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9-Hydroxybenfluron: effect on energy-yielding processes in Ehrlich ascites and P388 murine leukemia cells

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The main purpose of the present investigation was to study the effect of 9-hydroxybenfluron (HBF) on aerobic glucose consumption, lactic acid formation, content of total (T-SH) and non-protein thiol groups (NP-SH), endogenous respiration and levels of ATP in both Ehrlich ascites and P388 murine leukemia cells. The lowest concentrations of HBF significantly stimulated both glucose consumption and lactate formation in Ehrlich ascites cells. HBF decreased the level of both T-SH and NP-SH in Ehrlich cells. However, the decrease in the level of NP-SH was significantly higher. Both respiration and ATP levels were inhibited more markedly in Ehrlich than in P388 cells. In P388 cells a significant decrease in ATP level (67%) was noted only at the highest concentration of HBF (100 µmol/l).

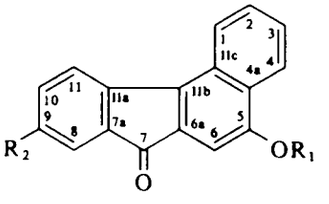
Key words: Aerobic glycolysis, ATP level, 9-hydroxybenfluron, respiration, thiol groups.

Introduction

Benfluron [5-(2-dimethylaminoethoxy)-7-oxo-7H-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 by thin-layer chromatography. Benfluron (500 mg/kg body mass) administered orally to rats yielded a phenolic metabolite in the feces. After chromatographic isolation and purification, 9-hydroxybenfluron (HBF) was obtained (Figure 1, compound **3**).[3]

In our previous paper,[4] it was shown that HBF was approximately twice as effective as a reference drug (benfluron). HBF inhibited biosynthesis of macromolecules indicated by the incorporation rate of [^{14}C]adenine (DNA,RNA), [^{14}C]thymidine (DNA), [^{14}C]uridine (RNA) and [^{14}C]valine (proteins) in both P388 leukemia and Ehrlich cells *in vitro*.

However, so far nothing is known about the action of HBF on energy-yielding processes in tumor cells. Further, as macromolecule biosynthesis is an energy-requiring process, we followed the effect of HBF on energy-producing processes, i.e. on aerobic glucose consumption, lactic acid formation, content of total (T-SH) and non-protein (NP-SH) thiol groups, endogenous respiration and levels of ATP in both Ehrlich ascites carcinoma and P388 murine leukemia cells. Linker *et al.*[5] calculated that Ehrlich cells, grown in standard medium, produced 60% of ATP via oxidative pathways and 40% via glycolysis. Beckner *et al.*[6] showed that the



Compound	R_1	R_2
1	$CH_2CH_2N(CH_3)_2$	H
2	H	H
3	$CH_2CH_2N(CH_3)_2$	OH

Figure 1. The chemical structure of [9-hydroxy-5-(2-dimethylaminoethoxy)-7-oxo-7H-benzo(*c*)fluorene] (compound **3**).

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motility of metastatic cells in the human melanoma line A 2058 depends in the presence of glucose primarily on energy from glycolysis. The findings suggest that the inhibition of glycolysis *in vivo* might reduce the tumour cells' ability to leave the primary mass and metastasize to secondary sites. Ehrlich ascites cells have been extensively used as an experimental model for biochemical investigation.[7,8] The P388 murine leukaemia cell line is widely used both in experimental oncology and chemotherapeutic screening.[9]

Materials and methods

Cells

Ehrlich ascites carcinoma (EAC) cells were maintained and propagated in strain H Swiss albino mice (Institute of Experimental Pharmacology, Czechoslovakia), about 10 weeks old and 20–25 g body weight, as described previously.[10] Ehrlich ascites cells were transplanted at 7-day intervals by i.p. injection of 0.2 ml of ascitic fluid collected under sterile conditions. The tumor cells were obtained from the peritoneal cavity of mice and were packed by low-speed centrifugation (600 × *g* for 10 min at 4°C). Mice with transplanted P388 cells were from Dr V. Ujházy, Cancer Research Institute, Bratislava. The cells were suspended in Krebs–Ringer phosphate buffer, pH 7.4, without calcium but with ascitic serum (2.5%, v/v) and glucose (final concentration, 3.0 mmol/l). The number of cells was adjusted to 5×10^6/ml of medium.[10] All operations were performed at 0–4°C.

Drugs

Chromatographically pure HBF was from the Research Institute for Pharmacy and Biochemistry, Prague. The substance was dissolved in dimethyl sulfoxide (DMSO) immediately before use as stock solution. After appropriate dilutions (1:1) each of these solutions was then added to the cell suspensions. The final concentration of DMSO was less than 1% which does not affect the metabolic processes studied.[11] The pH of the incubation mixture was not altered by adding DMSO. The chemicals and enzymes necessary for the determination of glucose consumption and lactate formation were purchased from Boehringer (Mannheim, Germany). DTNB for the determination of T-SH and NP-SH was purchased from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma Chemical Co.

Glucose uptake and lactic acid production

The kinetics of aerobic glucose uptake and lactic acid production by both cell types were determined by commercially available tests (Boehringer, Mannheim, Germany) as described earlier.[12] The concentration of glucose and lactate was determined enzymatically in the supernatant obtained after precipitation of suspensions of EAC and P388 cells with 1 ml 0.6 mol/l glacial perchloric acid in an ice bath. The precision of these measurements is ±3%.

Determination of T-SH and NP-SH groups

The determination was done according to the method of Ellman,[13,14] modified by Sedlak and Lindsay[15] in performance according to Drobnica *et al.*[16] The level of SH groups was determined concomitantly with glucose consumption and lactate formation. 5,5′-Dithiobis(2-nitrobenzoic)-acid is reduced by SH groups giving rise to 1 mol of 2-nitro-5-mercaptobenzoic acid per mole SH. This anion is of bright yellow color (E_{412} = 13 600 M^{-1} cm^{-1}).

Respiration

The effect of HBF on endogenous respiration of Ehrlich and P388 cells was determined on the basis of oxygen consumption in 154 mmol/l NaCl, 6.2 mmol/l, KCl, 11 mmol/l sodium phosphate buffer, pH 7.4.[17] HBF was mixed with 2.0 ml of this medium and 200 µl of the cell suspension in the same buffer solution was added. In control experiments, DMSO replaced the HBF solutions.

Assay of respiration

Cellular respiration was measured with a Clark-type oxygen electrode in a thermostatically controlled reaction vessel equipped with a stirring device. The reaction system and procedure were essentially as described previously.[18,19] Oxygen consumption was monitored for approximately 10 min and the linear portion of the oxygen consumption curve was used to calculate rate of oxygen consumption.

The respiratory rate was expressed as natoms (nanoatoms) of oxygen consumed per minute and dry weight of the cells.

ATP determination

ATP level determination was carried out according to the method described elsewhere.[20]

Protein determination

Determination of the protein concentration in the cell suspension was done according to the method of Lowry *et al.*[21]

Results

The effect of HBF on aerobic glycolysis of Ehrlich ascites cells was investigated in dependence on both time and concentration (Tables 1 and 2). Ehrlich cells consumed glucose from the medium linearly with the time, even if its concentration fell by more than 50% of its original value. A likewise proportional increase in lactate concentration in the medium was seen in control cells. Control Ehrlich ascites cells at the time interval of 15–120 min metabolized 0.9 mmol/l of glucose (Table 1) and 1.22 mmol/l of lactate was formed. The conversion of glucose to lactate in control Ehrlich cells was approximately 65% (calculated for the interval 15–120 min during the measurement of glycolysis). As shown in Tables 1 and 2, the effect of HBF depends on its concentration in the medium. The lowest concentrations of the drug significantly stimulated both glucose consumption (Table 1) and lactate formation (Table 2). The results appear to indicate that the stimulation takes place immediately on addition of the drug to the cancer cell suspension. However, the highest concentration of HBF (100 μmol/l) altered neither glucose consumption nor lactate formation. If the concentration of HBF were higher than 100 μmol/l, an inhibition of glycolysis would be expected to occur.

It is known that the key glycolytic regulation enzymes contain cysteine SH-groups which are essential for their catalytic activities.[22] In this relation we investigated the level of T-SH as well as NP-SH groups in Ehrlich ascites cells (Table 3) after the action of HBF. HBF decreased the level of both thiol groups in Ehrlich cells. However, the decrease in the level of NP-SH was significantly higher over the whole concentration range. The decrease in the level of T-SH groups was not probably large enough to affect the activity of key enzymes of glycolysis, i.e. hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12).

HBF at the lowest concentrations stimulated the aerobic glycolysis of Ehrlich ascites cells (Tables 1 and 2). Such a stimulation of glycolysis at low concentrations by HBF pointed to potential

Table 1. Kinetics (mmol/l) of aerobic glucose utilization by Ehrlich ascites cells in the presence of HBF

Time of incubation (min)	Concentration of the inhibitor (μmol/l)				
	0	12.5	25.0	50.0	100.0
15	2.91	2.91	2.85	2.84	2.89
30	2.75	2.70	2.70	2.68	2.76
60	2.48	2.38	2.41	2.36	2.49
90	2.22	2.09	2.07	2.06	2.25
120	1.96	1.76	1.76	1.74	1.98

The cells were incubated at 37 C in the presence of different concentrations of HBF. The initial glucose concentration was 3 mmol/l. At various times, 1 ml samples of suspension were analyzed for glucose and lactate. HBF was dissolved in DMSO just before the experiments.

Table 2. Kinetics (mmol/l) of aerobic lactate formation by Ehrlich ascites cells in the presence of HBF

Time of incubation (min)	Concentration of the inhibitor (μmol/l)				
	0	12.5	25.0	50.0	100.0
15	0.80	0.85	0.81	0.81	0.77
30	0.97	1.07	1.03	1.11	1.01
60	1.33	1.50	1.47	1.50	1.32
90	1.68	1.93	1.90	1.90	1.67
120	2.02	2.36	2.33	2.33	2.00

The experimental conditions are the same as in Table 1.

Table 3. Effect of HBF on T-SH and NP-SH content in Ehrlich cells after 120 min of incubation *in vitro*

μmol/l	T-SH		NP-SH	
	$A_{412\,nm}$	% of the control	$A_{412\,nm}$	% of the control
0	1.05	100	0.297	100
12.5	0.97	92.4	0.21	70.71
25	0.97	92.4	0.202	68.01
50	0.98	93.33	0.213	71.72
100	0.98	93.33	0.202	68.01

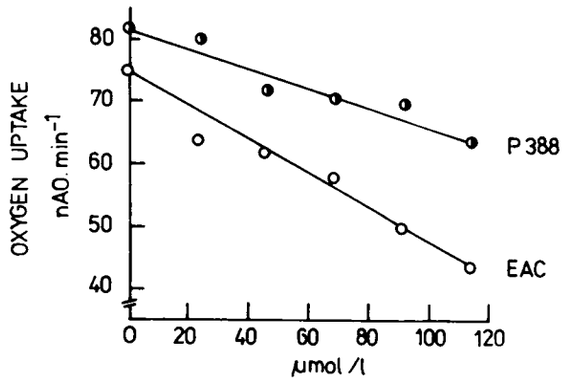

Figure 2. Effect of HBF on endogenous oxygen uptake by both EAC and P388 murine leukemia cells. The rate of oxygen uptake was determined immediately after the addition of inhibitor to the cells. Oxygen uptake was measured at 30°C. Cell suspension (0.2 ml) containing 14.8 mg (EAC) and 15.5 mg (P388), dry weight, was added to 2.0 ml of isotonic saline phosphate medium pH 7.4.

interference with the respiratory processes in cancer cells or in isolated mitochondria, respectively.[12] Experiments were carried out in order to verify this, results of which are presented in Figure 2. HBF inhibited oxygen uptake of both tumor cells, the extent of inhibition was dependent on the concentration of the drug in the incubation medium. Ehrlich cells were more 'sensitive' than P388 murine leukemia cells. The rate of oxygen consumed was calculated immediately after HBF addition.

To obtain direct evidence indicating interference of the drug with energy-generating systems, we studied the effect of HBF on the level of ATP in both Ehrlich ascites and P388 cells (Table 4). HBF decreased the level of ATP in Ehrlich cells to the same extent over the whole concentrations range, i.e. no concentration dependence of ATP level on treatment was observed. However, in P388 murine leukemia cells a significant decrease in ATP level (67%) was noted only at the highest concentration of HBF (100 µmol/l). This is in agreement with the results in Figure 2, where Ehrlich ascites cells were also more 'sensitive' than P388 murine leukemia cells.

Table 4. Effect of HBF on the level of ATP (nmol ATP/mg) in both Ehrlich ascites and P388 cells after 2 h incubation (the cells were incubated at 37°C in the presence of different concentration of HBF)

Cells	Concentration of the inhibitor (µmol/l)				
	0	12.5	25	50	100
EAC	5.16(0)[a]	4.14(20.1)	4.14(20.1)	4.14(20.1)	4.14(20.1)
P388	8.05(0)	8.05(0)	7.8(3.2)	7.5(6.4)	2.6(67.7)

[a] The numbers represent percentage of inhibition, in parentheses. HBF was dissolved in DMSO just before experiments.

Discussion

The results described here show that HBF interferes with energy-yielding processes in tumor cells. In our previous study we showed that benfluron and its metabolites are capable, at least partially, of releasing oligomycin-inhibited respiration of Ehrlich ascites cells.[23] The observed reversal of oligomycin inhibition of respiration provides evidence for the uncoupling activity of benfluron and its metabolites. 7-Dihydrobenfluron, one of the benfluron metabolites, stimulated the aerobic glycolysis of Ehrlich cells over the whole range of concentrations studied.[24] Diamond *et al.*[25] have also shown that agents such as dinitrophenol (an uncoupler of oxidative phosphorylation) and oligomycin (an inhibitor of oxidative phosphorylation), that interfere with ATP synthesis, markedly stimulated lactic acid production by intact quiescent 3T3 cells; however, the effect of oligomycin occurs at much lower concentrations than that of dinitrophenol. Increased glycolysis, for example, may be an appropriate response to increased energy demand resulting from cellular functions (e.g. ion movement, motility), but such conditions are commonly associated with decreased biosynthetic activities which may be supplied with reducing equivalents by the operation of the hexose monophosphate pathway.[26]

It has been shown that Ehrlich cells are glycolysis-dependent in support of their metabolism and growth. Therefore, they have an effective system for glucose transport. Cuppoletti *et al.*[27] showed that the density of glucose carriers in Ehrlich cells is 30 times higher than in erythrocytes. The inhibition of glucose uptake may result in the inhibition of growth in Ehrlich cells. The cytotoxic effects of methotrexate, for example, are at least partially ascribed to its ability to inhibit glucose uptake under *in vitro* conditions.[28] Recently, Medina *et al.*[29] have shown that if Ehrlich ascites cells can choose among different energy substrates (as it is the case in physiological conditions), they choose glucose preferentially.

The studies of several authors indicated that anti-cancer chemotherapy based on specific inhibitors of NADH-linked respiration may be worth

investigating.[30,31] Our results indicate that a substantial percentage of anti-cancer agents are active respiratory inhibitors.[30] The effects of anti-cancer drugs on respiration can, in some cases, provide information relevant to the mechanism of action, mechanisms of toxicity and biochemical side effects of the compounds.[31] HBF inhibited endogenous respiration of both tumor cells (Figure 2). P388 leukemia cells are less 'sensitive' than Ehrlich ascites. The use of P388 cells was not accidental since, as demonstrated by Miyamoto and Terasaki,[32] these cells have a different membrane composition. The differences in behavior of the two cell types can probably be attributed to the nature and composition of their cytoplasmatic membrane. Possible explanations could be that the intramitochondrial HBF concentration in intact P388 tumor cells is less than in the surrounding medium, that HBF does not easily enter into the cell at pH 7.4 or that it binds to other proteins. This is probably connected with the transport of HBF across the cell membrane, its distribution in cytoplasma, or biotransformation, as well as with transport across the inner mitochondrial membrane where the respiratory chain is located. Reduction of respiration by addition of respiratory inhibitors to aerobically incubated tumor cells cannot greatly reduce energy stores as glycolysis is released under those circumstances; it is well known that loss of ATP by diminished respiration is balanced by an approximately equal gain of ATP by an increased rate of lactate production.[33] This assumption could be applied to lower concentrations of HBF when glycolytic ATP is capable of making up for ATP losses caused by an inhibition of respiration (Table 4, P388 cells). At higher concentrations of HBF (100 μmol/l) such a compensation does not take place to an extent sufficient to maintain ATP at the required level (P388 cells). A different picture was obtained in the case of Ehrlich ascites cells. There is no dose-related relationship between the concentrations of HBF and ATP level in Ehrlich cells. Probably the losses of ATP caused by the inhibition of endogenous respiration can only be compensated for by aerobic glycolysis to a certain degree (Table 4, Ehrlich cells).

In most cell types, even in tumor cells, ATP is predominantly formed by oxidative phosphorylation. The energy-requiring processes cannot distinguish between the ATP formed by mitochondrial oxidative phosphorylation and that produced by cytosolic glycolysis. In Ehrlich ascites cells it was found that about 30% of total ATP produced was consumed by protein synthesis, 5–10% by ATP- and ubiquinone-dependent proteolysis, about 20% by the Na^+/K^+-ATPase, about 10% by the Ca^{2+}-ATPase and about 10% by the transcription processes.[34]

The results presented above show that HBF at the lowest concentrations does not interfere with ATP synthesis in P388 leukemia cells (Table 4). From the results, it further follows that the inhibition of macromolecular biosynthesis (DNA, RNA, proteins) in P388 cells[4] at the lower concentrations cannot be explained by an interference with the generation of utilization of high-energy phosphate bonds (ATP), but rather by a direct interference of HBF, mainly with DNA and consequently also RNA and protein synthesis. HBF decreased the level of both thiol groups (Table 3) in cancer cells. We must take into consideration that multi-target inhibitors, especially in the case of thiol reagents, apart from affecting bioenergetic processes, also directly inhibited nucleic acid precursors and the polymerization reactions themselves. A variety of sulfhydryl reagents have been evaluated for possible use as anti-tumor agents.[35]

The capacity of HBF to inhibit both respiration and ATP production (at higher concentrations) of tumor cells make this drug worthy of further interest. In fact, any attempt to inhibit tumor cell growth and survival by interfering with tumor cell energy production must take into account the ability of these cells to utilize equally well both oxidative phosphorylation and glycolysis to support cell growth.[36]

Although the majority of currently used anti-cancer drugs are cytotoxic, either by inhibiting DNA synthesis or by damaging the DNA template by alkylation or intercalation, Hill[37] emphasizes that this is an over-simplification. Most agents have multiple effective target sites within the cell, especially in the case of thiol reagents. The work by Farber[38] and others indicates that inability to synthesize ATP in a cell leads to multiple secondary derangements in cellular metabolism.

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