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Time schedule-dependent effect of the CK2 inhibitor TBB on PC-3 human prostate cancer cell viability

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Abstract. Inhibitors of CK2 kinase inhibit cell proliferation and induce apoptosis in numerous cancer cell lines. Due to these properties, they are considered potentially useful in anticancer therapy. In this study, we show that the exact effect of the specific CK2 inhibitor TBB on PC-3 human prostate cancer cell viability depends on the time schedule of administration: it was not observed when the treatment was directly followed by the viability assay but it appeared when the treatment and the assay were separated by a 24-h incubation without the inhibitor. Such a pattern was maintained when the TBB treatment was combined with either camptothecin or TRAIL. The time schedule-dependence of cell viability was not reflected by a similar dependence of induction of apoptosis. Despite this, the schedule in which a treatment with the CK2 inhibitor precedes that with an anticancer drug seems to be a good choice for a potential therapy against androgen-refractory prostate cancer.

Introduction

CK2 kinase promotes cell proliferation and protects cells from apoptosis (1). CK2 has been shown to be necessary for progression of cells through the cell cycle (2-4) because of phosphorylation of several cell cycle regulators and mitotic proteins (5-7). It has also been identified as a suppressor of apoptosis (8-11). The latter role of CK2 has been attributed to phosphorylation of proteins that are directly involved in apoptosis. CK2-driven phosphorylation protects proteins such as BID (12), Max (13), PTEN (14) or procaspase 2 (15) from proteolytic cleavage that is necessary for progression of apoptosis. It also promotes cell survival acting on c-FLIP expression (16), increasing the level of survivin (17), and enhancing degradation of tumor suppressors: PML (18) and inositol hexakisphosphate kinase-2 (19). Due to general pro-survival effects of CK2 activity, its inhibitors are seriously considered as potential anticancer drugs (20,21). Their application seems especially promising in combination with other anticancer agents whose effective concentration might be reduced this way. There is convincing evidence that inhibitors of CK2 simultaneously lower the activity of the kinase in the cancer cells and facilitate apoptosis induced by other agents (22-24). In the case of a death ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), its synergism with CK2 inhibitors has been demonstrated (8-10).

Despite a well-documented anti-survival effect of inhibitors of the CK2 activity, a pro-survival effect of 4,5,6,7-tetrabromobenzotriazole (TBB), an inhibitor specific for CK2 kinase (25), has been recently reported for the cellular response to camptothecin (CPT) treatment (26). It has been suggested as a result of inhibition of phosphorylation of the CPT target topoisomerase I that reduces its sensitivity to CPT (26). Such diverse effects of CK2 inhibitors may be explained by a highly pleiotropic character of CK2 kinase that targets few hundreds of protein substrates (27,28). Their serine or threonine residues are surrounded by residues that differently influence efficiency of phosphorylation (28,29). Moreover, phosphorylation of the substrates containing distinct target sequences is individually sensitive to changes of CK2 activity (5). Therefore, inhibition of the activity may differently influence phosphorylation of particular cellular substrates and the processes in which they are engaged.

Activity of CK2 kinase is elevated in tumor cells. In prostate cancer cells the activity is ~3-5 times higher than in a normal tissue (30), making them a promising target for drugs based on CK2 inhibitors. However, although CK2 inhibitors lower activity of the kinase both in androgen-sensitive and androgen-refractory prostate cancer cells (31), only in the first case it leads to apoptosis whereas the latter cells are not sensitive to the inhibitors (24,31,32). In this study, we demonstrate that viability of the androgen insensitive PC-3 cells may be diminished by TBB acting either alone or combined with anticancer agents CPT or TRAIL when a proper time schedule of the administration is applied. We also show that the time schedule-dependent activity of TBB does not come from its effect on apoptosis in PC-3 cells.

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Materials and methods

sTRAIL preparation. cDNA corresponding to human soluble TRAIL (sTRAIL; amino acids 114-281) was cloned into the

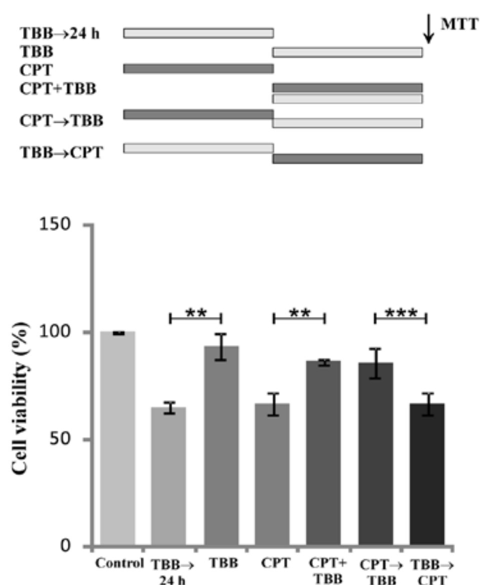

Figure 1. Viability of prostate cancer PC-3 cells treated according to different time schedules with either TBB or TBB combined with CPT. The schedules shown in the upper part of the figure: each bar corresponds to 24 h treatment. The histogram shown in the lower part of the figure: metabolic activity of cells measured by the MTT assay expressed as the percentage of mock treated cells (Control). The average ± SD (n=3) is shown and asterisks indicate a significant difference between presented schedules.

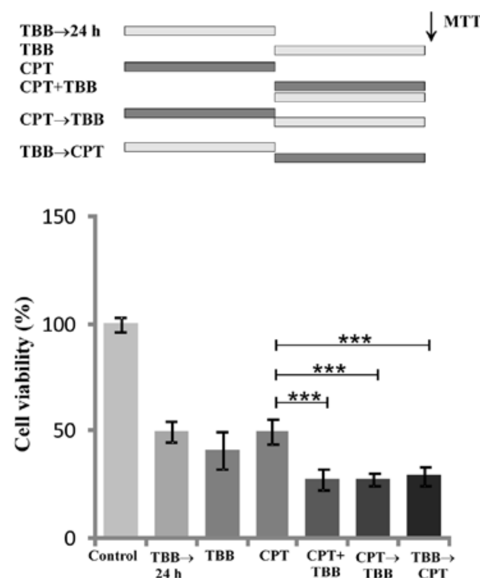

Figure 2. Viability of ovarian cancer HeLa cells treated according to different time schedules with either TBB or TBB combined with CPT. Details as in Fig. 1.

pQE30 bacterial expression vector (Qiagen) and expressed in *Escherichia coli* M15 [pREP4] (Qiagen). After induction of sTRAIL expression using isopropyl β-D-thiogalactopyranoside (final concentration 1 mM, 37˚C, 4 h) bacterial cells were harvested, and sTRAIL was purified on a Ni-NTA agarose resin (Sigma). A trimeric active form of purified sTRAIL was confirmed using gel-filtration chromatography on Superdex-200 (Amersham).

Cell culture and MTT assay. PC-3 and HeLa cells were purchased from the European Collection of Cell Cultures (ECACC). PC-3 or HeLa cells were cultured routinely in RPMI-1640 and DMEM media, respectively, which were supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humidified atmosphere of 5% CO_2. Cells were seeded at $5x10^4$ cells/well (PC-3) or $2x10^4$ (HeLa) in 24-wells plates and cultured for 72 h. TBB (final concentration 60 μM), CPT (final concentration 5.8 nM), 2-deoxyglucose (2-DG; final concentration 0.5 mM) or TRAIL (final concentration 13.3 ng/ml) were added to the medium individually or in a combination and the cells were cultured for additional time, indicated on each figure. After treatment, the medium with the agent was removed and 500 μl of MTT mixture (0.5 mg/ml for PC-3 and 5.0 mg/ml for HeLa cells in medium without phenol red) was added to each well and incubated for an additional 1 h at 37˚C. The formazan crystals were diluted in 250 μl of DMSO. The absorbance was measured at 570 nm (33).

Detection of apoptosis. PC-3 cells were cultured and treated with TBB and/or TRAIL as described above. Then, both floating and adherent cells were harvested. The floating cells were collected by centrifugation at 700 x g for 5 min at 4˚C. Adherent cells were first trypsinized and then collected by centrifugation at 700 x g for 5 min at 4˚C. Both cells were resuspended in the Annexin V binding buffer (BD Pharmingen) and pooled (34). Apoptotic cells were detected by flow cytometry using a FACSCalibur (Becton Dickinson) and analyzed with a CellQuest software. Cells were stained with an annexin V-FITC and propidium iodide using a Annexin V-FITC Apoptosis Detection kit I (BD Pharmingen) according to the manufacturer's instructions.

Statistical analysis. All experiments were carried out at least in triplicate, and for each individual point three independent measurements were done. The average from all measurements was expressed graphically ± SD. The differences between groups were calculated using One-Way ANOVA and Tukey HSD, Bartlett's test and Shapiro-Wilks W statistic. $p<0.05$ was considered statistically significant: $p<0.05$, $p<0.01$, and $p<0.001$. Statgraphics Plus software (StatPoint Technologies Inc., USA) was used for analysis.

Results

Similarly to that previously reported (24,31), we observed that a 24-h treatment of the PC-3 cells with 60 μM TBB did not significantly influence cell viability. However, if the treatment was followed by a 24-h period when cells were cultured without the inhibitor, the viability was considerably lowered (Fig. 1, TBB vs. TBB→24 h). The above time schedule-dependence of the effect was maintained when TBB was administered in combination with CPT. We observed reduction of CPT-induced drop in cell viability by TBB when both agents were administered simultaneously (Fig. 1; CPT vs. CPT+TBB). However, despite the earlier suggestion that the reduction might result from diminished phosphorylation of the CPT-target protein topoisomerase I (26), we observed a similar effect when the TBB treatment followed that with CPT (Fig. 1; CTP→TBB) and thus it could not influence sensitivity of topoisomerase I to CPT. Moreover, the reduction was not observed when TBB treatment

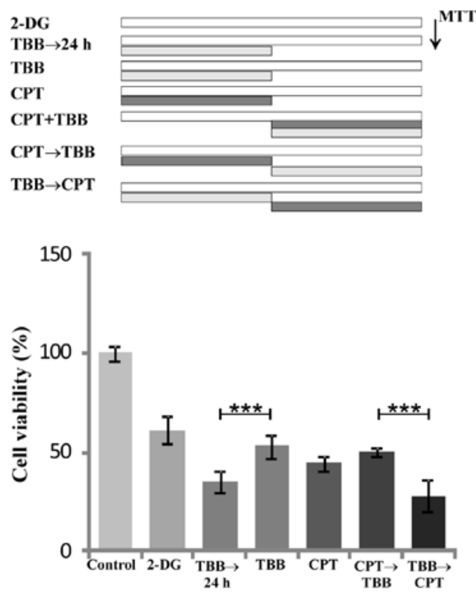

Figure 3. Effect of 2-deoxyglucose (2-DG) on viability of PC-3 cells treated with either TBB or TBB combined with CPT. Details as in Fig. 1.

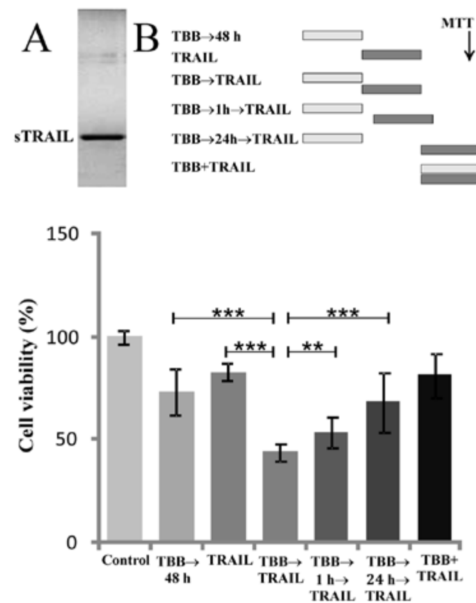

Figure 4. Effect of TRAIL on viability of PC-3 cells. (A) SDS-PAGE of purified preparation sTRAIL. (B) Viability of PC-3 cells treated according to different time schedules with either TBB or TBB combined with sTRAIL. Details as in Fig. 1.

preceded administration of CPT (Fig. 1; TBB→CPT). The above time schedule-dependence of the anti-survival effect of TBB combined with CPT was specific for PC-3 cells. In the case of HeLa cells no difference in cell viability was observed between schedules TBB→CPT and TBB+CPT (Fig. 2). We also noticed that for PC-3 cells the effects of TBB and CPT were not additive (Fig. 1; TBB→24 h, CPT and TBB→CPT) whereas for HeLa cells a clear additivity of the effects of TBB and CPT was observed (Fig. 2).

Two conclusions may be drawn from the above experiments. Firstly, the pattern of effects of TBB on the CPT-induced decrease in cell viability suggested that the anti-survival activity of the inhibitor, clearly visible for HeLa cells, was masked in PC-3 cells by a pro-survival component which was the most pronounced when a TBB treatment precisely preceded the cell viability assay. Next, the results indicated that a lowered efficiency of CTP exhibited in the presence of TBB did not result from inhibition of phosphorylation of the CPT target topoisomerase I (26), but was caused by reduction of phosphorylation of another target. Looking for a possible CK2 target that might act as an anti-survival factor we paid attention to the glycolytic enzyme phosphoglucose isomerase. Cell viability is critically dependent on glycolysis because of its direct link to some stages of apoptosis (35-39) and because of controlling the ATP concentration (35,36). Phosphorylation of phosphoglucose isomerase inhibits its activity and is suggested to be a factor that may influence glucose metabolism in the cancer cells (40). To examine whether the time schedule-dependence of the TBB effect might be linked with its effects on glycolytic pathway, we carried out a set of experiments similar to those described above but performed in the presence of 2-deoxyglucose (2-DG), a specific inhibitor of glycolysis. We found the pattern similar to that observed in the absence of 2-DG: decrease of cell viability induced by either TBB acting individually or together with CTP was the most pronounced when TBB treatment was followed by a period when cells were cultured without the CK2 inhibitor (Fig. 3). Therefore, we concluded that the anti-survival component of the activity of CK2 in PC-3 cells is not directly linked with phosphorylation of glycolytic enzymes.

We asked next whether the pro-survival component of TBB activity would be maintained when the inhibitor was combined with the anticancer agent that acts synergistically with it in inducing apoptosis. The synergy has been shown for a death ligand TRAIL (8-10) that in contrast to CPT activates an extrinsic pathway of apoptosis. We used a recombinant sTRAIL the purity of which is shown in Fig. 4A.

Similarly to the experiments performed with CPT, efficacy of sTRAIL combined with TBB depended on the time schedule of the administration of both agents (Fig. 4B). When both agents were administered simultaneously, TBB did not intensify the effect of sTRAIL acting individually (Fig. 4B; TRAIL vs. TBB+TRAIL). On the other hand, when a TBB treatment preceded that of sTRAIL, cell viability was significantly reduced (Fig. 4B; TBB→TRAIL).

According to the results presented above, when a TBB treatment was directly followed by administration of sTRAIL, the inhibitor was still able to improve the effect of sTRAIL but did not exert any pro-survival effect. However, the extent of reduction of cell viability decreased when a gap between the TBB and the sTRAIL treatments was gradually lengthened (Fig. 4B; TBB→TRAIL vs. TBB→1 h→TRAIL and TBB→24 h→TRAIL).

The lack of effects of the treatment of PC-3 cells with TBB on cell viability has been mainly attributed to defects in a signaling pathway that prevents induction of apoptosis (32). Therefore, we asked whether the time schedule-dependence observed in the cell viability test would also be found in the apoptosis assay. The Annexin V/propidium iodide double staining assay revealed a pattern different from that observed when cell viability was analyzed. An example of the FACS analysis carried out for different schedules of the treatment is shown on Fig. 5. In all experiments the number of apoptotic cells, expressed as a ratio to the number of that cells in the culture treated with sTRAIL

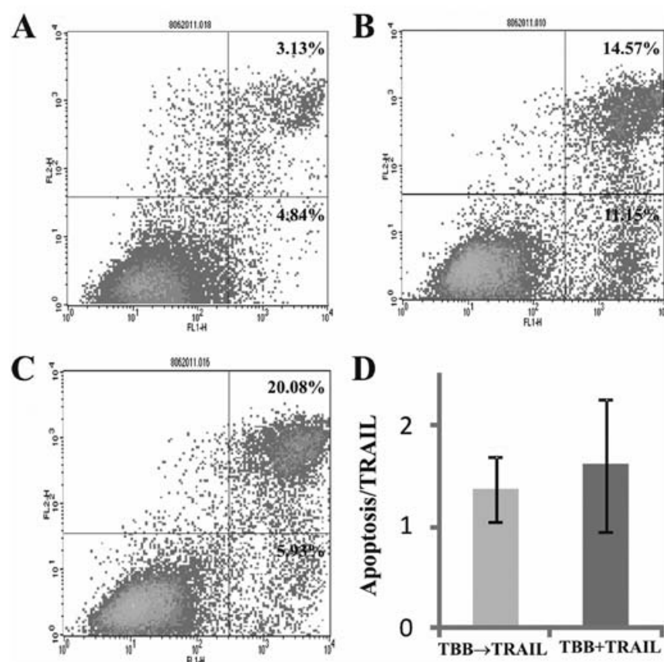

Figure 5. Apoptosis in PC-3 cells treated according to either TBB→TRAIL or TBB+TRAIL schedule. Quantitative analysis of the apoptotic cells was carried out using annexin-V/FITC and propidium iodide in exponentially growing cells. The flow cytometric analysis was performed and the percentage of apoptotic, live and necrotic cells was measured. The left lower quadrant represents live cells, the right lower quadrant early apoptotic cells, the top right quadrant late apoptotic cells, the top left quadrant necrotic cells. This experiment was repeated with similar results on at least three occasions. (A) Control cells; (B) TBB→TRAIL schedule; (C) TBB+TRAIL schedule. (D) The average number of apoptotic cells (± SD) determined in TBB→TRAIL or TBB+TRAIL schedule presented as a ratio to the number of apoptotic cells in the culture treated with sTRAIL only.

only, did not significantly depend on whether the TBB→TRAIL or TBB+TRAIL schedule was applied (Fig. 5).

Discussion

Specific feature of androgen-refractory PC-3 cells is that they do not respond to inhibition of CK2 activity and do not undergo apoptosis upon treatment with CK2 inhibitors (24,31,32). In this study, we described another specific effect of the CK2 inhibitor TBB on PC-3 cells which is hiding the reduction of cell viability resulting from apoptotic activity of CPT or TRAIL. An explanation of the pro-survival activity of TBB that is responsible for hiding has not been provided here. However, we showed that the inhibition of CK2 activity did not exert a dual effect on apoptosis induced in PC-3 cells by TRAIL. We also excluded a possibility that increased cell viability observed upon administration of TBB together with the CPT treatment could result from diminished phosphorylation of the CPT target. Lastly, we observed that reduction of the energy supply did not eliminate the pro-survival component of TBB activity.

The observation presented in this study has an impact on three issues concerning cellular activity of CK2. The first one is that the CK2 activity which is generally considered as a pro-survival factor (1) has also an anti-survival component, at least in the PC-3 cells. Identification of this component would improve our understanding of the complex regulatory network governed by activity of CK2 kinase. The other issue is linked with the potential use of inhibitors of CK2 in anticancer therapy (20,21) and concerns their efficacy as sensitizers to anticancer agents such as TRAIL. Although the schedule in which TBB treatment precedes administration of sTRAIL does not cause more cell death, it prevents elevated metabolic activity of the cancer cell that may overwhelm the apoptotic efficacy of the combination of TBB and TRAIL. Therefore, the TBB→TRAIL schedule seems to be a better choice for a potential application of this drug combination against androgen-refractory prostate cancer than simultaneous administration of both drugs. Lastly, the observation presented here pays attention to consequences of pleiotropic effects caused by the inhibitors of CK2 kinase. Although they generally reduce proliferation and induce or help apoptosis to proceed (1), they may also improve metabolic activity of the cancer cells as is the case in PC-3 cells. Therefore, the observation presented herein points to looking for drugs specifically targeted to apoptotic proteins impaired by increased activity of CK2 kinase as an alternative to inhibition of the enzyme itself.

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