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INHIBITION OF USP1, A NEW PARTNER OF BCR-ABL, RESULTS IN DECREASE OF BCR-ABL LEVEL IN K562 CELLS

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***Aim*: To analyze interaction of ubiquitin specific peptidase 1 (USP1) with Bcr-Abl and to assess the relation between USP1 functional activity and Bcr-Abl expression in K562 chronic myeloid leukemia cells. *Materials and Methods:* The interaction between USP1 and Bcr-Abl in K562 cells was analyzed by co-immunoprecipitation, Western blot analysis, and confocal microscopy. *Results:* A direct interaction between Bcr-Abl oncoprotein and USP1 protein in K562 cells was established by co-immunoprecipitation. Immunofluorescence analysis and confocal microscopy revealed that Bcr-Abl/USP1 protein complex is formed in the cell nucleus. The inhibition of USP1 protein activity by ML323 reduced the level of Bcr-Abl oncoprotein in K562 cells. *Conclusions:* USP1 protein has been identified as a new protein partner of Bcr-Abl oncoprotein in chronic myeloid leukemia. The relationship between the functional activity of USP1 protein and the level of Bcr-Abl oncoprotein has been demonstrated, suggesting that the targeted inhibition of USP1 activity could be a challenging approach for reducing Bcr-Abl expression.**

***Key Words*: chronic myeloid leukemia, Bcr-Abl oncoprotein, USP1 protein, ML323 inhibitor, K562 cells.**

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Chronic myeloid leukemia (CML) is a myeloproliferative disease that arises from a clonal pluripotent bone marrow stem cell [1–3]. CML cytogenetic marker is the Philadelphia chromosome resulting from t(9;22)(q34;q11.2) reciprocal translocation, where the *Abl* gene is fused with the *Bcr* gene of 22 chromosome [1–7]. A hybrid Bcr-Abl oncoprotein is expressed in several forms associated with different types of the disease, namely p190 (acute lymphoblastic leukemia), p210 (CML) and p230 (benign neurophilic form of myeloid leukemia) [2, 5, 8–13]. Despite similar tyrosine kinase activity, isoforms of Bcr-Abl oncoprotein are involved in different signaling networks in cell [12]. Imatinib is a specific targeted drug for CML treatment aimed to inhibit the tyrosine kinase activity of Bcr-Abl oncoprotein. Nevertheless, the acquired resistance to inhibitor that develops following the long-term administration renders the medications ineffective [1, 3, 6, 12, 14–16]. Usually, resistance develops due to amino acid substitutions within the kinase domain [13, 17, 18]. To overcome this problem, new generation drugs have been developed, such as dasatinib, nilotinib, bosutinib, and ponatinib [18, 19], but they only selectively inhibit the protein tyrosine kinase activity of Bcr-Abl without reducing the oncoprotein level, therefore not eliminating the main problem [14, 18, 19]. Bcr-Abl oncoprotein can act as a protein scaffold for the organization of signaling complexes that are not always dependent on kinase activity [16]. Therefore, alternative approaches are required to provide long-term effective treatment for CML patients [3, 6, 14–16, 19, 20].

According to our previous results of the mass spectrometric analysis, 23 proteins were identified as potential candidates for interaction with the Pleckstrin-homology domain of Bcr-Abl [13]. One of these proteins is ubiquitin-specific protease 1 (USP1) [6, 13]. The USP1 protein belongs to the cysteine proteases family of deubiquitination (DUB) proteins [21–25]. USP proteins catalyze the cleavage of isopeptide bonds between ubiquitin and substrate, or ubiquitin and ubiquitin, thus reversing the activity of E3 ligase and changing the type of protein ubiquitination affecting the function and localization of proteins, and preventing their degradation [21, 26, 27]. The dynamic balance between DUB activity and ubiquitin ligase E3 underlies the modulation of activity, degradation and localization of proteins, providing homeostasis and performing critical cell functions, such as gene expression, cell cycle progression, etc. [21, 22, 26–28]. DUB proteins control levels of methylases and demethylases, kinases, histone proteins and histone-binding partners, repressors and transcription factors [29]. Changes in DUB activity are associated with various diseases affecting significantly the growth and progression of various tumors [24, 26, 28, 30–32]. Here we show that USP1 protein binds to Bcr-Abl in K562 cells and that specific inhibition of DUB activity leads to a change in the localization of their interaction in the cell as well as decrease in Bcr-Abl level. We suggest that during the interaction between Bcr-Abl oncoprotein and USP1 protein, the latter, due to its DUB activity, may contribute to the accumulation of Bcr-Abl in the cell, promoting disease progression. Therefore, the approach with blocking of USP1 can potentially be used to decrease expression of Bcr-Abl oncoprotein.

MATERIALS AND METHODS

Cell culture. K562 cells (the Bank of Cell Lines of Human and Animal Tissues of RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, the NAS of Ukraine) are human myelogenous leukemia line expressing p210 isoform of Bcr-Abl oncoprotein. K562 cells were cultured in RPMI-1640 medium (Thermo Scientific, USA) supplemented with

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Abbreviations used: CML – chronic myeloid leukemia; DUB – deubiquitination; MOC – Manders overlap coefficient; PCC – Pearson correlation coefficient; USP – ubiquitin-specific protease.

10% fetal bovine serum (Biowest, France), 100 U/mL penicillin, and 100 µg/mL streptomycin (Arterium, Ukraine). Cells were cultured at 37 °C with 95% humidity and 5% CO_2. The activity of USP1 protein in K562 cells was inhibited by 52 nM and 76 nM ML323 (Axon Medchem, Netherlands).

Co-immunoprecipitation. Sepharose G (Sigma, USA) previously equilibrated with NP40 lysis buffer (150 mM NaCl, 1.0% Triton X100, 50 mM Tris-HCl pH 8.0) was incubated with 100 µl NP40 lysis buffer and 3 µl monoclonal anti-Bcr-Abl antibody (MA1-153, Thermo Fisher Scientific, USA). After that, cell lysates were added into the mixture of sepharose G with antibody and incubated for 3–4 h at 4 °C. As a control, only lysate of cells without antibody was added to sepharose G. Then the bead-protein mixtures were centrifuged and washed three times with NP40 lysis buffer. Loading buffer with β-MeEtOH (200 mM Tris-HCl, pH 6.8, 400 mM β-MeEtOH, 4% SDS, 0.01% bromophenol blue, 40% glycerol) was added to samples and incubated for 5 min in a water bath at 99 °C/100 °C. The immunoprecipitated samples were analyzed by Western blot analysis.

Western blot analysis. Immunoprecipitated samples were separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. The following antibodies were used at the indicated dilutions: anti-USP1 antibody (1:1000, PA5-55852, Thermo Fisher Scientific, USA), anti-Bcr-Abl antibody (1:1000; MA1-153, Thermo Fisher Scientific, USA), and anti-rabbit (1:10000, Thermo Fisher Scientific, USA), anti-mouse (1:5000, AS003, ABclonal Technology, USA). The membranes were treated with ECL buffer (1.5 M Tris-HCL pH 8.8, coumaric acid (14 mg/ml DMSO), luminol (44 mg/ml DMSO), 30% H_2O_2) and were analyzed via chemiluminescence detection (Chemidoc, Bio-Rad Laboratories, USA).

Immunofluorescence and confocal microscopy. K562 cells were fixed in with 4% paraformaldehyde and 2% Triton X-100 in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 20 min at room temperature, washed three times with PBS, and blocked in PBS containing 2.5% bovine serum albumin (BSA) for 1 h at room temperature. To determine the intracellular colocalization of the USP1 protein and Bcr-Abl oncoprotein cells were incubated with anti-USP1 antibody (1:200, PA5-55852, Thermo Fisher Scientific, USA) and anti-Bcr antibody (1:200, G2117, Santa Cruz Biotechnology, USA) and anti-Rabbit, DyLight550 (1:200, SB245608, Thermo Fisher Scientific, USA) and anti-mouse, DyLight488 (1:200, SA234709, Thermo Fisher Scientific, USA) for 1 h at room temperature. Cell nuclei were identified by staining with DAPI for 2 min. Then the samples were washed and embedded into non-hardening anti-fading/anti-bleaching mounting medium CitiFluor™ AF1. Microscopy studies were performed using Zeiss LSM 510 microscope (Germany). For visual analysis, the Zeiss LSM Image Browser software was used.

Visual and quantitative analyses. Analysis of the results was performed by Fiji ImageJ software using JACoP plugin. For quantitative assessment of colocalization, we used Pearson correlation coefficient (PPC) and Manders overlap coefficient (MOC) that represent the two major metrics of colocalization used in biomedical research [33–35].

Statistical analysis. The experimental results are presented as the mean ± standard deviation. The differences between groups were evaluated using Student's *t*-test. The p value < 0.05 was considered statistically significant.

RESULTS

Interaction of USP1 protein and Bcr-Abl oncoprotein in K562 cells. The interaction between USP1 and Bcr-Abl in K562 cells was studied using the coimmunoprecipitation method. The precipitation results were detected by Western blot analysis using anti-USP1 and anti-Bcr antibodies. Interestingly, only two of three isoforms of USP1 interact with Bcr-Abl (Fig. 1). One reason for this may be an alternative splicing that causes the formation of proteins with different structures and domain compositions, which affects specific molecular binding [36]. In addition, different isoforms of USP1 might be crucial players in various signaling pathways and perform diverse functions in the cell.

Colocalization of USP1 protein and Bcr-Abl oncoprotein in K562 cells. Next, we studied the colocalization of USP1 and Bcr-Abl proteins by immunofluorescence analysis and visualization using confocal microscopy. We detected overlapping of localization signals ("fusion point") of USP1 and Bcr-Abl oncoprotein by means of overlaying two images using the Zeiss LSM Image Browser software (Fig. 2). The quantitative indices of colocalization are presented in the Table.

It is clear that the localization of the USP1 protein almost completely overlaps with the localization of Bcr-Abl oncoprotein in K562 cell. At the same time, Bcr-Abl oncoprotein is only partially localized to USP1, this can be explained by the fact that the Bcr-Abl is localized not only in the nucleus of the cell, but also beyond its boundaries.

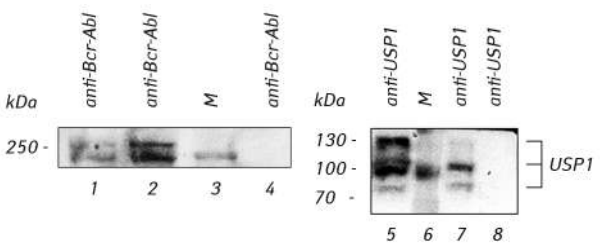

Fig. 1. Western blot analysis of co-immunoprecipitation in lysate of K562 cells: 1 — expression of Bcr-Abl (anti-Bcr-Abl antibody), 2 — co-immunoprecipitation results (anti-Bcr-Abl antibody), 3, 6 — prestained 10–250kDa protein ladder (Thermo Scientific), 4, 8 — negative control, incubation of sepharose G with lysate without antibodies, 5 — USP1 protein expression (anti-USP1 antibody), 7 — co-immunoprecipitation (anti-USP1 antibody)

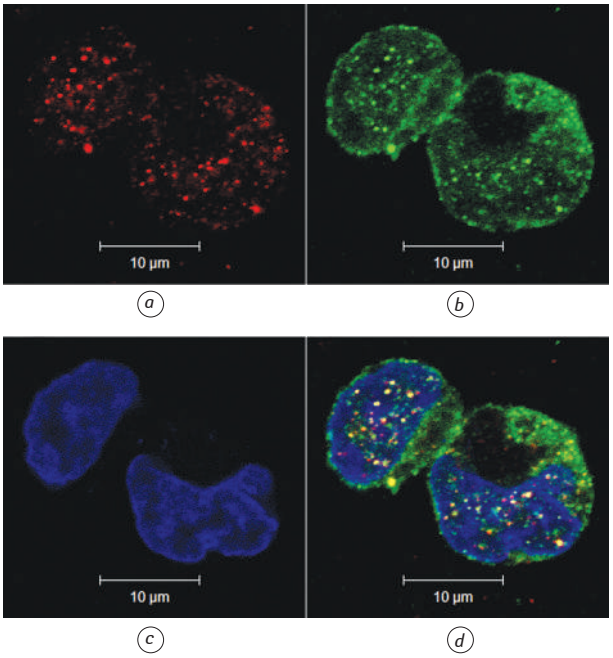

Fig. 2. Immunofluorescence analysis of colocalization of USP1 protein and Bcr-Abl oncoprotein in K562 cells: *a* — localization of USP1 protein, *b* — localization of Bcr-Abl proteins, *c* — DAPI fluorescence, *d* — fusion signals of localization of proteins USP1 and Bcr-Abl

Table. Quantitative indices of colocalization of USP1 protein and Bcr-Abl oncoprotein in K562 cells

Overlap coefficients	Without ML323	3 h incubation with ML323	24 h incubation with ML323
PCC	0.63	0.23	0.53
MOC			
M1	0.91	0.44	0.61
M2	0.58	0.05	0.35

Note: M1 – fraction of USP1 overlapping Bcr-Abl; M2 – fraction of Bcr-Abl overlapping USP1.

Effect of inhibition of USP1 protein on the localization and level of Bcr-Abl oncoprotein in K562 cells. To study the possible effect of the functional activity of USP1 on the expression of Bcr-Abl oncoprotein, K562 cells were incubated with 56 nM and 76 nM ML323 inhibitor for 24 h at 37 °C. ML323 is highly potent, reversible, nanomolar inhibitor of the USP1-UAF1 deubihivitanase complex, with high selectivity against human DUBs, deSUMOylase, deneddylase and unrelated proteases [37, 38]. Analysis of cells by immunofluorescence method and confocal microscopy showed that the ML323 inhibitor affects the localization of the USP1 protein (Fig. 3). Despite that USP1 protein is a nuclear protein, after 1.5 h incubation with ML323, we found not only nuclear but also cytoplasmic localization in some cells. By the third hour of incubation with ML323 protein USP1 was detected mainly in the cytoplasm, and in some cells, the protein was not detected at all. In this case, the PCC decreased from 0.63 to 0.23 as compared to the control. The MOC indices also decreased, both MOC M1 (fraction of USP1 overlapping Bcr-Abl) and MOC M2 indicating the almost complete absence of overlap of localization signals of Bcr-Abl oncoprotein with USP1 protein. After 24 h of incubation with ML323 only cytoplasmic localization of USP1 protein remains. It should be noted that at 24 h of incubation with the ML323 inhibitor, we did not detect Bcr-Abl oncoproteins in the cell nucleus. Thus, the action of the ML323 inhibitor alters the localization of USP1 protein and significantly reduces the level of colocalization with Bcr-Abl oncoprotein.

To observe the effect of USP1 protein on Bcr-Abl, we studied lysates of K562 cells after incubation with 52 nM or 76 nM ML323 inhibitor using Western blot (Fig. 4, 5).

Thus, we have observed that after 1.5 h incubation with ML323, the level of Bcr-Abl oncoprotein begins to decrease significantly. The lowest rates of Bcr-Abl oncoprotein were detected after 3 h incubation with the ML323. After 6 h, the level of the oncoprotein begins to increase and by 12 h reaches the level in the control variant.

DISCUSSION

Human DUB proteins are involved in important cellular processes, such as cell cycle control, apoptosis, or response to DNA damage, which are often deregulated in tumor cells [26, 28, 30]. Deubiquitinases prevent the degradation of substrates, leading to increased protein accumulation [29, 32]. A growing evidence of research indicates that USPs are crucial for the progression of cancer, and some are used as the targets for the development of inhibitors for cancer prevention [29, 31, 32, 37, 39]. USP1 is highly expressed in multiple myeloma cells compared to normal cells [23]. Increased expression of USP1 was observed in melanoma, sarcoma, cervical and gastric cancers [21, 23]. Hyperactivation of USP1 deubiquitinase promotes breast cancer metastasis [24]. USP1 expression correlates with poor prognosis in patients with multiple myeloma [23, 40]. Interestingly, mutations in USP1 also lead to certain types of human cancer, but the functional implications of these mutations for USP1 activity remain unclear [21]. Activity, localization and protein level of USP1 are modulated by several mechanisms, in particular interaction with UAF1 protein induces conformational changes of the active site of USP1, thus stabilizing the enzyme and increasing its catalytic activity [25, 37]. The USP1/UAF1 DUB complex is involved in various biological processes and cancer development [22]. Inhibition of the USP1/UAF1 complex sensitizes cancer cells to chemotherapy and is therefore a promising target for anticancer therapy [40]. USP1 protein is known to localize in the nucleus. The main localization site of Bcr-Abl oncoprotein in the cell is the cytoplasm [6, 7, 13, 17], but unlike the p190 isoform, which is uniformly distributed across the cytoplasm, the localization of the p210 isoform is related to the nucleus and Golgi complex [13].

In this work, we detected by immunofluorescence analysis and confocal microscopy that Bcr-Abl colocalizes with USP1 protein in the nucleus. ML323 is an allosteric inhibitor that selectively inhibits the USP1/UAF1 protein complex [37]. We have found

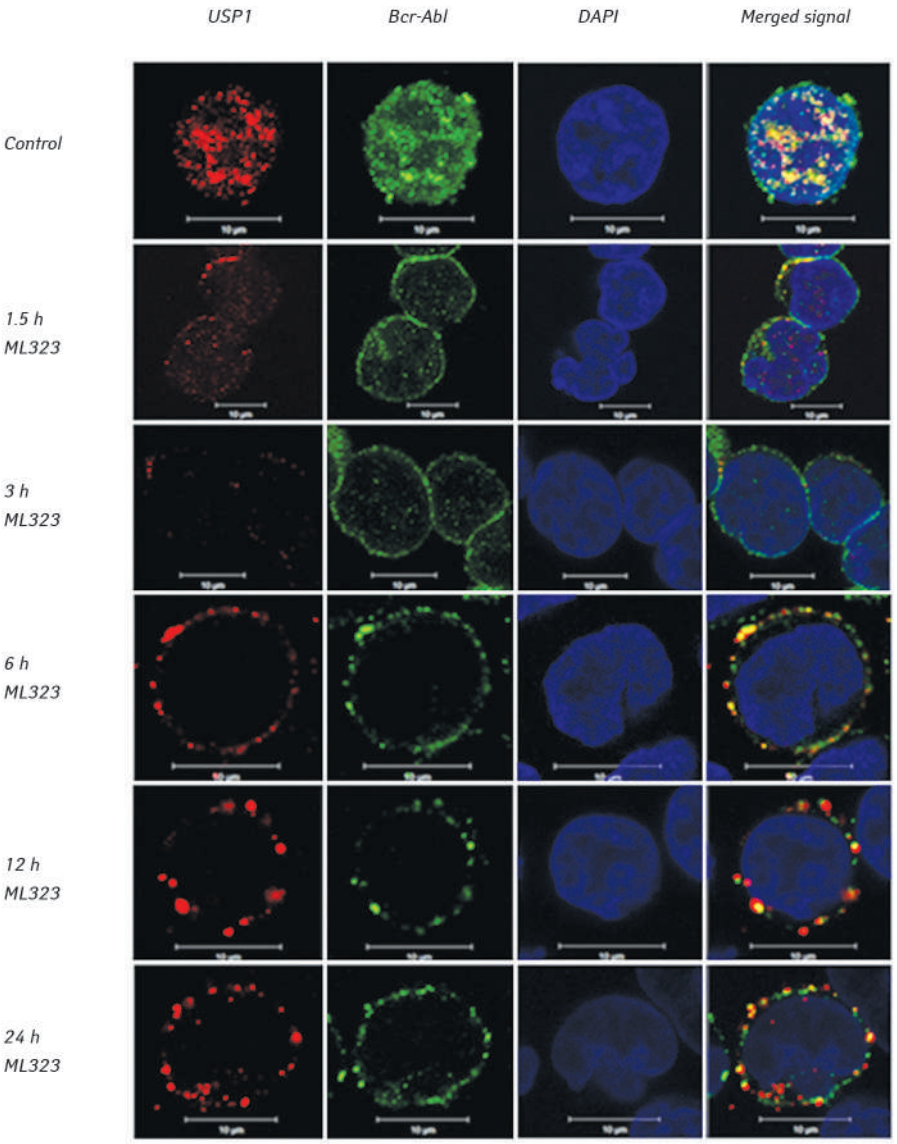

Fig. 3. Immunofluorescence analysis of colocalization of USP1 protein and Bcr-Abl oncoprotein in K562 cells after incubation with 76 nM ML323 inhibitor: localization of USP1 protein (red), localization of Bcr-Abl protein (green), DAPI fluorescence (blue), fusion signals of localization of proteins USP1 and Bcr-Abl (yellow)

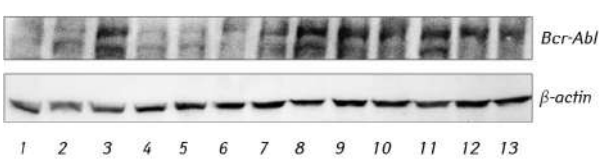

Fig. 4. Western blot of the lysates of K562 cells with anti-Bcr-Abl antibody. The cells were incubated with 52 nM ML323 (lane 1 — 1.5 h; lane 4 — 3 h; lane 6 — 6 h; lane 9 — 12 h; lane 12 — 24 h) or 76 nM ML323 (lane 2 — 1.5 h; lane 5 — 3 h; lane 7 — 6 h; lane 10 — 12 h; lane 13 — 24 h). Lanes 3, 8, 11 — control, lysate of cells without ML323 inhibitor

that after the action of ML323 inhibitor USP1 protein begins to change nuclear localization to cytoplasmic, which can be explained by the disruption of its interaction with the UAF1 cofactor that targets the anchoring of USP1 protein to its nuclear substrates. We found that ML323 inhibitor dramatically reduces the colocalization rate of USP1/Bcr-Abl proteins in K562 cells. Analysis of cell lysates by Western blot analysis showed that, together with a decrease in the colocalization rate of USP1/Bcr-Abl proteins, the overall level of the oncoprotein also decreases. It is known from literature that the p210 Bcr-Abl oncoprotein is characterized by a process of ubiquitination that occurs due to UBD within its NH2 end [14, 16] (residues 180–191). We assume that USP1 protein deubiquitates Bcr-Abl oncoproteins by removing ubiquitin chains, thus preventing its proteasomal degradation and promoting cellular accumulation. A similar mechanism of action is known for the protein kinase Akt (phosphoinositide 3-kinase), where the USP1 deubiquitinates Akt by removing polyubiquitin chains, thus affecting a number of signaling pathways [41].

It is known from literature that the interaction of USP1 protein with UAF1 cofactor is not the only mechanism for its activation. Large-scale phosphoproteomic studies of human deubiquitinases have found that most USPs are phosphorylated [42]. USP1 is no exception, as protein phosphorylation stimulates its activity [42, 43]. Bcr-Abl oncoprotein

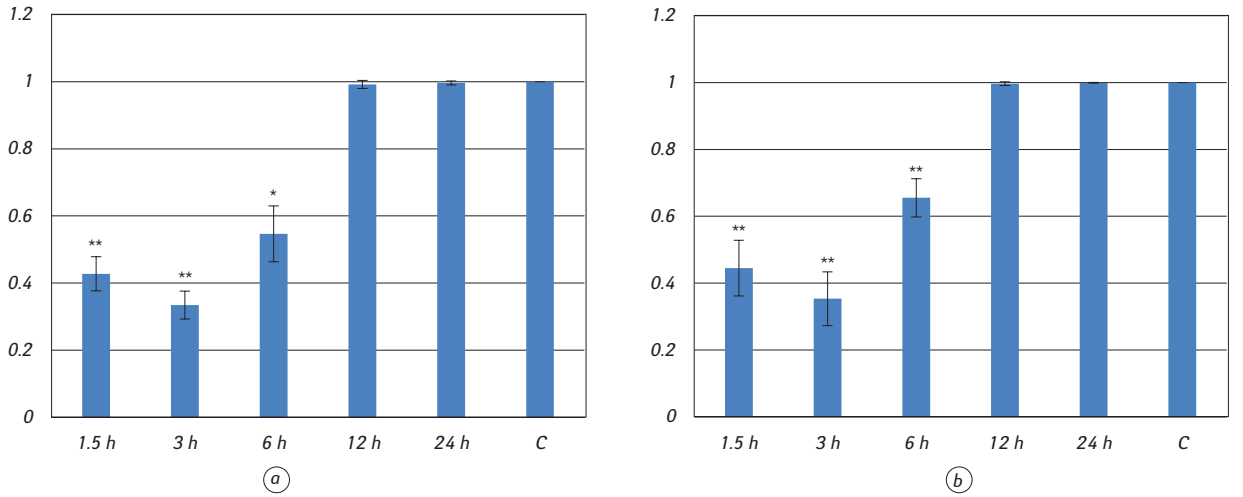

Fig. 5. Normalized values of band areas in Western blot of the lysates of K562 cells analyzed with anti-Bcr-Abl antibody: *a* — incubation with 52 nM ML323 inhibitor; *b* — incubation with 76 nM ML323 inhibitor . *$p < 0.01$, **$p < 0.001$ as compared to control

is a kinase that activates uncontrollably downstream signaling pathways. We assume that the oncoprotein can phosphorylate USP1 protein, thereby activating its DUB activity without cofactor involvement. This assertion may be the reason the inhibitor is reduced when USP1 protein accumulates in the cytoplasm and colocalization with Bcr-Abl oncoprotein is resumed. The restoration of the DAB activity of USP1 may result in an increase in Bcr-Abl level recorded after 6 h of incubation with ML323. Thus, USP1 is a deubiquitinating protein that forms a protein complex with an oncoprotein in the cell nucleus. Inhibition of the USP1 activity contributes to the decrease of Bcr-Abl in K562 cells. The involvement of USP1 protein in processes that are deregulated in cancer cells makes it a promising target for the development of novel anti-cancer inhibitors [23, 24, 40, 44]. Reducing Bcr-Abl levels due to cellular degradation systems is a new promising direction for CML therapy, as it is not only selectively reduces oncoprotein levels in cells but also allows overcoming drug resistance.

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