Biomedicine & Pharmacotherapy 168 (2023) 115635

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Proteasome inhibition potentiates Kv1.3 potassium channel expression as therapeutic target in drug-sensitive and -resistant human melanoma cells

Clemens Cammann [a,1], Jonas Kulla [b,1,2], Lüder Wiebusch [c], Christian Walz [d], Fang Zhao [e], Theresa Lowinus [b,3], Eylin Topfstedt [a], Neha Mishra [f], Petra Henklein [g], Ursula Bommhardt [b], Lukas Bossaller [f], Christian Hagemeier [c], Dirk Schadendorf [e], Boris Schmidt [d], Annette Paschen [e], Ulrike Seifert [a,*]

[a] Friedrich Loeffler - Institute of Medical Microbiology - Virology, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany
[b] Institute of Molecular and Clinical Immunology, Medical Faculty, Otto-von-Guericke-University, Leipziger Str. 44, 39120 Magdeburg, Germany
[c] Department of Pediatric Oncology and Hematology, Charité-Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany
[d] Clemens Schöpf-Institute for Organic Chemistry and Biochemistry, Technical University Darmstadt, Alarich Weiss-Straße 4–8, 64287 Darmstadt, Germany
[e] Department of Dermatology, University Hospital Essen, University Duisburg-Essen, Hufelandstr. 55, 45147 Essen, Germany
[f] Section of Rheumatology, Clinic and Policlinic of Internal Medicine A, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany
[g] Institute of Molecular Biology and Biochemistry, Charité-Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Campus Charité Mitte, Charitéplatz 1, 10117 Berlin, Germany

ARTICLE INFO

Keywords:
Melanoma
$BRAF^{V600E}$ mutation
BRAFi-resistance
Proteasome inhibitor
Kv1.3 potassium channel

ABSTRACT

Primary and acquired therapy resistance is a major problem in patients with BRAF-mutant melanomas being treated with BRAF and MEK inhibitors (BRAFI, MEKi). Therefore, development of alternative therapy regimes is still required. In this regard, new drug combinations targeting different pathways to induce apoptosis could offer promising alternative approaches. Here, we investigated the combination of proteasome and Kv1.3 potassium channel inhibition on chemo-resistant, BRAF inhibitor-resistant as well as sensitive human melanoma cells. Our experiments demonstrated that all analyzed melanoma cell lines were sensitive to proteasome inhibitor treatment at concentrations that are not toxic to primary human fibroblasts. To further reduce proteasome inhibitor-associated side effects, and to foster apoptosis, potassium channels, which are other targets to induce pro-apoptotic effects in cancer cells, were blocked. In support, combined exposure of melanoma cells to proteasome and Kv1.3 channel inhibitor resulted in synergistic effects and significantly reduced cell viability. On the molecular level, enhanced apoptosis correlated with an increase of intracellular Kv1.3 channels and pro-apoptotic proteins such as Noxa and Bak and a reduction of anti-apoptotic proteins. Thus, use of combined therapeutic strategies triggering different apoptotic pathways may efficiently prevent the outgrowth of drug-

Abbreviations: Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; β1i/LMP2, low molecular mass protein 2; β5i/LMP7, low molecular mass protein 7; β2i/MECL-1, multicatalytic endopeptidase complex like 1; β5/MB1, multicatalytic endopeptidase complex epsilon chain; Bcl-2, apoptosis regulator B-cell lymphoma 2; BRAF, v-Raf murine sarcoma viral oncogene homolog B1; BRAFi, BRAF inhibitor; BRAF-V600E, valine to glutamate mutation at position 600 in the *BRAF* gene; CTLL-2, Cytotoxic T cell line 2; IFN, interferon; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; KCa3.1 channel, calcium-activated potassium channel 3.1; Kv1.3 channel, voltage-gated potassium channel 1.3; $Ma\text{-}Mel_{PLX}$, PLX-4032/vemurafenib resistant melanoma cells; MAPK, mitogen-activated protein kinase; MAPKi, mitogen-activated protein kinase inhibitor; MCL-1, induced myeloid leukemia cell differentiation protein; MEK, mitogen-activated protein kinase-kinase; $MeWo_{Eto}$, etoposide-resistant MeWo cells; mitoKv1.3 channel, mitochondrial voltage-gated potassium channel 1.3; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NMDAR, N-methyl-D-aspartate receptor; Noxa, phorbol-12-myristate-13-acetate-induced protein 1; NSCLC, non-small-cell lung cancer; PARP-1, poly(ADP-ribose)-polymerase 1; PD-1, programmed cell death protein 1; ROS, reactive oxygen species; TNF, tumor necrosis factor; UPS, ubiquitin-proteasome-system; XIAP, X-linked inhibitor of apoptosis protein.

* Correspondence to: Friedrich Loeffler-Institut für Medizinische Mikrobiologie-Virologie, Universitätsmedizin Greifswald KdöR, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany.
E-mail address: ulrike.seifert@med.uni-greifswald.de (U. Seifert).
[1] These authors contributed equally to this work
[2] Present address: Dietrich-Bonhoeffer-Klinikum, Salvador-Allende-Straße 30, 17036 Neubrandenburg, Germany
[3] Present address: Clinic of Internal Medicine I, Hematology, Oncology, and Stem Cell Transplantation, Faculty of Medicine, Medical Centre, University of Freiburg, Hugstetter Straße 55, 79106 Freiburg, Germany

https://doi.org/10.1016/j.biopha.2023.115635
Received 7 July 2023; Received in revised form 24 September 2023; Accepted 3 October 2023
Available online 8 October 2023

resistant and -sensitive BRAF-mutant melanoma cells. In addition, this could be the basis for an alternative approach to treat other tumors expressing mutated BRAF such as non-small-cell lung cancer.

1. Introduction

Treatment of metastatic melanoma has been extraordinarily improved over the past decade by approval of small-molecule kinase inhibitors and checkpoint blocking antibodies [1,2]. Depending on the mutation status, e.g. oncogenic mutations at position V600 in the *BRAF* gene, BRAF and MEK inhibitor combinations are first-line treatments targeting the MAPK signaling pathway [3,4] or are administered as second-line therapy in patients with resistance to PD-1-based immunotherapy [5]. Limits are given due to primary and acquired therapy resistance as consequences of tumor heterogeneity and immune evasion [6,7]. In addition, and based on the underlying mechanism that checkpoint inhibitors target T cell activity, tumors with low T cell count cannot be efficiently controlled by immune checkpoint inhibitors [8].

To overcome drug resistance and therapy failure due to the absence of cellular target structures, i.e. tumors expressing wild-type BRAF and low numbers of infiltrating T cells, identification of new targeted therapy combinations is required. In this context, proteasome inhibitors have become important which block the activity of the proteasome complex, the proteolytic component of the ubiquitin-proteasome-system (UPS). The UPS plays a central role in regulating the degradation of damaged or short-lived proteins thereby interfering with cell cycle progression, gene expression, response to oxidative stress, cell proliferation, inflammation and apoptosis [9–11]. The proteasome complex contains a 20 S catalytic core particle, which is composed of four staggered rings, each containing seven non-identical subunits, three of which (β1, β2, and β5) express the active sites. In the presence of type I and/or type II interferons, the β1, β2 and β5 standard subunits are replaced by the immunosubunits β1i/LMP2, β2i/MECL-1 and β5i/LMP7, thereby forming *de-novo* synthesized immunoproteasome complexes [12]. In this regard, recently published data demonstrate that the expression of immunoproteasome subunits β1i/LMP2 and β5i/LMP7 in melanoma reflecting an IFN-γ signature correlates positively with the strength of patients' immune response to checkpoint inhibitors [13]. Furthermore, proteasome inhibition interferes with NFκB-activation by stabilization of its inhibitor IκBα. This seems to be of particular importance in melanoma where a pro-inflammatory microenvironment with an activated NFκB-pathway and high level of tumor necrosis factor (TNF)-α causes dedifferentiation and growth of melanoma cells [14].

Bortezomib was the first proteasome inhibitor approved for the first- and second-line treatment of multiple myeloma due to its efficiency in non-solid tumors. Immunoproteasome reactive inhibitors are part of phase II clinical trials for the treatment of chronic inflammatory diseases [15]. In solid tumors, however, therapeutic limitations are attributed to sub-optimal tumor penetration and neurotoxic side-effects [16]. To resolve the issue of inhibitor distribution, proteasome inhibitors containing a highly active reversible binding lead motif have been developed which have the potential to penetrate deep into tissue [17]. Several in vitro and in vivo studies have proposed the increased efficacy of inhibitor combinations that target proteasomes and other proteins important for melanoma cell survival. In this regard, combinations with MCL-1 inhibitor and bortezomib [18], proteasome and XIAP antagonists, which interfere with the inhibition of the apoptotic cell death mediated by XIAP [19] and combined treatment with proteasome inhibitor and IFN-α [20] or IFN-β [21] resulted in enhanced cell death in melanoma.

Another class of therapeutic targets for tumor regression are ion channels, in particular voltage-gated potassium channels representing the largest group. Kv1.3 potassium channels can be found in many normal and cancerous cell types. They are expressed in the plasma membrane and inner mitochondrial membrane of the cell [22]. Inhibition of mitochondrial Kv1.3 (mitoKv1.3) channels results in induction of apoptosis which can be mediated by binding of the pro-apoptotic protein Bax to the channel pore [23]. In addition, mitoKv1.3 channel inhibition leads to apoptosis of cancer cells even in the absence Bax and Bak [24,25].

In this study, we analyzed the biological effects of a combined target-based approach on drug-resistant and sensitive human melanoma cells using the proteasome inhibitor BSc2189 with a highly active α-ketoamide group [17,26] and the FDA approved Alzheimer's drug memantine which blocks N-methyl-D-aspartate type glutamate receptors (NMDARs) as well as Kv1.3 potassium channels [27,28]. As model systems we used chemoresistant and BRAFi-resistant as well as corresponding wild-type cell lines. Our data show that proteasome inhibitor treatment reduces cell viability of all melanoma cell lines at concentrations that are non-toxic to primary human fibroblasts. Furthermore, combined exposure of melanoma cells to both proteasome and Kv1.3 inhibitor significantly increases apoptosis compared to proteasome inhibition alone which results in synergistic reduction of melanoma cell growth.

2. Material and methods

2.1. Chemical reagents

BSc2189 [29], Bortezomib (Selleckchem, #S1013), Etoposide (Bristol-Myers Squibb), Vemurafenib (PLX-4032, Selleckchem, #S1267), PAP-1 (Sigma-Aldrich, #P6124) and TRAM-34 (Selleckchem, #S1160) were dissolved in DMSO. Memantine (Tocris, #0773) was dissolved in water.

2.2. Cell culture

Human melanoma cell lines MeWo (RRID:CVCL_0445), BRAF wild-type [30], etoposide-resistant $MeWo_{Eto}$ (RRID:CVCL_AZ62), BRAF-mutant ($BRAF^{V600E}$) Ma-Mel-63a (RRID:CVCL_A198), Ma-Mel-66a (RRID:CVCL_A201), Ma-Mel-86c (RRID:CVCL_C7TP)[31,32] and BRAFi PLX-4032-resistant Ma-Mel-63a$_{PLX}$, Ma-Mel-66a$_{PLX}$ and Ma-Mel-86c$_{PLX}$ were obtained by Westdeutsche Biobank Essen (WBE/SCABIO, University Hospital Essen). Melanoma cell lines were grown in RPMI-1640 Medium (Merck, #F1215) supplemented with 10% FCS, penicillin (100 U/ml, Merck, #A2212) and streptomycin (100 µg/ml, Merck, #A2212). Addition of 1 µg/ml etoposide to the $MeWo_{Eto}$ cells and 1 µmol/l (final concentration) PLX-4032 to the Ma-Mel-63a$_{PLX}$, Ma-Mel-66a$_{PLX}$ and Ma-Mel-86c$_{PLX}$ twice a week maintained inhibitor resistance. Cell lines were authenticated by genetic profiling on genomic DNA at the Institute for Forensic Medicine (University Hospital Essen) using the AmpFLSTR-Profiler Plus kit (Applied Biosystems). Primary human fibroblasts (PromoCell, #C-12352) were cultivated according to manufacturer's instructions. For stimulation cells were seeded in 6-well plates. After 24 h, IFN-γ (Peprotech, #300–02) or TNF (Peprotech, #300–01 A) was added for the indicated time periods. Rat glioma cell line C6 (RRID:CVCL0194) was a kind gift from A. Vogelgesang (Department of Neurology, University Medicine Greifswald). C6 cells were grown in RPMI-1640 Medium (Merck, #F1215) supplemented with 10% FCS. Murine cytotoxic T cell line CTLL-2 (RRID:CVCL0227) was a kind gift by B. Schraven (Institute of Molecular and Clinical Immunoogy, Magdeburg, ATCC #TIB-214) CTLL-2 cells were grown in RPMI-1640 Medium supplemented with 10% FCS and 10 IU/ml IL-2 (Peprotech, #200–02). All experiments were performed with mycoplasma-free cells and regularly tested with MycoAlert Mycoplasma Detection Kit (Lonza).

2.3. Cell viability assays

To assess the effects of proteasome inhibition, primary human fibroblasts, Mewo and $MeWo_{Eto}$ were analyzed in vitro using crystal violet assay as described before [33]. For combined treatment proteasome inhibitor BSc2189 was added 2 h prior addition of the potassium channel inhibitors.

Viability of Ma-Mel-63a, Ma-Mel-$63a_{PLX}$, Ma-Mel-66a, Ma-Mel-$66a_{PLX}$, Ma-Mel-86c, and Ma-Mel-$86c_{PLX}$ was examined by MTT assay. In brief, 24 h after seeding cells were incubated with the depicted concentrations of BSc2189 and/or memantine or vemurafenib in quadruplicates. After 24, 48 or 72 h incubation cells were stained with the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide] and incubated for 4 h followed by absorbance measurement at 570 nm. Calculation of the results of the crystal violet assay and MTT assay was performed as previously described [33].

2.4. Immunoblot analysis

Total cell lysates of melanoma cells were separated by SDS-Page and transferred to nitrocellulose membranes. Membranes were probed overnight with primary antibodies anti-LMP7 (Abcam, #ab3329), anti-MB1 (#12919), anti-phospho-STAT-1 (#7649), anti-IκBα (#4814), anti-PARP-1 (#9542), anti-Bak (#3814), anti-Bax (#2772), anti-Bcl-XL (#2762), anti-Bcl-2 (#15071), anti-GAPDH (#5174, all Cell Signaling Technology), anti-Kv1.3 (#APC-101, Alomone Labs), anti-Noxa (#PRS2437) and anti-β-actin (#A1978, all Sigma-Aldrich). The immunoblots were visualized with horseradish peroxidase–conjugated secondary antibodies (Dianova) and enhanced by chemiluminescent substrate ECL (Cell Signaling Technologies, #6883). Band intensities were semi-quantified using ImageJ software and normalized to loading control.

2.5. Cell cycle analysis

For cell cycle analysis, cells were treated for 24 or 48 h with BSc2189 (0–1 μmol/l). After incubation, melanoma cells were fixed in ice-cold PBS-80% ethanol and incubated at 0 °C for at least 12 h. After washing with PBS–0.5% BSA, cells were incubated in PBS supplemented with 50 μg/ml propidium iodide and 100 μg/ml DNase-free RNase A for 20 min at room temperature. Cells were analyzed with a FACSCanto II flow cytometer (BD Biosciences) using FACSDiva, CellQuest-Pro (BD Biosciences), FlowJo (FlowJo LLC) and ModFit-LT (Verity Software House) software packages as described before [33].

2.6. Flow cytometry

24 h after seeding inhibitors were added to the cells and incubated as indicated. Afterwards, cells were fixed and permeabilized with FOXP3 intracellular staining Kit (Ebioscience, #00–5523–00) according to manufacturer's instructions. Finally, cells were stained with anti-potassium channel Kv1.3-FITC antibody (Sigma-Aldrich, #P4247) and subsequently measured by MACSQuant flow cytometer (Miltenyi). To assess surface Kv1.3 channel expression cells were stained prior fixation and permeabilization. For NMDA-receptor staining cells were stained with primary anti-NMDA Receptor 2B antibody (Alomone Labs #AGC-003) for 20 min in the dark and subsequently stained with goat-anti-rabbit-Alexa 488 secondary antibody (Dianova #111–545–144) [28].

2.7. Statistical analysis

Experiments were presented as means and Student's *t* test (two-tailed) was used to compare differences between analyzed samples. Cell viability between fibroblasts and MeWo/$MeWo_{Eto}$ was compared by ordinary one-way ANOVA, Tukeys multi comparisons Test (Fig. 1A), and between MeWo and $MeWo_{Eto}$ as well as Ma-Mel-86c and Ma-Mel-$86c_{PLX}$ by two-way ANOVA (Fig. 1A and D). Intracellular potassium channel data was analyzed by ordinary one-way ANOVA, Tukeys multi

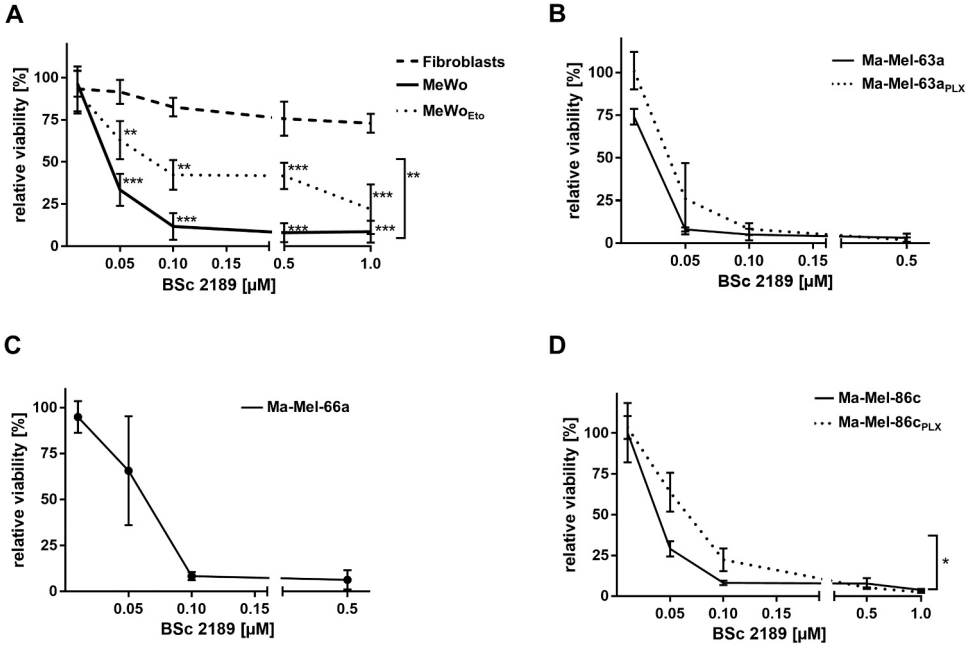

Fig. 1. Relative viability of human melanoma cells after 48 h exposure to BSc2189. (A-D) MeWo cells, etoposide-resistant MeWo cells ($MeWo_{Eto}$) and primary human fibroblasts (A) as well as Ma-Mel-86c and BRAFi PLX-resistant Ma-Mel-$86c_{PLX}$ cells (D) were treated with 0–1 μmol/l BSc2189. Ma-Mel-63a, Ma-Mel-$63a_{PLX}$ (B) and Ma-Mel-66a (C) were treated with 0–0.5 μmol/l BSc2189. Graphs depict the relative viability as percentage of the untreated control group. (A) n = 4, crystal violet assay (B), (C) and (D) n = 3, MTT assay. Values are the means ± SD of triplicate determinations. Significant difference between fibroblasts and MeWo/$MeWo_{Eto}$ at indicated concentrations (one-way ANOVA, Tukeys multi comparisons Test) and between MeWo, $MeWo_{Eto}$ and Ma-Mel-86c, Ma-Mel-$86c_{PLX}$ (shown as bracket on the right side, two-way ANOVA) *$P < 0.05$ * * $P < 0.01$ * ** $P < 0.001$.

comparisons test (Fig. 6A and B). All statistical analysis was done by GraphPad Prism software version 7.01 and considered significant at * $P < 0.05$ * * $P < 0.01$ and * ** $P < 0.001$.

The combination index (CI) was determined with CompuSyn1.0 software using the Chou-Talalay method [34].

2.8. Data Availability

Data will be made available on request.

3. Theory

One approach for treating highly proliferative cancers such as melanoma is to disrupt cell homeostasis using inhibitors to ultimately drive tumor cells into apoptosis. Many successful strategies have already been introduced to cure cancer. However, therapy resistance and individual differences between patients reduce therapeutic success. Therefore, chemically improved inhibitors, as demonstrated in our study for proteasome inhibition, as well as novel combinations with compounds such as memantine, already approved in the clinics, provide an opportunity to develop new strategies for triggering cancer cell death. The results obtained in our study analyzing BRAFi- and chemo-resistant human melanoma cells in comparison to their drug-sensitive counterparts may serve as a platform for further approaches to overcome potential resistances and to interfere with tumor growth.

4. Results

4.1. Proteasome inhibition reduces viability of human melanoma cells

To investigate the effect of proteasome inhibitor treatment on the viability of human melanoma cells, MeWo (BRAF-WT) and etoposide-resistant $MeWo_{Eto}$, as a model for chemo-resistant cells, and BRAF-mutant ($BRAF^{V600E}$) Ma-Mel-63a, Ma-Mel-66a, Ma-Mel-86c as well as BRAFi PLX-4032-resistant Ma-Mel-$63a_{PLX}$ and Ma-Mel-$86c_{PLX}$ cells were exposed to increasing concentrations of proteasome inhibitor BSc2189 [17,26,29]. Notably, Ma-Mel-66a cells display intrinsic resistance to BRAFi (Supplementary Fig. S1). After 48 h treatment with BSc2189 all melanoma cell lines showed a concentration-dependent reduction of cell viability (Fig. 1A – D; Supplementary Table S1), whereas viability of primary human fibroblasts could only be reduced in the presence of extremely high BSc2189 concentrations (0.5 μmol/l - 1 μmol/l BSc2189) (Fig. 1A). In contrast, exposure of fibroblasts to low concentration (10 nmol/l) of the clinically approved proteasome inhibitor bortezomib revealed enhanced cytotoxicity compared to BSc2189 (Supplementary Fig. S2A). Thus, cell viability is affected in drug-sensitive and -resistant BRAF-WT and $BRAF^{V600E}$ melanoma cells by the BSc2189 proteasome inhibitor using concentrations that are non-toxic to primary human cells.

Since proteasome inhibitor BSc2189 targets both the β5- and β5i-subunits of the cellular proteasome pool, we next analyzed the expression level of the β5/MB1 standard proteasome and β5i/LMP7 immunoproteasome subunit in all melanoma cell lines. Under non-stimulated conditions the β5/MB1 standard proteasome and the β5i/LMP7 immunoproteasome subunits were expressed in the BRAFi-sensitive and -resistant $BRAF^{V600E}$ melanoma cells, whereas the latter was below detection limit in MeWo and $MeWo_{Eto}$ cells (Fig. 2A - D). To resemble the pro-inflammatory environment of in vivo melanoma, we stimulated the cells with IFN-γ. Exposure of the melanoma cells to IFN-γ resulted in enhanced β5i/LMP7 expression except in etoposide-resistant $MeWo_{Eto}$ cells which turned out to be deficient for the β5i/LMP7 immunoproteasome subunit (Fig. 2A). This could be confirmed on mRNA level (Supplementary Fig. S2B). Since phosphorylation of the transcription factor STAT-1 in $MeWo_{Eto}$ cells was not altered upon IFN-γ treatment compared to MeWo cells (Supplementary Fig. S2C), we conclude that the absence of β5i/LMP7 in $MeWo_{Eto}$ is not caused by a defect in IFN-γ signaling.

4.2. BSc2189 differentially affects cell cycle progression and apoptosis in chemo-sensitive and -resistant melanoma cells

Proteasome inhibition interferes with cell cycle progression, induction of apoptosis and activation of NFκB-signaling by blocking the degradation of cyclins, pro- and/or anti-apoptotic proteins and by stabilizing the NFκB-inhibitor IκBα. To explain the differential response of the drug-resistant and sensitive melanoma cells to treatment with proteasome inhibitor (Fig. 1), we investigated the effect of BSc2189 on cell cycle progression in MeWo and $MeWo_{Eto}$ cells. Detailed analysis revealed a concentration-dependent G2-M arrest in both cell lines after 24 h and 48 h exposure to BSc2189. However, as observed in the experiments published before, chemo-resistant melanoma cells were considerably more resistant to proteasome inhibition than MeWo cells

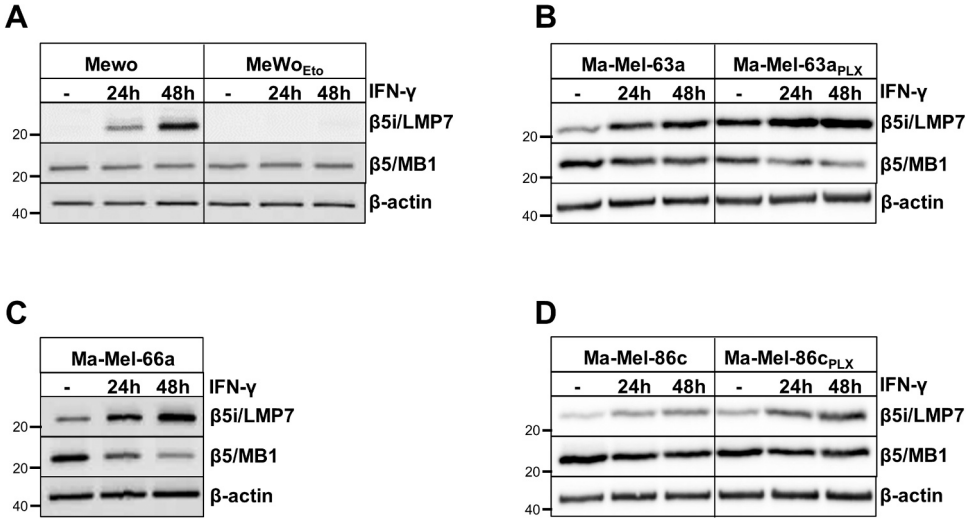

Fig. 2. Expression of immunoproteasome-subunits in drug-sensitive and drug-resistant human melanoma cells. (A–D) Induction of immunoproteasome subunit β5i/LMP7 and expression of standard proteasome subunit β5/MB1 upon addition of 200 U/ml IFN-γ in melanoma cells. Protein expression was analyzed 24 h and 48 h after IFN-γ treatment, β-actin served as loading control. Enhanced β5i/LMP7 expression in all cell lines except in $MeWo_{Eto}$. Compensatory down-regulation of β5/MB1, in Ma-Mel-66a and Ma-Mel-$63a_{PLX}$ cells.

[33]. A subG1-peak indicating accumulation of apoptotic cells could be observed after 48 h proteasome inhibition which was more prominent in MeWo than in $MeWo_{Eto}$ cells (Fig. 3A). These data were consistent with our findings of diminished induction of apoptosis in $MeWo_{Eto}$ cells compared to MeWo cells. Since apoptosis is accomplished by cleavage of several key enzymes such as Poly(ADP-Ribose)-Polymerase 1 (PARP-1) which is crucial for DNA-repair, we determined PARP-1 cleavage due to increasing BSc2189 concentrations in MeWo and $MeWo_{Eto}$ cells (Fig. 3B). Although both cell lines displayed an apoptotic phenotype, higher BSc2189 concentrations and/or longer incubation periods with BSc2189 were required to induce PARP-1 cleavage in $MeWo_{Eto}$ cells. In contrast, TNF-induced IκBα degradation could be stabilized by proteasome inhibition in both MeWo and $MeWo_{Eto}$ cells to the same extent (Supplementary Fig. S2D). Thus, drug-resistant melanoma cells are less affected by proteasome inhibitor treatment than chemo-sensitive melanoma cells displaying impaired induction of apoptosis and representing PARP-1 cleavage apparently at higher inhibitor concentrations.

4.3. *Synergistic effects of proteasome and potassium channel inhibition on melanoma cell viability and apoptosis*

Recent advances highlight potassium channels as a target in cancer therapy including melanoma [22]. In this context, direct inhibition of potassium channel Kv1.3, highly expressed in the mitochondria (mitoKv1.3) of various tumor cells, has been shown to play a fundamental, functional role in the induction of apoptosis in cancer cells [25].

To take advantage of a drug already approved in humans we chose memantine which blocks N-methyl-D-aspartate type glutamate receptors (NMDARs) on neurons [35] and inhibits Kv1.3 potassium channels in T lymphocytes as well as in acute lymphoid and myeloid leukemia cells as shown by electrophysical patch clamp analysis before [28,36,37]. To exclude possible side effects, we determined NMDAR expression on melanoma cells. Flow cytometry-based analysis revealed no NMDAR expression on the drug-sensitive melanoma cells studied here (Supplementary Fig. S3A). In order to determine the outcome of proteasome and/or Kv1.3 inhibition on the viability of melanoma cells, we treated MeWo, $MeWo_{Eto}$, Ma-Mel-63a and Ma-Mel-$63a_{PLX}$ cells (Fig. 4) as well as Ma-Mel-66a, Ma-Mel-86c and Ma-Mel-$86c_{PLX}$ cells (Fig. 5) with low (50 nmol/l) or high (500 nmol/l) BSc2189 concentrations or with different memantine concentrations.

As demonstrated in Fig. 1, viability of Ma-Mel-63a cells was already considerably impaired when exposed to low BSc2189 concentration, whereas higher BSc2189 concentrations were needed to significantly reduce the viability of the other melanoma cell lines (Figs. 4 and 5). Administration of memantine alone had neglectable effect on melanoma cell survival, except for Ma-Mel-66a where the viability was reduced by fifty percent when exposed to 100–200 μmol/l memantine. In contrast, co-incubation of the melanoma cells with BSc2189 and memantine resulted in significant impairment of cell viability (Figs. 4 and 5). This observation was supported by calculation of the combination index which revealed a synergistic effect of BSc2189 and memantine in all melanoma cell lines (Table 1).

To determine the induction of apoptosis in the presence of the inhibitors we analyzed PARP-1 cleavage in the different melanoma cell lines. After a short incubation period (24 h) with the combination of both inhibitors PARP-1 cleavage was clearly visible in MeWo cells, whereas long incubation (48 h) with the inhibitor combination led to pronounced PARP-1 cleavage even in chemo-resistant $MeWo_{Eto}$ cells (Fig. 4B). Analysis of the $BRAF^{V600E}$ melanoma cell lines Ma-Mel-63a, Ma-Mel-66a and Ma-Mel-86c and their BRAFi-resistant counterparts (Figs. 4D, 5B and D) displayed similar results with enhanced apoptosis due to addition of both inhibitors. Collectively, these results demonstrate that combined proteasome and Kv1.3 potassium channel inhibition synergistically aggravates reduction of viability of both sensitive and drug-resistant melanoma cells.

Since memantine also inhibits KCa3.1 channels in T cells [28], we intended to clarify its mode of action on melanoma cells. Therefore, we incubated sensitive and resistant melanoma cells with low concentrations of BSc2189, the known membrane-permeant Kv1.3 inhibitor PAP-1 [24] and the KCa3.1 channel specific inhibitor TRAM-34 [38] either alone or in combination (Supplementary Fig. S3B). In parallel, we confirmed the expression of both Kv1.3 and KCa3.1 channels in Ma-Mel- and MeWo- cell lines as well as in their drug-resistant counterparts (Supplementary Fig. S3C). As observed before (Fig. 4A), combined proteasome and Kv1.3 inhibition significantly reduced viability of MeWo/$MeWo_{Eto}$ cells, albeit to a lesser extend in $MeWo_{Eto}$ cells. Additionally, combining BSc2189 with the KCa3.1 inhibitor TRAM-34 (10 μM) had no effect on cell viability suggesting that KCa3.1 channels cannot be significantly inhibited in melanoma cells (Supplementary Fig. S3B). Moreover, analyzing Kv1.3 deficient murine CTLL-2 cells

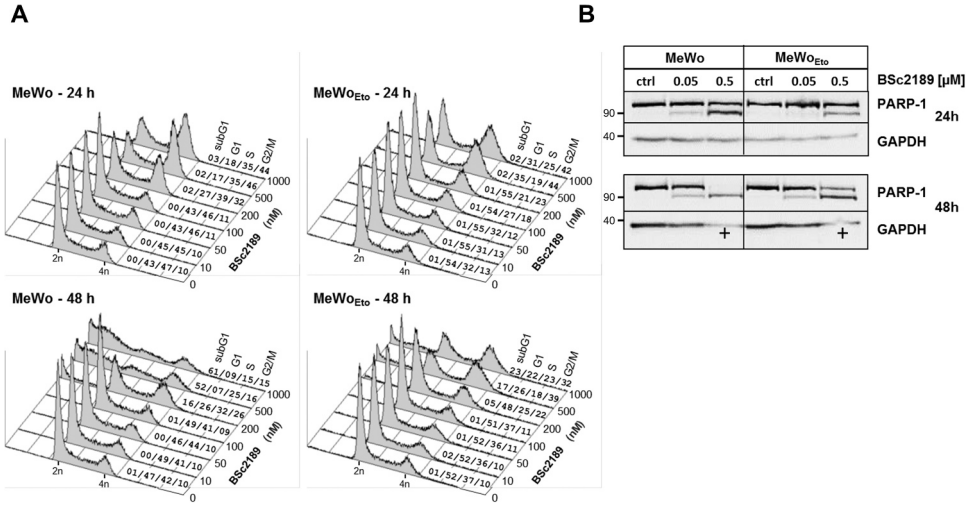

Fig. 3. The BSc2189-induced G2-M arrest is maintained in drug-resistant melanoma cells. (A) Proliferating MeWo and $MeWo_{Eto}$ cells were treated with BSc2189 at concentrations ranging from 0 to 1 μmol/l. At 24 h and 48 h post treatment, cell cycle distribution was analyzed by propidium iodide staining and flow cytometry. Shown are DNA histograms where cell number (y-axis) is plotted against DNA content (x-axis). *Numbers next to each histogram* represent percentages of cells in apoptosis (subG1-population), in G1 phase, S phase, and G2-M phase of the cell cycle, respectively. *Z-axis*, concentrations of BSc2189, n = 2. (B) MeWo and $MeWo_{Eto}$ cells were analyzed by immunoblot for cleavage of apoptotic marker PARP-1 upon 24 h and 48 h treatment with BSc2189, GAPDH served as loading control. n = 3. $^{+}$ signal loss due to long exposure with high inhibitor concentrations.

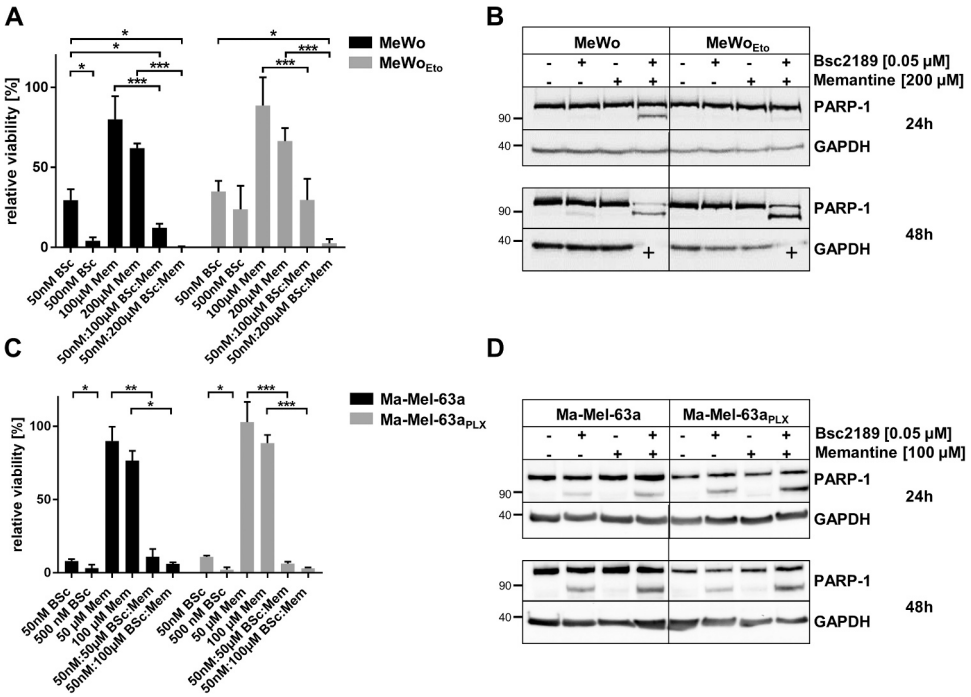

Fig. 4. Combined treatment of MeWo, MeWo$_{Eto}$, Ma-Mel-63a and Ma-Mel-63a$_{PLX}$ cells with BSc2189 (BSc) and memantine (Mem) enhances induction of apoptosis. (A-D) All cell types were treated with the depicted combinations of the inhibitors BSc2189 and memantine. MeWo and MeWo$_{Eto}$ cells were analyzed for cell viability after 72 h (A), relative viability was displayed as percentage of the untreated control group, n = 4; *bars,* SD, values are the means ± SD of quadruplicate determinations. MeWo and MeWo$_{Eto}$ cells were analyzed for activation of apoptotic marker PARP-1 (B), n = 3, $^{+}$ signal loss due to long exposure with high inhibitor concentrations. GAPDH served as loading control. Ma-Mel-63a and Ma-Mel-63a$_{PLX}$ cells were analyzed for cell viability after 48 h (C), n = 3; *bars,* SD. Values are the means ± SD of quadruplicate determinations. Ma-Mel-63a and Ma-Mel-63a$_{PLX}$ cells were analyzed for activation of apoptotic marker PARP-1 (D), n = 2. GAPDH served as loading control. Significant differences between indicated concentrations * $P < 0.05$ * * $P < 0.01$ * ** $P < 0.001$ (Student́s *t* test).

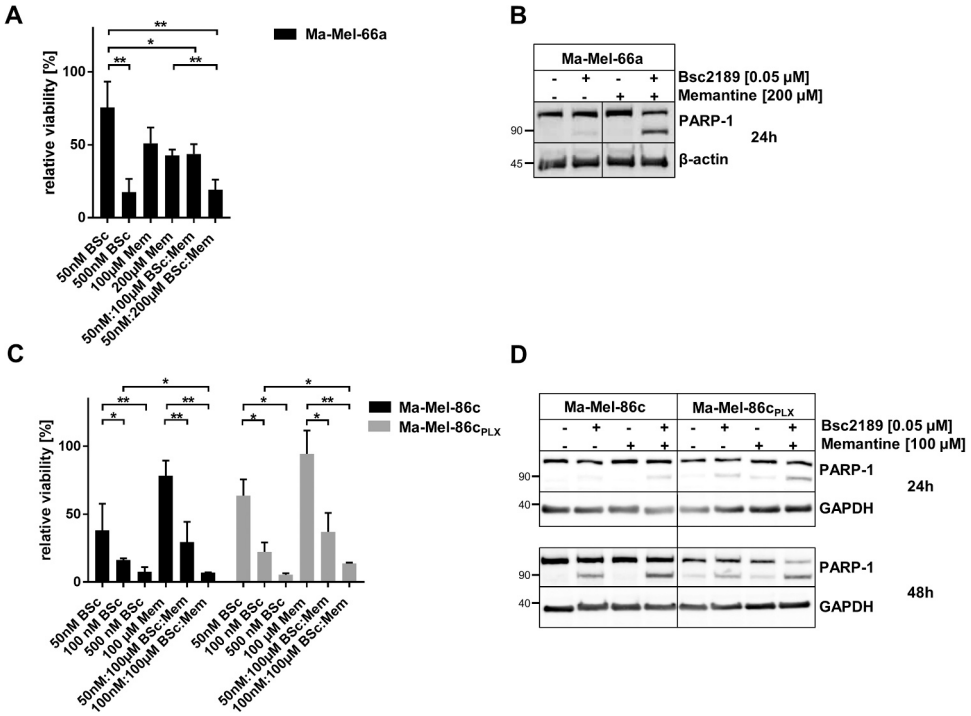

Fig. 5. Validation of the combinatory effect of BSc2189 (BSc) and memantine (Mem) in Ma-Mel-66a, Ma-Mel-86c and Ma-Mel-86c$_{PLX}$ cells. (A) and (C) Exposure of Ma-Mel-66a, Ma-Mel-86c and Ma-Mel-86c$_{PLX}$ cells to the depicted combinations of the inhibitors BSc2189 and memantine. Cells were analyzed for cell viability after 24 (Ma-Mel-66a) and 48 h, n = 3, *bars,* SD. Values are the means ± SD of quadruplicate determinations * $P < 0.05$ * * $P < 0.01$ (Student́s *t* test). (B) and (D) Ma-Mel-66a, Ma-Mel-86c and Ma-Mel-86c$_{PLX}$ cells were analyzed for activation of apoptotic marker PARP-1, n = 2. β-actin and GAPDH served as loading control.

Table 1
Combination index (CI) was determined with CompuSyn1.0 software according to the Chou-Talalay method [34].

	combination index (CI)
MeWo*	0.05
$MeWo_{Eto}$*	0.02
Ma-Mel-63a**	0.41
Ma-Mel-63a $_{PLX}$**	0.36
Ma-Mel-66a*	0.81
Ma-Mel-86c***	0.60
Ma-Mel-86c $_{PLX}$***	0.87

The depicted combinations of BSc2189 and memantine were shown to be synergistic for all investigated melanoma cell lines (CI > 1 antagonistic, CI = 1 additive, CI < 1 synergistic).
For Ma-Mel-63a, Ma-Mel-63a$_{PLX}$, Ma-Mel-86c, and Ma-Mel-86c$_{PLX}$ after 48 h treatment, Ma-Mel-66a after 24 h and MeWo as well as $MeWo_{Eto}$ cells after 72 h treatment CI was calculated for the combination of 50 nM or 100 nM BSc2189 and 100 μM or 200 μM memantine.
* 50 nM BSc2189 and 200 μM memantine
** 50 nM BSc2189 and 100 μM memantine
*** 100 nM BSc2189 and 100 μM memantine

showed no synergistic effect of BSc2189 and memantine which underlines the mechanistic link between proteasome inhibition and Kv1.3 channel blockers (Supplementary Fig. S3D and E).

4.4. Combination of BSc2189 and memantine increases intracellular $_{mito}$Kv1.3 potassium channels and fosters accumulation of pro-apoptotic proteins and ROS

Mitochondrial Kv1.3 channels are crucially involved in the induction of the intrinsic apoptosis pathway [24,39]. In agreement with previously published data we observed intracellular expression of Kv1.3 potassium channels within all melanoma cell lines but not at the cell surface (Supplementary Fig. S4A, 25). Therefore, we next analyzed the impact of both inhibitors on the potassium channel expression in the different drug-resistant and -sensitive melanoma cell lines. Upon treatment with BSc2189 and memantine alone no significant changes in intracellular potassium channel expression were observed in all melanoma cell lines tested (Fig. 6A and B, Supplementary Fig. S4B and C). Only the combination of both inhibitors induced a significant increase of Kv1.3 potassium channels in MeWo, $MeWo_{Eto}$, Ma-Mel-66a, Ma-Mel-86c and Ma-Mel-86c$_{PLX}$ cells and a tendency of an increase in Ma-Mel-63a and Ma-Mel-63a$_{PLX}$ cells indicating that under these conditions sensitivity of melanoma cells to apoptosis should be enhanced.

Since mitochondria-related pathways play an important role in triggering intrinsic apoptosis by stabilizing pro-apoptotic proteins like Noxa, Bak and Bax and degrading anti-apoptotic proteins such as Bcl-2 and Bcl-XL, we analyzed the expression level of these proteins in the presence of BSc2189 and memantine in MeWo cells. Co-incubation of MeWo cells with BSc2189 and memantine resulted in the strongest accumulation of the pro-apoptotic proteins Noxa and Bak (Fig. 6C-E), whereas expression of the anti-apoptotic proteins Bcl-2 and Bcl-XL was reduced (Fig. 6C and F-G). In contrast, expression analysis of the pro-apoptotic protein Bax revealed no differences upon BSc2189 and memantine treatment (Supplementary Fig. S4D) suggesting that Bax has only an indirect effect on mitoKv1.3 channel-induced apoptosis by its interaction with Bak as proposed before [40,41].

Oxidative stress has been associated with the development of melanoma and its progression to chemoresistance [42]. On the other hand, intracellular accumulation of reactive oxygen species (ROS) triggers ROS-mediated cell death and is known to be induced by exposure of tumor cells to proteasome or Kv1.3 inhibitor [25,43]. In our analyses, combined BSc2189 and memantine treatment of MeWo and $MeWo_{Eto}$ cells induced significant ROS formation which was higher than by each

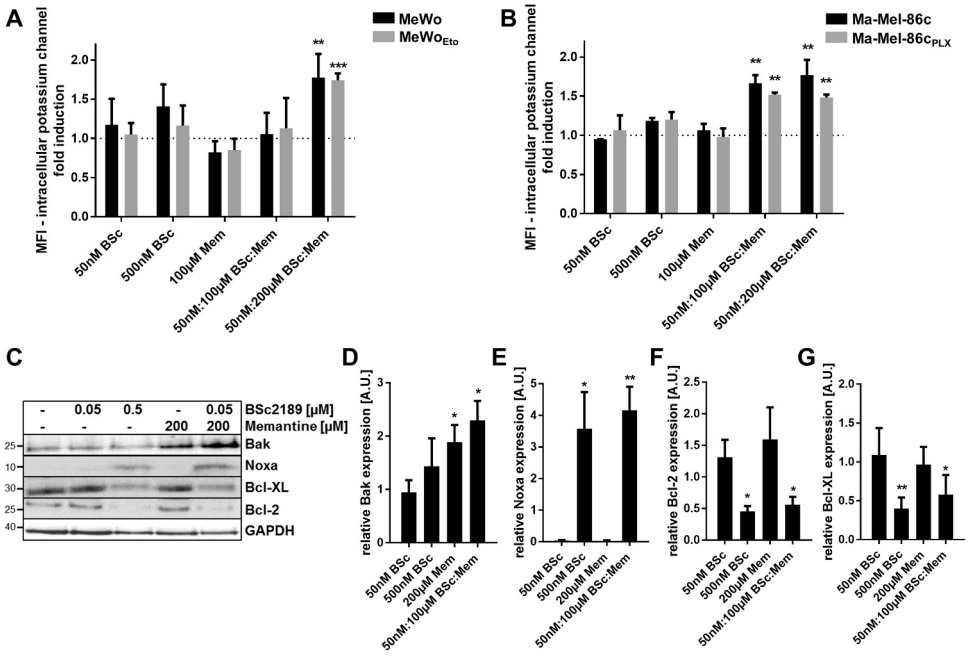

Fig. 6. Combined treatment of melanoma cells with BSc2189 (BSc) and memantine (Mem) leads to an increase of intracellular potassium channels, pro-apoptotic proteins and degradation of anti-apoptotic proteins. (A) and (B) MeWo, $MeWo_{Eto}$ cells were treated with the depicted combinations of the inhibitors BSc2189 and memantine for 48 h (A), Ma-Mel-86c and Ma-Mel-86c$_{PLX}$ cells were treated for 12 h (B). All cell types were analyzed for expression of intracellular potassium channels by flow cytometry shown as fold induction of the MFI compared to the untreated control group; (A) n = 4, (B) n = 3, *bars*, SD. Significant difference between untreated control and MeWo/$MeWo_{Eto}$ and Ma-Mel-86c/Ma-Mel-86c$_{PLX}$ at indicated concentrations * $P < 0.01$ * ** $P < 0.001$ (Student's *t* test). (C) MeWo cells were treated with depicted inhibitor concentrations followed by expression analysis of the pro-apoptotic proteins Noxa and Bak and anti-apoptotic proteins Bcl-XL and Bcl-2 after 48 h. (D-G) Densitometric analysis of three independent experiments for Bak (D), Noxa (E), Bcl-2 (F) and Bcl-XL (G) 48 h following inhibitor treatment, band intensities were normalized to their respective GAPDH loading control and changes were calculated in relation to untreated control. *bars*, SD. Significant differences at indicated concentrations compared to untreated control * $P < 0.05$ * * $P < 0.01$ (Student's *t* test).

inhibitor alone. However, this finding could not be confirmed in Ma-Mel-86c and Ma-Mel-86c$_{PLX}$ cells indicating that the underlying mechanism(s) is (are) cell-specific (Supplementary Fig. S5).

In sum, these results demonstrate that the combination of proteasome and Kv1.3 potassium channel inhibition enhances apoptosis of chemo-resistant, BRAFi-resistant and corresponding drug-sensitive melanoma cells which can be explained by an increase of mitoKv1.3 channels and pro-apoptotic proteins Bak and Noxa as well as by the reduced levels of anti-apoptotic proteins Bcl-2 and Bcl-XL.

5. Discussion

Melanoma progression is associated with a high degree of plasticity based on cell growth-promoting mutations. Activating MAPK-mutations most frequently affect the BRAF gene accounting for 33–47% of primary and 41–55% of metastatic melanoma [44]. Clinical application of BRAF inhibitors combined with MEK inhibitors significantly increases survival of patients [45]. However, prolonged inhibitor treatment has been associated with the development of secondary resistances such as amplification of BRAF in 20% of melanomas after treatment with BRAFi, or alternative splicing of BRAF which is present in 32% of melanomas resulting in tumor progression [46,47]. This indicates that acquired resistance poses a major challenge for melanoma therapy and still requires new therapeutic strategies [48]. Our present study demonstrates enhanced efficacy of combined treatment of BRAF-WT and BRAF mutant human melanoma cells both drug-sensitive and drug-resistant with two inhibitors targeting different pathways to trigger apoptosis. Our results show that simultaneous exposure of melanoma cells to proteasome and Kv1.3 inhibitor causes an increase of intracellular Kv1.3 channels. Enhanced potassium channel levels have been detected before due to pharmacological Kv1.3 inhibition in neural cells [49], stabilization of Kv1.5 channels by proteasome inhibition [50] and in non-small-cell lung cancer (NSCLC) contributing to tumor progression as well as in many other types of cancer [23,51]. Kv1.3 channels are expressed at the plasma membrane. Recently published data suggest that Kv1.3 channels are partially processed in the cytosol indicating a possible role of the proteasome in this process [52]. However, in cancer cells high expression of Kv1.3 channels has been identified at the inner mitochondrial membrane which offers a promising target for successful pharmacological intervention [25]. In this regard, mitochondrial potassium channel expression directly correlates with the sensitivity of tumor cells to drug-induced cell death [39], and inhibition of mitochondrial Kv1.3 channels leads to hyperpolarization of the mitochondrial membrane with enhanced ROS-production and apoptosis even in the absence of pro-apoptotic proteins Bax and Bak [24,25].

Induction of apoptosis can be mediated by binding of pro-apoptotic proteins to mitochondrial Kv1.3 channels in cancer cells and it could be shown that Bax oligomers directly insert into the channel pores [23,40]. Notably, in our experiments combined treatment of melanoma cells with proteasome and Kv1.3 inhibitor induced the expression of the pro-apoptotic Bak but not of Bax protein. This is consistent with earlier findings where only Bak was upregulated upon exposure of melanoma cells to bortezomib [53]. Functional studies suggested overlapping roles of Bak and Bax in the regulation of apoptosis [54], since Bak is able to oligomerize in the absence of Bax as well [55]. Interaction between Bak and Kv1.3 channels could mediate stabilization of each other, therefore contributing to increased induction of apoptosis.

We observed that proteasome inhibition as single treatment and in combination with potassium channel inhibitors caused an accumulation of the proteasomal substrate Noxa and destabilization of the anti-apoptotic proteins Bcl-XL and Bcl-2 in drug-sensitive BRAF-WT melanoma cells (Fig. 6C-G). This is in agreement with previously published data obtained in melanoma and multiple myeloma cells but not in melanocytes, indicating a Noxa-mediated apoptotic response exclusively in tumor cells [53,56].

6. Conclusions

We conclude that simultaneous activation of different pathways to induce apoptosis can inhibit growth of drug-resistant and -sensitive human melanoma cells both BRAF-WT and BRAF-mutant, thus potentiating the expression of pro-apoptotic proteins as well as mitochondrial Kv1.3 channels.

We suggest the combined proteasome and potassium channel inhibition as a novel approach for the treatment of patients especially with primary and acquired resistance to BRAF inhibitors and in patients who are unresponsive or intolerant to immune checkpoint inhibitors due to immune-related side effects. In addition, the synergistic outcome of the combined proteasome and potassium channel inhibition allows a reduction in the inhibitor concentrations used, attenuating possible adverse side effects due to high drug concentrations.

Based on the observation that NSCLC expressing the BRAF^{V600E} mutation are sensitive to BRAF inhibition and that potassium channel blocker display antitumor activity in alveolar epithelial cells combined proteasome and Kv3.1 inhibition could be also considered for targeted therapy of e.g. NSCLC [51,57].

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki principles. Biological samples and related data were provided by the Westdeutsche Biobank Essen (WBE/SCABIO, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; approval no. SCABIO_114715).

Funding sources

This work was supported by grants of the “Hiege Foundation against skin cancer” to U.S. and by the Comprehensive Cancer Center Mecklenburg-Western Pomerania, Germany (CCC-3900) to C.C. We acknowledge support for the Article Processing Charge by the German Research Foundation and the Open Access Publication Fund of the University of Greifswald.

CRediT authorship contribution statement

Clemens Cammann: conceptualization, methodology, visualization, funding acquisition, writing - original draft; Jonas Kulla: investigation, visualization; Lüder Wiebusch: methodology, investigation; Christian Walz: resources; Fang Zhao: methodology, investigation; Theresa Lowinus: investigation, writing - review & editing; Eylin Topfstedt: investigation; Neha Mishra: investigation; Petra Henklein: resources; Lukas Bossaller: resources; Ursula Bommhardt: conceptualization, writing - review & editing; Christian Hagemeier: writing - review & editing; Dirk Schadendorf: resources, writing - review & editing; Boris Schmidt: resources; Annette Paschen: conceptualization, writing - original draft; Ulrike Seifert: conceptualization, supervision, project administration, funding acquisition, writing - original Draft.

Declaration of Competing Interest

A.P. reports research grant support and provision of reagents from Bristol-Myers Squibb (BMS) and Merck Sharp & Dohme (MSD).

Data Availability

Data will be made available on request.

Acknowledgements

We thank Nadine Riemann and Dr. Xenia Gorny, both OVGU Magdeburg, Germany, for excellent technical assistance and PD Dr. Roland

Schönherr, Friedrich-Schiller-University Jena, Germany, for technical advice.

Consent for publication

All authors read and approved the final manuscript for publication.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115635.

References

[1] A. Paschen, D. Schadendorf, The era of checkpoint inhibition: lessons learned from melanoma, Recent Results Cancer Res 214 (2020) 169–187, https://doi.org/10.1007/978-3-030-23765-3_6.

[2] D. Liu, B. Schilling, D. Liu, A. Sucker, E. Livingstone, L. Jerby-Arnon, L. Zimmer, R. Gutzmer, I. Satzger, C. Loquai, S. Grabbe, N. Vokes, C.A. Margolis, J. Conway, M.X. He, H. Elmarakeby, F. Dietlein, D. Miao, A. Tracy, H. Gogas, S.M. Goldinger, J. Utikal, C.U. Blank, R. Rauschenberg, D. von Bubnoff, A. Krackhardt, B. Weide, S. Haferkamp, F. Kiecker, B. Izar, L. Garraway, A. Regev, K. Flaherty, A. Paschen, E. M. Van Allen, D. Schadendorf, Integrative molecular and clinical modeling of clinical outcomes to PD1 blockade in patients with metastatic melanoma, Nat. Med 25 (2019) 1916–1927, https://doi.org/10.1038/s41591-019-0654-5.

[3] G.V. Long, D. Stroyakovskiy, H. Gogas, E. Levchenko, F. de Braud, J. Larkin, C. Garbe, T. Jouary, A. Hauschild, J.J. Grob, V. Chiarion Sileni, C. Lebbe, M. Mandalà, M. Millward, A. Arance, I. Bondarenko, J.B. Haanen, J. Hansson, J. Utikal, V. Ferraresi, N. Kovalenko, P. Mohr, V. Probachai, D. Schadendorf, P. Nathan, C. Robert, A. Ribas, D.J. DeMarini, J.G. Irani, M. Casey, D. Ouellet, A. M. Martin, N. Le, K. Patel, K. Flaherty, Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma, N. Engl. J. Med 371 (2014) 1877–1888, https://doi.org/10.1056/NEJMoa1406037.

[4] R. Dummer, G.V. Long, C. Robert, H.A. Tawbi, K.T. Flaherty, P.A. Ascierto, P. D. Nathan, P. Rutkowski, O. Leonov, C. Dutriaux, M. Mandalà, P. Lorigan, P. F. Ferrucci, J.J. Grob, N. Meyer, H. Gogas, D. Stroyakovskiy, A. Arance, J.C. Brase, S. Green, T. Haas, A. Masood, E. Gasal, A. Ribas, D. Schadendorf, Randomized phase iii trial evaluating spartalizumab plus dabrafenib and trametinib for BRAF V600-mutant unresectable or metastatic melanoma, J. Clin. Oncol. 40 (2022) 1428–1438, https://doi.org/10.1200/jco.21.01601.

[5] S. Kreft, V. Glutsch, A. Zaremba, P. Schummer, P. Mohr, I. Grimmelmann, R. Gutzmer, F. Meier, C. Pföhler, M.M. Sachse, F. Meiss, A. Forschner, S. Haferkamp, J. Welzel, P. Terheyden, R. Herbst, J. Utikal, M. Kaatz, C. Weishaupt, A. Kreuter, D. Debus, P. Duecker, A. Sindrilaru, H. Löffler, G. Schley, M. Weichenthal, D. Schadendorf, S. Ugurel, A. Gesierich, B. Schilling, MAPKinase inhibition after failure of immune checkpoint blockade in patients with advanced melanoma – an evaluation of the multicenter prospective skin cancer registry ADOREG, Eur. J. Cancer 167 (2022) 32–41, https://doi.org/10.1016/j.ejca.2022.02.023.

[6] C. Robert, J. Schachter, G.V. Long, A. Arance, J.J. Grob, L. Mortier, A. Daud, M. S. Carlino, C. McNeil, M. Lotem, J. Larkin, P. Lorigan, B. Neyns, C.U. Blank, O. Hamid, C. Mateus, R. Shapira-Frommer, M. Kosh, H. Zhou, N. Ibrahim, S. Ebbinghaus, A. Ribas, Pembrolizumab versus Ipilimumab in advanced melanoma, N. Engl. J. Med. 372 (2015) 2521–2532, https://doi.org/10.1056/NEJMoa1503093.

[7] P. Savoia, P. Fava, F. Casoni, O. Cremona, Targeting the ERK signaling pathway in melanoma, Int J. Mol. Sci. (2019) 20, https://doi.org/10.3390/ijms20061483.

[8] P.A. Ascierto, K.D. Lewis, A.M. Di Giacomo, L. Demidov, M. Mandalà, I. Bondarenko, C. Herbert, A. Mackiewicz, P. Rutkowski, A. Guminski, B. Simmons, C. Ye, G. Hooper, M.J. Wongchenko, G.R. Goodman, Y. Yan, D. Schadendorf, Prognostic impact of baseline tumour immune infiltrate on disease-free survival in patients with completely resected, BRAF(v600) mutation-positive melanoma receiving adjuvant vemurafenib, Ann. Oncol. 31 (2020) 153–159, https://doi.org/10.1016/j.annonc.2019.10.002.

[9] X. Wang, T. Meul, S. Meiners, Exploring the proteasome system: a novel concept of proteasome inhibition and regulation, Pharm. Ther. 211 (2020), 107526, https://doi.org/10.1016/j.pharmthera.2020.107526.

[10] S. Narayanan, C.Y. Cai, Y.G. Assaraf, H.Q. Guo, Q. Cui, L. Wei, J.J. Huang, C. R. Ashby Jr., Z.S. Chen, Targeting the ubiquitin-proteasome pathway to overcome anti-cancer drug resistance, Drug Resist Updat 48 (2020), 100663, https://doi.org/10.1016/j.drup.2019.100663.

[11] P.M. Kloetzel, Antigen processing by the proteasome, Nat. Rev. Mol. Cell Biol. 2 (2001) 179–187, https://doi.org/10.1038/35056572.

[12] B. Strehl, U. Seifert, E. Krüger, S. Heink, U. Kuckelkorn, P.M. Kloetzel, Interferon-gamma, the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing, Immunol. Rev. 207 (2005) 19–30, https://doi.org/10.1111/j.0105-2896.2005.00308.x.

[13] S. Kalaora, J.S. Lee, E. Barnea, R. Levy, P. Greenberg, M. Alon, G. Yagel, G. Bar Eli, R. Oren, A. Peri, S. Patkar, L. Bitton, S.A. Rosenberg, M. Lotem, Y. Levin, A. Admon, E. Ruppin, Y. Samuels, Immunoproteasome expression is associated with better prognosis and response to checkpoint therapies in melanoma, Nat. Commun. 11 (2020) 896, https://doi.org/10.1038/s41467-020-14639-9.

[14] J. Landsberg, J. Kohlmeyer, M. Renn, T. Bald, M. Rogava, M. Cron, M. Fatho, V. Lennerz, T. Wölfel, M. Hölzel, T. Tüting, Melanomas resist T-cell therapy through inflammation-induced reversible dedifferentiation, Nature 490 (2012) 412–416, https://doi.org/10.1038/nature11538.

[15] E.M. Huber, M. Groll, A nut for every bolt: subunit-selective inhibitors of the immunoproteasome and their therapeutic potential, Cells 10 (2021) 1929, https://doi.org/10.3390/cells10081929.

[16] R. Bahleda, M.C. Le Deley, A. Bernard, S. Chaturvedi, M. Hanley, A. Poterie, A. Gazzah, A. Varga, M. Touat, E. Deutsch, C. Massard, H. Van De Velde, A. Hollebecque, M. Sallansonnet-Froment, D. Ricard, H. Taillia, E. Angevin, V. Ribrag, J.C. Soria, Phase I trial of bortezomib daily dose: safety, pharmacokinetic profile, biological effects and early clinical evaluation in patients with advanced solid tumors, Invest N. Drugs 36 (2018) 619–628, https://doi.org/10.1007/s10637-017-0531-3.

[17] D. Stubba, D. Bensinger, J. Steinbacher, L. Proskurjakov, Á. Salcedo Gómez, U. Schmidt, S. Roth, K. Schmitz, B. Schmidt, Cell-Based optimization of covalent reversible ketoamide inhibitors bridging the unprimed to the primed site of the proteasome β5 subunit, ChemMedChem 14 (2019) 2005–2022, https://doi.org/10.1002/cmdc.201900472.

[18] E.F. Lee, T.J. Harris, S. Tran, M. Evangelista, S. Arulananda, T. John, C. Ramnac, C. Hobbs, H. Zhu, G. Gunasingh, D. Segal, A. Behren, J. Cebon, A. Dobrovic, J. M. Mariadason, A. Strasser, L. Rohrbeck, N.K. Haass, M.J. Herold, W.D. Fairlie, BCL-XL and MCL-1 are the key BCL-2 family proteins in melanoma cell survival, Cell Death Dis. 10 (2019) 342, https://doi.org/10.1038/s41419-019-1568-3.

[19] M.A. Laussmann, E. Passante, H. Düssmann, J.A. Rauen, M.L. Würstle, M. E. Delgado, M. Devocelle, J.H. Prehn, M. Rehm, Proteasome inhibition can induce an autophagy-dependent apical activation of caspase-8, Cell Death Differ. 18 (2011) 1584–1597, https://doi.org/10.1038/cdd.2011.27.

[20] L.P. Suarez-Kelly, G.M. Kemper, M.C. Duggan, A. Stiff, T.C. Noel, J. Markowitz, E. A. Luedke, V.O. Yildiz, L. Yu, A.C. Jaime-Ramirez, V. Karpa, X. Zhang, W. E. Carson 3rd, The combination of MLN2238 (ixazomib) with interferon-alpha results in enhanced cell death in melanoma, Oncotarget 7 (2016) 81172–81186, https://doi.org/10.18632/oncotarget.12791.

[21] U.A. Rossi, L.M.E. Finocchiaro, G.C. Glikin, Bortezomib enhances the antitumor effects of interferon-β gene transfer on melanoma cells, Anticancer Agents Med Chem. 17 (2017) 754–761, https://doi.org/10.2174/1871520616666160923103849.

[22] A. Teisseyre, A. Palko-Labuz, K. Sroda-Pomianek, K. Michalak, Voltage-gated potassium channel Kv1.3 as a target in therapy of cancer, Front Oncol. 9 (933) (2019), https://doi.org/10.3389/fonc.2019.00933.

[23] I. Szabó, J. Bock, H. Grassmé, M. Soddemann, B. Wilker, F. Lang, M. Zoratti, E. Gulbins, Mitochondrial potassium channel Kv1.3 mediates Bax-induced apoptosis in lymphocytes. Proc. Natl. Acad. Sci. USA 105 (14861–6) (2008) https://doi.org/10.1073/pnas.0804236105.

[24] L. Leanza, B. Henry, N. Sassi, M. Zoratti, K.G. Chandy, E. Gulbins, I. Szabò, Inhibitors of mitochondrial Kv1.3 channels induce Bax/Bak-independent death of cancer cells, EMBO Mol. Med 4 (577–93) (2012), https://doi.org/10.1002/emmm.201200235.

[25] L. Leanza, M. Romio, K.A. Becker, M. Azzolini, L. Trentin, A. Managò, E. Venturini, A. Zaccagnino, A. Mattarei, L. Carraretto, A. Urbani, S. Kadow, L. Biasutto, V. Martini, F. Severin, R. Peruzzo, V. Trimarco, J.H. Egberts, C. Hauser, A. Visentin, G. Semenzato, H. Kalthoff, M. Zoratti, E. Gulbins, C. Paradisi, I. Szabo, Direct pharmacological targeting of a mitochondrial ion channel selectively kills tumor cells in vivo, Cancer Cell 31 (516–31) (2017), e10, https://doi.org/10.1016/j.ccell.2017.03.003.

[26] M.L. Stein, H. Cui, P. Beck, C. Dubiella, C. Voss, A. Krüger, B. Schmidt, M. Groll, Systematic comparison of peptidic proteasome inhibitors highlights the α-ketoamide electrophile as an auspicious reversible lead motif, Angew. Chem. Int Ed. Engl. 53 (2014) 1679–1683, https://doi.org/10.1002/anie.201308984.

[27] R. McShane, M.J. Westby, E. Roberts, N. Minakaran, L. Schneider, L.E. Farrimond, N. Maayan, J. Ware, J. Debarros, Memantine for dementia, *Cochrane Database Syst. Rev.* 3 (2019), Cd003154, https://doi.org/10.1002/14651858.CD003154.pub6.

[28] S. Kahlfuß, N. Simma, J. Mankiewicz, T. Bose, T. Lowinus, S. Klein-Hessling, R. Sprengel, B. Schraven, M. Heine, U. Bommhardt, Immunosuppression by N-methyl-D-aspartate receptor antagonists is mediated through inhibition of Kv1.3 and KCa3.1 channels in T cells, Mol. Cell Biol. 34 (820–31) (2014), https://doi.org/10.1128/mcb.01273-13.

[29] H.A. Braun, S. Umbreen, M. Groll, U. Kuckelkorn, I. Mlynarczuk, M.E. Wigand, I. Drung, P.M. Kloetzel, B. Schmidt, Tripeptide mimetics inhibit the 20 S proteasome by covalent bonding to the active threonines, J. Biol. Chem. 280 (2005) 28394–28401, https://doi.org/10.1074/jbc.M502453200.

[30] M. Li, D. Liu, D. Lee, S. Kapoor, K.N. Gibson-Corley, T.P. Quinn, E.A. Sagastume, S. L. Mott, S.A. Walsh, M.R. Acevedo, F.L. Johnson, M.K. Schultz, Enhancing the efficacy of melanocortin 1 receptor-targeted radiotherapy by pharmacologically upregulating the receptor in metastatic melanoma, Mol. Pharm. 16 (2019) 3904–3915, https://doi.org/10.1021/acs.molpharmaceut.9b00512.

[31] L. Such, F. Zhao, D. Liu, B. Thier, V.T.K. Le-Trilling, A. Sucker, C. Coch, N. Pieper, S. Howe, H. Bhat, H. Kalkavan, C. Ritter, R. Brinkhaus, S. Ugurel, J. Köster, U. Seifert, U. Dittmer, M. Schuler, K.S. Lang, T.A. Kufer, G. Hartmann, J.C. Becker, S. Horn, S. Ferrone, D. Liu, E.M. Van Allen, D. Schadendorf, K. Griewank, M. Trilling, A. Paschen, Targeting the innate immunoreceptor RIG-I overcomes melanoma-intrinsic resistance to T cell immunotherapy, J. Clin. Investig. 130 (2020) 4266–4281, https://doi.org/10.1172/JCI131572.

[32] N. Pieper, A. Zaremba, S. Leonardelli, F.N. Harbers, M. Schwamborn, S. Lübcke, B. Schrörs, J. Baingo, A. Schramm, S. Haferkamp, U. Seifert, A. Sucker, V. Lennerz, T. Wölfel, D. Schadendorf, B. Schilling, A. Paschen, F. Zhao, Evolution of

melanoma cross-resistance to CD8(+) T cells and MAPK inhibition in the course of BRAFi treatment, Oncoimmunology 7 (2018), e1450127, https://doi.org/10.1080/2162402x.2018.1450127.
[33] I. Młynarczuk-Biały, H. Roeckmann, U. Kuckelkorn, B. Schmidt, S. Umbreen, J. Gołab, A. Ludwig, C. Montag, L. Wiebusch, C. Hagemeier, D. Schadendorf, P. M. Kloetzel, U. Seifert, Combined effect of proteasome and calpain inhibition on cisplatin-resistant human melanoma cells, Cancer Res 66 (2006) 7598–7605, https://doi.org/10.1158/0008-5472.can-05-2614.
[34] T.-C. Chou, Drug combination studies and their synergy quantification using the chou-talalay method, Cancer Res. 70 (2010) 440–446, https://doi.org/10.1158/0008-5472.can-09-1947.
[35] S. Alam, K.S. Lingenfelter, A.M. Bender, C.W. Lindsley, Classics in chemical neuroscience: memantine, ACS Chem. Neurosci. 8 (2017) 1823–1829, https://doi.org/10.1021/acschemneuro.7b00270.
[36] T. Lowinus, F.H. Heidel, T. Bose, S.C. Nimmagadda, T. Schnöder, C. Cammann, I. Schmitz, U. Seifert, T. Fischer, B. Schraven, U. Bommhardt, Memantine potentiates cytarabine-induced cell death of acute leukemia correlating with inhibition of K(v)1.3 potassium channels, AKT and ERK1/2 signaling, Cell Commun. Signal 17 (2019) 5, https://doi.org/10.1186/s12964-018-0317-z.
[37] T. Lowinus, T. Bose, S. Busse, M. Busse, D. Reinhold, B. Schraven, U. H. Bommhardt, Immunomodulation by memantine in therapy of Alzheimer's disease is mediated through inhibition of Kv1.3 channels and T cell responsiveness, Oncotarget 7 (53797–807) (2016), https://doi.org/10.18632/oncotarget.10777.
[38] J. Schmidt, K. Friebel, R. Schönherr, M.G. Coppolino, A.-K. Bosserhoff, Migration-associated secretion of melanoma inhibitory activity at the cell rear is supported by KCa3.1 potassium channels, Cell Res. 20 (2010) 1224–1238, https://doi.org/10.1038/cr.2010.121.
[39] L. Leanza, P. O'Reilly, A. Doyle, E. Venturini, M. Zoratti, E. Szegezdi, I. Szabo, Correlation between potassium channel expression and sensitivity to drug-induced cell death in tumor cell lines, Curr. Pharm. Des. 20 (2014) 189–200, https://doi.org/10.2174/1381612811319990032.
[40] L. Leanza, E. Venturini, S. Kadow, A. Carpinteiro, E. Gulbins, K.A. Becker, Targeting a mitochondrial potassium channel to fight cancer, Cell Calcium 58 (2015) 131–138, https://doi.org/10.1016/j.ceca.2014.09.006.
[41] S. Iyer, R.T. Uren, M.A. Dengler, M.X. Shi, E. Uno, J.M. Adams, G. Dewson, R. M. Kluck, Robust autoactivation for apoptosis by BAK but not BAX highlights BAK as an important therapeutic target, Cell Death Dis. 11 (2020) 268, https://doi.org/10.1038/s41419-020-2463-7.
[42] S. Pizzimenti, S. Ribero, M.A. Cucci, M. Grattarola, C. Monge, C. Dianzani, G. Barrera, G. Muzio, Oxidative stress-related mechanisms in melanoma and in the acquired resistance to targeted therapies, Antioxidants 10 (2021) 1942, https://doi.org/10.3390/antiox10121942.
[43] X. Li, M. Liang, J. Jiang, R. He, M. Wang, X. Guo, M. Shen, R. Qin, Combined inhibition of autophagy and Nrf2 signaling augments bortezomib-induced apoptosis by increasing ROS production and ER stress in pancreatic cancer cells, Int J. Biol. Sci. 14 (2018) 1291–1305, https://doi.org/10.7150/ijbs.26776.
[44] A. Rossi, M. Roberto, M. Panebianco, A. Botticelli, F. Mazzuca, P. Marchetti, Drug resistance of BRAF-mutant melanoma: review of up-to-date mechanisms of action and promising targeted agents, Eur. J. Pharm. 862 (2019), 172621, https://doi.org/10.1016/j.ejphar.2019.172621.
[45] S. Stadler, K. Weina, C. Gebhardt, J. Utikal, New therapeutic options for advanced non-resectable malignant melanoma, Adv. Med Sci. 60 (2015) 83–88, https://doi.org/10.1016/j.advms.2014.12.002.
[46] R.B. Corcoran, D. Dias-Santagata, K. Bergethon, A.J. Iafrate, J. Settleman, J. A. Engelman, BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation, *Sci. Signal* 3 (2010) ra84, https://doi.org/10.1126/scisignal.2001148.
[47] P.I. Poulikakos, Y. Persaud, M. Janakiraman, X. Kong, C. Ng, G. Moriceau, H. Shi, M. Atefi, B. Titz, M.T. Gabay, M. Salton, K.B. Dahlman, M. Tadi, J.A. Wargo, K. T. Flaherty, M.C. Kelley, T. Misteli, P.B. Chapman, J.A. Sosman, T.G. Graeber, A. Ribas, R.S. Lo, N. Rosen, D.B. Solit, RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E, Nature 480 (2011) 387–390, https://doi.org/10.1038/nature10662.
[48] A. Roesch, M. Fukunaga-Kalabis, E.C. Schmidt, S.E. Zabierowski, P.A. Brafford, A. Vultur, D. Basu, P. Gimotty, T. Vogt, M. Herlyn, A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth, Cell 141 (2010) 583–594, https://doi.org/10.1016/j.cell.2010.04.020.
[49] T.M. Reeves, P.A. Trimmer, B.S. Colley, L.L. Phillips, Targeting Kv1.3 channels to reduce white matter pathology after traumatic brain injury, Exp. Neurol. 283 (188–203) (2016), https://doi.org/10.1016/j.expneurol.2016.06.011.
[50] M. Kato, K. Ogura, J. Miake, N. Sasaki, S. Taniguchi, O. Igawa, A. Yoshida, Y. Hoshikawa, M. Murata, E. Nanba, Y. Kurata, Y. Kawata, H. Ninomiya, T. Morisaki, M. Kitakaze, I. Hisatome, Evidence for proteasomal degradation of Kv1.5 channel protein, Biochem Biophys. Res Commun. 337 (343–8) (2005), https://doi.org/10.1016/j.bbrc.2005.09.053.
[51] F. Glaser, P. Hundehege, E. Bulk, L.M. Todesca, S. Schimmelpfennig, E. Nass, T. Budde, S.G. Meuth, A. Schwab, KCa channel blockers increase effectiveness of the EGF receptor TK inhibitor erlotinib in non-small cell lung cancer cells (A549, Sci. Rep. 11 (2021) 18330, https://doi.org/10.1038/s41598-021-97406-0.
[52] J. Capera, M. Navarro-Pérez, A.S. Moen, I. Szabó, A. Felipe, The mitochondrial routing of the Kv1.3 channel, Front. Oncol. 12 (2022), https://doi.org/10.3389/fonc.2022.865686.
[53] J.Z. Qin, J. Ziffra, L. Stennett, B. Bodner, B.K. Bonish, V. Chaturvedi, F. Bennett, P. M. Pollock, J.M. Trent, M.J. Hendrix, P. Rizzo, L. Miele, B.J. Nickoloff, Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells, Cancer Res 65 (2005) 6282–6293, https://doi.org/10.1158/0008-5472.can-05-0676.
[54] T. Lindsten, A.J. Ross, A. King, W.X. Zong, J.C. Rathmell, H.A. Shiels, E. Ulrich, K. G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V.M. Eng, D.M. Adelman, M. C. Simon, A. Ma, J.A. Golden, G. Evan, S.J. Korsmeyer, G.R. MacGregor, C. B. Thompson, The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues, Mol. Cell 6 (2000) 1389–1399, https://doi.org/10.1016/s1097-2765(00)00136-2.
[55] G. Dewson, T. Kratina, P. Czabotar, C.L. Day, J.M. Adams, R.M. Kluck, Bak activation for apoptosis involves oligomerization of dimers via their alpha6 helices, Mol. Cell 36 (2009) 696–703, https://doi.org/10.1016/j.molcel.2009.11.008.
[56] Y. Fernández, M. Verhaegen, T.P. Miller, J.L. Rush, P. Steiner, A.W. Opipari Jr., S. W. Lowe, M.S. Soengas, Differential regulation of noxa in normal melanocytes and melanoma cells by proteasome inhibition: therapeutic implications, Cancer Res 65 (2005) 6294–6304, https://doi.org/10.1158/0008-5472.can-05-0686.
[57] D. Planchard, B. Besse, H.J.M. Groen, P.J. Souquet, E. Quoix, C.S. Baik, F. Barlesi, T.M. Kim, J. Mazieres, S. Novello, J.R. Rigas, A. Upalawanna, A.M. D'Amelio Jr., P. Zhang, B. Mookerjee, B.E. Johnson, Dabrafenib plus trametinib in patients with previously treated BRAF(V600E)-mutant metastatic non-small cell lung cancer: an open-label, multicentre phase 2 trial, Lancet Oncol. 17 (2016) 984–993, https://doi.org/10.1016/s1470-2045(16)30146-2.