CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Organic and Bioorganic Chemistry

Synthesis of novel cardioprotectants and metabolites of potent anticancer drug Bp4eT

RIGOROSUM THESIS

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

2018 Tomáš Eisner

DECLARATION

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

Hradec Králové 2018 Tomáš Eisner

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This thesis is product of many hours spent in laboratory, extensive literature research, thorough discussion of obstacles that occurred during my work and of course trials and errors.

ABSTRAKT

Univerzita Karlova
Farmaceutická fakulta v Hradci Králové
Katedra organické a bioorganické chemie

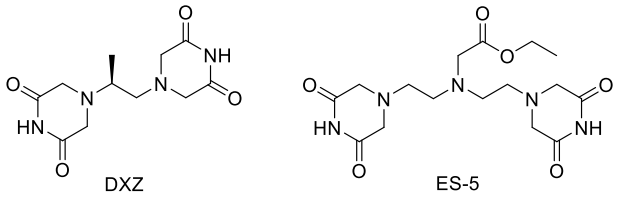
Student: Mgr. Tomáš Eisner

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

Název rigorózní práce: Syntéza nových kardioprotektiv a metabolitů potenciální protinádorové látky Bp4eT

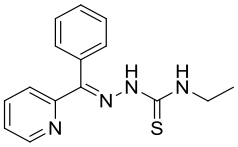
Anthracykliny (ANT) jako například doxorubicin, daunorubicin nebo epirubicin patří mezi nejefektivnější protinádorová léčiva. Avšak jejich hlavním vedlejším účinkem je chronická kardiotoxicita vedoucí až k nevratnému poškození myokardu a městnavému srdečnímu selhání. Předpokládá se, že tento vedlejší účinek je způsoben reaktivními kyslíkovými radikály, jejichž tvorba je katalyzována komplexy ANT s ionty železa. Jediná, klinicky používaná látka na prevenci ANT kardiotoxicity je dexrazoxan (DXZ, Obr. 1).

V této práci jsme se zaměřili na syntézu nových analogů DXZ, i proto, že zatím nebyly provedeny studie vztahu struktury a aktivity DXZ. Hlavní analog, nazvaný ES-5 (Obr. 1), byl úspěšně připraven a jeho kardioprotektivní účinky byly vyhodnoceny jak *in vitro*, tak *in vivo*.



Obr. 1. Struktury DXZ a ES-5

Zároveň se podařilo připravit protinádorový chelátor iontů železa, thiosemikarbazon Bp4eT (2-benzoylpyridin4-ethyl-3-thiosemikarbazon, Obr. 2), a jeho metabolity, což bylo další nedílnou součástí této práce. Tyto sloučeniny byly použity jako standardy v metabolických a farmakokinetických studiích. Byla hodnocena i jejich chelatační a protinádorová aktivita.



Obr. 2. Struktura chelátoru železa Bp4eT

ABSTRACT

Charles University
Faculty of Pharmacy Hradec Králové
Department of Organic and Bioorganic Chemistry

Student: Mgr. Tomáš Eisner

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

Title of rigorosum thesis: Synthesis of novel cardioprotectants and metabolites of potent anticancer drug Bp4eT

Anthracyclines (ANT) such as doxorubicin, daunorubicin, or epirubicin rank among the most effective anticancer drugs. However, their major side effect is chronic cardiotoxicity leading to irreversible cardiac damage and congestive heart failure. It is assumed that this side effect is caused by reactive oxygen species, whose formation is catalyzed by the complexes of anthracyclines with iron ions. The only clinically used drug preventing ANT cardiotoxicity is dexrazoxane (DXZ, Figure 1).

In this work we dealt with the synthesis of novel DXZ analogues, because the structure-activity relationship studies have not been performed yet. The main analogue named ES-5 (Figure 1) was synthesized and its cardioprotective effect was evaluated both *in vitro* and *in vivo*.

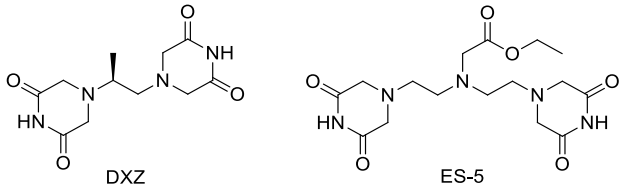


Fig. 1. Structures of DXZ and ES-5

Furthermore, thiosemicarbazone Bp4eT (2-benzoylpyridine 4-ethyl-3-thiosemicarbazone, Figure 2), a potent anticancer iron chelator and its metabolites were synthesized. These compounds were used as standards in metabolic and pharmacokinetic studies. The chelating and anticancer properties of these metabolites were also evaluated.

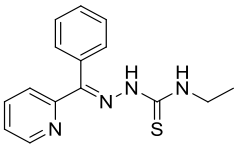


Fig. 2. Structure of iron chelating agent Bp4eT

Content

1. List of abbreviations

***Table 1**: Abbreviations*

ANT	Anthracycline
ATC group	Anatomical Therapeutic Chemical classification/group
Bp4eT	2-benzoylpyridine 4-ethyl-3-thiosemicarbazone
CDK	cyclin-dependent kinase
CHF	congestive heart failure
DAU	Daunorubicin
DOX	Doxorubicin
Dp44mT	di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone
DpT	Di-2-pyridylketone thiosemicarbazone
DXZ	Dexrazoxane
SPC	Summary of product characteristics
TOP2	Topoisomerase II

2. Introduction

Cardiotoxicity induced by anthracyclines (ANTs) therapy is a serious drawback of the group of ANT anti-cancer drugs. Dexrazoxane (DXZ) is the only cardioprotective agent with clinically established efficacy. DXZ was the main drug of focus for the first part of this research project. DXZ can prevent iron-mediated oxidative stress that causes cardiotoxicity by its open ring metabolite ADR-925 via mechanism of iron chelation in the oxidative cascade. However, DXZs cardioprotective properties are also attributed to catalytic inhibition of topoisomerase II (TOP2) and the exact mechanism of cardioprotection is not absolutely clear, which is one of the reasons for DXZ and its analogues research. What is even more interesting, cardioprotective effects of DXZ were discovered accidentally and only few structure-activity relationship studies have been carried out yet.[1]

In the first part of this work we dealt with the synthesis and afterwards more importantly optimization of the synthetic routes leading to novel DXZs analogues such as the main product 'ES-5' ("ring-closed" analogue of DXZ) (Fig. 1). 'ES-5'cardioprotective effect was evaluated both *in vitro* and *in vivo*. Studying the different analogues of the DXZ can potentially help to clarify and specify the exact mechanism of action of DXZ and elucidate its structure-activity relationships.

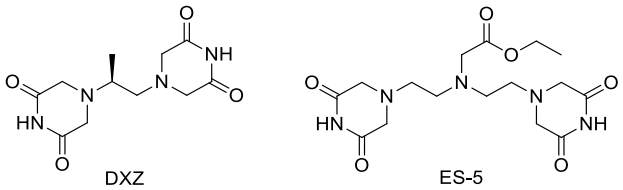


Fig. 1. Structures of DXZ and ES-5

Second part of this project consisted of synthesis of thiosemicarbazone Bp4eT (2-benzoylpyridine 4-ethyl-3-thiosemicarbazone, Fig. 2) and its metabolites, which are also potential chelating and anticancer agents. Novel thiosemicarbazone chelators are currently being extensively studied anti-cancer agents with marked and selective activity against a wide variety of cancer cells, as well as human tumor xenografts in mice.[2] The purpose of synthesis of the novel thiosemicarbazone anti-cancer agent Bp4eT and its metabolites was their simultaneous determination in plasma and application to a pilot pharmacokinetic study in rats. In case of novel thiosemicarbazone anti-cancer drugs, no quantitative data regarding the pharmacokinetics of the parent drug, as well as the predicted metabolites, were available. Quantitative information on metabolites may reveal their biological relevance in terms of activity and/or toxicity.[2]

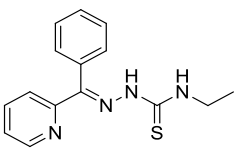


Fig. 2. Structure of iron chelating agent Bp4eT

3. Theoretical part

3.1 Anthracyclines (ANTs)

ANTs belong to the Anatomical Therapeutic Chemical (ATC) classification of antineoplastic and immunomodulating agents and subgroup of anthracyclines and related substances (under code L01DB, www.sukl.cz). ANTs, such as doxorubicin (DOX), daunorubicin (DAU), epirubicin and idarubicin (Fig. 3), are highly effective anti-neoplastic agents.[3] Although ANTs are more than 50 years old, they are still widely prescribed for the treatment of a number of hematological and solid malignancies.[4] Clinically, ANTs are associated with a significant risk of cardiotoxicity.[3] The most important are chronic forms of cardiotoxicity that develop months or years after the completion of chemotherapy and typically manifest as dilated cardiomyopathy and heart failure.[4] Despite numerous proposed theories, the exact pathophysiological mechanism of ANT cardiotoxicity remains not clear.

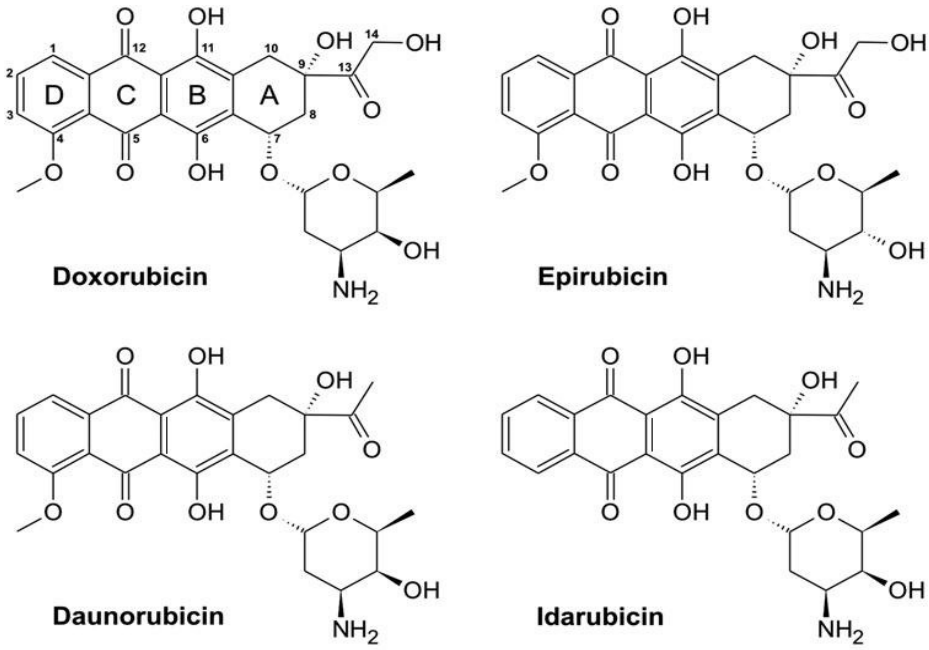


Fig. 3. Structures of four main anthracyclines

The dominant and most cited hypothesis implicates the iron (Fe)-mediated generation of reactive oxygen species (ROS).[5] ANTs can induce the production of superoxide radicals via the quinone/semiquinone redox cycling of their aglycone as shown in Fig. 4.[6, 7] The superoxide dismutates into hydrogen peroxide (H_2O_2), which may in turn enter the Fe-catalyzed Haber–Weiss reaction, yielding hydroxyl radicals. The hydroxyl radical is an extremely reactive and toxic form of ROS that damages DNA, alters proteins and lipids and promotes myocyte dysfunction and death.[8] Furthermore, ANTs are able to form complexes with free Fe ions and undergo a cascade of reactions resulting in further hydroxyl radical production.[5]

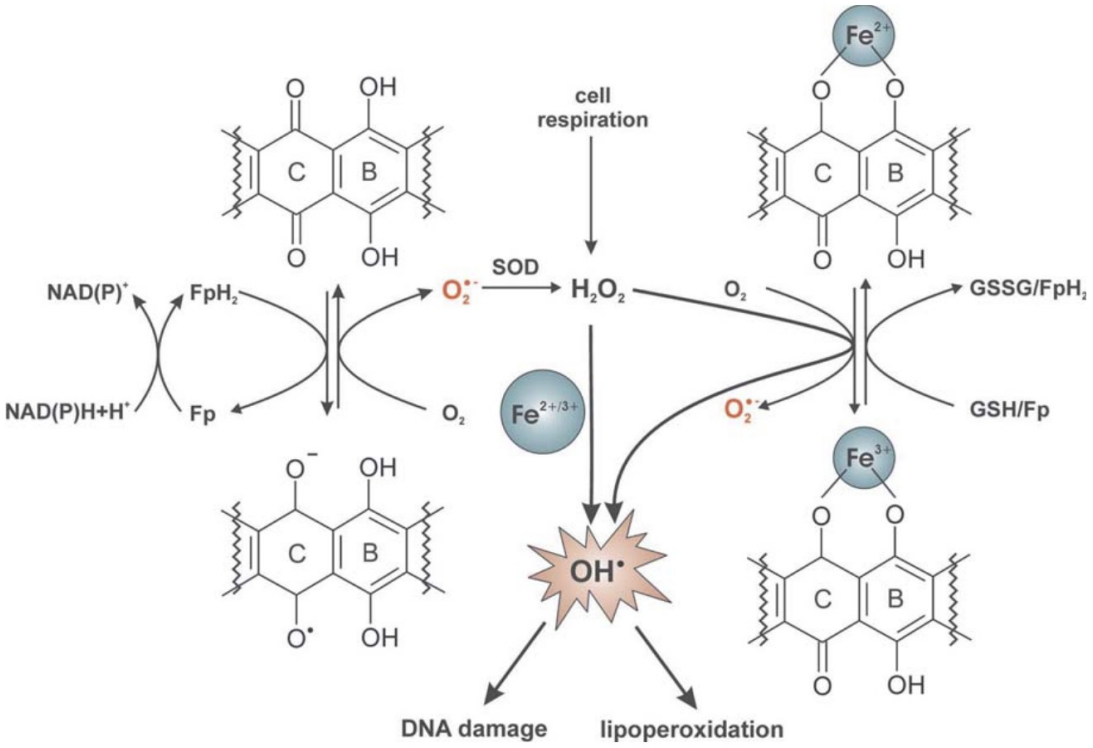


Fig. 4. Proposed mechanisms of ROS formation by ANTs that involve iron.
Fe – iron,$O_2^{\bullet}$ – superoxide radical, SOD– superoxide dismutase, H_2O_2 – hydrogen peroxide, $OH^{\bullet}$ – hydroxyl radical, NAD(P) – nicotinamide adenine dinucleotide (phosphate), Fp – flavoprotein, GSH/GSSG – reduced/oxidized glutathione[7]

3.1.1 **Doxorubicin**

The therapeutic indications and the undesirable effects are specified and can be demonstrated on the example of the SPC of the marketed and clinically used ANT doxorubicin, in the Czech Republic under commercial name DOXORUBICIN TEVA 2 MG/ML (DOX0.2% injection contains DOX hydrochloride 2 mg/ml, Solution for Injection).[9]

DOXORUBICIN TEVA 2 MG/ML has following therapeutic indications: in combination with other antineoplastic drugs, DOX is intended for the treatment of acute lymphocytic leukaemia, except acute lymphatic leukaemia of low risk in children, acute myeloid leukaemia (Hodgkin- and non-Hodgkin lymphomas), osteosarcoma, Ewing sarcoma, adult soft tissue sarcoma, metastatic breast carcinoma, gastric carcinoma, small-cell lung cancer, neuroblastoma, Wilms tumour and bladder carcinoma. DOX may be used intravesically as single agent for treatment and prophylaxis of superficial bladder carcinoma.[9]

Posology and method of administration section mentions the risk of development of cardiomyopathy that gradually increases with the dosage. A cumulative dose of 550 mg/m^2 should not be exceeded. The administration of DOX should be monitored by electrocardiography, echocardiography and carotid pulse curve: when the voltage of the

QRS wave decreases by 30% or at a fractional shortening of 5% it is recommended to stop the treatment.[9]

Under the 'undesirable effects' are specified dose limiting toxicities of therapy as myelosuppression and cardiotoxicity. Myelosuppression includes a transient leukopenia, anaemia and thrombocytopenia, reaching its nadir at 10 to 14 days after treatment. Cardiotoxicity may occur as arrhythmia directly following drug administration; ECG changes, including T-wave flattening and S-T depression, may last up to 2 weeks after administration. The risk of cardiomyopathy increases at cumulative doses higher than 550 mg/m^2. Age over 70 or below 15 years should be regarded as a risk factor. Cardiotoxicity may be encountered several weeks or months after discontinuation of DOX therapy.[9]

The anthracycline DOX is a topoisomerase inhibitor and blocks cell growth by preventing DNA replication.[10] At the molecular level, DOX (Fig. 3.) forms a 1:3 Fe(III)-$(DOX)_3$ chelate at neutral pH.[11] The Fe(III)-$(DOX)_3$ chelate can undergo reduction to give Fe(II)-$(DOX)_3$ chelate. The formation of hydroxyl radical via the Haber-Weiss reaction (Fe(III)-$(DOX)_3$ /Fe(II)-$(DOX)_3$ redox couple (Fig. 5), may result in oxygen-radical mediated lipid peroxidation in the cardiolipid rich inner mitochondrial membrane leading to irreversible destruction of cardiac myocytes and heart muscle failure.[12, 13]

$$O_2^{-\cdot} + H_2O_2 \xrightarrow{\text{Iron}} O_2 + OH^- + OH\cdot$$

Fig. 5. Haber-Weiss reaction via Fe(III)-$(DOX)_3$ /Fe(II)-$(DOX)_3$ redox couple

3.1.2 **Mechanism of action of doxorubicin**

The mechanism of action is not yet completely clear. DOX is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius var. caesius*. Animal studies have shown an oncolytic action in several solid and hematologic tumours. A major mechanism is probably inhibition of topoisomerase II, resulting in DNA breakage. Intercalation and free-radical formation is probably of minor importance. Drug resistance, due to increased expression of the MDR-1 (Multi-Drug Resistance-1) gene encoding for a multidrug efflux pump, has been reported regularly.[9]

3.1.3 **Different types of cardiotoxicity of ANTs**

Cumulative ANTs dose received by patients is the main factor of cardiotoxicity.[14] Cardiotoxicity can be divided into four types.

The first is "acute" cardiotoxicity that occurs during ANT administration or immediately afterwards. It occurs mainly with the drug administered as a bolus or rapid intravenous infusion, and it typically involves vasodilatation, hypotension and transient cardiac rhythm disturbances. Acute cardiotoxicity does not constitute a major clinical problem and it usually resolves shortly after the end of an infusion.[15]

Second but extremely uncommon is “subchronic” cardiotoxicity manifested as a pericarditis-myocarditis syndrome within 1–3 days after the ANT treatment.[16]

The third type of cardiotoxicity is “early chronic” ANT cardiotoxicity that develops later in the treatment course, or weeks to months after the completion of chemotherapy. It is defined by dilated (less often restrictive) cardio-myopathy, with subsequent development of left ventricular contractile dysfunction and congestive heart failure (CHF).[15]

The fourth and the last defined type is “delayed” cardiotoxicity, also called “late-onset chronic”. It is now well established that ANT cardiotoxicity may manifest even decades after the completion of anticancer treatment.[17]

The chronic types of toxicity are serious and clinically significant, substantially affecting overall morbidity and mortality and requiring long-term therapy. The incidence of chronic ANT-induced cardiotoxicity and CHF ranges between 1% and 16% in weeks to months after the ANT-containing chemotherapy, and it further increases with the length of the follow-up.[17] The “classical” study by von Hoff et al. in 1979 estimated that 7% of patients developed DOX-related CHF after a cumulative dose of 550 mg/m^2[14], and this dose was considered for many subsequent years to be the highest recommended for DOX and DAU. However, the meta-analysis by Swain et al. published in 2003 estimated a much higher cardiotoxicity incidence. 26% of patients are at risk of DOX-related CHF for a cumulative dose of 550 mg/m^2.[18] A retrospective analysis in 2006 revealed that compared to expected values, 30-year childhood cancer survivors had a 15-fold higher rate of heart failure, a 10-fold higher rate of other cardiovascular diseases, and a nine-fold higher rate of stroke.[7, 19]

Very important method for ANT cardiotoxicity prevention is pharmacological cardioprotection. Despite decades of research and testing of thousands of potentially protective agents, only one drug has been approved for use in clinical practice: dexrazoxane[7] (see detailed in following section).

3.2 Dexrazoxane

Interestingly, DXZ cardioprotective effects were discovered accidentally during its preclinical testing as a potential anticancer drug in combination with DAU.[20, 21] Analyses of clinical trials revealed that cardioprotection from DXZ did not compromise the antitumor action of ANTs.[22, 23] Thus, DXZ to this day serves as the reference drug for preventing ANT cardiotoxicity.[7]

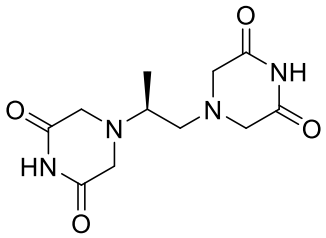


Fig. 6. DXZ (ICRF 187)

DXZ is used in conjunction with DOX [11] in cancer treatment.[12] DXZ is clinically administered in patients with cancer to prevent long-term toxic effects on the heart caused by treatment with the anticancer medicines as previously described DOX and also epirubicin.[10]

The way in which DXZ protects the heart is not entirely clear, but may be linked to the way the medicine attaches to charged iron particles that are involved in the processes that make ANTs toxic to the heart muscle. Medicines containing DXZ are authorized for the prevention of cardiotoxic effects in Austria, Czech Republic, Germany, Denmark, Finland, France, Greece, Hungary, Ireland, Italy, Lithuania, Luxembourg, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Spain and the United Kingdom under the following invented commercial names: Cardioxane, Cyrdanax, Dexrazoxane Cyathus, Enaxozar and Procard.[24]

DXZ, which belongs to the ATC classification group detoxifying agents for antineoplastic treatment (ATC group V03AF02), is registered under following commercial names in the Czech Republic - Cardioxane, Cyrdanax and Savene.

Cyrdanax (CYRDANAX 20 MG/ML, Powder for solution for injection, intravenous use) is the only currently marketed drug (from the three above) containing DXZ in the Czech Republic according to Czech national health authority (SÚKL) website (http://www.sukl.cz).

Indication of the use of DXZ-containing medicinal products 500 mg powder for solution for infusion is 'prevention of chronic cumulative cardiotoxicity caused by DOX or epirubicin use in advanced and/or metastatic adult breast cancer patients who have received a prior cumulative dose of 300 mg/m^2 of doxorubicin or a prior cumulative dose of 540 mg/m^2 of epirubicin when further anthracycline treatment is required.'[10]

3.2.1 **Mechanism of action and chemistry of dexrazoxane**

The exact mechanism by which DXZ exerts its cardioprotective effect has not been fully explained, however based on the available evidence the following mechanism has been suggested. The dose-dependent cardiotoxicity observed during ANT administration is due to ANT-induced iron-dependent free radical oxidative stress on the relatively unprotected cardiac muscle. DXZ, is hydrolyzed in cardiac cells to the ring-opened product ADR-925 (ICRF-198), an analogue of EDTA (ethylenediaminetetraacetic acid) (Fig. 7.). DXZ and mainly ICRF-198 are capable of chelating metal ions.[25]

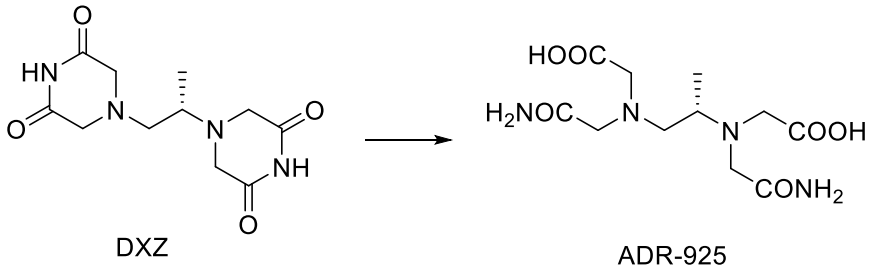


Fig. 7. DXZ (ICRF 187) is a prodrug and is either hydrolyzed or biotransformed to give the open form drug ADR-925

Dexrazoxane (ICRF 187), as shown in Fig. 6., is effective in blocking chronic cardiac toxicity of DOX. ICRF 187 is a prodrug and is either hydrolyzed or biotransformed to give the open form drug ADR-925 as shown in Fig. 7.[12, 26] ADR-925 quickly displaces Fe^{3+} from the Fe-$(DOX)_3$ complex via a shuttle mechanism because ADR-925 has a stronger binding affinity for Fe^{3+}. Hence, the iron chelator (Fig. 8.) provides a cardioprotective effect via the removal of Fe-$(DOX)_3$.[27]

The evidence from clinical trials to this date suggests increasing cardioprotective benefit from DXZ as the cumulative ANT dose is increased. DXZ does not protect against non-cardiac toxicities induced by ANT.[25]

The majority of controlled clinical studies were performed in patients with advanced breast cancer. Data collected from adults treated in 8 controlled randomized clinical studies have been reviewed, 780 patients received DXZ plus chemotherapy and 789 received chemotherapy alone. The rate of death on study was higher with the combination dexrazoxane plus chemotherapy (5.0%) compared to chemotherapy alone (3.4%). The difference was not statistically significant and no consistent cause was apparent, however a contribution of dexrazoxane to the difference cannot be ruled out. [25]

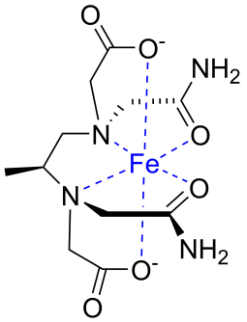


Fig. 8. Chemical structure of ADR-925 complex with Fe^{3+}; hexadentate chelator

Although DEX is used as a pure enantiomer, which is about five times more soluble in water than the racemic compound,[28] several studies showed that the cardioprotective effect of DEX is not associated with the (S)-configuration at its chiral carbon atom (Fig. 6).[29]

3.3 Thiosemicarbazone iron chelating agents

3.3.1 **Thiosemicarbazones**

Novel thiosemicarbazone metal chelators are currently extensively studied anti-cancer agents with marked and selective activity against a wide variety of cancer cells, as well as human tumor xenografts in mice.[2]

Thiosemicarbazones derived from di-2-pyridylketone (the DpT series; Fig. 9.) show potent and selective anti-cancer activity that overcome drug resistance and are currently in advanced preclinical development.[29, 30]

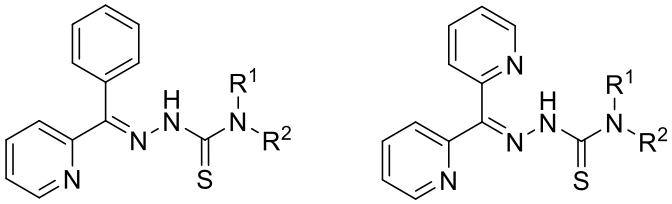


Fig. 9. General structures of di-2-pyridylketone thiosemicarbazones (DpT) and 2-benzoylpyridine thiosemicarbazones (BpT)

3.3.2 **Dp44mT (di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone)**

Thiosemicarbazone analogs derived from di-2-pyridylketone thiosemicarbazone (DpT) such as di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT, Fig. 10.) represent potent and selective anti-cancer agents that overcome drug resistance and which are currently under advanced preclinical development. Specifically Dp44mT is highly effective against wide variety of cancer cells *in vitro*, as well as a range of human xenografts in mice.[32] The *in vitro* and *in vivo* anti-cancer efficacy of Dp44mT was significantly higher relative to the thiosemicarbazone Triapine® (commercial name). Triapine has been investigated in more than 20 clinical trials, including multicenter studies.[32, 33]

However, Dp44mT showed high cardiotoxicity, which lead to further development of more selective analogs such as Bp4eT.[30, 29]

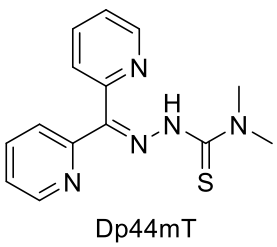


Fig. 10. di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone

3.3.3 **Bp4eTand its metabolites**

The replacement of the non-coordinating pyridyl moiety of the DpT group by a phenyl ring in the BpT series (shown in Fig. 9.) resulted in enhanced lipophilicity, increased anti-cancer activity, decreased Fe(II/III) redox potentials and increased redox activity of the Fe complexes.[34,35]

As the lead compound of the thiosemicarbazone substances for this research project was chosen the Bp4eT (2-benzoylpyridine 4-ethyl-3-thiosemicarbazone) due to the results of a systematic study focused on efficacy and cytoxicity of the thiosemicarbazones. Bp4eT was used for further advanced preclinical development.[2]

Bp4eT showed potent anti-cancer activity on one side but also potential anti-retroviral activity based on its ability to inhibit HIV-1 transcription and viral replication *in vitro* (via cellular targets of iron, cyclin-dependent kinase (CDK) 2, and CDK9) on the other side.[37]

Regarding the pharmacokinetics of the Bp4eT chelator, it has been observed that Bp4eT permeates cell membranes in order to enter cells by a temperature- and energy-independent process.[38] The Bp4eT might have a good intestinal absorption which is suggested by possibility of Bp4eT (*E*- and *Z*-isomericforms in aqueous solution, Fig. 11.) permeation in Caco-2 monolayers as a model of gut absorption demonstrated *in vitro.*[39] This observation is also supported by the fact that this compound satisfies the Lipinski's rules of having physical characteristics which facilitate its efficacy as therapeutic substance.[40]

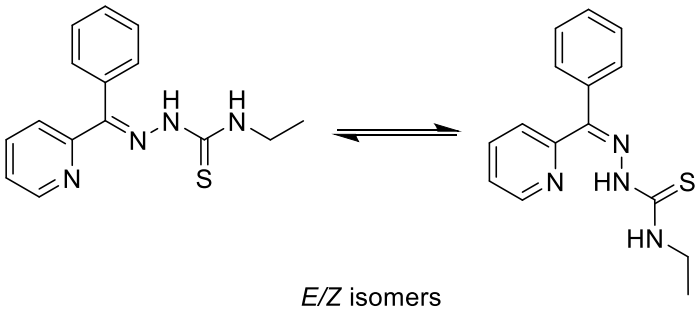


Fig. 11.*E*/*Z* isomers of 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT)

It was found that Bp4eT in the solid state consists predominantly of the *Z*-isomer.[35, 34] However as shown in the Fig. 11., both isomers coexist in aqueous media and common interconversion between the *E* and *Z* isomers occurs. The interconversion is solvent dependent.[41]

Previous bioanalytical study by Stariat et al on Bp4eT provided the first, qualitative data regarding the chemical structures of the main *in vitro* and *in vivo* phase I metabolites, in which the semicarbazone (2-benzoylpyridine 4-ethylsemicarbazone, Fig. 12.), amidrazone (N^3-ethyl-N^1-[phenyl(pyridin-2-yl)methylene]formamidrazone, Fig. 12.) and also hydroxylated amidrazone metabolites were suggested.[42]

Quantitative information on metabolites may reveal their biological relevance in terms of activity and/or toxicity. Thus, the aim of this research project was to synthesize the Bp4eT and its metabolites, which were subsequently used by the team of P. Kovaříková to develop and validate the LC-MS/MS method for the simultaneous quantification of Bp4eT and its main phase I metabolites (semicarbazone and amidrazone) in rat plasma. The group of P. Kovaříková indicated that the amidrazone derivatives were the main metabolites of this lead compound.[2]

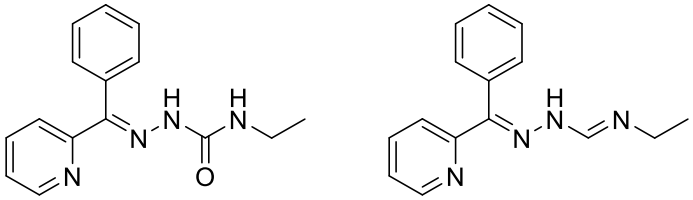


Fig. 12. Chemical structures of semicarbazone and amidrazone metabolites

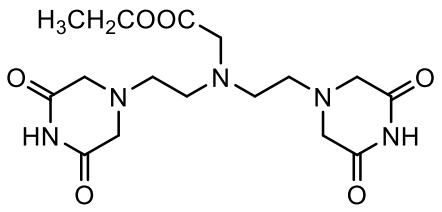
3.3.4 **Mechanism of action of thiosemicarbazones / Bp4eT**

There are three mechanisms of action, which contribute to the anti-tumor activity of the novel thiosemicarbazones such as Bp4eT. Thiosemicarbazone activity is mediated by inhibition of ribonucleotide reductase through binding Fe ion from its active site-inhibition of replication of DNA and suppression of tumor cells growth.[40] Second contribution to MoA is the redox-activity of thiosemicarbazone complexes with iron (Fe) and copper (Cu) forming cytotoxic free radicals (ROS) - which leads to necrosis of tumor cells.[42, 43] And the third is the up-regulation of the potent metastasis suppressor, N-myc downstream regulated gene-1 (NDRG1).[45]

4. Aim of the work

There were two separate purposes and pathways of this research project. The overall aim was firstly the large scale synthesis of the novel analogue of DXZ as a potential cardioprotectant and secondly metabolites of known anticancer drug Bp4eT. These compounds all together belong to the group of iron chelators and could be subsequently used by the cooperating pharmacological teams in further *in vitro* and *in vivo* studies.

The first part of this research project was the preparation of novel analogue ES-5 derived from DXZ (Fig. 13.). Intensive searching for possible ways and optimization of different synthesis routes for the large-scale synthesis (due to 10 weeks *in vivo* evaluation, compounds must have been produced in 15-30 g scales) was the main goal of this project. In each step of the synthesis routes several challenges occurred. By optimization of the small-scale synthesis routes, we were able to gain a significant yield of the desired product, which was necessary for *in vivo* studies of cardioprotective efficiency.



ES-5

Fig. 13. Structure of DXZ analogue ES-5 prepared in this work

The second part, which was of the same priority, was the synthesis of Bp4eT (Fig. 14.) and its metabolites, semicarbazone and amidrazone, which were used for the pharmacokinetic study of this potent anti-cancer chelator. The inherent chelating and antiproliferative activity of these matabolites were also studied in the follow up research.[46]

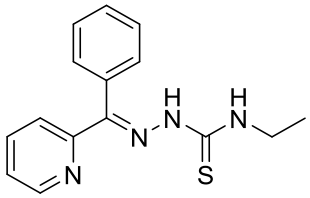


Fig. 14. Structure of iron chelator Bp4eT studied in this work

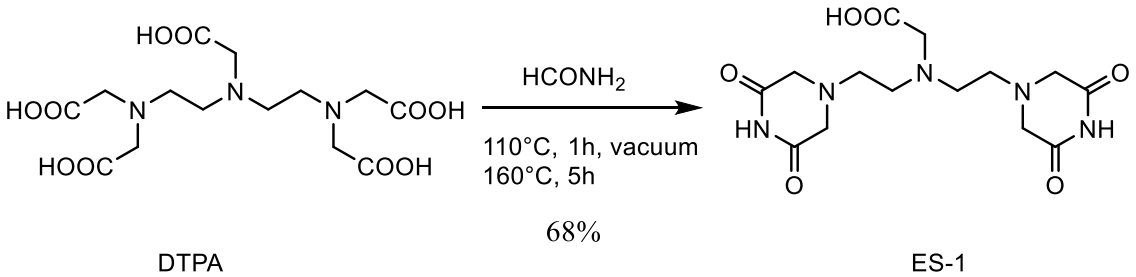
5. Experimental part

The structures of the prepared compounds were identified using ^{1}H-NMR and ^{13}C-NMR spectroscopy. All chemicals were purchased from Sigma-Aldrich (Germany), Fluka (Germany), Merck (Germany), or Penta (Czech Republic) and were of the highest available pharmaceutical or analytical grade. TLC was performed on Merck aluminum plates with silica gel 60 F_{254}. Merck Kieselgel 60 (0.040-0.063 mm) was used for column chromatography. Melting points were recorded with a Büchi B-545 apparatus (BUCHI Labortechnik AG, Flawil, Switzerland) and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded by VNMR S500 NMR spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts were reported as δ values in parts per million (ppm) and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal. The elemental analysis was carried out on an Automatic Microanalyser EA1110CE (Fisons Instruments S.p.A., Milano, Italy). Mass spectra were recorded using an Agilent 500 Ion Trap LC/MS (Agilent Technologies, Santa Clara, California, U.S.A.).

5.1 Synthesis of DXZ analogues

Most of previously described analogs of DXZ differ often in the linker between the two piperazine-2,6-dione cycles. Aim of our work was to synthesize analog of DXZ derived from diethylenetriaminepentaacetic acid (DTPA) and to prepare its lipophilic prodrug, which could be potentially activated *in vivo* to the active drug / chelator. See Attachment 1 for part of these results that were already published.

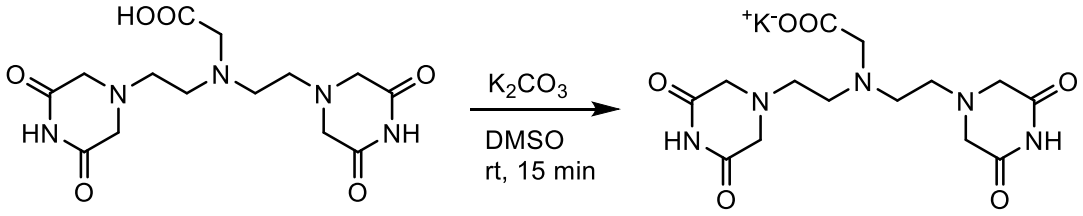
5.1.1 Step 1 –Large-scale preparation of diimide of DTPA (2-{Bis[2-(3,5-dioxopiperazin-1-yl)ethyl]amino}acetic acid, **ES-1**)



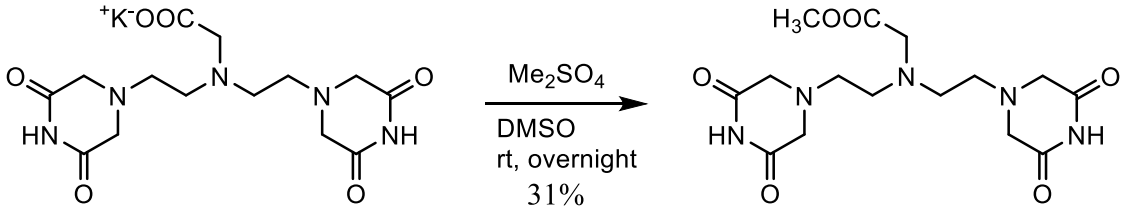
500 mL Oven-dried round bottomed flask was charged with DTPA (50.0 g; 0.14mol) and formamide (200 mL) and the reaction mixture was first stirred under vacuum at 100 °C for 1 h and then under Argon atmosphere at 160°C for 5 hours. Upon cooling, formamide was distilled off under vacuum and the residue was diluted with MeOH (150 mL) and placed into the refrigerator (5 °C) for 24 h. The precipitated product was filtered and washed with cold MeOH (100 mL).

Yield: 68% as a cream-colored solid; mp 254 °C.
^{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%).

5.1.2 **Step 2 a) Preparation of methyl ester ES-4** (Methyl-2-{bis[2-(3,5-dioxopiperazin-1-yl)ethyl]amino}acetate)



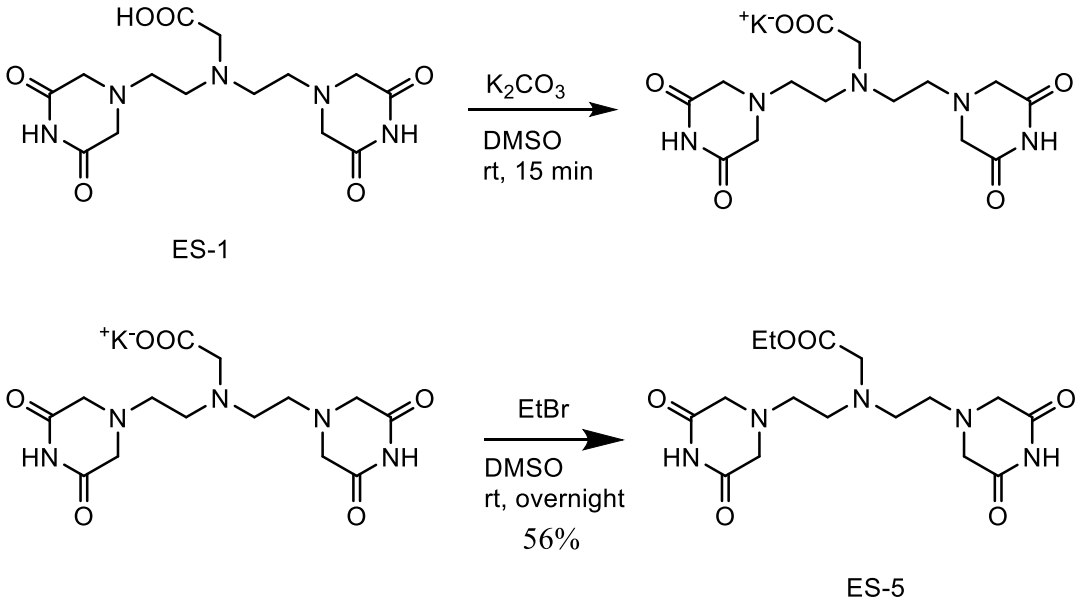
ES-1



20 mL Oven-dried round bottomed flask was charged with ES-1 (0.5 g; 1.4 mmol) and dissolved in DMSO (5 mL). K_2CO_3 (0.2 g, 1.44 mmol) was added and the reaction mixture was stirred at RT for 15 min. Dimethyl sulfate (0.13 mL, 0.17 g, 1.37 mmol) was added to previously created potassium salt of ES-1 and stirred at RT overnight. The reaction mixture was diluted with H_2O (50 mL) and extracted with ethyl acetate (3 × 50 mL). The organic extracts were dried and evaporated. The pure product was obtained using column chromatography on silica gel with mobile phase of ethyl acetate : acetone (1:1). Methyl ester ES-4 would release toxic metabolite MeOH after *in vivo* activation. Therefore the preparation of ES-4 was used as model reaction for the preparation of ethyl ester, which was intended as the main aim of our work.

Yield: 31%
LRMS m/z (APCI) 370.6 (100, M + H^+), 371.5 (17%).

5.1.3 **Step 3 - Preparation of ethyl ester ES-5 ("Eisovín",** Ethyl-2-{bis[2-(3,5-dioxopiperazin-1-yl)ethyl]amino}acetate**)**



EtBr was used as an alkylation agent (instead of dimethyl sulfate in model reaction ES-4). 50 mL Oven-dried round bottomed flask was charged with ES-1 (2.0 g, 5.6 mmol) and dissolved in DMSO (8 mL). After dissolution of ES-1, K_2CO_3 (0.5equiv., 0.4 g, 2.9 mmol) and EtBr (0.4 mL, 0.95 equiv., 5.35 mmol) was added. The reaction mixture was stirred at RT. After overnight stirring inorganic residues were filtered off and DMSO was evaporated under vacuum. The product was purified using column chromatography on silica gel with mobile phase of ethyl acetate.

This reaction was optimized for large-scale synthesis.

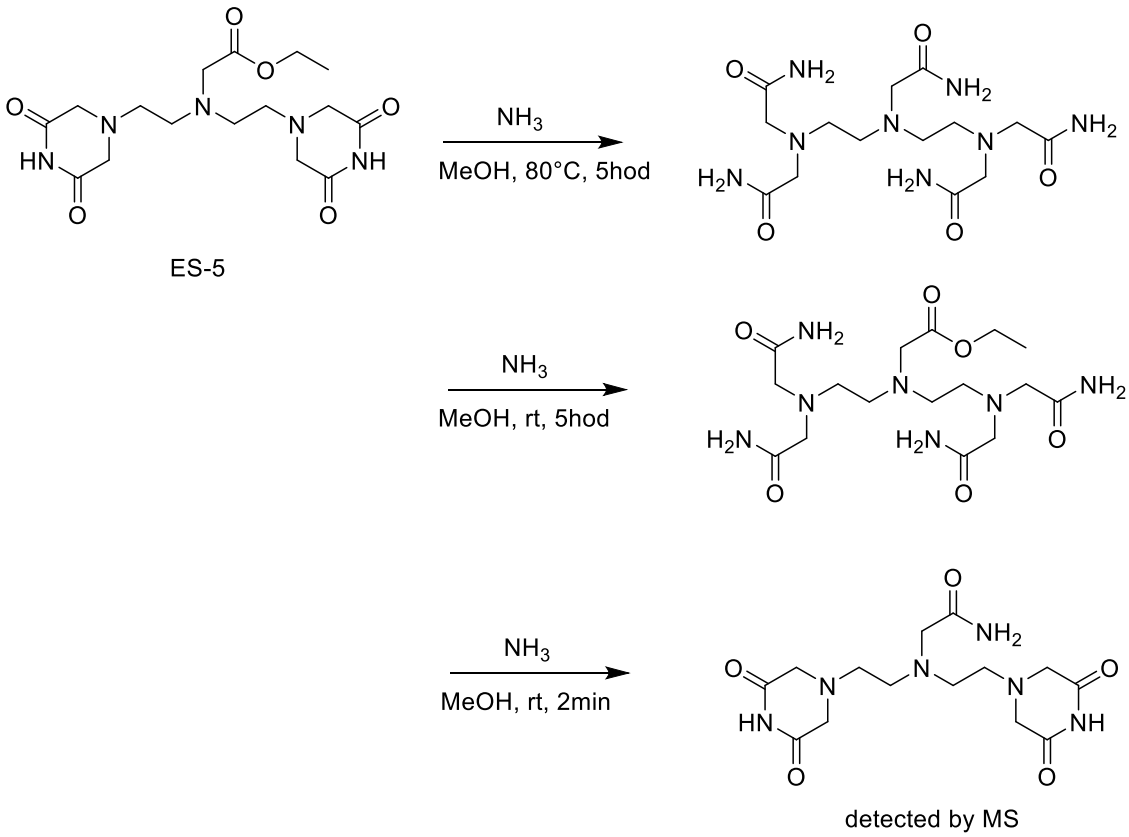
Yield: 56%as a yellowish solid

mp 110 °C

^{1}H NMR (300 MHz, DMSO-d_6) 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-d_6) 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%)

5.1.4 **Step 4 - Preparation of amide of ES-5**



MeOH was chosen as the best option to perform the reactions in different reaction times and temperatures. TLC was continuously performed during the reaction, however with poor results.

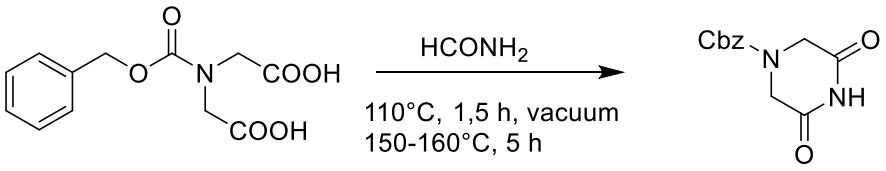
25 mL Oven-dried steel autoclave (due to reactions with boiling point of MeOH 64 °C) was charged with 100 mg of ES-5 dissolved in 10 mL of MeOH, bubbled with NH_3 for 5 min, closed and stirred at 80 °C for 5h.

Two 25 mL oven-dried round bottomed flasks were charged with 100 mg of ES-5 dissolved in 10 mL of MeOH and subsequently at RT bubbled with NH_3. First reaction was bubbled for 5 min and stirred at RT for 5h, second reaction was bubbled for 2 min at RT.

The reaction were monitored using MS, however, just the trace signal of target amide was detected in the last reaction.

5.1.5 **Preparation of Cbz-protected piperazine-2,6-dione (TE-27)**

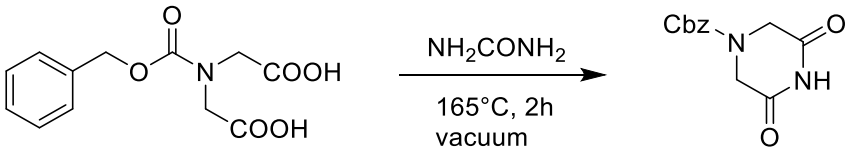
a) Heating with formamide



JR-220

100 mL oven-dried round bottomed flask was charged with formamide (40 mL) and Cbz-protected JR-220 (2 g, 7.5 mmol). The reaction mixture was stirred under vacuum at 110°C for 1 hour in order to prepare the anhydride *in situ*. Second step of this reaction was stirring at increased temperature of 155°C under reflux condenser under Argon atmosphere in order to gain protected piperazine-2,6-dione. Upon evaporation of the formamide under reduced pressure, black tar was obtained. The target product was not isolated from this reaction residue.

b) Heating with urea

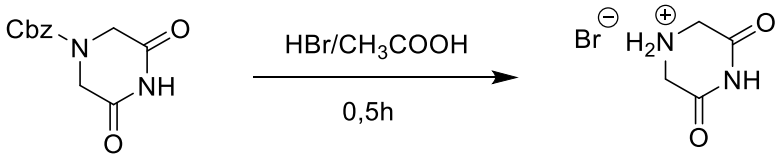


52%

100 ml Oven-dried round bottomed flask was charged with urea (13.5 g, 0.225mol) and Cbz-protected JR-220 (20.0g, 75.8 mmol). The reaction mixture was stirred at 165°C under vacuum for 2 hours. Methanol (15 mL) was added to the cooled melt, and the mixture was stirred for 2 days. The methanol was then evaporated, and the residue was partitioned between $CHCl_3$ (200 mL) and sat. $NaHCO_3$ (300 mL). The aqueous phase was then extracted with $CHCl_3$ (2 x 200 mL). The combined chloroform extracts were dried over Na_2SO_4 and evaporated.

Yield: 52%
mp: 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.

5.1.6 **Deprotection of Cbz-protected piperazine-2,6-dione**



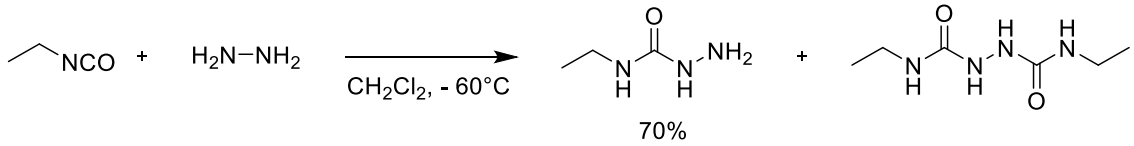
Cbz-protected piperazine-2,6-dione (10 g, 0.04mol) was added in several portions to a stirring solution of HBr (33%) in acetic acid (30 mL). The resulting suspension was stirred vigorously for 2 h at rt. Et_2O (50 mL) was then added, and the solid was filtered off, washed with Et_2O and left under vacuum over NaOH in a desiccator for 48 h.
Yield: 98% (7.6 g) as a white solid;
mp 290-300 °C (with decomposition)
^{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.

5.2 Synthesis of Bp4eT and its metabolites

The iron chelator, 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone (Bp4eT), was identified as a lead compound of the 2-benzoylpyridine thiosemicarbazone series, which were designed as potential anti-cancer agents. This ligand has shown to possess potent anti-proliferative activity with a highly selective mechanism of action. However, further progress in the development of this compound required data regarding its metabolism in mammals.[42] See Attachment 2 for part of these results that were already published.

5.2.1 Step 1 - Synthesis of oxygen analogue of Bp4eT chelator - Preparation of starting material 4-ethylsemicarbazide (a precursor for 2-benzoylpyridine 4-ethylsemicarbazone, M1)

4-Ethylsemicarbazide was prepared according to a published procedure (Beukers et al., 2003) and was obtained as colorless oil with 70% yield.



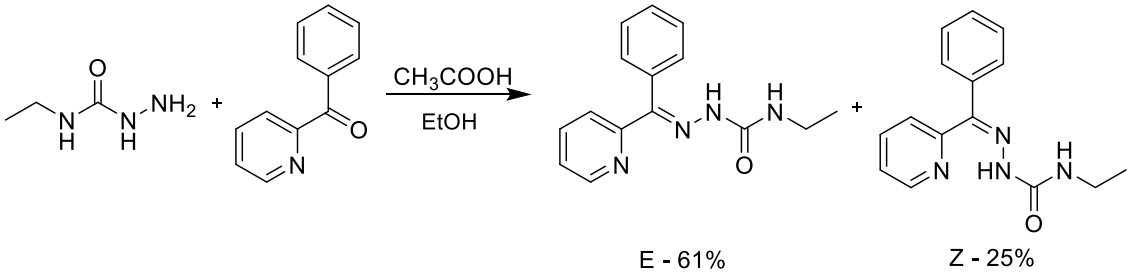
70%

20 mL Oven-dried round bottomed flask was charged with hydrazine hydrate (1 eq; 0.7 g; 0.014 mol) and DCM (30 ml). The solution was cooled down to -60 °C and ethyl isocyanate (1.0 g; 0.014 mol) was added dropwise. The mixture was stirred until reached room temperature. The resulting mixture was filtered and the resulting filtrate was evaporated under reduced pressure.
Yield: 70% as a colorless oil

^{1}H NMR (300 MHz, DMSO-d_6) δ 6.85 (s, 1H, NH), 6.28 (s, 1H, NH), 4.03 (s, 2H, NH_2), 3.09–2.94 (m, 2H, CH_2), 0.98 (t, J = 7.2 Hz, 3H, CH_3).
^{13}CNMR (75 MHz, DMSO-d_6) δ 160.36, 33.85, 16.05.

5.2.2 Step 2 - Synthesis of oxygen analogue of Bp4eT chelator - Preparation of 2-Benzoylpyridine-4-ethylsemicarbazone (M1)

4-Ethylsemicarbazide (0.27 g; 2.62 mmol) was used as starting material and combined with 2-benzoylpyridine (0.4 g; 2.18 mmol) in 96% ethanol (10 mL). Then, 5 drops of glacial acetic acid was added and the resulting mixture was refluxed for 8 hours. Analytically pure products – *E* and *Z*- (2-benzoylpyridine-4-ethylsemicarbazone) was obtained by column chromatography on silica gel with mobile phase of hexane:EtOAc (1:2) followed by pure ethylacetate. This procedure allowed for the isolation of both isomers.



M1-Z:(*Z*)-2-benzoylpyridine 4-ethylsemicarbazone.

Yield: 25% as a pale yellow solid.

R_f = 0.39 (ethylacetate).

^{1}H NMR (300 MHz, $CDCl_3$) δ 12.39 (s, 1H), 8.79–8.74 (m, 1H), 7.79–7.67 (m, 1H), 7.53–7.22 (m, 7H), 6.36 (s, 1H), 3.46–3.31 (m, 2H), 1.20 (t, *J* = 7.2 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 155.98, 152.87, 148.44, 141.30, 138.19, 136.93, 128.83, 128.69, 128.36, 125.52, 123.59, 34.51, 15.55.

M1-E:(*E*)-2-benzoylpyridine 4-ethylsemicarbazone.

Yield: 61% as white solid.

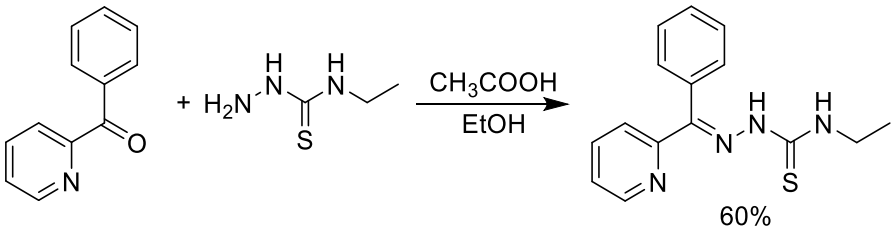
R_f = 0.17(ethyl acetate).

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.59–8.54 (m, 1H), 7.84–7.67 (m, 3H), 7.58–7.44 (m, 3H), 7.31–7.20 (m, 3H), 6.40 (s, 1H), 3.48–3.30 (m, 2H), 1.24 (t, *J* = 7.2 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 154.96, 149.30, 136.27, 131.22, 129.78, 129.65, 129.61, 128.50, 123.33, 121.58, 34.72, 15.51

5.2.3 **Step 3 - Preparation of parent compound, chelator Bp4eT**

Bp4eT was synthesized and characterized as described previously by Kalinowski et al. [34, 35]

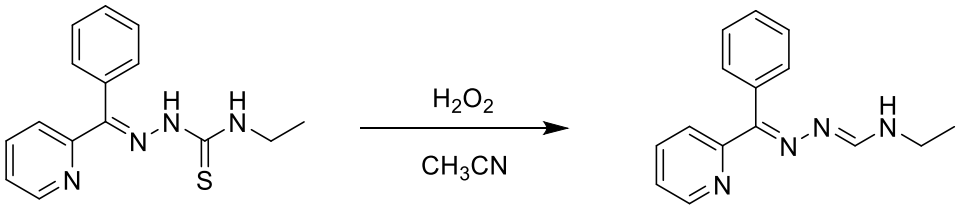


2-Benzoylpyridine (1.0 g, 5.5mmol) was combined with 4-ethylthiosemicarbazide (0.7 g, 5.9 mmol) and dissolved in EtOH (10 mL), 10 drops of acetic acid were added and the resulting mixture was stirred at 70 °C for 2 hours. The pure product (2-benzoylpyridine-4-ethylthiosemicarbazone) crystallized from the reaction mixture upon cooling in the refrigerator.

Yield: 60%.
^{1}H NMR (300 MHz, DMSO-d_6) δ 12.75 (s, 1H), 8.88 – 8.83 (m, 1H), 8.76 (t, J = 6.0 Hz, 1H), 8.00 (td, J = 7.8, 1.8 Hz, 1H), 7.65 – 7.55 (m, 3H), 7.49 – 7.42 (m, 3H), 7.33 (dt, J = 8.1, 1.1 Hz, 1H), 3.67 – 3.54 (m, 2H), 1.14 (t, J = 7.1 Hz, 3H).
^{13}C NMR (75 MHz, DMSO-d_6) δ 177.14, 151.66, 149.03, 143.03, 138.37, 137.13, 129.34, 129.13, 128.55, 126.24, 125.04, 38.90, 14.53.

5.2.4 **Step 4 - Preparation of amidrazone metabolite of Bp4eT - N^3-ethyl-N^1-[phenyl(pyridin-2-yl)methylene]formamidrazone** (M2)

N^3-ethyl-N^1-[phenyl(pyridin-2-yl)methylene]formamidrazone was prepared according to a previously published procedure [42].



14%

Oxidation of 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone (0.1 g) with 10 drops of hydrogen peroxide (H_2O_2 - 30%) was carried out at laboratory temperature (20°C) for 2 hours. There was many different products in the resulting mixture according to TLC (isomers and tautomers).

The product N^3-ethyl-N^1-[phenyl(pyridin-2-yl)methylene]formamidrazone was separated using column chromatography using silica with hexane–chloroform–triethylamine (12:5:1, v/v/v) as the mobile phase. This yielded 14% (62 mg) of M2 as a pale yellow viscous oil. $R_f = 0.1$ (hexane–chloroform–triethylamine). This metabolite was characterized by LRMS spectrum and used in the further studies.

Yield: 14%
LRMS m/z (APCI) 253.4 (100, $M + H^+$).

6. Results and conclusion

The main results of this work and the subsequent studies with synthesized molecules has already been published in two articles in the journals with impact factor (see Att. 1 and 2)

6.1 DXZ analogues

Preparation of diimide of DTPA (**ES-1**) was optimized for multigram-scale yields by optimizing the exact temperatures and times of stirring in each step. The yield of the optimized protocol was 68% that is satisfactory for these types of reactions.
ES-1 has free carboxylic group, which was used as starting material for synthesis of more lipophilic products that could permeate through biological membranes. Synthesis of methyl ester **ES-4** was used as model reaction for the later synthesis of target ethylester (**ES-5**), which resulted as the main compound of our research project.

ES-5 („Eisovín") - reaction was optimized for large scale synthesis.

- issues with solvent DMSO - optimization - evaporation of DMSO under high vacuum without destruction of ES-5
- yield 56% - recrystallization from acetone/ethylacetate
- *in vitro* study was performed with ES-5 on isolated neonatal rat cardiomyocytes
- *in vivo* study on rabbit model of chronic anthracyclines cardiotoxicity – combined administration of ES-5 with DAU

The following idea was to prepare **more stable amide** from ES-5. Aminolysis with ammonia in methanol did not proceed as expected, imide group was even more reactive than ester bond. Aminolysis at elevated temperature led to the preparation of DTPA pentaamide, while aminolysis at RT predominantly led to the formation of tetraamide with preserved ester bond. The target **monoamide with two end-cycles** was not isolated and the traces were detected using MS.

Resulting synthesized analogue of the DXZ, **ES-5**, was evaluated *in vitro* and *in vivo*, however, did not show any cardioprotection compared to parent DXZ. Cardioprotection was evaluated *in vitro* on isolated rat neonatal cardiomyocytes and *in vivo* on rabbit model of chronic anthracycline toxicity.

In contrast to DXZ, ES-5 had no anti-cancer effect of its own and the changes in the chemical structure resulted in a loss of **TOP2β inhibitory activity**. ES-5 also did not protect isolated cardiomyocytes and rabbits from daunorubicin-induced cardiotoxicity and heart failure.[1] This result suggested that the actual mechanism of action of DXZ can be connected with TOP2β inhibition and/or depletion rather than with Fe chelation. For more information see Attachment 1.

Another unsuccessful attempts of aminolysis of ES-5:

- reaction with NH_3 in THF
- reaction with diethylamine in MeOH
- reaction with methoxybenzylamine in MeOH
- reaction with hydroxylamine in MeOH

Another part of this research project was the synthesis of **piperazine-2,6-dione hydrobromide** as the building block in the DXZ analogues synthesis. We cyclized Cbz-protected iminodiacetic acid by its melting with urea. The main drawback of this method was the formation of glass like melt, which had to be ground or dissolved in some suitable solvent prior to further purification.

Resulting Cbz-protected piperazine-2,6-dione was cleaved using HBr in acetic acid with almost quantitative yield.

6.2 Bp4eT and its metabolites

Parent **Bp4eT** and its semicarbazone (**M1**) andformamidrazone (**M2**) metabolites were prepared and used as standards in metabolic and pharmacodynamics study.

The metabolites M1 and M2 of Bp4eT demonstrated poor iron chelation efficacy.

The reason for the low chelation activity of the semicarbazone, M1, could be because the amide moiety highly prevails over the imidol tautomer.[47] Sulfur atom in Bp4eT acts as a better donor atom than the carbonyl oxygen of M1.

M2 metabolite contains the formamidrazone moiety and it does not possess the sulfur atom of the original thiosemicarbazone, which appears to be crucial in terms of the iron chelation efficacy of Bp4eT.[46]

Bp4eT is metabolized into semicarbazone and formamidrazone which have at least a 300-fold decreased cytotoxicity against both cancer and non-cancerous cells,[2] this is very important for the positive pharmacological profile of the drug and its potential use as anticancer agent.

The findings of studies with Bp4eT and its metabolites semicarbazone and amidrazone are crucial for understanding the structure-reactivity relationships of these compounds. For more information see Attachment 2.

7. References

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8. Attachments

8.1 Attachment 1

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8.2 Attachment 2

Ján Stariat, Vlasta Suprunová, Jaroslav Roh, Vít Šesták, **Tomáš Eisner**, Tomáš Filipský, Přemysl Mladěnka, Milan Nobilis, Tomáš Šimůnek, Jiří Klimeš, Danuta S. Kalinowski, Des R. Richardson and Petra Kovaříková, **2014**. Simultaneous determination of the novel thiosemicarbazone anti-cancer agent, Bp4eT, and its main phase I metabolites in plasma: Application to a pilot pharmacokinetic study in rats. *Biomedical Chromatography*, *28*(5), pp.621-629.