



# The study of the apoptogenic effect of pyrimidine derivatives on murine leukemia cells

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## ABSTRACT

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**BACKGROUND:** *In the light of the recent findings concerning the role of apoptosis and of tumor cell enzymes in cancer chemotherapy, the interest in pyrimidine derivatives has greatly increased. Thio- and hydrazine- pyrimidines were synthesized as potential antimetabolites inhibiting the biosynthesis of nucleic acids. Some of them demonstrated biological activity, including antibacterial and antitumor action. The aim of this study was to analyze the cytotoxic activity and the ability of some derivatives to induce apoptosis in murine leukemia cells.*

**METHODS:** *Exponentially growing cells were incubated with compounds and after 24, 48, and 72 hours were stained with trypan blue and counted hemocytometrically. For detection of the cell fraction undergoing apoptosis, a morphological analysis was made using fluorescent dye propidium iodide.*

**RESULTS:** *Eight thio- and hydrazine- pyrimidine derivatives were investigated. 2-Thiouracil and 6-hydrazinouracil did not influence the cell growth. 2,4-dithiouracil, 2-thio-4-hydrazinouracil, 2-hydrazinouracil, and 2-thio-5-fluorouracil decreased cell proliferation, but even at the highest studied concentration (1000  $\mu$ M) had no cytostatic action. Only high concentrations of 2,4-dihydrazinouracil and 2-chloro-4-hydrazinouracil showed a strong cytotoxic activity. The treatment with 2,4-dihydrazinouracil as well as with 5-fluorouracil caused the appearance of apoptotic cells with typical fragmented condensed nuclei, ghosts and apoptotic bodies. In contrast, dead cells treated with 2-chloro-4-hydrazinouracil did not show apoptotic morphology.*

**CONCLUSION:** *Among studied eight thio- and hydrazine- pyrimidine derivatives only 2,4-dihydrazinouracil demonstrated strong apoptogenic activity. Its active concentrations were about 100 times higher than apoptogenic concentrations of 5-fluorouracil which points to different mechanisms of cytotoxic action.*

**KEY WORDS:** *Pyrimidines; Apoptosis; Leukemia, Experimental*

## INTRODUCTION

Increased proliferative activity of tumor cells is closely related to the increased activity of essential enzymes that participate in pyrimidine metabolism. The interest in studying the inhibitors of nucleic acid metabolism is connected to the fact that a large number of these compounds have found important application in the therapy of cancer, viral infections, and some other diseases (1). The most important representatives of these inhibitors are 5-fluorouracil and its nucleosides (2). In the light of the recent findings concerning the role of apoptosis and of tumor cell enzymes in cancer chemotherapy, the interest in pyrimidine derivatives has greatly increased (3).

Thio- and hydrazine- pyrimidines have been developed as potential antimetabolites and the compounds obtained are structurally very similar to the natural pyrimidine bases uracil and cytosine (4,5). Some of them demonstrate biological activity, including antibacterial and antitumor action (6,7). In the course of investigation of wide spectrum of pyrimidine derivatives it has been found that 2-thio-4-hydrazinouracil show the highest activity. This compound inhibits the growth of various microorganisms; it displays an inhibitory effect on the final stages of pyrimidine nucleotide synthesis, on the conversion of orotate into uridine nucleotides, on cytosine triphosphate synthase reaction, and on the maturation of 45 S pre-RNA (5,6). The mechanism of the last effect is not sufficiently clear; perhaps, it is due to

the incorporation of the antimetabolite into polynucleotide chain. It has been shown that the 2-thio-4-hydrazinouracil possesses a strong antitumor effect in transplantable mouse tumors myeloma P-8 and sarcoma 180. In the light of the recent results showing that 5-fluorouracil causes apoptosis (programmed cell death) in many kinds of tumor cells (9,10), it was interesting to study if hydrazine- and thio- derivatives of pyrimidines are able to induce apoptosis in tumor cells.

## MATERIALS AND METHODS

**Materials.** Thio- and hydrazine- derivatives of uracil (Table 1) were prepared as previously described (4,5). All compounds were of analytical grade. 5-Fluorouracil, thymidine, leucovorin, propidium iodide and all other chemicals were purchased from the Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany.

**Cell culture.** Murine erythro leukemia cells, clone F4N (virus-transformed erythroid precursor cells) (11) were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% calf serum, in 5% CO<sub>2</sub> atmosphere at 37°C, and passaged every day at a concentration of 5x10<sup>5</sup> cells/ml.

**Incubation with drugs and evaluation of cytotoxicity.** Exponentially growing cells (0.5 x 10<sup>6</sup> cells/ml) were incubated with compounds in 24-well microtiter plates. After 24, 48, and 72 hours of drug treatment, the cells were counted hemocytometrically and analyzed by fluo-

rescent microscopy. The number of dead cells was determined by staining with trypan blue. **Fluorescence microscopy.** The cells were fixed with methanol-acetic acid (3:1, v/v), stained with propidium iodide (6  $\mu\text{g/ml}$ ), and viewed using a Leitz orthoplan epifluorescence microscope.

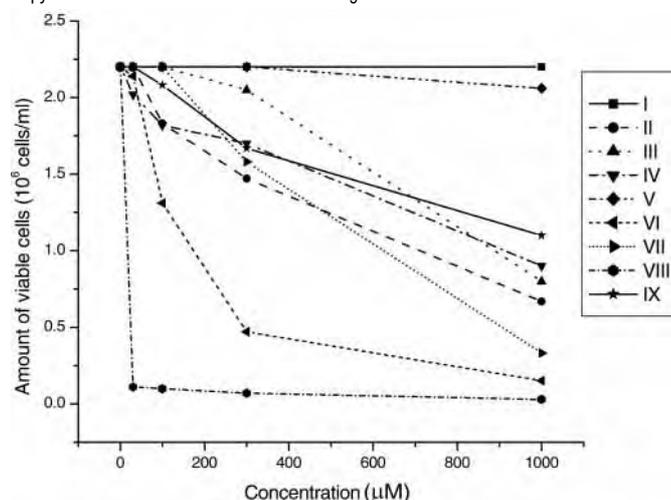
**Table 1.** Apoptogenic and growth inhibitory effect of pyrimidine derivatives; Murine leukemia cells were incubated with compounds for 72 h

R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	Compound	IC <sub>50</sub> ( $\mu\text{M}$ )	IC <sub>90</sub> ( $\mu\text{M}$ )	AM*
I	SH	OH	H	2-thiouracil	>1000	>1000	-
II	SH	SH	H	2,4-dithiouracil	410	1000	-
III	SH	NHNH <sub>2</sub>	H	2-thio-4-hydrazinouracil	860	1000	-
IV	NHNH <sub>2</sub>	OH	H	2-hydrazinouracil	630	>1000	-
V	OH	OH	H	6-hydrazinouracil	>1000	>1000	-
VI	NHNH <sub>2</sub>	NHNH <sub>2</sub>	H	2,4-dihydrazinouracil	100	260	+
VII	Cl	NHNH <sub>2</sub>	H	2-chloro-4-hydrazinouracil	450	850	-
VIII	OH	OH	F	5-fluorouracil	1	7	+
IX	SH	OH	F	2-thio-5-fluorouracil	700	>1000	-

AM\* - cells with apoptotic morphology

## RESULTS AND DISCUSSION

The structure of eight thio- and hydrazine- derivatives of pyrimidines studied is shown in Table 1. The activity of these compounds was compared to the activity of 5-fluorouracil and 2-thio-5-fluorouracil. In this study we used the mouse erythroleukemia cells, clone F4N, in which several antitumor drugs have already been shown to readily trigger apoptosis (12,13). The growth inhibitory effect of incubation of F4N cells with different concentrations of pyrimidine derivatives is demonstrated in Figure 1.



**Figure 1.** Effect of pyrimidine derivatives on growth of murine leukemia cells. Cells were exposed to various concentrations of compounds for 72 h

The quantitative comparison of the effects of each compound was made based on IC<sub>50</sub> and IC<sub>90</sub>, which were calculated as concentrations, reducing the number of viable cells by 50% or 90% respectively (Table 1). Because of displayed activity, the compounds were divided in three groups. First group included 2-thiouracil (I) and 6-hydrazinouracil (V), which did not influence the growth of murine leukemia cells. The second group contained 2,4-dithiouracil (II), 2-thio-4-hydrazinouracil (III), 2-hydrazinouracil (IV), and 2-thio-5-fluorouracil (IX), which decreased cell proliferation, but even at highest concentration studied (1000  $\mu\text{M}$ ) had no cytostatic action; their ID<sub>90</sub> was higher than 1000  $\mu\text{M}$ . The relatively low activity of 2-thio-4-hydrazinouracil was a surprise when having in mind the previous data, which prove

its antitumor activity (7). The compounds from the third group (2,4-dihydrazinouracil and 2-chloro-4-hydrazinouracil) were able to completely inhibit cell growth with ID<sub>90</sub> of 260 and 850  $\mu\text{M}$ , respectively. They demonstrated strong cytotoxic action at 1000  $\mu\text{M}$  concentration, where the amounts of trypan blue positive cells were 90% and 56% respectively. The most potent inhibitor was 5-fluorouracil with an IC<sub>50</sub> of 1  $\mu\text{M}$  and IC<sub>90</sub> of 7  $\mu\text{M}$ , which means that its active concentrations are about 100 times lower than the cytotoxic concentrations of other pyrimidine derivatives.

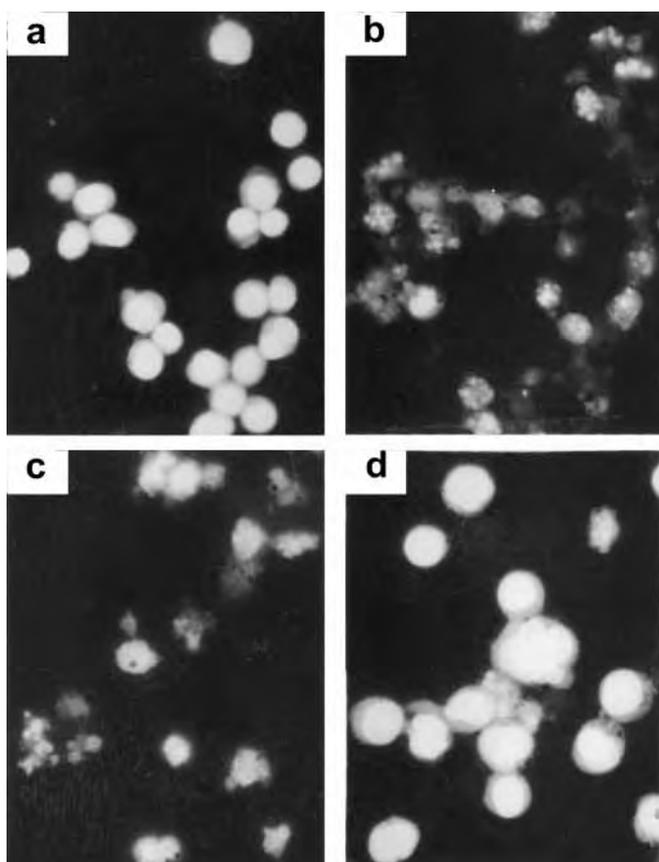
The analysis of data obtained demonstrated that the presence of thio-group in pyrimidine ring of 5-fluorouracil leads to the loss of its cytotoxic activity. Moreover 2-thiouracil did not show any activity. That is in agreement with recently published data demonstrating that 2-thio analogue of dUMP is a good substrate of thymidilate synthase (12) and that the free energies of binding in the binary complex with thymidilate synthase show qualitatively indistinguishable discrimination among the fluorinated and non-fluorinated 2-thio analogues of dUMP.

In contrast to 2-thio analogs, the including of second thio-group at fourth place resulted in increased activity. Possibly, it is related to the high aromaticity of its pyrimidine ring that leads to a change of enzyme interactions with 2,4-dithio-dUMP. For example, in the experiments with purified thymidilate synthase it has been shown that 2,4-dithio-dUMP is not a substrate of this enzyme, but is a competitive inhibitor, relative to dUMP (13).

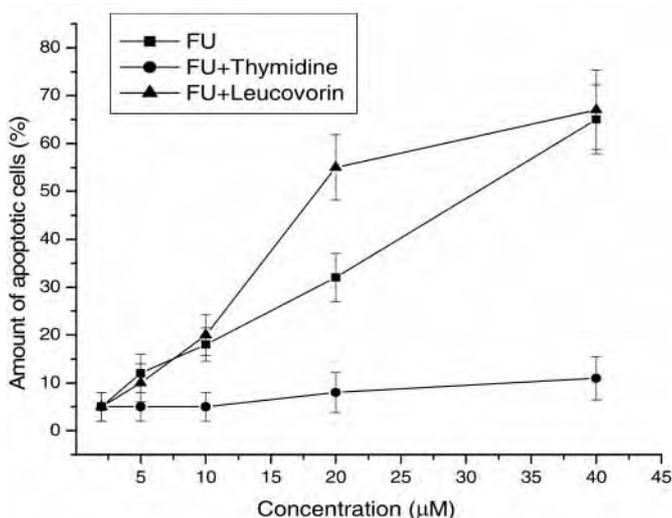
It is important to underline that hydrazino- derivatives, and especially 2,4-dihydrazinouracil, are significant more active than corresponding thio- derivatives. Having in mind these results, it would be interesting to synthesize and to study the hydrazino- derivatives of 5-fluorouracil.

To clarify if the main mechanism of cell killing by active derivatives is the induction of apoptosis, the comparative study of their apoptogenic effect on F4N cells was carried out. To detect the cell fraction that undergoes apoptosis a morphological analysis was made using fluorescent dye propidium iodide. Using this method as well as comet assay, we have previously shown (14,15) that apoptotic F4N cells exhibited changed morphology with fragmented condensed nuclei. Representative results are shown in Figure 2. The untreated control cells (panel a) had uniform size and morphology. 5-Fluorouracil (panel b) and 2,4-dihydrazinouracil (panel c) added to cells in cytotoxic concentrations caused the appearance of apoptotic cells with typical fragmented condensed nuclei, ghosts, and apoptotic bodies. The amount of apoptotic cells increased with increasing drug concentration and time of treatment. In contrast, the dead cells treated with 2-chloro-4-hydrazinouracil (panel d) did not show apoptotic morphology.

To gain more detailed insight into the cell death process, the ability of thymidine to rescuer cell from apoptogenic action of 5-fluorouracil was studied (Figure 3). The addition of 10  $\mu\text{M}$  thymidine to the medium did not affect the growth of control cells but significantly decreased the amount of apoptotic cells. This modulation was more pronounced in the interval of middle concentrations. Another biochemical modulator of 5-fluorouracil activity at the level of its target, thymidilate synthase, leucovorin was used. Leucovorin provides folate cofactor resulting in stabilization of the ternary complex with thymidilate synthase and fluorodeoxythymidinmonophosphate. This combination represents standard clinical treatment improving the therapeutic effect of 5-fluorouracil (16). In our experiments, leucovorin alone did not influence cell growth, but in combination with 5-fluorouracil increased the amount of apoptotic cells. So, both modulators were effective in combination with 5-fluorouracil, which points to the domination of DNA-directed apoptogenic action of fluoropyrimidine based on the inhibition of thymidilate synthase.



**Figure 2.** Morphological analysis of treated murine leukemia cells. Cells were incubated for 72 h with (a) control, 1% DMSO (b) 30  $\mu\text{M}$  5-fluorouracil, (c) 1000  $\mu\text{M}$  2,4-dihydrizinouracil, (d) 1000  $\mu\text{M}$  2-chloro-4-hydrizinouracil. Cells were fixed, stained with propidium iodide (6  $\mu\text{g}/\text{ml}$ ) for 20 min and analysed by fluorescent microscopy



**Figure 3.** Modulation of apoptotic effect of 5-fluorouracil (FU) by thymidine (10  $\mu\text{M}$ ) or leucovorin (10  $\mu\text{M}$ ). Murine leukemia cells were incubated with compounds for 72 h

## CONCLUSION

The most potent inducer of apoptosis was shown to be 5-fluorouracil. The pattern of modulation of its apoptotic action by thymidine and leucovorin points to the domination of DNA-directed apoptotic action of this fluoropyrimidine, based on the inhibition of thymidylate synthase. Our results show that two of the eight studied pyrimidine derivatives, 2,4-dihydrizinouracil and 2-chloro-4-hydrizinouracil, had strong cytotoxic action, but only

2,4-dihydrizinouracil was able to induce apoptosis in murine leukemia cells. Its active concentrations were about 100 times higher than apoptogenic concentrations of 5-fluorouracil which points to different mechanisms of cytotoxic action. It is important to underline that hydrazino- derivatives, and especially 2,4-dihydrizinouracil, are significant more active than pyrimidine thio- derivatives. Having in mind these results it would be interesting to synthesize and to study the hydrazino- derivatives of 5-fluorouracil.

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