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Bradykinin antagonists and thiazolidinone derivatives as new potential anti-cancer compounds

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ABSTRACT

Glioblastoma (GB), the most aggressive brain tumour, and mantle cell lymphoma (MCL), a rare but very aggressive type of lymphoma, are highly resistant to chemotherapy. GB and MCL chemotherapy gives very modest results, the vast majority of patients experience recurrent disease. To find out the new treatment modality for drug-resistant GB and MCL cells, combining of bradykinin (BK) antagonists with conventional temozolomide (TMZ) treatment, and screening of thiazolidinones derivatives were the main objectives of this work. As it was revealed here, BKM-570 was the lead compound among BK antagonists under investigation (IC_{50} was 3.3 μM) in human GB cells. It strongly suppressed extracellular signal-regulated kinases 1/2 (ERK1/2) and protein kinase B (AKT) phosphorylation. BK antagonists did not decrease the viability of MCL cells, thus showing the cell-specific mode, while thiazolidinone derivatives, a novel group of promising anti-tumour compounds inhibited proliferation of MCL cells: IC_{50} of ID 4526 and ID 4527 compounds were 0.27 μM and 0.16 μM, correspondingly. However, single agents are often not effective in clinic due to activation of collateral pathways in tumour cells. We demonstrated a strong synergistic effect after combinatorial treatment by BKM-570 together with TMZ that drastically increased cytotoxic action of this drug in rat and human glioma cells. Small proportion of cells was still viable after such treatment that could be explained by presence of TMZ-resistant cells in the population.

It is possible to expect that the combined therapy aimed simultaneously at different elements of tumourigenesis will be more effective with lower drug concentrations than the first-line drug temozolomide used alone in clinics.

1. Introduction

The tumours of central nervous system constitute from 2% to 5% of all human cancers, about half of them are the brain glial tumours of different malignancy grades of which glioblastoma (GB), the most malignant glioma, occupies up to 50%.[1] Mantle cell lymphoma (MCL) is a subtype of B-cell non-Hodgkin lymphoma characterized cytogenetically by the t(11;14)(q13;32) resulting in overexpression of cyclin D1.[2] GB and MCL patients exhibit an aggressive clinical course however, first-line cytotoxic chemotherapy is unable to induce long-term remission in the majority of patients, both GB and MCL are known to be highly therapy resistant and have the poor prognosis. The development of new treatment modalities, especially those based on multitargeted therapy, is desperately needed for these diseases.

The nonapeptide bradykinin (BK) has been shown to act as a growth factor in human lung, prostate, gastrointestinal, breast, and ovarian cancers.[3,4] Previously, Gera et al.[5,6] developed and synthesized a series of potent and metabolism-resistant BK antagonists, among which BKM-570 caused an impressive inhibition of human small cell lung cancer (SCLC) cell growth in vitro. Injection of this compound in nude mice displayed even higher inhibitory effects than the vascular endothelial growth factor receptor (VEGFR) inhibitor Semaxanib (SU5416) or the conventional chemotherapeutic drug cisplatin.[7] Among other BK antagonists, the inexpensive BKM-1800 derivative of BKM-570 had also valuable anti-cancer properties.[7] Interestingly, that orally active antagonist

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B10238 showed poor potency against cancer in vitro, however, was highly active in vivo.[5]

Last years, evaluation of anti-tumour activity appeared to be actual and promising for the thiazolidinone derivatives.[8,9] Several subgroups of these compounds, namely 5-arylidene-2-amino-4-thiazolones and pyrazoline substituted thiazolones, were evaluated for anti-cancer activity in vitro in a standard National Cancer Institute 60 cancer cell line assay. The majority of the tested substances have shown significant anti-tumour effects at the micromolar and submicromolar level.[10,11]

However, most tumours are driven by multiple molecular aberrations that cannot be controlled by a single targeted agent. The creation of smart schemes of multitargeted therapy aimed simultaneously at different elements of tumour formation mechanisms should be an effective strategy for cancer treatment.[12] The main purpose of this manuscript is to identify novel anti-cancer compounds and to develop modern approaches to complex glioma therapy based on the newly developed compounds and conventional chemotherapeutics used in clinics.

In this paper, we demonstrated that BKM-570 is the lead cytotoxic substance for glioma cells, while thiazolidinones ID 4526 and ID 4664 appeared to be efficient suppressors of MCL cell viability. Decreased viability of cells after BKM-570 treatment was probably associated with inhibition of ERK1/2 and AKT1 phosphorylation. Combination of BKM-570 with first-line drug temozolomide (TMZ) was much more effective even in much less concentration than anti-GB TMZ used alone.

2. Results and discussion

2.1. Compounds under investigation

Compounds synthesized for the first time for this investigation are described in the experimental section. Cytotoxic properties of tested substances are summarized in the Table 1. Chemical structures of compounds used in this study are depicted in Figure 1.

Design and synthesis of peptide B10238, non-peptide small mimetic BKM-570 and non-peptide small mimetic BKM-1800 were described previously.[5–7] B10238 is F5c-D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg, where F5c is (*E*)-1,2,3,4,5,6-penta-fluo-rocinnamoyl, Hyp is *trans*-4-hydroxyprolyl, Igl is α-(2-indanyl)glycyl, and Oic is octahydroindole-2-carbonyl); BKM-570 is N^{α}-[(*E*)-2,3,4,5,6-penta-fluorocin-namoyl]-*N*-(2,2,6,6-tetramethylpiperidin-4-yl)-*O*-(2,6-dichlorbenzyl)-L-tyrosin-amide; BKM-1800 is Fmoc-OC2Y-Atmp, where Fmoc is *N*-(fluorenyl-9-methoxycarbonyl), OC2Y is (*O*-2,6-dichlorobenzyl)tyrosyl, and Atmp is 4-amino-2,2,6,6-tetramethyl-piperidine. Compounds were prepared using solution or solid phase methods, purified by HPLC and characterized by thin layer chromatography (TLC) and laser desorption mass spectrometry (LDMS).

Table 1
Compounds under investigation

	IC_{50} (μM)						
	U373	C6	293_*CHI3L1*	Granta	Jeko	UPN1	Mino
Bradykinin antagonists							
BKM-570	3.3	4	3.8	63.3	49.3	*>100*	—[b]
BKM-1800	20	15	25.8	41.2	52.3	*>100*	—[b]
B10238	*>100*[a]	37	67.9	*>100*	50.5	*>100*	—[b]
Thiazolidinones							
ID 11	—[b]	—[b]	—[b]	*>100*	*>100*	*>100*	—[b]
ID 28	—[b]	—[b]	—[b]	*>100*	*>100*	*>100*	61.2
ID 28car*	15	4	0.13	—[b]	—[b]	—[b]	—[b]
ID 3166	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	55.3
ID 3643	—[b]	—[b]	—[b]	*>100*	*>100*	*>100*	—[b]
ID 4132*	—[b]	—[b]	—[b]	*>100*	*>100*	*>100*	—[b]
ID 4522*	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	68.2
ID 4523*	—[b]	1.22	—[b]	—[b]	—[b]	—[b]	80.7
ID 4523car.*	—[b]	0.13	—[b]	—[b]	—[b]	—[b]	1.58
ID 4524*	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	0.73
ID 4525*	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	10
ID 4526*	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	0.27
ID 4527*	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	7.4
ID 4664*	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]	0.16

* Newly synthesized compounds are marked by asterisk. Lead compounds are given in *italics* and underlined.
[a] Compound did not reach the IC_{50} value in corresponding cell line.
[b] Compound was not tested in corresponding cell line.

ID 28 and ID 3166 (Scheme 1) were synthesized starting from 2-(4-hydroxyphenylamino)thiazol-4-one **1** and 3-(benzothiazol-2-ylamino)-2-thioxo-thiazolidin-4-one **2**, respectively, using standard Knoevenagel reaction procedure (medium: acetic acid, catalyst: fused sodium acetate) and structurally analyzed as described elsewhere.[10,13]

The pyrazoline–thiazolidinone–isatin conjugates ID 3643, ID 4522, ID 4523, ID 4524, ID 4525, ID 4526, ID 4527, and ID 4664 were synthesized as described here by one-pot methodology involving reaction of 3,5-diaryl-1-thiocarbamoyl-2-pyrazolines **3** with chloroacetic acid and isatins in the presence of fused sodium acetate in refluxing acetic acid (Scheme 2).[14]

The oxadiazole–thiazolidinone hybrid ID 4132 (Scheme 3) was synthesized following the reaction of generated in situ potassium salts of 5-(4-dimethylamino-benzylidene)-thiazolidine-2,4-dione **4** with 2-chloro-*N*-[5-(4-methoxyphenyl)-[1,3,4]oxadiazol-2-yl]-acetamide.[15]

Synthesized compounds were characterized by NMR spectra, which are presented in the experimental part. ^{1}H NMR spectra of compounds ID 4522, ID 4523, ID 4524, ID 4525, ID 4526, ID 4527, and ID 4664 show characteristic patterns of the AMX system for CH_2–CH protons of pyrazoline fragment. The chemical shifts of the protons H_A, H_M, and H_X have been assigned to about δ ~3.47–3.52, δ ~4.18–4.20, and δ ~5.85–6.03 with coupling constants of J_{AM} = 18.0–18.6, J_{MX} = 11.0–11.5, and J_{AX} = 3.5–3.9 Hz, respectively. The chemical shift for the CH(4) of indoline cycle is insignificantly displaced in weak magnetic field (δ = 8.95–9.14) and clearly indicated that only *Z*-isomers were obtained. The protons of the methylene group (CH_2CO) of ID 4132 appear as broad singlet at δ = 4.60 ppm.

Compounds ID 28 and ID 4523, immobilized on a PEGylated carrier (PEGylated olygoelectrolyte polymers),[16] were kindly provided by Dr. A. Zaichenko (Lviv Polytechnic National University).

2.2. Cytotoxicity of synthesized compounds in cancer cell lines

Increased proliferation rate is a common feature of cancer cells. To analyse the anti-viability effects of synthesized compounds, we used several different cancer cell lines, namely rat glioma C6 cells, human GB U373 cells, 293 cells, overexpressing *CHI3L1* oncogene (293_*CHI3L1*),[17] MCL cell lines Granta, Jeko, UPN1, and Mino.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based) cell viability assay revealed significant dose-dependent cytotoxicity of BKM-570 and BKM-1800 in 293_*CHI3L1* and U373 cells 72 h after addition to the culture medium (Fig. 2, panels A and B). IC_{50} (molar concentration of compound leading to 50% cell death compared to control group treated with DMSO alone) for BKM-570 and BKM-1800 in 293_*CHI3L1* cells was 3.8 μM and 25.8 μM, respectively, and 3.3 μM and 20 μM, respectively, in U373 cells. B10238 did not suppress U373 cell viability at all and had only a partial cytotoxic activity in 293_*CHI3L1* cells, with IC_{50} 100 μM. In C6 rat glioma cells, BKM-570 was the most effective of BK antagonists under study, with IC_{50} 4 μM. The IC_{50} values for BKM-1800 and B10238 in C6 cells were 15 μM and 37 μM, respectively (Fig. 2, panel C). In our study we revealed very

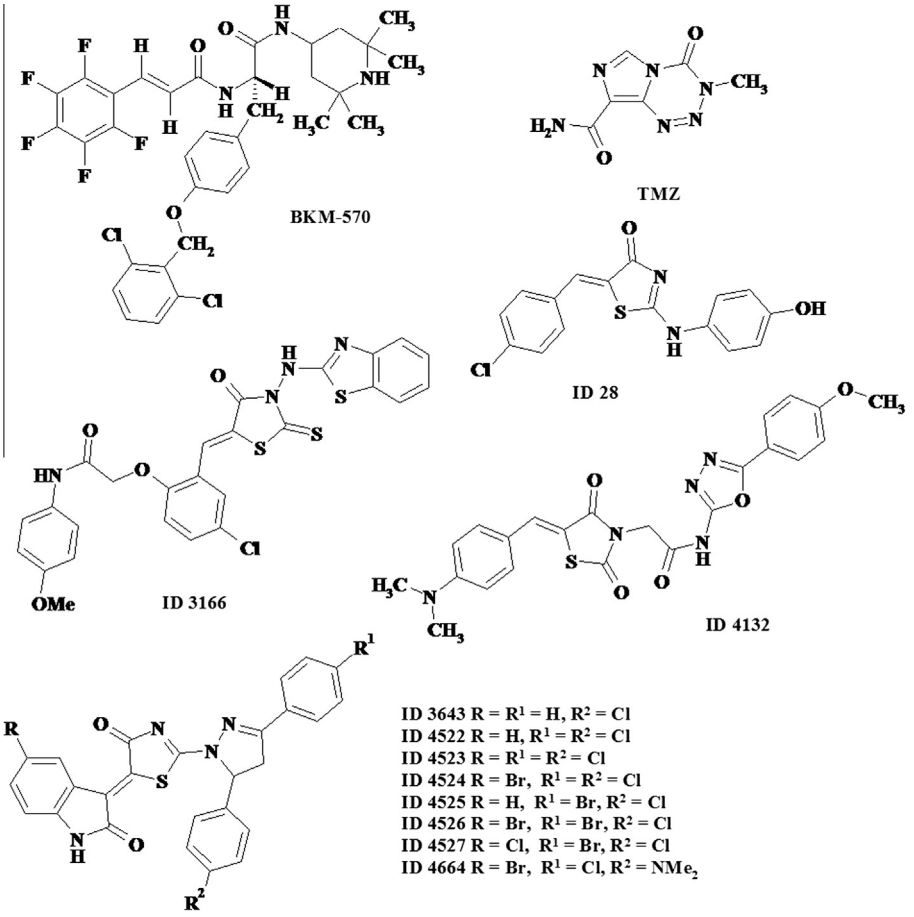
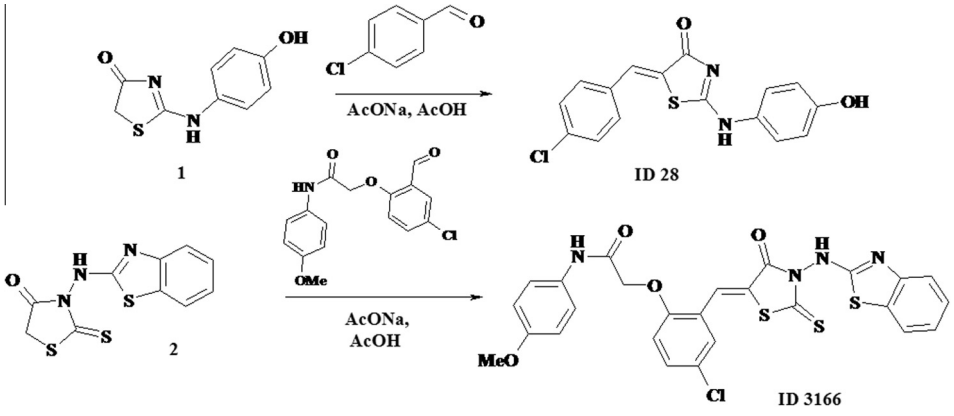


Figure 1. Structure of BKM-570, TMZ, ID 28, ID 3166, ID 3643, ID 4132, ID 4522, ID 4523, ID 4524, ID 4525, ID 4526, ID 4527, and ID 4664.

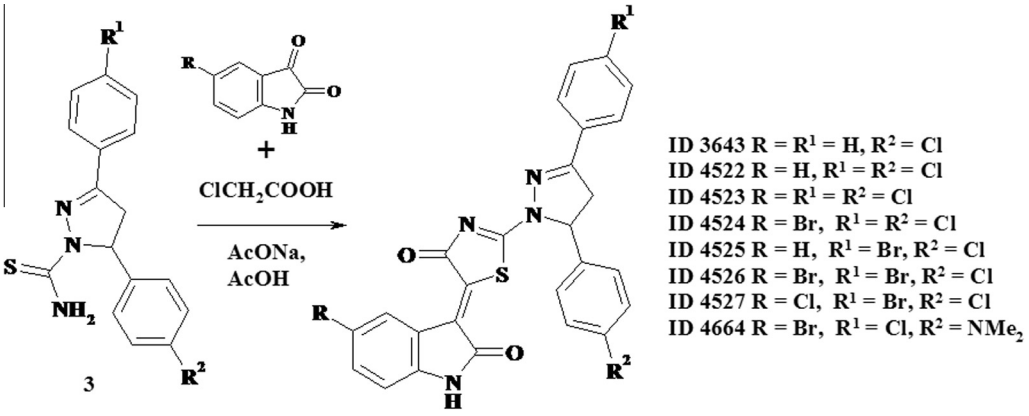


Scheme 1. Synthesis of ID 28 and ID 3166 compounds.

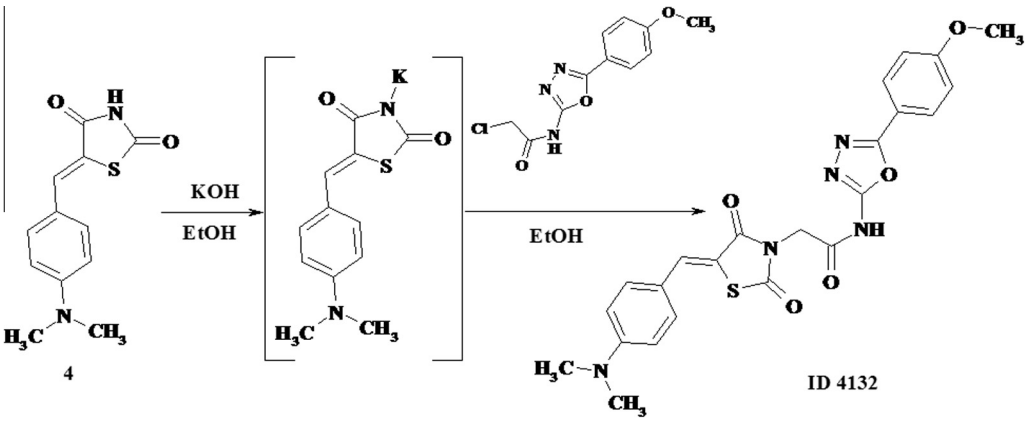
low cytotoxicity of B10238, same as it was reported previously, although it suppressed tumour growth in mice, what may suggest that other mechanisms, probably inhibition of angiogenesis, could come into play in vivo.[6] BKM-1800, a small non-peptide BK-antagonist, possessed higher activity in SCLC cells[7] than in 293_*CHI3L1*, U373, and C6 cells in this investigation (Fig. 2, panels A–C), while BKM-570 was shown to be the lead compound both in 293_*CHI3L1*, U373, and C6 (Fig. 2, panels A–C) and in SCLC cells (IC_{50} 1.8 μM), as well as in vivo (91% inhibition of tumour growth) in xenograft mice.[6]

Points in the graphs A and C, corresponding to the lowest concentration of the compound used varied from 90% to 70% that probably was due to some proportion of cells in the population, more sensitive to the used compounds.

BKM-570 inhibited ERK1/2 and AKT1 phosphorylation in 293_*CHI3L1* and U373 cells in a dose-dependent manner, whereas B10238 treatment did not decrease their level of phosphorylation. In 293_*CHI3L1* cells 10 μM BKM-570 suppressed efficiently the level of phospho-ERK1/2 and 50 μM BKM-570 led to the complete abolishment of ERK1/2 and AKT1 phosphorylation (Fig. 3, panel A). Same correlation was observed in MTT-test: 50 μM BKM-570 reduced number of viable cells to basal level. As it concerns AKT1 phosphorylation, even 10 μM BKM-570 was sufficient to strongly suppress the phospho-AKT1 signal (Fig. 3, panel A). Since



Scheme 2. Synthesis of ID 3643, ID 4522, ID 4523, ID 4524, ID 4525, ID 4526, ID 4527, and ID 4664 compounds. Reagents, conditions and yields: $ClCH_2COOH$ (1.0 equiv), AcONa (2.0 equiv), AcOH, reflux 5 h, 67–85%.



Scheme 3. Synthesis of ID 4132 compound.

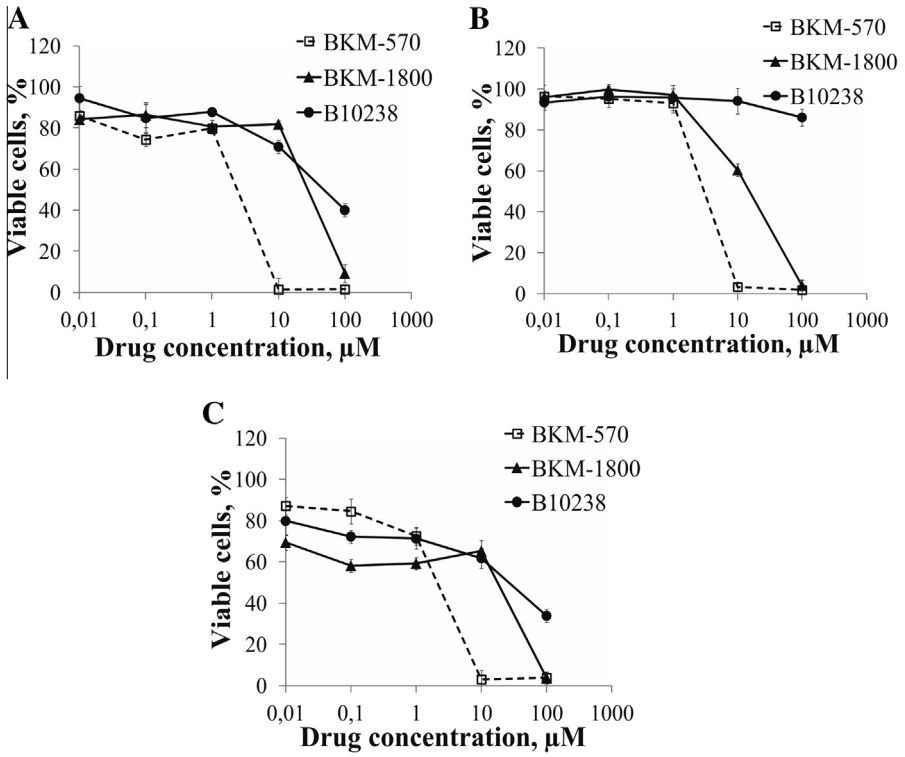


Figure 2. Cell viability after treatment with BK antagonists (MTT assay). 293_*CHI3L1* (A), U373 (B), and C6 (C) cells were cultured in the absence (vehicle control) or in the presence of increasing doses of BKM-570, BKM-1800, or B10238 for 72 h. Data are expressed as the percentage of viable cells compared to vehicle control (100%). Each point represents the mean of three experiments performed in triplicate.

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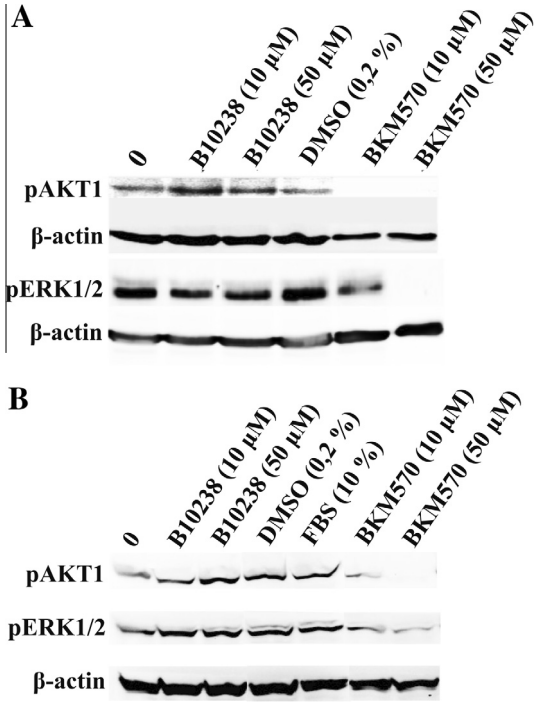

Figure 3. Impact of BK antagonists on AKT1 and ERK1/2 phosphorylation. Western blot analysis of phospho-AKT1 (pAKT1) and phospho-ERK1/2 (pERK1/2) in 293_*CHI3L1* (A) and U373 (B) cells after treatment with BKM-570 or B10238.

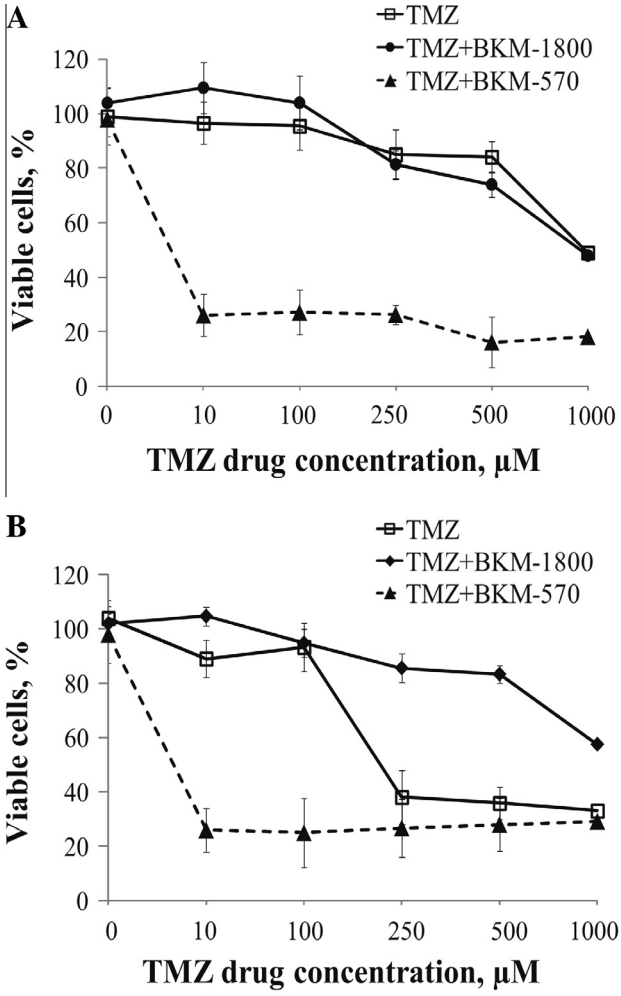

Figure 4. Effect of BK antagonists combination with temozolomide (TMZ) on cell viability (MTT assay). U373 (A) or C6 (B) cells were cultured in the medium with TMZ alone either TMZ with 1 μM of BKM-570, or TMZ with 1 μM of BKM-1800 for 72 h. Data are expressed as the percentage of viable cells compared to vehicle control (100%). Each point represents the mean of two experiments performed in triplicate.

non-stimulated U373 cells have much lower levels of ERK1/2 and AKT1 phosphorylation compared to 293_*CHI3L1* cells, we used 10% FBS to induce their phosphorylation before incubating cells with BK antagonists. Under such conditions, 10 μM BKM-570 significantly suppressed the phospho-AKT1 signals. However, in contrast to AKT1, even 50 μM BKM-570 did not inhibit completely phosphorylation of ERK1/2 (Fig. 3, panel B) that could be explained by the absence of phosphatase PTEN in these cells[18,19] which regulates ERK1/2 phosphorylation.[20] The inhibition of AKT1 and ERK1/2 phosphorylation by BKM-570 is probably involved in the mechanisms underlying the anti-proliferative activity of this compound, as it has been shown for other anti-cancer reagents.[21,22]

The first-line GB chemotherapy is the cytotoxic drug temozolomide (TMZ) but its effectiveness is temporary: the average survival expectancy is 14.6 months and the overall 5-year survival rate for GBM is only 9.8%.[23] Moreover, the high TMZ doses (75–200 mg per square meter of body-surface area per day) used for GB treatment are known to induce neurotoxicity and cognitive dysfunction as treatment-related side-effects.[24,25] Attempts to increase the anti-tumour TMZ efficacy by combining with different substances reported previously were not very successful.[26,27]

In our study, the addition of BKM-570 in non-cytotoxic concentration of 1 μM to as little as 10 μM TMZ-containing culture medium gave about 80% reduction of viable rat C6 and human U373 glioma cells compared to TMZ used alone (Fig. 4). The observed combinatorial effect of BKM-570 and TMZ was synergistic, rather than additive: it was much higher than simple arithmetic sum of their individual effects. However, there was no complete suppression of cell viability that could be explained by the presence of TMZ-resistant cells in the population. On the other hand, such combination with BKM-1800 did not induce increased cytotoxicity, and even reduced the effect of TMZ in U373 cells (Fig. 4, panel B). Suppression of AKT1 phosphorylation may contribute to the improved cytotoxicity seen after combining by BKM-570 with TMZ, since AKT1 activation has been shown to play a role in the down regulation of TMZ-induced checkpoint kinase 2 (Chk2) activity and G2 arrest, thus protecting cells from TMZ-mediated cytotoxicity.[28]

It was revealed that the cytotoxic activity of BK antagonists was restricted to the 293_*CHI3L1*, C6, and U373 cell lines and did not exist in MCL cell lines Granta, JeKo, and UPN1 (Fig. 5). BKM-570 and BKM-1800 produced only 50% inhibition of Granta and JeKo cells viability in concentrations range of 40–65 μM (Fig. 5, panels A and B), and did not suppress at all UPN1 cells viability (Fig. 5, panel C). B10238 had the lowest activity of BK antagonists under experiment. Cell specificity of BK antagonist cytotoxicity may reflect differences in BK pathways and innate sensitivity to BK antagonists between blood (MCL) cells and cells of other origin (293_*CHI3L1*, U373, and C6 cells).

To find out compounds suitable for inhibition of viability in MCL cells, we have screened a set of new promising compounds belonging to thiazolidinones family. Previously it was reported that in silico molecular modeling of thiazolidinone based conjugates revealed their increased affinity towards antiapoptotic proteins of the Bcl-2 family which are often overexpressed in leukemia and lymphoma cells.[14] These compounds were tested at the National Cancer Institute (NCI, USA) on a panel of 60 tumour cell

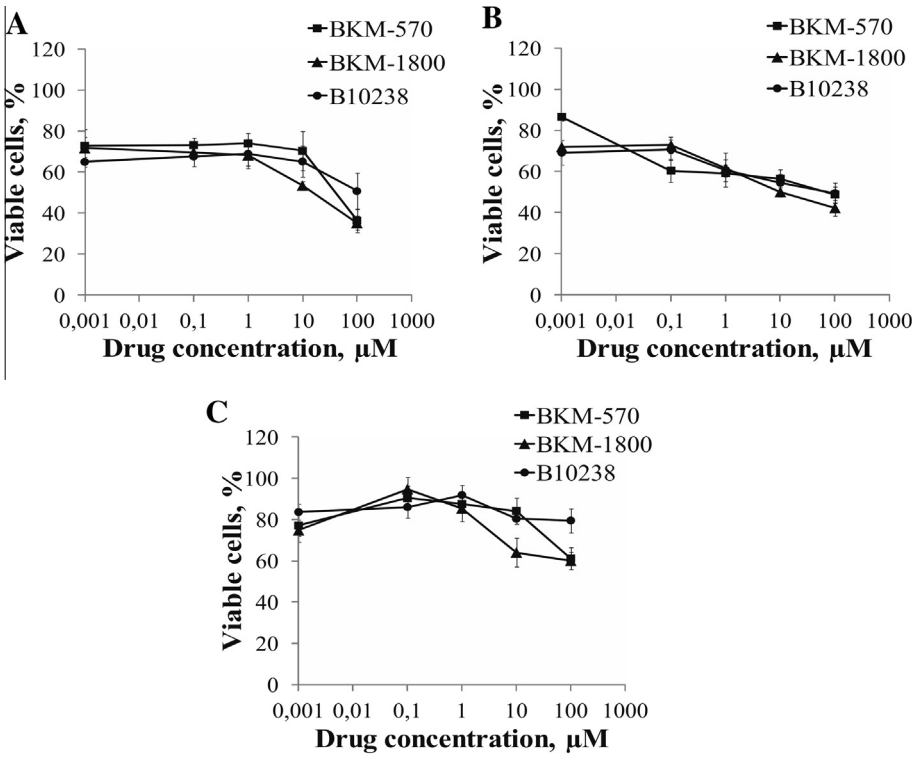

Figure 5. Effect of BK antagonists on MCL cells viability (MTT assay). Granta (A), JeKo (B), and UPN1 (C) cells were cultured in the absence (vehicle control) or in the presence of increasing doses of BKM-570, BKM-1800, or B10238 for 72 h. Data are expressed as the percentage of viable cells compared to vehicle control (100%). Each point represents the mean of three experiments performed in triplicate.

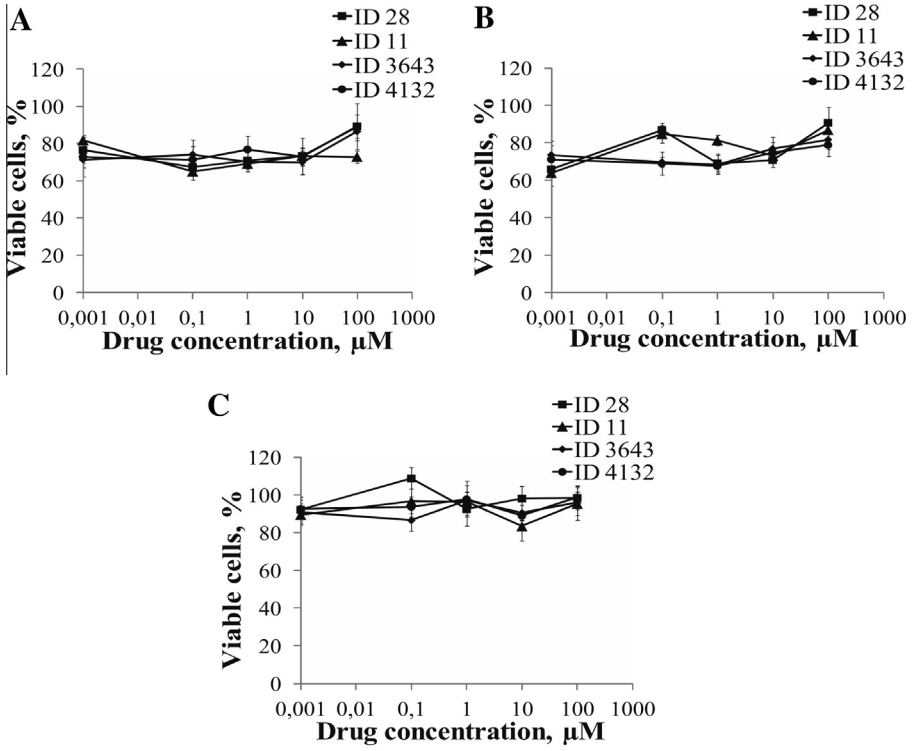

Figure 6. Effect of thiazolidinones on MCL cells viability (MTT assay). Granta (A), JeKo (B), and UPN1 (C) cells were cultured in the absence (vehicle control) or in the presence of increasing doses of thiazolidinones for 72 h. Data are expressed as the percentage of viable cells compared to vehicle control (100%). Each point represents the mean of three experiments performed in triplicate.

lines, and their high toxicity towards leukemia cell lines was revealed.[10] Twelve compounds of the thiazolidinone family were involved in our study (Table 1). Among them, ID 11, ID 28, ID 3166, and ID 3643 were already published,[10] while ID 4132, ID 4522, ID 4523, ID 4524, ID 4525, ID 4526, ID 4527, and ID 4664 were newly developed substances.

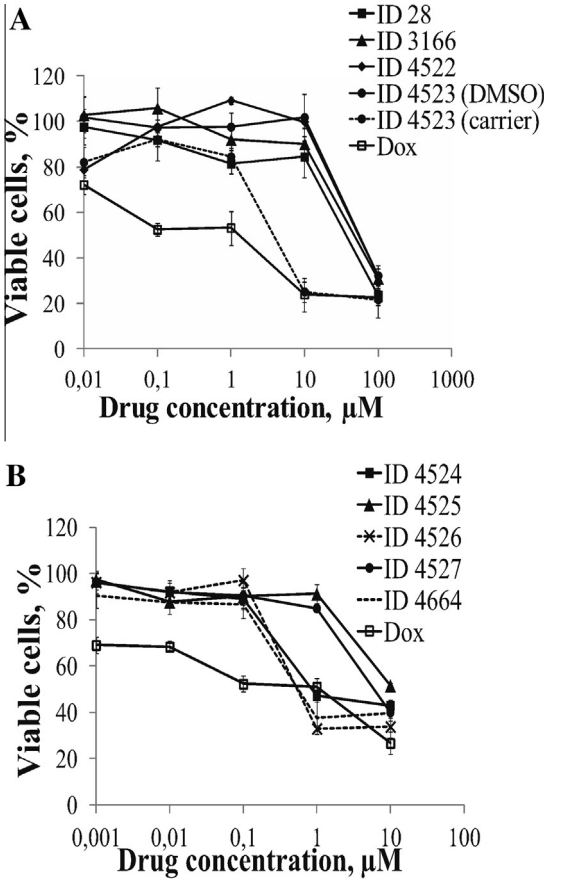

Figure 7. Inhibition of Mino cells viability by ID 4523 (A) and ID 4523-related thiazolidinones (B) (MTT assay). Cells were cultured in the absence (vehicle control) or in the presence of increasing doses of thiazolidinones for 72 h. Data are expressed as the percentage of viable cells compared to vehicle control. Each point represents the mean of three experiments performed in triplicate.

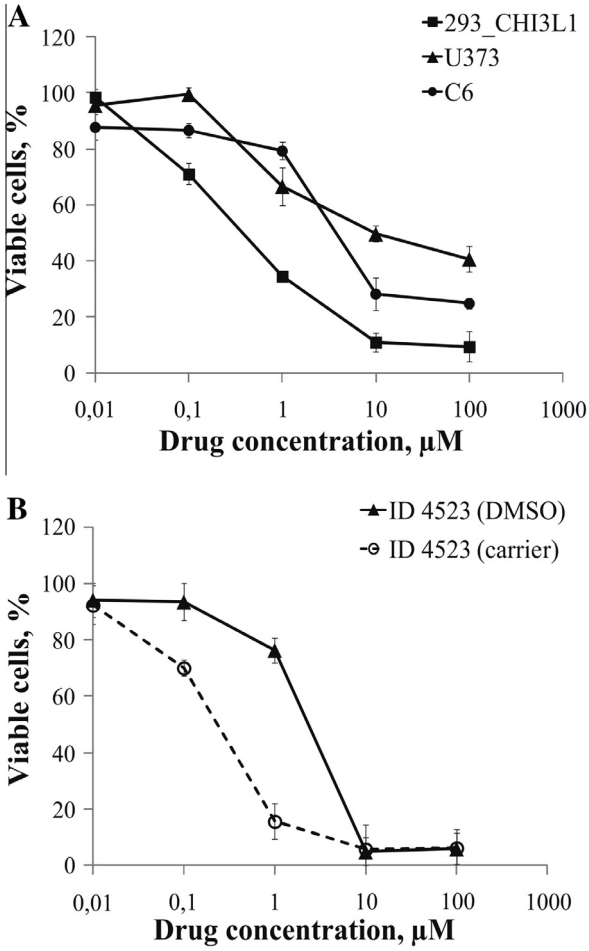

Figure 8. Cytotoxicity of thiazolidinones in 293_*CHI3L1*, U373, and C6 cells (MTT assay). (A) 293_*CHI3L1* or U373 cells were cultured in the absence (vehicle control) or in the presence of increasing doses of ID 28car for 72 h. (B) C6 cells were cultured as described above with ID28_car., ID 4523 and ID 4523car. Data are expressed as the percentage of viable cells compared to vehicle control (100%). Each point represents the mean of three experiments performed in triplicate.

Based on the NCI data, compounds ID 11, ID 28, ID 3166, ID 3643, and ID 4132 were selected as those with prominent activity. Nevertheless, we observed only slight decreased viability of treated cells, even with 100 μM concentrations of these substances, as well as with novel compound ID 4522 (Figs. 6 and 7, panel A). Although we did not observe valuable cytotoxicity of BK antagonists and thiazolidinone derivatives in the most MCL cells, same 60–70% of viable cells remained at the lowest and the highest concentration of compounds used. It is possible to suppose that treated cell lines contained a small subpopulation of cells, which were more sensitive to the applied compounds.

However, treatment of MCL cells Mino with ID 4523, immobilized on a PEGylated carrier (ID 4523car) was highly cytotoxic (IC_{50} 1.58 μM) (Fig. 7, panel A). Chemical analogues of ID 4523: ID 4524, ID 4525, ID 4526, ID 4527, and ID 4664 behaved even much better: IC_{50} was 0.73 μM for ID 4524, 10 μM for ID 4525, 0.27 μM for ID 4526, 7.4 μM for ID 4527, and 0.16 μM for ID 4664 (Fig. 7, panel B). The IC_{50} values for the compounds ID 4526 and ID 4664 were significantly better than that of doxorubicin, a conventional chemotherapeutic drug in clinical use, with IC_{50} of 0.37 μM.[29] Thus, such thiazolidinone derivatives possess a valuable cytotoxic activity in some MCL cells. Optimizing the effectiveness of these agents is a focus of ongoing research and could be a promising tool for future anti-MCL drug development.

To address the question whether thiazolidinones have cytotoxic properties against glioma cells, we tested compounds ID 28, immobilized on a PEGylated carrier (ID 28car), as well as ID 4523 and ID 4523car in C6 and U373 cell lines. ID 28car demonstrated the strongest cytotoxic properties (IC_{50} 0.13 μM) in 293_*CHI3L1* cells, significantly higher in comparison with C6 rat glioma cells (LC_{50} 4 μM) and human U373 cells (IC_{50} 15 μM) (Fig. 8, panel A). In C6 cells ID 4523 had IC_{50} 1.22 μM, while immobilization on a PEGylated carrier enhanced the growth inhibitory properties of ID 4523 (LC_{50} 0.13 μM) (Fig. 8, panel B). Thus, the cell specific modes of action were observed for certain members of bradykinin antagonists and thiazolidinone derivatives, indicating the involvement of different mechanisms to implement the cytotoxic properties of these compounds.

3. Conclusions

Here we described the cytotoxic activity for representatives of two chemically different classes of molecules, BK antagonists and thiazolidinone derivatives, using several lines of malignantly transformed cells. Among BK antagonists under experiment, BKM-570 appeared to be the lead compound in the 293_*CHI3L1*, U373, and C6 cell lines. BKM-570 strongly inhibited ERK1/2 and AKT phosphorylation in 293_*CHI3L1* and U373 cells, thus, the cytotoxic effect of BKM-570 could be mediated by modulation of MAPK- and PI3K-signaling cascades.

BK antagonists, prominently active in 293_*CHI3L1*, U373, and C6 cell lines did not affect MCL cells. Among five ID 4523-related

substances, ID 4524 and ID 4664 were shown to be the most effective in MCL cells. A significant cytotoxicity was observed also after treatment of human and rat glioma cells with thiazolidinones ID 28car and ID 4523car. This could indicate the cellular specificity of cytotoxic properties of BK antagonists and thiazolidinone derivatives.

Especially important result was the finding that simultaneous application of bradykinin antagonist BKM-570 drastically improved the effectiveness of TMZ in glial cells, which alone has only temporary therapeutic benefit and severe side-effects in clinics. In this paper we made a pilot attempt to create the smart drug combinations of newly synthesized compounds and conventional chemotherapeutics that will enable the development of therapeutic regimens with improved effectiveness and less toxicity. Further investigations of anti-cancer properties of BK antagonists, azolidinone derivatives, and their combinations with each other and with conventional therapeutic agents using animal models are needed for the evaluation of these compounds in a pre-clinical study.

4. Experimental section

4.1. Chemistry

4.1.1. Materials and methods

The starting 3,5-diaryl-1-thiocarbamoyl-2-pyrazoline was obtained according to the method described previously.[30,31]

Melting points were measured in open capillary tubes on a BŰCHI B-545 melting point apparatus and are uncorrected. The elemental analyses (C, H, N) were performed using the Perkin–Elmer 2400 CHN analyzer. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values. The ^{1}H NMR spectra were recorded on Varian Gemini 400 MHz in DMSO-d_6 using tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in ppm units with use of δ scale.

4.1.2. General procedure for synthesis of 3-{2-[3,5-bis-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl]-4-oxo-4*H*-thiazol-5-ylidene}-1,3-dihydroindol-2-ones[32]

A mixture of 3,5-diaryl-1-thiocarbamoyl-2-pyrazoline (10 mmol), chloroacetic acid (10 mmol), appropriate isatin (12 mmol), and anhydrous sodium acetate (10 mmol) was refluxed for 5 h in glacial acetic acid (10 mL). Precipitate obtained upon cooling was filtered off, washed with water and methanol and recrystallized with DMF/ethanol mixtures (1:2 vol).

4.1.2.1. 3-{2-[3,5-Bis-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl]-4-oxo-4*H* thiazol-5-ylidene}-1,3-dihydroindol-2-one (ID 4522). Yield 85%, mp 340–341 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 10.80 (s, 1H), 8.95 (d, J = 7.0 Hz, 1H), 7.92–7.94 (m, 2H), 7.53 (d, J = 8.2 Hz, 2H), 7.37–7.40 (m, 3H), 7.29 (t, J = 7.6 Hz, 1H), 7.00 (t, J = 7.5 Hz, 1H), 6.92 (d, J = 7.5 Hz, 1H), 6.00 (dd, J = 11.0, 3.7 Hz, 1H), 4.18 (dd, J = 18.1, 11.0 Hz, 1H), 3.47 (dd, J = 18.1, 3.7 Hz, 1H). Calcd for $C_{26}H_{16}Cl_2N_4O_2S$: C, 60.12; H, 3.10; N, 10.79; Found: C, 60.39; H, 3.25; N, 10.61.

4.1.2.2. 3-{2-[3,5-Bis-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl]-4-oxo-4*H*-thiazol-5-ylidene}-5-chloro-1,3-dihydroindol-2-one (ID 4523). Yield 80%, mp 355–356 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 10.96 (s, 1H), 9.00 (br s, 1H), 7.93 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.37–7.39 (m, 4H), 7.29 (d, J = 7.6 Hz, 1H), 6.92 (d, J = 7.5 Hz, 1H), 6.03 (dd, J = 11.2, 3.6 Hz, 1H), 4.20 (dd, J = 18.0, 11.2 Hz, 1H), 3.52 (dd, J = 18.0, 3.6 Hz, 1H). Calcd for $C_{26}H_{15}Cl_3N_4O_2S$: C, 56.38; H, 2.73; N, 10.12; Found: C, 56.22; H, 2.84; N, 10.20.

4.1.2.3. 3-{2-[3,5-Bis-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl]-4-oxo-4*H*-thiazol-5-ylidene}-5-bromo-1,3-dihydroindol-2-one (ID 4524). Yield 71%, mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 10.95 (s, 1H), 9.13 (br s, 1H), 7.85 (d, J = 8.1 Hz, 2H), 7.68 (d, J = 8.1 Hz, 2H), 7.36–7.40 (m, 4H), 7.28 (t, J = 7.0 Hz, 1H), 6.98 (t, J = 7.0 Hz, 1H), 6.91 (t, J = 7.6 Hz, 1H), 6.00–6.04 (m, 1H), 4.19 (dd, J = 18.2, 11.5 Hz, 1H), 3.46 (dd, J = 18.2, 3.8 Hz, 1H). Calcd for $C_{26}H_{15}BrCl_2N_4O_2S$: C, 52.20; H, 2.53; N, 9.36; Found: C, 52.11; H, 2.64; N, 9.50.

4.1.2.4. 3-{2-[3-(4-Bromophenyl)-5-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl]-4-oxo-4*H*-thiazol-5-ylidene}-1,3-dihydroindol-2-one (ID 4525). Yield 73%, mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 10.84 (s, 1H), 8.95 (d, J = 7.7 Hz, 1H), 7.34–7.85 (m, 8H), 6.92–7.28 (m, 3H), 6.00 (dd, J = 11.4, 3.8 Hz, 1H), 4.20 (dd, J = 18.1, 11.4 Hz, 1H), 3.46 (dd, J = 18.1, 3.8 Hz, 1H). Calcd for $C_{26}H_{16}BrClN_4O_2S$: C, 55.38; H, 2.86; N, 9.94; Found: C, 55.45; H, 2.72; N, 10.05.

4.1.2.5. 5-Bromo-3-{2-[3-(4-bromophenyl)-5-(4-chlorophenyl)-4,5-dihydro-pyrazol-1-yl]-4-oxo-4*H*-thiazol-5-ylidene}-1,3-dihydroindol-2-one (ID 4526). Yield 73%, mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 11.00 (s, 1H), 9.14 (s, 1H), 7.38–7.86 (m, 8H), 6.88–7.29 (m, 2H), 6.00–6.04 (m, 1H), 4.18–4.22 (m, 1H), 3.49 (dd, J = 18.0, 3.9 Hz, 1H). Calcd for $C_{26}H_{15}Br_2ClN_4O_2S$: C, 48.59; H, 2.35; N, 8.72; Found: C, 48.67; H, 2.47; N, 8.58.

4.1.2.6. 5-Chloro-3-{2-[3-(4-bromophenyl)-5-(4-chlorophenyl)-4,5-dihydro-pyrazol-1-yl]-4-oxo-4*H*-thiazol-5-ylidene}-1,3-dihydroindol-2-one (ID 4527). Yield 73%, mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 10.96 (s, 1H), 9.00 (br s, 1H), 7.37–7.86 (m, 8H), 6.92–7.29 (m, 2H), 6.01–6.05 (m, 1H), 4.18-4.22 (m, 1H), 3.49 (dd, J = 18.0, 3.6 Hz, 1H). Calcd for $C_{26}H_{15}BrCl_2N_4O_2S$: C, 52.20; H, 2.53; N, 9.36; Found: C, 52.41; H, 2.68; N, 9.17.

4.1.2.7. 5-Bromo-3-{2-[3-(4-chlorophenyl)-5-(4-dimethylaminophenyl)-4,5-di-hydropyrazol-1-yl]-4-oxo-4*H*-thiazol-5-ylidene}-1,3-dihydroindol-2-one (ID 4664). Yield 67%, mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 11.20 (s, 1H), 9.09 (br s, 1H), 7.91 (d, J = 7.9 Hz, 2H), 7.58 (d, J = 8.1 Hz, 2H), 7.48 (d, J = 8.5 Hz, 1H), 7.12 (d, J = 8.4 Hz, 2H), 6.87 (d, J = 8.3 Hz, 1H), 6.69 (d, J = 8.6 Hz, 2H), 5.85 (dd, J = 11.1, 3.5 Hz, 1H), 4.10 (dd, J = 18.6, 11.1 Hz, 1H), 3.50 (dd, J = 18.6, 3.5 Hz, 1H), 2.87 (s, 6H). Calcd for $C_{28}H_{21}BrClN_5O_2S$: C, 55.41; H, 3.49; N, 11.54; Found: C, 55.27; H, 3.38; N, 11.64.

4.1.3. Synthesis of 2-[5-(4-dimethylaminobenzylidene)-2,4-dioxo-thiazolidin-3-yl]-*N*-[5-(4-methoxyphenyl)-[1,3,4]oxadiazol-2-yl]-acetamide (ID 4132)[33]

A mixture 5 mmol of *N*-potassium salt of 5-(4-dimethylaminobenzylidene)-thiazolidine-2,4-dione and 6 mmol 2-chloro-*N*-[5-(4-methoxyphenyl)-[1,3,4]oxadiazol-2-yl]-acetamide as well as catalytic amount of potassium iodide and carbonate in 15 ml of mixture DMF–EtOH (vol. 1:1) were refluxed for 4 h. Reaction product was filtered off after cooling and pouring into water, washed by water, ethanol, and diethyl ether. Recrystallized with the mixture of DMF–ethanol (1:2). Yield 70%, mp 239–240 °C. ^{1}H NMR (400 MHz, DMSO-d_6+CCl_4): 7.83–7.85 (m, 3H), 7.48 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 8.5 Hz, 2H), 6.82 (d, J = 8.6 Hz, 2H), 4.60 (s, 2H), 3.82 (s, 3H). Calcd for $C_{23}H_{21}N_5O_5S$: C, 57.61; H, 4.41; N, 14.60; Found: C, 57.82; H, 4.52; N, 14.48.

4.2. Biological experiments

4.2.1. Cell cultures

U373 cells (human glioblastoma–astrocytoma cells) were kindly provided by Dr. M. Sanson (INSERM, U711, Biologie des

Interactions Neurones & Glie, Paris, France), MCL cell lines (Granta, JeKo, Mino, and UPN1) by Dr. V. Ribrag (Departement de Medecine, Institut Gustave Roussy, Villejuif, France), C6 cells by Dr. V. Baklaushev (State Research Center of Forensic and Social Psychiatry, Moscow, Russian Federation). 293_*CHI3L1* cells (293 cells stably produced CHI3L1 oncoprotein) were kindly gifted by Dr. O. Balynska (Institute of Molecular Biology and Genetics, Kyiv, Ukraine). 293_*CHI3L1*, U373, and C6 cells were grown in DMEM (Hyclone, USA), UPN1 cells in alpha-MEM (Gibco, USA), Granta, JeKo, and Mino cells in RPMI (Gibco, USA). All culture mediums were supplemented with FBS at 10%, penicillin at 100 units/ml, and streptomycin at 100 μg/ml final concentration. Cells were cultivated in the environment of 95% air/5% CO_2.

4.2.2. Antibodies

Antibodies were obtained from multiple sources and used at the specified dilutions for immunoblots: pERK (E-4) Mouse monoclonal IgG (Santa Cruz, USA) 1:2000 (anti-phospho-ERK1/2); Anti-phospho-AKT1/PKBα (Ser473), clone 11E6 (Millipore, USA) 1:1000; Monoclonal anti-β-actin antibody produced in mouse, clone AC-15 (Sigma–Aldrich Co, USA) 1:5000 (anti-β-actin). The reagents for enhanced chemiluminescence (ECL) from Sigma (USA) and Fluka (Switzerland) were used for the visualization of immunoreactive bands in Western blot analysis.

4.2.3. Analysis of ERK1/2 and AKT phosphorylation

293_*CHI3L1* and U373 cells were seeded in triplets into 6-well tissue culture plates at density 10^5 cells/well in DMEM/10% FBS and allowed to grow to near-confluence. Cells were serum-starved for 24 h and then exposed to BKM-570 or B10238 for 24 h. At the end of incubation periods, cell layers were washed twice in ice-cold PBS, lysed in 2× Laemmli sample buffer and boiled during 5 min. Proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose membrane, which was blocked for 1 h at room temperature with 5% powdered skim milk in TBS (Tris-buffered saline) and 0.05% Triton X-100 (TBST). Membrane was treated with anti-phospho-ERK1/2 or anti-phospho-AKT1 antibodies at 4 °C overnight and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG for 1 h. Blots were developed with an ECL detection system. Then membranes were incubated in stripping buffer (0.5 M NaCl, 0.2 M Gly, pH 2.5) for 15 min at 70 °C, washed twice in TBST and subsequently incubated with anti-β-actin antibodies (1 h at room temperature) and (HRP)-conjugated anti-mouse IgG, followed by ECL detection.

4.2.4. MTT assay

293_*CHI3L1*, U373, C6, Granta, JeKo, UPN1, or Mino cells were seeded in triplicates into 96-well plate at density 3×10^3 cells/well. Compounds were applied at final concentration 0.01, 0.1, 1, 10, and 100 μM, which were reached by serial dilution. For combinatorial treatment, TMZ was added to the final concentration of 10, 100, 250, 500, and 1000 μM combined with 1 μM BKM-570 or BKM-1800. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma), which is converted to dark blue, water-insoluble MTT formazan by mitochondrial dehydrogenases, was used to determine viable cells according to manufacturer's protocol (Sigma).

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