Acute myeloid leukaemia (AML) accounts for over 80% of all adult acute leukaemias (7) and is characterised by a clonal expansion of immature myeloid cells in all haematopoietic tissues. Many patients progress to AML from preleukaemic myelodysplastic syndrome (MDS) or from chronic myelogenous leukaemia (CML). AMLs show varied morphologic, cytochemical, immunologic and cytogenetic characteristics and varied sensitivity to conventional chemotherapeutic regimens. Sixty percent to 70% of patients with de novo AML initially achieve complete remission. However, the majority of these patients relapse and eventually die of the disease. The first described and best characterized mechanism of resistance is mdr1 gene product, Pgp. Pgp is the ATP-binding cassette (ABC) transporter family which is expressed on the surface of AML blasts and encodes the Pgp ATP-binding cassette transporter protein. Pgp expression is correlated with the chemoresistance of AML cells. The use of chemotherapeutic agents is the standard treatment for AML, but the development of drug resistance is a major clinical problem. The National Cancer Institute has identified two main mechanisms of drug resistance in AML: the overexpression of Pgp and the overexpression of another ABC transporter, P-gp-related protein (MRP), which is responsible for the multidrug resistance (MDR) phenotype.

The mode of induction of apoptosis is dependent upon the cell type and the type and concentration of cytostatic drug used. Three different routes to the induction of apoptosis have been described:

1. Early interphase apoptosis, where death occurs after arrest in G2 phase. 2. Delayed interphase apoptosis, where death occurs after arrest in G1 or S phase. 3. Mitotic/delayed mitotic death, where death occurs after one or more cell division. (6).

To investigate whether the sensitivity of leukaemias to chemotherapeutic agents depends on the abilities of leukaemia cells to respond to therapeutic insult by inducing apoptosis, we examined the biological effects of Idarubicin in HL-60 cells.

**Summary:** TP-53 deficient cells of human leukaemia HL-60 die by massive apoptosis after treatment by high (50-100 nmol/l) doses of DNA damaging agent Idarubicin, regardless of the cell-cycle phase, in which they are affected. In contrast, after relatively low dose 10 nmol/l the cells die after cell-cycle arrest in G2 phase. The results show, that apoptosis induced by idarubicin could appear independently of the cell-cycle phase and that period in which apoptosis is observed is related to the dose of Idarubicin.

**Key words:** HL-60; Idarubicin; Apoptosis; G2 cell-cycle arrest

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Variable response of malignant cells to cytostatic thera-
py could be explained first by proliferation status of these cells, second by the ability of the cytostatic to induce cell-
cycle arrest in a specified cell-cycle phase and third by the ability to induce apoptosis. In our work we analysed cell-
cycle status of HL-60 line, duration and intensity of cell-
cycle arrest and the ability of damage repair or apoptosis ini-
tiation after low-doses idarubicin treatment using flow-
cytometric DNA analysis.

Materials and Methods

Cell culture and culture conditions

Human leukaemia HL-60 cells were obtained from the
European Collection of Animal Cell Cultures (Porton
Down, Salisbury, UK) and were cultured in Iscove’s medi-
um (Sigma Inc.) supplemented with 20% fetal calf serum
(FCS) in a humidified incubator at 37°C and controlled 5%
CO_2_ atmosphere. The cultures were divided every 3rd
day by dilution to a concentration of 2x10^6 cells/ml. Cell count
was performed with a haemocytometer, cell membrane in-
tegrity was determined using the Trypan blue exclusion
technique. HL-60 cells in the maximal range of 20 passages
were used for this study.

Cell treatments

Exponentially growing HL-60 cells were suspended at a
concentration of 2x10^5 cells/ml in complete medium. 10
ml of aliquots were plated into 25 cm^2 flasks (Nunc) and
mixed with idarubicin (Zavedos, Pharmacia Upjohn S.p.A.
Laboratories) at desired concentrations. After 4 hours ida-
rubincontaining medium was removed and replaced with
fresh culture medium without idarubicin. Following 6,
24, 48 and 72 hours the cells were counted and cell viabili-
ty determined with the Trypan blue exclusion assay.

Cell morphology

For calculation of the percentage of cells showing mor-
phology of apoptosis, aliquots were removed from control
and drug-treated cell cultures at various times of incubation
and usually 400 cells were counted on Diff-Quik (DADE BE-
HRING) stained cytospin preparations. Apoptotic cells were
identified by the condensed and fragmented state of their
nuclei and focal protrusions of the cell surface.

Cell surface markers and cell size analysis

Flow cytometry was used for cell surface antigen analy-
sis and for apoptosis monitoring. Cells were washed twice
with PBS containing 5% FCS. Then, 1x10^6 cells were sus-
pended in 0.5 ml PBS with 5% FCS and 0.02% NaN_3 and
incubated with mAb APO2.7 (clone 2.7 A6A3) (obtained from
Immunotech) for detecting 7A6 antigen expressed by cells
undergoing apoptosis has been used. We used this method
without cell permeabilisation.

Flow cytometric analysis was performed on a Coulter
Epics XL flow cytometer. A minimum of 10 000 cells was
collected for each sample in a list mode format. List
mode data were analysed using Epics XL System II soft-
ware colour eventing (Coulter Electronic, Hialeah, FL,
USA).

Cell cycle analysis

Following 6, 24, 48 and 72 hours of incubation, the
cells were washed with cold PBS, fixed by 70% ethanol
and stained with propidium iodide (PI) in Vindelev’s solution
for 30 minutes at 37°C. Fluorescence (DNA content) was
measured with Coulter Electronic, Hialeah, FL, USA appa-
ratus. A minimum of 10 000 cells analysed in each sample
served to determine the percentage of cells in each phase of
cell cycle, using Multicycle AV software. Three indepen-
dent experiments were performed.

Results

Cell growth and viability

Fig. 1 shows the effects of idarubicin on the proliferati-
ve rate of HL-60 cell line. Cultivation with 5 nmol/l idaru-
binic induced high inhibition of the rate of HL-60 cell
growth. The decrease of the proliferative rate observed in
HL-60 cells after addition of higher concentration of ida-
rubin was observed. After 48 hours all cells with 100 and
50 nmol/l idarubicin were dead.

Morphologic changes

HL-60 cells were incubated in the presence 5, 10, 20,
50 and 100 nmol/l idarubicin for 72 hours. After 1, 6, 24,
48 and 72 hours cell morphology was examined on Diff-
Quik stained cytospin preparations. Morphologic evidence
of apoptosis was found in cells treated with idarubicin.
After addition of idarubicin dose dependent increase in
the proportion of apoptotic cells was detected in cultures
treated with 5 -100 nmol/l idarubicin. The maximal per-
centage of apoptotic cells was observed in cultures incubated
for 6 or 24 hours treated with 100 or 50 nmol/l idarubicin
respectively. (Fig. 2). During in vitro studies, where apo-
ptotic cells cannot be removed by fagocytosis, secondary
necrosis can be observed in later intervals (24 to 72 hours
in Fig.2).

Analysis of cell-cycle and sub-diploid DNA content

We assessed DNA cleavage in the afore mentioned
5 and 10nmol/l idarubicin-treated tumour cells. We have
observed that after 6 hours of incubation most of the live
idarubicin-treated cells were in S phase of cellcycle (62% or
71%, respectively), after 24 hours most of them moved to
G_2_ phase (64% or 75%) and after 48 hours the percentage
of cells in various cellcycle phases was comparable to con-
trol untreated cells (Fig.3). Results of one representative ex-
periment with idarubicin concentration 5 nmol/l are shown
in Fig. 4.
Variable response of malignant cells to cytostatic ther-apy could be explained first by proliferation status of these cells, second by the ability of the cytostatic to induce cell cycle arrest in a specified cell-cycle phase and third by the ability to induce apoptosis. In our work we analysed cell cycle status of HL-60 line, duration and intensity of cell cycle arrest and the ability of damage repair or apoptosis initiation after low-doses idarubicin treatment using flow-cytometric DNA analysis.

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Fig. 1 shows the effects of idarubicin on the proliferative rate of HL-60 cell line. Cultivation with 5 nmol/l idarub-inic induced high inhibition of the rate of HL-60 cell growth. The decrease of the proliferative rate observed in HL-60 cells after addition of higher concentration of idarubicin was observed. After 48 hours all cells with 100 and 50 nmol/l idarubicin were dead.

Fig. 2: Time course of apoptosis in HL-60 cells exposed to idarubicin as determined by cell morphology examined on Diff-Quik stained cytospin preparations. Data represent medium values from 3 independent experiments.

Fig. 3: Flow cytometric analysis of DNA content and cell-cycle after treatment with 5 and 10 nmol/l idarubicin.

Fig. 4: Flow cytometric analysis of DNA content and cell-cycle after treatment of HL-60 cells with 5 nmol/l idarubi-cin. Apoptotic cells are identified as cells with subdiploid DNA content (lower DNA content than cells in G0/G1 phase), i.e. subG1 peak. Representative results for single exper-iment are shown.

Fig. 5: Histograms for cell number versus APO2.7-PE fluorescence intensity of unprocessed HL-60 cells after treatment with 5 nmol/l idarubicin. Representative results for single experiment are shown.
phases. The fluorescence peak representing apoptotic cells. Our re-
2 phase in comparison to other cell-cycle
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**Flow cytometric detection of apoptotic cells using monoclonal antibody APO2.7** Since 7A6 antigen is selectively expressed on the mito-
choondrial membrane in cells undergoing apoptosis, we attemp-
ted to detect apoptotic cells using APO2.7 monoclonal anti-
body after 5 and 10nmol/l idarubicin treatment of HL-60 cells. As shown in Fig.5 in unpermeabilised cells APO2.7 antibody staining significantly increased from 6% at 6h to 24h to 50% at 72h after treatment with 10nmol/l idaruc-
bin, while increased only slightly after treatment with 5 nmol/l idarubicin (from 6% at 6h to 16% at 72h). (Fig. 6). 

**Discussion and Conclusions**

HL-60 line has amplified myc and activated ras onco-
gene, it is p53 negative and does not contain the charac-
teristic t(15;17) translocation seen in acute promyelocytic leukemia (3, 4). HL-60 cells were negative for expression of CD34 and AC133 antigens (0.6/0.7%), the antigens usu-
ally used for separation of haematopoietic progenitors from mobilised peripheral blood patients for autologous trans-
plantation. HL-60 cells expressed high levels of CD15 (93%) and CD13 (84%) antigens, 82% cells were CD15+/CD33+. Haematopoietic progenitor cells giving rise to monocytc and granulocytic lineages express nume-
rous surface antigens to varying degrees depending on the-
ir developmental stage. CD33 antigen is expressed prior to myeloid commitment and CD15 is expressed at later stages in myelomonocytic development. Hofmanova et al. (3) described that 90% HL-60 cells were promyelocytes and 8% myelocytes with no expression of CD14/CD11b antigens.

We show in this study that relatively high doses of ida-
rubcin (50-100 nmol/l) induce as soon as after 6 hours in leukaemic cell line HL-60 cell shrinkage, membrane blebbing and cytoplasmic and nuclear fragmentation leading to the formation of apoptotic bodies, as determined by evaluation of DiffQuik stained cytospin preparations using standard light microscopy at 1000 x magnification, as well as by flow-cytometric analysis of cell DNA content. During apo-
posis DNA becomes fragmented by endonucleases and these small DNA fragments can break out from the cells, resulting in a reduced total DNA content and hence a sub-G1 fluorescence peak representing apoptotic cells. Our re-
suits show that high doses of idarubicin induce apoptosis soon after treatment (6 hours). We presume that the cells die by rapid interphase apoptosis, where the apoptosis is triggered in all phases of cell cycle. Some studies (8) sug-
gest that apoptosis induction and G1 or G2 cell cycle arrest are two separate phenomena in Jurkat cells (T-cell line, mutated gene TP53, undetectable levels of TP53 protein) It has been shown (in accordance with thesis that cells with mutated TP3 are radioresistant) that apoptosis occurs in these cells during 24 hours after irradiation by high doses of ionising radiations (10-20 Gy). Regardless of the cell cyc-
le phase 20% of apoptotic cells have been detected 6 hours after irradiation dose 20 Gy. Apoptosis was lower when ear-
ly G1 subpopulation has been irradiated in comparison to other cell cycle phases. It seems that after irradiation of cells in G1 phase apoptosis occurs 2 hours later in compa-
rison with cells in other cell cycle phases. However, 24 hours following irradiation by 20 Gy all cells were apop-
totic regardless of the cell-cycle phase, in which they were irradiated.

Relatively lower doses of idarubicin (5-10 nmol/l) first inhibit proliferation of the cells and induce changes in cell cycle. We have observed that after 6 hours of incubation most of the live cells accumulate in S phase of cell-cycle af-
ter 24 hours we observed arrest in G1 phase. For 48 h fol-
lowing 10 nmol/l idarubicin treatment we observed significant apoptotic and the cells did not proliferate during 72 hours. Lower dose (5 nmol/l) induces only small per-
centage of apoptosis after cell-cycle arrest in G1 phase and the cells slightly proliferate during 72 hours following ida-
rubicin treatment. We suppose that after 10 nmol/l the cells die by delayed interphase apoptosis, which typically occurs after cell-cycle arrest in G2 phase. Similar results have been re-
gored after irradiation of HL-60 cells by 5-Gy, where the apoptosis occurs following cell-cycle arrest in G2 phase 36-48 hours after irradiation (9). Cell-cycle arrest in G2/M phase has been observed in Jurkat cells (8) following irradiation by relatively low dose 2 Gy, regardless of cell-
phase cycle, in which they were irradiated. Apoptotic cells were cumulated 24-50 hours after irradiation. It is inter-
esting that apoptosis occurred sooner in population of cells irradiated in G2 phase in comparison to other cell-cycle phases.
Flow cytometric detection of apoptotic cells using monoclonal antibody APO2.7

Since 7A6 antigen is selectively expressed on the mitochondrial membrane in cells undergoing apoptosis, we attempted to detect apoptotic cells using APO2.7 monoclonal antibody after 5 and 10 nmol/l idarubicin treatment of HL-60 cells. As shown in Fig.5 in unirradiated cells APO2.7 antibody staining significantly increased from 6% at 6h to 65% at 72h after treatment with 10nmol/l idarubicin, while increased only slightly after treatment with 5 nmol/l idarubicin (from 6% at 6h to 16% at 72h) (Fig. 6).

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It can be concluded that apoptosis induced by low doses of idarubicin in TP<sub>3</sub>-negative cells HL-60 as well as apoptosis induced by ionizing radiation in dose 2-10 Gy in various TP<sub>3</sub>-negative haematopoietic cell lines (Jurkat, HL-60) occurs following G<sub>1</sub> cellcycle arrest. Apoptosis was observed at HL-60 cells as soon as 6 hour after treatment with high idarubicin concentrations (50-100 nmol/l), similarly to Jurkat cells irradiated by supraletal doses (10-20 Gy), which also underwent apoptosis after 6 hours. Syujena and McBridge (8) proved that Jurkat cells treated by ionizing radiation could undergo apoptosis independently on cellcycle arrest and that period in which apoptosis is observed is related to the dose of radiation. Similar results from our experiments on idarubicin influence on HL-60 cells.

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MUDr. Martina Mareková, Charles University Prague, Faculty of Medicine Hradec Králové, Department of Medical Biochemistry, Sinkova 870, 500 01 Hradec Králové, Czech Republic.

e-mail: marekov@fhk.cuni.cz