Cellular mechanisms underlying complete hematological response of chronic myeloid leukemia to BRAF and MEK1/2 inhibition in a patient with concomitant metastatic melanoma.

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TRANSLATIONAL SIGNIFICANCE

Despite radically altering the treatment and prognosis of chronic myeloid leukemia (CML), BCR-ABL-targeted tyrosine kinase inhibitors are frequently limited by the development of resistance or intolerable toxicity. A number of pre-clinical studies have demonstrated a potential role for MAPK inhibition, principally in drug-resistant CML, but clinical data are lacking. We describe a complete hematological response to MEK inhibition in a patient with CML who notably did not receive conventional BCR-ABL kinase inhibitor therapy. We demonstrate the sensitivity of myeloid and erythroid progenitor outgrowth to MEK inhibition and place this in the context of what is currently known about BCR-ABL and MAPK pathway signaling. These findings support further clinical evaluation of the therapeutic role for MEK inhibition, not limited to the setting of conventional BCR-ABL kinase inhibitor resistance.

ABSTRACT

Purpose: Targeted MEK inhibition is an emerging therapy in a number of solid tumors. It holds particular promise in BRAF V600E mutation-positive malignant melanoma, where constitutive activation and cell growth through the MAP kinase (MAPK) pathway is well established. In vitro and pre-clinical research indicates that MAPK pathway activation is important in chronic myeloid leukemia (CML) leukemogenesis however the potential of MEK inhibition has not yet been investigated clinically in the setting of such hematological malignancies.

Experimental design: We report a case of complete hematological response of CML to MEK inhibition in a patient with synchronous metastatic melanoma, who received treatment with combination BRAF and MEK1/2 inhibitors. We studied the effects of
these agents on proliferation and outgrowth of myeloid precursors, and longitudinal shifts in peripheral blood phenotyping during the course of treatment. A model cell line system was used to examine the effects of dabrafenib and trametinib on MAPK and BCR-ABL1-signalling.

**Results:** After 35 weeks on treatment with BRAF and MEK inhibitors, complete hematologic response was observed without recourse to BCR-ABL1-targeted therapy. MEK inhibition was principally responsible for impaired proliferation of both mature and primitive myeloid precursors, as well as growth and hemoglobinization of erythroid precursors. Paradoxical activation of the MAPK pathway was seen in response to BRAF inhibitor therapy but this was easily overcome by clinically-relevant doses of concurrent MEK inhibitor.

**Conclusions:** These studies suggest that further evaluation of the optimal MAPK targeting approach is warranted to extend therapeutic options in CML.
INTRODUCTION

Chronic myeloid leukemia (CML) is almost universally characterized by a reciprocal translocation between the breakpoint cluster region (BCR) gene on chromosome 22, and the Abelson murine leukemia viral oncogene homolog 1 (ABL1) gene on chromosome 9, resulting in constitutive expression of a BCR-ABL1 fusion protein. Imatinib, a small molecule tyrosine kinase inhibitor that binds to and sterically interferes with the kinase domain of BCR-ABL1, effectively halts its constitutive and oncogenic cell cycle signaling activity. Kinase inhibition, as typified by imatinib, now forms a major part of the therapeutic options in molecularly-based subtypes of advanced lung, breast and medullary thyroid cancers, melanoma, gastrointestinal stromal tumors, and haematological malignancies such as CML.

Based on high rates of oncogenic activating BRAF mutations, current kinase inhibitor-based therapy in melanoma is centered upon targets within the MAPK signaling pathway. Due to the importance of MAPK signaling in virtually all tissues of the body, effects of these inhibitors on non-malignant cells has become a field of intense research.

The MAPK pathway is also known to be an important mitogenic signaling cascade in leukocytes, and its role in myeloproliferative disease is being increasingly established however the precise effects of MAPK inhibitors are highly dependent on the specific clinical context in which these agents are used. MEK inhibition has been demonstrated to rapidly correct aberrant myeloproliferative activity seen in murine models of both chronic and juvenile myelomonocytic leukemia, relating to MAPK hyperactivity induced by an activating KRAS mutation or inactivation of the NF1 tumour suppressor gene. Other studies have revealed that RAS-driven MAPK-
pathway dependence may be inadvertently intensified by BCR-ABL kinase inhibitors, leading to a synergistic anti-leukemic effect when used in combination with a MEK inhibitor(6). Similarly, a case of accelerated RAS-mutant leukemia was reported in a patient with metastatic melanoma receiving treatment with a BRAF inhibitor (7).

We report the case of a patient with BRAF V600E metastatic malignant melanoma who was treated with a combination of the V600-mutation-specific BRAF inhibitor dabrafenib, and the MEK1/2 inhibitor trametinib. Incidentally, he had a synchronous diagnosis of t(9;22) positive CML with an initial circulating leukocyte count of >100 x10^9/L. Despite not receiving BCR-ABL targeted kinase inhibitor therapy, he achieved hematologic complete remission while on dabrafenib and trametinib treatment. Correlative ex vivo and in vitro analyses confirmed a dominant effect of the MEK inhibitor rather than the BRAF inhibitor on the patient’s leukocytes, mediated by a relatively selective reduction in cell number and MAPK signaling within precursor (primitive) cell populations and reduced colony forming potential. We also demonstrate that in CML cells, in contrast to several solid tumour types, the addition of a MEK inhibitor is able to overcome the paradoxical MAPK activation induced by BRAF inhibition. These findings suggest that further investigation of the role of MAPK pathway inhibition in de novo CML in a clinical setting is warranted.

MATERIALS AND METHODS

Patient Details

The patient was enrolled in a phase I/II clinical trial of dabrafenib and trametinib in advanced melanoma (BRF113220, GSK, ClinicalTrials.gov number NCT01072175)(8), approved by the institutional review board of Austin Health,
Australia (Austin Health Human Research Ethics Committee, project number H2011/03955). All patient-derived tissue/blood samples were provided voluntarily under the auspices of the Cancer Biobanking and Research protocol approved by the institutional review board of Austin Health, Australia (H2012/04446), to which the patient had previously consented. Blood samples were collected at multiple timepoints prior to, during, and after the patient’s period of treatment with kinase inhibitors for metastatic melanoma. Baseline leukocyte samples were also obtained from peripheral blood leukapheresis. Clinical follow-up data was as per trial protocol and standard clinical care.

Methods

Hematologic Monitoring

Differential cell counts and blood films were obtained from diagnostic blood samples collected and analysed through the local pathology service provider (Austin Pathology). Cytogenetic analyses were performed by the Victorian Cytogenetics Service, and BCR-ABL1 counts performed at the Royal Melbourne Hospital (Parkville, Victoria, Australia).

Flow Cytometry

To estimate the effects of in vivo kinase inhibitor treatment on MAPK signaling in leukocyte subsets, peripheral blood mononuclear cells, (PBMCs) isolated from blood samples taken at baseline, week 8 and week 20, were subjected to multicolor flow cytometry. Two stains were applied: a “monocytic” stain, comprising CD45 (Cy5, BD Pharmingen Cat. 555484), CD34 (PE-Cy7, Beckman Coulter PN A51077 clone 581), HLA-DR (FITC, BD Pharmingen Cat. 555811), CD14 (APC, Beckman Coulter IM2580 clone RMO52), pERK (PE, Cell Signaling #5682S) or equivalent isotype
controls, and live/dead stain (Pacific Blue, LIVE/DEAD® fixable violet dead cell stain, Invitrogen L34955); and a “myeloid” stain, comprising CD45, CD34, HLA-DR, CD11b (APC, BD Pharmingen Cat. 550019), CD16 (APC-AlexaFluor750, Beckman Coulter PN A66330 clone 3G8), pERK, or isotype controls, and live/dead stain. All cells were gated on forward and side-scatter characteristics, live cells, and CD45+ cells. Subpopulations of interest included blast (CD34+/HLA-DR+), immature/promonocyte (CD34-/HLA-DR-/CD14-), mature monocyte (CD34-/HLA-DR-/CD14+), myelocytes (CD34-/HLA-DR-/CD11b+/CD16-) and metamyelocytes/mature neutrophils (CD34-/HLA-DR-/CD11b+/CD16+). MAPK pathway activation status was measured by both the proportion of cells staining positive for phosphorylated ERK1/2 (pERK) relative to isotype control, and comparison of relative pERK staining intensities between timepoints in monocytic and myeloid lineage cells.

Cell line culture

Three BCR-ABL harboring cell lines were used for in vitro studies. K562 (BCR-ABL b3-a2 fusion) was obtained from institutional stocks, MEG-01 (BCR-ABL b2-a2) was a gift from Dr Ashley Ng (Walter and Eliza Hall Institute, Parkville, Victoria, Australia), and KU-812 (BCR-ABL b3-a2 fusion) was obtained from Cell Bank Australia (Westmead, New South Wales, Australia); identity was confirmed by STR profiling. All cell lines were maintained in semi-adherent culture in RPMI 1640 media (Gibco®, Life Technologies, Mulgrave VIC, Australia) supplemented with 10% fetal bovine serum (Sigma, St Louis MO, USA), 1% glutamine (glutaMAX™) and 1% penicillin/streptomycin (both from Gibco®, Life Technologies) and incubated in 5% atmospheric CO2 at 37°C.

In vitro Proliferation Assays
In vitro analysis of kinase inhibitor effects on proliferation was performed using the K562 leukoerythroblastic cell line. Cells were plated out at seeding densities of 4000 cells per well in 96-well plates and treated with dabrafenib at doses of 0, 0.01, 0.1, 1 or 5 uM, trametinib at doses of 0, 0.1, 1, 10 or 100 nM, or their respective combinations, for a period of 72 hours. The doses used were chosen to cover the typically-achieved human plasma concentrations in vivo. Proliferation was determined colorimetrically by addition of MTS reagent (1 in 6 dilution of CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison WI, USA), incubation for 1.5 hours, measurement of the absorbance at 490nm, and comparison to the absorbance in untreated cells following background-correction.

Colony-forming Assays

DMSO-cryopreserved aliquots of leukapheresis samples collected prior to commencement of any TKI were thawed, resuspended in IMDM/10%FCS and then cultured, as previously described in detail(9). Cultures were set up at 5x10^4 cells/ml in 0.3% Bacto Agar (Becton Dickinson #214010), 25% fetal bovine serum (Gibco®, Life Technologies) and IMDM (Invitrogen, containing glutamine, 2-mercaptoethanol, asparagine and DEAE Dextran) in a fully humidified 5%CO2/air atmosphere before scoring. Day 7 colony forming cells (CFC; mature myeloid progenitors) were scored if >30 cells were present, and 14 day BFU-E and GM-CFC (immature erythroid and myeloid progenitors respectively) were scored if >50 cells were present. Day 7 cultures were stimulated with G-CSF (2000 U/ml (Neupogen, Amgen), and Day 14 cultures with GM-CSF (100ng/ml, R&D Systems Inc), IL-3 (100ng/ml,R&D Systems Inc), SCF (100ng/ml, R&D Systems Inc) and Erythropoietin (200ng equivalent to 4U/ml, Aranesp, Amgen). Cultures were in triplicate for initial titrations, and duplicate for mixture experiments. EC50s were calculated from log transformed
normalized data using GraphPad Prism software (GraphPad Software Inc. La Jolla
CA, USA).

Western Blotting

For \textit{in-vitro} phosphoprotein studies, K562, MEG-01 and KU-812 cells were
resuspended in serum-free media, allowed to equilibrate for 30mins, and subsequently
plated into 6 well plates (1.5x10^6 cells/well) containing imatinib mesylate, dabrafenib,
trametinib, or DMSO only (vehicle control, 0.1-0.2%) to the final doses as indicated
in results. Cells were incubated for 2 hours prior to harvest and lysis in RIPA buffer
(Pierce) containing the manufacturer’s recommended concentrations of PhosSTOP
phosphatase inhibitor and cOMplete ULTRA EDTA-free protease inhibitor (Roche
Diagnostics GmbH, Mannheim, Germany). Protein concentrations were estimated
using the BCA method (Thermo Scientific™ Pierce™ BCA™ Protein Assay Kit,
Cat#23225) and samples adjusted to uniform final concentrations, prior to the addition
of NuPAGE® LDS Sample Buffer (4X) and NuPAGE® sample reducing agent (10X)
(both from Novex®, Life Technologies, Cat#NP008 and Cat#NP009). Samples were
electrophoresed on NuPAGE® Novex® Bis-Tris 4-12% pre-cast gels with SeeBlue®
Plus2 pre-stained protein standard (Novex®, Life Technologies) prior to transfer to
nitrocellulose membrane by dry (iBlot® Dry Blotting System, Invitrogen™), or semi-
wet (Invitrogen™ XCell II™ Blot Module, Cat#EI9051) methods as necessary for
optimal transfer. Erk phosphorylation was determined using mouse anti-human
p44/42 MAPK (Erk1/2) (3A7) at 1:2000, rabbit anti-human phospho-p44/42 MAPK
(Erk1/2) (Thr202/Tyr204) (197G2) at 1:2000, and rabbit anti-human α/β-tubulin at
1:2000 (Cell Signaling Technology®, Cat#9107, Cat#4377, Cat#2148) followed by
IRDye® 680RD goat anti-mouse IgG and IRDye® 800CW goat anti-rabbit IgG
secondaries at 1:40,000 and 1:20,000 respectively (LI-COR®, Prod. Nos. 926-68070
and 926-32211). BCR-ABL1 activity was measured using the PathScan® Bcr/Abl Activity Assay cocktail (1:500, Cell Signaling Technology®, Cat#5300) consisting of rabbit antibodies raised against phospho-c-Abl (Tyr245), phospho-Stat5 (Tyr694), phospho-CrkL (Tyr207) and Rab11 (loading control), followed by anti-rabbit antibody as above. Wet-transfer combination treatment blots also contained mouse anti-human β-actin (1:3000) primary followed by goat anti-mouse secondary as above as an additional loading control. After staining, membranes were dried and scanned on the LI-COR® ODYSSEY® Infrared Imaging System and densitometry performed in the ODYSSEY® software. Densitometry results were normalized to Rab11 (ABL activity assay); ERK phosphorylation was expressed as the ratio of phosphorylated to total ERK1/2 and normalized to non-treated (DMSO) control.

For analysis of BIM isoform protein expression following kinase inhibitor treatment, K562, MEG-01 and KU-812 cells were incubated in full-serum media for 24 hours in the presence of imatinib, dabrafenib, and/or trametinib at doses indicated in the results, prior to harvest and processing as described above. Following transfer to nitrocellulose membranes using the iBlot® system, membranes were probed with mouse anti-β-actin (as above) and a rabbit monoclonal anti-BIM antibody [Y36] at 1:1000 (ab32158, abcam®), followed by secondary staining and visualization as above. For brevity, where blots have been displayed in figures, only relevant bands of confirmed correct size according to markers are shown.

RESULTS

Case report
A 26 year-old man with a history of moderately-severe psoriasis managed with topical steroids and prior PUVA therapy, was diagnosed with stage IIIB malignant melanoma (10) of unknown primary, involving the left inguinal lymph nodes. He underwent a left groin lymph node dissection, followed by resections of left upper thigh subcutaneous and left iliac lymph node recurrences within six months. He received local post-operative radiotherapy after the second recurrence. A further ten months later he underwent resection of a solitary right lower lobe pulmonary metastasis detected on routine surveillance imaging. Surveillance FDG-PET scan performed four months later confirmed relapsed disease with multiple metastases involving the left adrenal, bone, lymph nodes, subcutaneous sites, and liver. At that time the patient was asymptomatic, with an Eastern Cooperative Oncology Group (ECOG) performance status(11) of 0 (unrestricted function).

Genetic testing performed on the previously-resected lung metastasis identified a V600E mutation in the \textit{BRAF} gene. The patient was considered for enrolment in a phase I/II clinical trial of combination BRAF and MEK-inhibitors in advanced melanoma (BRF113220, GSK, clinical trial identifier NCT01072175). Screening investigations performed as per trial protocol demonstrated a markedly elevated white cell count (WCC) of \(109 \times 10^9/L\), with a left-shifted white cell differential. No eosinophilia or basophilia were reported per the automated analyzer differential. Hemoglobin was 106 g/L, and the platelet count was 145 \(x10^9/L\). The blood film showed a leukoerythroblastic picture with marked leukocytosis and occasional tear-drop cells, with no other specific features. Other than an elevated LDH of 579 (normal range 98-192 IU/L), biochemistry results including electrolytes, urea and creatinine and liver enzymes were within normal limits. The patient had no known medical history of blood dyscrasias and was unaware of any previous abnormal blood
investigations. Of note, a full blood count (FBC) sample taken at the time of the lung metastectomy, which had been performed at a different institution, was subsequently located. This showed normal hemoglobin and platelet counts, mild leukocytosis with total WCC 25 x10⁹/L, with a left-shifted differential, and no eosinophilia or basophilia.

The marked leukocytosis was initially considered to be a leukemoid reaction caused by paraneoplastic release of G-CSF secondary to metastatic melanoma(12), and the leukoerythroblastic film attributed to marrow involvement with melanoma. Peripheral blood was sent for molecular analysis, although results for this were anticipated to take at least one week. Given the rapidity with which the patient had relapsed and the high burden of metastatic disease, commencement on therapy was considered a higher clinical priority. The patient was enrolled in the BRF113220 clinical trial and randomized to receive both the BRAF-inhibitor dabrafenib (GSK2118436, 150mg twice daily), and the MEK-inhibitor trametinib (GSK1120212, 2mg daily). Treatment commenced immediately following a cytoreductive leukapheresis.

One week after starting on trial therapy, molecular analysis of peripheral blood was completed and demonstrated the presence of BCR-ABL1 t(9;22) fusion gene, with both a major b2-a2 (p210) transcript, and a minor e1-a2 (p190) transcript detectable. A subsequent manual review of the baseline blood film and repeat differential noted mild eosinophilia and basophilia. A bone marrow biopsy confirmed a diagnosis of CML in chronic phase, and did not show evidence of infiltration by melanoma. Cytogenetic analysis demonstrated t(9;22)(q34;q11.2) without additional abnormalities or evidence of deletion of the derivative chromosome 9.
Despite this concomitant diagnosis, as the patient’s overall prognosis was deemed to be more significantly impacted by metastatic melanoma than CML he was permitted to remain on trial protocol therapy, providing that he did not commence imatinib for treatment of the CML concurrently, given the lack of safety data for the combination of dabrafenib/trametinib with other kinase inhibitors. As CML was in chronic phase and was to be monitored closely with weekly FBC and regular Hematology review, he continued on trial.

The patient initially tolerated BRAF- and MEK-inhibitor therapy without any adverse effects and performance status remained excellent. Despite lack of specific therapy for CML, total WCC decreased within four weeks of commencing on trial treatment, and continued to decline over the subsequent six months (Figure 1). High resolution melt curve analysis of the patient’s leukocytes confirmed the absence of a BRAF V600 mutation.

Restaging for melanoma after 8 weeks on trial demonstrated a partial response by RECIST 1.1 criteria (13), and at week 16 he had a near-complete response on CT imaging together with a complete metabolic response on FDG-PET imaging. At week 24, the patient developed a solitary site of melanoma progression in a left adrenal metastasis, but remained on trial due to continuing clinical benefit with ongoing good control of melanoma at all other sites.

During enrollment on trial, the patient required two treatment breaks. The first occurred at week 24 due to development of asymptomatic reduction in left ventricular ejection fraction. Both inhibitors were withheld for four weeks as per study protocol. Left ventricular dysfunction resolved without specific intervention and dabrafenib and trametinib were restarted with a protocol-mandated 25% dose reduction of trametinib.
The second treatment break occurred at week 38, at the time that the patient underwent elective laparoscopic left adrenalectomy for treatment of the solitary progressing metastasis. After a period of 48 hours following surgery, both inhibitors were recommenced at the same doses.

Weekly FBC monitoring throughout treatment confirmed a normalized WCC of 8.4 x10^9/L after 35 weeks on trial, with associated resolution of the left-shifted myeloid series, eosinophilia and basophilia (Table 1), consistent with a complete hematological response. Nevertheless, repeat PCR in peripheral blood for the \textit{BCR-ABL1} fusion gene showed persistently detectable levels (Table 2). Following left adrenalectomy, total WCC increased, peaking at week 41 (Figure 1 and Table 2) with associated recurrence of a left-shifted differential. At the same time, the patient experienced a significant flare of psoriasis that required temporary intensification of topical agents (calcipotriol/betamethasone).

**Hematologic monitoring**

Temporal trends in the patient’s WCC, differential and blood/bone marrow \textit{BCR-ABL} levels are shown in Figure 1, and Supplementary Data Tables 1 and 2. Rather than a proportional cytoreduction in WCC, there was a greater reduction in myelocyte, promyelocyte, eosinophil and blast cell numbers than lymphocyte subtypes. Following the four-week break from dabrafenib/trametinib therapy (weeks 24-28), myeloid cells at varying stages of maturation increased in number. Total WCC again peaked approximately three weeks following adrenalectomy, consisting largely of myelocytes, metamyelocytes and band neutrophils, potentially suggesting a left-shift precipitated by perioperative stress.
The patient ceased kinase inhibitors late in week 48 due to clear progression of his melanoma. At that time, CML remained well-controlled with a circulating leukocyte count of 14.0 x10^9/L. The patient subsequently received several lines of cytotoxic chemotherapy with resultant multilineage cytopenias, confounding ongoing interpretation of CML behavior.

**Ex vivo analysis of leukocyte subsets and MAPK signaling**

Representative FACS plots and gates for the monocytic and granulocytic stains are shown in Supplementary Data Figure 1. Proportions of cells within each defined subpopulation are shown in Supplementary Data Table 3 (Table S3); due to the relative depletion of mature myeloid cells during standard Ficoll-based PBMC isolation, the constituent leukocyte sub-populations observed by flow cytometry differ markedly from those seen in contemporaneous peripheral blood films and are likely unreliable for more mature granulocytic populations. CD34+/HLA-DR+ blast numbers declined by over 6-fold from baseline to week 20 (p<0.0001, one-way ANOVA; Figure 2A). The proportion of blasts with measurable levels of phosphorylated ERK1/2 (pERK) declined at week 8 and remained low at week 20 (p=0.0014 across all timepoints, one-way ANOVA, Figure 2B, Table S3). Phosphorylated ERK1/2 staining intensity remained stable as measured by the mean fluorescence intensity (MFI) of pERK-PE (Table S3). Mature monocytes were almost entirely (>99.0%) positive for pERK at all timepoints (Table S3) however the mean intensity of pERK staining rose significantly from baseline to week 20 (3949 vs 4735). This may be due to the uneven separation of cells into pERK high- or low-sub-populations (Figure 2C).
Cell staining to define myeloid/granulocytic maturation stages demonstrated similar results for the CD34+/HLA-DR+ blast population as were seen in the monocytic stain series (Table S3). Myelocyte (CD34-/HLA-DR-/CD11b+/CD16-) pERK positivity reduced progressively from baseline to week 20 (p=0.0001, one-way ANOVA; Figure 2D, Table S3) together with a greater than 50% reduction of mean pERK signal intensity (Figure 2E). Metamyelocyte (CD11b+/CD16+) pERK positive proportions fluctuated moderately, with no sustained trend in pERK staining intensity over the observed timepoints (Figure 2F, Table S3).

**Effects of BRAF and MEK inhibitor therapy on colony formation.**

Next, the effect of each kinase inhibitor on hematopoietic progenitor cells from the patient was analyzed. Tested dose ranges of either drug were chosen to encompass typically-achieved maximal plasma concentrations *in vivo*, namely 1-2μM for dabrafenib and 10-30nM for trametinib. Both drugs inhibited the formation of colony formation from leukapheresis mononuclear cells in a dose-dependent fashion (Figure 3). Trametinib was significantly more potent than dabrafenib for each colony type analyzed. Both mature (Day 7 CFC) and more primitive myeloid progenitors (Day 14 GM-CFC) were inhibited by concentrations of trametinib achievable *in vivo*. Primitive erythroid progenitors (BFU-E) displayed similar sensitivity to trametinib; hemoglobinization and size of individual colonies was also significantly negatively affected in a dose-dependent fashion. Next, the effects of co-incubation of trametinib with dabrafenib at two fixed concentrations achievable *in vivo* were tested. Divergent behavior was observed according to the type of progenitor cell being assayed. Dabrafenib had no significant effect on BFU-E and GM-CFC sensitivity to trametinib (Figure 3B). However, for more mature Day 7 myeloid CFC, dabrafenib 1.25μM demonstrated an additive effect with trametinib on the inhibition of Day 7 CFC.
progenitor cell growth, with the calculated half-maximal effective concentration (EC50) for trametinib reducing from 17nM (95% CI 13-23) to 4nM (95% CI 3 – 6). In contrast, in the presence of 0.31μM dabrafenib, the calculated half-maximal effective concentration (EC50) of trametinib was higher, 32nM (95% CI 25 – 42) than for trametinib alone, suggesting partial antagonism (Figure 3B).

**Effect of BRAF and MEK inhibition on K562 cell line proliferation.**

Due to limited available patient-derived sample, the effect of the BRAF and MEK1/2 inhibitors was then evaluated further in a BCR-ABL-dependent leukemic cell line, K562. Proliferation curves at constant trametinib dose (Figure 4, panel A) indicate the relative lack of effect of dabrafenib on proliferation unless cells are co-treated with clinically-relevant doses of trametinib (≥1nM; blue and red lines). Very high doses of trametinib (100nM; red line) produced >50% inhibition of cellular proliferation with minimal additional contribution from dabrafenib at any dose.

Similarly, the effect of up-titrating trametinib in combination with each given dose of dabrafenib (Figure 4, panel B) clearly demonstrates the dominance of trametinib in driving a dose-dependent inhibition of proliferation.

Notably, at low dabrafenib doses (<1μM), the inhibitory effect of trametinib on proliferation was reduced relative to dabrafenib-untreated cells (Figure 4, panel A), whilst at higher dabrafenib doses of 1μM and 5μM there was additive anti-proliferative effect. This biphasic effect is consistent with the *in vitro* analyses of the patient’s mature myeloid progenitor cells (ie Day 7 CFC).

Overall, there was a dominant anti-proliferative effect of kinase inhibitor therapy mostly attributable to the effect of trametinib at clinically-relevant doses. This is
consistent with a MEK-mediated effect in this cell line which lacks an activating BRAF V600 mutation.

**Effects of BRAF and MEK inhibition on BCR-ABL1 and MAPK pathway activity.**

As expected, imatinib treatment of K562 cells markedly reduced phosphorylation of BCR-ABL1 with consequent downstream reduction of STAT5 phosphorylation and ERK1/2 phosphorylation, confirming that the MAPK pathway responds to upstream modulation of BCR-ABL1 activity (Figure 5A). Trametinib monotherapy for 2 hours in K562 cells inhibited ERK1/2 phosphorylation relative to basal (untreated) conditions in a dose-dependent manner. Consistent with potential paradoxical activation of MEK/ERK signaling in RAS-activated cells that lack activating BRAF V600 mutations, dabrafenib monotherapy markedly increased ERK1/2 phosphorylation with a maximal response between 0.5 and 1μM (Figure 5A and 5B). In combination with dabrafenib, trametinib doses attenuated (1nM) or abrogated (10-100nM) this paradoxical activation, and reduced ERK phosphorylation to below basal levels (Figure 5C and 5D). These findings were further confirmed in studies of the MEG-01 and KU-812 cell lines, both of which displayed even greater sensitivity to imatinib with an identical pattern of paradoxical ERK activation in response to dabrafenib, that could be overcome by trametinib co-treatment (Figure 5, panels E and F).

To assess for potential direct off-target effects of dabrafenib and/or trametinib on BCR-ABL1 activity, we analysed phosphorylation of BCR-ABL1, as well as the BCR-ABL1 interacting adaptor protein CRKL and the direct downstream target STAT5 in the same cells used to study ERK phosphorylation. No statistically
significant effect on activation of BCR-ABL1 was observed in response to dabrafenib or trametinib treatment, either alone (Figure 5A/B) or in combination (Figure 5C/D). Dabrafenib induced a dose-dependent attenuation of STAT5 phosphorylation which was statistically significant for 5μM dabrafenib (p<0.05 versus control) although was no longer significant with the addition of trametinib (Supplementary Data, Figure 2). Neither kinase inhibitor had significant effect on CRKL phosphorylation. Analogous studies in MEG-01 and KU-812 cells using the principal clinically-relevant doses demonstrated no significant off-target effects of dabrafenib or trametinib on downstream targets of BCR-ABL.

Effects of kinase inhibitors on pro-apoptotic molecule BIM expression

In all three cell lines tested (K562, MEG-01, and KU-812), BIM-EL was markedly more-abundantly expressed than the shorter isoforms (Supplementary Figure 3). Following 24 hours of exposure to imatinib, increased expression of BIM-EL became evident in K562 (marginally), of all three isoforms in MEG-01, with minimal change in expression of any BIM isoform seen in KU-812 cells (Supplementary Figure 3). No significant effects on BIM expression were seen with dabrafenib and/or trametinib in K562 or MEG-01 cells, however dabrafenib in combination with the higher-dose of trametinib (10nM) increased BIM isoform expression in KU-812 cells only.
DISCUSSION

Imatinib is an extremely effective therapy in chronic phase of CML, with over 65% of patients achieving complete cytogenetic response (CCR) after 12 months of therapy and 5-year overall survival rates of approximately 90% (14, 15). Data from the International Randomized Study of Interferon versus STI571 (IRIS) trial comparing interferon-alpha and imatinib (STI571), demonstrated impressive event-free, disease-specific and overall survival advantages for patients receiving first-line imatinib (16), together with both deep and durable molecular responses including major metabolic responses (MMR) in 85-90% at 5 to 6 years (17). Subsequent generations of inhibitors such as dasatinib and nilotinib, and more recently ponatinib, are also in clinical use(18). These agents are, however, susceptible to such set-backs as inadequate target specificity, unfavourable side-effect profiles, reduced efficacy due to intra- and inter-tumoral molecular and genetic heterogeneity, and development of resistance through a variety of mechanisms.

In the present case, a complete hematological response was seen after a 28-week period of treatment with BRAF and MEK1/2 kinase inhibitors. Concurrently, metastatic melanoma was well-controlled, with near-complete radiological response by week 16. Additionally, the patient ceased kinase inhibitors late in week 48 due to clear progression of his melanoma, despite well-controlled leukocyte counts at that time. Whilst a humorally-mediated effect secondary to the melanoma may have altered the patient’s CML disease course, the kinetics of the CML response were delayed and discordant from the melanoma disease course, suggesting a direct anti-leukemic effect of the BRAF inhibitor, the MEK inhibitor, or both. In particular, the gradual decline in circulating leukocytes and restoration of a normal mature differential count suggests an effect on a primitive or precursor cell population. In
support of this, *in vitro* analysis of the patient’s leukocytes revealed dose-dependent inhibition of colony formation across multiple precursor cell populations of varying maturity, with a dominant and potent effect of trametinib relative to dabrafenib.

Evaluation of phospho-ERK1/2 levels in patient-derived leukocytes as a surrogate for MAPK pathway activity revealed only modest differences over time in most subpopulations tested, with the exception of myelocytes and mature monocytes. Flow cytometric analysis of patient-derived leukocyte samples is subject to several potential confounding effects on subset enumeration and quantification of residual phosphoprotein levels introduced by sample collection, handling, purification and freeze-thaw, which largely depleted the dominant circulating cell type (neutrophils) in this patient. Nonetheless, these data suggest that differences in relative sensitivity to MAPK inhibition exist between leukocyte subclasses and maturation stages. Whilst mature subpopulations were relatively depleted in this analysis, the slow kinetics of response and breadth of cell types affected is most consistent with a more primitive target cell population being ultimately responsible for the observed hematologic response in this patient.

The majority of the anti-proliferative effect observed in treatment of the K562 CML cell line was secondary to MEK inhibition, consistent with other studies using MEK inhibitors in this cell line(6, 19-22). Whilst MEK inhibition appears to have a biphasic dose-response effect on erythroid differentiation in K562 cells (23), it demonstrated a dose-dependent inhibitory effect on erythroid precursors in our patient-derived cells, suggesting a difference in underlying MAPK and erythropoietin receptor (EPO-R) signaling in these differing contexts. Additionally, regardless of MEK inhibitor dose, intermediate doses of dabrafenib tended to antagonize the anti-proliferative effect of trametinib on K562 cells, resulting in a net proliferation either close to, or higher than
control. Western Blot analysis demonstrated the paradoxical activation of ERK phosphorylation in K562 cells treated with dabrafenib alone; this effect was dose-dependently reversed by trametinib co-treatment.

Several other studies have demonstrated that pharmacological inhibition of MAPK signaling can increase cell death and decrease cell proliferation in CML cells *in vitro* (6, 19-22, 24). In a competing-fates model of BCR-ABL1 inhibition by inhibitors such as imatinib and dasatinib, cells typically commit to apoptosis more rapidly than they can restore responses to growth-factor-induced survival signals (25). Accordingly, addition of the MEK-inhibitor PD184352 to imatinib increased apoptosis in *BCR-ABL1* positive human myeloid leukemia cells (22) and in another study, enhanced mitochondrial damage and caspase activation following dasatinib treatment, including in cells overexpressing *BCR-ABL1* and in some forms of *BCR-ABL1* point mutations (20). Another study reported a novel CRAF dysregulation mechanism that would, theoretically, be amenable to MEK/ERK inhibition (26). The pro-apoptotic molecule BIM is known to be an important mediator of the response to imatinib in CML; we did not find a consistent change in BIM expression in three model CML cell lines in response to dabrafenib/trametinib, suggesting that whilst differences exist between the cell lines, BIM is unlikely to represent a critical role in the beneficial hematologic effects of MAPK targeting in this setting(27, 28).

These findings are interesting considering what is known about the paradoxical MAPK-activating effects of BRAF inhibitors in cells that are wild-type for *BRAF*. As we and others have previously reported, MAPK-activated cells, commonly those harboring oncogenic *RAS* mutations, may in fact be stimulated to proliferate when exposed to V600 mutation-selective BRAF inhibitors (7, 29). Insofar as RAS appears to be a downstream target of BCR-ABL1, CML cells may thus be considered RAS-
activated and therefore susceptible to BRAF inhibitor-driven proliferation (Figure 6). Furthermore, CML cells with acquired resistance to BCR-ABL1-targeted kinase inhibitors may also display paradoxical MAPK activation. This has been observed in CML cells harboring the common BCR-ABL1 T315I secondary-resistance mutation during continued exposure to kinase inhibitors such as imatinib, due to weak off-target binding of inhibitor to BRAF. This phenomenon could be exploited by the addition of a MEK inhibitor, which appeared to induce synthetic lethality in this context (6).

Interacting partners and downstream modulators of BCR-ABL1 signaling are numerous and include RAC and RHO family members, STAT5, CRKL, and the PI3K/AKT pathway in addition to MAPK members. BCR-ABL1 activates MAPK signaling in a GRB2 and SOS-1-dependent manner (24, 30-36), but leads to marked MEK-dependent negative feedback of receptor-level growth factor signaling, as well as direct negative feedback on RAS itself (Figure 6) (25). Although we did not find a significant direct effect of dabrafenib or trametinib on BCR-ABL1 activation as measured by phosphorylation of ABL-Tyr245 and CRKL, possibly due to the kinetics of our assay, we found clear evidence that dabrafenib dose-dependently reduced STAT5 phosphorylation. This effect was likely due to paradoxical activation of MEK in the context of BRAF inhibitor monotherapy, with consequent intensification of MEK-dependent negative feedback on STAT5 phosphorylation, as has been described in other studies (25). Whilst trametinib co-treatment had a very small counter-effect on STAT5 phosphorylation, trametinib monotherapy had little effect on STAT5 phosphorylation relative to untreated cells (Supplementary Data, Figure 2). This suggests that the set-point of this feedback loop is such that negligible negative feedback on STAT5 occurs until MEK activity is raised above basal levels, in this
case by dabrafenib. Whether some of the functional differences observed between BRAF monotherapy and combined BRAF/MEK inhibition may relate to this divergent effect on STAT5, either in preference or in addition to divergent effects on MAPK pathway signalling, is unclear and warrants further investigation.

In our case, despite complete hematological response, there was no significant decline in *BCR-ABL1* gene expression either in blood or by repeat bone marrow cytogenetics, indicating that MEK inhibition was not effective at inducing molecular or cytogenetic response. Given the clear relationship between depth of molecular response and long-term outcome in CML (16), MEK inhibition as a single agent therapy would thus seem unsuitable, but could be considered in combination with other therapies, or potentially in cases of therapeutic resistance such as the *BCR-ABL1* T315I mutation, for which current first- and second-generation *BCR-ABL1* kinase inhibitors are ineffective (37-39). Whilst unintended MAPK activation may be undesirable in other cancer settings, it may paradoxically be advantageous in therapy-resistant CML by driving cells into the downstream pro-apoptotic block afforded by concurrent MEK inhibition. Exactly how dependent the efficacy of this approach is on the degree of upstream MAPK activation is unclear and may impact upon its therapeutic potential.

**CONCLUSION**

This case documents a unique observation of complete hematologic response to MEK inhibition in a patient with chronic-phase CML and concomitant metastatic melanoma. It confirms differential effects of MEK inhibition on defined leukocyte subsets, highlighting the inhibitory effects on CML cell proliferation and colony formation. Whilst previous pre-clinical work suggests a role for MEK inhibition in
acquired BCR-ABL1 inhibitor resistance, the response seen in our patient occurred without prior exposure to, or selection against, imatinib or related kinase inhibitors. These findings may support a more prominent therapeutic role for MAPK targeting in CML that is not limited to exploitation of paradoxical MAPK activation seen in some drug-resistant CML populations. MEK inhibition appears to affect a broad spectrum of hematopoietic lineages in CML, but our correlative analyses indicate that its primary clinically-relevant effect may be on a primitive subpopulation. Further clinical evaluation of MAPK targeting as add-on therapy to conventional BCR-ABL1 kinase inhibitors, and in the setting of resistance to these agents is warranted.

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FIGURE LEGENDS:

Figure 1. Evolution of peripheral blood total and differential white cell counts during treatment for metastatic melanoma. (A) total white cell count (WCC), and (B) differential cell counts from peripheral blood samples taken throughout the patient’s treatment period; mono+eos+baso indicates the combined count of monocytes, eosinophils and basophils. Critical clinical events and the upper limit of normal (ULN) of total WCC are indicated in panel A.

Figure 2. Flow cytometric analysis of leukocytes collected from the patient at baseline, prior to commencement of kinase inhibitor therapy (baseline), and again at week 8 and week 20 of therapy. (A) CD34+/HLA-DR+ blast-like cells decreased in number (expressed as a percentage of CD45+ cells) over time, coincident with a reduction in the percentage of cells bearing detectable amounts of phospho-ERK1/2 (B). Mature monocytic forms (CD14+) also appeared to split into two phospho-ERK1/2 positive sub-populations with a bias towards higher-intensity staining (C). Myelocytes (CD11b+/CD16-) showed a reduction in pERK positivity (D) due largely to a loss of right-skewing in the distribution of PE stain intensity over time (E). Metamyelocytes (CD11b+/CD16+) showed no consistent trend in pERK staining positivity over time.

Figure 3. Sensitivity of myeloid and erythroid colony formation to inhibition with trametinib or dabrafenib or a combination. (A) Fitted curves and mean ± SEM (n = 4 or 5 independent experiments) of normalized log-transformed colony data are shown according to progenitor type for each drug alone. (B) Effect of combining
dabrafenib at two fixed concentrations (0.31 and 1.25 μM) with trametinib at various concentrations is represented by fitted curves. Data are mean of two independent experiments. The half-maximal effective concentration (EC50) for the trametinib titrations were calculated for each fixed dabrafenib concentration and the vehicle control. The only significant differences from vehicle control were observed for Day 7 CFC.

**Figure 4. Effects of dabrafenib and trametinib on proliferation of K562 cells**

...grouped either by A) constant trametinib dose or B) constant dabrafenib dose to emphasise the relative contributions of each drug to effects on cellular proliferation. Data are shown as mean ± SEM for three independent replicates normalised to control (DMSO-only treated) cells. Proliferation was significantly influenced by both trametinib dose (67.4% of variance) and dabrafenib dose (19.9% of variance) with evidence of a positive interaction (10.2% of variance) by 2-way RM-ANOVA with Bonferroni correction for multiple comparisons; † p<0.01, * p<0.0001 within treatment-curves relative to no dabrafenib (A) or no trametinib (B).

**Figure 5. BCR-ABL activity and ERK phosphorylation** were assessed by Western Blot following treatment of K562, MEG-01 and KU-812 cells for 2 hours with dabrafenib (Dab) and trametinib (Tram) at the doses indicated. Imatinib (IMAT) was used as a positive control and DMSO-only as a non-treated control (NT); relevant bands from representative blots are shown. A) monotherapy treatments showing dose-dependent reduction of Stat5 phosphorylation by dabrafenib treatment and paradoxical activation of ERK phosphorylation. B) densitometry of
phosphorylated:total ERK1/2 shown relative to control (p<0.0001 overall, one-way ANOVA; Tukey’s multiple comparison method p-values versus control indicated). C) representative blot of combination treatments showing persistent paradoxical activation of ERK phosphorylation only at the lowest concurrent dose of trametinib, confirmed by D) densitometry. E) equivalent BCR-ABL activity and ERK phosphorylation blots using an abbreviated panel of doses in the BCR-ABL harboring cell lines MEG-01 and KU-812, demonstrating minimal effect of dabrafenib or trametinib on BCR-ABL activity, but clear dabrafenib-mediated paradoxical ERK phosphorylation which was ablated by trametinib co-treatment, confirmed by densitometry (F).

Figure 6. Selected interacting molecules and signal transduction pathways acting downstream of BCR-ABL1. Paradoxical activation of wild-type (wt) BRAF in the presence of activated RAS and a BRAF inhibitor (BRAFi) such as dabrafenib leads to BRAF-CRAF heterodimerisation (curved arrow), translocation to the cell surface and activation by RAS (dashed double-headed arrow) with consequent CRAF-mediated activation of MEK. MEK inhibition (MEKi) by drugs such as trametinib prevents MEK-directed activation of ERK and relieves MEK-dependent negative feedback on STAT5 (blunt-ended arrow).
A

Figure 1

B
Figure 2

A. CD34+ / HLA-DR+

B. CD34+ / HLA-DR+

C. HLA-DR+ / CD14+

D. CD34- / HLA-DR- / CD11b+

E. CD11b+ / CD16-

F. CD11b+ / CD16b+
Figure 3

(A) Percent Colony Formation vs. Trametinib (nM) for different conditions:
- Day 7 CFC
- Day 14 BFU-E
- Day 14 GM-CFC

(B) Percent Colony Formation vs. Trametinib (nM) for different Dabrafenib concentrations:
- Dab 0.31µM
- Dab 1.25µM
- no Dab

Legend:
- Dab 0.31µM
- Dab 1.25µM
- no Dab

Day 7 CFC
Day 14 BFU-E
Day 14 GM-CFC
Figure 4

A

B
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