Simultaneous Inhibition of Protein Kinase CK2 and Dihydrofolate Reductase Results in Synergistic Effect on Acute Lymphoblastic Leukemia Cells

PATRYCJA WIŃSKA¹, ŁUKASZ WIDŁO¹, KATARZYNA SKIERKA¹, ALICJA KRZYŚKO¹,
MIROŚŁAWA KORONKIEWICZ², JAROSŁAW M. CIEŚLA³, JOANNA CIEŚLA¹ and MARIA BRETNER¹

¹Chair of Drug and Cosmetics Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland;

²Department of Drug Biotechnology and Bioinformatics, National Medicines Institute, Warsaw, Poland;

³Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

Abstract. *Background/Aim:* Recently, we demonstrated the ability of inhibitors of protein kinase 2 (casein kinase II; CK2) to enhance the efficacy of 5-fluorouracil, a thymidylate synthase (TYMS)-directed drug for anticancer treatment. The present study aimed to investigate the antileukemic effect of simultaneous inhibition of dihydrofolate reductase (DHFR), another enzyme involved in the thymidylate biosynthesis cycle, and CK2 in CCRF-CEM acute lymphoblastic leukemia cells. *Materials and Methods:* The influence of combined treatment on apoptosis and cell-cycle progression, as well as the endocellular level of DHFR protein and inhibition of CK2 were determined using flow cytometry and western blot analysis, respectively. Real-time quantitative polymerase chain reaction was used to examine the influence of silmitasertib (CX-4945), a selective inhibitor of CK2 on the expression of DHFR and TYMS genes. *Results:* The synergistic effect was correlated with the increase of annexin V-binding cell fraction, caspase 3/7 activation and a significant reduce in the activity of CK2. An increase of DHFR protein level was observed in CCRF-CEM cells after CX-4945 treatment, with the mRNA level remaining relatively constant. *Conclusion:* The obtained results demonstrate a possibility to improve methotrexate-based anti-leukemia therapy by simultaneous inhibition of CK2. The effect of CK2 inhibition on DHFR expression suggests the important regulatory role of CK2-mediated phosphorylation of DHFR inside cells.

Acute lymphoblastic leukemia (ALL) is the most common cancer among children (1), and constitutes approximately 25% of cancer diagnoses among children and teenagers (2). The most common method of treatment of ALL is chemotherapy, divided into several phases, such as: induction phase, consolidation phase, and finally the maintenance phase, while physicians attempt to reduce the possibility of relapse (3). Methotrexate, frequently used in the final stage of ALL treatment, is a potent inhibitor of dihydrofolate reductase (DHFR) (4), a key enzyme in intracellular folate metabolism. After entering the cell, methotrexate undergoes polyglutamylation catalyzed by folylpolyglutamate synthetase. Polyglutamylation causes the retention of methotrexate within cells and consequently results in sustained inhibition of DHFR for long intervals (5). Due to the fact that in the last stage of treatment, stretching over up to several years in pediatric patients, the maintenance drugs should have the minimal possible side-effects. Unfortunately, methotrexate demonstrates potentially serious and life-threatening toxicities in a subset of patients (6). Therefore, new therapies are being sought to allow the dose of methotrexate to be reduced, these include drug combination treatments. It was demonstrated that simultaneous inhibition of DHFR and mammalian target of rapamycin kinase had a synergistic effect on inhibition of leukemia cell proliferation with combination index (CI) values in the range from 0.33 to 0.98 (7). Other studies have shown that the combination of methotrexate and suberoylanilide hydroxamic acid (a histone deacetylase inhibitor) has a synergistic effect in leukemia cells with combination index values in the range of 0.64-0.84, depending on the cell line used (for CCRF-CEM with line: CI=0.84) (8). The most recent research carried out on L1210 mouse lymphocytic leukemia cell line showed that combined treatment of pretubulysin, a potent tubulin-binding antitumoral drug, with methotrexate displayed strong anticancer effects in both *in vitro* and *in vivo* models (9).

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Correspondence to: Patrycja Wińska, Chair of Drug and Cosmetics Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Noakowskiego St. 3, 00-664, Warsaw, Poland. Tel: +48 222345573, e-mail: pwinska@ch.pw.edu.pl

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DHFR, with two other enzymes, namely thymidylate synthase (TYMS), and serine hydroxymethyltransferase (SHMT), constitute the thymidylate synthesis cycle, providing the substrate required for DNA synthesis and repair (10). DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate, which is subsequently converted into TYMS cofactor, N⁵,10-methylenetetrahydrofolate, by SHMT. Since this cycle is the only *de novo* source of thymidylate (dTTP), the inhibition of these enzymes induces thymidylate deficiency that affects DNA replication and repair, leading to thymine-less death of the cell (11). Here, we studied the effect of simultaneous inhibition of protein kinase 2 (casein kinase II; CK2) and DHFR on the viability of leukemia cells, cell-cycle progression, apoptosis and endocellular level of DHFR, CK2 α and phosphorylated serine 529 of nuclear factor kappa-light-chain-enhancer of activated B-cells p-65 subunit (NF- κ B P-Ser529-p65).

The aim of our study was the investigation of treatment of ALL cells (CCRF-CEM) using methotrexate together with a selective inhibitor of protein kinase CK2, CX-4945, which is under stage I/II clinical trial. The efficacy of CX-4945 has been evaluated in a broad range of human hematological cancer types, including T-cell ALL (12, 13). Additionally, it was demonstrated that combined treatment with CX-4945 and proteasome inhibitor bortezomib induced synergistic apoptotic effects in T- and B- ALL cell lines *via* reduction of chaperoning activity of heat-shock protein 90 (HSP90) and inhibition of NF- κ B signaling in T-ALL cell lines as well as in primary cells from patients with T-ALL (14). Recently, it was also shown that T-ALL cells express high levels of CK2 subunits, and that CK2 inhibition by CX-4945 had synergistic effects, promoting the inhibition of survival and interleukin-2 production in T-ALL cells (15). Of note, it was demonstrated that inhibition of CK2 by CX-4945 activated caspase-3 and caspase-7 in cancer cells, with no detectable change of caspase-3/7 activity in normal cells (16). Recently, we demonstrated synergistically induced apoptosis of CCRF-CEM T-ALL cells after simultaneous inhibition of TYMS, a key enzyme of thymidylate biosynthesis, and protein kinase CK2 (17). Our previous results showed that combining a newly obtained derivative, 4,5,6,7-tetrabromo-2-methyl-1*H*-benzimidazol-1-yl)acetonitrile (17), and 5-fluorouracil (5-FU) enhanced endocellular inhibition of CK2 in CCRF-CEM cells, leading to increased apoptosis.

Materials and Methods

Reagents and antibodies. Molecular biology grade dimethyl sulphoxide (DMSO), used as a solvent for all stocks of the agents in this study, was obtained from Roth (Karlsruhe, Germany). All reagents used in flow cytometry were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Primary polyclonal antibodies against NF- κ B P-Ser529-p65 were obtained from Biorbyt (Cambridge, UK), whereas those against NF- κ B p-65 (total p65)

and CK2 α were obtained from Cell Signaling Technology (Beverly, MA, USA). Monoclonal antibodies against DHFR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from BD Biosciences and Merck Millipore (Darmstadt, Germany), respectively. Secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (IgG-HRP) and anti-mouse IgG were purchased from DAKO (Santa Clara, CA, USA). Protease inhibitors were from Bioshop (Burlington, VT, Canada). High Capacity cDNA Reverse Transcription Kit was from Applied Biosystems™ (Foster City, CA, USA). Nitrocellulose membrane was from GE Healthcare Life Sciences (Freiburg, Germany), solvents for HRP reaction (Western Bright Peroxide and Western Bright Quantum) were purchased from Advanta (Menlo Park, CA, USA). CX-4945 was obtained from Biorbyt. Methotrexate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Other solvents, reagents and chemicals were purchased from POCH (Avantor Performance Materials, Gliwice, Poland) Merck and Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Cell culture and drug treatment. CCRF-CEM cell line was purchased from The European Collection of Authenticated Cell Cultures. Cells were cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 20% fetal bovine serum (EuroClone, Sizio, Italy) and antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin). Cells were grown in 75 cm² cell culture flasks (Sarstedt, Nümbrecht, Germany), in a humidified atmosphere of 5% CO₂/95% air at 37°C. All the experiments were performed in exponentially growing cultures. Stock solutions of the tested compounds were prepared in DMSO and stored at -80°C for no more than 1 month. Stock solutions of the tested compounds were diluted 200-fold with the culture medium to obtain the final concentration of the tested compounds at 0.5% DMSO. For the combination experiments, stock solutions of the tested compounds (400-fold) were diluted at a 1:1 ratio with DMSO (when used separately) or with the second compound (when used in a combination). For combination experiments CCRF-CEM were seeded at 4×10⁴ cells/well and treated with the compounds, as follows: six to eight concentrations of each compound or their combination, in the range of 0.125 × drug potency (Dm) to 8 × Dm, *i.e.* from 0.00275 to 0.176 μ M and from 0.3863 to 24.72 μ M for methotrexate and CX-4945, respectively in a constant ratio at two-fold dilution series were used.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based viability assay. After incubation with the test compounds, MTT test was performed as described previously (17). All measurements were carried out in a minimum of six replicates and the results expressed as the fraction of non-viable cells (Fa) relative to that of the control (cells without inhibitor in 0.5% DMSO). Fa values were calculated from the following equation $1-(T-B/C-B)$, where *T* and *C* were the mean absorbance obtained for the treated and untreated cells, respectively, whereas *B* was the absorbance for the blank well (without cells).

Detection of apoptosis. CCRF-CEM cells were cultured in 6-well plates at a density of 1.8×10⁵/ml and treated with the tested compounds. Twenty microliters of the tested drugs or their combination were added to each well in the following concentrations: 0.02 and 0.04 μ M methotrexate for 48 h treatment; 0.01 and 0.02 μ M methotrexate for 72 h treatment; and 1.5, 3 and 6 μ M CX-4945. The plates were then incubated for 48 or 72 hours. The cells were then

harvested by centrifugation at $200 \times g$ at 4°C for 5 min, washed twice in cold phosphate-buffered saline (PBS), and subsequently suspended in binding buffer at 1×10^6 cells/ml. Subsequently, 100- μl aliquots of the cell suspension were labeled according to the kit manufacturer's instructions. Briefly, annexin V-fluorescein isothiocyanate and propidium iodide (BD Pharmingen) were added to the cell suspension, and the mixture was vortexed and incubated for 15 min at room temperature in the dark. Cold binding buffer (400 μl) was then added and the cells were vortexed again and kept on ice. Flow cytometric measurements were performed within 1 h after labeling. Viable, necrotic, early and late apoptotic cells were detected by fluorescence-activated cell sorting using BD FACSCanto II and analyzed with BD FACSDiva software (BD Biosciences, San Jose, CA, USA).

Cell-cycle analysis. CCRF-CEM cells were cultured in 25 cm^2 culture flasks and treated with the tested compounds as described above. After treatment, the cells were washed with cold PBS and fixed at -20°C in 70% ethanol for at least 24 h. The subsequent procedures were carried out as described previously (17). Cellular DNA content and the distribution of the cells in different phases of the cell cycle were determined by flow cytometry employing FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA), and analyzed using the BD FACSDiva software. The obtained DNA histograms were analyzed using MacCycle (Phoenix Flow Systems, San Diego, CA, USA) software.

Western blotting. CCRF-CEM cells growing exponentially were seeded at 2×10^5 cells/ml in 75 cm^2 flasks. Subsequently, compounds were added in the following concentrations: 0.02 μM methotrexate and 3 μM CX-4945, separately or in combination. After 48 h treatment, cells were centrifuged ($200 \times g$, 1,700 rpm, 4°C for 5 min), washed ($3 \times$ with PBS), and pellets were collected and stored at -20°C . For the assay, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium orthovanadate and Protease Inhibitors Cocktail (Roche, Indianapolis, IN, USA). The protein concentration was determined using Bradford assay (18). Equivalent amounts of total protein (40 μg) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently western blotting was performed using primary antibodies to P-Ser529-p65 (1:500 dilution), total p65 (1:1,000), DHFR (1:500), CK2 α (1:1,000) and GAPDH (1:500 dilution) in Blocking Buffer: 3% bovine serum albumin in 10 mM Tris-HCl (pH 8) and 150 mM NaCl with 0.1% Tween 20 (TBST). After overnight incubation at 4°C with the primary antibodies, the membranes were washed with TBST and subsequently secondary goat anti-rabbit IgG-HRP or anti-mouse Ig-HRP antibodies were used at 1:5,000 dilution. ECL substrate (Advansta, Menlo Park, CA, USA) was used for detection and immunoblots were scanned using G Box Chemi (Syngene, Cambridge, UK).

Densitometry. For densitometry, immunoblots were scanned using G Box Chemi (Syngene), and the density of each lane of phosphorylated and total protein was quantified using GeneTools software. Phosphorylated protein densities were normalized to GAPDH densities, assuming 1 for untreated cells and then they were converted to a fraction of the appropriate control.

Caspase-3/7 activity assay. Caspase-3/7 activity was measured using the Apo-ONE[®] Homogeneous Caspase-3/7 Assay (Promega,

Madison, WI, USA) according to the manufacturer's protocol. The obtained data were normalized using MTT data.

Total RNA isolation, reverse transcription and real-time quantitative polymerase chain reaction (QPCR). Total RNA from treated and untreated CCRF-CEM cells was isolated and purified using commercial reagents Renzol TRI RNA Extraction Reagent (GenoPlast Biochemicals, Rokocin, Pomorskie, Poland) and The PureLink[®] RNA Mini Kit (Invitrogen[™], Carlsbad, CA, USA), respectively, according to manufacturer's protocols. Additionally, RNA preparations were cleaned thoroughly by propanol precipitation. The quality and quantity of RNA was assessed on 1% agarose gel and spectrophotometrically at 260/280/230 nm. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations, and cleaned by sodium acetate/ethanol precipitation. QPCR assays were carried out on LightCycler[®] 480 Instrument (Roche), 7500 Real Time PCR System (Applied Biosystems) or PicoReal 96 (Thermo Scientific, Waltham, MA, USA). Primer sequences for *DHFR* (F: 5' GCGTTCTGCTGTAACGAG 3' and R: 5' ACCAGATTCTGTTACCTTCTAC 3') were designed using Kalign tool available at www.ebi.ac.uk (19) and ordered from DNA Sequencing and Oligonucleotides Synthesis Laboratory at Institute of Biochemistry and Biophysics (Warsaw, Poland). QPCR was performed with the use of Real-Time 2xHS-PCR Master Mix SYBR[®]A (A&A Biotechnology Gdynia, Poland) virtually as it was previously described (20). The amount of target mRNA was calculated by the ΔCt method (21) with the geometric mean of Cts of two reference genes, β -glucuronidase (*GUSB*, NM_000181) and TATA-binding protein (*TBP*, NM_003194). Relative *DHFR* and *TYMS* gene expression in control cells (RE) was set at a value of 1 and the relative *DHFR* or *TYMS* gene expression after treatment with inhibitor(s) (RE_i) was calculated as the ratio RE_i/RE .

Statistical analysis. Values of experimental measurements were considered as independent variables. Rare outlying results were omitted in further calculations. In the next step, the ratio of each relative gene expression in sample from inhibitor treated cells *versus* each relative gene expression in sample from untreated cells was calculated. This created a two-dimensional matrix of values comparing gene expression in untreated and treated cells. If compared expressions were the same, these ratios should equal 1. Therefore, it was possible to apply chi-square test with 1 as the expected value and average of all results from the matrix as the observed value. Significantly outlying quantiles from the matrix were omitted in this test. The result of chi-square test is the probability that gene expression in treated cells is equal to gene expression in untreated cells. Expression in both groups of cells was considered as different when probability of this equality was lower than 0.05 ($p \leq 0.05$).

Results

CCRF-CEM cell line, representing ALL, was treated with combinations of CX-4945 (inhibitor of CK2) and methotrexate (inhibitor of DHFR). MTT-based assay and the combination index method (22) were used to determine the type of interaction (*i.e.* synergistic, additive or antagonistic) between tested anticancer agents. Additionally, the dose-reduction index (DRI) was determined on the basis of drug interaction

Table I. Combination index (CI) calculated at effective dose (ED)₅₀, ED₇₅ and ED₉₀, drug potency (Dm) and dose reduction index (DRI) for the tested drug combinations for CCRF-CEM cells after 48-h and 72-h treatment. CI values were generated by CalcuSyn software after fitting fraction affected (Fa) values obtained by MTT-based assay. Dm of the dose-response curves was obtained after fitting the MTT-based assay data to the median-effect equation using CalcuSyn Software. Six to eight data points at 2-fold dilution range were used for combinations.

Treatment (h)	CI at			Dm (μM)		DRI at Fa=95		
	ED ₅₀	ED ₇₅	ED ₉₀	MTX	CX-4945	MTX:CX-4945	MTX	CX-4945
48	0.86±0.12	0.74±0.09	0.84±0.04	0.028±0.00	3.41±0.05	0.011±0.001	12.2±3.2	1.2±0.3
72	0.44±0.03	0.44±0.06	0.45±0.09	0.055±0.006	4.42±0.54	0.0085±0.0004	12.0±4.8	3.1±0.9

data analysis. This parameter is inversely associated with CI and represents the number of times each single drug dose may be reduced in a combination setting without compromising the final therapeutic effect. To understand the mechanism of a synergistic interaction, the influence of selected compound combinations on cell cycle progression, apoptosis and endocellular level of CK2α protein and activity (phosphorylation of NFκB Ser529 p65) as well as level of DHFR mRNA and protein were investigated.

Influence of methotrexate and CX-4945 on viability of cancer cell lines. To optimize the compound ratio used in the combination treatment, the influence on the cell viability of each compound alone was investigated, and Dm values corresponding to the drug potency were calculated. The ratio of the tested compounds used in the combinations, specified by their Dm values and also by the preliminary results (data not shown) provided an Fa value in the range of 0 to 1. Six to eight concentrations of each compound, in the range of 0.125 × Dm to 8 × Dm at a constant ratio in a 2-fold dilution series according to the recommendation given by Chou (22) were used in combination experiments, as described in Materials and Methods. CI values were generated in CalcuSyn Software at ED₅₀, ED₇₅ and ED₉₀ after fitting Fa values obtained by MTT-based assay after 48 h and 72 h incubation time (Table I). As it is important to reduce toxicity in clinical treatment and methotrexate is an essential component of chemotherapy in ALL commonly used in consolidation therapy, DRI values are shown for tested combinations. The synergistic effect for the combination methotrexate:CX-4945, used at a 1:140 ratio, was observed after both 48 and 72 h incubation, with CI values in the range 0.65-0.78 and 0.44-0.45, respectively. Representative examples of the graphs generated from the analysis of two-compound combinations, demonstrating the synergistic effect for CX-4945 plus methotrexate are shown in Figure 1. Interestingly, the average DRI value for methotrexate was 12 at Fa of 0.95 for both treatment durations, and indicated that the dose of methotrexate may be reduced by about 12-fold when used in combination with CX-4945, and resulted in 95% of leukemia cells affected. DRI for

CX-4945 was lower than for methotrexate, with the highest value of 3.1 after 72-h combined treatment.

Induction of apoptosis and caspase 3/7 activation in CCRF-CEM cells. In order to better understand the observed synergism for combination of methotrexate and CX-4945, induction of apoptosis in CCRF-CEM cells was studied by annexin V and PI staining. The results, showing a pro-apoptotic influence of the tested compounds used separately and in combination are shown in Figure 2. methotrexate was used at two different concentrations per incubation time, 0.02 and 0.04 μM for 48 h and 0.01 and 0.02 μM for 72 h treatment, whereas CX-4945 was used at three concentrations per each treatment time, 1.5, 3 and 6 μM. The percentage of apoptotic cells induced using methotrexate was greater than in control samples at all tested concentrations after both incubation times and ranged from 19% to 26%, with the highest observed for 0.02 μM methotrexate after 72 h treatment. In contrast, for CX-4945 the percentage of apoptotic cells was greater than in control samples at the two highest concentrations after 48 h incubation, and at all tested concentrations after 72 h of treatment, and ranged from 14% to 33%, with the highest observed for 6 μM CX-4945. Interestingly, the percentage of apoptotic cells was higher after use of all tested combinations than for single drugs, and ranged from 28% to 68% of cells in early and late apoptosis, with the highest proportion observed for the combination of 0.04 μM methotrexate and 6 μM CX-4945 after 48 h of incubation. However, when the additive effect of the tested drugs is taken into account, the synergistic pro-apoptotic effect is clearly visible for all combinations after 48 h of incubation, with the best effect for 0.04 μM methotrexate and 3 μM CX-4945, which produced 25% more apoptotic cells than that resulting from the single agents (Figure 2C). After 72 h of incubation, the best effect was observed for 0.01 μM methotrexate and 3 μM CX-4945, which led to approximately 19% more apoptotic cells than that resulting from the additional effect.

Since it was demonstrated that inhibition of CK2 by CX-4945 activated caspase-3 and caspase-7 in cancer cells (16), Apo-ONE® Homogeneous Caspase-3/7 Assay was used to study the influence of CX-4945 and its combination with

Figure 1. Synergistic interaction of methotrexate (MTX) with protein kinase 2α subunit inhibitor CX-4945 in inhibition of viability of CCRF-CEM cells. Representative CalcuSyn software-simulated isobolograms (A and C) and fraction affected-combination index (Fa-CI) curves (B, D) after 48 h and 72 h of treatment, respectively, generated after fitting Fa obtained from the MTT assay.

methotrexate on caspase 3/7 activity in CCRF-CEM cells after 48 h of treatment. Based on the results obtained by flow cytometry, the concentrations of inhibitors with the highest pro-apoptotic effect were selected for this assay. The results were normalized by dividing the fluorescence by the fraction of living cells and the values obtained are shown in Figure 2D. The activity of caspases in cells cultured with 1.5 and 3 μM CX-4945 was very similar to that of the untreated cells, whereas in other cases, the results indicated increased activity of caspases compared to the control. The highest activity of caspases was observed in cells cultured with the combination of 0.04 μM methotrexate and 6 μM CX-4945. The data confirm the strongest pro-apoptotic effect of this combination on CCRF-CEM cells. The obtained data indicated a synergistic effect of the combination of methotrexate and CX-4945 on induction of apoptosis and are in a good agreement with the MTT-based drug-combination results.

The effect of the synergistically acting combinations on a cell-cycle progression in CCRF-CEM cells. Since it has been

shown by others that CX-4945 (23), as well as methotrexate (24) can affect cell-cycle progression of leukemia cells, the influence of methotrexate alone and in combination with CX-4945 on cell-cycle progression was tested by flow cytometry after 48 h and 72 h treatment of CCRF-CEM cells. Representative plots with the calculations of cell percentages in each phase of the cell cycle are shown in Figure 3. The results indicated that methotrexate led to G₂/M-phase arrest in the studied cells. The number of CCRF-CEM cells in the G₂/M-phase correlated with the duration of treatment, with the strongest effect leading to 12.8% cells in G₂/M phase after 72 h treatment at 0.02 μM methotrexate, with a small number of cells in sub-G₁-phase, suggesting apoptosis. On the contrary, the treatment of leukemia cells with 3 μM CX-4945 alone led to S-phase accumulation (56.6%) after 72 h and insignificant change after 48 h of treatment. Interestingly, more significant changes were observed only in the case of cells treated for 72 h with the combination of drugs, and for which cell accumulation was observed in both S-phase and G₂/M-phase. Furthermore, cells in the sub G₁-phase was

Figure 2. Continued

Figure 2. Pro-apoptotic activity of methotrexate (MTX) and protein kinase 2 α subunit inhibitor CX-4945 on CCRF-CEM, when used separately and in combination. The data were acquired by fluorescence-activated cell sorting after 48-h (A) and 72-h treatment (B). C: Representative cytograms for: Control [0.5% dimethyl sulfoxide (DMSO)], 0.04 μ M MTX, 3 μ M CX-4945, and their combination after 48-h treatment. D: Caspase 3/7 activation in CCRF-CEM after 48 h treatment with 0.04 μ M MTX, 1.5 μ M, 3 μ M and 6 μ M CX-4945 and respect combinations. Values in A and B are means $\pm$ standard error of mean, and represent three biological replicates. Statistical significance was determined by two-tailed t-test. PI: Propidium iodide.

observed, which indicates apoptotic cells and confirms the results obtained with annexin V and PI staining.

The effect of simultaneous inhibition of DHFR and CK2 on protein levels of DHFR, CK2 α and total p65, as well as p65 phosphorylation. In view of the observations that DHFR protein level increased after methotrexate treatment and contributed to the inefficiency of chemotherapy (25), we checked the influence of methotrexate and CX-4945 used separately and in combination on the level of DHFR and CK2 α proteins in cellular extracts obtained from CCRF-CEM. Additionally, NF κ B P-p65–Ser529 was used as a marker of intracellular CK2 activity. Both methotrexate and CX-4945 influenced the level of DHFR, which was significantly higher in cells treated than in control cells after 48 and 72 h treatment (Figure 4). Densitometric analysis showed that the DHFR level in cells treated with methotrexate or CX-4945 alone increased by up to 9- and 5-fold in 48-h cultures, and up to 7-

and 4-fold in 72-h cultures, respectively. Interestingly, the higher level of DHFR in cells treated with the combination of drugs did not result in an additive effect, but was similar to that observed with methotrexate used alone. An increase in DHFR in cells treated with methotrexate is well known and results most likely from unblocking suppression of DHFR translation (26). However, in the case of cells treated with CX-4945, such an increase is new evidence that may indicate the important role of phosphorylation in the regulation of DHFR protein expression. Taking into account that DHFR was found to be phosphorylated by CK2 *in vitro* (27), our results seem to confirm the physiological role of such phosphorylation. The level of phosphorylation of Ser529-p65, reflecting CK2 activity, was highest in control cells and lowest in cells treated with the drug combination. Interestingly, the strongest CK2 inhibition, corresponding to 20% of the activity in control cells, was observed for the combination after 72 h of treatment. The results correspond to those obtained with the

Figure 3. The effect of compounds on a cell-cycle progression in CCRF-CEM cells. Leukemia cells were treated with 0.02 μ M methotrexate (MTX), and 3 μ M protein kinase 2 α subunit inhibitor CX-4945 separately or in combination for 48 h (A) and 72 h (B), and then fixed and stained with propidium iodide. The cell-cycle distribution profiles of the cells were determined by flow cytometry. Representative graphs indicate the percentages of cells in the G₁, S and G₂/M phases of the cell cycle.

MTT assay, indicating a stronger synergistic effect after a longer incubation period.

The influence of CX-4945 on expression of DHFR and TYMS genes. QPCR was used to examine whether the observed increased level of DHFR protein in cells upon treatment with CX-4945 was correlated with increased transcription of *DHFR* gene. Additionally, the mRNA level of *TYMS* was determined. CCRF-CEM cells were treated with 3 μ M or 6 μ M CX-4945, or only DMSO as a control, and subsequently *DHFR* and *TYMS* mRNA levels were assayed after 24, 48, and 72 h. The relative *DHFR* and *TYMS* gene expression after inhibitor treatment as REI/RE is shown in Figure 5.

The results indicate that only 6 μ M CX-4945 enhanced *DHFR* gene expression, by 1.24-fold after 24 h cell culture, but lowered it after 72 h treatment by 1.70-fold, whereas no obvious changes in *DHFR* expression were detected after treatment with 3 μ M CX-4945. CX-4945 at 3 μ M caused 1.4-fold decrease of *TYMS* gene expression after 24 h and 48 h treatment, with no obvious changes after 72 h treatment.

Discussion

The effect of inhibitors directed against two different molecular targets, namely protein kinase CK2 and DHFR,

alone and in combination, was studied in leukemia cells. Our results demonstrated the synergistic interaction of methotrexate and CX-4945 combination on the CCRF-CEM cell line. Interestingly, we showed previously that CX-4945 combined with 5-FU (TYMS-directed prodrug) only affected the viability of cells in a synergistic manner in the MCF-7 breast cancer cell line, whereas in the CCRF-CEM cell line, the combination resulted in antagonism (17). It should be taken into account that methotrexate, in addition to having an inhibitory effect on DHFR, is also the inhibitor of another enzyme involved in thymidylate synthesis, TYMS, as well as certain other enzymes involved in the *de novo* biosynthesis of purines, e.g. glycylamide ribonucleotide formyltransferase (GARFT) and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranosyl-5'-monophosphate (AICART) (28). Because the polyglutamylated metabolites of methotrexate remain in cells much longer than the parent drug itself, polyglutamylation determines the efficacy of methotrexate as a cytotoxic agent. Therefore, simultaneous inhibition of CK2 and one of two enzymes involved in thymidylate biosynthesis, i.e. TYMS or DHFR, can have different effects within cells. However, it is worth noting that the clinically relevant parameter obtained for 5-FU and methotrexate, namely the DRI (Fa=0.95), was very similar for both drugs, with values of 12.25 and 12.0, respectively.

Figure 4. A: Western blot analysis of dihydrofolate reductase (DHFR), protein kinase 2 α subunit (CK2 α), total nuclear factor kappa-light-chain-enhancer of activated B-cells p-65 subunit (NF- κ B p65 and P-Ser529-p65) in the crude extracts obtained from CCRF-CEM cells after treatment for 48 h (upper panel) and 72 h (lower panel) with 0.02 μ M methotrexate (MTX) and 3 μ M CK2 α inhibitor CX-4945, separately or in combination. P-Ser529-p65 was used as a marker of cellular inhibition of CK2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control for each sample. B: Densitometric quantifications for each protein are shown for the representative blots shown in A relative to that in untreated control cells set as 1.

Considering that methotrexate causes unwanted side-effects, including leukoencephalopathy (29), lowering the therapeutic effective dose of this agent is desirable.

The obtained synergistic effect on CCRF-CEM cells after combination treatment with CX-4945 and methotrexate seems to be correlated with stronger inhibition of CK2 and consequently with the increase in the pro-apoptotic activity as shown by the increase in the proportion of annexin V-stained cells. Moreover, the sub-G₁ phase, indicating the presence of apoptotic cells, was detected on the basis of a cytometric analysis of the quantity of DNA after the combined treatment of cells with methotrexate and CX-4945. In addition, we showed that the combination of methotrexate and CX-4945 had a synergistic effect on the caspases 3/7 activation in CCRF-CEM cell line. The results are in

agreement with the literature data demonstrating that CK2 and its substrates such as serine/threonine kinase AKT protect cells from apoptosis by phosphorylation of a wide range of proteins involved in the apoptotic response (30, 31). Consequently, inhibition of CK2 leads to increased apoptosis in many types of cancer cells, including leukemia (32). Considering that CK2 inhibits apoptosis, increased apoptosis after the combined treatment corresponded with a lower level of phosphorylation of NF- κ B Ser529-p65, thereby indicating the strongest CK2 inhibition in cells after the use of the methotrexate and CX-4945 combination.

Additionally, we observed CCRF-CEM cell accumulation in both S- and G₂/M phases of the cell cycle after the combined treatment, which seems to have been an additive effect, since methotrexate and CX-4945 used individually led to G₂/M-

phase arrest and S-phase arrest, respectively. Interestingly, our results demonstrating G₂/M-phase arrest after methotrexate treatment are contrary to previous results obtained for L1210 mouse leukemia suggesting that methotrexate cytotoxicity is related to irreversible S-phase and G₁/S-phase arrest (9, 24). S-Phase arrest occurring after CK2 inhibition in the CCRF-CEM line is consistent with our previous data demonstrating the effect of another CK2 inhibitor, 4,5,6,7-tetrabromo-2-methyl-1*H*-benzimidazol-1-yl)acetonitrile, on cell-cycle progression in this cell line (17). The obtained results may be associated with DNA-repair mechanisms, since it was shown by others that CX-4945 blocks the DNA-repair response in ovarian cancer cells (33). It was established that CK2-mediated phosphorylation of mediator of DNA damage checkpoint protein 1 (MDC1), a key mediator of homologous recombination repair of double-strand breaks, is necessary for the formation of a multiprotein complex required for repair signaling (34). Taking into account that inhibition of DHFR affects *de novo* thymidylate biosynthesis and consequently leads to DNA damage, simultaneous inhibition of CK2 and DHFR might block DNA repair, thus contributing to the increased antiproliferative effect.

Importantly, we detected a significantly increased level of DHFR protein in cells treated with methotrexate and CX-4945 as single agents, as well as with the combination of both inhibitors. Methotrexate is a tight-binding inhibitor of DHFR, and the concentration of methotrexate required to achieve inhibition of enzyme activity increases in direct proportion to the amount of the enzyme in the target cells. Considering that the increase in the level of DHFR protein and its resulting activity in the cell contributes to a reduction in the effectiveness of methotrexate therapy (25), the results presented here seem to be important for the consideration of clinical use of both DHFR and CK2 inhibitors. Among the best-known mechanisms leading to the rise of DHFR level after methotrexate treatment is amplification of the *DHFR* gene after prolonged treatment (35, 36). However, in the case of our research conducted on the period from 48 to 72 h, other reasons for DHFR increase should be taken into account; the most likely mechanism may be related to unblocking of translation. It was demonstrated that DHFR protein itself has a suppressive effect on translation of its own mRNA, by binding a 100-base-long fragment in the coding region (37). When methotrexate binds to DHFR protein, this suppressive effect may be lost, allowing the synthesis of new enzyme molecules (35, 38). Moreover, in the case of the observed increase in DHFR protein level after CX-4945 treatment, the regulatory role of CK2 should also be taken into account, particularly in the light of our most recent study (27) showing the phosphorylation of DHFR by CK2 *in vitro*. Taking into account that DHFR-CK2 interaction is strong and specific, shown by means of quartz crystal microbalance (27), it is likely that such phosphorylation may also occur *in vivo*.

Figure 5. The effect of protein kinase 2α subunit inhibitor CX-4945 on the relative expression of dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS) genes in CCRF-CEM cells. Relative DHFR and TYMS gene expression in control cells (RE_i) was set at a value of 1 and the relative DHFR and TYMS gene expression after treatment with inhibitor (RE_t) was calculated as the ratio RE_t/RE_i . Data are median $\pm$ SE_{me} . SE_{me} was calculated according to the equation: $SE_{me} = 1.253 \cdot SD / \sqrt{n}$, where n is the number of results. *Statistically significantly different from that of untreated cells by chi-square test ($p \leq 0.05$) (see Materials and Methods).

Considering that CX-4945 at 3 μ M affected the expression of DHFR at the protein but not mRNA level, the possible regulatory role of CK2-mediated phosphorylation in translation/degradation of DHFR protein should be taken into account. Recently, it was demonstrated that phosphorylation of TYMS affected its catalytic and non-catalytic properties, including translation and binding of its own mRNA (39-41). CK2-mediated phosphorylation of DHFR might have a similar effect on enzyme properties. It should also be noted that phosphorylated TYMS bound and inhibited translation not only of its own mRNA, but also of other mRNAs encoding DHFR, SHMT, thymidine kinase and deoxycitidylate deaminase proteins (41). If we assume that *in vivo* lack of or diminished phosphorylation of TYMS due to inhibition of CK2 prevents the binding of TYMS protein to *DHFR* mRNA, this mechanism certainly might contribute to an increase of DHFR protein after treatment of cells with CK2 inhibitor.

Considering our previous results showing the effect of CK2 inhibition on the level of TYMS protein in the CCRF-CEM cell line (17), expression of *TYMS* gene was evaluated here after treatment with CX-4945. Interestingly, *TYMS* mRNA level in cells treated with CK2 did not changed after 72-h culture, and thus did not correspond to our previous data, demonstrating a decrease of TYMS protein to about

60% of that of the control after TBBi derivative, 4,5,6,7-tetrabromo-2-methyl-1H-benzimidazol-1-yl)acetonitrile treatment (17). However, the level of *TYMS* mRNA after 24-h and 48-h treatment slightly decreased.

In summary, the results obtained indicate that inhibition of DHFR may have a better therapeutic effect when combined with a drug that exerts a CK2-inhibitory effect. Moreover, they suggest the important regulatory role of CK2-mediated phosphorylation of both *TYMS* and DHFR in their translation/protein degradation within cells. However, considering that CK2 is a new potential target for anti-leukemia therapy (42), it should be taking into account that an increase of DHFR protein resulting from CK2 inhibition might contribute to chemoresistance, and therefore further studies need to be performed.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

PW and JC designed the experiments and wrote the article; PW performed western-blot analysis; ŁW performed the drug combination experiments; KS, AK and JMC performed real-time quantitative polymerase chain reaction experiments; MK performed flow cytometric analysis; MB reviewed the article. All Authors read and approved the final article.

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