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Ethyl 4-isothiocyanatobutanoate—antiproliferative activity *in vitro* and *in vivo*

Katarína Horáková, Soňa Jantová, Miroslav Stred'anský,[1] L'udmila Novotná[3] and L'ubomír Floch[2]

Department of Microbiology, Biochemistry and Biology, [1]Department of Biochemical Technology and [2]Department of Organic Chemistry, Faculty of Chemical Technology, Slovak Technical University, Radlinského 9, SQ-812 37 Bratislava, Slovakia. Tel: (+42) 7 506040; Fax: (+42) 7 49318.
[3]Cancer Research Institute, Slovak Academy of Sciences, SQ-812 32 Bratislava, Slovakia

A new isothiocyanate (ITC) derivate ethyl 4-isothiocyanatobutanoate (E-4IB) induces an immediate dose-dependent inhibitory action on the division of HeLa cells in the concentration range 1.0–0.1 mg/l. Concomitant with the decrease in cell proliferation which follows E-4IB treatment the protein:cell number ratio increases and DNA accumulates. Cells which have lost their ability to divide do not stop their glucose metabolism and only partly stop their glutamine metabolism. The increased content of DNA suggests that cells synthesize DNA without entering into mitosis and that dying cells are in late S or G_2 phases prior to death. E-4IB produces a significant growth inhibition of transplanted sarcoma cells B77-RF in rats (at 28 mg/kg). A 57% regression in tumor volume was observed for at least 30 days following the completion of the *in vivo* treatment. These findings support the presumption that E-4IB is a potential anti-cancer drug. However, further studies are needed for the optimization of its *in vivo* activity.

Key words: Ethyl 4-isothiocyanatobutanoate, interphase death, *in vitro* and *in vivo* interaction, unbalanced cell metabolism, unbalanced growth.

Introduction

The biological activity of isothiocyanates (ITC) is based on the isothiocyanate group (–N=C=S) which reacts with the thiol (–SH) groups of proteins. Inhibition of glycolysis in yeasts, bacteria and mammalian cells is elicited by inactivation of glyceraldehyde-3-phosphate dehydrogenase, hexokinase and other enzymes, which require free thiol groups for their activity. Inhibition of some functions in isolated mitochondria and inhibition of protein synthesis in cell-free extracts have also been explained by the reactions of ITC with protein –SH groups. They display various biological activities, depending on the chemical structure of the radicals.

In previous papers[2,3] we have summarized the data characterizing the cytotoxicity of naturally occurring ITC, their synthetic analogues as well as series of aromatic ITC and polycondensed aromatic hydrocarbons on HeLa cells. Esters of isothiocyanatocarboxylic acids rank among the most potent compounds in the group 228 ITC which have been tested for cytotoxic activity in HeLa cells and V79 cells.[4]

Ethyl 4-isothiocyanatobutanoate (E-4IB), formally a derivative of γ-aminobutyric acid (GABA), is the most potent ITC tested so far against the above mentioned cell lines. As it did not express any antimicrobial activity, E-4IB can be characterized as a compound with specific cytotoxic efficiency. The study of its mutagenicity using the Ames test provided negative results.[4]

We have investigated the relationship between the lethal effects of E-4IB and the mechanism of its cytotoxic action. Moreover the anti-cancer capacity of this drug was studied in an *in vivo* system.

Materials and methods

In vitro studies

Drug. E-4IB was synthesized from hydrochloride GABA ester with thiophosgene in the chloroform:water system.[5] The IR spectra were taken with a Philips analytical PV 980 FTIR spectrometer: IR (KBr, neat), [cm^{-1}]: 2187 (NCS), 1732 (CO_2Et) and NMR spectra with a Varian VXR 300 MHZ spectrometer for solution in deuterochloroform (internal ME_4Si): ^{1}H-NMR [δ, p.p.m.]: 1.27(t, 3H, $J = 7.1$), 2.01 (kv, 2H, $J_{3,2} = 6.4$, $J_{3,4} = 7.0$), 2.46 (t, 2H, $J_{4,3} = 7.0$), 3.63 (t, 2H, $J_{2,3} = 6.4$), 4.16 (q, 2H, $J = 7.1$) [t = triplet, q = quartet, kv = kvintet]. ^{13}C-NMR [δ, p.p.m.]: 172.06 (C-1), 130.29 (NCS), 60.64 (OCH_2), 44.26 (C-2), 30.87 (C-4), 25.12 (C-3), 14.10 (CH_3).

Correspondence to: K Horáková

Cell cultures. HeLa cells, kindly supplied by Dr P Veber (Institute for Virology, Slovak Academy of Sciences, Bratislava) were maintained in Basal Eagle medium (BEM, USOL Prague) supplemented with 10% heat-inactivated calf serum (ICS), non-essential amino acids (1 ampule/l), glutamine (300 mg/l) and antibiotics (10^5 U/l penicillin, 100 mg/l streptomycin). Trypsin–EDTA was used to prepare single cell suspensions.

HeLa cells, free from mycoplasma, were kept in 5% CO_2 solution in air in a high humidity atmosphere at 37°C. The cells used in the experiments were just in the exponential phase.

Cytotoxic assay. Growth curves were initiated by seeding 2.5×10^5 HeLa cells per 5 ml in Müller flasks. On the second day of cultivation 50 μl of E-4IB at the indicated concentrations was added. Control cells were treated with the same amount of vehicle alone [dimethylsulfoxide (DMSO)]. The final DMSO concentration never exceeded 0.5% (v/v) in either control or treated samples.

After specified time intervals, first the effect of the derivative on cell morphology was evaluated (using the light microscope), and then the cell sheets were washed once with phosphate buffered saline (PBS) to remove fragments and dead cells. The flasks were harvested in triplicate with 0.25% trypsin, washed once with PBS and resuspended in the later. One portion of this suspension was used for the determination of cell number using a counting chamber and protein content.[6] Another portion was alternatively frozen and thawed three times to disrupt cells and then the nucleic acids were extracted by a modified Schmidt–Tannhauser procedure.[7] The DNA content of the extracts was estimated by the diphenylamine method[8] and that of RNA by ultraviolet absorption measurement.[9] To standardize the variability in protein and nucleic acids cell content among the different control groups at the indicated time intervals, the relative amounts (RA) were estimated using the formula: $RA = C_1/C_0$, where C_1 is the mean DNA, RNA or protein cell content of a treated group and C_0 the control mean for that group at a given time. Simultaneously, the culture medium was gradually taken out of the culturing flasks and after centrifugation (20 min at 180 *g*) it was biochemically analyzed. The concentrations of glucose,[10] lactate[11] and glutamine[12] were determined by enzymatic methods. The utilization of substrates as well as the production of metabolites was expressed as mmol/μg protein/10^6 cells/24 h.

In vivo studies

Animals and tumors. The 2 month old rats, weighing about 180 *g*, used in the study were purchased from the Cancer Research Institute (Slovak Academy of Sciences, Bratislava) and maintained in standard conditions.

Rat fibrosarcoma cells (B77-RF) transformed by B77 avian sarcoma virus were used from a spontaneous chicken tumor.[13] This oncovirus exhibits a broad host range and can induce sarcomas in various species of animals. Solid B77-RF fibrosarcomas induced in rats can be easily passaged in the syngeneic system.[14]

Following excision, rat tumors were collected in PBS and minced with scissors. A suspension of single cells was washed once with PBS and adjusted to a concentration of 10^7 viable cells/ml as assessed by Trypan blue exclusion. Inbred Lewis (LW) rats were inoculated subcutaneously with 1 ml of the respective tumor cell suspension for line maintenance and for chemotherapy studies.

For toxicity studies Sprague-Dawley (SD) rats were injected intraperitoneally.

Toxicity. The drug was dissolved in 80% DMSO and administered intraperitoneally in a volume of 2.5 ml/kg body weight. Toxicity was studied in the range of concentrations from 7.5 mg/kg to 1.5 g/kg in five parallel experiments. The percentage of dead animals was determined 48 h following exposure to the drug. The LD_{50} value was calculated from the dose–survival curve with the least squares method.

Evaluation of antitumor activity. The chemotherapy course was started after 5 days of tumor cell transplantation. The drug was administered intraperitoneally every day. Single doses of E-4IB (7, 14 and 28 mg/kg), which represented 5, 10 and 20% of the LD_{50} value, were given for 5 days. Control rats received intraperitoneal injections of sterile 80% DMSO every day throughout the treatment period. The tumor size was measured 3 days after finishing the treatment. Tumor volume was calculated as the volume of the ellipsoid from the linear measurements using the formula:

$$V = 4/3\pi a^2 b$$

where a represents half of the tumor width (in cm) and b represents half of the length (in cm). The cytostatic effect *in vivo* was determined by comparing the retardation of tumor growth in the control and treatment groups. The experimental groups consisted of five rats each for the control

group and for each treatment group. Variability was expressed as the standard error of the mean and statistical significance of the results was determined with Student's *t*-test.

Results

In vitro studies

The proliferation of HeLa cells during permanent exposure to E-4IB concentrations ranging from 1.0 to 0.1 mg/l is shown in Figure 1. As can be seen from the growth curves after 24 h of culturing, all of the tested concentrations had an immediate cytotoxic effect which even led to degeneration in some part of the HeLa cell populations. The next 24 h interval brought a rise of cell number under the three lowest concentrations. After 72 h the cytotoxic effect of E-4IB in the concentrations monitored was directly proportional to the actual concentration and represented an inhibition as compared with the control.

To characterize the cytotoxic effect of E-4IB in greater detail, the changes in the amount of DNA, RNA and protein resulting from exposure of the cells to E-4IB were measured. The decrease in cell proliferation which followed E-4IB treatment at concentrations of 0.5 and 0.25 mg/l was accompanied by a considerable increase in the cellular content of DNA. At the same time a slight but statistically significant increase (at 48 h), followed by a decrease (at 72 h), in cellular RNA occurred (Table 1). The protein content showed a peak at 48 h of E-4IB exposure (Table 2).

Table 3 shows the effect of E-4IB on HeLa cell metabolism. The culture medium was analyzed at 24 h intervals simultaneously with the determination of cell number. The values of substrate uptake and metabolite production are expressed as mmol/μg protein/10^6 cells/24 h. Cells treated by 1.0 and 0.75 mg/l of the drug used 28.6–79% less glucose from the culturing medium during the whole incubation period than control cells. The three lowest E-4IB concentration tested (0.50, 0.25 and 0.10 mg/l) initiated, after 24 h, a 1.1–2.75 times higher glucose consumption, proportional to the drug concentration, as compared with the control. However, in the next 48 h interval the glucose uptake of the treated cells was nearly the same as

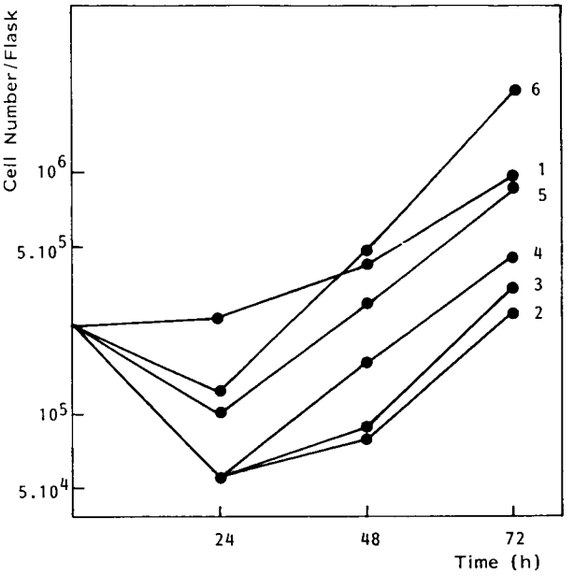

Figure 1. Cell proliferation in response to E-4IB at various times of exposure. Exponentially growing HeLa cells (2×10^4) were plated in Müller flasks in BEM medium containing 10% ICS. The drug was added 24 h after plating. At the indicated times cell proliferation was measured using a counting chamber. The cytotoxic values represent the mean of five replicates. Variations from the mean did not exceed 10%. Except at the lowest concentration tested the changes in the values are statistically significant at $p \leq 0.05$ as compared with the control. 1, control; 4-EIB in mg/l: 2, 1.0; 3, 0.75; 4, 0.5; 5, 0.25; 6, 0.1.

Table 1. Protein content of HeLa cells during exposure to 4-EIB

	Relative amount compared with control at the given time					
Drug concentration (mg/l)	0.00	1.00	0.75	0.50	0.25	0.10
Time (h)						
24	1.0	1.2	1.3	0.8	0.7	0.3
48	1.0	2.8	1.5	1.7	1.6	1.2
72	1.0	2.0	1.6	1.4	1.1	1.5

Average of three experiments is given. SD was less than 10% of the average values. The changes in the values compared with the control were significant ($p \leq 0.05$).

Table 2. Nucleic acid content of HeLa cells during exposure to 4-EIB

	Relative amounts compared to control at the given time					
Nucleic acids	DNA	DNA	DNA	RNA	RNA	RNA
Drug concentration (mg/l)	0.00	0.25	0.50	0.00	0.25	0.50
Time (h)						
24	1.0	1.3	1.4	1.0	1.1	0.9
48	1.0	1.5	1.8	1.0	1.1	1.2
72	1.0	1.7	1.9	1.0	0.8	0.9

SD averaged 10%. The changes in the values are statistically significant as compared with the control ($p \leq 0.05$).

Table 3. Effect of 4-EIB on the metabolism of HeLa cells

Exposure time (h)	Drug concentration (mg/l)	Consumption of glucose (mmol/l) $\times 10^{-1}$	Production of lactate (mmol/l) $\times 10^{-1}$	Conversion of glucose to lactate (%)	Consumption of glutamine (mmol/l) $\times 10^{-1}$
24	0.00	2.8 ± 0.2	4.2 ± 0.4	75.0 ± 6.0	0.7 ± 0.06
	1.00	2.0 ± 0.2	3.1 ± 0.3	77.5 ± 6.2*	0.4 ± 0.03
	0.75	1.4 ± 0.1	2.9 ± 0.2	103.5 ± 8.2	0.4 ± 0.03
	0.50	3.1 ± 0.3	4.5 ± 0.4*	72.5 ± 5.8*	0.3 ± 0.02
	0.25	3.8 ± 0.3	5.2 ± 0.5	68.4 ± 6.1	1.1 ± 0.10
	0.10	7.7 ± 0.6	8.3 ± 0.7	53.8 ± 4.3	2.7 ± 0.20
48	0.00	5.7 ± 0.5	8.5 ± 0.7	74.6 ± 6.7	0.7 ± 0.06
	1.00	1.2 ± 0.1	2.7 ± 0.2	128.5 ± 10.2	0.1 ± 0.01
	0.75	3.1 ± 0.3	5.8 ± 0.5	93.5 ± 7.4	0.5 ± 0.05
	0.50	1.8 ± 0.1	4.2 ± 0.4	116.6 ± 9.3	0.4 ± 0.04
	0.25	3.5 ± 0.3	7.9 ± 0.6	112.8 ± 9.0	0.2 ± 0.02
	0.10	5.3 ± 0.4*	10.0 ± 0.8	94.3 ± 7.4	1.0 ± 0.09
72	0.00	5.1 ± 0.4	6.0 ± 0.5	58.8 ± 4.6	0.9 ± 0.07
	1.00	1.4 ± 0.1	−0.1 ± 0.01*[a]	0.0	−0.08 ± 0.006*
	0.75	3.1 ± 0.3	−0.6 ± 0.06[a]	0.0	0.2 ± 0.02
	0.50	4.8 ± 0.4*	4.2 ± 0.4	67.7 ± 5.3	1.1 ± 0.10
	0.25	5.5 ± 0.5*	6.0 ± 0.5*	62.5 ± 5.0*	1.5 ± 0.10
	0.10	5.6 ± 0.5*	6.0 ± 0.5*	53.6 ± 4.3	0.2 ± 0.02

* The changes in the values are not statistically significant as compared with the control (significance level $p \leq 0.05$).
[a] Utilization of lactate.
[b] Production of glutamine.

in the control culture. At the same time the treated cells produced lactate in decreased proportions as compared with the control during the whole testing; however, the two lowest concentrations, after 24 h of treatment, caused a rise of 23.8 and 97.6%, respectively. After 24 h of cultivation in the presence of three concentrations of E-4IB used (1.0, 0.75 and 0.5 mg/l) treated cells utilized less glutamine compared with the control. The lowest concentrations of E-4IB increased glutamine uptake 1.6 and 3.9 times. In the next time interval, the first four concentrations of E–4IB induced inhibition of glutamine uptake. During 72 h, only concentrations of 0.5 and 0.25 mg/l increased glutamine uptake compared with the control.

In vivo studies

Because of the significant *in vitro* antiproliferative effects of E-4IB achieved, the antitumor activity *in vivo* was investigated. First of all the toxicity of the drug on Sprague-Dawley rats in the range of concentrations from 7.5 mg/kg to 1.5. g/kg was studied and the LD_{50} = 140.9 mg/kg (for intraperitoneal injection) value was determined. Then the cancerostatic activity *in vivo* was studied with the transplanted B77-RF line. The *in vivo* results are summarized in Figure 2.

The groups of five rats transplanted subcutaneously with 10^7 B77-RF cells were treated as described in Materials and methods. All of the

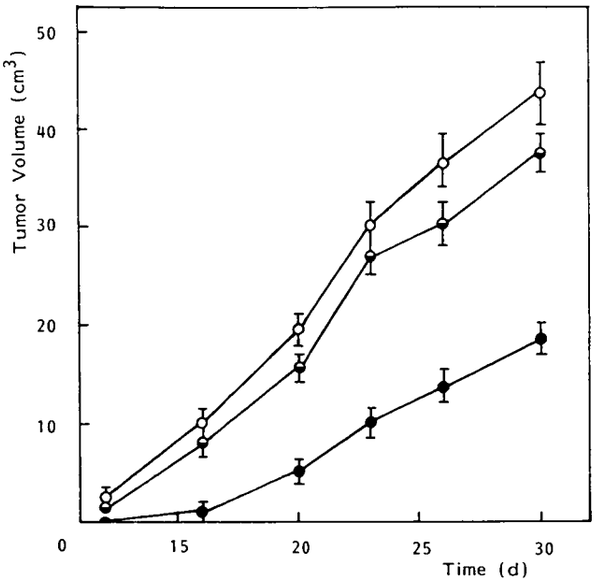

Figure 2. Antitumor effect of E-4IB in B77-RF. Ten million fibrosarcoma cells per rat were inoculated subcutaneously into the back of inbred Lewis rats. Treatment was initiated after 5 days of tumor cell transplantation. The E-4IB, 14 and 28 mg/kg, was administered intraperitoneally for 5 days. Results are expressed as the mean of tumor volume of a given group, each consisting of five rats, at a given time. Significance of difference from control values at 28 mg/kg $p \leq 0.001$: ○, control; ◒, 14 mg/kg; ●, 28 mg/kg E-4IB.

control animals grew well-circumscribed, spherically shaped, solid tumor masses during the 30 day study period. After five E-4IB injections of 7, 14 and 28 mg/kg, the tumor growth rate was significantly retarded ($p = 0.001$) at the highest doses and decreased by 57%, although total tumor regression did not occur. At all tested doses, E-4IB was well tolerated without apparent toxicity. Moreover much less damage of inner organs, induced by tumor growth, of treated animals (28 mg/kg) was observed as compared with the control at least 30 days following the completion of therapy.

Discussion

E-4IB induced a concentration-dependent acute cytotoxic reaction in HeLa cells. From the results presented, it is apparent that the initial aspect of the E-4IB cytotoxic reaction is characterized by unbalanced growth in which the protein:cell ratio as well as the DNA content increased (Tables 1 and 2). It can also be presumed that the surviving cells, not having the ability to divide, continued to synthesize proteins to a larger extent than the controls. Earlier data from our laboratory suggested that unbalanced growth (e.g. an increase in protein, DNA and RNA cell content) has to be considered as a possible mechanism of cell death induced during delayed (as compared with acute) cytotoxic expression of 6-mercaptopurine and 6-thioguanine (TG) toward HeLa cells.[15,16] Moreover, it was shown that the abnormal DNA (which was synthesized after incorporation of TG into DNA) accumulated in chromatin clusters and was not able to form chromosomal structures.[17] An increase in protein and the DNA:cell ratio is also typical in cells treated with other antitumor drugs which interfere with protein and nucleic acid synthesis.[18,19]

Studies on the cytotoxic activity of benfluron[20] have shown that unbalanced growth (which was characterized by a 1.5- to 1.8-fold increase and at the non-toxic concentrations by a 0.56- to 0.92-fold decrease of the cell protein content) induced by a cytostatic substance must be taken into account when expressing and interpreting the metabolism of influenced cells. Therefore the actual glucose and glutamine consumption and lactate production by HeLa cells is expressed as mmol/μg protein/10^6 cells/24 h (and not only by mmol/10^6 cells/24 h). The results reveal (Table 3) that at concentrations of 0.25 and 0.10 mg/l the treated cells utilize glutamine to a larger extent as the main source of energy throughout 72 h of E-4IB exposure. Simultaneously, there occurred a 1.4 and 2.8 times, respectively, higher glucose utilization only after 24 h, comparatively to the control. However, the three highest concentrations tested (0.1–0.5 mg/l) initiated an inhibition of glucose uptake (in the range 79.0–28.6%). A more pronounced inhibition in glutamine consumption was also apparent, particularly after 72 h (in the range 100–77.8%). Furthermore, the results listed in Table 3 show that at the same time re-utilization of lactate occurred in the treated cells. In addition it can be deduced from the glycolytic coefficient that after 48 h not only all of the glucose was converted to lactate by E-4IB-influenced cells (values about 100) but that some also have to use some other carbon sources besides glucose for lactate production. Evidence for this is proven by the fact that the obtained percentage of glucose transformation to lactate was higher than the theoretical rate of change (values above 100).

We conclude that cells, which partly or totally lose their ability to divide, continue to metabolize. As the surviving cells in fact do not divide and need energy for their metabolism, it can be expected that

E-4IB affects one of the phases of the cell cycle and only secondarily influences the energy processes in the cell. Since similar concentrations of the drug were suggested to interfere with DNA, RNA and protein synthesis, one cannot rule out the possibility that the observed decrease in cell proliferation and increase in protein, RNA and DNA amount imply that cells synthesized protein and DNA without entering into mitosis, and that the dying cells were in late S or G_2 phase immediately prior to death.

The significant cytotoxic activity of E-4IB *in vitro* suggested that this drug might have some cancerostatic potential. As its toxicity *in vivo* was not known, we first determined its LD_{50} value. The dose range of E-4IB was chosen on the basis of previous knowledge about the LD_{50} = 2350 mg/kg of an isothiocyanatocarboxylic acid ester derived from lysine (unpublished data). Because this ITC derivative was inactive *in vitro* (ID_{50} > 87.7 mg/l for HeLa cells) it could be expected that E-4IB would be more toxic *in vivo* (LD_{50} = 140.9 mg/kg).

With the use of B77-RF, therapeutic cures in rats were achieved when a transplanted fibrosarcoma was treated with E-4IB (28 mg/kg). During the first 8 days after the last treatment the tumor growth was inhibited with a greater than 80% reduction in tumor size. The observed decrease in tumor growth inhibition between day 15 and 30, i.e. 88–57% compared with the control group, can be explained by the metabolic transformation of the drug in the animal which could result in its inactivation. A degradation product of E-4IB could be GABA, which is normally present in organisms as part of the γ-aminobutyrate cycle (which acts as a neurotransmittor). It also has to be taken into consideration that the *in vivo* activity can vary with the anatomical site of drug administration[21] and that E-4IB was administered for only 5 days. However, our results indicate that E-4IB has cancerostatic properties which could probably be increased by knowing the period of its active action in the organism and further pharmacokinetic data.

Conclusion

Unbalanced growth and unbalanced metabolism (e.g. an increase in protein and DNA cell content, increase or decrease in RNA cell content, in glycolytic activity and in glutamine metabolism) are both an integral part of lethal E-4IB exposure to HeLa cells. The obtained results lead to the presumption that E-4IB primarily interferes with some of the cell cycle phases and only secondarily influences energy processes in the cell. More research is required to establish optimal therapy schedules in B77-RF and other animal tumor models, and in the synthesis and biological activity of E-4IB-related compounds.

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