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High tumor levels of IL6 and IL8 abrogate preclinical efficacy of the γ -secretase inhibitor, RO4929097

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ABSTRACT

Interest continues to build around the early application of patient selection markers to prospectively identify patients likely to show clinical benefit from cancer therapies. Hypothesis generation and clinical strategies often begin at the preclinical stage where responder and nonresponder tumor cell lines are first identified and characterized. In the present study, we investigate the drivers of *in vivo* resistance to the γ -secretase inhibitor RO4929097. Beginning at the tissue culture level, we identified apparent IL6 and IL8 expression differences that characterized tumor cell line response to RO4929097. We validated this molecular signature at the preclinical efficacy level identifying additional xenograft models resistant to the *in vivo* effects of RO4929097. Our data suggest that for IL6 and IL8 overexpressing tumors, RO4929097 no longer impacts angiogenesis or the infiltration of tumor associated fibroblasts. These preclinical data provide a rationale for preselecting patients possessing low levels of IL6 and IL8 prior to RO4929097 dosing. Extending this hypothesis into the clinic, we monitored patient IL6 and IL8 serum levels prior to dosing with RO4929097 during Phase I. Interestingly, the small group of patients deriving some type of clinical benefit from RO4929097 presented with low baseline levels of IL6 and IL8. Our data support the continued investigation of this patient selection marker for RO4929097 and other types of Notch inhibitors undergoing early clinical evaluation.

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1. Introduction

Human tumors are inherently heterogeneous possessing multiple molecular defects complicating the development of new targeted therapies (Marusyk and Polyak, 2010; Copeland and Jenkins, 2009). Many investigational drugs struggle to show clinical benefit early in development leading to a loss of interest or progressively more expensive clinical trials. One of the most important aspects of translational medicine is the ability to preselect patients likely to derive benefit from a new agent

early in clinical testing. Pre-selection enables the rapid collection of key data reducing the number of patients required for decision making. One of the important responsibilities of drug discovery research centers on the identification and preclinical validation of response hypotheses for testing in early clinical development.

Notch signaling is an area of intense research in oncology with several agents undergoing Phase I dose escalation, including Roche RO4929097, Merck MK-0752, and Pfizer PF03084014. Roche RO4929097 is a potent and selective

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inhibitor of γ -secretase, inhibiting Notch signaling in tumor cells leading to preclinical efficacy (Luistro et al., 2009). A multicenter Phase I dose escalation study in oncology is nearing completion designed to identify a well tolerated optimal dose and schedule. An important issue with this class of drugs remains the identification of response markers. The Notch axis presents additional complications since it plays a key role in three tumor survival processes: tumor cell transformation, cancer stem cell survival, and tumor angiogenesis (Tien et al., 2009; Fortini, 2009; D'Souza et al., 2008).

Notch signaling and tumor vascularization are the most compelling aspects of Notch tumor biology. Tumor endothelial cells utilize Notch signaling to build the tumor vasculature (Phng and Gerhardt, 2009; Dufraigne et al., 2008). The current understanding of Notch signaling during tumor angiogenesis suggests the Notch axis plays a determining role in helping tumor endothelial cells to adopt either a tip or tube phenotype at critical points during angiogenesis (Carmeliet et al., 2009). This cell–cell communication and cell signaling are carried out in conjunction with VEGF signaling to generate and finely tune the mature vessels. We have previously reported that xenografts responsive to RO4929097 treatment show reduced expression of genes associated with angiogenesis consistent with the ability of RO4929097 to inhibit tumor angiogenesis (Luistro et al., 2009). However, little change was observed in the angiogenic gene profile for the H460a xenograft, which is resistant to the efficacious effects of RO4929097. These differential angiogenic responses set up a discovery effort to define response markers.

In the present study, we investigate the drivers of *in vivo* RO4929097 resistance. Beginning at the tissue culture level using comparative antibody arrays, we identify clear IL6 and IL8 expression differences. We engineered the overexpression of IL6 and IL8 in the sensitive A459 xenograft converting it into a resistant xenograft. In addition, *in vivo* combination dosing of RO4929097 with IL8 shRNA knockdown or IL8 neutralizing antibodies sensitized the H460a xenograft to RO4929097. We applied this response hypothesis prospectively and successfully identified additional xenograft models resistant to the *in vivo* effects of RO4929097. Our data suggest that for IL6 and IL8 overexpressing tumors, RO4929097 no longer impacts angiogenesis or the infiltration of tumor associated fibroblasts. *In vivo* downregulation of the direct target of the Notch signaling, *Hes1*, by RO4929097 in host mouse cells is also ablated by high level of IL6 and IL8. These preclinical data provide a rationale for preselecting patients possessing low levels of IL6 and IL8 prior to RO4929097 dosing increasing the likelihood of deriving clinical benefit. Extending this hypothesis into the clinic, we monitored patient IL6 and IL8 serum levels prior to dosing with RO4929097 during Phase I. Interestingly, the small group of patients deriving some type of clinical benefit from RO4929097 preferentially presented with low baseline levels of IL6 and IL8.

2. Materials and methods

2.1. Reagents and cell lines

RO4929097 was used as described (Luistro et al., 2009). The human cancer cell lines were purchased from the American

Type Culture Collection. pLKO.1-based shRNAs against IL8 were purchased from Open Biosystems. Neutralizing anti-IL8 antibody (MAB208, clone 6217) was purchased from R&D systems. Cytokine arrays were purchased from RayBiotech, Inc. (Norcross, GA.) and used according to the manufacturer's protocol.

2.2. RNA isolation and qRT-PCR

RNA isolation, and reverse transcription-PCR (RT-PCR) were conducted using standard laboratory techniques. The catalog numbers for each probe set were human primers: *Hes1* (Hs00172878_m1), *ACTB* (4333762F), *18S* (4319413E); mouse primers: *Hes1* (Mm01342805_m1), *ACTB* (4352933E), *CD146/MCAM* (Mm00522397_m1), *TIE2/TEK* (Mm00443243_m1), *SMA/ACTA2* (Mm01546133_m1), *CD45* (Mm01293575_m1) and *CD68* (Mm03047343_m1). The tumor angiogenesis study used homogenized tumor for RNA purification.

2.3. Xenograft tumor models

The *in vivo* efficacy experiments were conducted as described (Luistro et al., 2009). RO4929097 was formulated as a suspension in 1.0% Klucel in water with 0.2% Tween 80 for oral administration.

2.4. ELISA measurement of secreted IL6 and IL8 in tissue culture medium and mouse serum

The human IL6 ELISA kits were purchased from Bender MedSystems (BMS213/2 or BMS213INST). The human IL8 ELISA kits were purchased from Bender MedSystems (BMS204/3INST) or R&D Systems (D8000C). Cells were seeded at a density of half a million in 35 mm plates to measure secreted IL6 and IL8 in tissue culture medium. Next day, cells were washed with 2 ml PBS and then replenished with 1 ml fresh medium. After 24 h, the medium was harvested and immediately used for ELISA analysis following the manufacturer's protocol.

2.5. ELISA measurement of secreted IL6 and IL8 in the plasma from human patients

Patient plasma was collected at the Phase I sites and sent to Rules Based Medicine for IL6 and IL8 ELISA assay using their Human CytokineMAP™ A v 1.0. All clinical investigations were conducted in accordance with the Declaration of Helsinki principles and received approval from individual institutional internal review boards prior to RO4929097 administration. Each patient received and signed an informed consent prior to entering the Phase I study.

3. Results

3.1. Elevated expression of IL6 and IL8 is associated with a lack of RO4929097 efficacy

The γ -secretase inhibitor, RO4929097, targets the Notch signaling pathway demonstrating broad preclinical activity against multiple xenograft models with the notable exception

Figure 1 – High expression of IL6 and IL8 correlated with resistance to RO4929097 treatment in preclinical models. (A) Expression analysis of 60 cytokines secreted from *in vitro* cultured RO4929097-resistant NCI-H460a cells and RO4929097-sensitive A549 cells. **(B)** mRNA (qRT-PCR) and protein expression (ELISA) of IL6 and IL8 in multiple cell lines used for preclinical efficacy studies. Expression of 18S was used as control for qRT-PCR. Tumor Growth Inhibition (TGI) by RO4929097 of respective xenograft tumors are indicated on the top.

of the H460a model (Figure 1B) (Luistro et al., 2009). H460a tumor cells demonstrate Notch signaling inhibition (*Hes1* down-regulation by qRT-PCR) in tissue culture (data not shown) suggesting H460a *in vivo* resistance may be driven by non-tumor cell processes including angiogenesis. We previously reported that RO4929097 treatment of the A549 xenograft model led to reduced expression of genes associated with angiogenesis. In contrast, the RO4929097-resistant H460a xenograft showed little change in expression of these genes, underscoring the *in vivo* anti-angiogenesis mechanism of action of RO4929097 (Luistro et al., 2009). We hypothesized that RO4929097's anti-angiogenic effects may be affected by extracellular cues arising from tumor cells during *in vivo* growth. We began by surveying cytokines secreted by H460a and A549 under tissue culture growth conditions using a cytokine

antibody array. Among 60 cytokines surveyed, IL6 and IL8 displayed strong differential expression between H460a and A549 (Figure 1A). This array analysis identifies a few other candidate cytokines. However, the expression differences were minor and did not warrant additional follow up. Interestingly, both cytokines have been implicated as proangiogenic modulators during tumor development. We investigated the possibility that high expression level of IL6 and IL8 in certain tumors antagonizes the anti-angiogenic mechanism of RO4929097.

Expression of IL6 and IL8 in H460a and A549 cells was further quantified by qRT-PCR for mRNA and ELISA for secreted proteins (Figure 1B). H460a cells express 12-fold higher IL6 mRNA (4-fold higher IL6 protein) and 8-fold higher IL8 mRNA (10-fold higher IL8 protein) than A549, consistent with published reports (Levina et al., 2008). We previously reported RO4929097 efficacy data utilizing eight xenograft tumors including A549 and H460a. Returning to these cell lines, we measured levels of IL-6 or IL-8 mRNA and protein (Figure 1B). Only the H460a resistant cell line expressed high levels of both IL-6 and IL-8 supporting the association of these proteins with *in vivo* resistance to RO4929097. MiaPaCa2, which expresses only high level of IL8, showed marginal efficacy (53% TGI, Tumor Growth Inhibition).

3.2. Engineered overexpression of IL6 or IL8 in A549 cells converts an RO4929097-sensitive model to an RO4929097-resistant model

In humans, IL6 signals through IL6R/gp130 receptor complexes, which are conserved in mouse. Human IL-6 has been shown active on mouse cells (Van Snick, 1990; Grötzinger et al., 1997). In humans, IL8 functions through two receptors, CXCR1 or CXCR2. There is no clear mouse ortholog for human IL8. Only CXCR2 is present in mouse. However, it has been clearly demonstrated that human IL8 can bind to mouse CXCR2 and function in mouse (Simonet et al., 1994; Lee et al., 1995). In order to directly test if expression of IL6 or IL8 modulates the efficacy of RO4929097, we used lentivirus to stably overexpress IL6 or IL8 in RO4929097-sensitive A549 cells and tested whether IL6 and/or IL8 overexpression alters RO4929097 *in vivo* sensitivity. These lentiviruses have GFP-tagged drug selection genes under IRES control. Therefore after drug selection, IL6 and IL8 overexpressing cells are positive for GFP expression, which can be clearly visualized by FACS analysis (Figure 2A). The overexpression of IL6 and IL8 protein was also quantified by ELISA (Figure 2B). IL6 overexpressing A549 cells secreted 11-fold higher IL6 compared to H460a. IL8-expressing A549 cells secreted about 40% less of IL-8 protein than H460a, but still about 9-fold higher than parental A549. A549 cells overexpressing IL6 or IL8 have a similar morphology as A549 vector control cells *in vitro* in tissue culture (data not shown). The *in vitro* growth kinetics of these new cell lines remained unchanged. We followed the growth of A549 vector control, A549 overexpressing IL6, A549 overexpressing IL8 and 1:1 mixture of A549 overexpressing IL6 or IL8 for 12 days by repetitive trypsinization/replating. All cell lines tested displayed a similar growth rate (Figure 2C).

Figure 2 – Exogenous overexpression of IL6 and IL8 is able to lead to RO4929097 tumor resistance in preclinical models. (A) FACS characterization of IL6 and IL8 overexpressing A549 cells. (B) Protein expression (ELISA) of IL6 and IL8 in IL6 and IL8 overexpressing A549 cells. (C) Characterization of growth *in vitro* of IL6 and IL8 overexpressing A549 cells. Equal numbers of cells from each cell line were seeded in 35 mm plates, then serially passed by trypsinization and dilution at the same ratio on the indicated days. Cells were counted during each passage and expressed as a percentage of vector control A549 cells each time. (D) *in vivo* efficacy following RO4929097 treatment of A549 parental, H460a and IL6 and IL8 overexpressing A549 tumors.

The *in vivo* effect of IL6 and IL8 overexpression on RO4929097 efficacy was evaluated for each of these cell lines using the parental A549 and H460a as controls. Xenograft tumors from H460a (high levels of both IL6 and IL8) treated with 10 mg/kg RO4929097 qd, produced a 9% TGI (Figure 2D). This resistance is in clear contrast to the 71% TGI ($P \leq 0.05$) observed for A549 tumors and 61% TGI ($P \leq 0.05$) for A549 vector control tumors (low levels of IL6 and IL8) and consistent with previous experiments. Overexpression of IL6 or IL8 individually in A549 reduced the TGI to 32% and 45% ($P \leq 0.05$), respectively. To effectively reconstruct the H460a resistant model, tumors were initiated following a 1:1 mixture of A549 overexpressing IL6 or IL8. These tumors demonstrate full resistance to RO4929097 (8% TGI), in line with the resistance of the parental H460a xenograft. These data demonstrate that both IL6 and IL8 are needed to completely suppress sensitivity to

RO4929097. Taxol was included as a positive drug control, and showed consistent TGI across all models (81–100% TGI, $P \leq 0.05$).

3.3. Reduced expression of IL8 in H460a cells partially reverses RO4929097 resistance

We used shRNAs to knock down IL6 and IL8 expression to address the question of whether IL6 and/or IL8 is necessary for the intrinsic resistance of H460a cells to our γ -secretase inhibitor. As shown in Figure 3A, one of five IL8 directed shRNA tested gave about 75% knockdown of IL8 expression (both mRNA and protein) compared to H460a parental cells. IL8-knockdown H460a cells still have more than 2-fold higher IL8 levels compared to A549 cells. We evaluated ten shRNAs targeting IL6. However, none yielded significant IL6 reduction (data not

Figure 3 – Reducing expression of IL8 sensitizes RO4929097-resistant tumors to treatment. (A) mRNA (qRT-PCR) and protein expression (ELISA) of IL6 and IL8 in engineered IL8 shRNA knockdown H460a cells. Expression of 18S was used as control for qRT-PCR. (B) *in vivo* efficacy of RO4929097 treatment of IL8 knockdown H460a tumors. (C) *in vivo* efficacy studies of RO4929097 treatment of H460a tumors in combination with anti-IL8 antibodies.

shown). Therefore we were only able to study IL8-knockdown H460a cells *in vivo*. As shown in Figure 3B, when IL8-knockdown H460a cells were implanted into mice, they grew slightly slower than parental H460a cells (12% TGI). This small growth inhibitory effect *in vivo* after IL8 knockdown is most likely due to IL8's role on tumor microenvironment since IL8-knockdown H460a cells have the same proliferation rate as parental H460a cells *in vitro* in tissue culture (data not shown). When treated with RO4929097, tumors derived from IL8-knockdown H460a cells show improved responsiveness compared to tumors from parental H460a cells (24% TGI (36–12%) vs 5% TGI) (Figure 3B). The incomplete sensitization is consistent with the degree of enhanced efficacy in Figure 2D and points to the need to disable both IL6 and IL8 for full sensitization.

We further addressed IL8 modulation using neutralizing antibodies to avoid misinterpretations of data generated from a single IL8 shRNA. A neutralizing antibody against IL8 was purchased from R&D Systems. This antibody was first tested *in vitro* in tissue culture. No growth inhibition was observed in H460a cells treated with this antibody (data not shown). The neutralizing IL8 antibody was dosed at 20 mg/kg with or without RO4929097. This antibody produced a small effect (21% TGI) on H460a tumor growth (Figure 3C), consistent with previous IL8 knockdown data (Figure 3B). The combination of the IL8 neutralizing antibody with RO4929097 improved the sensitivity of H460a tumors to RO4929097 (22% TGI (43–21%) vs 10% TGI). This modest anti-tumor effect is consistent with the previous data utilizing IL8 shRNA.

3.4. U87MG and LOX derived xenografts express high levels of IL6 and IL8 producing resistance to RO4929097

An important aspect of any response biomarker is the ability to prospectively identify responder and nonresponder tumors. We applied the IL6 and IL8 “signature” to identify additional xenograft models predicted to show *in vivo* resistance to RO4929097. Approximately one hundred cell lines across multiple tumor types were screened for expression of IL6 and IL8 by qRT-PCR. Thirteen cell lines had at least 10-fold higher expression of either IL6 or IL8 than A549 (data not shown). Two cell lines, U87MG (glioblastoma) expressing high levels of both IL6 (35-fold higher IL6 mRNA than A549) and IL8 (85-fold higher IL8 mRNA than A549), along with LOX (melanoma) expressing only high level of IL8 (50-fold higher IL8 mRNA than A549) and compatible level of IL6 (2.5-fold higher IL6 mRNA than A549) were selected for further *in vivo* assessment (Figure 4A). The higher expression level of IL6 and/or IL8 from these two cell lines was confirmed by ELISA (Figure 4B). Both cell lines showed clear *Hes1* mRNA downregulation after RO4929097 treatment in tissue culture (Figure 4C). When tumors from these two cell lines were treated with RO4929097, both models proved resistant to RO4929097 treatment (Figure 4D) as predicted, with a <0% TGI for U87 MG tumors and a 15% TGI for LOX tumors.

3.5. IL6 and IL8 antagonize the anti-angiogenic effect of RO4929097

IL6 and IL8 are well known to promote angiogenesis during tumor progression (Nilsson et al., 2005; Brat et al., 2005; Smith et al., 1994; Mizukami et al., 2005). A key driver of γ -secretase inhibitor activity in the clinical setting likely stems from the role Notch plays during tumor angiogenesis (Luistro et al., 2009; Phng and Gerhardt, 2009). In previous studies, RO4929097 treatment reduced the expression of several angiogenesis related mRNAs in the RO4929097-sensitive A549 xenograft tumors while having no effect on the RO4929097-resistant H460a model. This result suggests the lack of response observed in the H460a model is likely driven by the ability of high levels of IL6 and IL8 to overcome the anti-angiogenic effects of RO4929097. To test this hypothesis, we treated RO4929097-sensitive A549 tumors, NCI-H460a, and RO4929097-resistant IL6 and IL8 overexpressing A549 tumors with or without RO4929097. When the control tumors reached about 400 mm³,

Figure 4 – U87MG and LOX tumors, expressing high levels of IL6 and IL8, are resistant to RO4929097 treatment. (A) mRNA (qRT-PCR) and protein expression (ELISA) of IL6 and IL8 in U87MG and LOX cells. (B) *Hes1* expression (qRT-PCR) in U87MG and LOX cells in the absence and presence of RO4929097 for 24 h *in vitro*. Expression of 18S was used as control for qRT-PCR. (C) *in vivo* efficacy studies of RO4929097 treatment of U87MG and LOX tumors.

all tumors were harvested for mRNA analysis by qRT-PCR. We monitored mRNA expression changes in mouse genes uniquely expressed in endothelial cells, leukocytes, macrophages, and tumor associated fibroblasts to determine RO4929097 effects on mouse stroma (Figure 5A). We utilized qRT-PCR to quantify the infiltrated mouse cell types into xenografted human tumors and selected mouse cell-type specific markers. CD146 and TIE2 are specific for endothelial cells, whereas CD31, a widely-used endothelial marker used for tumor IHC is also expressed in platelets, monocytes and macrophages. We present our CD146 and TIE2 results in Figure 5. We also quantified the mouse CD31 mRNA and saw a similar trend as CD146 and TIE2 after RO4929097 treatment. However, the *p*-value missed our $p < 0.05$ cutoff due to an outlier in the A549 vector treatment group (data not shown). All mouse Taqman PCR primers showed more than 1000-fold specificity toward mouse genes over human genes (data not shown). As shown in Figure 5B, two mouse endothelial cell markers, CD146 and TIE2, are clearly downregulated (20–40%, $P < 0.05$) after RO4929097 treatment in both A549 parental and A549 vector control tumors. Both of these tumors express low levels of

IL6 and IL8. In contrast, IL6 and IL8 overexpressing A549 tumors and H460a tumors did not show downregulation of these two markers after RO4929097 treatment. This suggests that high IL6 and IL8 counter RO4929097's anti-angiogenesis effect leading to RO4929097 resistance. Interestingly, similar to CD146 and TIE2, expression of the mouse tumor associated fibroblast marker, SMA, decreased ($>20\%$, $P < 0.05$) following RO4929097 treatment in IL6 and IL8 low tumors, but not in IL6 and IL8 high tumors. We further investigated the change in infiltration of monocytes/macrophages and pan-leukocytes into xenograft tumors after RO4929097 treatment by checking expression of CD68 and CD45. No difference was observed. In order to better understand how overexpression of IL6 and IL8 affects Notch's function during angiogenesis, we further analyzed the expression of mouse *Hes1*, a direct Notch transcriptional target, in mouse stroma within xenograft tumors. Mouse *Hes1* is significantly downregulated in IL6 and IL8 low tumors after RO4929097 treatment as expected. However, mouse *Hes1* downregulation by RO4929097 is unexpectedly lost in IL6 and IL8 high expressing tumors, suggesting that the IL6 and IL8 signaling pathways *in vivo* may converge with the Notch signaling

Figure 5 – IL6 and IL8 are able to offset the anti-angiogenic effect of RO4929097 in preclinical models. (A) Selected mouse markers for mRNA expression analysis in xenografted human tumors. (B) mRNA expression analysis (qRT-PCR) of selected mouse markers in xenografted human tumors. Control for qRT-PCR of each marker is indicated in the figure. All groups have five tumors with the exception of the control-treated A549 parental group which has 4 tumors. For all A549-derived tumors, the average expression in A549 parental tumors was preset to one, then the expression in individual tumors was normalized accordingly. The same normalization approach was applied to H460a-derived tumors. Statistics: student's *t*-test. (C) *Hes1* expression (qRT-PCR) in vector control A549 and IL6 and IL8 overexpressing A549 cells in the absence and presence of RO4929097 for 24 h *in vitro*. Expression of 18S was used as control for qRT-PCR.

pathway at or above regulation of *Hes1* expression. This convergence has not been observed in cancer cell lines grown under *in vitro* tissue culture conditions. As shown in **Figure 5C**, human *Hes1* downregulation by RO4929097 is maintained in IL6 and IL8 overexpressing A549 cells *in vitro* in tissue culture. The same is true for U87MG and LOX which have a natural overexpression of IL6 and IL8. (**Figure 4C**), indicating a more complicated *in vivo* signaling network.

3.6. Patients with reduced baseline plasma IL6 and IL8 levels show a trend toward deriving clinical benefit from RO4929097 treatment

Our preclinical data suggest that tumor IL6 and/or IL8 secretion may reduce the effectiveness of treatment with RO4929097, while patients with tumors lacking IL6 and/or IL8 secretion may have a better chance of responding. We collected plasma from patients prior to dosing with RO4929097 as part of the Phase I dose escalation to begin validating our response hypothesis. The pre-treatment IL6 and IL8 plasma levels were quantified by ELISA and are expressed

in **Figure 6** divided by clinical benefit (CT response, PET response or stable disease after 4–8 cycles) using information from all doses tested. Based on previously published literature in healthy individuals, we set the baseline for normal plasma levels of IL6 (<10 pg/ml) and IL8 (<40 pg/ml). In patients without clinical benefit, 9 out of 40 (22.5%) have high plasma level of both IL6 and IL8. In contrast, in patients with signs of clinical benefit, only 1 out of 12 (8.3%) had a high plasma level of both IL6 and IL8. The patient population is small, so the *P* value (=0.42) is not significant. However, the trend that patients with low baseline plasma levels of both IL6 and IL8 are more likely to derive some kind of clinical benefit is encouraging and worth following up in larger clinical studies.

4. Discussion

Inhibition of the Notch signaling pathway is an area of great interest in oncology. The clinical investigation of γ -secretase inhibitors blocking the Notch pathway is gaining interest in

Figure 6 – Quantitation of pre-dose IL6 and IL8 plasma levels in patients treated with RO4929097 during Phase I dose escalation. Individual patients are shown in categories of clinical benefit with their corresponding IL6 and IL8 plasma levels. When patient data were treated categorically based on high or low IL8 and IL6 levels, 22.5% of patients (9 out of 40) without clinical benefit have both IL6 and IL8 at high levels ($IL6 \geq 10$ pg/ml and $IL8 \geq 40$ pg/ml), while only 8.3% of patients (1 out of 12) with clinical benefit have both IL8 and IL6 at high levels. This trend, although positive, is non-significant given the small patients populations (two-tailed Fisher's exact test, $P = 0.42$).

oncology with the entry of several small molecule inhibitors into Phase I. Additionally, several antibodies showing different selectivity for each of four Notch receptors are poised for clinical investigation (Wu et al., 2010). However, Notch signaling biology is quite complex and remains poorly understood. Analogous to Notch's role guiding cell fate decisions during development, inhibition of the pathway can impact tumor growth and survival in three ways: 1) reversing cellular transformation, 2) inhibiting cancer stem cell survival, 3) blocking tumor angiogenesis. The best characterized aspect of Notch and tumor cell biology comes from the angiogenesis field where it has been demonstrated by several labs that the Notch pathway plays a pivotal role in guiding the production of new tumor blood vessels (Phng and Gerhardt, 2009; Ridgway et al., 2006; Noguera-Troise et al., 2006). Given the increasing interest in inhibiting Notch in oncology and the complex signaling events, there is a critical need to identify predictive markers to guide patient selection. We present preclinical data characterizing the *in vivo* resistance of the H460a xenograft to the γ -secretase inhibitor, RO4929097. This compound is a selective and potent inhibitor of γ -secretase targeting the Notch signaling axis and is completing Phase I dose escalation (Luistro et al., 2009). We report here that one preclinical resistance mechanism is driven by the elevated expression of two important cytokines, IL6 and IL8.

IL6 and IL8 are powerful cytokines playing important roles in such diverse disease settings as infection and immunity, inflammation, autoimmune disease and cancer (Hong et al., 2007; Waugh and Wilson, 2008). The biological impact of these cytokines varies depending on the target cell. For example, IL6 and IL8 exhibit potent effects on vascular endothelial cells impacting tumor angiogenesis (Nilsson et al., 2005; Brat et al., 2005). Angiogenesis is the process giving rise to new vessels derived from existing ones and follows a series of cell signaling events initiated by both proangiogenic and anti-angiogenic factors. Angiogenesis begins as the parent

vessels become more permeable, releasing plasma and depositing a proangiogenic matrix. Local concentrations of proangiogenic growth factors and chemotactic factors attract new proliferating endothelial cells into this new matrix. Once relocated, these endothelial cells undergo a series of differentiation events forming tubes with a central lumen. The final stage is the establishment of a new basement membrane and recruitment of pericytes and smooth muscle cells that coat the mature vessel.

It is easy to understand the existence of many important signaling molecules that regulate such a complex staged process as tumor angiogenesis including VEGF, IL6, IL8 and Notch (Li and Harris, 2009). VEGF is a potent and well characterized mitogen for endothelial cells inducing the formation of new tumor vessels. VEGF is also capable of activating Notch signaling (Vecchiarelli-Federico et al., 2010). Liu et al. (2010) suggest that IL6 secreted from glioblastoma cells promotes endothelial cell migration during the early formation of new blood vessels. The role of IL8 centers on its ability to inhibit endothelial apoptosis, promote endothelial proliferation and chemotaxis (Li et al., 2004). Both IL6 and IL8 have the ability to stimulate VEGF expression in endothelial cells which contributes to their potent proangiogenic ability (Martin et al., 2009; Borg et al., 2005). The Notch pathway plays several important roles during angiogenesis but the exact function has been difficult to determine (Phng and Gerhardt, 2009). It is clear that Notch signaling directs specific endothelial cell changes giving rise to new functioning blood vessels. Disrupting endothelial Notch signaling produces defects in sprouting, branching, motility and proliferation inhibiting tumor angiogenesis (Phng and Gerhardt, 2009). This would be similar to the inhibitory effects of RO4929097. Similar to individual musical notes which only when organized in a particular manner produce music, the delicate balance and interplay of VEGF, IL6, IL8 and Notch signaling must be appropriately combined to produce new blood

vessels. We hypothesize that given the functional redundancy between the VEGF, IL6, IL8 and Notch pathways, overcoming the anti-angiogenic function of RO4929097 is accomplished through elevated expression of IL6 and IL8.

Using this IL6 and IL8 expression profile, we were able to successfully identify new preclinical tumor models resistant to the *in vivo* effects of RO4929097. We found that IL6 and IL8 expression may offset the anti-angiogenic effect of RO4929097 directly or indirectly through regulation of *Hes1* expression, converging at the central axis of the Notch pathway. Our study provides the preclinical rationale to clinically test the hypothesis that patients with high baseline levels of IL6 and IL8 are less likely to derive clinical benefit from RO4929097 treatment. In contrast, patients with low baseline levels are more likely to derive clinical benefit.

Importantly, this biomarker hypothesis was implemented in a small patient population during the dose escalation of RO4929097. We are encouraged by the observation that the majority of patients deriving some type of clinical benefit from RO4929097 present with low plasma levels of IL6 and IL8. This early clinical data warrant further study and refinement as the RO4929097 compound progresses into Phase II. Many patients presented with low levels of IL6 and IL8 prior to treatment did not derive clinical benefit. This is not surprising since the Notch pathway is involved in two other tumor survival processes namely tumor cell transformation and cancer stem cell survival. IL6 and IL8 likely reflect the Notch related angiogenesis aspect of RO4929097 inhibition. There may be Notch signaling alterations that could provide an additional RO4929097 resistance mechanism, such as Notch3 amplification status (Dang et al., 2000), expression or mutations of ADAM17 in NSCLC (Baumgart et al., 2010) and mutations of other γ -secretase complex components.

In conclusion, we present data supporting the idea that elevated tumor derived IL6 and IL8 expression is associated with the preclinical resistance of the γ -secretase inhibitor, RO4929097. Our working hypothesis proposes that elevated IL6 and IL8 expression functions to impair the anti-angiogenic effects of RO4929097 by-passing the RO4929097 mediated Notch inhibition in tumor stroma. This hypothesis has undergone early clinical testing during the Phase I dose escalation of RO4929097. These results show an encouraging trend supportive of our hypothesis and warrant further study.

Conflict of interest

All authors are employees of Hoffmann-La Roche Inc.

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