4 (2007).

19. Shankar, S. M. *et al.* Risk of therapy related myelodysplasia and acute myeloid leukemia (t-MDS/AML) in children exposed to alkylating agents, anthracyclines, platinum compounds and epipodophyllotoxins: a report from Children's Oncology Group (COG). *J. Clin. Oncol.* **26**, 10005–10005 (2008).

20. Giordano, S. H., Lin, Y. L., Kuo, Y. F., Hortobagyi, G. N. & Goodwin, J. S. Decline in the use of anthracyclines for breast cancer. *J. Clin. Oncol.* **30**, 2232–2239 (2012).

21. Nabhan, C. *et al.* Disease characteristics, treatment patterns, prognosis, outcomes and lymphoma-related mortality in elderly follicular lymphoma in the United States. *Br. J. Haematol.* **170**, 85–95 (2015).

22. Smith, L. A. *et al.* Cardiotoxicity of anthracycline agents for the treatment of cancer: Systematic review and meta-analysis of randomised controlled trials. *BMC Cancer* **10**, (2010).

23. von Hoff, D. D. *et al.* Risk factors for doxorubicin-induced congestive heart failure. *Ann. Intern. Med.* **91**, 710–717 (1979).

24. Lefrak, E. A., Piťha, J., Rosenheim, S. & Gottlieb, J. A. A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer* **32**, 302–314 (1973).

25. Scott, J. M. *et al.* Modulation of anthracycline-induced cardiotoxicity by aerobic exercise in breast cancer: current evidence and underlying mechanisms. *Circulation* **124**, 642–50 (2011).

26. Geisberg, C. A. & Sawyer, D. B. Mechanisms of anthracycline cardiotoxicity and strategies to decrease cardiac damage. *Current Hypertension Reports* vol. 12 404–410 (2010).

27. Santa-Maria, C. A. & Gradishar, W. J. Adjuvant and neoadjuvant systemic therapies for early-stage breast cancer. in *The Breast: Comprehensive Management of Benign and Malignant Diseases* 752-762.e4 (Elsevier Inc., 2018). doi:10.1016/B978-0-323-35955-9.00055-6.

28. Chatterjee, K., Zhang, J., Honbo, N. & Karliner, J. S. Doxorubicin cardiomyopathy.

Cardiology **115**, 155–162 (2010).

29. Barrett-Lee, P. J. *et al.* Expert opinion on the use of anthracyclines in patients with advanced breast cancer at cardiac risk. *Ann. Oncol.* **20**, 816–827 (2009).

30. Aapro, M. *et al.* Anthracycline cardiotoxicity in the elderly cancer patient: A SIOG expert position paper. *Ann. Oncol.* **22**, 257–267 (2011).

31. Rudzinski, T., Ciesielczyk, M., Religa, W., Bednarkiewicz, Z. & Krzeminska-Pakula, M. Doxorubicin-induced ventricular arrhythmia treated by implantation of an automatic cardioverter-defibrillator. *Europace* **9**, 278–280 (2007).

32. Bristow, M. R., Mason, J. W., Billingham, M. E. & Daniels, J. R. Doxorubicin cardiomyopathy: evaluation by phonocardiography, endomyocardial biopsy, and cardiac catheterization. *Ann. Intern. Med.* **88**, 168–175 (1978).

33. Hayek, E. R., Speakman, E. & Rehmus, E. Acute doxorubicin cardiotoxicity [4]. *New England Journal of Medicine* vol. 352 2456–2457 (2005).

34. Steinberg, J. S., Cohen, A. J., Wasserman, A. G., Cohen, P. & Ross, A. M. Acute arrhythmogenicity of doxorubicin administration. *Cancer* **60**, 1213–1218 (1987).

35. Whiteside, H., Nagabandi, A., Jyothidasan, A., Brown, K. & Thornton, J. Acute Anthracycline Induced Cardiotoxicity: a Rare and Reversible Cause of Acute Systolic Heart Failure. *J. Am. Coll. Cardiol.* **71**, A2356 (2018).

36. Ewer, M. S. & Lippman, S. M. Type II chemotherapy-related cardiac dysfunction: Time to recognize a new entity. *J. Clin. Oncol.* **23**, 2900–2902 (2005).

37. Felker, G. M. *et al.* Underlying Causes and Long-Term Survival in Patients with Initially Unexplained Cardiomyopathy. *N. Engl. J. Med.* **342**, 1077–1084 (2000).

38. Ewer, M. S. & Lenihan, D. J. Left ventricular ejection fraction and cardiotoxicity: Is our ear really to the ground? *J. Clin. Oncol.* **26**, 1201–1203 (2008).

39. Volkova, M. & Russell, R. Anthracycline Cardiotoxicity: Prevalence, Pathogenesis and Treatment. *Curr. Cardiol. Rev.* **7**, 214–220 (2012).

40. Groarke, J. D. & Nohria, A. Editorial: Anthracycline cardiotoxicity a new paradigm for an old classic. *Circulation* **131**, 1946–1949 (2015).

41. Von Hoff, D. D., Rozencweig, M., Layard, M., Slavik, M. & Muggia, F. M. Daunomycin-induced cardiotoxicity in children and adults. A review of 110 cases. *The American Journal of Medicine* vol. 62 200–208 (1977).

42. Swain, S. M., Whaley, F. S. & Ewer, M. S. Congestive heart failure in patients treated with doxorubicin: A retrospective analysis of three trials. *Cancer* **97**, 2869–2879 (2003).

43. Abu-Khalaf, M. M. *et al.* Long-term assessment of cardiac function after dose-dense and -

intense sequential doxorubicin (A), paclitaxel (T), and cyclophosphamide (C) as adjuvant therapy for high risk breast cancer. *Breast Cancer Res. Treat.* **104**, 341–349 (2007).

44. Cardinale, D. *et al.* Early detection of anthracycline cardiotoxicity and improvement with heart failure therapy. *Circulation* **131**, 1981–1988 (2015).

45. Kremer, L., Van Dalen, E., Offringa, M. M., Ottenkamp, J. J. & Voute, P. *UvA-DARE (Digital Academic Repository) Anthracyclinee induced clinical heart failuree in a cohort of 607 children: aa long-term follow-up study Introduction n*. *JournalJournal of Clinical Oncology* vol. 19 http://dare.uva.nl (2001).

46. Mulrooney, D. A. *et al.* Cardiac outcomes in a cohort of adult survivors of childhood and adolescent cancer: Retrospective analysis of the childhood cancer survivor study cohort. *BMJ* **339**, 34 (2009).

47. Steinherz, L. J., Steinherz, P. G., Tan, C. T. C., Heller, G. & Murphy, M. L. Cardiac Toxicity 4 to 20 Years After Completing Anthracycline Therapy. *JAMA J. Am. Med. Assoc.* **266**, 1672–1677 (1991).

48. Grenier, M. A. & Lipshultz, S. E. Epidemiology of anthracycline cardiotoxicity in children and adults. *Seminars in Oncology* vol. 25 72–85 (1998).

49. Lipshultz, S. E. *et al.* Late Cardiac Effects of Doxorubicin Therapy for Acute Lymphoblastic Leukemia in Childhood. *N. Engl. J. Med.* **324**, 808–815 (1991).

50. Gianni, L., Salvatorelli, E. & Minotti, G. Anthracycline cardiotoxicity in breast cancer patients: Synergism with trastuzumab and taxanes. in *Cardiovascular Toxicology* vol. 7 67–71 (Springer, 2007).

51. Champoux, J. J. DNA T OPOISOMERASES : Structure , FunctChampoux, J. J. (2001). DNA T OPOISOMERASES : Structure , Function , 369–413.ion ,. 369–413 (2001).

52. Menna, P. & Salvatorelli, E. Primary Prevention Strategies for Anthracycline Cardiotoxicity: A Brief Overview. *Chemotherapy* **62**, 159–168 (2017).

53. Ichikawa, Y. *et al.* Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *J. Clin. Invest.* **124**, 617–630 (2014).

54. Keizer, H. G., Pinedo, H. M., Schuurhuis, G. J. & Joenje, H. Doxorubicin (adriamycin): A critical review of free radical-dependent mechanisms of cytotoxicity. *Pharmacology and Therapeutics* vol. 47 219–231 (1990).

55. Free Radicals in Biology and Medicine - Barry Halliwell, John M. C. Gutteridge - Knihy Google. https://books.google.cz/books?hl=cs&lr=&id=3DlKCgAAQBAJ&oi=fnd&pg=PP1&dq=Halliwell+B+and+Gutteridge+JMC.+Free+Radicals+in+Biology+and+Medicine.+Oxford%3B+New+York:+Oxford+University+Press,+2007.&ots=bopG5-uspX&sig=klFkgvGNobOjfAeCLEoXNglHcC0&redir_esc=y#v=onepage&q&f=false.

56. Fisher, N. G. & Marshall, A. J. Anthracycline-induced cardiomyopathy. 265–268 (1999).

57. Wadugu, B. & Kühn, B. The role of neuregulin/ErbB2/ErbB4 signaling in the heart with special focus on effects on cardiomyocyte proliferation. *Am. J. Physiol. - Hear. Circ. Physiol.* **302**, 2139–2147 (2012).

58. Crone, S. A. *et al.* ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat. Med.* **8**, 459–465 (2002).

59. Ma, J. *et al.* Rac1 signalling mediates doxorubicin-induced cardiotoxicity through both reactive oxygen species-dependent and -independent pathways. *Cardiovasc. Res.* **97**, 77–87 (2013).

60. Huang, C. *et al.* Juvenile exposure to anthracyclines impairs cardiac progenitor cell function and vascularization resulting in greater susceptibility to stress-induced myocardial injury in adult mice. *Circulation* **121**, 675–683 (2010).

61. El-Kareh, A. W. & Secomb, T. W. A mathematical model for comparison of bolus injection, continuous infusion, and liposomal delivery of doxorubicin to tumor cells. *Neoplasia* **2**, 325–338 (2000).

62. Van Dalen, E. C., Van Der Pal, H. J. H., Caron, H. N. & Kremer, L. C. M. Different dosage schedules for reducing cardiotoxicity in cancer patients receiving anthracycline chemotherapy. *Cochrane Database of Systematic Reviews* (2009) doi:10.1002/14651858.CD005008.pub3.

63. Corremans, R., Adão, R., De Keulenaer, G. W., Leite-Moreira, A. F. & Brás-Silva, C. Update on pathophysiology and preventive strategies of anthracycline-induced cardiotoxicity. *Clin. Exp. Pharmacol. Physiol.* **46**, 204–215 (2019).

64. Salvatorelli, E. *et al.* The concomitant management of cancer therapy and cardiac therapy. *Biochim. Biophys. Acta - Biomembr.* **1848**, 2727–2737 (2015).

65. Vaage, J., Barberá-Guillem, E., Abra, R., Huang, A. & Working, P. Tissue distribution and therapeutic effect of intravenous free or encapsulated liposomal doxorubicin on human prostate carcinoma xenografts. *Cancer* **73**, 1478–1484 (1994).

66. Duncan, R. *et al.* Preclinical evaluation of polymer-bound doxorubicin. *J. Control. Release* **19**, 331–346 (1992).

67. Rahman, A. M., Yusuf, S. W. & Ewer, M. S. Anthracycline-induced cardiotoxicity and the cardiac-sparing effect of liposomal formulation. *Int. J. Nanomedicine* **2**, 567–583 (2007).

68. Vail, D. M. *et al.* Pegylated liposomal doxorubicin: Proof of principle using preclinical animal models and pharmacokinetic studies. *Semin. Oncol.* **31**, 16–35 (2004).

69. Safra, T. Cardiac Safety of Liposomal Anthracyclines. *Oncologist* **8**, 17–24 (2003).

70. Mordente, A. *et al.* Anthracycline secondary alcohol metabolite formation in human or rabbit heart: Biochemical aspects and pharmacologic implications. *Biochem. Pharmacol.* **66**, 989–998 (2003).

71. Vejpongsa, P. & Yeh, E. T. H. Prevention of anthracycline-induced cardiotoxicity: Challenges and opportunities. *J. Am. Coll. Cardiol.* **64**, 938–945 (2014).

72. Aminkeng, F. *et al.* Anthracycline-Induced Cardiotoxicity in Childhood Cancer. *Nat Genet.* **47**, 1079–1084 (2015).

73. Wojnowski, L. *et al.* NAD(P)H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity. *Circulation* **112**, 3754–3762 (2005).

74. Reichwagen, A. *et al.* Association of NADPH oxidase polymorphisms with anthracycline-induced cardiotoxicity in the RICOVER-60 trial of patients with aggressive CD20+ B-cell lymphoma. *Pharmacogenomics* **16**, 361–372 (2015).

75. Wang, Y., Lauer, M. E., An, S., Mack, J. A. & Maytin, E. V. Hyaluronan synthase 2 protects skin fibroblasts against apoptosis induced by environmental stress. *J. Biol. Chem.* **289**, 32253–32265 (2014).

76. Angelis, A. De, Cappetta, D., Berrino, L. & Urbanek, K. Doxorubicin Cardiotoxicity: Multiple Targets and Translational Perspectives. in *Cardiotoxicity* (InTech, 2018). doi:10.5772/intechopen.80057.

77. Kalay, N. *et al.* Protective Effects of Carvedilol Against Anthracycline-Induced Cardiomyopathy. *J. Am. Coll. Cardiol.* **48**, 2258–2262 (2006).

78. Kaya, M. G. *et al.* Protective effects of nebivolol against anthracycline-induced cardiomyopathy: A randomized control study. *Int. J. Cardiol.* **167**, 2306–2310 (2013).

79. Georgakopoulos, P. *et al.* Cardioprotective effect of metoprolol and enalapril in doxorubicin-treated lymphoma patients: A prospective, parallel-group, randomized, controlled study with 36-month follow-up. *Am. J. Hematol.* **85**, 894–896 (2010).

80. Gulati, G. *et al.* Prevention of cardiac dysfunction during adjuvant breast cancer therapy (PRADA): A 2 × 2 factorial, randomized, placebo-controlled, double-blind clinical trial of candesartan and metoprolol. *Eur. Heart J.* **37**, 1671–1680 (2016).

81. Seicean, S., Seicean, A., Plana, J. C., Budd, G. T. & Marwick, T. H. Effect of statin therapy on the risk for incident heart failure in patients with breast cancer receiving anthracycline chemotherapy: An observational clinical cohort study. *J. Am. Coll. Cardiol.* **60**, 2384–2390 (2012).

82. Schmidinger, M. *et al.* Cardiac toxicity of sunitinib and sorafenib in patients with metastatic renal cell carcinoma. *J. Clin. Oncol.* **26**, 5204–5212 (2008).

83. Zhao, Z. *et al.* Pre-treatment of a single high-dose of atorvastatin provided cardioprotection in different ischaemia/reperfusion models via activating mitochondrial KATP channel. *Eur. J. Pharmacol.* **751**, 89–98 (2015).

84. Ec, V. D. *et al.* Different anthracycline derivates for reducing cardiotoxicity in cancer patients (Review) Different anthracycline derivates for reducing cardiotoxicity in cancer patients. 4–6 (2015) doi:10.1002/14651858.CD005006.pub4.Copyright.

85. Ryberg, M. *et al.* New insight into epirubicin cardiac toxicity: Competing risks analysis of 1097 breast cancer patients. *J. Natl. Cancer Inst.* **100**, 1058–1067 (2008).

86. *Pharmaceuticals and Medical Devices Safety Information 2. Revision of PRECAUTIONS (No. 207)*. http://www.pmda.go.jp/english/index.html (2009).

87. Mimnaugh, E. G., Siddik, Z. H., Drew, R., Sikic, B. I. & Gram, T. E. The effects of α-tocopherol on the toxicity, disposition, and metabolism of adriamycin in mice. *Toxicol. Appl. Pharmacol.* **49**, 119–126 (1979).

88. Hermansen, K. & Wassermann, K. The Effect of Vitamin E and Selenium on Doxorubicin (Adriamycin®) Induced Delayed Toxicity in Mice. *Acta Pharmacol. Toxicol. (Copenh).* **58**, 31–37 (2009).

89. Reichardt, P., Tabone, M. D., Mora, J., Morland, B. & Jones, R. L. Risk-benefit of dexrazoxane for preventing anthracycline-related cardiotoxicity: Re-evaluating the European labeling. *Futur. Oncol.* **14**, 2663–2676 (2018).

90. Fulbright, J., Hamir, A., Lu, X., Cancer, J. C.-P. B. & & 2010, undefined. ROLE OF REACTIVE OXYGEN SPECIES IN AMRUBICIN'S DECREASED CARDIOTOXICITY AND ITS LEUKEMIA CELL CYTOTOXICITY:(POSTER 174).

91. Herman, E. H., Mhatre, R. M. & Chadwick, D. P. Modification of some of the toxic effects of daunomycin (NSC-82,151) by pretreatment with the antineoplastic agent ICRF 159 (NSC-129,943). *Toxicol. Appl. Pharmacol.* **27**, 517–526 (1974).

92. Herman, E., Ardalan, B., Bier, C., … V. W.-C. T. & 1979, undefined. Reduction of daunorubicin lethality and myocardial cellular alterations by pretreatment with ICRF-187 in Syrian golden hamsters. *books.google.com*.

93. Herman, E. H. & Ferrans, V. J. Influence of vitamin E and ICRF-187 on chronic doxorubicin cardiotoxicity in miniature swine. *Lab. Invest.* **49**, 69–77 (1983).

94. Herman, E. H., Ferrans, V. J., Young, R. S. & Hamlin, R. L. Effect of pretreatment with ICRF-187 on the total cumulative dose of doxorubicin tolerated by beagle dogs. *Cancer Res.* **48**, 6918–25 (1988).

95. Herman, E. H. *et al.* Use of cardiac troponin T levels as an indicator of doxorubicin-induced cardiotoxicity. *Cancer Res.* **58**, 195–7 (1998).

96. FDA. prescribing information for ZINECARD. (1995).

97. Inn, C. Assessment report Note : **44**, (2017).

98. Speyer, B. J. L. *et al.* Cumulative 187. **10**, 117–127 (2014).

99. Rhomberg, W. & Hellmann, K. (Kurt). *Razoxane and dexrazoxane - two multifunctional agents : experimental and clinical results*. *Razoxane and Dexrazoxane - Two Multifunctional Agents* (Springer, 2010).

100. CARDIOXANE 500mg powder for solution for infusion - Summary of Product Characteristics (SmPC) - (emc). https://www.medicines.org.uk/emc/product/1234.

101. EU/3/01/059 | European Medicines Agency. https://www.ema.europa.eu/en/medicines/human/orphan-designations/eu301059.

102. MINOTTI, G., CAIRO, G. & MONTI, E. Role of iron in anthracycline cardiotoxicity: new tunes for an old song? *FASEB J.* **13**, 199–212 (1999).

103. Jirkovská-Vávrová, A. *et al.* Synthesis and analysis of novel analogues of dexrazoxane and its open-ring hydrolysis product for protection against anthracycline cardiotoxicity in vitro and in vivo. *Toxicol. Res. (Camb).* **4**, 1098–1114 (2015).

104. Nitiss, J. L. Targeting DNA topoisomerase II in cancer chemotherapy. *Nature Reviews Cancer* vol. 9 338–350 (2009).

105. Yi, L. L. *et al.* Topoisomerase IIβ-mediated DNA double-strand breaks: Implications in doxorubicin cardiotoxicity and prevention by dexrazoxane. *Cancer Res.* **67**, 8839–8846 (2007).

106. Zhang, A. *et al.* A protease pathway for the repair of topoisomerase II-DNA covalent complexes. *J. Biol. Chem.* **281**, 35997–36003 (2006).

107. Štěrba, M. *et al.* Cardioprotective effects of a novel iron chelator, pyridoxal 2-chlorobenzoyl hydrazone, in the rabbit model of daunorubicin-induced cardiotoxicity. *J. Pharmacol. Exp. Ther.* **319**, 1336–1347 (2006).

108. Popelová, O. *et al.* Deferiprone does not protect against chronic anthracycline cardiotoxicity in vivo. *J. Pharmacol. Exp. Ther.* **326**, 259–269 (2008).

109. Hasinoff, B. B. Dexrazoxane (ICRF-187) protects cardiac myocytes against hypoxia-reoxygenation damage. *Cardiovasc. Toxicol.* **2**, 111–118 (2002).

110. Tebbi, C. K. *et al.* Dexrazoxane-associated risk for acute myeloid leukemia/myelodysplastic syndrome and other secondary malignancies in pediatric Hodgkin's disease. *J. Clin. Oncol.* **25**, 493–500 (2007).

111. Swain, S. M. *et al.* Cardioprotection with dexrazoxane for doxorubicin-containing therapy in advanced breast cancer. *J. Clin. Oncol.* **15**, 1318–1332 (1997).

112. Herman, E. H. *et al.* Comparison of the protective effects against chronic daxorubicin cardiotoxicity and the rates of iran (III) displacement reactions of ICRF-187 and other bisdiketopiperazines. *Cancer Chemother. Pharmacol.* **40**, 400–408 (1997).

113. Hochster, H., Liebes, L., Wadler, S., … R. O.-J. J. of the & 1992, undefined. Pharmacokinetics of the cardioprotector ADR-529 (ICRF-187) in escalating doses combined with fixed-dose doxorubicin. *academic.oup.com*.

114. Prudêncio, M. *et al.* A Caged Lanthanide Complex as a Paramagnetic Shift Agent for Protein NMR. *Chem. - A Eur. J.* **10**, 3252–3260 (2004).

115. Snapka, R. M., Woo, S. H., Blokhin, A. V. & Witiak, D. T. Inhibition of topoisomerase II by ICRF-193, the meso isomer of 2,3-bis(2,6-dioxopiperazin-4-yl)butane: Critical dependence on 2,3-butanediyl linker absolute configuration. *Biochem. Pharmacol.* **52**, 543–549 (1996).

116. J. R. Geigy: Manufacture of new poly-n-diacetic acid imides. GB 978724 (1962).

117. Dunetz, J. R., Magano, J. & Weisenburger, G. A. Large-Scale Applications of Amide Coupling Reagents for the Synthesis of Pharmaceuticals. *Organic Process Research and Development* vol. 20 140–177 (2016).

7. Attachments

7.1. Attachment 1

Jirkovska, J. Roh, O. Lenčová-Popelová, E. Jirkovsky, K. Hruskova, E. Potuckova, H. Jansova, P. Haskova, P. Martinkova, T. Eisner, M. Kratochvil, **J. Sus**, M. Machacek, L. Tichotova, V. Gersl, D. S. Kalinowski, M. Muller, D. Richardson, K. Vavrova, M. Sterba, T. Simunek. Synthesis and analysis of novel analogues of dexrazoxane and its open-ring hydrolysis product for protection against anthracycline cardiotoxicity in vitro and in vivo. *Toxicol. Res.* **2015**, *4*, 1098–1114. (IF_{2015} = 2.161)

Full text: https://pubs.rsc.org/en/content/articlelanding/2015/tx/c5tx00048c#!divAbstract