

Evaluation of Cardiac Effects of the New Antineoplastic Drug – Dimethoxybenfluron – in the Rabbit

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Summary

Cardiotoxicity ranks among the most serious adverse effects of some cytostatics. The cardiac effects of repeated i.v. administration of a new antineoplastic agent, dimethoxybenfluron (once a week, 10 administrations), were investigated in rabbits with respect to cardiac function and the release of cardiac troponin T (cTnT). Different doses of dimethoxybenfluron were administered to two groups of animals (12 mg/kg; n = 7 and 24 mg/kg; n = 6) and compared with either a control group (saline 1 ml/kg; n = 6) or a group with experimentally induced cardiomyopathy (daunorubicin 50 mg/m²; n = 13). In daunorubicin-induced cardiomyopathy, cTnT levels in animals with premature deaths were significantly higher (0.31±0.11 µg/l) in comparison with the surviving animals (0.04±0.03 µg/l). However, cardiac TnT levels after the administration of dimethoxybenfluron in both doses were within the physiological range (lower than 0.1 µg/l) during the whole experiment as it was in the control group. The lack of cardiotoxicity of this new antineoplastic drug was supported by the absence of alterations in PEP:LVET ratio, left ventricle dP/dt_{max} or histological heart examination as well as by the fact that no premature death of animals occurred following repeated administration of dimethoxybenfluron. It is possible to conclude that no signs of cardiotoxicity were observed following repeated i.v. administration of dimethoxybenfluron.

Key words

Cardiac troponin T • Daunorubicin • Dimethoxybenfluron • Cardiomyopathy • Antineoplastic drugs

Introduction

Benfluron (5-[2-(N,N-dimethylamino)ethoxy]-7-oxo-7H-benzo[c]fluorene – the compound of U.S. patent

application Ser. No. 417.834) and some of its new derivatives developed in the Research Institute of Pharmacy and Biochemistry in Hradec Králové, were shown to possess significant antitumor, immuno-

suppressive, antibacterial, and antiviral activity (Mělka *et al.* 1982, Křepelka *et al.* 1983, Mělka *et al.* 1987, Mělka and Křepelka 1987). The main mechanism of their effects is based on the inhibition of the biosynthesis of nucleic acids and the biosynthesis of proteins (indicated by lowering the incorporation rate of precursors, i.e. thymidine, uridine, valine) in both P388 leukemia and Ehrlich cells *in vitro* (Miko *et al.* 1991). Moreover, they inhibited both cellular respiration and ATP production, leading to multiple secondary rearrangements in cellular metabolism (Miko *et al.* 1992). Benfluron also exhibited a very favorable therapeutic index, LD₅₀ being 678 mg/kg in mice and 1300 mg/kg in rats (Mělka and Křepelka 1987). All these above mentioned features led to an effort to synthesize new derivatives. Dimethoxybenfluron (3,9-dimethoxybenfluron hydrochloride, compound Code NO-1-B, Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Hradec Králové) is considered to be a potent intercalating antineoplastic drug. Its activity in neoplastic diseases in animals has been reported (e.g. in leukemia – Mělka 1995). The molecular formula of dimethoxybenfluron is C₂₃H₂₄O₄NCl and molecular weight 413.9.

The aim of the present study was to evaluate possible signs of cardiotoxicity of 3,9-dimethoxybenfluron hydrochloride. The long-term intravenous administration of daunorubicin in rabbits is considered to be a satisfactory animal model for anthracycline-induced cardiomyopathy (Geršl and Hrdina 1994, Geršl *et al.* 1996). In our study, this model was used to compare possible cardiac effects of dimethoxybenfluron with those of daunorubicin and control groups using the determination of the functional parameters of the left heart ventricle and cardiac troponin T (cTnT). Furthermore, we tried to determine the diagnostic performance of cardiac troponin T for the evaluation of cardiotoxic effects of new drugs.

Methods

Experimental animals

Medium size Chinchilla male rabbits (Velaz, Prague, Czech Republic, average body weight 3 kg at the beginning of the experiment) served as experimental animals. All experiments performed in this study were approved by the Ethical Committee of Charles University in Prague, Faculty of Medicine in Hradec Králové.

The effects of dimethoxybenfluron were investigated in two groups of animals with different doses

of the drug. Dimethoxybenfluron was administered to 7 animals in a dose of 12 mg/kg (once weekly, 10 administrations) and to 6 animals in a dose of 24 mg/kg (once weekly, 10 administrations). The results were compared with a control group (n=6, saline, 1.0 ml/kg according to the same schedule) and a group with experimentally induced cardiomyopathy (n=13, daunorubicin 50 mg/m², once weekly, maximally 10 administrations) constituting the reference group.

The doses of dimethoxybenfluron were calculated as equieffective and equitoxic doses to the doses used in mice (80 mg/kg i.v. in leukemia L 1210 – Mělka 1995), the dose of daunorubicin corresponded to the data of Geršl and Hrdina (1994). Drugs were injected intravenously (over 30 s) into the marginal ear vein. At the end of the follow-up period, animals were killed by i.v. administration of pentobarbital and the heart was excised for the evaluation of morphological changes.

Biochemical analysis

The venipunctures for biochemical examination were performed at the following time intervals of the experiment: 1) the initial control value before the first administration of the drug, 2) before the 5th administration, 3) 24 h after the 5th administration, 4) before the 8th administration, 5) 24 h after the 8th administration, 6) at the end of experiment (5-7 days after the last administration of the drug).

The concentration of cardiac troponin T in heparinized plasma samples was measured using an Elecsys Troponin T STAT Immunoassay. The electrochemiluminescence immunoassay ECLIA was performed on an Elecsys 2010 Immunoassay Analyzer (Roche) with the detection limit <0.010 µg/l. The value below this detection limit was considered to be zero.

Polygraphic recordings

The polygraphic recordings of systolic time intervals were obtained in previously anesthetized and restrained supine rabbits (anesthesia with ketamine 50 mg/kg i.m.) at the beginning, before the 5th administration, and then during the experiment (at weekly intervals from the 7th administration). Phonocardiogram recordings, carotid pulse waveforms and standard limb leads for ECG detection were recorded using a polygraph Biomedica C6b (Italy) with adequate transducers (ECG Preamp. model KAG-Ec, High gain Preamp. model KAG-Eb, Pulse-PléthysmoPreamp. model KPL-Eb). The absolute values of PEP:LVET ratio were calculated as the

ratio of PEP (pre-ejection period – measured as a difference between electromechanical systole Q-2 and LVET) and LVET (left ventricular ejection time – interval between the beginning of the steep upstroke of the carotid pulse tracing and carotid dicrotic notch). The relative values were related to the initial value.

Invasive hemodynamic measurements

At the end of the experiment, blood pressure in the femoral artery and left ventricular dP/dt_{max} were measured invasively in rabbits anesthetized by pentobarbital (30 mg/kg i.v.). Left ventricular differential pressure was derived using a differentiator (VÚFB, Prague, Czech Republic).

Histological examination

After the autopsy, a routine histological examination of cardiac ventricular tissue was performed. Tissue blocks of the transversely sectioned left and right ventricles (the region under the atria) were fixed by immersion in 10 % formalin. Paraffin sections (7 μ m in thickness) were stained with hematoxylin-eosin and Masson's blue trichrome.

Drugs and dosage

Dimethoxybenfluron (3,9- dimethoxybenflurone hydrochloride), dissolved in saline, was given in doses 12 mg/kg and 24 mg/kg, whereas daunorubicin (Cérubidine, Bellon Rhone-Poulenc, France), dissolved in water at a concentration 4 mg/ml, was administered in a dose of 50 mg/m². Ketamine (50 mg/kg i.m., Narkamon 5 %, Léčiva, Czech Republic) and pentobarbital (30 mg/kg i.v., Nembutal, Abbott, USA) served as anesthetics.

Statistical analysis

Statistical evaluation of both absolute and relative values (i.e. values related to the initial control value) was performed. The values were compared within one group and in different groups at the corresponding time intervals for the level of significance $p \leq 0.05$ using the SigmaStat software program (revision STAT 32 2.0, Jandel GmbH, Erkrath, Germany). Values of individual parameters expressed in figures and tables are presented as mean \pm S.E.M.

Table 1. Cardiac troponin T (μ g/l) following repeated administration of drugs

Administration	Control	Daunorubicin with premature deaths	Daunorubicin without premature deaths	NO-1-B 12	NO-1-B 24
Before the 1 st	0.000	0.000	0.003 \pm 0.002	0.000	0.000
Before the 5 th	0.014 \pm 0.005	0.024 \pm 0.008*	0.012 \pm 0.003*	0.004 \pm 0.011	0.007 \pm 0.012
After the 5 th	0.024 \pm 0.010	0.036 \pm 0.002	0.010 \pm 0.004	0.016 \pm 0.031	0.000
Before the 8 th	0.002 \pm 0.002 [§]	0.193 \pm 0.044*	0.038 \pm 0.006* [§]	0.003 \pm 0.008 [§]	0.003 \pm 0.008 [§]
After the 8 th	0.022 \pm 0.007* [§]	0.328 \pm 0.130*	0.065 \pm 0.019 [§]	0.012 \pm 0.013 [§]	0.013 \pm 0.017 [§]
The end of experiment	0.017 \pm 0.009 [#]	–	0.122 \pm 0.038*	0.000 [#]	0.000 [#]

NO-1-B dimethoxybenfluron

The end of experiment – 5 to 7 days after the last administration of a drug

Daunorubicin with premature deaths – animals (n=6) of the daunorubicin group with premature death

Daunorubicin without premature deaths – animals (n=7) of daunorubicin group surviving till the end of experiment

* significant difference ($p < 0.05$) of values compared within one group

§ significant difference ($p < 0.05$) between the daunorubicin with premature deaths group and the other groups at the corresponding intervals of measurements

significant difference ($p < 0.05$) between the daunorubicin without premature deaths group and the other groups at the corresponding intervals of measurements

Results

Cardiac troponin T

In the control group, the levels of cTnT were lower than 0.1 µg/l during the whole experiment. In the daunorubicin group, cTnT levels after the 8th administration were significantly higher (0.31 ± 0.11 µg/l,

$n=6$) in all animals with premature deaths compared to the rest of surviving animals (0.04 ± 0.03 µg/l; $n=7$) (Table 1). The levels of cardiac TnT after the administration of dimethoxybenfluron in either dose were always within the physiological range (lower than 0.1 µg/l) during the whole experiment.

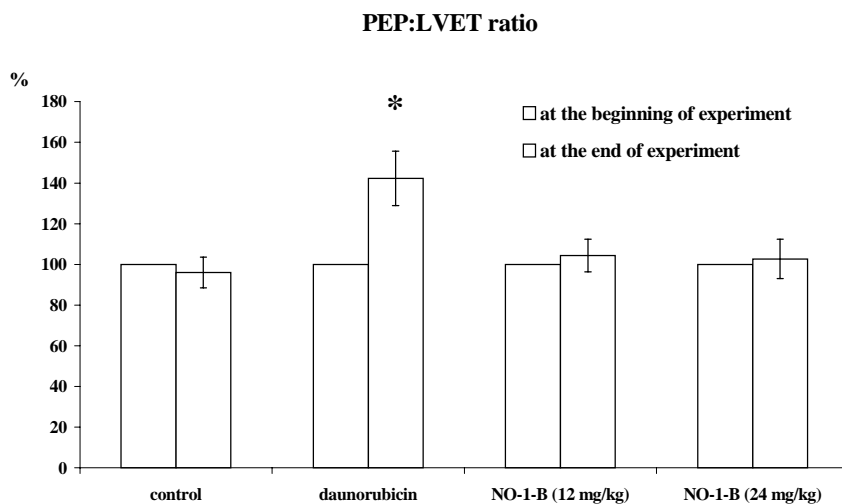


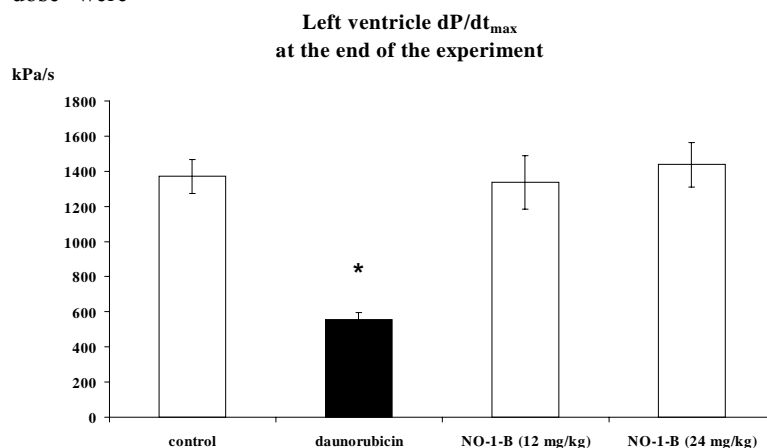
Fig. 1. PEP:LVET ratio, NO-1-B – dimethoxybenfluron, the relative values of PEP:LVET ratio are expressed in % of the initial value of PEP:LVET (100 %), * - significant difference ($p \leq 0.05$) between the control group and the daunorubicin group.

PEP:LVET ratio

No significant changes of PEP:LVET ratio during the experiment were usually found in the control group (values oscillated between 0.4070 and 0.4612, i.e. 100-114 %) and after both doses of dimethoxybenfluron. The values in the group treated with a lower dose oscillated between 0.4077-0.5025 (100-124 %), the values of PEP:LVET ratio after a higher dose were

0.4145-0.4333 (100-109 %). A progressive significant increase in the PEP:LVET ratio (0.3556 ± 0.0212 at the beginning and 0.5506 ± 0.0323 , i.e. 100-165 % at the end of experiment) was found in the daunorubicin group. The values of PEP:LVET ratio in the daunorubicin group were significantly higher in comparison with other groups at the end of the experiment (Fig. 1).

Fig. 2. Left ventricle dP/dt_{max} at the end of experiment, NO-1-B – dimethoxybenfluron, * significant difference ($p < 0.05$) between the control group and the daunorubicin group.



Invasive hemodynamic measurements

There were no significant differences between the control and dimethoxybenfluron-treated groups in the left ventricular dP/dt_{max} (1174.38 ± 103.26 kPa/s vs

1336.98 ± 149.81 kPa/s or 1438.17 ± 128.02) at the end of the experiment (Fig. 2). The values of dP/dt_{max} in all groups were significantly higher in comparison with the daunorubicin group (555.48 ± 41.65 kPa/s).

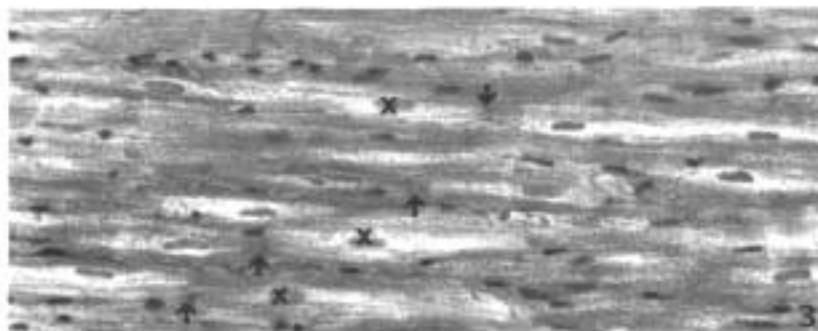


Fig. 3. Control group (Score 0). The intact myocardium: oval-shaped nuclei (x) of the cardiomyocytes are surrounded with pale-stained endoplasm; the cytoplasm is filled with the cross-striated myofibrils except for the region of the endoplasm. Intercalated discs (arrows) represent junctional complexes between adjacent cells. Masson's blue trichrome Direct magnification: 250x

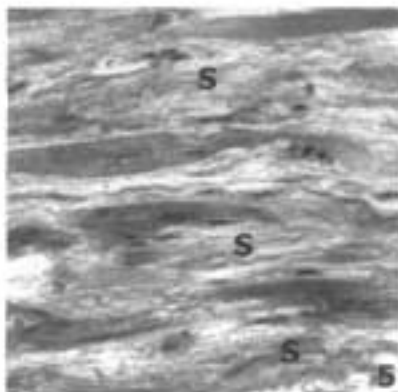
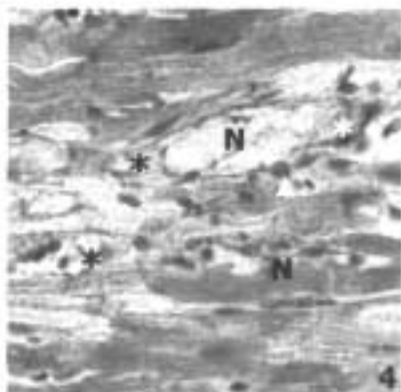


Fig. 4: Daunorubicin group (Score 3). Numerous and relatively large groups of degenerated or necrotic myocytes accompanied with the intensely eosinophilic (dark-stained) cells are typical. Necrotic cells (N) are gradually replaced by granulation tissue (*); macrophages (arrow-head) assist in scavenging of the cellular debris. Masson's blue trichrome Direct magnification: 312.5x

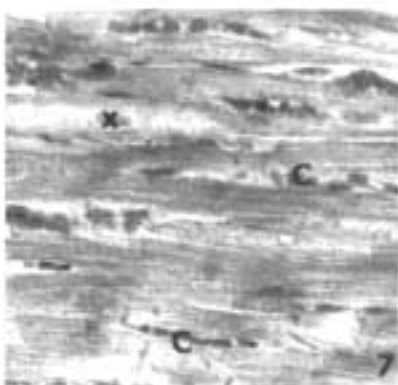
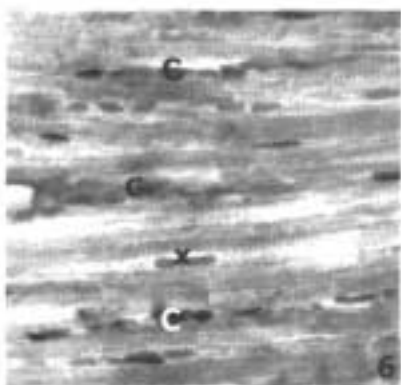


Fig. 5. Daunorubicin group (Score 5). Conspicuous disperse myofibrosis: Over several weeks the fibrovascular granulation tissue becomes more fibrous and these changes lead to the formation of numerous small fibrous scars (S).

Most spared cardiomyocytes exhibit highly intensive eosinophilia of their cytoplasm (dark-stained cells). Masson's blue trichrome. Direct magnification: 312.5x

Figs. 6, 7. Dimethoxybenfluron groups – DMB 12 and DMB 24 (Score 0-1). Nearly normal appearance of the myocardium: Scattered groups or strips of cells with intensely eosinophilic cytoplasm and rarely single necrotic cells are the only changes in these groups. x – nucleus of intact cardiomyocyte, C – capillary marked by erythrocytes. Masson's blue trichrome. Direct magnification: 312.5x

Premature deaths of animals

No premature deaths of rabbits occurred in either the control or dimethoxybenfluron-treated groups. The administration of daunorubicin induced premature deaths in six (i.e. 46 %) animals after the 8th administration of the drug.

Histological examination

The normal appearance of myocardial tissue after the repeated administration of dimethoxybenfluron prevailed (Figs. 6 and 7). The changes in staining (increased eosinophilia of the cytoplasm of some cardiomyocytes) and isolated cells with degenerated myofibrils were similar as in the control group (Fig. 3).

In the daunorubicin group, diffuse toxic damage was found in the whole myocardium (with a maximum in the ventral part of the left ventricle wall). Degenerated and necrotic cells (single or in groups), and numerous myocytes with intensely eosinophilic cytoplasm were present in both ventricles (Fig. 4); subsequent interstitial fibrosis developed as a reparative process in regions of the damaged myocardium (Fig. 5).

Discussion

The derivatives of benfluron rank among the prospective antitumor agents which exhibit considerable cytolytic activity (Mělka and Křepelka 1987, Miko *et al.* 1989). Besides antitumor activity, these substances have been studied from other aspects, e.g. the influence of dimethoxybenfluron on cell metabolic activity (Horáková *et al.* 1988a), biochemical and hematological parameters (Macháčková *et al.* 1999) or morphological changes caused by the derivatives of benfluron (Miko *et al.* 1989). The mechanisms of their effect probably include blockade of cells in S and G2 phases of the cell cycle (Horáková *et al.* 1988a). Dimethoxybenfluron was also reported to induce cell lysis. The cytotoxicity of these drugs is dose-related (Horáková *et al.* 1988b) and was shown in both *in vitro* and *in vivo* studies (Mělka and Křepelka 1987, Mělka *et al.* 1982).

The present study was aimed to evaluate the effects of repeated administration of dimethoxybenfluron on some cardiovascular parameters in rabbits as the changes of these parameters can often limit the possible therapeutic use of new derivatives with antitumor activity.

According to data in literature, no cardiac specific assays are available for diagnostic purpose in laboratory animals. Species-specific immunoassays for CK-MB are not commercially available and CK-MB immunoreactivity differs in various species (O'Brien *et al.* 1997). Our recent data (Adamcová *et al.* 1998, 1999) support the findings that cardiac troponin T is a valid marker of myocardial injury in laboratory animals (O'Brien *et al.* 1997). There is a considerable sequence homology between cardiac troponin T in different species, certainly in the higher vertebrates (Mesnard *et al.* 1993). Thus clinical assays for the cardiac forms of troponin T or troponin I can be used for samples from preclinical studies. Troponins represent a significant advance over the conventional markers in terms of specificity and sensitivity (Adamcová and Pelouch 1999).

Our previous study revealed that during the development of daunorubicin-induced cardiomyopathy the levels of cTnT were within the physiological range (cTnT lower than 0.1 µg/l) at the beginning of the experiment as well as before and after the 5th administration of daunorubicin. However, the pathological values of cTnT after the 8th administration correlated well with the premature deaths of animals and morphological changes. In the control group, the levels of cTnT were always lower than 0.1 µg/l (Adamcová *et al.* 1998, 1999). In several clinical studies that were carried out during the anthracycline therapy in both adults and children, very controversial cTnT values were reported. In some studies, no increase in cTnT was observed (Fink *et al.* 1995, Raderer *et al.* 1997), while in others a mild increase in cTnT was described in a subset of patients at higher risk of myocardial damage (Lipshultz *et al.* 1997, Missov *et al.* 1996, 1997). In animal studies, doxorubicin cardiotoxicity could be monitored by serum cTnT levels (Seino *et al.* 1993, Herman *et al.* 1999) and such cTnT elevations seem to be related to the loss of cTnT from damaged myocytes (Herman *et al.* 1999). These controversial results may be caused by the methodological differences (e.g. by the different cumulative dose of anthracyclines for different species, or by the different cut-off for cTnT). This will require further investigations. Cardiac markers are not routinely used to evaluate drug toxicities affecting the heart. As has been stressed by Wu (1998), with the development of sensitive and specific markers for minor myocardial injury, such as cardiac troponins, future drug trials for antineoplastic agents should include a biochemical assessment of myocardial damage.

Left ventricular dP/dt_{max} (a parameter of cardiac contractility) was one of the indices used for assessing the function of the cardiovascular system. Previous studies showed the decreased values of LV dP/dt_{max} accompanying the failure of cardiac contractility (Geršl 1987, Tang *et al.* 1993, Adler *et al.* 1996). In our study, no significant differences between the control and dimethoxybenfluron treated groups in the left ventricular dP/dt_{max} were found at the end of the experiment. Following the development of daunorubicin-induced cardiomyopathy, the values of LV dP/dt_{max} were low and the values of dP/dt_{max} in all the other groups were significantly higher than in the daunorubicin group.

Noninvasive polygraphic records were used for the measurement of the systolic time intervals to evaluate the myocardial function (Weissler *et al.* 1969,

Schoemaker and Smits 1990, Schott 1991, Geršl and Hrdina 1994). PEP:LVET ratio is considered to be an important parameter of systolic function of the heart. Depressed cardiac function is accompanied by a progressive increase in the PEP:LVET ratio due to a prolongation of the pre-ejection period (PEP), while left ventricular ejection time (LVET) may be shortened (Schott 1991, Geršl and Hrdina 1994). No significant changes of the PEP:LVET ratio during the experiment were found in the control group and after both doses of dimethoxybenfluron. A progressive significant increase in the PEP:LVET ratio was found during the experiment in the daunorubicin group. The values of the PEP:LVET ratio in the daunorubicin group were significantly higher in comparison with the other groups at the end of the experiment. These data have confirmed the adequacy of this model (daunorubicin-induced cardiomyopathy in the rabbit) and have indicated the lack of significant influence of the new antineoplastic drug – dimethoxybenfluron – on the followed parameters of the cardiovascular system.

Following administration of the new, potentially active antitumour drug, dimethoxybenfluron, the signs of cardiotoxicity were not observed. No significant increase in cTnT levels was found and also the changes in systolic time intervals and left ventricle dP/dt_{max} can be regarded as very discrete and significantly different from the cardiotoxic effects of daunorubicin. The histological examination of the heart suggests that the effect of repeated administration of dimethoxybenfluron (in the used doses, i.e. 12 mg/kg and 24 mg/kg) on the

myocardium was not significant in comparison with the daunorubicin group. The frequency of premature deaths of animals (none after dimethoxybenfluron, 46 % after daunorubicin) also corresponds with this conclusion as well as the fact that no significant changes in biochemical and hematological parameters following repeated administration of dimethoxybenfluron have been described (Macháčková *et al.* 1999).

It is possible to conclude that repeated i.v. administration of dimethoxybenfluron is well tolerated in rabbits from the viewpoint of its cardiovascular effects. This observation is considered to be important for possible further clinical use of this new antineoplastic agent. Furthermore, cardiac troponin T seems to be very useful for evaluation of cardiotoxic (and possibly cardioprotective) effects of new drugs. A similar biochemical marker has hitherto been missing.

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