PLX4032, a potent inhibitor of the B-Raf V600E oncogene, selectively inhibits V600E-positive melanomas

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KEYWORDS PLX4032/RG7204/B-Raf/melanoma/therapy/V600E

PUBLICATION DATA Received 10 August 2010, revised and accepted for publication 30 August 2010, published online 7 September 2010

Summary
Targeted intervention of the B-Raf V600E gene product that is prominent in melanoma has been met with modest success. Here, we characterize the pharmacological properties of PLX4032, a next-generation inhibitor with exquisite specificity against the V600E oncogene and striking anti-melanoma activity. PLX4032 induces potent cell cycle arrest, inhibits proliferation, and initiates apoptosis exclusively in V600E-positive cells in a variety of in vitro experimental systems; follow-up xenograft studies demonstrate extreme selectivity and efficacy against melanoma tumors bearing the V600E oncoproduct. The collective data support further exploration of PLX4032 as a candidate drug for patients with metastatic melanoma; accordingly, validation of PLX4032 as a therapeutic tool for patients with melanoma is now underway in advanced human (Phase III) clinical trials.

Introduction
Patients afflicted with metastatic melanoma display 5-year survival rates of approximately 10–15%. A major factor in this dismal prognosis is the relative absence of effective drugs to combat this deadly disease. Dacarbazine represents the sole FDA-approved drug for metastatic melanoma, but elicits durable responses in less than

Significance
Genotype-derived, patient-specific therapies are imminent. In malignant melanoma, the most frequent somatic mutation is the valine to glutamic acid base substitution at position 600 in BRAF (V600E); this mutation confers constitutive flux through the mitogen-activated protein kinase (MAPK) pathway and is believed to be an initiating event in malignant transformation. However, extensive efforts to pharmacologically inhibit the MAPK pathway in advanced melanoma for therapeutic benefit have largely failed. Next-generation inhibitors, tailored to inhibit only the mutant form of B-Raf, are now being investigated as alternatives to broadly acting inhibitors of the MAPK pathway. Here, we demonstrate the striking specificity of PLX4032 for melanoma cells expressing the V600E oncoproduct using a variety of experimental approaches; we also reveal that PLX4032 is highly efficacious in physiologically relevant, human-derived 3D-based platforms, which might better predict clinical success than more conventional methods. Collectively, our findings suggest that minimally toxic, but measurable responses can be achieved through tailored therapies that inhibit the mutant form of B-Raf, an oncogene product expressed in a majority of patients with melanoma.
5% of patients. Thus, most patients with melanoma are enrolled in clinical trials, where the promise of efficacy supersedes the poor response rates observed with dacarbazine.

Since the identification of a highly prevalent somatic mutation (V600E) of the BRAF gene in 2002 (Davies et al., 2002), several inhibitors of the Raf/MEK/ERK signal transduction cascade have been developed (Shepherd et al., 2010). Sorafenib represented a first-generation Raf inhibitor designed to inhibit aberrant MAPK signaling, but failed to display measurable responses in melanoma clinical trials (Eisen et al., 2006; Flaherty et al., 2008). Likewise, inhibitors of MEK also exhibited subpar results in the clinic (Dummer et al., 2008). These early failures led to the development of next-generation, oncogene-specific small molecules capable of inhibiting signals initiating from mutant, but not wild-type, B-Raf (King et al., 2006; Tsai et al., 2008).

PLX4032, a highly specific and potent inhibitor of the V600E mutant form of B-Raf, was developed from a scaffold-based screening platform (Tsai et al., 2008). This small molecule is currently under evaluation in advanced clinical trials for patients with metastatic melanoma. Results from these studies indicate that PLX4032 is effective in approximately 80% of patients harboring the V600E mutation while inducing minimal substantial toxicities (Flaherty et al., 2010). Phase III trials are currently under accrual.

Here, we report the preclinical characterization of PLX4032. PLX4032 displays potent anti-melanoma effects in a variety of in vitro and in vivo melanoma models and represents a highly exciting drug candidate for patients with few therapeutic alternatives.

Results and discussion

The BRAF oncogene is mutated in approximately half of the patients with melanoma (Nathanson, 2010) and is consequently a high-priority target for drug discovery and development. We previously published a scaffold- and structure-based discovery platform that identified PLX4720, a first-generation inhibitor from which PLX4032 was subsequently derived (Tsai et al., 2008). First, the specificity of PLX4032 on inhibition of B-Raf V600E was tested via immunoblotting analysis. A panel of melanoma cell lines expressing the V600E oncogene product, mutant NRAS, or wild-type BRAF/NRAS (Table 1) was assayed for activated ERK after treatment with varying concentrations of PLX4032. The compound displayed dose- and V600E-dependent inhibition of pERK for periods up to 72 h (Figure 1). As a comparison to a clinically relevant inhibitor of Raf, a panel of cells was also treated with Sorafenib; Sorafenib did not display the selectivity for mutant B-Raf that was observed with PLX4032 (data not shown).

Several groups have recently reported that mutant-specific B-Raf inhibitors will activate MAPK signaling in cells that do not express mutant BRAF (Halaban et al., 2010; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010), potentially initiating pro-tumorigenic effects in BRAF wild-type melanomas. Mechanistically, they demonstrate that the use of Raf inhibitors in tumors lacking Raf mutations can actually increase flux through the MAPK pathway through formation of Raf heterodimers. Our in vitro data do not demonstrate increased pERK levels after PLX4032 treatment in all cells expressing wild-type BRAF; we attribute this discrepancy to the substantially decreased amount of serum in our melanoma culture conditions (2%).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>B-Raf</th>
<th>N-Ras</th>
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<tbody>
<tr>
<td>WM35</td>
<td>V600E</td>
<td>Wild type</td>
</tr>
<tr>
<td>WM793</td>
<td>V600E</td>
<td>Wild type</td>
</tr>
<tr>
<td>1205Lu</td>
<td>V600E</td>
<td>Wild type</td>
</tr>
<tr>
<td>WM3434</td>
<td>V600E</td>
<td>ND</td>
</tr>
<tr>
<td>SbCln2</td>
<td>Wild type</td>
<td>Q61K</td>
</tr>
<tr>
<td>WM852</td>
<td>Wild type</td>
<td>Q61R</td>
</tr>
<tr>
<td>WM1361A</td>
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<td>Q61R</td>
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<tr>
<td>C8161</td>
<td>Wild type</td>
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<tr>
<td>WM3451</td>
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<td>ND</td>
</tr>
<tr>
<td>WM NCI1</td>
<td>Wild type</td>
<td>ND</td>
</tr>
</tbody>
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The BRAF and NRAS mutation status of all relevant melanoma cell lines is depicted. ND, not determined.

Figure 1. PLX4032 Inhibits Raf Signaling in a V600E-dependent Manner. A panel of melanoma cell lines with either mutant (left panel) or wild-type (right panel) B-Raf was treated with increasing doses of PLX4032 for 2 h before lysates were collected for immunoblotting. pERK levels were measured to determine activity of the Raf/MEK/ERK signaling cascade both pre- and post-treatment. β-actin serves as a loading control.
Figure 2. In vitro activity of PLX4032 in Melanoma. (A) Proliferation of V600E+ (left panel) or wild-type B-Raf (right panel) cells in increasing concentrations of PLX4032. (B) Colony formation assay; cells were seeded in 6-well dishes and exposed to 100 nM and 1 µM PLX4032 for 14 days. Surviving cells were stained in methylene blue and photographed. (C) Representative cell cycle analysis of mutant (1205Lu) and wild-type (C8161) melanoma cells in response to PLX4032 treatment. (D) Annexin V/PI staining of mutant and wild-type B-Raf cells at 24-h intervals after treatment with 1 µM PLX4032.
Figure 3. PLX4032 Exhibits Anti-melanoma Activity in 3D-based Cellular Models. (A) Collagen-embedded melanoma spheroids from established lines were treated with indicated doses of PLX4032 for 72 h and stained for viability with calcein-AM (green) and ethidium bromide (red). (B) Primary spheroids from freshly isolated human melanomas were embedded in collagen and exposed to PLX4032 for 72 h, followed by staining as described in 3A. (C,D) Artificial skin reconstructs were generated with either mutant or wild-type B-Raf cells and treated with 1 μM PLX4032 for 72 h before harvesting and immunostaining for the indicated protein markers; Ki67 indicates proliferation, TUNEL represents apoptosis.

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PI3K pathways is an effective therapy for advanced melanoma (Krasilnikov et al., 2003; Meier et al., 2007).

Because an overwhelming majority of preclinical drug candidates fail during clinical development, it would appear that preclinical characterization of drug potential merits improvement. For some time, we have argued that three-dimensional (3D) based assays are more predictive of clinical efficacy because these models incorporate many of the tumor microenvironmental features (i.e., stromal cells and extracellular matrices) otherwise not present in traditional 2D approaches. First, melanoma spheroids were embedded into a collagen matrix and overlaid with increasing concentrations of PLX4032. The results again demonstrate specificity against mutant B-Raf, while leaving wild-type B-Raf unperturbed (Figure 3A). Next, primary spheroids were generated from freshly harvested human tumor specimens to minimize genotypic artifacts associated with prolonged in vitro culture. These experiments demonstrated similar effects against the V600E oncogene product (Figure 3B).

The in vitro and ex vivo data from the spheroid experiments suggested that PLX4032 may be effective in complex, heterogenous tumors. Before testing the compound in vivo, we employed the use of human artificial melanoma skin reconstructs. This complex model incorporates extracellular matrices, as well as the primary cellular entities found within a melanotic tumor including fibroblasts, keratinocytes, and melanoma cells (Meier et al., 2000). Melanoma skin reconstructs were generated for approximately 21 days and subsequently overlaid with PLX4032 for 72 h before harvesting. The reconstructs were paraffin-embedded, sectioned, and stained for markers of proliferation (Ki67; Figure 3C) and apoptosis (TUNEL; Figure 3D). Results from this human model showed that PLX4032 is capable of decreasing proliferation, as well as inducing apoptosis in mutant B-Raf melanomas; non-transformed cells, however, appear to be unaffected by the compound indicating that therapeutic toxicities in patients may be minimal.

SCID mice were subsequently used in xenograft studies to ascertain the effects of PLX4032 in vivo. Highly tumorigenic B-Raf mutant (1205Lu) or B-Raf wild-type (C8161) metastatic melanoma cells were subcutaneously injected into the flanks of animals and tumors were allowed to reach palpability before drug intervention. PLX4032-treated animals displayed no visible signs of toxicity at regimens as high as 100 mg/kg per BID. In control-treated animals, tumor volume increased rapidly (Figure 4A,B). B-Raf wild-type xenografts treated with PLX4032 grew at similar rates to control tumors (Figure 4A). V600E-positive tumors, however, were largely abolished by PLX4032 treatment indicating that its activities are largely cytotoxic, not cytostatic (Figure 4B).

Tumors were harvested from mice, paraffin-embedded, and immunohistochemistry performed to determine the effects of PLX4032 on key indicators of cell
proliferation. As expected, pERK levels were significantly diminished, exclusively in mutant BRAF tumors (Figure 4C); likewise, Ki67 was equally reduced, suggesting that the proliferative index of these tumors was severely compromised (Figure 4D). Lastly, plasma drug levels were obtained at various time points on the first and last day of treatment to assess the pharmacokinetic profile of PLX4032 in this xenograft model. After 15 days of treatment, plasma drug levels were approximately 100 μM, a number congruent with the levels currently achieved in the ongoing clinical trials (Flaherty et al., 2010). Taken together, the data strongly support further development of PLX4032 for treatment of patients with melanoma expressing the mutant form of BRAF.

A number of Raf and MEK inhibitors have demonstrated promising preclinical results in vitro and in xenograft models, but subsequently failed in human trials. Functional redundancy between isoforms of these kinases had previously been implicated in the lack of clinical response (Montagut et al., 2008). More recent evidence suggests that broad inhibition of MEK impairs T-lymphocyte function, when compared against inhibition of the V600E oncoprotein (Boni et al., 2010); this phenomenon may explain why broadly acting inhibitors of Raf (sorafenib) and MEK (AZD6244) exhibit little activity in the clinic, where immunomodulation of disease is more prominent than in immune-deficient xenograft models. The recent success of ipilimumab in Phase-III clinical trials underscores the involvement of the immune system in achieving optimal therapeutic response in melanoma (Hodi et al., 2010). Future trials will likely implement both a small molecule arm and an immune effector component to determine whether synergistic activity is possible.

The ‘next wave’ of research in targeted therapy in melanoma will likely center on the emergence of Raf inhibitor-resistant clones. Indeed, data from clinical trials has indicated that, despite high response rates, nearly all patients eventually relapse. The mechanism(s) underlying this therapeutic escape will lead to next-generation
treatment regimens that may render Raf-resistant melanomas susceptible to alternative therapies. We recently reported two plausible resistance mechanisms including upregulation of the receptor tyrosine kinase, IGF1R (Villanueva et al., 2009), and emergence of a minor subpopulation of melanoma cells expressing a histone demethylase, JARID1B (Roesch et al., 2010). In both cases, it appears that melanoma cells are extremely plastic and can readily adapt to extreme selective pressures.

Acknowledgements

This work was supported by funding to M.H. from the NIH (RO1CA114046).

References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PLX4032 Induces Activation of mitogen-activated protein kinase Signaling in Primary Melanocytes. Foreskin-derived primary melanocytes (FOM180) were subjected to 1 μM PLX4032 for 24 h before immunoblotting analysis for pERK. β-actin levels indicate equal loading between sample lanes.

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