Sustained Inhibition of Receptor Tyrosine Kinases and Macrophage Depletion by PLX3397 and Rapamycin as a Potential New Approach for the Treatment of MPNSTs

Parag P. Patwardhan¹, Oliver Surriga¹, Michael J. Beckman³, Elisa de Stanchina², Ronald P. Dematteo³, William D. Tap¹, and Gary K. Schwartz⁴

Abstract

Purpose: Malignant peripheral nerve sheath tumor (MPNST) is a highly aggressive tumor type that is resistant to chemotherapy and there are no effective therapies. MPNSTs have been shown to have gene amplification for receptor tyrosine kinases (RTK), PDGFR and c-Kit. We tested the c-Kit inhibitor, imatinib, and PLX3397, a selective c-Fms and c-Kit inhibitor, to evaluate their efficacy against MPNST cells in vitro and in vivo.

Experimental Design: We tested the efficacy of imatinib or PLX3397 either alone or in combination with TORC1 inhibitor rapamycin in a cell proliferation assay in vitro and by immunoblotting to determine target inhibition. Immunoblotting and immunohistochemical analysis was further carried out using xenograft samples in vivo.

Results: Our in vitro studies show that imatinib and PLX3397 similarly inhibit cell growth and this can be enhanced with rapamycin with comparable target specificity. However, in vivo studies clearly demonstrate that compared with imatinib, PLX3397 results in sustained blockade of c-Kit, c-Fms, and PDGFRβ, resulting in significant suppression of tumor growth. Moreover, staining for Iba-1, a marker for macrophages, indicates that PLX3397 results in significant depletion of macrophages in the growing tumors. The combination of PLX3397 and rapamycin results in even greater macrophage depletion with continued growth suppression, even when the drug treatment is discontinued.

Conclusions: Taken together, our data strongly suggest that PLX3397 is superior to imatinib in the treatment of MPNSTs, and the combination of PLX3397 with a TORC1 inhibitor could provide a new therapeutic approach for the treatment of this disease. Clin Cancer Res; 20(12); 1–13. ©2014 AACR.

Introduction

Malignant peripheral nerve sheath tumors (MPNST) are soft-tissue tumors with a very poor prognosis (1). They are highly aggressive and therapeutically resistant tumors that arise in connective tissue surrounding peripheral nerves. MPNSTs occur in a subset of patients with Neurofibromatosis type 1 (NF1), an autosomal dominant genetic disorder (2, 3). There is a 10% chance that patients with NF1 will develop MPNSTs in their lifetime (4, 5). Despite advances in cancer treatment, MPNSTs typically remain fatal and there is an unmet need to develop new therapeutic strategies in this disease setting.

Growth factor–dependent pathways driven specifically by receptor tyrosine kinases (RTK) have been of particular interest due to their crucial role in tumor progression and survival. Targeting single or multiple RTK pathways using small-molecule inhibitors is an attractive chemotherapeutic treatment option for blocking sarcoma cell growth. Such inhibitors have been used in patients with sarcoma with some promising results (6, 7). In addition to the RTKs, mTOR protein plays a key role in AKT activation and downstream survival signaling. Blocking RTK signaling pathways such as c-Kit and PDGFR using multitargeted inhibitors like imatinib has been used with some positive results in vitro in MPNSTs (8–10). However, MPNSTs still remain one of the most challenging sarcoma subtypes to treat and novel therapeutic approaches are urgently needed to treat this disease.

MPNSTs have been shown to have gene amplification for receptor tyrosine kinases such as PDGFR as well as c-Kit (9, 11). The role of c-Kit oncogenic mutations in gastrointestinal stromal tumors (GIST) is well established (12, 13). PDGFRα has also been shown to be amplified in tumors...
and in cell lines from patients with MPNSTs. c-Kit was similarly amplified in 4 of the 5 tumors and patient-derived cell lines. MPNSTs can also carry PDGFRα mutations. No mutations in c-Kit have been reported. Imatinib mesylate (Imatinib), an inhibitor of c-Kit and PDGFR, which is approved for the treatment of GISTs, has been shown to be active in patients with plexiform neurofibromas, a slow-growing, chemotherapy-resistant tumor that develops in patients with neurofibromatosis (14). However, in clinical trials, response rates to imatinib in neurofibromatosis are only in the order of 17%, indicating that alternate signaling pathways must be involved in the growth and development of tumors associated with NF1 loss (14). Although imatinib has never been formally tested in MPNSTs, it has been evaluated in patients affected by NF1 loss who developed GISTs. Similar to patients with MPNSTs, the overall prognosis of this patient population is poor, and the response to imatinib in NF1-associated GIST is very low (15).

In this study, we tested the multitargeted tyrosine kinase inhibitor, PLX3397. PLX3397 selectively inhibits c-Fms and c-Kit receptor tyrosine kinases with biochemical IC_{50} values of 0.02 and 0.01 μmol/L, respectively (16). Recently, it has been reported that macrophage infiltration of both mouse and human neurofibromas correlates with disease progression. Macrophages account for almost half of the neurofibroma cells. In the Dhh-Cre/Nf1 mouse model of neurofibroma, PLX3397 has been shown to cause neurofibroma regressions and to block macrophage infiltration (17). In this study, we elected to compare imatinib with PLX3397 against MPNST cells. Our studies indicate that PLX3397 is a superior in vivo kinase inhibitor and results in significantly greater suppression of tumor volume and macrophage depletion.

Furthermore, inhibition of mTOR signaling, which is also active in this disease, with rapamycin further enhances this effect. Findings from our studies suggest that PLX3397 in combination with an mTOR inhibitor is biologically active in MPNSTs and should be explored for future clinical development in patients with MPNSTs.

Materials and Methods

Drugs

PLX3397 (Fms/Kit inhibitor) and vehicle carrier, were provided by Plexikon Inc. in the form of powder for in vitro studies, or chow containing PLX3397 or control chow, respectively, for in vivo studies. Imatinib and rapamycin were purchased from LC Laboratories.

Cell lines

MPNST and ST8814 cell lines have described previously (18). GIST882 cell line has also been described previously (19). Briefly, MPNST was derived from a patient with a high-grade peripheral nerve sheath tumor of the thigh. ST8814 was derived from a patient with NF1-associated MPNST of the thigh. Dr. Jonathan Fletcher (Dana Farber Cancer Institute, Boston, MA), graciously supplied both these cell lines. In ST8814, a nonsense mutation (C910T) in codon 304 of exon 7 of NF1 has been reported (Y. Kloog, Tel Aviv University, Israel; personal communication to Dr. Schwartz). This is a pathogenic mutation leading to exon 7 skipping. These cell lines have been authenticated using short tandem repeat DNA fingerprinting (20, 21). MPNST cells were able to form tumors when injected in nude mice; however, we were unable to grow ST8814 tumors in mice. All the three cell lines were maintained in RPMI-1640 media containing 10% FBS (HyClone, Thermo Scientific). For serum starvation, cells were plated in RPMI-1640 media without FBS overnight and then treated with the drugs in RPMI-1640 media containing 10% FBS overnight and then treated with the drugs in RPMI-1640 media containing 10% FBS for the indicated time.

Cell viability assays

Cell viability assays were carried out with the Dojindo Molecular Technologies Kit per manufacturer's instructions. Briefly, 2,000 cells were plated in 96-well plates in RPMI media with 10% FBS and then treated with the indicated drugs the next day. Media were replaced with 100 μL of media with 10% serum and 10% CCK-8 solution (Dojindo Molecular Technologies Kit). After 1 hour, the optical density was read at 450 nm using a Spectra Max 340 PC (Molecular Devices Corp.) to determine viability. Background values from negative control wells without cells were subtracted for final sample quantification. Data were plotted as % cell viability compared with dimethyl sulfoxide (DMSO; no drug) control.

Western blots

Western blots were carried out as described previously (18). Briefly, cell lysates were prepared by washing the cells once with sterile PBS followed by scraping in...
radioimmunoprecipitation assay (RIPA) lysis buffer. For xenograft tissues, lysates were prepared by cutting the snap-frozen tumor tissue into a small piece and then grinding it in RIPA lysis buffer using sample grinding kit (GE Healthcare). Protein concentrations were measured using Bio-Rad protein assay dye (Bio-Rad) and equal amounts of protein (20 or 30 μg) were loaded on 4% to 12% gradient gels (Invitrogen) and transferred to polyvinylidene fluoride membrane (Immobilon, Millipore) or nitrocellulose membrane (0.45 micron, Thermo Scientific) for the detection of phosphorylated c-Kit. After blocking with 5% milk, membranes were probed with primary antibodies overnight. Bound antibodies were detected with horseradish peroxidase secondary antibodies (GE Healthcare) and visualized by enhanced chemiluminescence reagent (GE Healthcare).

**Xenograft studies**

Briefly, MPNST xenografts were transplanted subcutaneously in the flank of ICR/SCID mice. Once tumors reached a volume of 80 to 100 mm³, the mice were randomized into different groups of 7 to 10 animals each and treated with the indicated drugs or vehicle control. Imatinib was used at a concentration of 30 mg/kg i.p. QD, 5 days a week and rapamycin was used at a concentration of 20 mg/kg i.p. on Monday, Wednesday, and Friday. For studies involving PLX3397, mice were fed a diet with either control chow or PLX3397 chow for 3 weeks. Tumor size was measured twice weekly by caliper. The average tumor volume in each group was expressed in cubic millimeter and calculated using the formula \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \). Approximately, 1 to 2 animals in each group were sacrificed after 3 weeks of drug treatment and the resected tumors were divided for formalin fixation (for hematoxylin/eosin staining and immunohistochemistry) and snap-frozen tissue (for western blot analysis). Experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol, and institutional guidelines for the proper and humane use of animals were followed.

**Analysis of tumor-associated macrophages**

Tumor-associated macrophages (TAM) were isolated from control and drug-treated xenograft samples \( (n = 3 \) animals per group) as described previously (22). Briefly, tumor cells were isolated by mincing the tumor in collagenase/DNAase in Hank’s balanced salt solution followed by flow cytometric analysis using indicated antibodies. Data were plotted using the GraphPad Prism software with relevant population percentages plotted with ANOVA and \( t \) test performed and indicated where appropriate.

**Iba-1 and Ki67 staining and quantitation**

Preparation of tissue sections for immunohistochemistry and staining for Iba-1 and Ki67 was carried out by Memorial Sloan Kettering Cancer Center (New York, NY) molecular cytology core facility. The immunohistochemistry detection of Iba1 and Ki67 was performed using Discovery XT processor (Ventana Medical Systems). Staining and quantitation details are included in the Supplementary Methods.

**Statistical analysis**

*In vitro* experiments were carried out at least three times unless otherwise indicated. Error bars shown in the graphs represent SD. All the graphs were plotted using GraphPad Prism software. Statistical analyses were carried out using Student \( t \) test with 95% confidence interval.

**Results**

**PLX3397 treatment downregulates RTK phosphorylation and inhibits cell proliferation in MPNST and GIST cells**

PLX3397 is a selective inhibitor of c-Fms and c-Kit tyrosine kinases with *in vitro* IC₅₀ values of 0.02 and 0.01 μmol/L, respectively. Out of a panel of 226 different kinases, including all the protein kinase subfamilies and lipid kinases, PLX3397 inhibited only five other kinases with low IC₅₀ values (16). As PLX3397 is a highly selective c-Fms/c-Kit inhibitor, we tested eight sarcoma cell lines to check for basal expression levels of c-Fms and c-Kit along with a GIST cell line, GIST882, as a positive control for c-Kit expression. Detectable levels of c-Kit expression were observed only in two MPNST cell lines, MPNST and ST8814, in addition to GIST882 (Supplementary Fig. S1). However, c-Fms expression levels were detectable in all the cell lines tested. GIST882 had the highest levels of c-Kit (Supplementary Fig. S1). On the basis of this finding, we carried out an *in vitro* cell proliferation assay using different doses of PLX3397 in the two MPNST cell lines, MPNST and ST8814 as well as in the GIST cell line, GIST882. As seen in Fig. 1A, both MPNST cell lines and GIST882 cell line were susceptible to inhibition of proliferation by PLX3397. The IC₅₀ value for the GIST cell line was approximately 0.1 μmol/L, whereas for the MPNST cell lines, IC₅₀ values were approximately between 0.5 to 1 μmol/L. The GIST cells, which harbor a c-Kit mutation and have a higher basal expression of p-KIT, were more susceptible to growth inhibition by PLX3397 than the MPNST cells. Western blot analysis using various concentrations of PLX3397 confirmed that p-KIT, a known target of the drug, was inhibited in all the three cell lines tested (Fig. 1B). In addition to p-KIT inhibition, AKT phosphorylation (p-AKT5473) was also inhibited (Fig. 1B) with increasing drug concentration suggesting a role for c-Kit in inducing AKT phosphorylation in these cell lines. Tyrosine phosphorylation of c-Fms (Y546), another target of PLX3397, was inhibited only in the GIST cell line but not in MPNST cell lines. GIST cells showed higher basal levels of p-KIT (Y703), whereas MPNST cell lines showed higher basal levels of p-Fms (Y546; Fig. 1B).

**Phosphorylated c-Fms kinase is not downregulated in MPNST cells in vitro**

As shown in Fig. 1B, phosphorylation of c-Fms at Tyr546 was not downregulated in MPNST cells compared with
Figure 1. Effects of PLX3397 in GIST, MPNST and ST8814. A, GIST, MPNST, and ST8814 cells were plated in 96-well plates and treated in triplicates with increasing doses of PLX3397 for 144 hours. Cell viability was measured using Dojindo Cell Counting Kit 8. B, the cells were grown to 60% confluence in 60 mm plates and treated for 24 hours with DMSO control or increasing concentrations of PLX3397 (0.5, 1, and 2 μmol/L). Thirty micrograms of RIPA lysates were loaded on SDS/PAGE and immunoblotted using indicated antibodies. C and D, cells were grown to 60% confluence in 100 mm plates and treated for 24 hours with DMSO control, 1 μmol/L imatinib, or 1 μmol/L PLX3397. Protein lysates were then extracted and used for p-Fms and total Fms sandwich ELISA following the manufacturer’s protocol for DuoSet IC ELISA Kit by R&D Systems.
GIST cells. To test whether PLX3397 can downregulate tyrosine phosphorylated c-Fms in MPNST cells, we carried out an in vitro ELISA assay to test the efficacy of PLX3397 in blocking overall c-Fms tyrosine phosphorylation instead of detecting phosphorylation at Tyr546 only. Imatinib has been shown to inhibit c-Fms at therapeutic concentrations (23, 24). Our ELISA results showed that tyrosine phosphorylation of c-Fms is inhibited neither by imatinib nor by PLX3397 in MPNST cells (Fig. 1C). However, in GIST cells, both imatinib and PLX3397 inhibited c-Fms tyrosine phosphorylation by 40% to 50% compared with no drug control (P < 0.01; Fig. 1C). This result is consistent with the results obtained using Western blot analysis (Fig. 1B). Total levels of c-Fms remained unchanged after the drug treatment in all the cell lines tested (Fig. 1D).

PLX3397 and imatinib inhibit MPNST cell proliferation and downregulate downstream targets effectively in vitro

As PLX3397 has recently been shown to impair tumor maintenance in mouse models of MPNSTs (17), we decided to focus our subsequent in vitro and in vivo experiments in the two MPNST cell lines.

RTK/P13K/AKT/mTOR pathways have been shown to be critical for oncogenic growth and survival in soft-tissue sarcomas. TORC1 downstream effectors, including S6 kinase (S6K) and S6 ribosomal protein (S6), a 40S ribosomal component, are critical in regulation of protein translation and cell proliferation (25). mTOR signaling has been shown to be particularly important for MPNST growth. However, mTOR inhibitors have shown minimal activity as single agents in sarcoma clinical studies (26, 27). PLX3397 does not inhibit TORC1 downstream targets including S6K and S6 ribosomal protein (S6; Fig. 1B). Therefore, we elected to test in the MPNST cells whether the combination of PLX3397 with the TORC1 inhibitor, rapamycin, could more effectively inhibit cell proliferation and to assess how this affected AKT and mTOR signaling. Results from the in vitro cell proliferation assay (Fig. 2A) show that either PLX3397 or imatinib, when combined with rapamycin, were more efficient in inhibiting MPNST cell proliferation than any single-agent drug treatment. Single-agent treatment resulted in approximately 40% to 45% decrease in cell viability compared with no drug control, whereas combination therapy resulted in approximately 70% inhibition of cell proliferation (P < 0.005; Fig. 2A). Western blot analysis confirmed inhibition of p-Kit (Y703) and p-PDGFRβ (Y751) with both single agent and combination therapy (Fig. 2B). Treatment with PLX3397 or imatinib alone did not inhibit p-S6 and treatment with rapamycin alone did not inhibit either p-Kit or p-PDGFRβ. Rapamycin alone was able to activate p-Kit, p-PDGFRβ, and p-AKT presumably through the release of negative feedback mechanisms (28). However, the combinations were effective in inhibiting phosphorylation of both RTKs as well as phosphorylation of AKT and S6. Similar to earlier results (Fig. 1B and C), p-Fms was not inhibited in MPNST cell lines with any of the drug treatments. Induction of apoptosis was confirmed by PARP cleavage upon treatment with either PLX3397 or imatinib (Fig. 2B, top) but it was more pronounced when either drug was combined with rapamycin. To test whether c-Kit or PDGFRβ or both play a role in inhibiting cell proliferation in vitro, we carried out siRNA-mediated knockdown of both the RTKs either alone or in combination. PDGFRβ knockdown resulted in approximately 50% inhibition, whereas c-Kit knockdown resulted in a modest 30% inhibition of cell proliferation (Supplementary Fig. S2) suggesting a greater role of PDGFRβ in cell proliferation. However, combined knockdown of both c-Kit and PDGFRβ resulted in approximately 70% inhibition of cell proliferation (Supplementary Fig. S2). PDGFRβ has not been evaluated as a target for PLX3397. To test whether PDGFRβ is a direct target of the drug, we carried out ligand stimulation experiment and tested for inhibition of p-PDGFRβ in MPNST and ST8814 cell lines. As expected, no p-PDGFRβ signal was detected under no serum conditions, but when cells were stimulated with PDGFB/B, p-PDGFRβ was inhibited only in cells treated with PLX3397 but not with rapamycin alone (Supplementary Fig. S3). Many of the small-molecule inhibitors that block RTK pathways such as sorafenib and lapatinib have been considered as cytokastic agents rather than cytotoxic drugs (29). Figures 1A and 2A suggest that the effects of PLX3397 appear to be cytokastic rather than cytotoxic. To test this, we carried out flow cytometric cell-cycle analysis after PLX3397 treatment either as single agent or in combination with rapamycin in MPNST and ST8814 cell lines. Treatment with PLX3397 but not rapamycin alone induced a G1 cell-cycle arrest (Supplementary Fig. S4A), which was more pronounced when the two drugs were combined. Cyclin D1 downregulation has been shown to play a role in G1 cell-cycle arrest in other cancer cell lines (30). Western blot analysis after treatment with imatinib or PLX3397 alone induced significant downregulation of cyclin D1 and a significant decrease in hyperphosphorylated form of retinoblastoma protein (p-Rb) (Supplementary Fig. S4B). Total levels of Rb and p53 remained unchanged.

PLX3397 treatment results in sustained inhibition of RTKs and inhibits macrophage infiltration by blocking c-Fms

Imatinib and PLX3397 both have overlapping target specificity in vitro and were similarly effective in blocking in vitro cell proliferation in MPNST cells (Fig. 2A). Therefore, we carried out mouse xenograft experiments to test whether the in vivo target inhibition profile of the two drugs was also similar. As shown in Fig. 3A and B, at the end of 3-week treatment, imatinib and rapamycin when used in combination resulted in enhanced suppression of tumor volume compared with either of the drugs alone (P < 0.005). However, at completion of treatment (week 3), when xenograft lysates were analyzed by Western blot analysis, none of the target tyrosine kinases or TORC1 effectors (S6) were effectively downregulated (Fig. 3C). As the animals were sacrificed approximately 24 hours after the last drug treatment, we hypothesized that the signaling pathways were
reactivated in the 24 hours after the treatment. To test this hypothesis, we carried out a time course experiment in MPNST xenografts, wherein the animals were sacrificed 8, 16, or 24 hours after the treatment on day 1. As shown in Fig. 3D, imatinib alone inhibited p-Kit and p-PDGFRβ but not p-S6. Phosphorylation of S6K, a known mTORC1 target, was downregulated effectively confirming that the mTOR pathway is downregulated by rapamycin in vivo. After 8 hours of treatment, p-Kit, p-PDGFRβ, p-AKT, and p-S6K were only inhibited in mice treated with imatinib in combination with rapamycin (Fig. 3D). A stronger signal for PARP cleavage was seen with the combination of imatinib and rapamycin as compared with imatinib alone (Fig. 3D). After 16 hours, p-Kit, p-PDGFRβ, and p-AKT were reactivated even in animals treated with the imatinib and rapamycin combination suggesting that the signaling pathways are only transiently inhibited after this drug treatment (Fig. 3D).

Next, we elected to repeat the mouse xenograft experiments using PLX3397. As shown (Fig. 4A and B), PLX3397 treatment resulted in significant tumor growth suppression when used either alone or in combination with rapamycin (P < 0.005). As long as the animals were treated with PLX3397, the tumor was maintained at a significantly lower volume than vehicle control or rapamycin alone (Fig. 4A and B). We did not observe any significant difference between PLX3397 alone versus PLX3397 and rapamycin combination in terms of tumor volume suppression (Fig. 4A and B). Therefore, we decided to test whether there was a
Figure 3. Effect of imatinib and/or rapamycin in MPNST xenografts. A and B, tumor growth of MPNST xenografts treated with the indicated drugs for a period of 3 weeks is shown. See Materials and Methods for drug dosing schedule. C and D, 30 μg of RIPA lysates obtained using sample grinding kit (GE Healthcare) from xenograft tissues at the end of 3-week treatment or at various time points posttreatment were loaded on SDS/PAGE and immunoblotted using indicated antibodies.
The difference between the tumor regrowth when the drug treatments were discontinued. The drug treatment was discontinued after 3 weeks, and the mice were monitored for increase in tumor volume for an additional 3 weeks. Surprisingly, tumors treated with PLX3397 alone grew back rapidly, whereas mice treated with PLX3397 and rapamycin in combination showed a slower increase in tumor regrowth (Fig. 4A and B). Western blot analysis was carried out to determine whether PLX3397 inhibited p-Kit and p-PDGFRβ. Similar to imatinib treatment, the animals were sacrificed approximately 24 hours after the completion of treatment on week 3. However, unlike imatinib, PLX3397 was able to achieve sustained downregulation of p-Kit and p-PDGFRβ signaling, even after 24 hours after the last drug treatment. Furthermore, in contrast with the effect of PLX3397 in vitro, p-Fms was significantly inhibited in vivo (Fig. 4C). We were unable to detect consistent expression levels of total S6 protein in these xenograft lysates; therefore, phosphorylation levels of S6 could not be determined. Nevertheless, p-AKT was inhibited significantly when animals were treated with PLX3397 alone and p-AKT was further downregulated when PLX3397 was used in combination with rapamycin (Fig. 4C). Ki67 staining indicated a greater inhibition of cell proliferation with PLX3397 than with rapamycin (Supplementary Fig. S5A and S5B). Treatment with PLX3397 and rapamycin resulted in even higher inhibition of cellular proliferation than when treated with PLX3397 alone \((P < 0.005)\). Xenograft lysates obtained from animals sacrificed 3 weeks after discontinuing the drug treatment showed that all the signaling pathways were reactivated when the drug treatments were discontinued (Fig. 4C, lanes 5 and 6). Staining for CD31, a known endothelial cell marker, did not show any significant change after PLX3397 treatment compared with control group.
Inhibition of RTKs and mTOR in MPNST

A

Vehicle  
Rapamycin  
Control diet  
Rapamycin

Imatinib  
Imatinib + rapamycin  
PLX3397  
PLX3397 + rapamycin

B

P < 0.05  
NS

P < 0.0001

C

3 weeks after the end of treatment

PLX3397  
PLX3397 + rapamycin

D

P < 0.05

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(Supplementary Fig. S5C). As reported previously (31), rapamycin treatment alone caused a reduction in CD31 signal indicating reduced tumor vasculature (Supplementary Fig. S5C).

Ionized calcium-binding adapter molecule 1 or Iba-1 is a protein expressed specifically by macrophage/microglia cells and is involved in tumor growth and maintenance mediated through c-Fms kinase (32, 33). To test whether macrophage population was affected by imatinib and PLX3397 drug treatment, we carried out immunohistochemical staining to detect Iba-1. As shown in (Fig. 5A and B, imatinib treatment alone did not affect Iba-1 signal as compared with vehicle control ($P = 0.365$; not significant). However, Iba-1 signal was decreased significantly compared with vehicle control, when mice were treated with rapamycin either as a single agent or in combination with imatinib ($P < 0.05$; Fig. 5A and B).

Similar to previously published reports (17, 34), PLX3397 significantly decreased macrophage population measured as Iba-1 signal ($P < 0.0001$; Fig. 5A and B). In contrast with imatinib, the effect of rapamycin and PLX3397 was greater than either agent alone ($P < 0.05$). Next, we determined Iba-1 signal in tumor samples obtained 3 weeks after stopping the drug treatment. As expected, Iba-1 signal increased (Fig. 5A and B vs. C) when PLX3397 treatment was discontinued. However, when compared with PLX3397 alone after discontinuation, Iba-1 signal remained significantly suppressed at least for the first one week with the combination therapy of PLX3397 and rapamycin (Fig. 5C and D).

**Macrophage depletion alone is not sufficient to suppress tumor growth in MPNST xenografts**

To test whether the effects of PLX3397 on tumor growth suppression are mediated by inhibition of RTKs and/or macrophage depletion, we carried out xenograft studies by treating the animals using c-Fms–specific drug (PLX5622) or a c-Fms–specific antibody (AFS98) either alone or in combination with rapamycin and compared it against PLX3397. As observed earlier, PLX3397 either alone or in combination with rapamycin suppressed tumor growth significantly (Supplementary Fig. S6A). Western blot analysis showed downregulation of target RTKs, including p-Kit, p-PDGFRβ, and p-AKT, was seen compared with PLX3397 (Supplementary Fig. S6B). As expected, c-Fms blockade by PLX5622 treatment resulted in macrophage depletion detected as reduced Iba-1 signal (Supplementary Fig. S6C). AFS98, a c-Fms–specific antibody, treatment also showed moderate p-Fms downregulation (Supplementary Fig. S7A) and, as expected, caused macrophage depletion detected as decreased percentage of live cells (Supplementary Fig. S7B). Though c-Fms blockade resulted in macrophage depletion (not comparable with PLX3397 however), it appeared that macrophage depletion alone was not sufficient to suppress tumor growth when xenografts were treated with PLX5622 or AFS98 alone (Supplementary Figs. S6A and S7C).

Combination with rapamycin further enhanced macrophage depletion (Supplementary Figs. S6C and S7B) and also resulted in enhanced tumor suppression (Supplementary Figs. S6A and S7C). Flow cytometric analysis showed that most of the TAM population was M2-like and rapamycin treatment alone caused M2-like TAMs to shift to more M1-like population (Supplementary Fig. S8). Such an effect of mTOR inhibition by rapamycin on macrophage polarization has been reported previously (35). Sufficient numbers of macrophages could not be isolated from other treatment groups to carry out flow cytometric M1-like versus M2-like sorting.

**Discussion**

MPNSTs that arise in patients with type 1 neurofibromatosis (NF1) are classically associated with activation of the Ras pathway by loss of function mutations in NF1, a gene which encodes the Ras-GTPase activating protein (GAP), neurofibromin (36). More recent evidence (9, 11, 37) has shown that several genes are amplified in a subset of MPNST cell lines along the 4q12 amplicon, some of which include genes for RTKs such as PDGFR, VEGFR, and c-Kit. Attempts have been made to identify "druggable" tyrosine kinases in MPNSTs (7, 38) and treatments have been targeted at downregulating pathways that include Src, PDGFR, IGF-1R, c-Kit, as well as c-Met and EGFR (9, 10, 20, 21, 39, 40).

PLX3397 is a novel multitargeted tyrosine kinase inhibitor that is selective for the M-CSF receptor kinase (c-Fms) and c-Kit. PLX3397 has recently been reported to have activity against breast cancer (16), acute myeloid leukemia (41), as well as neurofibromas (17). Although, it is a known c-Fms inhibitor, the exact mechanism of action of this drug.

**Figure 5.** Effect of imatinib and PLX3397 and/or rapamycin on Iba-1. A, xenograft tissues obtained from mice at the end of 3-week treatment with the indicated drugs were stained immunohistochemically using Iba-1 antibody. Scale bar (50 μm) is shown in the right hand corner of each image. Images from a tumor obtained at the end of 3-week treatment from a representative experiment reproduced at least three independent times are shown. B, Iba-1 signal (brown staining) was quantified using MetaMorph image analysis software (Molecular Devices). Iba-1 signal was quantified from at least 3 different high power fields and plotted as arbitrary intensity units. NS, not significant. Quantitation of Iba-1 signal from a tumor obtained from a representative experiment reproduced at least three independent times is shown. C, xenograft tissues obtained at the end of 1 week after stopping the drug treatment were stained immunohistochemically using Iba-1 antibody. Scale bar (50 μm) is shown in the right hand corner of each image. Images of a tumor obtained from a representative experiment reproduced at least two independent times are shown. D, Iba-1 signal (brown staining) was quantitated using MetaMorph image analysis software (Molecular Devices). Iba-1 signal was quantified from at least 3 different high power fields and plotted as arbitrary intensity units. Quantitation of Iba-1 signal from a tumor obtained from a representative experiment reproduced at least two independent times is shown.
especially in MPNST, has not been reported. In this study, we tested the effects of the drug on two MPNST cell lines that were derived from patients with MPNST tumors of the thigh, one of which was NF1 associated (18). Our results clearly demonstrate that PLX3397 inhibits cell proliferation in MPNST cells. When we tested the activity of the drug in vitro using Western blot analysis, we observed downregulation of c-Kit but not c-Fms even though both the MPNST cell lines express detectable levels of total c-Fms.

It has been recently demonstrated that tumor cell lines derived from NF1 patients, NF1-deficient cell lines as well as spontaneous tumors arising in a genetically engineered mouse model with NF1 and TP53 mutations were highly sensitive to the mTOR inhibitor rapamycin (42, 43), arguing NF1 loss mediates oncogenesis through mTOR activation. To explore the effect of combined inhibition of RTK pathways and TORC1 signaling, we carried out an in vitro proliferation assay as well as analyzed cell lysates by Western blot analysis confirmed p-Kit and p-PDGFRα downregulation irrespective of rapamycin combination. PDGFRβ has not been reported previously as a target for PLX3397. Rapamycin was able to downregulate TORC1 effectors such as S6 but activated p-AKT through release of negative feedback inhibition (28). Combined blockade of RTKs and TORC1 pathway resulted in significantly increased inhibition of MPNST cell proliferation compared with single-agent treatments. This may be in part due to the fact that both imatinib and PLX3397 block the reactivation of p-AKT induced by rapamycin presumably mediated by PDGFRβ and c-Kit signaling.

Imatinib has been shown to inhibit c-Fms phosphorylation at various therapeutic concentrations (23, 24). On the basis of our in vitro data, the target specificity of imatinib and PLX3397 is highly overlapping. To test this specificity in vivo, we carried out MPNST mouse xenograft experiments using single agent or combination treatments. Interestingly, imatinib as a single agent or in combination with rapamycin was unable to maintain sustained inhibition of the target tyrosine kinases as well as AKT phosphorylation compared with PLX3397. PLX3397 was clearly superior in terms of sustained target inhibition compared with imatinib and also resulted in significant tumor volume suppression when the animals were treated with the drug alone or in combination with rapamycin.

c-Fms (M-CSF1R) kinase is involved in macrophage/microglial activation (33). Iba-1 (Ionized calcium binding adapter molecule 1), is a macrophage/microglia-specific calcium-binding protein that has been shown to be involved in c-Fms-mediated tumor growth (32). Macrophages are typically divided into two types: classically activated or M1 and alternatively activated or M2 (44). M1 macrophages are considered antitumorigenic, whereas M2 macrophages are protumorigenic (45) and are required for tumor vascularization, invasion, and development. Novel therapeutic strategies aimed at targeting M2 or TAMs are currently being pursued with much interest (46). In a recent report (17), mouse models of neurofibromas showed that PLX3397 was highly effective in inhibiting macrophages in established tumors and therapeutically targeted macrophage depletion may result in impaired tumor maintenance.

Our immunohistochemical studies clearly demonstrated that PLX3397 was superior to imatinib in terms of macrophage depletion. Western blot analysis of xenograft lysates showed significant blockade of p-Fms as well as total c-Fms that was not seen in our in vitro studies strongly suggesting stromal down regulation of M-CSFR pathway in these tumors. Reduction in macrophage population can therefore be attributed to inhibition of c-Fms signaling in xenografts. Macrophage depletion could also have contributed to the reduction in total c-Fms levels detected on Western blot analysis. Surprisingly, mouse xenografts treated with a combination of PLX3397 and rapamycin showed decreased macrophage reimplantation when the drug treatments were discontinued. A recent study reported that TAMs (M2) are dependent on mTOR signaling and inhibition of mTOR signaling by rapamycin can block tumor growth and angiogenesis by preventing monocyte differentiation into M2 macrophages (47). Results from our Iba-1 staining and flow cytometric analysis indicated that rapamycin alone not only caused a decrease in macrophage population but also caused TAMs to shift from M2-like to more M1-like population partly explaining its potent antitumor effect in MPNST xenografts. Also, such a shift to M1-like population can possibly explain the observed lag time in tumor regrowth after PLX3397 and rapamycin treatment is discontinued. A recent study reported that PLX3397 in combination with anisited rapamycin treatment results in a switch to M1-like phenotype. This may cause a delay before macrophages become more M2-like again and support tumor growth when the drug treatments are discontinued. Studies targeting c-Fms inhibition resulted in macrophage depletion but such depletion alone was not sufficient to suppress tumor growth strongly suggesting a greater role played by RTKs in causing MPNST tumor growth and development.

Taken together, our data strongly suggest that sustained inhibition of RTK signaling pathways by PLX3397 in vitro makes it superior to imatinib, even though the target specificity of the two drugs is overlapping. Furthermore, even though neither imatinib nor PLX3397 inhibited p-Fms in vitro, only PLX3397 could inhibit p-Fms in vivo. This resulted in a marked depletion of macrophages and contributed to its significant antitumor effect. In addition, rapamycin was able to sustain this effect by decreasing cell proliferation and promoting further macrophage depletion as part of the combination therapy. PLX3397 is currently in clinical trials for various types of cancers, including lymphoma, glioblastoma, and metastatic breast cancer. Our results indicate that PLX3397 in combination with an
mTOR inhibitor should be considered the next step in drug development for patients with MPNSTs.

Disclosure of Potential Conflicts of Interest

W.D. Tap is a consultant/advisory board member of Plexxikon. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: P.P. Patwardhan, E.D. Stanchina, W.D. Tap, G.K. Schwartz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.P. Patwardhan, O. Surriga, M.J. Beckman, E.D. Stanchina, R. Dematteo, G.K. Schwartz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.P. Patwardhan, O. Surriga, M.J. Beckman, R. Dematteo, W.D. Tap, G.K. Schwartz
Writing, review, and/or revision of the manuscript: P.P. Patwardhan, M.J. Beckman, R. Dematteo, W.D. Tap, G.K. Schwartz

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.P. Patwardhan, O. Surriga, G.K. Schwartz

Study supervision: P.P. Patwardhan, W.D. Tap, G.K. Schwartz

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Parag P. Patwardhan, Oliver Surriga, Michael J. Beckman, et al.

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