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Declarations under Rule 4.17:

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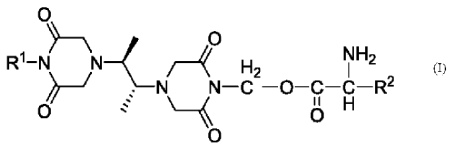
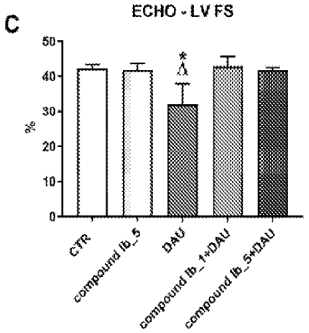
(54) Title: USE OF ICRF-193 DERIVATIVES AND PHARMACEUTICAL PREPARATIONS CONTAINING THEREOF FOR THE PREVENTION OF CHRONIC CUMULATIVE CARDIOTOXICITY CAUSED BY THERAPY WITH ANTHRACYCLINE ANTICANCER DRUGS



(I)

Fig. 6



(57) Abstract: Cardioprotective compounds based on analogues of the compound ICRF-193 of general formula I wherein R^1 = H, 2-R^2-2-aminoacetoxymethyl wherein R^2 is selected from the group consisting of H, a C_1-C_6 alkyl or a branched alkyl of benzyl. These compounds can be produced by simple syntheses and are characterized with low toxicity and high effectiveness of protection of the heart against chronic cumulative cardiotoxicit y caused by anthracycline anticancer therapy. The invention provides also a pharmaceutical preparation comprising the compound of general formula (I) as an active ingredient as well as use of this compound for the manufacture of a medicament for the prevention of chronic cumulative cardiotoxicity caused by therapy with anthracycline anticancer drugs.

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Use of ICRF-193 derivatives and pharmaceutical preparations containing thereof for the prevention of chronic cumulative cardiotoxicity caused by therapy with anthracycline anticancer drugs

Field of the Invention

The invention relates to the use of ICRF-193 derivatives for the preparation of a medicament for the prevention of chronic cumulative cardiotoxicity caused by repeated administration of anthracyclines within the treatment of various kinds of hematological or solid malignancies.

Background Art

Anthracyclines (ANTs), such as doxorubicin, daunorubicin (DAU), epirubicin or idarubicin, belong to highly effective and widely used anti-tumor drugs indicated for a number of hematological malignancies as well as solid tumors (Cortes-Funes and Coronado, Role of anthracyclines in the era of targeted therapy. *Cardiovasc Toxicol* **2007,** *7*, 56-60; Lenneman and Sawyer, Cardio-Oncology: An Update on Cardiotoxicity of Cancer-Related Treatment. *Circulation research* **2016,** *118*, 1008-1020). However, clinical use of these drugs is complicated and frequently also limited by their toxic effects on the myocardium. With respect to the clinical manifestation, its timing and clinical significance, it is important to distinguish several forms of ANT cardiotoxicity. Acute and subacute forms of ANT cardiotoxicity are manifested several hours or days after administration of ANTs, do not show clear dose dependency and are only rarely clinically significant (Gharib and Burnett, Chemotherapy-induced cardiotoxicity: current practice and prospects of prophylaxis. *European journal of heart failure* **2002,** *4*, 235-242; Zamorano *et al.*, 2016 ESC Position Paper on cancer treatments and cardiovascular toxicity developed under the auspices of the ESC Committee for Practice Guidelines: The Task Force for cancer treatments and cardiovascular toxicity of the European Society of Cardiology (ESC). *European Heart Journal* **2016,** *37*, 2768-2801; Allen, The cardiotoxicity of chemotherapeutic drugs. *Seminars in oncology* **1992,** *19*, 529). Acute forms of ANT cardiotoxicity are most frequently described as transient electrophysiological abnormalities that often occur only subclinically. Their relationship to chronic ANT cardiotoxicity remains unclear as well as their prognostic significance despite the long-term investigations. Subacute forms of ANT cardiotoxicity are considered to be very rare and are

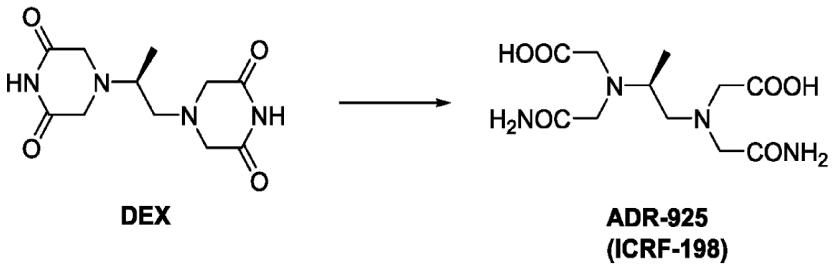
mainly associated with the syndrome of toxic myocarditis and/or pericarditis (Gharib and Burnett, Chemotherapy-induced cardiotoxicity: current practice and prospects of prophylaxis. *European journal of heart failure* **2002,** *4*, 235-242; Allen, The cardiotoxicity of chemotherapeutic drugs. *Seminars in oncology* **1992,** *19*, 529; Kantrowitz and Bristow, Cardiotoxicity of antitumor agents. *Progress in cardiovascular diseases* **1984,** *27*, 195-200). Histopathological findings are considerably different from chronic cardiotoxicity with typical presence of an inflammatory infiltrate (Kantrowitz and Bristow, Cardiotoxicity of antitumor agents. *Progress in cardiovascular diseases* **1984,** *27*, 195-200). Clinically most significant, and therefore also most dreaded, are chronic forms of cardiotoxicity that develop after the administration of multiple chemotherapeutic cycles comprising ANTs (i.e. after repeated exposure of myocardium to ANTs), and the risk primarily depends on the total cumulative dose of ANTs (Gharib and Burnett, Chemotherapy-induced cardiotoxicity: current practice and prospects of prophylaxis. *European journal of heart failure* **2002,** *4*, 235-242; Allen, The cardiotoxicity of chemotherapeutic drugs. *Seminars in oncology* **1992,** *19*, 529; Kantrowitz and Bristow, Cardiotoxicity of antitumor agents. *Progress in cardiovascular diseases* **1984,** *27*, 195-200). Chronic forms of cardiotoxicity are associated with the development of cardiomyopathy (most frequently of the dilatation type) and progressive heart failure. The chronic cardiotoxicity is clinically manifested in the course of months or years after completion of a therapy comprising ANTs (i.e. with an early or delayed onset of toxicity, respectively) (Bloom *et al.*, Cancer Therapy–Related Cardiac Dysfunction and Heart Failure: Part 1: Definitions, Pathophysiology, Risk Factors, and Imaging. *Circulation: Heart Failure* **2016,** *9*, e002661). Chronic cardiotoxicity is histopathologically characterized as a loss of myofibrils and vacuolar degeneration of cardiomyocytes (Kantrowitz and Bristow, Cardiotoxicity of antitumor agents. *Progress in cardiovascular diseases* **1984,** *27*, 195-200). Severely damaged cardiomyocytes are subsequently subject to unprogrammed and programmed cell death. Chronic ANT cardiotoxicity is considered to be largely irreversible and difficult to treat, which can principally affect morbidity, mortality and quality of life of cancer survivors who received ANTs as a part of cancer therapy. Thus, it is essential to prevent the onset of damage to the myocardium. This requirement may be particularly urgent in patients with pre-existent major morphological or functional damage to the myocardium (Zamorano *et al.*, 2016 ESC Position Paper on cancer treatments and cardiovascular toxicity developed under the auspices of the ESC Committee for Practice Guidelines: The Task Force

for cancer treatments and cardiovascular toxicity of the European Society of Cardiology (ESC). *European Heart Journal* **2016,** *37*, 2768-2801).

Effective protection of the myocardium from chronic cumulative ANT cardiotoxicity is hampered by insufficient understanding of its pathophysiological mechanisms. A traditional hypothesis suggests involvement of direct oxidative damage to the myocardium caused by redox active ANTs and their complexes with iron ions (Keizer *et al.*, Doxorubicin (adriamycin): a critical review of free radical-dependent mechanisms of cytotoxicity. *Pharmacology & therapeutics* **1990,** *47*, 219-231; Hasinoff *et al.*, Chemical, biological and clinical aspects of dexrazoxane and other bisdioxopiperazines. *Current medicinal chemistry* **1998,** *5*, 1; Šimůnek *et al.*, Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron. *Pharmacological reports* **2009,** *61*, 154-171). More recent theories point to the key importance of interaction of ANTs with topoisomerase IIβ (Top2b) in the heart with subsequent DNA damage and number of other events that lead to a toxic damage to the myocardium (Lyu *et al.*, Topoisomerase IIβ–mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane. *Cancer research* **2007,** *67*, 8839-8846; Zhang *et al.*, Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nature medicine* **2012,** *18*, 1639). Besides, there are numerous other theories that can replace or supplement the above-mentioned mechanisms. Some experts ascribe essential role to retention and specific toxic action of reduced metabolites of ANTs (e.g. doxorubicinol) in the myocardium (source: Menna *et al.*, Anthracycline cardiotoxicity. *Expert opinion on drug safety* **2012,** *11*, S21-S36). Other theories assume essential importance of i) induction of accumulation of iron in mitochondria (Ichikawa *et al.*, Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *The Journal of clinical investigation* **2014,** *124*, 617-630), ii) impairment of expression of sarcomeric proteins (Boucek Jr *et al.*, Persistent effects of doxorubicin on cardiac gene expression. *Journal of molecular and cellular cardiology* **1999,** *31*, 1435-1446), iii) induction of damage to mitochondrial DNA (Lebrecht *et al.*, Time-dependent and tissue-specific accumulation of mtDNA and respiratory chain defects in chronic doxorubicin cardiomyopathy. *Circulation* **2003,** *108*, 2423-2429), iv) a toxicity towards cardiac progenitor cells (Angelis *et al.*, Anthracycline Cardiomyopathy Is Mediated by Depletion of the Cardiac Stem Cell Pool and Is Rescued by Restoration of Progenitor Cell Function. *Circulation* **2010,** *121*, 276-292), or v) pathological signaling from TLR 9 mediated by phosphoinositide 3-kinase γ (Li *et al.*, Phosphoinositide 3-Kinase Gamma Inhibition

Protects From Anthracycline Cardiotoxicity and Reduces Tumor Growth. *Circulation* **2018,** *138*, 696-711). However, universal validity of any of these theories has not been proved so far.

Dexrazoxane (DEX, dextrorotatory enantiomer (+)-(*S*)-4,4'-(propane-1,2-diyl)bis(piperazine-2,6-dione, Scheme 1) is historically the first and only drug that has been approved as a cardioprotective drug for clinical use in prevention of chronic cumulative cardiotoxicity caused by ANT therapy (Reichardt *et al.*, Risk–benefit of dexrazoxane for preventing anthracycline-related cardiotoxicity: re-evaluating the European labeling. *Future Oncology* **2018,** *14*, 2663-2676). Its efficiency has been repeatedly proved in experimental models (with the use of various laboratory animals and also with various ANTs) (Herman *et al.*, A review of the preclinical development of dexrazoxane. *Progress in Pediatric Cardiology* **2014,** *36*, 33-38; Jirkovský *et al.*, Early and delayed cardioprotective intervention with dexrazoxane each show different potential for prevention of chronic anthracycline cardiotoxicity in rabbits. *Toxicology* **2013,** *311*, 191-204) and especially in randomized controlled clinical studies with more than 1000 patients (van Dalen *et al.*, Cardioprotective interventions for cancer patients receiving anthracyclines. *Cochrane Database of Systematic Reviews* **2011**). Though the cardioprotective effect of DEX has been proved beyond all doubt, its clinical use is limited - especially due to concerns about possible negative impact on outcomes of ANT anticancer therapy (Swain *et al.*, Cardioprotection with dexrazoxane for doxorubicin-containing therapy in advanced breast cancer. *Journal of Clinical Oncology* **1997,** *15*, 1318-1332) or about possible increased incidence of secondary malignancies (Tebbi *et al.*, Dexrazoxane-associated risk for acute myeloid leukemia/myelodysplastic syndrome and other secondary malignancies in pediatric Hodgkin's disease. *Journal of Clinical Oncology* **2007,** *25*, 493-500). Further studies largely disproved these concerns (Reichardt *et al.*, Risk–benefit of dexrazoxane for preventing anthracycline-related cardiotoxicity: re-evaluating the European labeling. *Future Oncology* **2018,** *14*, 2663-2676; van Dalen *et al.*, Cardioprotective interventions for cancer patients receiving anthracyclines. *Cochrane Database of Systematic Reviews* **2011**; Kim *et al.*, Risk factor analysis for secondary malignancy in dexrazoxane-treated pediatric cancer patients. *Cancer research and treatment: official journal of Korean Cancer Association* **2019,** *51*, 357), but recommendations for clinical use of DEX have been modified only partially (Reichardt *et al.*, Risk–benefit of dexrazoxane for preventing anthracycline-related cardiotoxicity: re-evaluating the European labeling. *Future Oncology* **2018,** *14*, 2663-2676).



DEX **ADR-925 (ICRF-198)**

Scheme 1. Formulae of DEX and its metabolite / decomposition product ADR-925

DEX has been traditionally considered to be a prodrug of the chelating substance named as ADR-925 (or ICRF-198, Scheme 1). Clinical use of the dextrorotatory enantiomer is not based on different pharmacodynamics of the optic isomers, but on significantly higher solubility of dextrorotatory enantiomer in an aqueous environment, which turned out to be the essential pharmaceutical limitation of the use of the racemic razoxane. Thus, the cardioprotective effect of DEX should be related to chelation of iron ions in the myocardium by its metabolite ADR-925, which should prevent oxidative damage to the myocardium induced by ANTs (Šimůnek *et al.*, Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron. *Pharmacological reports* **2009,** *61*, 154-171; Hasinoff, The interaction of the cardioprotective agent ICRF-187 ((+)-1, 2-bis (3, 5-dioxopiperazinyl-1-yl) propane); its hydrolysis product (ICRF-198); and other chelating agents with the Fe (III) and Cu (II) complexes of adriamycin. *Agents and actions* **1989,** *26*, 378-385). A valid Summary of product characteristics of Cardioxane 500 mg powder for solution for infusion says: *"The exact mechanism by which dexrazoxane exerts its cardioprotective effect has not been fully elucidated, however based on the available evidence the following mechanism has been suggested. The dose-dependent cardiotoxicity observed during anthracycline administration is due to anthracycline-induced iron-dependent free radical oxidative stress on the relatively unprotected cardiac muscle. Dexrazoxane, an analogue of EDTA (ethylene diamine tetra-acetic acid), is hydrolyzed in cardiac cells to the ring-opened product ICRF-198. Both dexrazoxane (ICRF-187) and ICRF-198 are capable of chelating metal ions. It is generally thought that they can provide cardioprotection by scavenging metal ions thus preventing the Fe^{3+}-anthracycline complex from redox cycling and forming reactive radicals."*

However, this theory has been questioned several times. Various antioxidants and free radical scavengers did exhibit certain protection against ANT toxicity in *in vitro* models and against acute or subacute toxicity of ANTs *in vivo*, but none of them was effective in protecting the myocardium from chronic cumulative ANT cardiotoxicity, which represents, as mentioned

above, an essential issue in the clinical practice. In experimental animal models, this type of cardiotoxicity is developed after repeated administration of clinically relevant doses of ANTs in the course of 1-3 months. A few antioxidative substances were studies in small clinical trials, and all of them with negative results (Štěrba *et al.*, Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. *Antioxidants & redox signaling* **2013,** *18*, 899-929). Similar results were obtained with more selective and more effective intracellular iron chelators than ADR-925, which, in addition, form redox inactive complexes with iron ions (unlike ADR-925). E.g. deferoxamine (DFO) protected the myocardium from acute toxicity induced by a high ANT dose in rats (Al-Harbi *et al.*, Prevention of doxorubicin-induced myocardial and haematological toxicities in rats by the iron chelator desferrioxamine. *Cancer chemotherapy and pharmacology* **1992,** *31*, 200-204), but did not show any effect against chronic ANT toxicity (Herman *et al.*, Comparison of the protective effects of desferrioxamine and ICRF-187 against doxorubicin-induced toxicity in spontaneously hypertensive rats. *Cancer chemotherapy and pharmacology* **1994,** *35*, 93-100). This finding contrasted with the robust cardioprotective efficiency of DEX in the same model. Although limited penetration of this hydrophilic chelating drug into cardiomyocytes can be used as an argument for failure of DFO as cardioprotectant, similar results were also obtained with a lipophilic selective chelator deferiprone, which easily penetrates directly into cardiomyocytes. Deferiprone has showed some protective effect *in vitro* in isolated cardiomyocytes (Barnabé *et al.*, Deferiprone protects against doxorubicin-induced myocyte cytotoxicity. *Free Radic. Biol. Med.* **2002,** *33*, 266-275), and *in vivo* in a model of acute cardiotoxicity induced by a high ANT dose in rats (Ammar *et al.*, Amelioration of doxorubicin-induced cardiotoxicity by deferiprone in rats. *Canadian Journal of Physiology and Pharmacology* **2011,** *89*, 269-276). However, when assessed in a model of chronic cumulative cardiotoxicity in rabbits, deferiprone did not show any protective effect (Popelová *et al.*, Deferiprone does not protect against chronic anthracycline cardiotoxicity in vivo. *J. Pharmacol. Exp. Ther.* **2008,** *326*, 259-269), which was again in contrast to the robust cardioprotective effect of DEX in the same model (Popelová *et al.*, Dexrazoxane-afforded protection against chronic anthracycline cardiotoxicity in vivo: effective rescue of cardiomyocytes from apoptotic cell death. *British journal of cancer* **2009,** *101*, 792). These results indicate the impossibility of reliable prediction of the cardioprotective effects of a drug against chronic cumulative ANT cardiotoxicity based solely on their chelating or antioxidative properties. Furthermore, relatively simple and rapid assessment of drug effects in *in vitro* or *in*

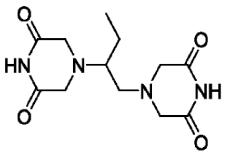
vivo on models of acute or subacute ANT toxicity is evidently insufficient. Limitations of *in vitro* experiments with respect to reliable prediction of the cardioprotective effect in clinical practice have also been discussed elsewhere (Lipshultz *et al.*, The relevance of information generated by in vitro experimental models to clinical doxorubicin cardiotoxicity. *Leukemia & lymphoma* **2006,** *47*, 1454-1458).

Recent studies ascribe the cardioprotective effect of DEX to its interaction with Top2b (Lyu *et al.*, Topoisomerase IIβ–mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane. *Cancer research* **2007,** *67*, 8839-8846; Lenčová-Popelová *et al.*, Cardioprotective effects of inorganic nitrate/nitrite in chronic anthracycline cardiotoxicity: comparison with dexrazoxane. *Journal of molecular and cellular cardiology* **2016,** *91*, 92-103). This hypothesis is in line with the results of experiments with conditional knockout of Top2b in the myocardium (Zhang *et al.*, Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nature medicine* **2012,** *18*, 1639; Vejpongsa and Yeh, Topoisomerase 2β: A Promising Molecular Target for Primary Prevention of Anthracycline-Induced Cardiotoxicity. *Clinical Pharmacology & Therapeutics* **2014,** *95*, 45-52). **However, no causal relationship between the ability to inhibit or deplete (i.e. to reduce the quantity) of Top2b in the myocardium and cardioprotection against chronic ANT cardiotoxicity has been reliably proved yet.** In addition, it is not clear whether both inhibition and depletion of Top2b in the myocardium would be necessary for cardioprotection. It should also be noted that various substances can inhibit Top2b in the myocardium by various mechanisms and it is not clear whether the way of inhibition is also important for the protective effects of DEX against chronic ANT cardiotoxicity. Moreover, there are also other theories explaining the cardioprotective effect of DEX. E.g. McCormack in his recent work (McCormack, The cardioprotective effect of dexrazoxane (Cardioxane) is consistent with sequestration of poly (ADP-ribose) by self-assembly and not depletion of topoisomerase 2B. *ecancermedicalscience* **2018,** *12*) completely refuted the importance of interaction of DEX with Top2b for achievement of cardioprotective effects and suggested instead an alternative hypothesis emphasizing the role of inhibition of poly(ADP-ribose) (PAR). Some authors emphasized the importance of direct antioxidative effects of the parent DEX (Junjing *et al.*, Scavenging effects of dexrazoxane on free radicals. *Journal of clinical biochemistry and nutrition* **2010,** *47*, 238-245) whereas others highlighted the role of protection of mitochondria from ANT-induced damage to mitochondrial DNA (Lebrecht *et al.*, Time-dependent and tissue-specific accumulation of mtDNA and respiratory chain defects in

chronic doxorubicin cardiomyopathy. *Circulation* **2003,** *108*, 2423-2429), or from overloading of mitochondria by iron (Ichikawa *et al.*, Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *The Journal of clinical investigation* **2014,** *124*, 617-630). **Thus, based on current state of knowledge, it is not possible to definitely claim that a compound inhibiting Top2b will be certainly effective against chronic ANT cardiotoxicity *in vivo*.**

Besides the above-mentioned chelating properties, DEX is, similarly to ANT, an inhibitor of topoisomerase IIα (Top2a), which also explains its certain anticancer a potential. Actually, bisdioxopiperazine compounds, where DEX belongs, were originally studied and developed as anticancer agents and their structure-activity relationships with respect to anticancer effect have been well studied and described (Creighton *et al.*, Antitumour activity in a series of bisdiketopiperazines. *Nature* **1969,** *222*, 384; Hasinoff *et al.*, A QSAR study comparing the cytotoxicity and DNA topoisomerase II inhibitory effects of bisdioxopiperazine analogs of ICRF-187 (dexrazoxane). *Biochemical pharmacology* **1995,** *50*, 953-958; Hellmann and Rhomberg, Razoxane and dexrazoxane-two multifunctional agents – Experimental and clinical results. **2010**). During these studies, the cardioprotective effect of DEX was discovered as a byproduct (Hasinoff *et al.*, Chemical, biological and clinical aspects of dexrazoxane and other bisdioxopiperazines. *Current medicinal chemistry* **1998,** *5*, 1; Patterson and Willis, Translational Cardiology: Molecular Basis of Cardiac Metabolism, Cardiac Remodeling, Translational Therapies and Imaging Techniques. **2012**). However, the structure-activity relationships of bisdioxopiperazines with respect to their cardioprotective effects against chronic ANT cardiotoxicity has not been clearly defined so far. It is mainly due to the unclear molecular mechanism of chronic ANT cardiotoxicity, and correspondingly also uncertain molecular target for the cardioprotective effect. However, even more important factor is a very limited number of experimental studies focusing on this matter with a **clear prevalence of negative results and absence of the cardioprotective effects of DEX derivatives** (Herman *et al.*, Comparison of the protective effects against chronic doxorubicin cardiotoxicity and the rates of iron (III) displacement reactions of ICRF-187 and other bisdiketopiperazines. *Cancer chemotherapy and pharmacology* **1997,** *40*, 400-408; Martin *et al.*, Evaluation of the topoisomerase II-inactive bisdioxopiperazine ICRF-161 as a protectant against doxorubicin-induced cardiomyopathy. *Toxicology* **2009,** *255*, 72-79; Jirkovská-Vávrová *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. *Toxicology Research* **2015,**

4, 1098-1114; Bures *et al.*, Investigation of novel dexrazoxane analogue JR-311 shows significant cardioprotective effects through topoisomerase IIbeta but not its iron chelating metabolite. *Toxicology* **2017,** *392*, 1-10). **Even slight modifications of the structure of DEX (or its racemate razoxane) led to the loss of the cardioprotective effect against chronic ANT cardiotoxicity** – e.g. substitution of methyl for ethyl in the aliphatic linker in the compound ICRF-192 (Herman *et al.*, Comparison of the protective effects against chronic doxorubicin cardiotoxicity and the rates of iron (III) displacement reactions of ICRF-187 and other bisdiketopiperazines. *Cancer chemotherapy and pharmacology* **1997,** *40*, 400-408), extension the linker with one methylene in ICRF-161, or substitution of one oxo-group in each of the cycles by hydrogen in MK-15 (Jirkovská-Vávrová *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. *Toxicology Research* **2015,** *4*, 1098-1114). The methylation of imide nitrogens of bisdioxopiperazines in ICRF-239 did partly reduce the score of chronic damage to the myocardium as compared to ANT alone, but at the same time it reduced the overall survival of the animals, which complicates the interpretation of this finding.



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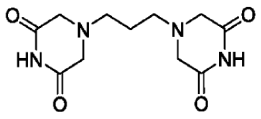

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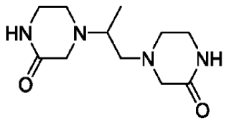

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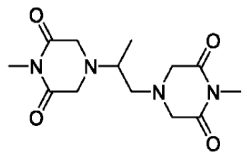
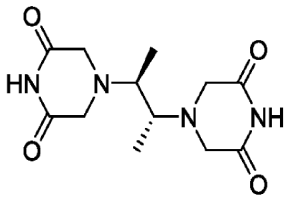


ICRF-239

These findings show that structurally similar compounds with bisdioxopiperazine structure may exhibit significantly different protective efficiency against chronic ANT cardiotoxicity. Thus, the bisdioxopiperazine structure is clearly not a guarantee of a clinically usable cardioprotective effect by itself. In addition, a study of Martin *et al.* (Evaluation of the topoisomerase II-inactive bisdioxopiperazine ICRF-161 as a protectant against doxorubicin-induced cardiomyopathy. *Toxicology* **2009,** *255*, 72-79) showed than an assessment of the protective potential of bisdioxopiperazine derivatives closely related to DEX in an *in vitro* model of isolated cardiomyocytes may exhibit false positive results, which were in sharp contrast to results obtained with the use of *in vivo* models of chronic ANT cardiotoxicity validated with DEX as a positive control. Furthermore, cardioprotective potential of DEX was not found in *in vitro* experiments with the use of human induced pluripotent stem cells (iPSC) cardiomyocytes (Burridge *et al.*, Human induced pluripotent stem cell–derived cardiomyocytes recapitulate the predilection of breast cancer patients to doxorubicin-induced

cardiotoxicity. *Nature medicine* **2016,** *22*, 547) However, in this cell model protective potential was observed in *N*-acetylcystein, whose cardioprotective inactivity in comparison with DEX have been proved in a randomized clinical study in 1983 (Myers *et al.*, A randomized controlled trial assessing the prevention of doxorubicin cardiomyopathy by N-acetylcysteine. *Seminars in oncology* **1983,** *10*, 53-55) and also in a chronic dog model (Herman *et al.*, Comparison of the effectiveness of (±)-1, 2-bis (3, 5-dioxopiperazinyl-1-yl) propane (ICRF-187) and N-acetylcysteine in preventing chronic doxorubicin cardiotoxicity in beagles. *Cancer research* **1985,** *45*, 276-281). Thus, only data obtained in *in vivo* models of chronic ANT cardiotoxicity validated with the use of clinically used DEX have principal conclusive importance for the evaluation of the cardioprotective potential of bisdioxopiperazine derivatives closely related to DEX. However, majority of DEX derivatives, including ICRF-193, are difficult to assess in these models due to their very low solubility in water - intravenous administration is frequently impossible and their absorption after intraperitoneal administration is uncertain.

One of the previously prepared and studied DEX analogue is ICRF-193 (optically inactive *meso*-4,4'-(butan-2,3-diyl)bis(piperazine-2,6-dione). This compound was studied with regard to the inhibitory activity against Top2a and became a lead compound for the preparation of further analogues with a potential cytostatic effect. The compound ICRF-193 and its analogues are included and described in several publications and patents, where the anticancer effect of ICRF-193 is described and claimed (e.g. A.M. Creighton *et al.* Nature, **1969**, *222*, 384-385; US3941790A, 1976; Kuan-Chun Huang, The Journal Of Biological Chemistry, **2001**, *276*, 44488-44494; N. Hajji, Mutation Research **2003**, *530*, 35–46).

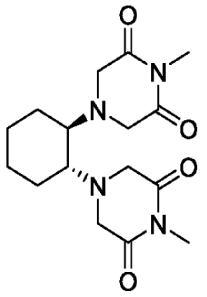


ICRF-193

ICRF-193 was screened for protective effects in an *in vivo* model of general acute toxicity induced by the administration of a high single dose of ANT (DAU, 25 mg/kg) (Herman *et al.*, Comparison of the protective effect of ICRF-187 and structurally related analogues against acute daunorubicin toxicity in Syrian golden hamsters. *Research communications in chemical pathology and pharmacology* **1985,** *48*, 39-55). The authors concluded that unlike DEX,

ICRF-193 does not have any protective effects against this massive multi-organ toxicity of ANT („ *no protective activity was found in any pretreatment doses of ICRF-158 /analogue 2/ ICRF-193 /analogue 8/....* "). However, no parameter that might directly or indirectly indicate the potential cardioprotective effect was described in this study.

The compound ICRF-193 falls within the general formula of the patent GB 2245832A 1992 (WO9200740), which protects roughly tens of thousands of compounds derived from bisdioxopiperazines. This patent generally claimed the ability of these compounds to protect against ANT cardiotoxicity. However, based on more recent data, it is obvious that the bisdioxopiperazine structure by itself is not a carrier of a clinically usable cardioprotective effect. In addition, the structure of ICRF-193 is not explicitly mentioned in this patent as an example and is not described there at all. Conversely, the authors cite the above-mentioned publication (Herman *et al.*, Comparison of the protective effect of ICRF-187 and structurally related analogues against acute daunorubicin toxicity in Syrian golden hamsters. *Research communications in chemical pathology and pharmacology* **1985,** *48*, 39-55) in the introduction and remark that unlike DEX, the compound with the formula of ICRF-193 did not show any protective effect against toxicity induced by a single dose of doxorubicin (page 2), which means that they expressed their negative opinion concerning the protective potential of this compound. The only example mentioned in the patent GB 2245832A 1992 is a completely different compound - *trans*-1,2-bis(4-methyl-3,5-dioxopiperazin-1-yl)cyclohexane, which was evaluated in a model of cardiotoxicity induced by a single dose of doxorubicin in rats. The key evaluated parameter was cardiac output, which was reduced by doxorubicin. **The evaluated bisdioxopiperazine derivative had very low (statistically insignificant) protective effect on the decrease of this parameter after single dose of ANT** (decrease by 59% after doxorubicin alone vs. decrease by 47% after a combination with this bisdioxopiperazine derivative) despite the employment of a relatively high dose of the bisdioxopiperazine derivative (100 mg/kg, *i.p.*). DEX/razoxane was not evaluated in this experiment and there are no reference data validating the use of this unusual experimental model. Moreover, the authors themselves indicate that there were no signs of a congestive heart failure in this model. Thus, no conclusion could be drawn from this example concerning the key ability of the potential cardioprotective agent to prevent the development of an ANT induced heart failure.



trans-1,2-bis(4-methyl-3,5-dioxopiperazin-1-yl)cyclohexane

Thus, from this example even an expert cannot come to the conclusion that the compound ICRF-193 would have significant cardioprotective effect against chronic cumulative cardiotoxicity induced by repeated ANT administration leading to cardiomyopathy and congestive heart failure, which are principal problems in the clinical practice. The protective effect against this type of cardiotoxicity was not evaluated in this patent, the compound ICRF-193 is not mentioned as an example in the patent and with respect to negative results of close DEX derivatives, even an expert could not predict the cardioprotective potential of compounds of the general formula in this patent against chronic cumulative ANT cardiotoxicity. The example presented in the patent concerns toxicity of single dose ANT administration, which is rarely an issue in the clinical practice, and in addition, the observed effect was very weak.

The substance ICRF-193 also falls within the general formula of the patent application US2008/0275036 A1. This application hypothetically protects thousands of compounds and one of the areas that is claimed is their protective ability against cardiotoxicity of anti-tumor drugs, including ANTs. The formula of the compounds ICRF-193 is presented in table 1 on page 30 of the said document, but its characteristic or any pharmacological effect is not explicitly mentioned or described. In addition, the authors assumed that the cardioprotective effects of DEX stems from the chelating properties of its metabolite and that it will be the case of the other bisdioxopiperazine derivatives. More recent data show that this assumption is not valid (Štěrba *et al.*, Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. *Antioxidants & redox signaling* **2013,** *18*, 899-929), which also questions the presented inventive step and validity of claiming the cardioprotective effect of bisdioxopiperazine derivatives. The provided evidence always concerned only DEX and did not have any relationship to cumulative chronic ANT cardiotoxicity leading to cardiomyopathy and a heart failure. Only acute effects on electrophysiological parameters were evaluated, including those that were pathologically

modified by supratherapeutic ANT concentrations, in various experimental models (an acute *in vivo* experiment, an experiment with isolated heart perfused according to Langendorff and with isolated cardiomyocytes *in vitro*). Even an expert cannot infer any conclusion about cardioprotective potential of ICRF-193 against cumulative chronic ANT cardiotoxicity from these data because this effect was not evaluated here and no data concerning this compound are mentioned.

The compound ICRF-193 is presented in the patent application WO2016/051213 A1 on page 4 and in figure 1 (Fig. 1). The entire application is focused on the ability of DEX, its metabolites B and C and DEX derivatives to inhibit formation of poly(ADP-ribose) (PAR) and related potential clinical application of these substances. One of the claimed potential applications of these compounds is protection against ANT toxicity. In this patent application, the authors completely rejected the importance of Top2b for the cardioprotective effect of DEX and suggested the interaction with PAR as the main mechanism of the cardioprotection. Although ICRF-193 is mentioned in the patent, no experimental data are provided for this compound as an example (the same is true for other DEX derivatives). Only data presented therein concerned DEX and its metabolites B and C , and were generated by *in silico* modelling of interaction of various substances with PAR and inhibition of AIF (Apoptosis Inducing Factor) release from isolated liver mitochondria induced by PAR. The entire patent application does not provide any direct evidence concerning ANT cardiotoxicity or DEX cardioprotection (no data concerning the effects of ANTs on the heart, cardiomyocytes or their organelles are presented here). The presented inventive step and the related attempt to claim the cardioprotective effect of DEX derivatives were based on speculative basis only. The above-presented patent application thus does not provide any evidence for the efficacy of ICRF-193.

The compound ICRF-193 is also disclosed in patent application WO2008/157358 A1, which protects methods of treating cancer with a combination of a Top2 inhibitor and Top2 (topoisomerase) poison, including ANT. As Top2 inhibitors, the most well-known representatives of bisdioxopiperazines ICRF-187 (DEX), ICRF-193, ICRF-159 (razoxane) and ICRF-154 are disclosed, together with their unspecified prodrugs and metabolites. The use of these compounds (including ICRF-193) as cardioprotective agents is mentioned in the text, but not explicitly claimed. The claims only generally stated the use of this combination of drugs but without description of a specific medical intention or purpose (i.e. without particular indication). The claim was exemplified by the *in vitro* reduction of doxorubicin-induced DNA

damage detected by the γH2AX marker in proliferating H9c2 cells by DEX and ICRF-193, which is associated with the inhibition and depletion of Top2b. However, as mentioned above, no direct evidence has been provided that pharmacological protection of cardiomyocytes against DNA damage detected as γH2AX is the sole or main prerequisite for effective protection of primary cardiomyocytes *in vitro* and particularly the heart against chronic cumulative cardiotoxicity caused by ANT therapy *in vivo*. The example performed on proliferating H9c2 cell line is also of unclear inventive value due to the fact that the cell line used, unlike cardiomyocytes, also expresses the Top2α isoform, which is indeed also significantly affected by studied substances (ANT and DEX/ICRF-193). Moreover, the molecular phenotype of these cells as well as the effects of doxorubicin on them are fundamentally different from primary cardiomyocytes (Lenčo J. *et al.* Proteomic investigation of embryonic rat heart-derived H9c2 cell line sheds new light on the molecular phenotype of the popular cell model. *Exp Cell Res*. **2015**; *339*: 174-186). In this patent, no evidence is provided for the ability of ICRF-193 or its prodrug or metabolite to protect either primary cardiomyocytes *in vitro* or the myocardium from chronic ANT cardiotoxicity *in vivo*.

The recent data from scientific journals and from the patent literature can be summarized as follows. Cardioprotective effects of ICRF-193 against chronic ANT cardiotoxicity have not been directly experimentally evaluated. There is only a negative finding from a study of overall acute toxicity induced by a high ANT dose, where the results of ICRF-193 were substantially different from those of DEX. A structure of ICRF-193 is included in patents protecting an anti-tumor effects of compounds and it is also one of the thousands of substances that can be generated from general formulae of two patents/patent applications (GB 2245832A 1992, US2008/0275036 A1), claiming the cardioprotective effects against ANT toxicity. But ICRF-193 and its potential protective effects against cumulative chronic ANT cardiotoxicity associated with cardiomyopathy and a heart failure is not mentioned as an example in a single case. More recent experimental data show that the cardioprotective effect is not related to chelation and antioxidative properties of metabolites of bixdioxopiperazine derivatives and that even a very small change of the structure of the parent compound DEX may lead to the loss of the effects against chronic ANT cardiotoxicity. Therefore, the inventive step and the related claims presented above with a very general formulae cannot be considered valid without further experimental data concerning a particular compound. There is the patent application WO2016/051213 A1, which mentions the compound ICRF-193 (together with a number of other DEX derivatives) as a potential cardioprotective agent against ANT

cardiotoxicity, and its cardioprotective effect against ANT toxicity is claimed herein. In fact, no protective effect of ICRF-193 against any type of ANT toxicity was demonstrated there. Therefore, this inventive step cannot be considered valid in association with chronic ANT cardiotoxicity either. Finally, patent application WO2008/157358 A1 claims the administration of ICRF-193 in combination with Top2 poison in the therapy of cancer, where possible cardioprotective efficacy is derived exclusively from the prevention of ANT-induced DNA damage on the H9c2 cell line *in vitro*. The inventive value of this work with respect to the prevention of chronic ANT cardiotoxicity is uncertain, as the cell model did not employ cardiomyocytes but a proliferating cell line of markedly different phenotype, and any association between evaluated *in vitro* protection against ANT-induced DNA damage and real protection against clinically relevant forms of ANT cardiotoxicity in *in vivo* has not been demonstrated so far. Even in this patent application, the protective effect of ICRF-193 against chronic cumulative cardiotoxicity of ANT is not demonstrated as an example.

Disclosure of the Invention

Firstly, a study of cytoprotective effects of DEX and a library of bisdioxopiperazines against clinically relevant concentrations of ANT was carried out *in vitro*. The results of this study showed the ability of compound ICRF-193 to protect primary cardiomyocytes against ANT toxicity *in vitro* at significantly lower concentrations than the parent drug DEX. As mentioned in the background art section, this *in vitro* test alone cannot reliably predict the cardioprotective potential against chronic cumulative ANT cardiotoxicity *in vivo*. However, a conclusive *in vivo* study with ICRF-193 could not be performed because this substance is practically insoluble in water, which hinders both its intravenous (*i.v.*) administration in to the organism in the required doses and effective absorption in case of different other routes of administration like oral (*p.o.*), intraperitoneal (*i.p.*) etc. This limitation may have influenced the results of evaluation of this substance against the general acute toxicity of DAU in the earlier study (Herman *et al.*, Comparison of the protective effect of ICRF-187 and structurally related analogues against acute daunorubicin toxicity in Syrian golden hamsters. *Research communications in chemical pathology and pharmacology* **1985,** *48*, 39-55).

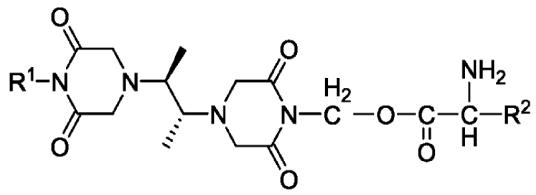
For this reason, derivatives of ICRF-193 of general formula I with higher solubility in water were prepared. These derivatives had been already described in the patent US 5393889A

(PCT/JP91/00894), claiming their use as anti-cancer drugs. The possible effects of compounds of general formula I against any form of ANT cardiotoxicity has not been described or mentioned in the literature yet.

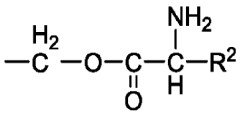
Disclosure of the invention is based on the discovery and unequivocal confirmation of the fact that the compounds of general formula I in the form of an ammonium salt with a pharmaceutically acceptable acid are able to protect primary cardiomyocytes against ANT toxicity *in vitro*, and, above all, to show excellent efficacy as cardioprotectants at very low doses against chronic cumulative ANT cardiotoxicity induced by repeated (10-week) administration of a clinically relevant dose of daunorubicin (once weekly i.v. at a dose of 3 mg/kg DAU) in a rabbit model.

This model shows functional, biochemical and morphological similarities to chronic ANT cardiotoxicity observed in the clinical practice. Moreover, the cardioprotective effect of DEX, the approved drug with demonstrated cardioprotective efficacy in the clinical practice, was repeatedly shown in this model (Jirkovský *et al.*, Early and delayed cardioprotective intervention with dexrazoxane each show different potential for prevention of chronic anthracycline cardiotoxicity in rabbits. *Toxicology* **2013,** *311*, 191-204; Popelová *et al.*, Dexrazoxane-afforded protection against chronic anthracycline cardiotoxicity in vivo: effective rescue of cardiomyocytes from apoptotic cell death. *British journal of cancer* **2009,** *101*, 792). Excellent cardioprotective effects of the compounds of general formula I were observed even with doses 30 times lower than the recommended doses of DEX (the only known and used drug against chronic ANT cardiotoxicity) studied under the same conditions in this model. The significant effect of the compounds of general formula I against chronic ANT cardiotoxicity was noted already at the dose of 1 mg/kg. Compared to known data from scientific literature this means that with respect to the required dose the compounds of general formula I are so far the most potent cardioprotective agents against chronic ANT cardiotoxicity.

An object of the invention is the use of the compounds of general formula I in the form of the ammonium salt with a pharmaceutically acceptable acid according to the invention for the manufacture of a medicament for the prevention of chronic cumulative cardiotoxicity (cardiomyopathy and a heart failure) caused by therapy with anthracycline anticancer drugs.



I

wherein $R^1 = H$,  ;

R^2 = H, a C_1-C_6 alkyl, benzyl or benzyl substituted at positions 2, 3, 4 and 5 with one or more electron-acceptor groups and/or one or more electron-donor groups, a phenyl or phenyl substituted at positions 2, 3, 4 and 5 with one or more electron-acceptor groups and/or one or more electron-donor groups.

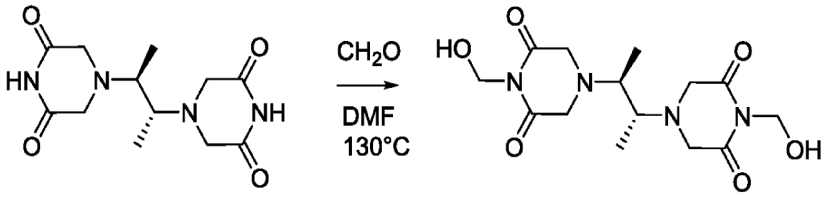
A pharmaceutically acceptable acid refers to such organic and inorganic acids whose anions do not exhibit a toxic effect on the human organism in clinically relevant concentrations. They are especially: chlorides, bromides, iodides, sulfates, nitrates, acetates, maleates, fumarates, methanesulfonates, citrates, succinates, phosphates and/or tartrates. (source: Lemke, Thomas *et al.*; (eds.).: *Foye´s principles of medicinal chemistry*. Philadelphia; Baltimore: Lippincott Williams & Wilkins, **2013**, 1500 s. ISBN 978-1-4511-7572-1.)

Electron-donor groups refer to such substituents that increase the electron density at the phenyl substituent of R^2. They are especially: $-NH_2$, -NHAlk, $-NAlk_2$, -OH, -OAlk, -OAr, $-NHCOCH_3$, -NHCOAlk; -NHCOAr; -Alk, -Ar, where Alk = Alkyl, especially having 1 – 4 carbon atoms, Ar = Aryl, where aryl = phenyl or phenyl substituted at positions 2, 3, 4 and 5 with one or more electron-acceptor groups and/or one or more electron-donor groups, naphthyl or pyridyl.

Electron-acceptor groups refer to such substituents that reduce the electron density at the phenyl substituent of R^2. They are especially: $-NO_2$, $-N^+Alk_3$, $-CF_3$, CCl_3, -CN, -COOH, -COOAlk, -COOAr, -CHO, -COAlk, -COAr, -F, -Cl, -Br, -I, where Alk = Alkyl, especially having 1 – 4 carbon atoms, Ar = Aryl, where aryl = phenyl or phenyl substituted at positions 2, 3, 4 and 5 with one or more electron-acceptor groups and/or one or more electron-

donor groups, naphthyl or pyridyl. (source: a) John McMurry: Organic Chemistry, Sixth edition, **2004**, Brooks/Cole, a Thomson Learning Company; b) L. G. Wade, Jr.: Organic Chemistry, Sixth edition, **2006**, Pearson Prentice Hall Inc.; c) J. Clayden *et al.*,: Organic Chemistry, **2001**, Oxford University Press).

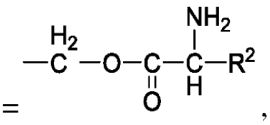
The compounds of general formula I can be obtained by routine methods of organic synthesis. For the synthesis of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) of formula II, the method described in the patent US 5393889A (PCT/JP91/00894) was used (Scheme 2).



ICRF-193 II

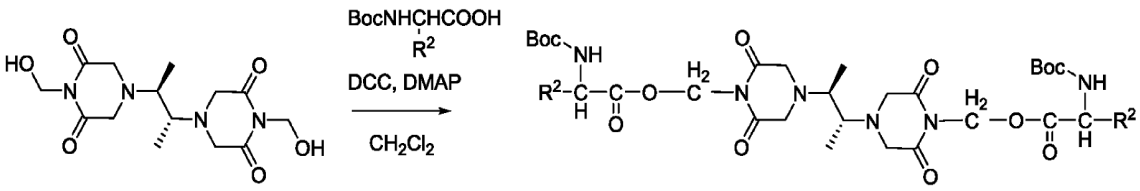
Scheme 2. Synthesis of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione of formula II (DMF = *N*,*N*-dimethylformamide)

Suitably protected precursors of the final compounds of general formula I,



where R^1 = ,

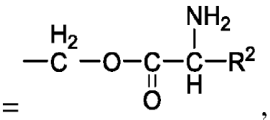
were then obtained by esterification of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl) piperazine-2,6-dione) of formula II with a suitably protected carboxylic acid with the use of dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine (Scheme 3).



II IIa

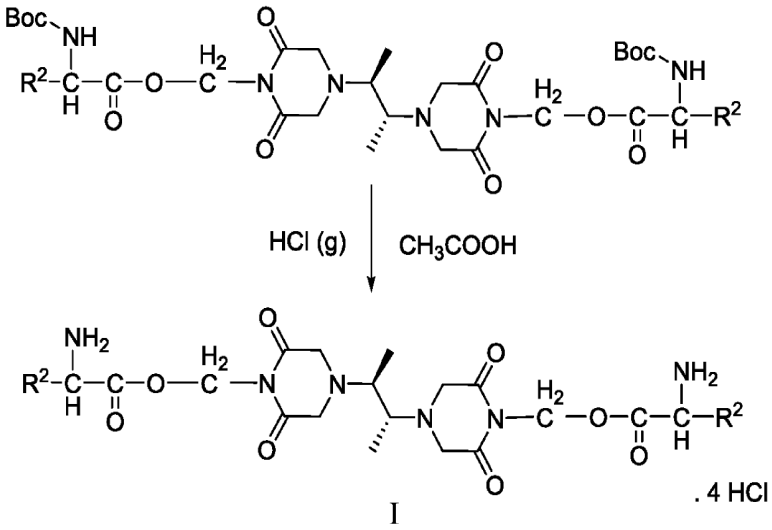
Scheme 3. Synthesis of Boc-protected precursors (of formula IIa) of the final substances of general formula I (DCC = dicyclohexylcarbodiimide; DMAP = 4-(dimethylamino)pyridine, Boc = *tert*-butyloxycarbonyl)

The final products of general formula I in the form of the ammonium salt with a pharmaceutically acceptable acid,



where R^1 = ,

were obtained from the precursors by deprotecting the Boc group with hydrogen chloride (Scheme 4).



IIa

I

Scheme 4. Preparation of the final compounds of general formula I in the form of hydrochlorides (Boc = *tert*-butyloxycarbonyl)

The simple assumptions made in the previous patent literature about the relationship of the chemical structure and cardioprotective activity of bisdioxopiperazine derivatives were refuted herein. This is demonstrated by an example of structurally very close derivatives of DEX or ICRF-193 not yet evaluated in the literature that were found completely free of any cardioprotective potential against chronic ANT cardiotoxicity *in vivo*. One of these examples is the *N,N′*-dimethyl derivative of DEX (ICRF-239, see page 8), mentioned in the background art, which, by substitution on imide nitrogen, is similar to the example in the patent GB 2245832A. The ineffectiveness of *N,N′*-dimethyl derivative of DEX (ICRF-239) against chronic ANT cardiotoxicity *in vivo* is demonstrated in example 10. In addition to what is mentioned above, only indirect further comparisons can be made. E.g. compounds of general

formula I show an order of magnitude higher cardioprotective potential than the example of another bisdioxopiperazine evaluated in the acute cardiotoxicity model using single administration of ANT in patent GB 2245832A (only marginal protective potential was observed at 100 mg/kg, page 20 of the patent).

The repeated administration of the compounds of formula I alone or in the combination with ANT was very well tolerated by animals (no signs of systemic or local toxicity at the site of administration were recorded), further supporting the potential of the compounds of general formula I as clinically appropriate drugs to prevent chronic cumulative cardiotoxicity caused by repeated administration of anthracyclines.

Brief description of the Drawings

Fig. 1. Protective effects of the compound ICRF-193 against DAU cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes.

Fig. 2. Protective effects of the compound Ib according to Example 1 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes.

Fig. 3. Protective effects of the compound Ic according to Example 2 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes.

Fig. 4. Protective effects of the compound Id according to Example 3 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes.

Fig. 5. Protective effects of the compound Ie according to Example 4 against DAU cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes.

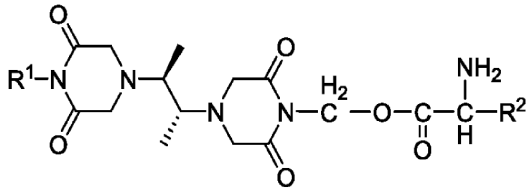
Fig. 6. Results of an evaluation of the cardioprotective effects of the compound Ib according to Example 1 in a rabbit model of chronic cumulative anthracycline cardiotoxicity.

Fig. 7. Results of an evaluation of the cardioprotective effects of the compound ICRF-239 in the rabbit model of chronic cumulative anthracycline cardiotoxicity.

Fig. 8. General formula I

Examples

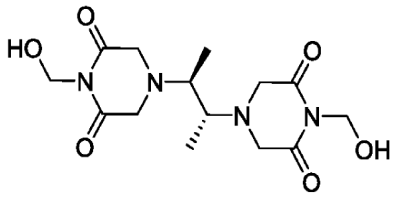
In the text below, compounds of general formula I will be introduced



I

where the symbols R^1, R^2 have the above-mentioned meaning.

Preparation of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione)

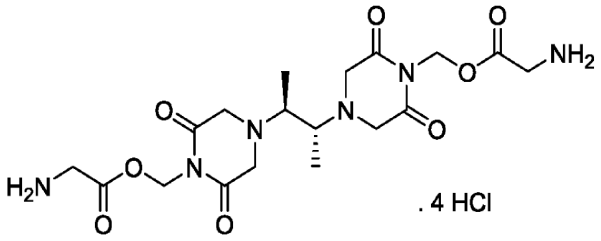


II

A suspension of the substance ICRF-193 (*meso*-4,4'-(butane-2,3-diyl)bis(piperazine-2,6-dione, 1 g, 3,54 mmol) in 5 ml of DMF was heated at 130 °C for 10 minutes. Then, an aqueous solution of formaldehyde (37%, 1.48 g, 1.43 ml, 18.23 mmol) was added to the reaction and the reaction was further heated to 130°C for 4 hours. After that, the solvent was evaporated, and 20 ml of ethanol was added. The produced precipitate was filtered and washed on the filter with 5 ml of ethanol and 10 ml of diethyl ether. Yield: 67% (0.26 g) as a white crystalline solid. Melting point: 319-321°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.17 (t, J = 7.3 Hz, 2H, O*H*), 5.01 (d, J = 6.3 Hz, 4H), 3.47 (dd, J = 16.4, 1.7 Hz, 4H), 3.36 (dd, J = 16.6, 1.8 Hz, 4H), 2.80 – 2.74 (m, 2H), 0.89 (d, J = 5.7 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.90, 61.22, 58.79, 52.53, 9.48

The substance ICRF-193 is commercially available. The substance can be prepared with the use of common synthetic procedures according to R.M. Snapka *et al.*, Biochemical Pharmacology, **1996**, 52, 543-549.

Example 1: *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-bis(2-aminoacetate) hydrochloride (**Ib**)



Ib

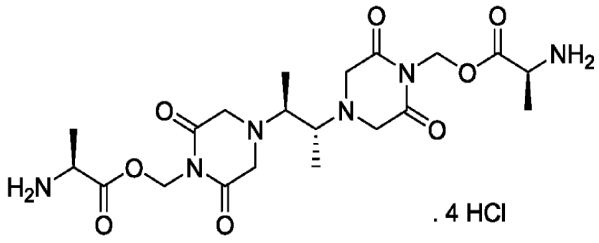
The compound *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-bis(2-aminoacetate) hydrochloride **Ib** is prepared according to Scheme 4 through a reaction of *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-bis(2-((*tert*-butoxycarbonyl)amino)acetate) (0.3 mmol) with an excess of hydrogen chloride in 5 ml of acetic acid. The reaction was stirred for 2 hours, then 50 ml of diethyl ether was added, and the resulting precipitate was filtered off and dried in a desiccator. Yield: 93% as a white crystalline solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.51 (t, J = 5.8 Hz, 6H), 5.75 (s, 4H), 3.79 (q, J = 5.7 Hz, 4H), 3.67 (d, J = 16.7 Hz, 4H), 3.59 (d, J = 16.8 Hz, 4H), 2.98 (s, 2H), 0.97 (d, J = 4.1 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.90, 166.92, 62.61, 59.17, 52.19, 9.75.

The starting *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-bis(2-((*tert*-butoxycarbonyl)amino)acetate) was prepared through a reaction of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) with Boc-glycine according to Scheme 3. A solution of dicyclohexylcarbodiimide (0.72 g, 3.45 mmol) in 30 ml of CH_2Cl_2 was added to a suspension of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) (0.6 g, 1.75 mmol), Boc-glycine (3.54 mmol) and 4-(dimethylamino)pyridine (6 mg, 0.049 mmol) in 50 ml of CH_2Cl_2 at 0°C under an argon atmosphere. The reaction was stirred for 48 hours. Then, the reaction was filtered, and the filtrate was extracted with 48 ml of a mixture of H_2O/CH_3COOH (15:1). The organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated. The product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH, 70:1). Yield: 50% as white powder; melting point 195-197 °C. ^{1}H NMR (500

MHz, DMSO-d_6) δ 7.21 (t, J = 6.2 Hz, 2H), 5.68 – 5.58 (m, 4H), 3.65 (d, J = 6.2 Hz, 4H), 3.58 – 3.51 (m, 4H), 3.48 – 3.41 (m, 4H), 2.84 – 2.76 (m, 2H), 1.37 (s, 18H), 0.90 (d, J = 5.1 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.52, 169.66, 155.94, 78.49, 62.20, 58.84, 52.29, 41.79, 28.31, 9.70.

The other substances are normally commercially available.

Example 2: *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-aminopropanoate) hydrochloride (**Ic**)



Ic

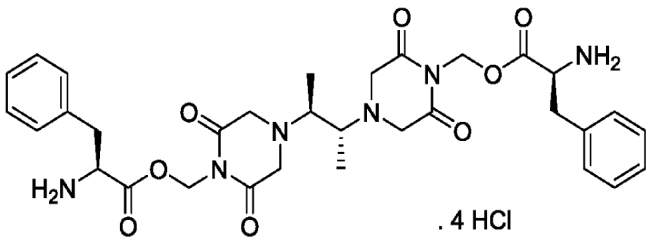
The compound *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-aminopropanoate) hydrochloride **Ic** is prepared according to Scheme 4 through a reaction of *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-((*tert*-butoxycarbonyl)amino)propanoate) (0.2 g, 0.3 mmol) with an excess of hydrogen chloride in 5 ml of acetic acid. The reaction was stirred for 2 hours, then 50 ml of diethyl ether was added, and the resulting precipitate was filtered off and dried in a desiccator. Yield: 86% as a white crystalline solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.65 (d, J = 5.5 Hz, 6H), 5.80 (dd, J = 9.8, 3.9 Hz, 2H), 5.71 (dd, J = 9.8, 4.5 Hz, 2H), 4.04 (d, J = 6.4 Hz, 2H), 3.69 (d, J = 16.9 Hz, 4H), 3.61 (d, J = 16.9 Hz, 4H), 2.99 (s, 2H), 1.36 (d, J = 7.2 Hz, 6H), 1.06 – 0.95 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.91, 169.25, 62.74, 59.24, 52.16, 47.85, 15.75, 9.18

The starting *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-bis(2-((*tert*-butoxycarbonyl)amino)propanoate) was prepared through a reaction of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) with Boc-L-alanine according to Scheme 3. A solution of dicyclohexylcarbodiimide (0.72 g, 3.45 mmol) in 30 ml of CH_2Cl_2 was added to a suspension of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) (0.6 g, 1.75 mmol), Boc-L-alanine (3.54 mmol) and 4-(dimethylamino)pyridine (6 mg, 0.049 mmol) in 50 ml of CH_2Cl_2 at 0°C under an argon atmosphere. The reaction was stirred

for 48 hours. Then, the reaction was filtered, and the filtrate was extracted with 48 ml of a mixture of H_2O/CH_3COOH (15:1). The organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated. The product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH, 30:1). Yield: 65% as a white crystalline solid. Melting point 190-192 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.29 (d, J = 7.4 Hz, 2H), 5.73 – 5.60 (m, 2H), 5.56 (dd, J = 9.5, 4.5 Hz, 2H), 3.95 (t, J = 7.3 Hz, 2H), 3.55 (d, J = 16.7 Hz, 4H), 3.45 (d, J = 16.8 Hz, 4H), 2.84 – 2.76 (m, 2H), 1.36 (s, 18H), 1.17 (d, J = 7.3 Hz, 6H), 0.90 (d, J = 4.7 Hz, 6H).

The other substances are normally commercially available.

Example 3: *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-amino-3-phenylpropanoate) hydrochloride (**Id**)



Id

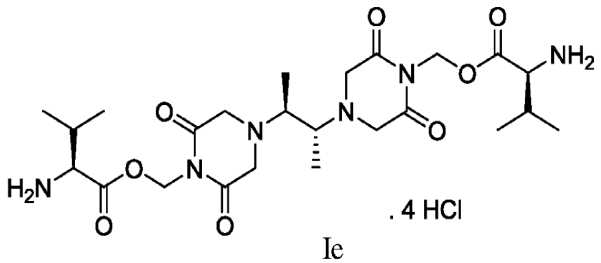
The compound *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-amino-3-phenylpropanoate) hydrochloride **Id** is prepared according to Scheme 4 through a reaction of *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate) (0.25 g, 0.3 mmol) with an excess of hydrogen chloride in 5 ml of acetic acid. The reaction was stirred for 2 hours, then 50 ml of diethyl ether was added, and the resulting precipitate was filtered off and dried in a desiccator. Yield: 86% as a white crystalline solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.76 (d, J = 5.3 Hz, 6H), 7.31 – 7.17 (m, 10H), 5.79 (dd, J = 9.8, 5.9 Hz, 2H), 5.62 (d, J = 9.8 Hz, 2H), 4.29 – 4.23 (m, 2H), 3.77 – 3.40 (m, 8H), 3.19 – 3.03 (m, 4H), 2.98 (s, 2H), 0.97 (d, J = 5.0 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.15, 169.80, 168.18, 168.15, 134.62, 134.55, 129.68, 128.73, 127.43, 62.74, 59.18, 59.11, 53.16, 53.11, 52.17, 35.73, 35.68, 33.47, 21.27, 9.73, 9.68.

The starting *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoate) was prepared through a reaction of

meso-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) with Boc-L-phenylalanine according to Scheme 3. A solution of dicyclohexylcarbodiimide (0.72 g, 3.45 mmol) in 30 ml of CH_2Cl_2 was added to a suspension of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) (0.6 g, 1.75 mmol), Boc-L-phenylalanine (3.54 mmol) and 4-(dimethylamino)pyridine (6 mg, 0.049 mmol) in 50 ml of CH_2Cl_2 at 0°C under an argon atmosphere. The reaction was stirred for 48 hours. Then, the reaction was filtered, and the filtrate was extracted with 48 ml of a mixture of H_2O/CH_3COOH (15:1). The organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated. The product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH, 100:1). Yield: 30% as a white crystalline solid. Melting point 156-158 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.34 – 7.20 (m, 6H), 7.20 – 7.12 (m, 4H), 5.82 (t, *J* = 3.2 Hz, 4H), 4.95 (d, *J* = 8.4 Hz, 2H), 4.60 (q, *J* = 6.8 Hz, 2H), 3.58 – 3.49 (m, 4H), 3.47 – 3.39 (m, 4H), 3.16 – 2.95 (m, 4H), 2.67 – 2.59 (m, 2H), 1.41 (s, 18H), 1.07 (d, *J* = 2.1 Hz, 6H).

The other substances are normally commercially available.

Example 4: *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-amino-3-methylbutanoate) hydrochloride (**Ie**)



Ie

The compound *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-amino-3-methylbutanoate) hydrochloride **Ie** is prepared according to Scheme 4 through a reaction of *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoate) (0.22 g, 0.3 mmol) with an excess of hydrogen chloride in 5 ml of acetic acid. The reaction was stirred for 2 hours, then 50 ml of diethyl ether was added, and the resulting precipitate was filtered off and dried in a desiccator. Yield: 25% as a white crystalline solid. LCMS *m/z* (ESI+) 541.15 (100, $M+H^+$), 442.04 (25%). ^{1}H NMR (500 MHz, D_2O) δ 6.00 – 5.84 (m, 2H), 5.78 – 5.70 (m, 2H), 3.95 (d, *J*

= 4.2 Hz, 2H), 3.82 – 3.60 (m, 8H), 2.96 (q, *J* = 5.7 Hz, 2H), 2.25 – 2.16 (m, 2H), 1.12 – 0.98 (m, 6H), 0.95 – 0.85 (m, 12H).

^{13}C NMR (126 MHz, D_2O) δ 176.59, 170.92, 170.89, 168.43, 63.04, 60.28, 60.19, 58.04, 52.19, 29.25, 17.02, 9.47, 9.42.

The starting *meso*-(butane-2,3-diylbis(2,6-dioxopiperazine-4,1-diyl))bis(methylene)-(2S,2'S)-bis(2-((*tert*-butoxycarbonyl)amino)-3-phenylbutanoate) was prepared through a reaction of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) with Boc-L-valine according to Scheme 3. A solution of dicyclohexylcarbodiimide (0.72 g, 3.45 mmol) in 30 ml of CH_2Cl_2 was added to a suspension of *meso*-4,4'-(butane-2,3-diyl)bis(1-(hydroxymethyl)piperazine-2,6-dione) (0.6 g, 1.75 mmol), Boc-L-valine (3.54 mmol) and 4-(dimethylamino)pyridine (6 mg, 0.049 mmol) in 50 ml of CH_2Cl_2 at 0°C under an argon atmosphere. The reaction was stirred for 48 hours. Then, the reaction was filtered, and the filtrate was extracted with 48 ml of a mixture of H_2O/CH_3COOH (15:1). The organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated. The product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH, 20:1). Yield: 50% as a white crystalline solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.16 (d, *J* = 8.1 Hz, 2H), 5.75 (dd, *J* = 9.7, 6.6 Hz, 2H), 5.55 (dd, *J* = 9.7, 3.8 Hz, 2H), 3.78 (t, *J* = 7.1 Hz, 2H), 3.59 – 3.39 (m, 8H), 2.86 – 2.74 (m, 2H), 1.92 (q, *J* = 6.7 Hz, 2H), 1.41 – 1.28 (m, 18H), 0.93 – 0.77 (m, 18H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.24, 170.44, 155.86, 78.45, 61.88, 60.27, 59.34, 58.79, 52.30, 29.70, 28.34, 18.86, 18.47, 9.61.

The other substances are normally commercially available.

Example 5. Protective effects of ICRF-193 against ANT cytotoxicity *in vitro* in a model of isolated rat neonatal ventricular cardiomyocytes

Methodology. The protective effects of ICRF-193 were assessed in a model of toxicity induced by administration of DAU into cultivation media of isolated neonatal rat cardiomyocytes. This bioassay was previously validated by DEX (Jirkovská-Vávrová *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. *Toxicology Research* **2015,** *4*, 1098-1114). The culture was obtained from heart ventricles of 3-5-day-old Wistar rats

via serial enzymatic digestion of intercellular structures by mixture of collagenase and pancreatin. The cell suspension obtained thereby was than enriched with cardiomyocytes through selective adhesion of other cells than myocytes on plastic surface. The myocyte-rich cell suspension was then cultivated for three days on plastic wells pre-coated with gelatin, to obtain a culture of spontaneously beating cardiomyocytes. Then, this culture was exposed to ICRF-193, DAU or their combination in serum-free media. In the case of the combination experiment, the culture was first exposed to ICRF-193 for 3 hours, then DAU was added to the cultivation media in the concentration of 1.2 μM for 3 hours, after which both the substances were removed through media replacement. The toxic effects of DAU and the protective effect of ICRF-193 were evaluated after 48 hours from the last media replacement by means of determination the activity of lactate dehydrogenase (LDH), an intracellular enzyme that is released into the cultivation media after a cell membrane damage. The activity of LDH in the cultivation media was compared to the total LDH activity obtained after lysis of all cells in the sample. The LDH activity was determined spectrophotometrically with the use of the lactate and NAD^+ as the substrates. The resulting increase of NADPH was determined by measurement of absorbance at 340 nm using a Tecan Infinite 200M spectrophotometer (Tecan Ltd., Switzerland).

Results. Pre-incubation with compound ICRF-193 for 3 hours before three-hour exposure to DAU statistically significantly reduced damage to the culture of isolated neonatal cardiomyocytes 48 hours after the exposure.

The protective effects of the compound ICRF-193 against DAU cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes is shown in Fig. 1. DAU induced significant toxicity in the culture of neonatal rat cardiomyocytes as determined by the LDH release. Pre-incubation with ICRF-193 statistically significantly reduced this damage in the range of the studied concentrations (hatched columns). Already at 10 μM of the agent, toxicity of DAU (expressed as LDH release into the media) was reduced by approximately 55% as compared to the control. Statistically significant differences ($p \leq 0,05$, one-way ANOVA) are shown as "c" (significant as compared with the control) and "d" (significant as compared with DAU).

Example 6. Protective effects of the substance **Ib** according to Example 1 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes

The protective effects of the substance **Ib** according to Example 1 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes was determined with the use of the procedure specified in Example 5.

Results. Pre-incubation with the substance **Ib** for 3 hours before three-hour exposure to DAU statistically significantly reduced damage to the culture of isolated neonatal cardiomyocytes 48 hours after the exposure.

The protective effects of the compound Ib according to Example 1 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes is shown in Fig. 2. DAU induced significant toxicity in the culture of neonatal rat cardiomyocytes as determined by the LDH release. Pre-incubation with the substance Ib statistically significantly reduced this damage in the entire range of the used concentrations (hatched columns, i.e. already from 1 μM) while the biggest effect was achieved at 10 μM, where the release of LDH into the media, i.e. the toxicity, was reduced by approximately 59%. Statistically significant differences ($p \leq 0,05$, one-way ANOVA) are shown as "c" (significant as compared with the control) and "d" (significant as compared with DAU).

Example 7. Protective effects of the substance **Ic** according to Example 2 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes

The protective effects of the substance **Ic** according to Example 2 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes was determined with the use of the procedure specified in Example 5.

Results. Pre-incubation with the substance Ic for 3 hours before three-hour exposure to DAU statistically significantly reduced damage to the culture of isolated neonatal cardiomyocytes 48 hours after the exposure.

The protective effects of the substance Ic according to Example 2 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes is shown in Fig. 3. DAU induced significant toxicity in the culture of neonatal rat cardiomyocytes as determined by the LDH release. Pre-incubation with the substance Ic (hatched columns) statistically significantly reduced this damage already in the concentration of 3 μM while the biggest effect was

achieved at 10 μM, where the release of LDH into the media, i.e. the toxicity, was reduced by approximately 52%. Statistically significant differences ($p \leq 0,05$, one-way ANOVA) are shown as "c" (significant as compared with the control) and "d" (significant as compared with DAU).

Example 8. Protective effects of the substance **Id** according to Example 3 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes

The protective effects of the substance **Id** according to Example 3 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes was determined with the use of the procedure specified in Example 5.

Results. Pre-incubation with the substance Id for 3 hours before three-hour exposure to DAU statistically significantly reduced damage to the culture of isolated neonatal cardiomyocytes 48 hours after the exposure.

The protective effects of the substance Id according to Example 3 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes is shown in Fig. 4. DAU induced significant toxicity in the culture of neonatal rat cardiomyocytes as determined by the LDH release. Pre-incubation with the substance Id (hatched columns) statistically significantly reduced this damage already in the concentration of 3 μM while the biggest effect was achieved at 10 μM, where the release of LDH into the media, i.e. the toxicity, was reduced by approximately 42%. Statistically significant differences ($p \leq 0,05$, one-way ANOVA) are shown as "c" (significant as compared with the control) and "d" (significant as compared with DAU).

Example 9. Protective effects of the substance **Ie** according to Example 4 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes

The protective effects of the substance **Ie** according to Example 4 against ANT cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes was determined with the use of the procedure specified in Example 5.

Results. Pre-incubation with the substance Ie for 3 hours before three-hour exposure to DAU statistically significantly reduced damage to the culture of isolated neonatal cardiomyocytes 48 hours after the exposure.

The protective effects of the substance Ie according to Example 4 against DAU cytotoxicity *in vitro* in a model of isolated neonatal cardiomyocytes is shown in Fig. 5. DAU induced significant toxicity in the culture of neonatal rat cardiomyocytes as determined by the LDH release. Pre-incubation with the substance Ie statistically significantly reduced this damage in the range of the used concentrations (hatched columns) while the biggest effect was achieved at 10 μM, where the release of LDH into the media, i.e. the toxicity, was reduced by approximately 43%. Statistically significant differences ($p \leq 0,05$, one-way ANOVA) are shown as "c" (significant as compared with the control) and "d" (significant as compared with DAU).

Example 10. Protective effects of the substance **Ib** according to Example 1 against chronic cumulative ANT cardiotoxicity *in vivo*

Methodology. For the study of cardioprotective effects of the substance **Ib** according to Example 1, the established model of chronic cardiotoxicity in rabbits was used. Adult male New Zealand White rabbits (Velaz, Czech Republic) were housed individually in cages under standard conditions and fed with a standard pelleted diet *ad libitum*. After acclimatization (2 weeks, weight 3.0-3.5 kg), the rabbits (n = 35) were randomized into 5 groups.

In the group 1 (DAU) (n = 7), chronic anthracycline cardiotoxicity was induced with daunorubicin (DAU) administered once a week *i.v.* into the marginal ear vein in the dose of 3 mg/kg.

The group 2 (control, n = 7) received saline (1 mg/kg) *i.v.* in the same manner. The groups 3 and 4 (n = 7 in each group): the substance **Ib** according to Example 1 was fully dissolved in the saline (concentration 1 and 5 mg/ml) immediately before drug administration and the solution was filtered through an antibacterial filter. The substance **Ib** according to Example 1 was subsequently administered in the doses of 1 and 5 mg/kg via slow *i.v.* injection (2 min) 30 min before each DAU administration (the same schedule of DAU administration as in the group 1 was adopted). The administrations of the compound **Ib** according to Example 1 and DAU were always conducted contralaterally (i.e. each administration was performed into the vein of a different ear).

The group 5 (n = 7) – the substance **Ib** according to Example 1 was administered alone in the dose of 5 mg/kg, in the same way as mentioned above in the group 4.

All the studied agents were administered to the rabbits once a week for 10 weeks. The experiment was completed 7 days after the last administration.

The body weight was monitored weekly; behavior changes were evaluated daily. The left ventricle (LV) systolic function was examined by echocardiography (Vivid 4, probe 10.5 MHz) before the beginning of the experiment and weekly from week 8 until the end of experiment. LV fractional shortening (LVFS) as the parameter of the systolic function was determined from the M-mode examination of LV. At the end of the experiment, the LV systolic function was also examined invasively under surgical anesthesia with pentobarbital (30 mg/kg). After preparation of *arteria carotis,* a Millar catheter (2.3F, Millar Instruments, USA) was introduced into LV and dP/dt_{max} as the parameter of systolic function was assessed. At the end of the experiment, a necropsy examination was conducted with a focus on signs of circulatory congestion (pleural effusion and effusion in the abdominal cavity), dilatation of heart chambers and macroscopic changes of internal organs.

Results: Chronic ANT cardiotoxicity induced by repeated DAU administration in an experimental rabbit model was associated with one premature death whereas all the animals in the both combination groups with the substance **Ib** according to Example 1 survived until the end of the experiment. Fig. 6A shows the body weight gain at the end of the experiment. In all groups except for the group 1 (DAU), a significant increase of the body weight was observed in the course of the experiment. At the end of the experiment, the body weight gain was significantly higher in all other groups (i.e. including the combination groups with the substance **Ib** according to Example 1), than in the group 1 (DAU). The administration of DAU led to slightly reduced consumption of food and water and slightly reduced motion activity. No other considerable changes of behavior or apparent signs of toxicity were observed in this study. Fig. 6B shows signs of blood congestion observed during the necropsy conducted at the end of the experiment or after the premature death. The necropsy examination performed in all the animals in this study identified signs of blood congestion in the group 1 (DAU), including pronounced forms of effusions in the thoracic and abdominal cavities with volumes exceeding 5 ml. The animal that died in the group 1 (DAU) exhibited very pronounced signs of blood

congestion and dilatation of the heart chambers. Conversely, in the combination groups 3 and 4 with the substance **Ib** according to Example 1, no effusion in the thoracic cavity and only one case of a mild effusion in the abdominal cavity were found. No macroscopically detectable anomalies were found in group 5 with the substance **Ib** according to Example 1 alone or in the both combination groups during the necropsy.

Fig. 6C shows results of echocardiographic (ECHO) examination of the left ventricle (LV) – fractional shortening (FS) was the evaluated parameter of the systolic function. The last measured values are presented. The echocardiographic examination of the LV systolic function revealed a significant decrease of LVFS as compared to the initial values in the group 1 (DAU) whereas in the both combination groups (the groups 3 and 4), the values of this parameter remained unchanged in the course of the experiment. The comparison of this parameter between the groups at the end of the experiment confirmed considerably lower values in the group 1 (DAU) as compared to the group 2 (the control group)), but also as compared to the both combination groups with the compound **Ib** according to Example 1 (the groups 3 and 4). The examination of LV systolic function via LV catheterization (Fig. 6D) showed a significantly lower value of the index dP/dt_{max} in the group 1 (DAU) as compared to all the other groups, including the both combination groups with the substance **Ib** according to Example 1 (the groups 3 and 4).

CTR- control group (group 2), compound **Ib**_5 - compound **Ib** according to example 1, administered alone in the dose of 5 mg/kg (group 5), DAU – daunorubicin (the group 1), compound **Ib**_1+DAU – the combination group with the compound **Ib** according to Example 1 administered in the dose of 1 mg/kg before each dose of DAU (the group 3), compound **Ib**_5+DAU – the combination group with the compound **Ib** according to Example 1 administered in the dose of 5 mg/kg before each dose of DAU (the group 4). The results are presented as the means ± S.D. Statistical significance ($p > 0,05$, SigmaStat 3.5 software) in comparison with: "*" – the values at the beginning of the experiment (paired t-test), „Δ" – with the values in all the other groups (ANOVA, Holm-Sidak post-hoc test).

Conclusions. This Example demonstrated a very pronounced cardioprotective potential of the substance **Ib** according to Example 1 against chronic cumulative ANT cardiotoxicity *in vivo*. Administration of the substance **Ib** according to Example 1 prevented the DAU-induced premature death of the animals associated with the development of cardiotoxicity and

advanced heart failure. The substance **Ib** according to Example 1 also effectively prevented DAU-induced blood congestion and systolic dysfunction, as determined by both echocardiographic and catheterization examinations. All these effects were significant already at the dose of 1 mg/kg, which makes this substance currently most effective cardioprotective agent against chronic cumulative cardiotoxicity (DEX is recommended at the dose ratio of 1:10 to 1:20 to ANT, i.e. in our model 30-60 mg/kg). The repeated administration of the substance **Ib** according to Example 1 was very well tolerated both when administered alone and in combination with DAU.

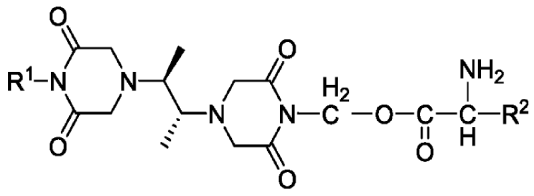
Figs. 7A-C show the results of evaluation of the cardioprotective effects of the compound ICRF-239 in the rabbit model of chronic cumulative ANT cardiotoxicity. In the same model, the compound ICRF-239 did not exhibit any protective effect.

Fig. 7A shows survival of the animals at the end of the experiment. Fig. 7B shows the results of echocardiographic (ECHO) examination of the LV systolic function – LV fractional shortening (LVFS). The last measured value is presented. Fig. 7C shows the results of LV catheterization examination of the animals surviving until the end of the experiment – the evaluated parameter was the index of the systolic heart function dP/dt_{max}. During the necropsy, signs of circulatory congestion and dilatation of cardiac chambers were observed in the ICRF-239+DAU combination groups, similarly to the DAU group.

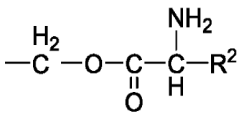
CTR – the control group (the group 2), ICRF-239 – compound ICRF-239 administered alone in the dose of 60 mg/kg, once a week for 10 weeks, DAU – daunorubicin (the group 1), ICRF-239+DAU – the combination group with the compound ICRF-239 administered in the dose of 60 mg/kg before each dose of DAU. The results are presented as the means ± S.D. (n = 7 in each group). Statistical significance ($p > 0.05$, SigmaStat 3.5 software) in comparison with: „#“ – the control (CTR) group, „†“ – the compound ICRF-239 alone (ANOVA).

CLAIMS

1. The use of the compounds of general formula I in the form of the ammonium salt with a pharmaceutically acceptable acid for the manufacture of a medicament for the prevention of chronic cumulative cardiotoxicity caused by therapy with anthracycline anticancer drugs



I

wherein R^1 independently refers to hydrogen or the group  ;

R^2 is selected from the group consisting of H, a C_1-C_6 alkyl or branched alkyl, or benzyl.

2. A pharmaceutical preparation for use to prevent chronic cumulative cardiotoxicity caused by therapy with anthracycline anticancer drugs characterized in that it contains the compound of general formula I according to claim 1 as an active ingredient.

Drawings

Fig. 1

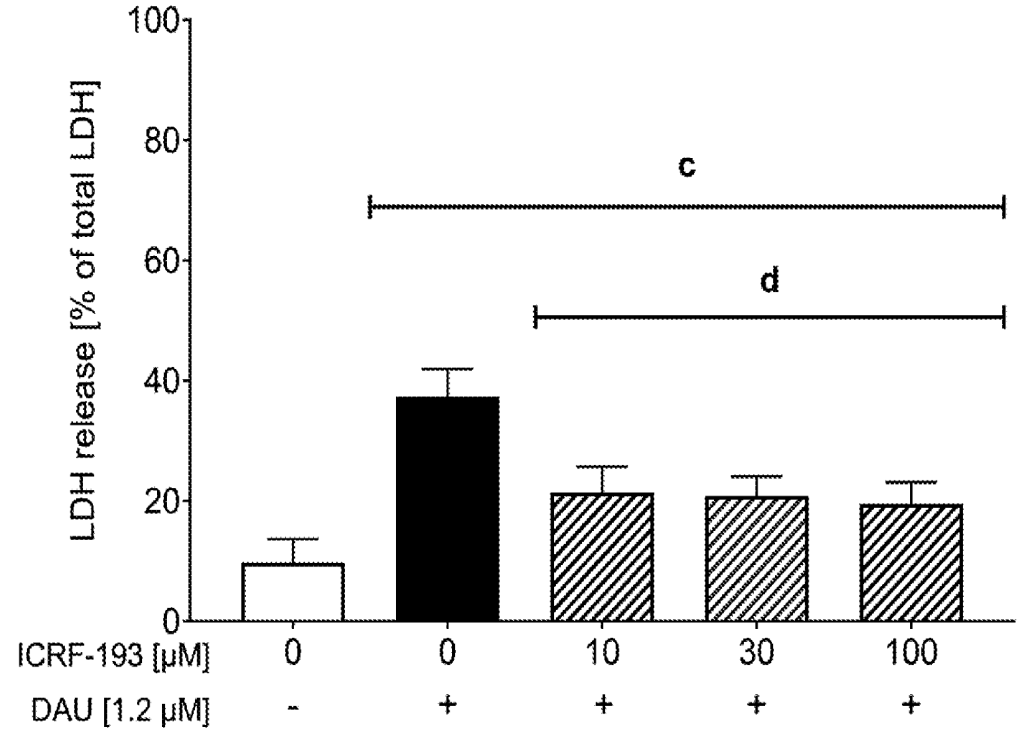


Fig. 2

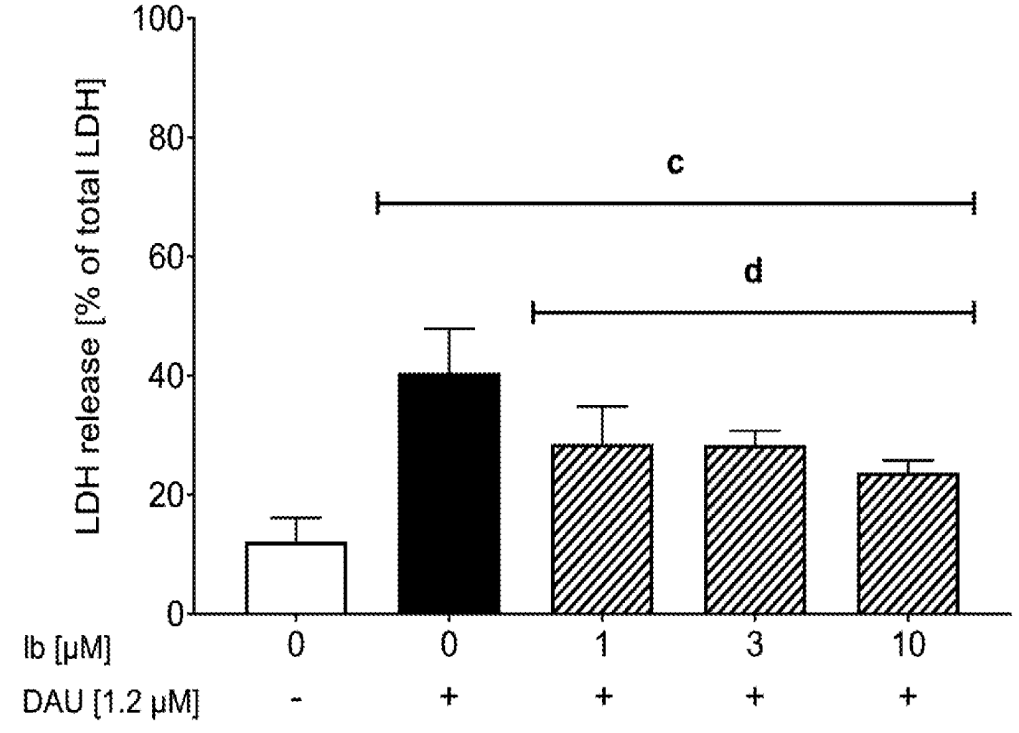


Fig. 3

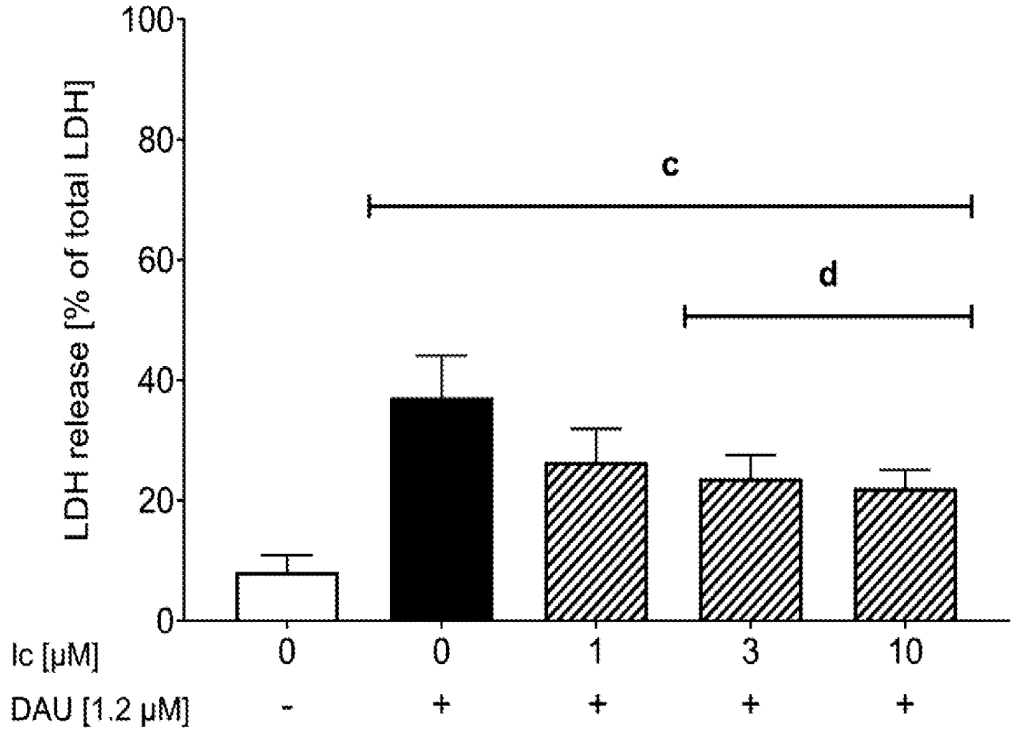

LDH release [% of total LDH]
100
80
60
40
20
0
c
d
Ic [µM]
0
0
1
3
10
DAU [1.2 µM]
-
+
+
+
+

Fig. 4

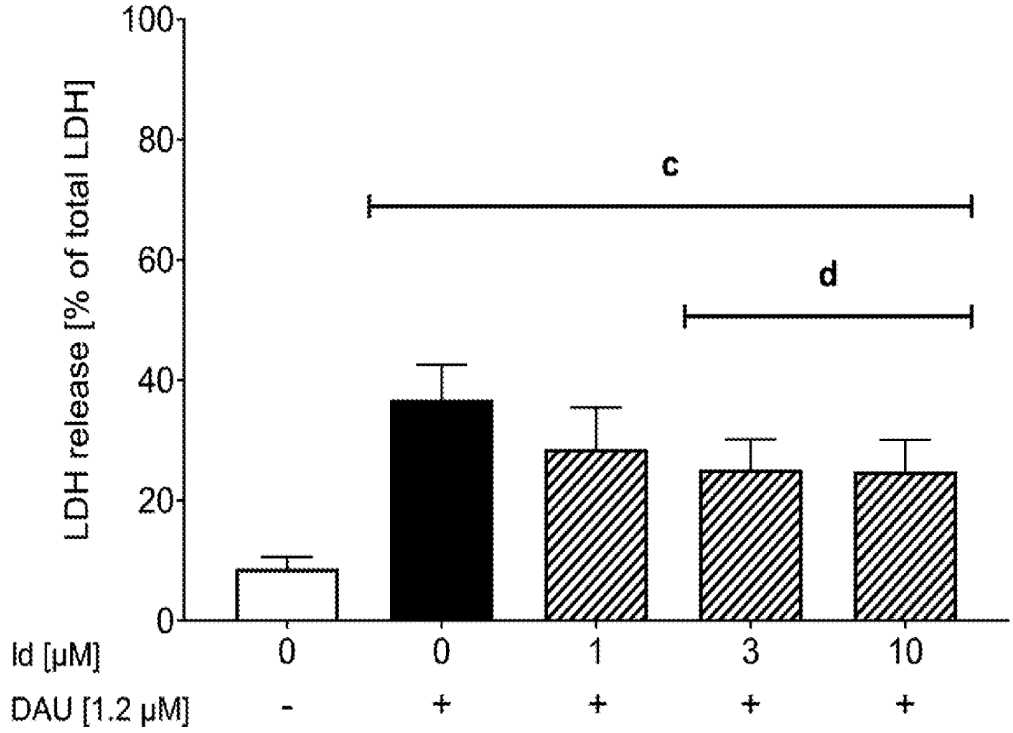

LDH release [% of total LDH]
100
80
60
40
20
0
c
d
Id [µM]
0
0
1
3
10
DAU [1.2 µM]
-
+
+
+
+

Fig. 5

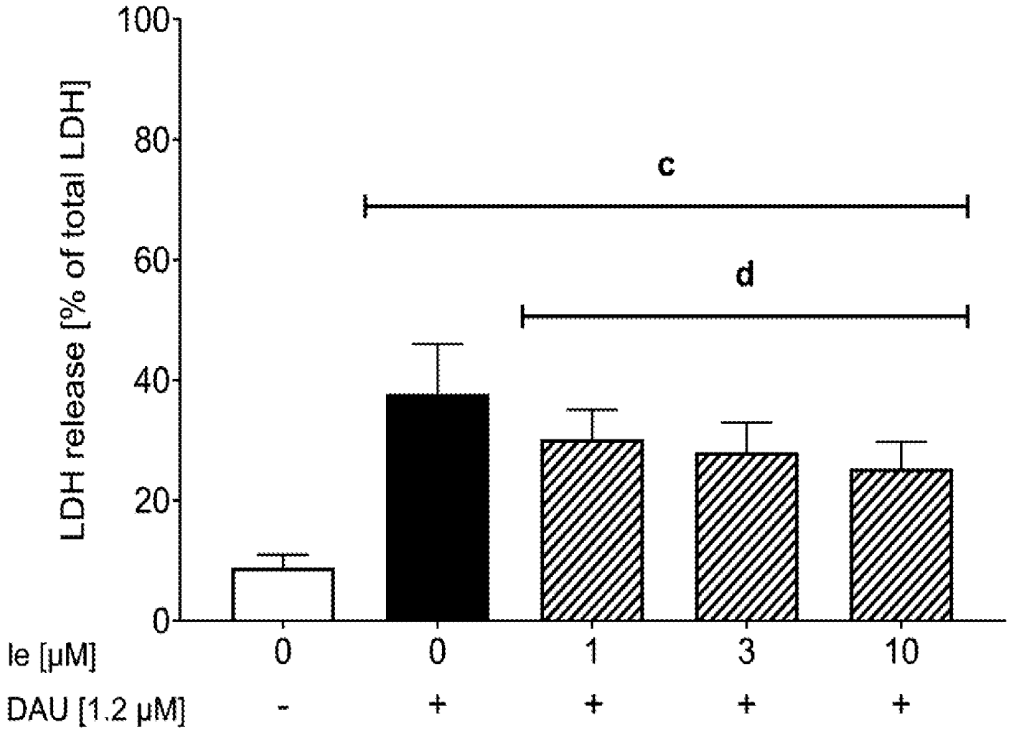

100
80
60
40
20
0
LDH release [% of total LDH]
c
d
Ie [µM]
0
0
1
3
10
DAU [1.2 µM]
-
+
+
+
+

Fig. 6

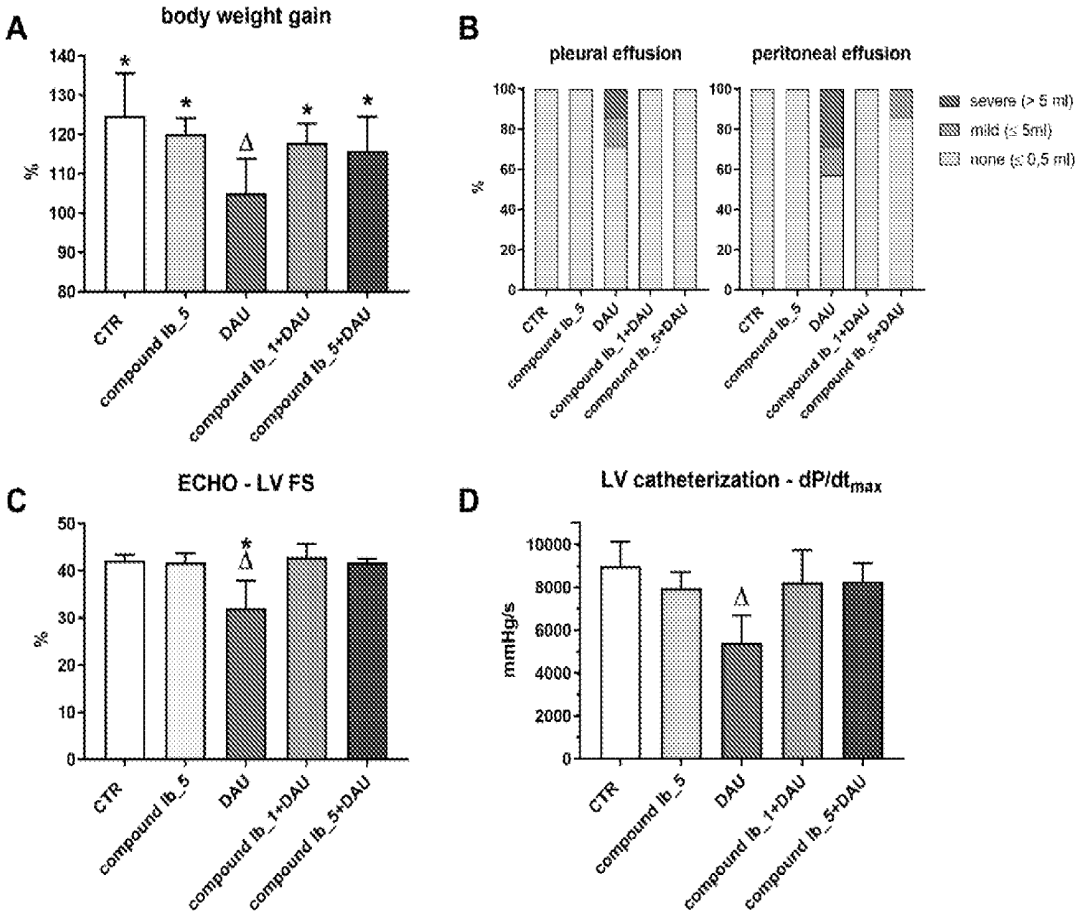

A
body weight gain
%
CTR
compound Ib_5
DAU
compound Ib_1+DAU
compound Ib_5+DAU
B
pleural effusion
peritoneal effusion
severe (> 5 ml)
mild (≤ 5ml)
none (≤ 0,5 ml)
C
ECHO - LV FS
D
LV catheterization - dP/dtmax
mmHg/s

Fig. 7

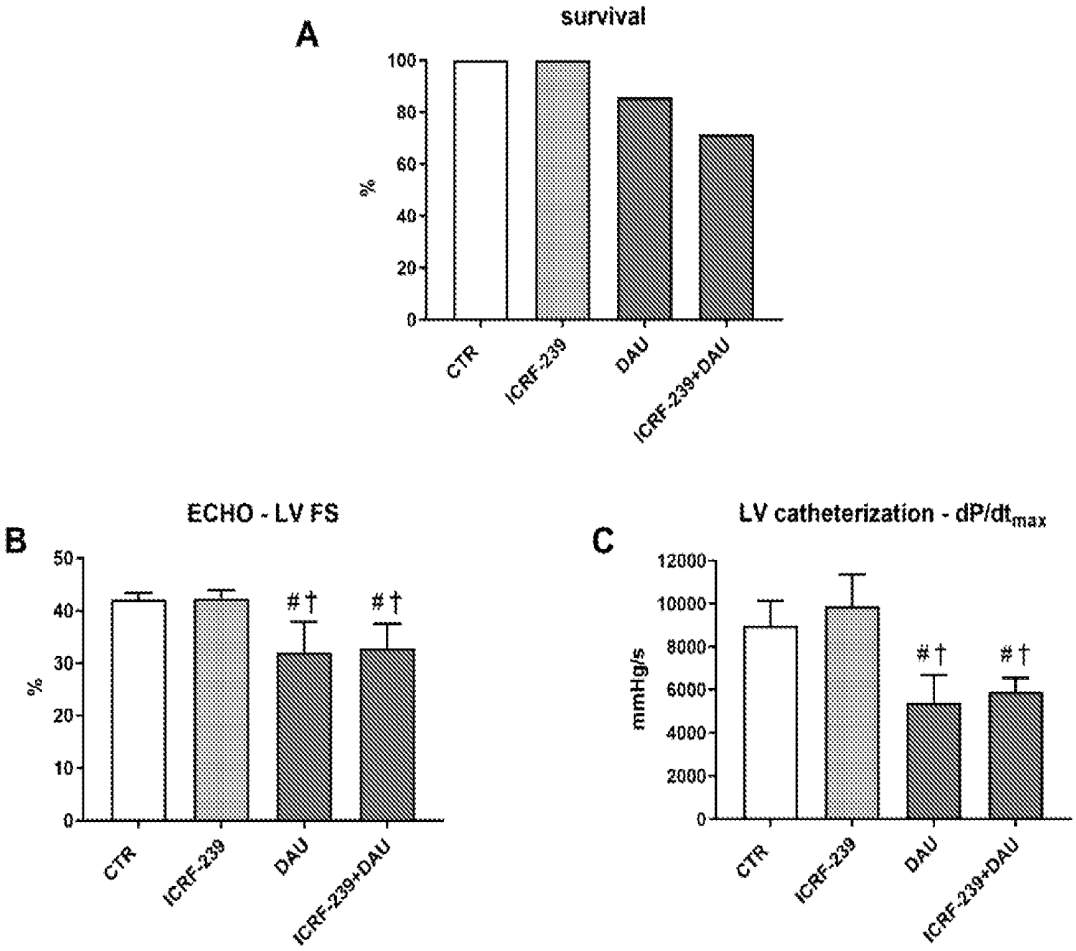
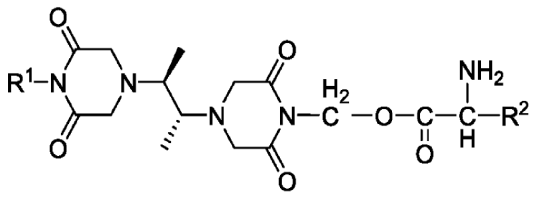

A
survival
%
100
80
60
40
20
0
CTR
ICRF-239
DAU
ICRF-239+DAU
B
ECHO - LV FS
%
50
40
30
20
10
0
#†
#†
CTR
ICRF-239
DAU
ICRF-239+DAU
C
LV catheterization - dP/dtmax
mmHg/s
12000
10000
8000
6000
4000
2000
0
#†
#†
CTR
ICRF-239
DAU
ICRF-239+DAU

Fig. 8


O
R1–N
N
O
N
O
N
C
H2
O
C
O
C
H
NH2
R2
O

I

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/050285

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/496 A61P43/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 393 889 A (TAKASE MUNEAKI [JP] ET AL) 28 February 1995 (1995-02-28) cited in the application claims 1-16	1,2
A	US 2015/150843 A1 (PETERSON RANDALL T [US] ET AL) 4 June 2015 (2015-06-04) claims 1-17; examples 1-10	1,2

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 April 2021	23/04/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hörtner, Michael

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/050285

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	POUILLART P: "Evaluating the role of dexrazoxane as a cardioprotectant in cancer patients receiving anthracyclines", CANCER TREATMENT REVIEWS, ELSEVIER, AMSTERDAM, NL, vol. 30, no. 7, 1 November 2004 (2004-11-01), pages 643-650, XP004623028, ISSN: 0305-7372, DOI: 10.1016/J.CTRV.2004.06.002 the whole document -----	1,2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2021/050285

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		EP 0491053 A1	24-06-1992
		ES 2082979 T3	01-04-1996
		JP 3050598 B2	12-06-2000
		US 5393889 A	28-02-1995
		WO 9200971 A1	23-01-1992
US 2015150843 A1	04-06-2015	US 2015150843 A1	04-06-2015
		WO 2013176955 A1	28-11-2013