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Effects of benfluron and its two metabolites on respiratory processes in P388 murine leukemia and Ehrlich ascites cells

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Benfluron [5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo(*c*)fluorene*] has cytostatic potential [1]. In the course of pre-clinical tests of benfluron, biotransformation of the compound has been studied both *in vitro* and *in vivo* [2]. The metabolites were isolated; two of them are shown in Fig. 1.

In our previous paper the effect of benfluron metabolites on the glycolysis of both P388 and Ehrlich ascites cells was investigated [3]. A significant inhibition of aerobic glycolysis in the presence of the NOBF was found in P388 cells. Linker *et al.* [4] calculated that Ehrlich cells, grown in standard medium, produce 60% of ATP via oxidative pathways and 40% via glycolysis. Beckner *et al.* [5] showed that in the presence of glucose the motility of metastatic cells in the human melanoma line A 2058 depends primarily on energy from glycolysis. These findings suggest that the inhibition of glycolysis *in vivo* might reduce the tumor cells' ability to leave the primary tumor mass and metastasize to secondary sites.

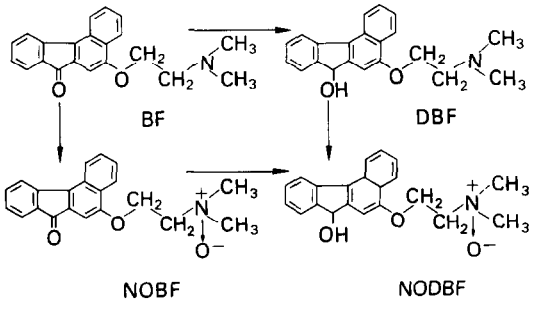

Fig. 1. The chemical structures of benfluron (BF) and its metabolites; DBF, NOBF and *N*-oxide of 7-dihydrobenfluron (NODBF).

* Abbreviations: benfluron, 5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo(*c*)fluorene; NOBF, *N* oxide of benfluron; DBF, 7-dihydrobenfluron; DMSO, dimethyl sulfoxide.

Up to now, however, little has been known about the action of benzo(*c*)fluorene derivatives on respiratory processes in intact tumor cells. This paper describes the effects of benfluron and its two metabolites on the endogenous and exogenous respiration, in the presence of succinate as substrate, of both P388 murine leukemia and Ehrlich ascites carcinomal cells.

Materials and Methods

Sources of most materials and methods of tumor cell preparation, and incubation have been reported previously [3]. Mice with transplanted P388 cells were from Dr V. Ujhazy, Cancer Research Institute, Bratislava. The cells were washed in a saline phosphate medium [6] and suspended in the same medium as described in earlier papers [7, 8]. Oxygen uptake was measured with a Clark oxygen electrode. The effect of benfluron and its metabolites on the endogenous respiration of both tumour cells was determined on the basis of oxygen consumption in a saline phosphate medium [6]. Respiration with succinate as exogenous substrate was studied in the MES medium [9], pH 6.2. For the inhibition of endogenous respiration, rotenone was present at a concentration of 3 μmol/L.

Benfluron was dissolved in DMSO and DBF, and NOBF in ethanol shortly before use as stock solutions of different concentrations. In control experiments, DMSO and/or ethanol replaced the benfluron-metabolite solutions. The final concentration of DMSO was less than 1% which does not affect the metabolic processes studied [10].

Results and Discussion

The effects of benfluron itself and its two metabolites on the endogenous respiration of both Ehrlich ascites and P388 murine leukemia cells are shown in Fig. 2. Comparing the results, it is evident that P388 cells are less "sensitive" than Ehrlich ascites cells. NOBF (3) did not interfere with the respiratory processes in Ehrlich ascites cells, even at quite high concentrations. The modification of the chemical structure of the molecule of benzo(*c*)fluorene (introduction of $N^+ \rightarrow O^-$) resulted in changes in its physicochemical properties (lipophilicity) and thus in its distribution in the intracellular space. Possible explanations could be that the intramitochondrial NOBF concentration in intact tumor cells is less than in the surrounding medium, that NOBF does not easily enter into the cell at pH 7.4 or that it binds to other proteins. In P388 cells up to a concentration of

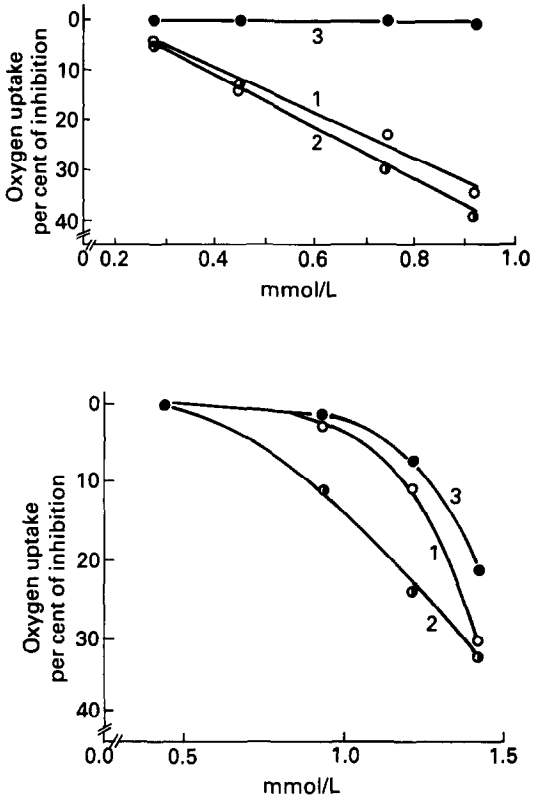

Fig. 2. Effects of BF (1), DBF (2) and NOBF (3) on endogenous oxygen uptake by both Ehrlich ascites cells (top) and P388 murine leukemia cells (bottom). The rate of oxygen uptake was determined immediately after the addition of inhibitors to the cells. Cell suspension (0.2 mL) containing 16.7 (1), 15.1 (2 and 3) mg (Ehrlich cells) and 15 mg (P388), dry weight, was added to 2.0 mL of isotonic saline phosphate medium pH 7.4. Oxygen uptake was measured at 30°. Rate of oxygen uptake for control was: 196 and 180 natoms of oxygen per min for Ehrlich and P388 cells, respectively.

500 μmol/L almost no inhibition of endogenous respiration was observed. The differences in the behaviour of the two cell types can probably be attributed to the nature and composition of their cytoplasmic membranes. The P388 leukemia cells were used because of the different composition of their cytoplasmic membrane [11]. The most effective inhibitor in P388 cells was DBF (2), just as in Ehrlich ascites cells.

Figure 3 demonstrates the effects of benfluron and its metabolites on the exogenous respiration of both Ehrlich ascites and P388 murine leukemia cells. As demonstrated by Spencer [9], succinate may be transported across the cell membrane by the organic anion carrier system. The uptake of succinate is maximal at pH 6.2. Therefore this pH value was maintained in these experiments. The rate of exogenous respiration is considerably lower than that of endogenous respiration. Benfluron and DBF exert an almost identical inhibitory effect on exogenous respiration, the least effective being NOBF (3, Ehrlich cells). A different picture was obtained in the case of P388 cells. The most effective inhibitor in this case was NOBF (3). Inhibition occurred at much lower concentrations than in the case of Ehrlich cells and oxygen uptake was completely inhibited at concentrations lower than 200 μmol/L. DBF (2) caused a stronger inhibition than benfluron (1). The differences between Ehrlich and P388 cells could probably be explained by differences in the composition of the cell membranes. As shown in Figs 2 and 3, benfluron and its metabolites inhibited dose-relatedly both the endogenous and exogenous respiration of both cancer cells. A reduction of respiratory rates is not a consequence of electron transport inhibition because these benzo(*c*)fluorene derivatives showed an effect similar to uncouplers of oxidative phosphorylation (results not shown). In a previous paper [12] we observed that benfluron and its two metabolites show considerable cytolytic activities, particularly at higher concentrations. Membranous effects were demonstrated by several methods. Hence, the decrease in the respiratory rates in cancer cells might also be due to the effects of benfluron and its metabolites on the cell membrane.

In conclusion, further studies, using isolated mitochondria and perhaps also reconstituted glycolytic systems, are required to elucidate the exact mechanism or mechanisms by which benfluron and its metabolites interfere with the energy metabolism of tumor cells.

In summary, this paper presents data on the effects of benfluron and its two metabolites DBF and NOBF on both endogenous and exogenous, respiration in the presence of succinate as substrate, of both P388 murine leukemia and Ehrlich ascites carcinoma cells. The most efficient inhibitors of endogenous and exogenous respiration were benfluron

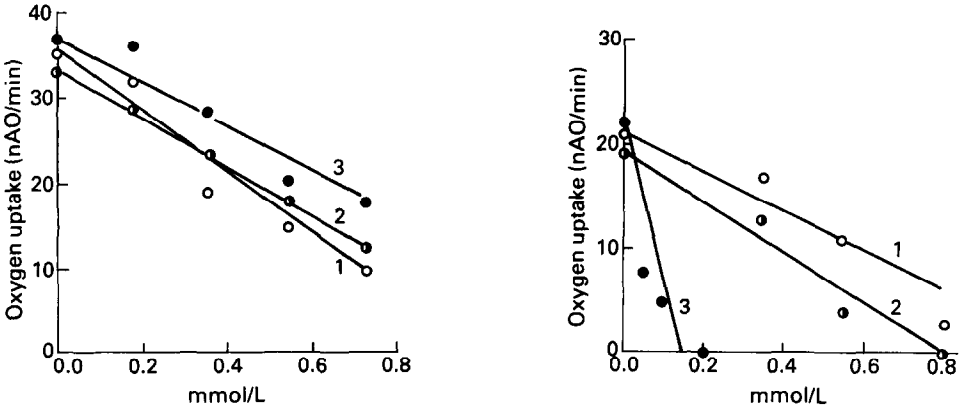

Fig. 3. Effects of BF (1), DBF (2) and NOBF (3) on the oxidation of succinate by both Ehrlich ascites cells (left) and P388 murine leukemia cells (right). Oxygen uptake was measured in 2.0 mL of isotonic 10 mmol/L MES medium pH 6.2 at 30°. Rotenone was present at a concentration of 3 μmol/L. Experimental conditions were the same as for Fig. 2.

and DBF. NOBF did not interfere with respiratory processes in Ehrlich cells, even at quite high concentration. BF and DBF exert an almost identical inhibitory effect on exogenous respiration, the least effective being NOBF (Ehrlich cells). The decrease in the respiratory rates in cancer cells might be due to the effects of benfluron and its metabolites on the cell membrane. P388 murine leukemia cells are less "sensitive" than Ehrlich ascites cells.

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Depolarization produces an acidification of adrenal gland perfusates

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Neuronal cell activity leads to considerable changes in the extracellular ionic composition in the vicinity of the cell membrane surface following the opening of ion channels. Changes in extracellular H^+ concentration can also be expected and have been detected by the use of pH sensitive electrodes, in the central nervous system, *in vivo* [1, 2], *in vitro* [3] or in brain slices [4, 5], and also in the peripheral nervous system ([6], see Ref. 7 for a review). External pH changes in the course of stimulation are characterized by an initial alkaline shift of short duration followed by a long lasting acid transient. However, the origin of these pH changes remains obscure. It has been proposed that the initial alkaline shift could result from a channel mediated transmembrane flux of proton equivalents [6]. The long lasting extracellular acid transient peaks at the end of stimulation, and returns to the original basal pH level. It could originate from the cellular release of metabolic products [1, 6], or from the exocytotic secretion of the highly acidic (pH 5.5) content of synaptic vesicles [5].

The bovine adrenal medulla has served as the most useful model tissue for the investigation of the exocytotic secretory processes [8, 9]. Much of the earlier progress on cholinergic receptor regulation of catecholamine secretion was accomplished by using perfused adrenal glands [10]. Stimulation of the adrenal gland with cholinergic agonists leads to the exocytosis of the vesicular content. By using various agonists and media of various buffering capacity, we have found that stimulation resulted in an acidification of the perfusion medium of a magnitude similar to that found in other systems. However, firstly the acidification could not be directly correlated with the amount of catecholamine released and then the time-course of both phenomena was different, suggesting that the acidification did not only result from the exocytosis of the acidic content of the chromaffin granules.

Materials and Methods

Bovine adrenal glands obtained from the slaughterhouse were removed within 20–30 min after the death of the animal and were kept in Locke solution during transportation to the laboratory. Retrograde perfusion were carried out essentially as described by Chubb and Smith [11] with some modifications. The composition of the perfusion fluids was (in mM): (a) regular Locke solution: 145 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 10 dextrose, 0.1 ascorbic acid, and 5 Hepes* buffer, pH 7.4; in some experiments, Hepes buffers at 0.5 mM and 1 mM were used. (b) High potassium solution was of the same composition as standard Locke solution except that NaCl was partially (56 mM) replaced with an equivalent concentration of KCl. (c) Ca^{2+}-free Locke solution was similar to the solution mentioned except that $CaCl_2$ was omitted. In the experiments in which acetylcholine (ACh) and nicotine were tested, they

* Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid).