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Evidence for upregulation of BIM and the splicing factor SRp55 in melanoma cells from patients treated with selective BRAF inhibitors

Running Head: Induction of apoptosis in melanoma by BRAF inhibitors

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Abstract

Relatively little attention has been given to the activity of selective BRAF inhibitors in the induction of apoptosis in melanoma, particularly in vivo. In the present study we have isolated cultures from biopsies taken from four patients prior to and during treatment of their melanoma. We report that the cell lines taken during treatment show varying degrees of upregulation of the proapoptotic BH3 protein Bim and its splice forms, downregulation of Mcl-1 and upregulation of the splicing factor SRp55 as reported in prior in-vitro studies. There was also evidence of ongoing apoptotic signalling despite the continued growth of the cultures. The cultures established during treatment were largely resistant to the selective BRAF inhibitor PLX 4720, consistent with the acquired resistance of melanoma in the treated patients. These results provide further insights into the mechanism of action of these agents against melanoma.

Key Words: Melanoma, BRAF inhibitors, apoptosis, Bim, splicing, in vivo

INTRODUCTION

Once melanoma has metastasized to distant sites it has proved difficult to treat by a variety of chemotherapeutic and biological agents [1, 2]. Significant advances have been made in identifying subgroups of patients with mutated proteins in signal pathways that preferentially activate individual pathways [3]. In particular, the finding that approximately 50% of melanoma have V600E mutations in the BRAF serine/threonine kinase has resulted in the generation of a number of new drugs which target the mutated active form of BRAF [4-6]. One of these drugs, Vemurafenib (PLX 4032/RG7204/ZelBoraf) has now been evaluated in phase II [7] and III [8] trials and shown to significantly prolong both progression free survival (PFS) and overall survival (OS) compared to standard chemotherapy with DTIC. The drug, GSK2118436, is also highly selective for the mutated forms of BRAF. In phase I studies, 60% of 30 patients had unconfirmed partial responses (PR) by RECIST criteria [9].

Unfortunately, with longer follow-up it is clear that recurrence of melanoma is common in patients treated with Vemurafenib [6, 10, 11]. Studies in vitro have suggested that this may be due to reactivation of the MEK/ERK pathway [12]. Mechanisms involved in the reactivation include a switch from BRAF to CRAF signalling [13], activating mutations in NRAS [14] or activation of MEK by alternate Kinases such as COT [15]. We have also reported that ERK can be activated by cross talk from the Akt pathway independent of MEK [16]. In addition, dimerisation of an aberrantly spliced form of BRAF that was resistant to Vemurafenib was identified in 6 of 19 patients with acquired resistance to Vemurafenib [17].

Activation of alternate pathways which bypass the MEK, ERK pathway has also evolved as a major mechanism of resistance. Studies by Nazarian et al identified one subset of melanoma cells with upregulation of the platelet derived growth factor receptor B (PDGFRB) that appeared to bypass the MEK/ERK pathway [14]. These results were applicable to five of

twelve patients who developed resistance to treatment with PLX4032. Increased expression of IGF -1R was associated with acquired resistance to Vemurafenib in 2 of 5 post relapse patient samples [18].

To gather more information on molecular determinants of apoptotic responses to the selective BRAF inhibitors in patients during treatment and particularly to acquired mechanisms of resistance to apoptosis, we have carried out studies on melanoma cultures established from four patients entered in a phase II study of PLX4032/RG7204 as second line treatment of patients with metastatic melanoma.

METHODS

Patient Studies

Primary melanoma cell cultures were established from 4 patients entered into the Roche “Brim2” phase II study of Vemurafenib in patients who had failed previous treatment and from one patient in the phase II GSK study of treatment with GSK 2118436. These trials were approved by the Hunter Area Research Ethics Committee.

Cell Culture and Reagents

Primary melanoma cell cultures were established from biopsies by the “spilling” technique or from suspensions of needle biopsy aspirates as reported elsewhere [19]. Cultures that were similar in appearance to fibroblasts were treated with Geneticin at 1ug/ml as described elsewhere [20]. They were cultured in DMEM containing 10% FCS (Commonwealth Serum Laboratories, Melbourne, Vic, Australia). The mouse MAbs against phospho-ERK (p-ERK) (Thr202/Tyr204), phospho-Akt (p-Akt) (Ser473), phospho-MEK (p-MEK), ERK, ERK1, ERK2, Akt, Akt3, MEK1 and B-Raf, were purchased from Cell Signalling Technology

(Beverly, MA). The rabbit polyclonal Ab against caspase-3 was from Stressgen (Victoria, BC, Canada). The rabbit polyclonal Ab against cleaved PARP was from Cell Signalling Technology (Beverly, MA). The mouse MAbs against Bcl-2, Bcl-X_L and Mcl-1, Bax, Bad and phosphorylated ERK1/2 were purchased from Santa Cruz Biotechnology. The rabbit polyclonal Abs against ERK1/2, MEK and PUMA were from Cell Signalling Technology. The MAb against Noxa and the polyclonal Ab against Bim were purchased from Imgenex. Isotype control Abs used were the ID4.5 (mouse IgG2a) MAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), the 107.3 mouse IgG1 MAb purchased from PharMingen (San Diego, CA), and rabbit IgG from Sigma Chemical Co. (Castle Hill, Australia). PLX4720 was provided from Plexxikon Inc. (Berkeley, CA). It was dissolved in DMSO and made up in stock solutions of 4mM.

The mutation phenotypes of the cell lines were assessed by the Sequenom Mass array system. The Oncocarta panel VI version 1 was extended to include screening for mutations in PTEN and MEK. The panel included assessment of mutations in BRAF, NRAS, KRAS, CDK4, EGRF, ERBB2, and ERBB4. BRAFV600E mutations were identified in all the cultures except from the culture established from patient 5 during relapse. Cultures from this patient were excluded from further studies. This culture was detected to have mutations in CDKN2A (E88) and CTNNB1 (β Catenin). Patient 3 also had mutations in ERBB4 (E452K) in the culture established during treatment. Patient 4 had mutations in AKT1 (E17K) in the treatment cultures.

Assessment of Doubling Times of Cultured Melanoma Cells

Cells were seeded in 24 well plates at 10^5 cells per well. At various time points - 24, 48, 72, 96 and 120 hours post plating cells were harvested and centrifuged for 5 minutes at 1800rpm. Pellets were resuspended in medium and cells counted using a “Countess” automated cell

counter (Invitrogen Catal. No. C10227). Doubling times were estimated from a line of best fit charting cell counts versus time (5-7 days) and using the “Forecast” Program in Microsoft Excel.

Apoptosis

Quantitation of apoptotic cells was carried out by measurement of sub-G1 DNA content using propidium iodide (PI) on a flow cytometer as described elsewhere [21-23]

Flow Cytometry

Immunostaining on intact and permeabilized cells was carried out as described previously [21, 22].

Western Blot Analysis

Western blot analysis was carried out as described previously [21, 22]. Labeled bands were detected by Immun-StarTM HRP Chemiluminescent Kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDocTM image system (Bio-Rad, Regents Park, NSW, Australia).

RESULTS

Melanoma Cultures Established During Treatment with PLX4032 Undergo Changes in Morphology and Growth Rates

Details of the patients in the study are shown in table 1. They had received multiple prior treatments and several had marked bulky cutaneous metastases. Patient 1 had a rapidly progressive form of the disease with low grade fevers, anorexia and fatigue.

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As shown in Figure 1, the four pre-treatment cultures had a rounded or epithelioid appearance whereas the cultures taken during treatment tended to have spindle cell morphology. The growth rates assessed in pre and post-treatment samples are shown in Table 2. Growth rates at successive passages are shown to illustrate that: (1) higher passages in all cultures tended to increase their growth rates; and (2) cultures established from treated patients tended to have increased growth rates. In particular the increase in growth rates from patient 1 was consistent with the rapid progression of the disease in this patient.

Stable Cell Lines Established During Treatment Show Evidence of Changes in Bcl-2 Family Proteins and Ongoing Apoptosis

We have shown previously that a similar selective BRAF inhibitor, PLX4720 induces upregulation of Bim and preferential splicing to produce Bim_S in cultured melanoma cells [21]. Increased Bim_S splicing was associated with upregulation of the splicing factor SRp55 (SFRS6). These changes were also seen in cultures established from patients (even though there was no PLX4720 in the media), as shown by the Western blots illustrated in Figure 2A. Several studies are shown for patient 1 at different passage numbers. Studies on passage 2 and 4 showed very strong upregulation of Bim_S but it was lower in subsequent studies on 10 (the latter not shown). Cultures from patient 3 also showed strong upregulation of Bim_S and Bim_{EL}. Upregulation of Bim_S, while evident, was not so marked in cultures from patient 6. There was also a more complex pattern of bands at the level of Bim_{EL} consistent with phosphorylation/dephosphorylation of Bim_{EL}. The splicing factor SRp55 responsible for splicing to produce Bim_S was upregulated in each of the cultures established during treatment but was less in cultures from patient 6 consistent with lower upregulation of Bim isoforms in this culture. The antiapoptotic proteins Mcl-1 and Bcl-2 were reduced in all of the cultures established during treatment.

Ongoing Changes of Apoptosis in Cultures from Treated Patients

Despite successive passage of the cells, as shown in Figure 2B, there was continuing evidence of ongoing apoptosis, as shown by the reduction of procaspase 3 levels and increases in cleaved poly (ADP-ribose) polymerase (PARP). Earlier passages from patient 1 had more marked signs of apoptosis but by passage 10 cleavage of PARP was no longer evident (data not shown). Cleaved PARP was detectable in cultures established during treatment of patient 6 but other changes of apoptosis were not as evident as in patients 1 and 3. There were insufficient cells to study patient 4.

Changes in ERK, MEK and Akt Vary Between Cell Lines

Patient 1 had marked remission of melanoma growth during treatment and equally rapid relapse of his melanoma during treatment. Figure 3 shows that pERK was weakly expressed and pMEK clearly expressed in cultures established prior to treatment. The culture from the progressing melanoma had strong pERK and barely detectable pMEK expression. We and others have previously reported MEK independent activation of ERK [16][24-27]. pAkt 3 was strongly upregulated in the cultures taken during relapse consistent with our previous report of MEK independent activation of ERK by cross talk from the AKT pathway [16].

Cultures from Patient 3 established pre-treatment had the expected activation of ERK and MEK. pAkt3 was also detected. Cultures established from samples during treatment had a similar pattern except pAkt3 levels were less than prior to treatment.

In patient 6, pERK and pMEK appeared to be upregulated in the cultures taken prior to treatment. Similarly Akt appeared to be upregulated in cultures taken prior to treatment. In the cultures taken during treatment, pMEK, pERK and pAkt were less evident.

Melanoma Cell Lines Established from Treated Patients Lose Sensitivity to PLX4720 induced apoptosis

As shown by the Western blot study in Figure 4A, MEK and ERK were inhibited by PLX4720 in cultures taken from patient 1 prior to treatment but the inhibitory effects were markedly reduced in cultures taken during relapse, indicating that the melanoma cells had reduced sensitivity to MEK inhibition by the selective BRAF inhibitor PLX4720. This was reflected in the apoptosis assays which showed relatively low levels of apoptosis in cell lines

established prior to but no significant apoptosis in cultures established during relapse during treatment with Vemurafenib (Figure 4A).

A similar study was carried out on cell lines from patient 3. pMEK and pERK were evident prior to treatment with PLX4720 but cultures taken prior to and to a lesser extent during treatment with Vemurafenib still appeared sensitive to PLX4720 with low levels of pMEK and pERK (Figure 4B). The pre-treatment cultures were partially sensitive to PLX4720 in apoptosis assays but those established during treatment were relatively resistant (Figure 4B), consistent with the partial inhibition of MEK and ERK in figure 4B.

Studies on cultures from patient 6 (treated with GSK2118436) showed only partial inhibition of pMEK and pERK in the presence or absence of PLX4720, and this was reflected in only low levels of apoptosis induced both pre and during treatment with this inhibitor.

DISCUSSION

The patients entered into these studies had been pretreated with various agents and in some cases had bulky advanced recurrent disease. Nevertheless, they all had good clinical partial responses that unfortunately were not durable. To better understand the response of these patients, cell lines were established from biopsies taken before and during treatment and subjected to analysis of changes in Bcl-2 family proteins and apoptotic pathways. We have previously reported that PLX4720, a similar selective BRAF inhibitor to PLX4032, induced apoptosis of BRAF^{V600E} melanoma largely by upregulation of Bim and particularly the Bim_S isoform [21]. Increased Bim_S splicing appeared dependent on the splicing factor SRp55 (SFRS6), which was shown in a previous study to be increased in melanoma with BRAF mutations [15]. The present study showed that similar upregulation of Bim_{EL}, Bim_L and Bim_S occurred in cell lines established from treated patients. Increases in SRp55 were also seen consistent with the changes seen in the Bim isoforms. The SR proteins have an amino-

terminal end which binds to RNA and a carboxy-terminal domain rich in serine and arginine residues which bind to other proteins in the spliceosome. The proteins are believed to be regulated by phosphorylation by kinases such as SR protein kinases, topoisomerase 1, Akt and GSK3 β [28]. The RNA binding exon splicing inhibitor, Sam68, was also reported to be a target for ERK [29] but whether SRp55 is a target of ERK remains to be established.

The Bim_S isoform was shown previously to be a key isoform for induction of apoptosis [21]. It was shown by others to bind directly to mitochondria [30, 31] and this was confirmed by showing relatively high concentrations of Bim_S in mitochondrial fractions taken from melanoma cells during treatment with PLX4720 [21]. As reviewed elsewhere, splicing of proteins appears to be an important regulator of apoptosis [32]. Changes in other Bcl-2 family proteins consisted of a decrease of Bcl-2 and reduction in Mcl-1 levels. The extent to which these changes in other Bcl-2 proteins contribute to apoptosis remains unclear. Bcl-2 was down-regulated in all cultures established during treatment. MITF is known to upregulate Bcl-2 expression [33] but whether changes in MITF levels were responsible for downregulation of Bcl-2 was not studied. MITF was reported to be transcriptionally upregulated in BRAF mutated melanoma but down-regulated by ERK-mediated phosphorylation [34]. We have reported previously that Bcl-2 levels in melanoma were strongly correlated with the levels of the transcription factor AP-2 [35] so that suppression of AP-2 by the BRAF inhibitors may also explain down regulation of Bcl-2.

Perhaps the most surprising results were those showing evidence of apoptosis in cultures established during treatment in the absence of the BRAF inhibitor in the culture for periods of 2-6 weeks. This was particularly evident in cultures from patient 1 and 3. These results imply that apoptotic events established by treatment such as upregulation of Bim and its isoform Bim_S persisted in the absence of the drug. Cultures from patient 1 at later passages (passage 10) had less evidence of Bim_S isoforms and of apoptosis compared to early passages.

These results would be consistent with gradual selection in culture of melanoma cells with less Bim and increased viability and may reflect similar changes in-vivo.

We have reported previously that culture of BRAF V600E melanoma in PLX4720 over prolonged periods resulted in outgrowth of melanoma cells with activated ERK in the absence of pMEK [16] ERK in these cells was activated by the PI3k/Akt pathway. In the present study all cultures taken from the treated patients had evidence of activated ERK. In two patients this was associated with activation of MEK but this was less evident in the cultures from patient 1, which also had marked activation of Akt. The results in patient 1 are therefore consistent with the results from studies on melanoma cells selected in vitro with PLX4720. To examine whether the reappearance of pERK in the cultures was associated with resistance to the BRAF inhibitors we exposed pre and post-treatment cultures to PLX4720. In patient 1 the post-treatment cultures did not show inhibition of pMEK or pERK by PLX4720. The basis for the resistance is the subject of ongoing studies. Pretreatment cultures from patient 1 had relatively low sensitivity in apoptosis assays and the cultures established during progression showed no apoptosis in response to PLX 4720. Cultures from the other patients appeared to be responsive to PLX4720 in assays of pMEK and pERK but cultures obtained from treated patients were resistant to PLX4720 in apoptosis assay, indicating that as yet unidentified factors were responsible for resistance to PLX4720.

Differences were noted in the morphology of the cells obtained during treatment with the inhibitors in that cultures obtained prior to treatment tended to have an epithelioid appearance whereas the 4 cultures obtained during treatment tended to have a spindle cell morphology. Similar morphological changes were described in studies by Hingorani [36] when mRNA for BRAF was knocked down by shRNA. Viros et al [37] reported that primary melanoma with BRAF mutations were more rounded, pigmented and formed nests which would be consistent with the morphology of the pre-treatment cultures in this study. Davidson et al [38] reported that desmoplastic melanoma in 12 cases did not have BRAF mutations, which again would be

consistent with the contrasting morphology reported by Viros et al [37]. Cultures taken from the patients during treatment had increased proliferative rates compared to those established pre-treatment which is of interest given that there have been anecdotal reports of increased growth rates in patients failing treatment with the selective BRAF inhibitors.

In summary, the present studies on melanoma cell lines from treated patients validate previous studies on established cell lines showing that treatment with PLX4720 was associated with upregulation of Bim, in particular Bim_S with upregulation of the splicing factor SRp55. In addition, the cell lines from treated patients have shown relatively prolonged signs of apoptosis in the absence of the drug, which gradually disappeared on prolonged culture. Activation of ERK was a common finding in cultures established during treatment and was associated with in vitro evidence of resistance to the selective BRAF inhibitor PLX4720. These results raise many questions which are the subject of ongoing studies, e.g. can therapy increasing Bim or the splicing factor SRp55 be complementary to the BRAF inhibitors? Do pro-apoptotic agents have a role with selective BRAF inhibitors? Further studies are needed to answer these questions.

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Table 1. Details of Patients in the Roche/Plexxikon NP22657 and GSK2118436 phase II study from which cell lines were established

Patient No.	Sex	Age	Primary Melanoma Thickness (mm)	Time from Diagnosis to First Metas. (mths)	Prior Treatment	Sites of Disease At Commencement Of PLX4032	Best Response	Duration of Rx (wks)	Duration to Death (wks)		Duration to Last Follow-Up (wks)
									From First Metas.	From Rx	
1	M	35	1	25	DTIC 10/9/2009	Lung, Adrenal, LNs, Bone	PR	11*	26	20	
2	M	66	Occult	206	DTIC +/- Bosentan 20/12/2005- 7/04/2006	Lung, Soft tissue	PR	36 (12 cycles)			>78
3	F	54	1.2	23	DTIC 12/11/2009- 17/12/2009	s.c., Lung, Bone, Peritoneum	PR	15* (5 cycles)	116	16	
4	F	59	2.7	53	Taxol Dec 2008 – March 2009	s.c., Lung	PR	15* (5 cycles)	100	24	
5	F	46	1.0	103	Temozolomide +/- ABT-888 Nov 2009 – Dec 2009	s.c., LNs, Pelvis	PR	17* (5.2 cycles)	52	19	
6 (GSK 2118436)	F	69	Occult	0 (Presented with s.c. metastases)	Fotemustine April 09-July09 Surgery s.i. [†] 09	s.c., LNs R Neck R Axilla Adrenal	SD	20*	138	24	

*Biopsies taken at 6 weeks (patients 3 and 6) and 4 weeks (patients 4 and 5) during treatment and last day of treatment (patient 1).

[†]s.i. = small intestine.

Table 2. Mean doubling times (days) for cultures established from biopsies* prior to and during treatment with Vemurafenib/GSK2118436

Passage Number	Patient 1		Patient 3		Patient 4		Patient 6	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
3	3.7		1.96	2	5.29	1.55		
4	3.4	0.45					6.3	4.5
5		1.05	1.23	1.02	3.97	1.94		
6	1.5	0.33		0.96		2.9		2.3
7	2.6		1.4	1.1	5.38	2.93		
8		0.21	1.8	0.58			1.6	6.3
9	1.94						2.0	
Mean	2.6	0.5	1.6	1.1	4.9	2.3	3.3	4.3
S.E.	0.42	0.19	0.17	0.23	0.46	0.35	1.3	1.44

*Biopsies taken at 6 weeks (patients 3 and 6) and 4 weeks (patients 4 and 5) during treatment and clinical PRs. Biopsies from patient 1 were taken on last day of treatment during clinical progression of melanoma.

Legends to Figures

Figure 1 - Melanoma cultures established during treatment with PLX4032 undergo changes in morphology

A. Representative microphotographs of melanoma cultures established from biopsies prior to and during treatment with the selective BRAF inhibitors PLX4032/RG7204/Vemurafenib (patient 1, 3, 4) and GSK2118436 (Dabrafenib) (patient 6).

Figure 2A – Changes in Bcl-2 family and SRp55 proteins in cultures taken prior to and during treatment with the selective BRAF inhibitors

Whole cell lysates from indicated melanoma cultures were subjected to Western blot analysis of Bim, Mcl-1, Bcl-2, SRp55, and GAPDH (as a loading control). The data shown are representative of three individual Western blot analyses. Pre: pretreatment; Post: during treatment.

Figure 2B – Cultures established from biopsies taken during treatment show evidence of apoptotic signalling

Whole cell lysates from indicated melanoma cultures were subjected to Western blot analysis of caspase-3, PARP, and GAPDH (as a loading control). The data shown are representative of three individual Western blot analyses. Pre: pretreatment; Post: during treatment.

Figure 3 – Changes in MEK/ERK and Akt signal proteins in cultures established from biopsies taken during treatment with the selective BRAF inhibitors

Whole cell lysates from indicated melanoma cultures were subjected to Western blot analysis of phosphorylated MEK (p-MEK), MEK, phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated Akt (p-Akt), and Akt. The data shown are representative of three individual Western blot analyses. Pre: pretreatment; Post: during treatment.

Figure 4 – Responses of the MEK/ERK pathway to PLX4720 in cultures established from biopsies taken during treatment

Whole cell lysates from indicated melanoma cultures with or without treatment with PLX4720 (10 μ M) for periods were subjected to Western blot analysis of phosphorylated MEK (p-MEK), MEK, phosphorylated ERK1/2 (p-ERK1/2), and ERK1/2. The data shown are representative of three individual Western blot analyses. Pre: pretreatment; Post: during treatment; p: passage.

Figure 4 – Cultures established from biopsies taken during treatment are less sensitive to apoptosis-induced by PLX4720

Melanoma cells from indicated cultures established from patient 1 (A), patient 3 (B), and patient 6 (C) were treated with PLX4720 (10 μ M) for 48 or 72 hours. Cell viability and apoptosis were quantitated by MTS assays and the PI method, respectively. The data shown are the mean $\pm$ SE of results from three individual culture wells.

Figure 1

A

B

Figure 2

Figure 3

Figure 4

Figure 4