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Antitumor Effect of Non-steroid Glucocorticoid Receptor Ligand CpdA on Leukemia Cell Lines CEM and K562

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Abstract—Glucocorticoids (GCs) are widely used in chemotherapy of hematological malignancies, particularly leukemia. Their effect is mediated by glucocorticoid receptor (GR), a well-known transcription factor. Besides their therapeutic impact, GCs may cause a number of side effects leading to various metabolic complications. The goal of immediate interest is testing glucocorticoid analogs capable of induction/enhancement of GR transrepression, but preventing GR dimerization and transactivation leading to side effects. In this work we have investigated effects of a promising new selective GR agonist, 2-(4-acetoxyphenyl)-2-chloro-N-methylethylammonium chloride (CpdA), on CEM and K562 leukemia cells. Both cell lines express functional GR. CpdA compared with the glucocorticoid fluocinolone acetonide (FA) exerted more prominent cytostatic and apoptotic effects on the cells. Both cell lines exhibited sensitivity to CpdA, demonstrating a good correlation with the effects of FA on cell growth and viability. In contrast to FA, CpdA did not induce GR transactivation evaluated by no obvious increase in expression of GR target (and dependent) gene FKBP51. At the same time, luciferase assay showed that CpdA efficiently activated transrepression of NF-κB and AP-1 factors. We also evaluated the effect of combined action of CpdA and the proteasome inhibitor Bortezomib. The latter induced a caspase-dependent apoptosis in both T-cell leukemia cell lines. By treatment of CEM cells with different CpdA/GC and Bortezomib doses, we have designed a protocol where CpdA shows potentiating effect on Bortezomib cytotoxic activity. Generally, the present work characterizes a novel non-steroid GR ligand, CpdA, as a promising compound for possible application in leukemia chemotherapy.

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Key words: glucocorticoid receptor, leukemia, selective GR agonist

Glucocorticoids (GCs) are important physiological and pharmacological regulators of blood cell growth and survival. These steroid hormones are widely used in combined chemotherapy of leukemias. Their biological effect is due to activation of glucocorticoid receptor (GR), a transcription factor capable of regulating gene expression via two mechanisms: transactivation and transrepression (Fig. 1) [1]. Therapeutic effect of GCs is mainly realized via DNA-independent transrepression, namely, protein–protein interaction between GR and transcription factors (NF-κB, AP-1, etc.). These transcription factors serve as effectors of inflammatory mediators, antiapoptotic genes, and cell adhesion molecules. As a result, a decrease in activity of these factors and their efferent pathways is observed, leading to the tumor cell death. Development of many complications, such as steroid diabetes, osteoporosis, Itsenko–Cushing syndrome, and other side effects observed in therapy with GC, is associated with transactivation mediated by interaction of the GR homodimer with responsive elements in promoters and enhancers of genes implicated in metabolic control [2-5]. So, a promising way for therapy of hemoblastoses is the search for selective agents activating transrepression mechanisms, namely selective GR agonists (SEGRAs) instead of GCs.

Like GCs, SEGRAs bind GR, but so activated receptor realizes its effect via a transrepression mechanism [4]. One such substance is 2-(4-acetoxyphenyl)-2-

Abbreviations: CpdA, 2-(4-acetoxyphenyl)-2-chloro-N-methylethylammonium chloride; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FA, fluocinolone acetonide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, glucocorticoid; GR, glucocorticoid receptor; HDAC-1, histone deacetylase-1; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; SEGRA, selective GR agonist.

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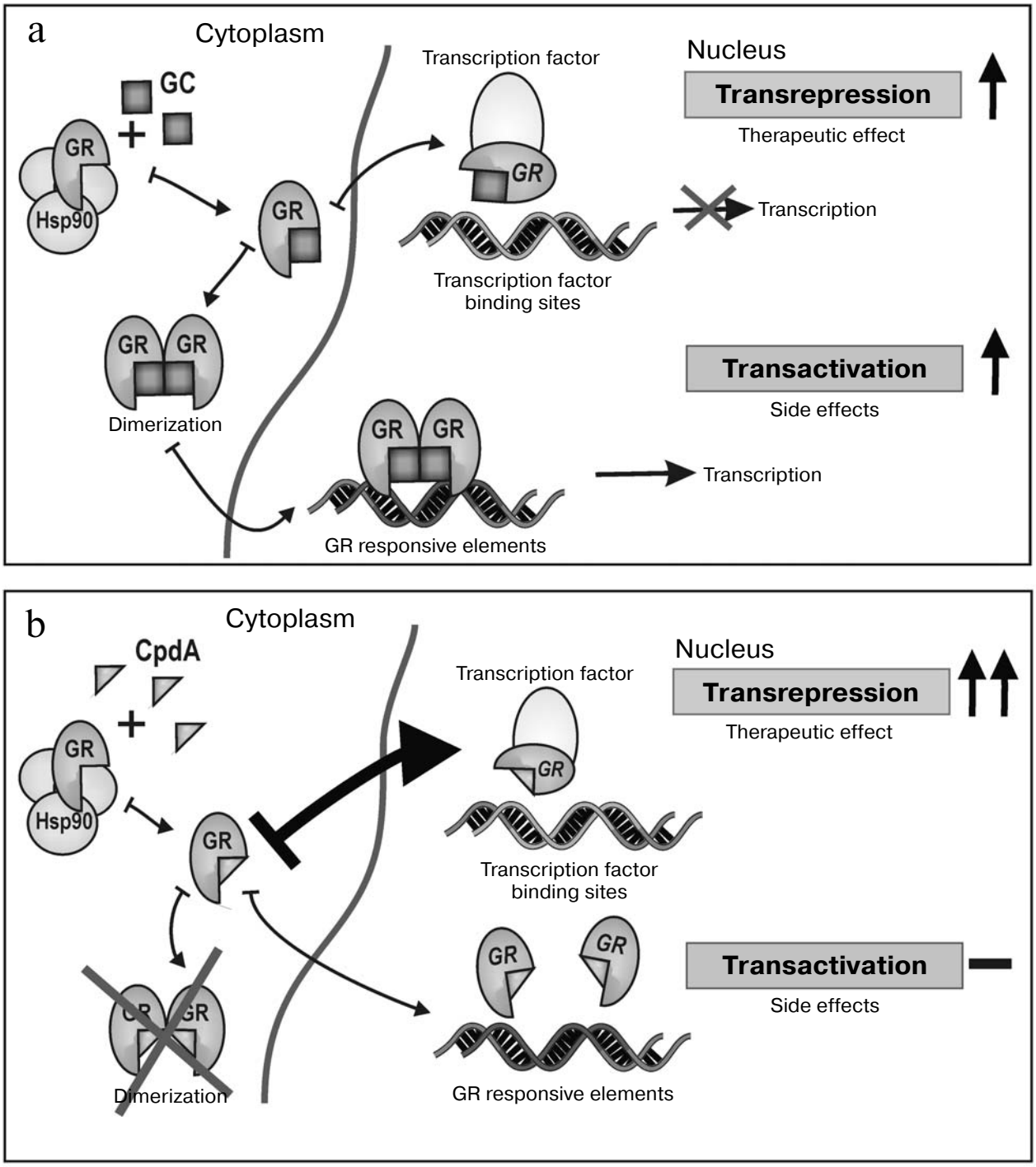

Fig. 1. Mechanism of GR activity when binding with GC (a) and CpdA (b).

chloro-N-methylethylammonium chloride (CpdA) (Fig. 2), a stable analog of aziridine precursor isolated from the African plant *Salsola tuberculatiformis* Botschantzev [6, 7]. In prostate cancer, CpdA activates transrepression, manifests antiinflammatory activity, inhibits growth, and decreases viability of the cells [7]. GR activated by CpdA does not form homodimers and, hence, cannot cause transactivation [8, 9]. Thus, the effect of this compound compared to standard GCs used in chemotherapy of leukemia seems very promising.

Contemporary therapy of cancer diseases commonly involves a combined chemotherapy in which a combination of two or more pharmaceuticals allows decreasing doses applied, and the use of certain administration

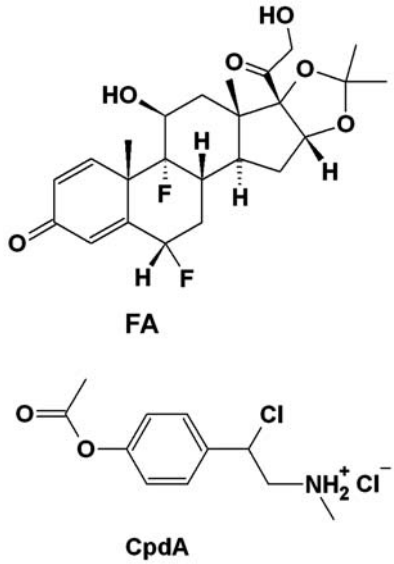

Fig. 2. Chemical structures of FA and CpdA.

regimes – achieving synergic or additive effects. In particular, potentiating effect of GCs was demonstrated in therapy of hemoblastoses with vincristine, adriamycin, morpholine, and other drugs. One of the most promising pharmaceuticals for chemotherapy of hemoblastoses is Bortezomib, which was approved for use in clinical practice in the USA and Europe in 2003-2004. This substance is actively used in therapy of multiple myelomas [10] and lymphomas [11, 12]. Also, it is effective in therapy of leukemias [13]. Bortezomib is a proteasome inhibitor. These inhibitors bind to the catalytic domain of the 26S proteasome to prevent cleavage of certain cell proteins, particularly of steroid hormone receptors [14]. It was found that proteasome inhibitors induce apoptosis in tumor cells without influencing normal tissue [15]. In this case, suppression of NF-κB activity is observed [16], as well as stress of endoplasmic reticulum and associated reactions [17]. Combined administration of GC and Bortezomib results in synergism of cytotoxic and proapoptotic effects observed in the cells of multiple myeloma, lymphomas, and leukemias [18]. Since we recognized CpdA as activating transrepression, particularly decreasing NF-κB activity, we anticipated that this selective GR agonist can potentiate chemotherapeutic effect of Bortezomib. This explains our interest in the combined effect of the GR modulator CpdA and Bortezomib.

In this work we aimed to study the cytostatic and proapoptotic effects of CpdA on leukemia cells expressing active GR and to determine the transrepression potential of this substance compared with the classic GR ligand fluocinolone acetonide (FA), as well as effect of combined administration with CpdA and Bortezomib on leukemia cells of the studied lines.

MATERIALS AND METHODS

Cell lines and cell treatment. The cells of acute lymphoblastic leukemia CEM and chronic myeloid leukosis K562 were used in the study. Cells were grown in a standard RPMI-1640 medium containing 5% fetal calf serum (PAA, Austria) and 50 U/ml of gentamicin (Paneco, Russia) at 37°C in an atmosphere of 5% CO_2. The cells were treated with FA (Sigma, USA), CpdA (synthesis was described in our previous work [7]), or Bortezomib (LC Chemicals, USA). Ethanol (0.1%) and DMSO were used as solvents.

Transient transfection and luciferase activity. To determine transcriptional activity of NF-κB, AP-1, Ets-1, Elk-1, SRF, CRE/ATF, and NFATc, we transfected K562 and CEM cells with plasmids containing a firefly luciferase reporter gene under a control of promoters containing regulatory elements of NF-κB, AP-1, Ets-1, Elk-1, SRF, CRE/ATF, and NFATc described in our previous works [5, 7]. Cells were transfected using Lipofectamine LTX (Promega, USA) according to the manufacturer's protocol. Efficiency and potential toxicity were controlled by cotransfection of the cells with a plasmid carrying a reporter Renilla luciferase gene under the control of minimal CMV promoter. Luciferase activity was measured according to the manufacturer's standard protocol (Promega) on a Turner Biosystems 20/20 luminometer (Turner Biosystems, USA).

Preparation of transgenic cell lines. Viral stocks of high titer expressing firefly luciferase under the control of promoters with binding sites of NF-κB and AP-1 were prepared by cotransfection of 293T cell line with vectors pGF1-AP1 or pGF1-NF-κB (System Biosciences, USA) together with pMD2.G and psPAX2 packing plasmids (Addgene, USA). Supernatant containing viral particles was collected 24-48 h after transfection and used for infecting K562 and CEM cells. The infection resulted in stable cell lines CEM-NF-κB-GFP-Luc, K562-NF-κB-GFP-Luc, CEM-AP1-GFP-Luc, and K562-AP1-GFP-Luc. Cells expressing GFP were selected using a flow cytofluorimeter (Beckman Coulter, USA). Efficacy and potential toxicity were controlled by preparation of CEM and K562 cells infected by lentivirus expressing the luciferase gene under control of CMV-promoter. Experiments with transgenic lines were performed 7-14 days after infection. Luciferase activity was determined as described above.

Sequencing. DNA fragments obtained from PCR of GR exons 2-9 of CEM and K562 cells were sequenced on an automated sequencer using Big Dye Sequencing Kit (Perkin-Elmer, USA) according to the manufacturer's protocol, and the samples were then analyzed on a ABI-377 DNA Sequencer.

Cytotoxic effect. Direct counting of cells using a Celigo Cytometer automated cell counter (Cyntellect, USA) was used to determine cytotoxic effect. Cells were

grown in a 24-well plate for 12 days in presence of FA, CpdA, Bortezomib, or solvent (0.1% ethanol or DMSO); cells were counted each 48 h. Each experimental or control group consisted of three wells.

Immunoblotting. Total cell lysates and cytoplasmic nuclear fractions were isolated as described in [19]. Protein was determined using Bio-Rad Protein Assay (BioRad, USA). Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (LI-COR, USA). Immunoblotting was carried out using antibodies against GR-α, p65, p50, c-Jun (Santa Cruz Biotechnology, USA), polymerase poly(ADP-ribose) (PARP) (Cell Signaling, USA), and FKBP51 (BD Pharmingen, USA). To prevent nonspecific sorption, membranes were incubated for 1 h in 5% Blotto blocking reagent (BioRad) in TBS-buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl) and then incubated for 6 h at room temperature. To control the load and transmission, the membranes were incubated with antibodies against actin and histone deacetylase-1 (HDAC-1) (Santa Cruz Biotechnology). Membranes were washed and incubated for 1 h with secondary antibodies IRDye 800CW (LI-COR); signals were visualized using an Odyssey infrared scanner (LI-COR).

PCR and reverse transcription (RT)-PCR. PCR was performed with a genomic DNA isolated using TRIzol Reagent (according to the manufacturer's protocol) and PCR mix (Invitrogen, USA). RT-PCR was performed using a MMLV Reverse Transcriptase kit, random primers, PCR mix (Invitrogen), and total RNA isolated using an RNeasy kit (Qiagen, USA). PCR primers for amplification of genomic DNA and cDNA were designed using the Primer-Bank database (http://pga.mgh.harvard.edu/primerbank/) and Oligo 6 program package. Primer sequences are given in the table. PCR products were separated by electrophoresis in 1.5% agarose gel, quantified using an Agilent 2001 Bioanalyzer, and normalized by the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR product.

The level of apoptosis was determined using immunoblotting by cleavage of PARP. We also used a flow cytofluorometry following staining with propidium iodide (PI) (Sigma); to do this, cells were centrifuged, the pellet was resuspended in 1 ml of solution containing 5 μg/ml of PI, 0.1% sodium citrate, and 0.3% surfactant NP-40. Thus prepared specimens were analyzed on a FACSCalibur flow cytofluorimeter (Becton Dickinson, USA). The portion of apoptotic cells (in percent) was determined as a pre-G1 peak on the DNA plot. The activation level of caspases was measured using an ApoAlert Caspase Assay kit (Clontech, USA) according to the manufacturer's protocol using total cell lysates, and activity of caspases was determined on a Victor Plate Reader (Perkin-Elmer).

Statistical data processing. All experiments were repeated three times. The means and standard deviations were calculated using Microsoft Excel software and compared with Student's paired *t*-test for the means. In all cases the data were regarded as significant at $P < 0.05$.

RESULTS

Characterization of CEM and K562 cell lines as models for human leukemia. The cell lines CEM and K562 are widely used in research into human leukemia and evaluation of mechanism and determination of therapeutic effectiveness of antitumor drugs in model systems *in vitro* [20, 21]. The cell line CEM (T-cell leukemia) was isolated from a woman with acute lymphoblastic leukemia [22, 23], and the line K562 (chronic myeloid leukemia, blast crisis) from a woman's polypotent neoplasia cells. A char-

Primer sequences

Primer	Sequence	
	forward	reverse
GREX2	5'-agctgcctcttactaatcgg-3'	5'-gctttaagtctgtttcccc-3'
GREX3	5'-agttcactgtgagcattctg-3'	5'-ggaaaataaactcttcaaaa-3'
GREX4	5'-ctgtgaaactttaatagtgcc-3'	5'-gaaacaattgctttcagata-3'
GREX5	5'-taaactgtgtagcgcagacctt-3'	5'-tgtattcacctgactctcccc-3'
GREX6	5'-ctgaagagtgttgcctcata-3'	5'-gggaaaatgacacacatacaa-3'
GREX7	5'-aaaatgtgctttttgggg-3'	5'-aggtgaaaaggtgttctacc-3'
GREX8	5'-cttttagttcctaaggacggtc-3'	5'-caagctatcaccaacatccaca-3'
GREX9	5'-actgaccaatttggaagcct-3'	5'-atttcaccatctactctccc-3'
FKBP51	5'-gaatggtgaggaaacgccgat-3'	5'-tgccaagactaaagacaaatggt-3'
IL1	5'-ctcgccagtgaaatgatggct-3'	5'-gtcggagattcgtagctggat-3'
IL6	5'-aacctgaaccttccaaagatgg-3'	5'-tctggcttgttcctcactact-3'

acteristic feature of the K562 cells is a translocation t(9;22), also known as Philadelphia chromosome [24]. This translocation leads to formation of a chimeric gene *BCR-ABL* activating a series of antiapoptotic genes (notably *NF-κB*) [25]. The CEM cells are polymorphous and can form clones with various chromosomal abnormalities leading to excessive activation of pro-proliferative and antiapoptotic signaling [26-28].

In studying effect of CpdA on leukemia cells, we have chosen these cell lines because of their GR-expressing capability [21, 29]. Therapeutic effectiveness of GCs depends on GR activity in tumor cells. Resistance to GCs is commonly associated with either the loss of GR expression or mutations affecting activity of GR as a transcription factor. In particular, ~40 mutations associated with resistance to GCs are known in GR exons 2-9 [30-34]. So, we have sequenced these exons in both cell lines and found no mutations leading to development of GC-resistance.

Immunoblotting of total cell lysates has shown that both cell lines express GR (Fig. 3). The level of GR was higher by 40.5 ± 9.3% in the CEM cells compared to the K562 cells, as is evident from the densitometry of blots. The treatment with FA for 24 h led to decrease of GR expression in the K562 cells compared to the control by 53.6 ± 6.8%. In contrast, GR expression elevated by 44.7 ± 8.8% in the CEM cells under the same conditions. When nuclear and plasmatic protein fractions were analyzed separately, it was found that FA caused considerable translocation of GR into the nucleus, thus suggesting high sensitivity of the receptor to activation. In CEM cells, GR level decreased by 73.7 ± 8.3% in plasmatic fraction and increased by 85.7 ± 5.7% in nuclear fraction. The same values for the K562 cells were 89.7 ± 6.9 and 74.8 ± 9.5%, respectively. Another index of GR functional activity is elevation of *FKBP51* gene expression in response to FA. The gene *FKBP51* is one of well-characterized GR targets, whose expression is directly controlled via GC responsive elements in the promoter [35]. Increase in expression of *FKBP51* in response to the introduction of FA was 1.9 ± 0.3 times for the CEM cells and 1.7 ± 0.2 times for the K562 cells. Note that effect of FA on expression of *FKBP51* gene correlates with GR expression level: it is more prominent in the CEM cells.

Thus, leukemia cells of the chosen lines exhibit stable expression of functionally active GR and so can serve as an adequate model for comparative analysis of CpdA and GC effects on leukemia cells.

Cytotoxic and proapoptotic effects of CpdA. Cytotoxic effect was determined for 12 days of incubation with FA or CpdA by direct cell count. The CEM cells compared to the K562 cells expressed more GR and were more sensitive to the cytotoxic effect of GC (Fig. 4a). While the total number of K562 cells at the 12th day was 5.8 ± 0.4 million in control, incubation with 10^{-6} M FA decreased this value to 3.0 ± 0.4 million (1.9-fold). The decrease was 2.6 times for the CEM cells treated under the same conditions.

Cytotoxic effect of CpdA on the CEM cells did not significantly differ from that of FA. The total number of CEM cells incubated with 10^{-6} M CpdA was 6.5 ± 0.8 million (2.8 times lower than in control). The cytotoxic effect of CpdA on the K562 cells was more prominent than that of FA (a 3.9-fold decrease was observed in comparison to control).

Using flow cytofluorometry, we showed that the cytotoxic effect of both CpdA and FA on the cells of both lines is associated with intensification of apoptosis (Fig. 4b). These data are in good agreement with observed increase in expression of proapoptotic proteins Bim and Bik and tumor suppressor p53 following the 24-h introduction of FA and CpdA (Fig. 4c), as well as with data on cleavage of PARP protein (Fig. 4d).

Effect of CpdA on expression and functional activity of GR. Analysis of molecular mechanisms underlying the effect of CpdA involved determining its effects compared to those of FA on GR expression, translocation into the nucleus, and transactivation and transrepression activities. Immunoblotting of total cell lysates showed that, unlike FA, CpdA does not influence the overall GR expression level (Fig. 3). As mentioned above, FA caused intensive nucleus-directed translocation of GR in both cell lines, whereas CpdA caused only insignificant translocation of GR into the nucleus. The index of GR transactivation activity is expression level of *FKBP51*. Using immunoblotting, we demonstrated that CpdA does not activate expression of *FKBP51* in cells of either line (Fig. 3). The absence of CpdA effect on expression of *FKBP51* was confirmed by RT-PCR (data not shown). Thus, CpdA significantly differs from FA in effect on expression of the GR-dependent gene *FKBP51*. This difference between CpdA and GC is most pronounced in the CEM cells.

DNA-independent transrepression triggered by GR is mainly determined by protein–protein interactions between GR and other transcription factors such as NF-κB, AP-1, Ets-1, Elk-1, SRF, CRE/ATF, and NFATc [5]. By means of transient transfection, reporter plasmids containing the luciferase gene under the control of promoters with binding sites of all above transcription factors were introduced into both cell lines. It is known that transfection of suspension cultures in not always effective. In our experiments this manner of transfection was absolutely ineffective for the CEM cells. The K562 cells were successfully transfected with the reporter plasmids carrying the luciferase gene under the control of promoters of NF-κB, AP-1, Ets-1, Elk-1, SRF, CRE/ATF, and NFATc. In the case of NF-κB, subsequent introduction of FA or CpdA resulted in a decrease of luciferase activity by 1.4 ± 0.2 and 1.7 ± 0.4 times, respectively. For AP-1 these values were 1.3 ± 0.3 times when the cells were treated with FA and 1.6 ± 0.2 times with CpdA. It is worth noting that the NF-κB major subunits, p65/RelA and p50/NF-κB1, are well expressed in both cell types. The factor NF-κB in these cells is a constitutively active protein, as is evident

from high level of p65 both in cell cytoplasm and nucleus (Fig. 5d). We also noted a relatively low expression level of AP-1 (c-Jun) in both cell lines (Fig. 5c).

To analyze effect of CpdA on the GR-mediated transrepression in the CEM and K562 cells, we constructed transgenic CEM and K562 cell lines carrying the luciferase gene under the control of promoters with binding sites for NF-κB and AP-1. It is worth noting that the effectiveness of infection of K562 cells with lentiviral reporter vectors appears to be considerably higher (70%) than that of CEM cells (30%). When studying effects of the tested GR ligands on activity of NF-κB, we showed that CpdA reproduces the inhibitory effect of glucocorticoids both in CEM and K562 cells with more prominent suppression of NF-κB activity with CpdA than with FA. In particular, NF-κB activity compared to the control decreased to 54.6 ± 2.7 and 61.8 ± 5.3% in CEM and K562 cells, respectively, treated with FA and to 28.3 ± 7.4 and 38.7 ± 3.6% when treated with CpdA (Fig. 5a). This is confirmed by results of RT-PCR of genes containing regulatory elements of NF-κB IL-1 and IL-6 in their promoters (Fig. 5e). CpdA also inhibited activity of AP-1 in CEM and K562 cells to 38.5 ± 2.5 and 41.4 ± 8.3%, respectively. This is in agreement with the data on GR-

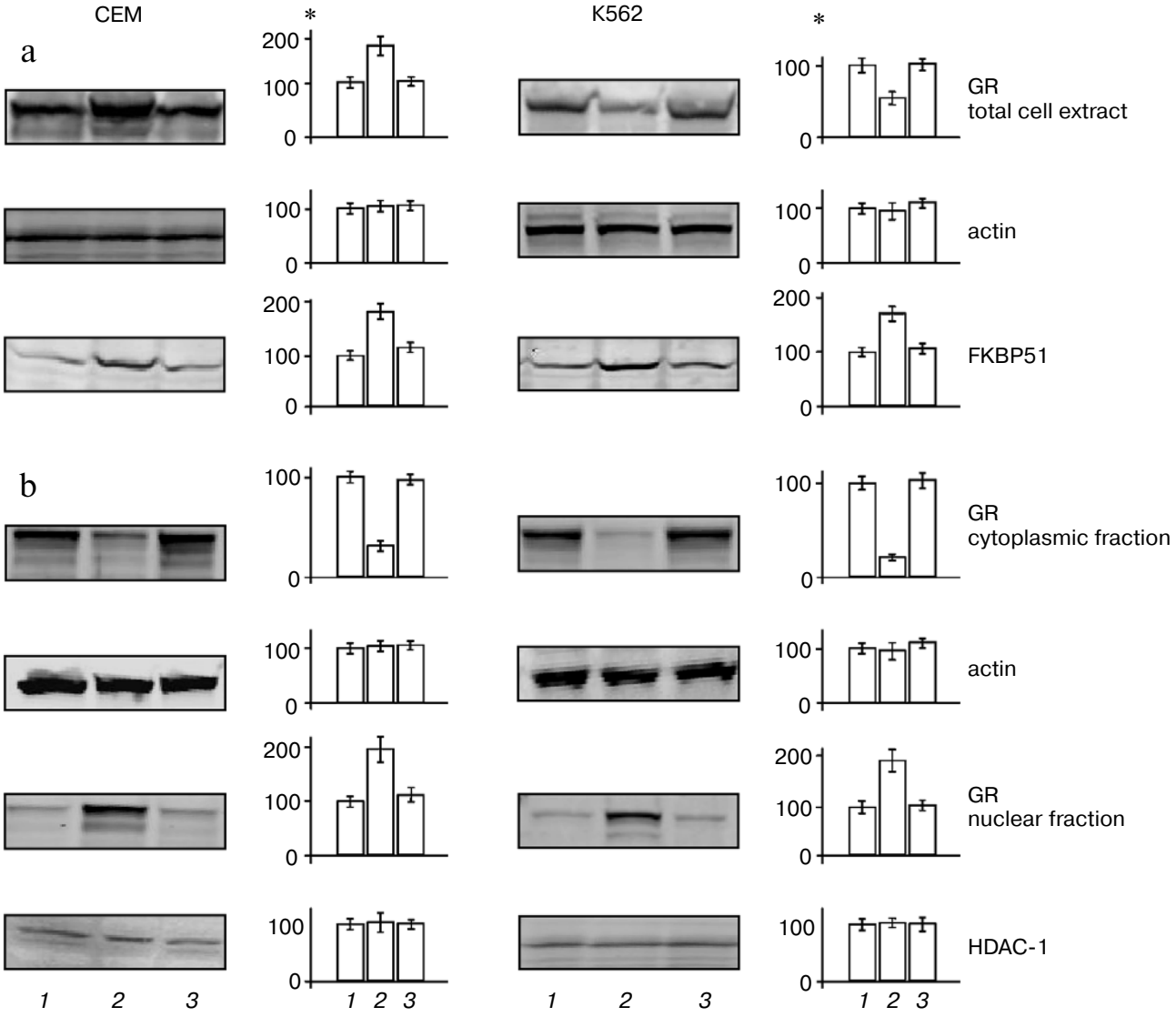

Fig. 3. Characterization of CEM and K562 cell lines by expression of GR and FKBP51 (a) and translocation of GR into the nucleus (b). * Densitometric analysis of blots was carried out using the Odyssey v.2.1 program package (LI-COR, USA). Plots represent band intensities (percent of control). a) Cells were grown for 24 h in the presence of solvent (*1*), 10^{-6} M FA (*2*), or 10^{-6} M CpdA (*3*). Expression levels of GR and FKBP51 in total extracts were determined by immunoblotting. Efficiency of protein binding with nitrocellulose membrane was estimated by immunoblotting with antibodies against actin. b) GR translocation into the nucleus determined by immunoblotting of nuclear and plasmatic fractions. Efficiency of protein binding with nitrocellulose membrane was estimated by immunoblotting with antibodies against actin and histone deacetylase-1 (HDAC-1).

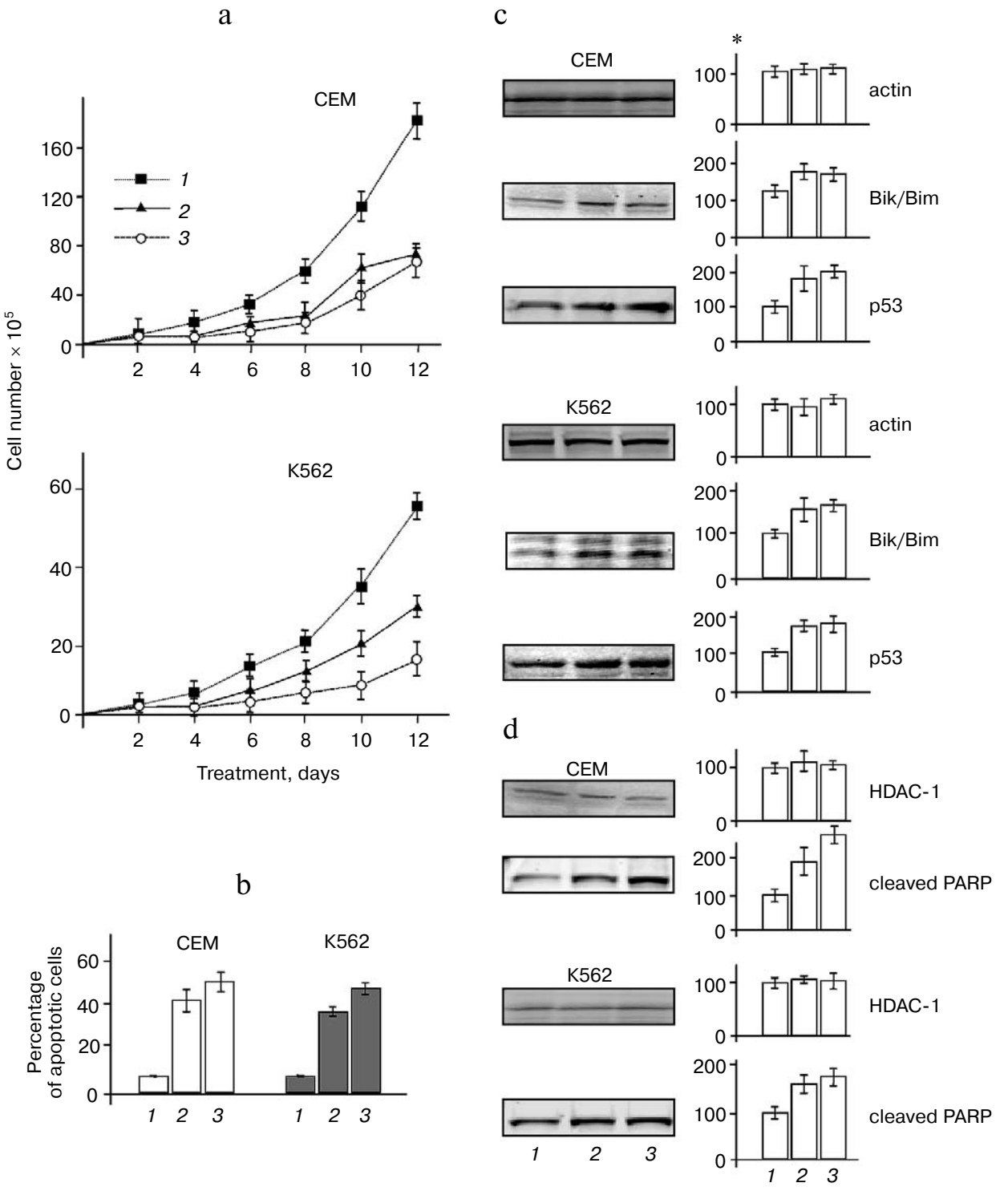

Fig. 4. Effects of FA and CpdA on the growth (a) and viability (b-d) of CEM and K562 cells. * Densitometric analysis of blots was carried out using the Odyssey v.2.1 program package (LI-COR, USA). Plots represent band intensities (percent of control). The cells were grown for 12 days (a), 48 h (b), or 24 h (c) in the presence of solvent (*1*), 10^{-6} M FA (*2*), or 10^{-6} M CpdA (*3*). a) Cells were counted each 48 h using an automated cell counter. b) The level of apoptosis was analyzed by flow cytofluorometry using staining with propidium iodide. c) Expression levels of Bim, Bik, and p53 were determined by immunoblotting. Efficiency of protein binding with nitrocellulose membrane was estimated by immunoblotting with antibodies against actin and HDAC-1. d) The level of cleaved PARP protein was determined by immunoblotting. Efficiency of protein binding with nitrocellulose membrane was estimated by immunoblotting with antibodies against HDAC-1.

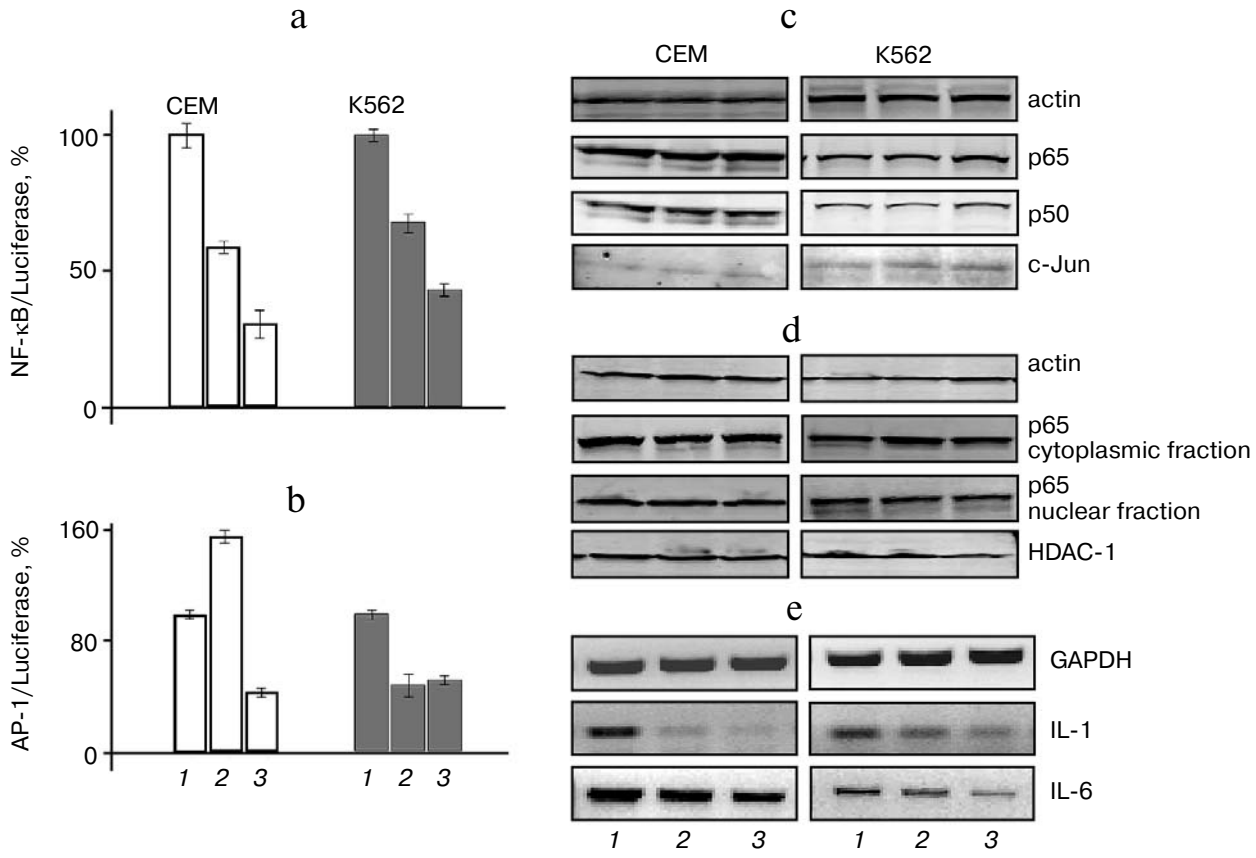

Fig. 5. Effects of FA and CpdA on expression levels of NF-κB and AP-1 and on regulation of the GR-dependent transcription activities of NF-κB and AP-1 in CEM and K562 cells. a, b) Effects of FA and CpdA on transcription activities of NF-κB and AP-1, respectively. The CEM and K562 cells were infected with lentiviral vectors pGF1-NF-κB and pGF1-AP-1 followed by growth for 8 h in presence of solvent (*1*), 10^{-6} M FA (*2*), or 10^{-6} M CpdA (*3*). Then luciferase activity was determined. c-e) Expression levels of NF-κB and AP-1. The cells were grown for 24 h in presence of solvent (*1*), 10^{-6} M FA (*2*), or 10^{-6} M CpdA (*3*). c) Expression levels of NF-κB and AP-1 were determined by immunoblotting. Efficiency of protein binding with nitrocellulose membrane was determined by immunoblotting with antibodies against actin. d) The level of p65 translocation into the nucleus determined by immunoblotting of nuclear and cytoplasmic fractions. Efficiency of protein binding with nitrocellulose membrane was quantified by immunoblotting with antibodies against actin and HDAC-1. e) Effects of FA and CpdA on expression of NF-κB-dependent genes. Expression levels of IL-1 and IL-6 were determined by RT-PCR. Amounts of PCR-products were estimated and normalized to the amount of GAPDH PCR-product.

mediated FA-dependent transrepression of AP-1 to 42.6 ± 1.9% in the K562 cells (Fig. 5b).

Combined effect of CpdA and Bortezomib on K562 and CEM cells. Bortezomib has demonstrated cytotoxic effect on both cell lines. The number of viable CEM cells decreased to 50.3 ± 5.2% after 48-h incubation in medium with 10^{-7} M Bortezomib. Rather less effect was observed in the case of K562 cells: the number of cells decreased to 66.4 ± 3.1% (Fig. 6a). Thus, more cytotoxicity was observed for the cells with higher GR level. The CEM cells demonstrated almost complete cleavage of PARP under the same conditions, thus suggesting intense induction of apoptosis by Bortezomib. In K562 cells, the PARP cleavage was not so expressed (Fig. 6d). This agrees with our data on activity of caspases 2, 3, 8, and 9. In CEM cells the level of caspase activity considerably increased following the introduction of Bortezomib, whereas in K562 cells caspase activity elevated only 1.5-2-fold in comparison with control.

Combined introduction of 10^{-6} M CpdA and 10^{-7} M Bortezomib did not result in synergic effect for 24- or 48-h incubation. We conducted a series of experiments with varied concentrations of these substances to achieve synergism. A potentiating effect of CpdA on cytotoxic and proapoptotic effects of Bortezomib was observed when the concentration of the former was increased to 10^{-5} M (Fig. 7).

DISCUSSION

A preliminary task of this work was to search for an adequate model for human leukemias to study effects of non-steroid GR ligand CpdA *in vitro*. Such a model

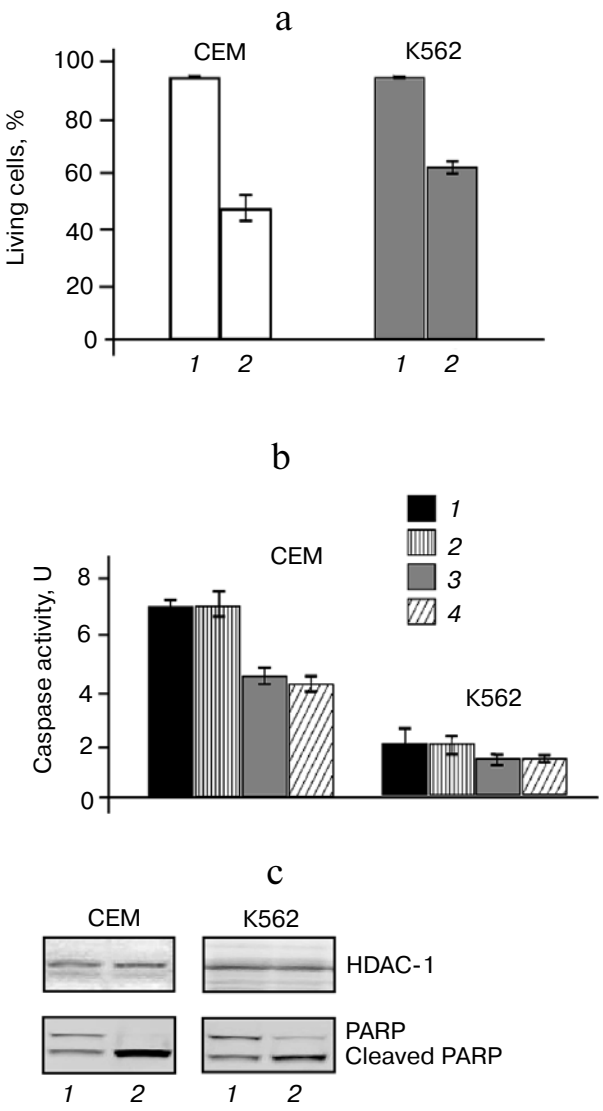

Fig. 6. Effect of Bortezomib alone and in combination with FA or CpdA on characteristics of CEM and K562 cells. a, b) Cytotoxic and caspase-activating effects of Bortezomib. Cells were grown for 48 h in presence of solvent (*1*) or 10^{-7} M Bortezomib (*2*) and then counted using an automated cell counter. b) Activation of caspases 2, 3, 8, and 9 (*1-4*, respectively) was analyzed using an ApoAlert Caspase Assay (Clontech) according to the manufacturer's protocol in total cell lysates. c) The level of PARP cleavage. Cells were grown for 24 h in presence of solvent (*1*) or 10^{-7} M Bortezomib (*2*). The level of cleaved PARP was determined by immunoblotting. Efficiency of protein binding with nitrocellulose membrane was estimated by immunoblotting with antibodies against HDAC-1.

might allow detailed investigation of CpdA effects and testing other selective GR activators.

We demonstrated that both studied cell lines steadily expressed the active non-mutated GR (Fig. 3). Thus, the chosen lines, CEM and K562, were appropriate for comparative estimating effects of CpdA and GC. Our data on GR degradation following the effect of GC on K562 cells agrees with the published data [36]. Elevation of GR expression in CEM cells following introduction of FA (Fig. 3) might be associated with stimulation of GR synthesis following degradation after a certain time after the beginning of incubation. This kind of effect of GC on GR in CEM cells is consistent with the published data [37]. The low capability of CpdA for initiating GR translocation into the nucleus corresponds to our published data of experiments on prostate cancer cells [5, 7].

An important parameter of GR functional activity is its pronounced nuclear translocation following the treatment with GC. CpdA did not cause GR degradation in cells. This fact is consistent with data that, unlike GC, CpdA does not cause desensitization of leukemia cells to its effect [38]. Nevertheless, although translocation of GR into the nucleus under the action of CpdA was insignificant, a considerable cytotoxic effect was observed under the same conditions. Experiments on prostate cancer cells suggested that some part of the cytotoxic effect of CpdA is associated with activated GR localized in the cytoplasm [7].

Cell treatment with GC and CpdA caused cytotoxic and proapoptotic effects correlating with GR level in each cell line and so, in our opinion, being GR-dependent

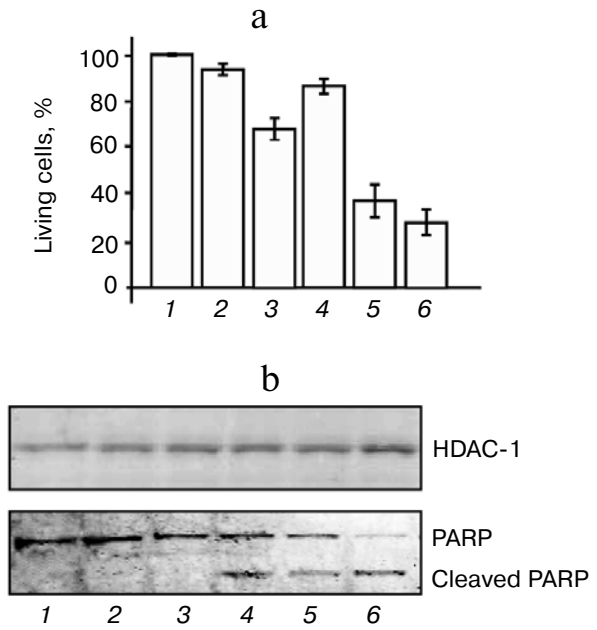

Fig. 7. Synergic effect of combination of Bortezomib and CpdA on the growth and viability of CEM cells. a) Cytotoxic effects of FA, CpdA, and Bortezomib. Cells were grown for 48 h in presence of solvent (*1*), 10^{-6} M FA (*2*), 10^{-5} M CpdA (*3*), 10^{-7} M Bortezomib (*4*), 10^{-6} M FA/10^{-7} M Bortezomib (*5*), or 10^{-5} M CpdA/10^{-7} M Bortezomib (*6*) and then counted using a Celigo Cytometer automated cell counter (Cyntellect, USA). b) The level of PARP cleavage. Cells were grown for 24 h in presence of solvent (*1*), 10^{-6} M FA (*2*), 10^{-5} M CpdA (*3*), 10^{-7} M Bortezomib (*4*), 10^{-6} M FA/10^{-7} M Bortezomib (*5*), or 10^{-5} M CpdA/10^{-7} M Bortezomib (*6*). Amount of cleaved PARP protein was determined by immunoblotting. Efficiency of protein binding with nitrocellulose membrane was estimated by immunoblotting with antibodies to HDAC-1.

(Fig. 4). This supposition was also confirmed by differential regulation of *FKBP51*, a known GR-dependent gene. Parameters characterizing regulation of *FKBP51* under the treatment of FA correlated well with those characterizing the cytotoxic effect. In our preliminary experiments FA and CpdA possessed cytotoxic activity at concentration of 10^{-6} M. This value corresponds to the previously determined effective concentrations of CpdA for PC-3 and DU145 prostate cancer cells [7].

An important part of our work was analysis of the mechanism of activation of transrepression, because it was demonstrated in a series of works that GR-dependent transrepression plays a key role in GC antitumor effect [2, 5, 7]. In particular, activity of transcription factors NF-κB and AP-1 is associated with tumorigenic and proliferative potentials of the cell. Antiapoptotic effect of NF-κB is also well known. Activity of these factors is suppressed under the action of GR by a transrepression mechanism. An aggregate effect of GC on cells or tissues is determined by actual degree of transrepression and transactivation mechanisms in given cell lines under given treatment conditions.

It was previously reported that CpdA suppresses activity of the above transcription factors [7] and mimics well the effect of GC. In particular, FA and CpdA near equally suppressed activity of the transcription factor AP-1 in K562 cells. In contrast, activity of AP-1 was elevated in response to FA in CEM cells (Fig. 5b). This effect can also result from a balance between AP-1 transactivation and transrepression under a given treatment protocol. AP-1 is a heterodimer composed of Fos- (c-Fos, FosB, Fra-1, and Fra-2) and Jun- (c-Jun, JunB, and JunD) subunits [39, 40]. Protein–protein interaction between GR and AP-1 subunits leads to inhibition of this heterodimer activity. Activated GR can also interact with MAP-kinases to inhibit their activity, which, in turn, leads to suppression of AP-1 activity [41, 42]. However, GR can interact with AP-1 via the transactivation mechanism – by the binding with GC regulatory elements in promoter regions of the *Jun* or *Fos* genes featuring a great amount. In this case, expression of these genes elevates, and the active AP-1 form predominates in the cell, as observed under the treatment of CEM cells with FA. In contrast, CpdA has virtually no effect on activity of MAP-kinases [42], and its complex with GR cannot bind with DNA. So, in this case the predominating mechanism is transrepression, which is consistent with our data.

CpdA compared to FA suppressed activity of NF-κB to a greater extent (Fig. 5a), which is in good agreement with results of our experiments with PC-3 prostate cancer cells and data from the literature [7, 8]. It is worth noting that NF-κB is constitutively active in CEM and K562 cells, and suppression of its translocation into the nucleus is only observed under brief (≤10 min) treatment, as described elsewhere [43]. In connection with this, NF-κB activity following the treatment with FA and CpdA was also tested using estimation of expression of well-known NF-κB-dependent genes *IL-1* and *IL-6* [44]. Expression of these genes decreased, thus indicating suppression of transcription capability of NF-κB.

It should be taken into account that suppression of NF-κB activity by glucocorticoids may be put together from transrepression itself and inhibition of MAP-kinase activity leading to inhibition of IKK-kinases, which play a key role in activation of NF-κB. Unlike CpdA, FA elevates expression of the main NF-κB inhibitor IκBα, which stabilizes NF-κB and prevents its translocation into the nucleus [45].

Thus, the integrated effect of GC on cells is an aggregate activation and/or inhibition of a great number of signaling pathways and depends on cell type and their treatment protocol. CpdA is a GR ligand and more selective activator of the transrepression mechanism. The cultures of CEM and K562 leukemia cells can be used as human leukemia models for studying combined effect of CpdA or other SEGRA and antitumor pharmaceuticals used in clinical practice. In our work, the proteasome inhibitor Bortezomib, which is already used in therapy of lymphomas and myelomas, was chosen for combining with CpdA or FA [10-13]. Moreover, a synergic effect of this pharmaceutical and GC was shown in therapy of hemoblastoses of various origins [18]. Such effect may be explained in several ways. Bortezomib suppresses a proteasomal cleavage of GR, resulting in its accumulation in the cell and enforcement of its function [14]. In their turn, GCs enhance expression of genes implicated in signaling pathways triggered at suppression of proteasomal activity, particularly genes implicated in development of ER-stress, such as ER-stress chaperones Grp78 (BiP) and Grp94 [17, 46], and so on. Moreover, negative effects of GCs and proteasomal inhibitors on NF-κB and antiapoptotic members of Bcl-2 family are described in literature [11, 16]. Since the effect of CpdA compared to GCs is more selective, it seemed important to us to test its combination with Bortezomib for the possibility of effectively combining selective GR agonists and proteasome inhibitors.

Our data evidences that Bortezomib possesses cytotoxic activity on CEM and K562 cells at concentration of 10^{-7} M and 5-8-fold elevates activity of effector and initiating caspases and induces apoptosis, which is in good agreement with published data [18].

We have demonstrated a potentiating effect of CpdA on activity of Bortezomib in CEM cells at some concentrations of both substances (Fig. 7). The treatment of cells with CpdA (or FA) and Bortezomib taken in combination considerably suppressed growth and viability of transformed cells. In connection with this, in further work we aim to study the combined effect of the given substances at concentrations providing synergic or additive cytotoxic effect.

Generally, our studies have shown that the use of CpdA is promising – both for development of new protocols for chemotherapy of leukemias and for detailed exploration of mechanisms underlying the cytotoxic

effect of GCs on tumor cells. The use of Bortezomib in combination with either GC or CpdA in therapy of leukemias also requires further investigation.

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