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CYTOSTATIC ACTIVITY AND METABOLIC EFFECTS OF AROMATIC ISOTHIOCYANIC ACID ESTERS

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Abstract—Several studies have revealed cytostatic activity and metabolic effects of isothiocyanic acid (ITC) esters. This was confirmed by the present study, in which the minimal dose of four aromatic ITC esters causing 100 per cent growth inhibition (min. ID_{100}) of ELD tumor cells propagated *in vitro* was determined.

The influence on cellular metabolism of the four ITC esters in concentrations equivalent to min. ID_{100} was also investigated. A decrease of the total O_2 uptake by 20–45 per cent was observed in the presence of ITC esters, and succinate dehydrogenase, glucose-6-phosphate dehydrogenase, and the oxidation of NADH by mitochondria from Ehrlich's ascites tumor cells were found to be inhibited. Under aerobic conditions glucose consumption and lactate accumulation were increased when ITC esters were added to the reaction medium. Under anaerobic conditions two of the compounds showed a moderate antiglycolytic effect while two were without any influence on glucose consumption and lactate production. The possibility of a causal relationship between cytostatic and metabolic effect is discussed.

NEMEC *et al.*[1] have shown that several heterocyclic and aromatic esters of isothiocyanic acid (ITC) inhibit the growth of Saccharomyces vini, Candida albicans, and Kloeckera brevis *in vitro*, and the Ehrlich's ascites tumor *in vivo*. An antiglycolytic effect of these compounds on Ehrlich's ascites carcinoma cells *in vitro* was demonstrated by the same authors, but whether this metabolic effect represented the mechanism of the cytostatic effect remained an open question.

The purpose of the present study was to elucidate the correlation between the cytostatic effect of four aromatic ITC esters on Ehrlich's ascites carcinoma cells propagated *in vitro* and their effect on cellular metabolism.

MATERIAL AND METHODS

The four aromatic ITC esters were synthesized at Katedra Technikej Mikrobiologie a Biochemie, Bratislava, Czechoslovakia, and kindly supplied by Professor Pavel Nemec. Their formulas and molecular weights are given in Table 1.

Two sublines of Ehrlich's ascites carcinoma were used for the present study, a hypertetraploid (ELT) and a hyperdiploid (ELD). These cells were propagated *in vitro* and *in vivo* as described elsewhere.[2] For their *in vitro* propagation Eagle's MEM medium[3] with twice the concentration of amino acids and four times the concentration of glutamine and vitamins recommended was used. This medium was fortified with 20 % fetal bovine serum (Fib. 14 B). The *in vivo* propagated cells used in this work were carried by inbred StA mice.

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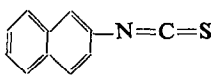
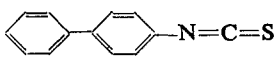
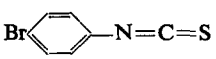
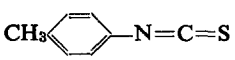
For the evaluation of the cytostatic effect *in vitro* of the four compounds 24-hr-old ELD cultures were treated for 72 hr with various concentrations of ITC esters dissolved in 99% ethanol. The final concentration of ITC esters varied from 0·5 μg/ml to 50 μg/ml, while that of ethanol was 0·5 per cent. The medium was changed daily. Initial cell counts of the cultures selected at random and final cell counts of each experimental group containing 10 cultures were made after trypsinization in a Bürker–Türk hemocytometer. Median values with 95 per cent confidence interval were calculated as described by Dean and Dixon.[4] The lowest dose causing complete growth inhibition and the highest dose causing significant, but incomplete inhibition indicate the interval within which the minimal dose causing complete growth inhibition (min. ID_{100}) is found.

TABLE 1. FORMULAS AND MOLECULAR WEIGHTS OF FOUR ITC ESTERS

β-naphthyl-ITC		185
p-diphenyl-ITC		211
p-brom phenyl-ITC		214
p-tolyl-ITC		149

The cytocidal effect *in vitro* of the 4 esters was measured by the trypan blue test as described by Pappenheimer.[5] ELD cells were cultured as described above with ITC esters added at concentrations corresponding to the lower limiting concentration of ID_{100}. For trypan blue staining the cells were removed mechanically from the culture vessels and suspended in Tyrode's solution. To 0·75 ml of this suspension 0·5 ml of a 0·25% trypan blue (Trypan blue BDH) solution in water was added. The percentage of trypan blue positive, i.e. dead cells was counted in a hemocytometer.

The influence of the 4 ITC esters on cellular respiration was investigated by the conventional Warburg technique. ELD cells were suspended in Krebs–Ringer phosphate buffer fortified with 45 mM Tris, and with 10 mM glucose. The ITC compounds were added in ethanolic solution about 15 min before the beginning of the experiment. The final ethanol concentration was 0·5 per cent which was found to have a very slight effect on glycolysis, but none on oxygen consumption. The effect on aerobic and anaerobic glycolysis was studied in Warburg vessels using specific enzymatic methods as described by Huggett and Nixon[6] and by Horn and Bruns.[7] Boehringer reagents were used for these tests. Aerobic glycolysis was measured in air while anaerobic glycolysis was measured after gassing the Warburg vessels for 3 min with nitrogen.

Additional investigations of the influence of ITC esters on the oxidative metabolism of tumor cells were performed with mitochondria prepared from ELT cells propagated *in vivo* by the technique described by Schneider and Hogeboom.[8]

The cells were suspended in 0·25 M sucrose (sucrose, Analar) and homogenized in a Potter–Elvehjem homogenizer. Further homogenization was obtained by freezing and thawing followed by repeated homogenization in the glass homogenizer.

The homogenate was centrifuged as described by Schneider and Hogeboom only omitting the final washing of the mitochondrial fraction. The mitochondria containing sediment was suspended in Krebs–Ringer phosphate buffer with 45 mM Tris and placed in the Warburg vessel.

The substrates used in these experiments were NADH or sodium succinate. NADH was administered from the side arm of the Warburg vessel after 10–15 min equilibration in amounts which gave a final concentration of 5 mM. Succinate was added directly into the vessel in a concentration of 2·5 mM.

Finally the influence of ITC esters on the glucose-6-phosphate dehydrogenase (G-6-PDH) activity was measured. The enzyme was obtained from ELT cells propagated *in vivo*. The cells were homogenized as described above using 0·4 M Tris as suspending medium and centrifuged for 30 min at 4° at 26,000 *g*. The G-6-PDH activity of the supernatant was measured as described in Methods of Enzymatic Analysis.[9] Glucose-6-phosphate was added at a concentration of 0·66 mM, and reduction of NADP to NADPH was followed spectrophotometrically at a wavelength of 366 mμ.

RESULTS

The cytostatic effect of the four ITC esters on ELD cells grown *in vitro* was studied as described. The results are shown in Tables 2 and 3 from which it appears that all four compounds were able to inhibit cell growth completely at concentrations about 0·2–0·3 mM. P-diphenyl ITC seemed somewhat more active than the other three compounds.

The cytocidal effect of the esters on ELD cells propagated *in vitro* was investigated by the trypan blue test. As shown in Table 4 the highest dose, which caused significant but incomplete growth inhibition, had no significant cytocidal effect except for *p*-diphenyl-ITC, which caused a slight increase in the percentage of trypan blue positive cells.

In the hope to elucidate the mechanism of the cytostatic effect of the ITC esters, their influence on the respiration, glucose consumption, and lactate production of the ELD cells at concentrations corresponding to min. ID_{100} was investigated. The results are presented in Tables 5–7B. It is seen that all four compounds had an antirespiratory effect, while the expected antiglycolytic effect could not be demonstrated under aerobic conditions. On the contrary, in the presence of all four compounds an increased glucose consumption and lactate accumulation were seen under aerobic conditions at the concentrations used in the present experiments. The effect on anaerobic glycolysis was less consistent. Two of the esters seemed to exert a moderate inhibition while two did not show any effect.

In order to obtain additional information about the antirespiratory activity of the ITC esters, their effect on NADH and succinate oxidation by mitochondria prepared from ELT cells propagated *in vivo* was investigated. As shown in Table 8 all 4 esters had a considerable inhibitory effect on NADH oxidation, while the effect on succinate oxidation was less pronounced and more variable (Table 9).

TABLE 2. CYTOSTATIC EFFECT OF ISOTHIOCYANIC ACID ESTERS ON ELD CELLS GROWN *IN VITRO*

	β-naphthyl-ITC		*p*-diphenyl-ITC	*p*-brom phenyl-ITC		*p*-tolyl-ITC	
Initial cel counts* in millions	0·35 ± 0·09	0·22 ± 0·04	0·18 ± 0·04	0·22 ± 0·04	0·18 ± 0·04	0·61 ± 0·10	0·16 ± 0·03
Final cell counts* in millions at different concentrations of compounds†							
0	3·50 ± 0·32	2·30 ± 0·30	2·62 ± 0·36	2·30 ± 0·30	2·62 ± 0·36	2·42 ± 0·41	0·76 ± 0·12
10 μg/ml	3·26 ± 0·36			2·46 ± 0·28		1·70 ± 0·31	
37·5 μg/ml		0·82 ± 0·14					0·48 ± 0·11
40 μg/ml			0·34 ± 0·08				
50 μg/ml	0·34 ± 0·02		0·04 ± 0·01	0·73 ± 0·18		0·13 ± 0·02	
66 μg/ml					0·26 ± 0·08		

* Median values with 95 per cent confidence interval.

† Values given for *p*-tolyl-ITC are in μl.

TABLE 3. MIN. ID_{100} OF FOUR ISOTHIOCYANIC ACID ESTERS ACTING ON ELD CELLS GROWN *IN VITRO*

	Min. ID_{100} interval
β-Naphthyl-ITC	0·203 mM < min. ID_{100} < 0·270 mM
p-Diphenyl-ITC	0·190 mM < min. ID_{100} < 0·237 mM
p-Brom phenyl-ITC	0·234 mM < min. ID_{100} < 0·309 mM
p-Tolyl-ITC	0·248 mM < min. ID_{100} < 0·370 mM

TABLE 4. PER CENT TRYPAN BLUE POSITIVE CELLS IN CELL CULTURES OF ELD CELLS TREATED WITH ITC ESTERS

	Trypan blue positive cells in per cent			
Duration of treatment	0 hr	24 hr	48 hr	72 hr
β-Naphthyl-ITC 0·20 mM	12·3 ± 3·2	29·5 ± 11·2	14·5 ± 6·8	13·5 ± 4·4
p-Diphenyl-ITC 0·18 mM	—	25·0 ± 5·6	22·5 ± 5·2	15·5 ± 4·9
p-Brom phenyl-ITC 0·23 mM	—	12·0 ± 6·8	13·5 ± 4·0	8·0 ± 2·9
p-Tolyl-ITC 0·25 mM	—	14·0 ± 10·0	11·0 ± 4·8	10·0 ± 0·1
Control	—	14·0 ± 4·8	7·0 ± 4·0	6·0 ± 3·8

(Each value representing the median value of 6 counts of 3 different cultures with 95 per cent confidence interval.)

TABLE 5. INFLUENCE OF ITC ESTERS ON OXYGEN CONSUMPTION OF ELD CELLS

Compound	Oxygen consumption $\mu l/10^6$ cells/hr				Per cent inhibition
Control	(1)	7·94	(2–5)	7·99	
(1) β-Naphthyl-ITC 0·20 mM		6·28			21
(2) *p*-Diphenyl-ITC 0·18 mM				6·06	24
(3) *p*-Brom phenyl-ITC 0·23 mM				5·03	37
(4) *p*-Tolyl-ITC 0·25 mM				4·39	45
(5) Ethanol 0·5 per cent v/v				7·50	6

TABLE 6A. INFLUENCE OF ITC ESTERS ON GLUCOSE CONSUMPTION OF ELD CELLS. AEROBIC CONDITIONS

Compound	Glucose consumption $\mu mole/10^6$ cells/hr				Per cent increase
Control	(1)	0·43	(2–5)	0·46	
(1) β-Naphthyl-ITC 0·20 mM		0·53			23
(2) *p*-Diphenyl-ITC 0·18 mM				0·60	30
(3) *p*-Brom phenyl-ITC 0·23 mM				0·75	63
(4) *p*-Tolyl-ITC 0·25 mM				0·53	15
(5) Ethanol 0·5 per cent v/v				0·45	2

TABLE 6B. INFLUENCE OF ITC ESTERS ON GLUCOSE CONSUMPTION OF ELD CELLS. ANAEROBIC CONDITIONS

Compound	Glucose consumption μmole glucose/10^6 cells/hr	Per cent decrease
Control	0·25	
(1) β-Naphthyl-ITC 0·20 mM	0·16	36·0
(2) *p*-Diphenyl-ITC 0·18 mM	0·26	−4·0
(3) *p*-Brom phenyl-ITC 0·23 mM	0·18	28·0
(4) *p*-Tolyl-ITC 0·25 mM	0·25	0·0
(5) Ethanol 0·5 per cent v/v	0·24	4·0

TABLE 7A. INFLUENCE OF THE ITC ESTERS ON LACTATE PRODUCTION OF ELD CELLS. AEROBIC CONDITIONS

Compound	Lactate production μmole/10^6 cells/hr			Per cent increase
Control	(1)	0·63	(2–5) 0·49	
(1) β-Naphthyl-ITC 0·20 mM		0·87		38
(2) *p*-Diphenyl-ITC 0·18 mM			0·90	84
(3) *p*-Brom phenyl-ITC 0·23 mM			0·84	71
(4) *p*-Tolyl-ITC 0·25 mM			0·79	61
(5) Ethanol 0·5 per cent v/v			0·63	29

TABLE 7B. INFLUENCE OF ITC ESTERS ON LACTATE PRODUCTION OF ELD CELLS. ANAEROBIC CONDITIONS

Compound	Lactate production μmole/10^6 cells/hr	Per cent decrease
Control	0·48	
(1) β-Naphthyl-ITC 0·20 mM	0·42	12·5
(2) *p*-Diphenyl-ITC 0·18 mM	0·50	−4·2
(3) *p*-Brom phenyl-ITC 0·23 mM	0·38	20·8
(4) *p*-Tolyl-ITC 0·25 mM	0·46	4·2
(5) Ethanol 0·5 per cent v/v	0·48	0

Finally a study was made of the influence of ITC esters on glucose-6-phosphate dehydrogenase. It appears from Table 10 that all 4 esters inhibited this enzyme, but the activity varied greatly from one compound to another.

DISCUSSION

The results of the present studies of the cytostatic effect of ITC esters are in agreement with those reported by Nemec *et al.*[1] However, they do not suport the assumption that this effect is due to an inhibition of glycolysis. Although two of the compounds studied showed a moderate inhibitory effect on anaerobic glycolysis, no

TABLE 8. THE EFFECT OF ITC ESTERS ON NADH OXIDATION BY MITOCHONDRIA PREPARED FROM EHRLICH'S ASCITES TUMOR CELLS

	Q_{O_2}*	Per cent inhibition
Control without NADH	0	
Control with NADH	9·95	
β-Naphthyl-ITC 0·20 mM	5·26	47
p-Diphenyl-ITC 0·18 mM	6·91	30
p-Brom phenyl-ITC 0·23 mM	4·65	53
p-Tolyl-ITC 0·25 mM	6·02	40

* Q_{O_2}: μl O_2 consumed/mg dry wt./hr.

TABLE 9. THE INFLUENCE OF ITC ESTERS ON OXIDATION OF SODIUM SUCCINATE BY MITOCHONDRIA PREPARED FROM EHRLICH'S ASCITES TUMOR CELLS

	Q_{O_2}	Per cent inhibition
Control without succinate	0	—
Control with succinate	3·66	—
β-Naphthyl-ITC 0·20 mM	3·14	14
Control with succinate	2·52	—
p-Diphenyl-ITC 0·18 mM	2·17	14
Control with succinate	2·30	—
p-Brom-phenyl-ITC 0·23 mM	2·09	9
p-Tolyl-ITC 0·25 mM	1·75	24
Control with succinate	1·63	—
Ethanol 0·5 per cent v/v	1·64	− 0·1

TABLE 10. EFFECT OF ITC-ESTERS ON REDUCTION OF NADP BY GLUCOSE-6-PHOSPHATE DEHYDROGENASE MEASURED SPECTROPHOTOMETRICALLY AT 366 mμ

	ΔE/min.	Per cent inhibition
Control	0·018	—
β-Naphthyl-ITC 0·20 mM	0·016	11
p-Diphenyl-ITC 0·18 mM	0·014	22
p-Brom phenyl-ITC 0·23 mM	0·009	50
p-Tolyl-ITC 0·25 mM	0·012	33

inhibition was seen under aerobic conditions. On the contrary aerobic glycolysis was found to be somewhat higher in the presence of ITC esters, which may be due to a decreased Pasteur effect. The discrepancy between the present results and those reported by Nemec *et al.* might be due to the fact that Nemec *et al.* used 5–6 times higher concentrations than were used in the present experiments, which shows that growth is more sensitive to these compounds than the glycolytic metabolism. This is not an unusual finding; similar observations have previously been made in this laboratory with 2-deoxyglucose and with monoiodoacetate.[2]

The antirespiratory effect, on the other hand, was obtained at concentrations corresponding to min. ID_{100}, suggesting that lack of respiratory energy or of essential metabolites derived from respiratory metabolism may be responsible for the cytostatic effect. However, these two explanations are perhaps not likely to be true, since

it has previously been shown[2] that the ELD cells are resistant to the cytostatic effect exerted by high CO_2 tensions, which have a greater inhibitory effect on cellular respiration than the present compounds.

The pronounced and consistent effect on NADH oxidation indicates that a disturbance of the NAD/NADH balance may be of primary importance for the anti-respiratory effect. The influence on succinate dehydrogenase was less convincing, which is in agreement with the results reported by Nemec *et al.*[1] At concentrations nine times higher than the concentrations used in the present experiments these authors using a colorimetric technique found that *p*-brom phenyl-ITC inhibited succinate dehydrogenase by 9 per cent, while the effect of β-naphthyl-ITC was only 2 per cent.

Nemec *et al.* also studied the effect of *p*-brom phenyl-ITC and β-naphthyl-ITC on glucose-6-phosphate dehydrogenase with the colorimetric technique and found an inhibition by 18 per cent and 68 per cent respectively. This effect was obtained at a concentration of ITC esters about nine times higher than the min. ID_{100} used in the present experiments which, however, showed an inhibitory effect even at this lower concentration. Thus, a decreased rate of pentose phosphate synthesis may contribute to the cytostatic effect of the ITC esters.

According to Nemec *et al.*[1] the biological and biochemical effect of the ITC esters is associated with the —N=C=S group. The small differences between the min. ID_{100} of the four compounds studied in the present experiments may support this claim, but the differences between their biochemical effects points in the opposite direction.

The cytostatic effect of the ITC esters raises the question whether they may be of any value as antineoplastic drugs. Further studies of their toxicity is required before this question can be answered. So far only the LD_{50} for β-naphthyl ITC is known. This being 0·87 m-mole/kg body wt. as compared to a min. ID_{100} between 0·20 and 0·27 mmoles suggests that the effective therapeutical dose is close to the upper limit of toxicity usually allowed for antineoplastic agents. However, other derivatives may be less toxic.

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