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Targeting B-Raf inhibitor resistant melanoma with novel cell penetrating peptide disrupters of PDE8A – C-Raf

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Abstract

Background: Recent advances in the treatment of melanoma that involve immunotherapy and B-Raf inhibition have revolutionised cancer care for this disease. However, an un-met clinical need remains in B-Raf inhibitor resistant patients where first-generation B-Raf inhibitors provide only short-term disease control. In these cases, B-Raf inhibition leads to paradoxical activation of the C-Raf – MEK – ERK signalling pathway, followed by metastasis. PDE8A has been shown to directly interact with and modulate the cAMP microdomain in the vicinity of C-Raf. This interaction promotes C-Raf activation by attenuating the PKA-mediated inhibitory phosphorylation of the kinase.

Methods: We have used a novel cell-penetrating peptide agent (PPL-008) that inhibits the PDE8A – C-Raf complex in a human malignant MM415 melanoma cell line and MM415 melanoma xenograft mouse model to investigate ERK MAP kinase signalling.

Results: We have demonstrated that the PDE8A – C-Raf complex disruptor PPL-008 increased inhibitory C-Raf-S259 phosphorylation and significantly reduced phospho-ERK signalling. We have also discovered that the ability of PPL-008 to dampen ERK signalling can be used to counter B-Raf inhibitor-driven paradoxical activation of phospho-ERK in MM415 cells treated with PLX4032 (Vemurafenib). PPL-008 treatment also significantly retarded the growth of these cells. When applied to a MM415 melanoma xenograft mouse model, PPL-008C penetrated tumour tissue and significantly reduced phospho-ERK signalling in that domain.

Conclusion: Our data suggests that the PDE8A-C-Raf complex is a promising therapeutic treatment for B-Raf inhibitor resistant melanoma.

Keywords: B-Raf, C-Raf, PDE8A, NRAS, PPL-008, CellPorter®, Disruption, Melanoma

Background

Malignant melanoma, an aggressive and lethal form of skin cancer, leaves metastatic patients with a 15–20% chance of surviving 5 years with the disease [1]. Over 50% of melanoma patients carry a mutation in their BRAF gene, with the V600E (valine to glutamic acid) missense mutation being responsible for 80–90% of BRAF mutations [2–4]. B-Raf is a serine/threonine protein kinase that is part of the RAS – RAF – MEK – ERK signalling axis, involved in regulating many cellular processes including: differentiation, proliferation, survival

and apoptosis [5]. This signalling pathway is believed to be crucial to melanoma progression, with the V600E mutation resulting in B-Raf protein conformational changes that constitutively activate B-Raf and downstream MEK – ERK signalling [5]. As a result, B-Raf-specific small molecule inhibitors (and eventually MEK inhibitors) were developed and found to dramatically improve patient prognosis, survival rate and lead to tumour regression through suppression of downstream ERK signalling [6–9]. Unexpectedly, B-Raf inhibitor resistance was developed in many patients through paradoxical activation of ERK; allowing the cancer to persist [10–14]. Pathway reactivation is believed to occur as a result of oncogenic mutations in a number of genes, including NRAS (20% of cases; Q61K/R/L most frequent)

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and KRAS (2% of cases) gain-of-function mutations [15] <http://www.sanger.ac.uk/genetics/CGP/cosmic/> [16, 17]. As B-Raf preferentially heterodimerises to C-Raf (vs. other A, B or C-Raf homo/heterodimers), B-Raf inhibition results in a negative feedback mechanism that switches from B-Raf to C-Raf activation by Ras and subsequent tumour invasion and metastasis [18, 19]. In light of this, C-Raf has become a key therapeutic target for the development of new treatments able to suppress RAS-mediated tumour progression in B-Raf inhibitor resistant melanoma.

Previously, we demonstrated an important role for the cAMP degrading enzyme, PDE8A, in protecting C-Raf from PKA-mediated inhibition [20] (reviewed [21]). PDE8A, believed to be responsible for regulating basal cAMP fluctuations, was found to directly interact with C-Raf. The association of C-Raf with a PDE markedly inhibited the ability of local PKA pools to phosphorylate and inhibit the kinase, increasing the likelihood of C-Raf activation. Peptide mapping of the PDE8A-C-Raf interface allowed for the rational development of a cell penetrating peptide disrupter based on the C-Raf binding site on PDE8A [22, 23]. This disrupter was found to inhibit the PDE8A – C-Raf protein-protein interaction (PPI) and significantly increase C-Raf-S259 phosphorylation while concomitantly suppressing phospho-ERK signalling. This concept was verified at an organismal level in both PDE8A knock out mice and a drosophila model, where basal ERK activation was attenuated compared to wild type [20].

Further verification of the PDE8A – C-Raf PPI inhibitor concept has been supplied by a recent study, which demonstrated that the disrupter was able to attenuate T-effector cell adhesion and migration in an auto-immune multiple sclerosis mouse model. The inhibition of T-effector cell function was a direct result of increased levels of inhibitory C-Raf-S259 phosphorylation and subsequent suppression of ERK activation [24]. The disrupter produced a more potent effect than highly-selective PDE8 enzyme inhibitors and highlighted a novel approach to targeting T-effector cells in inflammatory disorders. These observations convincingly demonstrate the disrupter's ability to attenuate ERK activation through PDE8A – C-Raf disruption.

Further development of the PDE8A-C-Raf disrupter has allowed us to conjugate the peptide to the patented Cell Porter® (Portage Pharmaceuticals Limited), a cell penetrating peptide based on the human HOXD12 protein. The Cell Porter® platform has successfully driven PPL-003, an NF-κB inhibitor designed to treat inflammatory disorders including dry eye syndrome, to pre-clinical success [25–27]. We report data from the testing of our novel PDE8A – C-Raf disrupter/HOXD12 conjugate (from here on, named PPL-008) in MM415, a B-Raf

inhibitor resistant malignant melanoma cell line (MM415; BRAF wt, KRAS wt, NRAS Q61L) and in an MM415 melanoma murine xenograft model. PPL-008 was efficacious in the attenuation of ERK signalling in both cases and suggests that the PDE8A – C-Raf complex is a promising therapeutic target for B-Raf inhibitor resistant melanoma.

Methods

All antibodies and chemical treatments are collated in Additional file 1: Table S1.

Animals

Generation of the MM415 melanoma murine xenograft model, and in vivo treatment was carried out by MI Bioresearch (Michigan, USA). All protocols involving animals used were approved by the Institutional Animal Care and Use Committee of the University of Washington in accordance with the National Institutes of Health. In vivo 5–6 week old female NSG – immunodeficient mice (Jackson Laboratory) were subcutaneously injected with 3.3×10^8 MM415 malignant melanoma cells, at the SC – axilla (high), and tumours were allowed to grow for 30 days (~200 – 400mm³). Mice were intraperitoneally injected at the site of tumour with PPL-008 peptide drug dissolved in a 5% dextrose – water solution at either 25 mg/kg or 100 mg/kg. Mice were euthanised via CO₂ inhalation (MI Bioresearch – AALAC accredited laboratory) and the tumours were harvested at varying time points post-treatment: 30 min, 1 h, 2 h, 4 h, 8 h, 12 h. Tumours were frozen down at –80 °C and sent to Baillie lab for preparation into lysates for follow-up western blot analyses.

MM415 cell culture and drug treatments

Both cell lines used in this study, A375 and MM415, were purchased from Sigma-Aldrich. A375 (BRAF V600E) and MM415 (BRAF wild-type, KRAS wild-type, NRAS Q61L) are human malignant melanoma epithelial skin cell lines. Cells were cultured with RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS, v/v), 1% L-glutamine (v/v), 1% penicillin-streptomycin (v/v) (all Sigma-Aldrich) and incubated at 37 °C, 5% CO₂ and 95% humidity. Cells were split at ~80% confluency, using 0.05% trypsin-EDTA, 1:5. Cells were tested regularly for mycoplasma contamination.

The original PDE8A – C-Raf disrupter, and its scrambled isoform, were synthesised with a C-terminal stearic acid group [CH₃(CH₂)₁₆COOH] (GenScript) [20]. PPL-008 (i.e. PDE8A – C-Raf disrupter, without stearic acid) was synthesised with Cell Porter® conjugated to the C or N-terminus via either thioester or disulphide bonds. All peptides were dissolved to the appropriate concentration in DMSO for in vitro experimentation. PLX4032 (Vemurafenib) was dissolved in DMSO to a final

concentration of 1 μM (Sellekchem). Peptides were added to cells for 2 h, and PLX for 1 h, before cells were harvested. In cases where co-treatments were administered, cells were first treated with peptides for 2 h, followed by 1 h PLX.

Western blotting

MM415 and A375 cells harvested from in vitro experiments were lysed in 3 T3 lysis buffer, whilst MM415 melanoma murine xenograft tissue was homogenised and lysed in 1X RIPA buffer (both supplemented with protease cocktail inhibitor tablets (Roche)). Soluble fraction of lysate was resolved via SDS/PAGE using 4–12% Bis-Tris gels (NuPAGE). Proteins were transferred at 30 V for 1 h onto 0.45 μm nitrocellulose membrane (Protran) and blocked for 1 h in 5% non-fat dry milk solution (Marvel, *w/v*) in 1x TBS-T (20 mM Tris-Cl pH 7.6, 150 mM NaCl, 0.1% Tween-20). Blocked membranes were incubated in primary antibody (diluted in 1x TBS-T, 1% marvel) overnight at 4 °C. Membranes were washed three times in 1x TBS-T before membranes were incubated in secondary antibody (diluted in 1x TBS-T, 1% marvel) for 1 h at room temperature. Membranes were washed a final three times in 1x TBS-T and fluorescent intensity of Li-Cor secondary antibody was measured using a Li-Cor Odyssey scanner.

xCELLigence: Measuring cell proliferation

Real-Time cellular growth analyses of MM415 cells, using the xCELLigence platform (Roche Applied Science), allowed for the label-free measurement of cell proliferation. 96 well E-plates, containing gold micro-electrode sensors on the bottom of the plate, were used to measure cellular impedance inside each well as per manufacturer's instructions. Cellular impedance measurements were translated into 'cell index', an arbitrary measurement that increases as MM415 cells adhere and spread-out/grow (and vice versa), giving quantitative information on cell proliferation and viability that were analysed using RTCA software (Roche). All protocols carried out using the xCELLigence platform were based on previous Baillie lab publications [20, 28–32]. Following MM415 cell adhesion, cells were treated with one of the peptide disrupters for 2 h, followed by PLX (1 μM). The slope (i.e. rate of cell proliferation/growth) was measured based on the normalised cell index from the point in which treatments were administered, until the response had plateaued appropriately.

Statistical analyses

Results from western blot analyses are represented as mean \pm SEM ($n \geq 3$). Results from xCELLigence cell proliferation assay are represented as mean \pm STDEV ($n \geq 3$). $P < 0.05$ indicates data are significant, with

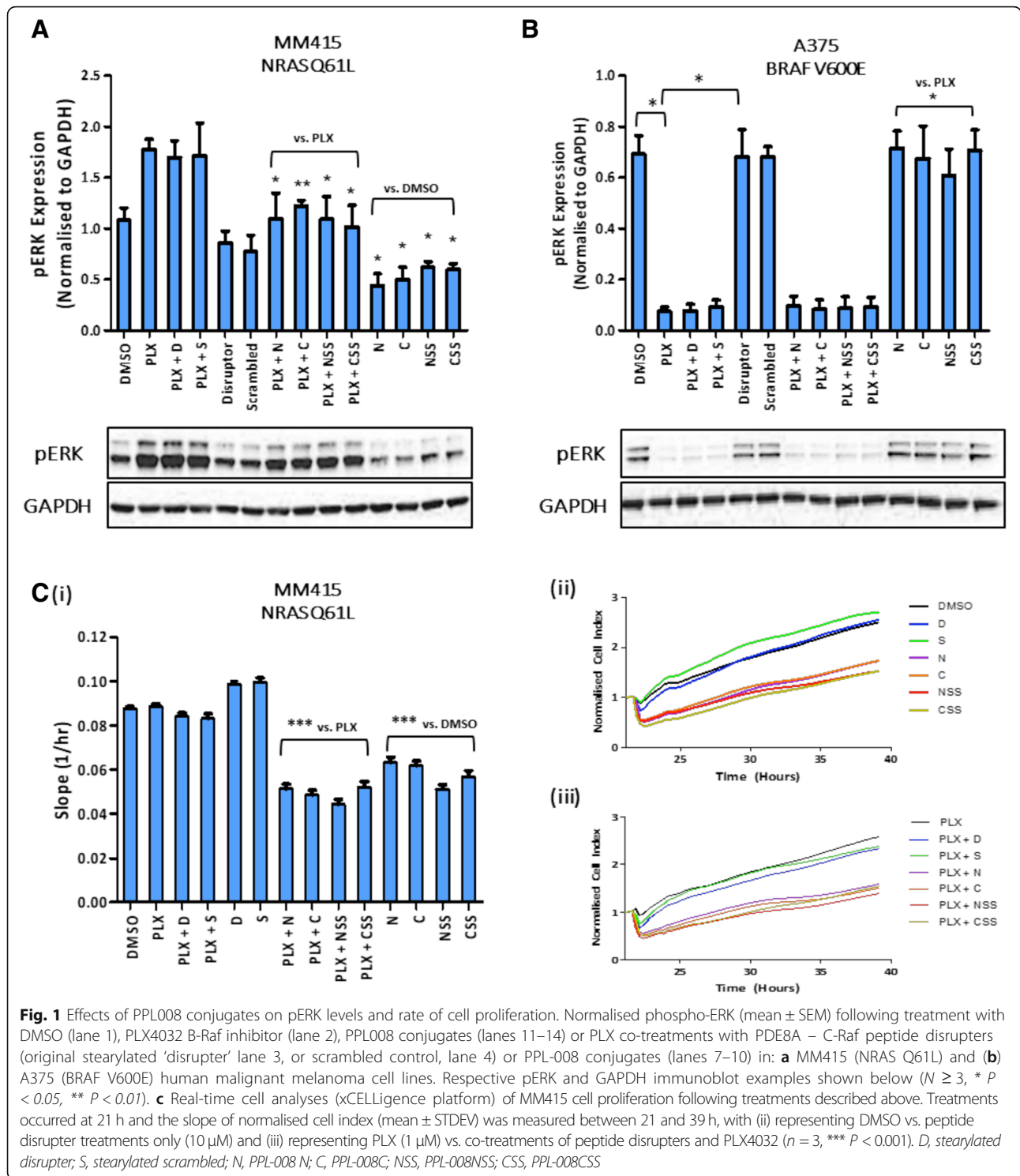
significance determined via unpaired t-test using Graph-Pad Prism software.

Results

PPL-008 attenuates paradoxical activation of pERK signalling and MM415 cell proliferation

Successful inhibition of the Raf – MEK – ERK signalling axis has previously been shown, using a PDE8A – C-Raf peptide disrupter based on the C-Raf binding site on PDE8A [20]. Here, we conjugated the Cell Porter[®] onto the C or N-terminal of the disrupter via either thioester (C or N giving PPL-008C or PPL-008N) or disulphide bonds (CSS or NSS giving PPL-008CSS or PPL-008NSS). We used the peptide conjugates to treat the B-Raf inhibitor resistant MM415 (human malignant melanoma, BRAF wt, KRAS wt, NRAS Q61L) cell line. To determine if PPL-008 conjugates (10 μM) could suppress phospho-ERK signalling in MM415 cells, pERK levels were determined via western blot (*pERK expression normalised to GAPDH, mean \pm SEM, $n = 4$, Fig. 1*). MM415 B-Raf inhibitor treatment (PLX4032, 1 μM) clearly induced a paradoxical activation of ERK and this was significantly reduced following treatment with all the analogues (PPL-008N, PPL-008C, PPL-008NSS and PPL-008CSS) (** $P < 0.01$ or * $P < 0.05$; Fig. 1a, lanes 7–10 inclusive). As expected, pERK was significantly reduced in the human A375 malignant melanoma cell line (BRAF V600E) following PLX treatment (1 μM) (Fig. 1b, lanes 2,3,4) with PPL-008 analogues providing no ERK inhibition as a mono-treatment (Fig. 1b, lanes 11–14 inclusive) or further ERK inhibition as a co-treatment with PLX (Fig. 1b, lanes 7–10 inclusive). This data reinforces how effective B-Raf inhibition can be in treating BRAF V600E mutant melanoma [6, 7]. As B-Raf inhibition sufficiently suppressed pERK expression in A375 cells, and as MM415 cells were resistant to PLX (resembling the clinical phenotype of interest), MM415 cells were used for the remainder of the study.

To assess the ability of PPL-008 conjugates to inhibit cell growth, real-time measurements of MM415 cellular impedance was recorded on the xCELLigence platform as an indicator of cellular proliferation (*slope (1/h), mean \pm STD, $n = 3$*). All PPL-008-conjugates (10 μM) significantly slowed cell proliferation in PLX treated MM415 cells (* $P < 0.05$, Fig. 1c, lanes 7–14 inclusive). Our data suggests treatment (10 μM) with each of the PPL-008 analogues suppressed both pERK and cell growth compared with DMSO treated control (Fig. 1a and c), indicating PPL-008 has potential as an effective therapy in this context. Surprisingly, PPL-008C and PPL-008CSS mono-treatments significantly attenuated A375 growth (Additional file 2: Figure S1) without affecting the phospho-ERK profile (Fig. 1b, lanes 11–14



inclusive). It is noteworthy that, the original stearylated PDE8A – C-Raf disrupter caused no significant reduction in pERK signalling (*D*; Fig. 1a, lane 5) or cell proliferation (*D*; Fig. 1c, lane 5) in MM415, similar to its scrambled control (*S*; Fig. 1a, lane 6 and Fig. 1c, lane 6), suggesting the stearic acid group was

insufficient in facilitating cell-penetration in MM415 cells. As all PPL-008 conjugates attenuated both pERK expression and cell proliferation, this indicates that Cell Porter® greatly improves intracellular delivery of PPL-008 conjugates compared with the original disrupter’s stearate group.

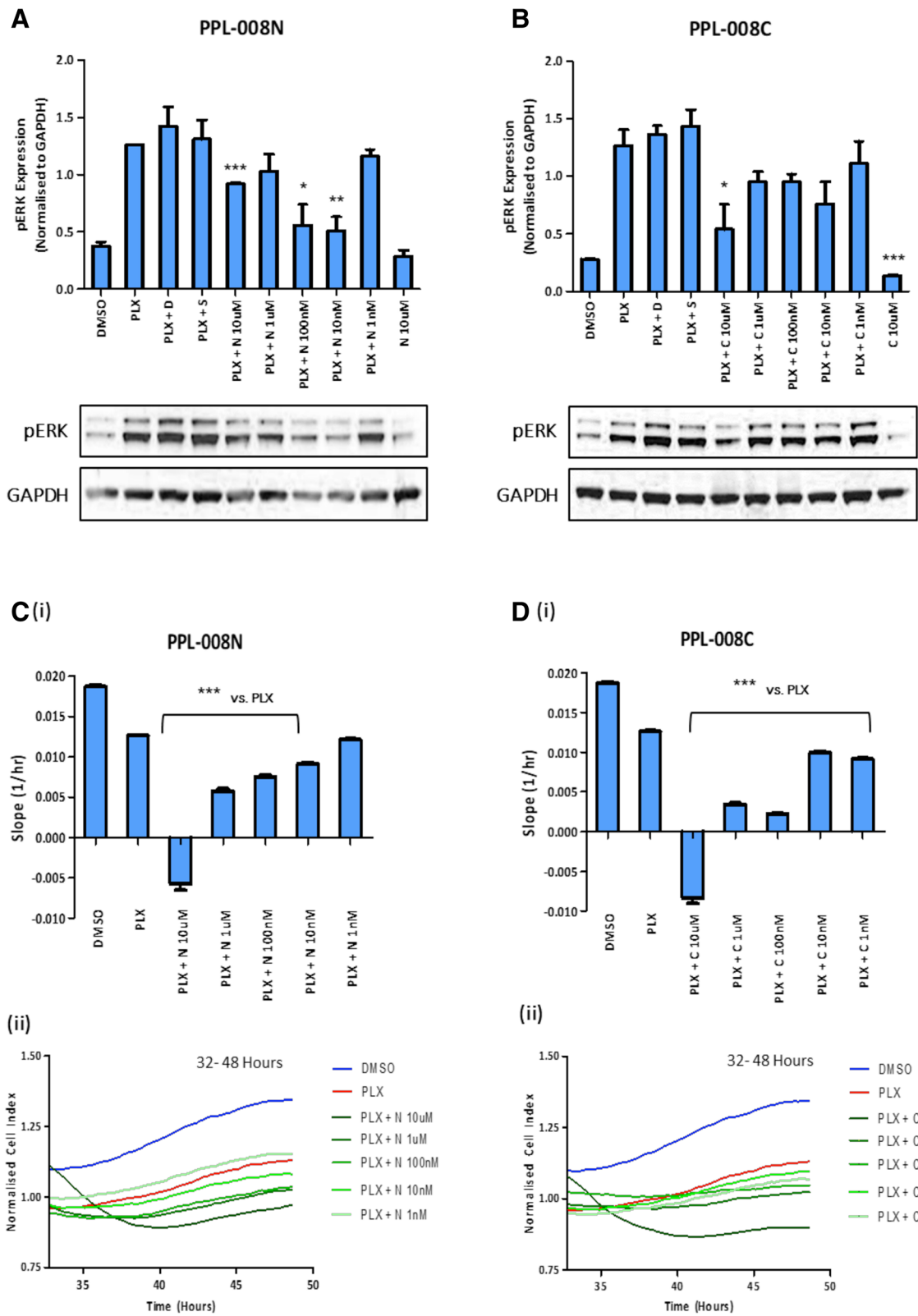


Fig. 2 (See legend on next page.)

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Fig. 2 PPL008-C/N dose response in MM415 (NRAS Q61L) human malignant melanoma cell line. **a & b:** Normalised pERK expression (mean \pm SEM) in MM415 cells following dose response co-treatment with PLX4032 (1 μ M) and PPL-008 N or PPL-008C (1 nM – 10 μ M). Respective pERK and GAPDH immunoblot examples shown below. ($N = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), DMSO vs. PPL008 only, PLX vs. PLX + PPL008). **c & d (i)** Real-time cell analyses (xCELLigence platform) of MM415 cell proliferation following dose response co-treatments with PPL-008 N or PPL-008C (1 nM – 10 μ M) and PLX4032 (1 μ M). Treatments occurred at 32 h and the slope of normalised cell index (mean \pm STDEV) was measured between 32 and 48 h ($n = 3$, *** $P < 0.001$). **c & d (ii)** Representative traces of normalised cell index of each treatment shown below. N, PPL-008 N; C, PPL-008C

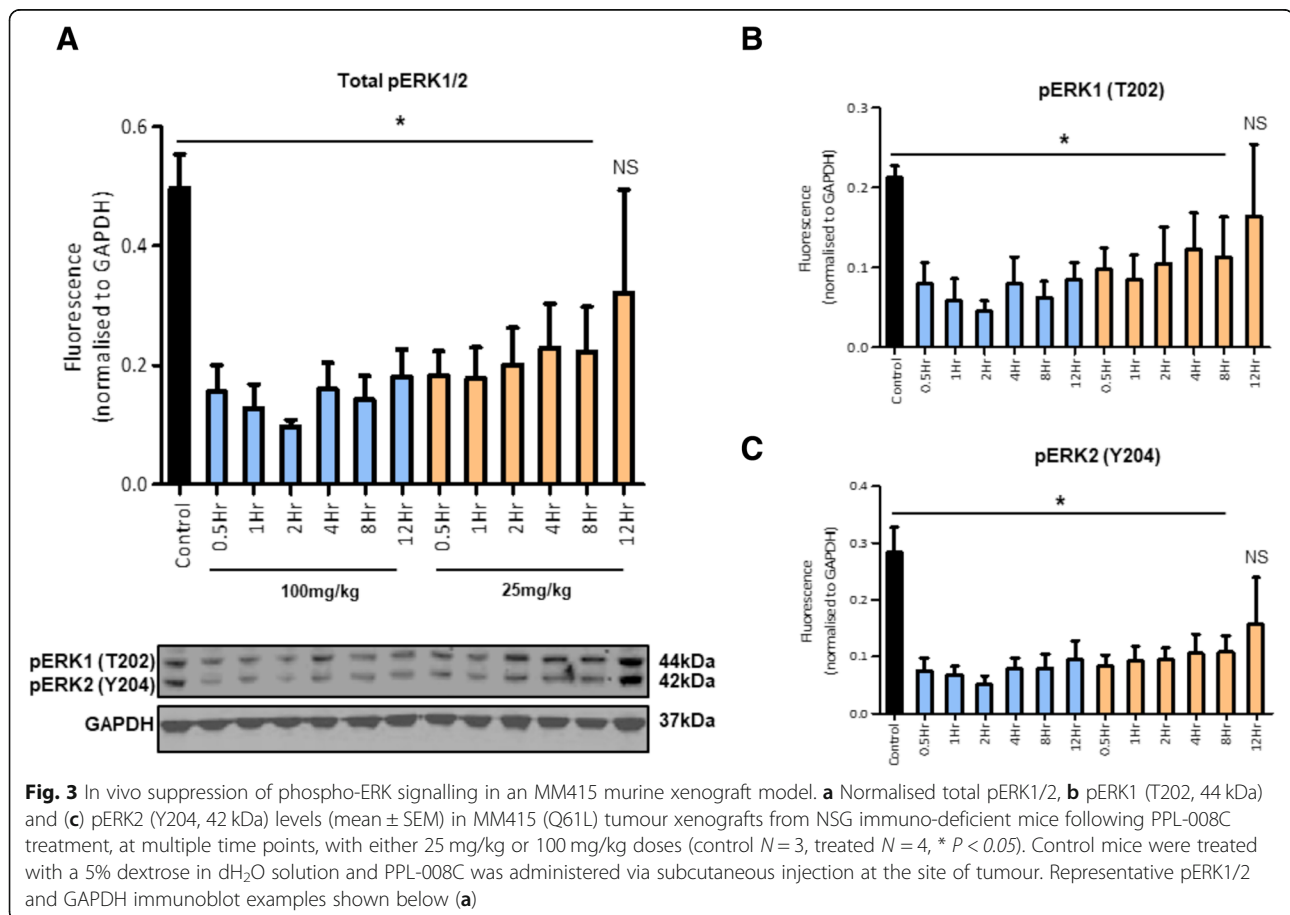
PPL-008C / N inhibits pERK expression and MM415 cell proliferation over multiple doses

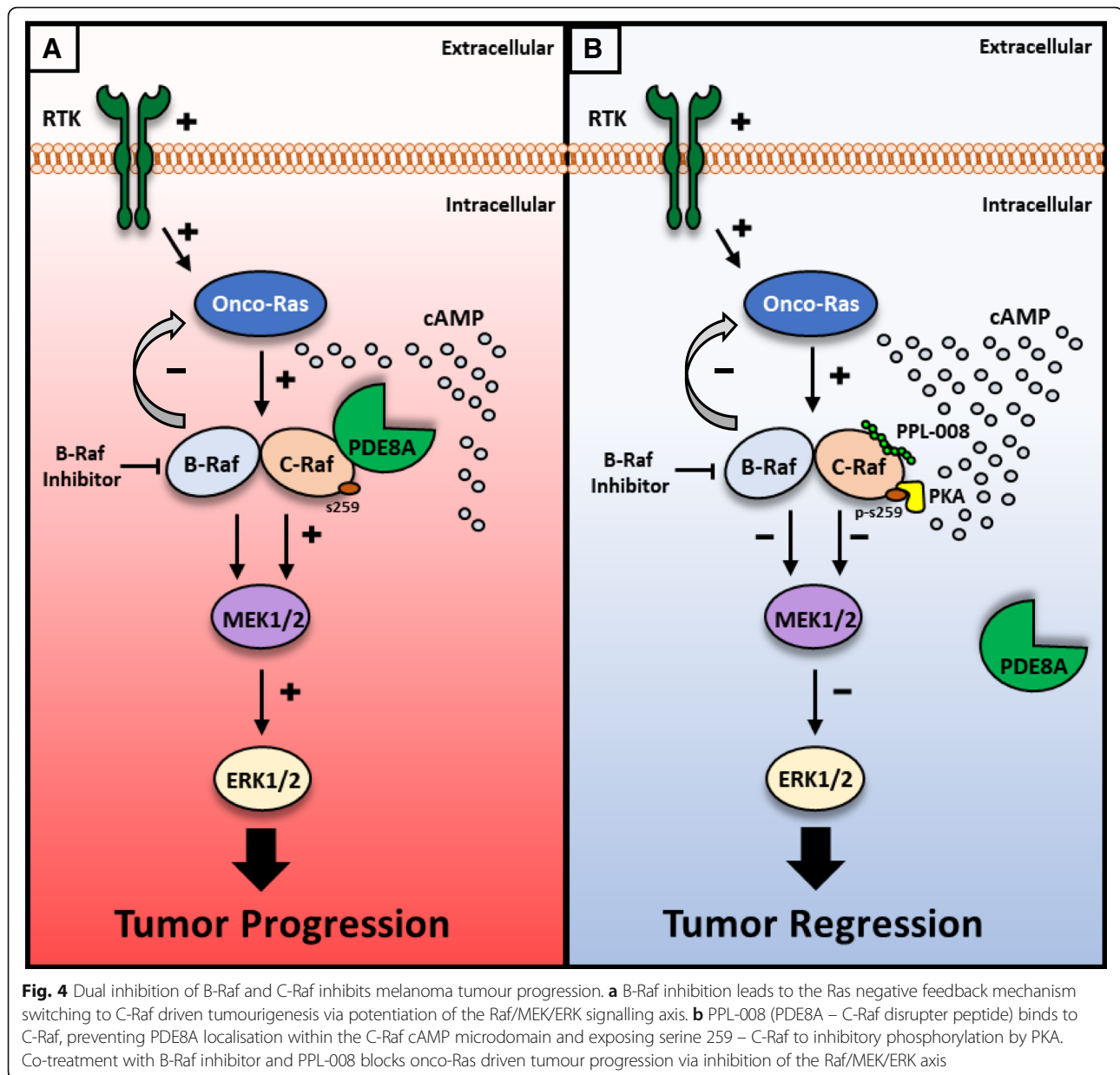
MM415 cells were co-treated with PLX (1 μ M), following pre-treatment with a dose range of PPL-008C or PPL-008 N (1 nM – 10 μ M). The levels of pERK and cell proliferation rates determined as before (Fig. 2). The levels of pERK triggered by PLX (Fig. 2a and b, lane 1 vs lane 2) were reduced at all concentrations following PPL-008 N treatment, with the higher [10 μ M] dose causing the most significant reduction (***) $P < 0.001$, Fig. 2a). This effect was recapitulated in the xCELLigence cell proliferation assay, where PPL-008 N reduced the rate of cell proliferation at all concentrations; most significantly at 10 μ M (***) $P < 0.001$, Fig. 2c (i and ii)). In addition, PPL-008C-conjugate reduced MM415 pERK levels and rate of cell proliferation at all concentrations,

with [10 μ M] producing the most significant inhibition (***) $P < 0.001$, Fig. 2b and d (i and ii)).

In vivo PPL-008C suppression of pERK in an MM415 melanoma murine xenograft model

Preliminary in vivo investigation of the effects of PPL-008C were carried out in an immuno-deficient NSG – MM415 melanoma murine xenograft model. PPL-008C was chosen as the lead peptide disrupter due to its consistency in attenuating pERK signalling and cell proliferation as both a single treatment and co-treatment with PLX (Figs. 2, 3 and 4). PPL-008C was administered subcutaneously at the site of the tumour as a single treatment. Tumours were removed at varying time points post-treatment and pERK expression was assessed via western blot ($N \geq 3$, Fig. 3).





PPL-008C significantly suppressed pERK1, pERK2 and total pERK levels over all time-points in the time course and at both doses (25 mg/kg and 100 mg/kg), excluding the 25 mg/kg – 12Hr treatment (* $P < 0.05$, Fig. 3). This shows that a single PPL-008C treatment can attenuate Raf – MEK – ERK signalling relatively quickly (within 30 min) and can maintain this inhibition for at least 12 h at higher concentrations (100 mg/kg, Fig. 3). Maximal pERK inhibition occurred 2 h post-treatment with 100 mg/kg PPL-008C.

Discussion

Melanoma is the most aggressive form of skin cancer, with a wide range of treatments currently available and

many more at pre-clinical and clinical phases [8, 22, 23]. First line B-Raf inhibitors are capable of managing the majority of melanoma patients that express the BRAF V600E mutation [23]. However, in patients expressing wildtype BRAF and NRAS or KRAS gain-of-function mutations, B-Raf inhibitors become ineffective and tumours persist – warranting the development of novel effective treatments [10–15] <http://www.sanger.ac.uk/genetics/CGP/cosmic/> [16, 17] (Fig. 4). We have identified the PDE8A – C-Raf complex as a point of cross-talk between the MAP kinase signalling and cAMP signalling systems, that can be manipulated by a disrupter peptide to promote the inhibition of C-Raf via increased S259 phosphorylation [20, 21, 24] (Fig. 4). This action can

counteract C-Raf driven paradoxical activation of ERK in B-Raf inhibitor resistant melanoma cell lines resulting in a retardation of cell proliferation (Figs. 1 and 2). Our specific approach of inhibiting C-Raf by targeting its binding to anchoring proteins rather than kinase activity is, to our knowledge, novel for this kinase and we aim to displace only a small percentage of total C-Raf that is in complex with PDE8A. Protein-protein interactions (PPIs) are increasingly being regarded as tractable molecular targets for the development of therapeutics, and peptides that mimic docking sites with protein complexes are often the ideal scaffold starting point for such agents [33].

The concept of developing protein-protein interaction inhibitors to treat melanoma, however, has previously been investigated with small molecule PPI inhibitors of the complex between bromodomain-containing protein 4 (BRD4) and acetylated histone having been developed [34]. Chromatin immunoprecipitation (ChIP) analysis highlighted the ability of these compounds to antagonise the interaction between BRD4 and chromatin at the MYC promoter in melanoma cells to effect the down regulation of oncogenic *c-myc*. This approach also demonstrated potent anti-proliferative *in vivo* activity in A375 xenografts. Another PPI inhibitor that targets melanoma growth, via the dual targeting of tumour and endothelial cells, is C4 [35]. This molecule targets the C-terminus of Focal Adhesion Kinases (FAK) to interdict the kinase's interaction with VEGF-receptor 3 and subsequently reduce vascularisation of B-RAF V600E xenograft tumour tissue to limit blood flow [36]. The inhibition of PPIs by small molecules as a therapeutic strategy is also being evaluated for other cancers. High content compound screening has identified Androgen receptor – Transcription Intermediary Factor 2 (TIF2) disrupters for prostate cancer [37], menin-mixed lineage leukemia 1 (MLL1) disrupters for leukemia [38, 39], B-cell lymphoma 6 (BCL6) – BCL6 corepressor (BCOR) disrupters for treatment of diffuse large B-cell lymphomas [40], Rictor – mTOR blockers for glioblastoma [41] and P53 – MDM2 inhibitors for a range of treatment resistant cancer types [42, 43].

However, although the above cases relate to small molecule inhibitors, instances of peptide PPI inhibitors as novel anti-cancer agents are also beginning to emerge. Recently, for example, cell permeable peptides have been developed against the NEMO – $\text{I}\kappa\text{B}$ kinase complex for the treatment of cisplatin-resistant ovarian cancer [44], stapled peptide disrupters that unhook β -Catenin from transcription factors have been produced as novel colon cancer agents [45] and colon cancer has also been the target of adenomatous polyposis coli (APC) – Asef disrupters [46] that inhibit the migration and invasion of colon cancer cell lines.

Cell delivery of therapeutic peptides in the cancer sphere has been undertaken by a variety of different routes involving liposomes [47], nanoparticles [48] and short cell-penetrating sequences (reviewed [49]). Our peptide is conjugated to Cell Porter® (Portage Pharmaceuticals Limited), a patented cell penetrating peptide based on the human HOXD12 protein [25–27]. Previously we have utilised stearate groups to good effect to deliver PPI inhibitor peptides into cellular [50, 51] and animal models of disease [52], however on this occasion the stearylated peptides had little effect on the levels of phospho-ERK in MM415 cells (Fig. 1a and c). Evidently, the effectiveness of peptide delivery systems is context specific and our data shows that CellPorter® has directed intracellular delivery of a novel C-Raf – PDE8A peptide disrupter leading to significant suppression of paradoxical ERK activation in a clinically relevant B-Raf inhibitor resistant human melanoma cell line and an apt xenograft model of the disease.

Conclusion

PPL-008 conjugates represent potential starting points for the development of co-therapies for resistant melanoma to be administered with an appropriate B-Raf inhibitor in order to overcome B-Raf inhibitor resistance and attenuate ERK activation in melanocytes.

Additional files

Additional file 1: Table S1. Chemicals and Antibodies used in study. (DOCX 67 kb)

Additional file 2: A375 growth data. Single-treatment of 10 μM PPL-008C and PPL-008CSS significantly attenuated A375 cell (V700E malignant melanoma cell line) growth although pERK levels are unaffected by these treatments (see Fig. 1.). (PPTX 118 kb)

Abbreviations

cAMP: cyclic adenosine monophosphate; CellPorter®: HOXD12 cell-penetrating peptide; C-Raf-S259: Inhibitory C-Raf serine residue 259; ERK: Mitogen-activated protein kinase; MEK: Mitogen-activated protein kinase of ERK; PDE8A: Phosphodiesterase 8A; PLX/Vemurafenib: Plexixikon developed B-Raf enzyme inhibitor; PPL008: PDE8A – C-Raf peptide disrupter; RAF: Serine/threonine protein kinase

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CMB and NMW undertook the work. CMB and GSB wrote the manuscript. CMB, NMW and GSB analysed the data. FWM and BHL contributed to supervision

and provided important intellectual input during manuscript revision. All authors have read and approved this manuscript.

Ethics approval

All protocols involving animals used were approved by the Institutional Animal Care and Use Committee of the University of Washington in accordance with the National Institutes of Health. No ethical approval was required for the cell lines used in this study.

Consent for publication

Not applicable

Competing interests

BHL and FWM have financial interests in Portage Pharmaceuticals Ltd. FWM, CMB and GSB are part of Portage Glasgow Limited.

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