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An adaptive Src–PDGFRA–Raf axis in rhabdomyosarcoma

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ABSTRACT

Alveolar rhabdomyosarcoma (aRMS) is a very aggressive sarcoma of children and young adults. Our previous studies have shown that small molecule inhibition of Pdgfra is initially very effective in an aRMS mouse model. However, slowly evolving, acquired resistance to a narrow-spectrum kinase inhibitor (imatinib) was common. We identified Src family kinases (SFKs) to be potentiators of Pdgfra in murine aRMS primary cell cultures from mouse tumors with evolved resistance *in vivo* in comparison to untreated cultures. Treating the resistant primary cell cultures with a combination of Pdgfra and Src inhibitors had a strong additive effect on cell viability. In Pdgfra knockout tumors, however, the Src inhibitor had no effect on tumor cell viability. Sorafenib, whose targets include not only PDGFRA but also the Src downstream target Raf, was effective at inhibiting mouse and human tumor cell growth and halted progression of mouse aRMS tumors *in vivo*. These results suggest that an adaptive Src–Pdgfra–Raf–Mapk axis is relevant to PDGFRA inhibition in rhabdomyosarcoma.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma in children [1]. This sarcoma of myogenic differentiation has two major subtypes, alveolar and embryonal. Alveolar rhabdomyosarcoma (aRMS) is frequently metastatic and even with the advent of intensified therapy, no significant improvement in outcome has occurred in nearly four decades [2,3]. In the majority of aRMS the chromosomal translocation t(2;13)(q35;q14) results in PAX3:FOX01A fusion gene [4]. This chimeric PAX3:FOX01A transcription factor has been shown to cause inappropriate activation of target genes including Pdgfra [5]. For this reason, molecularly-targeted therapies targeting Pdgfra are of great interest [5]. The conditional mouse model of aRMS expressing Pax3:Fox01A and having homozygous deleted *p53* is a useful tool our group has used for preclinical interrogation of PDGFRA as a molecular target [6].

Imatinib is a prototypic small molecule inhibitor of PDGFRA and PDGFRB [7]. Despite the tremendous success of this tyrosine kinase inhibitor therapy for CML and GIST, subsets of patients become resistant to imatinib [8,9]. Our previous studies firmly established PDGFRA as a prevalent target in aRMS, and to be functionally important by RNA interference *in vitro* and PDGFRA-specific antibodies *in vivo* [5]. Furthermore, we showed that imatinib causes tumor regression or halts the progression of tumors in our mouse model of aRMS through the inhibition of Pdgfra activity; however, nearly one-third of the mice slowly evolved resistance to imatinib therapy [5]. In the current study, we have investigated adaptive signaling mechanisms associated with narrow-spectrum PDGFRA inhibition in aRMS and explored the use of broader spectrum kinase inhibitors to overcome the molecular re-wiring and tumor progression encountered with imatinib in aRMS.

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2. Materials and methods

2.1. Western blotting

Immunoblotting was performed as described previously [10]. A summary and primary antibodies used is given in the Supplementary material.

2.2. Primary tumor cell cultures

Murine primary cell cultures were generated from fresh tumors as described previously [10].

2.3. Cell viability assays

Cell viability assays were performed as described previously [10]. A summary of the reagents (drugs and siRNAs) used is given in the Supplementary material.

2.4. Small molecule inhibitor panel

Two naïve (untreated) and two imatinib-resistant murine aRMS primary cell cultures from mice treated with 50 mg/kg/day imatinib were plated in 96-well plates at a seeding density of 4000 cells/well over graded concentrations of 66 small-molecule kinase inhibitors. A detailed summary of the drug screen is given in the Supplementary material.

2.5. In vivo studies

The mouse model for aRMS has been previously described [5,6]. Tumor-bearing mice were treated with sorafenib at the dose of 30 mg/kg/day by intraperitoneal injection for 14 days. Tumor dimensions were measured with digital calipers and volume was calculated by the formula $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. All the experiments were conducted in accordance with the institution-approved IACUC protocols. Conditional *Pdgfra* knockout mice [11] were bred to the established genetically-engineered mouse model of aRMS [5,6] to generate mice whose tumors were genetically ablated for *Pdgfra*.

3. Results

3.1. Narrow-spectrum PDGFRA inhibitor resistance is cell intrinsic

To investigate whether resistance to narrow-spectrum PDGFRA inhibitors is cell-autonomous, we treated both naïve (untreated) and mouse aRMS primary cell cultures from tumors slowly evolving imatinib resistance with varying concentrations of imatinib. Results of a 72 h cell viability assay showed the imatinib IC_{50} to be higher for primary cultures established from imatinib-resistant tumors (19 μM) than for the naïve primary cultures established from tumor bearing mice that had received no treatment (10 μM). Representative cell cultures are shown in Fig. S1A. Although imatinib effectively abrogated activation of Pdgfra in naïve (untreated) cells, imatinib did not alter activation (phosphorylation) of Pdgfra in resistant aRMS cultures (Fig. 1A).

3.2. Src is highly activated in resistant tumors

To functionally identify pathways associated with narrow-spectrum PDGFRA resistance, we screened a panel of 66 small molecule kinase inhibitors with imatinib-naïve and imatinib-resistant primary cell cultures. We identified Src family kinase (SFK) inhibitors (Table 1) as having increased activity towards the imatinib-resis-

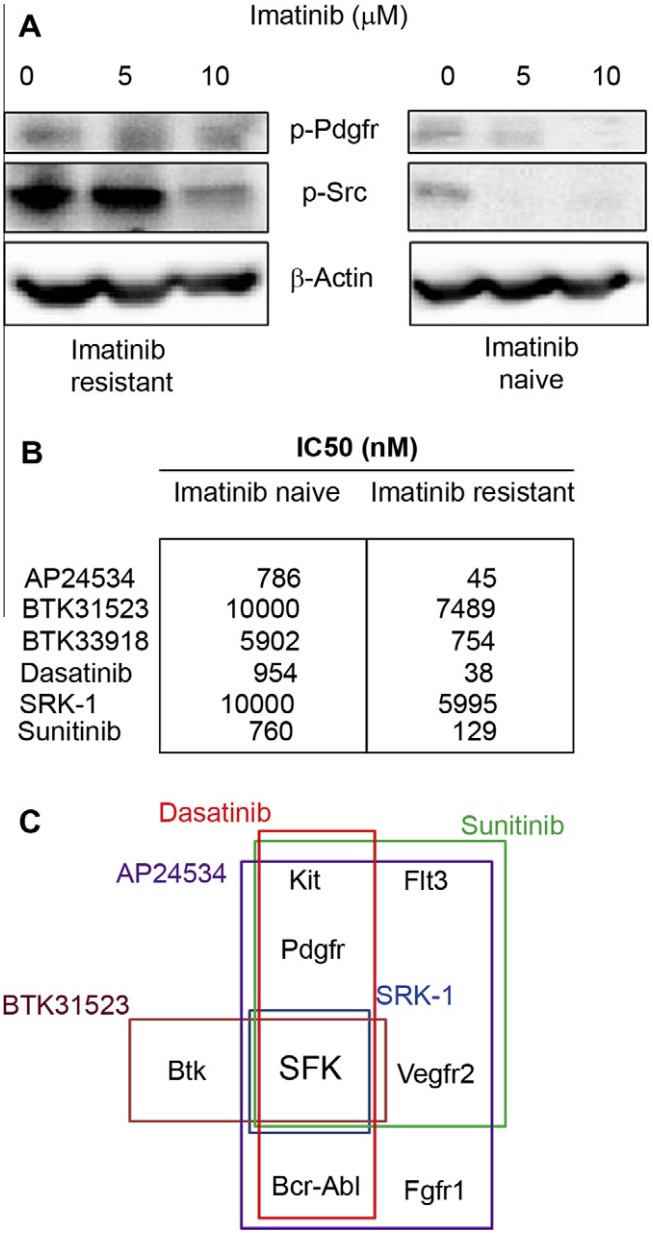

Fig. 1. Cell autonomous resistance to the prototypic PDGFRA inhibitor, imatinib. (A) Western blot analysis showing complete reduction in phospho-Src (p-Src) and phospho-Pdgfr (p-Pdgfr) levels upon imatinib treatment in imatinib-naïve primary cell cultures whereas phospho-Pdgfr and phospho-Src were still present at detectable levels upon imatinib treatment in imatinib-resistant primary cell cultures. (B) Src family kinase inhibitors that were found to be effective against imatinib-resistant primary cell cultures in a kinase inhibitor screen. (C) Venn diagram of specificities for drugs in (B) implicates Src family kinases (SFK).

Table 1
Inhibitory activity of select drugs from a screen on Src family kinases.

	C-Src	Lyn	Lck	Fyn	Hck
Src family kinases (IC_{50})					
AP24534	5.4 nM	0.24 nM	na	na	na
BTK31523	na	14 nM	97 nM	na	na
BTK33918	na	2.5 nM	20.5 nM	na	na
Dasatinib	0.21 nM	0.57 nM	0.2 nM	0.79 nM	0.35 nM
SRK-1	44 nM	na	88 nM	na	na
Sunitinib	2.1 μM	0.27 μM	0.23 μM	0.52 μM	0.88 μM

AP24534 [29]; BTK31523 and BTK33918 [30]; Dasatinib [7]; SRK-1 [31]; Sunitinib [29]; na, not available.

tant primary cultures compared to the naïve primary cultures (Fig. 1B and C). Overexpression of activated (phosphorylated) Src was also observed in imatinib-resistant primary cell cultures at baseline or with treatment by up to 10 μm imatinib (Fig. 1A). On the other hand, untreated aRMS cell cultures show no activation of Src (phospho-Src) in the presence of imatinib (Fig. 1A). These results suggested that a Src–Pdgfra signaling axis is active in the imatinib-resistant aRMS tumors in mice and that this axis might contribute resistance to narrow-spectrum PDGFRA inhibitors.

3.3. PDGFRA and Src inhibitors have an additive effect in murine alveolar rhabdomyosarcoma cells

To investigate the functional significance of Src activation in imatinib-resistant aRMS tumors we performed a cell viability assay for imatinib with or without the Src inhibitor, PP2. Results showed that the combination of imatinib and PP2 had an additive effect on cell viability compared to treatment with imatinib or PP2 alone (Fig. 2A). These functional data further raised the possibility that resistance to narrow-spectrum PDGFRA inhibitors in aRMS may be mediated by Src or SFKs.

3.4. Treatment with PP2 inhibits the Raf–Mapk signaling pathway

Previous reports have shown that SFKs can phosphorylate PDGFRA [12] and, conversely, evidence suggests SFK substrates may be the targets of PDGF-induced activation [13]. To investigate whether a Src/Pdgfra/Raf/Mapk signaling axis exists in aRMS, we treated the imatinib-resistant primary cell cultures with PP2 at 5 μM or 10 μM for 30 min. Western blot analysis of the lysates showed that PP2 treatment caused a reduction in the levels of phospho-Pdgfr, phospho-BRaf as well as phospho-Mapk (Fig. 2B). These results inferred that SFKs can activate Pdgfra thereby activating the Raf–Mapk signaling axis in imatinib-resistant aRMS cells. To investigate the functional significance of Raf activation, we treated the imatinib-resistant primary cells with the dual PDGFR-Raf inhibitor, sorafenib (Fig. 2C). After treating the cells with sorafenib for 72 hours, we performed a cell viability assay and found that sorafenib was more effective at inhibiting cell growth than imatinib (IC_{50} 4 μM vs. 19 μM). However, as expected, the combination of sorafenib and PP2 had no additive effect on cell viability (Fig. 2C). These results are consistent with Raf being downstream of Src in aRMS.

3.5. Pdgfra is important in Src–Raf–Mapk mediated cell growth

To validate the importance of persistent Pdgfra expression and activation in imatinib-resistant tumors, we performed a cell viability assay of imatinib, sorafenib and/or PP2 with primary cell cultures from a mouse model with both copies of *Pdgfra* genetically ablated by Cre-LoxP recombination. Remarkably, PP2 showed no effect on cell viability over 72 hours, implicating Pdgfra as an essential factor in Src–Pdgfra–Raf–Mapk mediated cell viability or growth. Treatment with PP2 had no effect whatsoever on tumor cell growth, confirming the functional importance of Pdgfra to mediate the effects of Src (Fig. 2D). The combination of imatinib and PP2 had no additive effect on cell viability in the *Pdgfra* knockout cells (Fig. 2D), which was strikingly different from aRMS cells which had intact alleles of *Pdgfra* (Fig. 2A). Nevertheless, while *Pdgfra* null tumor cells lacked Pdgfra protein, these cells had persistent BRaf activation (Fig. 2E). Furthermore, neither PP2 nor PP2 combined with imatinib had any additive effects with sorafenib on cell viability (Fig. 2D). To interrogate signaling, *Pdgfra* knockout cells were treated with 0, 5 or 10 μM PP2 for 30 minutes. Interestingly, a paradoxical increase in BRaf, CRaf and Mapk phosphorylation was observed (Fig. 2E) (see Supplemental Discussion). Notably, however, *Pdgfra* null cells were equally as sensitive to sorafenib as imatinib-resistant cells (IC_{50} = 4–5 μM for both), indicating that BRaf may be an important common downstream mediator of mitogenesis whether Pdgfra is present or not.

3.6. The dual PDGFRA-Raf inhibitor sorafenib is effective in vitro and in vivo

To investigate whether cross-resistance to sorafenib would occur in imatinib-resistant cell lines, we performed a cell viability assay using sorafenib in both naïve (untreated) and imatinib-resistant primary cultures. Results showed that sorafenib was equally effective on both naïve and the imatinib-resistant cells with no difference in the IC_{50} values (Fig. 3A). In addition, sorafenib had comparable activity *in vitro* for mouse and human RMS (embryonal and alveolar) (Fig. S1B). Futhermore, we affirmed that sorafenib conclusively inhibits Pdgfra phosphorylation without altering the total protein levels in a mouse aRMS primary cell culture (Figs. S1C and S1D). We then tested the efficacy of sorafenib for naïve (non-pretreated) tumors in our genetically-engineered mouse model of aRMS. In untreated mice, tumors grew 2–8-fold larger than their original size; however, when treated with sorafenib tumor growth *in vivo* was halted or slowed in nearly all mice (Figs. S2A and S2B). Sorafenib treatment resulted in a trend towards increased event-free survival (EFS) in mice bearing very small tumors ($p = 0.08$; Fig. 3B; some mice were sacrificed early secondary to overgrooming and bleeding of limb tumors) and a statistically significant increase in EFS was observed for mice bearing large tumors ($p = 0.032$; Fig. 3C).

4. Discussion

Rhabdomyosarcoma is a clinically challenging musculoskeletal system cancer of children and young adults [14]. Despite significant improvements in multimodality treatment, the survival rate for children with metastatic aRMS has been less than 20% for more than three decades [2,3]. Our previous studies have shown that the narrow-spectrum PDGFRA inhibitor, imatinib, can effectively inhibit tumor progression in a mouse model of aRMS [5]. However, one-third of the mice progressed on therapy. This phenomenon is not clinically uncommon as imatinib resistance has been observed in other types of cancer such as chronic myelogenous leukemia (CML) and gastrointestinal stromal (GIST) tumors [9,15]. Therefore, understanding the mechanisms of resistance to imatinib, as a prototypic narrow-spectrum receptor tyrosine kinase inhibitor, is critical for developing better therapies for aRMS and potentially other solid tumors.

In our present study investigating the mechanisms underlying narrow-spectrum PDGFRA inhibitor resistance in rhabdomyosarcoma, a chemical screen identified SFK inhibitors as having higher potency for inhibiting growth of imatinib-resistant cells compared to the naïve tumor cells. However, treating the imatinib-resistant cell cultures with the highly specific Src inhibitsor PP2 caused a significant decrease in phospho-Raf and phospho-Mapk levels suggesting that Src phosphorylates Pdgfra, resulting in the activation of a Raf–Mapk signaling axis. Src inhibition had no effect on a *Pdgfra* knockout aRMS primary cell culture confirming that Pdgfra is upstream of the Raf–Mapk signaling axis and that the action of Src is through an interaction with Pdgfra. When tested *in vivo*, the dual PDGFRA-Raf inhibitor sorafenib was effective at slowing or stopping tumor progression and extending survival.

A remarkable result of these studies was that aRMS tumors would form in the genetically-engineered mouse model when *Pdgfra* was conditionally deleted. In our prior studies, Pdgfra was validated as a target not only by pharmacological approach but also

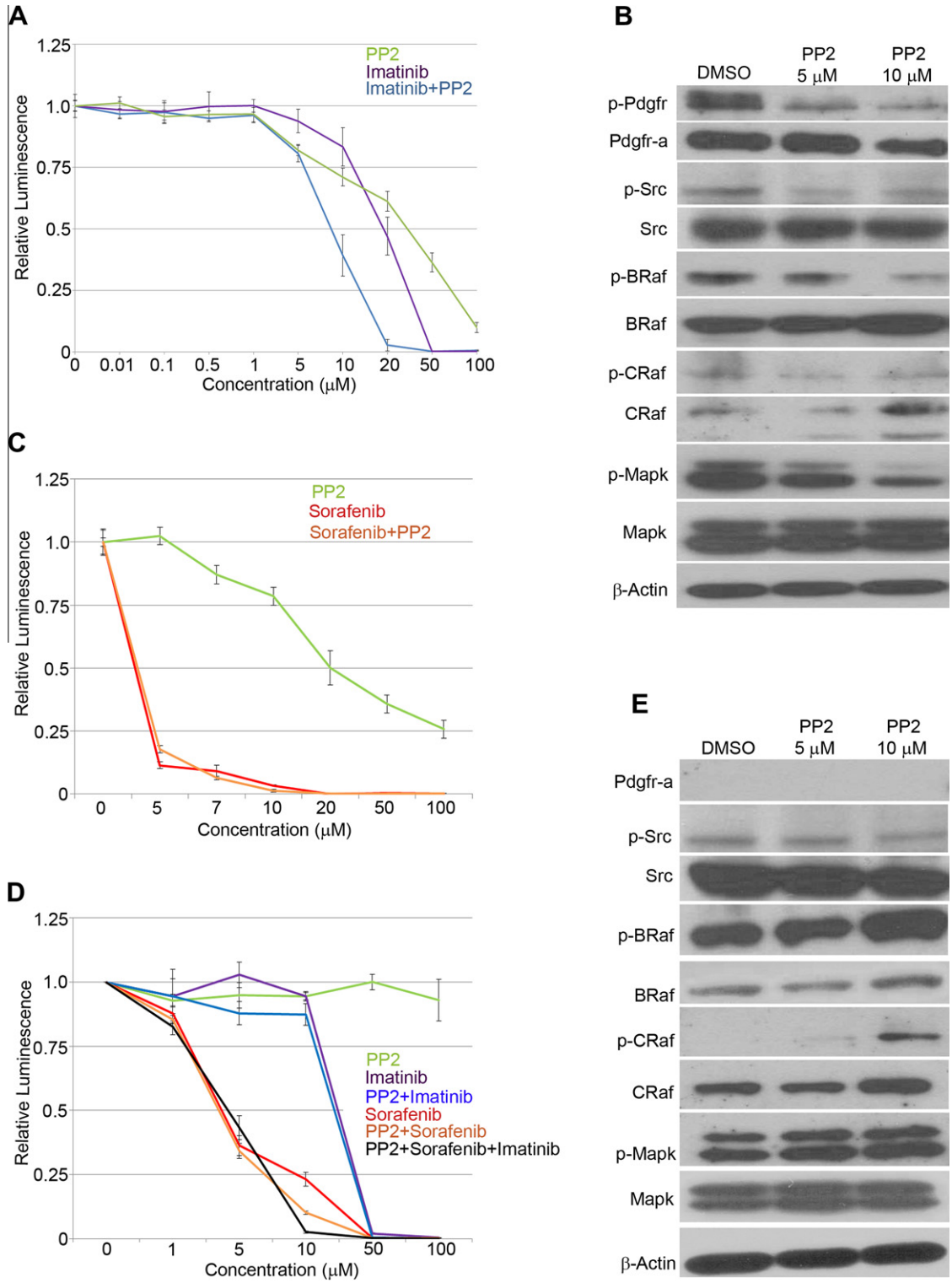

Fig. 2. B-Raf is downstream of Src and Pdgfra in Pdgfra expressing rhabdomyosarcoma. (A) Cell viability assay for an imatinib-resistant mouse aRMS primary cell culture showing the combination of imatinib and PP2 to have an additive effect compared to imatinib or PP2 alone. (B) Western blot analysis of an imatinib-resistant mouse aRMS primary culture lysates showing a decrease in the levels of phospho-Mapk (p-Mapk) and phospho-BRaf (p-BRaf) upon treatment with increasing amounts of PP2. (C) Cell viability assay for an imatinib-resistant aRMS primary cell culture revealing that the combination of sorafenib and PP2 has no additive effect on inhibiting cell growth. (D) *In vitro* cell growth assay for a *Pdgfra* knock-out mouse rhabdomyosarcoma primary cell culture showing that PP2 had no effect on cell viability. (E) Western blot analysis of a *Pdgfra* knockout mouse aRMS primary cell culture showing absence of Pdgfra but ongoing activation of BRaf.

using RNA interference *in vitro* and monospecifc PDGFRA blocking antibody *in vivo* - affirming importance of PDGFRA as a target in aRMS [5]. However, Pdgfra knockout tumors often had an early onset and faster progression than aRMS tumors with intact Pdgfra (unpublished observation) suggesting that Pdgfra loss moves aRMS tumor cells to a "second state" which is more aggressive that the

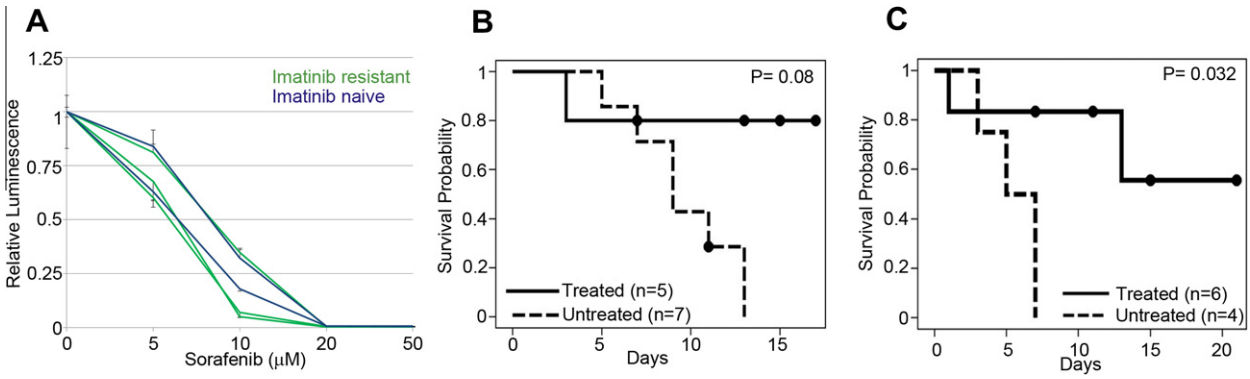

Fig. 3. Sorafenib efficacy for aRMS. (A) Cell viability assay showing sorafenib being equally effective in inhibiting the growth of naïve (not pretreated) or imatinib-resistant mouse rhabdomyosarcoma primary cell cultures. Sorafenib sensitivity was also similar for embryonal rhabdomyosarcoma (eRMS) and aRMS across species (Fig. S1B). (B,C) Kaplan–Meier survival curves showing sorafenib treatment prolonging event-free survival (EFS) in mice with tumors which measured (B) less than 0.25 cc at diagnosis and (C) larger than 0.25 cc at diagnosis. For EFS, the events were defined as tumor size growing >0.25 cc and >0.75 cc respectively. Tumor growth curves for B and C are presented as Supplementary Fig. S2A–B.

first. Neverless, nearly all human tumors express PDGFRA [5], and our results suggest that when PDGFRA can be expressed, tumors try to maintain its expression.

The occurrence of imatinib resistance is not uncommon to human cancers, including those driven by PDGFRA. Even for the treatment of patients with advanced GIST, imatinib has been found to be an effective therapy [16,17]. However, resistance to imatinib has been a significant clinical problem. The most common mechanism for imatinib resistance in GIST is the presence of secondary mutations in the kinase domains of KIT or PDGFRA [18–20]. We found no such *Pdgfra* mutations in our murine aRMS (data not shown). Similarly for CML, resistance to imatinib leading to disease progression is a major concern in a subset of patients. The BCR-ABL dependent mechanism of imatinib resistance is mediated either by *BCR-ABL* gene amplification or most frequently by mutations in the kinase domain [15]. Studies have shown that overexpression and/or activation of SFKs is one of the BCR-ABL independent mechanisms that contribute to imatinib resistance in CML [21–23]. A related mechanism appears to be at work in aRMS.

Is imatinib resistance in aRMS mediated by the same mechanism as in GIST or CML? A similar mechanism has recently been shown for GIST wherein *KRAS* or *BRAF* activating mutations accompany or substitute for activating mutations in *KIT* or *PDGFRA* [24]. Our results for aRMS show a unique specific mechanism involving a Src/Pdgfra interaction with downstream mitogenesis mediated by BRaf (and perhaps CRaf) under conditions where Pdgfra expression is maintained by necessity in certain rhabdomyosarcoma tumors. However, BRaf activation continues to be functionally important in a "second state" for other RMS tumors when Pdgfra expression is absent (Fig. 4). The use of a dual PDGFRA-Raf inhibitor to treat RMS appears to be a good approach by proof-of-principle, particularly because preclinical tests using our genetically-engineered mouse model of aRMS have shown sorafenib to very effective at inhibiting progression. When tested by the NCI Pediatric Preclinical Testing Program, sorafenib failed to achieve an objective response in multiple xenograft models of solid tumors except tumors with high expression of VEGFA for which greater growth inhibition was observed [25]. These results might have suggested that VEGFA (and PDGFRA) expression is required for sorafenib to be effective. A caveat, however, is that many human rhabdomyosarcoma cell lines lack PDGFRA expression despite strong PDGFRA expression being typical of the overwhelming

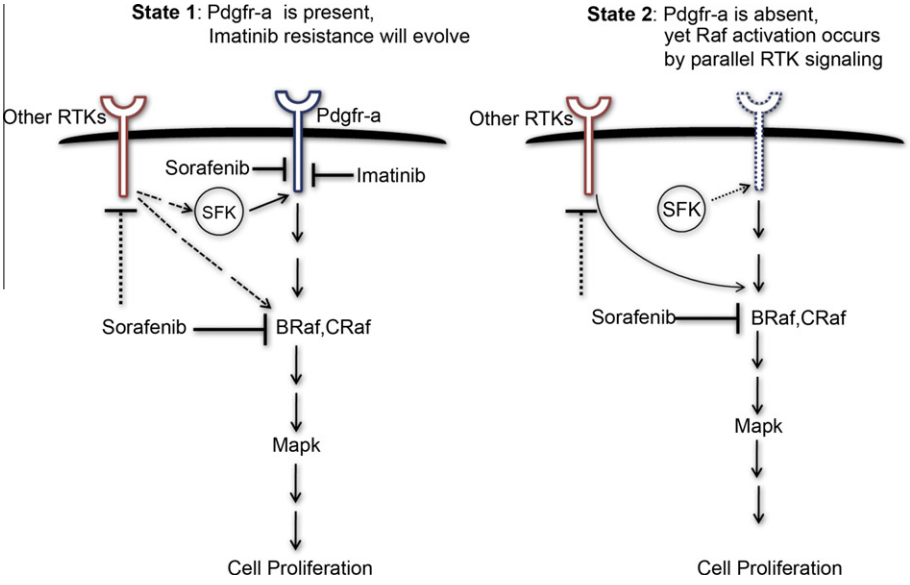

Fig. 4. Model of the Pdgfra/Src/Raf/Mapk axis in rhabdomyosarcoma. A model depicting the Pdgfra/Src/Raf/MAPK signaling axis in aRMS cells which express Pdgfra and in cells which do not express Pdgfra. We speculate that an RTK other than PDGFRA may activate Src or BRAF under conditions of evolving PDGFRA resistance. In the absence of PDGFRA, Raf signaling remains vital to tumor cell survival.

majority of clinical RMS cases [5]. For this reason, our genetically-engineered mouse models may be more representative. Yet is sorafenib a drug to be taken to the clinic? Sorafenib was an effective tool compound for our preclinical studies, and the 10 μM IC_{50} for rhabdomyosarcoma mouse and human cell lines are within the clinically-achievable maximum serum concentration (10.9 μM) reported in adult Phase I studies [26]. However, the RMS IC_{50}s here are well above the cell-free K_d values of sorafenib for BRAF (22 nM) or PDGFRA (62 nM). Because prior reports using RNA interference and blocking antibodies solidly implicate Pdgfra as a critical factor in tumor growth and progression [5], the high IC_{50}s for sorafenib may imply that intracellular accumulation is inhibited by drug efflux. Sorafenib is a substrate for both P-gp (*ABCB1*) and BCRP (*ABCG2*) [27], which are known to be overexpressed in RMS [28]. Future studies will explore whether co-administration of P-gp and BCRP inhibitors such as cyclosporine-A or mebendazole improve intracellular accumulation of sorafenib and its biological potency *in vitro* and *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.092.

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