Antiproliferative Action of Isatine-β-thiocarbohydrazone and N-Ethylisatine-β-thiocarbohydrazone on Human PBMC and on Two Neoplastic Cell Lines

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The antiproliferative action of two synthetic compounds, isatine-b-thiocarbohydrazone (IsTCH) and N-ethyl isatine-β-thiocarbohydrazone (N-Et-IsTCH), towards healthy human peripheral blood mononuclear cells (PBMC) and two neoplastic cell lines *in vitro*, was investigated. IsTCH and N-Et-IsTCH were dissolved in DMSO and then diluted with nutrient medium to desired final concentration. Target cells were PBMC, as well as human cervix carcinoma - HeLa cells, and murine melanoma B16 cells.
Five different concentrations (3 μM to 50 μM) of investigated agents were applied on target cells. Cell survival was determined 72 h after the agent's action using MTT test. Results obtained showed that both investigated compounds exerted a dose dependent antiproliferative action to neoplastic cell lines. Their action was only cytostatic; trypan blue exclusion test did not show any sign of direct drug cytotoxicity when drugs concentration were less than 50 μM. ICs50±SD for IsTCH antiproliferative action were 61.69 ± 4.25 μM for HeLa cells; 34.1 ± 7.15 μM for B16 cells: 17.62 ± 7.11 μM for nonstimulated and 30.0 ± 9.46 μM for stimulated (by 5mg/ml PHA) PBMC. ICs50 ±SD for the action of N-Et-IsTCH were 21.86 ± 1.77 μM for HeLa cells; 10.37 ± 1.55 μM for B16 cells; >47 μM for both, nonstimulated and for stimulated, PBMC. Nonstimulated human PBMC appeared to be the most sensitive to the cytostatic IsTCH action; while HeLa cells were the most resistant. N-Et-IsTCH showed more than two or five fold stronger antiproliferative effect toward B16 cells than on HeLa or PBMC cells, respectively, and more than three times intensive activity compared to IsTCH, indicating specificity of N-Et-IsTCH towards inhibition of melanoma cell growth. While increasing concentrations of IsTCH led to decrease in the the PBMC induced suppression of HeLa cell survival.; N-Et-Is-TCH in the difference from IsTCH, in dose dependent way contributed to the PBMC induced suppression of HeLa cell survival. In conclusion, the activity of N-Et-Istch on malignant melanoma cells deserves further investigation.

Key Words: Isatine-β-thiocarbohydrazone, N-ethyl isatine-β-thiocarbohydrazone, MTT, HeLa, B16

The seriousness of malignant diseases makes the search for new compounds that could be potential anticancer drugs as an imperative in modern medicine. In this sense the aim of our work was to test the action of two synthetic compounds, isatine-β-thiocarbohidrazone (IsTCH) and N-ethylisatine-β-thiocarbohidrazone (N-Et-IsTCH (chemical structures presented on Figure 1A and 1B) on the growth of two neoplastic cell lines. This study has been started since in the recent literature it was not possible to find any data on their biological action. As we have found that examined compounds affected proliferation of two neoplastic cell lines, we performed some additional experiments to see if there were any effect of these compounds on normal cells, on human PBMC, and what was their effect on PBMC mediated, suppression of HeLa cells survival.

Material and Methods

Synthesis of 3-thiocarbazono-2-indolinone (IsTCH) was reported earlier. (1): Aqueous solution of 1.47 g (0.1 mM) of indolin-2,3-dione, was slowly added into a hot solution of 3.2 g thiocarbo-hydrazide (TCH) in 50 cm^3 of water, acidifyed with 2-3 drops of HCl, during

half hour. Reaction mixture was refluxed for another half hour. Precipitate was filtered on Büchner funnel, washed several times with hot water. The crude product was dried in exicator. Yield is 2.9 g of yellow silky fine crystals, m.p. 240°C. After the recrystallisation from pyridine the small yellow crystal needles were obtained that decomposed by melting (2). Purity was proven by NMR and IR spectra.

Synthesis of 3-thiocarbazono-N-ethyl-2-indolinone (N-Et-IsTCH) (1): Aqueous solution of 1-ethylindolin-2,3-dione was added dropwise into the hot solution of TCH 0.6 g in 10 ml water, acidified with 2 drops of HCl. After the reflux, a precipitate was filtered and purified. M. p. >300°C (2). Purity was proven by NMR and IR spectra.

Stock solutions of investigated compounds were made in dimethyl sulfoxide (DMSO) at concentration of 6.5 mM IsTCH, and 5.7 mM N-Et-ITCH, and afterwards diluted by nutritient medium to various final concentrations needed (in the range 3-54 μM). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemicals (St. Luis, MO, U.S.A.). MTT was dissolved, 5 mg/ml in phosphate buffer saline pH 7.2, and filtered through milipore filter, 0.22 μm, before use. RPMI 1640 cell culture medium and fetal bovine serum, (FBS) were products of Gibco (Paisley, Scotland, U.K.)

Cell culture. Mouse melanoma B16 cells, and human cervix carcinoma HeLa cells were maintained as a monolayer culture in the same nutritient medium (RPMI 1640 medium supplemented with l-glutamine (3 mmol/L), strepto-mycin, and garamycin (100 μg/mL, each), 10% heat inactivated fetal bovine serum, FBS and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution). The cells were grown at 37°C in 5% CO2 and humidified air atmosphere, by twice weekly subculture.

Preparation of peripheral blood mononuclear cells. PBMC were separated from whole heparinised blood of healthy volunteers (age range 20-50 years) by Lymphoprep™ gradient centrifugation. Interface cells, washed three times with Haemaccel® aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM.$Ca2^+$, 145 mM Cl- and 35 g/L gelatine polymers, pH=7.4, were counted and resuspended in nutrient medium.

Treatment of PBMC. Target cells were seeded (100,000 cells per well) into nutrient medium and in nutrient medium enriched with 5mg/ml phytohaemaglutinin, PHA, (Welcome) in 96-well microtiter plates and two hours later, five different concentrations of investigated compounds were added to the PBMC in quadruplicates to final concentrations (3-54 μM), except to the control wells where a nutrient medium was added to the cells. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank.

Treatment of B16 and HeLa cells. Target cells were seeded (1,500 cells per well) into different 96-well microtiter plates and twenty hours later, five different concentrations of investigated compounds were added to the wells to final concentrations (3-54 μM), except to the control wells where a nutrient medium with was added to the cells. All analyses were done in triplicate. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank, in triplicate too.

Determination of PBMC, HeLa and B16 cell survival. Cell survival was determined by MTT test according to the method of Mosmann 1983 (3), modified by Ohno and Abe 1991 (4), and reported by Juranić et al.,1998 (5) 72 h after the drug addition. Briefly, 20 μl of MTT solution (5 mg/ml PBS) were added to each well. Samples were incubated for further four hours at 37°C in 5% CO_2 and humid-ified air atmosphere. Then, 100 μL of 10% SDS in 0.01M HCl were added to the wells. Optical density (OD) at 570 nm was red the next day. To get cell survival (%), optical density at 570 nm of a sample with cells grown in the presence of various concentration of investigated agent (OD), was divided with control optical density ODc, (the OD of cells grown only in nutritient medium) 100. (It was implied that OD of blank was always subtracted from OD of a corresponding sample with target cells). Concentration IC50 was defined as the concentration of a drug needed to inhibit cell survival by 50%, compared with vehicle-treated control.

B16 and HeLa cells treated for 72 hours with investigated compounds, in microtiter plates, corresponding to those assayed by MTT test, were fixed with ethanol and stained with hematoxilin-eosin for morphological examinations of drugs treated cells.

Viability and survival of HeLa and B16 cells treated for 72 hours with investigated compounds, in microtiter plates, were also analysed by trypan blue exclusion test.

Determination of PBMC induced suppression of HeLa cell survival. This test was performed according to the previously published procedure (6, 7). HeLa cells were seeded in into 96 microtiter plates (1,500 cells per well). After 20 hours to one series of wells without or with HeLa cells 30,000, or 15,000 PBMC were introduced. Than immediately, five different concentrations of IsTCH or N-Et-Is TCH were added to

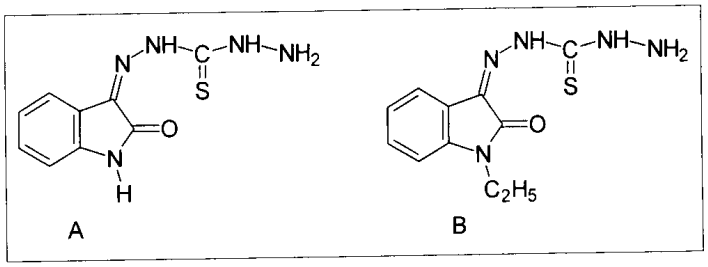

Fig. 1 - Structures of isatine-β-thiocarbohydrazone (A) and N-ethylisatine-β-thiocarbohydrazone (B)

the groups of wells with or without PBMC, with or without HeLa cells, and in samples with mixed cultures of HeLa and PBMC. In all cases nutrient medium with all corresponding additions, but void of target cells was used as blank. HeLa cells survival was determined assayed by MTT test, 24, 48 and 72 hours after the continuous agents and PBMC action. In this way we compared the effects of IsTCH, or N-Et-IsTCH, or of PBMC, as well as of their combinations with the proliferation of cells grown only in nutrient medium.

Results

The effect of IsTCH and of N-Et-IsTCH on the HeLa and B16 cell survival, 72 hrs after the agent actions was shown on Fig. 2 and Fig.3 respectively. It could be seen that investigated agents expressed the dose dependent antiproliferative action toward target cell lines; N-Et-IsTCH being stronger than IsTCH in the suppression of neoplastic cell survival. Data presented on Fig.4 (A) and (B) showed that IsTCH induced dose dependent suppression of nonstimulated and stimulated by (5mg/ml PHA) PBMC obtained from three different healthy volunteers. Contrary, data from diagrams in Fig.5 (A) and (B) indicate to some resistance of nonstimulated and stimulated PBMC, to the antiproliferative action of N-Et-IsTCH.

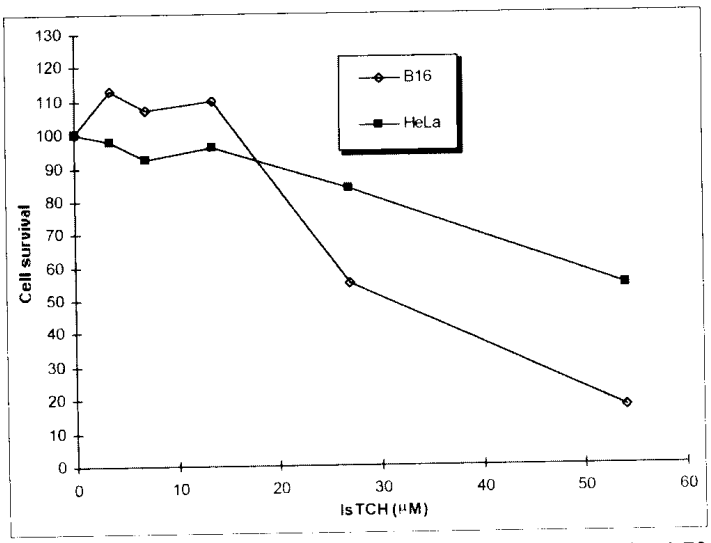

Fig. 2 - Survival of HeLa (■) and B16 cells (◇), determined 72 hours after the IsTCH action, is given as the function of different drug concentration. CV was less than 8%.

Data presented on Table I. showed IC50 for IsTCH antiproliferative action. These were (61.69 ± 4.25) μM for HeLa cells; (34.1 ± 7.15) μM for B16 cells: (17.62 ± 7.11) μM for nonstimulated PBMC and (30.0 ± 9.46) μM for stimulated by (5 μg/ml PHA) PBMC. ICs50 for N-Et-IsTCH action were (21.86 ± 1.77) μM for HeLa cells; (10.37 ± 1.55) μM for B16 cells: >47 μM for nonstimulated PBMC and for stimulated PBMC. Nonstimulated human PBMC appears to be the most sensitive to the cytostatic IsTCH action; while HeLa cells are the most resistent cell line. N-Et-IsTCH showed more than three times stronger antiproliferative effect toward B16 cells than on HeLa or PBMC cells, indicating specificity of N-Et-IsTCH towards inhibition of melanoma cell growth.

Morphological examination of target cells on inverted microscope did not show any signs of cytotoxic action of investigated compounds at the concentration lower than 50 μM. Cells were adherent to the surface of the wells, and more pigmented cells were observed in B16 cells treated with 48 μM N-Et-IsTCH, than in control, or in samples treated with the same concentration of Is-TCH. There were some phylopodia in B16 cells treated with high concentrations (up to 50 μM) of investigated agents; these were not seen in untreated B16 cells.

Data on HeLa and B16 cell survival and viability, obtained 72 hours after continuous agents action using

Table I - IC50 for the action of IsTCH and N-Et-IsTCH on various cell lines and human PBMC. Data were obtained from at least three independent experiments each done in triplicate.

Cell line	IsTCH (μM) Means	± SD	N-Et-IsTCH (μM) Means	± SD
B16	34.17	7.15	10.37	1.55
HeLa	61.68	4.23	21.86	1.77
PBMC	17.62	7.11	>47	
PBMC stimulated by 5 μg/ml PHA	30	9.46	>47	

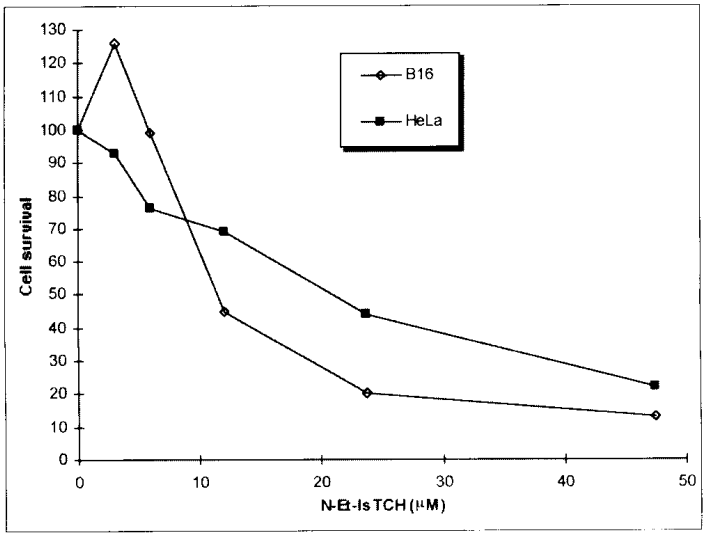

Fig. 3 - Survival of HeLa (■) and B16 cells (◇), determined 72 hours after the N-Et-IsTCH action, is given as the function of different drug concentration. CV was less than 9%.

trypan blue exclusion test are pesented on Table II. It could be seen that investigated compounds induced only reproductive cell death. Direct agents cytotoxicity was not observed since cell viability was very high (>95%) even in the highest applied agents concentration. The emergence of giant B16 cells, (8-18%) was observed under the action of N-Et-IsTCH, accompanied by varying number of melanin producing cells in comparison to control. The action of IsTCH produced only variable number if melanin producing B16 cells.

Results obtained 24, 48 and 72 hrs after the set up of mixed cell culture, using MTT test, showed that increased concentrations of IsTCH led to the slight increase in the percent (Fig. 6) and of the total number (Fig. 8) of survived HeLa cells grown in the mixed culture with human PBMC; abrogating the PBMC mediated suppression of target cell survival. Co-culture of HeLa cells with 15,000 PBMC and with N-Et-IsTCH led to the very pronounced decrease in HeLa cells survival and total number of survived HeLa cells, that was proportional to the N-Et-IsTCH concentration and to the incubation time (Figs. 7 and 9 respectively).

Therefore, N-Et-IsTCH, besides the direct antiproliferative action on neoplastic cells, as the difference from IsTCH, in dose dependent way contributed to PBMC induced suppression of HeLa cell survival.

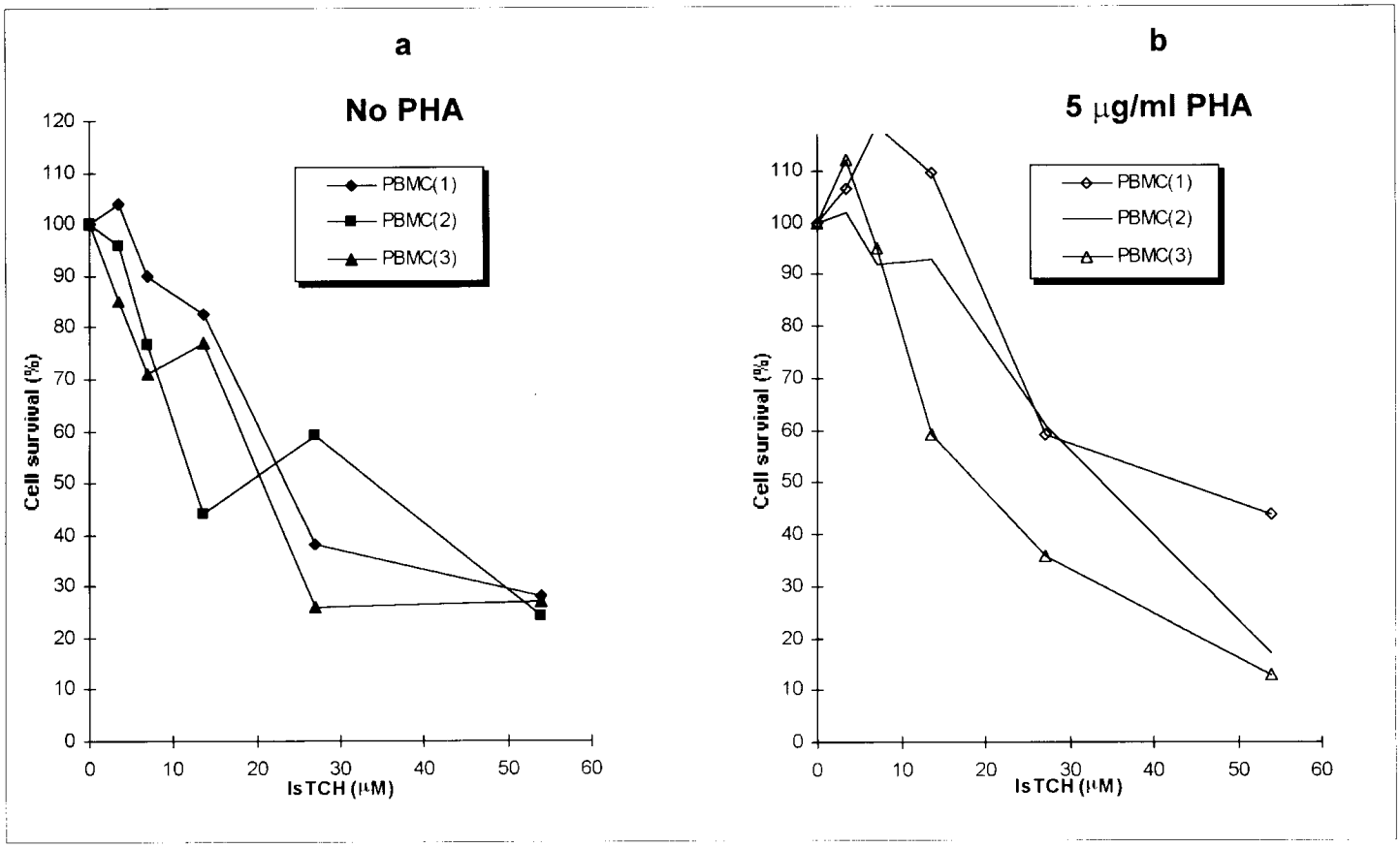

Fig. 4 - Survival of human PBMC, nonstimulated (a), or stimulated by 5 mg/ml PHA (b) determined 72 hours after the IsTCH action, is given as the function of different drug concentrations. Data represent means of quadruplicates of individual PBMC survivals. CV was less than 8%.

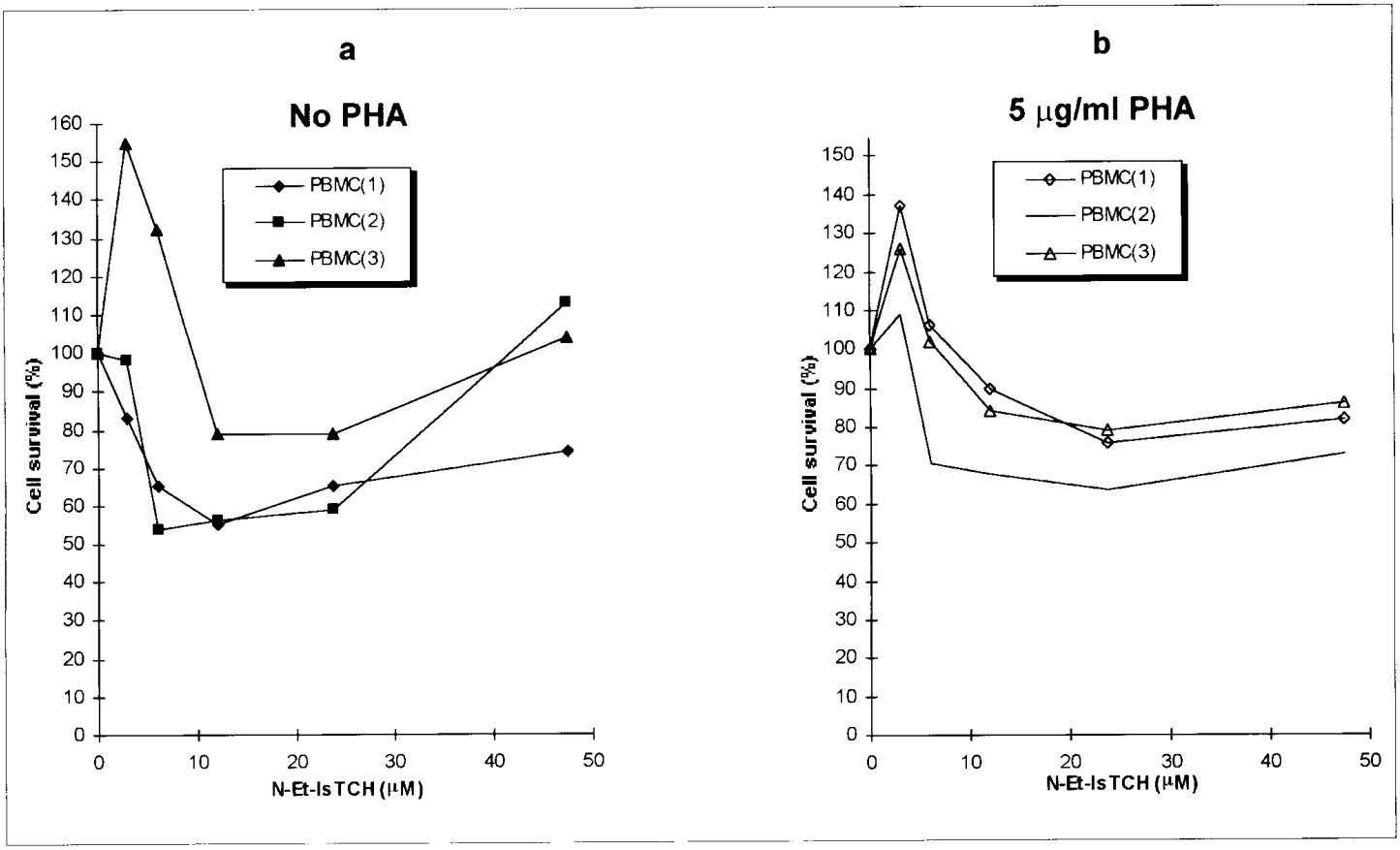

Fig. 5 - Survival of human PBMC, nonstimulated (a), or stimulated by 5 mg/ml PHA (b) determined 72 hours after the N-Et-IsTCH action, is given as the function of different drug concentration. Data represent means of quadruplicates of individual PBMC survivals. CV were less than 6%.

Discussion

The results obtained in this work clearly showed that IsTCH and N-Et-IsTCH suppressed survival of investigated neoplastic cells. While normal, immunocompetent cells, human PBMC, were the most sensitive to the IsTCH action; malignant melanoma B16 cells were the most sensitive cells towards N-Et-IsTCH antiproliferative action. This agent had the ability to induce production of melanin in B16 cells, and

Table II - Effect of IsTCH and N-Et-IsTCH on the survival and viability of HeLa and B16 cells determined 72 h after the continuous agents action by trypan blue exclusion. Results represent mean ± SD of two independent experiments performed in triplicate.

Cell line	IsTCH (μM)	N-Et-IsTCH (μM)	N/Nc ± SD (%)	V (%)
B16	0	0	100 ± 5	99
	54	0	26 ± 4	98
	0	48	2.8 ± 1.5	97
HeLa	0	0	100 ± 6	99
	54	0	45 ± 5	98
	0	48	11 ± 1	99

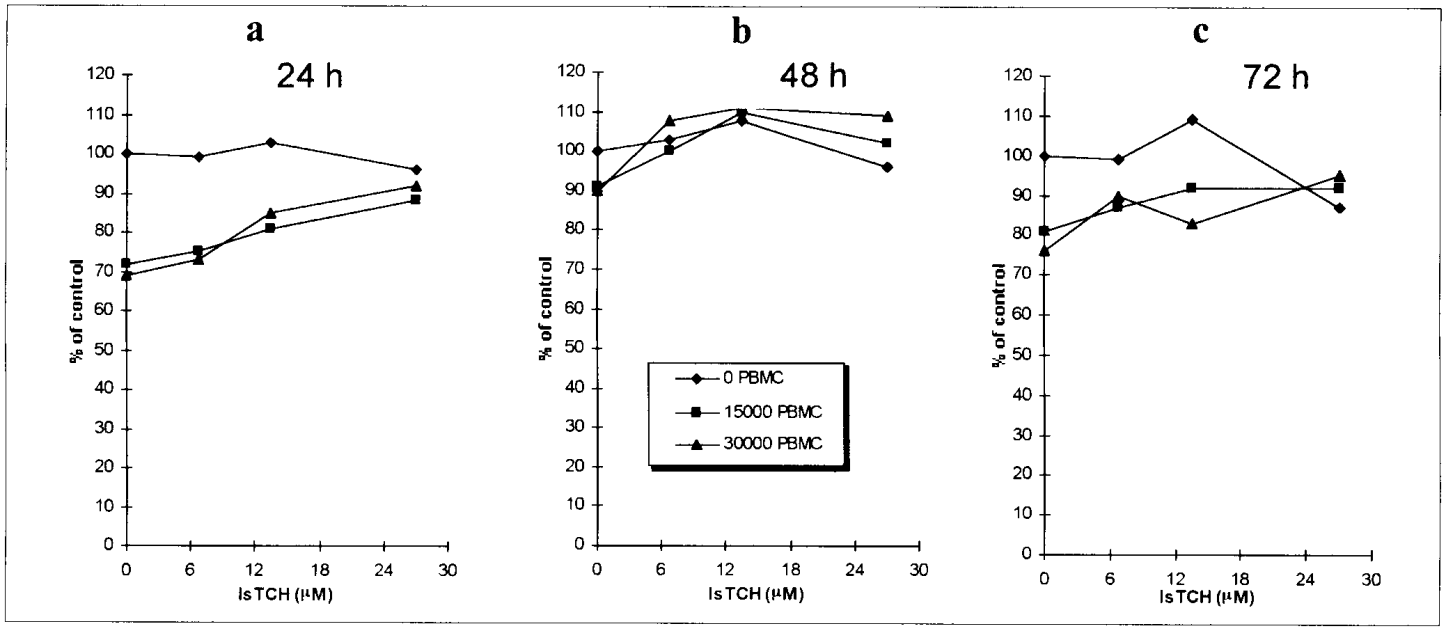

Fig. 6 - Survival (%) of HeLa cells grown alone, or co-cultured with different number of human PBMC is given as the function of IsTCH concentration, determined after 24 (a), 48 (b) or 72 hours(c). In all samples 1,500 of HeLa cells were seeded per well. (◆) HeLa cells; (■) HeLa cells co-cultured with 15,000 human PBMC; (▲) HeLa cells cocultured with 30,000 human PBMC. CV was less than 12%. Data are the mean of triplicate.

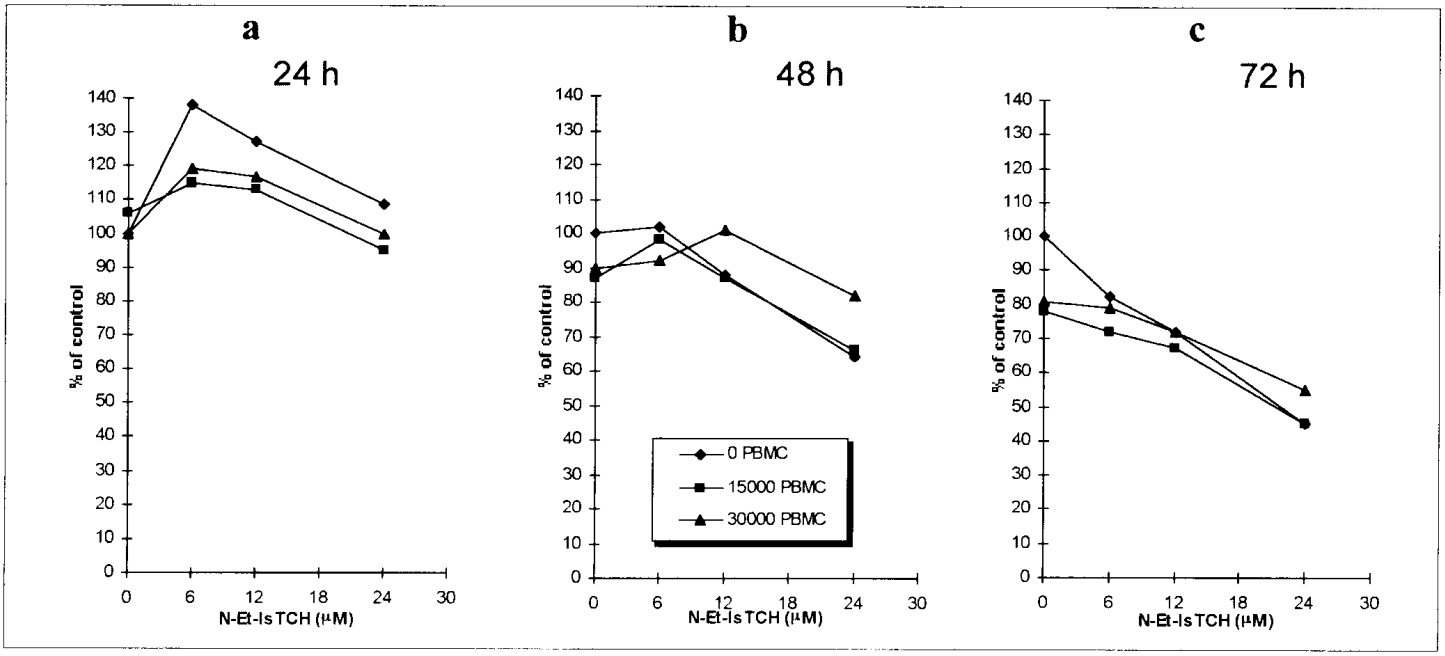

Fig. 7 - Survival (%) of HeLa cells grown alone, or co-cultured with different number of human PBMC is given as the function of N-Et-IsTCH concentration, determined after 24 (a) 48 (b) or 72 hours(c). In all samples 1,500 of HeLa cells were seeded per well. (◆) HeLa cells; (■) HeLa cells co-cultured with 15,000 human PBMC; (▲) HeLa cells cocultured with 30,000 human PBMC. CV was less than 14%. Data are the mean of triplicate.

induced after 72 hrs, in part, the formation of giant B16 cell. Investigated agents in the doses applied induced only reproductive cell death.

N-Et-IsTCH besides the direct antiproliferative action on neoplastic cells, as the difference from IsTCH, did not abrogated, but in dose dependent way contributed to PBMC induced suppression of HeLa cell survival. Looking for the explanation of the observed antiproliferative action of IsTCH and N-Et-IsTCH, in recent literature, we have not found any data on the antiproliferative action of this group of compounds toward malignant cells. There were only data on the bio-

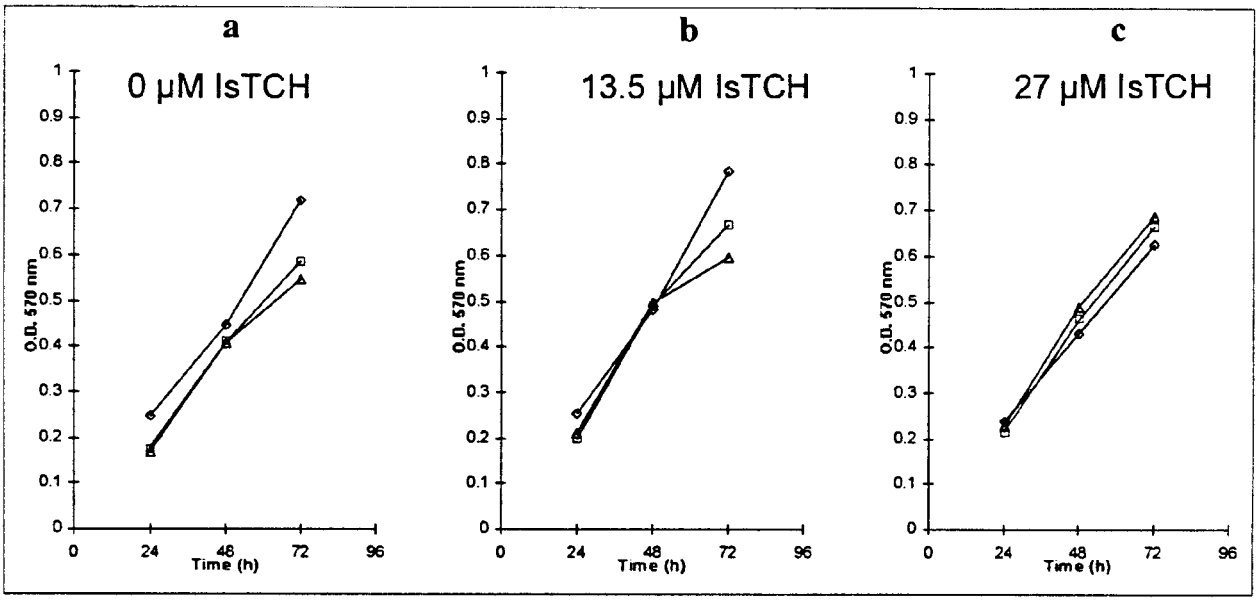

Fig. 8 - Optical density at 570 nm (OD570) of MTT assayed HeLa cells. (a) Cells grown alone (◇), or co-cultured with 15.000 of human PBMC (□); or 30.000 PBMC (△); at the end of 24, 48 and 72 hours of the setup of mixed cell culture. (b) OD570 of HeLa cells grown in the same conditions as in (a) and in the presence of 13.5 µM of IsTCH (b), or 27 µM of IsTCH (c). Data are the mean of triplicate. CV was less than 12%. (Representative experiment from three performed.)

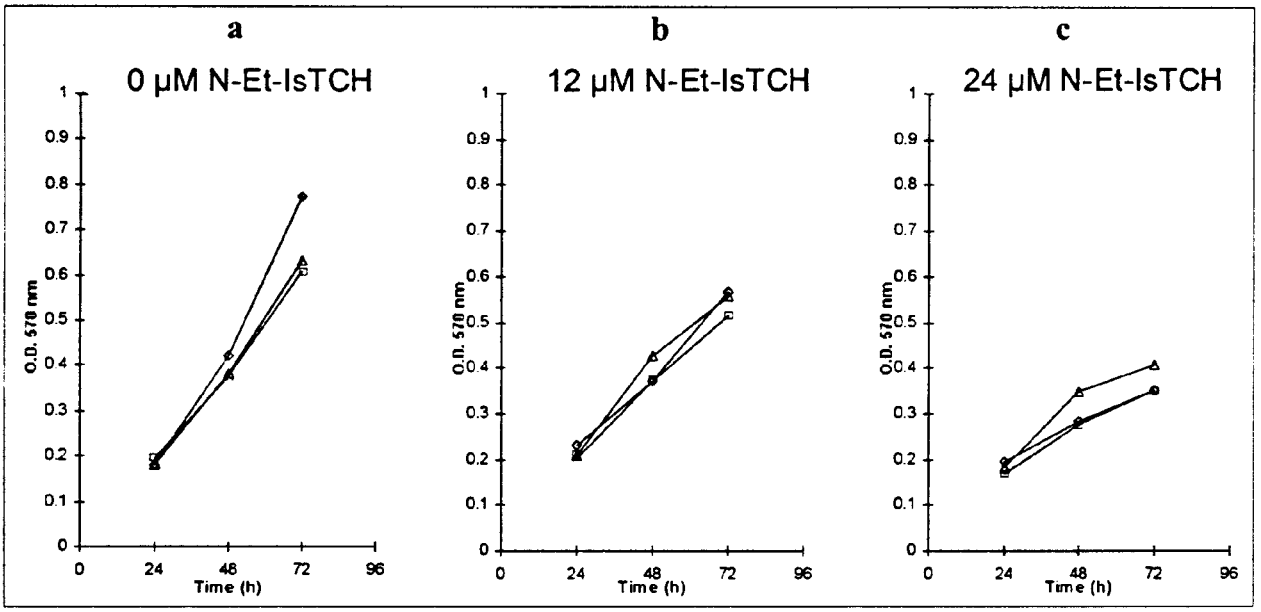

Fig. 9 - Optical density at 570 nm (OD570) of MTT assayed HeLa cells. (a) Cells grown alone (◇), or co-cultured with 15.000 of human PBMC (□), or 30.000 PBMC (△); at the end of 24, 48 and 72 hours of the setup of mixed cell culture. (b) OD570 of Hela cells grown in the same conditions as in (a) and in the presence of 12 µM of N-Et-IsTCH (b), or 24 µM of N-Et-IsTCH (c). Data are the mean of triplicate. CV was less than 12%. (Representative experiment from three performed.)

logical action of some chemicaly simpler substances, on antipoxviral drug, isatine-beta-thiosemicarbazide, whose action was to induce unique defect in late viral gene expression, so to disturb the regulation of the elongation potential of the viral RNA polymerase late during a *vaccinia* virus infection (8,9)

A lot of experiments need to be done to solve the exact molecular mechanisms of cytostatic action of these two drugs. The appearance of higher percent of pigmented B16 cells in relation to control samples in the presence of 54 μM of IsTCH or 48 μM N-Et-IsTCH, could indicate that additional antiproliferative potential for B16 cells, may be through the activation of dopamine oxidase and subsequently, through cell maturation.

Certainly, results from these preliminary experiments opened many ways for further research: on the mechanisms of these drugs action, on the specificity of action to neoplasic cell, on the nature of their immunostimulating or immunosuppressing properties.

In conclusion, the importance of the finding that N-Et-IsTCH has cytostatic activity towards malignant melanoma cells deserves special attention as it is known that there are no efficient drugs in clinics (chemotherapy) to this chemoresistant disease and new drugs are desperately needed.

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