Effect of Simultaneous Inhibition of Protein Kinase CK2 and Thymidylate Synthase in Leukemia and Breast Cancer Cells

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Abstract. *Background/Aim: Protein kinase CK2 was recently identified as a promising therapeutic target for combination therapy. Our study aims to investigate the anticancer effect of a simultaneous inhibition of thymidylate synthase (TS) and CK2 in MCF-7 breast cancer and CCRF-CEM leukemia cells. Materials and Methods: The type of interaction between CK2 inhibitors: CX-4945, 4,5,6,7-tetrabromo-1H-benzimidazole (TBBi), or recently obtained 4,5,6,7-tetrabromo-2-methyl-1H-benzimidazol-1-yl)acetonitrile (2b) and TS-directed anticancer drug, 5-fluorouracil (5-FU) was determined using the MTT assay and a combination index method. The influence of the combined treatment on apoptosis in leukemia cells, as well as on cell-cycle progression and the levels of TS, CK2α and P-Ser529-p65 were determined in both cell lines, using flow cytometry and western blot analysis, respectively. Results: The best synergistic effect was observed in CCRF-CEM cell line with the combination of 5-FU and 2b which correlated with a decrease in the endocellular CK2 activity and enhancement of the pro-apoptotic effect. Conclusion: The obtained results demonstrate the ability of CK2 inhibitors to enhance the efficacy of 5-FU in anticancer treatment, indicating a different molecular mechanism of the studied CK2 inhibitors interaction with 5-FU.*

Inhibition of protein kinase CK2 by small ATP-competitive inhibitors like 4,5,6,7-tetrabromo-1*H*-benzimidazole (TBBi) and its derivatives, or by clinical stage agent CX-4945 (Silmitasertib) in breast cancer cell lines caused a range of

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phenotypic changes in these cell lines, including decreased viability, cell cycle arrest, apoptosis and loss of migratory capacity (1, 2). 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 and caused arrest in G_2-M transition in breast cancer cells (2). The efficacy of CX-4945 has also been evaluated in a broad range of human hematologic cancers, including chronic lymphocytic leukemia (CLL), T-cell acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), and lymphomas (3-5). Moreover, it has been demonstrated (6, 7) that anti-proliferative activity of CX-4945 against chronic lymphocytic leukemia (CLL) was higher when used in a combination with several other inhibitors, including GS-1101, ibrutinib, and fludarabine, regulating B-cell receptor (BCR)-mediated signaling cascades or downstream mediators. It has been shown that the anti-proliferative effect of CK2 inhibition was caused by suppressed activation of CK2-mediated PI3K/Akt/mTOR signaling pathways that act downstream of BCR (8). Additionally, it was demonstrated that bortezomib (proteasome inhibitor)/CX-4945 combined treatment induced synergistic apoptotic effects in T- and B-ALL cell lines, *via* reduction of chaperoning activity of Hsp90 (9) and inhibition of NF-κB signaling in T-ALL cell lines as well as in primary cells from T-ALL patients. Recently, it has also been 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 IL-2 production in T-ALL cells (10). Other studies demonstrated that simultaneous treatment of A2780 and SKOV-3 ovarian cancer cells with CX-4945 and cisplatin or gemcitabine promotes synergistic induction of apoptosis. This synergistic effect was a result of inactivation of XRCC1 and MDC1, two mediator/adaptor proteins essential for DNA repair (11).

Recently, it has been reported that protein kinase CK2 phosphorylates *in vitro* human thymidylate synthase (TS) (12, 13) a key enzyme of a thymidylate biosynthesis cycle that catalyzes the conversion of dUMP to dTMP, which

subsequently undergoes phosphorylation to dTTP, a substrate for DNA polymerases. CK2 mediated phosphorylation of TS stabilizes it in an inactive conformation (13). Additionally, another study proved that the phosphorylated recombinant TS forms show decreased (at least by 3-fold) molecular activity, compared to the non-phosphorylated forms, and bind their cognate mRNA repressing their own mRNA translation (14). As inhibition of TS leads to the cessation of DNA replication and thymineless death of proliferating cells, the enzyme is an attractive target for cancer chemotherapy (15). 5-Fluorouracil (5-FU) is one of the most commonly used TS-directed anticancer drug applied in combination with other chemotherapy compounds for treatment of various cancers (16-19). 5-FU is converted inside cells into 5-fluoro-dUMP (F-dUMP), which forms stable complex with TS and thus inhibits deoxythymidine monophosphate (dTMP) production, essential for DNA replication and repair (20). Another important mechanism of 5-FU-induced cytotoxicity is its mis-incorporation into RNA and DNA in place of uracil or thymine, that consequently induces DNA repair and finally double strand breaks (DSB) induction and DNA damage (21).

Based on the assumptions, that CK2 inhibitors may influence the endocellular activity and protein level of TS, as well as inhibition of TS consequently leads to DNA damage (22), which can be repaired with the participation of CK2 (23), we have hypothesized that simultaneous inhibition of CK2 and TS may lead to a synergistic effect in anticancer therapy. Therefore, the aim of our studies was the investigation of the treatment of two cancer cell types, acute lymphoblastic leukemia (CCRF-CEM) and breast cancer (MCF-7), with compound combinations using 5-FU together with different inhibitors of protein kinase CK2: CX-4945, TBBi, or its previously obtained derivative (4,5,6,7-tetrabromo-2-methyl-1H-benzimidazol-1-yl)acetonitrile (2b) (1). We have previously shown that a new inhibitor of CK2α (4,5,6,7-tetrabromo-2-methyl-1H-benzimidazol-1-yl)acetonitrile (2b) (Ki=1.63 μM) demonstrated significantly better anticancer properties toward CCRF-CEM and MCF-7 cells, than the parental compound, TBBi (1). Additionally, the newly obtained derivative 2b induced endocellular CK2 inhibition in a higher extent than TBBi in both CCRF-CEM and MCF-7 cell lines and demonstrated better pro-apoptotic activity towards leukemia cells (~47% apoptotic cells at 10 μM 2b), than TBBi (5.1% apoptotic cells at 10 μM TBBi).

To evaluate the combination effect, we sought to determine CI (combination index values) for each compound combination. Our results show, that synergistic effect occurs in CCRF-CEM only with the combination of 5-FU with 2b, whereas in MCF-7 only with the combination of 5-FU with CX-4945. To explain the mechanism of the observed synergistic effect, the influence of the above mentioned combinations on pro-apoptotic properties, cell cycle progression, CK2 inhibition (phosphorylation extent of Ser529 p65), TS and CK2α protein level was studied. Our results show, that the combination of 2b and 5-FU synergistically enhances inhibition of CK2 in CCRF-CEM, leading to increased apoptosis, while a synergistic effect observed in MCF-7 cells after treatment with the combination of 5-FU and CX-4945 can be due to the delay of 5-FU-induced S-phase arrest recovery.

Materials and Methods

Reagents and antibodies. Dimethyl sulphoxide (DMSO), Molecular Biology grade, used as a solvent for all stocks of the chemical agents, 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 P-Ser529-p65, monoclonal antibodies against TS and CK2α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas polyclonal antibodies against total p65 and monoclonal GAPDH antibodies were from Cell Signalling Technology (Beverly, MA, USA), and Merck Millipore (Darmstadt, Germany), respectively. Secondary goat anti-rabbit IgG-HRP and anti-mouse IgG were purchased from DAKO (Santa Clara, CA, USA). Protease inhibitors were from Bioshop. Nitrocellulose membrane was from GE Healthcare Life Sciences (Freiburg, Germany), solvents for HRP reaction (Western Bright Peroxide and Western Bright Quantum) were purchased from Advansta. CX-4945 was obtained from Biorbyt. TBBi and its derivative – 2b, were obtained as it was described previously (1). 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 treatment with agents. MCF-7 adherent cells (human breast cancer cell line) were cultured in DMEM with high glucose medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (EuroClone), 2mM L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and 10 μg/ml of human recombinant insulin. CCRF-CEM suspension cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% fetal bovine serum (EuroClone) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Cells were grown in 75 cm^2 cell culture flasks (Sarstedt, Nümbrecht, Germany), in a humidified atmosphere of CO_2/air (5/95%) at 37°C.

All the experiments were performed in exponentially growing cultures. Stock solutions of the tested compounds were prepared in DMSO and stored in –80°C for maximum one month. For the cytotoxicity studies, stock solutions of the tested compounds were diluted 400-fold with the proper culture medium to obtain the final concentrations. Stock solutions were diluted in a 1:1 ratio with DMSO or the second compound for a single compound and combination tests, respectively, so the final concentration of vehicle was constant in each case, and the same stock solution was used in each experiment. For cytotoxicity studies 2-fold serial dilutions were prepared in the proper medium containing 0.5% DMSO.

MTT-based viability assay. Before treatment MCF-7 cells were trypsinized in 0.25% trypsin-EDTA solution (Sigma-Aldrich) and seeded into 96-well microplates (Sarstedt) at a density of 1.5-3×10^4 cells/well. Cells were treated with the tested compounds or DMSO (0.5%) at the appropriate concentrations 18 h after plating. CCRF-

CEM were seeded at $4×10^4$ cells/well and treated with the compounds. After 72 h incubation, leukemia cells were centrifuged and supernatants of CCRF-CEM and MCF-7 cells were discarded; subsequently MTT stock solution (Sigma-Aldrich) was added to each well to the final concentration of 0.5 mg/ml. After 2 h incubation at 37°C, water-insoluble dark blue formazan crystals were dissolved in DMSO (200 µl) (37°C/10 min incubation). Optical densities were measured at 570 nm using BioTek microplate reader. All measurements were carried out in a minimum of six replicates and the results expressed as fraction of not viable cells (Fa) relative to 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 mean absorbance obtained for the treated and untreated cells, respectively, whereas B means blank well (without cells).

Detection of apoptosis. CCRF-CEM cells were cultured in 6-well plates in a density of $1.8×10^5$/ml and treated with the tested compounds as it was described above. Then, the cells were harvested by centrifugation at $200 × g$ at 4°C/5 min, washed twice in cold PBS, and subsequently suspended in binding buffer at $1×10^6$ cells/ml. Then, 100-µl aliquots of the cell suspension were labeled according to the kit manufacturer's instructions. Briefly, annexin V-FITC and PI (BD Pharmingen) were added to the cell suspension, and the mixture was vortexed and incubated for 15 min at room temperature in the dark. Then, 400 µl of cold binding buffer was added, and the cells were vortexed again and kept on ice. Flow cytometry measurements were performed within 1 h after labeling. Viable, necrotic, early and late apoptotic cells were detected by flow cytometry using BD FACSCanto II and analyzed with a BD FACSDiva software.

Cell cycle analysis. The MCF-7 and CCRF-CEM cells were cultured in 25 cm2 culture flasks and treated with the tested compounds as it was described above. After exposure to the tested compounds, the cells were washed with cold PBS and fixed at −20°C in 70% ethanol for at least 24 h. Subsequently, the cells were then washed in PBS and stained with 50 µg/ml PI and 100 µg/ml RNase solution in PBS supplemented with 0.1% v/v Triton X-100 for 30 min in the dark at room temperature. 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 the MacCycle (Phoenix Flow Systems, San Diego, CA, USA) software.

Western blotting. The CCRF-CEM cells growing exponentially were seeded at $2×10^5$ cells/ml in 75 cm2 flasks. Subsequently compounds were added in a final concentration of 0.5% DMSO. After 48 h treatment, cells were centrifuged (200 × g, 1,700 rpm, 4°C for 5 min), washed (3 × 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% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium orthovanadate and Protease Inhibitors Cocktail, Roche). The protein concentration was determined using Bradford assay. Equivalent amounts of total protein (40 µg) were analyzed by SDS-PAGE and subsequently western blotting was performed using P-Ser529-p65 (1:500 dilution), total p65 (1:1,000), TS (1:1,000), CK2α (1:500) and GAPDH (1:500 dilution) primary antibodies in Blocking

Buffer: 3% BSA in TBST (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% Tween20). After overnight incubation at 4°C with the primary antibodies, the membranes were washed with TBST and subsequently the secondary goat anti-rabbit IgG-HRP or anti-mouse Ig-HRP antibodies were used at 1:5,000 dilution. ECL substrate (Biorad) was used for detection and immunoblots were scanned using G Box Chemi (Syngene).

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 Image J software. Phosphorylated protein densities were normalized to GAPDH densities, assuming 100% for untreated cells and then they were converted to a percent of the appropriate control.

Results

Two cell lines, CCRF-CEM and MCF-7, representing acute lymphoblastic leukemia (ALL) and breast cancer, respectively, were treated with the combinations of selected inhibitors of CK2 and TS. CX-4945, TBBi, 4,5,6,7-tetrabromo-2-methyl-1*H*-benzimidazol-1-yl)acetonitrile (2b), and 5-FU were used, respectively. Among these compounds CX-4945 is in stage I/II clinical trial, TBBi is established as a strong CK2 inhibitor, 5-FU is a well-known prodrug targeting TS, and 2b was recently synthesized (1) as a new compound which efficiently induced inhibition of CK2 in both CCRF-CEM and MCF-7 and demonstrated better anticancer properties against MCF-7 than TBBi. MTT-based assay and the combination index method (24) were used to determine the type of interaction (*i.e.*, synergistic, additive or antagonistic) between one of the CK2 inhibitors: TBBi, 2b or CX-4945 and 5-FU (inhibition of TS by 5-FU metabolite, FdUMP). Additionally, the dose reduction index (DRI) was determined on the basis of drug interaction 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 (24). To understand the mechanism of a synergistic interaction, the influence of selected compound combinations on cell cycle progression, apoptosis and cellular level of TS and CK2 α proteins as well as on activity of CK2 (phosphorylation extent of Ser529 p65) were investigated.

Influence of compounds on viability of cancer cell lines. To optimize the compounds ratio used in the combination treatment, the influence on the cell viability of each compound used alone, and the shape of the dose effect curve were determined by obtaining Dm values and m parameter, respectively. The results are summarized in Table I. Interestingly, the obtained Dm values (with the exception of 5-FU) were mostly higher for breast cancer cells (6.41-19.27 µM), than for leukemia cells (2.54-8.27 µM). The TBBi derivative 2b was more effective (Dm 2.54-6.41 µM), than CX-

Table I. *The drug potency (Dm) and steepness (m) of the dose-response curves obtained after fitting the MTT-based assay data to the median effect equation using CalcuSyn Software.*

Compound	Molecular target	CCRF-CEM		MCF-7	
		Dm*±SD (μM)	m**±SD	Dm±SD (μM)	m±SD
4,5,6,7-tetrabromo-1*H*-benzimidazole (TBBi)	CK2	11.29±0.57	8.50±2.26	19.27±2.69	1.68±0.24
(4,5,6,7-tetrabromo-2-methyl-1*H*-benzimidazol-1-yl)acetonitrile (2b)	CK2	2.54±0.34	3.95±1.86	6.41±0.63	2.66±1.38
CX-4945 (Silmitasertib)	CK2	3.09±0.50	3.43±0.42	8.36±0.35	1.12±0.18
5-Fluorouracil (5-FU)	TS	120±13.86	3.06±1.16	14.88±2.56	0.74±0.05

*Dm: Values were obtained after fitting the MTT-based assay data to median effect equation using the CalcuSyn software. **m: The slope of the median-effect plot.

Table II. *Combination index values calculated at ED_{50}, ED_{75} and ED_{90}, Dm and DRI values for tested drug combinations at constant ratio for CCRF-CEM and MCF-7 after 72 h treatment.*

Drugs in combination		CCRF-CEM				DRI for 5-FU at Fa=0.95	MCF-7				DRI for 5-FU at Fa=0.95
	Combination ratio*	Combination Index** at			Dm		Combination Index at			Dm	
		ED_{50}	ED_{75}	ED_{90}			ED_{50}	ED_{75}	ED_{90}		
5-FU:CX-4945	12:1	1.79±0.12	1.69±0.40	1.64±0.40	35.94±22.36	12.25±5.79	0.92±0.11	0.74±0.07	0.62±0.08	11.31±3.68	3.25±0.01
5-FU:TBBi	6:1	1.18±0.15	1.18±0.06	1.20±0.04	56.44±4.91	6.57±4.23	1.56±0.11	1.08±0.37	1.83±0.8	25.06±13.37	1.54±0.51
5-FU: 2b	12:1	0.81±0.19	0.65±0.19	0.52±0.18	13.27±5.78	20.60±5.67	1.00±0.11	1.57±0.25	1.32±0.29	10.24±0.03	4.65±2.21

*The combination ratios depended on previously obtained Dm for each tested drug alone; six to eight data points at 2-fold dilution range were used for each combination. **CI values were generated by CalcuSyn software after fitting Fa values obtained by MTT-based assay.

4945 (Dm 3.09-8.36 μM). For leukemia cells, all tested compounds showed sigmoidal dose effect curves (m>1), whereas for MCF-7, sigmoidal curves were obtained only for TBBi and 2b, while for CX-4945 and 5-FU dose-effect curves were hyperbolic (m=1) and flat sigmoidal (m<1), respectively.

Compound combination experiments
Combination and dose reduction index. 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 the fraction of not viable cells (Fa) in the range of 0 to 1. Six to eight concentrations of each compound, in the range of 0.125 × Dm to 6 × Dm in a constant ratio at 2-fold dilution series according to recommendation given by Chou (24) were used in combination experiments. Combination index values (CI) were generated in CalcuSyn Software at ED_{50}, ED_{75} and ED_{90} after fitting Fa values obtained by MTT-based assay (Table II). As it is important to reduce toxicity in clinical treatment and 5-FU is an essential component of chemotherapy in acute lymphoblastic leukemia (ALL) and breast cancer commonly used in consolidation therapy, dose reduction index (DRI) values are shown for tested combinations. The obtained CI values for CCRF-CEM were in

the range of 0.52 to 1.79 with the lowest Dm for 5-FU:TBBi. However, the best synergistic effect with CI values in the range of 0.52-0.81 (synergism/moderate synergism) was achieved for the 5-FU:2b combination and DRI for 5-FU was equal to 20.6 at Fa=0.95. The obtained data indicated, that the dose of 5-FU may be decreased about 20-fold at 95% of leukemia cells affected, when it is in a combination with 2b derivative. For 5-FU:CX4945 combination, the synergistic interaction was observed only in MCF-7 cells with CI values in the range of 0.62-0.92 indicating synergism/light synergism. The quite different combination scenario was clearly observed in CCRF-CEM cells for CX-4945 plus 5-FU combination, and in MCF-7 cells for 5-FU and TBBi, where antagonism occurred. Representative examples of the graphs generated from the analysis of two compound combinations, demonstrating the synergistic effect, *i.e.*, CX-494 plus 5-FU and for CX-4945 plus 2b in both cell lines are shown in Figure 1.

Induction of apoptosis of CCRF-CEM leukemia cells. In order to explain the observed synergism of 5-FU and 2b, induction of apoptosis in CCRF-CEM was studied. Due to a strong synergism of the 5-FU: 2b combination used for CCRF-CEM treatment, concentrations of 15 μM and 30 μM
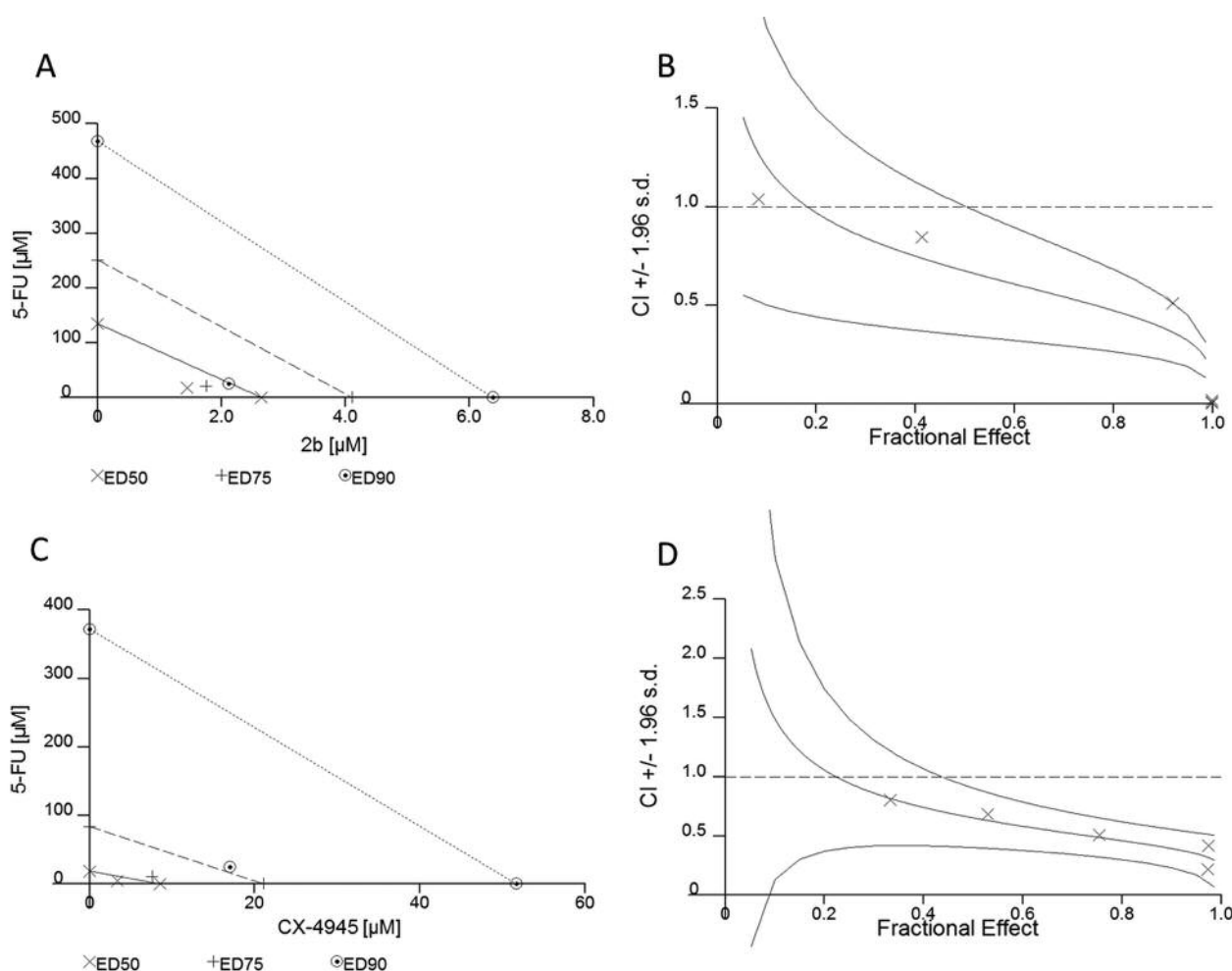
Figure 1. *Synergistic interaction of 5-FU with 2b or CX-4945 in inhibition of viability of CCRF-CEM (A, B) and MCF-7 (C, D). Representative CalcuSyn software-simulated isobolograms (A, C) and Fa-CI curves (B, D), generated after fitting Fa obtained from the MTT assay.*

5-FU, corresponding to 0.125 Dm and 0.25 Dm, respectively, and 3 μM 2b were used in this assay. The results, showing a pro-apoptotic influence of the tested compounds used separately or in two combinations are shown in Figure 2. The treatment of the leukemia cells with the combinations of 15 μM or 30 μM 5-FU with 2b, resulted in 57.1% and 70.1% total apoptotic cells, respectively. Treatment with 15 μM 5-FU, 30 μM 5-FU or 3 μM 2b separately resulted in 10.9%, 18.2% and 14.9% total apoptotic cells. The obtained data indicated synergistic effect of both combinations of 5-FU and 2b 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 and MCF-7 cell lines. Since, it has been shown by others that CX-4945 (2), as well as 5-FU

(25), can affect cell-cycle progression of leukemia and breast cancer cells, the influence of 5-FU in combination with 2b or CX-4945 on cell cycle progression was tested after 48 and 72 h treatment of CCRF-CEM and MCF-7 cells. Concentrations of 5-FU used in this experiment varied in the range of 0.125 and 0.7 Dm depending on the cell line (due to strong synergism in CCRF-CEM cells 15 μM and 30 μM 5-FU, corresponding to 0.125 and 0.25 Dm, respectively, were used, while for MCF-7 cells 5 μM and 10 μM 5-FU, corresponding to 0.3 and 0.7 Dm, respectively, were used). Inhibitors of CK2, 2b and CX-4945 were used at 3 μM and 4 μM concentrations, respectively. The cell-cycle distribution profiles after treatment with each compound used separately and with two compound combinations were determined by flow cytometry. The representative plots with the calculations of cell percentages in each phase of the cell cycle are shown in Figure 3.
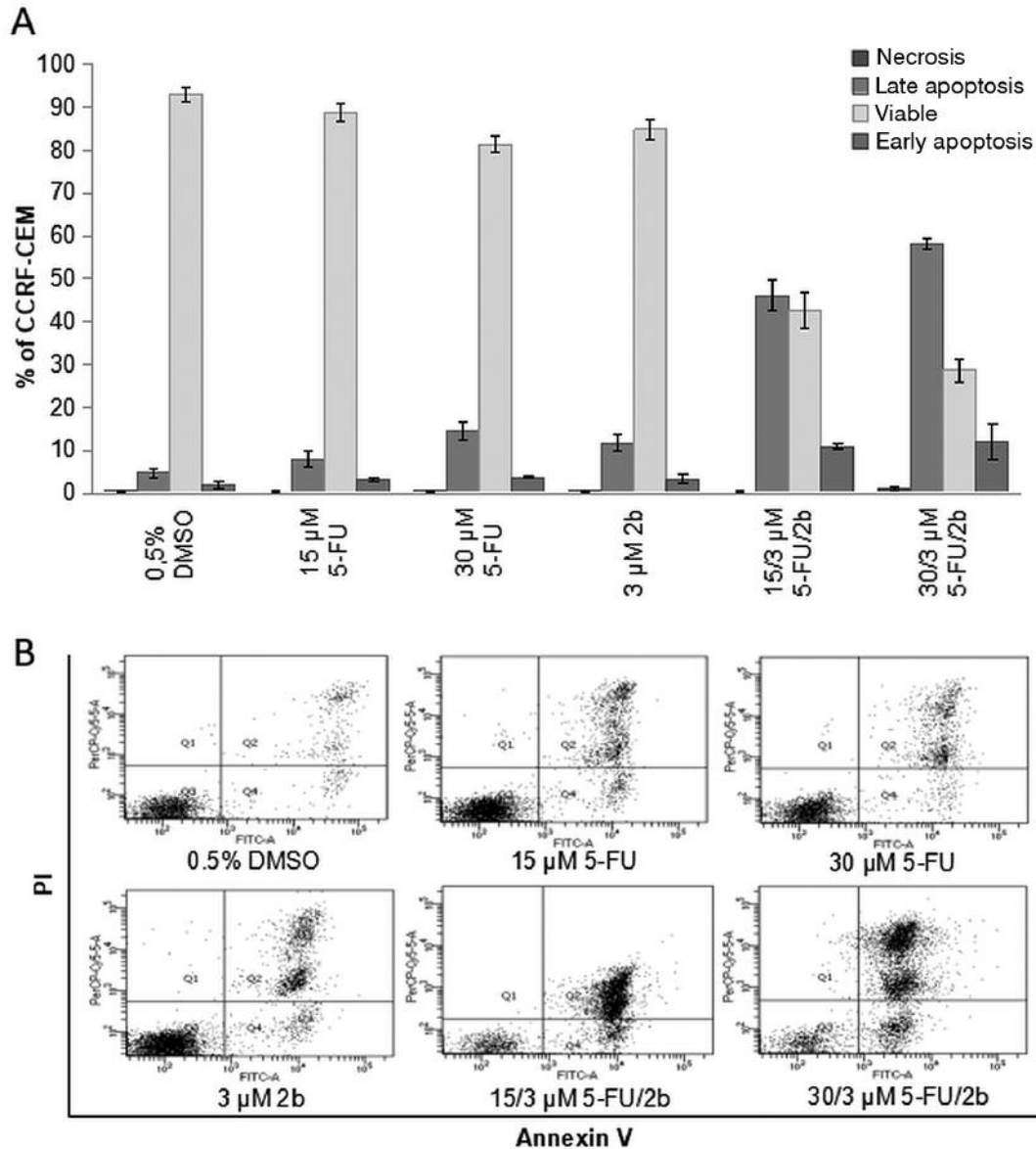
Figure 2. *Pro-apoptotic and anti-leukemic activity of 5-FU and 2b, when used separately or in combination. The data were acquired by FACS cytometer after 72 h treatment. Cells were stained with annexin V-FITC and PI. (A) Mean and standard deviation (SD) of total apoptosis as percent from three independent experiments each. (B) Representative graphs for: control (0.5% DMSO), 15 μM 5-FU, 30 μM 5-FU, 3 μM 2b and combinations of 5-FU (15 and 30 μM) and 2b, respectively.*

The obtained results indicated that 5-FU led to S-phase arrest in both studied cell lines. The number of cells in S-phase correlated with the time of the treatment in MCF-7 cells, with the strongest effect leading to 41% more cells in S phase at 10 μM 5-FU after 72 h treatment than in control cells. In contrary, although the treatment of MCF-7 cells with 4 μM CX-4945 alone led to S-phase accumulation (43%) after 48 h, G2/M arrest occurred (13% more cells, than in control) after 72 h. Interestingly, the observed effect of 5-FU:CX4945 combination in both concentrations seems to unblock the CX-4945 induced G_2/M-phase arrest in MCF-7 cells after 72 h treatment, leading to 12% and 9% less cells in this phase. Additionally, the recovery from S-phase arrest induced by 5-FU in both concentrations occurred after the combined treatment with 5-FU: CX-4945 for 72 h. However, 5-FU:CX4945 combination at 10 μM: 4 μM concentrations delayed replication recovery, leading to 17% more cells in S-phase relatively to control cells after 72 h than after 48 h treatment.
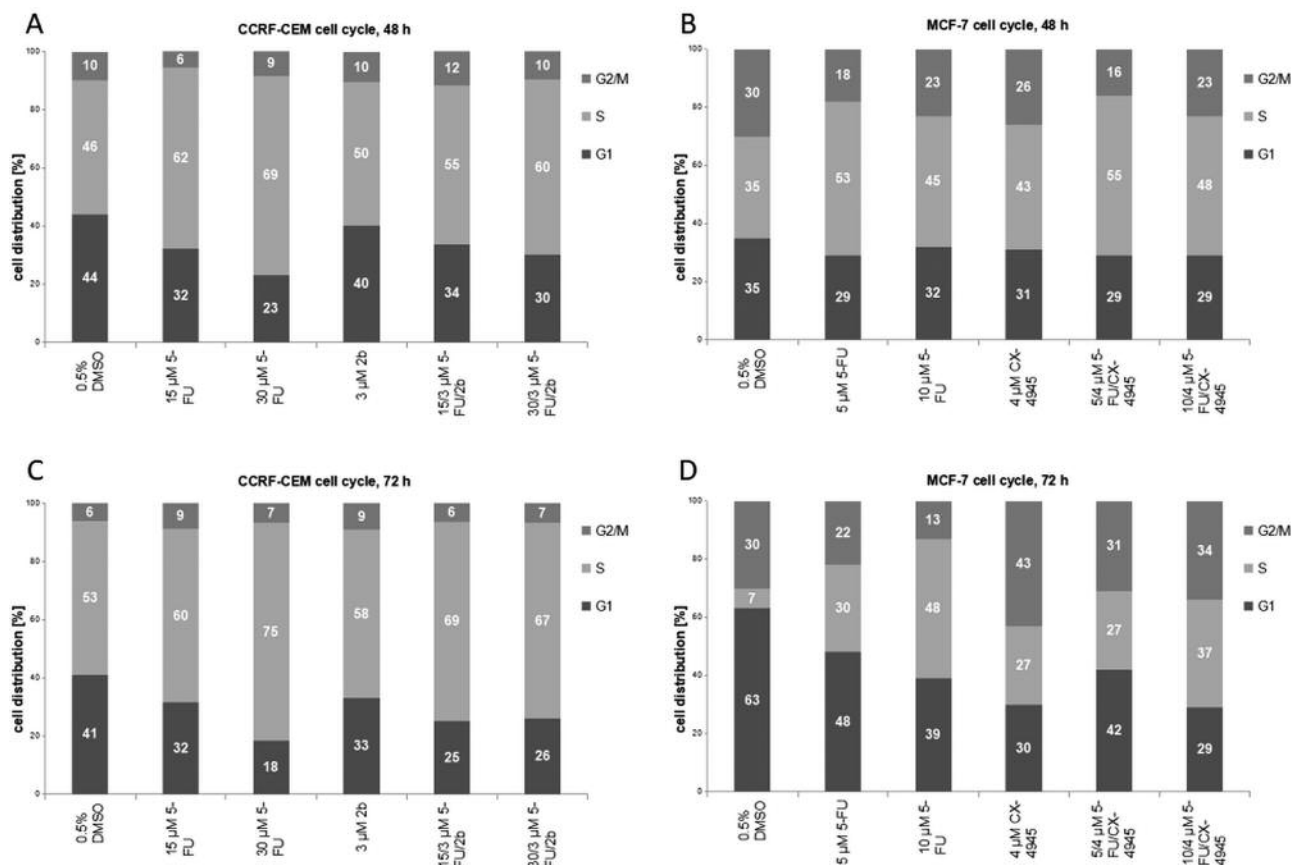
Figure 3. *The effect of compound combinations on a cell-cycle progression in CCRF-CEM and MCF-7 cell lines. CCRF-CEM cells were treated with 5-FU and 2b used separately or in combination for 48 h (A) or 72 h (C), MCF-7 cells were treated with 5-FU and CX-4945 used separately or in combination for 48 h (B) or 72 h (D), and then fixed and stained with PI. The cell cycle distribution profiles of the cells were determined by flow cytometry. Representative graphs indicate the percentages of cells in the G_1, S and G_2/M phases of the cell cycle.*

TBBi derivative 2b at 3 μM concentration induced S-phase arrest in CCRF-CEM after 48 h treatment, but to a lesser extent than CX-4945 in MCF-7 and did not induce G_2/M arrest. However, the similar recovery from 5-FU induced S-phase arrest was observed after 5-FU:2b combination treatment for 48 h and 72 h incubation time, as it was observed in MCF-7 after 72 h treatment with 5-FU:CX4945 (both combinations).

The effect of simultaneous inhibition of TS and CK2 on TS, CK2α, and total p65 protein levels and p65 phosphorylation. In view of the observations that TS protein level increased after inhibition of protein catalytic activity contributed to the inefficiency of chemotherapy (26), we checked the influence of 5-FU, 2b and CX-4945 used separately, and in combinations on the level of TS and CK2α proteins in cellular extracts obtained from both cell lines. The complex of TS with 5-fluoro-dUMP was detected

in CCRF-CEM and MCF-7 cell lines (Figure 4B and D, the upper line) after 5-FU treatment, as well as after treatment with combination of 5-FU with either 2b or CX-4945. Consequently, near 1.6-fold increase of TS protein level, compared to control cells, was observed after treatment with 5-FU and its combination with 2b in leukemia cells (Figure 4A and B) and about 3-fold and 3.5-fold increase of TS protein level after treatment with 5-FU and its combination with CX-4945 in MCF-7 cells, respectively (Figure 4C and D). Interestingly, whereas compound 2b decreased the TS level in CCRF-CEM (Figure 4 A and B), almost 2-fold increase of TS level was observed after CX-4945 treatment in MCF-7 (Figure 4 C and D). Additionally, the endocellular inhibition of CK2 was studied by assaying p65 (NF-κB factor) serine 529 phosphorylation. Inhibition of CK2 activity was detected in leukemia cells after treatment with 2b (68.5% phosphorylation of p65 *versus* control), whereas after combination treatment with 5-FU
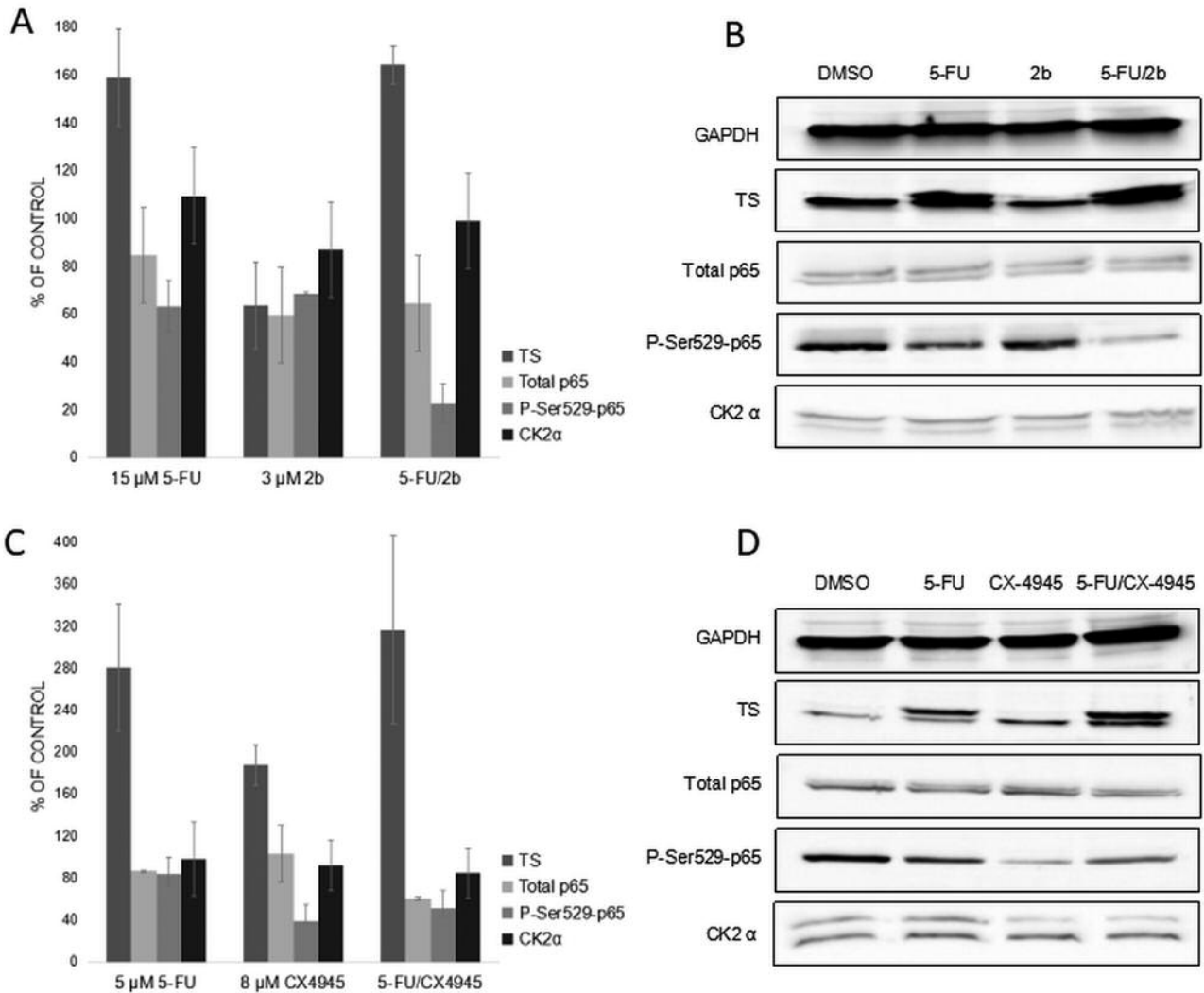
Figure 4. *Western blot analysis of TS, CK2α, total p65 and P-Ser529-p65 in the crude extracts obtained from CCRF-CEM (A, B) or MCF-7 (C, D) after 72 h treatment with 5-FU and 2b (CCRF-CEM) and 5-FU and CX-4945 (MCF-7), used separately or in combinations. 40 μg extract (in RIPA buffer) was loading per well. P-Ser529-p65 (NF-κB factor) was used as a marker of cellular inhibition of CK2. GAPDH was used as a loading control for each sample. Densitometry quantifications for each protein are shown in the graphs (A, C) with untreated cells serving as the reference point (100%), and the representative blots (B, D) are shown for CCRF-CEM and MCF-7, respectively. Preparation of cell extracts and protein detection is described in the Materials and Methods Section.*

and 2b phosphorylation of p65 reached only 22.7%, indicating a synergistic mechanism. As, no essential influence on CK2α protein level was observed after treatment with 2b or its combination, the decreased level of phosphorylated Ser529-p65 is due to inhibition of CK2 activity. In contrary, in MCF-7 cells the combination treatment of 5-FU and CX-4945 did not show a similar synergistic effect, as inhibition of CK2 after the combined treatment was even lower (51% of phosphorylated p65) than after CX-4945 treatment (39% of phosphorylated p65).

Discussion

The effect of inhibitors directed against two different molecular targets, *i.e.* protein kinase CK2 and TS, used alone or in combination, was studied on leukemia and breast cancer cells. Our results demonstrated that the effects of 5-FU, 2b and CX-4945 used in combination was depended on the type of cell line. Interestingly, 5-FU:CX-4945 affected the viability of cells in a synergistic manner only in MCF-7 cell line, whereas 5-FU:2b resulted in synergism only in CCRF-CEM cell line. It has been

shown by others, that drug interaction effect may be dependent on both cell line and concentration ratio (11, 27) and a combination of anti-casein kinase 2 CIGB-300 peptide and 5-FU provided antagonistic interaction in both the NCI-H125 lung cancer cell line and in the SiHa cervical cancer cell line (27). The obtained synergistic effect in CCRF-CEM after combination treatment with 5-FU and TBBi derivative 2b seems to be correlated with stronger inhibition of CK2 and consequently with increase in the pro-apoptotic activity. It has been shown that CK2 and its substrates such as kinase Akt protect cells from apoptosis by phosphorylating a wide range of proteins involved in the apoptotic response (28, 29) and consequently inhibition of CK2 leads to increase apoptosis in many types of cancer cells, including leukemia (7). The increase in the pro-apoptotic activity of 5-FU and 2b combination could not influence MCF-7 cells, taking into account that this line demonstrates caspase-3 deficiency and consequently undergoes cell death that lacked typical apoptotic properties (30).

The accumulation of cells in S-phase, that occurred in both studied cell lines after 48 h and 72 h 5-FU treatment, indicates the initiation of repair mechanisms (17), induced by DNA damage. By contrast to CCRF-CEM, the synergistic effect occurring in MCF-7 after simultaneous CX-4945 and 5-FU treatment, seems to be related to the delay of 5-FU-induced S-phase arrest recovery, leading to 17% more cells in S-phase *versus* control after 72 h, compared to 48 h treatment. The obtained results can be partially correlated to DNA repair mechanisms, as it was shown by others that CX-4945 blocks the DNA repair response induced by gemcitabine and cisplatin in ovarian cancer cells (11). It was demonstrated that phosphorylation of a key mediator of homologous recombination double strand break (DSB) repair MDC1 by CK2 (23) is necessary for the formation of a multiprotein complex, that is required for DSB repair signaling (31). Taking into account that DNA damage is an essential mechanism of cytotoxicity of 5-FU in different cell lines, and DSB induction exhibited a significant correlation with sensitivity of MCF-7 to 5-FU (22), inhibition of CK2 leading to block DNA repair, after 5-FU treatment seems to be a very likely mechanism of a synergistic interaction between 5-FU and CX-4945.

The G_2/M arrest that occurred in MCF-7 after CX-4945 treatment is in a good agreement with the literature data, showing that cell-cycle regulation by CK2 is cell type dependent and CX-4945 treatment induced different effect on cell cycle progression in BT-474 breast cancer and non-NSCLC -small cell lung cancer, BxPC-3 pancreatic cancer and leukemia stem cells, causing an arrest in G_2/M, G_1 or in the late S-G_2-M phases of the cell cycle, respectively (5, 32, 33). It was demonstrated that treatment of cells with CX-4945 resulted in reduced phosphorylation of a key cell-cycle inhibitor protein, p21 (T145) and increased the stability and levels of total p21 and p27 (34).

We detected increased levels of TS protein after 5-FU treatment in both studied cell lines with a higher value of 281% over the control in MCF-7 cell line. It was demonstrated that the clinical use of TS inhibitors is limited by the emerging tumor resistance which is caused by increased TS protein level. Among the mechanisms leading to the rise of TS levels are reduced turnover and increased stability of the protein in the presence of enzyme–inhibitor complexes, and the up-regulation of TS expression (35, 36). The increase in TS expression occurring during 5-FU chemotherapy is associated with an autoregulatory mechanism of translation control for the enzyme (36). Ligand-free TS protein binds its own mRNA and thereby represses translation (38, 39). Complex formation with the dUMP substrate or inhibitors, including FdUMP, abolishes mRNA binding of TS (40). Therefore, increased levels of TS expression are observed during chemotherapy with 5-FU despite inactivation of the enzyme, which ultimately results in emergence of tumor cells resistance. Many studies demonstrated that combination treatment of 5-FU with other agents such as paclitaxel or cisplatin is a promising regimen that is well tolerated in patients with advanced gastric cancer (41-43). Other studies showed, that combination of rapamycin (inhibitor of mTOR protein kinase) and pemetrexed (folate antimetabolite, inhibitor of thymidylate biosynthesis cycle enzymes) synergistically inhibits *in vivo* proliferation of non-small cell lung cancer (NSCLC) xenografts *via* decreasing mTOR activity and pemetrexed-induced TS expression (44). Our studies indicated almost 2-fold increase in TS levels in MCF-7 cells after CX-4945 treatment and 3-fold increase of TS levels after combined treatment with 5-FU. The observed effect at about 60% of CK2 inhibition seems to be in a good agreement with previous studies, demonstrating the influence of phosphorylation of TS on both catalytic and non-catalytic properties of the enzyme. It has been demonstrated by Luo (13) that phosphorylated TS is stabilized in an inactive conformation. Other studies indicated that phosphorylation of TS increased its binding to its own mRNA, causing a negative regulation of translation (14). Therefore, consequently inhibition of TS phosphorylation should lead to increased levels of TS protein in cells. By contrast to CX-45945, a TBBi derivative, 2b, induced a decrease of the TS levels to 64% of control in CCRF-CEM cell line. However, after combined treatment with 5-FU, the TS levels were similar to that observed after 5-FU used separately. The lack of increase of TS levels after 2b treatment in CCRF-CEM can be related to a lower extent of CK2 inhibition to about 30%, in comparison to inhibition of CK2 in MCF-7, although, both inhibitors were used in concentrations close to their Dm values, representing the summary effect of the inhibitor on viability of cells of each line (MTT assay). Therefore, differences in the selectivity of the tested

inhibitors should be taken into account. Interestingly, a decrease of p65 subunit of nuclear factor-kappa B (NF-κB) phosphorylation (Ser529) indicating CK2 inhibition in CCRF-CEM after 5-FU treatment was observed, whereas in MCF-7 p65 total and p65-P-Ser529 were only slightly decreased in compare to control. Recently, it was demonstrated that activation of NF-κB protected colon cancer cells against therapy-induced cytotoxicity (45) and consequently, suppressing PI3K/AKT and NF-κB/iNOS signaling pathways by melatonin synergized the chemotherapeutic effect of 5-FU in colon cancer (19).

In summary, the obtained results indicated that inhibitors of TS may have a better therapeutic effect when combined with a drug that exerts a CK2-inhibitory effect. However, the interaction mechanism can be different and depends on cancer cell line and drug combination. Moreover, taking into account that increase of TS protein levels could contribute to chemo-resistance, further studies need to be performed.

Acknowledgements

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