Published in final edited form as:

Mol Cancer Res. 2019 February ; 17(2): 348–355. doi:10.1158/1541-7786.MCR-18-0427.

Cyclooxygenase-2 inhibition potentiates the efficacy of vascular endothelial growth factor blockade and promotes an immune stimulatory microenvironment in preclinical models of pancreatic cancer

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Abstract

Resistance to standard therapy remains a major challenge in the treatment of pancreatic ductal adenocarcinoma (PDA). Although anti-VEGF therapy delays PDA progression, therapy-induced hypoxia results in a less differentiated mesenchymal-like tumor cell phenotype, which reinforces the need for effective companion therapies. COX-2 inhibition has been shown to promote tumor cell differentiation and improve standard therapy response in PDA. Here, we evaluate the efficacy of COX-2 inhibition and VEGF blockade in preclinical models of PDA. In vivo, the combination therapy was more effective in limiting tumor growth and metastasis than single-agent therapy. Combination therapy also reversed anti-VEGF–induced epithelial-mesenchymal transition, collagen deposition and altered the immune landscape by increasing tumor-associated $CD8^+$ T cells while reducing $FoxP3^+$ T cells and FasL expression on tumor endothelium.

Graphical Abstract

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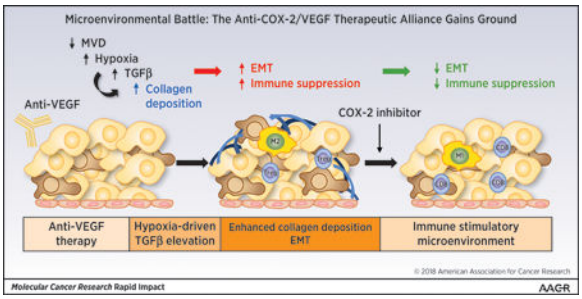
Author contributions

AK, MTD, and RAB designed the experiments; AK, YZ, HH, NBS, and MTD performed the experiments and collected data. YZ, AK, FJB, and RAB analyzed data and wrote the manuscript and RAB is the corresponding author.

Disclosure of potential conflicts of interest: R.A. Brekken declares research funding from Tragara Pharmaceuticals, Inc. Additionally, F.J. Burrows was an employee of Tragara Pharmaceuticals at the time of the majority of the studies. The remaining authors have declared that no conflict of interest exists.



Keywords

COX-2; VEGF; tumor microenvironment; pancreatic cancer

Introduction

Primary tumors and metastases require nutrients and oxygen delivered by blood vessels[1]. Although angiogenesis is complex, it is widely recognized that vascular endothelial growth factor-A (VEGF-A) is the predominant angiogenic factor that promotes tumor neovascularization[2,3]. Inhibitors of angiogenesis have become a central part of systemic therapy for a variety of malignancies[4,5]. However, angiogenesis inhibition has in general resulted in only modest gains in clinical outcomes in cancer patients as many patients treated with anti-angiogenic/anti-VEGF therapy either fail to respond or relapse on therapy[6,7]. Additionally, anti-angiogenic therapy has been implicated in promoting tumor progression and accelerating metastasis in preclinical models[8,9].

Pancreatic cancer, the third-leading cause of cancer-related death[10], is highly metastatic and poorly responsive to standard therapy[11,12]. It is also an immunologically “cold” tumor that has remained largely refractory to immune checkpoint blockade[12,13]. Anti-VEGF therapy has been studied in pancreatic cancer patients[4]; however, it has not provided significant clinical benefit in combination with gemcitabine, the standard chemotherapy for pancreatic ductal adenocarcinoma (PDA)[14–16]. Previously, we investigated the efficacy and biology of anti-VEGF therapy in preclinical models of PDA using the antibody mcr84[9,17]. We found that mcr84 alone or in combination with gemcitabine slowed the growth of PDA but induced hypoxia-induced epithelial plasticity that resulted in a less differentiated tumor cell phenotype and continued metastatic burden[9]. These observations reinforce the need to develop companion therapies that combat therapy-induced epithelial plasticity.

Inflammation is a pathological phenotype that facilitates the “hallmarks” of cancer[18]. Further, the incidence of several cancers is associated with inflammation, which contributes to tumor initiation and cancer cell survival by producing reactive oxygen species, cytokines, and proinflammatory mediators[19]. Among mediators of inflammation that are associated with tumor progression is cyclooxygenase-2 (COX-2), an inducible enzyme that catalyzes the rate-limiting step in the synthesis of the prostaglandin E_2 (PGE_2). COX-2 is induced at sites of inflammation and during the process of tumor progression[20]. Multiple studies have demonstrated that elevated COX-2 expression is prevalent in human malignancies, including

PDA[21,22]. In addition, elevated expression of COX-2 in tumors correlates with advanced stage and worse outcome by promoting chemoresistance, metastasis, and angiogenesis[23,24]. COX-2 has also been identified as a potential mediator of VEGF-independent tumor angiogenesis[25]. Thus, targeting COX-2 inhibition has been explored as a potential anticancer therapy[26]. Additionally, COX-2 blockade can enhance the efficacy of anti-angiogenic treatments in breast cancer, which supports the investigation of COX-2 inhibitors with VEGF blockade in other tumors[27].

Apricoxib is a selective COX-2 inhibitor that has shown significant anti-tumor activity in various xenograft models[28] and has been under clinical investigation. Previously, we demonstrated that apricoxib improved the efficacy of standard therapy in preclinical models of PDA[29]. Further, we found that inhibition of COX-2 reversed epithelial-mesenchymal transition (EMT), leading to a shift toward a more epithelial phenotype in xenograft models of PDA[29]. In the present study, we investigated the combination of anti-VEGF therapy and COX-2 inhibition as a therapeutic strategy in robust preclinical models of PDA with the hypothesis that apricoxib would prevent or reduce therapy-induced epithelial plasticity. We also investigated the effect of anti-VEGF and COX-2 inhibition on the immune landscape of PDA given that prior reports have demonstrated VEGF and PGE_2 can limit T cell infiltration into tumor cell nests[30] and reports that EMT can be a significant driver of immune suppression in tumors[31–34].

Materials and Methods

Cell culture

Human pancreatic cancer cell lines AsPC-1, HPAF-II were obtained from ATCC. Colo357 was obtained from MD Anderson Cancer Center. AsPC-1 was grown in DMEM, Colo357 and HPAF-II in MEM. All cell lines were grown in a humidified atmosphere with 5% CO_2, at 37˚C, and were DNA fingerprinted for provenance using the Power-Plex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained by ATCC. Cell lines were confirmed to be free of mycoplasma (e-Myco kit, Boca Scientific) before use. In vitro PGE_2 and VEGF response to apricoxib treatment was evaluated by enzyme-linked immunosorbent assay (ELISA; R&D Systems) of conditioned media over different time points. To induce EMT, cells were grown on collagen I-coated plates and treated with 50 ng/ml transforming growth factor-β (TGFβ) for 24 hours[35]. EMT changes were confirmed by probing cell lysates for E-Cadherin, N-Cadherin and Vimentin (Cell Signaling) (see Supplementary Table 1).

Animal studies

All animals were housed in a pathogen-free facility with 24-hour access to food and water. Experiments were approved by and conducted in accordance with the Institutional Animal Care and Use Committee at UT Southwestern (Dallas, TX). *Kras*$^{LSL\text{-}G12D}$*; Cdkn2a*$^{fl/fl}$*; Ptf1a*$^{Cre/+}$ (*KIC*) mice were generated as previously described[36]. At 3 weeks of age, mice were randomized to receive saline, mcr84 500 μg/dose i.p. once weekly, Apricoxib 10 mg/kg by oral gavage daily or mcr84 plus Apricoxib. All mice were sacrificed when they were 7 weeks old. Four-to-6-week-old female NOD/SCID mice were obtained from a campus

supplier. A total of 1×10^6 Colo357 cells were injected orthotopically and tumor growth was monitored by ultrasound. Mice with established tumors were randomized to receive therapy. Treatment groups were the same as described above. Mice bearing Colo357 tumors received 4 weeks of therapy prior to sacrifice. Tissues from all animal experiments were fixed in 10% formalin or snap-frozen in liquid nitrogen for further studies.

Histology and tissue analysis

Formalin-fixed tissues were embedded in paraffin and cut in 5 μm sections. Sections were evaluated by Masson's Trichrome staining and immunohistochemical analysis using antibodies specific for VEGF, COX-2 (Abcam), E-cadherin, N-cadherin, Slug, Snail (Cell Signaling), CD3 (Bio-Rad), CD8 (Bioss), FoxP3 (eBioscience), CD31 (dianova), FasL (Santa Cruz), CD11b (Abcam), iNOS, Arginase 1 (Thermo Fisher, Arg-1), endomucin (Santa Cruz), NG2 (Millipore) and F4/80 (Novus Biologicals). Fluorescent images were captured with Zeiss Aixoscan Z1 using ZenLite software. Color images were obtained with Hamamatsu Nanozoomer 2.0HT using NDPview2 software. Pictures were analyzed using NIS Elements (Nikon) and Fiji software.

Statistical analysis

Data were analyzed using GraphPad software. Results are expressed as mean ± SEM. Data were analyzed by ANOVA with the Dunn's test for multiple comparisons and results are considered significant at $p< 0.05$.

Results

Pharmacologic blockade of COX-2 and VEGF inhibits tumor growth and limits metastatic burden in pancreatic cancer models

To investigate the efficacy of COX-2 inhibition with apricoxib and VEGF blockade with mcr84[17] in preclinical models of PDA, we used a genetically engineered mouse model of PDA and SCID mice bearing established orthotopic pancreatic xenografts. Therapy was initiated in 3-week-old *KIC* mice. Mice were randomized to receive saline, mcr84, apricoxib, or mcr84 + apricoxib and were sacrificed after 4 weeks (7 weeks old). Therapy with mcr84 or apricoxib reduced primary tumor weight by ~30% whereas mcr84 + apricoxib reduced primary tumor weight by 62% compared to the control group ($P < 0.0001$; Fig. 1a). At the time of sacrifice, the extent of liver metastasis was determined based on gross metastasis. Seven out of 10 evaluable mice in the control group had at least 1 macroscopic metastasis; this number was reduced to 1/5, 2/7, 1/6 for the mcr84, apricoxib, and combination therapy groups, respectively (Fig. 1a). To further define the effect of COX-2 inhibition and anti-VEGF therapy on tumor burden and liver metastases, we established human PDA xenografts in mice by orthotopically injecting Colo357, a human pancreatic cancer cell line, into the pancreas of SCID mice. Similar to in vitro data published previously[29], Colo357 cells showed high COX-2 expression and were responsive to apricoxib (data not shown). Mice with established tumors, which was confirmed by ultrasound, were randomized to receive treatment as described above. After 4 weeks of therapy, we found that single-agent therapy had a minimal effect on primary tumor growth (Fig. 1b) and metastatic incidence, although the mean metastatic events per treatment group

was reduced by mcr84 or apricoxib (Fig. 1b). In contrast, combination therapy significantly reduced primary tumor weight ($P < 0.05$ vs. control) and substantially limited metastases ($P < 0.01$ vs. control; $P < 0.05$ vs. single-agent therapy; Fig. 1b). H&E analysis of livers confirmed metastatic lesions in Colo357 tumor bearing mice (Supplementary Fig. S1a). The effect of mcr84 + apricoxib on primary tumor growth compared favorably to the effect of gemcitabine + erlotinib in the same model reported in our prior study[37] (Supplementary Fig. S1b).

COX-2 activity has been implicated in promoting angiogenesis[25,38,39]. Previously, prostaglandins, products of COX-2 activity were shown to elevate VEGF expression and inhibition of COX-2 was shown to contribute to anti-angiogenic effects[40,41]. Furthermore, fibroblasts from *Cox-2-deficient* mice were reported to produce significantly less VEGF than fibroblasts from wild-type or *Cox-1-deficient* animals[42]. Additionally, treatment of wild-type fibroblasts with a selective COX-2 inhibitor resulted in a 90% reduction in VEGF production[42]. However, recently Xu et al[25] determined that PGE_2 can contribute to angiogenesis in a VEGF-independent manner in colon cancer models. Given these data, we sought to investigate the relationship between COX-2 activity and VEGF production in PDA cell lines, we selected a COX-2 negative cell line, AsPC-1, and 2 COX-2 positive cell lines, one with a high expression of COX-2, Colo357, the other with moderate COX-2 expression, HPAF-II[29]. Cells were treated with 500 nM apricoxib and the level of VEGF produced was determined by ELISA. Only in high COX-2 cell line, Colo357, COX-2 inhibition reduces VEGF production transiently. In HPAF-II and AsPC-1 cells, VEGF production was unaffected by apricoxib, with VEGF production in HPAF-II cells elevated over time (Fig. 1c). To determine if EMT induction altered VEGF production and/or the effect of apricoxib, we plated Colo357 and AsPC-1 cells under conditions that stimulate EMT. Under normal culture conditions we observed similar trends as shown before. However, under EMT-inducing conditions VEGF production was elevated significantly and was largely independent of COX-2 inhibition in Colo357 cells. In AsPC-1 cells, VEGF production increased faster over time under induced EMT conditions compared to normal conditions **(Fig. 1c)**. We also investigated the effect of apricoxib on PGE_2 production by Colo357 and Aspc-1 cells under normal and EMT-inducing culture conditions (Supplementary Fig. S2a, b). The induction of EMT was confirmed by evaluating the expression level of E-Cadherin, N-Cadherin and Vimentin (Supplementary Fig. S2c). The induction of EMT reduced the effect of apricoxib on PGE_2 production in Colo357 cells. In contrast, AsPC-1 cells produced minimal PGE_2 under either culture condition (Supplementary Fig. S2a, b). We corroborated these findings by examining the level of VEGF expression in Colo357 pancreatic xenografts by immunofluorescence staining and found that VEGF expression was not affected by apricoxib (Fig. 1d). Importantly, apricoxib did reduce COX-2 expression in Colo357 tumors supporting the pharmacodynamic activity of the drug. The induction of hypoxia by mcr84 is consistent with prior studies[9] and the reduction of microvessel density by mcr84 in Colo357 tumors (Supplementary Fig. S3a, b). We found that apricoxib alone did not reduce microvessel density in Colo357 tumors (Supplementary Fig. S3a, b), which further supports that apricoxib anti-tumor activity is not mediated by inhibition of angiogenesis. However, we did observe that apricoxib alone or in combination with mcr84 increased the percentage of pericyte-associated blood vessels in Colo357 tumors (Supplementary Fig. S3c). These data

suggest that COX-2 functions in a VEGF-independent manner in PDA to promote tumor progression.

Apricoxib in combination with mcr84 reverses anti-VEGF–induced EMT and collagen deposition

Although anti-VEGF therapy with mcr84 restricts tumor growth and improves the survival of *KIC* mice[9], therapy-induced hypoxia results in a less differentiated tumor cell phenotype[9]. We previously found that COX-2 inhibition with apricoxib reverses EMT in HT29 xenografts[28] and Colo357 tumor-bearing mice[29]. To determine whether apricoxib can prevent or reduce hypoxia-induced epithelial plasticity as a result of mcr84 treatment, we analyzed tumor tissue from *KIC* mice in each treatment group. Treatment with mcr84 alone increased the expression of N-cadherin, a common marker of mesenchymal cells and Slug, an EMT-inducing transcription factor (EMT-TF). Apricoxib alone or in combination with mcr84 significantly reduced N-cadherin expression and downregulated Slug expression to the same level of control group. Although the expression of Snail, another EMT-TF[43] was not affected by mcr84, treatment with apricoxib or apricoxib combined with mcr84 decreased Snail expression significantly (**Fig. 2a)**. We also observed that collagen deposition was increased in *KIC* and Colo357 tumors after treatment with mcr84, a feature we identified previously that is associated with epithelial plasticity[9]. This effect was attenuated by apricoxib alone or in combination with mcr84 (Fig. 2b).

Blockade of VEGF and COX-2 pathways promotes an immune stimulatory microenvironment

Eicosanoids, including PGE_2, contribute to the immune microenvironment of solid tumors[20]. For example, PGE_2 can induce a shift in cytokine expression in myeloid-derived suppressor cells (MDSCs) and macrophages towards an immune suppressive profile (e.g., IL-4, IL-10, IL-6) and PGE_2 can directly reduce T effector cell activity[44]. Furthermore, EMT is also associated with an immunosuppressive tumor microenvironment[31,32,34]. Thus, given our observations that COX-2 inhibition with apricoxib reduces PGE_2 production and decreases therapy-induced EMT, we investigated the immune landscape in *KIC* tumors from the different treatment groups shown in Figure 1. Tumors harvested from mice that received mcr84 or apricoxib alone had an increase in the number of tumor-associated $CD3^+$ and $CD8^+$ T cells. Combination therapy further elevated $CD3^+$ and $CD8^+$ T cell levels (Fig. 3a). Additionally, apricoxib alone and in combination with mcr84 significantly decreased $FoxP3^+$ regulatory T (T_{reg}) cells (Fig. 3a). Motz et al[30] previously reported that selective expression of the death mediator Fas ligand (FasL) on endothelial cells in human and mouse solid tumors was associated with scarce T-cell infiltration. They also identified that FasL was induced on endothelium by VEGF, IL-10, and PGE_2. Thus, we evaluated FasL expression in the vasculature of *KIC* tumors by dual staining of the endothelium for CD31 and FasL. We found that FasL was indeed present on $CD31^+$ endothelial cells in control-treated *KIC* tumors and that treatment with mcr84, apricoxib, or the combination significantly reduced endothelial FasL expression (Fig. 3b). To determine the effect of VEGF blockade and COX-2 inhibition on macrophages in the tumor microenvironment, we stained for CD11b, iNOS, and Arginase 1. We found that mcr84 alone and mcr84 in combination with apricoxib reduced $CD11b^+iNOS^+$ macrophages but apricoxib alone did

not. In contrast, mcr84 or apricoxib alone decreased $CD11b^{+}Arg1^{+}$ macrophages, while the effect was more significant with combination therapy (Fig. 3c). Although the number of total myeloid cells that were marked by CD11b was elevated in the combination treatment group, the total macrophage number (F4/80) was reduced with anti-VEGF and COX-2 inhibition (Supplementary Fig. S4a, b).

Discussion

Our data support that VEGF production by tumor cells is independent of COX-2, especially following COX-2 inhibition, and the data also strongly support that COX-2 activity on tumor cells is linked closely to the induction and/or maintenance of a less differentiated tumor cell phenotype. Epithelial plasticity is a common pathway exploited by tumors to resist therapeutic interventions, including chemotherapy and targeted therapy. Our data demonstrate that reducing hypoxia-induced epithelial plasticity by blocking COX-2 enhances the therapeutic activity of anti-VEGF in PDA. We have shown previously that anti-VEGF therapy (mcr84) of PDA induces hypoxia, which drives an increase of TGFβ and subsequent increase in collagen deposition. Furthermore we found that collagen and TGFβ in the tumor microenvironment stimulated tumor cell EMT[9]. Additionally, we previously reported that COX-2 inhibition (apricoxib) reduces EMT in models of GI cancer in vivo and TGFβ-induced EMT in vitro[28,37]. Therefore, we further investigated the effect of COX-2 inhibition on the level of active TGFβ in orthotopic Colo357 pancreatic tumors. We found that anti-VEGF (mcr84) increased active TGFβ levels, as anticipated but that this increase was blunted by COX-2 inhibition (data not shown), which suggests that COX-2 inhibition reduces EMT and immune suppression in part by reducing hypoxia-induced TGFβ expression (Supplementary Fig. S5). TGFβ, a multifunctional cytokine can drive tumor cell EMT and is also a potent immunosuppressive factor produced by tumor cells, fibroblasts and tumor-infiltrating lymphocytes[45]. TGFβ can inhibit innate and adaptive immune responses in the tumor microenvironment. For example, TGFβ can polarize macrophages towards an immunosuppressive phenotype, support regulatory T cell differentiation and directly inhibit effector T cell activity[46]. In addition, our results are consistent with reports that celecoxib, another selective COX-2 inhibitor, reduces hepatic expression of TGFβ thereby attenuating EMT of hepatocytes[47]. Furthermore, COX-2 has been shown to participate in TGFβ-driven EMT in human hepatocellular carcinoma[48]. Thus there are multiple examples of a connection between COX-2 activity and TGFβ-driven tumor progression.

We also found that COX-2 inhibition might reduce immune suppression in PDA. The immunosuppressive microenvironment is a major limitation for the efficacy of cancer immune therapy[49]. Our data are consistent with other studies that have shown that anti-angiogenic agents and COX-2 inhibitors have the potential to reduce the immunosuppressive tumor microenvironment and enhance immunotherapy[50,51,52]. Our results support the findings of Motz et al[30], who found that pharmacologic blockade of VEGF and COX-2 resulted in a significant increase in infiltrating $CD8^{+}$ T cells and a reduction in $FoxP3^{+}$ T_{reg} cells by downregulating FasL expression on tumor endothelial cells in multiple murine cancer models. Our data indicate that in *KIC* tumors, VEGF blockade or COX-2 inhibition alone could reduce FasL expression on the tumor endothelium but combination therapy

resulted in higher T effector cell recruitment and lower T_{reg} infiltration than single-agent therapy.

In summary, our data support the rationale of a combination of anti-VEGF and COX-2 inhibition in PDA patients and also provide evidence that this combination might prime PDA or other tumors for increased efficacy with immune therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge helpful discussions from members of the Brekken laboratory and editorial assistance by Dave Primm in the Department of Surgery at UT Southwestern.

Financial support: The work was supported by NIH grants R01 CA192381 and U54 CA210181 (PI: M. Ferrari) to R.A. Brekken, T32 CA136515 (PI: J. Schiller) to A. Kirane, F31 CA19603301 to N.B. Sorrelle, a sponsored research agreement from Tragara Pharmaceuticals, Inc., to R.A. Brekken, and the Effie Marie Cain Scholarship in Angiogenesis Research to R.A. Brekken. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Implications:

Together, these findings demonstrate that COX-2 inhibition enhances the efficacy of anti-VEGF therapy by reducing hypoxia-induced epithelial plasticity and promoting an immune landscape that might facilitate immune activation.

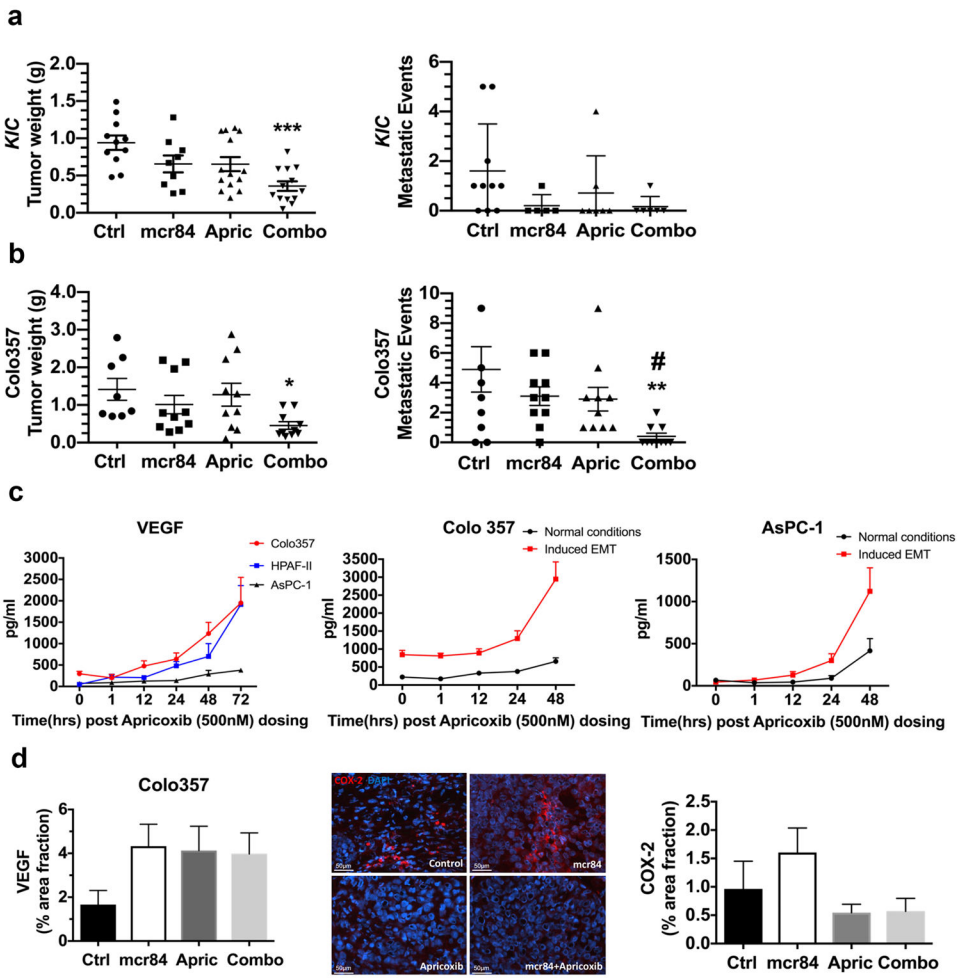

Figure 1. Combination therapy with apricoxib and mcr84 reduced tumor growth and metastasis in murine models of pancreatic cancer.

(a) At 3 weeks of age, *Kras*$^{LSL\text{-}G12D}$; *Cdkn2a*$^{fl/fl}$; *Ptf1a*$^{Cre/+}$ (*KIC*) mice were randomized to receive saline (n = 11), mcr84 (n = 10), apricoxib (n = 13), or mcr84 plus apricoxib (n = 13). All mice were sacrificed when they were 7 weeks old. Mean tumor weight and metastasis burden were compared. **(b)** A total of 1106 Colo357 cells were injected orthotopically into NOD/SCID mice. Treatment began when established tumors were visible by ultrasound and consisted of control (n = 8), mcr84 (n = 10), apricoxib (n = 10) or mcr84 plus apricoxib (n = 10) and continued for 4 weeks, after which mean tumor weight and metastasis burden were shown. Data are displayed in a scatter plot with mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ vs. control; $^{\#}P < 0.05$ vs. single-agent mcr84 or apricoxib by ANOVA with Dunn's MCT. **(c)** Human pancreatic cancer cell lines, HPAF-II, Colo357, and AsPC-1 were treated with 500 nM apricoxib and evaluated by ELISA for the production of VEGF. Colo357 and AsPC-1 were plated under normal conditions or conditions of forced EMT (50 ng/ml TGFβ on collagen I -coated plates for 24 hours). VEGF levels were evaluated by ELISA after 500 nM apricoxib treatment. Biological repeats have been performed (n=3) and data are displayed as mean ± SEM. Paraffin-embedded tumor sections from Colo357 tumor-bearing mice were analyzed for **(d)** VEGF and COX-2 expression by immunofluorescence.

Quantification of percentage area fraction is shown. Data are displayed as mean ± SEM and represent 5 images per tumor with 3 animals per group. Representative images (COX-2, red; DAPI, blue) are shown for Colo357 tumors. Total magnification is 400X. Scale bars are presented as indicated.

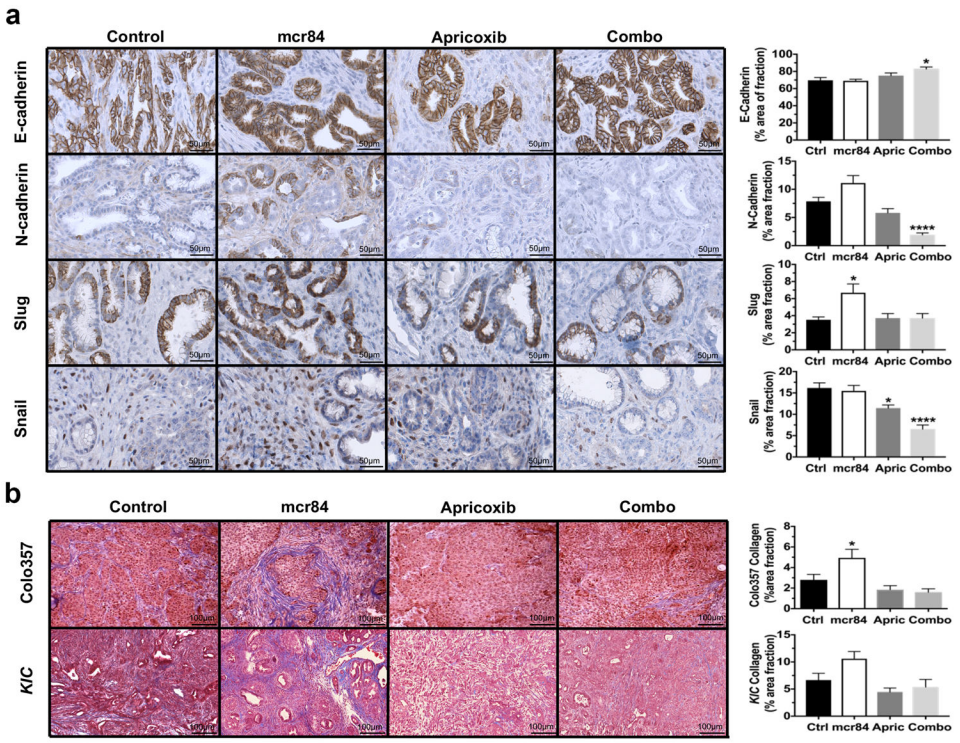

Figure 2. Apricoxib in combination with mcr84 reverses anti-VEGF–induced EMT and collagen deposition.

(a) *KIC* pancreatic tissues from the treated mice underwent immunohistochemistry for E-cadherin, N-cadherin, Slug or Snail. Images are shown at 400X. Scale bars are presented as indicated. **(b)** Pancreatic tissues from Colo357 tumor-bearing mice and *KIC* mice were stained with Masson's trichrome. Total magnification is 200X. Scale bars are presented as indicated. The whole tumor areas were scanned with Hamamatsu Nanozoomer 2.0HT. Images of whole tumor areas were analyzed using ImageJ software. Quantification of percentage area fraction is shown. Data are displayed as mean ± SEM with 3 animals per group analyzed. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ vs. control; by ANOVA with Dunn's MCT.

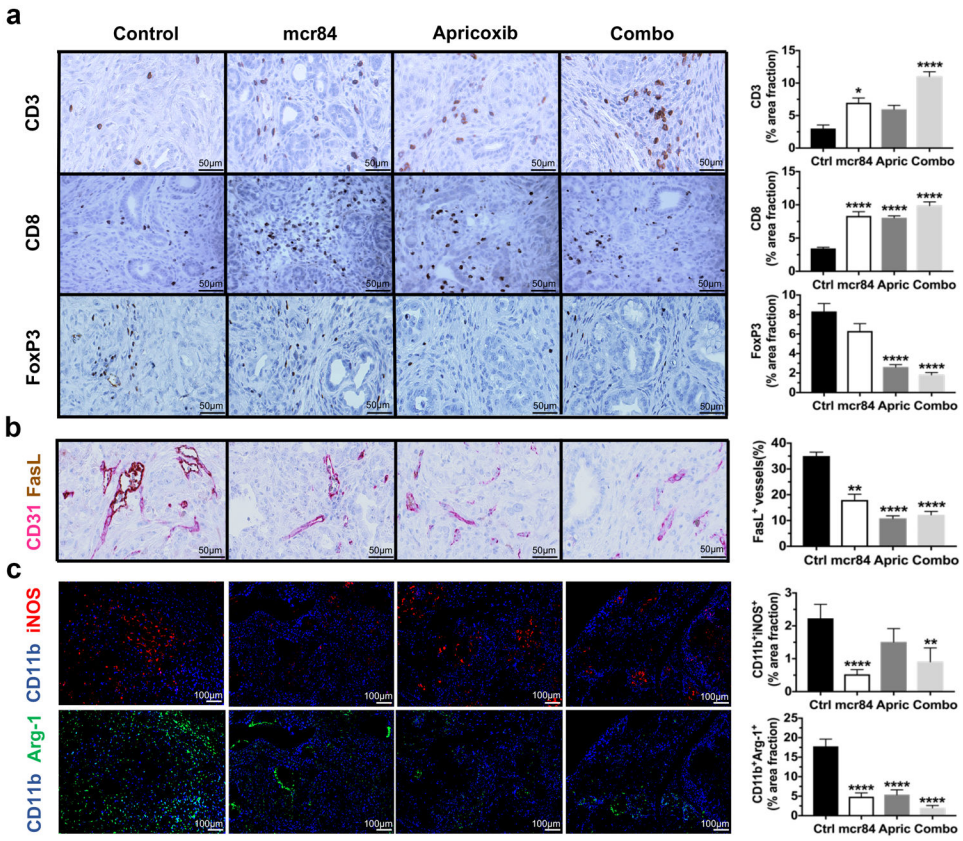

Figure 3. Combination blockade of VEGF and COX-2 pathway restores antitumor immunity.

KIC pancreatic tissue was subjected to immunohistochemistry for **(a)** CD3, CD8, FoxP3, **(b)** CD31 and FasL, **(c)** CD11b and iNOS, CD11b and Arginase 1. The whole tumor areas were scanned with Hamamatsu Nanozoomer 2.0HT and Zeiss Aixoscan Z1. Images of whole tumor areas were analyzed using NIS Elements (Nikon) and Fiji software. Representative images are shown at 400X in **(a)** and **(b)**. Scale bars are presented as indicated. Schematic Quantification of percentage area fraction for each target in each treatment group is shown. Data are displayed as mean ± SEM with 3 animals per group analyzed. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.0001$ vs. control, by ANOVA with Dunn's MCT.