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9-Hydroxybenfluron: cytostatic effects and inhibition of macromolecular biosynthesis in Ehrlich ascites and P388 murine leukemia cells

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Primary screening *in vitro* and study on the mode of action of 9-hydroxybenfluron (HBF) in both murine P388 leukemia and Ehrlich ascites carcinoma cells have been performed. Metabolite HBF is approximately twice as effective as a reference drug (benfluron). To elucidate the biochemical mode of action, the effect of HBF on the biosynthesis of macromolecules indicated by the incorporation rate of [^{14}C]adenine (in DNA and RNA), [^{14}C]thymidine (in DNA), [^{14}C]uridine (in RNA) and [^{14}C]valine (in protein) was studied in concentration and time dependence. HBF inhibited incorporation of all four precursors into the trichloroacetic acid-insoluble fraction of Ehrlich ascites cells. The fact that incorporation of these four precursors is inhibited suggests that the effect of HBF lies at an underlying level of energy generation or transfer rather than at specific reactions in the biosynthesis of DNA and proteins.

Key words: Biosynthesis of macromolecules, 9-hydroxybenfluron, mode of action, primary screening.

Introduction

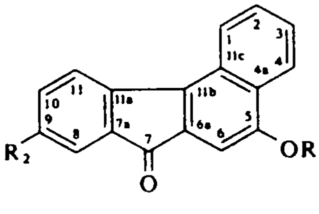
Benfluron [5-(2-dimethylaminoethoxy)-7-oxo-7H-benzo(c)fluorene] has cytostatic potential.[1] In the course of pre-clinical tests of benfluron, biotransformation of the compound has been studied both *in vitro* and *in vivo*.[2] The metabolites were isolated by thin-layer chromatography. Benfluron (500 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]

However, so far nothing is known about the cytotoxic activity and biochemical mode of action of HBF. The main objective of the present investigation was to screen HBF for cytotoxicity on P388 murine leukemia cells[4] and Ehrlich ascites cells,[5] used routinely for mass screening of candidate compounds for anti-neoplastic activity.[4] Ehrlich ascites tumor cells have been extensively used as an experimental model for biochemical investigations.[6,7] We have used Ehrlich cells also for the study of the mechanism of action of some antibiotics,[8] ethidium bromide,[9] isothiocyanate,[10] and other known anti-cancer drugs.[11]

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



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-dimethyl - aminoethoxy) - 7 - oxo - 7H - benzo(c)fluorene] (compound 3).

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body weight, as described previously.[12] 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.[12] 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. [8-^{14}C]Adenine sulfate (sp. act. 44 mCi/mmol), [U-^{14}C]valine (sp. act. 175 mCi/mmol), [2-^{14}C]thymidine (sp. act. 53 mCi/mmol), and [2-^{14}C]uridine (sp. act. 53 mCi/mmol) came from the Institute for Research, Production and Applications of Radioisotopes, Prague, Czechoslovakia. Other chemicals were supplied by Boehringer, Mannheim, Germany.

Primary biochemical screening (cytotoxic assay)

The procedure used in evaluating the cytotoxic effect of HBF was similar to that used when testing other metabolic inhibitors.[13] In short, cells were incubated for 1 h in the presence of at least four selected concentrations of the substance, under defined conditions *in vitro*, and the active synthesis of nucleic acids and proteins was followed. After 1 h of drug exposure, the test-tubes were transferred to an ice bath. [8-^{14}C]Adenine was added to the first series to a final concentration of 0.187μCi/1.02 μg and L-[U-^{14}C]valine was added to the second series to a final concentration of 0.165 μCi/2.64 μg. Both series were again incubated for 1 h at 37°C. In control experiments only DMSO was used. The final concentration of DMSO was less than 1% which does not affect the metabolic processes studied.[14] Incorporation was terminated by adding 1 ml of 5% trichloroacetic acid (TCA) to each test-tube in an ice bath. The samples were filtered through synpor membrane filters, pore size 4 μm (Synthesia, Prague), the precipitate washed with 10 ml of cold 2.5% TCA and 10 ml water and dried at 105°C. The radioactivity was measured on a methane flow counter (Frieseke und Hoepfner, Erlangen, Germany).

Kinetics of DNA, RNA and protein synthesis

To define further the mechanism of action of the selected drug, the kinetics of DNA, RNA and protein synthesis were examined using isotope incorporation.[15] The cells were incubated in a water bath at 37°C without shaking. At the indicated time intervals, samples of suspensions (1 ml) were analysed for radioactivity in acid-insoluble material. Radioactivity was measured on a methane flow counter as in primary biochemical screening. In some cases, the nature of the labeled material was checked by alkaline–acid hydrolysis. In the case of adenine incorporation, 60.6% of the incorporated radioactivity corresponds to the RNA fraction and 39.4% corresponds to the DNA fraction. In the case of thymidine, 90% of its incorporation was found in DNA. In uridine, 87.5% of the radioactivity was found in the RNA fraction.[12] All the data points are from duplicate determinations. The precision of these measurements is ±5%.

Results

Primary biochemical screening

Cytotoxic activity was assessed as a degree of incorporation inhibition of [^{14}C]adenine and [^{14}C]-valine into TCA-insoluble fraction of both Ehrlich ascites and P388 leukemia cells under defined *in vitro* conditions. These conditions ensured an active synthesis of nucleic acids and proteins for at least 2 h.[12] The cells were preincubated for 1 h in the presence of at least four concentrations of the drug. Then labeled precursors were added to the suspension, and after incubation for another 1 h the incorporation was stopped by adding TCA. The results from primary biochemical screening of the cytotoxic activity on both Ehrlich ascites and P388 cells are summarized in Table 1. The numbers represent cpm, with percentage of inhibition (or stimulation) in parentheses. The inhibitory effect was characterized by IC_{50} values (molar concentration of compound required for 50% reduction of

Table 1. Primary biochemical screening of cytotoxic activity of 9-hydroxybenfluron. The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine (a) and [^{14}C]valine (b) incorporation into TCA-insoluble fraction of both Ehrlich ascites carcinoma (EAC) and P388 cells after 2 h incubation in vitro. Inhibition of incorporation in cpm or % (in parentheses)

Cells	µmol/l					IC_{50} (µmol/l)	*R*
	0	12.5	25.0	50.0	100		
EAC	(a) 1571(0) (b) 1360(0)	1016(35.3) 1333(2.02)	729(53.6) 965(29.1)	403(74.3) 773(43.2)	316(79.9) 337(75.2)	22.5 60.0	0.37
P388	(a) 1017(0) (b) 1408(0)	576(43.4) NT	588(42.2) 1452(+3.1)	546(46.3) 1332(5.4)	397(60.9) 858(39.1)	65.0 >100.0	?

9-Hydroxybenfluron was dissolved in dimethyl sulfoxide just before experiments, *R* = IC_{50} adenine: IC_{50} valine.
NT = not tested.

the incorporation rate). HBF significantly inhibited incorporation of both ^{14}C-precursors into appropriate macromolecules of both cancer cells, according to concentration dependence (Table 1). This has been confirmed not only by percentage inhibition (given in parentheses) but also by IC_{50} values. The IC_{50} values of HBF were 22.5 for adenine (Ehrlich cells) and/or 65.0 µmol/l (P388 cells). The IC_{50} values of HBF for valine were higher than for adenine. This means that for the tested drug biosynthesis of nucleic acid is 'more sensitive' than is the biosynthesis of proteins. It is possible that the cell cycle is inhibited (cytostatic effect), resulting in less thymidine and uridine incorporation (Figure 3), whereas the synthesis of the household proteins are less affected. Compared to P388 cells, Ehrlich ascites cells showed a higher degree of inhibition of both precursors. The metabolite HBF is approximately twice as effective as a reference drug (benfluron).[16] The IC_{50} values of benfluron were 55 for adenine and 70 µmol/l for valine (Ehrlich cells).[16]

On the basis of our previous results, [4,13,15,16] it is convenient to use the IC_{50} adenine:IC_{50} valine ratio (*R*) as a suitable parameter to indicate the possible primary mode of action of the substance investigated; the ratio for Ehrlich ascites cells is 0.37 (Table 1). On the basis of our previous results, we can conclude that such a ratio is typical for compounds (see Table 2, group III) which interfere with the energy-generating system of cells.[4,15,16] Inhibition of energy metabolism may, for example, be due to direct interaction or through the disorganization of the membrane structure.[16]

In the case of P388 cells, HBF inhibited the incorporation rate of both precursors to a significantly lower extent that in Ehrlich ascites cells. Therefore, the IC_{50} value for valine could not be calculated.

Different R values for some selected antibiotics and metabolic inhibitors, for which the mode of action is known, are shown in Table 2 (from Ref. 4). They show the difference in the cytotoxicity of the substances, and indicate primarily the similarity or diversity of their mode of action (in the initial stages). From these R values it is possible to distinguish three types of mechanisms: (1) the substance inhibits the incorporation of adenine and valine to a similar degree; (2) the incorporation of the first precursor is inhibited more markedly; or (3) the incorporation of the second precursor is inhibited more markedly. R values in the first group are 6000 for pactamycin and more than 133 for cycloheximide. In the second group R values are several orders of magnitude different from that observed in the first group (0.0049 and 0.0057). In the third group R values are in the range 0.55–2.25. These R values are quite different from those indicated by the first two groups of inhibitors. In this way it is possible to deduce whether the agent affects more markedly energy metabolism or the synthesis of proteins or of nucleic acids.

Effect on macromolecule biosynthesis

In a first approach to determine the mode of action of the cytotoxically active compounds, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. The inhibitory effect of HBF upon the biosynthesis of macromolecules indicated by incorporation of [^{14}C]adenine and [^{14}C]valine into TCA-insoluble material of Ehrlich ascites cells is shown in Figure

Table 2. Primary biochemical screening of cytotoxic activity of some selected antibiotics and inhibitors of energy metabolism. The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine and [^{14}C]valine incorporation into TCA-insoluble fraction of Ehrlich ascites cells after 2 h incubation *in vitro*

Substance	Formula	Mol. wt	IC$_{50}$		*R*	Group
			(µg/ml) [^{14}C]adenine	(µmol/l) [^{14}C]valine		
Pactamycin	$C_{28}H_{38}O_8N_4$	558.58	120(214.80)	0.020(0.036)	6000	I
Cycloheximide	$C_{15}H_{23}NO_4$	281.30	>40(>142.2)	0.30 (1.07)	>133.3	
Nogalamycin	$C_{39}H_{49}NO_{17}$	803.78	0.98(1.22)	>200(248.82)	<0.0049	II
Cirolemycin (U-12,241)	?	?	1.15	200	0.0057	
Citrinin	$C_{13}H_{14}O_5$	250.24	34(135.86)	36(143.86)	0.94	III
Tubercidin	$C_{11}H_{14}N_4O_4$	266.25	5.35(20.1)	9.8(36.8)	0.546	
Iodoacetate	$CH_2ICO_2.Na$	207.9	15(72.1)	7.5(36.0)	2.0	
Cyanide	KCN	65.12	125(1919.5)	100(1535.6)	1.25	
2,4-Dinitrophenol	$C_6H_4N_2O_5$	184.1	18(97.8)	8(43.4)	2.25	
Lapachol	$C_{15}H_{14}O_3$	242.26	110(454.05)	90(371.50)	1.22	

All compounds were dissolved in Krebs–Ringer phosphate media shortly before experiments.
R = IC$_{50}$ adenine: IC$_{50}$ valine.

2. HBF inhibited incorporation of both precursors into appropriate macromolecules of cancer cells, the extent of inhibition being dependent on both time and concentration of the compound in the incubation medium. However, [^{14}C]adenine incorporation was inhibited more than [^{14}C]valine incorporation. At the highest concentration nearly complete inhibition of [^{14}C]adenine took place. These results are in agreement with those obtained with other metabolites of benfluron.[16] The results appear to indicate that the inhibition takes place immediately on addition of the drug to the cancer cell suspension, i.e. without the appearance of a lag phase.

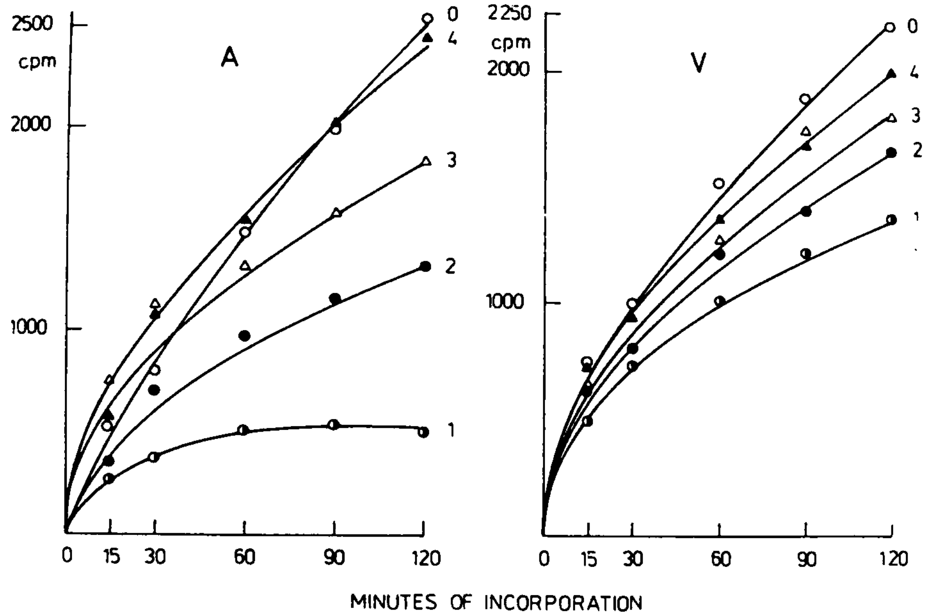

Figure 2. The effect of 9-hydroxybenfluron on macromolecule synthesis of Ehrlich ascites cells. Incorporation of radioactive precursors into acid-insoluble fractions was determined by incubating Ehrlich cells with [8-^{14}C]adenine sulfate (A) and L-[U-^{14}C]valine (V) in the presence of drug at various concentrations. Compound and precursors were added to the cell at the same time. 9-Hydroxybenfluron concentrations: 0 = none, 1 = 100, 2 = 50, 3 = 25, 4 = 12.5 µmol/l.

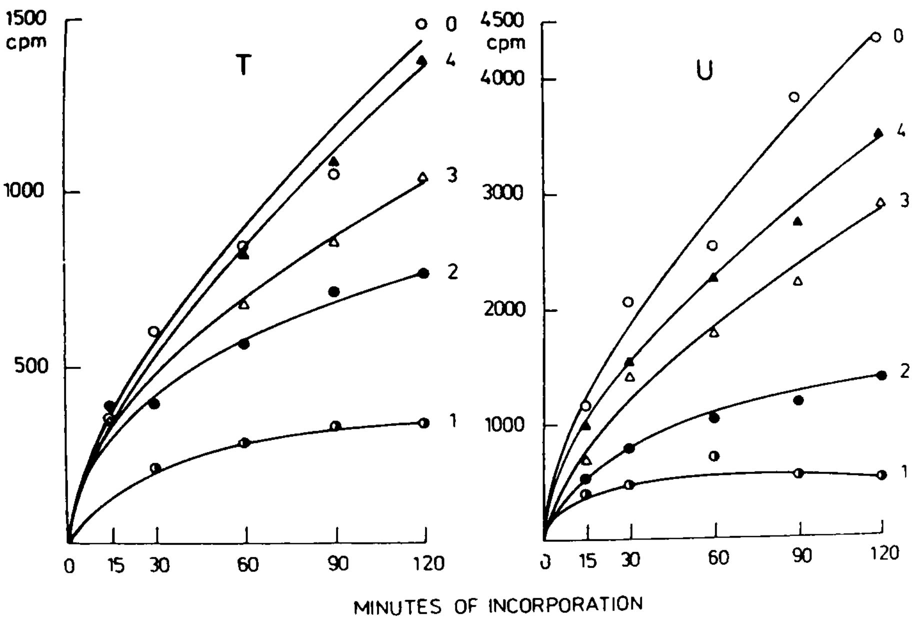

Figure 3. The effect of 9-hydroxybenfluron on dynamics of [2-^{14}C]thymidine (T) and [U-^{14}C]uridine (U) incorporation into TCA insoluble fractions of Ehrlich cells. Other experimental conditions and symbols are the same as for Figure 2.

As [^{14}C]adenine is incorporated into both DNA and RNA, we determined which of these nucleic acids was more sensitive by the experiments presented in Figure 3. HBF inhibited incorporation of both precursors into appropriate macromolecules of Ehrlich ascites cells. Among the labeled isotopes tested, the incorporation of [^{14}C]uridine (RNA synthesis) was inhibited most strongly by the compound. At the highest concentration tested (100 μmol/l), nearly complete inhibition of RNA biosynthesis occurred. The lower concentrations of HBF inhibited incorporation of both precursors in proportion to the tested concentrations. Similar results have also been obtained previously with other benfluron metabolites.[16]

The incorporation of all precursors was followed in the incubation medium containing glucose as a sole energy source. Therefore, it was interesting to study the effect of HBF on incorporation of [^{14}C]uridine in the absence of glucose. In this case, the cells utilized the energy produced only by oxidation of endogenous substrates. Endogenous production of ATP by oxidative phosphorylation is limited to approximately 30 min; after that the level of ATP rapidly decreases. Therefore, the effect of HBF on biosynthesis of RNA in the absence of glucose was followed only up to 30 min (Figure 4). From this figure, it is clearly evident that the

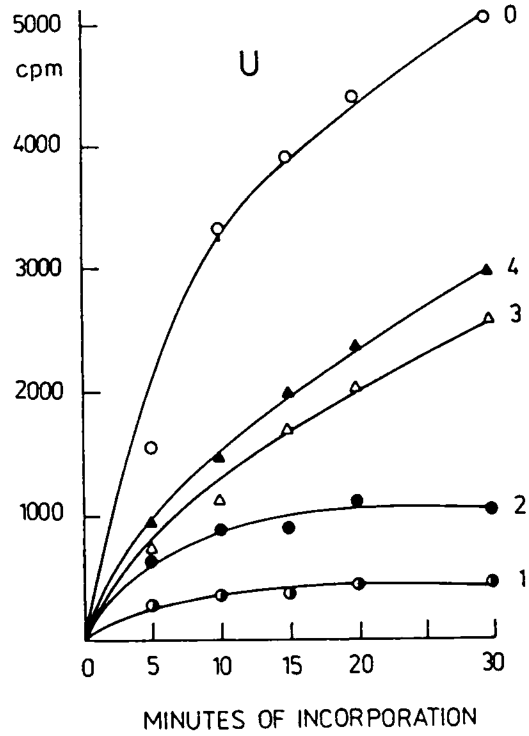

Figure 4. The effect of 9-hydroxybenfluron on dynamics of [^{14}C]uridine incorporation into TCA-insoluble fractions of Ehrlich cells in the absence of glucose in the incubation medium. Other experimental conditions and symbols are the same as for Figure 2.

biosynthesis of RNA, indicated by incorporation of [^{14}C]uridine, was inhibited from the beginning, on addition of HBF to the suspension of Ehrlich cells. If we compare Figures 3 and 4 it is evident that inhibition of uridine incorporation in the absence of glucose is much higher than in the presence of glucose.

Discussion

The values from biochemical screening represent the first fundamental information about the cytotoxic activity of HBF. The data obtained in a relatively short time indicate whether the tested substance has cytostatic activity at all, and perhaps also its possible mode of action (ratio). We have previously reported a rapid radiometric *in vitro* technique of primary screening for anti-cancer substances.[5,13–15] This method, which measures the drug-induced inhibition of [^{14}C]adenine and [^{14}C]-valine incorporation, is relatively simple, reliable and sensitive. In the Ehrlich carcinoma cells the degree of influence on metabolic activity is determined by uniformly selected concentrations of the substances, in defined conditions *in vitro*, ensuring the active synthesis of proteins and nucleic acids.

From the results presented in Table 1, it is evident that the new drug is approximately twice as active as benfluron (compare IC_{50} values).[16] In our screen, P388 leukemia cells are less 'sensitive' than those of Ehrlich ascites. The use of P388 cells was not accidental since, as demonstrated by Miyamoto and Terasaki,[17] 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 cytoplasmic membrane. For a reliable screening, human cells of different origin derived from cancer and from normal tissues have to be recommended. The ratio IC_{50} adenine:IC_{50} valine shows the difference in the cytotoxicity of the substances, and indicates primarily the similarity or diversity in the mode of action (in the initial changes). The ratio (R) of 0.37 for Ehrlich cells is typical for other biologically active compounds which interfere with energy-generating systems in cells (Table 2). All these drugs interfere with the generation or utilization of energy in cancer cells.[9,10,11,13,16]

Until recently, substances which are already present in their active form could be tested under *in vitro* conditions, whereas substances requiring activation metabolic steps (e.g. cyclophosphamide) could not be tested *in vitro*. Recently, however, by the addition of activating enzyme systems, it has been possible to use the latter substances in *in vitro* systems as well. Volm[18] found reasonably good correlations between a test based on the inhibition of radioactive nucleoside uptake and the *in vivo* chemosensitivity of several rodent tumors.

Recently, Von Hoff *et al.*[19] developed a radiometric system for the screening of anti-tumor agents. The index of cytotoxic effectiveness was based on the inhibition of transformation of [^{14}C]glucose into [$^{14}CO_2$]. This radiometric system (BAC-TEC 460) was optimalized with the aid of tumor cell lines of both human and animal origin. Scheithauser *et al.*[20] used this new screening system for the selection of anti-tumor agents for the treatment of human colorectal tumors.

Although the mechanism of action of HBF has not been determined, the present results show that HBF inhibited incorporation of all four precursors (Figures 2 and 3) into appropriate macromolecules of Ehrlich ascites cells. If we compare the degree of incorporation inhibition, it is evident that biosynthesis of nucleic acids is inhibited more markedly than that of [^{14}C]valine (compare IC_{50} values for adenine and valine in Table 1 and see Figure 2). Knowing that data from thymidine incorporation studies do not reflect absolute values of the extent of DNA synthesis, we still favor the assumption that HBF interferes with DNA processes in P388 cells (Table 1) and Ehrlich cells (Table 1, Figures 2 and 3). On the other hand, the fact that incorporation of the four precursors is inhibited suggests that the effect of the compound is at an underlying level of energy generation or transfer rather than at specific reactions in the biosynthesis of DNA and protein.

The process of DNA synthesis is actually the culmination of many synthetic pathways. In the intact cell, interference with any of these pathways, as well as alterations and variation in the pool size of precursors, can alter the apparent rate of DNA synthesis and obscure specific drug effects. The rate of DNA synthesis is rapidly affected by a decreased level of any of the four deoxyribonucleotide triphosphates. Interference with the generation of high-energy phosphate bonds is one of the mechanisms available for the induction of nucleotide deficiency. A depletion of nucleotide pools can serve as an efficient tool to inhibit cellular growth and to induce cell death under some circumstances.

Our preliminary results suggest that HBF significantly decreased the level of both ATP and thiol groups (protein and non-protein) in cancer cells. We must take into consideration that multitarget inhibitors, especially in the case of thiol reagents, besides affecting bioenergetic processes, also directly inhibit nucleic acid precursors and the polymerization reactions themselves. A variety of sulfhydryl reagents have been evaluated for possible use as anti-tumor agents.[21]

Results obtained by several groups during the past 5 years have shown that several of the more successful drugs in clinical use are compounds that cause potential breaks in the nuclear DNA of the cells as revealed by the alkaline elution method.[22] Typical examples are ellipticines, adriamycin and anilino-acridine derivatives. A considerable amount of evidence indicates that the drug-induced DNA breaks occur through alternation of topoisomerase II activity.[23] While DNA topoisomerases, an unique class of enzymes, participating in vital processes involving DNA, have been studied in eubacteria and eukaryotes over the past 15 years, it was recognized only a few years ago that some of the most valuable anti-cancer chemotherapeutics interact with topoisomerase II (for a review see NCI Monographs, No. 4, 1987). Whether HBF interacts with topoisomerase II activity is at present unknown.

Conclusion

It is very difficult to assign a definite causal relationship between the observed biochemical effects caused by a drug and physiological responses of neoplastic tissue to the drug. Although in the case of many anti-neoplastic agents, attention has been focused upon their effects on DNA, RNA and protein synthesis, work by Farber[24] and others indicate that the inability to synthesize ATP in a cell leads to multiple secondary derangements in cellular metabolism.

Further work is necessary to investigate the exact mechanism(s) of HBF action.

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