23814, an Inhibitory Antibody of Ligand-Mediated Notch1 Activation, Modulates Angiogenesis and Inhibits Tumor Growth without Gastrointestinal Toxicity

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Abstract

Dysregulation of Notch signaling has been implicated in the development of many different types of cancer. Notch inhibitors are being tested in the clinic, but in most cases gastrointestinal and other toxicities have limited the dosage and, therefore, the effectiveness of these therapies. Herein, we describe the generation of a monoclonal antibody against the ligand-binding domain of the Notch1 receptor that specifically blocks ligand-induced activation. This antibody, 23814, recognizes both human and murine Notch1 with similar affinity, enabling examination of the effects on both tumor and host tissue in preclinical models. 23814 blocked Notch1 function in vivo, inhibited functional angiogenesis, and inhibited tumor growth without causing gastrointestinal toxicity. The lack of toxicity allowed for combination of 23814 and the VEGFR inhibitor tivozanib, resulting in significant growth inhibition of several VEGFR inhibitor-resistant tumor models. Analysis of the gene expression profiles of an extensive collection of murine breast tumors enabled the successful prediction of which tumors were most likely to respond to the combination of 23814 and tivozanib. Therefore, the use of a specific Notch1 antibody that does not induce significant toxicity may allow combination treatment with angiogenesis inhibitors or other targeted agents to achieve enhanced therapeutic benefit. Mol Cancer Ther; 14(8): 1–10. © 2015 AACR.

Introduction

The Notch signaling pathway regulates many fundamental processes essential for normal development such as the control of cell differentiation, survival, proliferation, and angiogenesis (1–3). Signaling pathways necessary for embryonic development are often found reactivated during tumorigenesis, and inappropriate activation of Notch signaling has been implicated in the development of many different types of adult and pediatric cancers (4, 5). Interestingly, in some tissues, inactivation of Notch signaling may facilitate tumor development, particularly in the case of squamous cell carcinomas (6–8) and bladder cancer (9). This highlights the complexities of Notch signaling, and the importance of understanding the correct context in which Notch pathway inhibition will be beneficial.

In mammals, there are four Notch receptors (Notch1-4), all with similar functional domains and structures (10). The extracellular domains consist of a series of EGF-like repeats that contain the ligand-binding domain (LBD), followed by a negative regulatory region (NRR) that locks the receptor in the “off” conformation in the absence of ligand (11). There are five canonical Notch ligands, including three Delta-like (DLL1, DLL3, and DLL4) and two jagged (Jag1 and Jag2) proteins, which are also membrane bound. Receptor–ligand activation requires interaction between neighboring cells, leading to conformational changes that result in sequential cleavage of the receptor by ADAM and gamma secretase proteases, and release of the Notch intracellular domain (ICD). The Notch ICD translocates to the nucleus where it complexes with other transcription factors and coactivators to turn on downstream target genes (10).

Notch1 activation affects tumorigenesis through multiple mechanisms. Activating mutations of Notch1 occur in several different types of hematopoietic malignancies, including >50% of T-cell acute lymphoblastic leukemia (T-ALL; ref. 12) and approximately 10% of chronic lymphocytic leukemia (CLL; refs. 13, 14), where Notch1 signaling appears to promote resistance to apoptosis (15). Immunohistochemistry shows elevated levels of cleaved Notch1 ICD in several different primary human tumor types, suggesting that Notch1 activation is also a common occurrence in solid tumors (16, 17). Cancer stem cell maintenance is another area where Notch signaling is thought to play a key role (18, 19). Inhibition of Notch1 in...
mouse models can reduce cancer stem cell numbers in both hematopoietic and solid tumors, potentially decreasing resistance to chemotherapy and delaying tumor recurrence (20–22). Finally, Notch1 activity in the microenvironment keeps VEGF signaling in check to optimize tumor angiogenesis. Inhibition of Notch1–DLL4 signaling leads to uncontrolled vascular sprouting, and a significantly greater number of tumor vessels (23–27). These vessels, however, are not efficiently perfused (23, 28). Notch1 inhibition has also been shown to decrease production of endothelial nitric oxide, a vasodilating agent important for regulating blood flow (29). Thus, despite increasing vascular density, Notch1–DLL4 blockade results in impaired vascular function and tumor growth inhibition, likely due to increased hypoxia.

The multifaceted manner in which Notch1 can facilitate tumor growth underscores the tremendous potential of targeting this pathway. Gamma-secretase inhibitors (GSI), which inhibit all four Notch receptors as well as numerous other substrates, have been investigated in the clinic, where hints of antitumor activity have been observed. However, severe gastrointestinal toxicity due to goblet cell metaplasia, or reduced GSI exposure due to CYP3A4-mediated deactivation, has forced suboptimal dosing and schedule alterations, limiting their therapeutic utility (30–33). Inhibitory antibodies targeting Notch1 have been developed in an effort to limit toxicity, but gastrointestinal and other toxicities have still been reported in preclinical and clinical settings (32–35). Nevertheless, signs of clinical response have been seen in patients treated with Notch1 antibody (35), indicating that Notch1 inhibition can be efficacious. Recent preclinical studies demonstrate that a soluble decoy encompassing a subset of the Notch1 EGF-like repeats can block Notch1 signaling without inducing gut toxicity, suggesting that inhibition and toxicity can be uncoupled (36).

Here, we describe the in vitro and in vivo activity of 23814, a human antibody made against the Notch1 LBD that specifically inhibits ligand-induced activation of the Notch1 receptor. The 23814 antibody possesses cross-reactivity toward human and murine Notch1 with similar affinity, allowing us to examine the effect on both tumor and host tissue. At effective doses that allow inhibition of Notch1 signaling in both physiologic and pathologic contexts, no evidence of gastrointestinal or other toxicity is observed. The lack of toxicity allowed combination of 23814 with the pan-VEGFR inhibitor tivozanib to achieve significant tumor growth inhibition in several models that do not respond robustly to tivozanib monotherapy. These results suggest that treatment with Notch1-specific antibodies may be a viable therapeutic option in cancer settings resistant to anti-angiogenic treatment.

**Materials and Methods**

**Cell lines and reagents**

Karpas 45 was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ) cell bank. The HT-1080 cell line was acquired directly from the ATCC and stored frozen at early passage until use. No additional authentication was performed. Stable Notch ligand-expressing lines were established by transfecting CHO Flp-In cells (Life Technologies) with full-length Jagged1, Jagged2, DLL1, or DLL4 using Lipofectamine 2000 (Life Technologies). Notch receptor antibodies for FACS analysis were purchased from BioLegend.

**Tumor xenograft studies**

All mice were treated in accordance with the OLAW Public Health Service Policy on Human Care and Use of Laboratory Animals and the IARL Guide for the Care and Use of Laboratory Animals. All in vivo studies were conducted following the protocols approved by the AVEO Pharmaceuticals Institutional Animal Care and Use Committee.

Efficacy studies were performed with 8- to 10-week-old female NCR nude mice. Cells were resuspended in HBSS containing 50% Matrigel and inoculated s.c. in the flank of each mouse. Primary human tumors from surgical resection were obtained through the Cooperative Human Tissue Network (CHTN). For the Ki-168 patient-derived xenograft (PDX) study, mice were inoculated with 5 × 106 cells obtained from a human primary renal clear cell tumor propagated in vivo and stored frozen before use. When tumors approached approximately 200 mm3, mice were randomized and received 23814 or control hIgG (Xolair; Novartis AG) at 20 mg/kg three times weekly, ErbB3 antibody (AV-203) at 20 mg/kg twice weekly, or tivozanib [dissolved in 0.5% carboxymethylcellulose (CMC)] at 5 mg/kg daily. Mice were inoculated with 3.5 × 106 HT-1080, or 1 × 106 BH cells and randomized when tumor size reached 150 to 200 mm3. Animals received 23814 or tivozanib as described above, combination treatment with 23814 (20 mg/kg three times weekly) plus tivozanib (5mg/kg daily), or vehicle control (20 mg/kg hIgG three times weekly plus daily dosing with 0.5% CMC). Statistical analysis was performed using one-way ANOVA and two-tailed t tests using GraphPad Prism software version 6.

**CD31 staining**

Mice were cooled when tumors approached 250 mm3, and treated with 20 mg/kg 23814 or hIgG antibody on days 1 and 3, and/or dosed daily with 5 mg/kg tivozanib for 3 days. Tumors were collected on day 4, fixed, processed, and embedded in paraffin. Tumor sections were immunostained with antibodies to CD31/PECAM1 to detect the vasculature. The average number of vessels per tumor area (number/μm2) was determined using an Aperio ScanScope XT (Aperio) and Microvessel Analysis v1 Parameters Tool image analysis software (Aperio).

**Thymocyte depletion**

Four-week-old female mixed background mice (Taconic) were separated into groups of five, and treated with 20 mg/kg hIgG or 23814 antibody by i.p. injection. Mice were dosed 2 to 3 times weekly for 18 days. Thymuses were harvested in 5% FBS/PBS, pushed through a 100-μm filter, and spun. The cell pellet was resuspended in cold 1X RBC lysis buffer (eBioscience) for 2 minutes before addition of 5% FBS/PBS. After spinning for 5 minutes at 1,000 rpm, the cell pellet was resuspended in 0.5% BSA/PBS. Thymocytes were counted using the Countess Automated Cell Counter (Invitrogen).

**Alcian blue staining**

Mice were treated with 23814 or hIgG at 20 mg/kg 3× weekly, or with the GSI dibenzazepine (DBZ; Syncom) at 10 μmol/kg daily. All mice were weighed twice weekly. DBZ-treated animals were taken down after 4 weeks due to deteriorating health. 23814 and hIgG arms were treated for 8 weeks. For goblet cell detection, sections of small intestine from all...
treatment groups were harvested at end of study, fixed in 10% formalin, processed, and stained using the Alcian Blue pH 2.5 Stain Kit (American Master Tech Scientific) according to the manufacturer’s instructions.

**Immunooassay for 23814 detection**

Serum samples and standards were diluted in FBS, and 23814 captured using recombinant Notch1Fc (R&D Systems) bound to 96-well High Bind plates (Meso Scale Discovery). Captured 23814 was detected with a SULFO-TAG labeled anti-human F(ab')2 (Jackson ImmunoResearch) using electrochemiluminescence with the Meso Scale Discovery platform.

**Luciferase reporter assays**

Karpas 45 were infected with lentivirus containing the firefly luciferase gene under control of the RBP-Jk transcriptional response element (SABiosciences), and selected with puromycin. Assays were performed by preincubating reporter cells with antibody for 30 minutes, then coculturing with ligand-expressing CHO Flp-In cells that had been seeded in 96-well plates. Twenty-four hours later, cells were processed using the Bright Glo (Promega) reporter assay protocol per the manufacturer’s instructions. Lysates were transferred to white-walled 96-well plates (Greiner Bio-One) and read on a GloMax Luminometer (Promega).

**ICD cleavage and Western blotting**

Recombinant Jag1 mFc and Jag2 mFc were created by fusing cDNA encoding the extracellular domain of human Jag1 or human Jag2, respectively, to the murine IgG1 Fc fragment. Constructs were stably transfected into CHO-K1SV cells and fusion proteins was purified from the supernatant. Ninety-six-well plates were coated with anti-His tag (R&D Systems) or anti-mFc (Jackson ImmunoResearch) to capture recombinant DLL4 (R&D Systems). Jag1 mFc, Jag2 mFc, or mFc (Jackson ImmunoResearch). Karpas 45 was preincubated with 23814 or hlgG for 30’ before plating onto ligand-coated wells. Cells were harvested after 4 hours, and lysates analyzed by SDS-PAGE and Western blot analysis. Blots were probed with antibody specific for cleaved Notch1 ICD (Cell Signaling Technology) or β-tubulin (Cell Signaling Technology). Bands were detected using a ChemiDoc XRS+ imager and ImageLab software (Bio-Rad).

**Expression analysis**

RNA was prepared using Qiashredders and RNeasy miniprep columns (Qiagen), and cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCR was performed on Applied Biosystems HT7900 using Taq-Man Gene Expression assays (DTX1-Hs00269995_m1, HES4-Hs00368353_g1, and NRARP-Hs01104102_s1) and manufacturer-suggested PCR protocols (Life Technologies). Results were analyzed by the comparative CΔΔT method (ΔΔCt) using β-actin as the loading control, as detailed in Supplementary Materials and Methods.

**Hierarchical clustering**

Unsupervised hierarchical clustering was conducted using Euclidian distance and the complete linkage method on the BH expression data (37), selecting the top 1,000 most variant probes as defined by median absolute deviation. The analysis was performed using R version 3.0.2.

**Results**

**Identification and characterization of Notch1-specific antagonistic antibodies**

To assess the therapeutic potential of targeting the Notch pathway in cancer, we used phage display to generate monoclonal antibodies that specifically target EGF-like repeats 11 to 12, the LBD of the Notch1 receptor. Candidate clones were screened by ELISA and FACS for binding to human and murine Notch1, in an effort to identify antibodies that cross-react with the LBD of both species. To identify antibodies able to inhibit ligand-induced activation, we used a luciferase reporter under the control of an RBP-Jk transcriptional response element, which mediates Notch signaling. A stable reporter line was established using Karpas 45, a human T-ALL cell line—expressing high levels of endogenous Notch1 receptor, and used to screen for blocking antibodies. This led to the identification of 23814. This antibody was able to block activation induced by all ligands tested in a dose-dependent manner. Luciferase activity was inhibited by 23814 when the reporter line was cocultured with cells expressing Jag1, Jag2, DLL1, or DLL4 (Fig. 1A). Western blots confirmed that addition of 23814 to Karpas 45 cells robustly prevented ligand-induced Notch1 ICD cleavage, maintaining Notch1 ICD at background levels even in the presence of high amounts of ligand (Fig. 1B). Consistent with effective inhibition of Notch1 signaling, expression of downstream target genes was significantly decreased in the presence of 23814 (Fig. 1C).

FACS analysis shows that 23814 binds to human and murine Notch1 expressed on the cell surface, but not to Notch2 or Notch3 (Fig. 1D). Surface plasmon resonance also confirmed high-affinity binding of 23814 to the extracellular domains of human and murine Notch1 (K_D = 2.3 nmol/L and 3.7 nmol/L, respectively), but not to those of other Notch family members. These results demonstrate that 23814 is a neutralizing Notch1-specific antibody that recognizes both human and murine Notch1 with high affinity.

**Treatment of mice with 23814 results in sustained inhibition of Notch1 signaling in vivo**

To assess the ability of 23814 to inhibit Notch1 function in vivo, we examined its effect on thymocyte development, a well-characterized process dependent on Notch1 signaling. Active Notch1 signaling is required for thymic progenitor cells to commit to the T-cell lineage, and for normal progression through T-cell development. Inducible knockout of the Notchi gene in neonatal mice has been shown to result in a marked reduction in thymus size, as well as a 5-fold reduction in thymocyte number (38). Treatment of mice with 23814 resulted in a dramatic reduction in the number of thymocytes relative to isotype control-treated mice (Fig. 2A). The decrease in thymocyte number was dose-dependent and consistent with effective sustained inhibition of murine Notch1 activity by 23814. No weight loss or diarrhea was observed at any of the doses tested (Fig. 2B).

**23814 does not induce gut toxicity at efficacious doses**

Several Notch inhibitors have entered the clinic, but have limited effectiveness due to the development of goblet cell metaplasia, an overwhelming conversion of progenitor cells to secretory goblet cells at the expense of absorptive enterocytes (30, 39). The ability of 23814 to recognize murine Notch1
including liver, heart, spleen, kidney, and thymus, were examined twice weekly with 5, 20, or 40 mg/kg 23814 antibody for 4 weeks. Upon termination of the study, all major organs, treated animals at end of study (Fig. 2D). To further investigate the potential toxicity of Notch1 inhibition, mice were treated for some DLL4 and Notch1 inhibitors (34). 23814 was confirmed to be present at high concentration in the serum of treated animals after 23814 treatment, at doses that achieve functional inhibition of Notch1 signaling (41). Therefore, Notch1 activation might be expected in settings where VEGF signaling is high. Although VEGF is overexpressed in a number of different types of cancer, clear cell renal cell carcinoma (RCC) is uniquely dependent on VEGF signaling (41). Therefore, Notch1 activation might be expected in settings where VEGF signaling is high. Although VEGF is overexpressed in a number of different types of cancer, clear cell renal cell carcinoma (RCC) is uniquely dependent on.

Figure 1.
In vitro characterization of 23814 activity. A, Notch1 luciferase reporter line cocultured with stable lines expressing Jag1, Jag2, DLL1, and DLL4 ligands in the presence of 23814 or control hlgG antibodies. Results represented as percent inhibition of luciferase activity. B, Karpas 45 plated on wells coated with recombinant Jag1, Jag2, DLL4, or Fc (no ligand) in the presence of 23814 or control hIgG antibodies. C, Karpas 45 cocultured with ligand-expressing CHO lines in the presence of 23814 or hIgG. Western blots were probed with antibody specific for cleaved Notch1 ICD (Cell Signaling Technology). Equal amounts of lysate transferred to a separate blot were probed with β-tubulin antibody as a loading control. D, FACS analysis of 23814 binding to CHO cells expressing human or murine Notch1, human Notch2, or human Notch3.

allowed us to investigate its effect on gut toxicity at doses relevant to the inhibition of Notch1 signaling. To confirm the lack of goblet cell conversion after 23814 treatment, Alcian blue staining was performed on the small intestines of mice treated for 8 weeks with 20 mg/kg of either hlgG or 23814, or for 4 weeks with DBZ, a gamma-secretase inhibitor. In agreement with previously reported results, mice treated with DBZ developed severe goblet cell metaplasia (30, 40) as indicated by increased Alcian blue staining of mucin in intestinal crypts (Fig. 2C). In contrast, the small intestines of 23814-treated mice were histologically similar to those of control mice and did not show increased goblet cell numbers (Fig. 2C). Moreover, no signs of vascular neoplasm were observed, as has been reported for some DLL4 and Notch1 inhibitors (34). 23814 was confirmed to be present at high concentration in the serum of treated animals at end of study (Fig. 2D). To further investigate the potential toxicity of Notch1 inhibition, mice were treated twice weekly with 5, 20, or 40 mg/kg 23814 antibody for 4 weeks. Upon termination of the study, all major organs, including liver, heart, spleen, kidney, and thymus, were examined for histology. Again, no signs of vascular neoplasm or vascular proliferation were observed in any of the animals (Supplementary Fig. S1A–S1C). Histopathology showed rare instances of single cell necrosis or mitotic figures in the livers of some 23814-treated animals, but these were not dose-responsive and considered recoverable. Serum collected at termination showed that clinical pathology analysis was well within normal reference range for liver enzymes (Supplementary Table S1), and that 23814 exposure was maintained in a dose-dependent manner (Supplementary Fig. S1D). This suggests that 23814 treatment for 4 weeks, at doses that achieve functional inhibition of Notch1 signaling in vivo, does not produce severe toxicities.

23814 inhibits tumor growth in kidney PDX model
Expression of DLL4 in endothelial cells is directly induced by VEGF signaling (41). Therefore, Notch1 activation might be expected in settings where VEGF signaling is high. Although VEGF is overexpressed in a number of different types of cancer, clear cell renal cell carcinoma (RCC) is uniquely dependent on...
VEGF signaling due to the frequency of the von Hippel-Lindau (VHL) tumor-suppressor mutation (42). To assess the functional activity of 23814 in a tumor model, we used Ki-168, a PDX model established from a clear cell RCC tumor. PDX models are thought to retain key characteristics of the original donor tumor and, therefore, to possess superior potential to predict clinical outcome compared with traditional cell line–based models (43).

Ki-168 PDX tumors were treated with hIgG, 23814, AV-203 (a negative control antibody against ErbB3), or tivozanib, a potent and selective inhibitor of VEGFR-1, -2, and -3 that has demonstrated significant activity in the clinic (44). Blockade of VEGFR signaling due to tivozanib treatment effectively inhibited Ki-168 tumor growth relative to control. Similarly, treatment with the 23814 Notch1 antibody resulted in potent tumor growth inhibition of Ki-168. In contrast, inhibition of ErbB3 signaling with AV-203 had no effect in this model (Fig. 3). These results show that monotherapy treatment with 23814 is able to inhibit growth of a patient-derived RCC tumor, a type of tumor especially sensitive to inhibitors of angiogenesis.

Treatment with 23814 alters tumor vasculature and inhibits tumor growth in models that do not respond well to VEGFR inhibitors

It has previously been demonstrated that inhibition of the Notch pathway through blockade of DLL4 can negatively affect tumor growth in preclinical models that are resistant to anti-VEGF therapy (23, 24, 26, 28, 45). To see if specific inhibition of the Notch1 receptor can achieve similar results, we used the highly vascular HT-1080 xenograft model, which has been widely used to study tumor angiogenesis. HT-1080 tumors were treated with 23814, either alone or in combination with tivozanib. Treatment with either 23814 or tivozanib monotherapy resulted in partial tumor inhibition relative to control. Combination treatment using 23814 and tivozanib together resulted in significantly greater efficacy than either drug alone (P < 0.001; Fig. 4A).

To determine whether 23814 was affecting tumor angiogenesis, immunohistochemistry was performed on HT-1080 tumors. Established tumors that had been treated for 3 days with either 23814, tivozanib, or a combination of the two were collected and stained with anti-CD31 to examine vessel density. Quantification of CD31 staining demonstrates that treatment with 23814...
markedly increased tumor vascular density (Fig. 4B), consistent with specific inhibition of Notch1–DLL4 signaling (23–25, 27). As expected, inhibition of VEGF signaling with tivozanib resulted in greatly decreased tumor vasculature. Combination treatment with 23814 and tivozanib resulted in reduced tumor vessel density relative to vehicle control, similar to that observed with tivozanib alone (Fig. 4B).

To determine whether 23814 could inhibit tumor growth in other VEGF inhibitor-resistant models, we examined a collection of breast cancer tumors derived from chimeric mice engineered to inducibly express mutated HER2 (V659E; refs. 37, 46). More than 100 individual primary tumors from these breast Her2 (BH) mice have been propagated, characterized, and shown to have diverse characteristics across the collection by histopathology, microarray analysis, and variation in response to drug treatment due to their chimeric nature (37). Accordingly, tumors from BH models differ widely in their response to tivozanib monotherapy, ranging from complete regression to varying degrees of resistance (Fig. 5A and Supplementary Fig. S2; and data not shown). Several different BH tumor models that did not robustly respond to tivozanib monotherapy were chosen to examine whether increased efficacy could be achieved through blockade of Notch1 signaling using 23814. Although most of the models tested had a moderate response to either 23814 or tivozanib alone, all but one model exhibited significantly increased tumor inhibition when treated with a combination of tivozanib and 23814 (Fig. 5A and Supplementary Fig. S2; and data not shown). To confirm that 23814 and tivozanib were effectively inhibiting the Notch and VEGFR signaling pathways, respectively, expression of downstream genes was examined using TaqMan assays. Analysis of the treated tumors demonstrated that 23814 inhibited Notch1 signaling, as canonical Notch targets such as HEY1 and HES1 were downregulated (Fig. 5B). Tivozanib treatment decreased FLT4 (VEGFR3)
expression as expected, but 23814 significantly increased FLT4 expression (Fig. 5B), in accordance with findings, suggesting that increased VEGFR3 signaling is responsible for the deregulated angiogenesis observed upon Notch1 inhibition (47). Hypoxia-regulated gene expression also increased upon treatment with 23814, tivozanib, and combination therapy, consistent with inhibition of angiogenesis (Fig. 5C). CD31 staining demonstrated a marked increase in tumor vessels relative to vehicle control in 23814-treated tumors consistent with Notch1 blockade, whereas tivozanib and combination treatment greatly decreased vessel number (Fig. 5D). Interestingly, significant increases in vessel sprouting were also observed in some BH tumors treated with 23814 monotherapy whose growth was not robustly inhibited (Supplementary Fig. S2), suggesting that aberrant vessel density is a marker of Notch1 inhibition, but is not predictive of potent tumor growth inhibition. Although addition of 23814 to tivozanib treatment did not decrease the number of tumor vessels relative to tivozanib alone (Fig. 5D), the combination of the two inhibitors did significantly increase the amount of tumor necrosis observed in BH224 (Supplementary Fig. S3).

Hierarchical clustering predicts response to 23814 and tivozanib combination treatment

As not all BH tumors are equally inhibited by 23814 and tivozanib, it would be desirable to be able to predict which tumors would best respond to combination treatment. Because BH224 exhibited robust tumor inhibition in response to combination therapy (Fig. 5A), unsupervised, hierarchical clustering was used to determine which BH tumor models were most biologically similar to BH224. BH224 clustered in a distinct subgroup that did not include any of the other BH tumors tested in initial studies with 23814 (Fig. 6A). To see if tumors in this subgroup share a dependence on Notch1–VEGF signaling, two of the closest neighbors to BH224, BH220, and BH228, were assessed for response to combination treatment with 23814 and tivozanib. Both BH270 and BH228 tumor models responded strongly to combination therapy, and exhibited greater sustained inhibition with 23814/
tivozanib treatment than previously tested BH models that clustered in separate subgroups (Fig. 6B). These results suggest that there is an underlying biology in the tumors clustering within the BH224 subgroup that predisposes them to dependence on Notch1–VEGF signaling, making them especially susceptible to inhibitors of these pathways.

**Discussion**

Here, we report the identification of a Notch1-specific antibody that exhibits antitumor efficacy and modulates tumor angiogenesis, without causing gastrointestinal or other toxicities previously reported for Notch pathway inhibitors. Several pan-Notch inhibitors have entered the clinic, but a major obstacle in their progress has been the induction of dose-limiting toxicity due to intestinal goblet cell metaplasia. Studies in conditional knockout mice have suggested that goblet cell metaplasia requires inhibition of both Notch1 and Notch2 receptors (48). Therefore, much effort has gone into the development of antibodies that specifically target various epitopes of Notch1 (20, 21, 27, 49, 50), with the expectation that this would eliminate gut toxicity. Surprisingly, however, it was reported that treatment of mice with antibodies that target Notch1 through binding to the negative regulatory region (NRR) can induce some level of goblet cell metaplasia (27). One possible explanation for the difference in toxicity observed between 23814 and the previous report is the differing mechanisms of action. Although 23814 binds to the Notch1 LBD and prevents association of the receptor with its activating ligands, the NRR antibody allows ligand binding but keeps the receptor locked in an "off" state. It is possible that Notch1 receptors bound to an NRR antibody are able to bind and sequester ligands in this inactive conformation, thereby affecting signaling of other Notch family receptors. Other Notch1-specific antibodies detailed in the literature either do not cross-react with murine Notch1 or are not characterized in *in vivo* experiments, so it is unknown whether induction of goblet cell metaplasia is a feature of anti-Notch1 NRR antibodies in general, or a specific property of certain antibodies. Another possible explanation for the lack of toxicity for 23814 versus other Notch1 antibodies is simply that there is a difference in potency or antibody kinetics.

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**Figure 6.**
Unsupervised hierarchical clustering identifies tumors likely to respond to 23814/tivozanib combination therapy. A, cluster analysis demonstrates that BH tumors separate into distinct subgroups. BH413 and BH226 (labeled in blue), representing a nonresponder and partial responder, respectively, cluster in different subgroups from BH224, whereas BH228 and BH270 (labeled in green) are biologically similar. B, BH228 and BH270 tumor growth inhibition is significantly increased by combination treatment with 23814/tivozanib relative to tivozanib monotherapy (*, P < 0.01 and **, P < 0.05).
that leads to a larger therapeutic window. Perhaps for inhibitors of targets such as Notch, which regulate stem cell and normal tissue homeostasis, the key is not to treat at the maximum tolerated dose, but instead to treat at the minimum efficacious dose.

Our data suggest that Notch1 inhibitors can be effective in settings where antiangiogenesis therapies are indicated. We have demonstrated potent tumor inhibition in a patient-derived primary RCC model using 23814 monotherapy, as well as in combination with tivozanib in several models that exhibit partial resistance to VEGFR inhibitor alone. Although monotherapy treatment of the BH tumors with 23814 resulted in only modest resistance to VEGFR inhibitor alone. Although addition of 23814 to tivozanib treatment resulted in significantly greater tumor necrosis, as well as increased induction of hypoxia genes, compared with tivozanib monotherapy (Fig. 5C and Supplementary Fig. S3). This is consistent with an antiangiogenic mechanism of inhibition, and is similar to what has been observed when DLL4 and VEGF inhibitors are combined (28), further suggesting that inhibition of the Notch1 and VEGF pathways is not redundant (23, 24, 45). Use of a Notch1 antibody may have the added advantage of simultaneously blocking oncogenic activation of Notch1 signaling not only through DLL4 in the stroma, but also through other ligands such as Jag1 or Jag2 in the tumor itself, possibly affecting prosurvival signals in addition to angiogenesis.

Undoubtedly, there are many mechanisms leading to VEGF inhibitor resistance, and discovering which tumors are likely to respond to Notch1 inhibition is essential for success in the clinic. Our BH tumors, derived from chimeric primary mouse models, demonstrate a wide range of responses to combined tivozanib and 23814 therapy, likely reflecting what would be observed in the patient population. It is well established that the expression level of Notch1 receptor itself is not predictive of response to Notch inhibitors in the clinic, and response to combination therapy is unlikely to depend on Notch signaling alone. Therefore, instead of limiting our scope to differences in canonical Notch pathway genes, we used unsupervised hierarchical clustering in an attempt to predict a priori which tumor models would respond to tivozanib/23814 treatment. The two models selected for testing from the same subgroup as the best responder exhibited robust tumor inhibition in response to tivozanib/23814 combination treatment that significantly exceeded response to either therapy alone. This underscores the ability to enrich for responders based on the unique combination of biologic pathways that are activated in subgroups of tumors.

Our findings demonstrate that the 23814 Notch1 antibody exhibits antitumor efficacy that is not redundant with VEGF pathway inhibition, and that angiogenesis inhibitors with different mechanisms of action can be combined to achieve improved outcome. The lack of toxicity may allow combination of 23814 with VEGF inhibitors or other therapies at efficacious doses, and suggests that evaluation of specific Notch1 inhibitors as a treatment option for VEGF inhibitor-resistant tumors is warranted.

Disclosure of Potential Conflicts of Interest

A. Bell has ownership interest in a provisional patent filed, WO2012003472A1. R. Nicoletti has ownership interest (including patents) in Aveo Pharmaceuticals. W. M. Winston has ownership interest (including patents) in AVEO. S. Weiler has ownership interest (including patents) in AVEO. J. Lin has ownership interest (including patents) in AVEO Pharmaceuticals. J. Gyuris has ownership interest (including patents) in AVEO Oncology. No potential conflicts of interest were disclosed by the other authors.

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