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Antitumorigenic Effect of Combination Treatment with BRAF Inhibitor and Cisplatin in Colorectal Cancer In Vitro and In Vivo

Kassandra Koumaki, Salomi Skarmalioraki, Vivian Kosmidou, Lida Krikoni, Maria Goulielmaki, Vassilis Zoumpourlis, Alexander Pintzas, and Vassilis L. Souliotis**

In colorectal cancer (CRC), BRAF inhibitor (BRAFi) monotherapy appears ineffective, while cisplatin treatment is associated with adverse effects, drug resistance, and reduced efficacy. Herein, a combinatorial approach is being explored to increase the likelihood of effectively killing colorectal cancer cells. The combined effect of BRAFi (PLX4720, Vemurafenib, Dabrafenib, Encorafenib) and cisplatin treatment is examined in BRAFV600E-mutated (RKO, HT29, Colo-205) and BRAFwt (Caco-2) cell lines, as well as in mouse xenografts of RKO cells. Following cisplatin-only treatment, all cell lines show accumulation within subG1 (apoptotic cells) and G2/M phases, as well as phosphorylation of ERK1/2 and H2AX. Following BRAFi-only treatment, BRAFV600E-mutated cells show accumulation within G0/G1 phase, reduced distribution in the S and G2/M phases, inhibition of ERK1/2 phosphorylation, and increased phosphorylation of H2AX. Combined BRAFi and cisplatin treatment synergistically decrease RKO cells viability, reduce phosphorylation of ERK1/2, and increase phosphorylation of H2AX. Importantly, in mouse xenografts of RKO cells, combined PLX4720 and cisplatin treatment show superior therapeutic potential than each monotherapy ($P < 0.001$). Taken together, in in vitro and in vivo preclinical models, BRAFi and cisplatin combined treatment has shown an improved antitumor effect, rendering it a potential anticancer treatment strategy for BRAF-mutant colon cancer patients.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide.[1,2] Approximately 65% of CRC cases are sporadic and believed to be associated with environmental factors.[3] The remaining cases exhibit inheritance, of which ≈3% exhibit highly penetrant inheritance, whereas the remaining less penetrant.[3,4] Despite availability of early detection methods, it has been reported that up to 25% of patients with CRC have already metastases at initial diagnosis and nearly twice as many will develop metastases in time, resulting in low survival rates.[5] CRC incidence and mortality rates differ among males compared to females. In particular, CRC is the third most frequent cancer and the fourth most common cause of cancer death in men, while among females CRC is the second most frequent cancer and the third most common cause of cancer death.[6]

CRC comprises a complex and heterogeneous disease, resulting from genomic, proteomic, epigenetic, and other changes. Surgical resection of the tumor, chemotherapy, and radiotherapy are the key treatment options for CRC patients. However, as previously stated, up to a quarter of CRC patients are diagnosed at a late stage with metastases, limiting effective surgical control, leading to poor prognosis and increased mortality rates.[7] Although cisplatin remains the gold standard for solid tumors, in CRC cisplatin treatment is correlated with adverse effects, drug resistance, and reduced efficacy. Therefore, improving treatment options, especially for metastatic CRC patients, is crucial in order to prolong survival.

The RAS/RAF/MEK/extracellular signal-regulated kinase (ERK) mechanism, also known as the mitogen-activated protein kinase (MAPK) pathway, regulates cell proliferation, differentiation, and survival. Activating mutations in the genes involved in this pathway, including BRAF, transfer signals from the cell membrane to the nucleus through downstream components, resulting in deregulation of key cellular activities and tumor development by uncontrolled cell proliferation and survival.[8] BRAF mutations have been reported in 5–12% of patients with

K. Koumaki, S. Skarmalioraki, V. Kosmidou, L. Krikoni, M. Goulielmaki, V. Zoumpourlis, A. Pintzas, V. L. Souliotis
Institute of Chemical Biology
National Hellenic Research Foundation
Athens 11635, Greece
E-mail: apint@eie.gr; vls@eie.gr

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metastatic CRC and affect treatment and prognosis of these patients.[9,10] The most frequent mutation in BRAF, accounting for more than 95% of BRAF mutations, is a single substitution at nucleotide 1799, replacing valine with glutamic acid (V600E mutation). BRAF mutations have been correlated with a poor prognosis, with a median survival of <12 months. Treatment strategies for patients with BRAF-mutant metastatic CRC are inadequate, therefore efficient anticancer treatment is urgently needed.

Interestingly, the MAPK system is regarded as a key therapeutic target, since it is mutated in a variety of cancer types. Indeed, three BRAF inhibitors (Vemurafenib, Dabrafenib, Encorafenib) and four MEK1/2 inhibitors (Cobimetinib, Trametinib, Selumetinib, Binimetinib) have been approved by the United States Food and Drug Administration (FDA) for the treatment of various cancers.[11] Vemurafenib has been shown to inhibit the kinase activity of BRAFV600E mutation in patients with metastatic melanoma and was the first potent and selective tyrosine kinase inhibitor to inactivate the MAPK pathway, demonstrating antitumor activity.[12,13] Dabrafenib was the second kinase inhibitor approved by the FDA for the treatment of patients with metastatic melanoma harboring the BRAFV600E mutation.[12] It is also used for the treatment of patients with BRAFV600E-mutant metastatic non-small cell lung cancer (NSCLC) or anaplastic thyroid cancer (ATC).[14,15] Encorafenib is a potent BRAF inhibitor that has been shown to decrease ERK phosphorylation and inhibit proliferation in BRAFV600E-mutant melanoma, which accounts for 50% of all melanomas. It also induces senescence, rather than apoptosis, by down-regulating Cyclin D1 and arresting the cell cycle in the G1 phase.[16,17]

BRAF inhibitors have been reported to show improved rates of rapid response in patients with BRAF-mutant metastatic melanoma, ranging from 48% in a phase III study assessing Vemurafenib efficacy to 59% in a phase II study of the clinical activity of Dabrafenib.[13,18] Moreover, the phase II COLUMBUS trial showed superior efficacy data of Encorafenib over Vemurafenib monotherapy.[19,20] However, BRAFi monotherapy does not usually have an enduring anticancer effect, since patients have been reported to progress to more advanced stages 6 to 7 months after the beginning of treatment. This acquired resistance to BRAF inhibitor therapy has been attributed to MAPK pathway reactivation and the reported mechanisms include activating mutations in NRAS, KRAS, MEK1/2, and AKT1, BRAF amplification and splicing, and CDKN2A loss, along with PI3K-PTEN-AKT pathway mutations overlapping with the MAPK pathway.[21–23] Thus, further research is urgently needed to find new therapeutic options, in order to increase treatment efficacy and prevent the development of BRAFi-associated resistance.

Rational combinatorial treatment protocols of BRAF inhibitors have offered high potential against resistant tumors. The currently investigated strategies include among others, co-treatment protocols with inhibitors of EGFR, MEK, or PI3K/AKT pathway or immunotherapies. Particularly, combinatorial therapies involving BRAF and MEK inhibitors or multiple inhibitors, like Avutometinib (inhibitor of both Raf and MEK) and Tovorafenib (inhibitor of wild-type BRAF, BRAFV600E, and CRAF) have been developed.[24] The best results so far have been observed by combining Vemurafenib with the selective MEK inhibitor Cobimetinib, a combination approved by the FDA in 2015 for the treatment of patients with BRAF-mutant metastatic or unresectable melanoma.[25] A combination of Dabrafenib with Trametinib was also approved by the FDA for BRAFV600E-mutant anaplastic thyroid cancer in 2018[26] and for BRAFV600E-mutant non-small cell lung cancer (NSCLC), where a major favorable effect has been demonstrated.[27,28] The Encorafenib-Cetuximab (EGFR targeting antibody) combination was approved in April 2020 for the previously treated BRAFV600E metastatic CRC patients, after it was shown to significantly improve their overall and progression-free survival during the trial. The doublet therapy was found to be equally effective to the triplet protocol but with slightly less (MEKi-related) adverse effects.[29,30] Furthermore, combination therapy with Binimetinib and Encorafenib showed positive outcomes in multiple myeloma patients with a BRAF V600E activating mutation.[31] Even though the combined treatment of several malignancies with Bortezomib and Sorafenib showed promising results,[32] in another study for metastatic or unresectable renal cell carcinoma, the combination of these drugs showed progression-free survival (PFS) and response rates very similar to those obtained following Sorafenib monotherapy.[33] Interestingly, although BRAF- and MEK-targeted treatment was initially designed on the basis of their tumor-intrinsic effects, later on, it was found to have high immune-potentiating efficacy.[34] This immunomodulatory action of BRAF/MEK treatment has stimulated interest in therapies including these drugs in combination with immune checkpoint inhibitors (ICI) against PD-1 and CTLA-4. However, the outcomes of subsequent clinical trials examining this strategy had variable results. Indeed, the combined inhibition of BRAF, MEK, and PD-1/PD-L1 pathway showed better survival benefits compared with the two-drug combination or monotherapy.[35] However, in another study, the triplet combination of BRAF (Dabrafenib), MEK (Trametinib), and PD-1/PD-L1 (Spartalizumab) blockade did not improve response rate, progression-free survival or 24-month overall survival in BRAF V600–mutated melanoma patients.[36]

The present study examined whether combined treatment of BRAFi and cisplatin is more effective than each drug alone in human colon cancer cell lines both in vitro and in vivo. For this purpose, the effects of BRAFi and/or cisplatin treatment on the cell viability, the cell cycle, as well as the phosphorylation of critical molecular components of the MAPK pathway and the DNA damage response (DDR) network were investigated in BRAFV600E mutated and BRAFwt cell lines. The antitumor activity of the combined treatment of BRAFi and cisplatin was also studied in vivo, using a mouse xenografts model.

2. Results

2.1. Cisplatin-Only Treatment of Colon Cancer Cell Lines

First, we analyzed the cytotoxic effects of cisplatin on several human colorectal adenocarcinoma cells, such as the BRAFV600E-bearing RKO, HT29, and Colo-205 cell lines, as well as the BRAFwt colon adenocarcinoma Caco-2 cells (**Figure 1A–G**). For all cell lines tested, the decrease in cell viability was time- and concentration-dependent. All BRAFV600E-bearing cell lines were resistant to cisplatin 24 h after treatment with concentrations up to 10 μg mL^{-1}. In RKO and HT29 cells, cisplatin doses

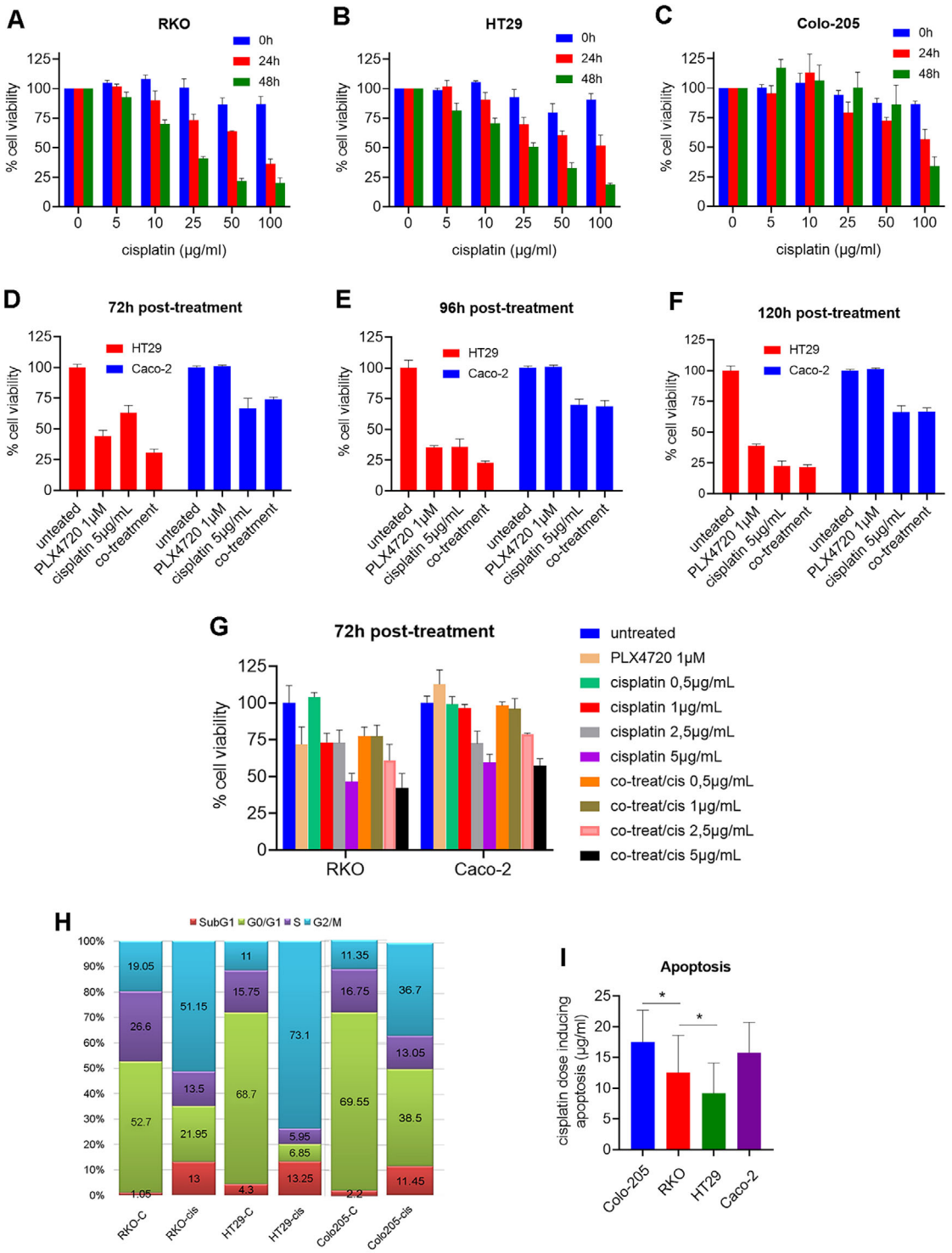


Figure 1. The effect of cisplatin-only treatment on CRC cells. Cytotoxicity and cell proliferation using the SRB assay at 0, 24, and 48 h after treatment of A) RKO, B) HT29, and C) Colo-205 cells with various doses of cisplatin (0—100 μg mL^{-1}) for 3 h. Cytotoxicity and cell proliferation were also measured in HT29 and Caco-2 cells at D) 72 h, E) 96 h, and F) 120 h following treatment with PLX4720 (1 μM), cisplatin (5 μg mL^{-1}) or co-treatment. G) Cytotoxicity and cell proliferation were measured in RKO and Caco-2 cells after treatment with PLX4720 (1 μM), various doses of cisplatin, or co-treatment. H) Cell cycle phase distribution for RKO, HT29, and Colo-205 cells treated with cisplatin. I) Bar charts showing distribution of the lowest concentrations of cisplatin required for the induction of apoptosis 48 h after cisplatin treatment. Error bars represent SD. $^*P < 0.05$.

>25 μg mL^{-1} led to increased cytotoxicity 24 h after treatment, while Colo-205 cells seemed more resistant, since reduction of cell viability was observed following treatment with drug doses >50 μg mL^{-1} (Figure 1A–C). Interestingly, the BRAFwt Caco-2 cell line was more resistant than HT29 cells at 72, 96, and 120 h time-points (Figure 1D–F) and more resistant than RKO cells, 72 h following treatment with various doses of cisplatin (Figure 1G).

Next, we studied the progression of the cell cycle following treatment with cisplatin (Figure 1H). In all cell lines tested, 48 h following treatment with 5 μg mL^{-1} cisplatin, an increase in the percentages of cells at the G2/M phase, combined with an increase in the percentages of cells at the subG1 phase (apoptotic cells) was observed. Driven by the reduction of the cell viability and the increase of the SubG1 phase upon treatment with cisplatin, we hypothesized that the apoptotic pathway is possibly triggered. Therefore, the apoptosis rates of all colorectal cell lines were evaluated 48 h after treatment with cisplatin using an ELISA assay. The lowest concentrations of cisplatin needed for induction of apoptosis were 17.5 ± 5.2 μg mL^{-1} for Colo-205 cells, 15.8 ± 4.9 μg mL^{-1} for Caco-2 cells, 12.5 ± 6.1 μg mL^{-1} for RKO cells and 9.2 ± 4.9 μg mL^{-1} for HT29 cells, indicating that the HT29 cell line showed the highest cisplatin-induced apoptosis rates (all $P < 0.05$; Figure 1I).

The effect of cisplatin on the phosphorylation of critical molecular components of the MAPK and DDR pathways, namely ERK1/2 and H2AX, respectively, was also evaluated 0, 24, and 48 h after treatment with 5, 10, 25, 50, or 100 μg mL^{-1} cisplatin. In all cell lines analyzed, we found that at the 48 h time-point cisplatin induced phosphorylation of ERK1/2 (Figure S1A—C, Supporting Information) and H2AX (Figure S1D—F, Supporting Information).

2.2. BRAFi-Only Treatment of Colon Cancer Cell Lines

The effects of various BRAF inhibitors, on the same colon cancer cell lines, were also analyzed. At first, the viability of the BRAF-mutant cell lines was evaluated 48 and 72 h following treatment with a BRAFi. After treatment of BRAFV600E-mutated cell lines with the Vemurafenib analog PLX4720, a dose-dependent decrease in the viability was observed in both RKO and HT29 cells; Colo-205 was the most sensitive and RKO the most resistant cell line (**Figure** 2A–C). As expected, treatment with PLX4720 was not effective on the viability of BRAFwt Caco-2 cells (Figure 2D). Moreover, 48 h after treatment all BRAF-mutant cell lines exhibited similar sensitivity to Dabrafenib or Encorafenib (Figure 2E).

The apoptosis rates of the BRAFV600E-mutated cell lines were also evaluated 48 h after treatment with PLX4720 using an ELISA assay. The lowest concentrations of PLX4720 needed for induction of apoptosis were 10.8 ± 5.8 μM for RKO cells, 4.5 ± 3.3 μM for HT29 cells, and 0.7 ± 0.3 μM for Colo-205 cells, indicating that Colo-205 cells showed the highest apoptosis rates (all $P < 0.001$; Figure 2F).

The progression of the cell cycle was also investigated following treatment with PLX4720 and Encorafenib. Both these BRAF inhibitors showed similar effects in all cell lines tested. That is, 48 h after treatment, increases were found in the percentages of cells at G0/G1 phase, combined with a reduction in their percentage at S and G2/M phases; no increase in the percentages of cells at the subG1 phase was observed (Figure 2G,H; Figures S2–S4, Supporting Information). The effect of PLX4720, Vemurafenib, Dabrafenib, and Encorafenib on ERK1/2 and H2AX phosphorylation was also evaluated by Western blot analysis (Figure 2I,J). It was found that 48 h after treatment, each one of these BRAFi inhibited phosphorylation of ERK1/2 and increased phosphorylation of H2AX.

2.3. The Effect of Cisplatin and BRAFi Combined Treatment on CRC Cell Lines

In order to examine the combined effect of BRAFi and cisplatin on CRC cell lines, we tested five different treatment schedules. In the 1st treatment schedule, cells were exposed for 3 h to 1 μM PLX4720, followed by 3 h treatment with 5 μg mL^{-1} cisplatin and 72 h post-incubation in drug-free medium. First, the effect of this combined treatment on the phosphorylation of ERK1/2 and H2AX was evaluated (**Figure** 3A–C). In all cell lines analyzed, compared with non-treated cells, the combined treatment inhibited the phosphorylation of the ERK1/2 kinase and increased the phosphorylation of H2AX at the 24 h time-point. Moreover, the combined treatment increased the percentage of all cells analyzed at the subG1 (apoptotic cells) and G0/G1 phases and decreased the percentage at the G2/M phase (Figure 3D–F). Using the SRB viability assay, RKO and HT29 cells exhibited synergistic effects 48 h after combined treatment, while Colo-205 at the 72 h time-point (Figure 3G–I).

In the 2nd treatment schedule, cells were exposed to 5 μg mL^{-1} cisplatin for 3 h, followed by 3 h treatment with 1 μM PLX4720 and 72 h post-incubation in drug-free medium, while in the 3rd treatment schedule, the simultaneous exposure to both 5 μg mL^{-1} cisplatin and 1 μM PLX4720 for 3 h, followed by 72 h post-incubation in drug-free medium was evaluated (Figure 3G–I). Both 2nd and 3rd treatment schedules showed similar viability results to those obtained following the 1st treatment schedule mentioned above.

In the 4th treatment schedule, cells were exposed for 24 h to 0.5 or 1 μM PLX4720, followed by 3 h treatment with 5 μg mL^{-1} cisplatin in the presence of the inhibitor and 72 h post-incubation in drug-free medium. When the 4th treatment schedule was validated experimentally, no synergistic effect was found (Figure S5, Supporting Information).

Finally, in the 5th treatment schedule, the simultaneous exposure to 5 μg mL^{-1} cisplatin and 1 μM PLX4720 or 1 μM Dabrafenib or 1 μM Encorafenib for 48 h was evaluated. In RKO cells, all BRAF-containing combinations exhibited synergistic effects, as shown by the SRB viability assay (**Figure** 4A). In Colo-205 cells, PLX4720- and Dabrafenib-containing treatments showed synergistic effects. No synergy on cell viability was found following treatment of Colo-205 cells with the Encorafenib-containing schedule and after exposure of HT29 cells with any of the combinations used. The effect of the 5th treatment schedule on the phosphorylation of H2AX and ERK1/2 was also evaluated (Figure 4B). In all BRAFi-containing treatments, phosphorylation of H2AX was observed, combined with a reduction in the phosphorylation of ERK1/2.

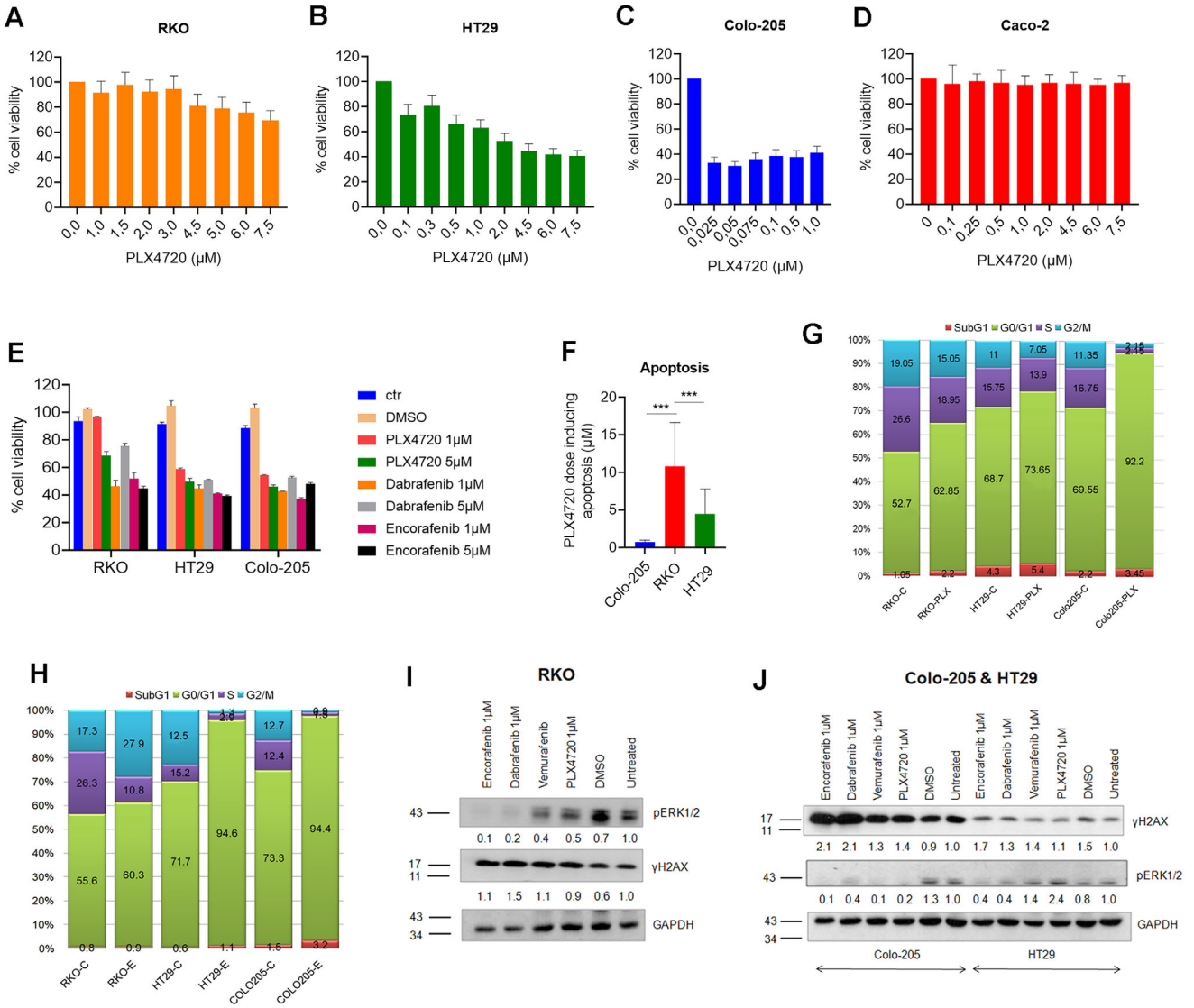


Figure 2. The effect of BRAFi-only treatment on human CRC cells. Cytotoxicity and cell proliferation using the SRB assay 48 h after treatment of A) RKO, B) HT29, C) Colo-205 cells, and D) Caco-2 with various doses of PLX4720. In E) cytotoxicity and cell proliferation of the same cell lines, 48 h following treatment with 1 and 5 μM of PLX4720, Dabrafenib or Encorafenib. F) Bar charts showing distribution of the lowest concentrations of cisplatin required for the induction of apoptosis, 48 h after cisplatin treatment. Error bars represent SD. ***$P < 0.001$. Cell cycle phase distribution of the same cell lines treated with G) PLX4720 or H) Encorafenib is also presented. Western blot analysis for the phosphorylation of ERK1/2 and γH2AX in I) RKO, J) Colo-205, and HT29 cells, following treatment with PLX4720, Vemurafenib, Dabrafenib or Encorafenib. Averaged densitometry data normalized to the control (GAPDH) at the same time points are also shown.

2.4. Combinatorial Treatment with Cisplatin and BRAFi Results in Strong Anti-Tumor Effects In Vivo

To shed light on the potential anti-tumorigenic effects of combined BRAFi and cisplatin treatment in vivo, xenografts of RKO cells were subcutaneously implanted into both flanks of severe combined immune-deficient (SCID) mice. When the tumors reached appropriate sizes, the mice were divided into five groups: control (untreated), DMSO-treated, cisplatin plus PLX4720-treated, cisplatin alone-treated and PLX4720 alone-treated. Tumor growth was monitored for 20 days after their formation, which was considered the reference point (Day 15) of the experiment. The combined treatment with 5mg kg^{-1} cisplatin and 10mg kg^{-1} PLX4720 caused a remarkable attenuation of colon cancer progression in mice bearing RKO xenografts, which was significantly higher than each separate monotherapy ($P < 0.001$; **Figure** 5A,B). Following a comparison of the excised tumor volume of the five experimental groups, cisplatin or PLX4720 monotherapy was found to reduce tumor sizes efficiently by 2.6 and 1.7 folds, respectively, as compared to untreated mice. Importantly, combined treatment with cisplatin plus PLX4720 resulted in ≈3.3-fold tumor size reduction, as compared to the control untreated mice by the end of the experiment (Figure 5C,D). Those results clearly indicate that the combination

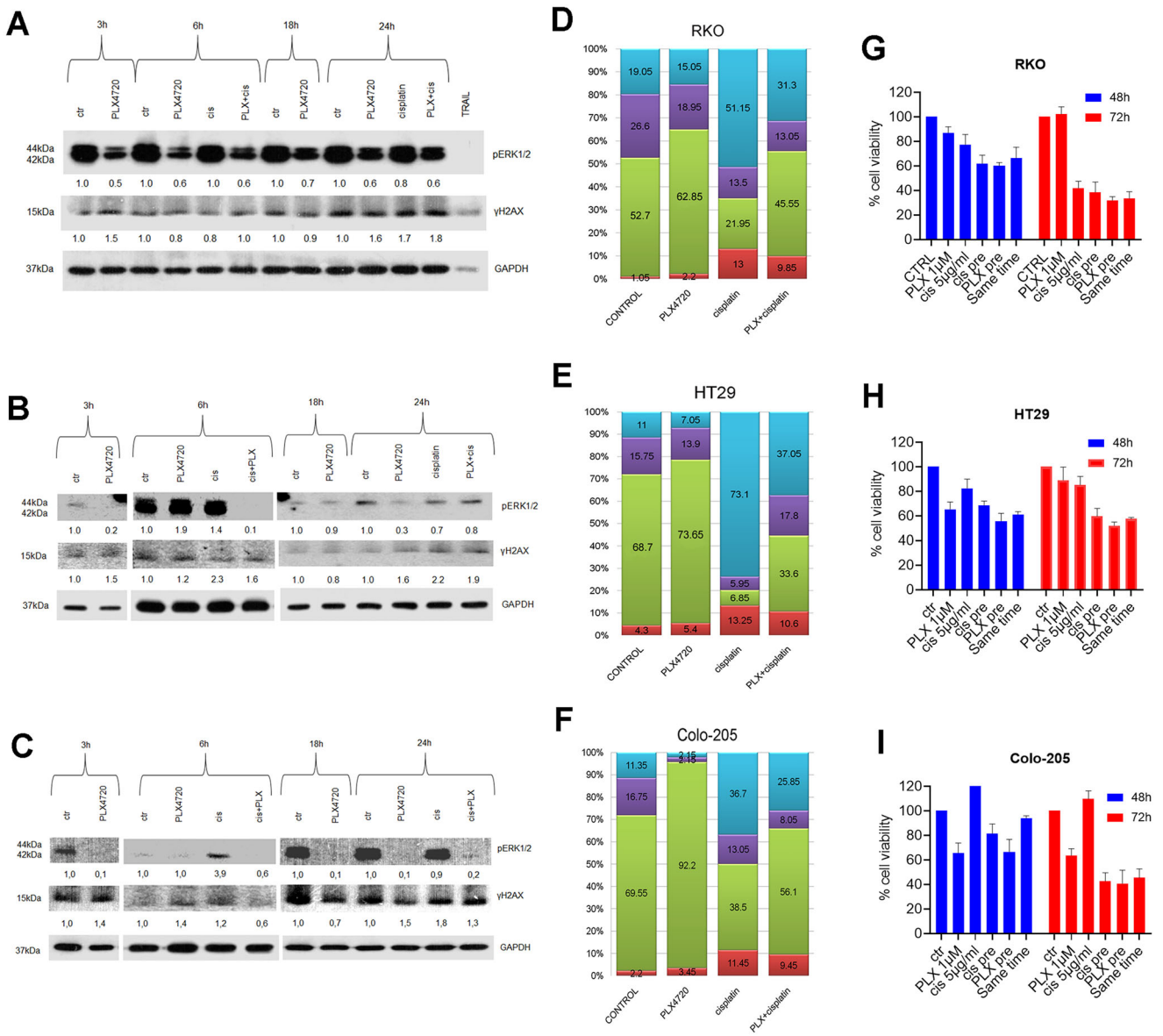


Figure 3. The effect of the combined treatment with BRAFi and cisplatin on CRC cells. Western blot analysis for the phosphorylation of A–C) ERK1/2 and γH2AX, D–F) cell cycle phase distribution, as well as G–I) cytotoxicity and cell proliferation for A,D,G) RKO, B,E,H) HT29 and C,F,I) Colo-205 cell lines following combined treatment with BRAFi and cisplatin. Averaged densitometry data normalized to the control (GAPDH) at the same time points are also shown.

of a BRAF inhibitor and cisplatin holds superior therapeutic potential than each monotherapy in vivo.

3. Discussion

Numerous investigations have demonstrated an interplay between the MAPK system and the DDR network.[37] In fact, the MEK/ERK pathway is also triggered after DDR activation, thus inducing DDR checkpoints to arrest cell division.[38] When DNA damage is present, inhibition of ERK/MAP kinase prevents cell cycle checkpoint activation and promotes cell proliferation, which in turn leads to the accumulation of mutations and the development of malignancies.[39] Interestingly, blocking checkpoint activation may also cause apoptosis or cell catastrophe, thus increasing the effectiveness of chemotherapy.[40] Guided by this notion, herein we examined the combined effect of BRAFi with cisplatin in colorectal cancer cell lines and a mouse xenograft model. First, the effect of cisplatin on CRC cell lines was analyzed. We found that following treatment of BRAF mutant cells with cisplatin, reduction of cell viability, G2/M phase arrest, and phosphorylation of H2AX were observed. Cisplatin is a genotoxic drug inducing the formation of single-nucleotide damage of guanine (monoadducts), intrastrand and interstrand cross-links.[41] Monoadducts and intrastrand cross-links are repaired by the nucleotide excision repair (NER) mechanism, while the removal of interstrand cross-links requires the activation of several DNA

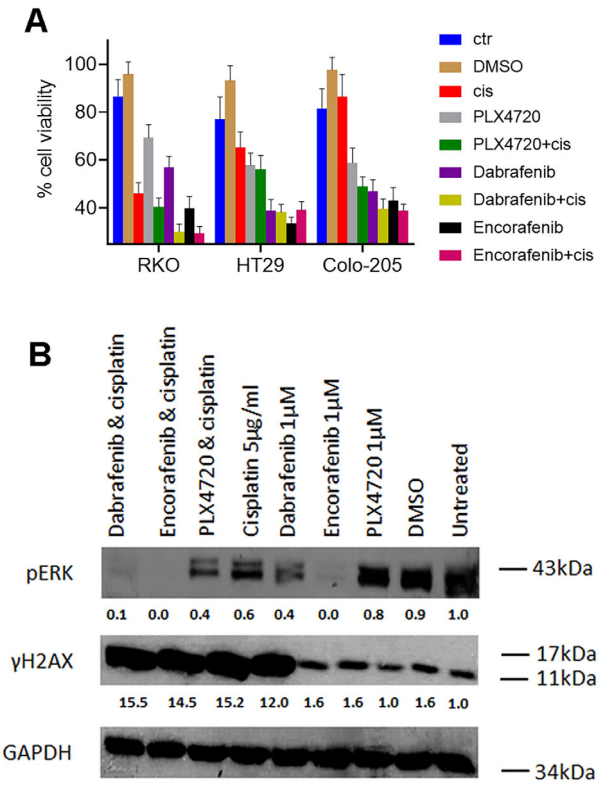


Figure 4. The effect of simultaneous exposure to cisplatin and BRAFi. A) Cytotoxicity and cell proliferation using the SRB assay after simultaneous exposure to 5 μg mL^{-1} cisplatin and 1 μM PLX4720 or 1 μM Dabrafenib or 1 μM Encorafenib for 48 h. B) Western blot analysis for the phosphorylation of ERK1/2 and γH2AX following simultaneous treatment with BRAFi and cisplatin. Averaged densitometry data normalized to the control (GAPDH) at the same time points are also shown.

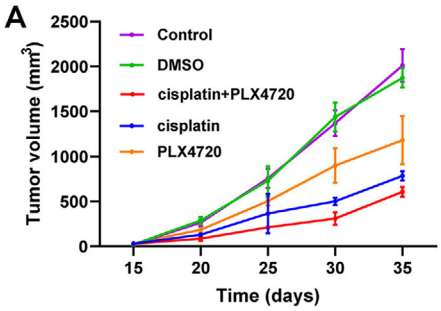
repair pathways, such as homologous recombination, NER, and translesion synthesis. Of note, DNA double-strand breaks (DSBs) are produced as intermediates in the interstrand cross-links repair process.[42] Following the detection of DSBs, cells induce the phosphorylation of histone H2AX on serine 139 (γH2AX), by the apical signaling kinases ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR).[43] Since the induction of γH2AX is an early event in the activation of the DNA damage response network, it is generally utilized as a marker of DSBs. Previous studies have also demonstrated that cisplatin impairs the synthesis of the DNA, induces S-phase slowdown and triggers a G2/M arrest through the inhibition of the CDK1/cyclin B activity.[44]

In addition, we found that following treatment of BRAF mutant cells with cisplatin, phosphorylation of the ERK1/2 kinase was observed. In accordance with our results, previous studies have shown that cisplatin treatment of HeLa cells caused a dose- and time-dependent phosphorylation, and therefore activation, of ERK and that activation of this kinase is important for the induction of the apoptosis pathway via the cytochrome c release from mitochondria.[45] In contrast, Wei et al.[46] have shown that ERK signaling inhibition enhanced the susceptibility of ovarian cancer cells to cisplatin. Another report has shown that various genotoxic insults, such as adriamycin, ultraviolet irradiation, etoposide, and ionizing radiation, induced activation of ERK1/2 in the MEF and IMR90 primary cells, the NIH3T3 immortalized cells and the transformed MCF-7 cells, thus leading to cell cycle arrest or induction of apoptosis, depending on the drug concentration used.[47]

Next, the effect of BRAF inhibition on CRC cells was analyzed. We found that treatment of BRAF mutant cells with a BRAFi resulted in decreased cell viability and reduction of ERK activation/phosphorylation. As expected, the inhibition of BRAF did not affect cell viability of BRAFwt cells. It is known that the pathway that leads to the activation of the two isoforms of ERK (ERK1 and ERK2) is initiated after ligand binding to a plasma membrane receptor tyrosine kinase and the activation of the GTP-binding protein Ras. Then, Ras activates the MAP3K kinase Raf, followed by the activation of the mitogen-activated protein kinase kinase (MEK or MAP2K), which in turn phosphorylates threonine and tyrosine residues in the Thr-Glu-Tyr (TEY) sequence of ERK1/2.[48] In several cancer types, such as melanoma, hairy cell leukemia, colon cancer, and papillary thyroid carcinoma, a mutation in codon 600 of exon 15 (V600E) has been reported. This mutation has been implicated in various mechanisms of cancer progression, such as stimulation of the MEK/ERK pathway, prevention of immune response, avoidance of apoptosis and senescence, angiogenesis, tissue invasion, and metastasis.[49] Interestingly, the inhibition of the BRAF kinase results in decreased pERK activity and the reduction of cell proliferation, indicating that the decreased pERK activity can be used as a pharmacodynamic biomarker of BRAF inhibition.

Moreover, we found that treatment of BRAF mutant cells with a BRAFi resulted in perturbation of cell cycle progression, inducing a G0/G1 arrest. An accumulating body of evidence suggests that the ERK kinases are involved in the cell cycle progression from G1 to S phase, which occurs immediately after growth factor stimulation. Indeed, previous studies have shown that ERK inhibition by a MEK inhibitor that was given even immediately prior to the beginning of the S phase, blocked cell cycle entry into the S phase.[50] That is, after growth factor stimulation, the activation of ERK causes the phosphorylation and thus activation of the ETS transcription factor Elk-1, resulting in the upregulation of immediate-early genes, such as the proto-oncogene c-fos, the expression of which is involved in the induction of delayed-early genes, such as cyclin D.[51] Then, the Cyclin D/Cdk4 complex initiates the phosphorylation of retinoblastoma, a protein known to activate the E2F family of transcription factors, and regulates the expression of several target genes, such as cyclin E. Next, the complex of cyclin E with Cdk2 further phosphorylates the retinoblastoma protein and activates the E2F transcription factors. These sequential events regulate the synthesis of various proteins that are involved in the entry of the cells in the S phase. Previous studies have revealed a set of genes whose expression levels were rapidly reduced after ERK inactivation. Interestingly, several of these genes were found to possess antiproliferative properties, i.e., they have the ability to suppress the entry of the cells into the S phase. Together, these data suggest that the BRAF inhibitor-induced ERK inactivation blocks S phase entry.[51,52]

Also, in accordance with our previous study,[53] herein we found that following treatment of BRAF mutant cells with a BRAFi, phosphorylation of H2AX was observed. In fact, evidence has accumulated that histone H2AX phosphorylated on Ser-139



B

	Statistic significance *vs* control group per time point				
Treatment group	Day 15	Day 20	Day 25	Day 30	Day 35
DMSO	ns	ns	ns	ns	ns
Cisplatin + PLX4720	ns	ns	****	****	****
Cisplatin	ns	ns	****	****	****
PLX4720	ns	ns	***	****	****

C

Mice	Treatment group				
	Control	DMSO	Cisplatin+PLX4720	Cisplatin	PLX4720
No. of sites injected	8				
Onset of tumor formation (days)	15				
Average size of primary tumors at 15 days (mm^3)	21.35 ± 8.91	24.89 ± 7.86	28.86 ± 6.44	30.47 ± 5.36	33.19 ± 11.67
Average size of primary tumors at 20 days (mm^3)	264.38 ± 35.35	287.56 ± 41.21	86.81 ± 25.31	129.88 ± 19.31	173.56 ± 56.11
Average size of primary tumors at 25 days (mm^3)	757.5 ± 105.94	731.00 ± 162.70	211.13 ± 23.43	366.50 ± 218.87	465.88 ± 119.31
Average size of primary tumors at 30 days (mm^3)	1369.88 ± 141.55	1438.75 ± 159.82	309.81 ± 70.86	500.50 ± 40.50	854.44 ± 294.31
Average size of primary tumors at 35 days (mm^3)	2011.75 ± 182.04	1874.00 ± 107.68	607.56 ± 55.21	785.31 ± 53.60	1162.25 ± 305.57

D

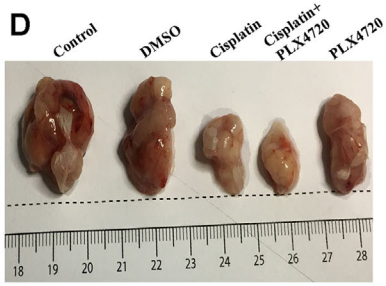


Figure 5. The effect of the combined treatment with BRAFi and cisplatin on colon tumors in vivo. A) Growth rates of RKO tumor xenografts in SCID mice. Twenty mice were equally divided into the following five groups based on the applied treatment: control (untreated), DMSO, a combination of 5mg kg^{-1} cisplatin and 10mg kg^{-1} PLX4720, 5mg kg^{-1} cisplatin alone, 10mg kg^{-1} PLX4720 alone. Chart lines represent tumor growth within a total period of 20 days after the first administration of the compounds to the developed tumors (Day 15). Standard deviation (SD) was used for error bar generation between all tumors of the same group (bars indicate standard deviation of tumor volumes, $n = 8$ sites injected). B) Statistical analysis was performed using two-way ANOVA, Bonferroni's multiple comparisons test. Statistical significance represents the comparison of each indicated sample with the control. ***$P < 0.001$, ****$P < 0.0001$, ns: non-significant. C) Size values of the tumors at the experiment onset and during the course of the administrations. D) Representative image of the tumors excised from each group of mice at the end of the in vivo experiment (Day 35).

(γH2AX), in addition to being a critical component of the DSB repair mechanism, is also implicated in many other biological processes. For example, Fragkos et al.[54] have shown that, in the absence of DNA damage, phosphorylation of H2AX is an early sign of replication stalling, inhibiting cell cycle progression from the G1 to the S phase. Interestingly, they found that after inhibition of the replication machinery, γH2AX is needed for increasing the levels of p21, thus resulting in checkpoint activation and cell cycle arrest. These results suggest that the phosphorylation of H2AX on Ser-139 that was observed in the present study correlated with the G0/G1 arrest after treatment of BRAF mutant cells with a BRAFi. Although the exact mechanism of the interaction between γH2AX and the p53/p21 pathway is still unknown, it is possible that H2AX phosphorylation affects the interaction of PCNA with chromatin, allowing the stable binding of PCNA to p21 and blocking its ubiquitination.[54]

Importantly, the antitumor activity of the combination treatment with cisplatin and PLX4720 was further validated in vivo in mouse xenografts of RKO cells, with the combinatorial treatment showing superior therapeutic potential than each drug alone. Combinatorial treatments of PLX4720 and other therapeutic compounds have proven efficient in growth suppression of distinct tumor types.[55] Especially in the case of colorectal cancer, in which Vemurafenib monotherapy is of no appreciable value for patients,[56] combination of the RAS/MAPK pathway inhibition and chemotherapy could potentially ameliorate the observed resistance mechanisms that contribute to reactivation of cancer cell proliferation. Finally, drug combinations can achieve higher therapeutic responses in lower individual doses, thus avoiding the complications of off-target toxicities induced by high drug concentrations. Herein, the in vivo confirmation of the remarkable in vitro results paves the way for the exploitation of similar therapeutic combinations in multiple preclinical and potentially, clinical settings. However, more in vivo studies regarding pharmacokinetics and other pharmacological parameters are required, in order to accurately characterize the therapeutic value of the proposed regime.

4. Conclusion

It is widely accepted that multicellular organisms are shielded against endogenous and external threats by a highly controlled system that combines the synergistic action of the MAPK signaling pathway and the DDR network. Indeed, a wide range of biological functions, including migration, differentiation, proliferation, inflammation, metabolism, and cell survival or death, are regulated by the MAPK signaling pathway. In addition, the repair of the DNA damage, the cell cycle, and the cell death are among the biological processes that appear to be significantly influenced by the DDR machinery. Guided by this notion, abnormalities in these networks might have a role in the onset and progression of several malignancies, including colorectal cancer.

Given that these changes could also play a role in the emergence of drug resistance, they might be exploited as new targets for treatment. In fact, recent reports have shown that several drugs that target the MAPK signaling pathway or the DDR network are at different phases of clinical validation. Notably, compared with monotherapy, combinatorial therapy provides a range of clinical advantages, such as improved treatment success, lower drug level, fewer adverse effects, reduced failure rates, and a decreased chance of relapse. Thus, the findings discussed herein potentially offer a new approach to boost the effectiveness of treatments for colorectal cancer by combining drugs that target the MAPK signaling pathway with those targeting the DDR network. Taken together, our study demonstrates that the combined treatment with BRAFi and cisplatin is more effective than single-drug treatment in preclinical models in vitro and in vivo, suggesting that these data, once further validated at the preclinical level, can be exploited for the design of new therapies for the treatment of CRC.

5. Experimental Section

Cell Lines: Human colorectal adenocarcinoma cell lines (Colo-205, HT29, RKO, and Caco-2) were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% nonessential amino acids, and 1% penicillin/streptomycin (all from Thermo Fisher Scientific) at 37 °C, 5% CO_2.

Inhibitors: The BRAF inhibitors PLX4720 (#S1152) and Vemurafenib (#S1267) were purchased from Selleckchem, while Encorafenib (#HY-15605) and Dabrafenib (#HY-14660) from MedChemExpress. Human recombinant SuperKiller cc-TRAIL (Alexis, ALX-522-020) was used as a control of apoptotic cell death.

Western Blotting: Whole-cell protein lysates were extracted with lysis buffer containing protease inhibitors separated in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, UK), as described previously.[53] The antibodies used were directed against pERK1/2 (Santa Cruz Biotechnology, sc-7383) (1:100 dilution), γH2AX (Cell Signaling Technology, #9718T) (1:1000 dilution) and GAPDH (Santa Cruz Biotechnology, sc-47724) (1:1000 dilution). The secondary antibodies used were mouse anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2357) (1:1000 dilution) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005) (1:1000 dilution). The antibody signal was enhanced with chemiluminescence and captured on X-ray film Super RX-N (Fujifilm Tokyo, Japan). Values were measured using Studio Lite software (LI-COR Biotechnology, Lincoln, NE, USA) and levels were normalized against the housekeeping GAPDH protein. The blots presented are representative of three independently repeated experiments.

Flow Cytometry: Cells were cultured and treated in 6-well plates. Upon the selected time-point, they were detached and fixed/permeabilized with ice-cold ethanol overnight. DNA was marked with propidium iodide (PI; BD Biosciences; #556 463) for 1 h at room temperature at a concentration of 50 μg mL^{-1}. RNA binding was avoided with the use of RNase A, 10 μg mL^{-1} (Thermo Fisher Scientific, #EN0531). Cell cycle was analyzed using a BD FACSAria II flow cytometer and the BD FACSDiva v8.0 software (BD Biosciences).

Cell Viability Assay: Cell viability was estimated with the Sulforhodamine B (SRB; Sigma–Aldrich, S1402) assay.[57] Cells were seeded for 24 h into 96-well microtiter plates. After completion of the treatment, fixation was performed with 10% trichloroacetic acid (TCA; Sigma–Aldrich, #T6399) and staining with 0.4% SRB in 1% acetic acid. Absorbance was measured using a TECAN microplate reader (TECAN, Mannedorf, Switzerland) and cell viability was estimated.

In Vivo Studies: A total of 1×10^6 RKO cells diluted in PBS were injected subcutaneously into the left and right flanks of 6-week-old female SCID mice. When the tumors became palpable, reaching an appropriate volume of 21–3 $3mm^3$ (Day 15), the mice were randomly assigned to five groups (four mice per group). The first group was used as a negative control (untreated) group. The second group was injected with DMSO (5% in distilled H_2O). The third group was treated intratumorally with a combination of 5mg kg^{-1} cisplatin plus 10mg kg^{-1} PLX4720 in 5% DMSO (100 μg cisplatin plus 200 μg PLX4720/mouse every five days). The fourth group was treated intratumorally with 5mg kg^{-1} cisplatin alone (100 μg/mouse every five days). The fifth group was treated intratumorally with 10mg kg^{-1} PLX4720 (200 μg/mouse every five days). The mice received 5 treatment doses in a total period of 25 days. During this period, tumor sizes were measured every 5 days using caliper, and tumor volumes were calculated using the formula V = (height x width x length)/2. The Standard Deviation (SD) was used for error bar generation between all tumors of the same group of animals. Statistical analysis of the data was performed using two-way ANOVA Bonferroni's multiple comparisons test in Graphpad Prism 9.0. At the end of the observation period, the mice were sacrificed due to tumor burden. Tumors were subsequently excised and photographed.

Apoptosis Assay: Cells were treated with 0—100 μg mL^{-1} cisplatin for 3 h, followed by 48 h post-incubation time in drug-free medium. The Cell Death Detection ELISAPLUS kit (Roche Diagnostics, Switzerland, #11 544 675 001) was used to determine apoptosis according to the protocol provided by the manufacturer.

Statistical Analysis: Continuous variables were compared among groups with Student's t-test, or Mann-Whitney U test when normal distribution did not apply, whereas paired comparisons were performed by paired t-test or Wilcoxon's test. Correlations were examined with Spearman's rank test. All statistical analyses were performed with SPSS v.24.0. Results were considered significant when $P < 0.05$.

Institutional Review Board Statement: The study was carried out in accordance with the EU Directive 2010/63/EU for animal experiments and was approved by the Ethics Committee of the National Hellenic Research Foundation (approval No. 431 956) after the license by the General Directorate for Agricultural Economy and Veterinary.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization was performed by V.Z., A.P., and V.L.S.; Data curation was performed by V.Z., A.P., and V.L.S.; Formal analysis was performed by K.K., V.K., M.G., V.Z., A.P., and V.L.S.; Funding acquisition by V.Z., A.P., and V.L.S.; Investigation was performed by K.K., S.S., V.K., L.K., and M.G.; Project administration was performed by A.P. and V.L.S.; Resources were acquired by V.Z., A.P., and V.L.S.; Supervision was performed by A.P. and V.L.S.; Visualization was performed by A.P. and V.L.S.; Original draft written by A.P. and V.L.S.; Review written and edited by all authors.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

BRAF inhibitor, BRAFV600E-mutated cells, cisplatin, colorectal cancer, combination therapy, mouse xenografts

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