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DRUG BIOCHEMISTRY

TEZACITABINE BLOCKS TUMOR CELLS IN G_1 AND S PHASES OF THE CELL CYCLE AND INDUCES APOPTOTIC CELL DEATH

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Abstract: Tezacitabine (FMdC) is a new cytostatic/cytotoxic agent widely investigated in clinical trials and on the cellular level. In a previous paper (3) we worked on human and murine leukemia (L-1210, HL-60, and MOLT-4) cells, and in this paper we investigated the influence of FMdC on the cell cycle and apoptosis *in vitro* of three other leukemias (CCRF-SB, KG-1, and Jurkat), and human solid tumor (carcinoma) cell lines (COLO-205, MCF-7, and PC-3). We found that FMdC induces the G_1 (at concentrations higher than 10 nM), and S-phase (at low concentration) leaky block of the cell cycle. FMdC also effectively induces apoptotic death of cells by the caspase 3/7 pathway. We found also that FMdC induces intensive changes in the protein metabolism. These changes are correlated with the cell death.

Keywords: cell cycle; apoptosis; flow cytometry; tezacitabine; FMdC; protein content

Tezacitabine ((E)-2'-deoxy-2'-(fluoromethylene)-cytidine, FMdC) is a new cytostatic and cytotoxic antimetabolite, fluorine derivative of cytidine. FMdC is now extensively tested in the 1st and the 2nd phase of clinical trials as drug acting against leukemias and solid tumors (carcinomas) (1, 2). The mechanism of its action is not, as yet, clearly understood, but it is known that it inhibits the ribonucleotide reductase (RR). It interferes with DNA replication and repair. In our previous work (3) we investigated the influence of FMdC on the cell cycle and the induction of apoptosis in murine and human (L-1210, HL-60, and MOLT-4) leukemia cells *in vitro*. We found that this substance has strong cytostatic and cytotoxic properties. We found also that cytotoxic effect reveals not only as apoptosis, but also as a change in protein metabolism. The intensity of these effects depended on the cell line, FMdC concentration and the exposure duration. To check whether these effects are cell line specific, in this paper we worked on the three other human leukemia cell lines of different origin – acute T- and B- lymphoblastic (Jurkat and CCRF-SB), and myelogenous (KG-1) leukemia cell lines. Taking into account the clinical trials on carcinoma treatment with FMdC we included also three solid tumor lines: colon carcinoma COLO-205, breast carcinoma MCF-7, and prostate carcinoma PC-3. Cytostatic effect was investigated using the mathematical analysis of the DNA (stained with DAPI) content. Cell proliferation was tested by protein staining with 5- (and-6-)-carboxylfluorescein diacetate succinimidyl ester (CFDA-SE). The cytotoxic effects were estimated using induction of apoptosis and protein content in cells. The former effect was tested using binding of Annexin V method and confirmed by cell morphology, measurements of caspase 3 activation (PARP cleavage), and the DNA cleavage (TUNEL).

EXPERIMENTAL

Chemicals

FMdC was synthesized by Drs. Maria Bretner and Krzysztof Felczak from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, according to the modified procedure of McCarthy et al. (4). The chemical identity of the substance obtained was confirmed in Nuclear Magnetic Resonance Lab at the NIPH, Warsaw, by proton NMR. 4', 6-diamidino-2-phenylindole (DAPI), sulforhodamine-101(SR-101), and propidium iodide (PI) were purchased from the Molecular Probes

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(Eugene, OR, USA). CFDA-SE, PIPES disodium salt, and Triton X-100 (Sigma-Aldrich) were used. Annexin V kit, and TUNEL kit (Apo-BrdU), were purchased from Pharmingen. Monoclonal antibody against cleaved PARP molecule (Anti-PARP CSSA FITC Apoptosis Detection kit) was purchased from BioSource Int. Tissue culture media and trypsin – EDTA solution were purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland. Fetal bovine serum (FBS) was purchased from Gibco. L-glutamine, D-glucose, non-essential amino acids, and antibiotic-antimycotic solution were purchased from Sigma-Aldrich. All other chemicals were of analytical or tissue culture grade.

Cells and cultures

CCRF-SB (human acute lymphoblastic B-cell leukemia, ATCC #CCL-120), diploid line; KG-1 (human acute myelogenous leukemia, ATCC #CCL-246), near-diploid line; Jurkat (acute T-cell leukemia, ATCC #TIB-152, clone E6-1), pseudo-diploid line; COLO-205 (human colon carcinoma, ATCC #CCL-222), hypertriploid line; MCF-7 (human breast adenocarcinoma, ATCC # HTB-22), hypertriploid to hypotetraploid line; and PC-3 (prostate adenocarcinoma, ATCC # CRL-1435), near triploid line, all were purchased from American Tissue Culture Collection, and were maintained routinely in Flow Cytometry Laboratory, NIPH as a suspension (CCRF-SB, KG-1, and Jurkat) or monolayer (COLO-205, MCF-7, and PC-3) culture, all in RPMI-1640, supplemented with 10% of FBS, 1% L-glutamine, 0.5 g/L D-glucose, 1% non-essential amino acids, and 1% antibiotic-antimycotic solution. The adherent cells were trypsinized (0.25% trypsin + EDTA) every third day. The suspension and adherent cultures were kept in an exponential phase of growth. All cultures were regularly checked against mycoplasma infection.

Fluorescence measurements

All measurements were performed using a FACS Vantage (Becton-Dickinson, San Jose, USA) flow cytometer equipped with a dual wavelength argon laser (Innova Enterprise, 351/488 nm). It had also an appropriate set of dichroic mirrors and interference filters to measure blue (FL4: emission light band centered at 424 nm and a bandwidth of 44 nm) for DAPI, green (FL1: 530/30 nm) for fluorescein isothiocyanate (FITC), and red (FL3: 630/22 nm) for sulforhodamine (SR 101) or propidium iodide (PI) fluorescence. At least 10 000 cells were measured per each sample. Data acquisition and analysis were done using CellQuest software (B-D). Cell cycle analyses were done using MacCycle (Phoenix Flow Systems, San Diego, CA, USA) software. S-phase estimations were performed using the 1st order polynomial model.

Methods

FMdC was dissolved (1 mg/mL) in 0.9% NaCl (stock solution) and next diluted in the culture medium to final concentrations of 0.01, 0.1, 1.0, and 10 μM. The cell cultures in the exponential phase of growth were exposed to this substance for 24 hours. All experiments were performed at least in triplicate.

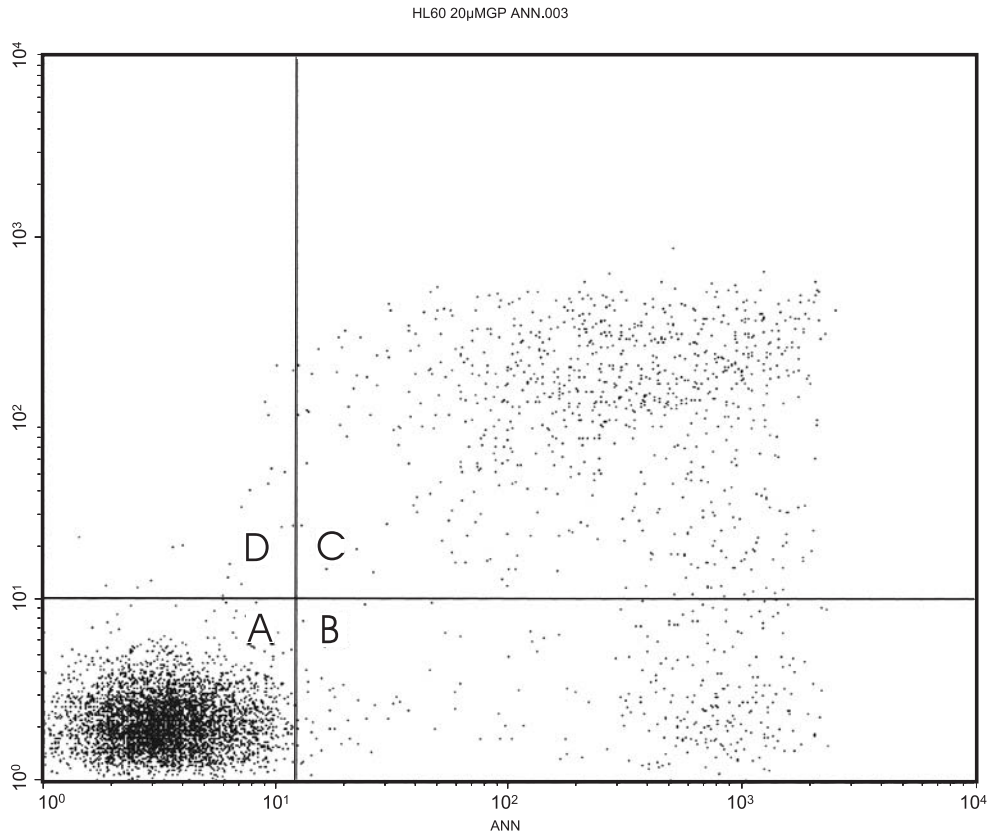
DNA/protein content

After incubation, adherent cells were trypsinized (using the trypsin/EDTA solution) and suspensions (together with the culture medium containing the detached mitotic cells and apoptotic cells) were spun down (300 x g, 5 min). The cell pellets were washed twice in PBS, resuspended in 1 mL of PBS, and fixed in the cold (-20°C) 80% ethanol by rapid injection under the surface of EtOH. The cells were fixed for at least 4 h, usually overnight. The cells growing in suspension (leukemia cells) were processed in the same way, except the trypsinization.

Fixed cells were twice washed out from ethanol by centrifugation in PBS (300 x g, 5 min), and the cell density was adjusted to 1 x 10^6/mL. 0.1 mL of this suspension was mixed with 1.5 mL of staining solution (10 mM PIPES buffer containing 1 mg/L of DAPI, 20 mg/L of sulforhodamine 101, 2 mM $MgCl_2$, and 0.1% Triton X-100). After 5 min blue fluorescence of DAPI bound to DNA (424 nm) and the red one of SR 101 bound to cellular proteins (630 nm) was measured using UV (351 nm) excitation. Single parameter (DNA) histograms were analyzed using the MacCycle software and the numerical values of the cell fractions in G_1, S, and G_2M phases of the cell cycle were plotted against the FMdC concentration (see Figures 2 and 3). The G_2M fraction is not an independent variable because the sum of G_1, S, and G_2M fractions is always equal to 100%. Thus this latter fraction was not shown on figures. Two parameter (bivariate) DNA/protein scattergrams (see Figures 4 and 5) were next analyzed for visible changes in shape.

Cell death (apoptosis) determination

The basic method used in this paper was the binding of fluorescein isothiocyanate (FITC) labelled Annexin V to the phosphatydylserine residues on the outer cell surface. The phenomenon of phosphatydylserine externalization is specific to



Quad	Events	% Gated	% Total	X Mean	YMean
UL	22	0.37	0.34	6.95	46.75
UR	859	14.38	13.12	509.75	181.88
LL	4804	80.40	73.39	3.72	2.22
LR	290	4.85	4.43	684.15	3.26

Figure 1. Bivariate distribution of PI fluorescence (ordinate) *vs*. FITC-labelled Annexin V (abscissa). Selected regions: upper left, necrotic cells; upper right, late apoptotic cells; lower right, early apoptotic cells; lower left, viable cells.

apoptotic process (5 – 7). Treated with FMdC, and untreated cells, were harvested as follows. The culture medium (containing the detached, apoptotic cells) was collected, cells on the flask surface washed with PBS (washings were also collected) and incubated with 2 mM EDTA until all the cells were detached. The combined cell suspension, culture medium and washings were centrifuged (300 x g, 5 min) and twice washed in PBS. The cells growing in suspension (leukemia cells) were only washed twice in PBS. After the last centrifugation, the cell pellet was suspended in the binding buffer (provided with the kit) containing the HEPES buffer, NaCl, and $CaCl_2$, pH = 7.4. 100 µL of this cell suspension ($2 – 3 \times 10^5$ cells) was next incubated with 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI) (50 µg/mL) for 15 min at room temperature in the dark. After this incubation, the cell suspension was diluted with 400 µL of binding buffer and the fluorescence of FITC (excited at 488 nm and measured at 530 nm), and that of PI (excited also at 488 nm and measured at 630 nm) were analyzed within 1 hour. The analyzed cells form the separate clusters on the bivariate scattergrams (PI *versus* FITC-Annexin V). The viable cells are double-negative (Figure 1, A). PI does not pass through the intact plasma membrane. The early apoptotic cells are Annexin V – positive, PI negative (Figure 1, B).

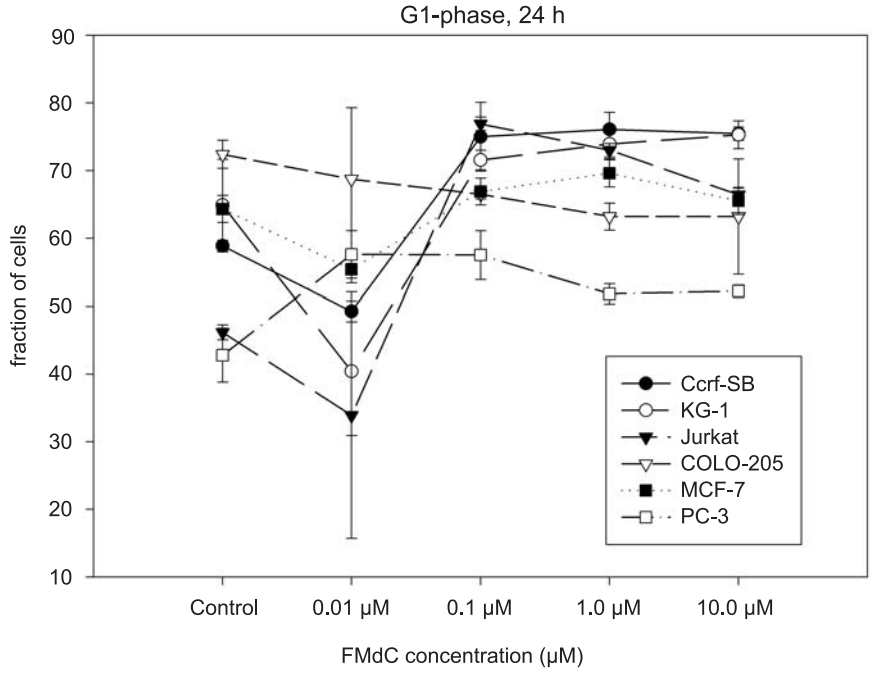


Figure 2. Fraction of cells in the G_1 phase of the cell cycle after 24 h of incubation, as a function of FMdC concentration.

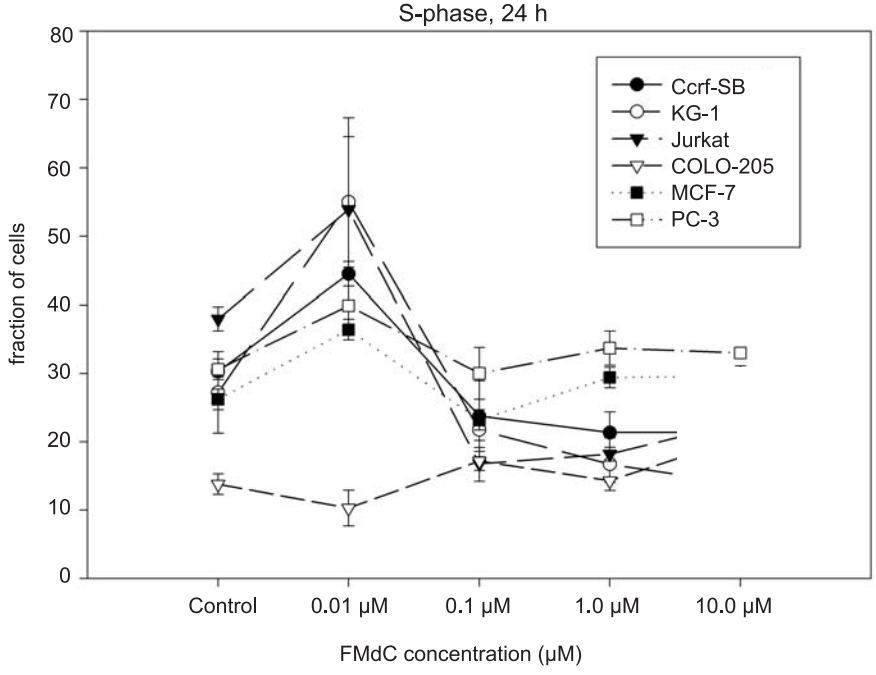


Figure 3. Fraction of cells in the S phase of the cell cycle after 24 h of incubation, as a function of FMdC concentration.

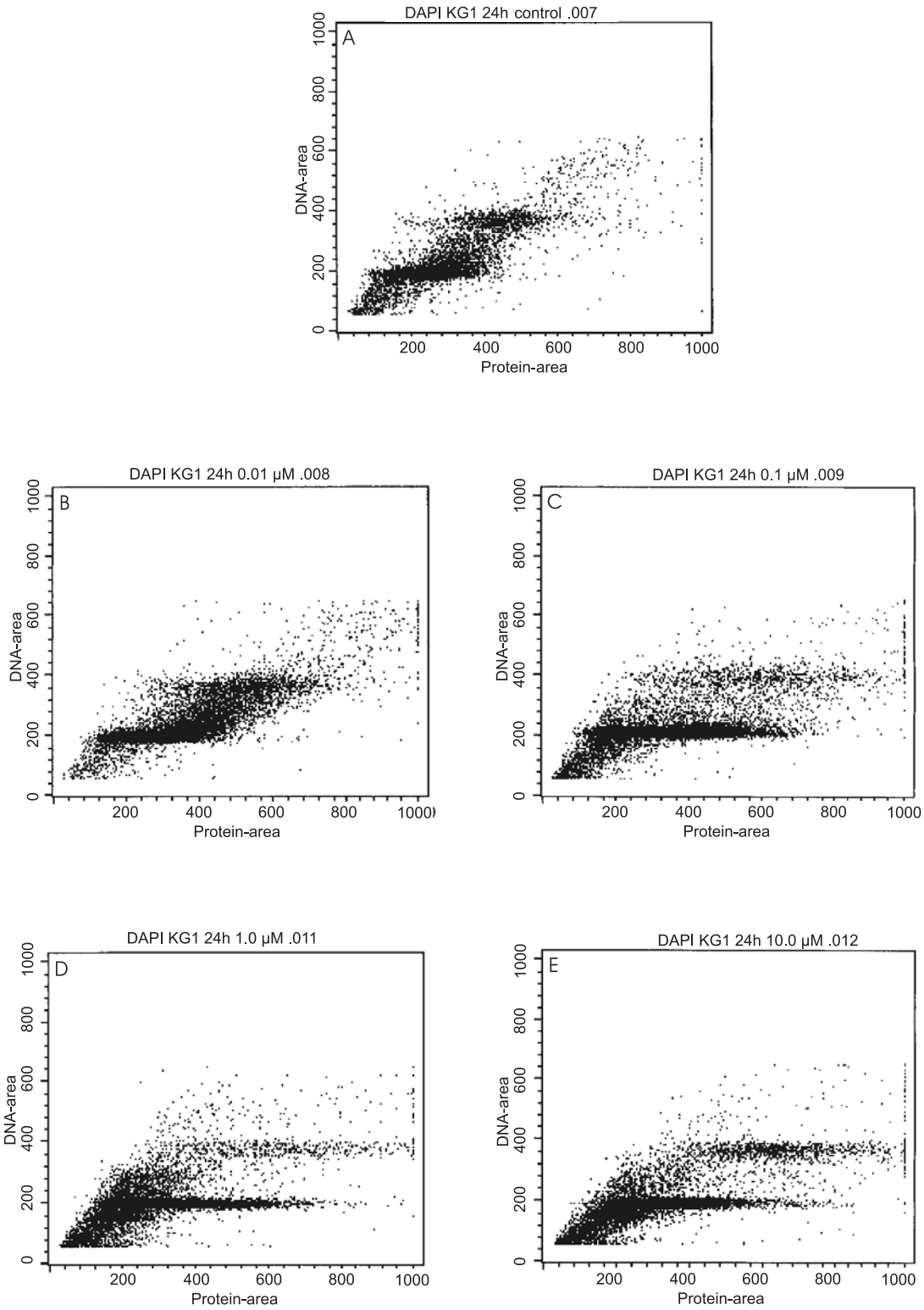


Figure 4. DNA/protein scattergrams of KG-1 cells incubated with various concentrations of FMdC for 24 h. Panel A – untreated population; panels B – E samples treated with 0.01, 0.1, 1,0, and 10.0 µM of FMdC, respectively.

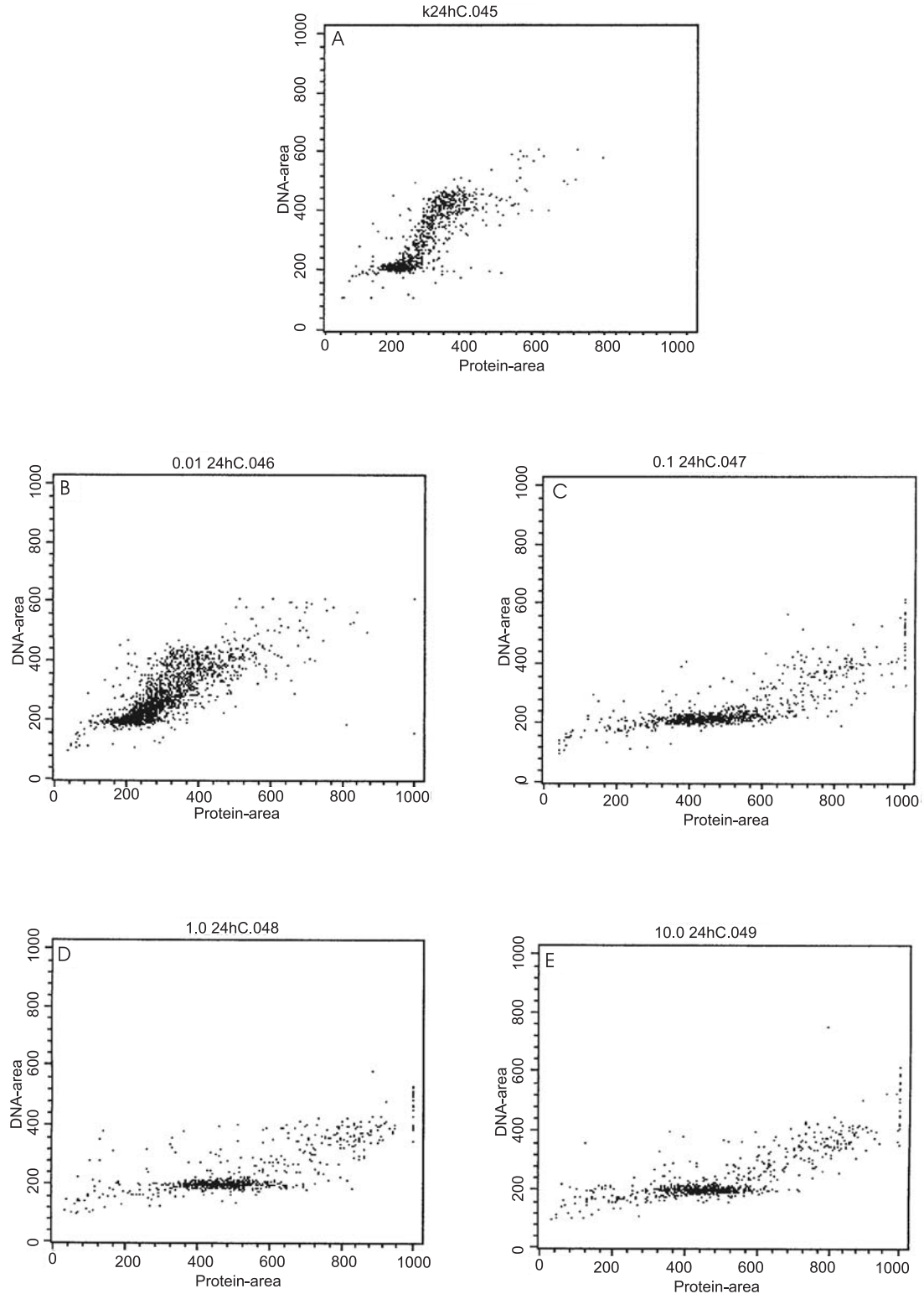


Figure 5. DNA/protein scattergrams of COLO-205 cells incubated with various concentrations of FMdC for 24 h. Panel A – untreated population; panels B – E samples treated with 0.01, 0.1, 1,0, and 10.0 μM of FMdC, respectively.

Late apoptotic cells have compromised plasma membrane and do not exclude PI, which stains nucleic acids. So, late apoptotic cells are double – positive (Figure 1, C). Necrotic cells with only damaged plasma membrane are PI – positive, Annexin V – negative (Figure 1, D). Setting the appropriate regions on the scattergram on the computer screen one can obtain the numerical values of viable, apoptotic and necrotic cell fractions (see Figure 1).

The apoptotic way of cell death was confirmed using two other, highly specific methods: PARP cleavage and DNA fragmentation (TUNEL). Poly (ADP – ribose) polymerase (PARP) is a 116 kDa protein located in the nucleus and is activated by DNA – strand breaks. During apoptosis active caspase-3 (and also caspase-7) cleaves PARP to yield an 85 kDa and a 25 kDa fragments. The FITC – labelled antibody specifically recognizes the bigger (85 kDa) fragment. Thus, the fluorescence intensity of FITC is a measure of the caspase 3/7 activity (8, 9). The PARP – positive cells undergo apoptotic death *via* the caspase pathway (not *via* mitochondrial – endonuclease G and AIF pathway).

For PARP – cleavage assays the cells were prepared as follows. The cell suspension, obtained as above, was centrifuged and the pellet fixed using the IC-Fix buffer (provided by the Apoptosis Detection Kit manufacturer, and containing the buffered paraformaldehyde) for 20 min. After that, the cells were centrifuged and washed twice in PBS. For intracellular staining, the cell membrane must be permeabilized. For that fixed cells were resuspended in IC-Perm buffer (also provided by the kit manufacturer and containing saponin), and the cell density adjusted to 1 x 10^6 cells/mL. 50 µL aliquots of the permeabilized cells were next incubated with 10 µL of FITC – conjugated anti-PARP antibody for 30 min at room temperature in the dark. After incubation, the cells were washed twice in the IC-Perm buffer and once in PBS. The FITC fluorescence was then measured under cytometer settings as above.

The last, apoptosis – specific method of cell death mechanism determination was DNA degradation (TUNEL assay). This acronym stands for Terminal deoxynucleotidyl transferase dUTP Nick End Labeling. The activated (at the end of apoptosis process) endonuclease cleaves the DNA producing the DNA strand breaks. In TUNEL assay, the terminal deoxynucleotidyl transferase (TdT) catalyses addition of bromolated deoxyuridine triphosphates (Br-dUTP) to the 3'OH termini of double- and single strand DNA. After incorporation, Br-dU molecules are identified by FITC-labelled monoclonal antibody. Thus, the FITC fluorescence is a measurement of the intensity of DNA fragmentation. The postlabelling of cells with PI (total DNA label) enables the correlation of DNA degradation and cell cycle phase (10, 11). The cells treated with FMdC and untreated (control population) were processed in the following way: the washed cell suspensions (adherent cells detached using trypsin – EDTA, as above) were fixed in 1% paraformaldehyde in PBS for 30 min and then postfixed in ice-cold 70% ethanol. Fixed cells were next washed twice in a wash buffer (from the kit) and the cell pellets were resuspended in the DNA-labelling buffer containing the enzyme (TdT) and the substrate (Br-dUTP). After the 30-min incubation at 37°C in the dark, cells were washed in the rinse buffer (from the kit) and the incorporated Br-dU detected by another 30-min incubation (in the dark, at room temperature) with anti-Br-dU FITC-labelled monoclonal antibody. After that, cell's DNA was counter-stained with PI by cell incubation in PI/RNA-se solution for another 30 min at room temperature in the dark. After this last incubation, the cell samples were ready for fluorescence determinations. The FITC and PI were excited by the blue line of argon laser (488 nm) and their fluorescence intensity measured at 530 nm (FITC) and 630 nm (PI).

Cell morphology was estimated on cell smears stained with DAPI and sulforhodamine, and analyzed under fluorescent microscope (Olympus BX60).

Cell proliferation was tested using the CFDA-SE method. Cells were labelled with this substance by short incubation in the culture vessel, washed, and then cells were incubated with various concentrations of FMdC for 24, and 48 hours. The green fluorescence was then measured (in conditions as above) and its intensity was compared to the fluorescence of the control sample taken at the same time. In this method cellular proteins are permanently stained with carboxylfluorescein diacetate succinimidyl ester (CFDA-SE). The fluorescence of the cell is halved each time the cells divide (12, 13).

RESULTS

Cell cycle

Stopping or slowing down the transition through particular phase of the cell cycle is recognized by comparing the numerical values of cell fractions to that of control population. Looking at Figure 2, one can see that for all three leukemia lines the G_1 fraction at the 10 nM concentration is lower than that for control. Thus cells from all leukemia lines (CCRF-SB, KG-1, and Jurkat) were stopped in

the S-phase at the lowest (10 nM) FMdC concentration. At the higher (100 nM – 10 μM) concentrations the G_1 block was apparent. The G_2M fraction (data not shown) in all samples was smaller than in the control populations, but never equal to zero, regardless the FMdC concentration (Figures 2 and 3).

Solid tumor cells reacted with FMdC in a different way than leukemia cells. Looking at Figure 3, it is obvious (for all cell lines, except COLO-205) that S-phase fraction at this lowest FMdC concentration is higher than that for the control. For COLO-205 cells, the results were not straightforward, although the fraction of G_1 cells was going down with FMdC concentration. For MCF-7 cells, the block in the S-phase at the 10 nM FMdC concentration was seen. This block moved to the G_1 phase at higher than 10 nM concentrations. For PC-3 cells the block in G_1 was independent of the FMdC concentration, with one exception: at 10 nM, the fractions of cells in both S- and G_1 phases were higher than in control population.

Figures 4 and 5 represent the typical DNA/protein scattergrams. The oval cluster of cells situated at the ordinate 200 is formed by the cells in the G_1 phase of the cell cycle. The next oval cluster, situated at the ordinate 400 is formed by the cells in the G_2 and M phases. Between those, parallelogram in shape, the S phase cells cluster is located. Looking at the cell (dots) density, changes in the cell cycle parameters can be identified. For example, at panel B (Figure 4) the S-phase cluster is denser than at panel A (control population). At panel C, both (S- and G_2M) clusters are substantially less dense, what tells about the G_1-phase block. Both the figures are representative for all cell lines investigated and all experiments. For this reason we included only one set of scattergrams representing leukemia cell line (KG-1) and one representing solid tumor (COLO-205), (Figures 4 and 5, respectively).

The most spectacular change in the shape of scattergrams is the change in the length and position of G_1 and G_2M clusters (cell protein content) on protein axis. The finding that FMdC influences the protein metabolism was unexpected. Moreover, the protein content is not only increased, but also cells are more heterogeneous. We did not identify the specific protein involved in this process, but we have some indications that those may be the heat shock proteins (HSP's).

The CFDA-SE staining showed that the block

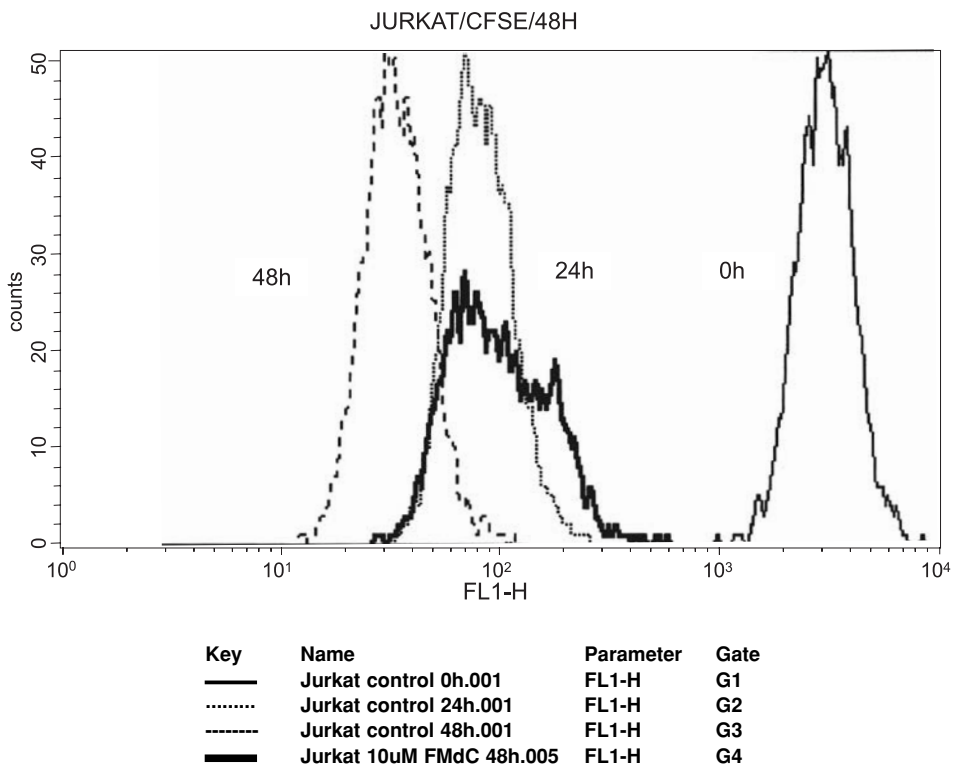


Figure 6. Proliferation of Jurkat cells measured using the CFDA-SE method.

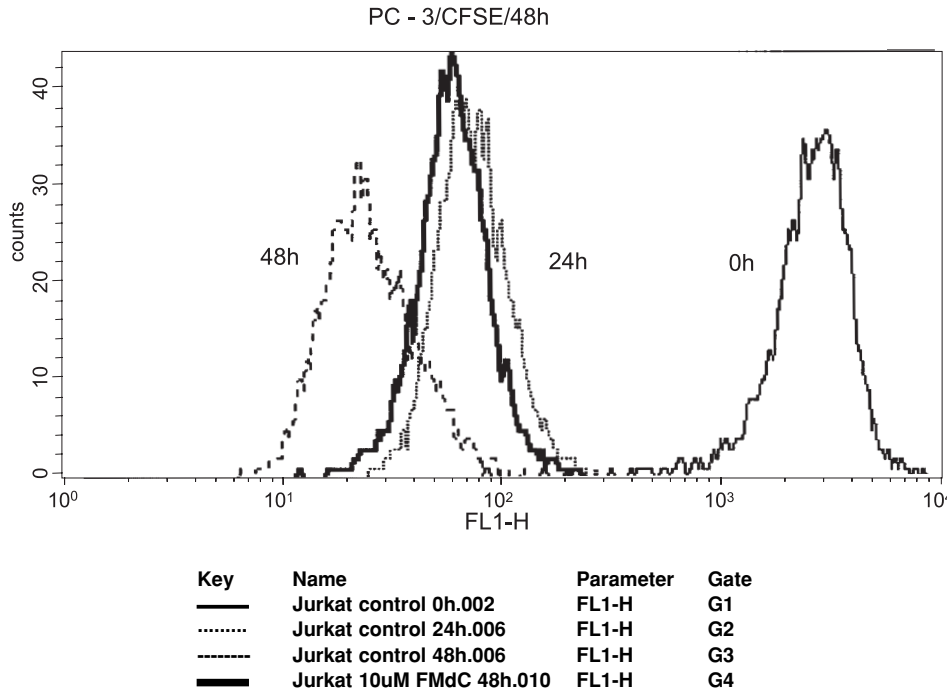


Figure 7. Proliferation of PC-3 cells measured using the CFDA-SE method.

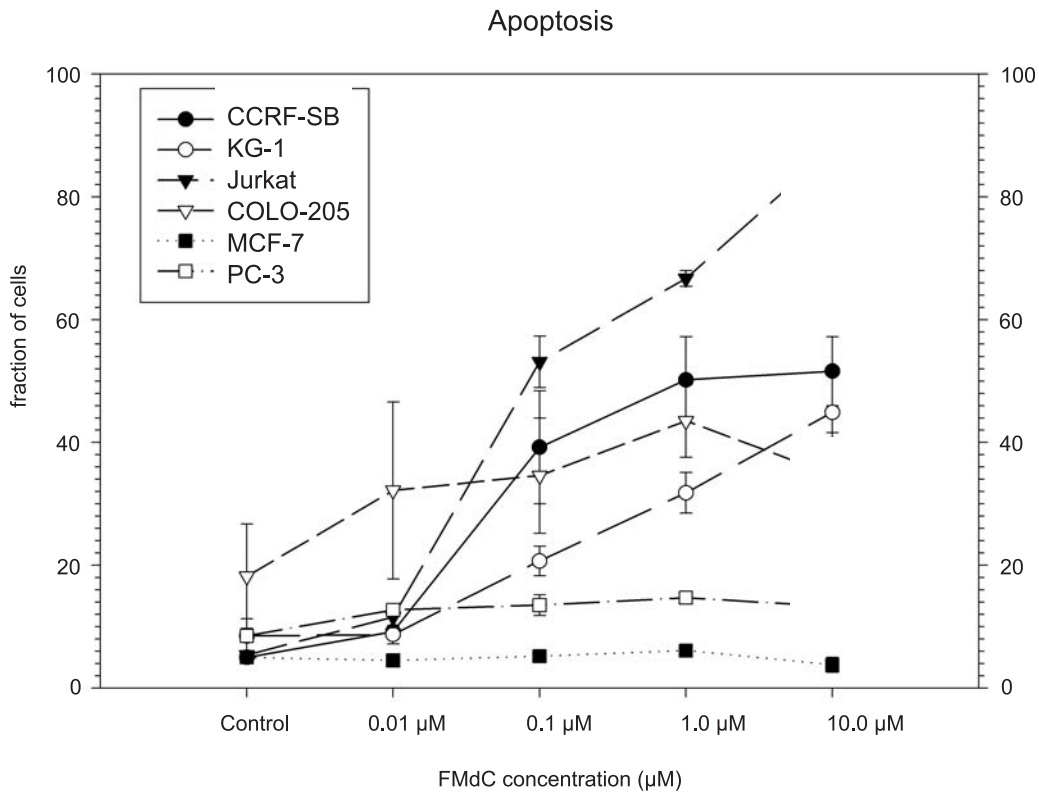


Figure 8. Fraction of apoptotic cells as a function of FMdC concentration.

in the cell cycle was incomplete, because cells of all cell lines did proliferate, although significantly slower (roughly two times) than control population. For example, the fluorescence of Jurkat cellular proteins stained with CFDA-SE (Figure 6) after 48 h of incubation with 10 μM of FMdC was roughly equal to that of the control sample cultured for 24 h. The proliferation rate of solid tumor lines incubated with FMdC was also lower than that of control samples (example – see Figure 7).

Cell death – leukemias

For all cell lines, the massive apoptotic cell death was apparent. The apoptotic cell fraction was concentration dependent. For KG-1 cells, at the FMdC concentration higher than 10 nM, the cell viability substantially decreased due to apoptosis. The Jurkat cells were the most susceptible to FMdC, their viability decreased in a concentration-dependent manner and the fraction of apoptotic cells rose to 88%. For CCRF-SB cells, this value was equal to 50% at the highest FMdC concentration (Figure 8).

Cell death – solid tumors

COLO-205 cells responded to FMdC by increasing the apoptotic cell fraction (to 40%). MCF-7 cells did not respond to FMdC (their viability was concentration independent). PC-3 cells reacted slightly – to approx. 13% of apoptotic cells at 10 μM of FMdC. We performed also experiments with MCF-7 and PC-3 cells in which the exposure time was extended to 48 and 72 hours. In these cases the apoptotic cell fraction was higher than that after 24 hours (Figure 8).

The apoptotic mode of leukemia and solid tumor cells death was demonstrated by three independent methods: cell morphology, Annexin V binding, and PARP cleavage. All the cells investigated were PARP-cleavage positive, what means that the apoptotic process went through caspase 3/7 pathway. We used also the TUNEL method. Analyzing the results of TUNEL, we always have seen the DNA fragmentation, but we have not seen any pattern indicating the phase-specific cell death. The fraction of necrotic cells in all cases was low and independent of FMdC concentration.

Protein content

For all cell lines investigated and all FMdC concentrations (examples in Figures 4 and 5) we observed substantial changes in cellular protein content. This cytotoxic effect depended on FMdC concentration, and was seen as a rise in protein content. Moreover, cells with this abnormal (comparing to control population) protein content did not proliferate, as it may be concluded from the density of dots in bivariate scatterplots.

DISCUSSION AND CONCLUSIONS

In our previous paper (3), we investigated the cytotoxic and cytostatic effects of FMdC on cells of three leukemia (mouse lymphoid L-1210, human promyelocytic HL-60, and acute lymphoblastic T-cell MOLT-4) lines with or without addition of deoxycytidine. We found that FMdC is a potent cytostatic and cytotoxic substance and that addition of 1 mM deoxycitidine completely reversed the cytostatic effect which means that phosphorylation is an important step in the intracellular FMdC activation. In this paper we worked on the other leukemia cell lines (acute lymphoblastic B-cell CCRF-SB, acute myelogenous KG-1, and acute lymphoblastic T-cell Jurkat). All these leukemia cell lines responded to FMdC in a very similar manner. The block in G_1-phase was evident, except for the lowest (10 nM) FMdC concentration. This finding is rather unexpected, because nucleoside derivatives should induce cell cycle block in the S-phase (14, 15). Even at the highest FMdC concentration DNA histograms showed that cells were in the cycle. Cell proliferation was confirmed by the CFDA-SE method. Analysis of DNA/protein cytograms indicated that FMdC, even at the very low concentrations, effected deep changes in the protein metabolism. These changes, common to all six cell lines investigated, consisted in that cycling cells had substantially less protein content than non-cycling cells. This is the reason that appropriate cytograms have the shape of letter "C", what was described by Darzynkiewicz (10) as "unbalanced growth of cells". The very similar effect was observed by us on human glioma cells (neurosurgical explants in the primary culture) treated with 2-CdA (16). It is known that inhibition of protein synthesis by cycloheximide decreases the cytotoxic effects of various drugs (17 – 19). We have seen the cytoprotection effect of cycloheximide in HL-60 cells treated with FMdC. All three, presently investigated, leukemia cell lines were much more susceptible to FMdC than those investigated before (L-1210, HL-60, and MOLT-4 cells) (3).

All six cell lines investigated responded to FMdC by apoptotic (caspase 3/7) cell death pathway. The fraction of necrotic cells was generally very low, in the range of 5 – 10%. Taking into account the duration of the exposition on FMdC, this apoptotic effect must be described as "delayed

apoptosis" (Halicka et al, 20), because immediate (during the first 6 h – data not shown) cell death was slightly pronounced. The incubation time required for effective induction of apoptosis for MCF-7 and PC-3 cells was longer than 24 hours.

We did not find any clear relationship between cell cycle phase and DNA fragmentation (using the TUNEL method) as seen in the case of HL-60 cell line (3).

We found that the CFDA-SE method cannot be used for precise estimation of the doubling time of at least cell lines mentioned in this paper. The drop of the fluorescence intensity is not proportional to the rise of cell number. For example (for Jurkat cells), median fluorescence (Figure 5) of stained control population at 0 time was equal to about 3000 units, whereas 24 h later it reached only about 80 units. During that time, the cell density increased only 4 times. Fluorescence measurements of cells and supernatant, performed in a spectrofluorimeter, confirmed that CFDA-SE leaks from cells to the medium. Nevertheless, this method can be used, in our opinion, for qualitative estimation of the cell proliferation, especially when the cell number (population density) is not a good measure, due to the cell death.

In conclusion – FMdC is a very strong antileukemic (and substantially weaker anticancer) agent, acting in the nano- to micromolar concentrations. It effectively induces the apoptotic cell death, mainly in the subsequent cell cycles (delayed apoptosis). This substance induces also distinct changes in protein metabolism. FMdC at low (10 nM) concentration blocks cells in the S-phase. Higher than 10 nM concentrations cause the G_1-phase block. The S- or G_1-phase blocks are not complete; G_2M-phase cells are visible on histograms and CFDA-SE experiments indicate that cells do proliferate with doubling time longer than that of the untreated population.

These findings suggest that the main mechanism of FMdC action does not depend on the inhibition of DNA synthesis, but on other, unknown as yet, molecular events, occurring during the G_1-phase of the cell cycle.

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