Augmentation of Radiation Response by Motesanib, a Multikinase Inhibitor that Targets Vascular Endothelial Growth Factor Receptors

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Abstract

Background: Motesanib is a potent inhibitor of vascular endothelial growth factor receptors (VEGFR) 1, 2, and 3, platelet-derived growth factor receptor, and Kit receptors. In this report we examine the interaction between motesanib and radiation in vitro and in head and neck squamous cell carcinoma (HNSCC) xenograft models.

Experimental Design: In vitro assays were done to assess the impact of motesanib on VEGFR2 signaling pathways in human umbilical vein endothelial cells (HUVEC). HNSCC lines grown as tumor xenografts in athymic nude mice were utilized to assess the in vivo activity of motesanib alone and in combination with radiation.

Results: Motesanib inhibited VEGF-stimulated HUVEC proliferation in vitro, as well as VEGFR2 kinase activity. Additionally, motesanib and fractionated radiation showed additive inhibitory effects on HUVEC proliferation. In vivo combination therapy with motesanib and radiation showed increased response compared with drug or radiation alone in UM-SCC1 (P < 0.002) and SCC-1483 xenografts (P = 0.001); however, the combination was not significantly more efficacious than radiation alone in UM-SCC6 xenografts. Xenografts treated with motesanib showed a reduction of vessel penetration into tumor parenchyma, compared with control tumors. Furthermore, triple immunohistochemical staining for vascular lature, proliferation, and hypoxia showed well-defined spatial relationships among these parameters in HNSCC xenografts. Motesanib significantly enhanced intratumoral hypoxia in the presence and absence of fractionated radiation.

Conclusions: These studies identify a favorable interaction when combining radiation and motesanib in HNSCC models. The data presented suggest that motesanib reduces blood vessel penetration into tumors and thereby increases intratumoral hypoxia. These findings suggest that clinical investigations examining combinations of radiation and motesanib are warranted in HNSCC.

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with a global yearly incidence of >500,000 new cases (1). Despite stepwise advances associated with combinations of radiation and chemotherapy (2), only 30% to 50% of advanced-stage patients are cured of their disease. Therefore, molecularly targeted agents are under investigation in conjunction with radiation and/or chemoradiation in HNSCC. A recent international phase III trial that combined the anti–epidermal growth factor (EGFR) monoclonal antibody cetuximab with radiation in HNSCC patients showed a near doubling of median survival for patients receiving the EGFR inhibitor (3). Furthermore, the addition of cetuximab to radiation did not seem to augment radiation-induced toxicities. This study highlighted the potential of targeted agents in HNSCC patients undergoing curative radiation; additional novel treatments are highly desired.

Targeting the vascular endothelial growth factor (VEGF) receptor has drawn interest in HNSCC. VEGF is a potent mitogen for vascular endothelial cells and acts through specific tyrosine kinase receptors: VEGFR1 (Flt-1), VEGFR2...
Translational Relevance

In this study we examine the interaction between radiation and motesanib, a potent, orally bioavailable inhibitor of vascular endothelial growth factor receptors 1, 2, and 3, platelet-derived growth factor receptor, and Kit receptors in xenograft models of head and neck squamous cell carcinoma (HNSCC). The intratumoral interactions between antiangiogenic agents and radiation remain poorly understood, and we show that motesanib can augment radiation response in HNSCC xenografts. By employing immunohistochemical techniques, low-dose radiation schedules are shown to induce accelerated repopulation in this model, and motesanib is found to alter the tumor microenvironment in a way that limits oxygen delivery to radiated xenografts, resulting in tumor necrosis. These findings highlight potential mechanisms of radiosensitization that warrant consideration in future preclinical studies and clinical trial design. Furthermore, the techniques utilized show that the interplay among tumor vasculature, oxygenation, and clonogenic proliferation can be studied in vivo in response to fractionated radiation with concurrent antiangiogenic therapy.

Cell lines and compounds

The human HNSCC lines UM-SCC1 and UM-SCC6 were provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) and SCC-1483 cells were provided by Dr. Jennifer Grandis (University of Pittsburgh, Pittsburgh, PA). SCC cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1 μg/mL hydrocortisone, and 1% penicillin/streptomycin. SCC cell culture media and supplements were obtained from Invitrogen. Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection. HUVECs were cultured in endothelial basal medium-2 supplemented with EGM-2 SingleQuots growth supplements (Lonza) and 1% penicillin/streptomycin.

Materials and Methods

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Immunoprecipitation and immunoblotting

HUVECs were grown to 70% confluence and treated with either motesanib or DMSO vehicle control for 24 hours. The final concentration in all plates of DMSO was 0.25%. Cells were exposed to either 50 ng/mL VEGF or vehicle for 45 minutes, and then whole cell lysates were obtained using Tween-20 lysis buffer. Immunoprecipitation of VEGFR2 was done by incubating 700 μg of HUVEC lysate with 1.5 μg of rabbit anti-FLK-1 (sc-504, SantaCruz Biotechnology [SCB] Inc.). After adding 30 μL of protein A/G agarose beads (sc-2003, SCB), lysates were incubated for another 2 hours at 4°C. The immunoprecipitates were pelleted by centrifugation and washed three times with Tween-20 lysis buffer. The captured immunocomplexes were then eluted by boiling the beads in 2× SDS sample buffer for 5 minutes and subjected to gel electrophoresis and transferred to Immobilon-P membrane (Millipore). Primary antibody for Western blot detection of VEGFR2 was murine anti-FLK-1 (sc-6251, SCB); primary pTyr antibody was murine anti-pTyr antibody (#05-321, Upstate). Secondary antibody for detection was horseradish peroxidase–conjugated goat anti-mouse IgG (sc-2005, SCB). Thereafter, proteins were detected via enhanced chemiluminescence (ECL+) detection system (Amersham Biosciences).

VEGFR2 kinase assay

VEGFR2 kinase activity was quantified in the presence of serial dilutions of motesanib using the HTScan VEGF Receptor 2 Kinase Assay Kit according to manufacturer’s instructions (Cell Signaling).

HUVEC proliferation assays

Crystal violet assay. Cells were grown in endothelial basal medium-2 with 2% fetal bovine serum, without exogenous growth factors (i.e., EGM-2 SingleQuots growth supplements were omitted). VEGF stimulation was 25 ng/mL every 24 hours × 4 days. Final DMSO concentration was 0.25% in all wells. Data points represented mean crystal violet staining intensity (6 wells/condition) ± SE.
**Augmentation of Radiation Response by Motesanib**

**HUVEC counting after motesanib and radiation.** HUVECs were plated at a density of 15,000 cells per p100 dish (27 dishes) at day 0. At day 1, three dishes were rinsed with PBS/0.02% EDTA, detached using 0.05% Trypsin/EDTA, and counted via trypan blue exclusion to establish a baseline number of cells/plate for the cohort. The remaining plates were treated with either motesanib (25 nmol/L) or vehicle control and 3 hours later half of the plates were irradiated at 2 Gy with a Shepherd & Associates Model 109 irradiator and a 137Cs cesium hotbox source. On day 3 the HUVECs on half of the plates were trypsinized and counted; the other half received fresh media plus motesanib and 2 Gy radiation where indicated. The remaining HUVEC plates were trypsinized and counted at day 4 to establish the final data points.

**Tumor growth in athymic nude mice**

Athymic nude mice (3- to 4-week-old males) were obtained from Harlan Bioproducts for Science. The care and treatment of experimental animals was in accordance with institutional guidelines. Cells (∼2 × 10⁷) from the respective human cancer lines were injected s.c. into the flank area on day 0. Tumor volume was determined by direct measurement with calipers and calculated by the formula \( \frac{\pi}{6} \times (\text{large diameter}) \times (\text{small diameter})^2 \). Motesanib or vehicle control was administered once daily by oral gavage at specified doses 5 days/week. Motesanib doses were chosen based on pilot experiments testing the in vivo efficacy of motesanib alone in the respective xenografts. Radiation treatment was delivered via a Philips RT-250 orthovoltage unit (Philips Medical Systems) using custom-designed mouse jigs, which specifically exposed the dorsal flank (harboring tumor xenografts) for irradiation.

**Immunohistochemistry**

Mice harboring UM-SCC1 xenografts were treated with vehicle control, motesanib, fractionated radiation, or the combination (see “Tumor growth in athymic nude mice” above for details), and harvested 3 hours after the last treatment for immunohistochemical examination.

**Von Willebrand Factor.** Von Willebrand factor (vWF) expression was detected in histologic sections of tumor xenografts. Briefly, excised tumor specimens were fixed in 10% neutral buffered formalin. Following embedding in paraffin, 5-μm sections were cut, and tissue sections were mounted. Sections were dried, deparaffinized, and rehydrated. vWF antigen was unmasked with Proteinase K at 37°C for 20 minutes. After endogenous peroxidase activity was quenched, slides were incubated at 4°C overnight with primary antibody (vWF, 1/150, Dako) followed by a 30-minute incubation of secondary antibody. Slides then were incubated with streptavidin peroxidase, visualized using the 3,3′-diaminobenzidine chromogen (Lab Vision Corp), counterstained with hematoxylin, dehydrated, and mounted. Quantitative analysis was done for the results of immunohistochemical examination by assessing the numbers of vessels per high-powered field (HPF), as well as the proportion of vWF expression at ×100 magnification. Five HPFs were randomly chosen and examined from each of vehicle and MSB tumor (n = 2 tumors per group), and vessels per HPF were counted. Percentage of vWF immunoreactive area was measured by ImageJ software. Statistical significance was analyzed by Student’s t-test.

**Hypoxia (pimonidazole), proliferation (Ki67), and vasculature (9F1).** One hour after administration of the final radiation dose, three mice per treatment group received 60 mg/kg of pimonidazole HCl (Hydroxyprobe-1, NPI, Inc.) i.p. injection. Two hours later the mice were euthanized, and tumors were excised and frozen. To minimize fracturing of tumor specimens during freezing, no embedding medium was used, and tumors were slowly frozen by placing them in small plastic boats floating in a mixture of isopentane and dry ice. The tissues were then wrapped in dry ice–chilled foil to minimize desiccation, and packaged in dry ice. Frozen sections (5 μm) were fixed in acetone (4°C), and the Hoechst 33342 signal was recorded on air-dried tissue sections. After rehydration, tumor sections were triple-stained for pimonidazole, vasculature, and Ki67.

Ki67 was visualized with rabbit anti-Ki67 (Calbiochem) and goat anti-rabbit FabCy3 (Jackson Immuno Research). In the same tissue section pimonidazole was detected with a polyclonal rabbit anti-pimonidazole and donkey anti-rabbit F(ab')2Alexa488 (Molecular Probes). Finally, again in the same section blood vessels were visualized using undiluted 9F1 supernatant (Calbiochem) and anti-rabbit Alexa647 (Molecular Probes).

**Image acquisition, processing, and analysis**

The tumor sections were mounted with Fluorostab (Euro Diagnostica) and quantitatively analyzed with a semiautomated image recording system as previously described (13). With the use of different filter sets, multiple scans at ×100 magnification yielded composite images of hypoxia (pimonidazole), proliferation (Ki67), and vasculature (9F1). The gray scale images were thresholded into binary images, which were used for quantitative analysis of the vasculature, hypoxia, and proliferative labeling indices. A consecutive H&E-stained tumor section was used to define the tumor area excluding nontumor tissue, necrosis, and staining artifacts.

**Results**

**Motesanib has specific activity on HUVECs in vitro**

In the absence of VEGF (HUVECs grown in basal medium with 2% fetal bovine serum, without exogenous growth factors) motesanib showed no significant antiproliferative effect on HUVECs; in the presence of VEGF stimulation, however, motesanib inhibited the promitogenic effect of VEGF in a dose-dependent manner (Fig. 1A). These dose-dependent antiproliferative observations are mirrored by the ability of motesanib to block both the
VEGF-stimulated kinase activity of VEGFR2 (Fig. 1B), as well as VEGF-stimulated phosphorylation of VEGFR2 (Fig. 1C). The nanomolar range of activity of motesanib observed is in line with previous studies (11).

To examine the effect of motesanib in combination with radiation on HUVEC proliferation in vitro, HUVECs were grown in medium fully supplemented with exogenous growth factors, including VEGF. Motesanib showed minimal antiproliferative activity on cell growth alone, whereas fractionated radiation significantly reduced the number of HUVECs present after four days of stimulation (Fig. 1D). The combination of motesanib and fractionated radiation showed an additive inhibition on HUVEC proliferation at the $P = 0.08$ level, two-sided t-test. Expression of VEGFR1, VEGFR2, VEGFR3, PDGFR, and c-Kit were not detectable by Western blot analysis in any of the HNSCC tumor lines tested (Supplementary Fig. S1), and motesanib had no activity on HNSCC cells in vitro using either proliferative assays or clonogenic radiation assays (data not shown).

**Motesanib augments in vivo radiation response**

To investigate the effects of combining motesanib and radiation in vivo, mice bearing established HNSCC xenografts were treated with vehicle control, radiation alone, motesanib alone, or radiation and motesanib combined. Treatment was initiated approximately 19 to 23 days post-implantation of cells once tumors had established. Mouse weights were measured weekly, and no discernible toxicity was observed in motesanib-treated groups.

In UM-SCC1 xenografts measured 24 days after initiation of treatment, motesanib 75 mg/kg daily alone resulted in a 15% reduction in tumor volume as compared with control tumors (Fig. 2A), but this was not significant ($P = 0.38$). Low-dose radiation alone resulted in a 45% reduction in tumor volumes ($P < 0.01$). The combination of radiation and motesanib resulted in a 72% growth reduction ($P = 0.0001$); this effect was significantly larger than that observed with either motesanib alone ($P = 0.0001$) or radiation alone ($P < 0.002$).

In SCC-1483 xenografts measured 21 days after initiation of treatment, motesanib 75 mg/kg daily alone resulted in a 46% reduction in tumor volume ($P < 0.02$; Fig. 2B). Treatment of xenografts with radiation alone resulted in a 43% reduction ($P = 0.004$) in tumor volume, whereas the combination of radiation and motesanib resulted in a 72% reduction in tumor volume when...
compared with drug alone ($P < 0.0001$). The combination of radiation and motesanib resulted in more pronounced tumor growth inhibition than either motesanib alone ($P = 0.008$) or radiation alone ($P = 0.001$).

In UM-SCC6 xenografts, motesanib 20 mg/kg daily alone resulted in a reduction in tumor volume (Fig. 2C), but this was not statistically significant. The combination of motesanib and radiation resulted in more pronounced tumor growth inhibition than motesanib alone ($P = 0.01$ at day 50), but the combined effect was not significantly greater than radiation alone in terms of antitumor effect.

Motesanib and radiation alter xenograft histomorphology

To examine the effect of combining radiation and motesanib on tumor tissue, we carried out immunohistochemical analyses to examine tumor vascularity and architecture. UM-SCC1 xenografts treated with vehicle control, radiation, motesanib, or the combination were harvested three hours after the last radiation treatment (as indicated in Fig. 2A). Tissue was fixed and stained for vWF, which is constitutively expressed in endothelium. Vehicle-treated tumors showed large areas of viable tumor

Fig. 2. Motesanib augments radiation response in tumor xenograft models. Mice bearing UM-SCC1 (A), SCC-1483 (B), or UM-SCC-6 (C) tumors were treated with either motesanib or vehicle by oral gavage 5× weekly (●). Twice-weekly radiation was also administered (*). Data points are expressed as mean tumor size ($n = 10$/group) ± SEM. Arrows, days that tumors were harvested for immunohistochemistry (as presented in Figs. 3 and 4).
cells with prominent vessels coursing through tumor parenchyma (Fig. 3A). To examine the effect of motesanib on tumor vasculature, we analyzed the vessels per HPF, as well as the proportion of tumor area staining positive for vWF. Motesanib treatment (75 mg/kg × 4 weeks) reduced the number of vessels per HPF (6.3 ± 1.4 versus 3.2 ± 0.7; \( P = 0.15 \); Fig. 3C). Additionally, control tumors had a significantly larger proportion staining positive for vWF (11.2 ± 1.4%) than motesanib-treated tumors (2.4 ± 0.9%; \( P < 0.01 \); Fig. 3D), suggesting that motesanib treatment reduced the number as well as the caliber of vessels coursing through the tumor, with little demonstrable impact on the tumor cell parenchyma. Radiation alone (3 Gy × 8) had a large effect on the tumor parenchyma, reducing the cellular content of tumors. However, vasculature could still be observed throughout the tumor. The combination of motesanib and radiation resulted in the emergence of large necrotic areas within the core of the tumors (Fig. 3A). Similar patterns were shown in SCC-1483 xenografts (data not shown).

**Motesanib and radiation impact the tumor microenvironment**

To explore the capacity of motesanib to augment antitumor efficacy (Fig. 2) and increase tumor necrosis (Fig. 3) in combination with radiation we examined the intratumoral relationships among tumor vascularity, proliferation, and hypoxia. Mice harboring UM-SCC1 xenografts treated with vehicle control, radiation, motesanib, or the combination (Fig. 2A) were injected with pimonidazole HCl (Hypoxyprobe-1), a validated *in vivo* marker of intracellular hypoxia (14), and harvested three hours after the last radiation treatment (day 47, at end of the 4th week of treatment). In addition, tumors were stained for markers of vascularity (9F1) and proliferation (Ki67). Vehicle-treated control xenografts showed prominent vessels throughout the tumor, with areas of hypoxia spatially related to the vascular distribution and tumor cell proliferation greatest at the periphery (Fig. 4A). A protracted 4-week schedule of fractionated radiation was shown to induce higher levels of proliferation (accelerated repopulation), both in the absence of motesanib (2.5-fold increase; \( P = 0.02 \)) and in the presence of motesanib (2.4-fold increase; \( P = 0.04 \); Fig. 4B). Motesanib alone was not shown to impact tumor cell proliferation.

Treatment with motesanib resulted in a 2-fold increase in hypoxic staining than that observed in control tumors \( (P < 0.02) \); Fig. 4C). Treatment with radiation (3 Gy × 8) significantly decreased hypoxia in the absence (83% reduction; \( P < 0.01 \)) and in the presence of motesanib.
(70% reduction; $P < 0.01$). Furthermore, although radiation treatment lowered levels of hypoxia compared with control, mice treated with motesanib and radiation had significantly higher levels of tumor hypoxia than mice treated with radiation alone (2.5-fold increase; $P < 0.01$). Therefore, motesanib consistently increased intratumoral hypoxia, both in the presence and the absence of fractionated radiation treatment. The impact of motesanib alone on tumor vasculature was not apparent in this small subset ($n = 3$ per group) of xenografts (Fig. 4C).

Discussion

This study presents evidence that motesanib, a potent inhibitor of the VEGF family of receptors, can augment the antitumor efficacy of radiation in tumor xenograft models of HNSCC. To examine mechanisms that may underlie this observed enhancement of radiation effect, we confirm that motesanib shows antiproliferative activity in VEGF-driven endothelial cell models (HUVECs) in vitro, both as a sole agent and in combination with radiation. We further confirm that motesanib blocks the VEGF-stimulated kinase activity and phosphorylation of VEGFR2. In vitro, we examined the effect of motesanib and radiation treatment on tumor histomorphology, and provide evidence that the combination may interact by altering the intratumoral distribution of vasculature, hypoxia, and proliferation.

The interaction between antiangiogenic therapeutic agents and radiation has been extensively studied (7, 8, 10, 15–17). In vitro, VEGF (7) and PDGF (10) have been shown to protect against the effects of radiation on endothelial cells, and receptor tyrosine kinase inhibitors of these signaling cascades, as well as VEGFR-specific antibodies, have shown efficacy in abrogating these radioprotective effects in vitro (10, 15, 16). We show that motesanib inhibited VEGF-stimulated HUVEC proliferation and VEGFR2 kinase activity in a dose-dependent fashion (Fig. 1). Additionally, in media containing VEGF, motesanib exerted mild antiproliferative activity on HUVECs, and when combined with fractionated radiation more potently inhibited HUVEC proliferation. Furthermore, in vitro data suggest that motesanib acts specifically on endothelial cells, as motesanib had no activity on HNSCC cells in either proliferative assays or clonogenic radiation assays.

![Fig. 4. Impact of motesanib on intratumoral hypoxia (pimonidazole), proliferation (Ki67), and vasculature (9F1).](image_url)
The interaction between antiangiogenic agents and radiation is most relevant in vivo, and remains an area of active investigation in both preclinical models and in clinical trials involving patients with solid tumors. When initially postulated (18), it was presumed that antiangiogenic agents held potential to limit the supply of oxygen and nutrients to tumors, thereby limiting proliferative potential. However, limiting tumor oxygenation could result in hypoxia-mediated radioresistance; indeed, some studies combining antiangiogenic agents and radiation have shown an antagonistic interaction (19, 20). However, our group (16, 17) and others have shown that antiangiogenic therapy can augment radiation efficacy in various tumor models. Although specific mechanisms of these interactions are difficult to isolate, effects on tumor cell repopulation and hypoxia are thought to underlie radiation resistance in HNSCC.

Accelerated repopulation of HNSCC tumors undergoing radiotherapy is a recognized clinical phenomenon that can limit the efficacy of radiotherapy in advanced HNSCC (21). Furthermore, the magnitude of accelerated repopulation during fractionated radiotherapy is thought to peak 3 to 5 weeks into the course of treatment (22). We carried out detailed histomorphologic and immunohistochemical examinations of xenograft tissue after four weeks of fractionated radiation and/or motesanib treatment. By staining for tumor vasculature, we identified that motesanib altered the penetration of vessels into tumor xenografts (Fig. 3), similar to previously published reports (11, 23). We also provide evidence that motesanib treatment results in significantly increased intratumoral hypoxia and that hypoxia is spatially related to tumor vessel distribution (Fig. 4). These findings in motesanib-treated xenografts have implications when considering the addition of fractionated radiation as an antitumor agent.

Some of the antitumor effects of radiation can be shown when examining tumor tissue microscopically (Fig. 3). Many tumor clonogens are killed by radiation, and subsequently the tumor parenchyma of irradiated xenografts shows reduced cellularity than control tumors or tumors treated with motesanib alone. However, irradiated tumors show more Ki67 staining than control xenografts, providing evidence for accelerated repopulation in this model (Fig. 4). When these rapidly proliferating cells were deprived of nutrients and oxygen by combining the antiangiogenic agent motesanib with radiation, we observed smaller tumors (Fig. 2) with increased regions of hypoxia (Fig. 4) and markedly enhanced areas of tumor necrosis (Fig. 3). A recent study in breast cancer xenograft models showed enhanced tumor necrosis after motesanib treatment (23). Radiation, by inducing accelerated proliferative activity of remaining clonogens and thereby increasing cellular oxygen and nutrient demand, may effectively prime tumor clonogens for the antiangiogenic effect of motesanib.

A limitation of the current study is that the molecular targets of motesanib were not readily detectable in our tested HNSCC tumor cell lines (Supplementary Fig. S1) and motesanib did not show demonstrable activity in the HNSCC tumor cell lines in vitro (data not shown). These data, however, do corroborate published reports showing that motesanib effects are mediated by inhibitory effects on endothelial cell signaling. Indeed, Polverino et al. (11) have shown that motesanib potently inhibits mouse VEGFR2, and provide data showing in vivo biological activity in mouse endothelial models.

The specific clinical implications of the current findings to human cancer patients remain unknown. Motesanib was shown to significantly induce hypoxia in tumor xenografts, and the prevalence of radioresistant cancer stem cells may be upregulated by hypoxia (24). These results suggest that the sequencing of radiation and antiangiogenic therapies warrant careful clinical evaluation, with reports suggesting that radiation followed by antiangiogenic treatment as the most logical approach (25). Only carefully conducted clinical trials can answer these questions in definitive fashion.

Motesanib is undergoing clinical evaluation in several solid tumor settings and has been shown to be well tolerated in phase I studies with promising early antitumor activity in phase II settings (26, 27). The present studies show that motesanib can augment radiation response in endothelial cells in vitro, and in HNSCC tumor xenograft model systems in vivo. These findings suggest that clinical investigations examining the combination of radiation and motesanib are warranted in HNSCC.

Disclosure of Potential Conflicts of Interest
Through the University of Wisconsin, P.M. Harari and K.R. Kozak hold laboratory research agreements with Amgen, Inc.

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