A karyometric study on ageing and butyrate or imatinib treated human leukemic myeloblasts represented by K562 cells originated from chronic myeloid leukaemia

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The present study was undertaken to provide more information on nuclear diameter in leukemic granulocytic early precursors – myeloblasts. These cells represented by K562 myeloblasts originated from the blastic phase of the chronic myeloid leukaemia (CML) carry characteristic bcr-abl fusion gene. They represent a convenient model for *in vitro* studies of CML myeloblasts and are sensitive to various agents which may induce ageing, differentiation and cell death. Mean nuclear diameter (MNuD) and largest nuclear diameter (Mx NuD) in stained cytospins of these cells were measured at a high light microscopic magnification by means of computer programme. Starving cultures were used for induction of ageing without preceding differentiation, sodium butyrate was used as a cytostatic agent or differentiation inducer and imatinib mesylate represented a cytostatic agent for CML. The largest shift of MNuD to smaller values was noted in ageing cultures or cultures treated with butyrate. The decrease of MNuD was less apparent in resistant cells treated with imatinib. This drug, however, produced a very large incidence of necrotic or apoptotic cells or bodies. From the methodical point of view it should be mentioned that values of maximal nuclear diameter (MxNuD) followed similar trends as MNuD and thus provided similar information. The measurement of both nuclear diameters, i.e. MNuD and MxNuD might be a complementary and simple tool to evaluate the cell state in cytological preparations because of their decrease in ageing cells or cells treated with antiproliferative drugs of different mode of action.

Key words: Karyometry of K562 myeloblasts; effect of ageing, sodium butyrate and imatinib mesylate

The karyometry was one of important approaches to study the biology of blood cells in the past and contributed to the morphological definition of their developmental stages [1 – 4]. However, the numerical information on the mean nuclear diameter (MNuD) or maximal nuclear diameter (MxNuD) in granulocytic precursors is still limited [2, 5]. On the other hand, previous studies on various cell types, including differentiating blood cells, indicated that the nuclear size is apparently related to the stage of the cell cycle and differentiation or maturation [4, 6, 7, 8]. In addition, nuclear karyometry in solid malignancies seem to be a very useful marker of cell state with a potential prognostic value [9 – 12].

The present study was undertaken to provide more and quantitative information on the MNuD and MxNuD in leukemic granulocytic precursors – myeloblasts. Leukemic myeloblasts were represented by K562 cells which originated from the blastic phase of the chronic myeloid leukaemia and carry characteristic bcr-abl fusion gene [13, 14]. These cells also represent the mostly used model for in vitro studies of CML myeloblasts and are sensitive to various agents which may induce their ageing, maturation, differentiation and apoptotic process [15 – 17]. Moreover, cultured K-562 cells are present in cytospins in satisfactory amount and are not "contaminated" by other cell types which are present in bone marrow of leukemic patients.

In the present study, starving cultures were used for induction of premature ageing without preceding differentiation [18, 19]. Sodium butyrate representing a histone deacetylase inhibitor was used as a cytostatic agent and differentiation inducer [13]. Imatinib mesylate represented in the present study a cytostatic agent for CML the effect of which is still discussed although its inhibitory effect on tyrosine kinase activity is clearly established and results in the induction of

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the cell cycle arrest as well as apoptotic process [16, 20]. The preceding flow cytometry studies on K562 cells in this laboratory suggested a shift of cells in S+G2 phase in favour of G0+G1 cells in ageing and imatinib treated cultures. However, the incidence of cells in S+G2 cells in butyrate treated cultures for 24 and 72 hrs was not substantially different [see 13, 16, 19]. Therefore, it appeared that karyometry of these cells under the same conditions might provide additional and useful information.

Material and Methods

K562 cells originated from chronic myeloid leukaemia (European Collection of Animal Cell Cultures, United Kingdom) were cultured in RPMI 1640 supplemented with 10% foetal calf serum, 100U/mL penicillin and 50 μg/mL streptomycin at 37°C in 5% CO_2 humidified atmosphere. In the first group, K562 were harvested after 24 and 72 hrs of cultivation without adding any anti-proliferation and/or differentiation agent. The cultivation of granulocytic precursors for 72 hrs is known to induce cell ageing and proliferation arrest [19, 21]. In the second group, K562 cells were cultured in the presence of 1.5 mM sodium butyrate (Sigma, Czech Republic) and in the third group, K562 cells were cultured with 1μM imatinib mesylate (Novartis, Switzerland). In both these groups, similarly as in the first group, cultured cells were harvested after 24 and 72 hrs. The used concentrations of added drugs are known to induce anti-proliferation as well as differentiation effect which is more pronounced after 72 hours [13, 16, 17].

Cytospins of harvested K562 cells were prepared using a Shandon II cytocentrifuge (Shandon Southern Products, UK) – 6000 RPM for 10 min. Then air dried cytospins were stained with May-Grünwald – Giemsa (MG-G) panoptic procedure which stains both the nuclear and cytoplasmic components (Fig. 1) [22]. The chromatin structure was visualised by a simple but sensitive cytochemical method for demonstration of DNA (Fig. 2, ref. 23). Micrographs of K562 cells were taken with a Camedia digital photocamera C-4040 Zoom (Olympus, Japan) placed on Jenalumar microscope (Zeiss, Germany) equipped with two special mechanical adapters. The resulting micrographs were processed with Quick Photoprogram (Olympus, Japan) in combination with L-view and Power Point Microsoft programs (Microsoft, USA) to see clearly the nuclear structure and outline. It must be also mentioned that MNuD and MxNuD were measured only in myeloblasts with a fine chromatin structure and not in apoptotic or necrotic cells or bodies which were characterised by nuclear chromatin condensation or fragmentation or swelling (see Fig. 1, 2) [18, 24, 25].

Both nuclear diameters, i.e. MNuD and MxNuD were measured directly on the monitor screen at magnification 4 300x using Quick Photoprogram. MNuD in each K-562 mononuclear cell representing leukemic myeloblast was calculated from two diameters, i.e. from long (major) and short (minor) axis diameters [9 – 12] (see Fig 2a). MxNuD represented the single largest diameter of the nucleus in each measured cell (see Fig 2b). All reported measurements (about 200 for each experimental group) were expressed by mean values and standard deviations (±).

Results

General description of cultured cells. Cultured mononuclear K562 cells appeared as myeloblasts with a fine chromatin structure (Fig. 1a, 2a, b, d). On the other hand, the cytoplasm of some cells was enlarged and in the proximity of the cell nucleus, in the Golgi region, possessed a distinct and large light area (Fig. 1b). These regions are generally known to represent sites of the formation of primary granules in promyelocytes. However, in such cells stained with MG-G method, characteristic primary azurophilic granules were absent (Fig. 1b). Mitotic cells (Fig. 1c and 2c) considered as morphological proliferation markers [26] were noted in all experimental groups but in different number. In 24 hours old cultures without any treatment the number of mitotic cells was high, i.e. 8 ± 2 per cent. In butyrate treated 24 hours old cultures the number of mitotic cells was smaller but still relatively large, i.e. 4 ± 1 per cent . In 24 hours old cultures treated with imatinib the incidence of mitotic cells was similar, i.e. 3 ± 1) per cent. In contrast, in 72 hours old cultures the number of mitotic cells markedly decreased to 1 (cultures with the presence of butyrate) or less than 1 per cent (ageing cultures without any treatment or cultures treated with imatinib).

As it was described in several previous studies [18, 24, 25], apoptotic cells and bodies, regardless of their morphological variability, were characterised by a distinct chromatin condensation and fragmentation (see Fig 1d,e and 2e,f). In 24 hours old cultures without any treatment or in cultures treated with butyrate the incidence of apoptotic cells or bodies did not exceed 5 per cent. In contrast, 24 hours old cultures treated with imatinib possessed about 10 per cent of apoptotic cells and bodies. In 72 hours old cultures without any treatment or in cultures treated with imatinib, the percentage of apoptotic cells or bodies with characteristic chromatin condensation and fragmentation increased to 12 ± 2 or 13 ± 1 per cent. In 72 hours old cultures with butyrate, the incidence of apoptotic cells was smaller (4.5 ± 5.0 per cent) and did not differ substantially from 24 hours old cultures. Swollen nuclei (see Fig. 1f and 2g), presumably representing necrotic nuclei, were present in all cultures but their incidence did not exceed 2 per cent except those in cultures with imatinib. In the latter their incidence reached 10 per cent regardless of the cultivation time. It seems to be interesting that the perinucleolar chromatin was more distinct in such nuclei especially after staining for DNA (Fig. 2g). Some of swollen nuclei (Fig. 1f) exhibited nuclear pulverisation – granular chromatin condensation which was visible particularly at higher magnifications (Fig. 1f). It should be mentioned that such phenomenon was considered as a special form of the apoptotic process [27].

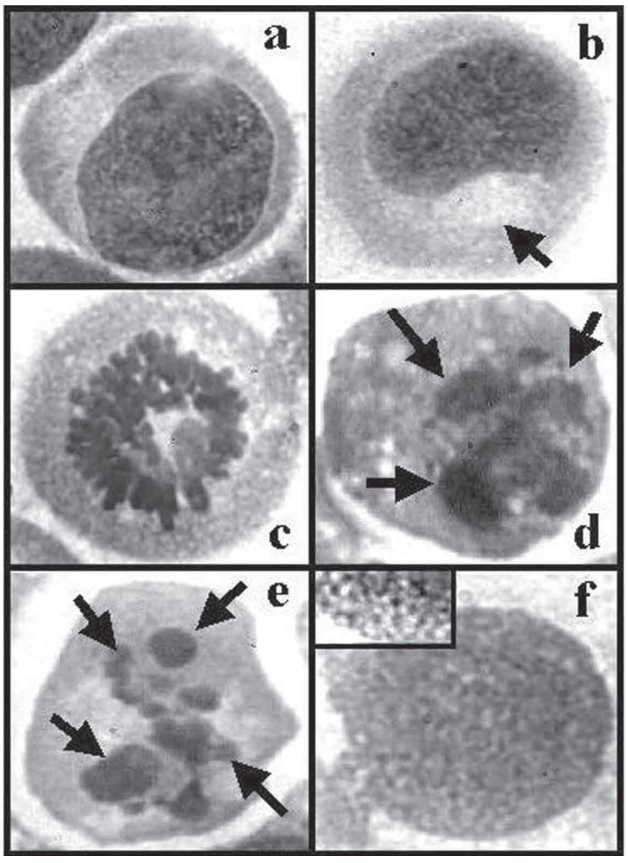


Fig. 1. K562 cells stained by MG-G method. (a, b) Cells with a fine chromatin structure but with a variable size and shape of the nucleus one of which (b) appeared to be more „differentiated". Such cell possessed a kidney-shaped nucleus in a larger cytoplasm with a distinct perinuclear clear area (arrow) but without azurophilic granules characteristic for promyelocytes. (c) A mitotic cell in the metaphase. (d) An apoptotic cell with a characteristic and highly condensed chromatin structure (arrows). (e) An apoptotic body with characteristic fragments of condensed chromatin (arrows). (f) A swollen nucleus with chromatin pulverisation which is better visible at larger magnification in the insert. Magnification approx. 1 700x; insert in the Figure f, 2 400x.

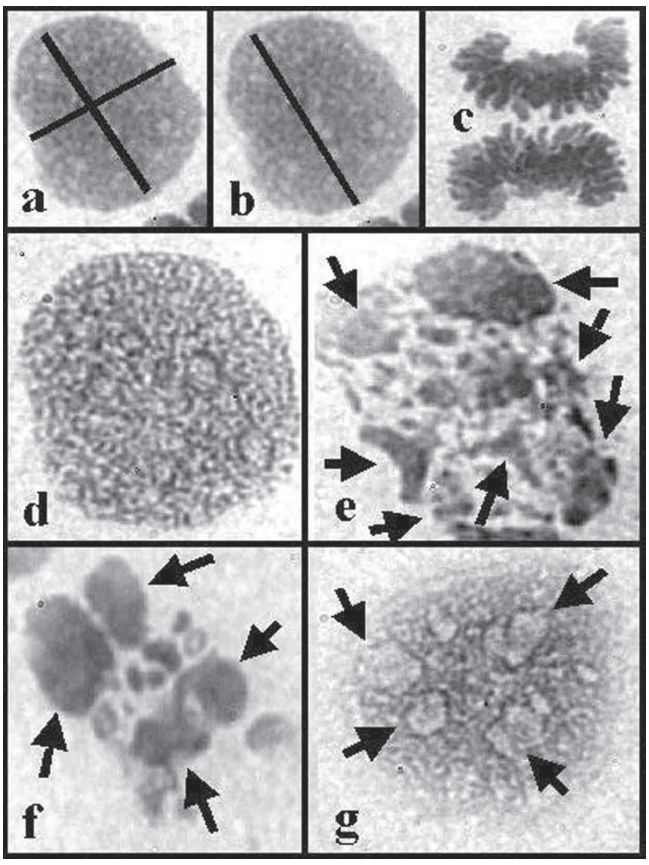


Fig. 2. K562 cells stained by a cytochemical method for demonstration of DNA for a selective visualisation of the chromatin. (a, b) The fine chromatin structure in nuclei with black lines which exhibit how MNuD (a) and MxNuD were measured. (c) A mitotic cell in the anaphase. (d) A larger magnification which shows a fine nuclear structure consisting of fine chromatin fibrils. (e) Chromatin condensation and fragmentation characteristic for an apoptotic cell. (f) Condensed and fragmented chromatin in an apoptotic body. (g) A swollen nucleus exhibiting fine chromatin filbrils which are more distinct in the perinucleolar regions (arrows). Magnification approx. 1 400x (a, b), 2 000 (c, d), 2 600 (e), 2 000 (f, g).

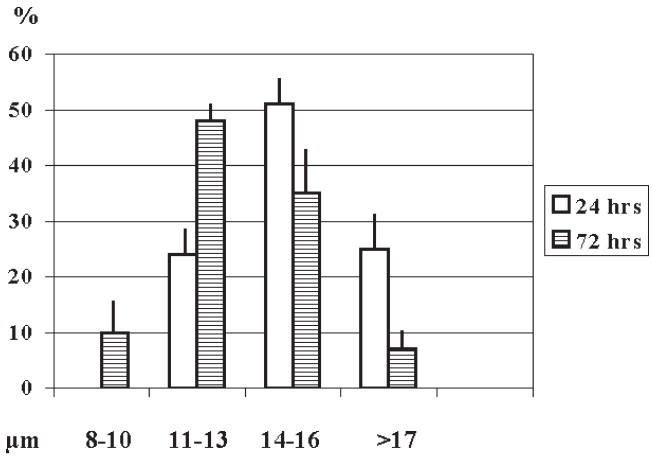
Nuclear diameters in cultures without any treatment. In K562 mononuclear myeloblasts cultured for 24 hours, MNuD was 14.6 μm in specimens stained for DNA and 13.6 μm after staining with MG-G method. After 72 hours of cultivation, MNuD significantly decreased to 12.6 μm in specimens stained for DNA and 12.7 μm in those stained with MG-G method (see Table 1).

The histograms indicated that the largest percentage of cells possessed nuclei with MNuD ranging between 14 and 17 μm regardless of the method used for their visualisation. In contrast, after 72 hours of cultivation, most cells were characterised by the presence of smaller nuclei the MNuD of which mostly ranged from 11 to 14 μm (Graph 1).

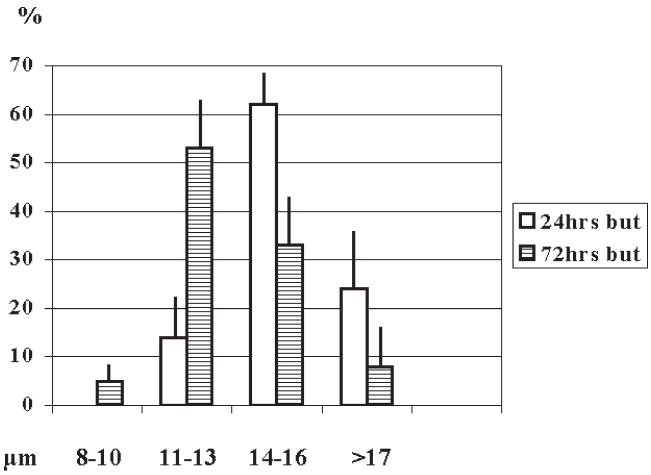
MxNuD was naturally larger (about 17 μm) than MNuD and significantly decreased after 72 hours regardless of the staining procedure (Tab. 2).

Nuclear diameters in cultures treated with butyrate. The cultivation of K562 myeloblasts with butyrate resulted in a small but significant increase of MNuD (see Table 1). In 24 hours old cultures, MNuD was 16.5 μm in specimens stained for DNA and 15.3 μm in specimens stained with the MG-G method. On the other hand, similarly as in cultures without any treatment, MNuD after 72 hours of cultivation decreased regardless of the method used for the nuclear visualisation. MNuD was 13.7 μm in specimens stained for DNA and 13.5 μm in MG-G stained specimens. However, it should be mentioned that MNuD was larger than that in ageing cultures without butyrate.

Similarly as in cultures without butyrate, the largest percentage of cells possessed nuclei with MNuD ranging between 14 and 17 μm in 24 hours old cultures. In contrast, similarly



Graph 1. The percentage of K 562 myeloblasts classified according to MNuD



Graph 2. The percentage of K 562 myeloblasts classified according to MNuD which were treated with butyrate

as in ageing cultures without butyrate, the cultivation with butyrate for 72 hours produced the shift of the largest percentage of cells to the smaller MNuD which mostly ranged between 11 and 14 µm (Graph 2).

Similarly as MNuD, MxNuD significantly decreased in butyrate treated myeloblasts cultured for 72 hours (Tab. 2) On the other hand it should be mentioned that values of the MxNuD after 72 hours were slightly larger in specimens stained for DNA and smaller in specimens stained with MG.G method (Tab. 2).

Nuclear diameters in cultures treated with imatinib. In cultures 24 hours old, MNuD of K-562 myeloblasts appeared to be larger, i.e. 15.5 µm (in specimens stained for DNA) and 15.8µm (in specimens stained with MG-G staining) than in untreated cultures. After 72 hours, MNuD decreased (Table 1). However, this decrease was smaller and less distinct than that in cultures without any treatment or treated with butyrate (Table 1). Therefore, most of K-562 myeloblasts possessed nuclei with MNuD ranging between 14 and 16 µm in both 24 and 72 hours old cultures (Graph 3). However, at this occasion it should be noted that these cells did not show any signs of apoptotic or necrotic process.

In contrast to MNuD, the differences of larger values of MxNuD between myeloblasts cultured with imatinib for 24 and 72 hours were more distinct (Tab. 2).

Discussion

It seems to be clear that both MNuD and MxNuD less or more markedly decreased in simply ageing myeloblasts as well as in myeloblasts treated with differently acting drugs, i.e. sodium butyrate and imatinib mesylate with the anti-proliferative and differentiation effect. In all experimental group such effect was accompanied by slightly or markedly decreased mitotic activity indicating the decrease of the proliferation activity [26] which was also noted in previous preceding studies under the same experimental conditions [see 13, 16, 19]. Thus both MNuD and MxNuD might serve as very useful morphological markers of the cell state in cytological preparations even before apparent chromatin changes

Table 1. Mean nuclear diameter (MNuD) in K-562 leukemic myeloblasts under various experimental conditions in DNA and MG-G stained specimens

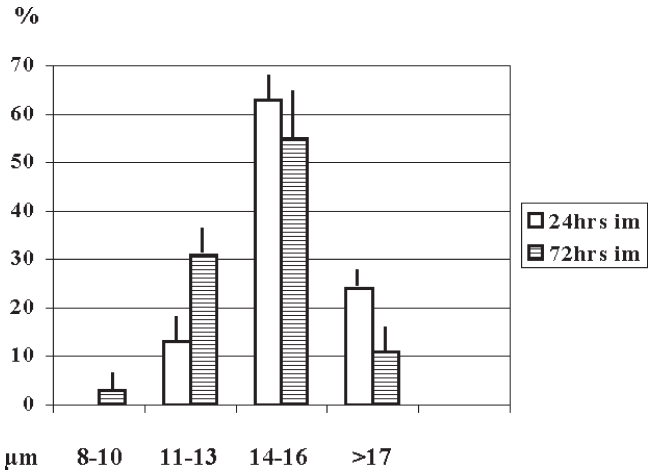
Treatment	Untreated and Ageing*		Butyrate		Imatinib	
Staining	DNA	MG-G	DNA	MG-G	DNA	MG-G
24 hrs	**14.6** (1.0)[a]	**13.6** (1.5)	**16.5** (2.2)	**15.3** (2.1)	**15.5** (2.1)	**15.8** (2.2)
72 hrs	**12.6** (1.2)[b]	**12.7** (1.0)[b]	**13.7** (2.4)[b,c]	**13.5** (1.6)[b.,c]	**14.8** (2.4)[c]	**14.5** (1.9)[b,c]

Legend
* – Cultures without adding butyrate or imatinib served as controls
[a] – **Mean** and standard deviation
[b] – Significant difference in comparison with untreated myeloblasts cultured for 24 hours and stained with the same procedure using the t-test ($p<0.005$)
[c] – Significant difference in comparison with untreated myeloblasts cultured for 72 hours and stained with the same procedure using the t-test ($p<0.005$)
MG-G – May-Grünwald – Giemsa panoptic staining

characteristic for differentiation, apoptotic or necrotic process. However, at this occasion it should be mentioned that in *vivo*, in patients suffering from CML and treated with imatinib, MNuD of myeloblasts did not change significantly [5] possibly due to the known presence of both not-leukemic and leukemic clones in the bone marrow. In the present study, the decrease of MNuD in myeloblasts treated with imatinib was also less apparent but was accompanied by a marked incidence of apoptotic or necrotic cells and apoptotic bodies.

The decreased values of both MNuD and MxNuD in ageing and butyrate or imatinib treated myeloblasts might reflect the shift of cells to G0/G1 phase of the cell cycle and to non-cycling state. In all these events the shift to G0/G1 or to non-cycling myeloblasts was reported in preceding studies of this laboratory [13, 16, 19]. However, a direct relationship of cells classified according to MNuD in the present study and previously reported cell cycle stages under the same experimental conditions was not detected. Thus, it seems to be likely that myeloblasts with smaller MNuD might correspond not only to cells in G0/G1 phase but might also indicate the initiation of maturing or ageing and apoptotic process since the incidence of mitotic cells was markedly reduced. Such speculation is supported by the generally known decreasing of nuclear size in ageing or maturing and differentiating precursors of blood cells. On the other hand, myeloblasts measured in the present study, possessed a fine chromatin structure without any apparent signs of condensation which is characteristic for maturing and differentiating or apoptotic cells [18, 24, 25, 28]. At this occasion it should be also mentioned the arrest of the cell cycle in other than G0/G1 phase of the cell cycle which was also reported [29 – 31]. Nevertheless, regardless of the interpretation, the decrease of MNuD and MxNuD reflected the effect of ageing and butyrate or imatinib treatment of studied K562 leukemic myeloblasts resulting in the decreased proliferation activity. The reduced incidence of mitotic cells indicating the decreased cell proliferation [26] is in harmony with such supposition.

From the methodical point of view it seems to be important that the observed trends in all experimental groups were seen after both cytochemical and panoptic staining. Therefore, in the future studies, the panoptic staining with MG-G method is satisfactory for nuclear measurements. At this occasion it should be mentioned that this method is a routine one and widely used procedure in most of clinical cytological and haematological laboratories. In addition, the more simple measurement of MxNuD is easier than that of MNuD and may be accomplished using less complicated and time consuming approach.



Graph 3. The percentage of K 562 myeloblasts classified according to MNuD which were treated with imatinib

At the end of the discussion it should be mentioned that the results of the present study, regardless of interpretation, also indicated that the karyometry is still an useful and complementary tool for evaluation of the effect of various treatment approaches on leukemic myeloblasts. The measurement of MNuD or MxNuD is relatively easy and reproducible especially when using a suitable method for the nuclear visualisation and cell identification at large magnifications in combination with a suitable computer program. In addition, according to studies on other malignant cells, both karyometric measurements appeared to be useful complementary diagnostic and prognostic markers for a variety of human

Table 2. Largest nuclear diameter (MxNuD) in K 562 leukemic myeloblasts under various experimental conditions in DNA and MG-G stained specimens

Treatment	Untreated and ageing*		Butyrate		Imatinib	
Staining	DNA	MG-G	DNA	MG-G	DNA	MG-G
24 hrs	**17.3**(2.0)[a]	**17.2** (2,2)	**17.9**(2.4)	**17.7** (2.0)	**17.1** (1.7)	17.6 (1.5)
72 hrs	14.4 (2.4)[b]	15.7 (2.0)[b]	15.4 (2.4)[b,c]	14.7 (2.1)[b,c]	16.2 (2.3)[b, c]	14.3 (2.1)[b,c]

Legend
* – 24 hours old cultures without adding butyrate or imatinib served as controls
[a] – **Mean** and standard deviation
[b] – Significant difference in comparison with untreated myeloblasts cultured for 24 hours and stained with the same procedure using the t-test ($p<0.005$)
[c] – Significant difference in comparison with untreated myeloblasts cultured for 72 hours and stained with the same procedure using the t-test ($p<0.01$)
MG-G – May-Grünwald – Giemsa panoptic staining

malignant solid tumours [9 – 11]. However, MNuD or MxNuD were not reported.

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