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Mechanisms of action of the anti-VEGF monoclonal antibody bevacizumab on chronic lymphocytic leukemia cells*

Mechanizmy działania bewacyzumabu, przeciwciała monoklonalnego anty-VEGF, na komórki przewlekłej białaczki limfocytowej

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Summary

Introduction: Chronic lymphocytic leukemia (CLL) remains incurable; therefore searching for new therapeutic strategies in this disease is necessary. An important mechanism of tumor development is neoangiogenesis. A potent antiangiogenic factor, bevacizumab (Avastin, AVA), has been poorly explored in CLL so far. In the current study we assessed cytotoxic activity of AVA alone or in combinations with drugs routinely used in this disease.

Materials and methods: Cells isolated from 60 CLL patients were treated with AVA alone or in combination with anti-CD20 monoclonal antibody (MoAb), rituximab (RIT), anti-CD52 MoAb, alemtuzumab (ALT), 2-CdA (2-chlorodeoxyadenosine), FA (fludarabine), MAF (mafosfamide) or RAPA (rapamycin). Cytotoxicity was assessed by propidium iodide staining. Apoptosis was evaluated using annexin-V and TUNEL assays. Additionally, a drop of mitochondrial potential (DYm) as well as expression of apoptosis-regulating proteins Bax, Bak, Bid, Bad, Bcl-2, Mcl-2, XIAP, FLIP, Akt and Bcl-2-A1 were determined by flow cytometry.

Results: At the dose of 40 μg/ml, after 48 hours of incubation, AVA induced significant cytotoxicity against CLL cells. The drug triggered apoptosis, with activation of caspase-3 and -9, but not caspase-8, along with a drop of DYm. Incubation with AVA induced significant overexpression of proapoptotic Bak and Bad as well as downregulation of antiapoptotic Mcl-2 and Akt proteins. Combination of AVA with RIT, ALT or RAPA significantly increased cytotoxicity when compared with the effects of single drugs.

Discussion: In conclusion, this is the first report showing proapoptotic activity of AVA against CLL cells. Combination of AVA with RIT, ALT or RAPA may be a promising therapeutic strategy, which requires confirmation in further studies.

Keywords: chronic lymphocytic leukemia • CLL • angiogenesis • bevacizumab • apoptosis

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Streszczenie

Wprowadzenie: Przewlekła białaczka limfocytowa (PBL) wciąż pozostaje chorobą nieuleczalną, dlatego wysiłki skierowane na opracowanie nowych strategii leczniczych są w pełni uzasadnione. Ważnym mechanizmem rozwoju nowotworów jest neoangiogeneza. Znany od kilku lat czynnik antyangiogenny, bewacyzumab (avastin - AVA), aktywny w niektórych nowotworach litych, jest wciąż stosunkowo słabo zbadany w PBL. Celem podjętych badań była ocena aktywności cytotoksycznej AVA w odniesieniu do komórek PBL, zarówno po zastosowaniu w monoterapii jak i w skojarzeniu z lekami rutynowo stosowanymi w tej chorobie.

Metody: Komórki izolowane od 60 chorych na PBL były inkubowane z AVA pojedynczo lub z przeciwciałami monoklonalnymi anty-CD20 (rytuksymab - RIT) i anty-CD52 (alemtuzumab - ALT), a także z kladrybiną (2-chlorodeoxyadenozyna - 2-CdA), fludarabiną (FA), mafosfamidem (MAF - aktywny biologicznie metabolit cyklofosfamidu) lub rapamycyną (RAPA). Cytotoksyczność badano za pomocą testu z jodkiem propydyny. Apoptozę oceniano za pomocą testów z Anneksyną V (Ann-V) oraz TUNEL. Ponadto badano spadek potencjału mitochondrialnego (DYm) oraz ekpresję białek regulujących apoptozę (Bax, Bak, Bid, Bad, Bcl-2, Mcl-2, XIAP, FLIP, Akt i Bcl-2-A1). Badania te wykonywano techniką cytometrii przepływowej.

Wyniki: W dawce 40 μg/ml, po 48 godzinach inkubacji, AVA indukował znaczącą cytotoksyczność w odniesieniu do komórek PBL. Lek ten indukował apoptozę poprzez aktywację kaspaz 3 i 9, ale nie kaspazy 8; zanotowano przy tym wyraźny spadek DYm. Inkubacja z AVA powodowała znamienny wzrost ekspresji Bak i Bad oraz obniżenie ekspresji białek Mcl-2 i Akt. Skojarzenie AVA z RIT, ALT lub RAPA znamiennie zwiększało cytotoksyczność w porównaniu do efektu poszczególnych leków stosowanych pojedynczo.

Dyskusja: Podsumowując, jest to pierwsze doniesienie wykazujące proapoptotyczną aktywność AVA w odniesieniu do komórek PBL. Skojarzenie AVA z RIT, ALT czy RAPA może być obiecującą strategią terapeutyczną, co wymaga jednak potwierdzenia w dalszych badaniach, w tym prowadzonych *in vivo*.

Słowa kluczowe: **przewlekła białaczka limfocytowa • PBL • angiogeneza • bewacyzumab • apoptoza**

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Introduction

Chronic lymphocytic leukemia (CLL) is one of the most frequent hematologic malignancies. Despite the progress in CLL treatment, the disease still remains incurable. Therefore, it is necessary to search for new therapeutic strategies.

Introduction of monoclonal antibodies (MoAbs) anti-CD20 (rituximab, RIT) and anti-CD52 (alemtuzumab, ALT) [5, 18] to CLL therapy gave some promise for improvement of outcome in this disease. The main mechanisms of antineoplastic action are CDC (complement-dependent cytotoxicity) and ADCC (antibody-dependent cellular cytotoxicity). Still the main drugs active in CLL are purine nucleoside analogues (PNA), including fludarabine (FA) and cladribine (2-chlorodeoxyadenosine; 2-CdA). Their cytotoxicity, similarly to many other routinely used cytostatics, is based mostly on triggering tumor cell apoptosis [19].

Neoangiogenesis is one of the most important mechanisms leading to development of neoplastic diseases, including hematologic malignancies. Therefore, inhibition of this process may have therapeutic potential in these disorders. Among clinically potent antiangiogenic factors, the best known is humanized MoAb against VEGF (vascular endothelial growth factor), bevacizumab (Avastin, AVA) [27]. AVA binds VEGF, leading to its inhibition. In turn it blocks vessel proliferation and inhibits tumor growth [14]. The drug was approved for treatment of metastatic colon cancer and then for therapy of disseminated breast cancer and non-small cell lung cancer.

The potential mechanism of antitumor action of AVA is ADCC or CDC immunological cytotoxicity, with the suggestion of eventual proapoptotic action. This latter mechanism would be very important, because of the possibility of antitumor strategy intensification by combination of AVA with other drugs, either cytostatics or other MoAbs.

Antitumor AVA activity in CLL has not been comprehensively explored so far, although the problem may be of clinical value. Namely, neoangiogenesis is probably important in pathogenesis of CLL [16]. Expression of VEGF receptors (VEGFRs) on leukemic cells and the presence of circulating serum VEGFRs have already been reported in this disease. In this study, we performed the first reported complex analysis of cytotoxic potential of AVA on CLL cells in *ex vivo* conditions. Cytotoxicity and its mechanisms were measured either for AVA alone or for its combinations with routinely used cytostatics or biological drugs active in CLL or other malignant lymphoproliferations.

Material and methods

The *ex vivo* studies were performed on peripheral blood mononuclear cells (PBMCs) obtained from 55 previously untreated CLL patients. In this group were 24 women and 31 men, aged 43-85 years (median 69 years). The control group consisted of 18 healthy volunteers.

PBMC isolation

PBMCs were isolated from heparinized blood samples by centrifugation in Histopaque-1077 (Sigma Diagnostic, St Louis MO, USA) density gradients. A 1:1 (v/v) mixture of blood and Hanks' Balanced Salt Solution (HBSS; Biomed, Poland) was layered on top of the Histopaque media in centrifuge tubes and centrifuged for 30 minutes at 200xg. The interphase region containing PBMC was collected and then washed twice, in HBSS and RPMI 1640 medium. Next, the cells were divided; a portion of them was resuspended in phosphate buffered saline (PBS; Sigma Aldrich Chemie GmbH, Germany) for the immunophenotypic study. The remaining cells were resuspended in RPMI 1640 at a cell density of about 0.5x106 cells/ml, and 1000 ml aliquots of cell suspensions were placed into 24 culture well dishes (Nunc, Denmark).

Cell immunophenotyping

Immunophenotyping of leukemic cells was performed routinely in the whole peripheral blood (the "lysed – not washed" method). A routine panel of MoAbs (CD3, CD5, CD10, CD11c, CD19, CD23, FMC7, IgK and Igλ), fluorescein isothiocyanate (FITC), R--phycoerythrin (R-PE) or cyanine-5 (Cy5) conjugated (all BD Pharmingen, San Diego, CA, USA) was evaluated using triple-color flow cytometry. The diagnosis was confirmed based on detecting the leukemic CD5+/CD19+/CD23+ clone. The rate of B-CLL cell population ranged from 59% to 98% of peripheral blood white cells (mean 84.1%). Based on this staining CD5+/CD19+ B-CLL cells could be distinguished from normal PBMCs for further multi-color analyses by flow cytometry.

PBMC culture design

Drugs

The following sets of cultures were prepared: controls containing PBMCs collected before treatment and cells treated with the study drugs. There were: AVA (Avastin, ROCHE, Switzerland; the drug was kindly provided by ROCHE Poland, Sp. z o.o.), RIT (Mabthera, ROCHE, Switzerland), ALT (CAMPATH-1H, ILEX Oncology, Inc., USA), 2-CdA (Biodrybin, Bioton, Poland), FA (Fludarabine, Bayer-Schering, Germany), MAF (kindly provided by the Department of Biochemistry, University of Lodz, Poland) and RAPA (Sigma, Aldrich, Denmark) alone. Moreover, combinations of AVA with RIT, ALT, 2-CdA, FA, MAF or RAPA were tested.

After a series of preliminary experiments (AVA in concentrations from 1 to 100 μg/ml) the dose of AVA 40 μg/ml was chosen for further studies. That was the minimal drug concentration inducing significant cytotoxicity compared with the untreated samples after 48 h of incubation. This action was independent of additional usage of complement, healthy control PBMCs containing cytotoxic cells, as well as of the presence of cross-linking with IgG antibodies.

Doses of other drugs were chosen based on previous experience and set at 10 μg/ml for RIT, and 20 μg/ml for ALT. 2-CdA was used in a final concentration of 20 μg/ml, as 10 times lower than in serum *in vivo*. Moreover, the dose of FA was 20 μg/ml, whereas that of MAF and RAPA was 8 μg/ml and 10 μg/ml, respectively.

Culture settings

All cultures were maintained for up to 24 h in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS) and antibiotics (streptomycin 50 mg/ml, penicillin 50 IU/ml; Life Technologies, Sco-

tland), at 37°C, 5% CO2, fully humidified. At the 0 h time point, after 24 h and 48 h PBMCs were subjected to a simultaneous assessment of viability, apoptosis or protein expression analysis by flow cytometry, using standard emission filters: green (FL1) for FITC, orange FL2 for R-PE and red (FL3) for Cy5.

Moreover, incubation in the presence of secondary IgG cross-linking antibody, complement or healthy PBMCs was used to potentiate cytotoxicity of AVA. Namely, for cross-linking studies the goat anti-human IgG, Fcg fragment specific (Jackson Immuno Research, West Grove, USA) secondary antibody was used at a concentration of 20 μg/ml. The secondary antibody was added 30 minutes after application of RIT or CAM. To mimic the CDC effect *in vitro*, additional incubation with complement was performed in two separate settings. Thus, for complement-mediated lysis pooled human serum from healthy donors or serum obtained from the examined patient was used. Human serum was added to a 5% final concentration together with the MoAb. Finally, to mimic the in vivo ADCC effect, PBMCs isolated from healthy volunteers were added to the examined sample. Patients and control cells were mixed in the proportion 1:14.

In all experiments with, cytotoxicity of RIT and ALT was tested with addition of secondary IgG cross-linking antibody. Calculation of exact cell number in the sample before and after incubation was performed using the Z1 Coulter Particle Counter/Beckman Coulter, when necessary.

Evaluation of VEGF level and VEGFR expression

In the series of experiments, the VEGF Human ELISA Kit (Lyfe Technologies, Grand Island, NY, USA) was used to quantify VEGF in cell culture medium before and after incubation with AVA. Similarly, the presence and expression of VEGFR on CLL cells was assessed, using anti-VEGFR-1 and anti-VEGFR-2 anti-human antibodies (VEGFR2/KRD-APC and VEGFR1/Flt1-PE; both R&D systems, UK).

Assessment of drug cytotoxicity

Cytotoxicity (cell viability) was assessed based on propidium iodide (PI) staining. After incubation with drugs, cells were washed twice in PBS and then re-suspended in 0.5 ml of 10 mg/ml PBS/PI solution. After 10 minutes of staining (room temperature, in the dark) cell fluorescence was measured by flow cytometry, using the FL3 (red) standard fluorescent filter. The viable cells were defined as PI-negative.

Assessment of apoptosis and its mechanisms

Apoptosis was evaluated based on annexin V (Ann-V) and TUNEL assays. Additionally, activation of caspase-3, -9 and -8, drop of mitochondrial transmembrane potential (DYm) as well as expression of apoptotic/proliferation regulatory proteins such as Bax, Bak, Bid, bad, Bcl-2, Mcl-2, XIAP, FLIP, Akt and A1 were determined.

Assessment of apoptosis by Ann-V assay

The rate of apoptosis was determined by Ann-V assay after 24 h of incubation with the drugs. In brief, after incubation cells were washed twice with cold PBS and then resuspended in 100 ml of binding buffer, containing 2 ml of FITC conjugated Ann-V and 10 mg/ml of PI (Becton-Dickinson, San Jose, CA, USA). Next, the samples were incubated for 15 minutes, at room temperature, in the dark. The fluorescence was measured immediately after staining by flow cytometer using FL1 (green, Ann-V) and FL3 (red, PI) standard fluorescent filters.

Assessment of apoptosis by TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids.

The APO-DIRECT™ assay (BD Pharmingen, San Jose, CA, USA), used in this study, is a single-step method for labeling DNA breaks with FITC-dUTP, followed by flow cytometric analysis. Before fixation in ethanol, the cells are fixed in 1% methanol-free paraformaldehyde, in order to crosslink the DNA fragments into the cell and to maximize the number of broken DNA ends. In the APO-DIRECT™ assay, TdT catalyzes addition of bromolated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl (OH) termini of double- and single-stranded DNA. After incorporation, these sites are identified by staining the cells with a FITC-labeled anti-BrdU MoAb. The fluorescence was measured by flow cytometry using an FL1 filter for detecting the green fluorescence.

Detection of active caspase-3

Active caspase-3 was detected using FITC-conjugated monoclonal rabbit anti-active caspase-3 antibody (BD Pharmingen, San Diego, CA, USA). After incubation cells were fixed and permeabilized using Cytofix/CytopermTM (BD Pharmingen, San Diego, CA, USA) solution (20 minutes, on ice), then washed twice and resuspended in Perm/WashTM buffer (BD Pharmingen, San Diego, CA, USA). The antibody was added in the amount of 60 ml per 300 ml of cell suspension (30 minutes incubation, at room temperature). The fluorescence was measured directly after staining and washing in Perm/WashTM buffer by flow cytometry using an FL1 filter for detecting the green fluorescence of anti-active caspase-3 antibody.

Assessment of caspase-8 and caspase-9 activation

Synthetic fluorochrome labeled fluoromethyl ketone peptides that are cell permeant and bind to the active catalytic site of caspase proteases were developed by Immunochemistry Technologies LLC (Bloomington, MN, USA). Commercially available FAM-LETD--FMK FLICA™ Caspase 8 Assay Kit and FAM--LEHD-FMK Reagent-9 FLICA™ Caspase 9 Assay Kit were used for assessment of caspase-8 and caspase-9 activation. According to the insert protocol it was initially prepared as a 150x concentrated solution in dimethylsulfoxide (DMSO; SIGMA Aldrich, USA). Aliquots were stored at -20°C, protected from light. Directly before use, they were diluted in PBS (1:5), and then added to 300 ml of culture to obtain a 10 mM concentration of FAM-LETD-FMK (caspase-8 detection) or FAM-LEDH-FMK (caspase-9 detection), respectively, for the final hour of incubation. Cultures were terminated by washing the cells twice (5 minutes, 140xg) with the "wash buffer" (component of both kits). After centrifugation, the pellets were resuspended in 1 ml of wash buffer and the samples were placed on ice. Cell green fluorescence derived from FAM--LETD-FMK or FAM-LEDH-FMK was measured during the next 15 minutes by flow cytometry.

Collapse of ΔΨm assessment

Dissipation of ΔΨm occurs early during apoptosis and is often considered as a marker of apoptosis activated by the mitochondrial pathway. As a probe of ΔΨm we used MitoTracker Red 580 dye (Molecular Probes, Eugene, Oregon, USA), which accumulates in active mitochondria of living cells. The stock solution of MitoTracker Red (1 mM) was diluted to a working concentration of 50 nM by adding to the growth culture medium (20 minutes incubation, at room temperature). The drop of ΔΨm was visualized by the decrease in red fluorescence of the dye, and detected by flow cytometry using an FL3 fluorescence filter.

Expression of apoptosis-regulating proteins

For assessment of the cellular expression of proteins involved in mechanisms of apoptosis regulation, cells were fixed with 1% methanol-free paraformaldehyde and permeabilized with 0.1% polysorbate 20 (Tween-20) in PBS (Amersham Biosciences, Freiburg, Germany). These were proteins acting on different levels of apoptotic pathways: promoters of apoptosis from the Bcl-2 protein family (Bax, Bak, Bid, Bad), and apoptosis inhibitors (Bcl-2, Mcl-1, XIAP and FLIP). Anti-human Bax primary rabbit Ab (DAKO, Glostrup, Denmark) was used in the dilution 1:100 (60 minutes). Anti-Bak, anti-Bid and anti-Bad (all Abcam, Cambridge, UK) primary rabbit anti-human MoAbs were used in dilutions of 1:10 (60 minutes). Anti--Bcl-2 MoAb (DAKO, Glostrup, Denmark) was used at a concentration of 1:15; the time of incubation was 30 minutes. Mouse anti-Mcl-1 MoAb (Abcam, Cambridge, UK) was used at a concentration of 1:30 (30 minutes). Rabbit anti-human FLIP antibody (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) was used at a concentration of 1:30 (60 minutes). Mouse anti-human XIAP MoAb (Oncogene Res. Products, San Diego, CA,) was used in 1:100 dilution (incubation 60 minutes). MoAbs anti-Akt (Becton Dickinson, USA) and anti-Bcl-A1 (Abcam, Cambridge, UK) were used in concentrations of 1:15 and 1:30, respectively, for 30 minutes of incubation. All antibody dilutions were made in 1% PBS-BSA. All stainings were performed at room temperature, in the dark. Increase or decrease in protein expression compared to the parallel control samples was defined as upregulation or downregulation, respectively.

Fluorescence measurements

All fluorescence measurements were performed by flow cytometry (FACScan; Becton-Dickinson, San Jose, CA, USA), using standard emission filters: green (l=530±20 nm; FL1), orange (l=560-600 nm; FL2) and red (l>600 nm; FL3), where necessary. Ten thousand cells per sample were calculated routinely in all experiments.

Statistics

For calculation of the apoptotic index (AI) the percentage of Ann-V-positive cells was estimated. For the statistical analysis of data obtained, the range of the measured variable, mean, median and standard deviation (SD) were calculated, using statistical software (STATISTICA v.7.0, Tulsa, OK, USA). The data are presented as mean ± SD values. The differences between values were evaluated with the non-parametric Mann-Whitney test, where the distribution of data was normal. The correlation between features was evaluated using the Spearman rank coefficient r. P values less than 0.05 were considered statistically significant.

Results

Cytotoxic effect of AVA on CLL

In the preliminary experiments AVA was tested in concentrations of 1-50 μg/ml, in 24 and 48 hour cultures. For further studies 40 μg/ml of AVA and 48-hour incubation were chosen as a minimal dose inducing significant increase in cytotoxicity compared to the parallel, untreated controls (Figure 1).

Addition of cross-linking Ig antibodies did not further increase AVA cytotoxicity in comparison with cells cultured in standard conditions. Also simulation of CDC (addition of complement from either pooled controls or from examined patients) and ADCC (sample in-

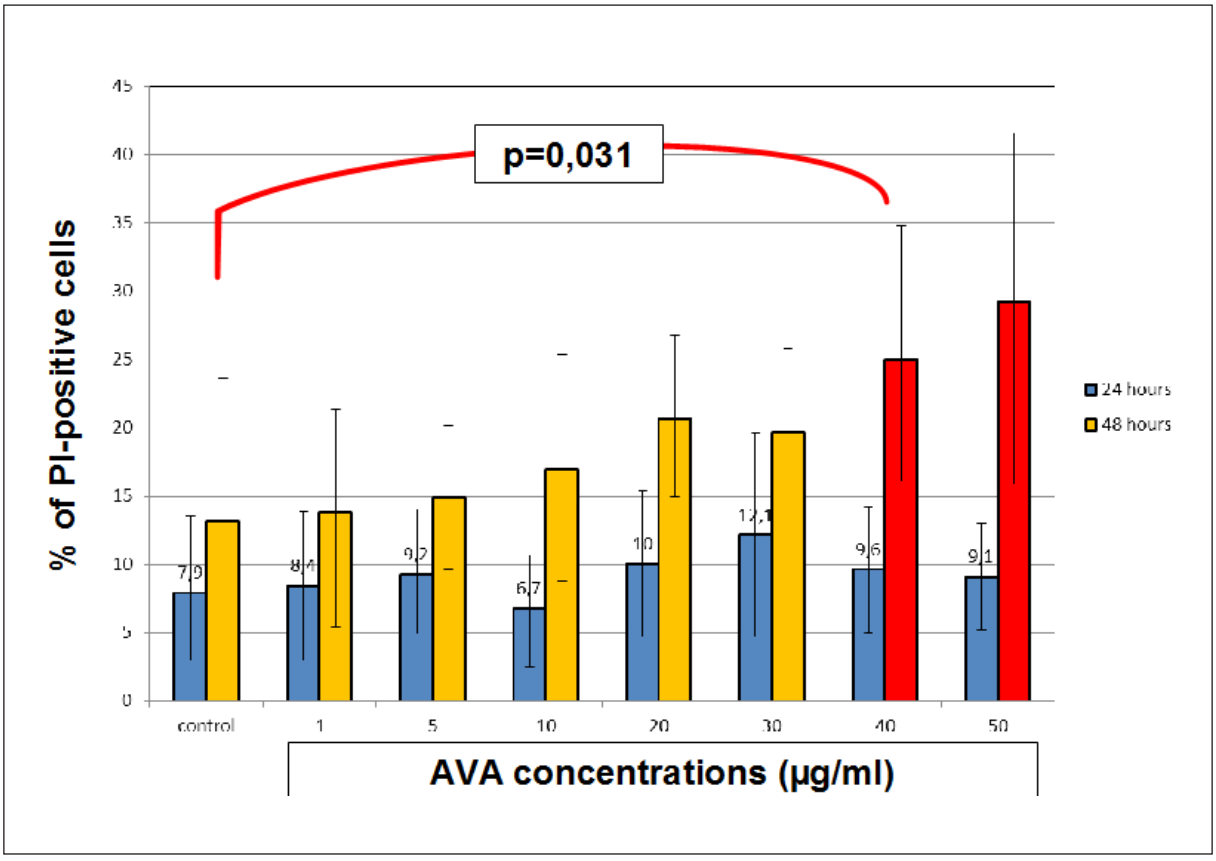

Fig. 1. Cytotoxicity of AVA, measured as a percentage of propidium iodide-positive cells (% of PI+). The lowest concentration inducing significant increase in cytotoxicity after 48 hours of incubation was 40µg/ml (*vs.* control - p=0.031) (marked in red). Mean values and standard deviations are presented

cubation with addition of control PBMCs) effects did not significantly potentiate cytotoxic AVA activity.

Influence of incubation with 40 µg/ml of AVA on VEGF levels and VEGFR expression on CLL cells was also investigated. There was a significant decrease in VEGF concentration in culture medium after incubation with AVA (p=0.002). In contrast, there were no statistically significant changes in either VEGFR-1 or VEGFR-2 expression levels before and after incubation with the study drug (both p>0.05).

Effect of AVA on healthy lymphocytes

During the experiments no significant cytotoxicity against healthy PBMCs was found. Namely, after 48 hours of incubation the median percentage of PI+ CLL cells was 25.0% (9.8-32.5%), whereas in samples with healthy PBMCs the value was 9.1% (0.9-13.1%; vs. control; N.S.).

Assessment of proapoptotic activity of AVA

AVA-induced apoptosis was examined using two markers: phosphatidylserine externalization (Ann-V) and DNA fragmentation (TUNEL assay). The percentage of Ann-V+ cells significantly correlated with the TUNEL+ cell rate (r=0.93. p<0.0001). Therefore Ann-V assay was used in further experiments for apoptosis evaluation. The median percentage of Ann-V+ cells after 48-hour incubation was 22.5% (14.1-28.1%) (vs. control – p=0.019).

Mechanisms of proapoptotic activity of AVA

Caspase activation

After 48 hours of incubation with AVA at the dose of 40 µg/ml significantly elevated caspase-3 and -9 activation in comparison with control samples was found. Median percentages of cells with caspase activation were 23.4% (11.3-29.1%) vs. 11.3% (5.6-19.1%) in the control (p=0.022) and 19.7% (12.6-27.9%) vs. 9.3% (6.3-15.9%) in the parallel control (p=0.009). Caspase-8 activation did not increase significantly after AVA treatment; median 8.3% (3.1-11.8%) (p>0.05) (Figure 2A).

Mitochondrial potential

The drop of DYm after 48-hour incubation with AVA was distinctly higher than in parallel, untreated cultures. The median percentage of cells with a drop of DYm was 20.7% (8.7- 27.1%) vs. 12.4% (3.1-19.8%) (Figure 2B).

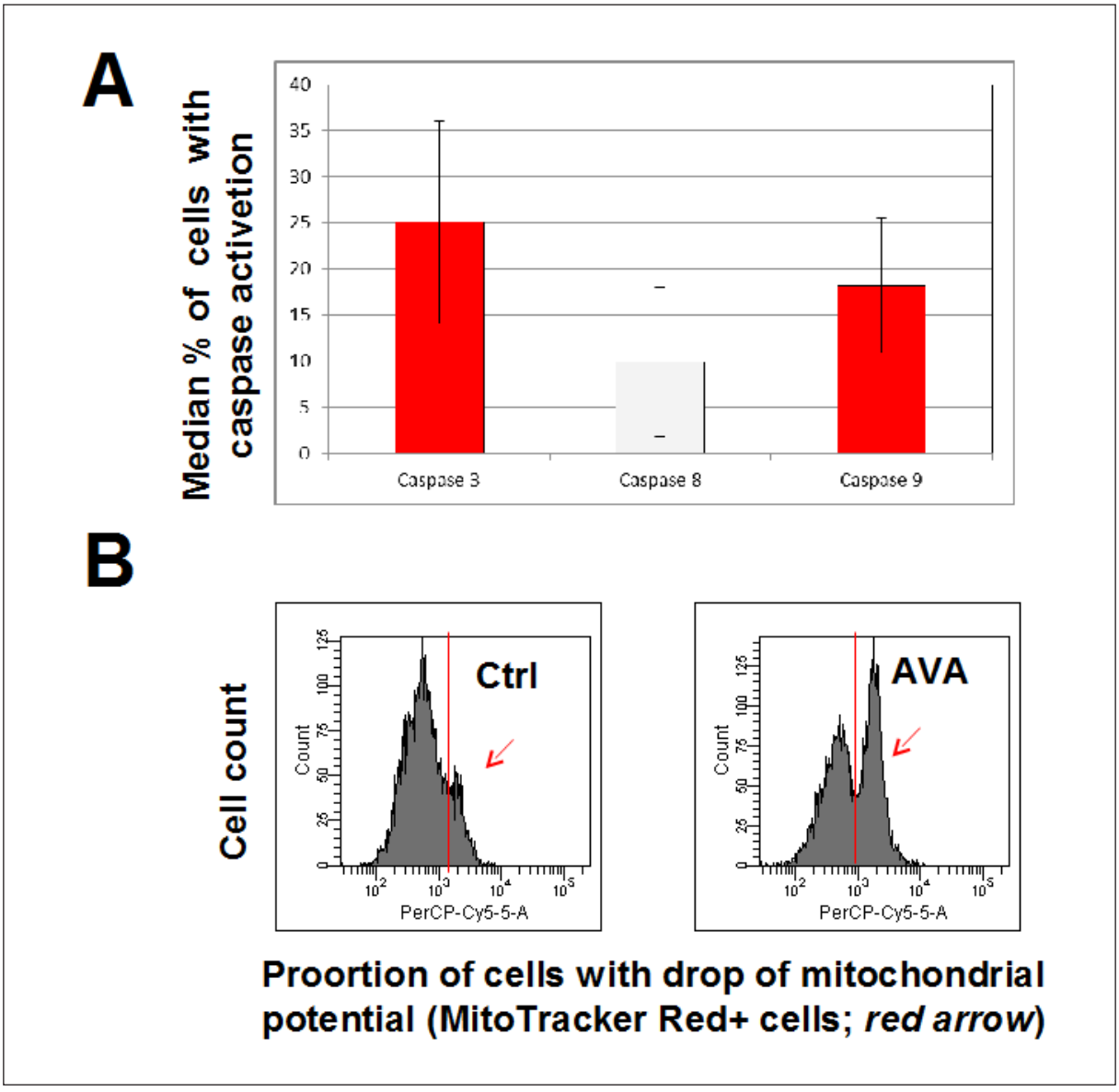

Fig. 2. Caspase activation (A) and drop of mitochondrial potential (B) after 48 hours of incubation with AVA. Percentages of cells with caspase activation or drop of mitochondrial potential were shown on Y axis. Mean values and standard deviations are presented

Expression of apoptosis-regulating proteins

After 48-hour incubation with AVA a significant increase in expression of Bax (p=0.031), Bad (p=0.014) and Akt-P (p=0.027) proteins was observed as well as a decrease in Mcl-1 expression (p=0.007) (Table 1, Figure 3). The level of other examined proteins did not significantly change in response to treatment with AVA.

Activity of AVA in combination with other drugs

Cytotoxicity of drug combinations

After 48-hour incubation with AVA or RIT used as a single drug, the median percentage of PI+ cells was 25.0% and 21.3%, respectively (Table 2). The median rate of PI+ cells in samples treated with AVA+RIT combination was 38.7% and was significantly higher than with AVA or RIT used separately (p=0.037 and p=0.041, respectively).

ALT used as a single drug induced a median percentage of PI+ cells of 27.3%. Combination of AVA and ALT exerted increased cytotoxicity (median percentage of PI+ cells 50.5%), significantly higher than AVA or ALT alone (p=0.008 and p=0.001, respectively).

Table 1. Expression of apoptosis-regulating proteins after 48 hours of incubation with AVA

Protein	Control	AVA		
	Median MFI* (range)		N**	p=
Bax	81.2 (41.4-167.3)	151.2 (67.2-211.8)	1.86	0.031
Bid	116.2 (50.5-176.3)	131.9 (30.2-209.5)	1.14	N.S.
Bak	75.2 (29.7-130.9)	85.7 (34.6-163.4)	1.14	N.S.
Bad	99.5 (32.1-158.6)	190.1 (49.2-269.3)	1.91	0.014
Bcl-2	204.9 (70.4-391.2)	179.9 (65.0-281.3)	0.88	N.S.
Mcl-1	165.1 (51.6-270.5)	75.1 (20.0-141.7)	0.45	0.007
XIAP	127.3 (40.1-190.6)	147.3 (32.5-206.7)	1.16	N.S.
FLIP	131.9 (51.7-211.1)	150.1 (37.7-202.1)	1.14	N.S.
Bcl-2-A1	98.3 (54.9-174.9)	58.3 (20.3-197.8)	0.59	N.S.
Akt-P	174.5 (44.7-271.3)	64.5 (29.0-169.2)	0.37	0.001

*** MFI - mean fluorescence intensity**

**** N - proportion of protein expression (MFI) in examined sample vs. control**

N.S. – not significant

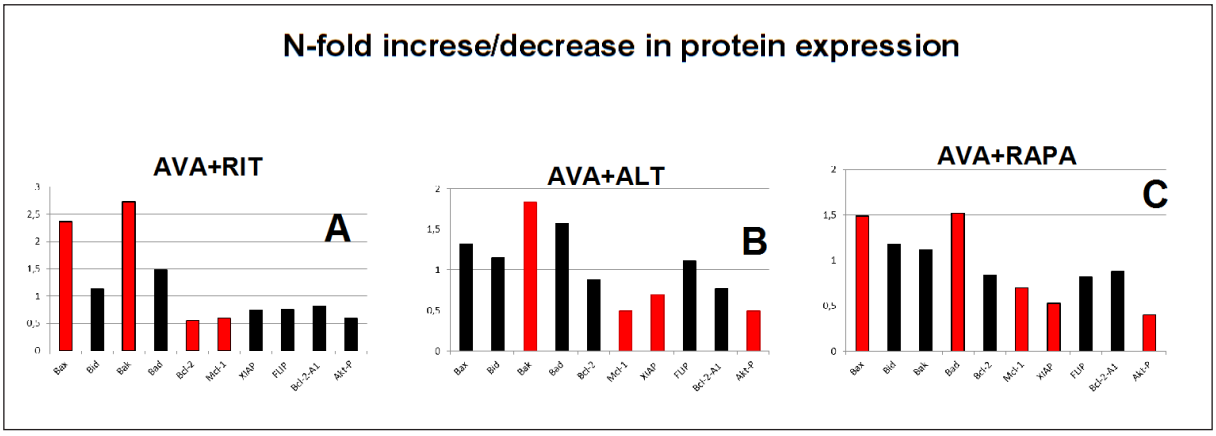

Fig. 3. Expression of apoptosis-regulating proteins after 48 hours incubation with AVA + RIT (A), AVA + ALT (B) and AVA + RAPA (C). On Y axis n-fold proportions of mean fluorescence cytotoxicity (MFI) between cell treated with the study drugs and untreated, control samples. Proteins with significant increase or decrease in expression are marked in red

Cytotoxicity of PNA: 2-CdA and FA alone and in combinations with AVA. After 48 hours of incubation in samples treated with AVA or 2-CdA alone the median percentage of PI+ cells was 53.4%. The median percentage of PI+ cells after treatment with AVA+2-CdA was 64.8% and was significantly higher only in comparison to the effect of AVA ($p<0.001$) (AVA+2-CdA *vs.* 2-CdA - N.S.). After 48 hours of incubation with FA alone the percentage of PI+ cells was 46.1%. The median percentage of PI+ cells after AVA+FA treatment was 67.3%, and, similarly to 2-CdA, was significantly higher only in comparison to the effect of AVA alone ($p<0.001$) (AVA+FA vs. 2-CdA - N.S.).

After incubation with MAF alone the median percentage of PI+ cells was 38.1%. In samples treated with the combination AVA+MAF it was 52.2% and did not differ from the effect of MAF (N.S.), but was higher than after treatment with MAF alone ($p=0.010$).

Treatment with RAPA used as a single drug induced a median of 36.8% of PI+ cells. The median rate of cytotoxicity in response to AVA+RAPA was 60.3%, and was significantly higher when compared with the effect of both AVA and RAPA used as single drugs ($p=0.034$ and $p=0.006$, respectively).

Table. 2. Cytotoxicity of AVA in combinations with other drugs. Cytotoxicity was measured as a percentage of propidium iodide-positive cells (% PI+). Data obtained after 48 hours of incubation are presented

Drugs/combinations	% of PI+ cells	Statistics (p=)
AVA alone	25.0 (9.8-32.5)	
RIT alone	19.2 (7.6-25.5)	
AVA + RIT	38.7 (9.3-52.4)	*vs*. AVA - 0.041, *vs*. RIT - 0.037
ALT alone	27.3 (11.3-38.7)	
AVA + ALT	50.5 (17.4-72.5)	*vs*. AVA - 0.008, *vs*. ALT - 0.001
2-CdA alone	53.4 (17.2-77.9)	
AVA + 2-CdA	64.8 (25.6-80.1)	*vs*. AVA - <0.001, *vs*. 2-CdA – N.S.
FA alone	46.1 (22.7-75.5)	
AVA + FA	67.3 (19.4-84.2)	*vs*. AVA - <0.001, *vs*. FA - N.S.
MAF alone	38.1 (15.8-59.3)	
AVA + MAF	52.2 (24.9-75.3)	*vs*. AVA - 0.010, *vs*. MAF - N.S.
RAPA alone	36.8% (18.9–71)	
AVA + RAPA	60.3% (45.1–98.3)	*vs*. AVA - 0.006, *vs*. RAPA - 0.034

AVA – bevazicumab. RIT – rituximab. ALT – alemtuzumab. 2-CdA – cladribine. FA - fludarabine. MAF – mafosfamide. RAPA - rapamycin
N.S. – not significant

Changes in apoptosis-regulating protein expression in response to combined treatment

Expression of apoptosis-regulating proteins was examined in samples treated with AVA in combinations with other drugs. Results of effects on protein expression are presented here only for drug combinations which exerted significantly higher cytotoxicity than single agents. As mentioned, such effective combinations were AVA+RIT, AVA+ALT and AVA+RAPA.

In samples incubated with AVA+RIT there was a significant elevation of Bax ($p<0.040$) and Bak ($p<0.018$) and decrease in Bcl-2 ($p<0.027$) and Mcl-2 ($p<0.025$) protein expression (Figure 3A).

The AVA+ALT combination triggered overexpression of Bak ($p<0.030$) and Akt-P ($p<0.003$) as well as Mcl-1 ($p<0.017$) and XIAP ($p<0.007$) downregulation (Figure 3B).

In samples cultured with AVA+RAPA significant elevation of Bax ($p<0.037$), Bad (<0.001) and Akt-P ($p<0.001$) was observed as well as decrease in Mcl-1 ($p<0.030$) and XIAP ($p<0.006$) protein expression (Figure 3C).

Discussion

The crucial role of neoangiogenesis in survival of neoplastic infiltration has been proved for many solid tumors. However, this process probably plays a role also in pathogenesis of hematological malignancies, including acute leukemias [28, 29, 30].

Several studies have indicated the importance of neoangiogenesis in CLL. Namely, Molica et al. [17] found that increased serum VEGF levels can be predictors of risk of CLL progression. VEGFR-1, VEGFR-2 and VEGFR-3 are the high-affinity receptors of mVEGF, which play an important role in *de novo* blood vessel formation and hematopoietic cell development. All of them are expressed on CLL cells [3]. A correlation between VEGFR-2 expression and patient survival was reported in CLL [7]. Moreover, Gora-Tybor et al. [12] found a strong, positive correlation between the serum level of VEGF and VEGFR-2 in CLL. VEGFR-2, but not VEGF R1, also correlated with lymphocyte count and clinical course. It was shown that VEGF/VEGFR2 interaction down-regulates matrix metalloproteinase-9 through STAT1 activation and inhibits CLL cell migration [25]. Moreover, circulating endothelial cells were reported as a noninvasive marker of angiogenesis in patients with CLL [13]. Altogether,

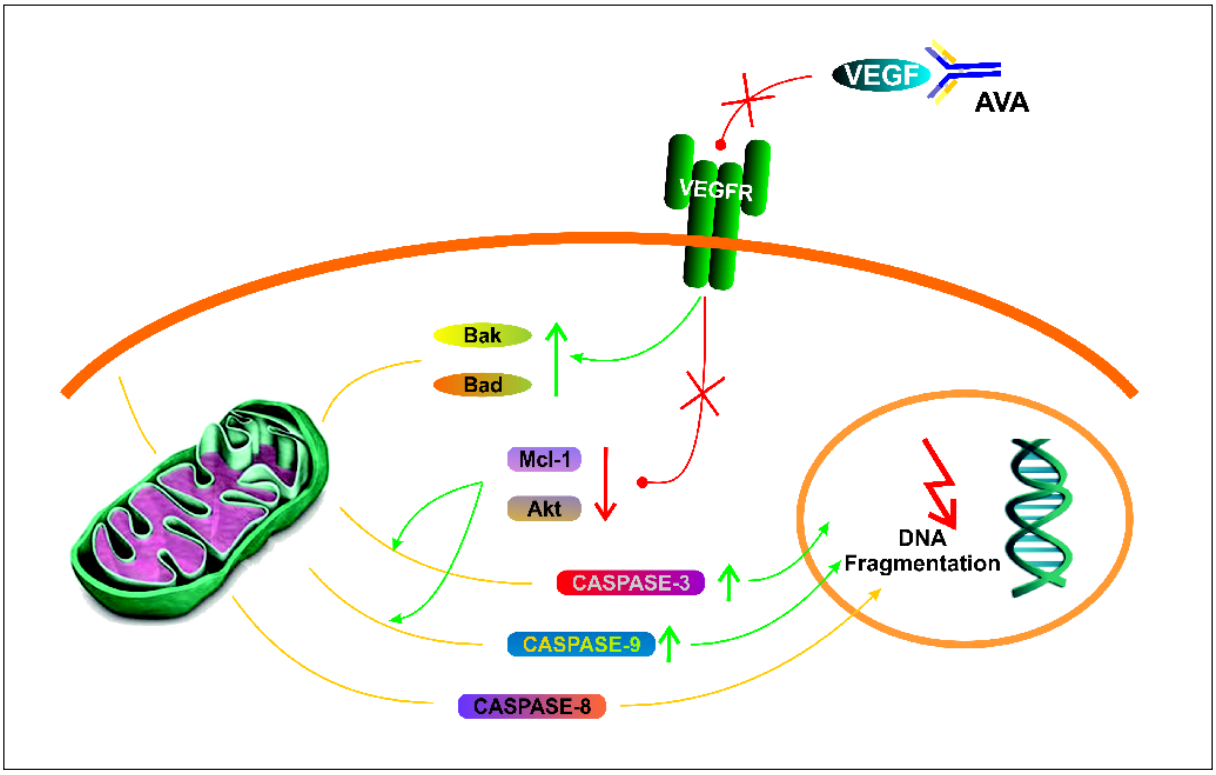

Fig. 4. Proposed mechanism of AVA action in CLL cells. Block of VEGF availability for its receptors (VEGFRs) leads to overexpression of Bak and Bad with downregulation of Mcl-1 and Akt proteins. This, in turn, activates mitochondrial pathway of apoptosis, with caspase-9 and -3 activation, DNA fragmentation and programmed cell death

these observations suggest that neoangiogenesis may have an important role in CLL development and progression.

AVA is a MoAb with antiangiogenic capacity, approved for treatment of solid tumors. The drug showed high anti-tumor activity of AVA in combinations with routine cytostatics in colon [11], breast [6], lung [4], and pancreatic [15] cancers. So far, there are only a few reports concerning the use of AVA in hematologic malignancies. A promising phase II study with AVA as a single drug was performed in 52 relapsed patients with aggressive non-Hodgkin lymphomas, including diffuse large B-cell or mantle cell lymphoma [22]. Moreover, the RIT, AVA and CHOP regimen (RA-CHOP) in untreated diffuse large B-cell lymphoma patients showed high activity and good tolerability in early clinical studies [8]. However, the further phase III study with RA-CHOP was finally stopped because of unacceptable side effects.

In CLL, only a phase II study was performed in 13 patients, showing weak activity of AVA used alone [21]. However, exact effects of antiangiogenic treatment, including AVA activity, have been poorly explored in this disease so far.

In this study we found significant cytotoxicity of AVA against *ex vivo* CLL cells in a dose of 40 μg/ml, after 48 hours of incubation. Importantly, AVA did not influence survival of normal lymphocytes from healthy donors.

AVA specifically binds VEGF, thus decreasing its level in serum or culture medium, which was observed in our *in vitro* experiments. AVA limits accessibility of VEGF to its receptors [27]. In fact, we did not find any effects of AVA on VEGFR-1 and VEGFR-2 expression.

AVA, similarly to other IgG1 monoclonal antibodies, has potential to bind Fcγ receptors and complement elements. However, in our studies cytotoxicity of AVA was independent from CDC or ADCC mechanisms. This was in concordance with the observations of Wang et al. [26] on AVA in *in vitro* cell lines Calu-6, DLD-1 and WIL2-S.

Our study is the first to show that the main mechanism of the cytotoxic effect on CLL cells is apoptosis, which was confirmed by both Ann-V and TUNEL assays. The drug triggered activation of caspase-3 and -9, with no significant changes in caspase-8 activity. Moreover, AVA caused a distinct drop of DYm in CLL cells. Thus, AVA-induced apoptosis of CLL is triggered through the mitochondrial, but not the receptor pathway of caspase activation.

VEGF is known to bind to its receptors and trigger a signal transduction cascade, which leads to proliferation [1] and migration [1, 2, 20] of vascular endothelial

cells. A possible mechanism of this activity is induction of the inhibitor of antiapoptotic survivin and XIAP, from the apoptosis protein (IAP) family [24]. VEGF acts as an apoptosis inhibitor *via* activation of Akt kinase [10], within the PI3K (phosphatidylinositol 3-kinase) signal transduction pathway [23]. Finally, VEGF increases expression of antiapoptotic proteins Bcl-2 and Bcl-2-A1 in endothelial cells [9]. AVA, by binding VEGF, inhibits the intracellular transduction pathway, thus activating apoptosis of endothelial cells.

As mentioned, so far there have been no reports on mechanisms of AVA activity in CLL cells. We found that 48-hour incubation with AVA induced changes in expression of several apoptosis-regulating proteins, with overexpression of Bak, Bad and Akt and downregulation of Mcl-1 and Akt. Taking these data together with knowledge on AVA activity in endothelial cells, we propose possible mechanisms of its cytotoxicity in CLL cells. A simplified scheme is shown in Figure 4.

To our best knowledge our study is the first to address cytotoxicity of AVA in combination with drugs already used in CLL – RIT, ALT, PNA and MAF. The latter drug was used in this study as a biologically active metabolite of cyclophosphamide in *in vivo* conditions, which also shows *in vitro* activity. Additionally, RAPA was tested as a member of the promising mTOR kinase inhibitors, agents with antiproliferative and cell migration-inhibiting effects and high antitumor potential. Combination of AVA with RIT, ALT or RAPA significantly increased cytotoxicity compared with these drugs used separately. A similar tendency was observed for combinations with 2-CdA or FA, but the differences were not statistically significant.

In conclusion, this is the first study to show that AVA has selective cytotoxic potential against CLL cells, inducing apoptosis through the mitochondrial pathway of caspase activation, with no influence on healthy lymphocytes. Lack of VEGF receptor inhibition due to AVA-induced neutralization of VEGF triggers overexpression of Bak and Bad, with downregulation of Mcl-1 and Akt proteins. Simultaneous incubation with AVA and RIT, ALT or RAPA potentiates efficacy of particular drugs used as single agents. Thus, such combined treatment may be a promising therapeutic strategy; further studies for confirmation of these results are warranted.

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