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# PRIMARY SCREENING AND INHIBITION OF MACROMOLECULAR BIOSYNTHESIS IN EHRLICH ASCITES CELLS BY BENZO(C)FLUORENE DERIVATIVES

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**SUMMARY**

The main objective of the present investigation was to screen a series of new benzo(c)fluorene compounds for *in vitro* activity. It can be stated that each of the 9 newly synthesized benzo(c)fluorene derivatives was about 10 times as active as tilorone. To elucidate the biochemical mode of action, the effects of 2 new compounds (13468 and 14200) on 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 (protein) were studied in concentration and time dependence. Both compounds inhibited the incorporation of the 4 precursors into the TCA-insoluble fraction of Ehrlich ascites carcinoma cells.

## INTRODUCTION

During the past 3 decades, more than 600,000 natural and synthetic products have been tested in a variety of antitumor screens /1/ to produce 30 to 40 established drugs for cancer chemotherapy /2/. The preclinical screening of synthetic compounds or natural products for evidence of antitumor activity has largely relied on *in vivo* tests. The development of an *in vitro* method to measure the sensitivity of tumor cells to cytotoxic drugs, so that the most effective drugs may be chosen for treatment, is a major goal in cancer chemotherapy /3/. An ideal test should be simple, statistically acceptable, easily standardized, cheap, rapid, flexible, capable of taking various methods of drug action into account, and should offer reasonable correlations with *in vivo* effects /4/. Possibilities for the measurement of cytotoxic effects include tests examining changes in cell morphology (cellular damage in monolayer or organ culture), tests measuring changes in cell viability (exclusion of vital stains or $^{51}Cr$ release), tests measuring inhibition of cell metabolism (inhibition of metabolic co-operation, cofactor depletion, e.g. ATP), tests utilizing measurement of uptake or incorporation of radioactive precursors, and tests measuring reproductive capability (cloning of tumors on plastic or in soft agar /4-7/. Recently Von Hoff *et al.* /8/ described a rapid, semi-automated radiometric system which utilizes inhibition of conversion of [$^{14}C$]glucose to $^{14}CO_2$ as an index of cytotoxicity. For detailed reviews of the use of *in vitro* tests as predictors of clinical response, see the excellent reviews by Venditti /9/, Weisenthal /10/, Hamburger /4/, Knock *et al.* /11/, Volm /12/, Kraemer and Sedlacek /13/. As reviewed previously /14/, *in vitro* screens have advantages over rodent tumor models in speed, accuracy, and quantities of new drugs required.

Recently, Krepelka *et al.* /15-18/ have described derivatives of benzo(c)fluorene with various substituents attached to positions 3, 5 and 7 (Fig. 1), and the results of preliminary tests of biological activity. These showed /16/ that derivatives with an ω-(N,N-dialkylamino)alkyl substituent bound via oxygen to position 5 were effectual antineoplastic substances so long as an oxo group was bound to position 7. From this series the compound 5-[2-(N,N-dimethylamino)ethoxy]-7-oxo-7H-benzo(c)fluorene (benfluron) was found to exert considerable activity against a group of transplantable tumors /16/ and against murine leukemia LA and L1210 /19/. Benfluron was selected for detailed preclinical and

clinical research on the basis of screening tests for anticancer agents /20,21/.

In our laboratory, a new system has been developed and is being used routinely for mass screening of candidate compounds for antineoplastic activity /21/. The main objective of the present investigation was to screen a series of new benzo(c)fluorene compounds for *in vitro* activity. The measure of the cytotoxic effect was the degree of inhibition of incorporation of [$^{14}$C]-labelled adenine and valine into the TCA-insoluble fraction of Ehrlich ascites carcinoma (EAC) cells after 2 h incubation *in vitro*. To validate this model, we tested a range of "standard" agents that had already been evaluated clinically. On the basis of the primary screening results, the effects of two selected compounds on the kinetics of DNA, RNA and protein synthesis inhibition were

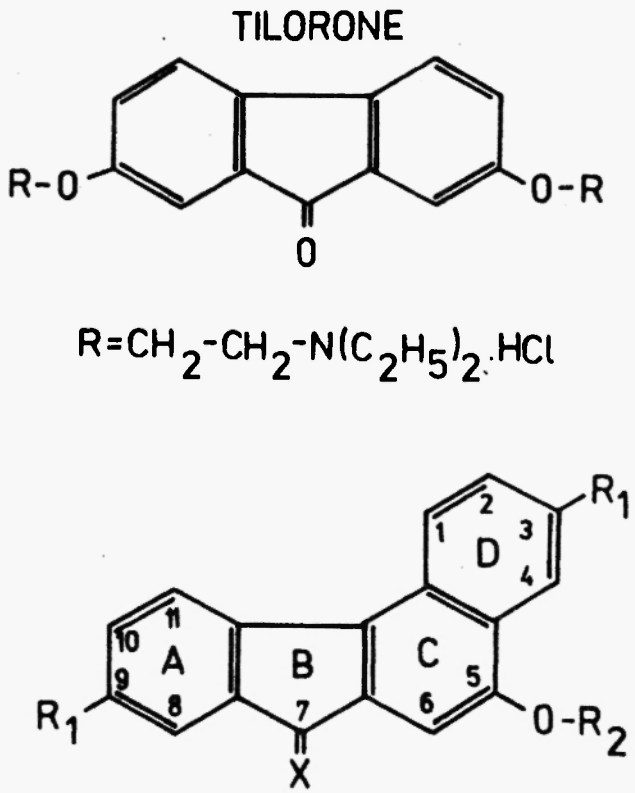



**Fig. 1:** The chemical structures of tilorone and benzo(c)fluorene derivatives.

examined using isotope incorporation. Only when the time course is known is it possible to state at what time and concentration the inhibitory effect appears.

In our previous papers, Ehrlich ascites cells have been used in primary screening for potential anticancer agents /22-25/ and to study the mechanisms of action of some antibiotics /26/, ethidium bromide /27/, isothiocyanates /28/, and other known cancerostatics /29-31/.

## MATERIALS AND METHODS

Ehrlich ascites carcinoma cells were maintained and propagated in albino mice, strain H (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Czechoslovakia), about 10 weeks old and 20 to 25 g body weight, as described previously /32,33/. Ascitic plasma was poured off and an incidental layer of erythrocytes was removed /34/. 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 $5x10^6$/ml of medium /33/. All operations were performed at 0-4 °C.

### *Materials*

Derivatives of new benzo(c)fluorene derivatives and tilorone were from the Research Institute for Pharmacy and Biochemistry, Prague. All the tested substances were chromatographically pure. The substances were dissolved in Krebs-Ringer phosphate medium or in dimethylsulfoxide (DMSO) shortly before use. Cancerostatic agents were from NCI, Bethesda, USA. [8-$^{14}$C]Adenine sulfate (specific activity, 44 mCi/mmol), [U-$^{14}$C]valine (specific activity, 175 mCi/mmol), [2-$^{14}$C]thymidine (specific activity, 53 mCi/mmol), and [2-$^{14}$C]uridine (specific activity, 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 (cytotoxicity assays)*

In our laboratory, a new system has been developed and is being used routinely for mass screening of candidate compounds for antineoplastic activity /23-25/. The procedure used in evaluating the cytotoxic effect of the compounds was similar to that used when

testing other metabolic inhibitors /24,35/. In short, cells were incubated 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 into an ice bath. [8-$^{14}$C]Adenine was added to the first series to a final concentration of 0.187 $\mu$Ci per 1.02$\mu$g and L[U-$^{14}$C]valine was added to the second series to a final concentration of 0.165 $\mu$Ci per 2.64 $\mu$g. Both series were again incubated for 1 h at 37°C. In control experiments only Krebs-Ringer phosphate medium or DMSO were used. The final concentration of DMSO was less than 1% which does not affect the metabolic processes studied /36/. Incorporation was terminated by adding 1 ml of 5% TCA to each test-tube in an ice bath. The samples were filtered through synpor membrane filters, pore size 4 $\mu$m (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 mechanisms of action of selected drugs, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. This method has been described in detail /35/. The cells were incubated in a water bath at 37°C without shaking. At the indicated time intervals, samples of suspensions (1 ml) were analyzed 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 that of DNA. In the case of thymidine, 90% of its incorporation was found in DNA. In the case of uridine, 87.5% of the radioactivity was found in the RNA fraction /33/. All the data points are from duplicate determinations. The precision of these measurements is ± 5%.

## RESULTS

*Biochemical screening of cytotoxic activity*

For the chemical structures of the substances studied, see Figure 1 and Table 1. The results from primary biochemical screening of

**TABLE 1**

Derivatives of benzo(c)fluorene (see Fig. 1) investigated in the primary screening

| Compound | $R_1$ | $R_2$ | X | M.W. |
|---|---|---|---|---|
| 134 68 | H | $CH_2CH_2N(CH_3)_2$ | 0 | 353.8 |
| 134 66 | H | $CH_2CH_2CH_2N(CH_3)_2$ | 0 | 367.8 |
| 134 67 | H | $CH_2C(CH_3)_2CH_2N(CH_3)_2$ | 0 | 395.9 |
| 141 67 | $CH_3$ | $CH_2CH_2N(CH_3)_2$ | 0 | 381.8 |
| 141 68 | $C_2H_5$ | $CH_2CH_2N(CH_3)_2$ | 0 | 407.9 |
| 142 01 | H | $CH_2CH(OH)CH_2NHCH(CH_3)_2$ | 0 | 361.4 |
| 141 81 | H | $CH_2CH_2N(CH_3)_2$ | N-$NHCNHN_2$ | 446.3 |
| 142 00 | H | $CH_2CH_2N(CH_3)_2$ | N-$NHCONH_2$ | 410.8 |
| 142 81 | H | $CH_2CH(OH)CH_2NHCH(CH_3)_2$ | N-$NHC(NH)NH_2$ | 489.9 |
| Tilorone | | | | 483.9 |

the cytotoxic activity on Ehrlich cells are summarized in Table 2. 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 the incorporation rate). As is seen from the results in Table 2, all the substances studied significantly inhibited incorporation of both $^{14}C$-precursors into appropriate macromolecules of Ehrlich cells, according to concentration dependence. This has been confirmed not only by percentage inhibition (given in parentheses) but also by $IC_{50}$ values. On the basis of the established $IC_{50}$ figures, it can be stated that each of the 9 newly synthesized benzo(c)fluorene derivatives was about 10 times as active as the parent compound (tilorone). The $IC_{50}$ values of tilorone were about 730 for adenine and 600 for valine, whereas the $IC_{50}$ values of the most active benzo(c)fluorene derivative (1346-VUFB, benfluron) were about 60 $\mu$mol/l for each of the two precursors. The most effective derivatives are substances VUFB 13468, 14168, 14201, 14181 and 14281. It is interesting to note that values of $IC_{50}$ for both precursors are approximately the same. However, $IC_{50}$ for adenine is in most cases lower than that of

valine. This means that the biosynthesis of nucleic acids, indicated by the incorporation of [$^{14}$C]adenine, is more sensitive than the biosynthesis of proteins. Modifications of both benzo(c)fluorene chemical structure at position 7 (see Table 1), as well as the side-chain bound via oxygen to position 5, did not significantly change cytotoxicity (Table 2). If the oxygen atom in position 7 is substituted by N, the cytotoxicity remains approximately the same (compounds Nos. 7 - 9, Table 2).

On the basis of our previous results /21,24,25,35/, it is convenient to use an $IC_{50}$ adenine:$IC_{50}$ valine ratio (R), which is a suitable parameter to indicate the possible primary mode of action of the substance investigated. All ratios, as demonstrated in Table 2, are in the range 0.50 to 1.36. Such ratios are typical also for other biologically active compounds which interfere with energy-generating systems in the cells. Inhibition of energy metabolism may be due to direct interaction or through the disorganization of the membrane structure.

Different $IC_{50}$ adenine:$IC_{50}$ valine ratios for some selected antibiotics and metabolic inhibitors, for which the mode of action is known, are shown in Table 3. These results show the difference in the cytotoxicity of the substances, and they indicate primarily the similarity or diversity of the mode of action (in the initial stages). From these ratios, it is possible to differentiate three cases, i.e., the substance inhibits the incorporation of adenine and valine to a similar degree; the incorporation of the first precursor is inhibited more markedly, or the incorporation of the second precursor is inhibited more markedly. On the basis of this phenomenon it is possible to deduce whether the substance affects more markedly energy metabolism or the synthesis of proteins and nucleic acids.

As "model substances" in such a screening, we used some selected anticancer antibiotics, known metabolic inhibitors and inhibitors of energy metabolism. All these inhibitors can be divided into 3 groups, according to their $IC_{50}$ adenine:$IC_{50}$ valine ratios. The first group is represented by 2 antibiotics, which are known inhibitors of protein biosynthesis (cycloheximide and pactamycin). This is well documented not only by the $IC_{50}$ values (compare 120 and more than 40 for adenine and 0.02 and 0.3 $\mu$g/ml for valine, respectively), but also by the $IC_{50}$ adenine:$IC_{50}$ valine ratios (6000 for pactamycin and more than 133 for cycloheximide). The second group is represented by 2 antitumor antibiotics, which primarily interfere with the biosynthesis of nucleic acids (nogalamycin and cirolemycin). Again the $IC_{50}$ values for adenine are much lower (0.98 and 1.15) in comparison with the $IC_{50}$ values for valine (more

**TABLE 2**

Primary biochemical screening of benzo(c)fluorene derivatives. 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 Ehrlich ascites cells after 2 h incubation *in vitro*.

| Substance No | Number of VUFB | M W | μmol/l | | | | | | $IC_{50}$ μmol/l | R |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 37.5 | 75 | 150 | 300 | 600 | | |
| | | | Incorporation in cpm (percent inhibition in brackets) | | | | | | | |
| 1 | 13 468 | 353 86 | (a) 1067(0)<br>(b) 2994(0) | 741(30.6)<br>2603(13.07) | 165(84 55)<br>689(77 0( ) | 57(94 67)<br>248(91.72) | 40(96.26)<br>40(98.70) | 66(93 8))<br>52(98 27) | 52<br>59 | 0.88 |
| 2 | 13 466 | 367.88 | 1313(0)<br>1859(0) | 964(26 59)<br>1522(18 13) | 520(60 40)<br>1142(38.57) | 68(94.83)<br>163(91 24) | 47(96 42)<br>30(98 39) | 49(96.27)<br>46(97.53) | 57.5<br>87.5 | 0.65 |
| 3 | 13 467 | 395.91 | 1313(0)<br>1859(0) | 935(28 79)<br>1564(10 49) | 834(36 49)<br>880(52.67) | 250(80.96)<br>788(57.62) | 56(95 74)<br>72(96.13) | 54(95.89)<br>50(97.31) | 98<br>72 | 1.36 |
| 4 | 14 167 | 381.8 | 943(0)<br>1505(0) | 724(23 23)<br>1299(13 69) | 476(49 53)<br>982(34.75) | 42(95.55)<br>70(95.35) | 48(94.91)<br>28(98.14) | 60(93.64)<br>30(93.01) | 71<br>90 | 0.78 |
| 5 | 14 168 | 407.9 | 943(0)<br>1505(0) | 633(32.88)<br>1014(32.63) | 353(62 57)<br>514(65 85) | 36(96.19)<br>27(98.21) | 32(96.61)<br>32(97 88) | 48(94.91)<br>39(97.41) | 56.5<br>55 | 1.02 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 14 201 | 361.4 | 943(0)<br>1505(0) | 776(17.71)<br>1882(+25) | 73(97 26)<br>186(87.65) | 43(95.44)<br>21(98.61) | 35(96.29)<br>49(96.75) | 47(95 02)<br>23(98 65) | 52<br>59 | 0.88 | |
| 7 | 14 181 | 446 37 | 851(0)<br>1737(0) | 838(1.53)<br>1815(+1) | 351(58.76)<br>– | 65(92 37)<br>99(94.49) | 62(92.72)<br>29(98 39) | 82(90 37)<br>57(96 83) | 63 5<br>56 | 1.13 | |
| 8 | 14 201 | 410.89 | 851(0)<br>1737(0) | 630(25 97)<br>1963(+9.57) | 201(76 38)<br>1607(10 58) | 42(95.07)<br>41(97.72) | 39(95.42)<br>30(93.33) | 45(94 72)<br>51(97.17) | 53<br>106 | 0 50 | |
| 9 | 14 281 | 489.9 | 851(0)<br>1737(0) | 610(28.32)<br>1680(6 51) | 273(67.92)<br>474(73.63) | 36(95.77)<br>43(97.61) | 41(95 19)<br>53(97 06) | 54(93.66)<br>49(97.28) | 55 5<br>58.5 | 0.94 | |
| 10 | Tilorone | 483 48 | 1060(0)<br>2455(0) | 1160(+9.39)<br>2395(2.45) | 1252(+18 1)<br>2374(3 30) | 1096(+3 36)<br>2247(8 48) | 979(7 68)<br>2443(0 49) | 563(46.91)<br>1944(20 82) | = 730<br>> 600 | ? | |

+ stimulation over 100% against control sample. Substances Nos. 1 to 3 and tilorone were dissolved in Krebs-Ringer phosphate medium and substances 4 to 9 in dimethylsulfoxide shortly before experiments R = $IC_{50}$ adenine:$IC_{50}$ valine.

**TABLE 3**

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 | MW | $IC_{50}$ μg/ml (μmol/l) [$^{14}$C]adenine | [$^{14}$C]valine | R | Group |
|---|---|---|---|---|---|---|
| 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. |
| Chromomycin (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 | $CH_2ICO_2Na$ | 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.

than 200 and 200 $\mu$g/ml respectively). However, the ratio R is several orders of magnitude different from that observed in the first group (0.0049 and 0.0057). In the third group are included 2 antitumor antibiotics (citrinin and cirolemycin) and known inhibitors of energy metabolism. If we compare first the $IC_{50}$ for adenine and valine, it is evident that both values are of approximately the same order of magnitude (compare citrinin, cyanide, lapachol, tuberidin, iodoacetate, and 2,4-dinitrophenol). These data also support the concept that the inhibition of biosynthesis by these inhibitors is the result of their primary action on energy-yielding processes in Ehrlich ascites cells. The same is true for the $IC_{50}$ adenine:$IC_{50}$ valine ratio, which is a suitable parameter indicating the possible primary mode of action of the substance investigated. The R for inhibitors is in the range 0.55 to 2.25. These ratios are quite different from those indicated by the first 2 groups of inhibitors. We can conclude that such ratios are typical also for other biologically active compounds which interfere with the energy metabolism of Ehrlich ascites cells /23-25,35/.

To validate this model, we tested a range of "standard" agents, some of which have already been evaluated clinically, and we extended the study to newer drugs, some of which may be currently undergoing phase I clinical trials (Table 4). As is seen from the results in Table 4, all substances affected the incorporation of both precursors. This was shown not only by the percentage inhibition (given in parentheses) but also by $IC_{50}$ values. However, the degree of incorporation inhibition is different. Emetine is a typical inhibitor of protein synthesis ($IC_{50}$ for valine is less than 75 $\mu$mol/l). On the other hand, cordycepin inhibited biosynthesis of nucleic acids more than the biosynthesis of protein. The most active inhibitors of incorporation of both precursors were CCNU, Me-CCNU and ethidium bromide. Other compounds listed in Table 4 were less effective in depressing the incorporation rate of both precursors. It was impossible to calculate $IC_{50}$ values because they are higher than the concentration tested. It is interesting to note that $IC_{50}$ values of the most effective agents are very similar for both precursors (CCNU, Me-CCNU and ethidium bromide). CCNU and Me-CCNU interfere with synthesis of nucleic acids ($IC_{50}$ values for adenine are less than 75 $\mu$mol/l), therefore it was impossible to calculate the ratio $IC_{50}$ adenine:$IC_{50}$ valine. Ethidium bromide, an uncoupler of oxidative phosphorylation /27/, inhibited incorporation of both precursors at the same level, therefore R=1, as for other inhibitors of energy metabolism (Table 3).

**TABLE 4**

Primary biochemical screening of cytotoxic activity of "standard" agents and other metabolic inhibitors. The measure of the cytotoxic effect was the degree of inhibition of [$^{14}$C]adenine (a) and [$^{14}$C]valine (b) incorporation in to TCA-insoluble fraction of Ehrlich ascites cells after 2 h incubation *in vitro*

| No. | Substance | M.W. | μmol/l | | | | | $IC_{50}$ μmol/l | R |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 75 | 150 | 300 | 600 | | |
| | | | Incorporation in cpm (percent inhibition in brackets) | | | | | | |
| 1 | Emetine | 553.56 | (a) 2141(0) | 1822(25.4) | 1807(26.0) | 1640(32.8) | 1699(30.4) | > 600 | ? |
| | | | (b) 3159(0) | 401(87.3) | 411(87.0) | 425(86.5) | 375(88.1) | < 75 | |
| 2 | ICRF | 129.94 | 2280(0) | 1832(19.6) | 1619(29.0) | 1722(24.5) | 1769(22.4) | > 600 | ? |
| | | | 3289(0) | – | 2389(27.4) | 2345(28.7) | 2141(25.8) | > 600 | |
| 3 | CCNU | 233.71 | 2280(0) | 960(58.0) | 710(68.9) | 383(83.20) | 352(84.6) | < 75 | ? |
| | | | 3283(0) | 1769(46.2) | 978(70.3) | 475(85.6) | 345(89.5) | 80 | |
| 4 | Me-CCNU | 247.70 | 2280(0) | 963(57.8) | 1031(54.8) | 334(85.3) | 132(94.2) | < 75 | ? |
| | | | 3289(0) | 1569(52.3) | 1005(69.4) | 361(89.0) | 403(87.7) | 75 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 | Diamin-dichloro-platinum | 300.1 | 2441(0)<br>3159(0) | 1791(26.6)<br>2805(11.2) | 1403(42.5)<br>2350(25.6) | 1372(13.8)<br>2169(31.3) | 1375(43.7)<br>1632(48.3) | > 600<br>> 600 | ? | |
| 6 | Picolin-aldehyde-5-OH-thiosemicarbazone | 196.2 | 2441(0)<br>3159(0) | 1953(20.0)<br>2511(20.5) | 2223(8.9)<br>2558(19.0) | 2079(14.8)<br>3143(0.5) | 2136(12.5)<br>4499(+42.4) | > 600<br>> 600 | ? | |
| 7 | Ethidium bromide | 391.9 | 2441(0)<br>3159(0) | 1285(47.4)<br>2351(25.6) | 1060(56.6)<br>1009(68.1) | 482(80.2)<br>768(75.7) | 252(89.7)<br>686(78.3) | 105<br>105 | 1.0 | |
| 8 | Hydroxyurea | 66.0 | 2441(0)<br>3159(0) | 3003(+23.0)<br>3019(4.4) | 2218(9.1)<br>2952(6.5) | 2108(13.6)<br>3132(0.9) | 2449(+0.3)<br>2436(22.9) | > 600<br>> 600 | ? | |
| 9 | Cordycepin | 251.25 | 2441(0)<br>3159(0) | 1873(23.3)<br>2789(11.7) | 1472(39.7)<br>2535(19.7) | 1120(54.1)<br>2506(20.7) | 530(78.3)<br>– | 270<br>> 600 | ? | |

+ stimulation over 100% against control sample. All compounds were dissolved in dimethylsulfoxide shortly before experiments.
R = $IC_{50}$ adenine:$IC_{50}$ valine

*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. Only when the time course is known is it possible to state at what time and concentration the inhibitory effect appears. The inhibitory effects of compounds No. 13468 (benfluron) and 14200 (Table 2) upon the biosynthesis of macromolecules indicated by incorporation of [$^{14}$C]adenine and [$^{14}$C]valine into TCA-insoluble material of Ehrlich ascites cells are shown in Figure 2. Both selected compounds inhibited incorporation of both precursors. The extent of inhibition was dependent on the concentration of each compound in the incubation medium. Benfluron is more effective than compound 14200. The complete inhibition of [$^{14}$C]adenine incorporation was reached at the highest concentrations of benfluron (150 and 75 $\mu$mol/l). At the same time compound 14200 completely inhibited incorporation of [$^{14}$C]-adenine only at the highest concentration (150 $\mu$mol/l). Incorporation of [$^{14}$C]valine is inhibited proportionally to the concentration of benfluron; however, it is less sensitive than that of adenine. On the other hand, compound 14200 inhibited incorporation of [$^{14}$C]valine at the same level as [$^{14}$C]adenine. This difference can be explained on the basis of the different chemical structures of the two compounds despite the fact that the mode of action is essentially the same.

As is known, [$^{14}$C]adenine is incorporated into both DNA and RNA. In order to differentiate which of the nucleic acids was more sensitive, experiments were carried out, the results of which are presented in Figure 3. Again, benfluron is a more effective inhibitor of [$^{14}$C]thymidine incorporation than compound 14200. At the highest concentrations tested (150 and 75 $\mu$mol/l), nearly complete inhibition of DNA biosynthesis takes place. Incorporation of [$^{14}$C]uridine is completely inhibited only at the highest concentration (150 $\mu$mol/l). The lower concentrations of benfluron inhibited incorporation of both [$^{14}$C]thymidine and [$^{14}$C]uridine proportionally to the tested concentrations. Similar results were also obtained with the compound 14200. However, this compound is less effective in comparison with benfluron. 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 benfluron on incorporation of both [$^{14}$C]thymidine and [$^{14}$C]uridine in the absence of glucose. In such a

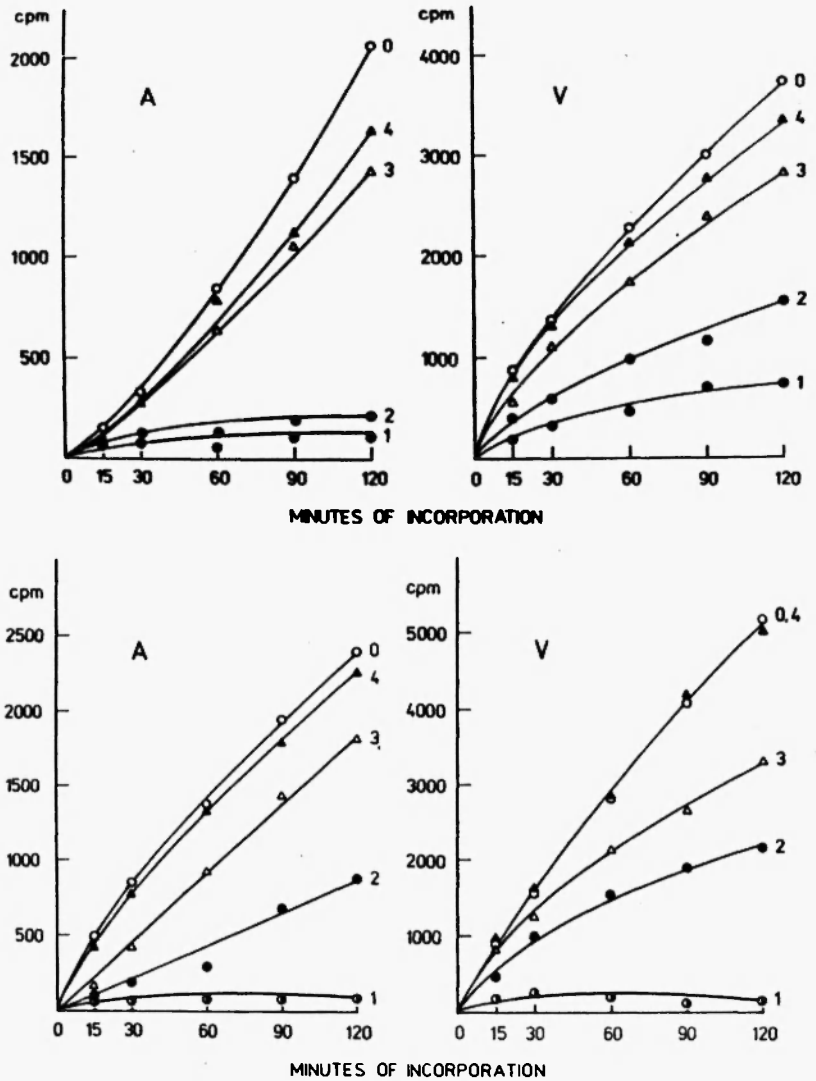
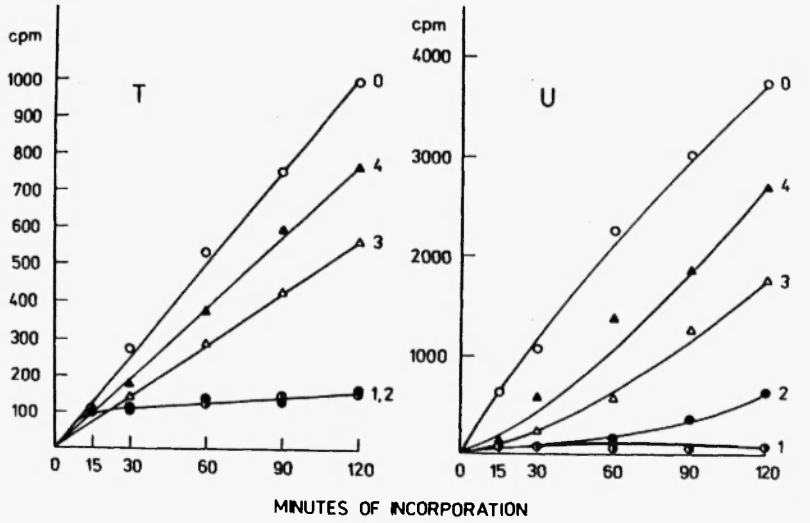



**Fig. 2:** The effects of benfluron (top) and compound 142 00 (bottom) on macromolecule synthesis of Ehrlich ascites cells. Incorporation of radioactive adenine (A) and valine (V) into acid-insoluble fractions was determined by incubating cells with appropriate $^{14}$C-precursors. Radioactive precursors and inhibitors were added to the cells at the same time. The test-tubes were incubated at 37°C, and 1 ml samples of each suspension were analyzed for radioactivity in acid-insoluble material. The results are expressed as cpm/5x10$^6$ cells. Concentrations: 0 = none, 1 = 150, 2 = 75, 3 = 37.5, 4 = 18.7 $\mu$mol/l.



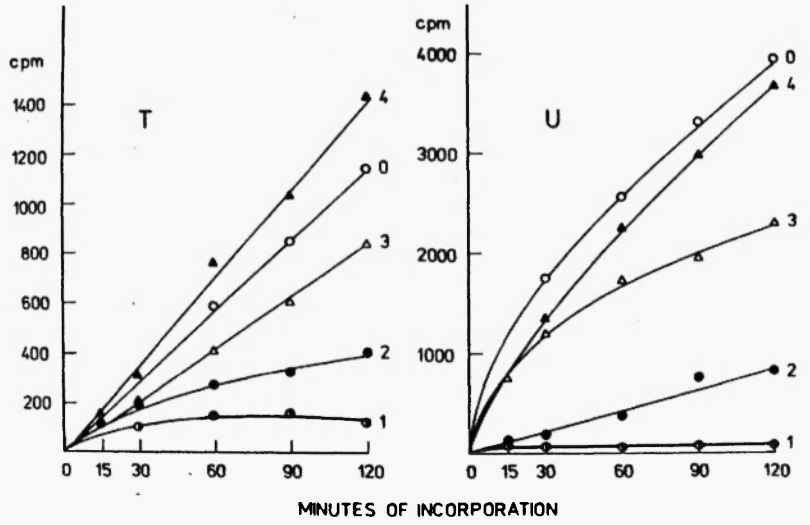




**Fig. 3:** The effects of benfluron (top) and compound 142 00 (bottom) on dynamics of [$^{14}$C]thymidine (T) and [$^{14}$C]uridine (U) incorporation into TCA-insoluble fractions of Ehrlich cells. Other experimental conditions are the same as for Fig. 2.

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 benfluron on biosynthesis of DNA and RNA in the absence of glucose was followed only up to 30 min (Fig. 4). From this figure, it is clearly evident that biosynthesis of DNA, indicated by incorporation of [$^{14}$C]thymidine, is inhibited from the beginning, from the addition of benfluron to the suspension of Ehrlich cells. These findings suggest that DNA synthesis was preferentially inhibited, followed by RNA and protein synthesis.

## DISCUSSION

The values from biochemical screening represent the first fundamental information about cytotoxic activity of new derivatives of benzo(c)fluorene derivatives. The data obtained in a relatively

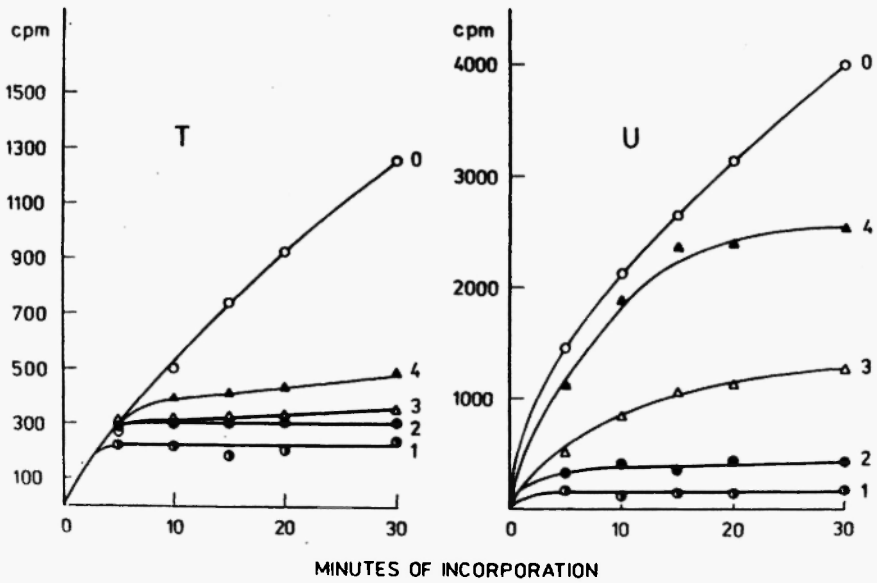



**Fig. 4:** The effect of benfluron (compound 134 68, Table 1) on dynamics of [$^{14}$C]thymidine (T) and [$^{14}$C]uridine (U) incorporation into TCA-insoluble fractions of Ehrlich cells in the absence of glucose in the incubation medium. Other experimental conditions are the same as for Fig. 2.

short time inform exactly whether the tested substance shows cytotoxic activity at all, and perhaps also indicate the possible mode of action. We have previously reported a rapid radiometric *in vitro* technique of primary screening for anticancer substances /23,24,35/. 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 ascites carcinoma cells the degree of influence on metabolic activity is identified by uniformly selected concentrations of the substances, in definite conditions *in vitro*, ensuring the active synthesis of proteins and nucleic acids.

From the results presented in Table 2, it is evident that the new drugs are approximately 10 times more active than the parent compound tilorone (compare $IC_{50}$ values). The ratios $IC_{50}$ adenine:$IC_{50}$ valine are in the range 0.5 to 1.13. Such ratios are typical for other biologically active compounds which interfere with energy-generating systems in cells (Table 3). Inhibition of energy metabolism may be due to direct interaction or through the disorganisation of membrane structure. The ratios of the benzo(c)fluorene derivatives are very similar to the ratios for CCNU, Me-CCNU and ethidium bromide presented in Table 4. All these drugs interfere with the generation or utilization of energy in cancer cells /27,29,30,31/.

Only substances which are already present in their active form can be tested under *in vitro* conditions. Substances requiring activating metabolic steps (e.g. cyclophosphamide) cannot be tested under *in vitro* conditions. Recently, however, by the addition of activating enzyme systems, it has also been possible to use such substances in *in vitro* systems. Volm /12/ found reasonably good correlations between a test based on the inhibition of radioactive nucleoside uptake and *in vivo* chemosensitivity of several rodent tumors.

Although the mechanism of action of benfluron has not been determined, our present results show that both benzo(c)fluorene derivatives inhibited incorporation of all 4 precursors (Figs. 2 - 4) 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 approximately at the same level as incorporation of [$^{14}$C(U)]valine. Among the different types of compounds synthesized within the cell (protein, polysaccharide, DNA, RNA) the greatest part of the total free energy released by ATP is used in protein synthesis (in E.coli nearly 90%) /37/.

Knowing that data from thymidine incorporation studies do not reflect absolute values of the extent of DNA synthesis, we still favor

the assumption that benzo(c)fluorene derivatives interfere with DNA processes in cancer cells (Figs. 2 and 3). On the other hand, the fact that incorporation of 4 precursors is inhibited suggests that the effect of both compounds lies at an underlying level of energy generation or transfer rather than at specific reactions in the biosynthesis of protein and DNA. 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 variations 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 the lowering of the level of any of the 4 deoxyribonucleotide triphosphates. Interference with the generation of high-energy phosphate bonds is one of the mechanisms available for 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 benfluron 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 antitumor agents /39/.

Results obtained by several groups during the past five 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. Typical examples are ellipticines, adriamycin and anilino-acridine derivatives. A considerable amount of evidence indicates that the drug-induced DNA breaks occur through alteration of topoisomerase II activity. While DNA topoisomerases, a unique class of enzyme participating in vital processes involving DNA, have been studied in eubacteria and eukaryotes over the past fifteen years, it was recognized only a few years ago that some of the most valuable anticancer chemotherapeutics interact with topoisomerase II (for a review see NCI Monographs, No. 4, 1987).

Nucleophilic groups play an important role in the chemistry of biological systems. Thiol groups are in a special position /40-42/. There are several enzymes whose catalytic activity is dependent on the presence of SH-groups (mainly cysteine). These enzymes participate in important processes such as glycolysis, oxidative and photosynthetic phosphorylation, transport of nutrients, microsomal

hydroxylation, protein and nucleic acid synthesis, etc. /41/. Non-protein thiols such as cysteine, glutathione, dihydrolipoic acid, coenzyme A, and other low-molecular thiol groups, are of great importance in metabolism. Thus, it is conceivable that the effect of many biologically active compounds is attributable to the ability to modify thiol groups chemically /43/. Recently, Arrick *et al.* /44/ reported that the interruption of the redox cycle of glutathione by the inhibition of glutathione reductase or glutathione peroxidase considerably sensitized tumor cells to peroxidase-catalyzed lysis. The role of thiol groups in topoisomerase II activity is not fully understood at present.

In conclusion, it is very difficult to assign a definite causal relationship between observed biochemical effects caused by a drug and physiological responses of neoplastic tissue to the drug. Although in the case of many antineoplastic agents, attention has been focused upon their effects on DNA, RNA and protein synthesis, work by Farber /45/ and others indicates 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 benfluron action.

## REFERENCES

1. Staquet MJ, Byar DP, Green SB, Rosenzweig M. Clinical predictivity of transplantable tumor systems in the selection of new drugs for solid tumors: rationale for a three-stage strategy. Cancer Treat Rep 1983; 67: 753-765.
2. Devita VT, Oliverio VT, Muggia FM et al. The drug development and clinical trials programs of the Division of Cancer Treatment, NCI. Cancer Clin Trials 1979; 2: 195-216.
3. Bird MC, Godwin UAJ, Antrobus JH et al. Comparison of in vitro drug sensitivity by the differential staining cytotoxicity (DiSC) and colony-forming assays. Br J Cancer 1987; 55: 429-431.
4. Hamburger AW. Use of in vitro tests in predictive cancer chemotherapy. J Natl Cancer Inst 1981; 66: 981-988.
5. Hamburger AW, Salmon SE. Primary bioassay of human tumor stom cells. Science (Wash DC) 1977; 197: 461-463.
6. Roper PR, Drewinko B. Comparison of in vitro methods to determine drug-induced cell lethality. Cancer Res 1976; 36: 2182-2188.
7. Von Hoff DD, Weisenthal L. In vitro methods to predict patients' response to chemotherapy. Adv Pharmacol Chemother 1980; 17: 133-156.
8. Von Hoff DD, Forseth B, Warfel LE. Use of a radiometric system to screen for antineoplastic agents: correlation with a human tumor cloning system. Cancer Res 1985; 45: 4032-4038.

9. Venditti JM. Preclinical drug development. Rationale and methods. Semin Oncol 1981; 8: 349-361.
10. Weisenthal, L.M. In vitro assays in preclinical antineoplastic drug screening. Semin Oncol 1981; 8: 362-376.
11. Knock FE, Galt RM, Oester YT, Sylvester R. In vitro estimate of sensitivity of individual human tumors to antitumor agents. Oncology 1974; 30: 1-22.
12. Volm M. Use of tritiated nucleotide incorporation for prediction of sensitivity of tumors to cytostatic agents. Behring Inst Mitt 1984; 74: 273-284.
13. Kraemer HP, Sedlacek HH. A modified screening system to select new cytostatic drugs. Behring Inst Mitt 1984; 74: 301-328.
14. Teatle R, Koziol J. In vitro drug testing using hemopoietic cells: goals and limitations. CRC Crit Rev Hematol Oncol 1985; 4: 169-201.
15. Krepelka J, Roubik J, Holubek J, Vancurova I. 5-, 6- and 7-substitution derivatives of 7-oxo-7H-benzo(c) fluorene. Coll Czech Chem Commun 1982; 47: 1258-1266.
16. Krepelka J, Vancurova I, Holubek J, et al. Derivatives of benzo(c)fluorene: II. Synthesis and biological effect of basic ethers of 7-oxo-7H-benzo(c)fluorene. Coll Czech Chem Commun 1982; 47: 1856-1866.
17. Vancurova I, Krepelka J, Smejkal F. Derivatives of benzo(c)fluorenes. III. Synthesis and biological action of some dibasic derivatives of 7-oxo-7H-benzo(c)fluorene. Coll Czech Chem Commun 1982; 47: 1867-1987.
18. Krepelka J, Vancurova I, Holubek J, Roubik J. Synthesis and antineoplastic effects of some N-substituted imides of 1-substituted 4-aryl-naphthalene-2,3-dicarboxylic acid. Coll Czech Chem Commun 1982; 47: 304-314.
19. Pujman V, Cernochova S. Antileukemic effects of 5-[2-(N,N-dimethylamino)ethoxy]-7-oxo-7H-benzo(c)fluorene, substance VUF B13468, on L1210 and La leukemia. Neoplasma 1981; 28: 715-720.
20. Melka M, Krepelka J. Benfluron hydrochloride. Drugs of the Future 1987; 12: 745-748.
21. Miko M, Krepelka J, Melka M. Screening of cytotoxicity of tilorone and benzo(c)fluorene derivatives. 3rd NCI-EORTC Symp. New Drugs Cancer Ther (October 15-17, Brussels) 1981; 80.
22. Fuska J, Miko M, Nemec P, Drobnica L. Screening of the cytotoxic action of fungus filtrates on Ehrlich's ascites carcinoma, utilizing $^{14}$C-labelled precursors. Neoplasma 1971; 18: 631-636.
23. Miko M, Drobnica L. Rapid in vitro technique of primary screening for anti-cancer substances. In: Abstracts of Twelfth Int. Cancer Congress, Buenos Aires, Argentina, October 5 to 11. Vol. 1, 1978; 287-288.
24. Miko M, Drobnica L, Jindra A, et al. Effect of chloro- and bromo-derivatives of isocrotonic acid on bioenergetic processes in Ehrlich ascites cells and isolated mitochondria. Neoplasma 1979; 26: 449-460.
25. Miko M, Skarka B, Porjanda J. Screening and mode of action of 4-alkylmorpholine-N-oxides. In: Letnansky K, ed., Biology of the Cancer Cell. Amsterdam: Kugler Publications, 1980; 63-71.
26. Miko M, Drobnica L. Effects of antibiotics nogalamycin, cirolemycin and tubercidin on endogenous respiration of tumor cells and oxidative phosphorylation of mammalian mitochondria. Experientia (Basel) 1975; 3L: 832-833.

27. Miko M, Chance B. Ethidium bromide as an uncoupler of oxidative phosphorylation. FEBS Lett 1975; 54: 347-352.
28. Miko M, Chance B. Isothiocyanates. A new class of uncouplers. Biochim Biophys Acta 1975; 396: 165-174.
29. Gosalvez M, Blanco M, Hunter J, Miko M, et al. Effects of anticancer agents on the respiration of isolated mitochondria and tumor cells. Eur J Cancer 1974; 10: 567-574.
30. Miko M, Chance B. Effects of 10 cancerostatics on endogenous respiration of tumor cells and oxidative phosphorylation of mammalian mitochondria. In: Abstracts of the Eleventh Int. Cancer Congress, Florence, October 20 to 26. Vol. 2, 1974; 128.
31. Miko M, Chance B, White FR. Effects of some cancerostatic agents on energy-yielding and energy-requiring processes in tumor cells and isolated mitochondria. In: Abstracts of the Twelfth Int. Cancer Congress, Buenos Aires, October 5 to 11. Vol. 1, 1978; 286.
32. Miko M, Drobnica L. Metabolic activity of the Ehrlich ascites carcinoma cells and significance of ascites serum addition. Neoplasma 1969; 16: 161-169.
33. Miko M, Drobnica L. Metabolic activity of the Ehrlich ascites cells in synthetic media and the significance of ascites serum addition. Neoplasma 1972; 19: 163-173.
34. Chance B, Hess B. Metabolic control mechanism. I. Electron transfer in the mammalian cells. J Biol Chem 1959; 234: 2404-2412.
35. Miko M, Drobnica L, Chance B. Inhibition of energy metabolism in Ehrlich ascites cells treated with dactylarin in vitro. Cancer Res 1979; 39: 4242-4251.
36. Drobnica L, Augustin J, Miko M. The influence of dimethyl sulfoxide on metabolic activity of Ehrlich ascites carcinoma cells and microorganisms. Experientia 1970; 26: 506-508.
37. Renger G. Biological energy conservation. In: Hoppe W, Markl H, Ziegler H, eds., Biophysics. Berlin-Heidelberg: Springer-Verlag, 1983; 347-371.
38. Weigel PH, Englund PT. Inhibition of DNA replication in Escherichia coli by dibromophenol and other uncouplers. J Biol Chem 1977; 252: 1148-1155.
39. Knock FE, Galt RM, Oester YT, Sylvester R. Inhibition of DNA polymerases and neoplastic cells by selected SH inhibitors. Oncology 1972; 26: 515-528.
40. Friedman M. The chemistry and biochemistry of sulfhydryl groups in amino acids, peptides and proteins. Oxford: Pergamon Press, 1973; 485 pp.
41. Jocelyn PC. Biochemistry of the SH Groups. New York: Academic Press, 1972; 404 pp.
42. Torchinskii YM. Sulfhydryl and disulfide groups of protein. New York: Plenum Press, 1974; 275 pp.
43. Zsolnai, T. Chemotherapeutischen und Pestiziden Wirkungen der Thiolreagenzien. Budapest: Akademia Kiado, 1975; 415 pp.
44. Arrick BA, Nathan CF, Griffith OW, Cohn ZA. Glutathione depletion sensitizes cells to oxidative cytolysis. J Biol Chem 1982; 257: 1231-1237.
45. Farber F. ATP and cell integrity. Fed Proc 1973; 32: 1534-1538.