XL888 limits vemurafenib-induced proliferative skin events by suppressing paradoxical MAPK activation

Manali Phadke1, Geoffrey T. Gibney2, Carolyn J. Rich2, Inna V. Fedorenko1, Y. Ann Chen3, Ragini R. Kudchadkar2, Vernon K. Sondak2, Jeffrey Weber2, Jane L. Messina2,*, and Keiran S. M. Smalley1,2,*

1Department of Tumor Biology, The Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL, 33612, USA
2Department of Cutaneous Oncology, The Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL, 33612, USA
3Department of Biostatistics, The Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL, 33612, USA

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To the Editor

Great progress has been made treating patients with disseminated BRAF-mutant melanoma with BRAF inhibitors (Hauschild et al., 2012). Despite this, responses tend to be short-lived and the majority of patients fail therapy. We recently demonstrated that most of the proteins involved in escape from BRAF inhibitor therapy were clients of the heat shock protein (HSP)-90 family of chaperones (Paraiso et al., 2012; Smyth et al., 2014). In cell culture models, the HSP90 inhibitor XL888 overcame acquired BRAF inhibitor resistance, an effect associated with decreased expression of receptor tyrosine kinases (RTKs) PDGFR-β and IGF1R and inhibition of signaling through the mitogen activated protein kinase (MAPK) and PI3K/AKT signaling pathways (Paraiso et al., 2012). In vivo, XL888 treatment decreased growth of BRAF inhibitor-resistant melanoma xenografts (Paraiso et al., 2012). When used as frontline therapy, the combination of another HSP90 inhibitor (AT13387) with vemurafenib prevented escape from BRAF inhibition in melanoma xenograft models (Smyth et al., 2014). On the basis of these data we recently opened a phase I dose-escalation
study of XL888 in combination with vemurafenib (960mg PO, BID) in patients with unresectable \textit{BRAF}-mutant melanoma (NCT01657591).

An initial analysis demonstrated the combination to be well tolerated, with early indications of efficacy. Similar to the experience with single-agent BRAF inhibitor therapy, patients developed hyperproliferative/neoplastic cutaneous lesions, with some notable differences in incidence. These secondary tumors, which arise as a result of the paradoxical activation of the MAPK pathway in keratinocytes, can be at least partly abrogated by the BRAF/MEK inhibitor combination (Flaherty \textit{et al.}, 2012). Activating \textit{HRAS} mutations are found in up to 71\% of these lesions, which can take the form of benign tumors (“BRAF inhibitor-associated verrucous keratosis”), verruca vulgaris (VV), keratoacanthoma-like squamous cell carcinoma (KA-SCC) and typical SCC (Su \textit{et al.}, 2012). Under physiological conditions, MAPK signaling follows the activation of Ras, with the GTP-bound form of Ras inducing RAF to form dimers, which in turn leads to phosphorylation and activation of MEK and ERK (Roring \textit{et al.}, 2012). In cells with upstream Ras activity, the formation of RAF dimers also plays a role in paradoxical MAPK signaling (Gibney \textit{et al.}, 2013; Halaban \textit{et al.}, 2010; Poulikakos \textit{et al.}, 2010). Mechanistically this proceeds through the binding of the BRAF inhibitor to one RAF protomer, which induces the binding and activation of its partner RAF molecule, and an increase in MAPK signaling (Gibney \textit{et al.}, 2013; Poulikakos \textit{et al.}, 2010). Recently, next generation BRAF inhibitors have been developed that limit the paradoxical activation of ERK signaling (Le \textit{et al.}, 2013), but as yet their incidence of associated secondary neoplasms in humans has not been quantified.

We report herein the incidence of biopsy-proven skin lesions occurring in the first 24 weeks of therapy when vemurafenib was combined with XL888 in increasing doses (Figure 1; Supplemental Figure 1; Supplemental Table 1). Lesions suspicious for malignancy were biopsied in routine dermatologic follow up. All tissue studies took place as part of an IRB-approved protocol after patients gave written, informed consent for tumor biopsies to be performed and used for research purposes. The incidence and number of skin lesions per patient decreased as the dose of XL888 increased in each of the 4 cohorts. In cohort 1 (35mg PO BIW), 3/3 patients (100\%) developed a total of 6 lesions (2 melanoma, 1 SCC, 3 VV). In cohort 2 (45mg PO BIW): 2/3 (66\%) patients developed 6 lesions (2 KA, 2 SCC, 2 VV). In Cohort 3 (90 mg PO BIW), 2/3 (66\%) patients had 3 total lesions (2 melanoma, 1 VV). In cohort 4 (135 mg PO BIW) 2/6 (33\%) developed 3 VV (Figure 1). All secondary melanomas were negative by immunohistochemistry for mutant \textit{BRAF} (VE-1 antibody, Ventana) and were \textit{NRAS}-wild-type by sequencing (not shown). The differences in incidence of SCC/KA between cohorts 1–3 and cohort 4 were not statistically significant, likely a reflection of the small sample size. The finding that no patients in cohort 4 developed SCCs/KAs compares favorably to the 26\% incidence of SCC development previously reported in patients on single agent vemurafenib therapy (Sosman \textit{et al.}, 2012).

We next determined whether XL888 prevented the growth of vemurafenib-induced skin lesions through the suppression of paradoxical MAPK activation. Previous studies have shown that BRAF inhibitors activate phospho-ERK signaling in \textit{NRAS}-mutant melanoma cell lines as well as in SCC cell lines that are driven through mutant \textit{HRAS} (Kaplan \textit{et al.}, 2010; Su \textit{et al.}, 2012). The administration of a BRAF inhibitor has been shown to shorten...
the latency of SCC induction in a two-step carcinogenesis model, an effect reversible through MEK inhibition (Su et al., 2012). In our studies, treatment of 3 NRAS mutant melanoma cell lines with vemurafenib (1 μM) increased levels of phospho-ERK, an effect that was suppressed following the addition of XL888 (300nM) (Figure 2A). These effects were concentration-dependent, with levels of phospho-ERK being inhibited at concentrations of XL888 >100nM (Supplemental Figure 2A). The ability of BRAF inhibitors to activate MAPK signaling is dependent upon CRAF transactivation (Poulikakos et al., 2011; Wan et al., 2004). As CRAF is an HSP90 client protein, we next asked whether XL888 decreased the expression of CRAF. As expected, the decrease in paradoxical ERK activation observed following combined vemurafenib+XL888 treatment was also associated with decreased CRAF expression in three NRAS-mutant melanoma cell lines (Figure 2A). Similarly, XL888 also suppressed vemurafenib-driven MAPK signaling in NIH3T3 cells transformed by HRAS Q61L (Supplemental Figure 2B), an effect associated with decreased CRAF expression (Figure 2B). Taken together, these data suggest that XL888 prevented the emergence of vemurafenib-associated skin lesions through the inhibition of both CRAF transactivation and paradoxical MAPK signaling. Our clinical findings agree with recent mouse model data showing that topical HSP90 inhibition inhibits UVR-induced SCC development (Singh et al., 2015).

Our conclusion is that the HSP90 inhibitor XL888 suppresses paradoxical MAPK activation in NRAS and HRAS-driven in vitro models and is associated with fewer hyperproliferative cutaneous lesions in melanoma patients receiving the combination of vemurafenib and XL888. These data suggest that the reduced incidence of cutaneous adverse events could be used as a biomarker of HSP90 inhibition in this setting.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Incidence of secondary skin lesions by dose cohort of XL888

Chart shows the incidence of hyperproliferative/neoplastic skin lesions (melanoma, SCC: squamous cell carcinoma, KA: keratoacanthoma, and VV: verucca vulgaris, stratified by XL888 dose cohort (cohort 1: 30mg; cohort 2: 45mg; cohort 3: 90mg; cohort 4: 135mg). Data shows number of lesions of each type for each individual patient. Cohort 1 had 7 total events (3/3 patients), cohort 2 had 6 total events (2/3 patients), cohort 3 had 3 total events (2/3 patients) and cohort 4 had 3 total events (2/6 patients).
Figure 2. XL888 suppresses paradoxical MAPK signaling in NRAS-mutant melanoma and HRAS-transformed NIH3T3 cells

a: XL888 prevents the paradoxical MAPK signaling in 3 NRAS mutant melanoma cell lines. IPC-298, M245 and WM1366 cell lines were treated with vemurafenib (1 μM, 72 hrs) in the absence and presence of XL888 (300 nM). Western blots show total CRAF, phospho-ERK and total ERK.

b: XL888 (300 nM) suppressed vemurafenib-driven (1 μM, 72 hrs) paradoxical MAPK signaling in NIH3T3 cells transduced with HRAS Q61L.