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BRAF paradox breakers PLX8394, PLX7904 are more effective against BRAFV600E CRC cells compared with the BRAF inhibitor PLX4720 and shown by detailed pathway analysis

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ABSTRACT

PLX7904 and PLX8394 are novel BRAFV600E inhibitors-BRAFi that are designed to evade the paradoxical MAPK activation, a trait for the name "paradox breakers"-PB. Current FDA approved inhibitors (Vemurafenib, Dabrafenib, Encorafenib) although improved progression-free survival of mtBRAF melanoma patients suffer from this treatment related side effect. mtBRAF Colorectal Cancer (CRC) is resistant to the approved BRAF inhibitors, although combinatorial treatment co-targeting BRAF and EGFR/MEK is offering a promising prospect. In an effort to explore the potential of the novel BRAF inhibitors-PB to impede CRC cell proliferation, they were tested on RKO, HT29 and Colo-205 cells, bearing the BRAFV600E mutation. This study shows that the BRAF paradox breakers PLX7904 and PLX8394 cause a more prolonged MAPK pathway inhibition and achieve a stronger blockage of proliferation and reduced viability than PLX4720, the sister compound of Vemurafenib. In some treatment conditions, cells can undergo apoptosis. Genomic analysis on the more resistant RKO cells treated with PLX7904, PLX8394 and PLX4720 showed similar gene expression pattern, but the alterations imposed by the PB were more intense. Bioinformatic analysis resulted in a short list of genes representing potential master regulators of the cellular response to BRAF inhibitors' treatments. From our results, it is clear that the BRAF paradox breakers present a notable differential regulation of major pathways, like MAPK signalling, apoptosis, cell cycle, or developmental signalling pathways. Combinatorial treatments of BRAFi with Mcl-1 and Notch modulators show a better effect than mono-treatments. Additional pathways could be further exploited in novel efficient combinatorial treatment protocols with BRAFi.

1. Introduction

The RAF family of protein kinases are key signalling intermediates of the mitogen-activated protein kinase/MAPK cascade acting as a central link between the membrane-bound RAS GTPases and the downstream kinases MEK and ERK. In mammalian cells, there are three RAF proteins, A-RAF, B-RAF and C-RAF. Under normal signalling conditions, the RAF proteins are dragged from the cytoplasm to the membranes and bind activated RAS. Upon binding, the release of the RAF N-terminal domain (NTD) inactivates auto-inhibition of the protein and allows the formation of homo- and heterodimers that can function as the initiating enzyme in the three-tiered ERK kinase cascade. Physiological RAS signals mainly via BRAF dimers [1–3].

The MAPK pathway is normally involved in the regulation of cell proliferation and survival, while oncogenic mutations of its components are frequently observed in several types of cancer. Specifically about BRAF, the first report on an oncogenic mutant was in 2002 [5] and over 45 mutations have been described since then [1]. Oncogenic BRAF

Abbreviations: MAPK, Mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; EGFR, Epidermal growth factor receptor; Mcl-1, Induced myeloid leukemia cell differentiation protein; AP-1, Activator Protein-1; CRC, colorectal cancer; DMSO, Dimethyl Sulphoxide; PBS, Phosphate buffered saline; SRB, Sulforhodamine; PI, Propidium iodide; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RNA-seq, RNA-sequencing; ATM, ataxia-telangiectasia mutated.

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signalling is implicated in approximately 50% of melanomas, 30%–70% of papillary thyroid tumours, 30% of low-grade serous ovarian tumours, and 8%–10% of colorectal cancers (CRC) [6].

The most common mutation of the BRAF gene is a point mutation at nucleotide 1799 in which valine is substituted with glutamic acid at codon 600; this results in the synthesis of the BRAFV600E oncoprotein. Other BRAF mutations (V600K, V600D and V600R) exist, but the prevalent (90%) form is V600E [7]. The BRAFV600E mutation causes BRAF to signal as a monomer and independently of its upstream activator protein-RAS and the ERK dependent negative feedback mechanism, resulting in increased stimulation of its downstream effector proteins-MEK and ERK via phosphorylation. Uncontrolled amplification of downstream signalling is linked with transformation, increased proliferation and finally tumourigenesis [1,2].

Vemurafenib, Dabrafenib and Encorafenib are currently the FDA approved drugs against BRAFV600E positive metastatic melanomas. These inhibitors, although exhibiting some degree of preferential inhibition of BRAFV600E, they generally bind to all RAF kinases. Their efficacy is ascribable to the inhibition of BRAFV600 monomeric mutants present and active in the mutant cells versus normal cells where RAF signals as a dimer [8].

The objective response rate for patients with BRAF mutant melanoma receiving Vemurafenib treatment is about 50%; however, initial response may be later lost due to the development of acquired resistance [9]. Resistance is mostly related to the non-effectiveness of the administered drugs against RAF dimeric forms, since binding to one monomer induces allosteric transactivation of the second monomer, while at the same time the binding of the drug at the second monomer is sterically prevented [8,10]. Thus, in the presence of alterations which increase RAF dimerization, in tumour cells bearing V600 mutants dimerize with mutant or wtBRAF and wtCRAF conferring resistance. Moreover, in cells bearing wtBRAF, the drugs may also induce the paradoxical activation of ERK signalling leading to secondary skin malignancies and keratoacanthomas [9,11,12].

Unlike melanoma, the effect of Vemurafenib in CRC patients with a BRAF mutation is often negligible, resulting in a clinical response in only 5% of patients [13,14]. The resistance of mtBRAF CRC to BRAFi seems to be elicited by feedback reactivation of the MAPK cascade through the receptor tyrosine kinase (RTK) EGFR signalling. It has been observed that EGFR is overexpressed mainly in epithelial tumours and probably this is why this particular reactivation mechanism is not triggered in melanoma cancers that originate from the neural crest [15,16]. Another possible cause of BRAFi resistance is the co-existence of mutations in several signalling pathways, allowing CRC cells to proliferate, independently of the MAPK signalling [17].

A combination of encorafenib (BRAFi), cetuximab (EGFRi), and binimetinib (MEKi) resulted in significantly longer overall survival and a higher response rate than standard therapy in patients with metastatic colorectal cancer bearing the BRAF V600E mutation [18,22]. FDA recently approved encorafenib (BRAFi) in combination with cetuximab (EGFRi) for metastatic colorectal cancer with a BRAFV600E mutation, as a therapy for these tumours for the first time. Other rational combinatorial treatments of BRAF targeting drugs with specific inhibitors of cancer pathways have been successfully examined at the preclinical stage in CRC and have entered the clinical trials with promising results [4,19–21].

The development of the next generation BRAFV600E inhibitors PLX7904 and its analogue PLX-8394, known as paradox breakers (PBs), the latter being in clinical trials in adult and paediatric patients with advanced BRAF-mutated tumours (NCT02428712), has opened new avenues in overcoming several mechanisms of resistance and controlling the paradoxical activation of the MAPK pathway [23].

PLX8394 displays a unique profile acting as a BRAF specific dimer breaker and selectively inhibiting ERK signalling in tumours driven not only by BRAFV600E monomers but also in tumours harbouring dimer-dependent BRAF mutants including fusions and splice variants [24]. Structural data of the analogue compound PLX7904 in complex with BRAFV600E prompt to a further shift of amino acids sidechains of the aC-helix, a hotspot region for RAF dimer formation, as compared with Vemurafenib respective complex which might be critical in causing disruption to the dimer interface in the biological environment [23]. PLX8394 is effective in disrupting BRAF homo- and BRAF-CRAF heterodimers but is ineffective against CRAF homodimers due to structural differences in the respective dimer interfaces [24]. Highly important is the fact that in wtRAS/RAF cells, where all isoforms are expressed, PLX8394 administration results in neither inhibition nor enhancement of ERK signalling thus inducing no toxicity [23–25].

Kawakami et al., revealed that in BRAFV600E colorectal cancer, the BRAF mutant phosphorylates and as a result stabilizes the anti-apoptotic protein Mcl-1 [26]. They also demonstrated that this mechanism of resistance to apoptosis can be suspended by simultaneous inhibition of Mcl-1 and components of the MAPK pathway. Similar results were obtained also using melanoma cells, where enhanced expression of Mcl-1 is associated with resistance not only to Vemurafenib but also to its combined administration with MEK inhibitors [27]. These data indicate that simultaneous inhibition of Mcl-1 and BRAFV600E might have synergistic effect in the treatment of BRAFV600E colorectal cancer.

The Notch pathway is a developmental pathway and it is found to be deregulated in many cancer types. For example, increased expression of the Notch-1 receptor is associated with tumorigenesis and metastasis [28]. This pathway has been studied as potent target for cancer therapy and many antibodies that bind the Notch receptors or drugs against the γ secretase have been tested [29]. γ secretase is the main activator of the pathway as it cleaves the active Notch receptor releasing its intracellular domain that acts as transcription factor. Monotherapy with RO4929097, a γ secretase inhibitor, in patients with metastatic colorectal cancer was not efficient although well tolerable from the patients [30].

In the current study, we examined the effect of the novel PBs on CRC tumour cells bearing the BRAFV600E mutation. It is shown that PB PLX7904 and PLX8394 cause a more prolonged inhibition of the MAPK pathway and achieve a stronger proliferation blockage and reduced cell viability than PLX4720, a sister compound to Vemurafenib, which is commonly used in preclinical studies. Genomic and bioinformatic analysis of PB PLX7904, PLX8394 vs PLX4720 treatments, showed differential gene expression changes by PB. Bioinformatic analysis resulted in a short list of genes representing possible master regulators of the cellular response to the treatment with BRAF inhibitors. Notably, the BRAFi treatments result in a remarkable differential regulation of major pathways-genes, like MAPK signalling, apoptosis, cell cycle, or developmental signalling pathways, thus proposing rational combinatorial treatments of BRAFi with modulators of these pathways. Here, we have selected combinatorial treatments of BRAFi with Mcl-1 inhibitor and Notch pathway modulator and a first indication of a potential benefit is presented.

2. Materials and methods

2.1. Cell lines

The BRAFV600E bearing Colo-205, HT29 and RKO human colorectal adenocarcinoma cell lines, as well as Caco-2 colon intermediate adenoma cell line and HCT116 colon adenocarcinoma cell line were obtained from the American Type Culture Collection (ATCC). Cell lines have been originally purchased from ATCC. Presence of BRAF mutations and/or main non-BRAF driver mutations from those listed in the table on p. 10 (in agreement to https://cancer.sanger.ac.uk/cell_lines) have been previously confirmed.

All cell lines were grown in DMEM medium supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin and amino acids (all from Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C, 5% CO_2.

2.2. Inhibitors

The BRAF inhibitor PLX4720 was purchased from Selleckchem, Houston, TX, USA and PLX7904 and PLX8394 from MedChem Express, Monmouth Junction, NJ, USA. RO4929097 – γ secretase inhibitor (γ secretase is component of the Notch pathway) and A-1210477 – MCl-1 inhibitor were purchased from (MedChemExpress, Monmouth Junction, NJ, USA). The A-1210477 inhibitor was dissolved in DMSO using ultrasound for 30 min at 37 °C. Before the experiment the inhibitors were diluted in DMEM medium.

2.3. RNA extraction, reverse transcription and real time-PCR

RKO cells were seeded in 6-well plates and left to attach overnight. Upon treatment, total RNA was extracted using the Trizol reagent (Ambion by Life Technologies, Foster City, CA, USA). Total RNA was reverse transcribed into cDNA using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Real-time quantitative PCR was performed for the determination of CDNK2B, ETS1, HEY1, JUN, SMAD7 and MCL1 mRNA expression levels in treated RKO cells. The assay was carried out in 96-well plates using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a Bio-Rad iCycler with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermal protocol consisted of a 3 min polymerase activation step at 94°C, followed by 40 cycles of denaturation at 95°C for 25 s and the primer annealing and extension at 60°C for 45 s. Each sample was amplified in duplicates and the average CT values were calculated for the expression analysis. The mRNA expression of all genes was normalized to the GAPDH reference gene. The primers used for each gene were the following: *CDKN2B* F: 5′–AGGGATATTTAGGAGTGTGTGAC-3′, R: 5′-CCATCGGAAGATTCGTAGCC-3′, *ETS1* F: 5′-ATACCTCGGATTACTTCATTAGC-3′, R: 5′-GGATGGAGCGTCTGATAGG-3′, *HEY*1 F: 5′-CGAAATCCCAAACTCCGATA-3′, R: 5′-CGAGGTGGAGAAGGAGAGTG-3′, *JUN* F: 5′-GAACGCCTGATTGTCCCC-3′, R: 5′-CGAAGCCCTCCTGCTCCTC-3′, *SMAD7* F: 5′-CATCTTCATCAAGTCCGCC-3′, R: 5′-ATAAACTCGTGGTCATTGGG-3′, *MCL1* F: 5′-GGACATCAAAAACGAAGACG-3′, R: 5′-GCAGCTTTCTTGGTTTATGG-3′ and *GAPDH* F: 5′-GAAGGTGAAGGTCGGAGT-3′, R: 5′CATGGGTGGAATCATATTGGAA-3′.

2.4. Western blotting

Whole cell protein lysates were extracted with lysis buffer containing protease inhibitors, separated in an SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, Amersham, UK) as described previously [20]. Membranes were then incubated overnight with specific antibodies at 4 °C, washed with TBS-Tween20 and incubated with the proper secondary antibody for 1 h, at room temperature. The antibodies used were directed against p-ERK 1/2 (sc-7383-Santa Cruz Biotechnology, Inc., Dallas, TX, USA), caspase-3 (#9662-Cell Signaling Technology, Danvers, MA, USA), γH2AX (Ser139; #9718, Cell Signaling Technology, Danvers, MA, USA). The secondary antibodies used were mouse anti-rabbit IgG-HRP (sc-2357) and goat anti-mouse IgG-HRP (sc-2005) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). 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 housekeeping proteins (GAPDH-sc-47724-Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The blots presented are representative of 3, or more, independently repeated experiments.

2.5. Cell viability assay

Cell viability was estimated with the Sulforhodamine assay. Cells were seeded for 24 h into 96-well microtiter plates. After completion of the treatment, fixation was performed with 10% trichloroacetic acid 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. IC50 values were calculated using the GraphPad8 software.

2.6. Flow cytometry

Cells were cultured and treated in a 6-well plate for 24 and 48 h. Upon the selected time point, they were detached and fixed/permeabilized with ice-cold ethanol overnight. DNA was marked with propidium iodide (BD, Franklin Lakes, NJ, USA) for 1 h at room temperature at a concentration of 50 μg/ml. RNA binding was avoided with the use of RNAase A, 10 μg/ml. Cell cycle was analysed using a BD FACSAria II flow cytometer and the BD FACSDiva v8.0 software (BD, Franklin Lakes, NJ, USA).

2.7. Immunofluorescence

Fluorescent cleaved caspase-3 and Hoechst stain were used for the detection of apoptotic cells under confocal microscopy. Cells (2×10^4 cells/well) were grown on cover slips in 24-well plates in medium at 37 °C and let to attach overnight. After the selected treatment, cells were fixed with methanol-acetone solution 7:1 and permeabilized with 0,25% Triton X-100 in PBS. The cells were incubated overnight with the primary antibody for cleaved caspase-3 (#9661, Cell Signaling Technology, Danvers, MA, USA) and for 1 h with the secondary fluorescent antibody (# A-21428, Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555, Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were stained with Hoechst No. 33342 (Sigma, B2261). Photographs of the cells were taken under light and confocal microscope (Leica 626 TCS SPE confocal laser scanning microscope) and acquired using the LAS AF software (Leica Lasertechnik, Heidelberg, Germany).

2.8. RNA sequencing and bioinformatics analysis

RNA sequencing (RNA-seq) was performed by BGI-Europe (Copenhagen, Denmark) on a BGI-500 sequencer, as paired-end reads (100 bp; minimum 62 million clean reads/sample). Quality control of raw sequencing data was performed using fastp [31]. Sequence alignment to the human genome (GRCh.38) was performed with STAR [32]. Transcript assembly and expression quantification was performed by featureCounts [33]. Genes with low expression in less than two samples were filtered out, using counts per million (CPM) to account for differences in library sizes. Normalisation was performed using the trimmed mean of M-values (TMM) and differential expression analysis by edgeR [34,35] on R programming environment [36]. To extract the lists of differentially expressed genes between DMSO (control) and the three substances, the quasi-likelihood method was used, and a double cut-off of FDR (false discovery rate) p-value < 0.05 and absolute $\log_2$ fold-change > 1 (logFC) was applied. Functional analysis was performed using the BioInfoMiner tool [37], exploiting Reactome [38] and Gene Ontology Biological Processes [39,40]. RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-9014.

A

IC50 values (μM)			
	RKO	HT29	Colo-205
PLX7904	2,08	0,48	0,05
PLX8394	7,79	0,32	0,04
PLX4720	24,87	2,63	0,04

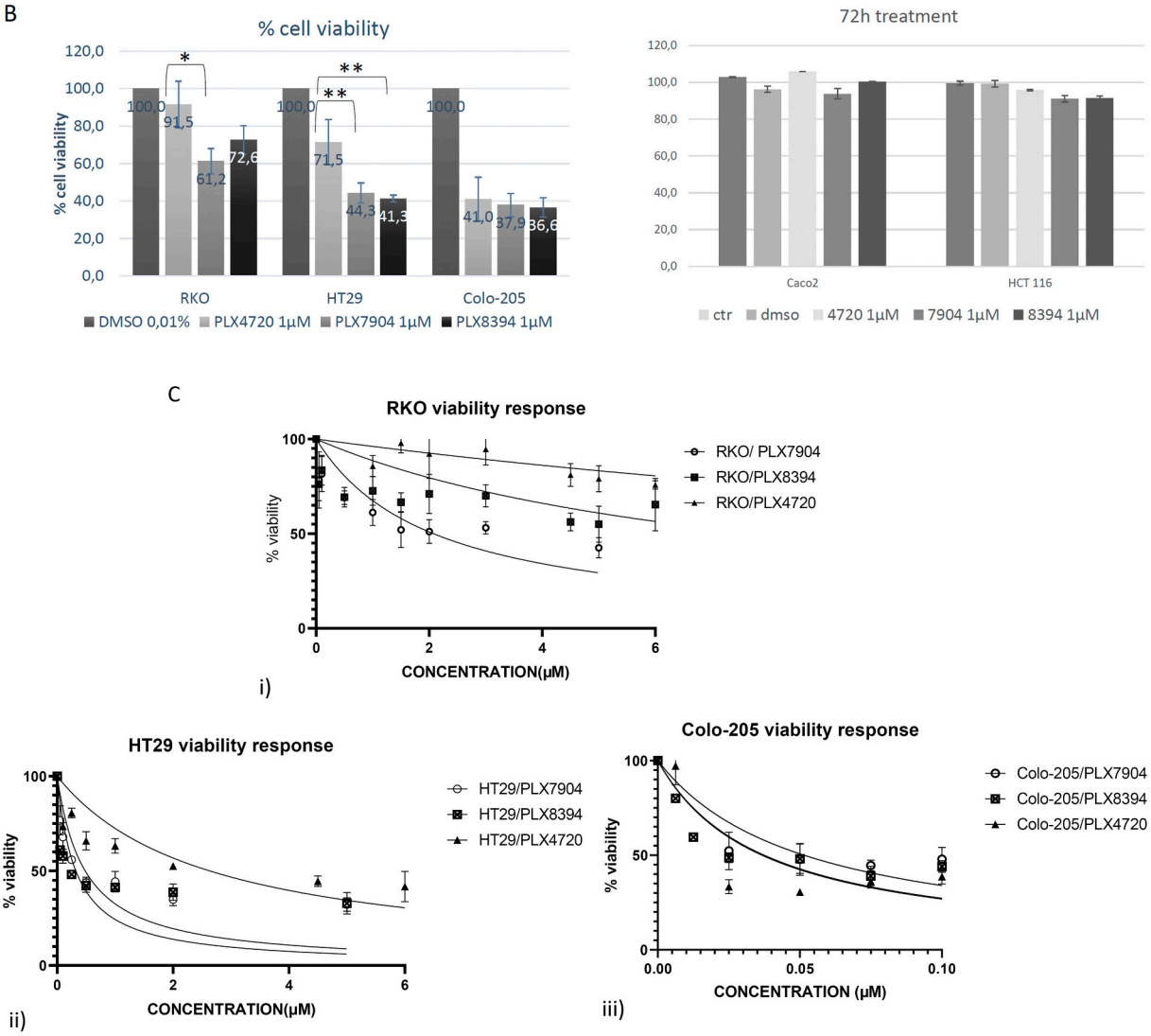


Fig. 1. Cell survival for a panel of BRAFmt colon cancer cell lines treated with BRAF inhibitors paradox breakers (PBs) vs PLX4720. Effect on viability, 72 h post treatment with paradox breakers PLX7904 or PLX8394 vs. PLX4720 for the BRAFmut cell lines: RKO, HT29 and Colo-205, according to SRB assay A) IC50 values (μM) for 72 h treatment with the three BRAFi B) Viability percentage 72 h post treatment with 1 μM of each compound. The diagram in Fig. 1B represents the viability effect on cells treated with 1 μM of each compound for 72 h, versus control cells treated with DMSO 0,01% *v*/v. The (*) indicates the significantly different results when compared to PLX4720, $P < 0{,}0001$ the ** C) Normalized curves of the viability effect on the cells after 72 h treatment with several concentrations of each compound: i) RKO cells ii) HT29 cells iii) Colo-205 cells.

3. Results

3.1. BRAF paradox breakers (PB) display a stronger inhibition of the MAPK pathway and cell growth, as compared to PLX4720 treatment in BRAFmut colon cancer cell lines

3.1.1. Cell lines for treatments and their main mutations

	MSI/MSS status	KRAS status	BRAF status	PIK3CA status	P53 status
RKO	MSI	WT	V600E	H1047R	WT
HT-29	MSS	WT	V600E	P449T	R273H
Colo-205	MSS	WT	V600E	WT	Y103fsX37 (−/−)/G266E
Caco-2	MSS	WT	WT	WT	G204T (stop codon)
HCT116	MSI	G13D (+/)	WT	H1047R	WT

The evaluation of the new BRAF inhibitors-paradox breakers (PBs) PLX7904 and PLX8394 was performed in cultures of colon cancer cell lines RKO, HT29 and Colo-205 that bear an heterozygous BRAFV600E mutation. Cytotoxic/cytostatic effect, MAPK inhibition potential, effect on cell cycle regulation and induction of apoptosis were examined. BRAFV600E mutant cells treated with PBs exhibit reduced viability, stronger G0/G1 arrest as compared to the PLX4720 treated cells and in some treatment conditions undergo apoptotic procedure.

At first, the treatment effect was tested of PB as compared to Vemurafenib analogue PLX4720on the viability of the colorectal cancer cell lines. Treatments by PB PLX7904 and PLX8394 significantly reduced the viability of the more resistant cell lines RKO and HT29 in comparison to PLX4720, 72 h post treatment (Fig. 1B, left panel). Particularly, the estimated IC50 values are 2,08 μM, 7,79 μM and 24,87 μM for the RKO cells and 0,48 μM, 0,32 μM and 2,63 μM for the HT29 cells, for PLX7904, PLX8394 and PLX4720 respectively (Fig. 1A). It is evident that the PBs can be as efficient as PLX4720 at almost 10fold lower concentration. In Colo-205, the relatively sensitive to BRAFi cell line, treatment effect was profound but not significantly different among the three compounds. The IC50 values for this cell line were estimated 0,05 μM, 0,04 μM and 0,04 μM for the PLX7904, PLX8394 and PLX4720 respectively (Fig. 1A). The enhanced effect caused by PLX7904 and PLX8394 is evident particularly in RKO and HT29 cells (Fig. 1C). Notably, treatments with all tested BRAFV600E targeting compounds had a marginal if at all effect on cell viability of either intermediate colon adenoma cells Caco-2, (BRAFwt, non tumourigenic properties, slow proliferation) or on cell viability of HCT116 colon adenocarcinoma cell line (bearing BRAFwt) (Fig. 1B, right panel).

In order to discern whether the reduction of the cell population was due to cytostatic or cytotoxic action after the corresponding drug treatments, we performed cell cycle analysis using flow cytometry and PI staining (24 h post treatment is presented, similar cell cycle effects were recorded 48 h post-treatment, data not shown). An inhibition at the G0/G1 phase was revealed with all BRAFi at a concentration of 1 μM at all cell lines treated. A significant enhancement of the G0/G1 inhibition with both PB PLX7904 and PLX8394 as compared to PLX4720 was observed in RKO and HT29 cell lines (Fig. 2A & B). Specifically, RKO cells exhibited weak and not statistically important G0/G1 inhibition after PLX4720 treatment (Fig. 2A, Lane 2). On the other hand, upon PLX8394 treatment, the effect is significant both in the increase of RKO G0/G1 cells and in the reduction of the S phase cells (Fig. 2A, Lane 4). In Colo-205 cell line, in accordance to the viability test, the effect was strong but similar among the three compounds (Fig. 2C). These results provide strong evidence for cell cycle regulation by PBs. It appears that the BRAF PBs PLX7904 and PLX8394 treatments have an enhanced ability to block cell proliferation in the more BRAFi resistant cell lines at lower concentration than PLX4720.

Driven by the significant viability reduction and the slight but repetitive increase of the SubG1 phase upon treatment with PLX8394, we hypothesized that apoptotic pathways are possibly triggered. Positive apoptotic signal appeared indeed (ARROWS) at 72 h post-treatment. Some apoptotic cells were detected in RKO, HT29 and Colo-205 cultures upon treatment with PLX8394, while absent in RKO cells treated with PLX4720 or PLX7904 (Fig. 3). Treatment with PLX4720 triggered apoptotic signals in Colo-205 cells, but not in other cell lines (Fig. 3). These results strongly suggest that the reduction of cell viability when treated with PLX8394 is caused not only by inhibition of cell proliferation, but also by induction of cell death pathways, possibly following a prolonged cell cycle arrest.

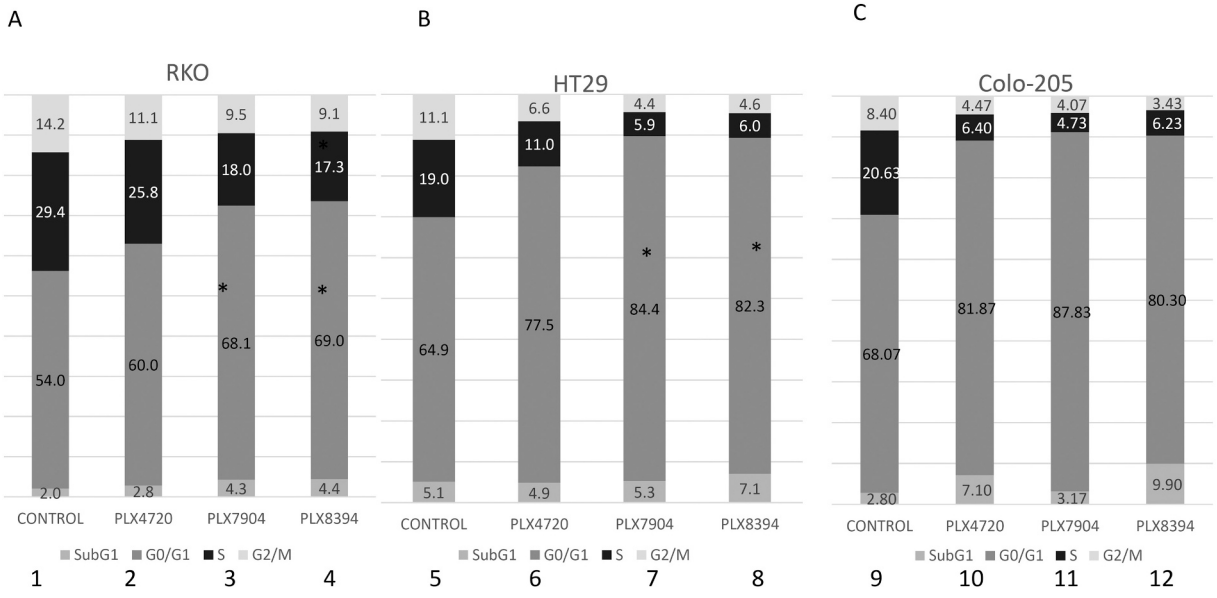


Fig. 2. BRAF paradox breakers (PBs) induce cell cycle arrest at G0/G1 phase in colorectal carcinoma cells A–C) Cell cycle phase distribution for cells treated for 24 h with DMSO 0,01% v/v (control, Lanes 1, 5 and 9), PLX4720 1 μM (Lanes 2, 6 and 10), PLX7904 1 μM (Lanes 3, 7 and 11) and PLX8394 1 μM (Lanes 4, 8 and 12). The (*) indicates values significantly different to PLX4720, $P < 0{,}0001$.

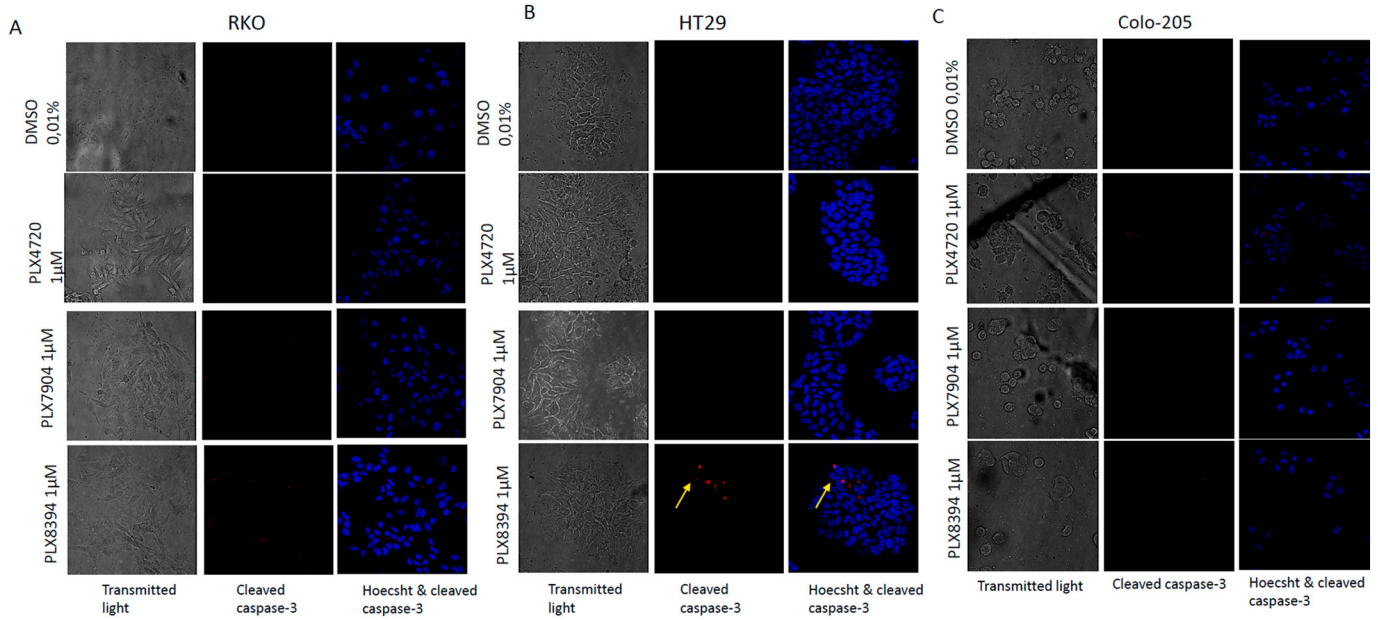


Fig. 3. PBs can induce apoptosis in CRC cells. Detection of cleaved caspase-3 with immunofluorescent confocal microscopy for cells treated with DMSO 0,01% v/v (control), PLX4720 1 μM, PLX7904 1 μM or PLX8394 1 μM for 72 h. Cleaved caspase-3 (RED) is detected at RKO and HT29 cells treated with PLX8394 and Colo-205 cells treated with PLX4720 and PLX8394. Nuclei are stained with Hoechst (cyan).

3.2. BRAF paradox breakers achieve p-ERK1/2 inhibition with a longer duration at low dose concentration. BRAFi interfere with the DDR pathway mediated by H2AX histone phosphorylation

In order to estimate the BRAFV600E inhibition potency, we performed Western blot analysis of p-ERK1/2, a MAP kinase downstream of BRAF, 24 h and 48 h after treatment with several concentrations of the PBs (0, 0.1, 0.5 and 1 μM). A negative control (DMSO 0,01% *v*/v) and a positive control (PLX4720, 1 μM) were evaluated at each case. At 24 h, the phosphorylation of ERK 1/2 was significantly inhibited (>80% inhibition) in all combinations of cell line-BRAFi, with the exception of the weak inhibition (60%) caused by PLX4720 on the RKO cells (Fig. 4A, RKO Lane 2). The effect caused by PBs PLX7904 and PLX8394 was at least twice as strong as the one caused by PLX4720 even at a lower dose, at the RKO and HT29 cells (Fig. 4A). Regarding the BRAFi sensitive cells Colo-205, inhibition of the p-ERK 1/2 was similar for all tested inhibitors ranging at about 90% of the control sample at concentrations as low as 0,1 μM (Fig. 4A). Diverse findings were observed at 48 h post treatment where the p-ERK 1/2 inhibition was maintained on Colo-205 cells with all compounds, while at HT29 cells p-ERK 1/2 inhibition was maintained with the paradox breakers (Fig. 4B).

Interestingly, we discovered an increase in the phosphorylation of the H2ax histone, at the RKO and Colo-205 cells, 48 h post treatment with all 3 compounds (Fig. 4B). The phosphorylation of the H2AX histone is known to be an important step of the DNA damage response and according to our knowledge, it has not been reported before as a result of BRAFi treatment.

Taking under consideration the results given by the viability assay and the detection of apoptotic cells, it appears that the duration of the p-ERK 1/2 inhibition may indicate the overall sensitivity of the cell lines to the BRAFi and the ability to cause strong proliferation blockage and cell death.

3.3. BRAF paradox breakers present differential effect compared to PLX4720 on gene expression and major pathway regulation as shown by next-generation sequencing

In an effort to explore the mechanisms underlying the phenotypic changes imposed by the inhibitors, we examined the gene expression profile of RKO cells treated for 48 h with PLX4720, and with either PBs PLX8394 or PLX7904 as compared to DMSO-treated cells. PLX8394 had the most pronounced effect on gene expression affecting a total number of 876 genes, followed by PLX7904 with 753 genes and, finally, PLX4720 altering the expression of 258 genes (Supplementary Tables 1–3). The number of common genes found as differentially expressed (DE) among the three treatments is presented in the Venn diagram of Fig. 5A.

Hierarchical clustering analysis of the union of DE genes, meaning those that were found significant at least once (959 genes), revealed many similarities among the differential gene expression alterations for the three compounds, with PBs PLX7904 and PLX8394, however, showing a stronger effect on a larger number of genes (Fig. 5B). Pathway analysis using as input the 959 genes, employing the BioInfoMiner tool [37] revealed key biological processes as significantly enriched, including, developmental processes, cell adhesion, cytokine-mediated signalling and angiogenesis (Fig. 5C).

With the scope of discriminating putatively causal genes, we focused on those with inference in diverse cross-talking biological mechanisms, indicating genes with a pivotal role in cellular physiology. Topological analysis exploiting BioInfoMiner was performed, where semantic information is used to prioritise genes based on their centrality, as described in knowledge networks, such as the Gene Ontology. This analysis resulted in a short list of genes (Table 1) representing possible master regulators of the cellular response to the treatment with BRAF inhibitors.

3.4. BRAF paradox breakers have a remarkable effect on major pathways-genes as MAPK (JUN, ETS), cell cycle (CDKN2B), apoptosis (MCL-1), developmental signalling (HEY1, SMAD7), as validated by RT-qPCR

In order to validate the results of the RNA-sequencing for the RKO cells, we performed qPCR for 6 DE genes that are involved in cell cycle regulation, apoptosis regulation and proto-oncogenes, as being described in the table on Fig. 6A. The up-regulation of the CDKN2B gene, which was more intense after treatment with the PBs, validates our cell cycle analysis results regarding G0/G1 inhibition. Specifically, the NGS analysis showed a 0.94 logFC overexpression at the PLX4720 treated

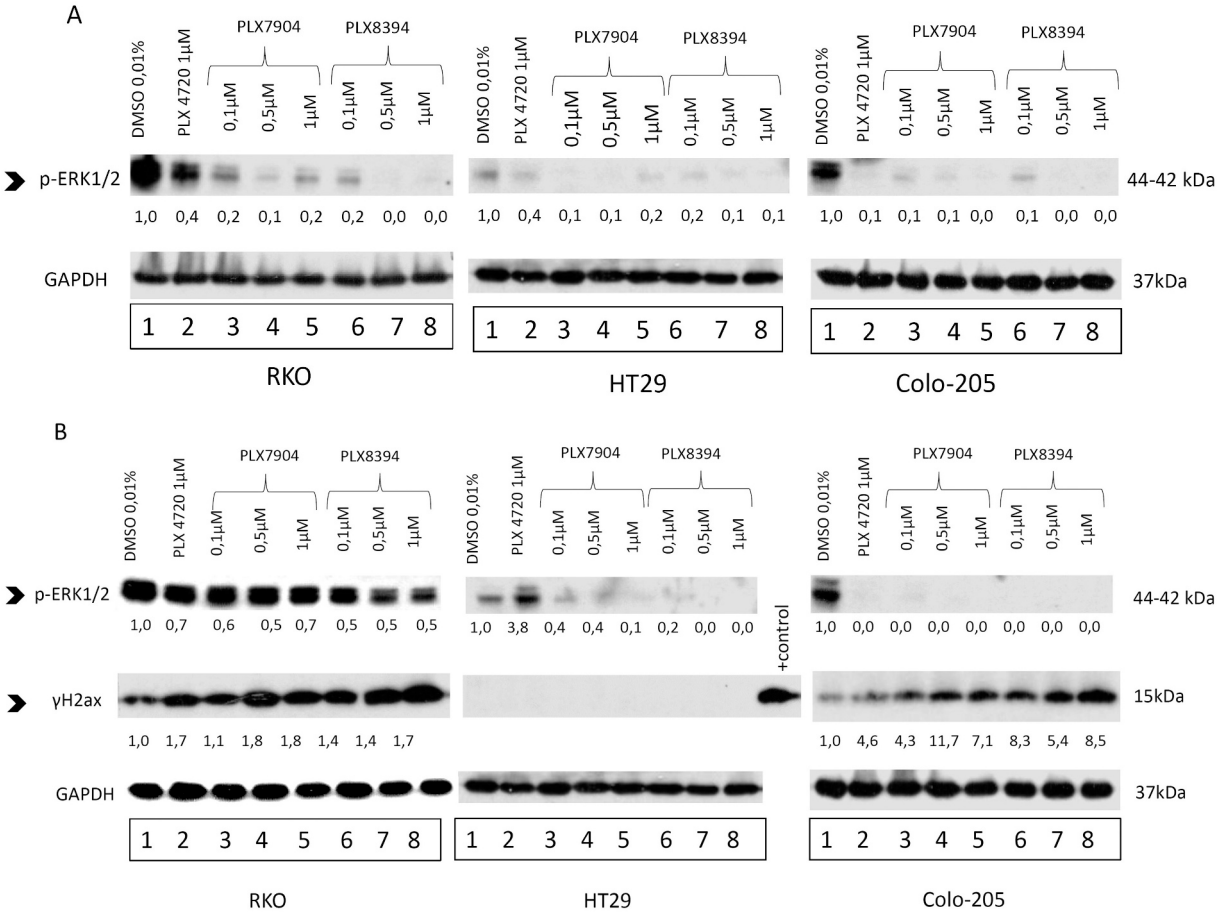


Fig. 4. PBs PLX7904 or PLX8394 in low concentrations achieve prolonged p-ERK1/2 inhibition, and induce a DNA damage response.
A) Western blots for ERK 1/2 phosphorylation, 24 h post treatment with DMSO 0,01% (Lanes 1), PLX4720 1 μM (Lanes 2), PLX7904 0,1 μM–1 μM (Lanes 3–5) and PLX8394 0,1 μM–1 μM (Lanes 6–8) for RKO, HT29 and Colo-205 cell lines. GAPDH is used as a reference protein. Data shown is representative of 3 independent experiments.
B) Protein levels of p-ERK 1/2 and γH2AX, 48 h post treatment with DMSO 0,01% (Lanes 1), PLX4720 1 μM (Lanes 2), PLX7904 0,1 μM–1 μM (Lanes 3–5) and PLX8394 0,1 μM–1 μM (Lanes 6–8) for RKO, HT29 and Colo-205 cell lines. GAPDH is used as a reference protein. Data shown is representative of 3 independent experiments.

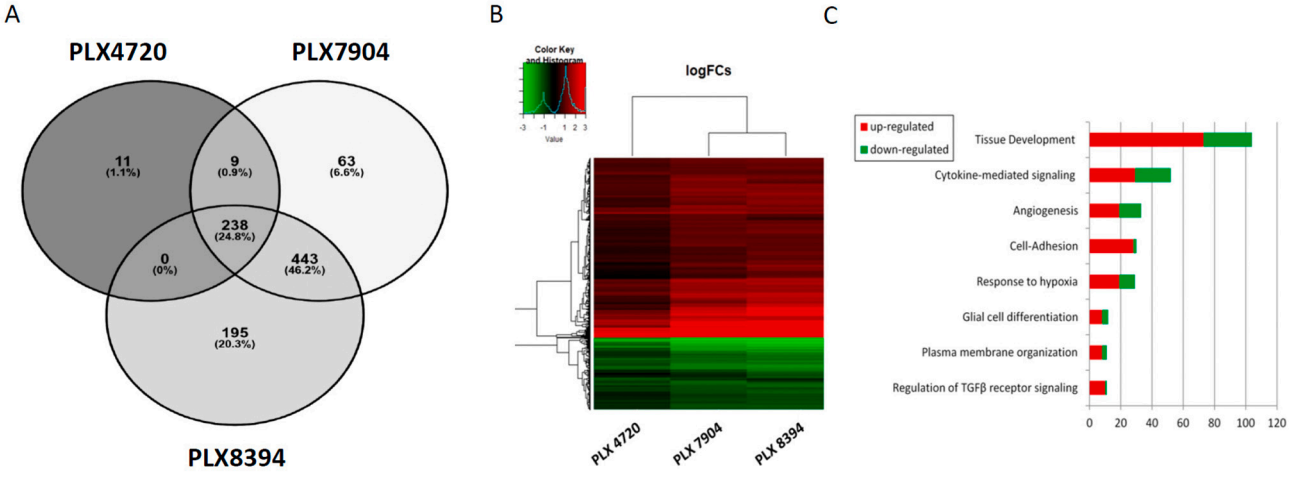


Fig. 5. Differentially expressed (DE) genes of CRC cells treated with BRAFi are revealed by NGS and hierarchical clustering analysis are shown to regulate major MAPK, developmental and apoptotic signalling pathways.
A) Venn diagram for the differentially expressed genes after treatment with the indicated inhibitors, as compared to DMSO. B) Expression heatmap of genes found as differentially expressed in at least one treatment condition, based on the $\log_2$FC value as compared to DMSO. Red colour represents up- and green down-regulated genes. c) Top ranked biological processes indicated as significantly altered by pathway analysis, horizontal axis depicts the number of relevant genes.

Table 1
Top-prioritised genes using the Gene Ontology vocabulary on BioInfoMiner, with their corresponding logFC per inhibitor.

Rank	Symbol	Definition	PLX4720	PLX7904	PLX8394
1	NOTCH1	notch 1	0.705	1.114	1.054
2	RBPJ	Recombination signal binding protein for immunoglobulin kappa J region	−0.358	−0.840	−1.141
3	HES1	Hes family bHLH transcription factor 1	2.715	3.735	4.168
4	SMAD3	SMAD family member 3	2.473	3.638	4.011
5	CAV1	Caveolin 1	2.349	3.332	4.016
6	ID2	Inhibitor of DNA binding 2	1.678	3.775	4.467
7	SMAD6	SMAD family member 6	1.606	4.437	5.404
8	LDLR	Low density lipoprotein receptor	−0.841	−1.454	−1.534
9	TGFBR3	Transforming growth factor beta receptor 3	−0.685	−1.068	−1.040
10	ID1	Inhibitor of DNA binding 1, HLH protein	3.307	4.432	4.713
11	WNT11	Wnt family member 11	0.578	1.090	1.056
12	SMAD7	SMAD family member 7	0.807	1.593	1.396
13	LAMA5	Laminin subunit alpha 5	0.921	1.303	1.150
14	EGR1	Early growth response 1	−1.540	−1.639	−1.768
15	HEY1	Hes related family bHLH transcription factor with YRPW motif 1	1.535	3.549	5.002
16	KLF2	Kruppel like factor 2	−1.066	−2.282	−1.157
17	EPAS1	Endothelial PAS domain protein 1	0.789	2.008	2.352
18	CLU	Clusterin	0.434	1.568	2.165
19	JUN	Jun proto-oncogene, AP-1 transcription factor subunit	−0.531	−1.114	−1.448
20	FOS	Fos proto-oncogene, AP-1 transcription factor subunit	−0.944	−1.512	−1.736
21	TBX1	T-box 1	0.579	1.029	0.931
22	LAMB2	Laminin subunit beta 2	0.450	0.977	1.183
23	IGFBP5	Insulin like growth factor binding protein 5	5.979	7.496	8.518
24	EP300	E1A binding protein p300	0.662	1.016	0.869
25	CAV2	Caveolin 2	0.529	1.387	2.032
26	PKD1	Polycystin 1, transient receptor potential channel interacting	0.752	1.062	0.928
27	TP53	Tumour protein p53	1.351	1.911	1.945
28	EFNA1	Ephrin A1	0.534	0.687	1.149
29	LRP1	LDL receptor related protein 1	0.959	1.711	1.611
30	SOX8	SRY-box 8	−0.442	−0.799	−1.010
31	ANXA1	Annexin A1	1.376	2.085	3.070
32	CPS1	Carbamoyl-phosphate synthase 1	0.579	1.022	0.924
33	BTG2	BTG anti-proliferation factor 2	0.602	1.071	1.234
34	ADM	Adrenomedullin	0.817	1.495	1.212
35	ID3	Inhibitor of DNA binding 3, HLH protein	3.335	4.578	4.944
36	SHANK1	SH3 and multiple ankyrin repeat domains 1	−0.609	−1.252	−1.669
37	ITGAX	Integrin subunit alpha X	−1.721	−1.825	−2.419
38	MYOF	Myoferlin	2.607	3.364	3.805
39	AJUBA	Ajuba LIM protein	0.401	0.704	1.015
40	TIMP1	TIMP metallopeptidase inhibitor 1	−0.680	−1.061	−1.354
41	LPIN1	Lipin 1	−1.112	−1.479	−1.714
42	TRPM4	Transient receptor potential cation channel subfamily M member 4	0.611	1.137	1.306
43	OAS2	2-5-oligoadenylate synthetase 2	0.793	2.202	3.652
44	ETV5	ETS variant 5	−0.540	−1.190	−1.659
45	CDH4	Cadherin 4	0.720	1.060	0.757
46	ZNF488	Zinc finger protein 488	−0.927	−1.428	−0.994

sample versus the DMSO treated one. The respective values for PBs PLX7904 and PLX8394 were 1.25 and 1.46. The qPCR revealed similar results as shown in Fig. 6b. The proto-oncogenes ETS1 and c-Jun that can be activated by the RAS-ERK pathway in tumours, were found down-regulated with a smaller logFC value after PB treatment, both with NGS and qPCR tests (Fig. 6B & Supplementary Table). The expression of the anti-apoptotic MCL1 was limited in a similar way. HEY1 and SMAD7, factors that participate in developmental processes and are typically found under-expressed in tumours, were up-regulated in samples treated with the PBs followed by the samples treated with PLX4720. PCR analysis of the selected genes confirmed the RNA sequencing results, suggesting several alterations in gene expression involved in cell differentiation, anti-proliferation and apoptotic regulation. It is clear that the BRAF paradox breakers result in a remarkable differential regulation of major pathways-genes, like MAPK signalling, apoptosis, cell cycle, or developmental signalling pathways which regulate cancer properties and are proposed for combinatorial treatments.

BRAFV600E bearing colon adenocarcinoma cells treated by BRAFi are partially resistant to BRAFi mono-treatments, as shown in the current as well as in other studies and clinical trials. Therefore, the potential benefit of combinatorial treatment of BRAFi with either the MCl-1 inhibitor A-1210477 or with RO4929097 – γ secretase inhibitor (γ secretase is component of the Notch pathway) in cell viability of colorectal cancer cells was tested. Co-treatments of A-1210477 Mcl-1 inhibitor with BRAFi provided a better efficiency in reducing tumour cell viability than that observed after individual BRAFi or Mcl-1 inhibitor mono-treatments (Fig. 6C). This effect is more evident in combinatorial treatments of RKO cells (Fig. 6C, lanes 8–9). Moreover, Co-treatments of RO4929097 γ-secretase inhibitor with BRAFi provided a better efficiency in reducing tumour cell viability than of individual BRAFi or γ-secretase inhibitor mono-treatments (Fig. 6D). This effect is more evident in combinatorial treatments of RKO and HT29 cells (Fig. 6d, RKO and HT29 panels, lanes 8–9), especially when 5μM of RO4929097 was used. Therefore, the benefit of BRAFi co-treatments with either Mcl-1 inhibitor or Notch inhibitor was shown in these preliminary experiments at the cellular level. More detailed exploitation of the pathways affected may provide novel combinatorial anti-cancer protocols.

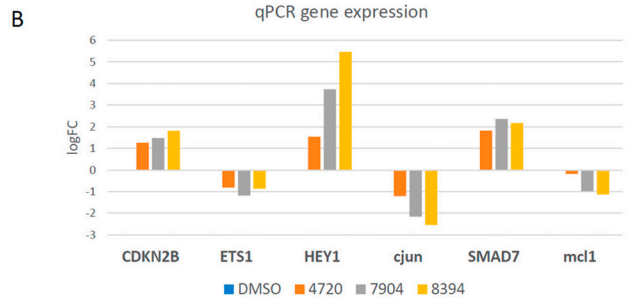
4. Discussion

BRAF mutated oncoproteins are targeted by specific inhibitors Vemurafenib, Dabrafenib or Encorafenib (BRAFi), which have shown high efficiency in melanomas bearing a BRAFV600E/K mutation, and have been approved in recent years by FDA and European authorities as monotherapies. Other tumour types bearing BRAF mutation, including CRC and NSCLC, have been shown to be resistant to the abovementioned drugs as monotherapies. Following intense investigation, major resistance mechanisms leading to reactivation of MAPK pathway in the presence of the BRAFi called “The BRAF paradox”, have been revealed in melanoma and other mtBRAF cancers.

Resistance due to the “BRAF paradox” is currently an intense area of basic, translational and clinical research. On one hand, new combinatorial treatment protocols of mtBRAFi with MEK inhibitors have entered clinical trials and have either been approved by FDA –as in mtBRAF melanoma [41,42]; and in mtBRAF NSCLC [43], or have recently shown very promising results as a triple therapeutic combination with EGFR

A

gene	Definition	Tr4720_logFC	Tr7904_logFC	Tr8394_logFC
CDKN2B	cyclin dependent kinase inhibitor 2B (p15)	0.94	1.25	1.46
ETS1	ETS proto-oncogene 1, transcription factor	-0.80	-1.11	-1.01
HEY1	hes related family bHLH transcription factor with YRPW motif 1	1.53	3.55	5.00
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	-0.53	-1.11	-1.45
SMAD7	SMAD family member 7	0.81	1.59	1.40
MCL1	MCL1, BCL2 family apoptosis regulator	-1.10	-1.56	-1.57



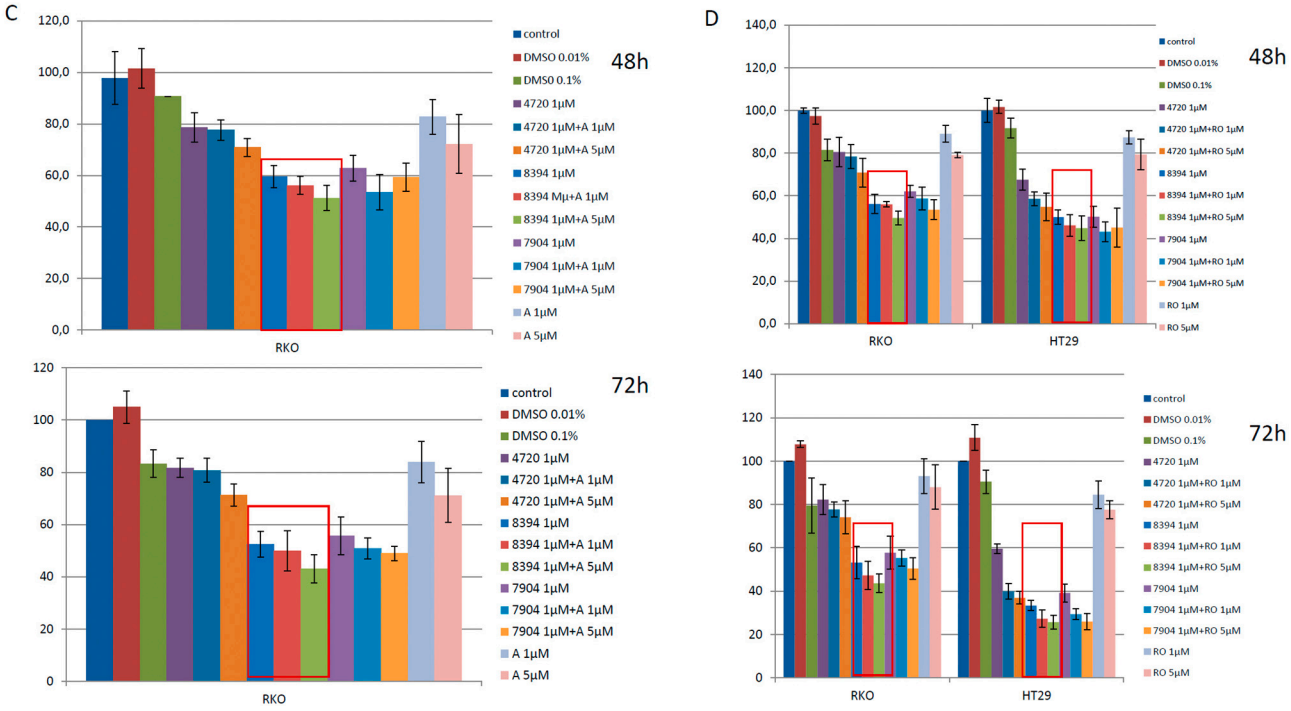


Fig. 6. Validation of selected genes by qPCR and selected combinatorial protocols. Gene expression of selected genes, involved in developmental pathways, apoptosis or cell cycle regulation that were found differentially expressed at RKO cells, 48 h after treatment with BRAFi versus DMSO treatment: A) expression logFC according to NGS analysis B) expression logFC according to qPCR. C) Cell viability after co-treatments with the Mcl-1 inhibitor A-1210477 with BRAFi. Cells were either left untreated (ctr = control) or treated with A-1210477 and 1 μM BRAFi and their combinations for 48 and 72 h. The average of three independent experiments is presented as fold change of the absorbance of treated/untreated cells, for each condition. Columns = % percentage of cell viability, bars = SD. D) Cell viability after co-treatments with the γ-secretase inhibitor RO4929097 with BRAFi. Cells were either left untreated (ctr = control) or treated with RO4929097 and 1 μM BRAFi and their combinations for 48 and 72 h. The average of three independent experiments is presented as fold change of the absorbance of treated/untreated cells, for each condition. Columns = % percentage of cell viability, bars = SD.

inhibitor in mtBRAF CRC [22], where EGFR is overexpressed [44]. For this protocol, recent development refers to a promising combination of encorafenib (BRAFi), cetuximab (EGFRi), (and binimetinib (MEKi)), which resulted in significantly longer overall survival and a higher response rate than standard therapy in patients with metastatic colorectal cancer with the BRAFV600E mutation and has recently been approved by FDA for BRAFV600E metastatic CRC [22]. On the other hand, the development of the next generation BRAFV600E inhibitors PLX7904 and its analogue PLX8394, known as paradox breakers (PBs) has opened new avenues in overcoming several mechanisms of resistance and controlling the paradoxical activation of the MAPK pathway [23]. PLX8394 has entered clinical trials involving patients with advanced BRAF-mutated solid tumours, including CRC and hairy cell leukemia (NCT02428712). The present study investigates the treatment effects of PBs PLX8394 and PLX7904 on colorectal cancer cells by the detailed analysis of changes in global gene expression and major cellular

pathways, as compared to PLX4720, the sister compound of Vemurafenib. Novel combinatorial promising protocols of BRAFi with apoptosis or developmental pathways modulators, proposed from the bioinformatic analysis, are also presented in this study.

4.1. BRAF inhibitors-paradox breakers partially overcome resistance in CRC by inhibition of cell proliferation, cell cycle arrest and induction of apoptotic markers

PLX8394 has been tested in preclinical models of melanoma, lung and colorectal cancer [25,45,46] with encouraging outcomes for further exploitation. In the current study, evaluation of BRAF PBs PLX7904 and PLX8394 indicated a stronger inhibition of cell growth, compared to PLX4720 treatment in BRAFV600E colon cancer cell lines. BRAFV600E bearing cells treated with PBs exhibit reduced viability, stronger G0/G1 arrest, and in some cases undergo apoptotic procedure in lower concentrations compared to the PLX4720 treated cells. The enhanced effect caused by PLX7904 and PLX8394 is particularly evident in RKO and HT29 cells. These results provide strong evidence for cell cycle regulation by PBs and are in line with previous data showing that Vemurafenib induces cell cycle arrest at G0/G1 phase in Vemurafenib-sensitive melanomas [47]. Our study has revealed through the detailed analysis of global expression, several changes in key cell cycle/apoptosis genes: the up-regulation of the CDKN2B gene, which was more intense after treatments with PBs, can be related to the observed G0/G1 inhibition of treated CRC cells. Moreover, the expression of the anti-apoptotic MCL-1 was remarkably reduced after PB treatments, which may contribute to the induction of apoptotic cell death in cancer cells. These results strongly suggest that the decrease in cell viability observed after PB PLX8394 treatment is caused not only by inhibition of cell proliferation, but also by induction of cell death pathways, possibly following prolonged cell cycle arrest.

In BRAFV600E colorectal cancer, the BRAF mutant induces through the MAPK signalling the phosphorylation and subsequent the stabilization of the anti-apoptotic protein MCL-1 [26], and this mechanism of resistance to apoptosis can be suspended by simultaneous inhibition of MCL-1 and components of the MAPK pathway. This data indicate that simultaneously inhibition of MCL-1 and BRAFV600E might have synergistic effect in the treatment of BRAFV600E colorectal cancer. Indeed, co-treatments of BRAFi with the MCL-1 inhibitor A-1210477 were here more efficient in reducing colorectal cancer cell viability, than the mono-treatments under the same conditions. Towards this direction, more detailed efforts in the near future should demonstrate if inhibition of MCL-1 would be effective in a combination with agents against the BRAFV600E in colorectal cancer.

4.2. Prolonged MEK-ERK signalling inhibition by PLX8394 and PLX7904 is associated with anti-cancer biological activity. Crosstalk of MEK-DDR pathways

As reported in the literature, surprisingly, MAPK suppression by BRAF inhibitor alone in BRAF mutant CRC cells is transient, and rapid reactivation of MAPK signalling and re-accumulation of phosphorylated ERK (p-ERK) is observed beginning roughly 6 h after initiation of BRAF inhibitor treatment, despite continued presence of the drug [48]. BRAF mutant CRCs (both in cell lines and human tumour specimens) express higher levels of total and phosphorylated EGFR (p-EGFR) than BRAF mutant melanomas, perhaps explaining why BRAF mutant CRCs are more prone to exhibit EGFR-dependent resistance and MAPK reactivation [15]. Therefore, the level of ERK1/2 reactivation in the presence of drug has become a measure of resistance of tumour cells to BRAF inhibitors.

In this study, the p-ERK inhibition caused by BRAF PBs, PLX7904 and PLX8394, even at a lower dose, was longer maintained than the one caused by Vemurafenib analogue PLX4720, more prominent in HT29 cells, possibly explaining the significant reduction in the viability of the cells. Regarding treatments of BRAFi sensitive cells Colo-205, inhibition of the p-ERK1/2 was estimated as high as 90% of the control sample at concentrations as low as 0,1μM. Further evidence for the efficiency of the prolonged inhibition of PBs treatment on ERK activity is provided here, as shown by the significant reduction in the expression of *ETS1* and *CJUN*, members of ETS and AP-1 families of transcription factors, two of the immediate downstream targets of RAS-MEK-ERK pathway in CRC [49]. This effect on *ETS-JUN* expression by PBs is again markedly stronger than in the case of Vemurafenib analogue. Taking under consideration the results presented here regarding the detection of apoptotic CRC cells, it appears that the duration of the p-ERK1/2 inhibition may indicate the overall sensitivity of the cell lines to the BRAFi and the ability to cause strong proliferation blockage and cell death. Thus, PBs treatments in CRC cells cause a prolonged inhibition of MEK-ERK pathway and reduce the appearance of "BRAF paradox".

Crosstalk between MAPK and DNA Damage Response pathways has been shown through several studies, although mechanistic details are not known yet in many cases. Activation of MEK pathway has been observed after cisplatin treatment, which may be then inhibited by MEK inhibitors [50,51]. It is of interest that MEK inhibitors could block growth of lung tumours with mutations in ataxia-telangiectasia mutated *ATM* [52]. As shown here, treatment with BRAF inhibitors resulted in the phosphorylation of H2AX at Ser139, referred to as γH2AX. Gamma H2AX is considered to be a sensitive marker of DNA damage and increased cancer risk [53]. However, γH2AX does not always indicate the presence of DNA damage [54]. DNA-Protein Kinase plays a dominant role in the regulation of H2AX phosphorylation in response to DNA damage and cell cycle progression [55]. As shown in the current study, treatment with BRAFV600E inhibitors may induce DNA damage response, which may be exploited in the near future for efficient rational combinatorial treatments.

4.3. PLX8394 regulates cell cycle profile of tumour cells critical to growth arrest and apoptotic cell death

The data presented in this study provide strong evidence for cell cycle regulation by PBs and are in line with previous data showing that BRAFi induce cell cycle arrest at G0/G1 phase in melanomas [47]. It appears that the PLX7904 and PLX8394 treatments have an enhanced ability to block cell proliferation in the more BRAFi resistant cell lines at lower concentration than PLX4720. Positive apoptotic markers were detected in colorectal tumour cells treated with PLX8394. Similar data have been presented in melanoma cells, where PLX8394 suppresses p-ERK1/2 and elicits apoptotic markers in patient samples as efficiently as combination treatment [46]. These results strongly suggest that the reduction of cell viability when treated with PLX8394 in colorectal adenocarcinoma cells is caused not only by inhibition of cell proliferation, but also by induction of cell death pathways, possibly following a prolonged cell cycle arrest.

4.4. PLX8394 treatments in CRC strongly affect developmental pathways related to cancer

BRAF inhibitors induce epithelial differentiation in BRAF-mutant colorectal cancer cells. Herr et al. reported that PLX4720-treated colorectal adenocarcinoma cells promote a more segregated and adhesive state by upregulating genes linked to epithelial differentiation [56]. Moreover sonic hedgehog (SHH) pathway activation has been involved in BRAF inhibitor resistance of melanoma cells with BRAF mutation [57]. The pathway analysis presented in the current study revealed that treatments of CRC cells with BRAF inhibitors strongly affect developmental pathways and cytokine signalling related to cancer. Gene prioritization analysis highlighted genes involved in NOTCH signalling are indicated as central regulators of the cellular response to BRAF inhibitors treatment. In particular, NOTCH1 and its transcriptional targets *HES1* and *HEY1* are overexpressed in all cases after BRAFi treatments,

with a remarkable effect in the case of BRAF PBs, while RBPJ, a constitutive repressor of Notch signalling, was downregulated, implying Notch pathway activation after PB treatments. High NOTCH activity in CRC has been linked to the cancer stem cell phenotype, to epithelial-mesenchymal transition and poor survival, suggesting that NOTCH contributes to tumour progression. Interestingly, previous transcriptomic studies in colorectal cancer and melanoma cells treated with Vemurafenib have shown similar overexpression of NOTCH1. Specifically, in HT29, Colo-205 [56], A375 [58] and RKO (this study) NOTCH1 was overexpressed, though only in RKO cells *HES1* and *HEY1* were upregulated, suggesting Notch pathway activation. Notch targeting in combination with MEK inhibitors has provided encouraging results in colon cancer [59]. It is also of interest, that treatment of BRAFV600E mutated thyroid cancer cells with Vemurafenib lead compound PLX4720, indicated that at least some anti-tumour activities, like cell migration were reduced through a lowering in the thyroid-cancer-microenvironment of CXCL8, a chemokine secreted by normal and thyroid cancer cells with proven tumour-promoting effects [60]. In the present study, co-treatments of BRAFi with RO4929097, a γ secretase inhibitor were more efficient in reducing colorectal cancer cell viability than the mono-treatments under the same conditions. Further detailed ex vivo and in vivo studies in the near future should be performed to prove that inhibition of the Notch pathway would be effective in a combination with agents against BRAFV600E colorectal cancer.

4.5. Other pathways of interest altered by PB treatments

Our gene expression analysis relieved that *PIK3IP1* and *PIK3R3*, two subunits of the PI3K that are responsible for the negative regulation of the *PIK3CA* are upregulated upon treatment with PBs with a log fold change over 2, while the *PIK3R3* subunit is upregulated with a logFC 1,25 and less than 1 for *PIK3IP1*, upon treatment with PLX4720 (Supplementary Tables 1–3). Likewise, 4 members of the SMAD gene family (*SMAD3*, *SMAD6*, *SMAD7* & *SMAD9*) were found upregulated with a logFC higher than 1 following treatment with PLX8394, higher than 3 following treatment with PLX7904 and higher than 2 following PLX4720 treatment. Tumour suppressor proteins (TP53 family) were affected similarly: TP53 and TP53INP1 were overexpressed in all samples and TP53I3 only in paradox breakers treated-samples. Apoptosis inducers *PARP3* & *PARP10* were upregulated at PLX8394 treated samples, *PARP10* at PLX7904 samples and none of the two were drastically affected in PLX4720 treated-samples. A parallel pattern is observed with the upregulation of several apoptotic and developmental genes as at the downregulation of tumorigenic (e.g. *BCL*, *FOS*) gene families where more members appear affected upon treatment with the paradox breakers than with PLX4720. These, in conjunction with the fold change expression FC itself, may explain the differentiation of the overall outcome in cell fate upon each treatment.

In conclusion, it is clear that PLX8394 (and PLX7904) PB treatments in BRAFV600E colorectal adenocarcinoma cells under several treatment conditions can evade paradoxical ERK pathway activation presented by BRAF inhibitors and may induce cancer cell death. Notably, as commented in [61], both PLX7904 and its analogue PLX8394 are more potent inhibitors of BRAFV600E than vemurafenib, and unlike vemurafenib, they do not promote paradoxical ERK activation in WT BRAF cells or squamous-cell carcinomas in preclinical models [23,61] Thus, the 'paradox breakers' are predicted to have fewer on-target toxicities than second-generation RAF inhibitors. Recent evidence from phase 1/2 precision medicine study of the next-generation BRAF inhibitor PLX8394 has shown that PLX8394 + cstat has been well tolerated and shows promising activity in refractory solid tumours with BRAF mutations [62]. Furthermore, detailed analysis of pathways in colon cancer cell lines has shown a remarkable differential regulation of major pathways and genes, like MAPK signalling, apoptosis, cell cycle, or developmental signalling pathways. It is proposed that PLX8394 and modulators of specific apoptotic/developmental pathways could be exploited further in novel efficient rational combinatorial anti-cancer treatment protocols.

CRediT authorship contribution statement

Conception and design of the study: **O. Papadodima, M. Zervou, V. L. Souliotis, A. Pintzas**; Acquisition of data: **K. Koumaki, G. Kontogianni, V. Kosmidou, F. Pahitsa, E. Kritsi**; Analysis and interpretation of data: **K. Koumaki, G. Kontogianni, V. Kosmidou, F. Pahitsa E. Kritsi, M. Zervou, A. Chatziioannou, V.L. Souliotis, O. Papadodima and A. Pintzas**; Drafting the article and final approval of the version to be submitted: **K. Koumaki, G. Kontogianni, V. Kosmidou, F. Pahitsa, E. Kritsi, M. Zervou, A. Chatziioannou, V.L. Souliotis, O. Papadodima and A. Pintzas**.

Declaration of competing interest

Aristotelis Chatziioannou is the founder and CEO of e-NIOS Applications PC. No potential conflicts of interest were disclosed otherwise.

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Appendix A. Supplementary data

Supplementary Tables 1–3. The differentially expressed genes (FDR ≤ 0.05 & $|\log_2$ fold change$| \geq 1$), with the corresponding FDR and fold change values, for the tree treatments under investigation. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2020.166061.

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