Gamma-Secretase Inhibitor Treatment Promotes VEGF-A-Driven Blood Vessel Growth and Vascular Leakage but Disrupts Neovascular Perfusion

Mattias Kalén¹, Tommi Heikura²*, Henna Karvinen²*, Anja Nitzsche¹, Holger Weber³, Norbert Esser³, Seppo Ylä-Herttuala², Mats Hellström¹*

¹Department of Immunology, Genetics, and Pathology, Uppsala University, Uppsala, Sweden, ²Department of Biotechnology and Molecular Medicine, A. I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland, ³ProQinase GmbH, Freiburg, Germany

Abstract
The Notch signaling pathway is essential for normal development due to its role in control of cell differentiation, proliferation and survival. It is also critically involved in tumorigenesis and cancer progression. A key enzyme in the activation of Notch signaling is the gamma-secretase protein complex and therefore, gamma-secretase inhibitors (GSIs)—originally developed for Alzheimer’s disease—are now being evaluated in clinical trials for human malignancies. It is also clear that Notch plays an important role in angiogenesis driven by Vascular Endothelial Growth Factor A (VEGF-A)—a process instrumental for tumor growth and metastasis. The effect of GSIs on tumor vasculature has not been conclusively determined. Here we report that Compound X (CX), a GSI previously reported to potently inhibit Notch signaling in vitro and in vivo, promotes angiogenic sprouting in vitro and during developmental angiogenesis in mice. Furthermore, CX treatment suppresses tumor growth in a mouse model of renal carcinoma, leads to the formation of abnormal vessels and an increased tumor vascular density. Using a rabbit model of VEGF-A-driven angiogenesis in skeletal muscle, we demonstrate that CX treatment promotes abnormal blood vessel growth characterized by vessel occlusion, disrupted blood flow, and increased vascular leakage. Based on these findings, we propose a model for how GSIs and other Notch inhibitors disrupt tumor blood vessel perfusion, which might be useful for understanding this new class of anti-cancer agents.

Introduction
In the last decade dozens of new cancer drugs of the “targeted therapy” class have been introduced. These drugs, for example trastuzumab and imatinib, are based on efforts in basic research to understand the cellular mechanisms underlying cancer development. The majority of these drugs are designed to interfere with growth promoting signaling pathways, hijacked by the tumor cells. More recently, drugs affecting tumor growth indirectly, through inhibition of VEGF-A-driven angiogenesis, have also been introduced in the clinic [1,2]. Based on the success of the first wave of targeted therapies, there is now a rapid development of novel modulators of cell signaling pathways, DNA-repair, proteolysis etcetera [3,4,5].

For more than a century, the Notch signaling pathway has been known as a critical regulator of fundamental cell fate decisions during development in Drosophila. Notch mainly exerts its functions through lateral inhibition, a process by which a cell - having acquired a certain phenotype - inhibits neighboring cells to take the same path of differentiation. The result is two distinct cell types, where both stem from cells with the same developmental potential [6]. In vertebrates there are five known Notch ligands (Delta-like 1, -3 and -4, Jagged 1 and -2), and four Notch receptors (Notch1-4). As in Drosophila, the Notch system controls development at multiple steps in almost all vertebrate tissues. Both the ligands and receptors are cell surface bound and require cell-cell interaction to trigger signaling. Furthermore, ligand-receptor interaction leads to a series of proteolytic cleavages, where the last step is mediated by the gamma-secretase membrane protein complex, which minimally consists of the four proteins Presenilin, Nicastrin, APH-1, and PEN-2. The series of proteolytic cleavages leads to translocation of the Notch intracellular domain (NICD) into the nucleus and initiation of transcription [7]. Apart from being essential for normal development, the Notch signaling system has been implicated in human hereditary cardiovascular diseases and cancer [8,9]. For example, the Notch gene is translocated, mutated and over-expressed leading to gain of function in a large number of
GSI Treatment Disrupts Neovascular Perfusion

A. CX induced endothelial sprouting in vitro

B. Vehicle

C. CX 5mg/kg

D. CX 10mg/kg

E. CX 20mg/kg

F. Tip cell filopodia per vessel length

G. Vehicle

H. GSI 10mg/kg
malignancies including cervical, head and neck, endometrial, renal, lung, pancreatic, ovarian, breast and prostate carcinoma [10,11,12] and reviewed in [8].

Recently, our understanding of the role of Notch signaling in angiogenesis in general, as well as in tumor angiogenesis, has improved. During normal development, Delta-like 4 (Dll4) is expressed by endothelial tip cells at the front of the angiogenic sprout and signals via Notch1 to neighboring stalk cells trailing behind, thereby suppressing differentiation of new tip cells and sprouts. The disruption of this signal, by targeting Dll4 or Notch1 either genetically or pharmacologically, leads to increased formation of endothelial tip cells, and in turn excessive angiogenic sprouting [13,14,15,16,17], reviewed in [18]. In the tumor setting the same mechanism operates so that Dll4 or Notch1 inhibition drives extreme angiogenic sprouting, which generates non-functional vascularule, in turn leading to inhibited tumor growth [19,20,21,22,23], reviewed in [24].

Thus, there are multiple reasons for designing drugs disrupting the Notch signaling pathway and various targeting approaches are under way ranging from antibodies that selectively inhibit specific Notch ligands or receptors, to low molecular weight compounds disrupting the signaling of all four receptors [25]. The latter class of compounds is based on inhibition of the gamma-secretase protein complex, and is consequently termed gamma-secretase inhibitors (GSIs). Apart from being essential for Notch signaling, the gamma-secretase facilitates catalytic cleavage of several other membrane proteins such as amyloid polypeptide (APP), crbB-4, CD44, E-cadherin etcetera [26]. The metabolic activity and nutrient supply of the tumor cell might also be affected by GSI treatment since mammalian target of rapamycin (mTOR) and the nutrient supply of the tumor cell might also be affected by GSI treatment since mammalian target of rapamycin (mTOR) and the glucose transporter Glu1 have been shown to be downstream of Notch signaling [27,28]. The first GSIs in drug development were aimed at treatment of Alzheimer’s disease based on the amyloid hypothesis. However, there is now an increased interest in GSIs as a potential treatment for hematological malignancies and cancer since they have been shown to be efficacious in several tumor models [29,30].

Despite thorough investigation of GSIs in tumor models, surprisingly little effort has been directed at analyzing its effect on tumor vessel morphology and functionality. We therefore analyzed the effect of Compound X (CX), a GSI previously reported to potently inhibit Notch signaling in vitro and in vivo [31]. Here we report that CX promotes VEGF-A-driven angiogenic sprouting in vitro and during developmental angiogenesis. CX also suppresses tumor growth in a renal carcinoma model, leads to the formation of abnormal vessels and an increased tumor vascular density. In a model of VEGF-A-driven angiogenesis in skeletal muscle, we then demonstrate that CX promotes abnormal blood vessel growth characterized by vessel occlusion, disrupted blood flow, and increased vascular leakage. These findings might suggest that GSIs act as anti-tumoral agents, not only via direct effects on tumor growth, survival and differentiation, but also through action on the tumor vasculature.

Results

Compound X promotes angiogenic sprouting both in vitro and during mouse development

CX has previously been shown to potently inhibit gamma-secretase activity and Notch signaling in vitro and in vivo [31]. To test whether CX directly promotes angiogenic sprouting in vitro, we titrated CX in a cellular angiogenesis assay where clustered human umbilical vein endothelial cells (HUVECs) form sprouts in a threedimensional collagen type I gel [32]. CX induced HUVEC sprouting dose-dependently with an EC50 of 8.8 μM (Figure 1). To ascertain that GSI inhibitors affect angiogenesis similarly, we also tested four other compounds; LY450.139, Sulfonamide 565763, WPE-III-31C and DAPT, pertaining to different classes of GSI inhibitors. All compounds promoted angiogenic sprouting dose-dependently (data not shown).

We and others have previously shown that genetic or pharmacological inhibition of Notch signaling leads to increased angiogenic sprouting in vivo during developmental angiogenesis of the retina, reviewed in [18]. As expected, CX dose-dependently increased the vascular density of the early postnatal retina at doses between 5 and 20 mg/kg/day (Figure 1). Treatment with CX between postnatal day (P) three and five led to increased vascular density in the outermost two thirds of the retina, accompanied by super numerous endothelial tip cells at the sprouting vascular front in the periphery. In line with this, treatment with CX (10 mg/kg) between P 3 and P 5 led to a 68% increase in the number of endothelial filopodial protrusions at the vascular front, compared to control (Figure 1). Thus, the pattern of vascular formation after CX treatment was essentially morphologically indistinguishable from treatment with other GSIs such as DAPT [13]. The in vitro and in vivo doses of CX, used to potently stimulate angiogenic sprouting, were similar to the doses used to inhibit Notch signaling in prior work by Scarfoss et al. [31].

Compound X modulates the angiogenic response in a model of renal cell carcinoma

To assess the effect of GSI treatment on tumor growth and vessel formation in the tumor setting, we treated mice with CX (10 mg/kg/day) in a model of renal cell carcinoma. Balb/C RENCA cells were orthotopically implanted into syngenic mice at day 0 and tumors were analyzed at day 21. Prior to animal experiments we assessed the effect of CX on RENCA cell viability at escalating doses in vitro, and found that it had no significant effect even at the highest dose tested (10 μM), data not shown. Hence, at the doses used in the study, CX affected angiogenic sprouting but not cell viability. As seen previously in the RENCA tumor model, vehicle-treated animals lost weight over time due to their tumor burden and at day 20 the reduction was 3.5% of the initial weight. CX-treated animals did not show a significant weight reduction (Figure 2). At the end of the study, the primary tumor volume and wet weight were both reduced after CX treatment; 53% and 58% of control, respectively (Figure 2). The
GSI Treatment Disrupts Neovascular Perfusion

A

Animal Weight

Weight index

Vehicle

CX 10 mg/kg

Days

0 5 10 15 20

1.2

1.1

1.0

0.9

0.8

B

Tumor volume

Vehicle

CX 10 mg/kg

Volume (mm³)

3000

2000

1000

0

C

Tumor weight

Vehicle

CX 10 mg/kg

Weight (g)

3

2

1

0

D

MVD

Vehicle

CX 10 mg/kg

No. of vessels (n/mm²)

400

300

200

100

0

E

Filopodia

Vehicle

CX 10 mg/kg

Filopodia/vessel

1.5

1

0.5

0

F

Vessel perimeter

Vehicle

CX 10 mg/kg

CD31-perimeter (µm)

1500

1000

500

0

G

Mural coverage

Vehicle

CX 10 mg/kg

Fraction of ASMA⁺ vessels

0.3

0.2

0.1

0.0

H

Occluded Vessels

Vehicle

CX 10 mg/kg

CD31-filled vessels (n/mm²)

2.5

2

1.5

1

0.5

0

I

Vehicle

J

CX 10 mg/kg

K

Vehicle

L

CX 10 mg/kg

M

Vehicle

N

Vehicle

O

CX 10 mg/kg

P

Vehicle

CX 10 mg/kg
Figure 2. CX treatment is anti-tumorigenic and promotes angiogenic sprouting in mouse renal cell carcinoma model. At day 0 twelve mice were orthotopically inoculated with RENCA cells under the kidney capsule. (A) Mean animal weight plotted in relation to their initial weight. There was a weight reduction in the control group, 1.0 g (a loss of 5.5% of initial weight), P = 0.049 between day 1 and 20. There was no significant weight reduction in the CX-treated cohort. At day 21 the study was terminated. (B) Tumor volume was reduced in the CX-treated group by 53% of control, P = 0.0055. (C) Tumor wet weight was reduced in the CX-treated group by 38% of controls, P = 0.041. (D) Microvascular density (MVD) was measured as the number of CD31 positive structures per area, and was increased by 29% of control after CX treatment, P = 0.0080. (E) The number of filopodia-like protrusions from the vessels' profiles were increased by 130% in CX 10 mg/kg treated tumors compared to vehicle treated tumors (CX 10 mg/kg: 0.84 versus vehicle; 0.37 endothelial protrusions per vessel profile, P = 0.017). (F) Vessel perimeter was calculated as the length surrounding the CD31+ blood vessel areas and there was a significant increase in the mean vessel perimeter in the CX-treated tumor as compared to control, 107 μm versus 85 μm, P<0.001. (G) The mural cell coverage was quantified as the fraction of CD31+ vessels with associated ASMA+ cells, and was 0.16 in the vehicle group and 0.06 in the CX-treated group, P = 0.048. (H) The number of occluded vessels per area was increased 1.93-fold in CX-treated tumors as compared to controls, P = 0.010. (I and J) Representative tumor fields from control and CX-treated tumors stained with CD31. Black arrowheads point at filopodia-like protrusions. (M) Occluded vessels were identified as large vessel structures completely filled with CD31-positive structures (arrowheads), clearly distinguished from small, presumably, tangentially sectioned vessels. (N) Control tumors stained for CD31, white structures. (O) CD31-stained CX-treated tumors form long continuous vessels structures that seem to arise from fusion of several previously distinct vessels, e.g. center of image. (P) Example of CD31 (green) and ASMA (red) stained tumor section. Error bars represent standard deviation. Compound X: CX. Scale bars are 100 μm, except K-L which are 25 μm. Significance level were indicated * = <0.05, ** = P<0.01, *** = P<0.001.

doi:10.1371/journal.pone.0018709.g002

microvascular density (MVD) in CX-treated animals, measured as the number of CD31+PECAM1-positive structures per unit area, was increased by 29% of control. The blood vessel morphology was also altered. The vessel perimeter surrounding the CD31-positive structures in the CX-treated group was increased by 33% of control. However, the vessels were not dilated, but rather appeared as if several blood vessels were in the process of merging into larger blood vessel structures (Figure 2). At the level of endothelial cells, they appeared different in CX-treated tumors compared to control. The endothelial surface was uneven and the number of filopodia-like protrusions from endothelial cells were more than doubled after CX treatment, compared to control (Figure 2). The mural cellular coverage of the tumor vasculature was quantified as percentage of CD31-positive blood vessels that were associated with a smooth muscle actin (ASMA) positive cells. In the vehicle treated group the ASMA-positive blood vessels were few (16%) and most of them were only partially covered. The mural cellular coverage was reduced by 61% of control in the CX-treated group (Figure 2). Interestingly, we also observed a higher frequency of vessels filled with a CD31-positive content. In trying to differentiate such vessels from the ones having merely been tangentially sectioned, we quantified only large vessels with CD31-positive content as being “occluded vessels”, exemplified in Figure 2. Occluded vessels were seen in proximity to areas undergoing necrosis and were 2-fold more common in the CX-treated tumors compared to vehicle control (Figure 2). Moreover, the intraluminal occlusions were positive for the endothelial markers CD31 and podocalyxin, as well as the nuclear stain DAPI, suggesting that they are viable endothelial cells and not membrane or matrix material (Figure S1).

Albeit we observed significant changes in microvascular density and tumor size between vehicle and CX-treated animals, we failed to quantify any differences in proliferation (BrdU-incorporation), apoptosis (TUNEL and cleaved Caspase 3-staining), or area of necrosis, data not shown.

Compound X disrupts VEGF-A induced neovascular perfusion

To study the effect of CX on vascular morphogenesis and functionality in a relevant, yet less complex milieu than that of a tumor, we turned to the rabbit hind limb model. In this model, adenoviral [Ad] VEGF-A165 gene transfer to the semimembranosus muscle led to vessel dilation and proliferation, not seen in skeletal muscle transduced with a control gene, LacZ (Figure 3). Contrast pulse sequence (CPS) ultrasound was used to determine the perfusion in living animals [33]. Compared to the control leg, VEGF-A165 gene transfer resulted in a more than 10-fold increase of vascular perfusion (Figure 4). To determine the dose of CX to be administered, several clinical chemistry markers were analyzed. Even at low doses (1–2 mg/kg), there was a dose-dependent increase in markers for liver stasis and toxicity including serum total bilirubin (s-TB), serum alkaline phosphatase (s-ALP) and serum alanin aminotransferase (s-ALT). In addition, we observed an increase in the levels of serum creatinin, indicating kidney damage (Table 1). Histological analysis of the liver, using CD31/PECAM-1 staining, revealed dose-dependent severe dilation of the sinusoids upon CX-treatment, as well as increased staining intensity for CD31. Furthermore, intrahepatic bile ducts were occluded by epithelial structures, a finding in line with the increased s-ALT (Figure 5). Consequently, low doses of CX (1–2 mg/kg/day) were chosen for further studies. In comparison to AdVEGF-A-treated skeletal muscle, the combined administration of CX and VEGF-A resulted in dose-dependent blood vessel dilation and most notably an increased number of CD31/PECAM1-positive structures filling the lumen of the vessels (Table 2).

Staining for another endothelial marker, podocalyxin, also identified the intraluminal structures as endothelial cells (Figure 3). As the vast majority of the vessels follow the fibers of the skeletal muscle, there is little risk of mistaking an occluded vessel from a tangentially sectioned vessel. CX treatment also led to a dose-dependent decrease in vascular perfusion, measured as a reduced CPS signal. Doses of 1 or 2 mg/kg/day of CX reduced CPS-signal by 48% and 66% of control, respectively (Figure 4). In addition, blood vessel leakage was determined using the modified Miles assay where extravasated Evans blue (intravenously injected and subsequently bound to plasma proteins) was quantified. Adenoviral gene transfer of VEGF-A led to a 35-fold induction of Evans blue leakage. 2 mg/kg/day of CX treatment resulted in a 76-fold increase in the leakage of the newly formed vessels (Figure 4). Importantly, there were no differences in permeability between vehicle treated and CX-treated muscles in the absence of AdVEGF-A treatment.

Discussion

We noted a pronounced change in vascular architecture after CX treatment in several model systems. During developmental angiogenesis and in the tumor model, the endothelial cell surface was rich in cytoplasmic protrusions and tip cell-like protrusions...
CX-mediated inhibition of the gamma-secretase is blockade of Notch signaling which leads to hypersprouting and the formation of an immature vessel network that coalesce into larger vessel structures. Furthermore, an interesting observation in our study was the CD31- or Podocalyxin-positive structures that partially or completely filled out the blood vessel lumen. These structures were very notable in the rabbit hind limb model, where the capillaries are cross-sectioned, but were also evident in the mouse tumor model. Our use of two distinct markers for endothelial cells strongly suggest that the cellular structures filling the vessel lumens are of endothelial origin, but does not rule out contribution from non-endothelial cells. Furthermore, others have reported vessel occlusion following Dll4-inhibition in an in vitro model, where HUVEC sprouting in fibrin gels led to the loss of lumen-like structures, which were replaced by densely packed endothelial cells [21]. Similarly it has been shown in the presenilin-1 knockout mice that the brain capillaries are occluded by abnormally shaped endothelial cells, which occasionally form multilayered stacks of endothelial cells completely filling the vessel lumen [36]. We speculate that the loss of Notch signaling leads to hyperproliferation of endothelial cells, which fail to organize properly and thereby occlude the newly formed blood vessels. Moreover, we observed that vascular leakage increased after GSI treatment. This could be explained by the known inhibitory effect of Notch on VEGF-A activity, since VEGF-A is known to potentiate vascular leakage, reviewed in [37]. Furthermore, CX had no effect on vascular leakage on its own further arguing that inhibition of Notch signaling rather potentiate the effects of VEGF-A. Alternatively, the abnormal vascular structures triggered by GSI treatment could lead to reduced vascular integrity and increased vascular leakage. Taken together, the abnormal vessel architecture in combination with increased vascular leakage, could explain the loss of vascular perfusion after CX treatment. Notably, we administered 10 mg/kg/day to the mice without overt toxicity, and the doses used in rabbits (1–2 mg/kg) were thus likely in the low range in terms of modulating vessel growth. Therefore, had we not observed symptoms of toxicity in rabbit model, higher dosing would have been possible, with potentially even more dramatic changes in vascular morphology.

In the tumor model, it is interesting to note that the occluded vessels were most commonly observed in proximity of necrotic areas. This could suggest that vessel occlusion led to loss of blood vessel perfusion, which deprived the tumor of oxygen and nutrients. However, we were not able to quantify any differences in proliferation, apoptosis, or necrosis between CX and vehicle treated tumors at the end of the study (day 21). Small additive differences in the measured parameters could still lead to the end result i.e. smaller tumors in the GSI treated group. Another plausible explanation is a progressive tumor adaptation to the treatment, in turn leading to immeasurable differences at day 21.

Early work by Paris and co-workers indicated that GSIs might have an anti-angiogenic effect on endothelial cells in vitro and in vivo, and inhibited tumor growth [38]. However, in contrast to that study, but in line with the recent genetic and pharmacological studies mentioned above, we found that CX promoted angiogenic sprouting and vessel growth in vitro and during developmental angiogenesis. The contrasting findings between our study and the one by Paris and co-workers could be explained by the use of different tumor models or different GSIs - where one of them does not even inhibit Notch processing (JLK6) [39]. There were also significant differences in administration, dosing of the GSIs, and finally the fact that Paris et al. analyzed smaller sized tumors than in the present study.
GSI treatment is unfortunately associated with side effects. One of the better-studied side effects is increased mucus production leading to diarrhea, which stems from increased proliferation of mucus-producing Goblet cells, due to Notch inhibition [31]. In addition, blockade of Notch results in altered lymphocyte development including decreased number of mature B-cells [40]. Recently, it has also been shown that Dll4 or combined Notch1 and -2 inhibition can lead to development of vascular tumors and hepatotoxicity [23,41]. Similarly, we found that CX treatment led to severe hepatotoxicity in rabbits. This effect is likely triggered in part by abnormal activation of liver endothelial cells as suggested by Yan et al and Wu et al [23,41]. In addition, we observed abnormal bile ducts and increased ALP levels, indicative of cholestasis. Interestingly, Notch signaling is important not only for endothelial cells, but also for bile duct epithelial cells. For example, Notch2, but not Notch1, has been shown to be indispensable for normal perinatal and postnatal intrahepatic bile duct development [42]. In addition, we found signs of renal toxicity, which could be a GSI-class effect, alternatively toxicity specific for CX. Despite these side effects, GSIs is still an interesting class of drugs when compared to more selective agents as therapeutic antibodies. GSIs have been shown to affect not only tumor cell growth via Notch, but also other targets e.g. erbB-4, mToR and Glut1 and thereby affecting other signaling pathways important for tumor growth, nutritional status of the tumor and possibly cancer stem cells [27,28]. Importantly, the gastrointestinal side effects mediated by increased Goblet cell number have been shown to be manageable using altered dosing regiments or concomitant glucocorticoid treatment [30,43].

In conclusion we have shown that the GSI Compound X, promotes angiogenic sprouting and neovascular formation during development and tumor growth. Using a model of VEGF-A-driven angiogenesis in the rabbit hind limb, we showed that CX treatment resulted in vessel occlusion, reduced perfusion and increased vascular leakage. We hypothesize that GSI treatment leads to endothelial hyperproliferation and subsequent occlusion of newly formed blood vessels, which leads to reduced vascular perfusion. This vascular hypersprouting, in combination with

**Figure 4.** CX treatment leads to decreased perfusion and increased leakage after VEGF-A induced angiogenesis. Contrast pulse sequence (CPS) ultrasound was used to measure vascular perfusion in the semimembranosus muscle. The CPS ratio between the VEGF-A adenovirus gene transferred leg and the non-treated leg was compared. (A) VEGF-A induced a 10.3-fold increase in perfusion. There was a significant dose-dependent reduction in the CPS ratio between VEGF-A transferred leg and the control leg when CX was systemically administered. The CPS ratio was 5.5 and 3.6 after administration of 1 and 2 mg/kg CX, respectively, ANOVA P = 0.0040. (B) Evans blue was injected into the blood stream prior to extraction of the muscle tissue. The signal between the VEGF-A treated muscle versus the non-treated leg was compared. There was a 35-fold increase in Evans blue leakage after VEGF-A gene transfer, which was further increased by administration of CX. Doses of CX at 1 mg/kg or 2 mg/kg resulted in a 40- or 76-fold increase in Evans blue leakage, respectively, ANOVA P = 0.041. (C) Biopsies from the semimembranosus muscle from either AdVEGF-A165 treated or intact leg with the systemic treatment as indicated in the figure. The blue dye comes from extravasated Evans blue that is trapped in the tissue. Compound X: CX. Error bars represent standard deviation. Significance level were indicated * = P<0.05, ** = P<0.01, *** = P<0.001.

doi:10.1371/journal.pone.0018709.g004
Table 1. Clinical chemistry parameters of VEGF-A and CX-treated rabbits.

| Gene Transfer | Day 0 | Day 6 |
|---------------|-------|-------|
|                | VEGF-A | VEGF-A | VEGF-A | VEGF-A | VEGF-A | VEGF-A |
| Systemic treatment | vehicle | CX 1 mg/kg | CX 2 mg/kg | vehicle | CX 1 mg/kg | CX 2 mg/kg |
| S-ALP (U/L) 71±34 | 125±24 | 105±16 | 119±20 | 50±19 | 197±37** | 288±7*** |
| S-ALT (U/L) 48±13 | 37±14 | 84±22 | 70±32 | 17±7 | 232±67** | 282±107** |
| S-TB (U/L) <2.5 | <2.5 | <2.5 | <2.5 | <2.5 | 8±2.6** | 30±1.8*** |
| S-CRP (mg/L) <5 | <5 | <5 | <5 | <5 | <5 | <5 |
| S-Creatinin (mM) 95±21 | 79±10 | 79±9.2 | 88±9.2 | 89±12 | 149±26* | 155±26* |
| S-LD (U/L) 107±44 | 73±21 | 69±11 | 98±16 | 127±42 | 120±19 | 140±14 |
| S-Na* (mM) 143±3 | 132±6.5 | 133±3.8 | 131±13 | 138±1.0 | 136±0.81 | 141±14 |
| S-K* (mM) 4.2±0.3 | 4.6±0.24 | 3.7±0.19 | 4.2±0.14 | 4.3±0.14 | 4.2±0.35 | 3.6±0.57 |

Serum markers of toxicity and inflammation were assayed using blood samples from the rabbit at the beginning (day 0) and end of the study (day 6). Changes in serum levels of the indicated markers were analyzed for statistically significant changes at day 6 between CX and vehicle-treated animals. In CX-treated rabbits there were significant increases of alkaline phosphatase, alanine aminotransferase and total bilirubin indicating liver stasis and toxicity. There was also an increase in creatinin after compound X treatment, suggesting renal toxicity.

Compound X: CX, S: serum, ALP: alkaline phosphatase, ALT: alanine aminotransferase, TB: total bilirubin, CRP: C-reactive protein, LD: Lactate dehydrogenase. Analyte levels are given +/- standard deviations. Reference values for rabbit are from Hewitt et al., and Ypsilantis et al.[48,49]. Significance level using students t-test.

* = p<0.05,
** = p<0.01,
*** = p<0.001.

doi:10.1371/journal.pone.0018709.t001

Materials and Methods

Ethics statement

The developmental angiogenesis study was performed under the license number: 354–04, granted by the Animal Ethics Committee, Göteborg, Sweden.

The study consisted of 2 groups containing 12 female BALB/c mice each. At day 0, RENCA cells were orthotopically implanted into the left kidney of all mice. Starting the following day (day 1) animals in the treatment group received 10 mg/kg CX per os (p.o.) once a day for the duration of 21 days. Animals of the control group received vehicle (10% Ethanol, 90% corn oil) also once a day p.o. starting day 1 until day 21.

At day 21 the study was terminated and a necropsy performed. At necropsy, primary tumor weight and volume was determined. For histological examination of the tumor vasculature, cryosections of primary tumor tissues (thickness = 5–10 μm) were taken from all animals. For the visualization of the blood vessels, immunohistochemical staining for CD 31 (PECAM-1 Cat. 553370, PharMingen, San Diego, CA) was performed, and vessels were counted microscopically using a defined magnification (x200), control treated (n = 11) and CX-treated (n = 9). For fluorescent images, Alexa Fluor 488 donkey anti-rat IgG, (1:200, A21208, Molecular Probes) was used. To stain for podocalyxin, goat anti-podocalyxin (1:100, AF1556, R&D Systems, Minneapolis, MN) and donkey anti-goat IgG Alexa 488 (1:200, A-11055, PromoCell, Heidelberg, Germany, and cultured according to the manufacture's instructions. HUVECs clustered into spheroids in cell culture media over night and dispensed in a 3-dimensional collagen type I matrix. Spheroids were assayed for sprouting in the presence of different concentration of CX for 24h and the ten longest sprouts from 10 spheroids were summarized as cumulative sprout length (CSL).

RENCA tumor model

The study consisted of 2 groups containing 12 female BALB/c mice each. At day 0, RENCA cells were orthotopically implanted into the left kidney of all mice. Starting the following day (day 1) animals in the treatment group received 10 mg/kg CX per os (p.o.) once a day for the duration of 21 days. Animals of the control group received vehicle (10% Ethanol, 90% corn oil) also once a day p.o. starting day 1 until day 21.

At day 21 the study was terminated and necropsy performed. At necropsy, primary tumor weight and volume was determined.

Increased vascular leakage, then leads to oxygen and nutrient deprivation of the tumor.

VEGF-A driven angiogenesis is important for tumor oxygen and nutrient supply. We have shown in models of VEGF-A-driven angiogenesis that a GSI, inhibiting the Notch pathway, can strongly affect vascular density, perfusion, and leakage. These observations warrant further investigations of the role GSIs might have in modulating tumor angiogenesis. Several compounds targeting Notch signaling are under development for treatment of a diverse array of malignancies [19,23,29,30,34,44]. Each of the compounds has its pros and cons in terms of selectivity and efficacy in blocking the Notch pathway. Further research will determine which one of these compounds that ultimately can be used effectively in the clinic.

Spheroid-based cellular angiogenesis assay

The Gamma-secretase inhibitor CX was synthesized as described in [31]. The Spherogenex assay was performed as described in [32]. Briefly, HUVECs were purchased from...
In addition, the proliferation index of the tumor tissue was examined by BrdU labeling of the cryosections. For this purpose, BrdU (500 mg/kg) was administered 12 h before sacrificing the animals. Apoptotic index was determined using TUNEL (Cat. 12 156 792 910, Roche, Mannheim, Germany) and cleaved Caspase 3 stainings of tumor sections. Cleaved caspase-3 (1:500 #9661, Cell Signaling Techn, Danvers, MA) and Alexa Fluor 555 donkey anti-rabbit IgG (1:200 # A-31572, Molecular Probes, Eugene, OR) were used. The determination of the necrotic areas within the primary tumor was performed using the CD 31-stained tissue samples. Necrotic areas were identified as areas in which no vessels could be detected.

Rabbit hind-limb model

Gene transfer and GSI administration. New Zealand White (NZW) rabbits (3.0–3.5 kg) were used for gene transfers. Intramuscular gene transfers with adenoviral human VEGF-A (n = 9) and AdLacZ (n = 1) were done into the hindlimb semimembranosus muscle with method as previously described [45,46]. Rabbits were anesthetized with medetomidine (Domitor 0.3 mg/kg, Orion, Finland) and ketamine (Ketalar 20 mg/kg, Pfizer, Finland) and the total dose of 1×10^11 virus particles in 1 ml was divided to ten 100 μl injections. CX (1 mg/kg (n = 3) or 2 mg/kg (n = 3)) or vehicle (n = 3) was administered by subcutaneous injections once a day. Animals were euthanized six days after gene transfer.

Ultrasound imaging. Skeletal muscle perfusion was measured with Acuson Sequoia and Cadense Pulse Sequence (Siemens) ultrasound imaging using 15L8 transducer of gene transduced semimembranosus muscle and the contralateral intact muscle, respectively[33]. Images were taken from transversal sections after a single bolus (0.5 ml) of SonoVue contrast agent (Bracco, The Netherlands). Measurements were performed six days after gene transfer. Perfusion ratio between gene transduced muscle and intact muscle was quantified with DataPro 2.13 software (Noesis) using the maximum signal intensities.

Modified Miles Assay. Modified Miles assay was used to evaluate tissue edema [47]. Evans Blue dye (30 mg/kg) was injected 30 min before euthanasia via ear vein. Animals were perfusion-fixed with 1% paraformaldehyde in 0.05 M citrate buffer (pH 3.5) via left ventricle after euthanasia. Muscles were photographed, and samples were taken and weighted from both gene transferred muscle and contralateral intact muscle. Evans Blue dye was eluted in formamide and measured with spectrophotometer of absorbance at 610 nm. Absorbance was normalized to the sample weight and ratio between gene transferred and intact muscle samples was calculated.

Histology of rabbit skeletal muscle. 7 μm thick paraffin sections were stained with monoclonal antibody against CD31 (clone JC/70A, Dako, Glostrup, Denmark) and against podocalyxin-like 1 (1:200, sc-23903, Santa Cruz Biotechnology, Santa Cruz, CA). Signal detection was done with avidin-biotin-horse radish peroxidase (HRP) system with diaminobenzidine (DAB) (Vector Laboratories/LabVision). Microphotographs were taken with an Olympus AX70 microscope (Olympus Optical, Tokyo Japan) and with a ZEISS AxioImager.M2 (Zeiss, Jena, Germany).

Quantification of occluded capillaries. A capillary was considered occluded, and thus counted, if more than 50% of the luminal area was filled with CD31 positive cells. Ten microscopy fields (200×magnification) per animal were captured. Altogether 30 microscopy fields from each treatment group were used for the quantification. Occluded capillaries were counted from the proximity of the needle track in each muscle biopsy.

Figure 5. CX treatment alters liver morphology. (A–C) Liver samples from rabbits were stained with CD31/PECAM1 (brown) and counter-stained with hematoxylin (blue). (A) AdVEGF-A + vehicle, (B) AdVEGF-A + 1 mg/kg CX and (C) AdVEGF-A + 2 mg/kg CX. Microphotographs show representative liver portal triads. (B–C) Note the dose dependent dilation of the liver sinusoids (arrowheads in C) as well as the increased staining intensity of CD31 in CX-treated livers. The bile ducts seemed occluded by intraluminal epithelial cells (asterisks in B and C). A: Artery, V: Vein, B: bile duct. Scale bars are 100 μm.

doi:10.1371/journal.pone.0018709.g005

Molecular Probes) were used. Monoclonal Anti-Actin, α-Smooth Muscle – Cy3 antibody, (1:100, C6198 Sigma, Saint Louis, MO, USA) was used to visualize mural cells.
Table 2. Quantification of occluded capillaries in rabbit skeletal muscle upon VEGF-A and CX treatment.

| Number of microscopy fields grouped according to number of occluded capillaries |
|---------------------------------|---------------------------------|---------------------------------|
|                                  | 1st–33rd percentile (few occluded capillaries) | 34th–66th percentile | 67th–100th percentile (many occluded capillaries) |
| VEGF-A + vehicle                 | 17                               | 6                               | 7                               |
| VEGF-A + 1 mg/kg CX              | 11                               | 6                               | 13                              |
| VEGF-A + 2 mg/kg CX              | 2                                | 16                              | 12                              |

The number of occluded capillaries per mm² was determined from photographs of microscopy fields from all treatment groups. These numbers ranged from 3.5 to 88.3 and were used to categorize the microscopy fields into three groups: Microscopy fields within 1st–33rd percentile, 34th–66th percentile and 67th–100th percentile, respectively. The 33rd percentile was at 15.6 and the 66th percentile at 34.6. The number of microscopy fields within each category was then determined and are displayed in the table. VEGF-A + vehicle versus VEGF-A + CX (1 mg/kg), Chi-square test \( P = 0.0002 \); VEGF-A + vehicle versus VEGF-A + CX (2 mg/kg), Chi-square test \( P = 0.0033 \).

doi:10.1371/journal.pone.0018709.t002

Statistical analysis

Student’s t-test was performed for comparison of two groups and ANOVA was performed for analysis of three groups. The t-test was calculated as unpaired and two-tailed, except for the analysis of ASMA-coated vessels that was calculated as one-sided. This was based on the assumption that there would be a reduction in the ASMA-positive cells in the treatment groups based on the publication by Scehnet et al.[22]. Alpha values less than or equal to 0.05 were regarded as significant. The Chi-square test was used when analyzing the frequency of occluded vessels. The GraphPad Prism software v. 5.01 was used for all statistical analysis.

Supporting Information

Figure S1: High resolution confocal images of vessels in the CX treated tumors. RENCA tumors stained for Podocalyxin or CD31 (green), and DAPI (blue or white). (A) A vehicle treated vessel as reference to B–D. (B) An example of a CX-treated tumor vessel filled with podocalyxin-positive cells with blue DAPI-stained nucleus, white asterisks. (C) Same image as B, where the green channel was removed to more clearly see the nuclei, black asterisks (DAPI in white). (D) A CX-treated tumor showing a partially occluded lumen, filled with CD31-positive cells. Scale bars 25 μm. (TIF)

Author Contributions

Conceived and designed the experiments: MK TH HK HW NE SY-H MH. Performed the experiments: MK TH HK AN HW NE MH. Analyzed the data: MK TH HK AN HW NE SY-H MH. Contributed reagents/materials/analysis tools: TH HK HW NE SY-H MH. Wrote the paper: MK MH.

References

1. Grothey A, Galanis E (2009) Targeting angiogenesis: progress with anti-VEGF treatment with large molecules. Nat Rev Clin Oncol 6: 507–518.
2. Ivy SP, Wick JY, Kaufman BM (2009) An overview of small-molecule inhibitors of VEGFR signaling. Nat Rev Clin Oncol 6: 569–579.
3. Schuilk RL (2010) Personalized medicine in oncology: the future is now. Nat Rev Drug Discov 9: 363–366.
4. Schrama D, Riefler RA, Becker JC (2006) Antibody targeted drugs as cancer therapeutics. Nat Rev Drug Discov 5: 147–159.
5. Thang VD (2006) Targeting proteases: successes, failures and future prospects. Nat Rev Drug Discov 5: 785–799.
6. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284: 770–776.
7. Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: unifying the activation mechanism. Cell 137: 216–233.
8. Miele L, Golda T, Osborne B (2006) Notch signaling in cancer. Curr Mol Med 6: 905–918.
9. Niessen K, Karusan A (2008) Notch signaling in cardiac development. Circ Res 102: 1169–1181.
10. Ellison LW, Bird J, West DC, Soreng AL, Reynolds TC, et al. (1991) TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 66: 649–661.
11. Malyskova A, Dohla T, von der Lehr N, Akhoondi S, Corcoran M, et al. (2007) The tumor suppressor gene hICD4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. Cancer Res 67: 3511–3516.
12. Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, et al. (2004) Activating mutations of NOTCH1 in human T-cell acute lymphoblastic leukemia. Science 306: 269–271.
13. Efferson CL, Winkelmann CT, Ware C, Sullivan T, Giampaoli S, et al. (2010) Downregulation of Notch pathway by a gamma-secretase inhibitor attenuates AKT/mammalian target of rapamycin signaling and glucose uptake in an ERM22 transgenic breast cancer model. Cancer Res 70: 2476–2484.
29. Luistro L, He W, Smith M, Paekman K, Vlencek M, et al. (2009) Preclinical profile of a potent gamma-secretase inhibitor targeting notch signaling with in vivo efficacy and pharmacodynamic properties. Cancer Res 69: 7672–7680.

30. Wei P, Walls M, Qu M, Ding R, Denlinger RH, et al. (2010) Evaluation of selective gamma-secretase inhibitor PF-03084014 for its antitumor efficacy and gastrointestinal safety to guide optimal clinical trial design. Mol Cancer Ther 9: 1618–1628.

31. Searfoss GH, Jordan WH, Calligaro DO, Gablebreath EJ, Schirringer LM, et al. (2003) Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. J Biol Chem 278: 46107–46116.

32. Korff T, Augustin HG (1999) Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. J Cell Sci 112 (Pt 19): 3249–3256.

33. Rissanen TT, Korpisalo P, Karvinen H, Liimatainen T, Laidinen S, et al. (2008) High-resolution ultrasound perfusion imaging of therapeutic angiogenesis. JACC Cardiovasc Imaging 1: 83–91.

34. Noguer-Trouix I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, et al. (2006) Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature 444: 1032–1037.

35. High FA, Lu MM, Peer WS, Loomes KM, Kaestner KH, et al. (2008) Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proc Natl Acad Sci U S A 105: 1955–1959.

36. Nakajima M, Yuasa S, Ueno M, Takakura N, Koseki H, et al. (2003) Abnormal blood vessel development in mice lacking presenilin-1. Mech Dev 120: 657–667.

37. Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF (2008) Vascular permeability, vascular hyperpermeability and angiogenesis. Angiogenesis 11: 109–119.

38. Paris D, Quadros A, Patel N, Dell’Donne A, Humphrey J, et al. (2005) Inhibition of angiogenesis and tumor growth by beta and gamma-secretase inhibitors. Eur J Pharmacol 514: 1–15.

39. Petit A, Pasini A, Alves Da Costa C, Ayral E, Hernandez JF, et al. (2003) JLK isocoumarin inhibitors: selective gamma-secretase inhibitors that do not interfere with notch pathway in vitro or in vivo. J Neurosci Res 74: 370–377.

40. Wong GT, Manfra D, Poulet FM, Zhang Q, Josien H, et al. (2004) Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoeisis and intestinal cell differentiation. J Biol Chem 279: 12876–12882.

41. Yan M, Callahan CA, Beyer JC, Allamani KP, Zhang G, et al. (2010) Chronic Dll4 blockade induces vascular neoplasms. Nature 463: E6–7.

42. Geisler F, Nagl F, Mazur PK, Lee M, Zimmer-Srtob U, et al. (2008) Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48: 607–616.

43. Real PJ, Posello V, Palomero T, Castillo M, Hernandez E, et al. (2009) Gamma-secretase inhibitors reverse glucocorticoid resistance in T cell acute lymphoblastic leukemia. Nat Med 15: 50–58.

44. Ante-Amerazga M, Zhang N, Lineberger JE, Arnold BA, Toner TJ, et al. (2010) Characterization of Notch1 antibodies that inhibit signaling of both normal and mutated Notch1 receptors. PLoS One 5: e9094.

45. Korpisalo P, Karvinen H, Rissanen TT, Kilpijoki J, Marjomaki V, et al. (2008) Vascular endothelial growth factor-A and platelet-derived growth factor-B combination gene therapy prolong angiogenic effects via recruitment of interstitial mononuclear cells and paracrine effects rather than improved pericyte coverage of angiogenic vessels. Circ Res 103: 1092–1099.

46. Rissanen TT, Korpisalo P, Markkanen JE, Liimatainen T, Orden MR, et al. (2005) Blood flow remodels growing vasculature during vascular endothelial growth factor gene therapy and determines between capillary arterIALIZation and sprouting angiogenesis. Circulation 112: 3937–3946.

47. Rissanen TT, Markkanen JE, Arve K, Rutanen J, Kettunen MI, et al. (2003) Fibroblast growth factor-4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. Faseb J 17: 100–102.

48. Hewitt CD, Innes DJ, Savory, J, Wills MR (1989) Normal biochemical and hematological values in New Zealand white rabbits. Clin Chem 35: 1777–1779.

49. Ypsilantis P, Politou M, Mikroulis D, Pitakoudis M, Lambropoulos M, et al. (2007) Organ toxicity and mortality in propofol-sedated rabbits under prolonged mechanical ventilation. Anesth Analg 105: 155–166.