Cardioprotective effects of a novel iron chelator – pyridoxal 2-chlorobenzoyl hydrazone – in the rabbit model of daunorubicin-induced cardiotoxicity.

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ANT, anthracycline; DAU, daunorubicin; LV, left ventricle; ROS, reactive oxygen species; o-108, pyridoxal 2-chlorobenzoyl hydrazone; PIH, pyridoxal isonicotinoyl hydrazone; SIH, salicylaldehyde isonicotinoyl hydrazone; LVEF, left ventricular ejection fraction.

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Abstract

Iron chelation is the only pharmacological intervention against anthracycline cardiotoxicity whose effectiveness has been well documented both experimentally and clinically. In this study we aimed to assess whether pyridoxal 2-chlorobenzoyl hydrazone (o-108, a strong iron chelator) can provide effective protection against daunorubicin (DAU)-induced chronic cardiotoxicity in rabbits. First, using the HL-60 leukemic cell line it was shown that o-108 has no potential to blunt the antiproliferative efficacy of DAU. Instead, o-108 itself moderately inhibited cell proliferation. *In vivo* chronic DAU treatment (3 mg/kg weekly for 10 weeks) induced mortality (33%), left ventricular (LV) dysfunction, a troponin T rise and typical morphological LV damage. In contrast, all animals treated with 10 mg/kg of o-108 prior to DAU survived without a significant drop in the LV ejection fraction (63.2±0.5% vs. 59.2±1.0%, beginning vs. end, n.s.) and their cardiac contractility (dP/dt_{max}) was significantly higher than in the DAU-only group (1131±125 vs. 783±53 kPa/s, $p<0.05$), which corresponded with histologically assessed lower extent and intensity of myocardial damage. Although higher o-108 dose (25 mg/kg) was well tolerated when administered alone, in combination with DAU it led to rather paradoxical and mostly negative results regarding both cardioprotection and overall mortality. In conclusion, we show that shielding of free intracellular iron using a potent lipophilic iron chelator is able to offer a meaningful protection against chronic anthracycline cardiotoxicity. However, this approach lost its potential with the higher chelator dose, which suggests that iron might play more complex role in the pathogenesis of this disease than previously assumed.

Introduction

Although introduced more than 40 years ago, anthracycline antineoplastic drugs (ANT) have remained among the most effective and widely used anticancer chemotherapeutics in clinical practice (Yee et al., 2005). Their clinical utility is, however, largely limited by adverse reactions accompanying their use. Besides reversible and often well manageable adverse effects typical for most of the anticancer drugs (e.g. nausea, myelosuppression) there is a well-documented risk of severe complication which legitimately warrants the highest vigilance – anthracycline cardiotoxicity (Hrdina et al., 2000, Minotti et al., 2004). Both the chronic (von Hoff et al., 1979) and delayed (Lipshultz et al., 1991) types of ANT cardiotoxicity are associated with cardiomyopathy and irreversible damage of left ventricular myocardium, which functionally manifests itself as congestive heart failure.

Although the precise mechanisms involved in the chronic ANT cardiotoxicity still remain to be determined, there is a general agreement that reactive oxygen species (ROS) play an important role there (Hrdina et al., 2000). Therefore, numerous experimental cardioprotective interventions have been focused on ROS scavengers, including the “classic” antioxidants like vitamin E or acetylcysteine. After some promising initial experience, obtained mostly in acute experimental settings, mixed, contradictive or solely negative outcomes were reported from both chronic experimental models and clinical studies (Herman et al., 1985, Berthiaume et al., 2005, Legha et al., 1982, Myers et al., 1983). From the numerous agents tested so far, only very few are currently in further development (Abou-El-Hassan et al., 2003, Iliskovic et al., 1999, Oliviera et al., 2004, Fisher et al., 2005). At present, the only drug with a well-evidenced cardioprotective effect, in both experimental and clinical settings, is dexrazoxane (ICRF-187), pro-drug yielding metal-chelating metabolites (Herman et al., 1986, Speyer et al., 1992, Swain et al., 1997, Marty et al., 2006). These active metabolites supposedly shield the so-called “labile iron pool” inside the cardiomyocytes

and/or replace iron from complexes with ANTs, and thereby prevent excessive production of ROS and particularly hydroxyl radicals (Hasinoff et al., 1998). Importantly, in most of recent clinical studies dexrazoxane did not interfere with anticancer efficacy of ANTs (Marty et al., 2006, Swain et al., 2004), which is in agreement with a recent experimental study, showing that ROS are not among the main mediators of their anticancer effect (Wu et al., 2005). Thus, intracellular iron chelation is the only well-established and successful strategy for cardioprotection in patients treated with higher cumulative doses of ANTs (Cvetkovic et al., 2005). Unfortunately, due to the myelosuppressive potential and high costs, the use of dexrazoxane is limited to selected groups of patients – those obtaining more than 300 mg/m^2 of doxorubicin (or equivalent). It is estimated that only 6-7 % of patients receiving ANTs are treated with dexrazoxane in Europe (Swain et al., 2004).

Apart from dexrazoxane, the data on possible cardioprotective properties of other iron chelators are surprisingly limited. Desferrioxamine (DFO), the most widely used iron chelator for the treatment of iron overload, failed as a cardioprotectant in a chronic *in vivo* model of ANT cardiotoxicity (Herman et al., 1994). This observation is explainable by a hydrophilic nature of this drug, which is responsible for a limited entry of this agent into the cardiomyocytes. *In vitro*, bidentate iron chelator deferiprone was shown to have a cardioprotective potential (Barnabe et al., 2002), whereas another strong novel chelator ICL670 was ineffective under similar conditions (Hasinoff et al., 2003). In part, iron chelation may be also involved in the cardioprotective effects of flavonoid monoHER (Bruynzeel et al., 2006). We have previously reported that pyridoxal isonicotinoyl hydrazone (PIH), an aroylhydrazone iron chelator (Ponka et al., 1979; Baker et al., 1992), is capable to improve survival and slightly ameliorate the cardiotoxicity induced by chronic ANT treatment in rabbits (Simunek et al., 2005b). On the other hand, PIH is nowadays understood to be rather a "parent compound", from which a number of novel analogues are being derived. These

advanced chelators are strong, selective for iron, and have improved cell penetration as well as excellent antioxidant properties (Simunek et al., 2005a). Pyridoxal 2-chlorobenzoyl hydrazone (o-108, Fig. 1) is among the most promising candidates (Link et al., 2003); and it was shown to be safe and well-tolerated after repeated administration to rabbits (Sterba et al., 2005). Therefore, the main goal of the present study was to assess whether o-108 has cardioprotective properties against chronic daunorubicin (DAU) cardiotoxicity without compromising its antiproliferative efficacy.

Methods

Animals

Adult Chinchilla male rabbits of an average initial weight of 3.44 ± 0.04 kg were housed under a 12h light cycle, constant temperature and humidity. The animals had free access to water and a standard laboratory pellet diet. Before experimental procedures the animals were fasting overnight. All experiments were performed under ketamine anesthesia (50 mg/kg, i.m.). Final invasive hemodynamic measurements were carried out under pentobarbital anesthesia (30 mg/kg, i.v.). All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication, 1996) and under the supervision of the Ethical Committee of the Faculty of Medicine in Hradec Králové.

Chemicals

Pyridoxal 2-chlorobenzoyl hydrazone (o-108) was synthesized in-house by a Schiff-base condensation of pyridoxal and 2-chlorobenzoylhydrazide as previously described (Link et al., 2003). The structure and purity of the compound was confirmed employing ^{1}H and ^{13}C NMR, IR spectroscopy and HPLC with UV detection (Kovarikova et al., 2004). Cremophor

EL (Sigma-Aldrich, Czech Republic), daunorubicin (Daunoblastina, Pharmacia, Italy), ketamine (Narketan inj., Gedeon Richter, Hungary), Aqua pro injectione (Biotika, Slovakia), saline (Natrium Chloratum, Biotika, Slovakia) and pentobarbital (Nembutal Sodium, Abbott, U.S.A.) were used in the experiment.

Experimental design

First, a study addressing potential effects of o-108 on the antiproliferative properties of DAU was performed, as this compound may only have value when it does not compromise the anti-cancer effect of anthracyclines. Thereafter, chronic anthracycline cardiomyopathy was induced in rabbits in a standard and previously validated schedule (DAU 3 mg/kg, i.v., once weekly for 10 weeks, n=15) (Gersl et al., 1994, Simunek et al., 2004). Control animals received saline (1 ml/kg, i.v., n=11) and another group was injected with the vehicle for the chelator (10% Cremophor EL, i.p., n=5) in the same scheme. The investigated iron chelator o-108 was partially dissolved in 10% Cremophor EL and administered intraperitoneally either alone (25 mg/kg, i.p., n=5), or in two doses (10 or 25 mg/kg, n=8 each) 30 minutes before each DAU administration (3 mg/kg, i.v.).

Body weight was recorded weekly, while mortality, general appearance and behaviour were observed daily. Non-invasive echocardiographic measurements were performed at the beginning of the study and later in weeks 9 and 10 and finally at the end of experiment (5-7 days after the last administration of drugs). Blood for cardiac troponin T (cTnT) determination was sampled before the 1^{st}, 5^{th}, 8^{th} and 10^{th} administration as well as at the end of the study. Standard biochemical and hematological parameters were determined from blood sampled before the 1^{st} and 5^{th} administrations and at the end of study. Five to seven days after the last administration, invasive hemodynamic measurements were performed.

Thereafter, the animals had been overdosed with pentobarbital and autopsy was performed. Heart and selected organs were excised and prepared for histological examination.

Proliferation studies with HL-60 cells

HL-60 human acute promyelocytic leukemia cell line was obtained from ATCC (USA). Cells were maintained in RPMI-1640 medium (Sigma Aldrich, Czech Republic) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich, Czech Republic), 1% penicillin/streptomycin (PAA, Austria) and grown in humidified atmosphere at 37 °C in 5% CO_2. Medium was renewed every 2-3 days. For proliferation studies, the cells were seeded at a density of 10^5 cells/mL. Tested substances (o-108 and/or DAU) had been added and cells were allowed to proliferate under standard conditions. The test concentrations of o-108 were chosen after pilot experiments and ranged from 0.1 to 300 μmol/L. For combination assays, 2.5 nmol/L DAU concentration was used, which was shown in our previous experiments to induce partial growth inhibition. In order to dissolve o-108, DMSO (0.2%, v/v) was used and it was present in the culture medium of all groups. At this concentration DMSO had no effect on cellular proliferation. To quantify the number of viable cells after each treatment, at 48 and/or 72 hours of incubation, trypan Blue unstained cells were counted using a Bürker's hemocytometer under a light microscope.

Cardioprotection studies in rabbits

Echocardiography

Non-invasive LV systolic function measurements were carried out in rabbits using a GE Vingmed CFM 800A echocardiograph (Horten, Norway) equipped with a pediatric 7.5 MHz probe. The LV long axis view was obtained through the left parasternal approach and a guided M-mode measurement at the tips of the mitral valve was performed. The left

ventricular ejection fraction (LVEF) was calculated from the LV end-diastolic and end-systolic dimensions determined from at least four heart cycles in each measurement. Individual LVEF values were determined as means of at least three independent examinations.

Invasive hemodynamic measurements

In pentobarbital anesthesia, the left carotid artery was prepared and a PE catheter (length 300 mm, inner diameter 1.0 mm), filled-in with heparinized (10 IU/mL) saline was introduced into the left heart ventricle. After a 15 minute-equilibration period, the maximum of the first derivative of LV pressure rise in the isovolumic phase of the systole (dP/dt_{max}, an index of LV contractility) was obtained together with heart rate (HR). For the arterial blood pressure (BP) measurement, a PE cannula was inserted into the right femoral artery. The ADI PowerLab/8SP (Adinstruments, Australia) with appropriate transducers and the software Chart for Windows 3.4.11 were used for pressure measurements, their differentiation and recording.

Cardiac troponin T determination

Cardiac troponin T, as a selective and sensitive marker of heart damage induced by chemotherapeutics (Adamcova et al., 2005), was determined in heparinized plasma using an Elecsys Troponin T STAT Immunoassay (Roche, Switzerland) and an Elecsys 2010 (Roche, Switzerland) immunoassay analyzer with the detection limit of 0.010 ng/mL. The values below this detection limit were considered to be zero.

Standard biochemical and hematological analyses

Routine biochemical parameters were determined in plasma/serum using an automatic analyser Modular (Japan) at the Institute of Clinical Biochemistry and Diagnostics; hematological parameters were measured using an automatic analyser Coulter T890 (U.S.A.) at the Institute of Clinical Hematology, University Teaching Hospital in Hradec Králové.

Histological examination

Tissue blocks of the transversely sectioned left and right cardiac ventricles, left kidney, left liver lobule, left caudal lung lobule and duodenum (approx. 3 cm below the pyloric sphincter) were fixed by immersion in 4% neutral formaldehyde for 5 – 7 days. Paraffin sections (6 μm thick) were stained with hematoxylin-eosin (H&E) and Masson's blue trichrome. Photomicrographs were made with a Lucia G software version 4.51 (Laboratory Imaging, Prague, Czech Republic) at the Department of Medical Biology and Genetics, Faculty of Medicine in Hradec Králové.

Statistical analysis

The statistical software SigmaStat for Windows 3.0 (SPSS, U.S.A.) was used in this study. All data are expressed as mean ± S.E.M. Significances of the differences were estimated using One Way ANOVA unpaired test (comparison between groups) or Paired t-test (comparison with the initial value within each group). Data without a normal distribution were evaluated using the nonparametric tests: Kruskal-Wallis ANOVA on Ranks and Wilcoxon Signed Rank Test. Correlation analysis was performed using Spearman's method and regression analysis. $P \leq 0.05$ was used as the level of statistical significance unless indicated otherwise.

Results

Proliferation studies with HL-60 cells

As seen in Fig. 2A, o-108 dose-dependently inhibited the proliferation of HL-60 cells. At the end of 72-hour incubation, the chelator concentration required for 50% growth inhibition (the IC_{50} value) was shown to be ≈ 30 μmol/L. Further dose escalation resulted in

pronounced cytotoxic action. DAU (2.5 nmol/L) significantly inhibited proliferation of HL-60 cell to 62 % of control values. At low doses (0.1 – 10 μmol/L), o-108 did not significantly influence the DAU action, while at higher concentrations an additive antiproliferative effect was detected (Fig. 2B).

Cardioprotection studies in rabbits

General toxicity

All animals from control, vehicle and o-108 (alone) groups survived until the end of the experiment. In contrast, repeated 10-week administration of DAU induced overall mortality of 33 %, which was preceded with reduced food intake and signs of lethargy. On the other hand, all animals treated with DAU together with a 10 mg/kg dose of o-108 survived until the scheduled end of experiment and no apparent changes in appearance or behaviour were observed. Combination of DAU with a higher dose of o-108 (25 mg/kg) led to premature death of a half of animals. Nevertheless, the timings of death occurrences varied significantly in this study. While DAU alone induced mortality in the last two weeks of the experiment (a similar pattern as previously reported - Simunek et al., 2004), in the group treated with a combination of o-108 in the higher dose, the mortality occurred earlier - around the middle of the study. The details on survival during the study are shown in Fig. 3.

Body weight changes in the course of the study are shown in Fig. 4. In most groups, body weight gain was significant commencing with the 3rd week, as compared with the initial values within each group. In contrast, only insignificant weight gain was witnessed in a combination of daunorubicin with the 25 mg/kg dose of the chelator, which eventually turned into a significant decrease between weeks 4-6. At the end of the experiment, the final mean body weights were in the daunorubicin group significantly lower in comparison with the control group, while in 10 mg/kg o-108+DAU group the body weights were close to those

observed in the vehicle group. On the other hand, the body weight of animals treated with 25 mg/kg o-108+DAU was significantly lower in comparison with most other groups under study, including the daunorubicin alone group (Fig. 4).

Echocardiography

Echocardiographically determined LVEF revealed a progressive decline in LV systolic function in DAU-treated animals (Fig. 5). In contrast, there was no significant change in this parameter during the whole study in the 10 mg/kg o-108+DAU group. Furthermore, LVEF values in this group did not statistically differ from those determined in controls and the LVEF was mostly also significantly higher than in the daunorubicin group. On the other hand, administration of 25 mg/kg o-108+DAU led to a fall in the LVEF resembling the administration of DAU alone.

Invasive hemodynamic measurements

LV contractility (dP/dt_{max}), assessed at the end of the experiment was significantly reduced in daunorubicin treated animals (Fig. 6). In contrast, significantly higher values were obtained with DAU in combination with the lower dose of o-108. Moreover, these results did not statistically differ from those determined in the control and vehicle groups. On the other hand, a combination of DAU with the higher dose of the chelator induced changes in the LV contractility similar to those seen in the DAU only group. Furthermore, correlation analysis of both parameters of the LV systolic function (dP/dt_{max} and LVEF) showed a significant positive relationship between these parameters (Fig. 7). Arterial blood pressure and heart rate values, as determined together with contractility are shown in Table 1.

Cardiac troponin T plasma concentrations

Repeated treatment with DAU induced a significant elevation in plasma concentration of cardiac troponin T commencing with the 8^{th} week (Fig. 8). Cardiac troponin T elevations were also determined in the combination group treated with 25 mg/kg of o-108. On the other

hand, markedly suppressed elevation of this marker was observed in the group treated with daunorubicin together with o-108 in the lower dose. At the end of the experiment, slightly increased levels of troponin T were detectable in animals treated with o-108 alone; nevertheless, these values did not reach the significance with respect to either the control or vehicle groups.

Biochemical and hematological analyses

The follow up of serum biochemistry (Table 2) in the DAU group revealed a significant elevation in creatinine, cholesterol, and triglycerides, while a significant decrease was observed in total protein, ALP and serum iron. The co-administration of either dose of o-108 mostly did not significantly change the trends observed in the group receiving DAU alone, although with the 10 mg/kg dose the most of changes were generally slightly less pronounced.

With respect to hematological parameters, repeated administration of DAU induced significant decreases in the counts of leukocytes and erythrocytes, hematocrit and hemoglobin, whereas, at the same time, the mean cell volume and red cell distribution width tended to increase (Table 3). Co-treatment with either dose of o-108 led to similar results; the only significant difference appeared in the value of the mean cell volume, which increased at the end of experiment in the 10 mg/kg o-108+DAU group.

Post-mortem examination

Pleural effusion (hydrothorax), often accompanied with pericardial effusion (hydropericardium), was present in 10 out of 15 (67 %) of daunorubicin receiving animals, while ascites was less frequent (4/15 - i.e. 27 %) and usually also less severe. The combination of DAU with 10 mg/kg of o-108 caused hydrothorax in only 25 % of animals (2/8) and no ascites was observed. The same treatment employing the higher dose of the chelator induced hydrothorax in 75 % animals surviving until the end of experiment (3/4),

whereas no distinguishable effusion was apparent in four prematurely dying animals. No other abnormalities were observed.

Histological examinations

In comparison with control animals (Fig. 9a) as well as other groups under study (Fig. 9b,c,e,f) DAU treatment induced a massive focal damage of the left-ventricular myocardium (Fig. 9d). The large groups of degenerating to necrotic cells frequently accompanied with a mononuclear infiltration were observed (Fig. 9d). The resulting damage was so profound that the extent of healing process (gradual replacement of necrotic cells by the fibrotic tissue) was insufficient which resulted into the partial disintegration of myocardium in these areas.

Basically similar features of myocardial damage were also observed in the LV in animals treated with 10 mg/kg o-108+DAU. Nevertheless, both the extent and intensity of this injury, as well as the amount of the fibrotic scar tissue were apparently less expressed (Fig. 9e). Therefore, the overall integrity of the myocardial tissue was not markedly altered. On the other hand, the combination of DAU with higher (25 mg/kg) dose of the chelator (Fig. 9f) led to myocardial injury broadly comparable with that after the treatment with DAU alone. However, a difference could be found in the less prominent disintegration of the myocardial tissue in this case. In contrast, only mild changes were detected in the prematurely died animals of this group. In comparison with the LV, the myocardium of the right ventricle was always less damaged. Interestingly, in the both groups treated with DAU in combination with o-108, these changes were less expressed than in DAU alone group.

With respect to the biochemical changes (namely the increased creatinine level) suggesting impaired glomelular filtration, our interest was also directed to the histopathological examination of the kidney. In comparison with controls (Fig. 10a), a severe damage of parenchyma was found in the kidney of group treated with DAU alone (Fig. 10d). Toxic damage developed, particularly of the cortical tubules, mostly under the picture of

tubular nephritis. The whole range of changes, i.e. from hyaline degeneration to the necrosis of the epithelial cells of many proximal and distal convoluted tubules in the cortex, less of the collecting tubules in the medulla were found, which documented the subsequent development of the damage. The volume of hyaline casts within the lumina of tubules varied from case to case (Fig. 10d).

No substantial differences in kidney morphology were found in animals treated with the vehicle for o-108 (10% Cremophor) and o-108 alone (Fig. 10b,c), these mild changes were only more focal and slightly less expressed in the o-108 only group. In comparison with DAU treatment, the less severe damage was observed similarly in both doses (10 and 25 mg/kg) of o-108 in combination with DAU (Fig. 10e,f). Hyaline degeneration of different intensity in most of the proximal, less distal convoluted tubules, and scattered hyaline cast in the lumina of these tubules were characteristics of the above mentioned groups. Only small number of necrotic epithelial cells in cortical tubules was present.

No abnormalities were observed in other evaluated organs in either group.

Discussion

Lack of interference with anticancer efficacy of ANTs is considered to be a principal prerequisite for a perspective cardioprotective agent. Hence, in the first part we have focused on this using the promyelocytic leukemia cell line HL-60. It is clearly shown that o-108, in a concentration range which might be expected *in vivo* (Kovarikova et al., 2006), does not have potential to blunt the extensive antiproliferative efficacy of DAU. Moreover, o-108 itself was shown to have moderate antiproliferative potential, which is in line with previous observations (Richardson et al., 1995). It should be noted that some other aroylhydrazones (particularly those derived from salicylaldehyde, 2-hydroxy-1-naphthylaldehyde or di-2-pyridylketone) has been shown to be significantly more efficient from this point of view

(Richardson et al., 1995, Kalinowski et al., 2005, Le et al., 2004, Yuan et al., 2004). The present findings are in concert with latest experimental and clinical data which conclude that chelator-based cardioprotective approach employing dexrazoxane does not have an impact on the anticancer efficacy of ANTs (Wu et al., 2005, Swain et al., 2004, Marty et al., 2006), despite some previous concerns (Swain et al., 1997). With this in mind o-108 certainly remains to be a very good candidate for further cardioprotective studies.

The main aim of the present study was to explore possible cardioprotective effects of o-108 against anthracycline-induced chronic cardiac toxicity. The model used in the present study was previously analyzed and has been shown as appropriate and suitable for this purpose (Simunek et al., 2004). The use of DAU instead of doxorubicin is based on our previous study, in which DAU administered weekly to rabbits was shown to induce less severe extracardiac toxicity and mortality along with well-reproducible cardiac injury (Klimtova et al., 2002). Dexrazoxane, as a positive control, was previously capable to afford effective cardioprotection in our model which again supports its relevance. Furthermore, it was recently pointed out that in rabbits the cardiomyocytes calcium handling as well as structure and function of myocardial sarcomere reflect both in normal and failing heart the human system more accurately than in rodents (Marian et al., 2005).

In this study the chelator o-108 was administered to rabbits in two doses (10 and 25 mg/kg) 30 min before each DAU injection. The timing and route of administration was adopted from previous successful studies with dexrazoxane whereas the dosage was derived from the study on the safety and tolerability of the repeated administration of this drug (Sterba et al., 2005). The results of the present study showed that 10 mg/kg of o-108, administered prior to DAU was able to completely abolish the daunorubicin-induced mortality. The well-being of the animals in this group was evidenced by significant body weight gain similar to that determined in the vehicle group and without significant differences

as compared to controls. Furthermore, the echocardiographically-determined LVEF showed no significant drop in the LV systolic function during the whole study. These values were significantly improved as compared with the group treated with DAU alone. The even more sensitive invasive measurement of the cardiac contractility (dP/dt_{max}), performed at the end of experiment, clearly revealed significantly better LV performance in the o-108 (10 mg/kg) treated animals. These findings are even more encouraging in the light of 100% survival in this group, since all animals under study were invasively examined; including those with potential cardiac deterioration. This is not the case of the DAU-only group, where those animals with the most severe cardiac failure died prematurely. Furthermore, at the end of study, both parameters of systolic function (LVEF and dP/dt_{max}) have shown a very good correlation. Cardiac troponin T plasma rise, as a result of myocardial injury, was also less pronounced in the group treated with the chelator, which corresponds with the lower extent and intensity of the LV myocardial damage, as assessed by histological examination.

With this in mind, rather surprising, nevertheless unequivocal results were obtained when the same co-treatment was realized with the 2.5-fold higher dose (25 mg/kg) of the chelator. First, the mortality was worse than in animals treated with DAU alone. However, the early timing of the premature mortality (in relatively low cumulative doses of DAU) is unlikely to be attributed to cardiac damage. This hypothesis was also evidenced by the absence of marked histopathological changes in myocardium together with the minimal levels of troponin T determined before the death cases. The most conspicuous biochemical finding in the 25 mg/kg o-108+DAU group was progressively increasing creatininemia, particularly at the end of the study - more pronounced than in DAU alone and even more marked in comparison with combination of DAU with the lower dose of the chelator. Surprisingly, these biochemical changes were not followed by corresponding morphological findings in surviving animals (only modest to medium changes of the similar pattern as in the DAU

group). Unfortunately, the kidneys as well as most of other organs could not be appropriately examined in prematurely died animals due to the autolysis. Therefore, it is not feasible to draw definitive conclusions on the cause of the mortality observed in this group from the present study and this is supposed to be addressed in further experiments designed for this purpose.

While the occurrence of premature deaths unrelated to heart injury was quite surprising, the absence of marked cardioprotection with an increased dose of the chelator was even more curious. With respect to these findings it should be noted that repeated administration of o-108 alone to rabbits in the same schedule had no distinct impact on either the morphology or function of the heart or other organs even in the dose of 100 mg/kg (Sterba et al., 2005). The findings from the present study cannot be attributed to inter-individual or seasonal variability, since these animals were randomized for o-108 co-treatment and the same experimental conditions were employed. Moreover, the model used in this study is experienced to be well-reproducible (Gersl et al., 1994, Simunek et al., 2004). The authors rather suggest that it is a causal observation, since a similarly unusual pattern of dose-response relationship was also witnessed previously during an extension of our study of cardioprotective effect of another aroylhydrazone iron chelator - pyridoxal isonicotinoyl hydrazone (PIH). While 25 mg/kg of PIH in our model appeared to have positive effects on DAU-induced mortality and cardiac function (Simunek et al., 2004), further escalation of the PIH dose to 50 mg/kg resulted in negative outcomes in the term of both overall mortality and cardioprotective effects (Gersl et al., 2004).

Several hypotheses might be proposed to explain the findings described above. *E.g.*, a very recent study on the pharmacokinetics of o-108 (Kovarikova et al., 2006) and salicylaldehyde isonicotinoyl hydrazone (SIH, another aroylhydrazone iron chelator with high antioxidative potential (Šimůnek et al., 2005a)) suggested that these chelators generally

possess relatively short terminal half-lives of elimination (Kovarikova et al., 2005). This might be a limitation in the light of the relatively long stay of anthracyclines and their metabolites in myocardium (Cusack et al., 1995). Our results suggest that the chelator administered at the optimal dose may chelate the intracellular labile iron pool inside the cardiomyocytes and thereby afford meaningful cardioprotection. Nevertheless, further boosting of the cardioprotective efficacy might rather require a longer intracellular half-life instead of its higher peak concentrations, as in this case some perturbations in the cellular iron metabolism in the cardiomyocytes already compromized with an anthracycline may appear.

Recent studies suggest that anthracyclines (and/or their 13-OH metabolites) are able to significantly impair cellular iron homeostasis (Kwok and Richardson, 2004, Xu et al., 2005). Kwok and Richardson (2003) demonstrated that anthracyclines are capable to cause marked perturbations in iron storage in ferritin and its subsequent release. It is plausible that very intensive chelation, such as that induced with ICL670 treatment (Hasinoff et al., 2003) or with high doses of aroylhydrazones, might “overshoot” the optimal degree of chelation and subsequently contribute to the iron metabolism misbalance caused by anthracyclines. As this effect might be associated with the peak concentrations, perhaps it could be helpful to administer aroylhydrazones by longer infusions or in smaller doses before and possibly also after anthracyclines. Furthermore, even better might be to improve the pharmacokinetic properties of o-108 through mild modification of its chemical structure.

In conclusion, the present study has shown that the novel iron chelator o-108 does not have any negative impact on the antiproliferative efficacy of daunorubicin. Moreover, the chelator itself has moderate antiproliferative effects which are additive to those of DAU. Furthermore, besides dexrazoxane, this study is the first to support iron chelation concept as an effective cardioprotective strategy against chronic type of anthracycline cardiotoxicity: Administration of o-108 (10 mg/kg) was able to completely overcome the daunorubicin-

induced mortality along with marked improvement in cardiac function and morphology. However, the surprising dose dependency experienced in this study suggests that the role of iron in this process might be more complex than originally supposed. Hence, further studies of iron chelation-based cardioprotection are needed in order to determine the potential of this approach as well as for obtaining deeper insight into the pathogenesis of the anthracycline cardiotoxicity.

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Footnotes

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Legend for figures

Fig. 1. Chemical structure of pyridoxal 2-chlorobenzoyl hydrazone (o-108).

Fig. 2. Cancer cell proliferation studies. A. Effect of o-108 on proliferation of HL-60 promyelocytic leukemia cell line. Absolute and relative cell numbers at the end of 72h incubation. B. Effects of o-108 on antiproliferative action of daunorubicin (2.5 nmol/L). Statistical significance (ANOVA; $P< 0.05$) c: comparison with control group; d – comparison with DAU 2.5 nM group; n=4 independent experiments for each group.

Fig. 3. Kaplan-Meier survival analysis. DAU – daunorubicin.

Fig. 4. Body weight changes during the study. Statistical significance within each group ($P<0.05$, paired t-test): (↓) a statistically significant decrease or (i) an insignificant body weight change in comparison with the initial values. Where no symbol is given: a significant increase. Statistical significance (ANOVA, $P<0.05$) in comparison with: (c) control group, (v) vehicle group, (o) o-108 25 mg/kg (alone), (d) daunorubicin group, (1) o-108 10 mg/kg+daunorubicin. DAU – daunorubicin.

Fig. 5. Left ventricular ejection fraction (LVEF) during the experiment. Statistical significance ($P<0.05$), paired t-test: (*) comparison with the initial values within each group. Statistical significance ($P<0.05$, ANOVA) in comparison with: (c) control group, (v) vehicle group, (o) o-108 25 mg/kg (alone), (1) o-108 10 mg/kg+daunorubicin, DAU – daunorubicin.

Fig. 6. Left ventricular contractility (dP/dt_{max}) at the end of the experiment. Statistical significance ($P<0.05$, ANOVA): comparison with groups: (c) control, (v) vehicle, (o) o-108 25 mg/kg (alone), (d) daunorubicin, (2) o-108 25 mg/kg+daunorubicin. DAU - daunorubicin.

Fig. 7. Scatterplot of the left ventricular contractility (dP/ dt_{max}) versus left ventricular ejection fraction (LVEF). dP/dt_{max} index of contractility obtained invasively, LVEF – left ventricular ejection fraction obtained non-invasively through echocardiographic examination, DAU – daunorubicin, R – correlation coefficient, p - statistical significance determined by the Spearman correlation analysis.

Fig. 8. Cardiac troponin T plasma concentrations during the experiment. Statistical significance (P<0.05, ANOVA) in comparison with: (c) control group, (v) vehicle group, (o) o-108 25 mg/kg (alone), (1) o-108 10 mg/kg+daunorubicin, DAU – daunorubicin.

Fig. 9. The myocardium of the left-ventricular wall.

a – ***Control group:*** Intact myocardium; typical cross-striated myofibrils fill in the cytoplasm of cardiomyocytes except for the endoplasm (a pale-stained region) around the nucleus; rich capillary network around the columns of cardiomyocytes with chains of erythrocytes marked the boundaries between adjacent cells.

b – ***Vehicle:*** Very mild signs of myocardial damage come through the presence of myocytes with intensely eosinophilic (dark-stained) cytoplasm, in which here and there the pyknotic nucleus appears; necrotic cells are present only rarely.

c – ***o-108 (25 mg/kg):*** Similar changes a in the previous group – scattered groups of cardiomyocytes with intensely eosinophilic (dark-stained) cytoplasm represent only fine changes within the myocardium.

d ***– Daunorubicin (3 mg/kg)****:* Massive focal toxic damage of the heart is depicted by large groups of degenerating cells (the majority of cells in the picture) to necrotic cells (N) frequently accompanied with a mononuclear infiltrate (M); this damage is so profound that the following formation of the granular tissue (G) with vessels, which subsequently becomes mature fibrotic scar tissue (F), is not sufficient and the process results in partial focal disintegration of the myocardium (compare it with all other groups).

e – ***o-108 (10 mg/kg) + DAU:*** Although the character of myocardial damage is similar to the DAU group, the extent and intensity of this injury, as well as the amount of the granular (G) and following fibrotic scar tissue (F) are markedly less expressed, and thus, the integrity of the myocardial tissue is not compromized; D - degenerating cell; N - necrotic cell.

f – ***o-108 (25 mg/kg) + DAU:*** The myocardial injury in this group is more comparable with that after the treatment with DAU alone, i.e. a large number of degenerating and necrotic (N) cells, and the fibrotic tissue (F) are observed; on the other hand, the granular tissue (G) is of a smaller amount here; the basic difference, compared with the previous group, is that the disintegration of the myocardial tissue is less prominent in this case; darker-stained cells are those with intensely eosinophilic cytoplasm.

Masson's blue trichrome; Bar 30 μm.

Fig. 10. The cortex of the kidney.

a – ***Control group:*** Normal appearance of the cortex – a part of the glomerulus (G) surrounded by Bowman's capsule with a slit for primary urine between its both layers; proximal (P) and distal (Di) convoluted tubules.

b – ***Vehicle:*** The hyaline degeneration of epithelial cells, depicted by increased eosinophilia of the cytoplasm (because of small eosinophilic granules - the remnants of the mitochondria) is present in most of the proximal tubules (P) but also in many distal tubules (Di) – here, all tubules reveal hyaline degeneration; here and there larger groups of necrotic cells are present in the epithelium and their remnants appear within the lumina of the tubules; G – glomerulus.

c – ***o-108 (25 mg/kg):*** The character of morphological changes is comparable with the previous group but they are less expressed; here and there the hyaline casts (H) are present within the lumina of the tubules (here in the proximal tubule); Di – distal tubules lined with undamaged epithelium; G – glomerulus.

d – ***Daunorubicin (3 mg/kg):*** Acute focal tubular nephritis characterized by the presence of numerous necrotic cells in both proximal (P) and distal (Di) tubules; the remaining lining epithelia reveals hyaline degeneration (dark-stained cells); G – glomerulus.

e – ***o-108 (10 mg/kg) + DAU:*** Protective influence of o-108 in co-administration with DAU is well-visible, despite the presence of hyaline degeneration of different intensity in most of the proximal, and less in the distal (not shown) convoluted tubules; only a small number of necrotic cells (x), and scattered hyaline cast (H) are present; G – glomerulus.

f – ***o-108 (25 mg/kg) + DAU:*** Similar extent of the damage as in the previous group; most of the tubules reveal the hyaline degeneration of the epithelium, scatter dark pyknotic nuclei are also present in the epithelial cells; P - proximal tubule, Di – distal tubule, G – glomerulus.

Masson's blue trichrome; Bar 20 μm

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Table 1. Invasive hemodynamic measurements: blood pressure (BP) and heart rate (HR).

Group	BP (mmHg)	HR (min^{-1})
Control	105.1 ± 3.0	306.7 ± 6.3
Vehicle	99.2 ± 4.1	309.2 ± 6.1
o-108 25 mg/kg	87.1 ± 3.5	320.5 ± 6.5
Daunorubicin	79.0 ± 4.0 **cv1**	270.4 ± 10.4 **cvo**
o-108 10 mg/kg+DAU	94.0 ± 3.0	277.4 ± 5.6 **vo**
o-108 25 mg/kg+DAU	84.6 ± 10.7 **c**	262.2 ± 6.8 **cvo**

Statistical significance ($P<0.05$), ANOVA: comparison with groups: (c) control, (v) vehicle, (o) o-108 25 mg/kg (alone), (1) o-108 10 mg/kg+daunorubicin, DAU – daunorubicin.

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Table 2. Selected biochemical parameters.

Parameter/group	Beginning of the study	Before the 5th administration	End of the study
Iron (μmol/L)			
Control	40.9 ± 3.4	34.9 ± 2.6	46.1 ± 1.8
Vehicle	34.2 ± 1.7	32.1 ± 2.1	43.7 ± 2.7*
o-108 25 mg/kg	36.5 ± 4.2	35.5 ± 4.6	42.8 ± 3.6
DAU	29.8 ± 3.7	24.2 ± 2.2c	15.4 ± 2.6*cvo
10 mg/kg o-108+DAU	28.2 ± 2.3	19.9 ± 1.9*cvo	22.8 ± 2.0*cvo
25 mg/kg o-108+DAU	54.0 ± 2.4vd1	20.9 ± 3.0*cvo	17.2 ± 2.1*cvo
Creatinine (μmol/L)			
Control	112.7 ± 4.5	112.6 ± 6.6	119.6 ± 8.1
Vehicle	109.1 ± 4.8	106.4 ± 4.8	111.3 ± 3.8
o-108 25 mg/kg	105.0 ± 1.5	115.0 ± 5.6	150.8 ± 8.7*
DAU	99.4 ± 4.4	108.8 ± 4.9	163.8 ± 25.6*
10 mg/kg o-108+DAU	117.4 ± 3.9	128.5 ± 3.6*	134.4 ± 4.2*
25 mg/kg o-108+DAU	98.1 ± 8.3	136.4 ± 15.8*	213.8 ± 24.3*cv
Protein (g/L)			
Control	60.8 ± 1.4	61.3 ± 1.6	62.7 ± 1.2
Vehicle	62.8 ± 1.4	62.9 ± 1.1	60.1 ± 0.9*
o-108 25 mg/kg	60.1 ± 1.1	60.9 ± 1.6	57.3 ± 4.4
DAU	62.3 ± 0.9	62.7 ± 1.2	47.5 ± 1.5*cv
10 mg/kg o-108+DAU	60.2 ± 0.7	66.4 ± 1.8*2	51.8 ± 1.1*c
25 mg/kg o-108+DAU	63.7 ± 1.0	58.6 ± 1.0	42.2 ± 1.8*cv
Albumin (g/L)			
Control	55.0 ± 0.9	53.9 ± 0.9	55.3 ± 0.9
Vehicle	56.5 ± 0.7	55.5 ± 0.8	53.4 ± 0.9*
o-108 25 mg/kg	51.0 ± 2.8	52.6 ± 2.9	49.2 ± 3.3
DAU	57.2 ± 1.0	57.3 ± 0.9	33.1 ± 3.6*cv
10 mg/kg o-108+DAU	57.2 ± 0.7	58.3 ± 1.4d2	41.9 ± 1.7*c
25 mg/kg o-108+DAU	61.0 ± 1.3	50.3 ± 2.3*	27.2 ± 1.6*cv
Cholesterol (μmol/L)			
Control	1.81 ± 0.49	1.53 ± 0.31	0.97 ± 0.17*
Vehicle	1.51 ± 0.15	1.16 ± 0.16*	1.15 ± 0.17
o-108 25 mg/kg	1.73 ± 0.42	1.71 ± 0.37	1.19 ± 0.25
DAU	1.16 ± 0.16	1.44 ± 0.26	2.50 ± 0.35*c
10 mg/kg o-108+DAU	1.81 ± 0.30	2.90 ± 0.43v	3.04 ± 0.43*cv
25 mg/kg o-108+DAU	1.89 ± 0.20	4.59 ± 0.78*cvd	7.65 ± 3.22cvo
TAG (mmol/L)			
Control	0.95 ± 0.11	1.18 ± 0.14v	0.92 ± 0.05
Vehicle	0.85 ± 0.09	0.63 ± 0.05*	0.79 ± 0.06
o-108 25 mg/kg	0.85 ± 0.14	0.77 ± 0.04	1.14 ± 0.27
DAU	0.84 ± 0.07	0.91 ± 0.07	2.11 ± 0.42*v
10 mg/kg o-108+DAU	0.63 ± 0.06	1.12 ± 0.27*	1.77 ± 0.29*v
25 mg/kg o-108+DAU	0.86 ± 0.15	2.33 ± 0.53*v	8.78 ± 4.68cv

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ALT (µkat/L)			
Control	1.19 ± 0.15	1.22 ± 0.18	0.99 ± 0.12
Vehicle	1.51 ± 0.24	1.19 ± 0.12	0.92 ± 0.05*
o-108 25 mg/kg	1.91 ± 0.31	1.40 ± 0.13	1.13 ± 0.13*
DAU	1.10 ± 0.10	1.09 ± 0.12	0.88 ± 0.19
10 mg/kg o-108+DAU	1.08 ± 0.13	0.94 ± 0.14	0.74 ± 0.08*
25 mg/kg o-108+DAU	1.05 ± 0.11	0.80 ± 0.07	0.73 ± 0.11*
AST (µkat/L)			
Control	0.60 ± 0.11	0.75 ± 0.17	0.68 ± 0.06
Vehicle	0.67 ± 0.09	0.52 ± 0.05	0.68 ± 0.04
o-108 25 mg/kg	0.70 ± 0.13	0.56 ± 0.09	0.70 ± 0.05
DAU	0.66 ± 0.11	0.64 ± 0.06	0.85 ± 0.22
10 mg/kg o-108+DAU	0.46 ± 0.07	0.59 ± 0.16	0.52 ± 0.04
25 mg/kg o-108+DAU	0.82 ± 0.09	0.45 ± 0.11*	0.48 ± 0.03*
ALP (µkat/L)			
Control	2.16 ± 0.32	2.09 ± 0.26	1.93 ± 0.21
Vehicle	2.73 ± 0.46	2.10 ± 0.32*	1.73 ± 0.23*
o-108 25 mg/kg	2.28 ± 0.23	2.11 ± 0.30	1.76 ± 0.26
DAU	2.78 ± 0.32	2.35 ± 0.17	0.86 ± 0.12*
10 mg/kg o-108+DAU	2.53 ± 0.44	2.27 ± 0.36	1.79 ± 0.37*
25 mg/kg o-108+DAU	3.40 ± 0.40	1.49 ± 0.26*	0.73 ± 0.03*

Statistical significance ($P<0.05$): Paired t-test): (*) paired comparison with the initial values within each group. ANOVA: comparison with: (c) control group, (v) vehicle group, (o) o-108 25 mg/kg (alone), (d) daunorubicin group, (1) o-108 10 mg/kg+DAU, (2) o-108 25 mg/kg+DAU, DAU – daunorubicin.

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Table 3. Hematological parameters.

Parameter/group	Beginning of the study	Before the 5th administration	End of the study
Leukocytes (x 10^9/L)			
Control	7.1 ± 0.5	6.5 ± 0.7	5.1 ± 0.5*
Vehicle	6.6 ± 0.3	6.0 ± 0.3*	5.5 ± 0.7
o-108 25 mg/kg	6.9 ± 0.2	6.3 ± 0.6	5.1 ± 0.7
DAU	5.4 ± 0.3	4.4 ± 0.4	3.3 ± 0.5*v
10 mg/kg o-108+DAU	6.8 ± 0.9	6.2 ± 0.9	2.7 ± 0.3*cv
25 mg/kg o-108+DAU	5.1 ± 0.5	5.3 ± 0.8	3.0 ± 0.7
Erythrocytes (x 10^{12}/L)			
Control	5.72 ± 0.18	6.00 ± 0.10	5.83 ± 0.12
Vehicle	5.84 ± 0.18	5.56 ± 0.14*	5.68 ± 0.12
o-108 25 mg/kg	5.68 ± 0.19	5.60 ± 0.15	5.34 ± 0.18
DAU	6.13 ± 0.19	4.49 ± 0.13*cvo	4.00 ± 0.23*cv
10 mg/kg o-108+DAU	6.11 ± 0.20	5.13 ± 0.28*	4.51 ± 0.24*cv
25 mg/kg o-108+DAU	6.38 ± 0.20	3.98 ± 0.43*cvo1	4.01 ± 0.27*cv
Hemoglobin (g/L)			
Control	125.3 ± 3.4	132.9 ± 1.8*	130.4 ± 1.8
Vehicle	128.1 ± 3.1	126.7 ± 2.9	129.5 ± 2.2
o-108 25 mg/kg	120.3 ± 5.5	118.8 ± 2.7	118.3 ± 5.2
DAU	131.5 ± 2.8	100.9 ± 2.9*cv	83.7 ± 4.7*cvo1
10 mg/kg o-108+DAU	130.0 ± 3.8	108.7 ± 6.7*	98.9 ± 4.8*cvo
25 mg/kg o-108+DAU	136.8 ± 3.4	87.8 ± 9.9*cv	83.0 ± 4.9*cvo
Hematocrit			
Control	0.385 ± 0.012	0.417 ± 0.007*	0.402 ± 0.007
Vehicle	0.399 ± 0.009	0.393 ± 0.008	0.391 ± 0.008
o-108 25 mg/kg	0.379 ± 0.013	0.374 ± 0.009	0.373 ± 0.017
DAU	0.393 ± 0.010	0.317 ± 0.008*c	0.273 ± 0.016*cvo1
10 mg/kg o-108+DAU	0.413 ± 0.010	0.351 ± 0.023*	0.334 ± 0.016*cv
25 mg/kg o-108+DAU	0.429 ± 0.008	0.294 ± 0.034*c	0.279 ± 0.013*cvo
MCV (fL)			
Control	67.3 ± 0.9	69.6 ± 1.1	69.0 ± 0.7
Vehicle	68.8 ± 1.1	70.9 ± 0.8*	69.0 ± 1.0
o-108 25 mg/kg	66.8 ± 0.4	66.8 ± 1.1	69.9 ± 1.3
DAU	60.0 ± 4.5	70.7 ± 0.9*	68.3 ± 1.5*
10 mg/kg o-108+DAU	67.6 ± 0.9	68.4 ± 0.8	74.1 ± 1.2*d
25 mg/kg o-108+DAU	67.5 ± 1.1	67.2 ± 6.0	70.1 ± 2.4
RDW (%)			
Control	15.6 ± 0.4	13.7 ± 0.3*	13.9 ± 0.3*
Vehicle	13.8 ± 0.6	13.4 ± 0.3	14.4 ± 0.5
o-108 25 mg/kg	13.6 ± 0.8	14.9 ± 0.8*	15.1 ± 0.2
DAU	16.2 ± 0.6v1	21.1 ± 0.9*cv	24.0 ± 0.7*cv
10 mg/kg o-108+DAU	13.7 ± 0.2	19.3 ± 1.2*	21.7 ± 1.2*cv
25 mg/kg o-108+DAU	14.9 ± 0.4	20.7 ± 1.5*cv	26.4 ± 1.2*cv
Trombocytes (x 10^9/L)			

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Control	469 ± 57	399 ± 28	445 ± 38
Vehicle	330 ± 21	352 ± 19	370 ± 31
o-108 25 mg/kg	327 ± 23	448 ± 73	355 ± 29
DAU	394 ± 44	331 ± 44	306 ± 65
10 mg/kg o-108+DAU	230 ± 27c	221 ± 60	287 ± 36
25 mg/kg o-108+DAU	205 ± 15cd	189 ± 81	252 ± 60

Statistical significance (P<0.05, paired t-test): (*) paired comparison with the initial values. Statistical significance (P<0.05, ANOVA) in comparison with: (c) control group, (v) vehicle group, (o) o-108 25 mg/kg (alone), (d) daunorubicin group, (1) o-108 10 mg/kg+DAU. MCV – mean cell volume, RDW - Red blood cell distribution, DAU – daunorubicin.

Fig. 1

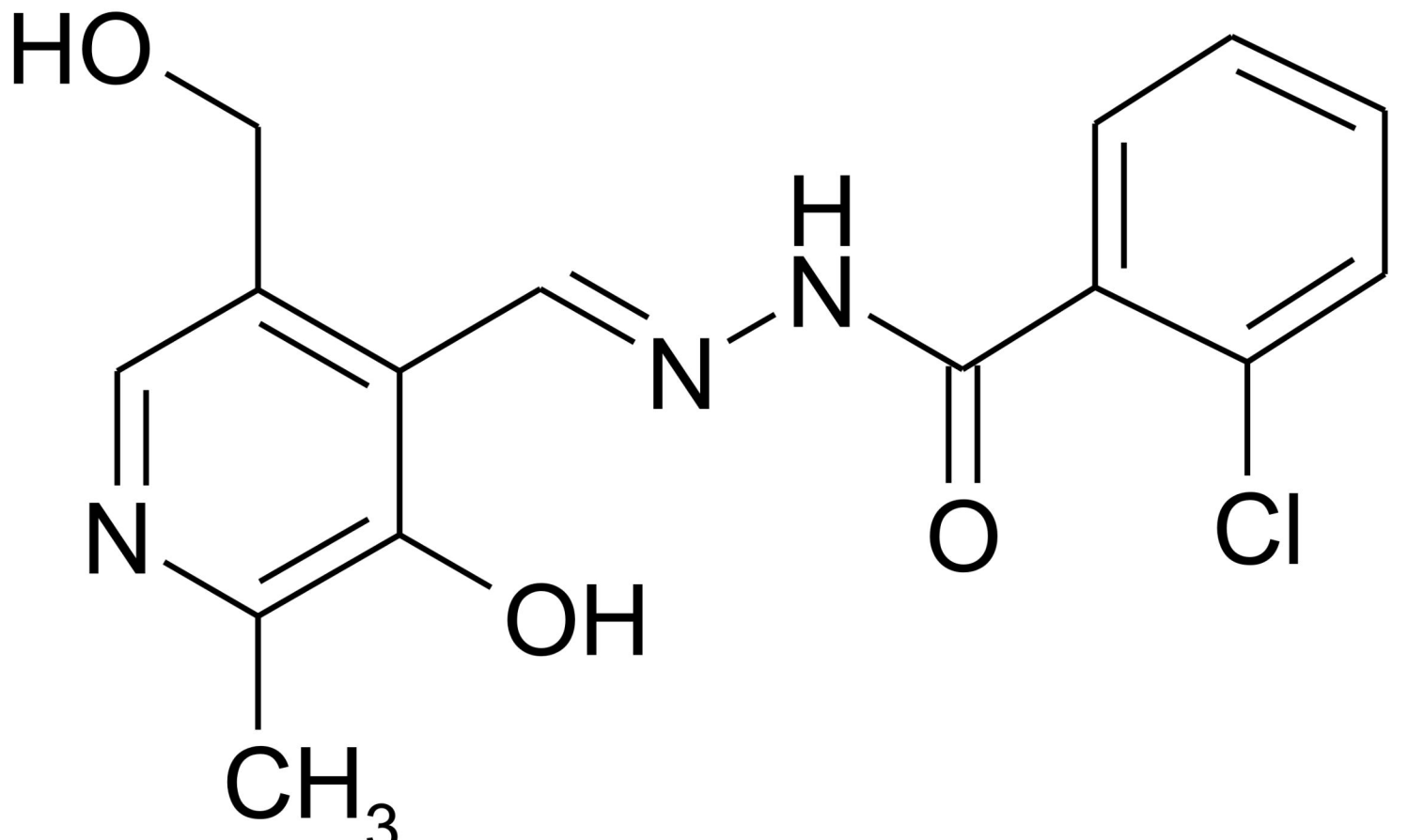
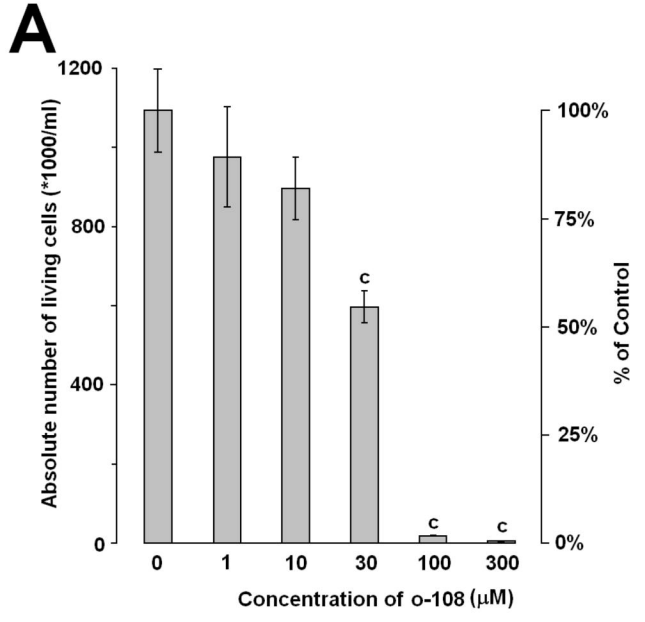


Fig. 2


A
1200
800
400
0
Absolute number of living cells (*1000/ml)
100%
75%
50%
25%
0%
% of Control
c
c
c
0
1
10
30
100
300
Concentration of o-108 (μM)

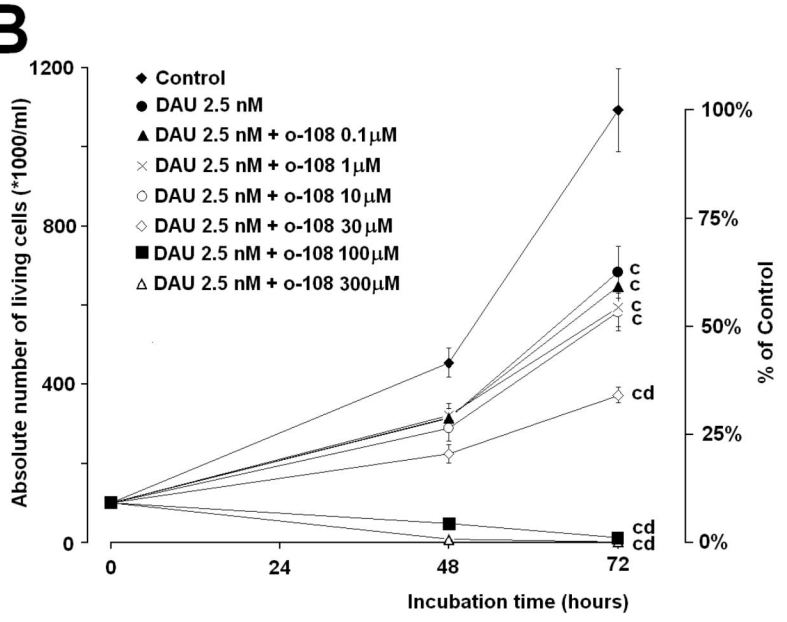

B
Control
DAU 2.5 nM
DAU 2.5 nM + o-108 0.1μM
DAU 2.5 nM + o-108 1μM
DAU 2.5 nM + o-108 10μM
DAU 2.5 nM + o-108 30μM
DAU 2.5 nM + o-108 100μM
DAU 2.5 nM + o-108 300μM
1200
800
400
0
Absolute number of living cells (*1000/ml)
100%
75%
50%
25%
0%
% of Control
c
c
c
c
cd
cd
cd
0
24
48
72
Incubation time (hours)

Fig. 3

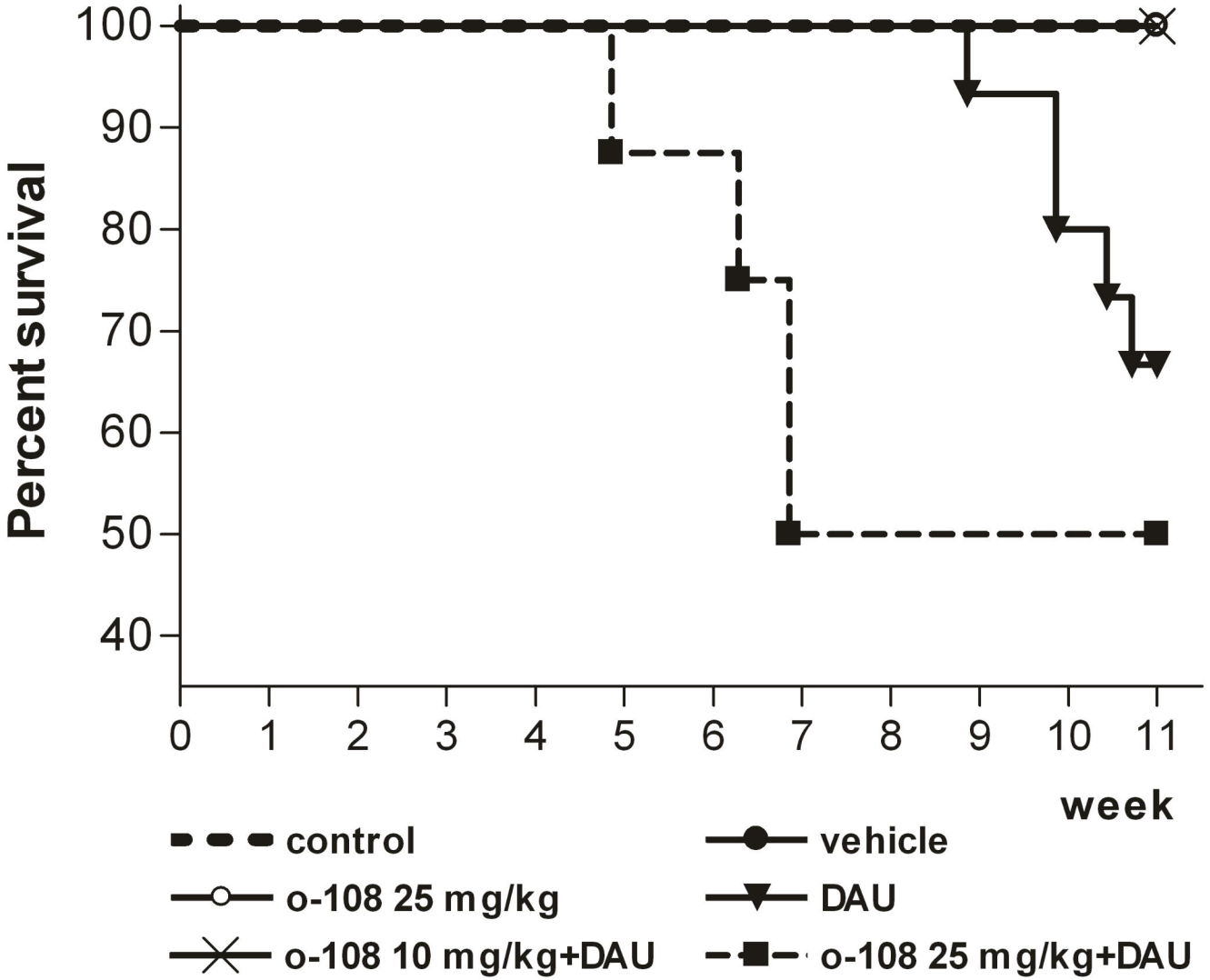

100
90
80
70
60
50
40
Percent survival
0
1
2
3
4
5
6
7
8
9
10
11
week
control
vehicle
o-108 25 mg/kg
DAU
o-108 10 mg/kg+DAU
o-108 25 mg/kg+DAU

Fig. 4

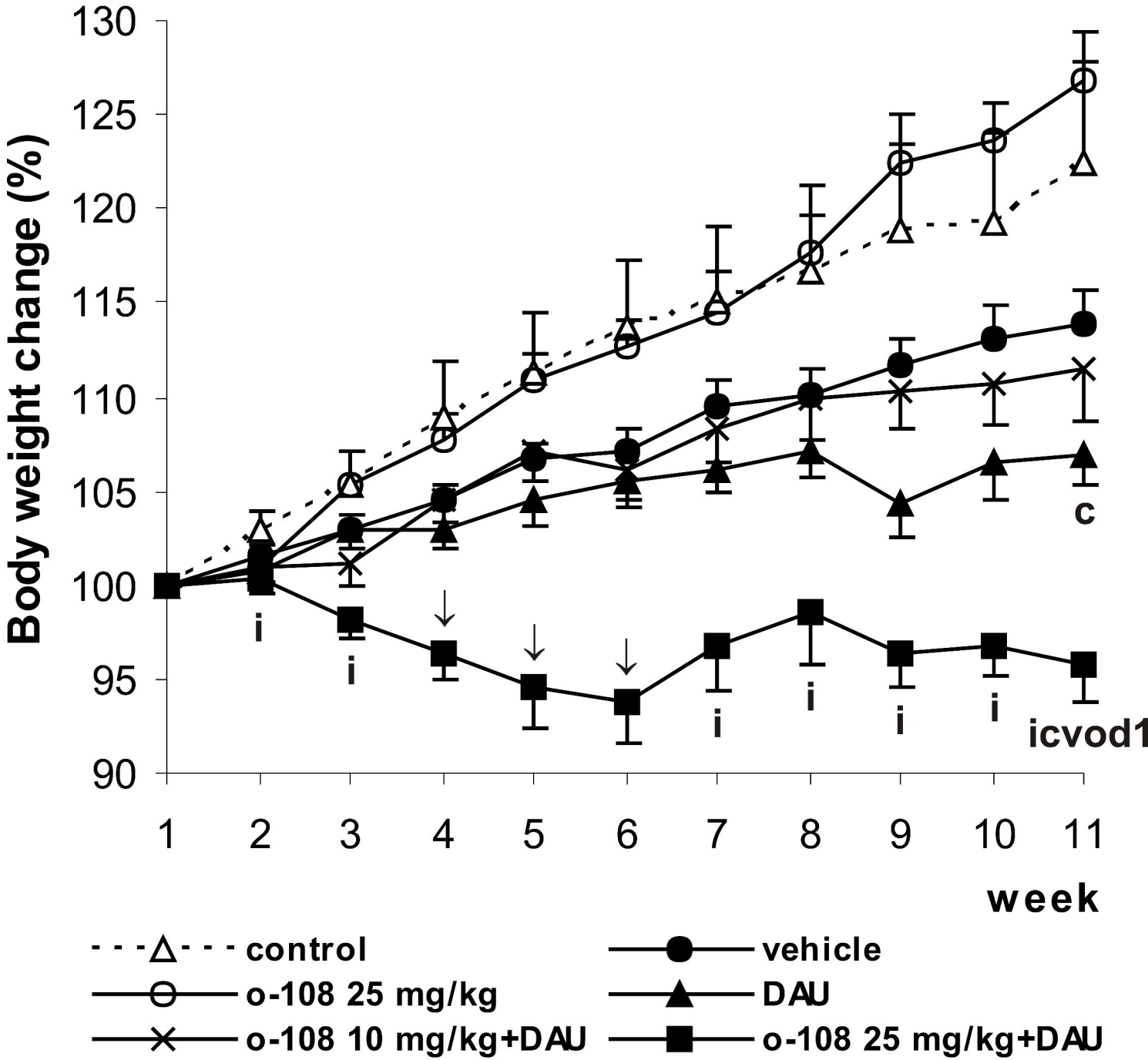

Body weight change (%)
130
125
120
115
110
105
100
95
90
1
2
3
4
5
6
7
8
9
10
11
week
i
i
i
i
i
i
c
icvod1
control
vehicle
o-108 25 mg/kg
DAU
o-108 10 mg/kg+DAU
o-108 25 mg/kg+DAU

Fig. 5

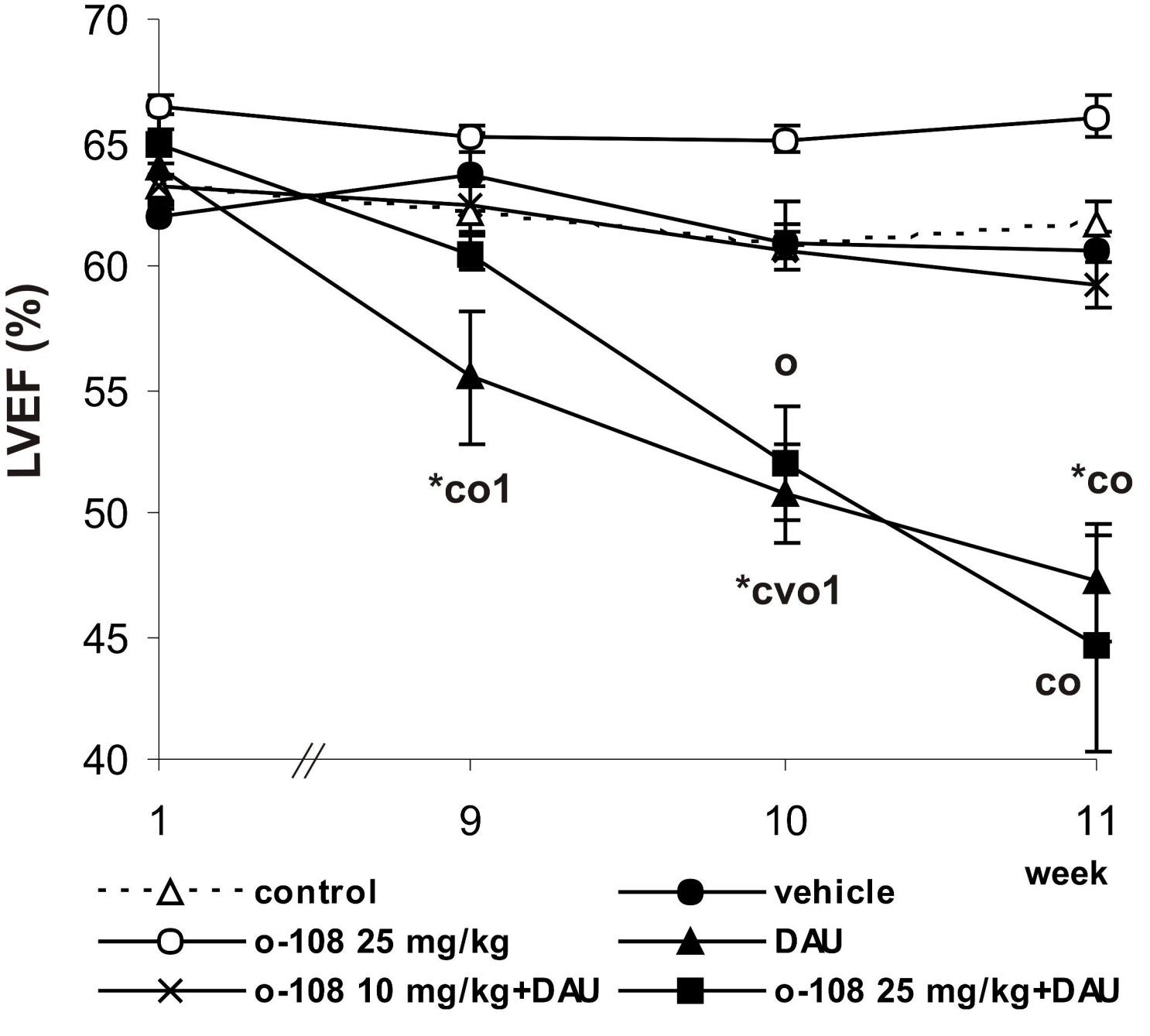

70
65
60
55
50
45
40
LVEF (%)
1
9
10
11
week
*co1
o
*cvo1
*co
co
control
vehicle
o-108 25 mg/kg
DAU
o-108 10 mg/kg+DAU
o-108 25 mg/kg+DAU

Fig. 6

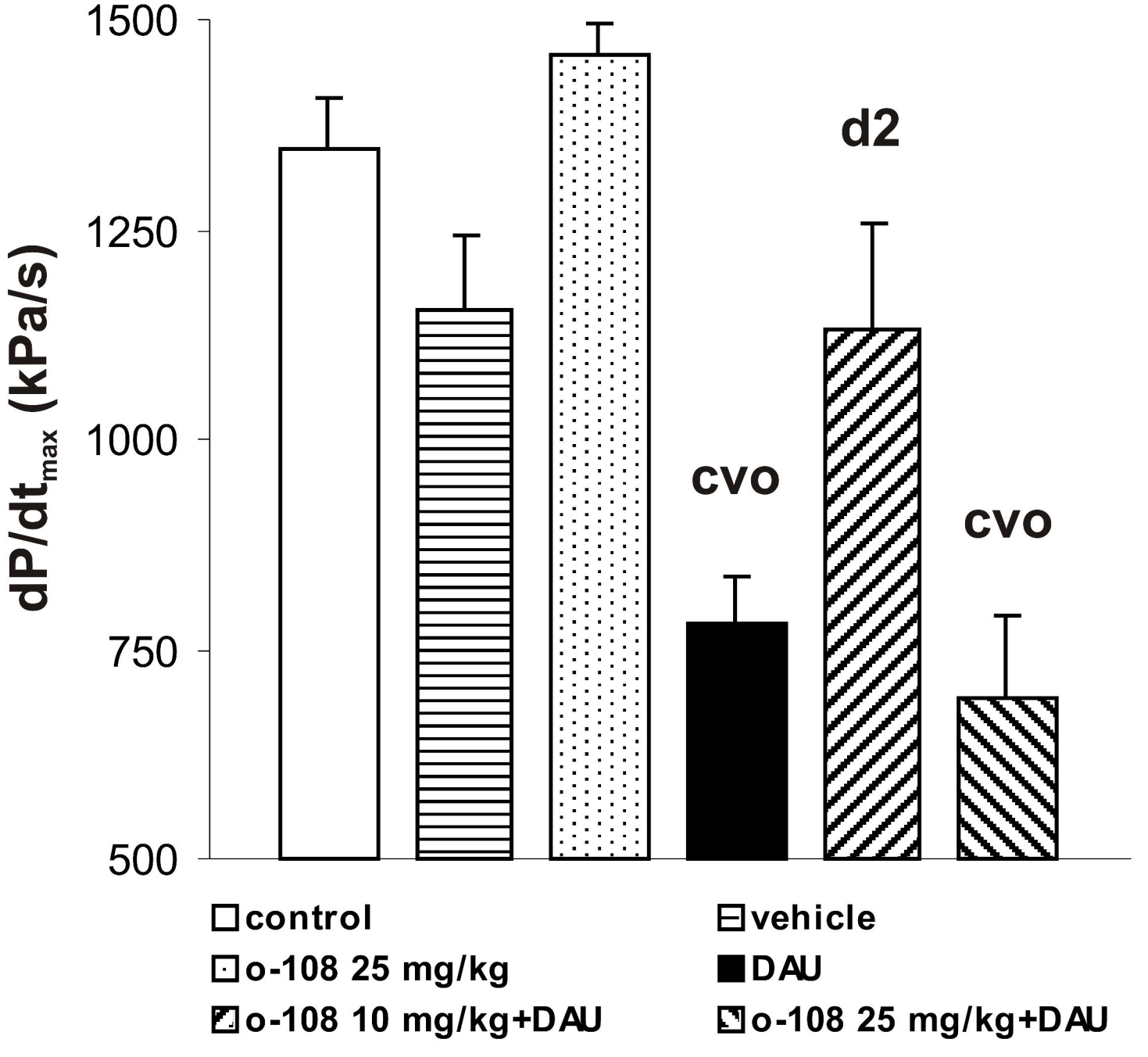

1500
1250
1000
750
500
dP/dt$_{max}$ (kPa/s)
d2
cvo
cvo
control
vehicle
o-108 25 mg/kg
DAU
o-108 10 mg/kg+DAU
o-108 25 mg/kg+DAU

Fig. 7

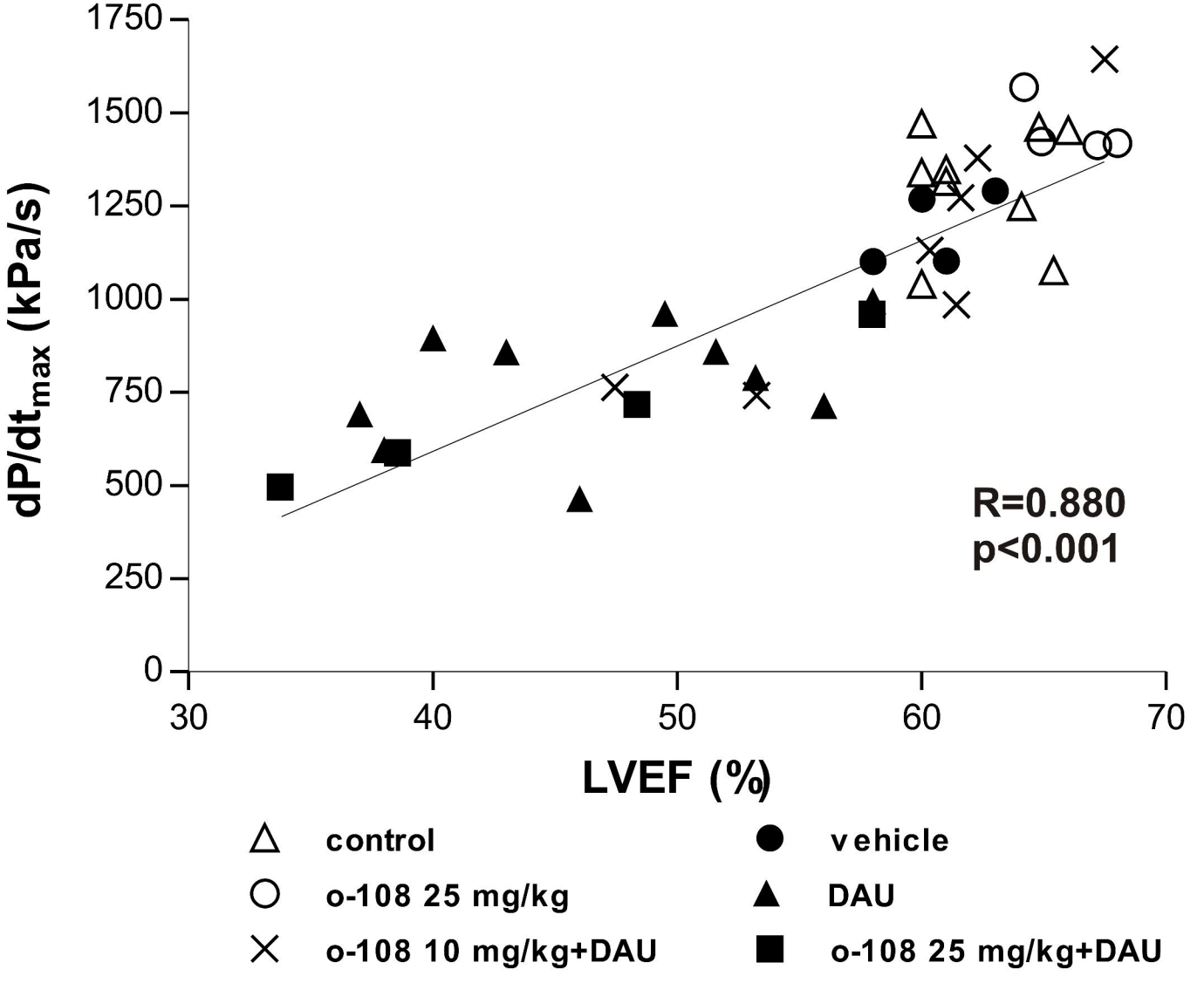

1750
1500
1250
1000
750
500
250
0
dP/dtmax (kPa/s)
30
40
50
60
70
LVEF (%)
R=0.880
p<0.001
control
vehicle
o-108 25 mg/kg
DAU
o-108 10 mg/kg+DAU
o-108 25 mg/kg+DAU

Fig. 8

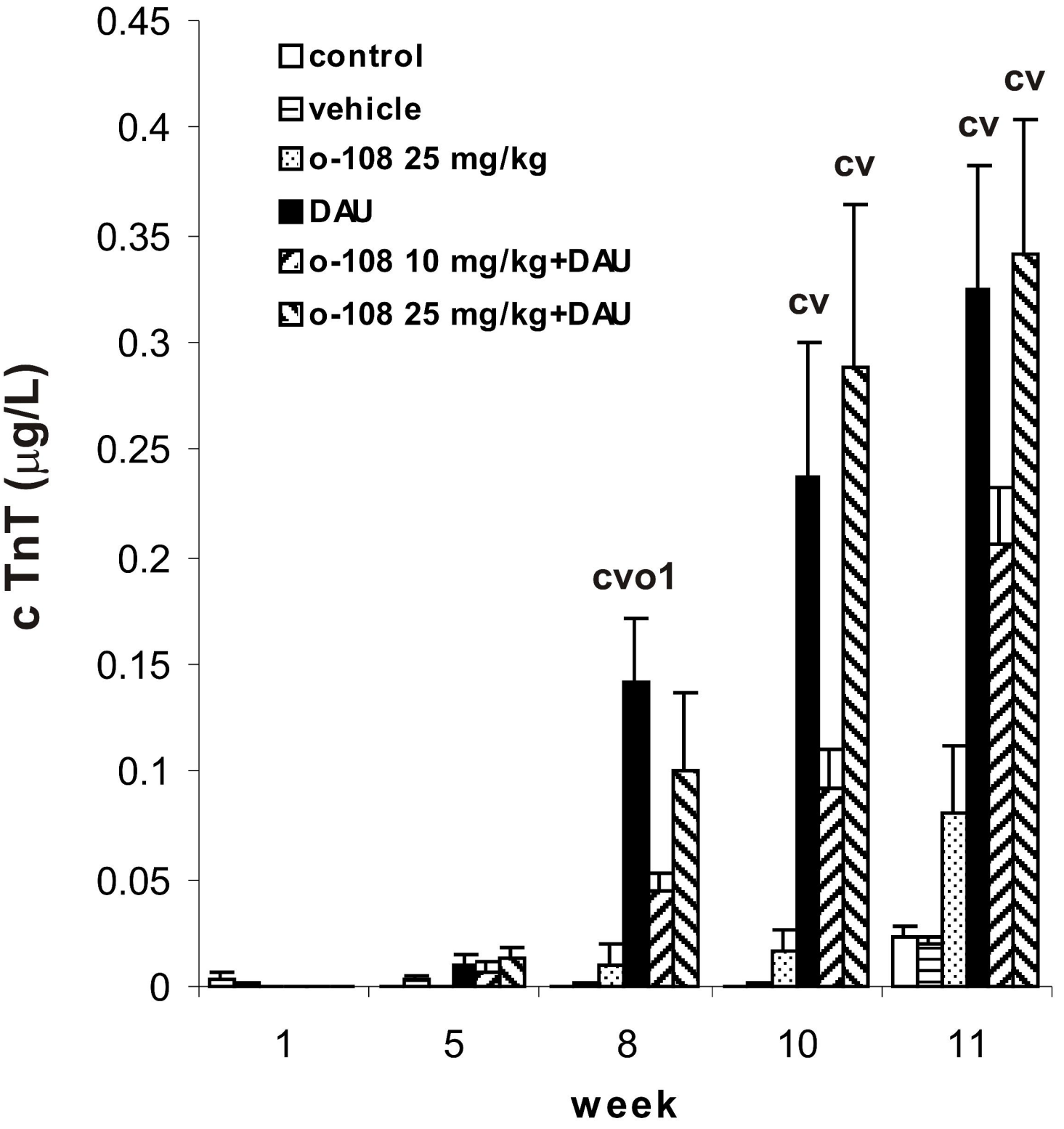

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
c TnT (μg/L)
control
vehicle
o-108 25 mg/kg
DAU
o-108 10 mg/kg+DAU
o-108 25 mg/kg+DAU
cvo1
cv
cv
cv
cv
1
5
8
10
11
week

Fig. 9

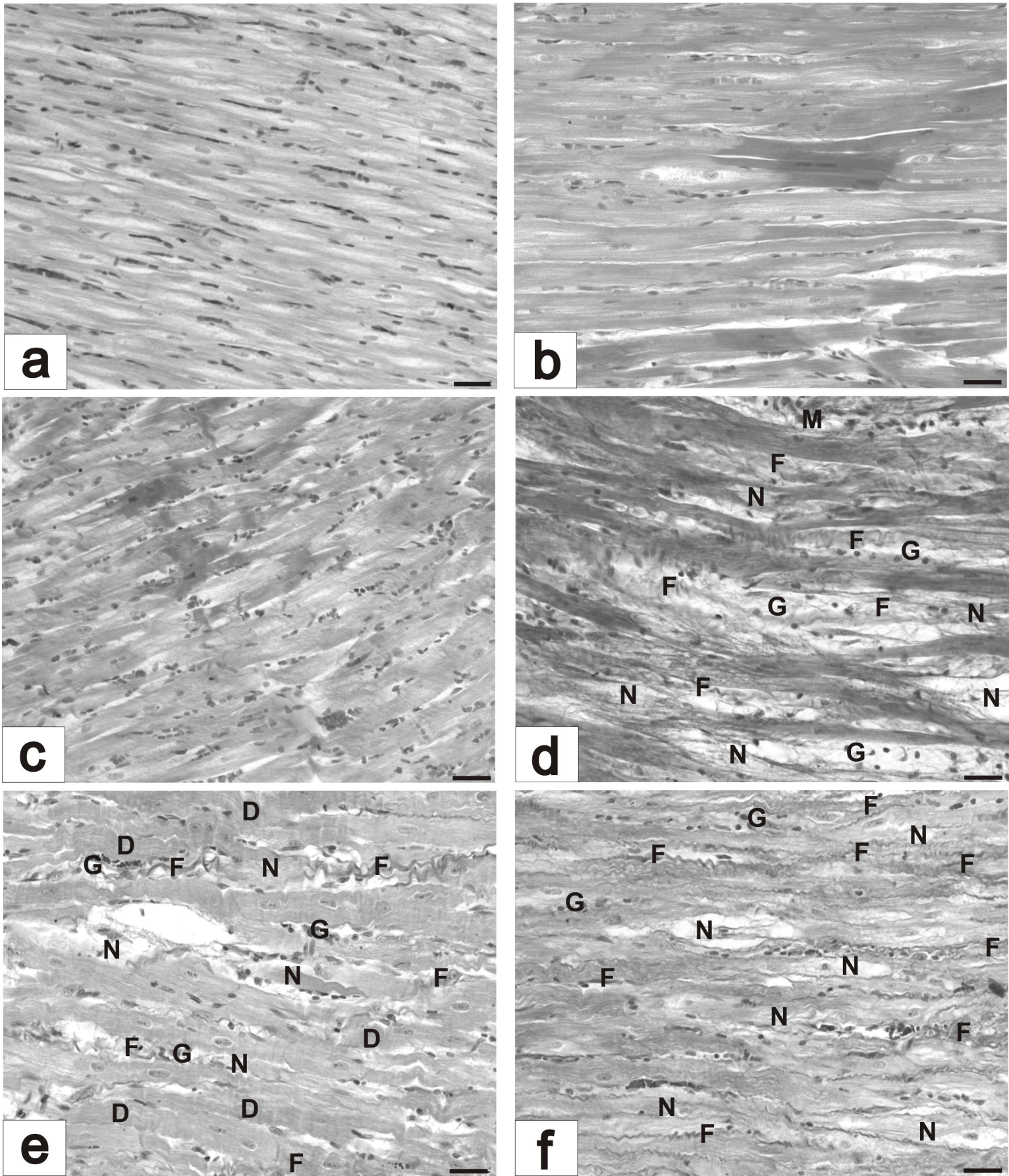
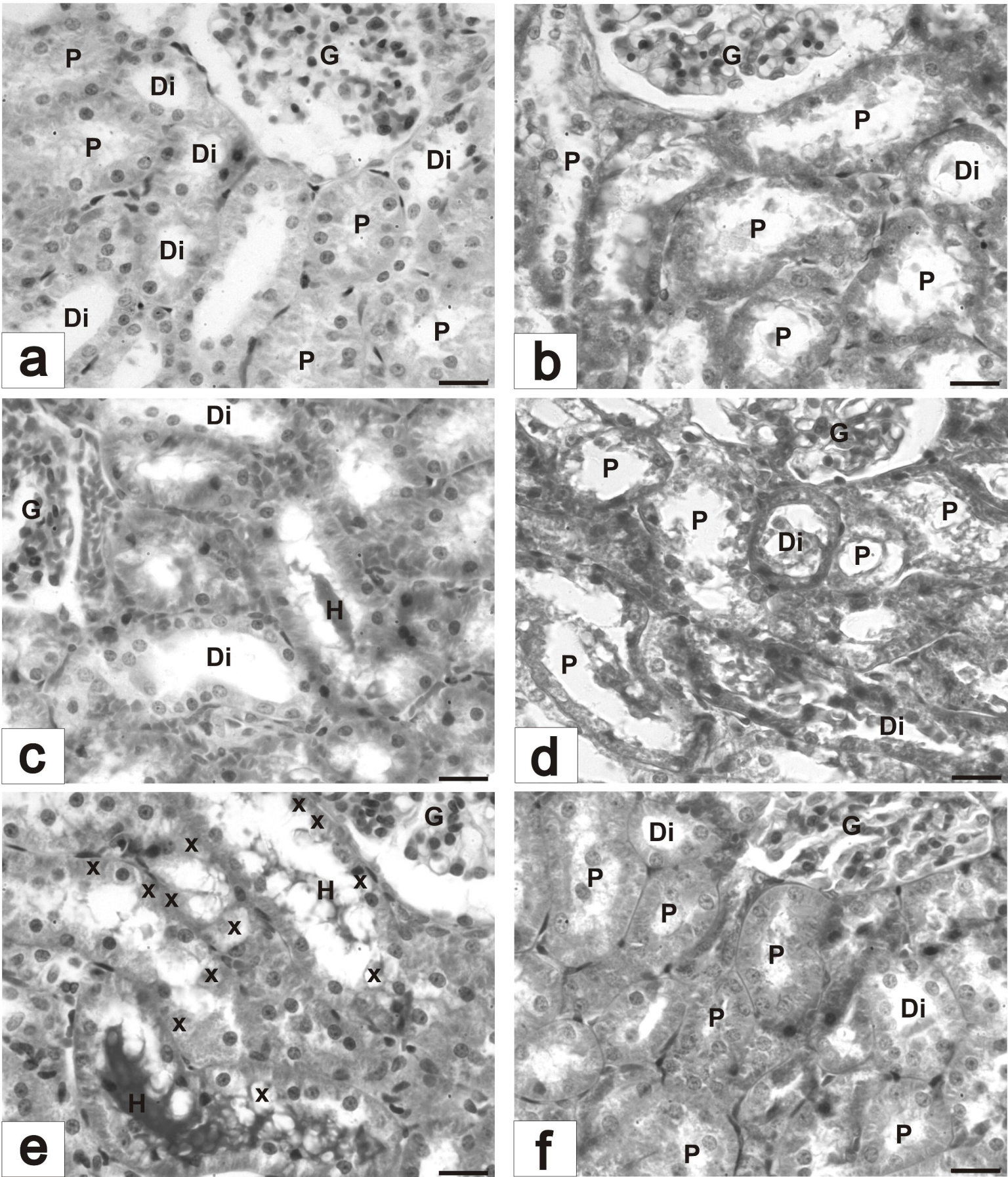


Fig. 10


P
Di
G
P
Di
Di
P
Di
Di
P
P
a
G
P
P
Di
P
P
P
P
b
Di
G
H
Di
c
P
G
P
P
Di
P
P
Di
d
x
x
G
x
x
H
x
x
x
x
x
x
x
H
x
e
Di
G
P
P
P
P
Di
P
P
f