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Original Research

Cytotoxicity of combinations of the pan-KRAS inhibitor BAY-293 against primary non-small lung cancer cells

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ABSTRACT

KRAS is mutated in approximately 25% of Non-small Cell Lung Cancer (NSCLC) patients and first specific inhibitors showed promising responses that may be improved by concurrent interference with downstream signaling pathways. Cell lines exhibiting KRAS mutations show specific sensitivities to modulators affecting glucose utilization, signal transduction and cell survival. Novel SOS1-directed inhibitors with a broader anticancer coverage such as BAY-293 and BI-3406 inhibit KRAS through the hindrance of SOS1-KRAS interactions. The aim of this study was to check the putative synergy of BAY-293 with modulators having revealed specific vulnerabilities of KRAS-mutated cell lines. The present investigation tested the cytotoxicity of BAY-293 combinations against a series of Osimertinib-resistant primary NSCLC cell lines using MTT tests and calculation of combination indices according to the Chou-Talalay method. The results show that BAY-293 synergizes with modulators of glucose metabolism, inhibitors of cellular proliferation, several chemotherapeutics and a range of diverse modulators, thus corroborating the chemosensitivities of cells expressing mutated KRAS. In conclusion, BAY-293 exerts cytotoxicity with a wide range of drugs against Osimertinib-resistant primary NSCLC cell lines. The administration of pan-KRAS inhibitors alone may be limited *in vivo* by toxicity to normal tissues but made feasible by its use as part of suitable drug combinations. This study shows that BAY-293 combinations are active against NSCLC cells not further amenable to mutated EGFR-directed targeted therapy and results likewise hold relevance for pancreatic and colon cancer.

Introduction

RAS comprising KRAS, NRAS and HRAS is the most frequently mutated family of genes in cancers and these mutations are drivers for a significant fraction of lung, colorectal (CRC) and pancreatic ductal adenocarcinomas (PDAC) [1,2]. RAS is a small GTPase that toggles between its GTP- bound active and the GDP-bound inactive state. RAS proteins show both intrinsic GTP hydrolysis and nucleotide exchange activity but their signaling state depends on activation by guanine exchange factors (GEFs) that execute the loading of GTP such as SOS1/2 and deactivation by GTPase-activating proteins (GAPs) that catalyze the hydrolysis of GTP [3,4]. In its GTP- bound state, RAS activates several downstream pathways and mutations in RAS result in constitutive activation and high cell proliferation [5,6].

KRAS mutations in lung cancer, CRC and PDAC occur predominantly at codon 12 and covalent inhibition of the KRAS- G12C mutation by inhibitors targeting the active site cysteine fix KRAS in its inactive GDP-bound state [7–10]. G12C comprises approximately 50% of KRAS mutations in lung cancer and G12D is most common in PDAC and CRC [11]. A series of mutated KRAS inhibitors has been developed that trigger cell death across various tumor models [12–14]. Two novel drugs, namely AMG510/Sotorasib [15] and MRTX849/Adagrasib [16], have half-maximal growth inhibitory concentration (IC_{50}) values in the low nanomolar range and have shown clinical activity. Furthermore, AMG 510 exhibited synergistic antiproliferative effects when combined with inhibitors of proteins that modulate RAS and RAS signaling, such as MEK, AKT, PI3K, SHP2 and EGFR family members [15,17]. AMG 510/Sotorasib was the first KRAS inhibitor to reach clinical trials revealing promising phase I trial results in NSCLC with responses in half of the patients of limited duration [18,19].

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The results of the first KRAS inhibitor trials indicate that these inhibitors may need to be combined with other therapeutics to achieve high and durable clinical responses. However, targeting effector signaling downstream of KRAS has not yielded significant clinical benefits in previous attempts [20,21]. To target native KRAS, its interaction with GTP loading can be inhibited and the best characterized GEF for RAS are the Son of Sevenless (SOS) proteins [22,23]. Nanomolar SOS1 inhibitors, such as BI-3406 and BAY-293, that disrupt the KRAS interaction and abolish GTP reloading of native and mutated KRAS resulting in antiproliferative activity were found in compound screens [24–26]. For mutated KRAS a number of pathways have been found to complement in synthetic lethality screens [27–29]. The inhibition of native KRAS is limited by toxicity to normal tissues but combination therapy with modulators of special vulnerabilities of KRAS mutated cells may help to improve the efficacy of pan-KRAS targeting compounds. The present investigation aimed at checking the efficacy of effectors found in these screens in combination with the pan-KRAS inhibitor BAY-293 for the treatment of NSCLC.

Materials and methods

Chemicals

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) or from Selleck Chemicals (Houston, TX, USA). Dulbecco's phosphate buffered saline (PBS) was purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Compounds were prepared as stock solutions of 10 mM in DMSO or 0.9% NaCl for cisplatin and aliquots stored at −20 °C. Equivalent concentrations of DMSO were supplemented for medium controls.

Cell culture

One commercial permanent cell line, NCI-H23, was obtained from the American Type Culture Collection (Rockville, MD, USA) and the primary Osimertinib-resistant lung cancer lines used were established in our lab. Collection of pleural effusions of lung cancer patients, isolation of tumor cells and generation of cell lines was done according to the Ethics Approval 366/2003 by the Ethics Committee of the Medical University of Vienna, Vienna, Austria. In brief, pleural effusions were centrifuged and the tumor cells washed with tissue culture medium consisting of RPMI-1640 medium, supplemented with 10% FBS (Seromed, Berlin, Germany) and antibiotics (final concentrations: 50 U/mL of penicillin, 50 μg/mL of streptomycin, and 100 μg/mL neomycin). When required, erythrocytes were removed by Histopaque®−1077 (Sigma-Aldrich) gradient centrifugation. Primary NSCLC cell lines were established in tissue culture medium and cultures split by trypsination. Mutational status of the patients has been established using the Oncomine Focus 52-genes NGS Array (ThermoFisher Scientific, Waltham, MA, USA).

In detail, NCI-H23 was derived from a lung adenocarcinoma carrying a KRAS G12C mutation and the IVIC-A, BH837 and BH828 lung adenocarcinoma cell lines are all established from pleural effusions of patients carrying EGFR Del19 mutations (no T790M or KRAS mutations present) progressing under Osimertinib therapy. The three PDAC cell lines used for comparison exhibit mutated G12C/Mia-Pa-Ca2, G12D/ASPC1 or wildtype KRAS/BxPC3.

Cytotoxicity assay

Aliquots of 1×10^4 cells in 200 μL medium were treated for four days with twofold dilutions of the test compounds in 96-well microtiter plates in quadruplicate (TTP, Trasadingen, Switzerland). The plates were incubated under tissue culture conditions and cell viability was measured using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (EZ4U, Biomedica, Vienna, Austria). Optical density was measured using a microplate reader at 450 nm and values obtained from control wells containing cells and media alone were set to 100% proliferation. For the assessment of the interaction of the compounds, tests were performed comprising the individual drugs alone and in combination, followed by analysis using the Chou-Talalay method with help of the Compusyn software (ComboSyn Inc., Paramus, NJ, USA). The combination index CI <0.9 indicates synergism, CI >1.1 indicates antagonism and 0.9< CI <1.1 indicates an additive effect.

Statistics

Statistical analysis was performed using Student's *t*-test for normally distributed samples (* $p < 0.05$ was regarded as statistically significant). Values are shown as mean ± SD.

Results

Cytotoxicity of BAY-293 against lung cancer and pancreatic cancer cell lines

The cytotoxic activity of BAY-293 was tested for 4 NSCLC and 3 PDAC cell lines in regular 2D culture MTT tests (Fig. 1). The IC_{50} values ranged from 1.7 for BH828 to 3.7 μM for BH837. The KRAS mutated NCI-H23 cell line exhibited a low BAY-293 sensitivity and, for comparison, for the PDAC cell lines the wildtype KRAS line BxPC3 was more sensitive to the inhibitor compared to the KRAS mutated MiaPaCa2 and ASPC1 lines.

Examples of the cytotoxicity against the NSCLC lines of selected BAY-293 combinations

Examples of the cytotoxic activity combinations of BAY-293 with 2-deoxyglucose (2-DG) and the Polo-like kinase inhibitor PLK1i/BI-2536 in MTT tests are shown for NCI-H23, BH828 and BH837, respectively (Fig. 2 A/B and Fig. 3 A/B). The PLK1i was synergistic with BAY-293 for BH837 but not for IVIC-A with a combination index of 1.33 ± 0.69 The combination of BAY-293 with 2-DG exhibited high synergy in KRAS-mutated NCI-H23 and in KRAS wildtype BH828 cells.

The initial concentrations of the test compounds were diluted twofold in 7 steps. Values represent mean values ± SD.

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The initial concentrations of the test compounds were diluted twofold in 7 steps. Values represent mean values ± SD. The CI for this combination was 0.66 ± 0.024 (Fig. 3).

Combination of BAY-293 with modulators of cellular glycolysis

BAY-293 was tested for antiproliferative effects in combination with 2-deoxy-D-glucose (2-DG), dichloroacetate (DCA), metformin (MET) and linsitinib that modulate glucose utilization and the signaling of insulin/insulin-like receptors, respectively (Fig. 4). Cyclosporin (CsA) was included as control and to test for the possible inhibition of a P-glycoprotein-mediated transport. All four cell lines revealed high synergy of BAY-293 in combination with 2-DG, whereas addition of DCA showed no significant effect. Metfomin was synergistic with the wildtype KRAS inhibitor, with exception of BH837 cells. Linsitinib or CsA were either ineffective or yielded an antagonistic effect in combination with BAY-293.

Values present mean values ± SD. Differences to a summary CI of inactive compounds (1.023 ± 0.018) are significantly different for all combinations except DCA, METF/BH837, Lins and CsA/BH837.

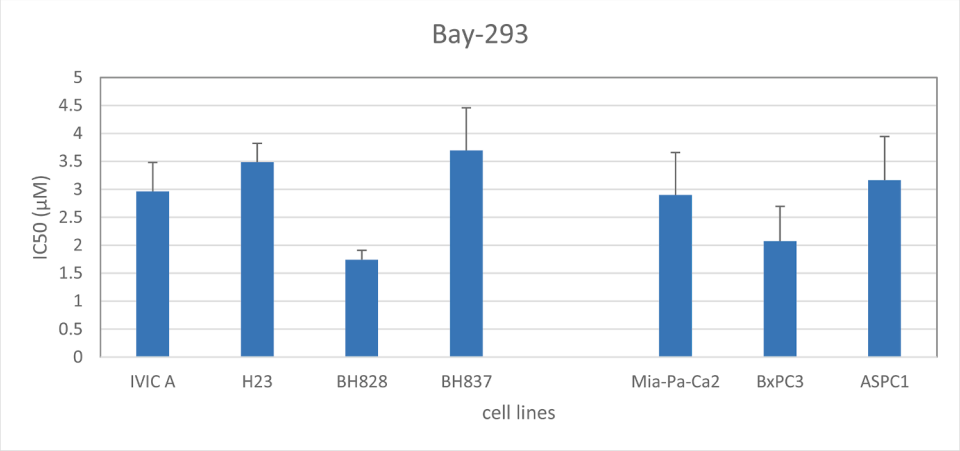

Fig. 1. IC_{50} values of BAY-293 for a panel of NSCLC and PDAC cell lines (mean values ± SD).

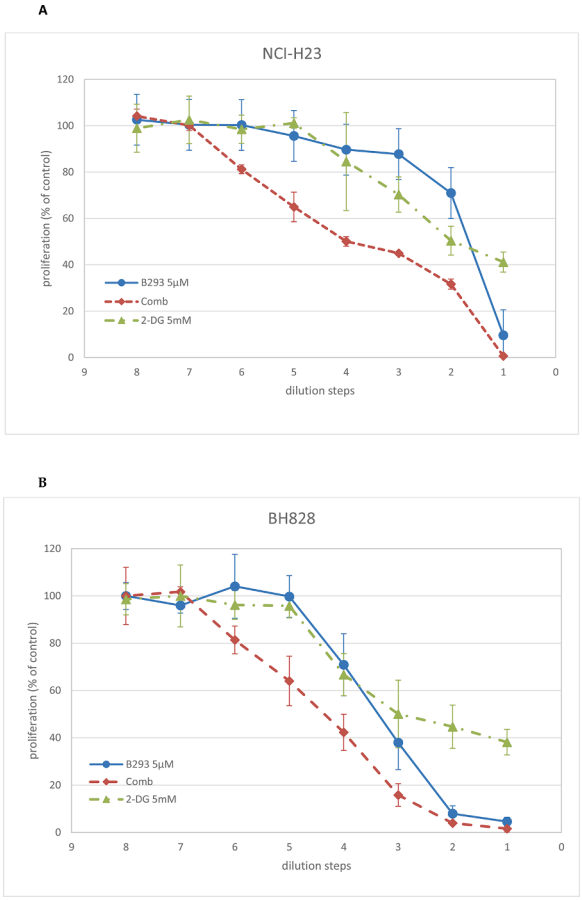

Fig. 2. A. Combination test of BAY-293 with 2-DG against H23 cells. B. Combination test of BAY-293 with 2-DG against BH828 cells.

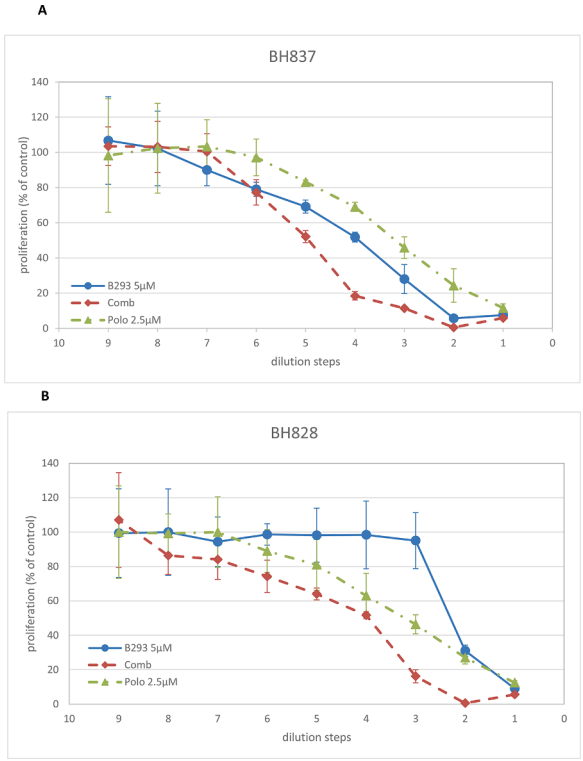

Fig. 3. A Combination of BAY-293 with inhibitor PLK1i BI-2536 (Polo) for BH837 cells. B. Combination of BAY-293 with inhibitor PLK1i BI-2536 (Polo) for BH828 cells. The initial concentrations of the test compounds were diluted twofold in 7 steps. Values represent mean values ± SD. The CI for this case was 0.58 ± 0.027.

Combination of BAY-293 with cell proliferation and cell cycle inhibitors

BAY-293 was tested for antiproliferative effects in combination with MAPK inhibitors trametinib and PD98059, AKT inhibitor rapamycin and CDK inhibitors Palbociclib and flavopiridol (Fig. 5).

Trametinib (TRAM; Mekinist) is an oral selective mitogen-activated extracellular signal regulated kinase 1/2 (MEK1/2) inhibitor, PD98059 is a potent and selective inhibitor of MAP kinase kinases (MAPKK), specifically MEK1, rapamycin is a specific mTOR inhibitor, Palbociclib is a highly selective inhibitor of CDK4/6 and flavopiridol competes with ATP to inhibit CDKs including CDK1, CDK2, CDK4 and CDK6. BAY-293 synergy tests show high efficacy with trametinib, PD98059 with exception of BH837 and flavopiridol. Rapamycin is significantly active in combination for IVIC-A and BH828 and Palbociclib for IVIC-A and BH837, respectively.

BAY-293 in combinations with chemotherapeutics

A range of chemotherapeutic drugs were tested for possible synergy with BAY-293 (Fig. 6). Cisplatin (cisPt) proved to be synergistic with the KRAS inhibitor, except for the BH837 cell line. Whereas the combination with pemetrexed (Peme) showed no enhanced cytotoxicity, doxorubicin

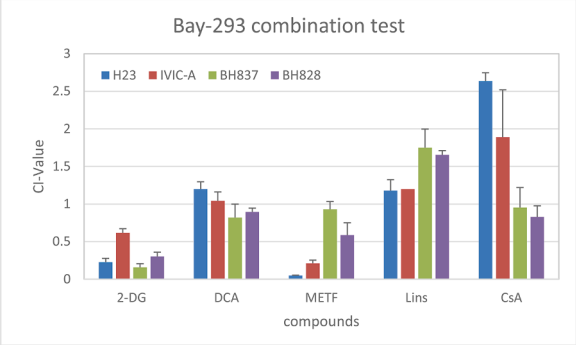

Fig. 4. Cytotoxicity of combinations of BAY-293 with modulators of glycolysis and CsA.

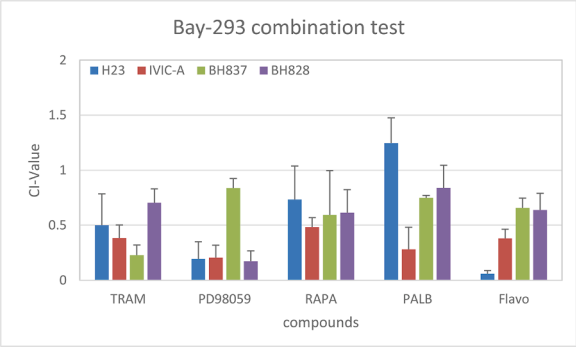

Fig. 5. BAY-293 combination with trametinib (TRAM), PD98059, rapamycin (RAP), Palbociclib (PALB) and flavopiridol (Flavo). Values present mean values ± SD. Differences to a summary CI of inactive compounds (1.023 ± 0.018) are significantly different for all combinations except RAPA/H23 and BH837 as well as PALB/BH828.

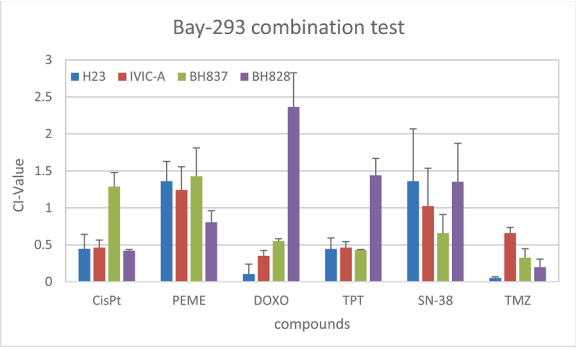

Fig. 6. Cytotoxicity of BAY-293 in combination with a range of chemotherapeutics. Values present mean values ± SD. Differences to a summary CI of inactive compounds (1.023 ± 0.018) are significantly different for all combinations except for PEME/H23, BH837, BH828, TPT/BH828 and SN-38/H23, IVIC-A, BH828.

(Doxo) and topotecan (TPT) were synergistic with BAY-293, except for the BH828 cell line. 7-Ethyl-10-hydroxycamptothecin (7ET), that represents the active ingredient of irinotecan, SN38, lacks synergy but temozolomide revealed high activity in combination with BAY-293.

BAY-293 combinations with diverse modulators

The covalent EGFR inhibitor afatinib exhibited high synergy with BAY-293 for H23 and IVIC-A but not for BH828 (Fig. 7). The ALK inhibitor crizotinib showed low activity, except for BH837. The two BET

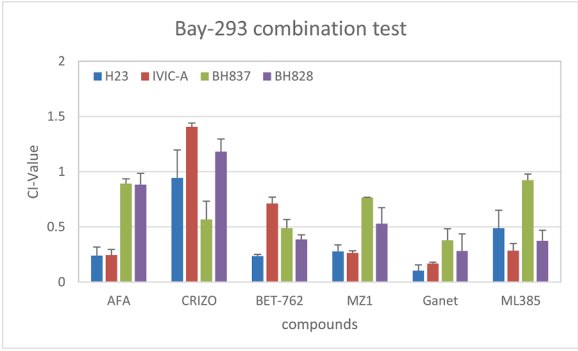

Fig. 7. Cytotoxicity of BAY-293 in combination with a range of modulators. Values present mean values ± SD. Differences to a summary CI of inactive compounds (1.023 ± 0.018) are significantly different for all combinations except for AFA/BH828 and Crizo/IVIC-A,.

inhibitors, namely I-BET762 and MZ1 yielded high synergy with the KRAS inhibitor. With exception of BH837, the three other lines were highly sensitive for the synergistic combination of BAY-293 with the HSP90 inhibitor Ganetespib and the NRF2 inhibitor ML385, respectively.

Conclusions

Although moving KRAS inhibitors into the clinic had been a major advance, the molecular background that predict response and resistance and their combination with other therapeutics remain to be fully exploited [2]. So far, responses to mutation-specific KRAS inhibitors were variable and of shorter duration [16–18]. The purpose of the present study was to investigate which therapeutics may be combined with the pan-KRAS inhibitor BAY-293 to achieve maximal synergistic effects against lung cancer cells. BAY-293 was described as a valuable chemical probe and test compound for investigations but not optimized for clinical use and in patients the dosage applied of such wildtype KRAS inhibitors may be limited by toxicity to normal tissues [30]. The cell lines used for the present study represent KRAS-mutated NSCLC NCI-H23 (G12C) cells and three primary KRAS wildtype NSCLC lines derived from patients progredient under EGFR inhibitor Osimertinib therapy [31]. Genomic investigations of NSCLC have indicated that KRAS mutations are mutually exclusive with other oncogenic drivers, including those derived from EGFR, ALK, ROS1, RET and BRAF because co-expression leads to oncogene-induced senescence [32]. KRAS activates several effector proteins including RAF kinases and PI3K which trigger signaling through MEK and ERK that drive proliferation [5,6]. Furthermore, PI3K-KRAS activates AKT and mTOR, which regulate metabolism and apoptosis [2]. CRISPR–Cas9 screen found that loss of several cell cycle and mTOR pathway genes as well as *SHP2* and *MYC* further reduced tumor growth in the presence of MRTX849 [15]. However, indirect targeting of KRAS-driven tumors by inhibition of downstream effectors of KRAS has shown limited clinical benefit and other useful combinations have to been formulated. For example, the EGFR inhibitor afatinib and the CDK4/6 inhibitor palbociclib increased the response of KRAS-G12C xenografts to MRTX849 [15].

KRAS-driven cancers are characterized by changes in metabolic pathways comprising increased uptake of nutrients, enhanced glycolysis, elevated glutaminolysis, and increased catabolism of fatty acids and nucleotides [33–35]. KRAS mutant CRCs exhibit increased expression of glycolytic and glutamine metabolic enzymes and inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was demonstrated to result in cell death [36,37]. *In vivo*, the hypovascularity of the tumor niche must be compensated by upregulation of the glucose transporter GLUT1 and hexokinase II (HK2), respectively [38,39]. The HK2 inhibitor 2-DG impairs cell growth through apoptosis and

autophagy [40]. CRC cell clones with mutant KRAS are more sensitive to glucose deprivation or metformin (inhibitor of mitochondrial complex I) than their wild-type KRAS counterparts [41]. Our results show that 2-DG is highly synergistic in combination with BAY-293 for all cell lines tested. Cancer cells show reduced mitochondrial oxidation, regardless of oxygen availability, the well-known Warburg effect and dichloroacetate (DCA) reverses the metabolism from anaerobic glycolysis to aerobic glucose oxidation by inhibiting mitochondrial pyruvate dehydrogenase kinase 1 (PDK1) [42,43]. DCA increases mitochondrial reactive oxygen species thereby inducing apoptosis in tumor cells [43,44]. However, for the four NSCLC lines tested here, DCA exhibited no synergy with BAY-293. Metformin inhibited cell proliferation, induced apoptosis with the greatest response in cells carrying activating mutations in KRAS [45, 46]. Metformin synergizes with cisplatin against KRAS/LKB1 co-mutated tumors, and may prevent the onset of resistance to cisplatin [47]. Actually, metformin showed high synergy with BAY-293 except for BH837 NSCLC cells. Linsitinib is a potent dual inhibitor of the IGF-1 receptor and insulin receptor (IR) that are critical for cell proliferation, growth, and survival [48,49]. This inhibitor proved to be antagonistic with 2/4 cell lines used. Cyclosporin A (CsA) was tested to check for possible P-glycoprotein effects but showed no sign of synergy with BAY-293, instead, antagonism was observed for NCI-H23 and IVIC-A. CsA was the first immunosuppressor that have been shown to modulate P-gp activity in laboratory models and entered very early into clinical trials for reversal of MDR [50]. In conclusion, direct interference with cellular glucose utilization by 2-DG and metformin acts synergistic with BAY-293.

Mutations in RAS oncogenes result in activation of the RAS-RAF-MEK-ERK pathway, leading to cell proliferation and survival [51]. Combined mTOR by Rapamycin and MEK inhibition produced tumor regression in KRAS-driven lung cancer models [49]. Replacing the MEK inhibitor with the KRAS-G12C inhibitor ARS-1620 was associated with greater efficacy and specificity. Clinical benefits achieved by BRAF and MEK inhibitors constitutes a promising approach for therapies of KRAS mutated cancers drug resistance is frequently observed [52,53]. The first MEK1/2 inhibitor PD098059 was reported in 1995 and Trametinib (MEKinist™) is the first MEK inhibitor approved for BRAF-mutated melanoma that is used in combinations [54–56]. Furthermore, tumors with KRAS mutations were more sensitive to CDK inhibitors relative to KRAS wild-type tumors [57]. Combined inhibition of both MEK and CDK4/6 inhibitor Palbociclib was effective in preclinical models of KRAS mutant CRC and NSCLC [58,59]. The pan-CDK inhibitor Flavopiridol proved highly cytotoxic in all KRAS mutant NSCLC cells at nanomolar concentrations [60]. Furthermore, silencing of CDK4 induced senescence in NSCLC with KRAS-activating mutations [61]. Our combination experiments using BAY-293 and ERK/mTOR as well as CDK inhibitors confirm the results showing synergism of a pan-KRAS inhibitorand the same modulators. In detail, trametinib, PD098059, rapamycin and the CDK inhibitors Palbociclib and flavopiridol exhibited synergistic cytotoxic effects with BAY-293, except for Palbociclib in case of NCI-H23 cells.

Other possible combinations with wildtype KRAS inhibitors would include cytotoxic drugs currently in use for lung cancers. In wildtype EGFR NSCLCs, a poor outcome on platinum/pemetrexed was reported for KRAS-mutant patients and activation of the KRAS pathway may drive resistance to platinum/pemetrexed [62]. Activation of the cell stress response gene NRF2 by KRAS is responsible for its ability to promote drug resistance [63]. RNAi-mediated silencing of NRF2 was sufficient to reverse resistance to cisplatin elicited by ectopic expression of oncogenic KRAS in NSCLC cells. With exception of BH837 cell line, cisplatin proved synergistic with BAY-293 but pemetrexed was not active. NSCLC cells with a KRAS mutation were highly sensitive to treatment with TRAIL and 5-fluorouracil (5FU) [64]. Sequential administration of 5-FU plus selumetinib would be a promising strategy for patients having KRAS or BRAF mutant colon cancers [65]. Oncogenic KRAS sensitized colorectal tumor cells to oxaliplatin and 5-FU in a p53-dependent manner [66]. Several of these agents induced a selective loss of viability in the KRAS mutant cells, most notably drugs that are known to inhibit DNA topoisomerases, either directly or indirectly, such as camptothecin, daunorubicin and doxorubicin [28]. Cancer cells with mutant KRAS showed selective addiction to proteasome function, as well as synthetic lethality with topoisomerase inhibition [28]. Combination targeting of these functions caused improved killing of KRAS mutant cells relative to wild-type cells. The topoisomerase II inhibitor doxorubicin showed synthetic lethality with mutant KRAS inhibitors. Whereas the topoisomerase I inhibitor topotecan proved to be synergistic with BAY-293, SN-38, the active derivative of irinotecan, was largely inactive which would have important consequences for the treatment of CRC. KRAS-mutated mCRC had higher protein expression of c-MET and lower MGMT, suggesting consideration of c-MET inhibitors and temozolomide [67,68].

The pan-ERB inhibitor afatinib effectively impairs KRAS-driven lung tumorigenesis and the use of ERB inhibition was suggested for the treatment of KRAS-mutated NSCLC [69]. AFR1 lung cancer cells exhibited afatinib resistance as a result of wild-type KRAS amplification [70]. In this study, two cell lines showed synergy for the BAY-293-afatinib combination and the EGFR inhibitor-resistant cell lines BH828 and BH837 exhibited minor effects. The ALK inhibitor crizotinib showed synergism with BAY-293 in case of BH837 cells most likely due to inhibition of c-MET that may be upregulated in resistance to EGFR inhibitors. Small-molecule inhibitors of the bromodomain and extra terminal domain (BET) family, such as JQ1, I-BET762 and OTX-015, are active in a wide range of different cancer types, including lung cancer. In a panel of 12 KRAS-mutated NSCLC models, cell lines responsive to BET inhibitors underwent apoptosis [71]. Targeting MYC by BET inhibition for the treatment of a murine KRAS mutant NSCLC showed therapeutic efficacy [72,73]. The PROTACs (proteolysis targeting chimeras) dBET1 and MZ1 induced degradation of BRD4 followed by a reduction in MYC expression and inhibition of CRC cell proliferation [74]. In our experiments, both inhibitors revealed marked synergy with BAY-293 for all 4 NSCLC cell lines tested. The HSP90 inhibitor ganetespib sensitized mutant KRAS NSCLC cells to chemotherapeutics of the antimitotic, topoisomerase inhibitor, and alkylating agent classes [75,76]. Dual inhibition of the HSP90 and MEK signaling pathways was proposed to treat KRAS-mutant NSCLC with intrinsic resistance to MEK inhibition [77]. These results correspond to the marked synergy of ganetespib with BAY-293 against the four NSCLC lines tested. Approximately 20% of KRAS-mutant NSCLCs carry loss-of-function mutations in KEAP1, a negative regulator of NRF2, which is the principal regulator of the cellular antioxidant response [78]. In KRAS-mutant NSCLC, LKB1 loss results in redox stress, that is ameliorated through KEAP1/NRF2-dependent metabolic adaptations and sensitivity to glutaminase inhibition [79]. With exception of the BH837 line, the NRF2 inhibitor ML385 proved to be synergistic with BAY-293.

In summary, BAY-293 exhibit synergy with a wide range of compounds and its anticancer activity is suggested to be amenable to combination with drugs discussed for KRAS G12C and mutation-specific inhibitors [80]. In a first clinical phase pan-KRAS and mutation-specific KRAS inhibitors are to be combined but the efficacy may be further enhanced by addition of inhibitors targeting characteristic vulnerabilities of KRAS-triggered cells. Inhibition of the common receptor tyrosine kinase (RTK) signaling intermediate SOS1 by BAY-293 was demonstrated to exert marked synergy with osimertinib in 3D spheroid-cultured EGFR-mutated NSCLC cells [81]. Furthermore, KRAS combination therapy using BAY-293 may be active in NSCLC patients after failure of targeted therapy employing Osimertinib.

Ethics approval

All procedures performed in this study involving human specimen were in accordance with the Declaration of Helsinki (as revised in 2013).

Patients signed informed consent forms before drawing of pleural effusions according to the Ethics Approval 366/2003 by the Ethics Committee of the Medical University of Vienna, Vienna, Austria.

CRediT authorship contribution statement

Adelina Plangger: Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Barbara Rath:** Investigation, Software, Data curation, Writing – original draft, Writing – review & editing. **Maximilian Hochmair:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Martin Funovics:** Conceptualization, Methodology, Writing – original draft. **Gerhard Hamilton:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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References

[1] A.R. Moore, S.C. Rosenberg, F. McCormick, et al., RAS-targeted therapies: is the undruggable drugged? Nat. Rev. Drug. Discov. 19 (2020) 533–552, https://doi.org/10.1038/s41573-020-0068-6.
[2] D. Kim, J.Y. Xue, P. Lito, Targeting KRAS(G12C): from inhibitory mechanism to modulation of antitumor effects in patients, Cell 183 (2020) 850–859, https://doi.org/10.1016/j.cell.2020.09.044.
[3] G.A. Hobbs, C.J. Der, K.L. Rossman, RAS isoforms and mutations in cancer at a glance, J. Cell Sci. 129 (2016) 1287–1292, https://doi.org/10.1242/jcs.182873.
[4] J.L. Bos, H. Rehmann, A. Wittinghofer, GEFs and GAPs: critical elements in the control of small G proteins, Cell 129 (2007) 865–877, https://doi.org/10.1016/j.cell.2007.05.018.
[5] J. Downward, Targeting RAS signalling pathways in cancer therapy, Nat. Rev. Cancer 3 (2003) 11–22, https://doi.org/10.1038/nrc969.
[6] M. Malumbres, M. Barbacid, RAS oncogenes: the first 30 years, Nat. Rev. Cancer 3 (2003) 459–465, https://doi.org/10.1038/nrc1097.
[7] J.M. Ostrem, U. Peters, M.L. Sos, et al., K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions, Nature 503 (2013) 548–551, https://doi.org/10.1038/nature12796.
[8] M. Gehringer, S.A. Laufer, Emerging and re- emerging warheads for targeted covalent inhibitors: applications in medicinal chemistry and chemical biology, J. Med. Chem. 62 (2019) 5673–5724, https://doi.org/10.1021/acs.jmedchem.8b01153.
[9] D.K. Simanshu, D.V. Nissley, F. McCormick, RAS proteins and their regulators in human disease, Cell 170 (2017) 17–33, https://doi.org/10.1016/j.cell.2017.06.009.
[10] K. Scheffzek, M.R. Ahmadian, W. Kabsch, et al., The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants, Science 277 (1997) 333–338, https://doi.org/10.1126/science.277.5324.333.
[11] S. Li, A. Balmain, C.M. Counter, A model for RAS mutation patterns in cancers: finding the sweet spot, Nat. Rev. Cancer 18 (2018) 767–777.
[12] M.P. Patricelli, M.R. Janes, L.S. Li, et al., Selective inhibition of oncogenic KRAS output with small molecules targeting the inactive state, Cancer Discov. 6 (2016) 316–329, https://doi.org/10.1158/2159-8290.CD-15-1105.
[13] M. Zeng, J. Lu, L. Li, et al., Potent and selective covalent quinazoline inhibitors of KRAS G12C, Cell Chem. Biol. 24 (2017) 1005–1016, https://doi.org/10.1016/j.chembiol.2017.06.017, e3.
[14] M.R. Janes, J. Zhang, L.S. Li, et al., Targeting KRAS mutant cancers with a covalent G12C-specific inhibitor, Cell 172 (2018) 578–589, https://doi.org/10.1016/j.cell.2018.01.006, e17.
[15] J. Canon, K. Rex, A.Y. Saiki, et al., The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity, Nature 575 (2019) 217–223, https://doi.org/10.1038/s41586-019-1694-1.
[16] J. Hallin, L.D. Engstrom, L. Hargis, et al., The KRASG12C inhibitor, MRTX849, provides insight toward therapeutic susceptibility of KRAS mutant cancers in mouse models and patients, Cancer Discov. 10 (2019) 54–71, https://doi.org/10.1158/2159-8290.CD-19-1167.
[17] D. Bar-Sagi, E.H. Knelson, L.V. Sequist, A bright future for KRAS inhibitors, Nat. Cancer 1 (2020) 25–27, https://doi.org/10.1038/s43018-019-0016-8.
[18] D.S. Hong, M.G. Fakih, J.H. Strickler, et al., KRAS^{G12C} inhibition with sotorasib in advanced solid tumors, N. Engl. J. Med. 383 (2020) 1207–1217, https://doi.org/10.1056/NEJMoa1917239.
[19] J.G. Christensen, P. Olson, T. Briere, et al., Targeting Kras^{g12c} -mutant cancer with a mutation-specific inhibitor, J. Intern. Med. 288 (2020) 183–191, https://doi.org/10.1111/joim.13057.
[20] Z. Karoulia, E. Gavathiotis, P.I. Poulikakos, New perspectives for targeting RAF kinase in human cancer, Nat. Rev. Cancer 17 (2017) 676–691, https://doi.org/10.1038/nrc.2017.79.
[21] P. Lito, N. Rosen, D.B. Solit, Tumor adaptation and resistance to RAF inhibitors, Nat. Med. 19 (2013) 1401–1409, https://doi.org/10.1038/nm.3392.
[22] L. Bonfini, C.A. Karlovich, C. Dasgupta, et al., The son of sevenless gene product: a putative activator of Ras, Science 255 (1992) 603–606, https://doi.org/10.1126/science.1736363.
[23] D. Vigil, J. Cherfils, K.L. Rossman, et al., Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? Nat. Rev. Cancer 10 (2010) 842–857, https://doi.org/10.1038/nrc2960.
[24] P. Lito, M. Solomon, L.S. Li, et al., Allele-specific inhibitors inactivate mutant KRAS G12C by a trapping mechanism, Science 351 (2016) 604–608, https://doi.org/10.1126/science.aad6204.
[25] R.C. Hillig, B. Sautier, J. Schroeder, et al., Discovery of potent SOS1 inhibitors that block RAS activation via disruption of the RAS-SOS1 interaction, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 2551–2560, https://doi.org/10.1073/pnas.1812963116.
[26] M.H. Hofmann, M. Gmachl, J. Ramharter, et al., BI-3406, a potent and selective SOS1:KRAS interaction inhibitor, is effective in KRAS-driven cancers through combined MEK inhibition, Cancer Discov. (2020), https://doi.org/10.1158/2159-8290.CD-20-0142 ahead of print.
[27] X. Pang, M. Liu, Defeat mutant KRAS with synthetic lethality, Small GTPases 8 (2017) 212–219, https://doi.org/10.1080/21541248.2016.1213783.
[28] M. Steckel, M. Molina-Arcas, B. Weigelt, et al., Determination of synthetic lethal interactions in KRAS oncogene-dependent cancer cells reveals novel therapeutic targeting strategies, Cell Res. 22 (2012) 1227–1245, https://doi.org/10.1038/cr.2012.82.
[29] A.J. Aguirre, W.C. Hahn, Synthetic lethal vulnerabilities in KRAS-mutant cancers, Cold Spring Harb. Perspect. Med. 8 (2018), a031518, https://doi.org/10.1101/cshperspect.a031518.
[30] J. Gillson, Y. Ramaswamy, G. Singh, et al., Small molecule KRAS inhibitors: the future for targeted pancreatic cancer therapy? Cancers (Basel) 12 (2020) 1341, https://doi.org/10.3390/cancers12051341.
[31] C. Little, M. Nau, D. Carney, et al., Amplification and expression of the c-myc oncogene in human lung cancer cell lines, Nature 306 (1983) 194–196, https://doi.org/10.1038/306194a0.
[32] J.D. Campbell, A. Alexandrov, J. Kim, et al., Distinct patterns of somatic genome alterations in lung adenocarcinomas and squamous cell carcinomas, Nat. Genet. 48 (2016) 607–616, https://doi.org/10.1038/ng.3564.
[33] K. Kawada, K. Toda, Y. Sakai, Targeting metabolic reprogramming in KRAS-driven cancers, Int. J. Clin. Oncol. 22 (2017) 651–659, https://doi.org/10.1007/s10147-017-1156-4.
[34] J.R. Mayers, M.E. Torrence, L.V. Danai, et al., Tissue of origin dictates branched-chain amino acid metabolism in mutant KRAS-driven cancers, Science 353 (2016) 1161–1165, https://doi.org/10.1126/science.aaf5171.
[35] K.L. Bryant, J.D. Mancias, A.C. Kimmelman, et al., KRAS: feeding pancreatic cancer proliferation, Trends Biochem. Sci. 39 (2014) 91–100, https://doi.org/10.1016/j.tibs.2013.12.004.
[36] J.E. Hutton, X. Wang, L.J. Zimmerman, et al., Oncogenic KRAS and BRAF drive metabolic reprogramming in colorectal cancer, Mol. Cell. Proteom. 15 (2016) 2924–2938, https://doi.org/10.1074/mcp.M116.058925.
[37] J. Son, C.A. Lyssiotis, H. Ying, et al., Glutamine supports pancreatic cancer growth through a KRAS regulated metabolic pathway, Nature 496 (2013) 101–105, https://doi.org/10.1038/nature12040.
[38] H. Ying, A.C. Kimmelman, C.A. Lyssiotis, et al., Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism, Cell 149 (2012) 656–670, https://doi.org/10.1016/j.cell.2012.01.058.
[39] C.J. Halbrook, C.A. Lyssiotis, Employing metabolism to improve the diagnosis and treatment of pancreatic cancer, Cancer Cell 31 (2017) 5–19, https://doi.org/10.1016/j.ccell.2016.12.006.
[40] H. Wang, L. Wang, Y. Zhang, et al., Inhibition of glycolytic enzyme hexokinase II (HK2) suppresses lung tumor growth, Cancer Cell Int. 16 (2016), https://doi.org/10.1186/s12935-016-0280-y.
[41] Z.D. Wang, S.Q. Wei, Q.Y. Wang, Targeting oncogenic KRAS in non-small cell lung cancer cells by phenformin inhibits growth and angiogenesis, Am. J. Cancer Res. 5 (2015) 3339–3349, eCollection 2015.
[42] S. Kankotia, P.W. Stacpoole, Dichloroacetate and cancer: new home for an orphan drug? Biochim. Biophys. Acta 1846 (2014) 617–629, https://doi.org/10.1016/j.bbcan.2014.08.005.
[43] G. Sutendra, E.D. Michelakis, Pyruvate dehydrogenase kinase as a novel therapeutic target in oncology, Front. Oncol. 3 (2013), https://doi.org/10.3389/fonc.2013.00038.
[44] H.R. Christofk, M.G. Vander Heiden, M.H. Harris, et al., The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth, Nature 452 (2008) 230–233, https://doi.org/10.1038/nature06734.
[45] D.A. Iglesias, M.S. Yates, D. van der Hoeven, et al., Another surprise from Metformin: novel mechanism of action via K-Ras influences endometrial cancer response to therapy, Mol. Cancer Ther. 12 (2013) 2847–2856, https://doi.org/10.1158/1535-7163.MCT-13-0439.

[46] D. Xiao, M. Yue, H. Su, et al., Polo-like kinase-1 regulates myc stabilization and activates a feedforward circuit promoting tumor cell survival, Mol. Cell. 64 (2016) 493–506, https://doi.org/10.1016/j.molcel.2016.09.016.
[47] M. Moro, E. Caiola, M. Ganzinelli, et al., Metformin enhances cisplatin-induced apoptosis and prevents resistance to cisplatin in co-mutated KRAS/LKB1 NSCLC, J. Thorac. Oncol. 13 (2018) 1692–1704, https://doi.org/10.1016/j.jtho.2018.07.102.
[48] W.T. Iams, C.M. Lovly, Molecular pathways: clinical applications and future direction of insulin-like growth factor-1 receptor pathway blockade, Clin. Cancer Res. 21 (2015) 4270–4277, https://doi.org/10.1158/1078-0432.CCR-14-2518.
[49] M. Molina-Arcas, C. Moore, S. Rana, et al., Development of combination therapies to maximize the impact of KRAS-G12C inhibitors in lung cancer, Sci. Transl. Med. 11 (2019) eaaw7999, https://doi.org/10.1126/scitranslmed.aaw7999.
[50] H. Morjani, C. Madoulet, Immunosuppressors as multidrug resistance reversal agents, Methods Mol. Biol. 596 (2010) 433–446, https://doi.org/10.1007/978-1-60761-416-6_19.
[51] S. Okumura, P.A. Jänne, Molecular pathways: the basis for rational combination using MEK inhibitors in KRAS-mutant cancers, Clin. Cancer Res. 20 (2014) 4193–4199, https://doi.org/10.1158/1078-0432.CCR-13-2365.
[52] F. Liu, X. Yang, M. Geng, et al., Targeting ERK, an Achilles' heel of the MAPK pathway, in cancer therapy, Acta Pharm. Sin. B 8 (2018) 552–562, https://doi.org/10.1016/j.apsb.2018.01.008.
[53] E. Manchado, S. Weissmueller, J.P. Morris 4th, et al., A combinatorial strategy for treating KRAS-mutant lung cancer, Nature 534 (2016) 647–651, https://doi.org/10.1038/nature18600.
[54] D.R. Alessi, A. Cuenda, P. Cohen, et al., PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*, J. Biol. Chem. 270 (1995) 27489–27494, https://doi.org/10.1074/jbc.270.46.27489.
[55] I. Lugowska, H. Koseła-Paterczyk, K. Kozak, et al., Trametinib: a MEK inhibitor for management of metastatic melanoma, Onco. Targets Ther. 8 (2015) 2251–2259, https://doi.org/10.2147/OTT.S72951.
[56] A.W. Tolcher, K. Khan, M. Ong, et al., Antitumor activity in RAS-driven tumors by blocking AKT and MEK, Clin. Cancer Res. 21 (2015) 739–748, https://doi.org/10.1158/1078-0432.CCR-14-1901.
[57] H. He, C. Xu, Z. Cheng, et al., Drug combinatorial therapies for the treatment of KRAS mutated lung cancers, Curr. Top Med. Chem. 19 (2019) 2128–2142, https://doi.org/10.2174/1568026619666190902150555.
[58] M.S. Lee, T.L. Helms, N. Feng, et al., Efficacy of the combination of MEK and CDK4/6 inhibitors *in vitro* and *in vivo* in KRAS mutant colorectal cancer models, Oncotarget 7 (2016) 39595–39608, https://doi.org/10.18632/oncotarget.9153.
[59] Z. Tao, J.M. Le Blanc, C. Wang, et al., Coadministration of trametinib and palbociclib radiosensitizes KRAS-mutant non-small cell lung cancers *in vitro* and *in vivo*, Clin. Cancer Res. 22 (2016) 122–133, https://doi.org/10.1158/1078-0432.CCR-15-0589.
[60] I. Dogan Turacli, F. Demirtas Korkmaz, T. Candar, et al., Flavopiridol's effects on metastasis in KRAS mutant lung adenocarcinoma cells, J. Cell Biochem. 120 (2019) 5628–5635, https://doi.org/10.1002/jcb.27846.
[61] M. Puyol, A. Martín, P. Dubus, et al., A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma, Cancer Cell 18 (2010) 63–73, https://doi.org/10.1016/j.ccr.2010.05.025.
[62] B. Ricciuti, G.C. Leonardi, G. Metro, et al., Targeting the KRAS variant for treatment of non-small cell lung cancer: potential therapeutic applications, Expert Rev. Respir. Med. 10 (2016) 53–68, https://doi.org/10.1586/17476348.2016.1115349.
[63] S. Tao, S. Wang S, S.J. Moghaddam, et al., Oncogenic KRAS confers chemoresistance by upregulating NRF2, Cancer Res. 74 (2014) 7430–7441, https://doi.org/10.1158/0008-5472.CAN-14-1439.
[64] H. Wang, T. Yang, X. Wu, 5-Fluorouracil preferentially sensitizes mutant KRAS non-small cell lung carcinoma cells to TRAIL-induced apoptosis, Mol. Oncol. 9 (2015) 1815–1824, https://doi.org/10.1016/j.molonc.2015.06.003.
[65] H.Y. Jang, D.H. Kim, H.J. Lee, et al., Schedule-dependent synergistic effects of 5-fluorouracil and selumetinib in KRAS or BRAF mutant colon cancer models, Biochem. Pharmacol. 160 (2019) 110–120, https://doi.org/10.1016/j.bcp.2018.12.017.
[66] M.T. de Bruijn, D.A. Raats, F.J. Hoogwater, et al., Oncogenic KRAS sensitises colorectal tumour cells to chemotherapy by p53-dependent induction of Noxa, Br. J. Cancer 102 (2010) 1254–1264, https://doi.org/10.1038/sj.bjc.6605633.
[67] W.S. El-Deiry, N. Vijayvergia, J. Xiu, et al., Molecular profiling of 6,892 colorectal cancer samples suggests different possible treatment options specific to metastatic sites, Cancer Biol. Ther. 16 (2015) 1726–1737, https://doi.org/10.1080/15384047.2015.1113356.
[68] F. Pietrantonio, F. Perrone, F. de Braud, et al., Activity of temozolomide in patients with advanced chemorefractory colorectal cancer and MGMT promoter methylation, Ann. Oncol. 25 (2014) 404–408, https://doi.org/10.1093/annonc/mdt547.
[69] H.P. Moll, K. Pranz, M. Musteanu, et al., Afatinib restrains K-RAS-driven lung tumorigenesis, Sci. Transl. Med. 10 (2018) eaao2301, https://doi.org/10.1126/scitranslmed.aao2301.
[70] T. Yamaoka, T. Ohmori, M. Ohba, et al., Distinct afatinib resistance mechanisms identified in lung adenocarcinoma harboring an EGFR mutation, Mol. Cancer Res. 15 (2017) 915–928, https://doi.org/10.1158/1541-7786.MCR-16-0482.
[71] O. Klingbeil, R. Lesche, K.A. Gelato, et al., Inhibition of BET bromodomain-dependent XIAP and FLIP expression sensitizes KRAS-mutated NSCLC to pro-apoptotic agents, Cell Death. Dis. 7 (2016) e2365, https://doi.org/10.1038/cddis.2016.271.
[72] A. Bouillez, H. Rajabi, S. Pitroda, et al., Inhibition of MUC1-C suppresses MYC expression and attenuates malignant growth in KRAS mutant lung adenocarcinomas, Cancer Res. 76 (2016) 1538–1548, https://doi.org/10.1158/0008-5472.CAN-15-1804.
[73] T. Shimamura, Z. Chen, M. Soucheray, et al., Efficacy of BET bromodomain inhibition in KRAS-mutant non-small cell lung cancer, Clin. Cancer Res. 19 (2013) 6183–6192, https://doi.org/10.1158/1078-0432.CCR-12-3904.
[74] C. Otto, S. Schmidt, C. Kastner, et al., Targeting bromodomain-containing protein 4 (BRD4) inhibits MYC expression in colorectal cancer cells, Neoplasia 21 (2019) 1110–1120, https://doi.org/10.1016/j.neo.2019.10.003.
[75] J. Acquaviva, D.L. Smith, J. Sang, et al., Targeting KRAS-mutant non-small cell lung cancer with the Hsp90 inhibitor ganetespib, Mol. Cancer Ther. 11 (2012) 2633–2643, https://doi.org/10.1158/1535-7163.MCT-12-0615.
[76] P. Tomasini, P. Walia, C. Labbe, et al., Targeting the KRAS pathway in non-small cell lung cancer, Oncologist 21 (2016) 1450–1460, https://doi.org/10.1634/theoncologist.2015-0084.
[77] K.S. Park, B. Oh, M.H. Lee, et al., The HSP90 inhibitor, NVP-AUY922, sensitizes KRAS-mutant non-small cell lung cancer with intrinsic resistance to MEK inhibitor, trametinib, Cancer Lett. 372 (2016) 75–81, https://doi.org/10.1016/j.canlet.2015.12.015.
[78] R. Romero, V.I. Sayin, S.M. Davidson, et al., Keap1 loss promotes Kras-driven lung cancer and results in dependence on glutaminolysis, Nat Med. 23 (2017) 1362–1368, https://doi.org/10.1038/nm.4407.
[79] A. Galan-Cobo, P. Sitthideatphaiboon, X. Qu, et al., LKB1 and KEAP1/NRF2 pathways cooperatively promote metabolic reprogramming with enhanced glutamine dependence in KRAS-mutant lung adenocarcinoma, Cancer Res. 79 (2019) 3251–3267, https://doi.org/10.1158/0008-5472.CAN-18-3527.
[80] A. Chakraborty, KRASG12C inhibitor: combing for combination, Biochem. Soc. Trans. 48 (2020) 2691–2701, https://doi.org/10.1042/BST20200473.
[81] P.L. Theard, S. Sheffels, N.E. Sealover, et al., Marked synergy by vertical inhibition of EGFR signaling in NSCLC spheroids shows SOS1 is a therapeutic target in EGFR-mutated cancer, Elife 9 (2020), https://doi.org/10.7554/eLife.58204.