Abstract. Malignant gliomas are highly resistant to current therapeutic approaches due to genetic alterations rendering them resistant to cell death. CK2, a ubiquitous and constitutively active serine/threonine kinase, frequently elevated in tumors, contributes to enhanced cell proliferation and resistance to apoptosis. Inhibition of CK2 expression or treatment with inhibitors of CK2 affected survival or induced apoptosis in various cancer cells. Here we compared cytotoxic effects of well-known and new CK2 inhibitors: 4,5,6,7-tetrabromo-1H-benzotriazole (TBB), 4,5,6,7-tetrabromo-1H-benzimidazole (TBI), 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT), the related 3-(4,5,6,7-tetrabromo-1H-benzimidazol-1-yl)propan-1-ol (MB001), 3-(4,5,6,7-tetrabromo-1H-1,2,3-benzotriazol-1-yl)propan-1-ol (MB002), 3-(4,5,6,7-tetrabromo-2H-1,2,3-benzotriazol-2-yl)propan-1-ol (MB003) and also structurally similar to above compounds pentabromobenzylisothiourea (ZKK1) and its derivatives (ZKK2-8) on cultured malignant glioma cells. TBI, ZKK1 and MB001-3 were more effective than TBB in inducing growth arrest and cell death in glioma cells. TBI and ZKK1 strongly induced apoptotic death involving caspase 3 and 7 activation followed by PARP cleavage. DMAT strongly upregulated the expression of cytotoxic ligand and its receptor Fas. Structural modifications of ZKK1 largely affected its efficacy: exchange of Br- to Cl- or F-substituents on the pentabromophenyl ring and inclusion of the bulky N-phenyl substituent in thiourea fragment of ZKK1 diminished cytotoxic activity, while N-substitution with short alkyl groups or an allyl group had opposite effects. Interestingly, TBI at moderate dose did not affect viability of non-transformed astrocytes, suggesting some specificity toward tumor cells in cytotoxic action. TBI, DMAT and ZKK1-induced apoptosis associated with caspase cascade activation in human malignant glioblastoma cells with mutated PT53 and PTEN genes. The reported data demonstrate that suitably modified polybromobenzene molecules exhibit a significant cytotoxic potential towards malignant glioblastoma cells.

Introduction

Gliomas are the third most frequent cause of cancer-related deaths in adults and the second most in children. Despite numerous advances in chemotherapeutic, radiation and surgical procedures, the survival rate for patients with gliomas/glioblastomas has remained unchanged for the past 30 years and a chance of two-year survival for patients with WHO grade IV glioblastoma multiforme is less than 30% (1,2). Even combination of treatment regiments is ineffective, because resistant tumor cells infiltrate normal brain tissue, so that tumor recurrence is inevitable. Frequent deficiencies in death ligand/receptor signaling, cyclin dependent kinase inhibitors, mutations in genes coding for tumor suppressors: PTEN, TP53 greatly limit an efficacy of current treatments and call for the development of novel therapeutic approaches overcoming the resistance of glioblastoma cells (4-6).

CK2 (casein kinase 2) is the most pleiotropic of all protein kinases with more than 300 substrates implicated in a wide variety of cellular functions in gene expression, signal...
transduction, proliferation and cell survival (7-9). Antisense oligonucleotides against CK2α or CK2β (10), microinjection of anti-CK2 antibodies or inhibitors of CK2 (11-14) inhibit cell cycle progression. Elevated CK2 activity has been demonstrated in a number of cancers and shown to regulate the activity of various oncoproteins and tumor suppressor proteins. Development of CK2 inhibitors is ongoing in preclinical studies, resulting in the generation of a number of CK2-directed compounds (reviewed in refs. 9,14,15). Some of these inhibitors such as 4,5,6,7-tetramethoxy-1H-benzimidazole (TBI) and 2-dimethylamino-4,5,6,7-tetramethoxy-1H-benzimidazole (DMAT) inhibited the growth of malignant lymphoblastic leukemia cells with a better efficiency than Imitinib, the well-known tyrosine kinase inhibitor (16-18). Dowregulation of CK2 activity with specific inhibitors or silencing of CK2α expression induced cell death in drug-resistant R-CEM cells, overcoming the multidrug resistance phenotype (19,20).

Inhibition of CK2 with 100 μM TBB or 20-40 μM DMAT significantly decreased viability and induced apoptosis of three human colon cancer and breast cancer cells in vitro (21). Furthermore, the CK2 specific inhibitors 5,6-Dichloro-1H-benzimidazole and Apigenin induced apoptosis in pancreatic cancer cells (22), and hormone-sensitive prostate cancer cells (23). DMAT induced caspase-mediated killing of human breast cancer MCF-7 cells (24) suggesting a broad spectrum of vulnerable cancer cells.

The two most widely used cell permeable CK2 inhibitors are TBB and DMAT, the latter one displays the lowest K(i) value reported for a CK2 inhibitor (40 nM) and its efficacy in endogenous CK2 inhibition was reported several-fold higher than TBB (25-27). Protein kinase CK2 is abundant in rat brain when compared with other rat tissues (28) and our unpublished data suggest elevated level of CK2 in glioma cells vs. non-transformed astrocytes. CK2 is involved in phosphorylation of the tumor suppressor PTEN in vitro and in glioblastoma cells, DMAT (20 μM) reduced PTEN phosphorylation in glioblastoma cell lines (29).

In the present study, we evaluated the cytotoxic efficacy of widely used CK2 inhibitors (25-27,30), as well as newly synthesized compounds of MB001-3 (31) and ZKK1-6 series on cultured glioblastoma cells and non-transformed glial cells. We determined molecular pathways involved in the induction of cell death following treatments. Furthermore, we determined drug effects on different human malignant glioblastoma cell lines with mutated TP53 and/or PTEN tumor suppressors, highly resistant to radiation and majority of anticancer drugs. The results described here demonstrate for the first time the promising antitumor efficacy of newly synthesized CK2 inhibitors and provide insights into molecular mechanism of their toxic action.

Materials and methods

Synthesis. Chemicals: The 4,5,6,7-tetramethoxy-1H-benzimidazole (TBI) and 4,5,6,7-tetramethoxy-1H-benzotriazole (TBB) were obtained by excessive bromination of 1H-benzi-

2,3-Dichlorobenzothiazole was obtained according to Iwai et al (35), the compounds ZKK1-6 and ZKK8 were synthesized according to the following procedure: to the hot solution of respective thiourea (1 mmol) in anh. ethanol (20 ml) pentabromobenzyl-(for ZKK1-6) or pentafluorobenzyl-(for ZKK8) bromide (1.1 mmol) was added. The reaction mixture was stirred and refluxed for 20 min. Next, it was concentrated to 15 ml and left to crystallize. White crystals of chromatographic pure compounds were obtained. For analytical and biological purposes small samples were crystallized from anh. ethanol. Melting points and yields were as follows: ZKK1, 273-276˚C (73%); ZKK2, 266-268˚C (88%); ZKK3, 242-244˚C (85%); ZKK4, 229-231˚C (77%); ZKK5, 250-252˚C (75%); ZKK6, 243˚C (72%) and ZKK8, 201-203˚C (84%). 1H-NMR spectra and elemental analyses are available from the authors upon request.

Cell cultures and treatments. The rat C6 glioma cell line and the human cell lines: T98G (derived from a glioblastoma multiforme, WHO grade IV), LN229 (derived from an astrocytoma WHO grade III) were from ATCC. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin). Cells were grown in 24-well plates or 10-cm diameter culture plates (Corning) in a humidified atmosphere of CO2/air (5/95%) at 37˚C. Primary cultures of cortical astrocytes were prepared from cortices of newborn rats as previously described (36). Astrocytes were plated on poly-L-lysine-coated 24-well culture plates (7x10^4 cells per well) and maintained in DMEM with Glutamax (Sigma) containing 10% fetal bovine serum and 1% antibiotics. Cells were treated with specific compounds dissolved in DMSO or DMSO at the corresponding concentrations 18 h after plating. The effects of the compounds were monitored at various time points by phase-contrast microscopy.

Proliferation assay. BrdU incorporation test was used to determine the cell proliferation rate. Briefly, C6 glioma cells (1x10^6 cells/well) were seeded onto a 24-well plate, cultured for 18 h, then the treatment (DMSO or various drugs) was applied for 18 h. BrdU (10 μM) was added to the culture medium and cells were incubated for 6 h. Subsequently, cells were fixed and the level of BrdU incorporation was determined according to the manufacturer’s protocol (Cell Proliferation ELISA BrdU assay, Roche Diagnostics GmbH Mannheim, Germany).

Cell viability (MTT metabolism assay). Cells were cultured in 96-well plates with the addition of the indicated concentrations of CK2 inhibitors or DMSO. MTT stock solution (Sigma-Aldrich) was added to each well to a final concentration of 0.5 mg/ml. After 4 h of incubation at 37˚C water-insoluble dark blue formazan crystals were dissolved in lysis buffer containing 20% SDS and 50% DMF. Optical densities were measured at 570 nm using a scanning multiwell spectrophotometer. All measurements were carried out in triplicate.

Western blot analysis. Cells were collected in PBS (phosphate-buffered saline) with protease inhibitors (1 mM
PMSF, 10 μg/ml aprotinin and leupeptin, 0.7 μg/ml pepstatin A) and total protein extracts were prepared mixed with 2X Laemmli sample buffer followed by boiling for 15 min. After centrifugation at 15,000 rpm for 15 min at 4˚C, protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham).Equal protein loading was confirmed by staining the membranes with Ponceau Red (Sigma). Phosphorylation of Rb (Retinoblastoma protein) was determined with antibody recognizing Ser780 phospho-Rb (Cell Signaling, USA). Caspase activation was evaluated by Western blot analysis in cell extracts using specific antibodies recognizing total (p57) and cleaved (p43/41) caspase 8, total (p59) and cleaved (p37) caspase 9, cleaved caspases: p20 (caspase 7), p19/p17 (caspase 3) and p116 and p89 for PARP (Cell Signaling) as described (37,38). Membranes were incubated with primary antibodies diluted in TBS-T (10 mM Tris/HCl, pH 7.6, 0.12 M NaCl, 0.1% Tween-20 and 0.05% sodium azide) containing 5% skimmed milk. Antibody recognition was detected with the anti-rabbit IgG secondary antibody linked to horseradish peroxidase (Cell Signaling). β-actin was detected using monoclonal anti-β-actin, peroxidase-linked antibody (Sigma). Immuno-complexes were visualized using enhanced chemiluminescence detection system (ECL, Amersham).

RNA isolation and PCR analysis. Total RNA was isolated according to manufacturer’s protocol (Quagen), including a DNase digestion step. cDNAs were synthesized by extension of oligo(dT)15 primers with 200 units of M-MLV reverse transcriptase in a mixture containing 1 μg total RNA in 20 μl. The expression of Fas and FasL was evaluated by a quantitative real-time PCR using the 7500 real-time PCR System (Applied Biosystems); amplification of rat 18S rRNA in the same samples served as the internal control. Reaction volume (20 μl) consisted of cDNA equivalent to 50 ng RNA, 1x SYBR Green PCR master mix (Applied Biosystems) and 0.9 μM of each primer. The following primers were used: rat 18S sense (5'-GTAACCCTTGGAAACCCCATT-3'); antisense (5'-CACCCAATCGGATAGCCG-3'); rat FasL sense (5'-CACCCACAGCCTTAGAGTATCATC-3'), antisense (5'-CGTTCTTTTTCCTCCTTACG-3').
The thermal cycling conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 15 sec at 95˚C for denaturation and 1 min at 60˚C for annealing and extension. The specificity of the PCR reaction was confirmed by a single peak in a dissociation curve. Ct, the threshold cycle, was determined after automatic setting the threshold in the linear amplification phase of the PCR reaction and averaged for each sample assayed in duplicates. Fold change was calculated as $2^{-\Delta\Delta C_t}$.

Statistical analysis. Data are presented as mean ± SEM and were analyzed by ANOVA followed by Newman-Keuls test using Statistica software as indicated. Differences were considered statistically significant for $p<0.05$.

Results

Novel CK2 inhibitors potentiate apoptosis and reduce cell death in cultured glioma cells. Fig. 1 shows the structure of tested compounds. We compared the biological effects of well known CK2 inhibitors: 4,5,6,7-tetrabromo-1H-benzotriazole [TBB, IC50 0.5 μM, (39)] and the related 4,5,6,7-tetramethylbenzotriazole [TBI IC50 0.5 μM, (39)] with an influence of recently developed 2-dimethylamino-4,5,6,7-1H-tetrabromobenzimidazole (DMAT IC50 0.14 μM, (39)), N-hydroxypropyl analogs MB001-3 [IC50 0.3-0.5 μM (31)] and pentabromobenzylisothioureas (ZKK1 IC50 7-50 μM, unpublished results) on growth and survival of cultured glioblastoma cells (Fig. 1). Cytotoxic effects of newly developed compounds were determined using MTT metabolism test 24 h after the treatment. While TBB did not affect the number of living cells, TBI and new reagents effectively reduced the viability of C6 glioma cells. The most effective was ZKK1 treatment leading to cell death of approximately 50-70%. Treatment for 48 h resulted in a massive cell death in over 70% of the cells. MTT metabolism test allows to quantify changes in the number of living cells without resolving if drug treatment affects cellular proliferation or viability, thus next we determined the effects of compounds at concentration 50 μM or 0.2% DMSO on the proliferation of C6 glioma cells using BrdU incorporation test. As shown in Fig. 2A, TBI, DMAT and ZKK1 with a similar efficacy reduced the BrdU incorporation during DNA synthesis.

Phase-contrast micrographs showed that C6 cells exposed for 24 h to TBI, DMAT and ZKK1 showed typical apoptotic changes in cellular morphology: retraction of cell extensions, cell shrinkage, membrane blebbing consistent with ongoing
apoptotic cell death. The enhanced apoptosis by TBI, DMAT and ZKK1 was confirmed by nuclear staining with DAPI visualizing morphological alterations of cell nuclei (not shown). The apoptotic cell-death is executed through activation of intracellular proteinases, known as caspases that results in cleavage of caspase substrates, and finally cell demise (42-44). We evaluated activation of caspase 7, caspase 3 and PARP proteolysis by immunodetection of cleaved proteins. C6 glioma cells were treated with 50 μM TBI, DMAT and ZKK1 for various times (Fig. 3). As shown in Fig. 3, a strong activation of executory caspases 3 and 7, as well as accumulation of cleaved PARP occurred in TBI and DMAT-treated cells 30 h post-treatment. ZKK1 induced activation of caspases much faster than other compounds: accumulation of cleaved caspases 3 and 7, and PARP was observed at 12-24 h.

Determining of mechanisms of cell death induced by novel CK2 inhibitors in glioblastoma cells. There are two major and characterized apoptotic pathways leading to caspase cascade; ‘intrinsic’ and ‘extrinsic’ (45-47). In the extrinsic pathway, the apoptotic machinery is triggered by the interaction of death ligands of the tumor necrosis factor (TNF) family (such as Fas/CD95 ligand and TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L) with their corresponding death receptors (such as Fas/CD95, DR4 and DR5) (45). The intrinsic apoptotic pathway involves alterations in mitochondrial membrane, release of cytochrome c to the cytosol, apoptosome formation, activation of initiatory caspases and further activation of executory caspases.

Figure 4. Analysis of mitochondrial transmembrane potential in drug-treated C6 glioma cells. (A) Mitochondrial membrane potential was evaluated by flow cytometry 24 h after treatment. Representative histograms of control (DMSO, 0.2%), TBB and ZKK1-treated cells. Glioma cells were stained with the fluorescent probe JC-1 after the treatment with inhibitors; cells treated with the uncoupling agent CCCP for 10 min served as a positive control for mitochondrial depolarization. An enhancement of the green fluorescence and reduction of the red fluorescence, are presented as shifts in FL-1 and FL-2 channel intensities, respectively. (B) Change of the red/green fluorescence intensity ratio, normalized by the values from untreated controls (relative FL-2/FL-1), indicates a statistically significant loss of mitochondrial membrane potential after 24 h exposure (‘p<0.05 and ‘’p<0.01) as compared to control, DMSO treated cells. Bars represent the mean ± SEM from 3 independent experiments.

Figure 5. Expression of fasl and fas in drug-treated C6 glioma cells. Rat C6 glioma cells were treated with different drugs at concentration 50 μM or corresponding dose of DMSO. Twenty-four hours after treatment cells were lysed and total RNA was isolated. The expression level of fasl and fas was determined using real-time PCR and relative quantification; determination of 18S RNA expression served as an internal control. Bars represent the mean ±SEM from 2 independent experiments, each in duplicate.
Flow cytometric analysis was employed to study alterations of mitochondrial transmembrane potential in control and CK2 inhibitors-treated C6 glioma cells stained with the fluorescent probe JC-1. Cells treated with the uncoupling agent CCCP for 10 min served as a positive control for mitochondrial depolarization. Fig. 4 shows representative histograms of DMSO, TBB and ZKK1 treated cells. Change of the red/green fluorescence intensity ratio indicates a loss of mitochondrial membrane potential 24 h after exposure. Treatment with 10 and 50 μM ZKK1 resulted in statistically significant alterations of mitochondrial membrane potential, while in TBB-treated cells, only at the 50 μM a loss of mitochondrial membrane potential was observed (Fig. 4). TBI and DMAT showed similar potency to modulate a mitochondrial membrane potential as ZKK1 (not shown).

To elucidate extrinsic apoptotic pathways we determined the expression of Fas and its ligand in C6 glioma cells 24 h after treatment with 50 μM TBI, TBB, ZKK1 and DMAT. The levels of fas and fasl mRNA were determined by quantitative real-time PCR. In contrast to other compounds, DMAT induced a strong increase of both fas and fasl mRNA in treated C6 glioma cells (Fig. 5).

Generation of ZKK derivatives to improve its inhibitory properties. Since ZKK1 showed strong cytotoxic and pro-apoptotic effects, we sought to determine whether its inhibitory properties can be improved. The ZKK1 derivatives were obtained in the reaction of pentabromobenzylbromide with N-substituted thiourea derivatives (Fig. 6A). The most efficient inhibitors were isothiourreas carrying methyl or dimethyl (ZKK2-3) and ethyl or allyl groups (ZKK4-5). The viability of cells treated with ZKK2-5 dropped on average by 30-60% at doses 10 and 50 μM, respectively, after 24 h of treatment (Fig. 6B). The viability of treated cells was further reduced after 2 days of treatment with ZKK2-5. Isothiourrea carrying Cl or F substituents on the phenyl ring showed low or hardly any cytotoxic activity (ZKK7-8) when compared to ZKK1 (Fig. 6B and C).

Effects of novel CK2 inhibitors on proliferation and survival of non-transformed glial cells. Furthermore, we studied effects of ZKK1 and TBI at the concentration range of 0.1-50 μM on viability of non-transformed, primary astrocyte cultures 24 and 48 h after the treatment (Fig. 7). While 10 and 50 μM ZKK1 strongly reduced a viability of astrocytes by over 50%, these cultures were relatively resistant to exposure to 10 and 50 μM TBI for 24 h. A prolonged treatment with 50 μM TBI resulted in 20% reduction of astrocyte viability. The results demonstrate that non-transformed glial cells show similar vulnerability to treatment with ZKK1 as C6 glioma cultures, but are more resistant to TBI.

Cytotoxic efficacy of novel CK2 inhibitors towards cultured human glioblastoma cells. Multiple molecular mechanisms involved in drug resistance of human glioblastoma cells have been described, including frequent alterations in genes coding for tumor suppressors: PTEN and TP53, that result in reduced sensitivity to anticancer drugs (48,49). To study drug effects on human glioblastoma cells, we selected two cell lines: T98G deficient in functional PTEN and TP53 and LN229 with mutated TP53.

We demonstrate that human LN229 glioblastoma cells were moderately resistant to TBB and DMAT treatment, while TBI and ZKK1 exerted cytotoxic effect and reduced cell viability by 40%, when applied at concentration of 50 μM. T98G cells were totally resistant to TBB and DMAT, but TBI and ZKK1 were effectively cytotoxic at concentration of 50 μM. Interestingly, ZKK1 was effective even at 10 μM (Fig. 8A). Immunoblots demonstrate that TBI, DMAT and ZKK1 strongly activate executory caspases 3 and 7, and the regulatory caspase 8 in cells treated for 12-24 h. In T98G cells, TBI and DMAT clearly activated caspases 3, 7 and 8, while ZKK1 did not activate these caspases. In contrast to other tested compounds, in both cell lines TBI induced accumulation of active caspase 9, known to be involved in
Figure 7. Effects of various CK2 inhibitors on viability of non-transformed astrocytes. Primary glial cultures were developed as described in Materials and methods, and pure astrocytic cultures were kept in the presence of 10% fetal bovine serum. Cytotoxic effects of TBI and ZKK1 at the concentration range of 0.1-50 μM were determined 24 and 48 h after the treatment; corresponding doses of DMSO were added to control cultures. MTT metabolism was measured as optical density at 570 nm. Results are expressed in values relative to DMSO-treated control cells and correspond to the mean ± SEM of three independent experiments, each in triplicate. Note the relative resistance of non-transformed astrocytes to TBI in contrast to ZKK1.

Figure 8. Apoptotic alterations of human malignant glioblastoma cells exposed to TBI and ZKK1. (A) Human malignant glioblastoma T98G and LN229 cells were exposed for 24 h to TBB, TBI, DMAT or ZKK1, corresponding doses of DMSO were added to control cultures. MTT metabolism was measured as optical density at 570 nm. Results are from a representative experiment, performed in triplicate and are expressed in values relative to DMSO-treated control cells. For TBI and ZKK1 treatment the results were reproduced in three independent experiments and the changes at 50 μM were highly statistically significant. (B) Representative immunoblots show activation of caspases and PARP proteolysis after drug treatment. Human glioblastoma cells were cultured in the presence of 10% foetal bovine serum and cytotoxic effects of different compounds were determined 12 and 24 h after the treatment; 0.2 or 1% DMSO were added to control cultures. Western blot analysis was performed using antibodies recognizing total and cleaved caspases 8 and 9, total caspase 3, cleaved caspase 3 and 7 and total and cleaved PARP. Blots were re-probed with antibody recognizing β-actin to ensure equal protein loading.
the initiation of mitochondrial apoptotic pathway. Caspase 9 activation was also observed in LN229 cells exposed to ZKK1. In cultures treated with the compounds, decreased cell viability was accompanied by typical apoptotic changes in cell morphology: retraction of cell extensions, cytoplasm condensation, cell membrane blebbing and formation of apoptotic bodies (not shown).

Discussion

In the present study we demonstrate for the first time cytotoxic effects of known and recently developed 2-(dimethylamino)-4,5,6,7 tetrabromobenzimidazolide (DMAT) and pentabromobenzylisothioureas (ZKK1-8) on cultured rat and human malignant glioma cells. The cytotoxic efficacy of novel compounds DMAT and ZKK1 is higher than the well-known CK2 inhibitor 4,5,6,7-tetrabromo-1H-benzotriazole (TBB) and is similar to 4,5,6,7 tetrabromo-1H-benzoimidazole (TBI). The new compounds were more effective than TBB in induction of growth arrest and cell death in glioma cells. Analysis of caspase cascade activation demonstrated an activation of caspase 3 and 7 followed by PARP cleavage (Figs. 1-3, and 8). The cytotoxic activity of ZKK1 derivatives could be further improved by generation of thiourea derivatives N-substituted with short alkyl groups or an allyl group. The most bulky N-phenyl substituent in thiourea fragment of the molecule reduced cytotoxic activity of the parent compound ZKK1. Isothioureas carrying Cl or F substituents on the phenyl ring showed much lower activity than any of the tested drugs from pentabromobenzyl series. The reported data demonstrate that suitably modified tetrabromobenzene molecules may be prospective agents against glioma cells.

Although putative CK2 inhibitors were studied in many different cancer cell lines, a mode and mechanisms of cell death were not evaluated. Kinetics and morphological alterations during CK2 inhibitor-induced cell death as well caspase activation indicated an induction of apoptotic cell death. Caspase activation can be triggered by induction of ‘intrinsic’ and ‘extrinsic’ apoptotic pathway, involving the interaction of death ligands with their corresponding death receptors or activation of mitochondrial pathway, respectively (45-47). We demonstrate that cell death induced by ZKK1 and TBI was associated with a disruption of mitochondrial membrane potential, implicating the ‘intrinsic’ apoptotic pathway in cell death. DMAT upregulated fasL and fas expression and impaired the mitochondrial membrane potential suggesting activation of both receptor-mediated and mitochondria-mediated proteolytic pathways for apoptosis in glioblastoma cells.

Interestingly, TBI and ZKK1 at higher concentration were very effective in the induction of cell death in chemoresistant human glioblastoma cells, even those having mutations in the tumor suppressor PTEN or/and TP53 genes. T98G cells, lacking both functional PTEN and TP53 tumor suppressors, are resistant to most treatments, thus their vulnerability to TBI and ZKK1 suggest an ability to overcome classical drug resistance (Fig. 8). In T98G cells DMAT induced similar activation of caspases 3, 7 and 8 as TBI, but with any measurable cytotoxicity. It may indicate that T98G cells express inhibitors of apoptotic pathway, which are downstream of caspases. The expression of inhibitor of apoptosis proteins (IAPs), which suppress caspase activity, was described in tumor cells (50), including T98G glioblastoma cells expressing large amounts of XIAP, cIAP1 and cIAP2 (51). Another interesting finding is the strong cytotoxic effect of ZKK1 (even at 10 μM) towards T98G cells in the absence of caspase activation, that may suggest the ability to activate programmed, non-apoptotic cell death. ZKK1 activated the caspase cascade in C6 cells having functional PTEN and p53, but not in T98G cells with the mutated tumor suppressors.

TBB, DMAT and ZKK1 impaired cell survival of non-transformed astrocytes at similar concentration range as they did in glioblastoma cells. Only TBI at moderate concentration (10 μM) did not affect the viability of non-transformed astrocytes suggesting some specificity toward tumor cells.

Development of CK2 inhibitors resulted in the generation of a number of CK2-directed compounds (reviewed in refs. 8,9,15,25). Some of these inhibitors such as 4,5,6,7-tetrabromobenzimidazolide (TBI) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzoimidazolide (DMAT) inhibited the growth of malignant lymphoblastic leukemia cells with a better efficiency than Imatinib (17,18). Pharmacological down-regulation of CK2 activity with specific inhibitors induced cell death in drug resistant R-CEM cells (19,20), human colon cancer cells and a breast cancer cell line (21). In the present study we demonstrate that TBI, DMAT and newly synthesized inhibitors MB01-3 and ZKK series have better efficacy towards malignant glioblastoma cells than the previously studied TBB. These inhibitors are cytotoxic at relatively high concentrations but it is well-known that glioblastoma cells have elevated activity of many anti-apoptotic and pro-survival signalling pathways resulting in their high resistance to apoptosis inducers.

The exact mechanism by which newly synthesized inhibitors MB01-3 and ZKK series induce apoptosis remains largely unknown. Selectivity is one of the major issues of ATP-competitive inhibitors and it is relevant for the ATP-competitive inhibitors of CK2 such as TBB, TBI and DMAT (25,26). A study of a panel of 113 kinases revealed that none of the ATP competitive inhibitors in clinical trials or clinically used at the time was totally selective (52). Recent studies by Pagano et al (26) demonstrated that DMAT and its parent compound TBI are potent inhibitors of several other kinases, with a special preference to PIM (provirus integration site for Moloney murine leukemia virus), PIM2, PIM3, PKD1 (protein kinase D1), HIPK2 (homeodomain-interacting protein kinase 2) and DYRK1a (dual-specificity tyrosine-phosphorylated and-regulated kinase 1a). In contrast, TBB is significantly more selective toward CK2, although it also inhibits PIM1 and PIM3 (26).

On the other hand, imatinib mesylate (Gleevec or STI571), the first and probably the best known kinase inhibitor on the market, approved by the FDA 10 May 2001, lacks absolute selectivity. In the present study, TBB which is the most specific CK2 inhibitor known to date, was found to be less effective than other compounds. The concept of ‘one drug-many targets’ implying that non-specific drugs are sometimes useful and even desirable, is getting recently more
attention. Thus, a lack of absolute selectivity should not preclude the use of new CK2 inhibitor-based compounds as potential anticancer drugs in preclinical trials and deserves further studies.

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References


