INFLUENCE OF 5-[2-(N,N-DIMETHYLAMINO) ETHOXY]-7-OXO-7H-BENZO(C)FLUORENE HYDROCHLORIDE (BENFLURONE) ON THE ACTIVITY OF RAT LIVER ASPARTATE AND ALANINE AMINOTRANSFERASES

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SUMMARY

The influence of repeated s.c. administration of the cytostatic 5-[2-(N,N-dimethylamino)ethoxy]-7-oxo-7H-benzo(*c*)fluorene hydrochloride (benflurone, 25 mg/kg body weight daily) on the activities of aspartate and alanine aminotransferases (AST, ALT) per g of tissue, and protein concentration in the liver of adult male rats was studied. During the first week of benflurone administration, the activities of ALT and AST decreased by 2/3 and 1/3, respectively, in comparison with controls while the protein concentration did not show any substantial change.

No *in vitro* influence of benflurone on AST and ALT was found even at the highest concentration tested (10^{-4} M).

The significance of the aminotransferase decrease after treatment with benflurone and possible participation of these changes in the side- or cytostatic effects of the compound are considered.

KEY WORDS

5-[2-N,N-dimethylamino)ethoxy]-7-oxo-7H-benzo(*c*)fluorene hydrochloride, benflurone, alanine aminotransferase, aspartate aminotransferase, rat liver

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INTRODUCTION

5-[2-N,N-dimethylamino) ethoxy]-7-oxo-7H-benzo(*c*)fluorene hydrochloride (benflurone), the compound synthetized by Krepelka *et al.* /1/, has shown antitumour activity against a wide spectrum of experimental rodent tumours /2/. Results of *in vitro* experiments on human leukemic cell lines have demonstrated that the compound might be a prospective antileukemic agent /3/. A great advantage of benflurone is its low toxicity, confirmed in clinical studies /4/. The mechanism of its anti-neoplastic effects is probably complex: an intercalation effect, inhibition of DNA, RNA and protein synthesis, as well as interference with energy-generating or energy-transfer systems have been described /4/.

Benflurone passes rapidly from the blood into various animal organs and, after i.v. administration to rats, high levels have been recorded in the heart, lungs, kidneys, brain and liver. The compound is partly biotransformed in the organism and about 70% of i.v. administered ^{3}H-benflurone was excreted in faeces, about 54% of the radioactivity being excreted in the bile within 12 h /5/.

Considering the above-mentioned effects and kinetics of the compound, we decided to investigate the influence of benflurone on liver enzymes. After looking at ornithine decarboxylase /6/, we studied aminotransferases as representatives of the intermediary metabolism of the liver.

Aminotransferases are enzymes widespread in animal tissues and, in higher animals and plants, the most active transaminating enzymes are aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) /7/. With respect to the key metabolic position of their oxo- and amino acid substrates, aminotransferases play an important role in the metabolism of amino acids, in its link to gluconeogenesis, in the citrate cycle, and in the nitrogen flow through the urea cycle in the hepatocytes of ureotelic organisms.

The metabolic functions of these two aminotransferases have not yet been clearly delineated, but their importance in the regulation of metabolic pathways is indisputable. Since these transaminating reactions are reversible and close to equilibrium in most tissues, and because there are no known allosteric controls of aminotransferases, their regulatory role is probably passive, primarily by acting as channels for the flow of nitrogen or carbon as dictated by metabolic

processes that are themselves under rigid control /7/.

In addition, AST is part of the malate-aspartate shuttle which, together with the glycerol phosphate shuttle, is considered to be the most important mechanism for the transfer of reducing equivalents from cytosol to mitochondria /8/. This has been proved in particular in the case of the rat liver /7, 9/.

MATERIALS AND METHODS

Adult male rats of the Wistar strain (mean weight 275 g) from the Breeding Centre of the Faculty of Pharmacy, Hradec Králové, were used in the experiments. The animals were kept under standard conditions during the experiments (pelleted diet [DOS 2-B, Velaz Prague] and water *ad libitum*).

5-[2-(N,N-dimethylamino) ethoxy]-7-oxo-7H-benzo(*c*)fluorene hydrochloride (benflurone, Research Institute for Pharmacy and Biochemistry, Prague) was administered s.c. in one dose of 25 mg/kg body weight daily, the longest period of administration being 13 days, according to the experimental schemes. The dosage was chosen with regard to our previous experience /6/. The solution of benflurone (10 g/l in aqua pro inj.) was prepared immediately before use. The experimental animals were killed by cervical disruption and subsequent exsanguination 24 h after the last dose of benflurone.

The 20,000 *g* supernatant of the liver homogenate (1 g of tissue in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4) was used for enzyme assays. The dinitrophenylhydrazine method (/10/, Bio-La Test Lachema, Brno) was applied for the aminotransferase assays in the *in vivo* experiments. The incubation mixture (0.6 ml) contained 4.2 x 10^{-2} M L-aspartate (in the case of AST assay) or L-alanine (in the case of ALT assay), 2-oxoglutarate 8.3 x 10^{-4} M, and 1200x diluted supernatant in 0.1 M sodium phosphate buffer pH 7.4. After 60 min incubation followed by the dinitrophenylhydrazine reaction, the absorbance was measured at 515 nm using a Spekol EK-1 (Carl Zeiss Jena) spectrophotometer against the blank.

In preliminary *in vitro* experiments on inhibition of aminotransferases by benflurone, higher concentrations caused false increases in the results due to interactions of benflurone with the reagents. For this reason, a UV-method based on the coupling of the aminotransferase reaction with that catalyzed by lactate dehydrogenase (/11, 12/ Bio-La Test Lachema, Brno) was used in the definitive *in vitro*

experiments. In this case, the final concentrations of the constituents of the incubation mixture (1.6 ml) for the AST assay were as follows: 2×10^{-1} M L-aspartate, 1.2×10^{-2} M 2-oxoglutarate, 1.8×10^{-4} M NADH, 14.0 μkat/l lactate dehydrogenase, 9.8 μkat/l malate dehydrogenase, 1×10^{-4} M pyridoxal-5'-phosphate, and 500x diluted supernatant of the rat liver homogenate in 0.1 M sodium phosphate buffer pH 7.4. The incubation mixture for ALT assay contained 4×10^{-1} M L-alanine, 1.5×10^{-2} M 2-oxoglutarate, 1.8×10^{-4} M NADH, 42 μkat/l lactate dehydrogenase, 1×10^{-4} M pyridoxal-5'-phosphate and 500x diluted supernatant in 1.6 ml of 0.1 M sodium phosphate buffer pH 7.4. The absorbance of 340 nm was measured using a Specord M40 (Carl Zeiss Jena) spectrophotometer against the blank.

The liver protein concentrations were determined by the method of Lowry *et al.* /13/.

RESULTS AND DISCUSSION

The results of the study of liver aminotransferase activities in male rats after repeated s.c. administration of benflurone (one dose of 25 mg/kg body weight daily) are shown in Figure 1, and those of the tissue protein concentrations in Figure 2. The liver protein concentrations do not show any substantial changes except the results of the 3rd day after starting benflurone administration, where there was a slight but significant ($p = 0.05$) decrease in comparison to the control group. Since other authors /4/ found no hepatotoxicity of benflurone in chronic toxicity tests, this transient decrease in the liver protein concentration might be caused by possible stress reaction. On the other hand, the activities of both aminotransferases decreased gradually during the first week of the experiment, while the activities after 13 days were similar to those on the 7th day after starting benflurone administration. The changes in ALT activity were more pronounced. As there was no change in aminotransferase activities 24 h after the first dose of benflurone, the decrease in the activities of both enzymes seems to show a certain delay. In order to find out whether direct interactions of benflurone molecules with the enzymes are responsible for the decrease observed in aminotransferase activities after benflurone administration, we looked at the *in vitro* influence of benflurone on aminotransferase activities. Because of the poor solubility of benflurone in the phosphate buffer used in the enzyme

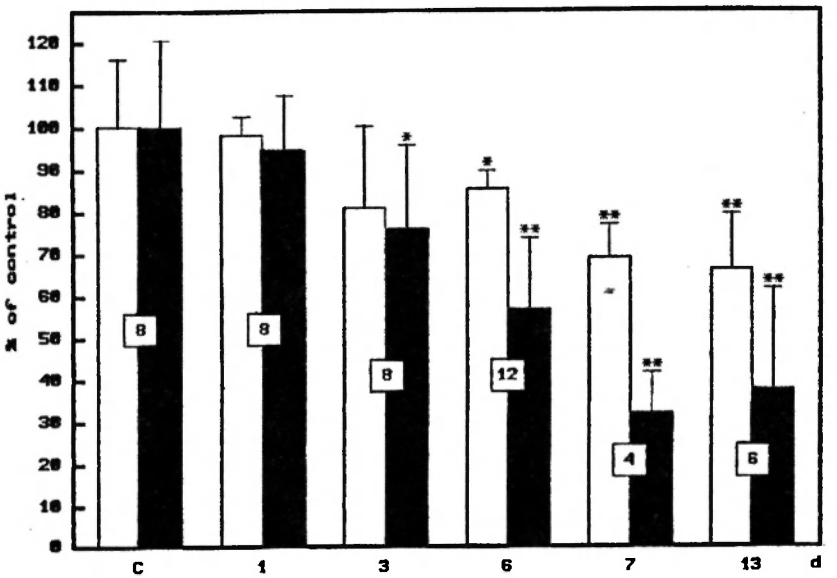

Fig. 1: Influence of s.c. administration of 5-[2-(N,N-dimethylamino) ethoxy]-7-oxo-7H-benzo(*c*)fluorene hydrochloride (benflurone, 25 mg/kg body weight daily) on the activities of hepatic aspartate and alanine aminotransferases (AST, ALT) per g tissue in adult male rats. The results are expressed as percentages of controls (untreated animals - mean values of activity 0.423 and 0.187 μkat/g tissue in AST and ALT, respectively). Open columns = AST, solid columns = ALT. Results are given as means; figures in columns are numbers of animals; bars = S.D.; *p = 0.05, **p = 0.01.

assays, the highest benflurone concentration tested was only 10^{-4} M. This concentration failed to show any influence on either ALT or AST *in vitro*.

Using other authors' data on the kinetics of benflurone in the rat /5/, we estimated the benflurone concentration in the liver during our *in vivo* experiments, and it did not seem to exceed that used in our *in vitro* experiments (i.e., 10^{-4} M) at any experimental interval, even under assumed uniform distribution of the compound throughout the hepatic tissue.

In addition, benflurone, when administered parenterally, is rapidly eliminated in the bile /5/ and its concentration can therefore be expected to be higher in the bile ducts than in the cytosol or mitochondria where aminotransferases are present.

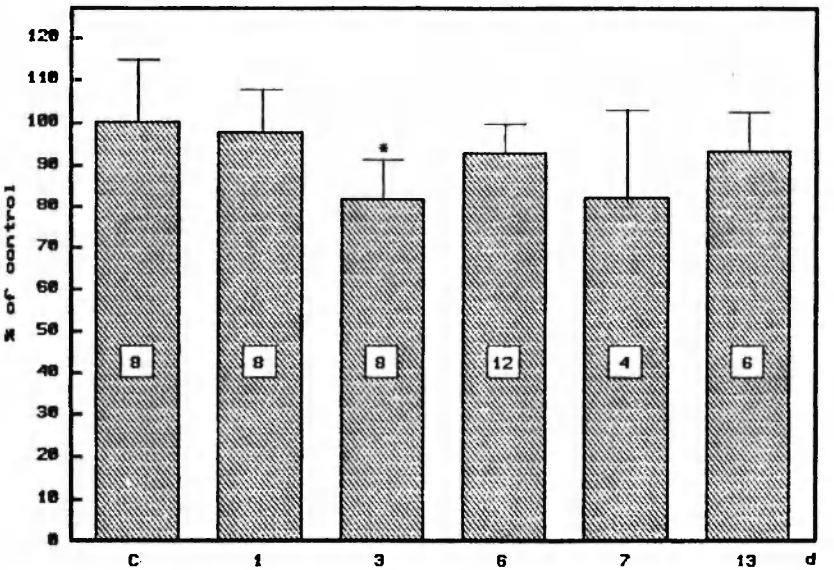

Fig. 2: Protein concentrations in the liver of adult male rats following s.c. administration of 5-[2-(N,N-dimethylamino) ethoxy]-7-oxo-7H-benzo(c)fluorene hydrochloride (benflurone, 25 mg/kg body weight daily). Control group (untreated animals) = 150 mg protein/g tissue. For other conditions see Fig. 1.

The direct influence of benflurone present in the samples during the enzyme assay after *in vivo* experiments can be excluded even more easily since the concentration of the compound in the incubation mixture did not exceed 10^{-10} M.

We therefore suppose that the decrease found in the activities of liver aminotransferases after benflurone administration was not caused by a direct interaction of the compound with the tissue enzymes but more probably by the effect(s) of benflurone and/or its metabolites on aminotransferase synthesis or degradation. The fact that the biological half-life of aminotransferases in hepatic tissue is several days /14/ is in good agreement with the profile found for both enzymes in the *in vivo* experiment, i.e., with a delayed and gradual decrease during the first week of benflurone treatment. It is of interest that the decrease of ALT after benflurone administration is more pronounced than that of AST: Changes in the hepatic ALT

activity were generally found to exceed those in AST activity. The relative stability of AST levels in the human liver in the course of various types of liver disease and nutritional or toxic liver damage has been repeatedly described, while ALT has usually shown a considerable decrease under such conditions. A suggestion has been made /15/ that this phenomenon might be related to the rather different roles of these two aminotransferases in liver cell metabolism. AST behaves as expected for an enzyme of basal cell metabolism, while ALT, like other enzymes with organ-specific functions, seems not to be synthetized to a sufficient extent or is decompensated more rapidly.

The interference of benflurone with DNA, RNA and protein synthesis in normal as well as tumour cells has been proved /4/ and the results of the present paper demonstrate a primary cytostatic effect on the activity of enzymes of intermediary metabolism. The question arises whether and how this effect can result in changes of the actual metabolic pathways, and whether it contributes to the cytostatic effect or side-effects of the compound. It should be taken into account here that the dose used in our experiments exceeded that intended for human therapy (2.5 mg/kg, see also /4/).

It is possible to speculate, in connection with the results of Miko *et al.* /16, 17/, who described the inhibition of the energy-generating or transfer system under the influence of benflurone (and its 9-OH derivative), and considering the key role of AST in the malate-aspartate shuttle, whether the decrease in AST activity found in normal hepatic tissue will also occur in various tumour cells. The results obtained with intact liver tissue cannot necessarily be applied to tumour cells, nor can the interference of a compound with aminotransferases alone be responsible for its cytostatic effect. Nevertheless, the interesting, though ambiguous, changes found in the specific activities of aminotransferases of tumour cells as compared with their original tissues should be mentioned (see /15/): The observed change depends on the type of the original tissue, the tumour and the degree of malignancy. The relative extent of the difference in enzyme activity seems to be determined by the cell metabolism of the original tissue and its aminotransferase activity rather than by the tumour. If the proliferation in the original tissue is weak, increased aminotransferase activities are found in the tumour, and *vice versa*. Greater changes are found in more dedifferentiated metastases. The highest increase in comparison to the original tissue has been observed in the

aminotranferases of juvenile cells in acute and chronic leukemias /15/. When compared with the size of changes in other tumour types, the changes in the mentioned leukemias are remarkably dramatic. This may suggest that the decrease in aminotransferase activities in the case of leukemia might participate in the therapeutic effect of benflurone.

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