

**MODELING OF INTERACTIONS BETWEEN VASCULAR ENDOTHELIAL GROWTH
FACTOR FAMILY MEMBERS AND THEIR RECEPTORS KDR AND FLT-1.**

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ABSTRACT

The vascular endothelial growth factor (VEGF) family is prominently involved in angiogenesis, the formation of new vasculature. Overexpression has been linked to pathological conditions including solid tumor growth. Responses to VEGF are mediated by Flt-1 and KDR, two cell-surface receptors selectively expressed by vascular endothelial cells. A secreted form of Flt-1 (sFlt1) retains high affinity for VEGF and can compete for VEGF binding, thereby inhibiting angiogenic responses. In this study, a computational model of this ligand-receptor binding system is used to quantitatively describe the activation of KDR signaling by VEGF and its inhibition by sFlt-1. The model consists of a system of 12 ordinary differential equations, based on mass action kinetics, which describe, based on mass-action kinetics, the formation and disappearance of receptor-ligand complexes competent to transduce a VEGF signal. Cell-based assays were used to test predictions of the *in silico* model by measuring dynamics of 1) KDR tyrosine phosphorylation and 2) generation of intracellular second messengers in response to VEGF stimulation in the presence and absence of sFlt-1. Results indicate that the model predicts well the maximal activation in the presence of VEGF-A or the KDR-selective agonist VEGF-E. Subsequent characteristics of binding, such as time-to-peak activations, were less realistically modeled, suggesting that the mathematical descriptions of certain receptor fates (receptor internalization, sequestration or externalization) may need refinement. Also presented are the results of KDR activation experiments in the simultaneous presence of VEGF-A, sFlt1, and the Flt1-selective agonist placental growth factor (PIGF). Here, the model predicts accurately the percentage of inhibition of maximal activation when sFlt-1 is present in the system. Finally, a revised model that includes receptor heterodimerization is presented that suggests sFlt-1 can more effectively inhibit VEGF binding and activation when acting in a dominant negative fashion. Overall, the findings support the idea that the early events in VEGF signaling can be modeled successfully. A natural extension of the model, refined based on experimental testing, will be its application to normal physiological systems.

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REVIEW OF LITERATURE, HYPOTHESIS, & AIMS

INTRODUCTION

The formation and maintenance of blood vessels are complex processes that begin early in embryogenesis and continue into the postnatal period with normal physiologic roles in reproduction, wound repair and adaptation to ischemic stress. [1-3]. These same events play a significant part, and are often altered, in some pathologic conditions. In both cases, normally inactive endothelial cells are stimulated by a number of factors to grow, replicate, and migrate. Areas of hypoxic tissue can lead to the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival [3, 4]. There are additional mechanisms that can stimulate new blood vessel growth including various cytokines and growth factors (e.g., basic fibroblastic growth factor); however, evidence suggests that vascular endothelial growth factor (VEGF) is involved in both embryonic vasculogenesis and pathological and adaptive angiogenesis in adults. It regulates multiple facets of the angiogenic processes, as well as vascular permeability and inflammation [5]. The overexpression of VEGF has been linked to tumor angiogenesis where high blood vessel density is necessary for tumor growth and metastasis [6-8].

Responses to VEGF are mediated by Flt-1 and KDR, two cell-surface receptors located primarily on endothelial cells. There is also a secreted form of Flt-1 (sFlt-1) that is derived from Flt-1 RNA transcripts by alternative splicing. This variant has a high affinity for VEGF and can compete for VEGF binding to Flt-1 or KDR, thereby inhibiting angiogenic responses. The relative abundance of these receptors can vary physiologically, and the nature of their interactions with VEGF and related ligands is still unclear. Angiogenic inhibitors like sFlt-1 are of particular interest in anti-cancer therapy, but, in order to optimize their use, the details of their binding and inhibition must be more clearly understood.

For part of my proposed research, I will describe quantitatively the inhibition of normal Flt-1 and KDR signaling by sFlt-1 using a mathematical model of ligand-receptor binding. This model was used to examine the inhibitory effect that sFlt-1 binding to various VEGF isoforms may have on the formation of receptor-ligand complexes on the

cell surface. Cell-based experiments that study the effects of altered VEGF binding require the variation of numerous parameters in a systematic manner. This requires both time and resources. With the application of modeling techniques that have characteristically been employed in engineering, similar experiments where multiple parameters are varied at the same time can be carried out *in silico*. Similarly, parameters that may be difficult to modify with the use of cell-based experiments may now be easily simulated in a computational model. There are many potential scenarios for how each component can be modified and, therefore, might influence the others components or ultimate predictions made by the model. For example, the conclusions drawn from a computational model of VEGF binding can be helpful for understanding how sFlt-1, or other competing molecules, affects VEGF activation in this complex, multiple ligand, multiple receptor system. That is, in part, why modeling can be such a powerful tool in cellular studies. It is my hope that this modeling technique will highlight the contributions made by each extracellular component on VEGF binding and signaling. With simple assumptions being applied to formulate this model, the predictions may aid in the direction of future experiments.

The *in silico* model will be used to simulate an *in vitro* system, and the predictions will be tested with various cell-based assays. The resulting conclusions will be valuable for application to normal physiological systems. The results of the cell-based experiments will be compared to simulations from the model. In addition, any assumptions and hypotheses that were necessary for developing the mathematical model will be discussed. Finally, after critically looking at the results of the model, ways of improving it will be explored.

LITERATURE REVIEW

Angiogenesis

Angiogenesis, the complex process by which new blood vessels arise from the pre-existing vasculature, is important in normal physiologic events from reproductive cycles and embryonic development to wound repair [1-3]. Similar events play a significant role, and are often altered, in pathologic conditions such as arthritis [9],

diabetic retinopathies [10], and neoplasias [2]. In both the cases, normally quiescent endothelial cells are stimulated by a number of factors to grow, replicate, and migrate. This process begins with the breakdown of the extracellular matrix (ECM), followed by the proliferation and migration of capillary endothelial cells [2]. In order to form newly functioning capillary structures, the infiltrating cells have to be remodeled back into vessels, and a new ECM must be deposited.

Because most mammalian tissues depend on the supply of oxygen and nutrients provided by a functional vasculature, one of the common initiating factors in angiogenesis is the occurrence of tissue ischemia and hypoxia. In order to counteract the effects of hypoxia, transcriptional changes are often induced whereby a series of genes that participate in angiogenesis are activated [3, 4]. The primary factor mediating this response is the hypoxia-inducible factor-1 (HIF-1), an oxygen-sensitive transcriptional activator [11]. This facilitated adaptation enables an organism to maintain homeostasis because the growth of new blood vessels will provide an adequate supply of O₂ to cells. Among the genes that may be stimulated are those that encode the production of various angiogenic cytokines, and proteins like angiogenin [12], acidic fibroblastic growth factor (aFGF), basic fibroblastic growth factor (bFGF), and VEGF.

Angiogenesis in tumor growth and development

The existence of a correlation between the growth rate or metastatic potential of tumors and their blood vessel density has considerable support [6, 13]. The theory that linked these two processes was introduced in 1971, when Folkman described the association between angiogenesis and the malignant potential of solid-phase neoplasms [14]. He proposed that without the development of new vasculature into and around tumors, they would reach a maximum diameter of 2-3 mm³—the maximum distance for passive oxygen diffusion. It was further hypothesized that, by releasing soluble mediators, tumors could effectively recruit new blood vessel formation. This would result in a new supply of the necessary oxygen and nutrients for continued growth and malignant transformation. Folkman proposed that by attempting to block

this tumor-angiogenesis factor, there might one day be therapeutics that could halt neoplastic growth at an early stage.

Much has been learned in the almost 40 years since Folkman's initial study. When a primary tumor arises, the proliferation of cancer cells may be balanced by apoptosis, and the tumor may remain undetectable for years [15]. The onset of neovascularization in primary tumors can be relatively sudden, described as the angiogenic switch [15, 16]. In general, the process of blood vessel growth to supply tumors is the same as previously described for physiologic angiogenesis. As a tumor grows, the supply of oxygen that is able to reach neoplastic cells gradually decreases, and this low oxygen tension stimulates the activation of HIF-1. Upon nuclear translocation, HIF-1 activates a variety of hypoxic response elements. It is now known that tumor growth and expansion is dependent not only on a single "tumor-angiogenesis factor" that stimulates the formation of new vasculature, but rather many positive and negative angiogenic factors released and acting in a sort of "tug-of-war". The overall degree of angiogenesis associated with a tumor hangs in the balance of these factors [9]. VEGF is the best characterized pro-angiogenic factor in tumor angiogenesis, and it is virtually ubiquitous in mammalian tumors [17-19]. Higher levels of VEGF from over-expression have been correlated with a more aggressive disease type, where high blood vessel density supports tumor growth and metastatic potential [6-8, 20].

Vascular Endothelial Growth Factor-A

Evidence suggests that VEGF is centrally involved in embryonic vasculogenesis as well as pathological and adaptive angiogenesis in adults. It regulates multiple facets of the angiogenic process, as well as the induction of vascular permeability and inflammation [5]. Initially identified as a heparin-binding vascular permeability factor (VPF) [21, 22], VEGF-A displays a high selectivity for endothelial cells and is identified as the most important regulator of endothelial cell physiology [23-25]. At the cellular level, VEGF-A stimulates cell growth and division [26] in endothelial cells *in vitro* and is up-regulated by hypoxia both *in vitro* and *in vivo* [27, 28]. It affects vessel development in two main ways: by stimulating vascular endothelial cell proliferation [23], and by increasing vascular permeability [22].

Since its discovery, several isoforms of VEGF-A have been identified, all produced from a common gene and forming active disulfide-linked homodimers [29]. Alternative splicing of the eight exons in human VEGF-A RNA transcripts gives rise to five known isoforms containing 121, 145, 165, 189, and 206 amino acid residues [30-32]. VEGF₁₆₅ is widely regarded as the most abundant and biologically significant form [33]. Despite the presence of the signal peptide identical to that found in other isoforms, VEGF₁₈₉ and VEGF₂₀₆ are poorly secreted. They are primarily cell-associated, and there are implications that they are less active than the secreted isoforms [30].

All isoforms of VEGF-A, with the exception of VEGF₁₂₁, bind heparin to measurable degrees (physiologically present as heparan sulfate proteoglycan [HSPG]). Besides acting as a structural component of the extracellular matrix, HSPGs have been recognized as secondary regulators of growth factors' activities. When HSPG binds VEGF, the proteoglycans can alter the concentration, and thereby the effect, of VEGF by storing the growth factor at the cell surface. In this case, a low concentration of HSPG could augment the biological activity of VEGF-A by allowing for more efficient binding to cell-surface receptors. This could generate stronger mitotic signals than in the absence of heparan [34]. VEGF₁₂₁ lacks a basic heparin-binding region, which could account for its inability to produce endothelial cell mitotic activity observed from other isoforms [34]. Its heparin-binding capability is thought to be one of the central reasons for strong angiogenic signaling via VEGF₁₆₅--the most abundant subtype *in vivo*, with a strong affinity for the cell surface and the ECM [35, 36].

VEGF Receptors

Responses to VEGF-A are mediated by cell-surface receptors, Flt-1 (fms-like tyrosine kinase, VEGFR-1), and KDR (kinase insert domain containing receptor, VEGFR-2) [37-39] (Figure 1). These receptors belong to the receptor tyrosine kinase (RTK) family and are structurally similar to one another. They are expressed on vascular progenitor cells during early embryonic development and thereafter remain essential components of signal transduction pathways that affect cell proliferation, differentiation, migration, and metabolism of endothelial cells. The VEGF receptors are characterized by seven extracellular immunoglobulin (Ig)-like domains, followed by a

membrane spanning region and intracellular tyrosine kinase domain [40-43]. Many of the signals that regulate embryonic cell growth and differentiation and regulation of adult tissue remodeling are mediated by this class of receptors. X-ray crystallography of Flt-1 complexes revealed that only Ig domains two and three are in close contact with the ligand and that domain 2 is sufficient for ligand binding [41]. Flt-1 and KDR become activated when they bind both receptor-specific and common ligands, thereby propagating specific signaling events that have various outcomes related to angiogenesis. The VEGF receptor family includes VEGFR-1, VEGFR-2, and VEGFR-3. Neuropilins 1 & 2 (NRP-1/-2) are another class of receptors residing on endothelial and neuronal cell surfaces, which are non-tyrosine kinase in nature but have a high-affinity for the VEGF ligands [44, 45]. Although there has been extensive analysis of Flt-1 and KDR, revealing regions in their structure that allow binding and signaling, the very earliest steps their signal transduction are just starting to be clarified.

VEGF-Related Ligands

In addition to different forms of VEGF-A, genes encoding distinct VEGF-related proteins have been identified. Currently, the VEGF family is described to include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) [46]—all of which bind and activate cell surface receptor tyrosine kinases. All of these polypeptides are endogenously expressed in mammals except VEGF-E, which is encoded by the double stranded DNA virus, orf [47, 48]. Most of these growth factors are structurally similar to VEGF-A, but in some cases, their functional roles remain to be elucidated [33, 49].

PlGF. Recent studies have revealed that, even though PlGF and VEGF-A share only 42% amino acid sequence identity, the structure of PlGF is quite similar to the structure of VEGF-A [50, 51]. Based on this homology with VEGF, it was proposed that PlGF was a possible angiogenic factor. This was confirmed in studies where conditioned media from cells transfected with PlGF cDNA had a weakly mitogenic effect on bovine arterial endothelial cells [52]. Like VEGF-A, PlGF is present physiologically as a homodimer, covalently linked by two disulfide bonds [50]. PlGF also contains the common feature, the cysteine-knot motif, which is found in all growth factors similar in

nature to VEGF-A [53]. The biggest differences between VEGF-A and PlGF reside at the amino- and carboxyl-terminal residues and at many of the residues that are involved in receptor-ligand binding. These differences provide a possible structural explanation for the specific binding characteristics of PlGF—homodimers of the protein bind preferentially to Flt-1 [54].

PlGF has direct effects on endothelial cells, both by binding and activating its own signaling through receptors and by amplifying angiogenesis which occurs subsequent to VEGF binding [55]. The proposed mechanisms by which PlGF may enhance VEGF induced angiogenesis include: intracellular signal transduction through VEGF receptors; increasing the pool of VEGF-A available to activate KDR by displacing VEGF-A from the 'Flt-1 sink' [55]; activation of Flt-1, which results in intermolecular transphosphorylation of KDR that could amplify VEGF-A induced angiogenesis [18]; and the formation of PlGF/VEGF-A heterodimers, which may bind and activate both Flt-1 and KDR [18]. Whatever its mechanism of action, the angiogenic potential for PlGF has been clearly demonstrated with the overexpression of PlGF during a number of pathological conditions where new blood vessel growth is required.

VEGF-B. VEGF-B, in many ways, mirrors the structure and function of PlGF. It is structurally similar to VEGF-A and binds preferentially to Flt-1 [49]. It is able to bind to heparan sulfates and NRP-1 [56], and is able to form heterodimers with VEGF-A [33, 57]. It is proposed that the heterodimerization of VEGF-B, as well as PlGF, can add to the diverse roles of these growth factors through a variety of cellular signaling transduction pathways [33]. VEGF-B is often produced in large quantities by the developing myocardium and by muscle, bone, pancreas, adrenal gland, and the smooth muscle cell layer of several large vessels; it has not been shown to be produced by endothelial cells. Because of this, VEGF-B is likely to act in a paracrine fashion, as Flt-1 is almost exclusively located on these cells [58].

Aase et al. (1999) were able to demonstrate that VEGF-B is highly expressed in many tissues; however, there are with two differentially spliced transcripts generating two secreted isoforms, VEGF-B₁₆₇ and VEGF-B₁₈₆. Li et al. (2001) took this investigation one step further and looked at the expression of VEGF-B in various tissues and cell lines in a manner that was able to distinguish the two isoforms [59]. Their

results showed that the VEGF-B₁₆₇ isoform was the predominant form, expressed in most tissues, and it accounted for more than 80% of the total VEGF-B transcripts. The VEGF-B₁₈₆ isoform, on the other hand, was expressed at lower levels and only in a limited number of tissues—among them mouse and human tumor cell lines and primary tumors [59]. Their data was able to demonstrate the genetic control for the expression of the two VEGF-B isoforms, showing that their expression can be very tissue and cell specific. Because it is able to bind to Flt-1, VEGF-B may act as a very weak endothelial cell mitogen, but otherwise its biological role is still unclear [49]. Much like PlGF, it is possible that VEGF-B may be acting by displacing VEGF-A from Flt-1 and thereby increasing the signaling pool of that growth factor for activation of KDR. Lastly it could, like PlGF, produce some biological activity in mammalian cells by forming VEGF-A/VEGF-B heterodimers.

VEGF-C and VEGF-D. VEGF-C & -D are both synthesized as preproteins, subsequently undergoing processing which produces mature forms of the growth factors that have different receptor-binding capabilities. Specifically, they are able to bind to KDR and to a third VEGF receptor, VEGFR-3 [8, 60]. Like VEGF-A, VEGF-C & -D have been shown to stimulate the migration and proliferation of endothelial cells, but these processes are not restricted to blood vascular endothelial cells. VEGF-C & -D have been shown to play significant roles in lymphangiogenesis, the formation of new lymphatic vessels [61, 62]. Activation of these processes likely occurs through KDR in blood vascular endothelial cells, and generally via VEGFR-3 in lymphatic endothelial cells.

VEGF-E. The newly-isolated VEGF-E shows strong angiogenic signaling. An early study of endothelial cells revealed the ligand selectively bound and activated KDR. This resulted in receptor autophosphorylation and a biphasic rise in free intracellular Ca²⁺ concentration, while, in contrast to VEGF-A, VEGF-E did not bind to Flt-1 [63]. More importantly, the physiologic effects of VEGF-E were recently discussed in a study by Kiba et al. (2003) [64]. In this study, VEGF-E transgenic mice demonstrated a significant increase in subcutaneous blood vessel formation, with only minor effects on proedematous and proinflammatory responses when compared with VEGF-A-transgenic mice. Both types of mice were K14-promoter-driven, which essentially

means that the epidermal basal cell layer was activated around or after birth causing endogenous over-expression of VEGF-E that remained active up to adulthood. VEGF-A-transgenic mice showed an increased vascularization that was coupled with edema. This was due, at least in part, to much higher vascular permeability—resulting in significant subcutaneous hemorrhaging. The VEGF-E-transgenic mice, on the other hand, also showed a significant increase in vascularization—about 10-fold compared to control mice—but this occurred without any clear edematous lesions or hemorrhagic spots on the skin. On the microscopic level, it seemed that the VEGF-E-induced capillary networks appeared well-organized. Ultimately, it was concluded that the physiologic differences observed between the two types of mice could be attributed to the different binding specificities for the two ligands. VEGF-A binds and activates both Flt-1 and KDR, whereas, VEGF-E binds only to KDR.

Therapeutic possibilities of VEGF-E. Since it was observed that VEGF-E induced significant angiogenesis *in vivo* with few side effects, it was also concluded that this ligand should be carefully studied as a candidate for pro-angiogenic therapies and other clinical uses [64]. This theory was proposed in a second study conducted by Kiba et al. (2003) in which a series of chimeric VEGF-E proteins with VEGF-E and PlGF were constructed and demonstrated sufficient specific binding and activation of KDR. These chimeric proteins essentially retained the strong activities as seen in wild type VEGF-E; however, they had human PlGF amino acid residues at the amino and/or carboxyl termini instead of the normal viral amino acid residues of present in VEGF-E. The authors proposed that the removal of these highly immunogenic amino- or carboxyl-terminal peptide sequences was a way of generating a “humanized VEGF-E family” that is potentially less immunogenic compared with the wild type VEGF-E [65]. These results suggest that VEGF-E may be a useful tool for proangiogenic therapy to treat patients with ischemic diseases. There is a need to further investigate the binding characteristics of this so-called humanized VEGF-E. If more information is known about the pharmacologic and kinetic profiles of its binding and activation, more techniques can be employed that may facilitate its use in more clinical studies.

VEGF Signaling

Much of the available literature suggests that when VEGF binds to either Flt-1 or KDR, there is a conformational change in the dimerized receptors, resulting in activation of intrinsic tyrosine kinase domains and autophosphorylation of tyrosine residues on the cytoplasmic region [66, 67]. The phosphorylation events that occur upon VEGF binding trigger intracellular signaling pathways. The events leading to angiogenic responses that occur downstream of each receptor can be quite diverse. For example, an early study by Waltenburger et al. (1994) aimed to determine the functional role of the two VEGF receptors and the effects of their different signaling pathways when VEGF bound to porcine aortic endothelial (PAE) cells overexpressing KDR or Flt-1 [67]. When stable lines of Flt-1 & KDR-expressing cells were established, receptor binding analyses were performed to determine that the transfected cells would be viable experimental models for what occurs after the initial ligand binding events.

One of the first assays performed on the cells measured changes in cellular morphology upon ligand stimulation. Within 5 minutes of the addition of VEGF₁₆₅ to KDR-expressing cells, the formation of cytoplasmic protrusions and ruffled edges were observed. These protrusions and edges gave way to the formation of pili and much more dramatic changes in the cell shape after 15 minutes of stimulation. These observations were indicative of cytoskeletal reorganization—the cells were stimulated in a way leading to motility. The first major difference observed in this experiment was that, under identical conditions, the Flt-1 expressing cells (along with the control PAE cells) did not respond with any changes in cellular shape or cytoskeletal reorganization [67].

With observed changes in cell morphology, it was necessary to quantify VEGF-induced chemotaxis in the cell lines. For this, cells expressing KDR or Flt-1 were examined using a modified Boyden chamber technique. A solution containing the VEGF₁₆₅ was placed in chamber and a cell suspension of either cell type was placed in another chamber above—separated by a collagen-coated membrane. Cells were able to migrate through the pores, across the thickness of the filter in a chemotactic manner toward the VEGF-A. The results for cell chemotaxis followed suit for those observed from cellular morphology assays. There was an efficient chemotaxis of KDR expressing

cells achieved using various concentrations VEGF-A; however, all attempts to induce cell migration in Flt-1 expressing cells failed [67].

Implications of VEGF receptors on embryonic development. There is general agreement throughout the literature that KDR is the major positive signal transducer of the mitogenic, angiogenic and permeability-enhancing effects of VEGF—in both physiologic and pathologic conditions. Early studies revealed that disruption of the gene encoding KDR results in severe abnormalities of blood vessel formation in homozygous animals. KDR^{-/-} homozygous mice die at approximately embryonic day 8.5 (E8.5) because of a severe deficiency in endothelial cell number along with a strong hematopoietic impairment [68]. This indicates that KDR signaling is essential for the proper differentiation and proliferation of endothelial cells during development. While the role of KDR as the primary signaling receptor in VEGF binding is established, the precise role of Flt-1 in endothelial cell function is yet unclear.

Flt-1 intracellular signaling. It is believed that there is some amount of similarity between KDR signaling pathways and the Flt-1 signaling pathways; however, because both activated receptors trigger different cellular responses, it is unlikely that the signaling pathways are identical [67]. Flt-1 does become phosphorylated when it binds VEGF; however, there are conflicting accounts as to what sort of intracellular signaling occurs downstream of this phosphorylation. While Flt-1 has a very high affinity for VEGF-A (at least one order of magnitude higher than that of KDR) [37, 69], the tyrosine kinase activity of Flt-1 is relatively weak [67, 70]. Furthermore, in a study by Seetharam et al. (1995), VEGF-A did not stimulate the proliferation of NIH3T3 cells overexpressing Flt-1. When PlGF and VEGF-B (Flt-1-specific ligands) did not stimulate proliferation of endothelial cells in culture, which express both receptor types, it was concluded that that Flt-1 activation is insufficient to trigger a mitogenic responses after VEGF binding [71].

Because this receptor shows such little activity in culture in response to VEGF-A stimulation, the downstream signaling of Flt-1 is not fully understood. Like KDR, the residues that are affected during autophosphorylation have been identified and they include: Tyr-1169, -1213, -1242, -1327 and -1333 [72, 73]. While the MAPK pathway is associated with Flt-1 at Tyr-1169 in a similar manner to Tyr-1175 of KDR,

phosphorylation of this analogous residue on Flt-1 is less readily detectable. This pathway is only mildly activated due to VEGF-A binding to Flt-1 [71, 73]. Altogether, the available literature indicates that in a defined experiment, when Flt-1 is activated by a ligand to form homodimers of Flt-1 (no cross-talk with KDR), Flt-1 is not capable of promoting biological responses such as cell migration and proliferation. In addition to the evidence of MAPK activation, Cunningham et al. (1995) were able to demonstrate binding of the p85 subunit of PI3-kinase to activated, phosphorylated Flt-1 [72]. This is one of the few accounts of secondary intracellular signaling events associated with Flt-1 activation, and the literature is inconclusive when it comes to linking activation of Flt-1 to any real biological response in endothelial cell line models. The uncertainty surrounding actual intracellular signaling events with Flt-1 binding and activation has given rise to the argument that this protein primarily acts as a “decoy receptor” that can bind VEGF--acting as a VEGF-trap to modulate KDR function.

Inhibitory role of Flt-1 in angiogenesis. Several lines of evidence in the available literature suggest Flt-1 may play primarily a negative role in angiogenesis. This theory is based on early studies that attempted to determine how a targeted deletion of the receptor in developing mouse embryos would affect their development. Mice lacking the complete Flt-1 gene die *in utero* at approximately embryonic day 9, and there were noted phenotypic abnormalities that caused these problems. The blood vessels in Flt-1^{-/-} mice were disorganized, and endothelial-like cells often overgrew the lumens of the developing vasculature [74]. The theory of a negative role for Flt-1 in angiogenesis was also bolstered by a report by Hiratsuka et al., (1998) in which the kinase region of the Flt-1 gene was deleted, resulting in Flt-1 receptors which lacked the intracellular tyrosine kinase domains but were still able to bind VEGF. Surprisingly, these mutations but did not result in embryonic lethality or any noticeable defects in vascular development of mice [75]. Taken together, these studies reinforced the idea that Flt-1 plays an inhibitory role in angiogenesis. The hypothesis is that Flt-1 is indirectly involved in VEGF binding and activation of KDR. Essentially, VEGF binds to Flt-1, whereby it acts as a “trap” to modulate KDR function—Flt-1 plays a negative role by suppressing pro-angiogenic signals in the early embryo to establish a balance essential for physiological angiogenesis.

Clearly, Flt-1's role in physiological events is not limited to its VEGF-trapping function in angiogenesis. There also has been evidence of Flt-1 activity in non-endothelial cells. The migration of monocytes in response to VEGF, for example, requires the tyrosine kinase domain of Flt-1 [75]. More recently, Flt-1 signaling has been linked to the induction of matrix metalloproteinase-9 in lung endothelial cells—a process that facilitated the metastasis of tumor cells [76]. These results, along with the phenotypic changes occurring in knockout mice, indicate a different role for Flt-1 than for KDR. In particular, recent studies have emphasized that Flt-1 is a complex receptor that, although it appears to have conflicting functions, undoubtedly plays important roles in hematopoiesis and recruitment of mononuclear cells [69, 77]. These conclusions are quite interesting considering that most of focus has been placed on the downstream effects of VEGF binding and activation of KDR.

KDR intracellular signaling. The body of literature devoted to KDR signaling is much greater and there has been considerable headway made in determining the events secondary to its activation with VEGF. Upon binding of VEGF-A, the KDR dimer is autophosphorylated at the kinase-insert region of the carboxyl terminal intracellular domain [78]. In human KDR, several tyrosine residues become phosphorylated including: 951, 1054, 1059, 1175, and 1214 (Figure 2). The phosphorylation sites in the second kinase domain of KDR (Y1054 and Y1059) have been implicated in positive regulation of the kinase (Table 1) [78-80]. Additionally, Y1305, Y1309, and Y1319 of the C-terminal tail are thought to be minor phosphorylation sites whose functional roles have yet to be elucidated [81]. One of the most important residues that becomes phosphorylated on KDR is Y1175, which serves as the binding site for the Src-homology 2 (SH2) binding domain of phospholipase C γ 1 (PLC γ 1) [79]. When PLC γ 1 associates with the phosphorylated KDR receptor at this residue, it is in turn tyrosine phosphorylated leading to the stimulation of the phosphatidylinositol-specific phospholipase activity. This leads to inositol trisphosphate (IP₃) generation, intracellular calcium mobilization, and protein kinase C (PKC) activation. In turn, the activated PKC leads to stimulation of the c-Raf-MEK-MAP-kinase cascade resulting in DNA synthesis and gene transcription [79].

These findings strongly suggest that autophosphorylation of Y1175 on KDR is crucial for endothelial cell proliferation. Interestingly, Y1175 on KDR serves a dual-role in that it also constitutes a binding site for Shb, a signaling adaptor which has been implicated in activation of phosphatidylinositol 3-kinase (PI3-kinase), downstream of the receptor. In this way, KDR can also regulate VEGF-induced formation of focal adhesions and cell migration [82]. This is not the only pathway through which cell motility is affected. Another important KDR site is the Y951 residue in the kinase-insert region. Phosphorylation of Y951 allows binding and tyrosine phosphorylation of the signaling molecule, T cell specific adapter (TSA_d), which also employs a SH2 binding domain [81]. This protein then binds the cytoplasmic tyrosine kinase, Src, which ultimately contributes to the regulation of actin fiber organization and migratory responses of endothelial cells [81].

The role of Y1214 phosphorylation has only recently started to be determined. It had previously been established that another major signaling pathway that is activated by VEGF binding to KDR is the stress-activated protein kinase (SAPK)2/p38 mitogen-activated protein kinases (MAP kinases). Prior to the most recent study by Lamalice et al., (2004) the relevant tyrosine residues on KDR, as well as the adapter molecules that couple the receptor to SAPK2/p38, were unknown. In this study, the expression of a dominant-negative form of Cdc42 (Cdc42 N17) was used to inhibit the activation of SAPK2/p38 in response to VEGF-A binding to KDR, thus providing some evidence of the upstream adaptor protein that links the phosphorylated receptor and the intracellular signaling pathway. The necessary tyrosine residue on KDR was subsequently identified with a site-specific mutant of Y1214 (a nonphosphorylatable residue), which inhibited the activation of both Cdc42 and SAPK2/p38 in response to VEGF. So, it was concluded that phosphorylation of Y1214 on KDR is required to trigger the series of events that aid in the cytoskeletal remodeling that is required to drive actin-based motility as part of the angiogenic process [83].

Flt-1 and KDR dimerization. Because Flt-1 and KDR are synthesized as monomers, and there is significant structural similarity between species, there exists the possibility that receptors can form heterodimers [84]. Thus far, truncated, soluble forms of both receptors have been shown to heterodimerize with full-length Flt-1, documented

as a ~200-kDa molecule that coimmunoprecipitated with Flt1 in endothelial cells transfected with both receptors [85]. Prior to an investigation by Autiero et al. (2003), evidence for the presence, function and ligand-dependent regulation of Flk1/Flt1 heterodimers in nontransfected endothelial cells was lacking, however [86]. In this study, Flt-1 and KDR were found to spontaneously heterodimerize in the absence of any ligand, and heterodimerization was increased in the presence of both VEGF/PlGF heterodimers and VEGF homodimers. The VEGF/PlGF ligand dimer increased heterodimerization of the receptors in a significant manner, but the biologic relevance of these heterodimers has yet to be elucidated. Could the heterodimers simply be another source of biologically inactive receptor species, thereby adding another moiety to the ligand “sink”, or is there a biologically relevant signaling function that is different than what occurs for ligand-bound homodimers of each respective receptor? These questions have yet to be clarified.

Differences in ligand binding affinities for Flt-1 and KDR

Not only are the cellular responses different for the two receptor types, but the two receptors bind ligands of the VEGF family with different affinities. The literature reports that Flt-1 binds VEGF-A with a K_D ranging from of 9 -130 pM, whereas the K_D for KDR binding has been measured over a higher range, from ~37-900 pM [25, 67, 87]. A higher K_D indicates a lower affinity for the ligand. These values vary depending on the type of binding experiments performed to measure the respective ligand binding. For example, in one case, the reported K_D values for VEGF binding to Flt-1 and KDR on colonic human microvascular endothelial cells were 130 and 675 pM, respectively [88]. Whether measured on specific receptor expressing cells or in a cell-free environment, the trends are generally the same for VEGF-A binding—it binds to Flt-1 with an average affinity that is an order of magnitude greater than KDR binding VEGF-A. This has the potential to greatly affect the activation of KDR. If VEGF is being sequestered by Flt-1, it may not be available in sufficient quantities for the binding and activation of KDR. This effect will be discussed in greater detail in ensuing chapters.

As stated previously, Flt-1 and KDR also have different ligand selectivities (Figure 1). Flt-1 interacts with VEGF-A and with two other members of the VEGF

family, PlGF and VEGF-B [55]. Direct measurements of the binding affinities for these ligands have been made, though they are not commonly found in the existing literature. Park et al. (1994) measured the K_D of PlGF in human umbilical vein endothelial cells (HUVECs) and determined that it fell within a similar range as the binding affinity of VEGF-A for these cells. The value of 230 pM lies within the same range as VEGF-A binding to Flt-1 and is within an order of magnitude for previously measured K_D values [54]. Another interesting observation made in the same study was that PlGF was able to compete with ^{125}I -VEGF₁₆₅ for binding to Flt-1 IgG but was unable to displace ^{125}I -VEGF bound to KDR IgG. This provides further evidence for the specificity of PlGF binding to Flt-1 [54]. Because the affinity of this ligand is so similar for Flt-1, it has the potential to compete for open binding sites on the cell surface.

Flt-1 binding PlGF. Depending on the concentration that is present, PlGF has the potential to completely inhibit VEGF-A binding to Flt-1. This can have major effects on the “ligand sink” function proposed for Flt-1 and VEGF-A. The likelihood of redistributing ligands from receptor to receptor has not been fully explored in cell-based experiments. Park et al. (1994) were among the first to probe these ligand-shifting effects, and they were only able to show that radiolabeled PlGF effectively competed with radiolabeled VEGF from Flt-1 binding assays. In another study by Carmeliet et al. (2001), the authors further investigated the interplay between PlGF and VEGF-A through *in vivo* studies [89]. To examine the role of PlGF in angiogenesis, they inactivated the gene expressing PlGF in mice. Interestingly, while the absence of PlGF had a negligible effect on vascular development, its deficiency reduced pathological angiogenesis, permeability and collateral growth in ischemia, inflammation and cancer. Not even exogenous supplementation with VEGF-B was able to rescue the mice from these effects—the pathological effects remained suppressed. The authors concluded that a unique synergism exists between PlGF and VEGF in pathological angiogenesis, whereby PlGF and Flt-1 are upregulated in endothelial cells. Because of the possibility for PlGF to shift VEGF-A to KDR, the net effect would be to amplify the responsiveness to VEGF in many pathological disorders. Their proposed “angiogenic switch” may have therapeutic implications for either stimulating or inhibiting angiogenesis [89].

Flt-1 binding VEGF-B. Though it is sometimes thought to mimic the action of PlGF, initial binding studies of VEGF-B indicate that it cannot play the same role as the other Flt-1 specific ligand. Measured K_D values for VEGF-B are not widely available in the literature. Scotney et al. (2002), using surface plasmon resonance, determined that two naturally occurring splice isoforms (VEGF-B₁₆₇ and VEGF-B₁₈₆) and one mutant isoform of VEGF-B₁₀₋₁₀₈—all differing in length but still retaining receptor binding regions—had higher K_D values than that of VEGF-A binding to Flt-1. They observed K_D values ranging from 0.8-2.0 nM for VEGF-B binding to Flt-1 while their measured values for VEGF-A binding were 10 pM [90]. It was proposed that several residues located near critical binding areas in VEGF-B differ from VEGF-A, and PlGF, in such a way they change the structural flexibility of the ligand and lead to less affinity for Flt-1 [91]. Of all the ligands that are capable of binding to Flt-1, VEGF-B is the least studied. It is not clear whether VEGF-B binding could potentially have the same effect as altering the balance of the ligand sink. Based solely upon the scarcity of data available for its binding to Flt-1, it is unlikely that the effects would be as dramatic, or it would have to be present in vastly larger quantities to elicit the same response.

KDR binding VEGF-E. Just as Flt-1 has ligands that are specific to that receptor, KDR interacts selectively with VEGF-E in addition to its normal binding of VEGF-A [63, 64]. VEGF-E binds to KDR but not Flt-1 [63, 92]. While VEGF-E family members share only 20 to 25% amino acid identity with VEGF-A, the K_D for VEGF-E/KDR is almost the same as that of VEGF₁₆₅ for KDR. A Scatchard analysis, performed by Ogawa et al. (1998) [25] of VEGF-E binding to KDR-expressing NIH3T3 cells indicated that the K_D for the binding of VEGF-E to KDR was about 330 pM [49]. This measurement is higher than some literature values gathered for VEGF-A binding to KDR, but it should be noted that the K_D of VEGF-A for KDR as measured in this same study, for the same cell type, was approximately 300 pM. These results indicate that VEGF-E binds to KDR with the same affinity as VEGF-A, making it an important ligand that is able to compete for KDR occupancy.

Soluble Flt-1 (sFlt-1), an endogenous VEGF antagonist

While the concept that Flt-1 acts to sequester VEGF is widely accepted, the physiological consequences of this was not well understood. For this reason, Hiratsuka et al. (2005) attempted to demonstrate how Flt-1 sequesters VEGF at the cell surface. To do this, the transmembrane (TM) domain of Flt-1 was deleted in mice that were also tyrosine-kinase (TK) deficient in Flt-1 [93]. These were similar to the mice used in previous studies performed by this group to determining the functional value of Flt-1 in the delivering VEGF to KDR for signaling. When the transmembrane domain was deleted, surprisingly about half of the Flt-1 (TM-TK)-deficient mice succumbed *in utero* due to a poor development of blood vessels—all other mice were healthy. Another interesting observation was that in Flt-1(TM-TK)^{-/-} mice, VEGF associated with the cell membrane was reduced. With the reduction of VEGF available at the cell surface, there was a decrease in KDR phosphorylation—an indicator of binding and activation for this receptor.

In a last experiment performed, the normal expression of KDR in the Flt-1 (TM-TK)^{-/-} mice was altered from homozygous (+/+) for KDR to heterozygous (+/-). In this case, the incidence of embryonic lethality was significantly increased to 80-90% from the previous 50% [93]. The conclusions drawn from these experiments were that transmembrane region of Flt-1 is necessary for anchoring the receptor in order that the ligand-binding capabilities of Flt-1 can trap VEGF and deliver it to KDR. The researchers determined that this is necessary for the regulation of VEGF signaling in vascular endothelial cells during early embryogenesis. These conclusions are well-founded, but this recent publication highlighted another important component in the regulation of physiologic angiogenesis—the potential role of the soluble form of Flt-1 (sFlt-1).

It is a commonly held belief that tumors must become vascularized if they are to grow larger than a certain size, and that their metastatic potential hinges on the development of new blood vessels feeding those tumors [94]. It follows that anti-angiogenic therapies for cancer has become a major focus in cancer research. Recent studies have revealed that inhibition of VEGF activity by neutralizing antibodies or by transfection of dominant negative VEGF receptors into the endothelium of supporting tumor blood vessels resulted in the inhibition of tumor growth and even tumor

regression [6]. Findings like these have triggered efforts to design drugs capable of interfering with VEGF signaling and various endogenous inhibitors of angiogenesis have been characterized.

One of the most potent inhibitors identified to date is the naturally-occurring, secreted, soluble form of Flt-1, sFlt-1. This receptor variant is derived from Flt-1 RNA by alternative splicing and retains the 1st-6th Ig domains of full-length Flt-1 receptors—the necessary loop for ligand binding, loop 2, is retained in this soluble receptor, and a unique C-terminus replaces the transmembrane and intracellular regions (Figure 1). The product is a heparin-binding protein that complexes VEGF with the same high affinity and presumably equivalent specificity of full-length Flt-1. It is expressed by vascular endothelial cells and can inhibit their mitogenic response to VEGF in culture by directly sequestering VEGF. In 1990, Shibuya et al. reported that human placenta expresses large amounts of sFlt-1 [41]. Although sFlt-1 is expressed preferentially in the placenta in mammals, it is also conserved in other species. For example, the avian genome encodes full-length Flt-1—therefore also sFlt-1 [95]. It is not only expressed, but when compared to the sequence of mammalian sFlt-1, a large portion of the carboxyl terminal in the soluble receptor is identical—approximately 74% identity between human and chicken. The six Ig loops are approximately 65% identical. The simple fact that it is so highly conserved across species suggests the importance of this gene product in physiologic angiogenesis.

Soluble Flt-1 in pathophysiologic conditions. While its importance in normal physiology has been demonstrated, abnormal regulation of sFlt-1 levels can likewise lead to pathophysiologic conditions. A recent study by Koga et al., (2003) in observed abnormally high levels of sFlt-1 in serum from preeclamptic patients [96]. In a similar study published the following year, it was reported that sFlt-1 was already being overexpressed in the middle of pregnancy in preeclamptic patients prior to the onset of clinical symptoms. Furthermore, the actual level of sFlt-1 in maternal serum was strongly correlated to the degree of preeclampsia present in the pregnant patients [97]. Collectively, these results suggest that preeclamptic symptoms on the maternal side of the placenta are due to an abnormal suppression of endogenous VEGF-A by this anti-angiogenic factor.

There have been attempts made to determine the extent to which sFlt-1 inhibits responses to VEGF. Many of these studies have involved the addition of exogenous sFlt-1 at various concentrations then subsequently looking at DNA synthesis or inhibited growth of endothelial cells [98-100]. Chen et al. (2000) investigated whether sFlt-1, derived from conditioned medium of transfected human embryonic kidney (HEK)-293 cells, could inhibit VEGF-induced angiogenesis in HUVECs. Serum-starved HUVECs were exposed to sFlt-1 in conditioned media in the presence or absence of VEGF. DNA synthesis (their primary marker of cell proliferation) was measured by the incorporation of [³H]thymidine into cultured HUVECs [100]. The result of this experiment was an observed increase in DNA synthesis of HUVECs induced by VEGF and a significant suppression of this proliferation by sFlt-1 as well as a neutralizing anti-VEGF antibody. This suppression was dose-dependent, and sFlt-1 treatment alone did not effect cell proliferation at all [100]. The results of this experiment were useful for demonstrating that VEGF-induced DNA synthesis of HUVECs could effectively be blocked by sFlt-1; however, the conclusions that one can draw from it are somewhat limited in that they do not demonstrate how sFlt directly affects vessel development or the acute signaling events that occur in response to VEGF binding. More experiments are needed to address the ultimate question of the role sFlt-1 plays in the actual events of angiogenesis.

sFlt-1 in physiologic avascularity. In a recent study by Ambati et al. (2006), it was demonstrated that sFlt-1 is essential for preserving the avascular nature of the mouse cornea by specifically sequestering VEGF-A [101]. Their model, which has historically been used for studies of vascular development, is ideal for understanding the ability of tissues to control vascular ingrowth and further to investigate how sFlt-1 preserves corneal avascularity in mice. One strategy the authors employed was to inject a neutralizing antibody against Flt-1 into one cornea of the test mice, while at the same time injecting an isotype control antibody in the other eye. This theoretically had the effect of neutralizing sFlt-1 and thereby inhibiting its binding VEGF-A in the eyes. They then observed the amount of vascular ingrowth in the corneas of the mice. They found that the eyes that were treated with the Flt-1-neutralizing antibody consistently developed corneal vascularization within 1 day of treatment, while the other eyes that

served as controls did not develop new vessel growth at all. Interestingly, the corneas treated with anti-Flt-1 antibodies contained more free VEGF-A, than did control-treated corneas [101]. One possible conclusion that can be drawn from these observations is that the VEGF-A promoting vascular ingrowth is normally sequestered by sFlt-1 to such an extent that there is a significant decrease in the growth of new blood vessels in the corneas. Another approach that the authors employed was the targeted knockdown of sFlt-1 expression in mouse corneas using RNA interference (RNAi). They injected plasmids into the corneas that expressed a short hairpin RNA that would target a sequence in the sFlt-1 mRNA. They found that sFlt-1 mRNA and protein was significantly reduced in the corneas of test animals (compared to control plasmid injections), indicating that knockdown was achieved through RNAi. With the knockdown, there was a corresponding increase in free VEGF-A in the corneas. These results further support the hypothesis that sFlt-1 sequesters VEGF-A in such a way to maintain physiological avascularity [101].

A similar approach was taken by Machens et al. (2003) to address the question of sFlt-1's role in functional angiogenesis. This study aimed to examine the angiogenic effects of VEGF₁₆₅ when VEGF₁₆₅ and sFlt-1 were employed in different experimental settings of an ischemic rat flap model [99]. The flap model was used because it allowed for VEGF-mediated mechanisms that could account for the improvement of wound healing and flap survival to be studied—the efficient rescue of the flap tissue from necrosis might imply that angiogenesis occurred. Ultimately, the most vital flap tissue of all the treatment groups was observed in those test animals that received exogenous injections of VEGF₁₆₅ at the wound site. Those animals receiving injections of VEGF₁₆₅ and sFlt-1 at the wound sites showed a significantly lower percentage of flap tissue maintained. The angiogenic events occurring *in vivo* were significantly altered when sFlt-1 was exogenously injected at the wound site [99]. This supports the theory that sFlt-1, in high enough levels, is capable binding available VEGF and preventing the normal angiogenic actions of this growth factor.

Dimerization of sFlt-1 with membrane receptors. The conclusions drawn in much of the literature is that sFlt-1 is inhibiting VEGF action by simply binding and sequestering free VEGF from the overall pool of available growth factor. Another

possible mode of inhibition by sFlt-1 is the formation of inactive heterodimers with KDR or Flt-1 [84]. DiSalvo et al. (1995) successfully demonstrated that PlGF could exist in a heterodimeric form with VEGF, and this VEGF/PlGF ligand, when purified, was able to elicit mitogenic responses in HUVE cells [102]. This discovery preceded the discovery of a VEGF-A/VEGF-B heterodimer ligand by Olofsson et al. (1996) [33]. With the discovery of naturally-occurring ligand heterodimers, it was reasonable to assume that the membrane-bound receptors could also heterodimerize. Kendall et al. (1996) demonstrated that sFlt-1, produced by HUVE cells in culture, formed VEGF-stabilized ternary complexes with KDR. To test their hypothesis human sFlt-1 was incubated, along with human recombinant VEGF₁₆₅ and the purified extracellular region of KDR, and a chemical crosslinker was added to the incubation [84]. Western blotting was performed with these samples and it was determined that, in the presence of sFlt-1 and VEGF, KDR had successfully formed complexes with VEGF and sFlt-1. It appears that heterodimeric sFlt-1/KDR complexes are stabilized by VEGF and only a small amount of heterodimers are observed when VEGF is absent [84].

This is an important factor when considering the extent to which sFlt-1 may act in a dominant-negative role. If the formation of heterodimers between membrane-bound receptors and sFlt-1 is VEGF-dependent, this could dramatically alter its heterodimeric, inhibitory role. It could be limited to forming these heterodimers only when it can diffuse to the cell surface and bind to the receptors with VEGF. If VEGF is also diffusing to the cell surface, it could be successfully binding to receptor homodimers and activating signaling events. If it were not dependent on the presence of VEGF, then the “pool” of available receptor homodimers could be significantly reduced. This observation is of particular importance when trying to model the interactions of VEGF with the Flt-1 or KDR. One of the limitations of the current literature is that this lone study by Kendall et al. (1996) is the only one to examine the possibility of sFlt-1 forming heterodimers with cell-based receptors.

Soluble Flt-1 as a therapeutic agent

Results like those reviewed above have led to sFlt-1 being proposed as a anti-angiogenic agent, by virtue of its ability to alter the availability of VEGF released from

tumors [98]. It is possible that a change in sFlt-1 expression could influence the overall vascularization of tumors—thus altering tumor growth. Goldman et al. (1998) were among the first to identify the usefulness of sFlt-1 to suppress tumor growth. In their study, VEGF-dependent tumor growth was selectively blocked in HT-1080 fibrosarcoma cells) by stably transfecting them with cDNA encoding sFlt-1. HT-1080 transfectants that expressed higher levels of sFlt-1 grew very slow as xenograft tumors in the mice. Furthermore, the ability of the cells to metastasize and implant in other areas of the mice (e.g., the lungs) was significantly inhibited. In the same study, when mice were administered intracranial injections of human glioblastoma cells stably transfecting with sFlt-1 cDNA, the mice demonstrated significantly longer survival times than mice injected with non-transfected controls. Brain tumor size could not be readily measured as a function of time, but the authors concluded that sFlt-1 cannot only inhibit tumor growth and metastatic implantation but may also increase host survival when challenged with cancerous cells [98].

Therapeutic agents similar to sFlt-1. The results of Goldman et al. (1998) are interesting because they not only support the targeting of VEGF receptors antiangiogenic therapies, but they also indicate that sFlt-1 might be employed as a feasible approach for inhibiting tumor angiogenesis and growth. Studies like these, that demonstrate the usefulness of soluble receptor decoys, laid the groundwork for the development of other therapeutics based on similar modes of action as sFlt-1. In December 2004 the first agent targeting angiogenesis, bevacizumab (Avastin®; Genentech, Inc., South San Francisco, CA), was approved to be given intravenously as a combination treatment along with standard chemotherapy drugs for metastatic colorectal cancer. Avastin is a humanized, mouse monoclonal antibody that binds VEGF thereby decreasing its availability for receptor binding and inhibiting angiogenesis and tumor growth. Although it is not a soluble receptor, it still acts to target VEGF and, in combination with other chemotherapy drugs, it has caused increasing response rates, progression-free survival, and overall survival of patients, with limited toxicity due to Avastin treatment [103]. Currently in clinical trials is a novel new therapeutic protein, called VEGF Trap, which works in a similar fashion as sFlt-1 in that it binds VEGF and its related ligands [104]. It was engineered as a fully human soluble decoy receptor that

consists of a fusion of the second Ig domain of human Flt-1 and the third Ig domain of human KDR with the constant region (Fc) of human Ig IgG1. Because it is a chimeric protein, it demonstrates advantages of both receptor types and retains a very high affinity for all isoforms of VEGF-A, as well as PlGF [104, 105]. By forming stable, inert complexes with VEGF, VEGF Trap shows great anti-tumor potential [106].

Current gaps in sFlt-1 literature. There is presently little information on the measurement of events that happen in response to VEGF signaling. There have been no studies to date that have directly measured the level of VEGF activation achieved by Flt-1 or KDR in the presence of sFlt-1 as an antagonist. Most of the published studies have probed events that happen long after the binding of VEGF (e.g., cell proliferation), but none have attempted to directly quantify the ligand-induced phosphorylation of Flt-1 or KDR in the presence of sFlt-1. An investigation like this would be important because it would directly measure receptor activation in the presence of a potent antagonist. Further, perturbations in downstream intracellular signaling events by sFlt-1 have not been investigated. To what extent does sFlt-1 act to sequester VEGF and/or inhibit receptor activation? *In vitro* experiments that attempt to directly measure receptor activation in the presence of sFlt-1 would be novel.

Early attempts at computational models of VEGF binding

The potential for using VEGF or anti-VEGF therapies to treat human diseases is an exciting one. In order to effectively understand these potentials, there must be an improved understanding of the mechanisms of VEGF binding and activation and the signal transduction pathways that mediate VEGF's complex biological functions. When examining inhibition of ligand binding due to the presence of competing soluble receptors, mathematical modeling can provide a quantitative examination of these processes. The solutions to these models can aid in interpretation of experimental observations and objectively suggest a direction for further experimental studies as well as and possible application to human disease states.

First VEGF modeling attempt. To date, there have been few models that have attempted to simulate VEGF binding in a multiple receptor state *in silico*. These originate primarily from a single group of researchers at the School of Medicine at Johns

Hopkins University. The first model by Mac Gabhann & Popel (2004), focused primarily on how PlGF competed with VEGF for binding to Flt-1 and KDR and the resultant VEGF-PlGF synergy that is proposed to be established between these two ligands. For the model, a set of coupled reaction-diffusion equations were constructed which described the secretion, transport, binding, and internalization of ligands in a multiple-ligand, multiple-receptor situation specific to the VEGF family and its receptors [107]. The primary receptors and ligands to be investigated were VEGF-A, PlGF, Flt-1 and KDR with the endpoint being predicted concentrations of ligand-receptor complexes formed on the surface of endothelial cells [107]. In their model, the authors compared the following two cases: (1) when exogenous VEGF was added without PlGF and (2) when exogenous VEGF and PlGF were added together. Ultimately, they aimed to simulate and quantify the ligand-shifting effect of PlGF—the theory originally introduced by Park et al. (1994) in which VEGF is displaced from Flt-1 to KDR by active binding of PlGF.

The computational simulations, carried out for ~24 hours, predicted the formation of Flt-1 and KDR complexed with growth factors in the presence of both ligands. Further, they observed a predicted increase in the formation of endothelial surface growth factor-Flt-1 complexes in the presence of PlGF. This was coupled with a less-dramatic increase in the number of KDR signaling complexes—indicating that VEGF-A was not effectively being “shifted” to KDR when PlGF was present in the system. It was, therefore, their conclusion that increases in angiogenic events, observed in an earlier experiment by Carmeliet et al. (2001) [89] appear to be less likely due to VEGF displacement to KDR but to an increase in the total Flt-1 signaling complexes. This is a curious conclusion, considering the lack of evidence of Flt-1 as a primary signaling receptor in the angiogenic process. In any case, Mac Gabhann & Popel (2004) were successful in developing a model that simulated VEGF binding to these membrane-bound receptors.

Only in an appendix of this study were the antagonistic effects of sFlt-1 explored. The authors included this moiety as a volumetric species and the simulations suggested that, at reported physiologic levels, the concentration of sFlt-1 in the medium would cause only a slight increase in the KDR complex formation and no significant change in

Flt-1 complex formation. The authors concluded that it is unlikely that cells are secreting sFlt-1 at the rates required to build up to a concentration that would significantly affect complex formation. This conclusion is at variance with the considerable evidence that sFlt-1 occurs naturally at levels sufficient to inhibit VEGF binding.

What then is the reason for the differences in the model predictions and experimental evidence? One limitation of this first model of VEGF binding may be the source of quantitative parameters used for modeling the binding processes. The existing experimental literature was searched for specific or representative values for the model parameters, but no values were experimentally obtained by the authors. The fidelity of the model is contingent on the use of correct model parameters, and inference of these values can lead to incorrect predictions in complex formation over time. This was confirmed in the sensitivity analysis that was performed with their values. A mathematical model that attempts to simulate observations of cell-based experiments, and is predicated on kinetic values obtained experimentally in those same cells, would add internal consistency to the modeling process. It could give a truer picture of the accuracy one can attain in modeling this or any system.

Subsequent modeling attempts of VEGF binding. Since the introduction of this original model, Mac Gabhann & Popel (2005) extended their previous model of VEGF binding to include NRP1 and its coupling to KDR. As before, all the parameters for the model were obtained from the literature, and the new coupling rate was inferred from previous diffusion-limited rate constants developed for bFGF and interleukin-2 receptors [108]. Additionally, they simulated the inclusion of VEGF-A₁₂₁, which has been shown to bind to KDR with a similar affinity of VEGF-A₁₆₅. More recently, this notion of receptor coupling was used to further expand the model with the inclusion of coupling rates for homo- and hetero-dimers of Flt-1 and KDR. Because the dominant mechanism of receptor dimerization has not yet been determined, it is possible that VEGF receptors may be present in an inactive pre-dimerized form or as monomeric moieties that, upon ligand binding, become dimerized when the two independent species diffuse and bind to available unligated receptor monomers. Both processes were included, and the impact of this dimerization mechanism on the binding of VEGF to the cell surface and on the formation of active signaling receptor complexes was

demonstrated [109]. Again, the authors focused primarily on cell surface receptors and neglected the possibility of sFlt-1 dimerizing with these receptors. As previously stated, this interaction has the potential to significantly inhibit Flt-1 and KDR complex formation and warrants inclusion in a model of sFlt-1 inhibition.

Gaps in VEGF modeling literature. An accurate model of VEGF binding is a necessary step toward a quantitative description, at a molecular level, of VEGF binding that can be extended to *in vivo* situations. Such a model would have applications in further research with pro-angiogenic, anti-angiogenic, or anti-tumorigenic therapies, as well as in tissue engineering which often employs growth factor stimulation in culture. With the inherent complexity of this biological system—containing multiple ligands and receptors—there must be a method developed to understand the interactions among these components. The current VEGF modeling literature contains significant gaps that can be addressed with a new modeling attempt. First there have been no attempts made to model binding and signaling events that occur specifically with Flt-1 and KDR-expressing cells, based on the determination of kinetic parameters using those same cells. It stands to reason that the *in silico* predictions made by a model, in this case, would more closely resemble experimental data obtained for *in vitro* experiments. If the model simulations and the experimental data do not qualitatively resemble one another, in this case, it would provide greater evidence of the need for alterations in the current model setup. The inhibition of VEGF binding by sFlt-1 is a second area that needs to be studied in greater detail. Only one previous attempt was made to incorporate this important molecule in a VEGF model, and the predictions made with regard to its mechanism of action were not informative. With the available literature repeatedly citing the importance of this molecule in the angiogenic process, sFlt-1 inhibition needs to be modeled in much greater detail. Lastly, much emphasis has been placed on modeling PlGF and its role in the system of VEGF binding, but one of the more interesting ligands in this family of proteins that has yet to be included: VEGF-E. This member of the VEGF family induces significant angiogenesis *in vivo* with few side effects; therefore, it is being carefully studied as a candidate for pro-angiogenic therapies and other clinical uses. One method by which it has not yet been studied is via computational modeling. If a model including this ligand is developed and tested, it can be expanded upon to

include even more biologically active components of a larger system. It can be of great value for better understand what is occurring *in vivo*—saving resources and lessening the need for *in vitro* experimentation.

I hypothesize that a mathematical model can predict how sFlt-1 influences, in a biologically significant manner, signaling through VEGF receptors Flt-1 & KDR. To accomplish this, I aim to formulate a testable, mathematical model that incorporates multiple receptor and ligand states and ultimately mimics VEGF binding and activation that occurs *in vivo*. Because of the obvious limitations to *in vivo* experimentation in this regard, I will focus on determining experimentally how sFlt-1 alters binding of VEGF, activation of receptors, and subsequent signaling through KDR and then compare these results to the simulations produced by the model.

Site	Signaling Effect	Physiologic Effect	Reference
Y951	Binds adapter molecule, T-cell-specific adapter protein (TSAd) via Src homology 2 (SH2) domain	Actin reorganization and cell migration	[81]
Y1054	Regulation of the receptor tyrosine kinase	Positive regulatory site	[78, 110]
Y1059	Regulation of the receptor tyrosine kinase	Positive regulatory site	[110, 111]
Y1175	(1) Binding (via SH2 domain), phosphorylation, and activation of PLC- γ , (2) also binds adapter molecules Shb & Sck for activation of PI3-kinase	(1) Ca ²⁺ release from internal stores, (2) mitogenic signaling via the Raf-MEK-MAPK pathway, (3) Shb-dependent focal adhesion turnover and cell migration	[79, 80, 82]
Y1214	Binding and activation of Cdc42 and p38 mitogen-activation protein kinase	Actin reorganization and cell migration	[83]
Y1305	Minor phosphorylation site	yields phosphopeptide spots visible after prolonged VEGF exposure	[81]
Y1309	Minor phosphorylation site	yields phosphopeptide spots visible after prolonged VEGF exposure	[81]
Y1319	Minor phosphorylation site	yields phosphopeptide spots visible after prolonged VEGF exposure	[81]

Table 1. A listing of the major & minor tyrosine phosphorylation sites located on the intracellular, C-terminal portion of KDR. Also included are the signaling and physiologic effects of phosphorylation at these specific residues.

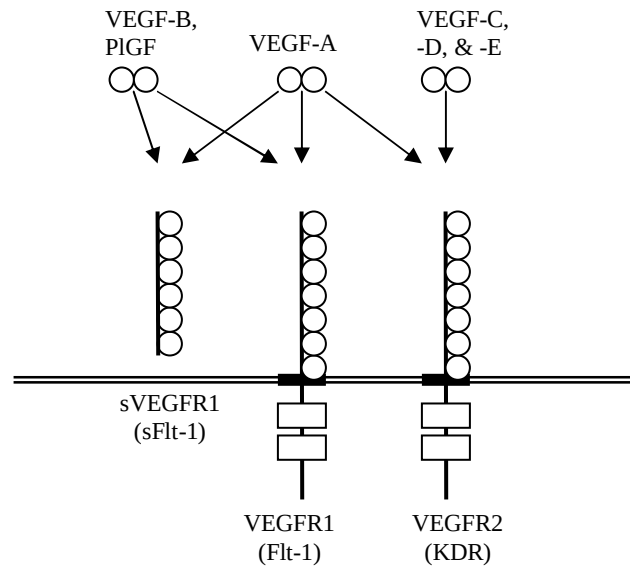


Figure 1. VEGF family of growth factors and primary VEGF receptors. VEGF-A, identified as the major growth factor contributing to angiogenesis, binds and activates two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR). Other ligands in this family of proteins can bind to the receptors. Soluble VEGFR-1 (sFlt-1) is also capable of binding VEGF-A and is thought to inhibit binding and signaling in the angiogenic process (Figure adapted from [112]).

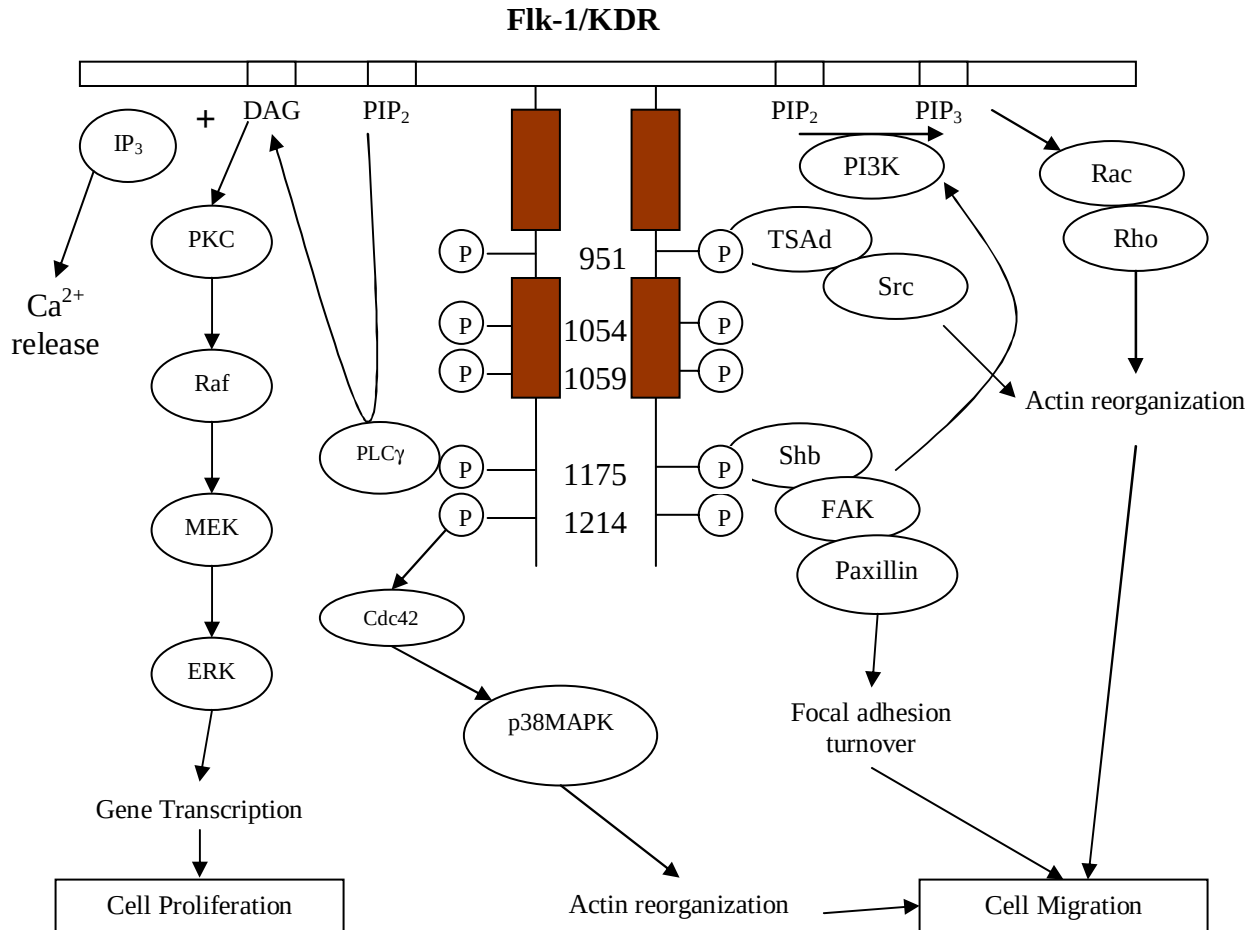


Figure 2. Schematic representation of KDR signaling in response to VEGF binding. The phosphorylation of key residues on the C-terminal region of the intracellular portion of KDR are necessary for the docking of adaptor proteins that trigger subsequent signaling pathways. The ultimate results of VEGF binding and signaling through KDR are cell proliferation and cell migration (Figure adapted from [113]).

CHAPTER 2: MODEL DEVELOPMENT

INTRODUCTION

A mathematical model for VEGF binding can include many different levels of detail. A full model could include all ligands of the VEGF family of proteins, multiple receptors, and the thermodynamic/kinetic parameters that define their interaction. It could also include the kinetic interactions regulating each of the individual trafficking processes (i.e., internalization, recycling, degradation, etc.) for the unoccupied receptors and complexed receptors—leading to a huge system with dozens of equations governed by numerous parameters. At the opposite end of the spectrum, a minimal model could include only one VEGF ligand binding to a single receptor. With the rate thermodynamic interactions that govern that singular event, the model would be quite small, and similarly uninformative. I have chosen to take an approach that would fall somewhere in the middle of the spectrum for modeling VEGF binding.

This computational model was originally developed to investigate the competitive inhibitory effects that sFlt-1 may have on Flt-1 ligand binding, KDR ligand binding, and any subsequent signaling processes that may occur. In addition, it was designed in such a way as to be compatible with *in vitro* experiments, providing a way of testing potentially interesting observations about the VEGF system, such as shifting effects that may take place in the presence of sFlt-1 and other ligands that may compete for receptors. Overall, this method of modeling is useful to understand how the kinetic properties of this receptor system influence ligand binding and receptor function. These parameters can be varied by wide margins *in silico* in ways that would be virtually impossible *in vitro*, due to time and resource limitations for experiments.

Originally developed as a way to simulate an *in vitro* environment, the simulated experiments all share a similar geometry. Basically, there are monolayers of cells, which express the respective receptor types, contacting a fluid layer containing serum and growth factors. These could be the wells of a multi-well plate or Petri dishes—it makes no difference. To start the simulations, various concentrations and combinations of volumetric species (ligands and sFlt-1 which occupy the entire fluid volume) are added to the media in contact with the cultured cells and, for the duration of the assay, the growth factors bind to the cell surface receptors or sFlt-1, from which they were able to be either re-released into the medium or internalized. All of these kinetic processes

were modeled. For the simulations, the predicted concentration of ligand-receptor complexes formed at the cell surface is of primary concern because it is then possible, with these solutions in hand, to interpret their relationship to the outcome of the experimental assays. This model is useful for describing the dynamic *in vitro* situations with the hope that it can be extended to even more complex, dynamic *in vivo* systems.

METHODS

Modeling Approach and Assumptions

The model consists of a system of 12 ordinary differential equations, based on mass action kinetics, which describe secretion, transport, binding, and internalization of receptors and VEGF in a multi-receptor situation. The model includes several other receptor-specific ligands that may be present in the system to get a more comprehensive picture of its overall behavior. This method for modeling receptor binding and internalization is adapted primarily from previously established methods that model binding and trafficking of receptor-ligand systems such as epidermal growth factor (EGF) [114-119]. The equations formulated, describing the change in species concentration with time, are conceptually similar to those formulated from earlier models [107, 119] but are extended to the VEGF binding kinetics model illustrated in Figure 3. In general, within the model, receptors are assumed to be produced at a specific rate by a cell source and added to a pre-existing population. Ligand is present in a volume of medium where it may reversibly bind free surface receptors with forward “on” rate and reverse “off” rate to form a receptor–ligand complex. The empty and occupied receptors are internalized based on characteristic rate constants and this act of internalization consumes the receptors and ligands. For the purpose of my general analysis, and for comparison-value to cell-based assays, the most important output of simulations will be the level of occupied receptors at the cell surface. One inherent assumption used in this modeling approach is that the dynamics of the system are inherently predictable given sufficient knowledge of the “state” of the system. This system is viewed as essentially deterministic in nature. This approach may leave out some aspects of the state of the system—such as position, orientation, and spatial

diffusion—that would require a stochastic approach to modeling the system dynamics. A stochastic approach would allow for greater understanding of the distribution associated with the time to extinction of species in the system (e.g., disappearance of free receptors), but the inference for stochastic models is at least an order of magnitude more difficult than inference for a deterministic model, in terms of the mathematics required, algorithm complexity, and computational time [120].

The main volumetric species represented in the model is VEGF-A, whose molar concentration is denoted as **Va**. Other growth factors used in the model are PlGF and VEGF-E; the concentrations of these ligands are denoted as **PI** and **Ve**, respectively. I assumed that the surface species (receptors and ligand-receptor complexes) Flt-1 (denoted **R1**) and KDR (denoted **R2**) are the only species capable of binding the growth factors at the cell surface. Further, it is assumed that, within the simulated experimental “wells” there is symmetry, such that the concentrations of ligands and receptors are spatially uniform throughout the plane of the cell surface. Other VEGF receptors (*i.e.*, neuropilin-1) [29, 121] or soluble extracellular matrix proteins normally present on and around endothelial cells are not included in this model. As previously stated, one of the goals in developing this model was to provide an *in silico* environment that may as closely as possible mimic experimental conditions. For example, I will be using transfected Human Embryonic Kidney (HEK) 293 cells (ATCC, Manassas, VA), for the determination of kinetic parameters and cell-based signaling experiments. For this reason, I attempted to include only those receptor subtypes that are present in these cells and would participate in the binding events of the VEGF system. There is little evidence available in the literature about the presence of other VEGF receptors (e.g., neuropilins, etc.) that may be naturally expressed, in high levels, in these cells. Without high basal expression levels of other receptors in my system, it follows that I elected to not include these receptors in the overall mathematical model—understanding that the model can easily be extended to include these, and other, types of membrane associated proteins that would bind and sequester VEGF at the cell surface.

In addition to the two cell-surface receptors, sFlt-1 (denoted **R3**) is the only moiety interacting with **Va** or **PI** in the extracellular space. Just as it is assumed that receptor concentration is uniform over the cell surface, it is also assumed that **R3** is

uniformly distributed throughout the cell medium. It is assumed that all receptors are dimerized and exist as homodimers—with sFlt-1 dimers forming in the extracellular space. Though there is experimental evidence supporting the phenomenon of heterodimerization [84], it is assumed that it does not occur in the absence of ligand [55]. Furthermore, preliminary data collected thus far has been inconclusive for determining if heterodimerization of sFlt-1 with cell surface receptors occurs on these cell types (see Chapter 5 for further discussion on dimerization). Any interactions with ligands and receptors are, therefore, approximated as one-to-one reactions. In this base model of receptor/ligand binding, VEGF binds and activates pre-dimerized receptors in a single step, and dissociates from those receptors in a single step with no further process modifying this interaction. With these constraints in mind, four surface complexes are included in the model: **C1**, formed from Flt-1 and VEGF-A; **C2**, formed from Flt-1 with PlGF; **C3**, KDR and VEGF-A; and **C4**, KDR and VEGF-E. Other complexes included are formed in the extracellular space: **C5**, sFlt-1 with VEGF-A; and **C6**, sFlt-1 and PlGF. One of the inherent assumptions in this model is a well-mixed assumption--neglecting of fluid phase ligand/soluble receptor diffusion. In essence, it is assumed that every ligand has an equivalent opportunity to bind with receptors—soluble or cellular—and no depletion zone is formed.

Because they have both of the necessary intracellular tyrosine kinase domains, it is assumed that only homodimeric Flt-1 complexes, **C1** & **C2**, and KDR complexes, **C3** & **C4**, can become activated and produce intracellular signaling cascades—for example, hydrolysis of phosphatidylinositol-1,4,5-trisphosphate (PIP_3) by $\text{PLC}\gamma$ upon binding VEGF isoforms [66, 67, 84]. The effect of heparan sulfate proteolysis, which has been noted to alter VEGF-binding to its receptors, is assumed to be included in the effective rate constants [122]. Predictive simulations with this model were carried out for very short time periods (15 minutes or less) and it is therefore, appropriate to assume that synthesis rates (V_{SR1} , V_{SR2} , & V_{SR3}) are essentially equivalent to internalization of unbound receptors. Terms describing these rates are included in the model, but the receptor number will not change enough to warrant inclusion of a separate, measured, rate constant.

The last term that is included in the model is receptor/complex internalization. After activation, receptor trafficking occurs in multiple steps including: endocytosis, endosomal sorting of receptors, receptor degradation through lysosomal targeting, and even receptor recycling back to the plasma membrane. While internalization can occur relatively quickly, there is normally a lapse in time while these receptors are sorted and either recycled to the cell surface for binding or degraded within the cell. Wiley & Cunningham (1982) performed binding studies for the EGF receptor and found that there was a significant chance that occupied EGF receptors would be internalized in 1 minute at physiological temperatures; however, after internalization, there was a delay of approximately 15 minutes before the initiation of degradation [123]. Degradation of internalized receptors will normally occur over time; however, the model is designed to simulate only the dynamic process of receptor activation, with the end-point of receptor phosphorylation. Because these processes occur quickly it is assumed that there is no degradation of receptors or ligand bound in complexes.

Elaboration of Rate Equations

The time evolutions of the receptors and complexes are represented by equations 1-9. The effective on rate and off rate constants for Va and $R1$ interactions ($M^{-1} \cdot min^{-1}$ and min^{-1}) are represented as k_1 and k_{-1} , respectively. The effective association and dissociation rate constants for Va and $R2$ interactions are represented as k_2 and k_{-2} , respectively. The on/off rates for Pl and $R1$ are represented as k_3 and k_{-3} , respectively, and the on/off rates for Ve and $R2$ are represented as k_4 and k_{-4} . The internalization rates (min^{-1}) for receptors and ligand-bound complexes are represented as the k_e values.

$$\frac{dR1}{dt} = V_{SR1} - k_{eR1}R1 + k_{-1}C1 - k_1VaR1 + k_{-3}C2 - k_3PlR1 \quad (1)$$

$$\frac{dR2}{dt} = V_{SR2} - k_{eR2}R2 + k_{-2}C3 - k_2VaR2 + k_{-4}C4 - k_4VeR2 \quad (2)$$

$$\frac{dR3}{dt} = V_{SR3} + k_{-1}C5 - k_1VaR3 + k_{-3}C6 - k_3PlR3 \quad (3)$$

$$\frac{dC1}{dt} = -k_{eC1}C1 + k_1VaR1 - k_{-1}C1 \quad (4)$$

$$\frac{dC2}{dt} = -k_{eC2}C2 + k_3P_L R1 - k_{-3}C2 \quad (5)$$

$$\frac{dC3}{dt} = -k_{eC3}C3 + k_2VaR2 - k_{-2}C3 \quad (6)$$

$$\frac{dC4}{dt} = -k_{eC4}C4 + k_4VeR2 - k_{-4}C4 \quad (7)$$

$$\frac{dC5}{dt} = k_1VaR3 - k_{-1}C5 \quad (8)$$

$$\frac{dC6}{dt} = k_3PlR3 - k_{-3}C6 \quad (9)$$

The evolution over time of the volumetric species concentrations (e.g., dV/dt) is represented by equations 10-12. Because one of the inherent assumptions in this model is a well-mixed assumption, the fluid phase diffusivities of ligand/soluble receptor are neglected. The equations include terms for $R3$, which is present in the fluid and can bind the ligands. The interactions with these volumetric moieties with cell-surface receptors necessitates additional terms not present in other time evolution equations.

Since ligand concentrations in the system are expressed in units of moles/L (M) and the receptors are expressed in sites/cell, it is necessary to include another term in front of each ODE describing the ligands in the system to preserve the units used in the model and those that will be used experimentally—it allows for the typical units for the association rate of $M^{-1} \cdot \text{min}^{-1}$ and K_D in M units. Therefore, equations for the each ligand have the term V (L/cell) * Na (Avagadro's number in sites/mol) in front of each ODE. Because the simulated assays are carried out for such a short duration, it is not necessary to include a term for ligand degradation in the medium.

$$VNa \times \frac{dVa}{dt} = [-k_1VaR1 + k_{-1}C1 - k_2C3 - k_1VaR3 + k_{-1}C5] \quad (10)$$

$$VNa \times \frac{dPl}{dt} = [-k_3PlR1 + k_{-3}C3 - k_3PlR3 + k_{-3}C6] \quad (11)$$

$$VNa \times \frac{dVe}{dt} = [-k_4VeR2 + k_{-4}C4] \quad (12)$$

The initial conditions for the simulations are listed in equation 13. The model was intended to help in understanding the inhibitory effects of sFlt-1 on binding and subsequent signaling of VEGF family members. For this reason there were many simulated conditions that included, but were not limited to: (1) where only Va is added to the system; (2) where both Va and $R3$ are added as volumetric species; (3) where Va , Pl , and $R3$ were added together; and (4) where all growth factors were added to the system together. The differences in predicted complex formation were compared between these cases. The numbers of receptors/cell used in the model were estimated from B_{max} values derived from competitive binding experiments (see below).

$$\begin{aligned}
Va(t = 0) &= Va_0 \\
Pl(t = 0) &= Pl_0 \\
Ve(t = 0) &= Ve_0 \\
R1(t = 0) &= R1_0 \\
R2(t = 0) &= R2_0 \\
R3(t = 0) &= R3_0 \\
C1(t = 0) &= C2(t = 0) = C3(t = 0) = C4(t = 0) = C5(t = 0) = C6(t = 0) = 0
\end{aligned} \tag{13}$$

Initially (time = 0), there are no preformed complexes $C1$ - $C6$, and the ligands are all added as a step change at this point. Recycling is not included in the model and synthesis of receptors is constant and based on steady-state measurements of surface levels of these receptors.

The equations were solved on a PC in Matlab version 7.5.0.342 R2007b (The Mathworks, Inc.) using ODE15S, a stiff differential equation solver, with a relative error tolerance of 1×10^{-3} and an absolute tolerance of 1×10^{-6} . Parameter values used are listed in Table 2. Every attempt was made to experimentally determine binding rate constants, and it should be noted that some experimentally determined constants were obtained at 4°C. This, in theory, eliminated membrane trafficking that would skew the rates. All simulations were carried out in experimental conditions that mimic physiological temperatures (37°C)—and some values were not available at these temperatures.

Transfection & expression of VEGF receptors

In order to generate a renewable source of the two transmembrane VEGF receptors, HEK293 cells previously transfected with the relevant expression constructs (Figure 4) were used. For Flt-1 expression, PCR-amplified cDNA encoding the full length transmembrane mouse Flt-1 protein was previously inserted into a vector (pcDNAIntA) containing the cytomegalovirus (CMV) intermediate-early promoter (Huckle and Roche, 2004); inclusion of the CMV-intron A permitted a higher level of expression (Goldman, 1998) [98]. The vector also contained a region encoding neomycin (neo) resistance for gentamicin (G418) selection. The vector, referred to as pFlt-1, has been shown to produce Flt-1 protein and mRNA when transiently transfected into HEK293 cells [124]. Prior to this investigation, pFlt-1 had been stably transfected into HEK293 cells using TransIT-293 (Mirus Inc., Madison, WI), and pools of cells resistant to 500 µg/ml G418 had been stored in liquid nitrogen. Here, clonal cell lines expressing Flt-1 were isolated from the pooled population by limiting dilution and screening colonies for Flt-1 protein by immunoblotting. In order to express KDR, an analogous expression plasmid was constructed in pcDNAIntA and transfected into HEK293 cells; a high-expressing clonal line, designated C57, was isolated previously (Huckle, unpublished). Pooled, G418-resistant HEK293 transfectants containing an empty pcDNAIntA vector served as control cells in experiments. All stable transfectants were maintained in T75 flasks in Dulbecco's Modified Eagle Medium containing 10% Fetal Bovine Serum and 50 µg/ml Gentamycin (DMEM-FBS-Gent) plus 500 µg/ml G418.

Western blotting for receptors

In order to test for the presence of each receptor type in the clonal cell lines, whole cell lysates were made by adding 1x Laemmli Sample Buffer (BioRad, Hercules, CA, USA) to cell monolayers, mechanically scraping the samples with a rigid plastic policeman, and heating the crude homogenates for 5 minutes at 95°C. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on whole cell lysates using the Criterion™ Precast Gel system (Bio-Rad). Thirty microliters of each sample were loaded into 8.7x13.3 cm precast 7.5% polyacrylamide gels

(8.7x13.3 cm; Criterion™ Precast Gels). ProSieve® color protein markers (Cambrex Bio Science, Rockland, Inc.) were also loaded, and the gels were run at 200 V until the dye front reached the bottom of the gel (approximately 55 minutes). Once completed, gels were equilibrated in 1x transfer buffer (25 mM Tris base, 192 mM glycine and 10% MeOH), and the fractionated proteins transferred electrophoretically to Immobilon-FL membranes (Millipore) 30 minutes at 100 with a cooling unit in place and the entire transfer cell submerged in ice-water. After the transfer was complete, the membranes were washed in 1x PBS for 5 minutes then agitated overnight at 4°C in 50 ml blocking buffer for near infrared fluorescent western blotting (Rockland, Gilbertsville, PA).

Following the blocking incubation, membranes were rinsed with 1x PBS for 5 minutes and then transferred to a clean container and incubated at room temperature for 1 hour with 0.1 µg/ml primary antibody in 0.5X blocking buffer in PBS containing 0.02% Tween-20. For Flt-1, the primary antibody was goat anti-mVEGFR-1 (R&D Systems, Minneapolis, MN), while KDR was probed with a mouse anti-KDR antibody (sc-6251, Cell Signaling Technology®). After the primary incubation, membranes were washed 4 times for 5 minutes/wash in PBS with 0.02% Tween-20 at room temperature with orbital shaking. The membranes were then incubated in the appropriate secondary antibody (IRDye800 Conjugated Affinity Purified Donkey Anti-goat or Anti-mouse IgG (H&L); Rockland®, 1:12,500 in blocking buffer supplemented with 0.02% Tween-20) for 1 hour at room temperature while shaking. The membranes were washed 4 times as above. Finally, membranes were scanned with an Odyssey® Infrared Imaging System (LI-COR, Lincoln, NE).

Determination of equilibrium dissociation constants

Attempts were made to experimentally obtain as many model parameters as possible. The on/off rates of ligands complexing with receptors were determined by radioligand binding assays, but first, the relative affinity of VEGF-A for Flt-1 and KDR were determined by measuring the equilibrium dissociation constant (K_D) for the ligand-receptor interaction. Protocols were modified from previously published methods that are well-established for these sorts of measurements [125-127]. Flt-1 and KDR over-expressing cells were grown in 24-well poly-L-lysine coated plates (BD BioCoat™, BD

Biosciences) to ~80% confluence ($\sim 8.0 \times 10^5$ – 1.0×10^6 cells/well; plated at $\sim 4 \times 10^5$ cells/1 ml DMEM-FBS-Gent/well). The cells were gently washed 2 x 1 mL PBS/BSA on ice to remove serum without dislodging cells from the plate. The PBS was removed from the wells and replaced with 0.4 ml/well ice-cold assay medium (Hank's Balanced Salt Solution plus 25mM Hepes, pH 7.5, and 0.1% BSA) containing [^{125} I]VEGF-A (~ 2000 Ci/mmol; $\sim 50,000$ cpm/well; 15-30 pM; Amersham). For homologous competition binding of VEGF, radioligand solutions were prepared containing four-fold serial dilutions (2.4 pM to 1 nM) non-labeled VEGF prior to application to the cells in triplicate. For the determination of non-specific binding (NSB), separate sets of three wells containing excess non-labeled VEGF (10 nM) were included. Additional wells were left with buffer only for determination of cell counts. The cells were incubated for 4 hrs at 4°C to allow binding to reach steady state. The binding incubation was terminated by removing assay medium containing radioligand and washing the cells rapidly with 2 x 1 mL ice-cold PBS/BSA to remove unbound radioligand. After washing, bound radioligand was solubilized by lysing the cells using 0.5 ml per well of 1% SDS/0.1 M NaOH. This lysate was transferred from the wells to 75 x 100mm polystyrene tubes followed by counting in a Cobra II Gamma Counter (Packard Instrument Company, IL, USA) [126].

Specific binding was determined by subtracting mean NSB cpm from total bound cpm at each concentration of competing ligand. Specific binding data were transformed and fit to linear mathematical expressions from which the K_D and maximal number of binding sites ($B_{\max}$) were extracted. The K_D values were determined by Scatchard analysis [118] and by non-linear regression using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). To the wells intended for cell counting, 0.25 mL of PBS containing 0.025% trypsin/0.22 mM EDTA was added and incubated for 10 minutes. Cells were dislodged and counted on a hemocytometer. For each separate binding experiment, cell counts were performed in triplicate and averaged for determinations of receptor density per cell.

Heterologous competition experiments were used to determine K_D values for the receptor-specific ligands PlGF and VEGF-E, using a protocol analogous to that described above. Dilutions of 2.4 pM to 9.5 nM VEGF-E (Cell Sciences, Inc., Canton,

MA) or PIGF (R&D rmPIGF-2, Minneapolis, MN) were applied; NSB was determined as above. The K_D values were determined by non-linear regression using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Determination of dissociation "off" rates

Dissociation binding experiments were used to measure the "off rate" for VEGF-A radioligand (the only commercially-available labeled ligand under study) dissociating from pre-occupied receptors. Initially, [^{125} I]VEGF-A allowed to bind Flt-1 and KDR-expressing cells for 4 hrs at 4°C as described above. After steady-state binding was established, the assay medium containing the radioligand was removed and the cells rapidly washed with 2 x 1 mL PBS/BSA at 4°C to remove non-bound ligand. Wash medium was then replaced with 37°C assay medium containing no radioligand and excess unlabeled VEGF-A at a concentration of ~2.5 nM to block re-binding of dissociated radioligand to unoccupied receptors. New binding of radioligand was expected to be negligible, owing to the low concentration of free radioligand generated by dissociation [126]. Specific binding was measured after 0, 15, 30, & 60 minutes of incubation at 37°C to determine the dissociation rate. The respective dissociation constants (K_D) were used to calculate the association rates ($k_1 = k_{-1} / K_D$) for VEGF-A binding to KDR and Flt-1.

Determination of internalization rate constants

To determine internalization rates of receptors and receptor-ligand complexes, radioligand binding was again employed for both cell types. Cells were grown in 24-well poly-L-lysine coated culture dishes as described above. Again, [^{125} I]VEGF-A (added at ~15-50 pM) and receptors were allowed to establish a binding steady-state at 4°C (~4 hrs). After this pre-occupation incubation, the 4°C radioligand solution was rapidly replaced with [^{125} I]VEGF-A solution prewarmed to 37°C to initiate internalization. NSB and cell counts were determined as above. After the indicated times (0-30 min), the medium was aspirated, and the cell monolayers were washed rapidly three times with ice-cold binding buffer to remove unbound ligand. The cells were then incubated for 10

min on ice in either (1) a low pH wash (50 mM glycine, 100 mM NaCl, pH 2.5) to remove acid-labile (presumed to be surface) radioligand, or (2) the neutral PBS/BSA wash to leave both surface and internalized radioligand in place. Following a second brief low or neutral pH wash, residual cell-associated specific radioligand binding was determined as described above.

RESULTS

Characterization of clonal cell lines

In order to express the two VEGF receptors, expression constructs were transfected into HEK293 cells (Figure 4). The KDR-expressing clone C57 was isolated previously (Huckle, unpublished). In the present studies, eleven G418-resistant clones from the pooled Flt-1 transfectants were isolated by limiting dilution. Flt-1 protein expression in these isolates was probed by western blotting with an N-terminal Flt-1 antibody (Figure 5). Most of the cell lines showed detectable expression of Flt-1, migrating as a species of $M_r \sim 180\text{-}190$ kDa [71]. Clone 3.1 was selected based on relatively high Flt-1 expression level and a growth rate comparable to the KDR and empty-vector transfectants.

A representative western blot containing samples from both KDR- and Flt-1 overexpressing cell lines, as well as control HUVEC and the pcDNAIntA empty-vector cells, is shown in Figure 6. Both cell types showed evidence of correct receptor expression—C57 cells expressed KDR and 3.1 cells expressed Flt-1—with no evidence of basal levels of expression for the other VEGF receptors. The lack of basal VEGF receptor expression in these cells was further confirmed by the absence of detectable KDR or Flt-1 in pcDNAIntA empty vector control cells. Curiously, there were not measurable amounts of Flt-1 or KDR in the HUVEC cell sample, but this may be due, in part, to the higher cell passage number from which these samples were obtained. Taken together, these results indicate that cell lines with the desired receptor expression characteristics had been successfully isolated, and suggest that these cell lines were candidates for use in binding studies for the determination of kinetic parameters for VEGF and related ligands. Furthermore, it holds the potential that a

model system of cells had been successfully developed which can be used for comparison to computational simulations in *in silico* experiments.

Determination of rate constants for radioligand binding

The ratio of the dissociation and association rate constants for a 1:1 binding reaction at equilibrium, the equilibrium dissociation constant (K_D), can be used in conjunction with either the dissociation rate or the association rate to determine the other rate constant. To determine the K_D for VEGF binding with Flt-1 and KDR, the receptor overexpressing cell lines were incubated with [^{125} I]VEGF-A, with varied concentrations of non-labeled VEGF-A (Figure 8). From these homologous competition experiments, the specific binding of [^{125} I]VEGF-A was used to generate Scatchard plots from which the K_D values were determined with simple linear regression in Excel (Microsoft Corp., Redmond, WA). For Flt-1 and KDR, the calculated K_D values were 110 ± 19 pM and 110 ± 13 pM, respectively. $B_{\max}$ values were obtained from the Scatchard analysis and, using average cell counts from each experiment, the average number of receptors/cell was determined. For Flt-1, it was determined that the 3.1 cell line contained an average of 6400 ± 2100 sites/cell. For KDR that number was less than half with an average of 4900 ± 1300 sites/cell present on KDR cells. Alternatively, the data were analyzed by performing non-linear regression with a least-squares fit using Graph Pad Prism Software (GraphPad Software, San Diego, CA). The K_D and $B_{\max}$ values were determined by performing a four parameter global fitting based on following equation for total binding in homologous competition experiments:

$$Y = \frac{B_{\max} \times [VEGF^*]}{([VEGF^*] + [VEGF] + K_D)} + NSB$$

where, $[VEGF^*]$ is the fixed concentration of radioligand used in the assay (in nM), and $[VEGF]$ is the concentration of competitor, which varies. The K_D values obtained from a global fitting for three independent experiments, performed in triplicate, were 120 ± 1.1 pM for Flt-1; and 124 ± 1.1 pM for KDR (Figure 9). This method produced best-fit values for Flt-1 and KDR with average R^2 values of 0.9811 and 0.9707, respectively. These equilibrium dissociation values are comparable to those obtained from Scatchard

analysis and within the range of values found in the literature for VEGF-A binding to each of these receptors. Because of the high value for the coefficient of determination for each fitting, the K_D values obtained by non-linear regression analysis were selected for use as model parameters (Table 2).

One difference between these measured values and the literature is that the measured K_D for VEGF-A binding to KDR is not higher than that observed for Flt-1—in fact, they are virtually indistinguishable in both methods of quantification. The reasons for these apparent similarities have not yet been identified. These differences should become more apparent in the measurement of “off” rates for VEGF-A from each respective receptor. B_{max} values were also obtained from the non-linear fitting and, using average cell counts from each experiment, the average number of receptors/cell was determined (Table 2). For Flt-1, it was determined that the 3.1 cell line contained an average of 8500 ± 270 sites/cell. For KDR that number was less than half with an average of 3900 ± 840 sites/cell present on KDR cells. These numbers are slightly lower when compared to the range of literature values for Flt-1 and KDR expression [25, 88, 128], but because these are stably-transfected cell lines that do not normally express these receptor types, these numbers are well within reason for levels of expression. Furthermore, it is unclear whether these estimated values represent dimeric or monomeric receptors. Both are capable of binding VEGF, but there is no way of distinguishing, with this method, which form of the receptors is binding the ligand. These numbers could, very well, be underestimated if there is a large number of monomeric receptors binding the radioligand.

To determine the K_D for PIGF and VEGF-E binding to Flt-1 and KDR, the clonal cell lines were incubated with [^{125} I]VEGF-A in the presence of varied concentrations of the heterologous competing ligands (Figure 10). The general binding profiles observed are as anticipated: PIGF binds competes preferentially against binding to Flt-1 and VEGF-E for binding to KDR—with each ligand showing no evidence of specific binding to the other receptor. From these heterologous competition experiments, the previously-determined K_D values for [^{125} I]VEGF-A binding to each receptor were used to determine K_D values for the competing ligands. First, specific radioligand binding at each concentration of non-labeled competitor was plotted. Next, the 50% inhibitory

concentration (IC_{50}) was determined using a one site-log(EC_{50}) model by fitting the equation below in Graph Pad Prism Software:

$$Y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{(X - \log IC_{50})})}$$

where Y is the specific binding, X is the log concentration of competitor, and Top and $Bottom$ are the Y values at the top and bottom plateaus of the curve. Lastly, the equilibrium dissociation constant for the unlabeled compound (K_i) was determined by solving the Cheng-Prusoff/Chou equation[129, 130] in Excel (Microsoft Corp., Redmond, WA):

$$K_i = \frac{IC_{50}}{\left(1 + \left(\frac{[L]}{K_D}\right)\right)}$$

where K_i is the equilibrium dissociation constant for the non-labeled competitor;

IC_{50} is the concentration causing 50% inhibition of binding;

$[L]$ is the concentration of radioligand; and

K_D is the previously determined equilibrium dissociation constant for the radioligand.

The K_i value (K_D) for PIGF obtained using this method of analysis for three independent experiments, performed in triplicate, was 170 ± 96 pM. Here, the trend observed in the literature is replicated, where this value for PIGF is similar—albeit slightly higher—than the K_D for VEGF-A binding to Flt-1 [54]. The K_D value for VEGF-E, 430 ± 55 pM, is also similar to the literature values for this ligand [48], and as expected, it is also higher than the experimentally determine K_D for VEGF-A binding to KDR.

As for the K_D determination for VEGF-A, the data were analyzed in an alternative fashion by performing non-linear regression with a least-squares fit using Graph Pad Prism. The K_i values were determined by fitting the following equations:

$$\log EC_{50} = \log(10^{\log K_i * (1 + [VEGF] / K_D)})$$

$$Y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{(X - \log EC_{50})})}$$

where, $\log K_i$ is the log of the molar equilibrium dissociation constant of unlabeled ligand;

K_i is the equilibrium dissociation constant in Molar.

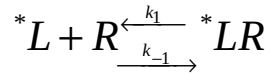
$[\text{VEGF}^*]$ is the concentration of radioligand;

K_D is the previously determined equilibrium dissociation constant for the radioligand

This model fits the K_i of the unlabelled ligand directly, without reporting the EC_{50} , so the application of the Cheng and Prusoff/Chou correction is unnecessary. Instead the concentration of radioligand is entered, along with the K_D , as constants, and the algorithm directly fits the K_i of the non-labeled competitor. The non-linear regression analysis of PIGF determined a K_D for this ligand of approximately 1200 ± 320 pM. The non-linear regression analysis likewise revealed a K_D for VEGF-E of approximately 2000 ± 600 pM. Both of these values are higher, by an order of magnitude, than the corresponding K_D values for VEGF-A. Furthermore, it should be noted that the non-linear fitting, according to the aforementioned model did not produce a best-fit values for PIGF and VEGF-E, as the average R^2 values for the fittings were 0.7656 and 0.8101, respectively. For this reason, the decision was made to use the K_D values as calculated using the Chen and Prusoff/Chou equation. These values are listed in Table 2.

Dissociation

Quantification of VEGF-A interactions with both cell surface receptors was performed as described in the methods section of this chapter. The analysis of the dissociation data demonstrated a deviation from the single reversible binding model which is inherent in this model; therefore, fitting the dissociation data for the determination of these rate constants was problematic. Classically, the determination of radioligand dissociation from a receptor incorporates “fixed” experimental conditions such that the re-association of the radioligand and the receptor are negligible, and only dissociation is measured [131]. The radiolabeled growth factor ($*L$) associates with the receptor of interest (R), and can be defined by the following relationship:



where k_1 = association rate constant (“on” rate)

k_{-1} = dissociation rate constant (“off” rate)

In this case, the rate of formation of $*LR$ over time can be expressed as:

$$\frac{\partial[*LR]}{\partial t} = k_1[*L][R] - k_{-1}[*LR]$$

In order to experimentally fix the conditions, and therefore make association negligible, a large excess of unlabeled competing ligand (L) is added during dissociation in order to diminish the effects of the radioligand rebinding of dissociated ($*L$) [131]. Normally, this would allow the association term to drop out of the expression that represents formation, and the expression would simplify to:

$$\frac{\partial[*LR]}{\partial t} = -k_{-1}[*LR]$$

This would allow the dissociation rate constant to be determined with relative ease, by fitting the dissociation data to:

$$\ln \frac{[*LR]}{[*LR]_0} = -k_{-1}t$$

where $[*LR]_0$ = the amount of radioligand bound at time = 0

$[*LR]$ = the amount of radioligand bound at any time, t

By integrating both sides of the equation one obtains a new exponential equation that defines $[*LR]$ as a function of time, the rate constant k_{-1} , and the value of $*LR$ at time zero, $[*LR]_0$, $[*LR] = [*LR]_0 e^{(-k_{-1}t)}$. The problem that arose with the present data was that the dissociation data did not reflect the exponential (first-order) decay one would expect for of a single $*LR$ complex with one affinity state. The R^2 values for a simple exponential fit of all time points for Flt-1 and KDR were 0.647 and 0.728, respectively (Figure 11). The exponential fitting was performed in Matlab using FMINSEARCH, a multidimensional unconstrained nonlinear minimization tool, with a termination tolerance on the function of 1×10^{-16} . While this method may not have

produced the best-fit for the data, it still produced useable parameter values that were ultimately chosen for the model. The off rates of VEGF-A for Flt-1 and KDR were determined to be 0.0056 min^{-1} and 0.0142 min^{-1} , respectively (Table 2). These rates compare quite favorably to previously determined rates of dissociation for VEGF from KDR and Flt-1 [87, 132], and the trend in the data is as expected, with a higher rate of dissociation observed for KDR. Because off-rate constants for PlGF and VEGF-E were not directly measured for this study, these rate constants determined for the dissociation of VEGF-A were also used for each receptor-specific ligand.

The low R^2 value suggested that the simple exponential fitting may not be optimal and that rebinding might have been a more prominent issue during data collection that ultimately led to the deviation from expected results for ligand dissociation. In view of the apparent non-exponential dissociation, an additional fitting was attempted, using the complementary error function (erfc) used by Gopalakrishnan *et al.* (2005). The erfc fitting was performed using FMINSEARCH as described above. The data were fit with the equation containing the erfc function that solves for the Bound receptor fraction at time t :

$$p(t) = p(0)e^{ct} \text{erfc}(\sqrt{ct}), \text{ where } c = \frac{4k^2\delta}{\theta^2} \text{ and } \text{erfc}(z) = \frac{2}{\sqrt{\pi}} \int_z^\infty e^{-x^2} dx$$

This equation is dependent on c , the key fitting parameters and the rate constant for the effective time scale of binding.

The fitting using erfc was marginally better than the exponential fit. For Flt-1 and KDR, the erfc function fitting procedure yielded R^2 values of approximately 0.6547 and 0.9093, respectively (Figure 11) and, by this measure, provided a more effective fit VEGF-A dissociation data for KDR. While this second method of fitting proved to be a better overall method for fitting the dissociation data, the dissociation rates cannot be calculated from the fitting parameter, c , with the available experimental data. Thus the dissociation rates from the simple exponential fitting are the rates that will be used for dissociation from Flt-1 and KDR.

Internalization

Internalization data were analyzed with the method first introduced by Wiley and Cunningham (1982) [123]. In brief, because the binding of a ligand to its cell surface receptor is the first step in receptor-mediated endocytosis, then the pool of complexed cell surface receptors can be considered the substrate of the internalization process. Because internalization is a first-order process with respect to the occupied receptors, then:

$$\frac{d[LR]_i}{dt} = k_e [LR]_s$$

where LR_s and LR_i are the concentrations of the ligand/receptor complex at the surface of the cell and in the interior of the cell, respectively, and k_e is the endocytotic rate constant. Rearranging and integrating the above equation yields the following equation for a straight line:

$$\frac{[LR]_i}{[LR]_s} = k_e t$$

where t = time after the addition of the ligand. With this in mind, a plot of LR_i/LR_s versus time will yield a slope of k_e [123]. These parameter determinations proved to be the most difficult to determine because the apparent inability of the low-pH washes to completely remove specifically-bound radioligand that should have remained at the cell surface. After 3-4 hours of equilibrium binding at 4°C (to establish steady-state), little or no internalization should have occurred. However, there was evidence of specifically bound ligand that could not be removed with the acid wash procedure (Figure 12A). This was not only observed in Flt-1 and KDR-expressing cells but also in control cells (pcDNAIntA) as well. It is unclear whether internalization had truly occurred when it should not have, during the equilibrium binding portion of the assay, or whether there exists a sequestered site of surface binding that meets the definition of specific binding but is acid stable. Attempts were still made to glean k_e values from Flt-1 and KDR experiments (performed in triplicate). Only after the data sets were normalized by expressing them in terms of pmoles/cell and the corresponding control values at 0 & 15 minutes were subtracted from the data was a successful internalization rate calculated (Figure 12B)—even then, a useful value for KDR only was determined.

The internalization rate for KDR complexes, as determined by interpolation between the 0 and 15 minute time points was 0.186 min^{-1} . In the case of Flt-1, the trend in the data was counterintuitive—the internalization rate ended up being negative and unable to be used. With regards to experimentally determined internalization rates of KDR bound to a ligand, there are gaps in the literature. This value is greater than those obtained by estimation of instantaneous internalization rate (0.0258 min^{-1}) of VEGF in HUVECs [25] by approximately a factor of 10. Another study by Dougher and Terman (1999) reported that, with a similar acid wash procedure, 293 cells expressing native KDR receptor, required 25 min for 50% of cell-associated [^{125}I]VEGF to be internalized at 37°C [78]. This equates to an approximate internalization rate of 0.02 min^{-1} for complexed KDR. In spite of the difference between the measured value for KDR internalization and literature values, 0.186 min^{-1} is still the value that is used for KDR complex internalization in the model. Because no internalization rate was determined for Flt-1 complexes, this rate is also used as the internalization rate for Flt-1 complexed with the available ligands. A value for the internalization rate of unoccupied VEGF receptors is not available in the literature, although in other receptor systems, the rate of internalization of unoccupied receptors typically is lower than that of ligand-receptor complexes. For example, for epidermal growth factor receptors, measurements of unoccupied receptors have been 10 times slower than occupied receptors [133]. In this case, a value of $\sim 0.0186 \text{ min}^{-1}$ for the internalization rate of both unoccupied VEGF receptors was used, making note that alterations in this parameter can greatly affect the predictions of the model. This is not only due to the decrease in viable binding sites, but because, as previously mentioned, the rate of insertion is directly linked to the rate of internalization.

MODEL SIMULATIONS

Base model of receptor binding successfully predicts complex formation

Before investigating the effect of sFlt-1 on VEGF binding, it was important to verify that the base model captures the critical feature of ligand binding and complex formation *in silico*. In its simplest form, with only KDR present in the model system to bind growth factor, the number of KDR complexes predicted by the model to be present

on the cell surface during an experiment is shown in Figure 13. With a starting concentration of 595 pM (25 ng/ml), Va binds rapidly to unoccupied KDR early in the time course and the number of complexes reaches its peak (787 complexes/cell; 20% of $B_{\max}$) at approximately 8.5 min. After this maximal level is achieved, the numbers start to decrease as complexes are gradually internalized. At the end of the simulated 15 minute time course, the number of KDR/Va complexes has decreased to 89% of the maximal value achieved during the experiment.

When Va is replaced with Ve in the system, at a starting concentration of 714 pM (25 ng/ml), the formation of KDR/Ve complexes occurs less rapidly than the formation of KDR/Va complexes. The formation of complexes, in this case, reaches its peak at ~12 minutes with no measurable decrease in complex number at the end of the 15 time course. The delay in complex formation is not the only difference observed in the binding of these two ligands, as the peak number of KDR/Ve complexes (362 complexes/cell) represents only 45% of the maximal number of KDR complexes formed with Va present as the only ligand for binding. While VEGF-E may not normally be present in a physiologic system, if it is introduced, it is unlikely that it will be the only ligand present; therefore, this warrants inclusion of both VEGF-A and VEGF-E in the system simultaneously. In this case, with both ligands competing for KDR, the dynamics of the system are only slightly altered. The maximal number of KDR/Va complexes formed over time is decreased to 90% of the original prediction, to 709 complexes/cell, while KDR/Ve complex numbers are more dramatically affected with a decrease in the maximal number of complexes to 69% of the original prediction (252 complexes/cell). Interestingly with the simultaneous inclusion of both ligands, the time to peak complex formation, for both types of complexes, occurred at the same time.

When a similar, simplistic approach was taken to model Flt-1 as the only receptor in the system, the predictions were slightly different than for KDR (Figure 14). With a starting concentration of 595 pM (25 ng/ml), Va binds less rapidly to unoccupied Flt-1 early in the time course, with the number of complexes reaching its peak (908 complexes/cell; ~11% of $B_{\max}$) at approximately 12 min with no measurable drop in the number of complexes at the end of the 15 minute time span. This can be understood

because the off rate for the dissociation of these complexes is lower than that for KDR (Figure 12).

When Va is replaced with PlGF in the system, at a starting concentration of 658 pM (25 ng/ml), the formation of Flt-1/Pl complexes occurs slightly less rapidly than the formation of Flt-1/Va complexes. The formation of Flt-1 complexes, in this case, reaches its peak at ~13 minutes with no decrease by the end of the 15 minutes. It should be noted that the peak number of Flt-1/Pl complexes (736 complexes/cell) represents approximately 81% of the maximal number of Flt-1 complexes formed with Va present as the only ligand for binding. With both ligands as part of the naturally-occurring physiologic system it is unlikely that it will be the only ligand present; therefore, a subsequent testing of the base model included both ligands in the system simultaneously. In this case, with both ligands competing for Flt-1, the dynamics of the system are only slightly altered. The maximal number of Flt-1/Va complexes formed over time is decreased to 87% of the original prediction, to 791 complexes/cell. Likewise, Flt-1/Pl complex numbers are only slightly affected, with a decrease in the maximal number of complexes to 611 complexes/cell, or 83% of the original prediction. As observed with KDR, the simultaneous inclusion of both ligands led to peak complex formation occurring at the same time.

Modeling of sFlt-1 inhibition

Having established that the model is able to simulate complex formation with the initial addition of ligand into the system, I sought to determine its effectiveness in predicting the inhibition on ligand binding by sFlt-1. Because I was interested in the dynamic process of complex formation over a short time-span, this set of simulations was also carried out over a 15 minute period. Two main questions were addressed with the model simulations that aid in understanding the primary mode of action of sFlt-1. First, how do perturbations in the ratio of receptors to sFlt-1 affect its inhibitory action? Second, how sensitive is this system to alterations in the ratio of sFlt-1 to VEGF? The results of simulations that address the first question are shown in Figure 15, where the ratio of sFlt-1 to KDR (#/cell) was increased from 1:1 to as high as 100,000:1. The predicted number of complexes/cell was scaled to the maximal number of KDR/Va in

the absence of sFlt-1—the value in its absence is 100%. Surprisingly, no measurable difference in predicted complex formation was noted until the ratio reached 100:1 and the peak was decreased to 81% of the max. Ratios of 1:1 and 10:1, sFlt-1 to KDR, only reduced maximal complex formation by 0.2% and 2.3%, respectively. With 1,000 times the amount of sFlt-1 to KDR, peak formation only reached 31% of maximal levels, and at 10,000 to 100,000 times, there was virtually no detectable complex formation with only 5.0% and 0.50% complexes formed. At these last two maximal levels, the concentration of sFlt-1 added to the system approaches 13.1 nM and 131 nM, respectively, and this represents addition of the soluble receptor at a concentration 22 and 220 times the starting concentration of Va (595 pM), respectively.

In view of this result, the next set of simulations was an attempt to investigate the dimensionless ratio of sFlt-1 to Va. Assuming a starting concentration, Va_0 , of 595 pM, the amount of sFlt-1 was altered such that the ratio of sFlt-1 to Va was changed in ten-fold increments. Complex formation was then scaled to the maximal number (without the addition of sFlt-1), and the percentage in this change was plotted as a function of the sFlt-1 to Va ratio. Similar to the sFlt-1 to KDR ratio, this ratio resulted in a sigmoidal change in maximal complex formation (Figure 16). The linear portion of this curve, falling roughly between 10% and 90%, indicate a window for the sFlt-1 to Va ratio that produces a large change in maximal complex formation. These results demonstrate that predicted KDR/Va complex formation is equally sensitive to alterations in the sFlt-1/Va ratio and the ratio of sFlt-1 to KDR.

Ligand-shifting in a multiple ligand, multiple receptor environment

The interplay between ligands and receptors is well documented, but one hypothesis that warrants further investigation is that regarding ligand-shifting. The rationale for this idea is based solely on an increase in the number of KDR/Va—which ultimately become signaling complexes—formed when PlGF and VEGF are present in a system with Flt-1 and KDR together. This has been demonstrated *in vitro* [54, 89] but was addressed subsequently in a mathematical model of VEGF binding [107]. In particular, this model investigated the addition of VEGF alone and then with the addition of both VEGF and PlGF. The authors noted the synergistic effects of adding PlGF,

which effectively “shifted” Va from Flt-1 to KDR, leading to more KDR/Va complex formation. While the focus of these earlier studies was to look at effective shifting of VEGF to KDR from membrane bound Flt-1, a detailed description of similar effects for sFlt-1 needs to be further investigated. In order to simulate this occurrence *in silico*, predictions about complex formation were made in a multiple ligand, multiple receptor system. PIGF (PI_0) was added in increasing amounts to the system where sFlt-1 was present in quantities (3.94×10^6 soluble receptors/cell) that decreased KDR/Va complex formation by ~70% (Figure 17). MacGabhann & Popel (2004) performed similar simulations with KDR and Flt-1 present in a system and found that the addition of equimolar concentrations of Va and PI resulted in the Va displacement which gave a transient, ~5%, increase in KDR complex formation [107]. The results of our new simulations are consistent with those previous predictions, in that the addition of equimolar concentrations for Va_0 and PI_0 resulted in a ~3% increase in predicted KDR/Va from basal sFlt-1 inhibitory levels. From there, the ratio of PIGF to Va was continuously increased by a factor of four until PI_0 present in the system was approximately 256 times the amount of Va_0 . At this level, predicted KDR/Va was increased to 213% above basal sFlt-1 inhibitory levels, at which point it also represented 98% of the KDR/Va predicted with no sFlt-1 present. These results demonstrate that the inhibitory effects of sFlt-1 can be successfully reversed with the addition of PIGF to bind to the soluble receptor and liberate Va to bind to KDR. One observation of note with these predictions is that the surprising amount of PIGF that is necessary for these results (152 nM) does not likely represent a level that is physiologically relevant for circulating levels of PIGF. Furthermore, this amount of PIGF necessary to restore the formation of KDR/Va complexes is in excess to the 1.31 nM concentration of sFlt-1 that produced the inhibition in the first place.

Modeling VEGF-E activation of KDR

The unique nature of responses to VEGF-E activation has only recently become the subject of study in physiologic systems. Transgenic mice that over-express this ligand have demonstrated significant increases in subcutaneous blood vessel formation without the normal proedematous and proinflammatory responses observed in VEGF-A-

transgenic mice [64]. Ultimately, these differences can be attributed to the binding specificities for the two ligands, where VEGF-A binds and activates both Flt-1 and KDR and VEGF-E binds only to KDR. The interest in this ligand as a candidate for pro-angiogenic therapies and other clinical uses [64] suggests that a model of its binding would be beneficial to better understand its mechanism of action. For this reason, the current model setup was extended to include VEGF-E in the presence of Flt-1 and KDR, as well as other ligands—to determine an effective way to increase the number of KDR/Ve signaling complexes that form over time. The results of these simulations are shown in Figure 18. With equal numbers of Flt-1 and KDR (#/cell), and Va_0 at the standard 595 pM, the maximal number of KDR complexes formed over 15 minutes (798, #/cell) is roughly double that of the maximal Flt-1/ Va complexes/cell. With the introduction of equimolar Ve_0 , predicted KDR/ Va drops slightly to 731 complexes/cell—with the corresponding formation of KDR/Ve at a level of 217 complexes/cell. Flt-1/ Va complexes remain unchanged. The question remains, what is an effective strategy to increase the number of signaling KDR/Ve complexes while decreasing the number of VEGF-A/receptor complexes that may form, leading to unwanted physiological effects (e.g., proedematous & proinflammatory effects). The ultimate power of a model like this is displayed in Figure 19, where slight increases in Ve_0 can be paired with the introduction of high levels of sFlt-1, ultimately increasing the predicted number of KDR/Ve complexes over time. When the level of Ve_0 was only doubled (1.2 nM) and $[sFlt-1]_0$ was introduced at a ratio of 1,000:1 to KDR, there was a dramatic increase (~180%) in the number of predicted KDR/Ve complexes over 15 minutes. This was coupled with a decrease in the number of Flt-1/ Va and KDR/ Va complexes of 75% and 61%, respectively. Strategies like this with VEGF-E have not yet been tested in any physiologic systems, so the ability to mathematically model these interactions prior to *in vitro* or *in vivo* experimentation is quite beneficial. The power of the model resides in its ability to simulate various combinations of ligands and receptors to optimize experimental conditions.

DISCUSSION

There is a growing interest in identifying potential inhibitors of VEGF-A-mediated actions in the microenvironment for many angiogenic processes. From the vascularization of tumors to events such as wound healing, there is a need to better understand the interplay of the many ligands and receptors that govern these physiologic processes. Thus far, sFlt-1 is considered to be one of the only naturally-occurring inhibitors that can specifically and effectively block VEGF-A binding and signaling. More recently, soluble forms of KDR [134] and neuropilin-1 [135] were identified in mouse and human plasma and in various mouse tissue samples. They may serve as functional antagonists for VEGF-mediated angiogenesis, but more details about their expression and biological action need to be determined. sFlt-1, on the other hand, is well-classified. It has been described in human endothelial cells, in various cancers, and different biological fluids like serum, but its significance is still unclear. sFlt-1 has been proposed to be a decoy receptor by binding VEGF-A and thereby regulating the availability of VEGF-related ligands for the activation of KDR [75, 136]. In the case of preeclampsia, for example, there is a strong correlation between the level of sFlt-1 in maternal serum and the degree to which preeclamptic symptoms exist [96, 97]. The theory is that increased sFlt-1 levels play a critical role in establishing a regulatory barrier against VEGF between the maternal and fetal blood vessel networks; therefore, it may be a cause of preeclampsia rather than a consequence of the condition.

In spite of the role of sFlt-1 in preeclampsia, its potential anti-angiogenic actions should not only be viewed in a negative light. One of the first targeted therapies to show a survival advantage, in certain cancers, when used in combination with standard chemotherapy has been bevacizumab--a monoclonal antibody directed against VEGF-A that mimics the action of sFlt-1 [137]. Whether they are used to understand how sFlt-1 causes certain pathologic conditions or how it may be used as an anti-angiogenic therapy, there is a need for methodologies that are useful for predicting the action of sFlt-1 *in vitro* and *in vivo*.

The results here support the hypothesis that a mathematical model simulating VEGF binding in a multiple ligand, multiple receptor system could be developed which

mimics *in vitro* experiments. One of the primary aims in developing this model was its use in investigating the competitive inhibitory effects that sFlt-1 may have on the ability of Flt-1 and KDR to bind ligands. It was designed with an eye toward *in vitro* experiments so as to provide a way of testing potentially interesting physical mechanisms of regulation in the VEGF system—such as the competitive shifting of ligands from one receptor to another in the presence of sFlt-1. Specifically, I sought to determine what mechanisms could lead to the inhibitory effects of sFlt-1 on VEGF-related ligands binding to cells expressing receptor populations that would normally be present. I was able to use the model to simulate a series of experiments in which the ratio of sFlt-1 to KDR and sFlt-1 to VEGF-A was varied.

In a series of recent studies, the kinetics of sFlt-1 secretion in normal healing wounds was observed to resemble the patterns of those described for VEGF-A/PIGF expression during normal wound repair, where physiologic levels of both ligands were elevated. This suggested a correlation between sFlt-1 and VEGF expression during wound angiogenesis that potentially controls over-stimulation of the angiogenic process locally that may occur with the increasing presence of VEGF-related ligands [138, 139]. This is in contrast to other studies—with chronic non-healing wounds—that have observed that the induction of sFlt-1 expression to supraphysiological levels could cause a disturbance in the balance between VEGF ligands and sFlt-1. It was concluded that dysregulation in this ratio may attenuate vessel growth to a level that disturbs normal granulation tissue formation, ultimately impairing wound closure (reviewed in [140]). These conclusions make reference to ratios of sFlt-1 to VEGF and VEGF-related ligands, but there are few studies, if any, that have investigated these ratios directly. Using my model, experiments were carried out *in silico* that measured the ratios of sFlt-1 and VEGF. Valuable information was generated about the necessary ratios of sFlt-1 to VEGF that produce maximal or sub-maximal levels of inhibition.

The predicted molar sFlt-1/VEGF ratio that produced a 50% maximal response was ~1:1. This means that when the starting concentration of 595 pM VEGF-A, approximately 595 pM of sFlt-1 was necessary to inhibit KDR complex formation by 50%. The next question is whether these ratios are physiologically relevant. Average serum levels of sFlt-1 in the healthy adults have been reported to be 28 ng/ml, or 250

pM [141], and so it can be concluded that 595 pM sFlt-1 is within physiologic limits. In this same study, however, that level of sFlt-1 was observed at a molar excess of approximately 140 times the measured VEGF-A concentration (1.8 pM). The present model predicts, that at a molar excess like this, there would be no measurable KDR complex formation in the system. This may be indicative that the model cannot be used to simulate VEGF complex formation due to circulating levels of ligands and receptors. Instead, it is more likely that this model is applicable at a microenvironmental level *in vivo* where physiologic levels of ligands and receptors may be locally elevated or altered from those readily measured in serum. For example, the present model could be used to investigate levels of sFlt-1 in a tumor microenvironment.

Along with the inhibition lent by sFlt-1 on VEGF binding, another interesting result to come from modeling simulation was the demonstration of the reversal of sFlt-1 inhibition by exogenous addition of PlGF. The theory of ligand shifting was first introduced by Park et al. (1994) where PlGF was demonstrated to potentiate the action of low, marginally efficacious, concentrations of VEGF in an *in vivo* vascular permeability assay. The authors noted that an effect like this required approximately a 10-20-fold molar excess of PlGF over VEGF. Using the current model, a significant reversal of sFlt-1 inhibition—where approximately 60% more KDR/Va complexes were formed—required a 16-fold molar excess of PlGF over VEGF-A. This is within the range observed by Park et al. (1994), so it may be concluded that the model represents a good predictor of ligand-shifting events that may occur. This conclusion is cautiously optimistic as there is no current way to determine how an increase in KDR complex formation translates to “potentiation” of an *in vivo* angiogenic response. Assuming that the potentiation of the action of VEGF means the formation of more KDR/Va complexes *in vivo* that would lead to a greater angiogenic response, a general comparison between these data and model predictions can be made. The model is quite effective at predicting how the upregulation of PlGF may stimulate angiogenic signaling via displacement of VEGF from sFlt-1 to KDR. Success on this initial level is encouraging, but one question that remains is, of what value are these simulated experiments to physiologic events? Drawing conclusions about these observations and extending them to *in vivo* situations is more difficult.

One novel result to come from this modeling attempt was the investigation of interplay between VEGF-E and sFlt-1 for complex formation. Because of the interest in VEGF-E ligand as a candidate for pro-angiogenic therapies and other clinical uses [64], the initial results from this model are valuable for understanding its mechanism of action in a physiologic system. The dramatic increase in KDR/Ve complexes with the introduction of a sFlt-1 (1.31 nM) was quite surprising. This level of sFlt-1 is well within the measured physiologic limits and this provides some insight as to why VEGF-E may be such an effective stimulator of angiogenic events. With its inclusion in a system, it is able to preferentially form complexes with KDR, and with levels of sFlt-1 considered “normal” for a physiologic system, the maximal amount of complex formation is increased in dramatic fashion. This sheds light on a potentially new corollary of the VEGF ‘sink’ theory. If VEGF-E is introduced into a system that also contains sFlt-1, it likely forms more KDR complexes when it acts in synergy with sFlt-1-mediated VEGF-A inhibition. In this case the VEGF sink potentiates VEGF-E binding and causes more VEGF-E to be shifted to KDR—thus eliciting a stronger angiogenic response from VEGF-E. This new theory of sFlt-1 dependent ligand-shifting has not yet been tested in any physiologic systems, so the ability to mathematically model this interaction prior to *in vitro* or *in vivo* experimentation is quite beneficial.

One problem with the few VEGF models that have previously been formulated is that the kinetic parameters are gleaned from disparate literature sources. This is also one of the more powerful aspects of the present study—the formulation of a model that includes as many experimentally determined kinetic parameters as possible. The power that resides in a model’s ability to make accurate predictions is dependent on the accuracy of the parameter values on which it is based.

Every attempt was made to measure these values in a controlled manner in both clonal cell lines expressing specific receptor types. One unexpected result in these measurements was that there were no apparent differences in the equilibrium dissociation constants in both cell types for VEGF-A. The literature suggests that the Flt-1 cells would have a lower K_D value—and therefore a higher affinity—for VEGF-A than for the KDR cells. One complicating factor for a measurement like this in clonal cell lines would be the presence of other receptors that may compete for binding of the

radioligand. When whole cell lysates of both cell types were probed, via western blotting, for the presence of neuropilin-1, there was evidence that the Flt-1 expressing cells also contained this additional receptor (Figure 7). Because neuropilin-1 may also bind VEGF-A, the determination of a K_D for Flt-1 in these cells may be complicated by this additional receptor competing for the available ligand. Furthermore, with no estimation of numbers of neuropilins on the Flt-1 cells, it is unclear to what extent the measured K_D values for Flt-1 were affected by these potential, additional binding events.

There were also difficulties in determining two additional parameter values: the off rates and internalization rates. It seems that the inability to accurately fit the dissociation data may have been complicated by ligand rebinding, which led to a decay of the bound fraction that was non-exponential in nature. This phenomenon where a ligand, following dissociation from a bound protein on the surface, may diffuse in the extracellular fluid environment for some brief time period and may be reabsorbed later at one of the free binding sites was of particular interest for the analysis of this data. The effect of rebinding may not have simply reduced the effective dissociation rate, but rather it may have led to a non-exponential temporal decay of the bound fraction. This topic has been addressed in detail in recent studies by Gopalakrishnan et al. (2005a & b) where the effect of rebinding of insulin-like growth factor-I (IGF-I) to one of its receptors was noted to cause a reduction in the effective dissociation rate [142, 143]. Their solution was to fit the data using the complementary error function (erfc) which ultimately led to a complete solution of their rebinding problem. The expression used for the fitting is characterized by a single effective time scale $1/c$, which is proportional to the inverse of the square of the dissociation rate [142]; however, because this fitting method represents a function of the rate and a number of other factors, it was impossible to directly calculate an off rate using this method. Ultimately, fitting the data with the complementary error function simply demonstrated that re-binding may play a significant role in ligand dissociation. In this case, almost by default, the values derived from exponential fitting of the dissociation data were used in the model. Because these values are subsequently used to calculate on rates from K_D values, inaccuracies in

measuring these rates are magnified. They can affect both parameters that dictate ligand binding.

Alternative methods exist for determining on and off rates (e.g., surface plasmon resonance), and perhaps in future studies these methods can be employed for the determination of ligand binding parameters. In lieu of these methods, a sensitivity analysis is helpful for determining how alterations in these parameters affect the model predictions. Such an analysis is discussed, in detail, in an ensuing chapter. A method of optimization, such as a Monte Carlo simulation [144], may be a beneficial method for varying binding parameters and making predictions of complex formation over a range of values; however, no such analysis will be included in this document.

Based on the predictions utilizing the current model: (1) sFlt-1 inhibition can be successfully simulated, but the model may be most useful at predicting local changes to a receptor-ligand microenvironment; (2) PlGF ligand shifting can be simulated with the model, and the levels needed for these observed effects are well-within those levels observed physiologically; (3) the binding of VEGF-E and formation of complexes with this ligand is upregulated with sFlt-1 present in the system to “shift” more VEGF-E onto KDR.

Parameter	Value	Source
Initial ligand concentrations, pM		
Va_0	600, varied accordingly	Based on M.W. of V_A
Pl_0	660, varied accordingly	Based on M.W. of PIGF
Ve_0	710, varied accordingly	Based on M.W. of V_E
Initial receptor populations, #/cell		
$R1_0$	8500	Experimental
$R2_0$	3900	Experimental
$R3_0$	varied	
Equilibrium dissociation constants (K_D), pM		
$K_D, Va, R1, R3$	120	Experimental
$K_D, Va, R2$	124	Experimental
$K_D, Pl, R1, R3$	170	Experimental
$K_D, Ve, R2$	430	Experimental
Association “on” rates, $M^{-1} \cdot min^{-1} \times 10^7$		
$k_1, Va, R1, R3$	4.68	“off”/ K_D
$k_2, Va, R2$	11.4	“off”/ K_D
$k_3, Pl, R1, R3$	3.27	“off”/ K_D
$k_4, Ve, R2$	3.34	“off”/ K_D
Dissociation “off” rates, min^{-1}		
$k_{-1}, Va, R1, R3$	0.0056	Experimental
$k_{-2}, Va, R2$	0.0142	Experimental
$k_{-3}, Va, R1, R3$	0.0056	Experimental
$k_{-4}, Ve, R2$	0.0142	Experimental
Internalization rates, min^{-1}		
$k_{eC1}, k_{eC2}, k_{eC3}, k_{eC4}$	0.186	Experimental
k_{eR1}, k_{eR1}	0.0186	Literature [133], based on k_{ec}
Insertion rate of receptors, receptors*cell⁻¹*sec⁻¹		
$V_{SR1}, V_{SR2}, V_{SR3}$	$k_{eR} \cdot R(t)$	Based on k_e
Translation of ligand conc, L/cell, $\times 10^{-9}$		inversely related to cell density and volume of well,
V	0.5	0.5 ml and 1×10^6 cells per well
Avagadro’s number, sites/mol		
Na	6.02×10^{23}	

Table 2. Parameter values used in mathematical model of VEGF system. Most values were determined experimentally, as indicated, or estimated from the available literature.

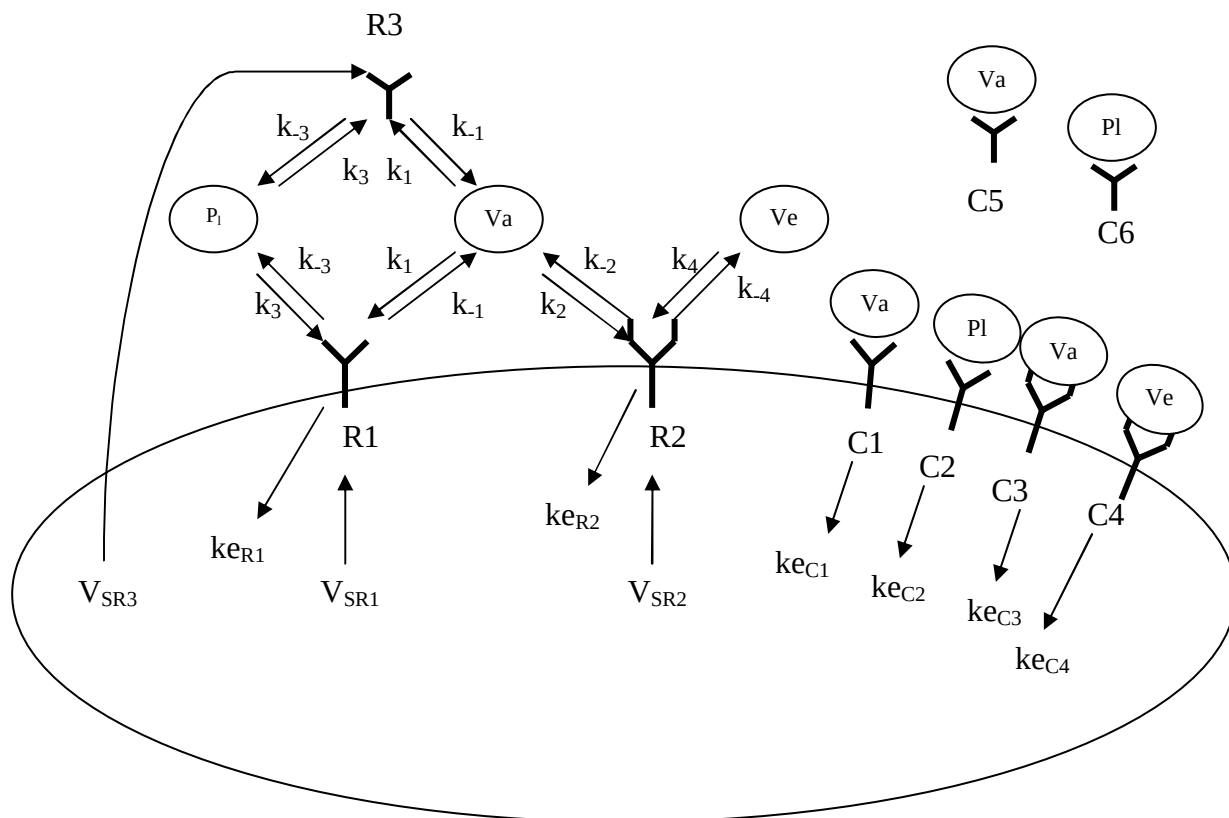


Figure 3. Schematic diagram of receptor-ligand system used to model VEGF binding.

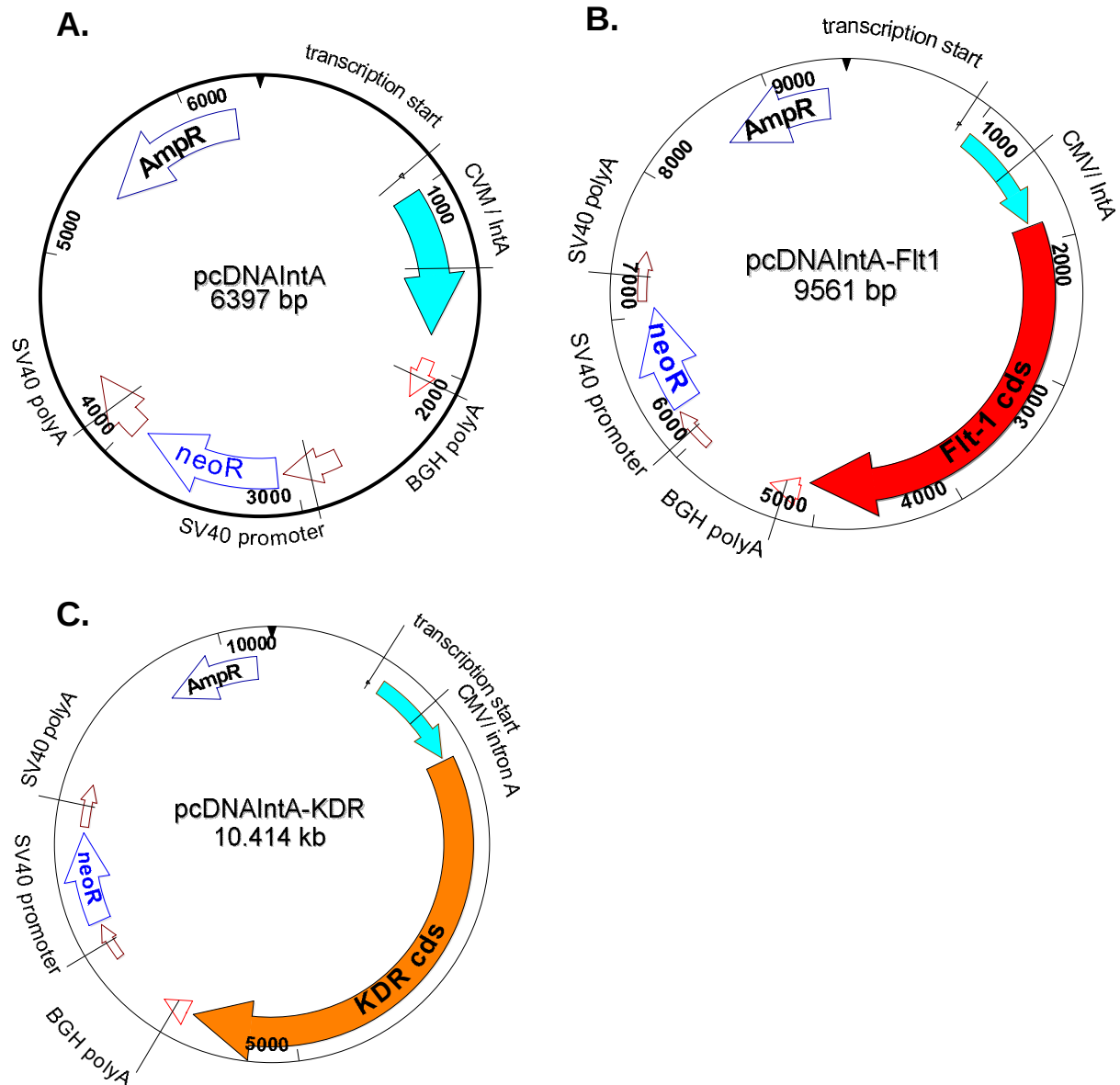


Figure 4. Plasmids used for development of clonal cell lines. These plasmids are presented a map format ease of visualization and show the components necessary for the establishment of stable lines of cells that overexpress Flt-1 or KDR, or contain the empty vector only. **(A)** pcDNAIntA contains the cytomegalovirus (CMV) intermediate-early promoter plus intron A and a cassette encoding the neomycin (neo) resistance marker for gentamicin (G418) selection. pcDNAIntA-Flt1 **(B)** and pcDNAIntA-KDR **(C)** contain all the aforementioned components as well as the full-length receptor coding sequences (CDS) for Flt-1 and KDR, respectively.

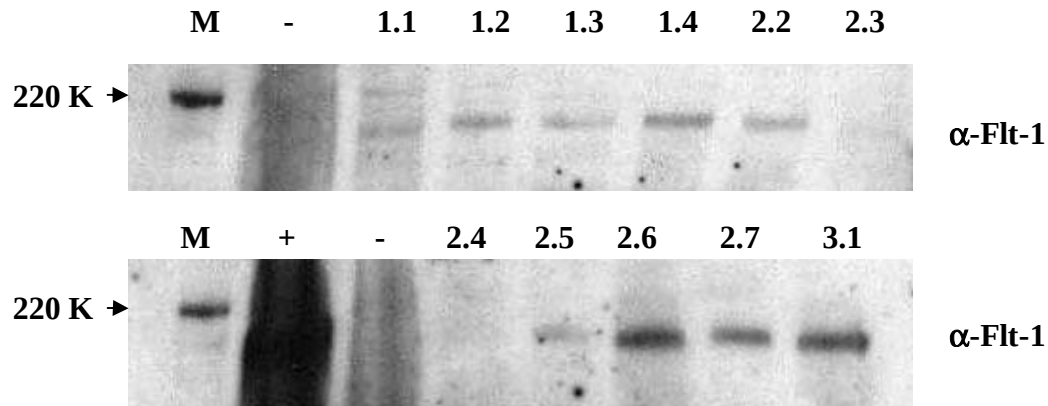


Figure 5. Flt-1 expression in clonal cell lines. Individual cell lines obtained by limiting dilution in a 96-well plate were labeled 1.1 – 3.1. Molecular weight markers (M), a negative and positive control (transiently transfected Flt-1 cells) are included. Flt-1 immunoreactivity (not normalized for protein loading) was probed with an N-terminal Flt-1 antibody as described in the text. Cell lines 2.6 and 3.1 showed the highest apparent level of Flt-1 expression.

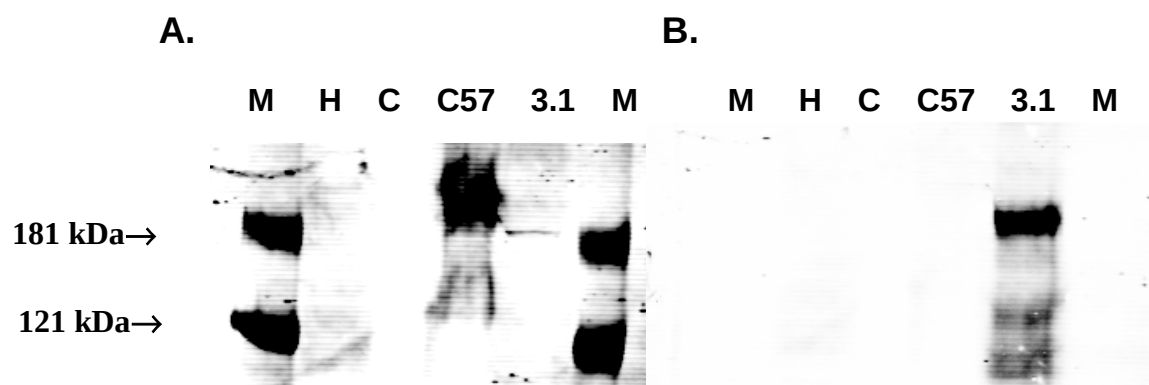


Figure 6. Representative western blots demonstrating the presence of KDR (A) and Flt-1 (B) in the respective clonal cell lines. Western blot images were generated with Odyssey[®] Licor imaging system software. These separate images are actually the same blot probed with two separate antibodies, but are separated for ease of viewing. The blot contains molecular weight markers (M), passage 8 HUVEC control (H), a pcDNA/IntA cell sample (C), C57 (KDR) cell sample, and 3.1 (Flt-1) cell sample. (A) When the blot was probed with α -KDR antibody, the C57 cells correctly displayed KDR expression with a large protein band that migrated to ~200 kDa. Likewise, when the blot was probed with α -Flt-1 antibody, the 3.1 cells correctly displayed Flt-1 expression with a protein band that migrated to ~190 kDa. There was no measurable level of KDR or Flt-1 content in the HUVEC or control cells.

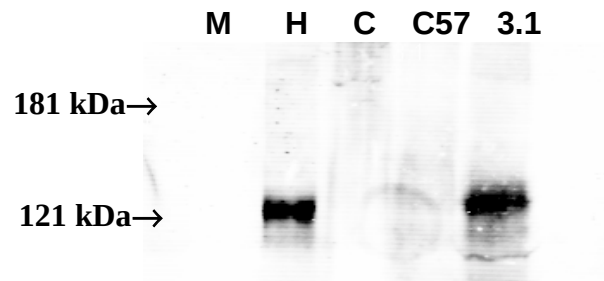


Figure 7. Western blot demonstrating the presence of neuropilin 1 in the respective clonal cell lines. Western blot image was generated with Odyssey® Licor imaging system software. The blot contains molecular weight markers (M), passage 8 HUVEC control (H), a pcDNA/IntA cell sample (C), C57 (KDR) cell sample, and 3.1 (Flt-1) cell sample. (A) When the blot was probed with α -neuropilin antibody (sc-7239, Santa Cruz Biotechnology, Inc.), the HUVEC cells correctly displayed NRP-1 expression with a large protein band that migrated to ~130 kDa. Likewise, the 3.1 cells displayed NRP-1 expression, but there was no measurable level of the receptor the control or KDR cells.

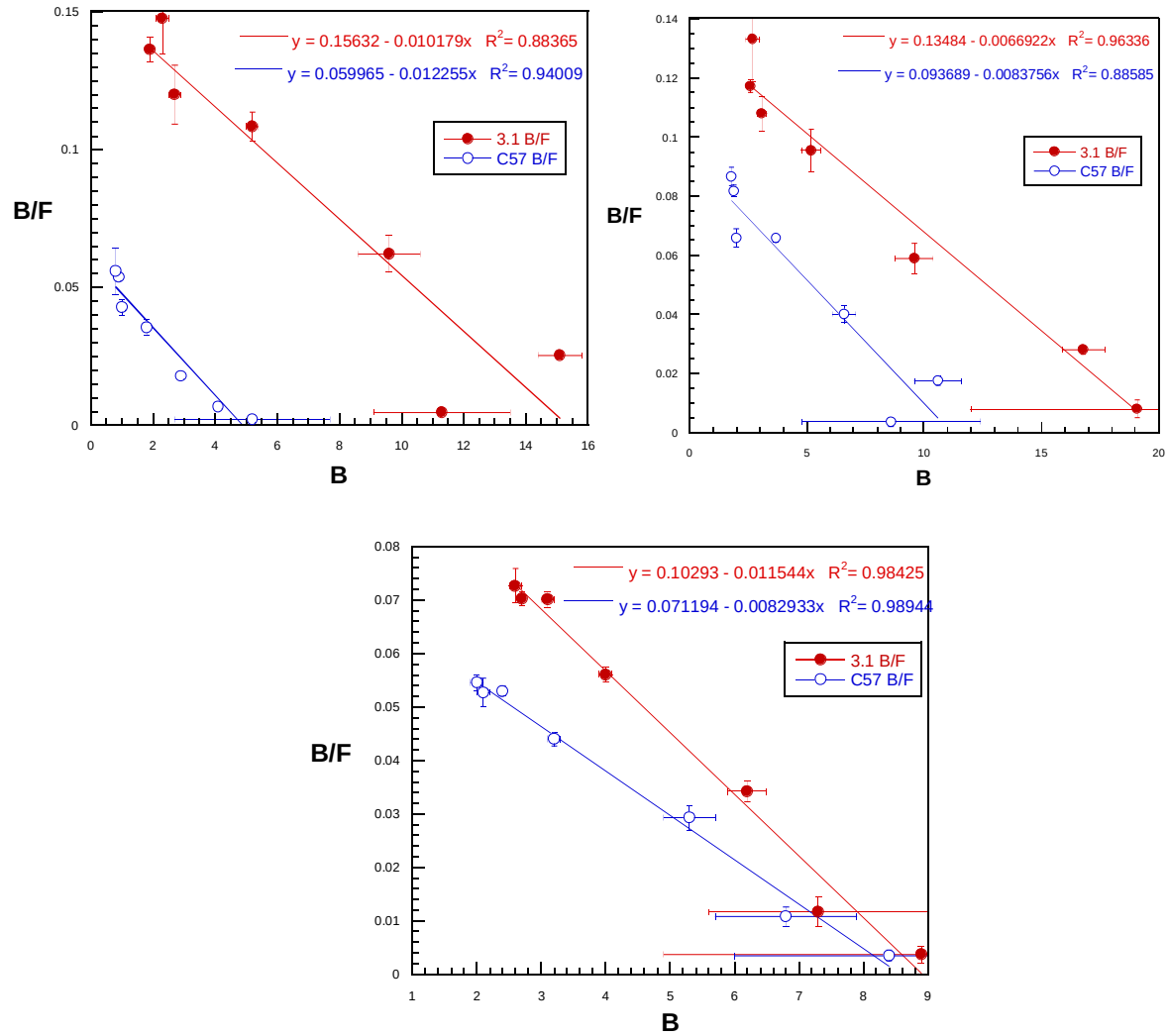


Figure 8. Scatchard analysis of VEGF-A binding to Flt-1 (3.1 cells) and KDR (C57 cells). Plotted as the ratio of Bound/Free (B/F) versus Bound (B), the linear regression analysis of the data yields a straight line with slope = $-1/K_D$. The x intercept is an estimate of Bmax and the y intercept = B_{max}/K_D . Scatchard plots with these characteristics are indicative of reversible binding to a single population of receptors governed by one dissociation constant (single affinity state for ligand). The average K_D values for VEGF-A binding to Flt-1 and KDR were 110 ± 24 pM and 110 ± 16 pM, respectively (n=3 separate experiments).

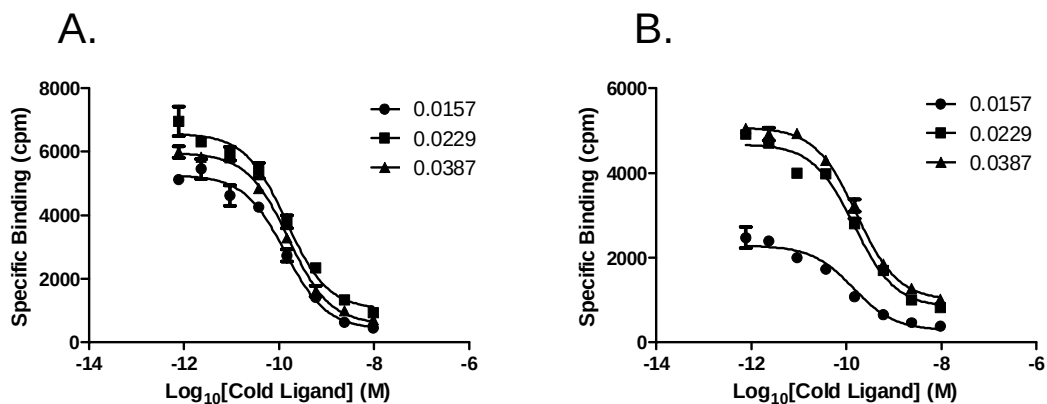


Figure 9. Non-linear regression analysis of VEGF-A binding to Flt-1 **(A)** and KDR **(B)**. Data are plotted as mean values from duplicate measures. The results of the global fitting, where a K_D value was shared between the non-linear fits, produce K_D values for Flt-1 and KDR of 120 ± 1.1 pM and 124 ± 1.1 pM, respectively. Average R^2 value for Flt-1 and KDR global fitting are 0.9811 and 0.9707, respectively.

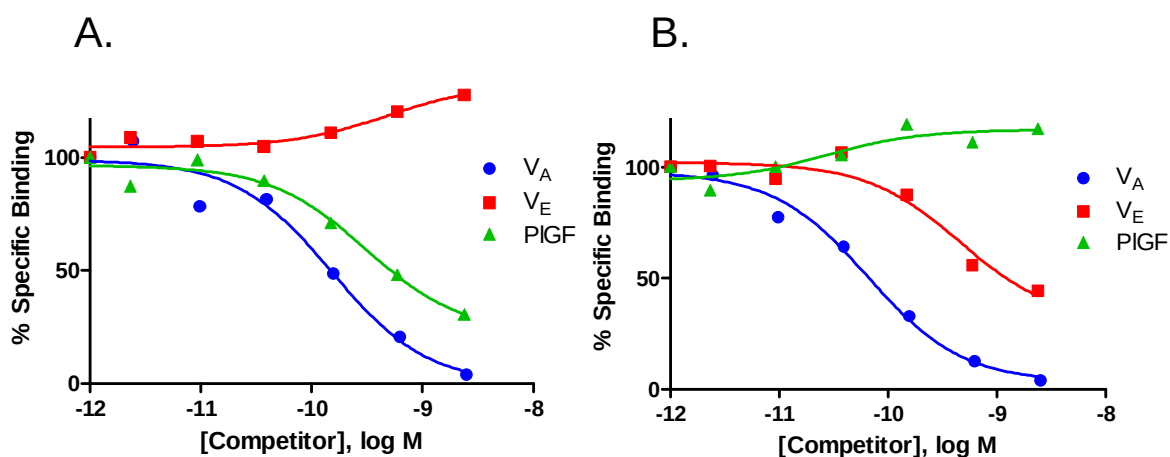


Figure 10. Example of pharmacologic profiles for VEGF-A, PIGF, & VEGF-E binding to Flt-1 (A) and KDR (B). Data (representative of 1 replicate experiment) represent mean of duplicate measures. Data are normalized by expressing specific binding at each competitor concentration as a percentage of specific binding in the absence of competitor. As anticipated, PIGF behaved as a Flt-1 selective competitor and VEGF-E as a KDR-selective competitor.

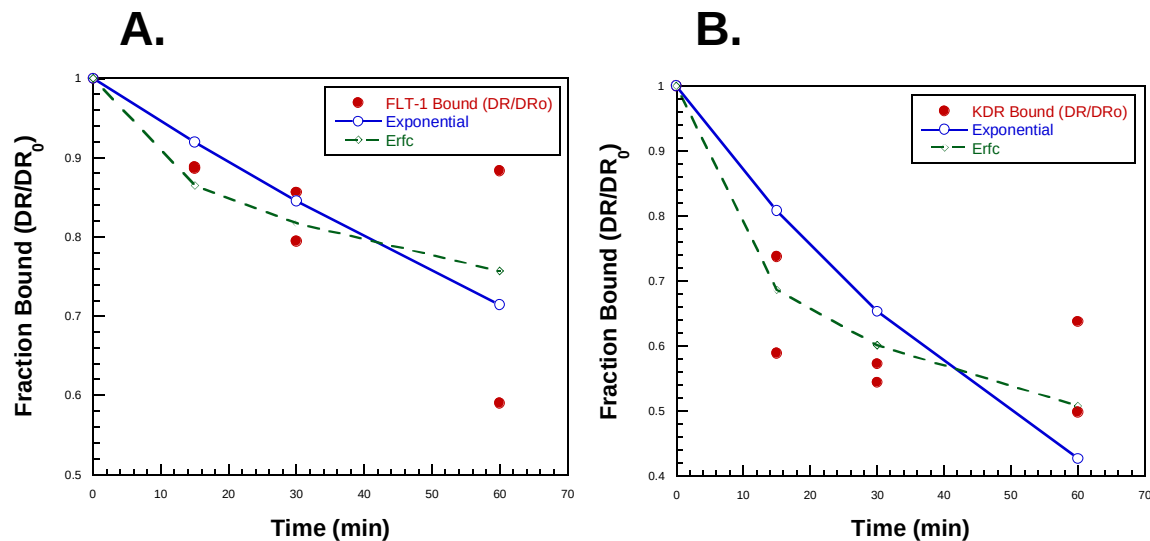


Figure 11. Comparison of different fitting methodologies with experimental radioligand data (solid circles) for VEGF-A dissociation from Flt-1 (A) and KDR (B). (A) For Flt-1, the solid blue curve represents the best exponential fit, with a dissociation rate $k_{-1} = 0.0056 \text{ min}^{-1}$. The green dashed curve represents the best fit using the erfc corrected equation. (B) For KDR, the solid blue curve represents the best exponential fit, with a dissociation rate $k_{-1} = 0.0142 \text{ min}^{-1}$, and likewise, the green dashed curve represents the best fit using the erfc equation. The experimental represents to two different experiments with triplicate measures at each point within each experiment.

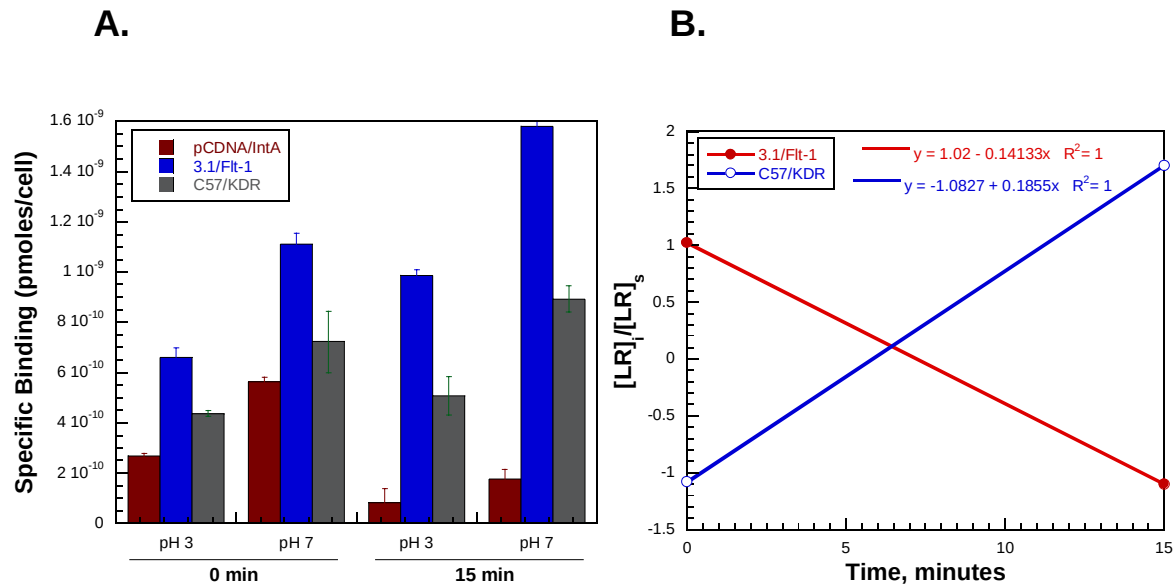


Figure 12. Internalization of [¹²⁵I]VEGF by control and KDR or Flt-1 expressing cells and the corresponding In/Sur analysis of [¹²⁵I]VEGF for obtaining internalization parameters. **(A)** Cells were preincubated with radiolabeled VEGF at 4°C for 3-4 hours to establish steady-state, equilibrium binding, after which the cells were quickly warmed to 37°C to allow for receptor internalization. The acid (pH 3) or neutral (pH 7.4) wash steps allow for internal (acid-stable) and surface bound (acid labile) ligand to be distinguished, thereby allowing for the data to be transformed. Values shown in the figure represent averaged values from experiments with control, Flt-1, and KDR expressing cells performed 1, 2, and 3 times, respectively. **(B)** A linear interpolation of the LR_i/LR_s versus t , time data yields a slope (k_e) of 0.1855 min⁻¹ for KDR. A negative slope for Flt-1 indicates that no internalization was measured for Flt-1 complexes.

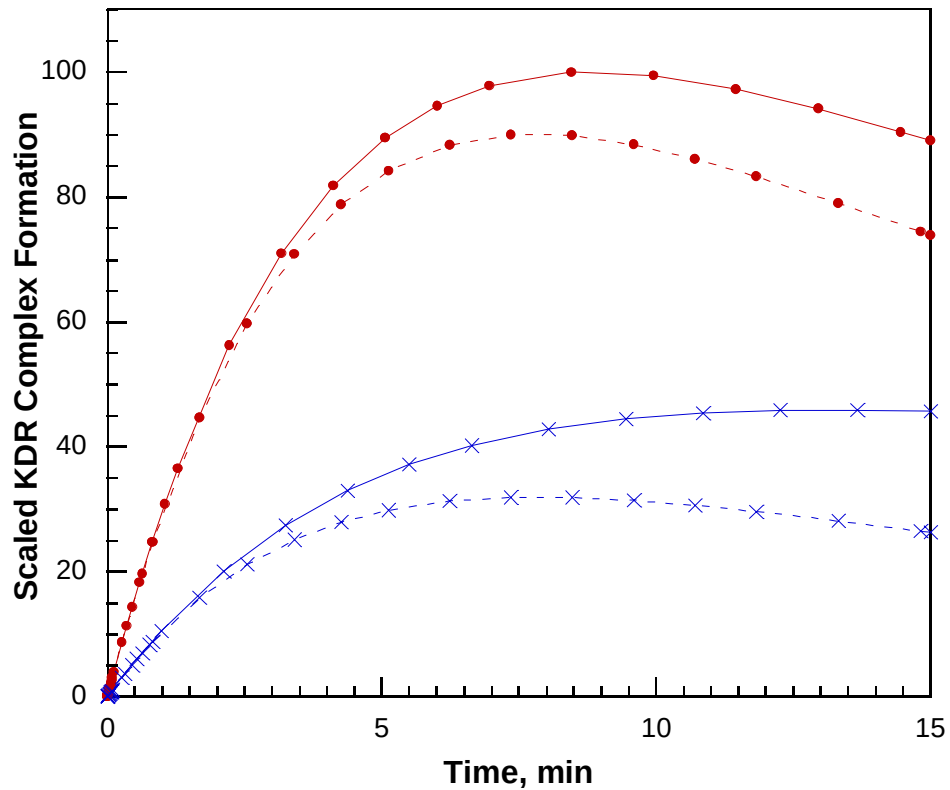


Figure 13. KDR binding levels for base model. (●) KDR-Va and (x) KDR-Ve complexes are shown as a function of time following 15 min of Va (595 pM) and Ve (714 pM) incubation. Solid lines (—) represent complex formation when each ligand is incubated with KDR separately, *in silico*, while dashed lines (- - -) represent complex formation when both ligands are incubated simultaneously. Values are scaled to maximal KDR/Va complex formation.

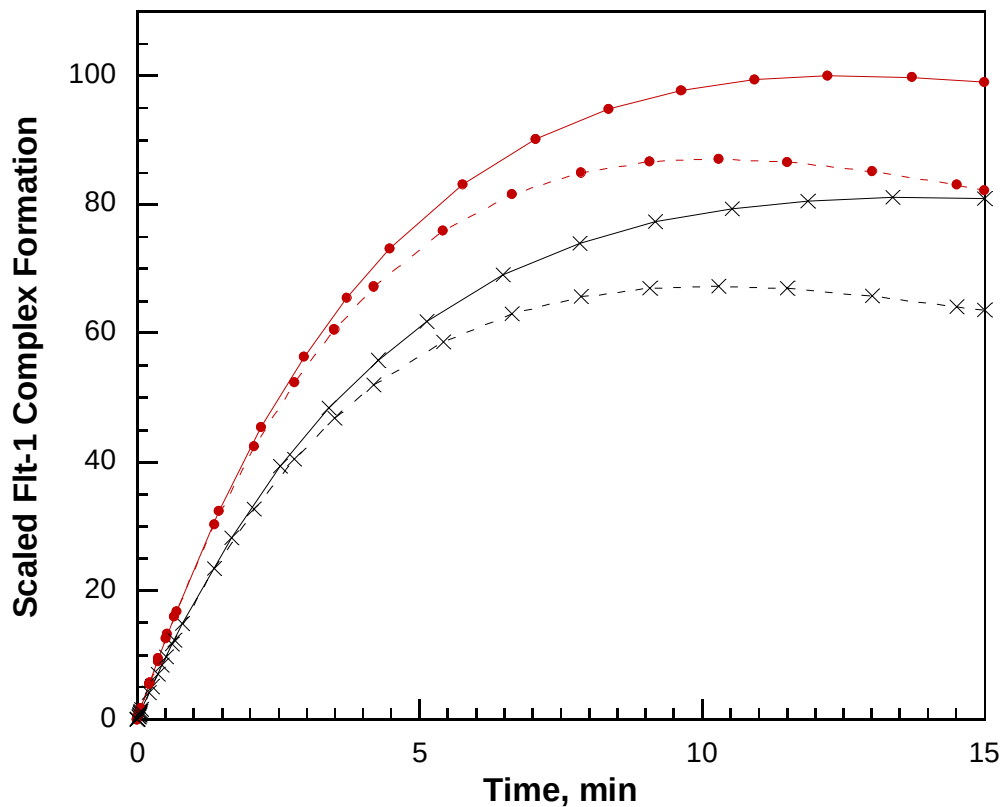


Figure 14. Flt-1 binding levels for base model. (●) Flt-1/Va and (x) Flt-1/PI complexes are shown as a function of time following 15 min of Va (595 pM) and PI (658 pM) incubation. Solid lines (—) represent complex formation when each ligand is incubated with Flt-1 separately, *in silico*, while dashed lines (- -) represent complex formation when both ligands are incubated simultaneously.

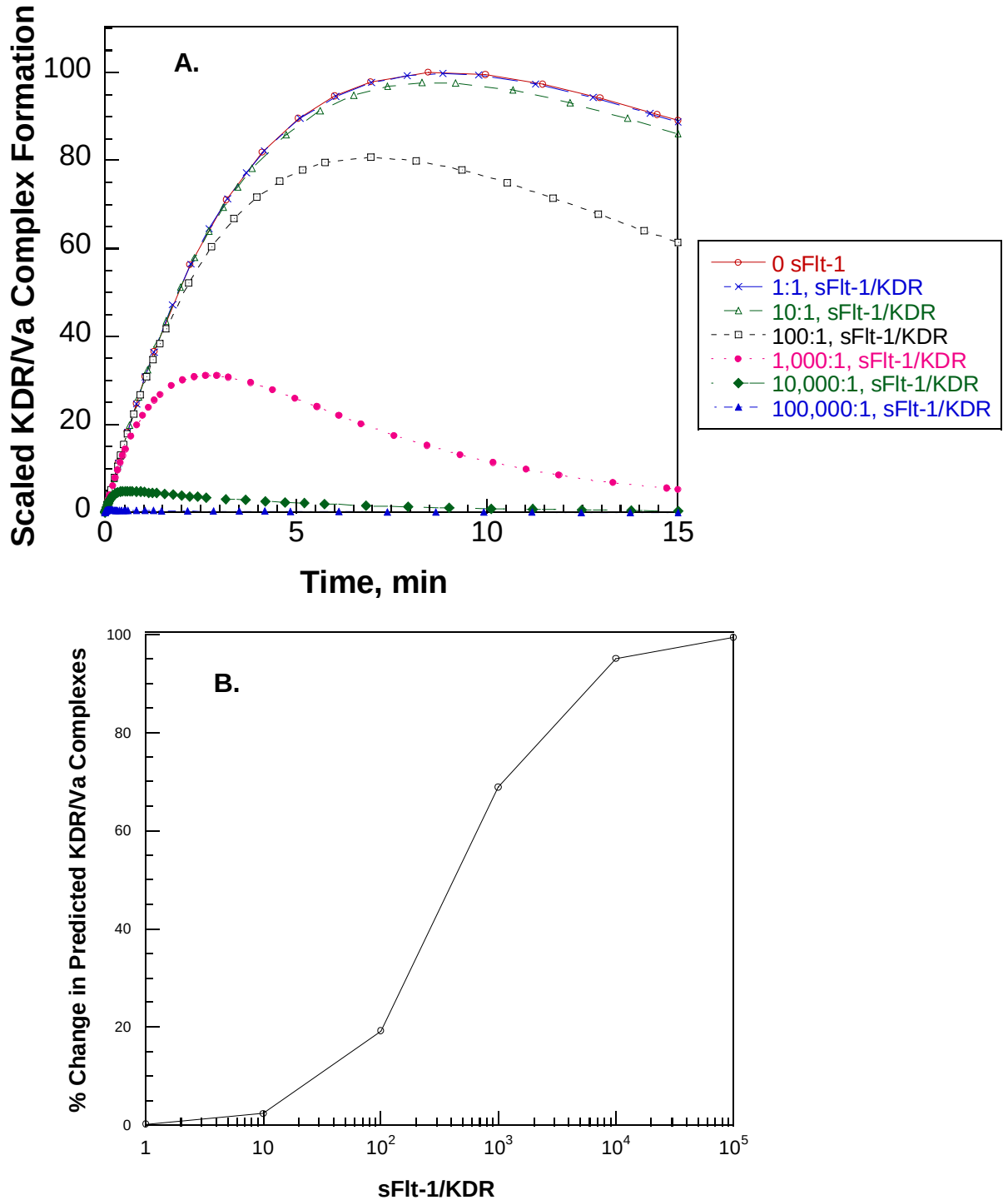


Figure 15. Effect of sFlt-1 addition to KDR/Va complex formation. **(A)** Stepwise increases in the ratio of sFlt-1 to KDR did not result in measurable inhibition until the ratio reached 100:1 (#/cell), at which there was an ~20% decrease in peak complex formation. **(B)** The ratio of sFlt-1/KDR—a dimensionless quantity when expressed as sites/cell—produces a sigmoidal response in the percentage change in maximal complex formation.

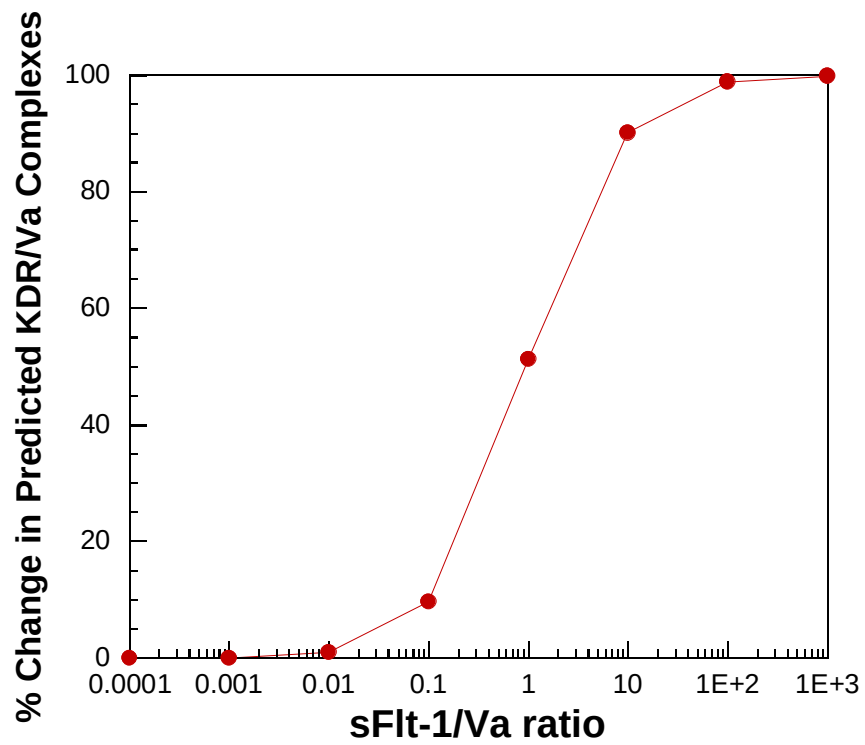


Figure 16. Effect of sFlt-1:Va ratio on maximal KDR/Va complex formation. The ratio of sFlt-1/Va produces a sigmoidal response in the maximal change in complex formation.

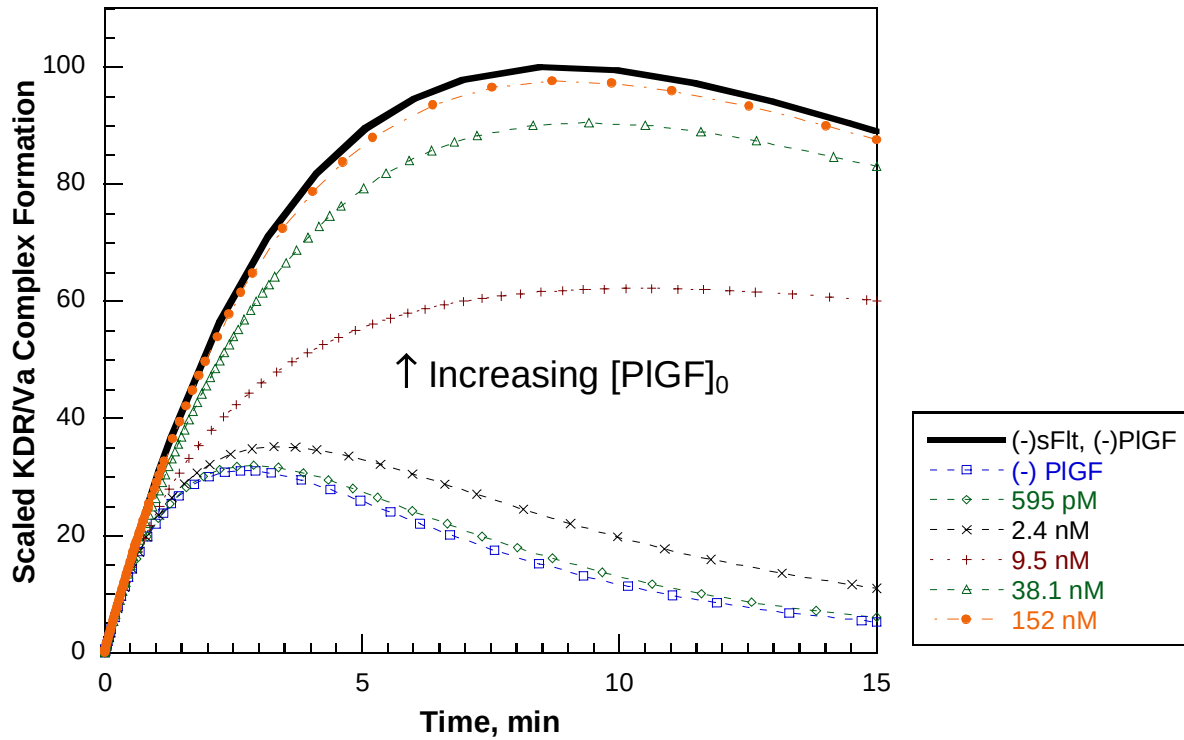


Figure 17. Predicted reversal by PIGF of sFlt-1 inhibition of the formation of KDR/Va complexes. The number of KDR/Va complexes/cell in the absence of either sFlt-1 or PIGF is depicted by the thick black line. With the addition of sFlt-1 at a ratio of 1,000:1 to KDR, the predicted complex formation is decreased by over 70%, but this trend is reversed when PIGF is increased. With PIGF at 152 nM (256 x [Va]₀), virtually all the Va has effectively “shifted” to KDR and original KDR/Va complex predictions are virtually restored.

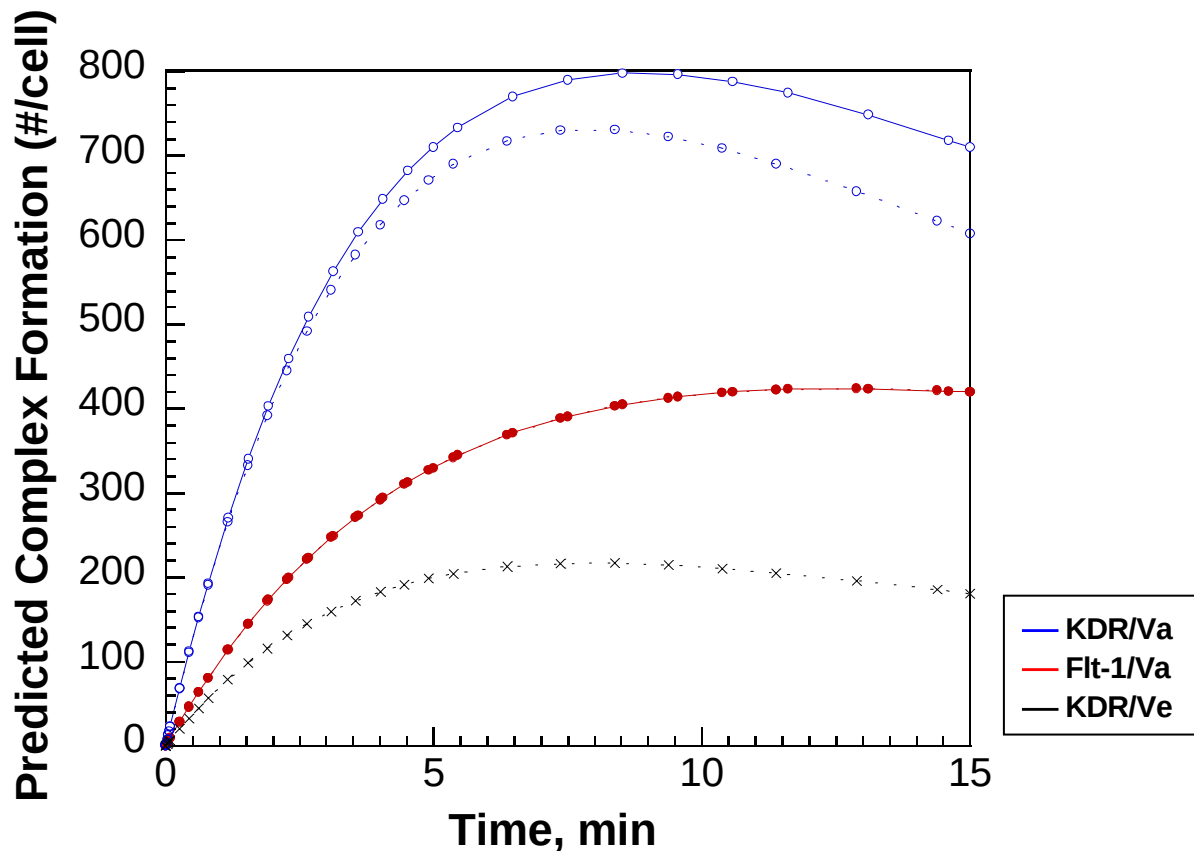


Figure 18. Ligand-receptor complex formation due to VEGF-A plus VEGF-E, or VEGF-A alone. The number of KDR/Va complexes (o) and Flt-1/Va complexes (•) per cell when only VEGF-A is added are shown with the solid (—) blue and red lines, respectively. When equimolar Ve_0 is included together with Va, the change in the number KDR/Va and Flt-1/Va complexes per cell is indicated with the dashed (--) lines. Also shown, in this case, are the newly formed KDR/Ve complexes (x) as indicated by the dashed black line. Note, the lines representing Flt-1/Va complex formation simulations are superimposed.

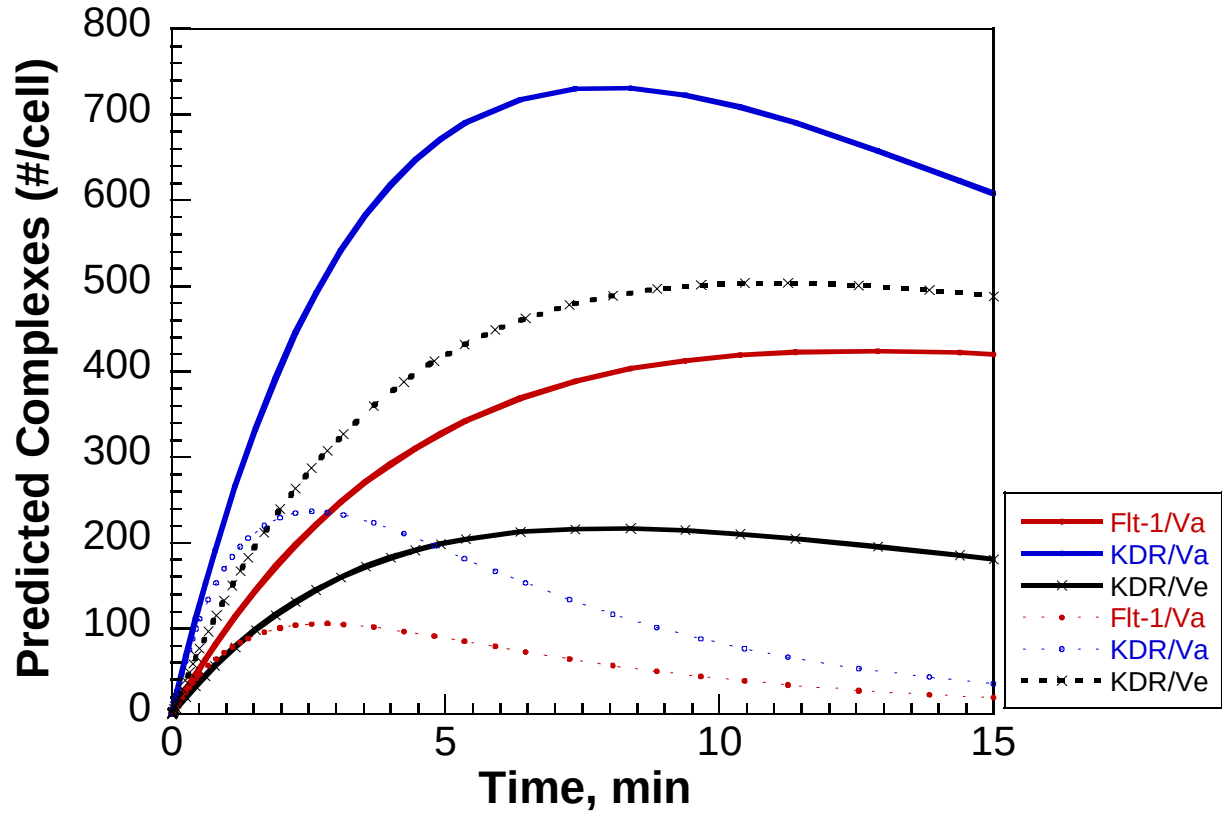


Figure 19. Optimizing predicted KDR/Ve complex formation with the addition of sFlt-1. With no sFlt-1, and equimolar concentrations of Va_0 and Ve_0 present in the system, the predicted number of KDR/Va (o), Flt-1/Va (•), and KDR/Ve (x) complexes per cell are shown with the solid (—) blue, red, and black lines, respectively. The predicted number of KDR/Ve complexes increases dramatically when Ve_0 is doubled along with a corresponding introduction of sFlt-1 to bind available VEGF-A. The predicted complex formations, in this case, are indicated by the dashed lines (---).

CHAPTER 3: TESTING MODEL PREDICTIONS

INTRODUCTION

There are many studies in the available literature that discuss the targeting of VEGF receptors for antiangiogenic therapies, and many also indicate that sFlt-1 might be employed as a feasible approach for inhibiting tumor angiogenesis and growth. While many have employed *in vivo* approaches to look at the inhibition of angiogenic events, there are gaps in the literature when it comes to studying the role sFlt-1 plays in events that happen in response to VEGF signaling. There have not been any studies, to date, that have directly measured the level of VEGF activation achieved by KDR when sFlt-1 is competing within an *in vitro* system to bind available ligand. Most of the published studies have probed events that happen long after the binding of VEGF (e.g., cell proliferation), and none have attempted to directly quantify the ligand-induced phosphorylation of KDR in the presence of sFlt-1. The goals of the experiments presented in this ensuing chapter are two-fold. First, the studies presented here directly measure receptor activation and signaling in the presence of a potent antagonist. The perturbations in intracellular signaling events are quite dramatic when sFlt-1 is inhibiting VEGF receptor activation. Second, an overall aim of this study was to develop a mathematical model that was testable with an *in vitro* system. This represents a unique attempt at modeling the VEGF system because efforts are made to replicate, as closely as possible, the *in silico* modeling system with the *in vitro* experimental conditions. The same clonal cell lines used to determine model parameters are used to test the model with *in vitro* experimentation. This is of great value for determining the accuracy of the model predictions.

METHODS

Expression and purification of rmsFlt-1

In this study, recombinant mouse sFlt-1 (rmsFlt-1) was expressed in the baculovirus expression system (Pharmingen, San Diego, CA) in order to obtain a large amount of rmsFlt-1 and allowing its inhibitory role to be explored.

Construction of msFlt-1 transfer vector. Approximately 0.5 µg of purified pcDNAIntA-msFlt-1 (Fig. 20, previously prepared [Huckle & Roche, 2004]) was digested with *EcoRI* (20 units with 1x NEBuffer *EcoRI*) in a reaction volume of 20 µl at 37°C for 1 hour. Separately, 0.6 µg of pVL1392 (Pharmingen baculovirus transfer vector) containing a stuffer fragment was digested with *EcoRI* and dephosphorylated by addition of 10 units of Calf Intestinal Alkaline Phosphatase (CIP) and further incubation at 37°C for 1 hour (Fig. 21). These digests were fractionated on a 0.7% agarose gel (0.7% agarose [w/v] in 1x Tris-Borate-EDTA [TBE]), stained with ethidium bromide (EtBr; 1.5 µg/ml in ddH₂O), and viewed by a ultra-violet transilluminating light box. Molecular size markers (λ -HindIII plus ϕ X 174-HaeIII, Life Technologies™) were also included on the gel for size comparison purposes. Fragment groups corresponding to the desired sized vector fragments were then cut from the gel, purified by adsorption to silica beads (QIAEX II Gel Extraction Kit), and eluted with 20 µl ddH₂O.

Purified msFlt-1 cDNA (approximately 200 ng) was combined with gel-purified, linearized, dephosphorylated pVL1392 (approximately 60 ng) and ligated with 1 unit of T4 DNA Ligase (Life Technologies) in a 20 µl reaction volume overnight at room temperature. A ligation mixture was also set up containing only the linearized, dephosphorylated pVL1392 transfer vector. Ligation mixtures (1.5 µl) were then transformed into a 25 µl suspension of chemically competent DH5 α cells (Subcloning Efficiency™, Life Technologies) on ice for 30 minutes. The cells were then heat-shocked at 37°C for 20 seconds before returning to ice for 2 more minutes. S.O.C. medium (475 µl; Life Technologies) was added to the cells and incubated at 37°C for 1 hour. Following this step, 100 µl of this mixture was plated onto a 100 mm LB-amp plate (Luria-Bertani [LB] agar plus ampicillin at 100 µg/ml) and incubated overnight at 37°C.

Multiple colonies (5 vector + insert, 1 vector only) were picked from the plates and inoculated into a 1 ml LB-amp broth culture (LB broth with ampicillin at 100 µg/ml). These were then incubated overnight at 37°C while shaking at 200 rpm. After incubation, cells were pelleted from 200 µl of each LB-amp culture (14,000 x g, at room temperature), resuspended in 100µl 1x TE and heated at 95°C for 5 minutes. Aliquots

(1 µl) of the TE lysates were screened by polymerase chain reaction (PCR) for the presence of the full msFlt-1 insert (primers BH436/BH437; see Appendix for primer sequences) and for the orientation of the insert (primers BH436/BH261). Each PCR reaction (20 µl) contained primers at 0.25 µM, and 2X *Taq* PCR Master Mix (200 µM dNTPs, 1.5 mM MgCl₂, 0.5 units *Taq* DNA polymerase, and Tris HCl based buffer; QIAGEN). After amplification, the products were fractionated on 2% agarose gel and stained with EtBr as previously described.

One of the 5 subcloned cultures that gave rise to products of the predicted size and desired orientation was streaked onto an LB-amp plate and incubated overnight at 37°C. One well-isolated colony from this plate was used to inoculate a 100 ml LB-amp culture and was incubated overnight at 37°C with shaking (170 rpm). For purification and elution of the plasmid, an adapted protocol was used with the QIAGEN Plasmid Midi/Maxi purification kit. The culture was centrifuged at 6,000 x g for 15 minutes. The pellet resuspended in 4 ml of 50 mM Tris-Cl (pH 8.0), 10 mM EDTA, and 100 µg/ml RNase A (Buffer P1). Resuspended cells were lysed with 4 ml Buffer P2 (200 mM NaOH and 1% SDS [w/v]), and protein and bacterial genomic DNA precipitated with 4 ml chilled neutralization Buffer P3 (3.0 M potassium acetate, pH 5.5). This mixture was centrifuged at 15,000 x g for 30 minutes at 4°C. The supernatant, containing plasmid DNA, was recentrifuged at 20,000 x g for 15 minutes at 4°C. Plasmid was adsorbed to a silica resin (QIAGEN-tip 100) pre-equilibrated with 4 ml Buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol [v/v], and 0.15% Triton® X-100 [v/v]), washed twice with 10 ml Buffer QC (1.0 M NaCl, 50mM MOPS, pH 7.0, and 15% isopropanol [v/v]). The DNA was eluted with 5 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, and 15% isopropanol [v/v]) and precipitated by adding 3.5 mls of room-temperature isopropanol. The DNA pellet collected by centrifugation (15,000 x g for 30 minutes at 4°C) was gently washed with 1.2 mls of 70% ethanol and recentrifuged. The supernatant was decanted, and the pellet was left to air dried for approximately 10 minutes. The DNA pellet was redissolved in 150 µl of DNase/RNase-free H₂O. The approximate concentration and purity of the DNA was estimated by measuring the absorbance at 260 nm and 280 nm ([DNA] µg/ml = $A_{260} \times \text{dilution factor} \times 50 \text{ µg/ml}$ [extinction coefficient]). This plasmid was designated pVL1392-msFlt1 #MR3705.

Generation of recombinant baculovirus. In order to generate recombinant baculovirus, 2×10^6 Sf9 insect cells were seeded onto two 60-mm tissue culture plates (Falcon). Initial cell confluence was 50–70%. Next, 0.5 µg BaculoGold™ DNA (Pharmingen) and 4 µg of pVL1392-msFlt1 was combined in a microcentrifuge tube. This mixture sat for ~5 min at room temperature before adding 1 ml of Transfection Buffer B, containing 25 mM Hepes, pH 7.1; 125 mM CaCl₂; 140 mM NaCl. The old medium was aspirated from the cells on the experimental co-transfection plate and replaced with 1 ml of Transfection Buffer A, containing Grace's Medium supplemented with 10% FBS, pH 6.0–6.2. Similarly, the old medium was aspirated from the negative control plate and replaced with 3 mls of fresh TNM-FH medium (BD Baculogold™). Nothing else was added to this plate. One ml of Transfection Buffer B/DNA solution was added, drop by drop, to the experimental co-transfection plate. After every 3–5 drops, the plate was gently rocked back and forth to ensure complete mixture of the transfection solution and medium. Both plates were then incubated at 27°C for 4 h. After 4 h, the medium was removed from the experimental cotransfection plate, and 3 ml of fresh TNM-FH medium was added to gently wash away residual Transfection buffer solution. Again, the medium was removed and 3 ml of fresh TNM-FH medium was added to the plate and the plate was incubated at 27°C. The plates were checked daily for signs of infection, and the experimental co-transfection plate was compared to the negative control. Infected cells characteristically appear much larger than uninfected ones, stopped dividing, and will often detach from the plate.

After 5 days, the supernatant of the experimental co-transfection plate was collected, and recombinant viral titer was determined by an end-point dilution assay. In brief, Sf9 cells were seeded at approximately 1.3×10^6 cells per well on a 6-well plate and allowed to attach firmly. The medium in each well was replaced with fresh TNM-FH, and serial dilutions of recombinant virus supernatant were added to each well. To separate wells, 200, 20, 2, 0.2, 0.02 or 0 µl of the supernatant were added. The cells were incubated at 27°C for three days and examined for signs of infection. Conditioned medium was recovered for analysis of sFlt-1 protein by western blot. The first transfection supernatant was amplified to produce high titer virus stocks that could be used for optimal recombinant protein production. After amplification, another end-point

dilution assay was performed to determine the viral titer. After two rounds of viral amplification, the desired viral titer was reached (3×10^8 plaque forming units/ml) and the supernatant containing the amplified viral stock was harvested and centrifuged at $260 \times g$ to remove the cellular debris. The virus supernatant was filter-sterilized (0.2μ) and stored in a sterile tube at 4°C .

sFlt-1 protein production. For production of rmsFlt-1, two 15 cm tissue culture plates (Falcon 3025) were seeded with $\sim 1.6 \times 10^7$ cells per plate. Fresh TNM-FH medium was added to each plate to make up a total of 30 ml media per plate. The seeded Sf9 cells were inoculated with high titer recombinant viral stock at a multiplicity of infection of 10. Cells were incubated for 3 days at 27°C . After three days, the supernatants from the plates were collected and centrifuged at $260 \times g$ for 5 min to remove cells and cellular debris.

The rmsFlt-1 present in the supernatant was purified batchwise by adsorption to immobilized Heparin (Heparin agarose resin, Pierce, Rockford, IL). Approximately 2 ml of the 50% bead slurry was washed twice in 10 mls of ddH₂O, centrifuged for 5 minutes at $150 \times g$, and resuspended in 1 ml ddH₂O. The suspension was added to the ~ 60 ml of clarified supernatant from the viral inoculation and then incubated overnight at 4°C with end-over-end mixing. After this incubation, the bead suspension was transferred to a 5 ml disposable chromatography column (Evergreen Scientific, Los Angeles, CA) and washed 4 times with 5 ml of PBS. The recombinant protein was eluted from the column with 4 ml fractions of PBS that contained increasing concentrations of NaCl (100-1000 mM in 100-mM increments). Elution fractions were saved at 4°C for analysis of rmsFlt-1.

Western Blotting for rmsFlt-1. SDS-PAGE was performed using Criterion precast gels as described in Chapter 2. - Aliquots (15 μ l) of each heparin-agarose fraction were mixed with an equal volume of 2x Laemmli Sample Buffer, heated for 5 minutes at 95°C , and loaded into precast 7.5% SDS-polyacrylamide gels along with ProSieve[®] color molecular weight. After electrophoresis, separated proteins were transferred to Immobilon FL and blocked as already described. Membranes were probed for 1 hour

with 0.1 µg/ml anti-mVEGF R1; R&D Systems), followed by (IRDye800 Conjugated Affinity Purified Anti-GOAT IgG (H&L) secondary antibody prior to imaging in the Odyssey® system.

After determining the two elution fractions that contained the highest concentration of rmsFlt-1, the fractions were pooled, ~0.1% BSA was added to the stock, and the recombinant protein was stored at 4°C. The final concentration of rmsFlt-1 was determined using the Quantikine® Mouse Soluble VEGF R1 Immunoassay (MVR100; R&D Systems, Minneapolis, MN).

Phospholipid metabolite assay

With the literature suggesting that the receptors for VEGF can associate with and phosphorylate the gamma-isozyme of phosphoinositide (PtdIns)-specific phospholipase C γ [43], it is likely that there is a mechanistic link between the activation of PtdIns hydrolysis and VEGF binding to these receptors. This link has been demonstrated in previous studies with similar RTK activation by growth factors such as epidermal growth factor [145]. Therefore, the coupling between PtdIns hydrolysis and VEGF activation in the transfected HEK293 cells was examined. The methodology was adapted from a study by Huckle et al., 1989 which examined gonadotropin-releasing hormone (GnRH) and how it regulated pituitary gonadotropin release by receptor-mediated PtdIns hydrolysis. [146].

KDR- or Flt-1 expressing cell lines were washed once with 10 ml of Dulbecco's Phosphate Buffered Saline without Ca²⁺ or Mg²⁺ (DPBS), trypsinized and re-plated on BD BioCoat™ 24 well poly-L-lysine plates at ~1 x 10⁵ cells/well. Cells were grown to ~70% confluence (1-3) days. Labeling of cellular inositol phospholipids was initiated by supplementing modified culture medium (0.5 ml/well inositol-free DMEM [ICN], 10% FBS, 50 µg/ml gentamycin) with [³H]inositol (20 Ci/mmol; Amersham) at a concentration of 10 µCi/ml. The cells were then incubated in an appropriate 5% CO₂/37°C incubator for 24 hrs. The pre-labeling medium was replaced with 0.5 ml/well assay medium (Hank's balanced salt solution containing 0.1% BSA, 25 mM Hepes pH 7.5, and 10 mM LiCl) and incubation continued at 37°C for 30 min. Depending on the experimental design, this assay medium may have been supplemented with various

concentrations of previously purified rmsFlt-1. Phosphoinositide hydrolysis was stimulated by direct addition of appropriate agonists (55 μ l/well of 10X concentrated working stocks), and incubation was continued at 37°C for up to 1 hr. The stimulation period was terminated by removing the assay medium and lysing the cells by the addition of 0.5 ml ice-cold 0.1 M formic acid and freezing at –20°C overnight.

[³H]Inositol phosphates in cell lysates were isolated by anion exchange chromatography and measured by liquid scintillation counting [146]. For this, plates were thawed and the contents of all wells were transferred to minicolumns packed with 0.3 ml Dowex 1-X8 anion exchange resin, formate form (BioRad). Flow-through from the columns was collected into a common reservoir, and the columns were washed (3 x 1ml) with 0.1 M formic acid. [³H]Inositol phosphates were eluted directly into 6 ml scintillation vials by addition of three sequential 1 ml additions of 0.1 M formic acid/1.2 M ammonium formate. Three mls of Ultima-Flo™ AF scintillation cocktail (PerkinElmer, Boston, MA) were added to each vial. The vials were shaken vigorously, and ³H cpm was measured in a scintillation counter (Beckman Coulter LS6500, Fullerton, CA).

Phosphorylated receptor experiments

The transfected clonal cell lines described in the preceding chapter were used to test receptor activation. Receptor activation, as indicated by tyrosine phosphorylation, was quantified by western blotting in order to determine the success with which the model can predict this indicator of receptor binding. First, dose response measurements were performed on homogeneous cell populations (either Flt-1 or KDR expressing cells) to determine the amount of ligand necessary to achieve adequate activation (via phosphorylation). In addition, the empty vector control cells were tested with the ligands to determine if there was background that may be attributable to endogenous receptor expression. All measurements were made at 37°C. After dose response evaluations for the cell populations, time-course experiments were performed to determine the optimal time for receptor activation. A similar approach was also taken with sFlt-1 present in the system to determine the level of inhibition that occurs for phosphorylated receptors.

SH2 binding assay: Cell lines, antibodies, and GST fusion proteins. In cells that respond to growth factors through RTKs, among the most frequently phosphorylated proteins are the kinases themselves. Autophosphorylation of the intracellular kinase domains on these receptors is critical for downstream signaling after growth factor binding. Earlier studies have suggested that many of the molecules involved in intracellular signaling stably bind to tyrosine-phosphorylated RTKs via “src homology 2” (SH2) domains but not to non-phosphorylated receptors [147]. Phospholipase Cy isoforms contain SH2 domains, which enable protein/protein interactions and mediate the association of phospholipase Cy with autophosphorylation sites on activated RTKs [148]. It follows that activated KDR should be able to be precipitated with proteins that contain certain SH2 domains. This is the approach that was taken to measure activated VEGF receptors *in vitro*.

Prior to the activation experiment, a protein composed of glutathione S-transferase (GST) fused to the C-terminal SH2 binding domain (SH2_C) derived from mouse phospholipase Cy was expressed and purified from *E. coli*. In brief, a 10 ml LB/Amp culture of cells transformed with pGEX-SH2_C #21 (Huckle and Earp, unpublished) was grown overnight at 37°C with shaking at 170 rpm. The culture was then diluted to 100 ml with LB without ampicillin and grown at 37°C with shaking at 170 rpm for 4 hrs. One ml of this culture was removed and added to a tube for later protein analysis. For this, the 1 ml sample was spun in a microcentrifuge (Eppendorf 5415C) for 5 minutes at 14,000 xg. The supernatant was aspirated and the cell pellet was resuspended in 100 µl ddH₂O. One hundred µl of 2x Laemmli buffer (BioRad) was added and the sample was stored at -20°C for later use. After removal of the 1 ml sample, protein expression was induced for one hour with the addition of 0.1 mM (final concentration) isopropyl thiogalactoside (IPTG), an artificial inducer of the Lac operon. Again, 1 ml of this culture was removed for later protein content analysis. The entire 100 ml culture was spun at ~6,000 x g in a GSA rotor for 15 minutes at 4°C. The supernatant was discarded and the pellet was held on ice. Cells were resuspended in 10 ml of B-PER[®] Reagent (Pierce, Rockford, IL) and gently agitated for 10 minutes. The suspension was centrifuged at 15,000 × g for 15 minutes, and the supernatant containing soluble GST-fusion protein was transferred to a new tube. Approximately

100 μ l of this supernatant was removed and mixed with 100 μ l of 2x Laemmli buffer (BioRad®) for later use to determine protein content. The pellet was then transferred to a 15 ml glass Dounce homogenizer (Kontes Glass Company, Vineland, NJ) and homogenized with 10 strokes with the B pestle. The homogenate was centrifuged at $15,000 \times g$ for 15 minutes, the supernatant saved and the pellet rehomogenized with an additional 10 ml P-BER. The supernatants were pooled for purification over immobilized glutathione.

Glutathione agarose bead prep Glutathione-agarose beads (Pierce) were thoroughly mixed to resuspend the resin, and ~500 μ l of Glutathione beads was washed twice with DPBS. Washed beads were added to the pooled supernatants and incubated overnight at 4°C with end-over-end mixing on a Labquake® (Barnstead Thermolyne). After this incubation, the mixture was centrifuged at $\sim 1,250 \times g$ for 5 minutes, the supernatant was removed, the resin was washed twice with 10 ml DPBS and resuspended with ~500 μ l of DPBS. The total volume of the 50% slurry was approximately 1 ml and it was stored at 4°C until later use. A representative SDS-PAGE gel showing the various stages of purification of the GST fusion protein—along with protein content in final bead samples—is shown in Figure 28.

VEGF binding and precipitation of activated KDR

General methods. KDR-overexpressing cells (C57) were seeded and grown to confluence in 60-mm poly-L-lysine-coated dishes. Cells were activated at 37°C with 25 ng/ml of VEGF isoforms in the presence or absence of rmsFlt-1 at a final concentration of 50 ng/ml. Activations were terminated by lysing the cells with ice-cold cell lysis buffer containing, among other components, Triton X-100 (for solubilization of transmembrane VEGF receptors) and the tyrosine phosphatase inhibitor sodium-orthovanadate (1 mM final) which preserved the phosphorylation states of intermediates of interest. Lysates were mixed for ~1 hour at 4°C with end-over-end mixing, and centrifuged at $14,000 \times g$ for 10 minutes. For precipitation of activated KDR, 10 μ l of GST-SH2_C beads (20 μ l of bead slurry) was added to the cell lysate supernatants and agitated overnight at 4°C. The bead suspensions were centrifuged at $8,000 \times g$ for 10 minutes and the

supernatants were removed. The samples were washed 3 x 1 ml in the ice-cold lysis buffer (containing Na⁺-vanadate). The packed bead were resuspended after addition of 30 µl of 2x Laemmli Sample Buffer and heated for 5 minutes at 95°C.

Western Blotting for receptors

SDS-PAGE was performed using 30 µl of each sample loaded into precast 7.5% SDS-polyacrylamide Criterion™ gels as described previously. Proteins were transferred to PDVF membranes (Immobilon-FL) and the membranes blocked overnight at 4°C. The activated, phosphorylated KDR was probed by sequential incubation with antiphospho-tyrosine mouse monoclonal antibody P-Tyr-100 (#9411, Cell Signaling Technology) and the IRDye800 conjugated donkey anti-mouse IgG (H&L; Rockland®, 1:2,500 in blocking buffer supplemented with 0.02% Tween-20). After final washing steps, the membranes were scanned with the Odyssey® Imaging System, and integrated intensity analysis of protein bands of purified protein was performed by Odyssey® imaging software.

RESULTS

Characterization of receptor-effector coupling of KDR cells

One of the primary objectives for this study was to effectively measure the amount signaling KDR complexes formed over time with the addition of VEGF *in vitro*. While it was demonstrated that the clonal cells expressed their receptors in measurable levels (Chapter 2), it was still desirable to characterize the integrity of receptor-effector coupling in both the KDR and Flt-1 cells. Measuring the amount of VEGF-promoted accumulation of inositol phosphates proved to be a very useful method for this purpose. Membrane phosphoinositides prelabeled with [³H]inositol were cleaved in the ligand-stimulated signaling events immediately following VEGF binding. Due to the direct inhibition of enzymes required for recycling of these inositol phosphates, they accumulated in the cells over the duration of the experimental procedure (10-60 mins).

Only the KDR-expressing cells showed a measurable response to VEGF stimulation. Concentration-effect curves for 60-minute stimulation VEGF-A promoted accumulation were considered characteristic of data fitting models for law of mass

action interaction at a single receptor site (Figure 22). Similarly, the concentration curve for VEGF-E paralleled that of VEGF-A, albeit at a lower potency, and was consistent with the interaction of this ligand with a single receptor site in the KDR cells (Figure 22). There was no evidence of biphasic interaction on these cells. From these data, it is logical to conclude that KDR is the only receptor present on these cells that generates a signal in response to VEGF stimulation. These data were normalized by subtracting off basal values, and within each experiment the values were expressed as a percentage of the maximal elicited response. In this way the data could be directly compared between experiments. The plotted data represent the averaged normalized values for VEGF-A & -E of three experiments performed in triplicate. The averaged values were analyzed using GraphPad Prism, by fitting a one-site, log(agonist) vs. normalized response, nonlinear regression model with a variable slope that does not assume a standard slope but rather fits the Hill Slope from the data. The nonlinear regression model indicated that VEGF-A and VEGF-E were agonists with average EC₅₀ values of 14 ± 1.2 ng/ml (340 pM) and 21 ± 1.1 ng/ml (610 pM), respectively. The EC₅₀ value for VEGF-A is well within the physiologically relevant concentration range for a variety of VEGF-induced physiologic processes [149, 150]. From these experimental calculations, it was determined that, in all remaining experiments, VEGF-A & -E would be used at a concentration of 25 ng/ml. This was approximated to be slightly higher than the calculated EC₅₀ values—hence eliciting a readily-measurable response—while still residing on the linear dynamic portion of the concentration curves for each respective ligand. Because this method of investigating VEGF binding and signaling is novel in these cell types, there is a lack of published values to which our findings may be compared. VEGF-A is generally observed to be a more potent ligand for cellular activation than VEGF-E, and these results were no exception to those observations. Furthermore, the stimulation of phosphatidylinositol hydrolysis demonstrated that the KDR-expressing cells are undoubtedly generating intercellular signals in response to KDR ligands. It was concluded that they are adequate cells for use in receptor phosphorylation experiments.

Characterization of receptor-effector coupling in Flt-1 cells

To ensure that the cell lines were devoid of other VEGF receptors capable of generating an inositol phosphate signal, the Flt-1 cells and pcDNAIntA cells were screened with various ligands including, but not limited to, VEGF-A & -E. There was no significant growth factor-promoted inositol phosphate accumulation observed in either cell type when normalized by subtracting basal levels (Figure 23). Both cell lines, however, responded robustly to 0.1mM ATP [151], suggesting physiological responses through activation of a family of G protein-coupled receptors and the ability to generate signaling events upon ligand binding. The cells behaved in the manner anticipated—with no significant signaling response to the KDR-activating growth factors. There was a small, measurable response in the Flt-1 cells to both PlGF and bFGF, with total inositol phosphate accumulation representing approximately 29% and 20% of the maximal ATP response, respectively. With the likelihood of some small amount of signaling occurring in response to VEGF-related ligands binding to Flt-1, it is entirely possible that these small responses can be accounted for. These results should be qualified by recognizing that, when stimulated with 0.1 mM ATP, the KDR cells also demonstrated a response similar in magnitude to that in the Flt-1 and pcDNAIntA cells; however, the response to VEGF-A stimulation was approximately 14 times greater than that which was caused by ATP stimulation (data not shown). When compared to the maximal responses of the KDR cells, the Flt-1 & pcDNAIntA cells simply do not respond in any significant way to stimulation by these ligands. In light of these initial screening results the Flt-1 expressing cells and pcDNA/IntA cells were not used for further testing of VEGF-stimulated signaling, and all subsequent experiments were limited to the KDR cells.

Expression and purification of rmsFlt-1

Because the commercially available recombinant sFlt-1 proteins typically are prepared as chimeric proteins that may display altered binding characteristics, the decision was made to express and purify a recombinant mouse form of sFlt-1 (rmsFlt-1) in insect cells. This recombinant protein is expressed, glycosylated, and secreted by the Sf9 cells, and use of this protein in binding and inhibitory studies circumvents any

differences in binding that may be present in the chimeric form of the commercially available product. The rmsFlt-1 transfer vector was successfully subcloned into DH5 α cells, sequenced to determine the orientation of the sFlt-1 insert, and purified using a commercially available plasmid purification kit (data not shown). After two rounds of viral amplification, Sf9 cells were inoculated and rmsFlt-1 was expressed and purified from the cell supernatant. A western blot for sFlt-1 was carried out using samples from each of the serial elution fractions from immobilized heparin (Figure 24). The rmsFlt-1, when compared against the molecular weight markers, migrated to ~86 kDa—a reasonable size when compared to literature values for this protein. The elution fractions containing the most rmsFlt-1 (500 & 600 mM NaCl) were combined, and the amount of rmsFlt-1 was quantified using a commercially available Immunoassay kit for soluble mouse VEGFR1 (R&D Systems, Minneapolis, MN). The final concentration of the Sf9-produced rmsFlt-1 was calculated to be 5.33 μ g/ml, when quantified against the kit control (Figure 25). After determining the concentration of purified rmsFlt-1, it was concluded that this stock solution was adequately concentrated for use in binding and activation experiments for the remainder of this study.

Inhibition of VEGF-stimulated PtdIns hydrolysis

With confidence that the KDR cells responded correctly to ligand stimulation and the other clonal cell lines would not be useful in these assays, the next set of experiments were designed to specifically investigate sFlt-1 inhibition on KDR-mediated signaling events. The rmsFlt-1 should not have an effect on signaling that is stimulated by VEGF-E because it does not bind VEGF-E. The results of a single experiment to profile this effect are shown in Figure 26. VEGF-E, at 25 ng/ml, produced a robust signaling response that was not inhibited by rsFlt-1 (50 ng/ml). No further testing was required to screen the sFlt-1 effect on VEGF-E-stimulated inositol phosphate accumulation.

While sFlt-1 did not adversely affect VEGF-E binding and signaling, it should have a marked inhibitory action on VEGF-A binding and signaling. A series of dose-response experiments were performed, over various time intervals, to determine the IC₅₀ value for the rsFlt-1 on VEGF-A binding. The results of those experiments carried

out over 10 or 60 minutes are shown in Figure 27. The curves were analyzed using GraphPad Prism, by fitting a one-site, log(inhibitor) vs. normalized response, nonlinear regression model with a variable slope that does not assume a standard slope but rather fits the Hill Slope from the data. The results are interesting as there seems to be a time-dependence in the calculated IC_{50} values. The 10 minute incubations produced an IC_{50} value for rmsFlt-1 of approximately 56 ± 1.1 ng/ml (651 pM). In contrast, when incubated over 60 min, the inhibitory effect of rmsFlt-1 on VEGF-A binding demonstrated an IC_{50} value of approximately 16 ± 1.1 ng/ml (180 pM). What could be the cause for differences in the observed IC_{50} values at different time points? First, it could be that the 10 minutes of incubation is just not long enough to produce the measured signaling responses to VEGF-A binding and, therefore, the inhibitory effects of rmsFlt-1 on its binding and activation could be muted in some way. It is clear from the data before it is normalized that total inositol phosphate accumulation over 60 minutes is significantly greater than that for a 10 minute incubation, so perhaps the longer incubation with rmsFlt-1 coupled with VEGF-A provides a more accurate assessment of its true inhibitory nature. While the true reason for the time-sensitive differences in calculated IC_{50} values remains unclear, what can be gleaned from this data is the fact that rmsFlt-1 is a potent inhibitor of VEGF-A binding and signaling and that this is a powerful method for quantifying potential interactions of multiple ligands and receptors as an *in vitro* model system. An interesting observation is that for the 60 minute incubation, with sFlt-1 present at its calculated IC_{50} value (180 pM), the theoretical free VEGF-A present would be 460 pM. This value is slightly higher than the 340 pM EC_{50} value determined for VEGF-A in the previous experiments and demonstrates that the primary inhibitory role that sFlt-1 is playing in the system is by binding up the free VEGF-A.

KDR phosphorylation as a primary end-point to link model predictions

One of the chief aims of the series of *in vitro* experiments performed in this study was to develop a method with which the simulations from the mathematical model could be tested. Because, ultimately, the computational model will produced predictions about the formation of ligand/receptor complexes over time, a similar endpoint had to be

chosen that is quantifiable with a series of cell-based assays. Clonal cell lines that selectively over-express Flt-1 or KDR served as the model systems. The endpoint used to measure complex formation over time was tyrosine-phosphorylated receptors, the content of which were quantified via western blotting. This was chosen as the closest measure of complex formation as, upon ligand binding, the receptors are activated and rapidly become phosphorylated.

In order to attempt to enrich the signal from the phosphorylated receptors present in each sample, a number of different methods were considered. Immunoprecipitation was viewed as a viable option, but upon testing many of the commercially available antibodies, concern was raised that the unphosphorylated receptors were being preferentially immunoprecipitated over the phosphorylated forms (data not shown). To circumvent these difficulties, the method chosen for receptor enrichment was the use of a GST fusion protein which contained a portion of the carboxyl SH2 domain from mouse PLC γ . This protein was adsorbed to glutathione agarose which permitted precipitation of phosphorylated (activated) receptors in each sample. The western blots produced from these samples were probed with antibodies against phospho-tyrosine residues. While this is an effective method to determine the content of phosphorylated KDR in a sample of KDR cells, it serves as a poor method of measuring Flt-1 complexed with any one of various ligands. This is, in part, because Flt-1 is poorly phosphorylated, and it is unclear whether the SH2 binding region of PLC γ even associates with the activated receptor. The only method for directly measuring phosphorylated Flt-1 is with a commercially available antibody directed against phospho-Flt-1 (Oncogene Research Products, Inc., Boston, MA), but the signal produced on western blots was weak, cross-reactive with KDR (data not shown), and the cost prohibitive.

With the preferred method of receptor enrichment identified, a series of experiments were designed for KDR cells to gain a relative measure of the amount of phosphorylated receptors (*i.e.*, complex formation) at various time points with VEGF-A and -E. Each ligand was added to cell monolayers and allowed to activate the cells for a predetermined amount of time. After this the cells were lysed and precipitated with the SH2-agarose beads. Activations at all time points were performed in duplicate and each ligand was tested, independently three times. All resultant western blots were

scanned with the Odyssey[®] imaging system, and the integrated intensities of bands representing phosphorylated KDR were quantified. Basal levels—integrated intensities from the zero time points, with no ligand added—were subtracted from each duplicate measure and the data were normalized to the maximal ligand-stimulated response on each blot. The averaged integrated intensities from each of the three experiments were plotted, and the results of these experiments are shown in Figure 29.

Both ligands demonstrated the formation of activated ligand/receptor complexes, as evidenced by the strong phosphorylated KDR signal. For VEGF-A there was a steady increase in the amount of phosphorylated KDR in each sample that reached its peak at 4 minutes. This was followed by a steady decrease of the phospho-KDR signal to 15 minutes where its level had dropped to 45% of the maximal response. For VEGF-E, there was a slight delay in the formation of VEGF/KDR complexes when compared to activation by VEGF-A. The gradual increase in phosphorylated KDR peaked at 8 minutes, after which the signal dropped off by 27%. It is yet unclear whether this characteristic of the receptor time-course curves can be accounted for by a dephosphorylation event, receptor internalization and degradation, or another unknown mechanism. These options will be discussed further in the ensuing section. This down-slope in the curves representing phosphorylated receptor content poses a particularly interesting challenge when modeling, computationally, the binding and activation of these ligands.

It should be noted that the difference between the two ligands was not only the delayed development of ligand/receptor complexes, but there was a marked difference in the content of phosphorylated KDR receptor complexes formed in response to growth factor stimulation. In all cases, maximal KDR activation (as determined by phospho-KDR) was observed in VEGF-A-stimulated KDR cells. Using this assumption to normalize all the data, the peak levels of activation for VEGF-A and -E, at 4 and 8 minutes respectively, differed by $48\% \pm 19\%$. An unpaired, two-tailed t-test, using GraphPad Prism revealed a significant difference between these means ($P < 0.001$).

Successful inhibition of KDR phosphorylation by rmsFlt-1

One important aim of this study was to quantify the amount of inhibition lent by sFlt-1 on VEGF binding and activation. By controlling the amount of VEGF and sFlt-1 added to *in vitro* cell experiments, the system can be more readily compared to *in silico* simulations of binding and activation. A similar set of time-course experiments was carried out with VEGF-A stimulation in the presence and absence of sFlt-1. The results of these experiments are summarized in Figure 30. As before, basal levels were subtracted from each duplicate measure, for each of three experiments, and the data were normalized to the maximal ligand-stimulated response on each blot. There was evidence of the formation of activated ligand/receptor complexes in the presence and absence of sFlt-1. Without sFlt-1 there was a steady increase in the amount of phosphorylated KDR in each sample that reached its peak at 2 minutes and was followed by a steady decrease of the phospho-KDR signal to 15 minutes where its level had dropped to 15% of the maximal response. When rmsFlt-1 was included in the assay, there was a slight delay in the formation of VEGF/KDR complexes, when compared to VEGF-A alone. The gradual increase in phosphorylated KDR peaked at approximately 4 minutes, after which the signal dropped off by 16%. Not only was there a difference in the delayed development of ligand/receptor complexes, but there was a significant difference in the content of phosphorylated KDR receptor complexes formed +/- rmsFlt-1. The peak levels of activation for VEGF-A +/- rmsFlt-1 differed by $60 \pm 19\%$. An unpaired, two-tailed t-test, using GraphPad Prism revealed a significant difference between these means ($P < 0.001$)

PIGF-dependent ligand shifting evidenced by PtdIns hydrolysis

One of the more intriguing theories about the interplay of VEGF binding is that of ligand-shifting. First introduced by Park *et al.* (1994), the original concept suggested that the addition of PIGF in the presence of VEGF-A and multiple receptors could impact VEGF-induced effects by binding to Flt-1 and thereby displacing it from Flt-1 to KDR [54]. These effects were computationally modeled in a recent paper by Mac Gabhann & Popel (2004) where, at low initial ligand concentrations, PIGF was to displace large quantities of VEGF from Flt-1, raising the effective VEGF concentration in

the medium, which in turn increased binding to the any available unoccupied KDR [107]. Ultimately, an increase in VEGF-A binding to KDR, with a simultaneous decrease in its binding to Flt-1, could potentially be a simultaneous shift in the receptor-binding profile of VEGF that is synergistic in terms of downstream signaling. One of the current gaps in the literature is that there is a general lack of *in vitro* evidence supporting the ligand-shifting theory. To address this issue, a series of experiments were designed using the aforementioned inositol phosphate accumulation assay to determine the extent to which PIGF could reverse the inhibitory action of sFlt-1 on VEGF-A signaling. For these experiments, KDR-expressing cells were stimulated with VEGF-A at a final concentration of 25 ng/ml—a concentration slightly higher than the calculated EC_{50} value for the ligand. This should have elicited a readily-measurable response. Cellular activation was initiated in the presence or absence of rmsFlt-1 at a final concentration of 50 ng/ml. This was also slightly higher than the confirmed IC_{50} value for rmsFlt-1 in these cells, depending on the length of time for ligand-induced activation. In either case, there should be pronounced inhibition of VEGF-A signaling. The final component included in the system was PIGF, at a final concentration of 100 ng/ml. The independent experiments, performed in triplicate for 10 and 60 minute incubations, respectively, are shown in Figure 31. These experiments again demonstrate the inhibitory effect that sFlt-1 has on VEGF-A binding to KDR as its presence in the *in vitro* system significantly decreased total inositol phosphate accumulation. In the 10 minute experiments, the average accumulation of phospholipids metabolites reached only 33% of the maximal value caused by VEGF-A alone. Similarly, in the 60 minute experiments, that average value was reduced to 12% of maximal VEGF-A stimulation. PIGF alone proved to be an insignificant activating factor for KDR—as it should be because PIGF does not bind to KDR—and did not significantly alter the maximal response attained by VEGF-A stimulation. The most profound result occurred when PIGF was included in the assay along with VEGF-A and sFlt-1. When PIGF was added, creating a multiple receptor, multiple ligand situation, the previous inhibition provided by sFlt-1 was virtually completely reversed. With PIGF present, at 10 minutes, VEGF-A stimulated inositol phosphate accumulation was restored to 85% of maximal values. At 60 minutes, the restoration was 83% of maximal VEGF-A stimulated accumulation.

While the 60 minute result was the only value demonstrated to be significantly different ($P < 0.05$) from the rmsFlt-1 inhibitory values, both of these results are impactful, nonetheless. These are the first *in vitro* results of their kind, to demonstrate reversal of sFlt-1 inhibition by another naturally occurring growth factor. It provides direct evidence that inhibition of binding and signaling events can be affected by ligand shifting. This was only previously theorized by other authors, and these results cannot be overstated.

DISCUSSION

VEGF has been demonstrated to induce many biological actions in endothelial cells, from the stimulation of cellular proliferation and migration to increasing vascular permeability (reviewed in [152]). In endothelial cells, VEGF mediates mitogenic signals by activating Flt-1 and KDR [153]. These receptors contain tyrosine kinase activities and are autophosphorylated at tyrosine residues. Upon activation, diverse intracellular signaling components will associate with the phosphorylated receptors to elicit different cellular responses to ligand binding. These interactions are mediated in part through SH2 domains, resulting in the tyrosine phosphorylation of downstream substrates. I show in the present document that inhibition of VEGF-promoted activation of KDR by sFlt-1 can be quantified in novel ways which utilize these intracellular events—from direct measurement of receptor phosphorylation to intracellular signaling via PLC γ -1.

The transfected cell lines 3.1/Flt-1 and C57/KDR express functional VEGF receptors, assessed both by western blotting, and in the case of KDR cells, an assay of ligand-dependent phosphorylation of activated KDR. With the current method of measuring receptor activation, the extent of detectable phosphorylation was only measurable in KDR-expressing cells. This confirmed observations made throughout the literature that identify Flt-1 as a receptor that is only weakly phosphorylated [67, 70]. Furthermore, the Flt-1 cells were unable to generate inositol phosphate responses to VEGF and VEGF-related ligands via the activation of Flt-1. This is not to say that Flt-1 is incapable of generating intracellular signals, but it does provide convincing evidence that Flt-1 does not signal through the activation of PLC γ -1. The KDR cells, on the other hand, were effectively activated with VEGF as demonstrated by functional assessment of ligand-receptor interaction through inositol phosphate generation and receptor

phosphorylation assays. These assays provided a good baseline for comparison of VEGF-E activation and inhibition of signaling by sFlt-1. It was concluded that these *in vitro* results provide good evidence of ligand-receptor complex formation and can, therefore, be effectively compared to model predictions made earlier in this document.

Using the GST-SH2 fusion protein was an effective method for determining the amount of VEGF-promoted receptor activation of KDR. Furthermore, the results were quantified in such a way that allow for the comparison of these results to model predictions. There have been many studies in the past that have investigated KDR activation (via phosphorylation) due to VEGF-A stimulation, but there are no studies, to date, that have looked at the function of VEGF-E from this angle. I have identified unique characteristics about the kinetics of VEGF-E binding and activation whereby it activates KDR, but its activation is delayed (Figure 29) compared to that of VEGF-A. Furthermore, the level of receptor phosphorylation due to stimulation with the same concentration of VEGF-E is approximately half of maximal VEGF-A stimulation. With growing interest in VEGF-E as a therapeutic agent [154], this information about the kinetics of its binding and activation may be helpful in guiding future pharmacologic studies with this ligand. The characteristic differences observed in activation by these two ligands provides a distinct feature that, if it can be replicated *in silico*, can be beneficial for determining the effectiveness of the mathematical model.

One very interesting observation from the western blotting experiments was the observed drop-off of the phospho-tyrosine signal with stimulation from either ligand (Figure 29). The signal was decreased to <50% maximal activation in the case of VEGF-A stimulation. One possible reason for this characteristic of the receptor time-course curves may be receptor internalization & degradation that occurs during the time course. The internalization and trafficking of ligand-activated cell-surface receptors has long been reported as one of the main mechanisms of down-regulation for many receptors [155]. This seems highly unlikely that the short duration of these experiments would allow adequate time for these multiple-step processes to occur. Of the few existing studies that have looked at VEGF-induced KDR internalization, one study concluded that 293 cells expressing the native KDR receptor required approximately 25 minutes for 50% of cell-associated [¹²⁵I]VEGF to be internalized [78]. If only 50% of

ligand-bound receptors were internalized in a time-course that is 66% longer than the activations in these experiments, it is highly unlikely receptors would be internalized and degraded in this time. In another study, by Ewan et al. (2006), antibodies to either extracellular or cytoplasmic domains were used to analyze spatial and temporal processing of KDR in response to VEGF-A stimulation in endothelial cells [156]. In this manner, VEGF-A-mediated loss of KDR was measured over time in order to implicate trafficking to an endocytic compartment for proteolytic degradation. While a 69% decrease in the levels of KDR (~220-kDa) occurred 180 min after VEGF-A stimulation, there were only insignificant losses of the receptor over shorter periods of stimulation (e.g., 10 min). Receptor internalization and degradation cannot account for the decrease in VEGF-promoted KDR phosphorylation over the 15 minute duration of these experiments.

A more likely cause of these characteristic decreases in phospho-KDR is receptor dephosphorylation. While the kinetics of VEGF receptor dephosphorylation has not been investigated, there are studies that indicate that the phosphorylation state of receptor tyrosine kinases is dynamically regulated, with dephosphorylation playing an important role in the signaling process [157, 158]. In one particular study in which the authors were able to accurately measure dephosphorylation reactions in rat liver subcellular endosomal fractions by stopping kinase activity, the dephosphorylation of both insulin and EGF receptors was quite rapid. The receptors were inhibited at their respective times of maximal phosphorylation (insulin, 5 min; EGF, 2 min), after which there was a rapid loss of receptor phospho-tyrosine content at a $t_{1/2}$ of 1.6-1.7 min [157]. With these results in mind, it is entirely possible that the loss in phospho-KDR signal over the 15 minute time period could be accounted for by receptor dephosphorylation. This is an area that could be further investigated in the future. Whatever the cause for these observations, this down-slope in the maximal activation poses a particularly interesting challenge when attempting to computationally model binding and complex formation with these ligands.

When executing the *in vitro* cellular activation experiments, there was some difficulty encountered in attaining an intense phospho-KDR signal from the activated cell lysates. After activation, and upon imaging the western blots, there were some

instances when the phospho-tyrosine signal was weak—much weaker than expected for a method intended to enrich the signal for phosphorylated KDR. It is unlikely that proteases were present in the samples that were degrading proteins because protease inhibitors were included in the lysis buffer. It is also unlikely that KDR was being dephosphorylated during the subsequent incubations and washing steps because Na_3VO_4 was included at all times to inhibit phosphatase enzymes. With these potential problems ruled out, what could be the cause for variability observed in this assay? The problem could lie in the chosen method for binding and precipitating activated KDR—with glutathione S-transferase fused to the C-terminal SH2 binding domain (SH2_C) derived from mouse phospholipase C γ . There are few key components of PLC- γ that are involved in its membrane recruitment and phosphorylation upon activation. These components are: two Src homology 2 (SH2) domains and a Src homology 3 (SH3) domain [159]. The N-terminal SH2 domain is a necessary portion of the catalytic domain for binding either tyrosine-phosphorylated receptor kinases or other adaptor molecules that mediate PLC- γ 1 trafficking to the plasma membrane. The function of the C-terminal SH2 domain is less clear but is thought to be essential for PLC- γ 1 activation. In a recent study by DeBell et al. (2007) the authors presented evidence that indicated that basal activity of PLC- γ 1 is repressed by its C-terminal SH2 domain, thus producing a unique autoinhibitory control mechanism. Furthermore, they concluded that, in addition to its function in mediating recruitment to the plasma membrane, binding activated receptors, and phosphorylation of PLC- γ 1, the N-terminal SH2 domain is required to release PLC- γ 1 from its inhibitory function [159]. Essentially, what this means is that the C-terminal SH2 domain used in GST-fusion proteins for precipitation of activated KDR has the potential to bind and precipitate phosphorylated PLC- γ 1 from activated cell lysates along with phosphorylated KDR. This occurrence played out in the VEGF activation experiments where a lower molecular weight band—later identified by western blotting as phosphorylated PLC- γ 1—routinely appeared on western blots when probing for phosphorylated KDR (data not shown). This is not to say that this method for precipitating receptors is the wrong method for enriching phospho-KDR in activated samples. In fact, the argument could be made that the presence of PLC- γ 1 binding to KDR is further evidence that activated ligand-receptor complexes have been

captured. This shows a mechanistic link for the activation of KDR cells by VEGF and the generation of inositol phosphates. Utilizing GST fused to the N-terminal SH2 binding domain could also be an effective method for enriching the signal of phosphorylated KDR, and this is a direction that should be explored in future experiments for VEGF activation. However, the current method was also effective, and I am confident that this method was a valid method for investigating sFlt-1 inhibition on VEGF binding and signaling.

While there have been attempts made to determine the extent to which sFlt-1 inhibits responses to VEGF, many of these studies focused primarily on mitogenic effects or inhibited growth of endothelial cells [98-100]. These endpoint measurements only allow for inferred changes in the state of signaling molecules in the presence of exogenous sFlt-1. The published studies have probed events that happen long after the binding of VEGF, but none have attempted to directly quantify the ligand-induced phosphorylation of KDR in the presence of sFlt-1. Furthermore, there have been few attempts to measure perturbations in intracellular signaling events with sFlt-1 inhibiting VEGF receptor activation. These experiments, which focus directly on the extent to which sFlt-1 acts to sequester VEGF and inhibit receptor activation, are novel. The results from western blotting experiments demonstrate that the concentration of rmsFlt-1 that is necessary for inhibiting maximal VEGF-A response by 50% is twice that (50 ng/ml) of VEGF-A used for activation (25 ng/ml). However, the molar equivalents, based on the respective molecular weights of each protein, are approximately equal, with rmsFlt-1 and VEGF-A used at 602 pM and 595 pM, respectively. This apparent 1:1 molar ratio of sFlt-1 to VEGF could be indicative that the inhibition lent by rmsFlt-1 in these time-course/activation assays was based solely on binding and sequestering of the volumetric ligand species. This is not to say that sFlt-1 cannot bind KDR and form heterodimers, thereby adding another level of inhibition to VEGF activation. These observations only indicate that, in 15 minutes of activation, sFlt-1 is likely inhibiting receptor phosphorylation due to binding the available ligand. This study reinforces the idea that sFlt-1 (like Flt-1) may function in a negative fashion in the process of angiogenesis. The hypothesis is that it is indirectly involved in VEGF binding and activation of KDR. Essentially, VEGF binds to sFlt-1, whereby it acts as a “trap” to

modulate KDR function—sFlt-1 plays a negative role by suppressing pro-angiogenic signals to establish a balance essential for physiological angiogenesis.

The inositol phosphate experiments were able to quantify sFlt-1 inhibition of VEGF-A phospholipid metabolite accumulation. The same level of inhibition was not replicated in these experiments—there was, in fact, more inhibition observed when VEGF-A and rmsFlt-1 were included at the same concentration. In the case of the 60 minute signaling experiments, average VEGF-A promoted inositol phosphate accumulation was reduced to 12.1% of maximal levels. In light of the fact that, again, roughly equimolar concentrations of the ligand and soluble receptor were included, some other mechanism of inhibition should not be ruled out in this case. This does not negate the aforementioned observations, and it is still very likely that sFlt-1 is acting as a “trap” receptor to inhibit KDR function; however, the almost complete ablation of KDR signaling may be indicative that other inhibitory mechanisms are working in concert with ligand binding. The longer incubation time used in this experiment may allow for the dimerization process to play a more effective role in inhibition. In addition, the presence of VEGF-A may have been necessary for these dimerizations to take place. Now, with the conditions optimal for these events, the total inhibition of signaling may have been more pronounced.

With KDR being the primary focus for signaling processes in VEGF-stimulated angiogenesis, until recently other molecules have been largely overlooked as possible targets for treating angiogenesis-driven disorders. The elucidation of the synergistic role of PlGF and Flt-1 was facilitated through gene targeting and inhibition studies in mice [89]. In these studies, it was concluded that the loss of PlGF, while not causing any vascular defects during development, reproduction, or normal adult life, impaired angiogenesis and plasma extravasation. Most notably, in one study by Lutun et al. (2002), it was reported that *Plgf*^{-/-} fibrosarcomas and embryonic stem cell-derived tumors were smaller and had less vascular ingrowth than wild type tumors, implanted in nude mice [160]. These observations underlined the role of PlGF in tumor angiogenesis and provided further evidence supporting the theory of PlGF ligand shifting. By displacing VEGF from Flt-1, PlGF is expected to make more VEGF available to bind and activate KDR and thereby enhance VEGF-driven angiogenesis.

Until now, the majority of the experimental evidence had been focused on PlGF displacing VEGF from membrane-bound Flt-1. Here I present novel findings whereby the inhibitory effects of sFlt-1 on VEGF-promoted signaling through KDR were significantly reversed when PlGF was added exogenously to the system. In fact, only a 4-fold molar excess of PlGF over VEGF-A was required to shift VEGF-A from sFlt-1 to KDR to stimulate signaling and generate more inositol phosphates. In the original study where the theory of ligand shifting was introduced, Park et al. (1994) noted that an effect of this type required approximately a 10-20-fold molar excess of PlGF over VEGF. The difference is that in their study, and most subsequent studies, attempts were made quantify ligand-shifting effects by only looking at *in vivo* angiogenic assays. The disparity in the necessary molar ratios may be attributed to the fact that, in this study, the signaling processes were directly probed. This is the first study to quantify the magnitude of ligand-shifting effects and one of the first to extend the theory to include the inhibitory interactions of sFlt-1. I provide definitive evidence that the inhibition lent by sFlt-1 on VEGF binding and activation of KDR can be almost completely restored with PlGF present in the system. Several questions remain after these initial conclusions because it is unlikely that the amounts of PlGF, or VEGF for that matter, are physiologically relevant to circulating levels of the ligand. Plasma concentrations of PlGF have been measured at 11 pM [161], and serum concentrations have been measured in the range of 1–11 pM [107, 162]. It is unlikely that the levels used in this study can be directly applied to an *in vivo* situation. So the conditions presented here may only be applicable at a very localized level in the tissue—where sFlt-1 and PlGF may be generated and released as paracrine agents. Targeting PlGF or sFlt-1 may be beneficial in the development of alternative angiogenic therapies, and the insights here may be a first step in making new anti-angiogenic drugs a reality.

Based on the current *in vitro* experiments: (1) sFlt-1 inhibition can be successfully quantified by measuring its inhibition of the levels of phosphorylated KDR in whole cell lysates; (2) the binding of VEGF-E and may also be quantified with the aforementioned methodology. It may prove to be an attractive alternative as a slightly less potent stimulator than VEGF-A for signaling through KDR. This information about the kinetics of VEGF-E binding and activation may be helpful in guiding future pharmacologic

studies with this ligand; (3) The effects of PIGF “shifting” VEGF-A from sFlt-1 to KDR can be directly measured via signaling intermediates. These results are novel and providing convincing *in vitro* evidence of this extension of the ligand shifting theory.

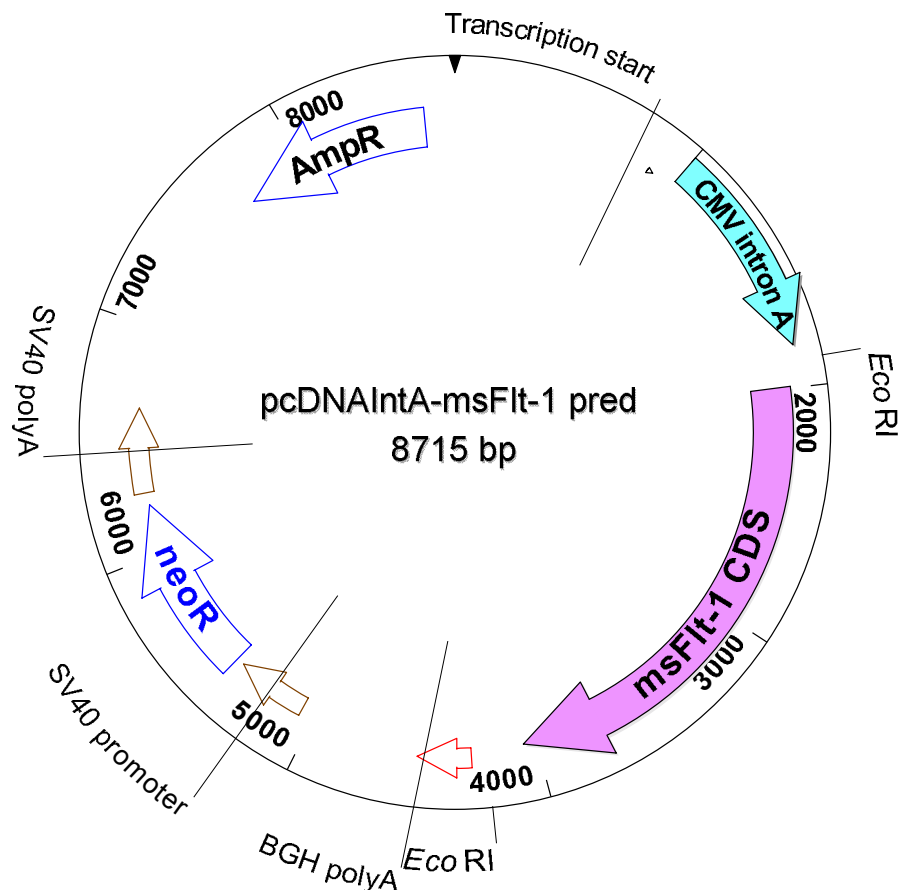


Figure 20. Plasmid used for development of rmsFlt-1 baculovirus vector. This plasmid is presented a map format ease of visualization and show the components necessary for the establishment of stable lines of cells that overexpress msFlt-1. The plasmid, pcDNAIntA-msFlt-1, contains the cytomegalovirus (CMV) intermediate-early promoter plus intron A and a cassette encoding the neomycin (neo) resistance marker for gentamicin (G418) selection as well as the full-length receptor coding sequences (CDS) for msFlt-1. The msFlt-1 coding sequence was removed from the plasmid by digestion with *EcoR*I and ligated with the baculovirus transfer vector, pVL1392.

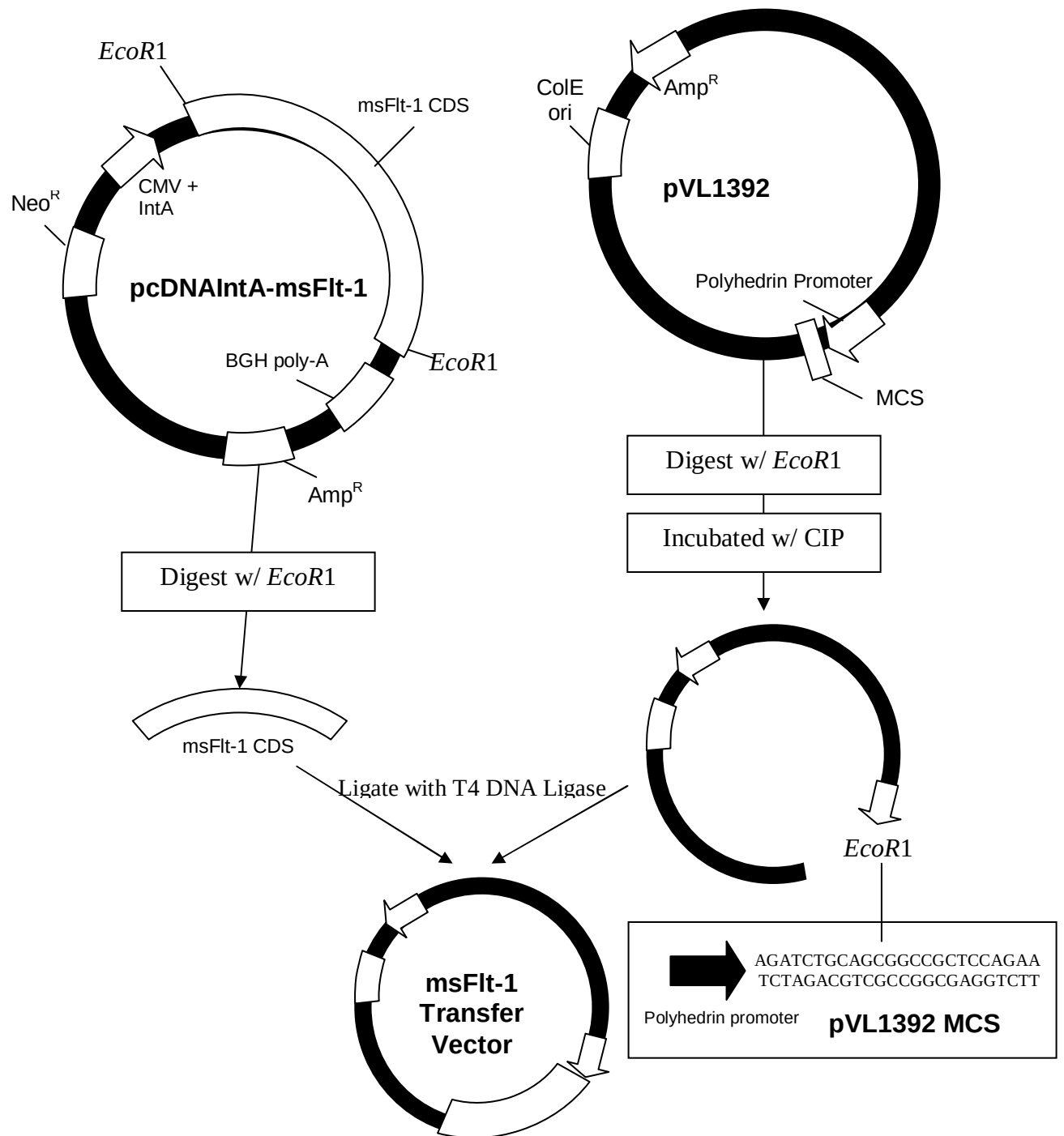


Figure 21. Strategy for directly cloning msFlt-1 cDNA into the pVL1392 transfer vector. On the left, the msFlt-1 foreign gene product with flanking *EcoRI* sites is digested with *EcoRI*. The resultant overhanging ends are compatible with the digested viral transfer vector, pVL1392 (on right). Heterologous DNAs were combined, ligated, and subcloned into competent *E. coli*. The resulting plasmid was sequenced to confirm correct orientation of the insert and finally transfected into insect cells (Figure modified from Baculogold manual).

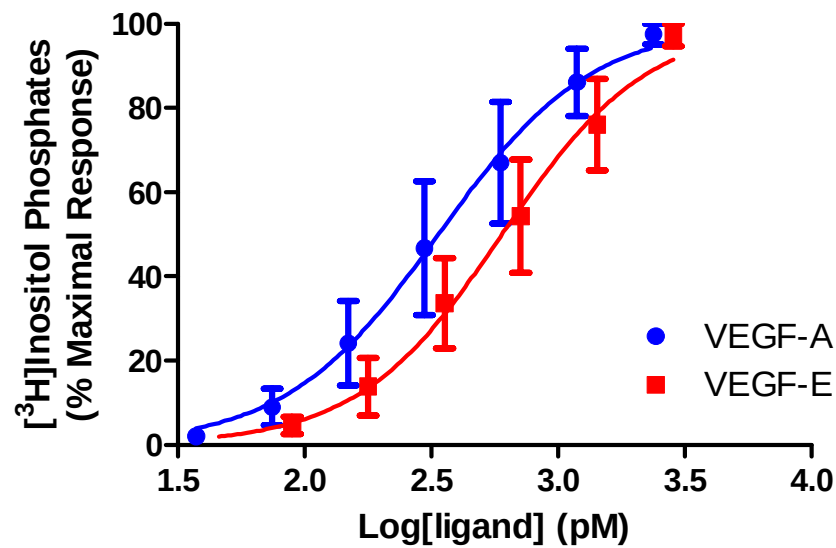


Figure 22. Concentration effect curves for VEGF-stimulated inositol phosphate accumulation in KDR-expressing cells. Inositol phosphate accumulation was quantified as described in the text. Average percent maximal responses are shown, calculated by subtracting of basal inositol phosphate accumulation (no added agonist) and normalized to the maximal stimulation within each experiment. The plotted data represent the averaged normalized values for VEGF-A & -E of three experiments performed in triplicate. Curves were fit to a one-site nonlinear regression model.

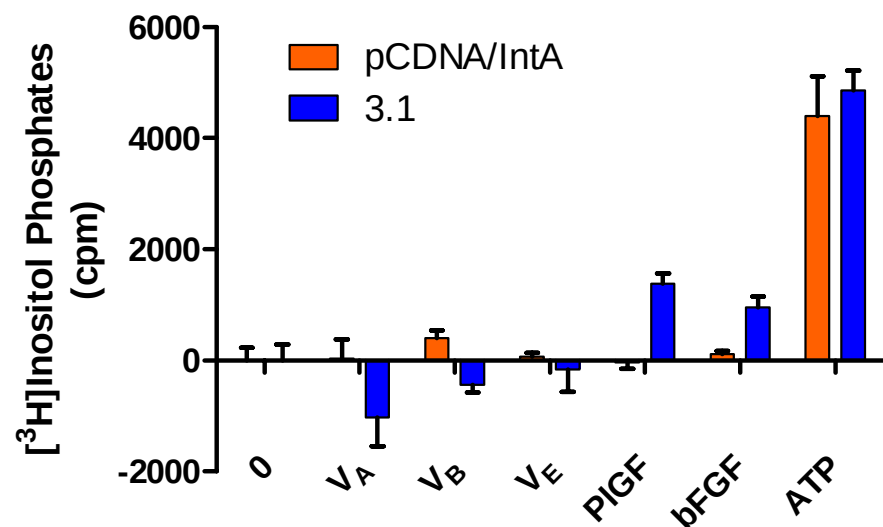


Figure 23. Screening Flt-1-expressing cells (3.1) and negative control transfectants (pCDNAIntA) for activation/signaling responses to various ligands. All ligands were added at a final concentration of 50 ng/ml and ATP was added at a final concentration of 0.1 mM. Inositol phosphate accumulation was quantified as described in the text, where the average values are shown after subtraction of basal inositol phosphate accumulation (no added agonist). The data, therefore, represent the averaged normalized values (in triplicate) for the various ligands from one experiment for each cell type.

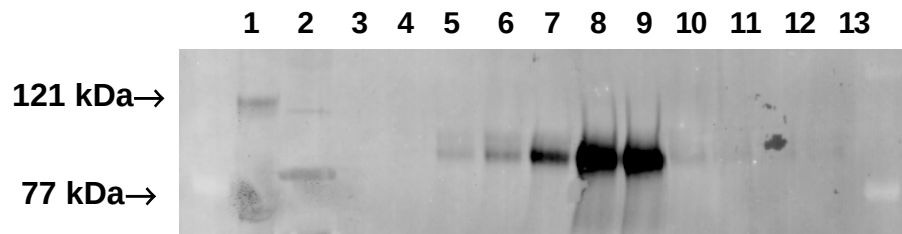


Figure 24. Purification of rmsFlt-1 from immobilized heparin with increasing concentrations of NaCl. When probed with an N-terminal α -Flt-1 antibody, the western blot demonstrated successful elution of sFlt-1 protein from the heparin-agarose beads. Lanes 1-3 contain molecular weight markers, a control sFlt-1 protein sample (R&D Systems, Minneapolis, MN), and heparin bead wash samples, respectively. Lanes 4-13 represent increasing concentrations from (100 mM to 1 M) of NaCl used to elute the rmsFlt-1 from the beads. Recombinant sFlt-1 migration revealed a protein of ~86 kDa in size, with peak elution—evidenced by the large protein bands in lane 8 and 9—occurring at 500 and 600 mM NaCl, respectively.

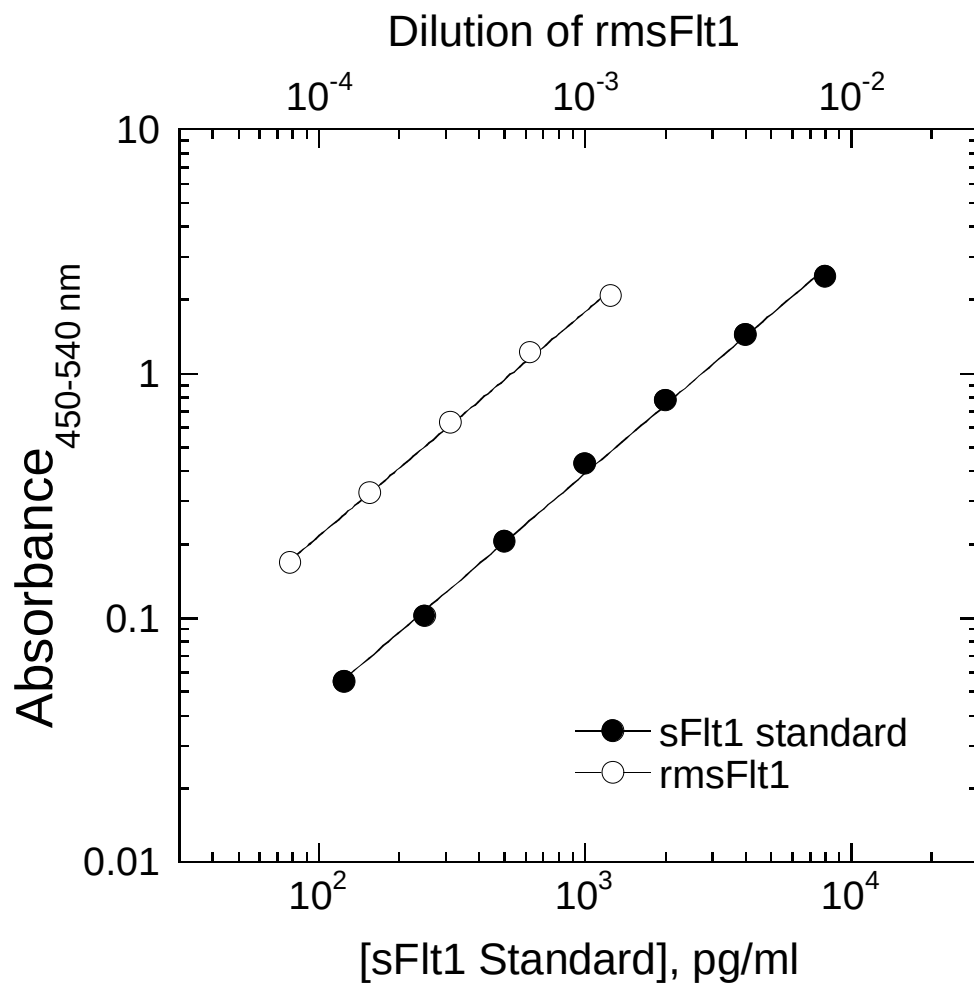


Figure 25. Absorbance curves generated from ELISA dilution series for kit standard and *Sf9*-purified sFlt-1. These curves are used to estimate the final concentration of the rmsFlt-1 protein stock that will be used for latter experiments.

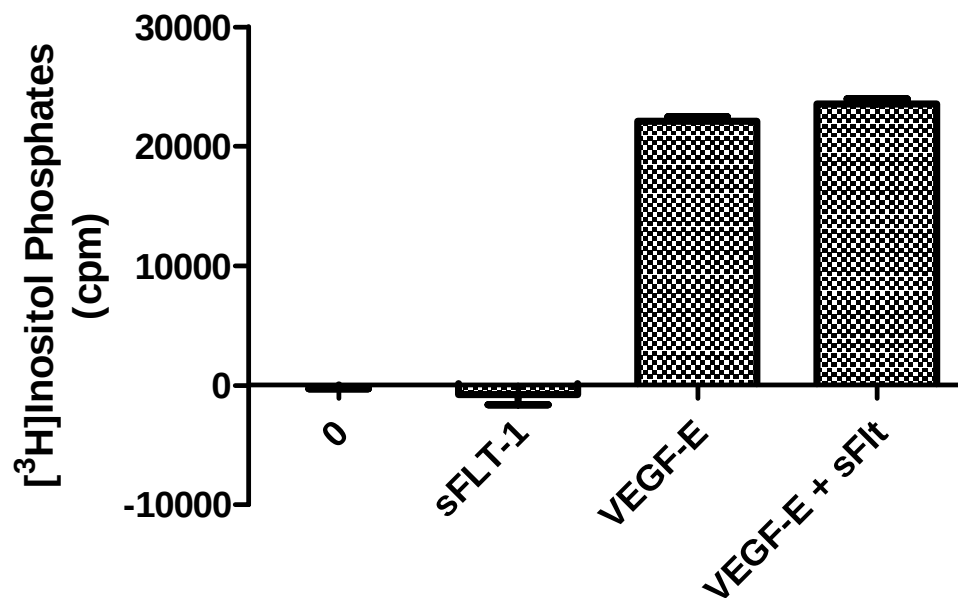


Figure 26. Screening rmsFlt-1 inhibition of VEGF-E binding and signaling in KDR cells. Inositol phosphate accumulation was quantified as described in the text. Average values are shown after subtraction of basal inositol phosphate accumulation (no added agonist). The plotted data represent the averaged normalized values for VEGF-E of one experiment performed in triplicate. No differences were observed between VEGF-E-stimulated groups; therefore, no inhibition was lent by rsFlt-1.

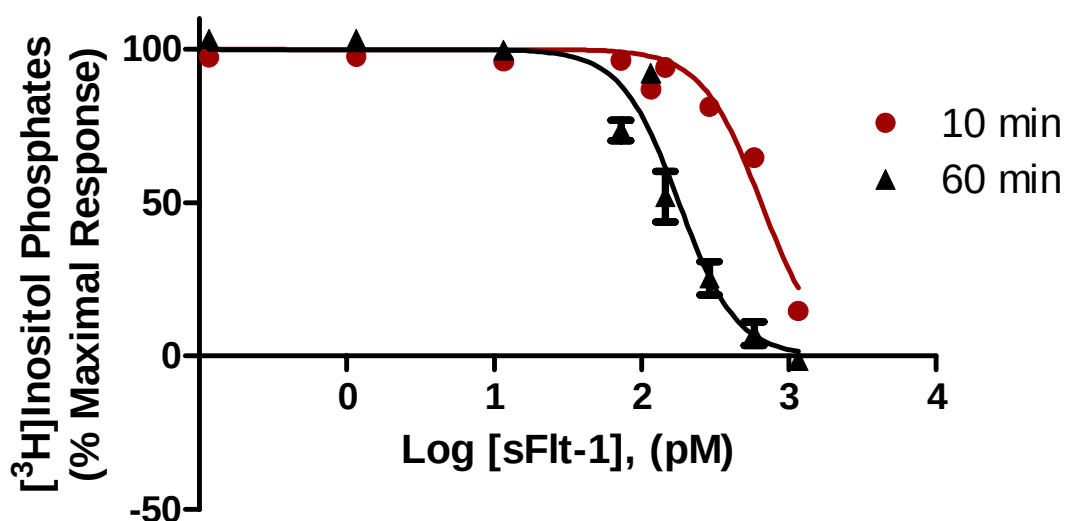


Figure 27. Response vs. log (inhibitor) curves for the inhibitory effects of rmsFlt-1 on VEGF-A stimulated inositol phosphate accumulation in KDR cells. Average values plotted after subtraction of basal inositol phosphate accumulation (no added agonist) and normalized to the maximal stimulation within each experiment. The plotted data represent the averaged normalized values for two separate experiments, performed in triplicate, for each time point. Curves were fit to a one-site nonlinear regression model. For the 60 minute incubation, with sFlt-1 present at its calculated IC_{50} value (180 pM), the theoretical free VEGF-A present would be 460 pM.

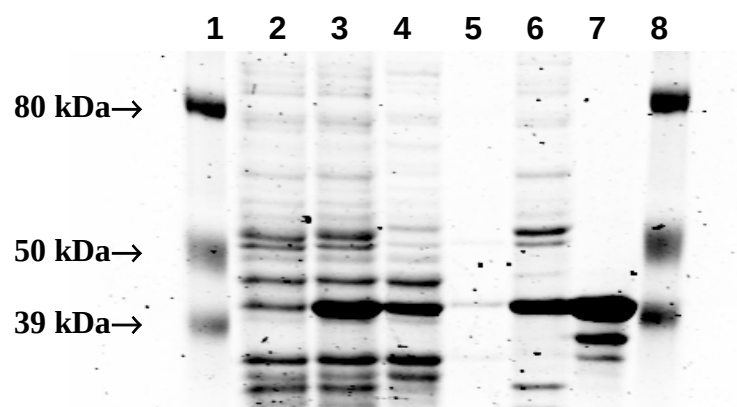


Figure 28. Purification of GST fused to the C-terminal SH2 binding domain (SH2_C) derived from mouse phospholipase C γ expressed and purified from *E. coli*. Shown here are the results of SDS-PAGE on multiple samples from the purification process, stained with Imperial Protein Stain (BioRad). Lanes 1-3 contain molecular weight markers, a sample of non-induced *E. coli*, and a sample of *E. coli* 1 hour after induction with IPTG, respectively. Lanes 4 & 5 represent two rounds of purification of the protein with B-Per protein extraction reagents. Lane 6 represents the protein of interest left in the cell pellet after two rounds of homogenization and purification. Lane 7 represents the purified GST fusion protein from 10 μ l of agarose beads after adsorption of the protein to the beads. Lane 8 contains molecular weight markers.

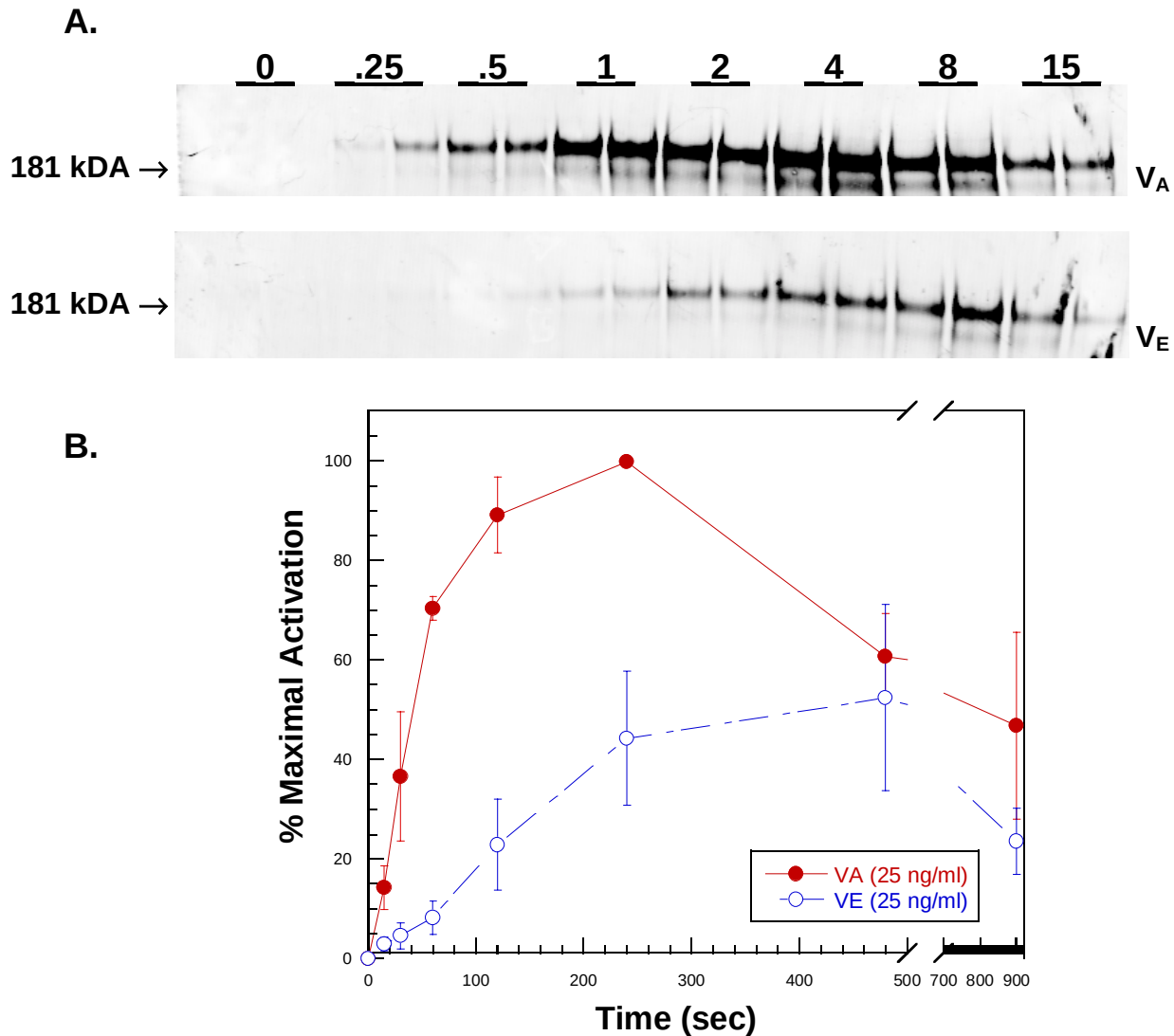
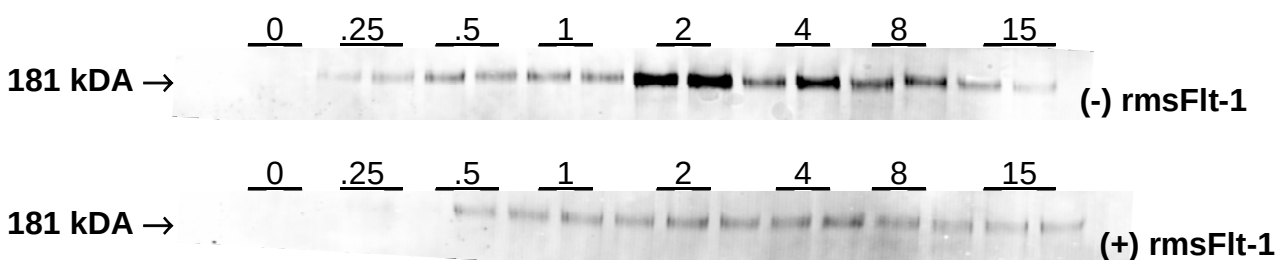


Figure 29. Representative blots and quantification of P-Tyr-KDR images for time-course activation assays of KDR cells stimulated with VEGF-A or -E. **(A)** Shows images representative for KDR stimulation with 25 ng/ml of VEGF-A or -E from 0 to 15 minutes of activation. Blots were probed with α -P-Tyr antibodies and imaged with Odyssey[®] imaging software. **(B)** When quantified, the mean values for percentage maximal activation revealed the highest amount of VEGF-A-stimulated phospho-KDR at 4 minutes of activation. In contrast, VEGF-E stimulation led to a slightly smaller percentage of maximal activation that reached its peak at 8 minutes. In both cases, a steady decrease in the phospho-tyrosine signal was observed after maximal activation was attained.

A.



B.

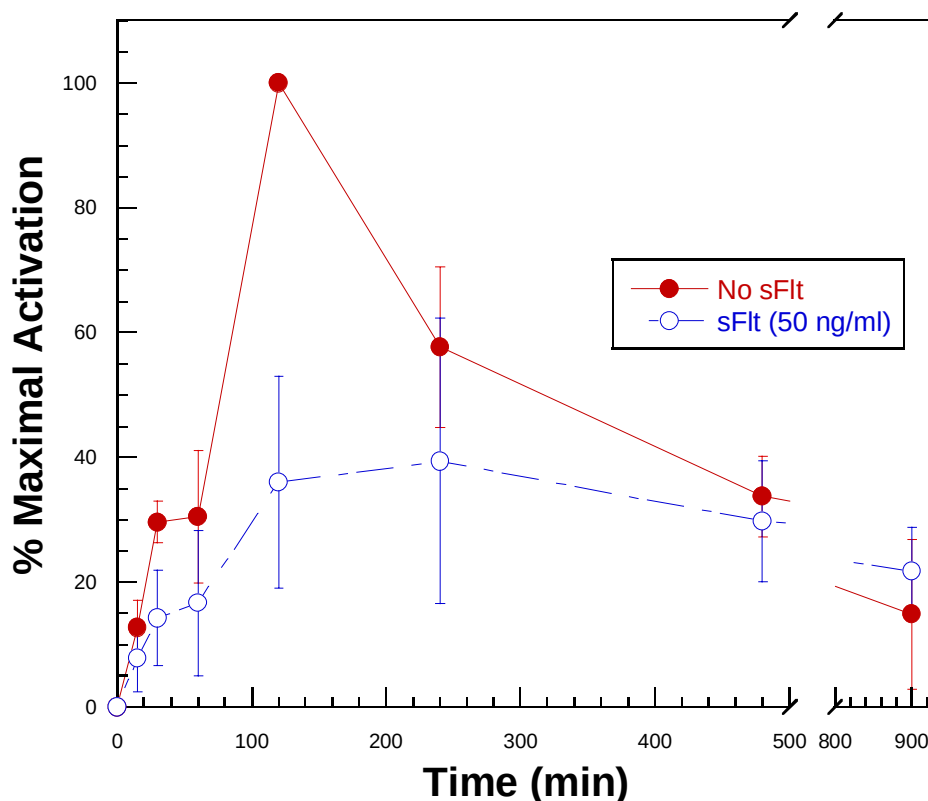


Figure 30. Representative blots and quantification of P-Tyr-KDR images for time-course activation assays of KDR cells stimulated with VEGF-A, +/- rmsFlt-1. **(A)** Shows images representative for KDR stimulation, from 0 to 15 minutes of activation, with 25 ng/ml of VEGF-A, +/- rmsFlt-1 at a concentration of 50 ng/ml. Blots were probed with α -P-Tyr antibodies and images with Odyssey[®] imaging software. **(B)** When quantified, the mean values for % maximal activation revealed the highest amount of VEGF-A-stimulated phospho-KDR at 2 minutes of activation. In contrast, with rmsFlt-1 present, stimulation lead to a much smaller % of maximal activation that reached its peak at 4 minutes. In both cases, the characteristic decrease in the phospho-tyrosine signal was observed after maximal activation was attained.

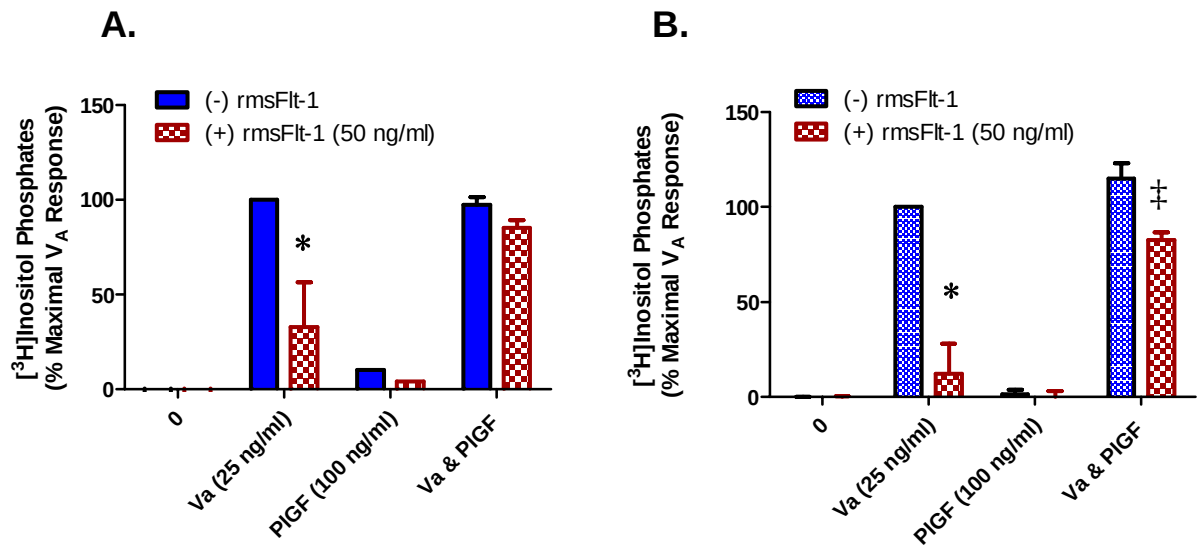


Figure 31. Ligand-shifting effects of PIGF on rmsFlt-1 inhibition of VEGF-A signaling. Average values are shown after subtraction of basal inositol phosphate accumulation and normalized to the maximal stimulation by VEGF-A within each experiment. The plotted data represent the averaged normalized values for two 10 minute incubation experiments **(A)** (each treatment performed in triplicate) and three 60 minutes experiments **(B)**, also performed in triplicate. *Indicates statistically significant difference from VEGF-A stimulation ($P < 0.05$), and ‡ indicates significant difference from rmsFlt-1 inhibition ($P < 0.05$). One-way ANOVA with Tukey's Multiple Comparison post-test was performed using GraphPad Prism.

**CHAPTER 4: DISCUSSION AND RECONCILITATION OF MODEL PREDICTIONS
WITH *IN VITRO* RESULTS**

SENSITIVITY ANALYSIS AND RECONCILIATION WITH *IN VITRO* EXPERIMENTS

Alterations in Rate of Internalization

The uncertainty in some of the experimentally determined model parameters was noted earlier in this document. The questions that arose during data analysis, due in part to biological variability or simple uncertainty in experimental measurements, suggest that some of these parameters should be varied when comparing the model predictions to *in vitro* experiments. A direct comparison of the scaled predictions of KDR complex formation over time and *in vitro* cellular activation assays (from western blotting results) is shown in Figure 32. While the predictions in the maximal complex formation numbers match quite well to the *in vitro* western blotting results, there is an obvious delay in the peak formation of both predicted KDR/Va and KDR/Ve. The time to peak formation for KDR/Va occurs at approximately 8.5 minutes, while the same value for KDR/Ve falls at approximately ~12 minutes—this compared to western blotting results of 4 and 8 minutes for VEGF-A and VEGF-E activation, respectively.

The parameter with the most uncertainty during its determination was the internalization rate of KDR complexes. This was, therefore, one of the primary targets of the sensitivity analysis, and the results of variation in complex internalization rate is shown in Figure 33. Increasing the rate of internalization for complexes by 2 and 3 times the original value did affect the time at which KDR/Va and KDR/Ve formation peaked. When complex internalization rates were doubled (0.372 min^{-1}), the time to peak formation for KDR/Va and KDR/Ve occurred at approximately 5.6 minutes and 8.1 minutes, respectively. At triple the rate of internalization (0.553 min^{-1}), these times to peak formation drop to 4.8 and 6.1 minutes, respectively. Increasing the rate at which complexes were internalized did bring the time to peak complex formation down to numbers resembling the *in vitro* values; however, as you would expect, this was coupled with a corresponding decrease in the maximal formation of complexes. If the maximal number of complexes predicted to form is decreased significantly, these predictions will eventually become obsolete for comparing them to *in vitro* results. The phospho-KDR signal for western blots depend on a certain level of activated receptors and unfortunately, there is no way of knowing the number of phosphorylated receptors

(#/cell) that are necessary to generate a strong signal via western blot. Intuitively, it seems that altering the rate at which complexes are internalized does not remedy the differences observed between the simulations and *in vitro* results. Other rate constants should be varied in order to sync the two sets of experimental results.

Altering On and Off Rates

The next logical variable to change in the experimental parameters is the off rate for ligands binding to KDR, but changing this variable alone produces predictions that mirror alterations in internalization (Figure 34). The time to peak complex formation is decreased to times more representative of the *in vitro* western blotting results; however, this is coupled to a dramatic decrease in the maximal number of complexes predicted to form. This approach results in two distinct problems. First, increasing the off rate to the point that produces time-to-peak predictions more representative of *in vitro* results (16 times the original rate) ultimately leads to a K_D that is not representative of KDR binding to VEGF-A (1.92 nM). Nor has a K_D for this interaction been experimentally measured this high. Second, the increase in off rate may solve the problem of time-to-peak complex formation, but it introduces another problem whereby there is less of a decrease in the surface formed KDR-ligand complexes. In the case of KDR/Ve predictions, there is no decrease noted at all—simply a constant, steady increase in formation of ligand-receptor complexes. With an increase in association rate comes a faster peak time to complex formation, but this is coupled to dramatic increases in the maximal number of complexes formed (Figure 35). This alteration may, ultimately be the main problem for the observed differences between both experimental approaches. In the case of KDR binding both ligands, predictions for maximal complex formation are much more sensitive to increases in on rates than for changes in the off rate of ligands and complexes. Because of this sensitivity, this alteration does not result in K_D values that are out of the range of measured values for these ligands. In light of these difficulties, a last set of simulations were carried out with increasing off rates for the ligands, while simultaneously rescaling the on rates for these ligands based on the measured K_D values for each receptor type (Figure 36). In this case, the time to peak formation of complexes is much more sensitive to alterations in off rate for the ligands.

An interesting observation is that with these simultaneous changes made, within the boundaries of the measured K_D values, the time-to-peak complex formation is not the only change of not in the simulation curves. There is also a distinct drop-off in predicted complexes after peak formation, over time. Much like the *in vitro* phosphorylation results for activation with these cell types, there the decrease in receptor complexes over time.

Changing these parameter values may further highlight the usefulness of the model in predicting interesting behaviors in the system but, to include more representative on/off rates in the current base model, either: (1) there must be a more correct determination of the off rate made; or (2) attempts can be made to directly measure the on rate for these receptor ligand interactions. Neither of the alterations produced the desired results where peak complex formation is optimized with time to peak formation, and it is likely that some combination of alterations for multiple parameter values will produce the desired optimization.

Comparing sFlt-1 Experiments—*In Silico* vs. *In Vitro*

Comparing *in vitro* western blot results from the series of rmsFlt-1 inhibition studies to model simulations poses a similar challenge as those previously discussed. Because quantifying a phospho-tyrosine signal from a western blot does not directly give rise to numbers of complexes, the results from these experiments must be scaled in such a way that they are represented as a percentage of maximal, uninhibited VEGF-A activation. In this way, the time course data from western blotting can be compared to scaled model predictions that are also scaled to maximal KDR/Va complex formation in the absence of sFlt-1 (Figure 37). When plotted together the model predictions correspond reasonably well to the results from *in vitro* cellular activation assays. In these model simulations the same issue seen in the previous simulations is observed, with a delay in time to peak complex formation. The time to peak signal development for western blotting results occurred at roughly 2 minutes, but this value, for the development of maximal KDR/Va complexes is delayed to 8.5 minutes for modeling experiments. Surprisingly, this same trend is not observed for the sFlt-1 inhibition experiments, where the times to peak signal/complex formation for both experiments

are virtually indistinguishable. In both cases, when starting conditions for Va_0 and $[sFlt-1]_0$ are the same, maximal cellular activation/complex formation occurs at ~4 minutes. In addition to this similarity, the percentage of maximal VEGF-A-dependent activation/complex formation for the *in vitro* and *in silico* experiments compare reasonably well, with values of 39% and 49%, respectively.

Making the same sort of comparisons with the *in vitro* phospholipid turnover experiments is quite challenging because the nature of these experiments is prohibitive for the development of time-course data. In these experiments, ligand stimulation (*i.e.*, the formation of signaling receptor/ligand complexes) promotes the accumulation of [^{3} H]inositol phosphates over time; therefore, any complexes that are formed over the duration of the assay are capable of generating active phospholipase and enhancing [^{3} H]inositol phosphate accumulation for the entire 60 minute duration. The model can be extended to simulate KDR/*Va* generation for longer periods of time, and the results of this experiment are shown in Figure 38. The conditions for the *in vitro* ligand-shifting assay—50 ng/ml sFlt-1 and 100 ng/ml PlGF—were simulated over 60 minutes. Assuming that each predicted KDR/*Va* complex that forms on the surface of simulated cells is able to generate a signal (*e.g.*, promote the accumulation of [^{3} H]inositol phosphates over time) it follows that the most direct method for determining the a net signaling effect over time would be to calculate the area under each curve representing KDR/*Va* formation over time. In this manner, the integrated total area under each curve would represent a summation of the “accumulation” of signaling complexes for 60 minutes. When these areas are scaled to represent a percentage of the curve area generated upon uninhibited VEGF-A binding, they can then be compared to similar results determined from the *in vitro* experiments (Fig. 39). When [^{3} H]inositol generation was measured for 60 minute activations with VEGF-A (25 ng/ml) and rmsFlt-1 present (50 ng/ml) together, average values were reduced by 88% of maximal VEGF-A stimulation with VEGF-A alone. In contrast, when PlGF was included to preferentially bind sFlt-1, VEGF-A promoted generation of inositol phosphates was restored to 83% of maximal VEGF-A stimulated accumulation. Using the aforementioned method of determining net signaling complex formation for model predictions, similar trends were observed, and a similar level of ligand shifting was demonstrated. With the same

conditions simulated *in silico*, sFlt-1 inhibition revealed a net “signaling effect” that was approximately 20% of the maximal VEGF-A promoted response. With the addition of PlGF at the same concentration as that used *in vitro*, the net signaling effect of KDR/Va complex formation was restored to 65% of that for VEGF-A stimulation alone. The results of these model simulations are similar to those from previous experiments in that the level of sFlt-1 inhibition does not reach the maximal values observed in *in vitro* experimentation.

DISCUSSION

The results here support the hypothesis that a mathematical model simulating VEGF binding in a multiple ligand, multiple receptor system could be developed which mimics *in vitro* experiments. I was able to use the model to simulate a series of experiments in which the ratio of sFlt-1 to KDR and sFlt-1 to VEGF-A was varied. Successful inhibition was demonstrated, and predictions from the mathematical model, upon initial visual inspection, the *in vitro* results from inhibition of VEGF-A activation by sFlt-1 looked quite similar to model solutions (Figure 37). With the *in vitro* conditions replicating those simulated *in silico*, the level of sFlt-1 inhibition predicted was estimated to be within 10% of those observed *in vitro*. Furthermore, these were well within the range for experimental error from the *in vitro* cell activation assays. The model works quite well at predicting the level of inhibition imposed on KDR complex formation.

Another interesting result to come from modeling attempts was the demonstration of the reversal of sFlt-1 inhibition by exogenous addition of PlGF. The current model demonstrated a significant reversal of sFlt-1 inhibition—where approximately 60% more KDR/Va complexes were formed—required a 16-fold molar excess of PlGF over VEGF-A. Subsequent attempts to extend the model to make predictions over a longer time span allowed the solutions to be tested with *in vitro* phospholipid turnover experiments. This has not been attempted previously, but results of this comparison was equally encouraging. The model predicted a level of sFlt-1 inhibition that was very similar to *in vitro* results, and well within the range of experimental error (Figure 39). An 18% difference in restoration of PlGF-dependent

VEGF-A signaling was noted between the two experimental methods. In both cases, only a 4-fold molar excess of PlGF over VEGF-A was required to shift VEGF-A to KDR for the formation of signaling complexes. The model is quite effective at predicting the how the upregulation of PlGF may stimulate angiogenic signaling via displacement of VEGF from sFlt-1 to KDR.

This is one of the only existing models describing how VEGF and VEGF-related ligands interact in multiple receptor systems. Its usefulness for making predictions about complex formation has effectively been demonstrated, but what challenges are faced in comparing a model like this to the *in vitro* experiments laid out earlier in this document? The base model (without the addition of sFlt-1) compares reasonably well to experimental data (Figure 32), although there are obvious challenges in comparing simulated complex formation to experimental data obtained from western blotting. Because the method of western blotting introduced earlier in this document only provides a measurement of the content of phosphorylated tyrosine residues from activated, precipitated KDR it is difficult to directly compare these results to *in silico* predictions of the number of complexes formed over time. An inherent assumption that must be made about model predictions is that upon complex formation, the receptors become phosphorylated and that these activated receptors could then be precipitated with the method of SH2 precipitation described earlier. Beyond that both data sets must be scaled either to: (1) maximal VEGF-A promoted receptor phosphorylation, in the case of western blotting, or (2) predicted maximal number of VEGF-A promoted complex formations, in the case of the mathematical model. Only then can the data sets be compared.

The differences observed in the time to peak activation with these two methods could be the result of incorrect parameter values used in the modeling process. One of the most novel aspects of this study—formulating a model from and measuring as many kinetic parameters as possible—may have introduced some error. While every attempt was made to measure these values with the same cells used for the *in vitro* experiments being modeled, there were difficulties in determining two key parameter values—off rates and internalization rates—used in the model. These issues were addressed in the

sensitivity analysis, but ultimately, the problem may not simply be inaccurate kinetic parameters, but rather the setup of the model itself.

The method of comparing *in silico* and *in vitro* experiments relies on an assumed relationship between the formation of the ligand-receptor complexes and the instantaneous generation of phosphorylation signals into the cell. For the simulations, the formation of the complexes is used as a marker for the quantity of receptor phosphorylation, but this relationship may not be linear or instantaneous. It may be affected by other parameters that should be included in the model—like a term describing the rate of phosphorylation or dephosphorylation. One observation of note from the western blotting results was the decrease in the intensity of the phospho-KDR signal over time. I tried to more accurately simulate these results *in silico*, where I believed the decrease in peak numbers of complexes formed over time was a result of incorrect internalization rates (Figure 33). This sensitivity analysis was helpful for shifting the time to peak activation for *in silico* predictions, but the decrease in the maximal predicted number of complexes formed does not reconcile the differences between model predictions and cellular activation experiments. What if this decrease observed in the phosphorylation of receptors was due to dephosphorylation of cell-surface KDR? A term like this could likely be included in the model that may provide more accurate predictions of complex formation over time—where the geometry of KDR complex curves more accurately estimate those observed via western blotting.

There may still be more modifications that can be made to this base model. Since the experimental data to which I am comparing the model do not distinguish between ligands binding to monomeric or dimeric receptors, I did not include dimerization in the base model. However, dimerization of VEGF receptors has been demonstrated to be important [84, 163] and, with a few appropriate modifications, dimerization could be included. If dimerization—specifically the formation of sFlt-1/KDR heterodimers—were included in the model, another level of complexity would be added to the ability of the soluble receptor to inhibit complex formation. Currently, the model is able to simulate sFlt-1 inhibition well, and experimental evidence for the inclusion of sFlt-1 heterodimerization was inconclusive (addressed in the ensuing chapter). This is not to imply that dimerization is not important, only that it is not distinguished within my

model and no clear consensus has emerged about whether its inclusion would improve the effectiveness of model predictions. To be truly physiologically relevant, and to make the most accurate comparisons to *in vivo* situations, the model can be modified to include dimerization and heterodimerization of membrane bound and soluble receptors. The model presented here can be extended to include this and many other players in the VEGF system of ligand binding. More information is necessary about processes like ligand depletion and receptor recycling before they are included in the model, but with its application to longer periods of ligand binding, these processes will have to be included.

Based on the predictions utilizing the current model: (1) sFlt-1 inhibition can be successfully simulated, but the model may be most useful at predicting local changes to a receptor-ligand microenvironment; modifications including receptor dimerization may represent an improvement in its predictive nature; (2) PlGF ligand shifting can be simulated with the model and, more specifically, the method for analysis of *in vitro* results provides a novel method for comparing them to model predictions; (3) along with dimerization, the model may need to include a separate term that defines receptor phosphorylation and dephosphorylation to account for the “down-slope” observed from cellular activation experiments. This term may be similar to a coupling and uncoupling rate.

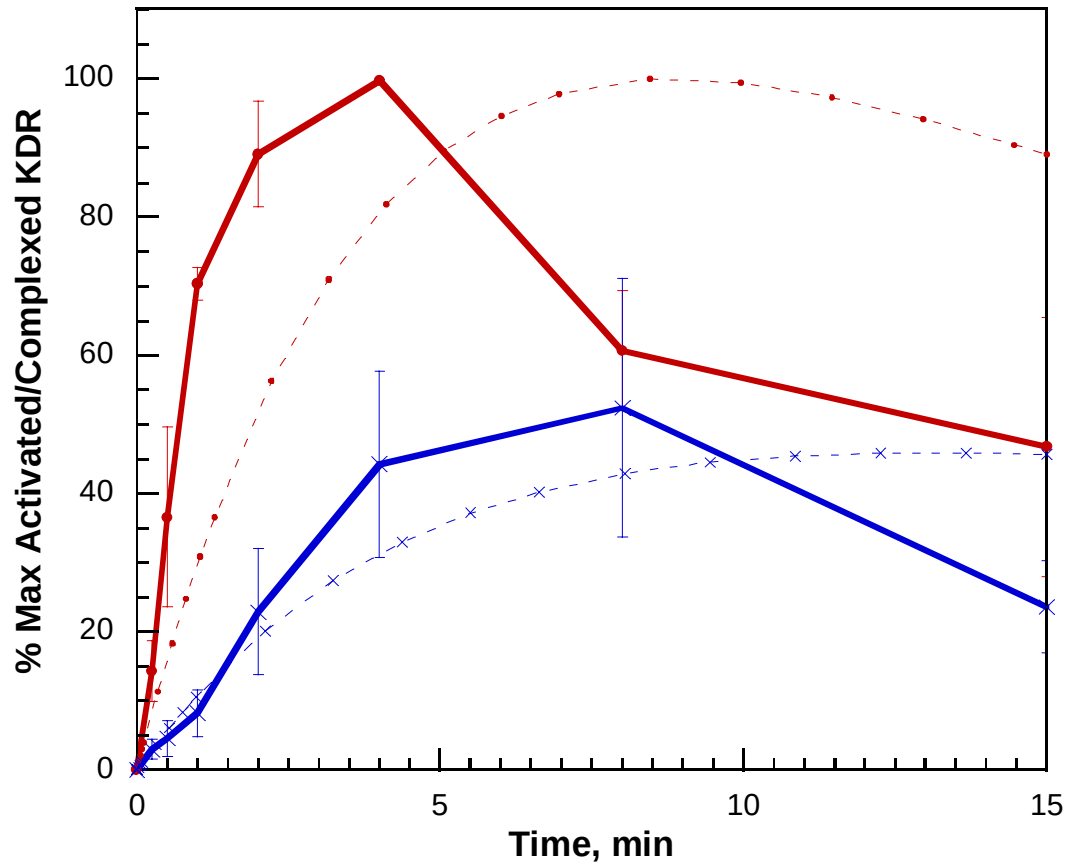


Figure 32. Comparison of scaled time-course activation data for VEGF-A (●) and VEGF-E (x). Results are scaled to peak activation/complex formation for exogenous VEGF-A addition (Va_0). Normalized, averaged values for activated KDR, as determined by phospho-tyrosine residues on precipitated KDR from western blotting, are depicted with solid lines (—), and model results of simulated KDR complex formation over time is depicted with dashed lines (---).

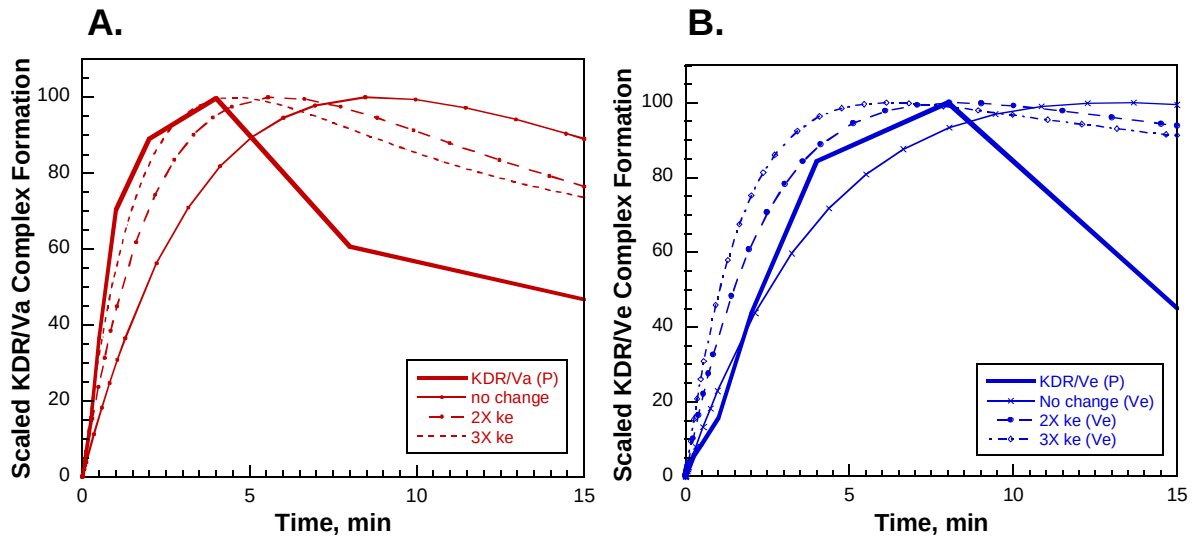


Figure 33. Effect of altering internalization rates on KDR complex formation with exogenous addition of **(A)** VEGF-A, and **(B)** VEGF-E. The darker, solid lines (—) represent the normalized, averaged values for activated KDR, as determined by phospho-tyrosine residues on precipitated KDR from western blotting. The thinner solid lines marked with (x) represent the original model predictions. Subsequent model predictions with internalization rates (•) 2 times and (◊) 3 times the original value are indicated by dashed lines.

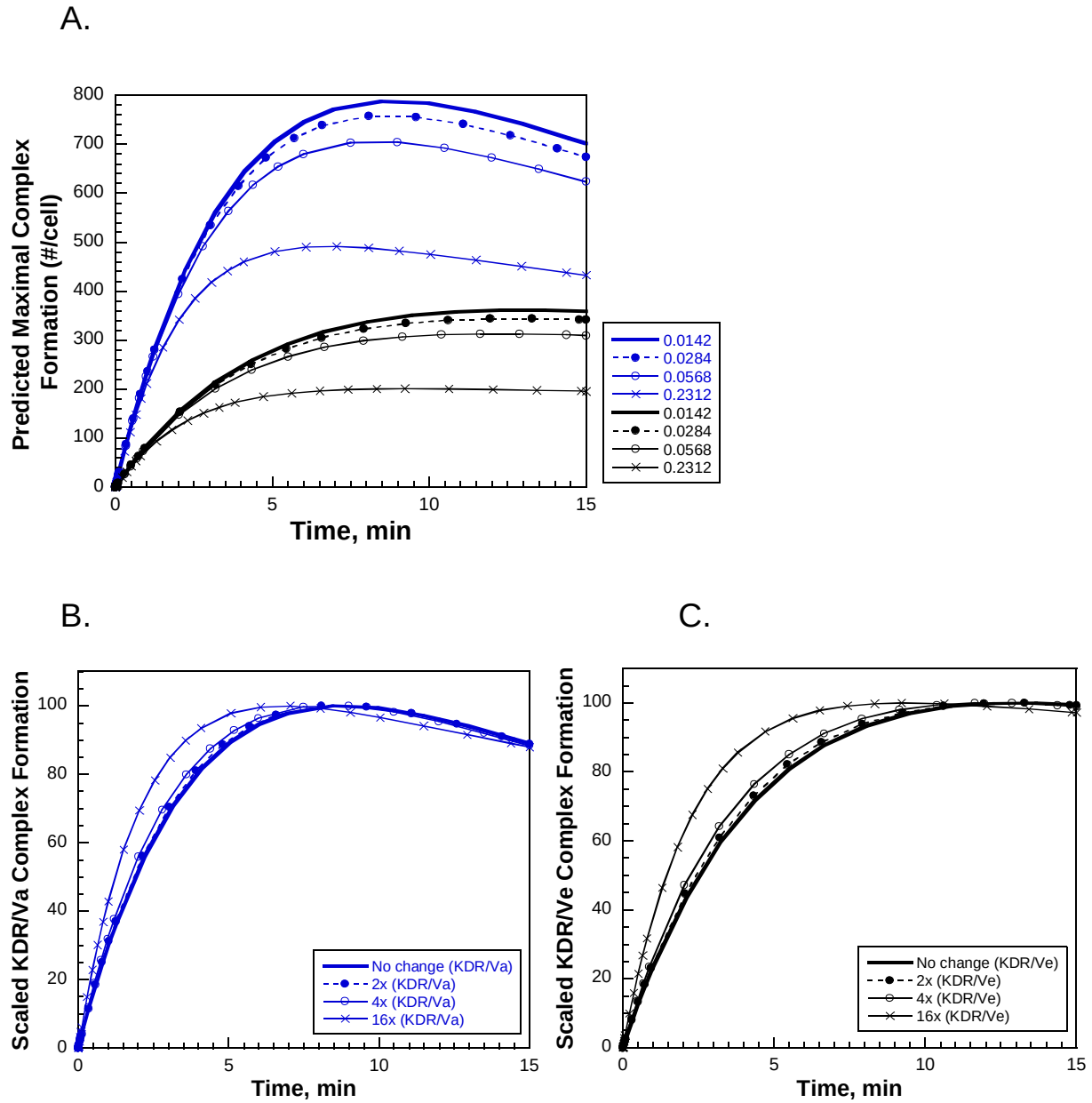


Figure 34. Sensitivity analysis for alterations in “off” rates. Original model predictions for KDR/Va & KDR/Ve complex formation are indicated by the thick blue and black lines, respectively. **(A)** Off rates are increased by 2, 4, and 16 times and the corresponding time course predictions are indicated by (•), (o), and (x), respectively. A gradual decrease in time to peak complex formation is coupled to a greater decrease in maximal complex formation, overall. When scaled to the maximal complex formation, the effects of increasing off rates on time-to-peak complex formation are shown for **(B)** KDR/Va complexes and **(C)** KDR/Ve complexes.

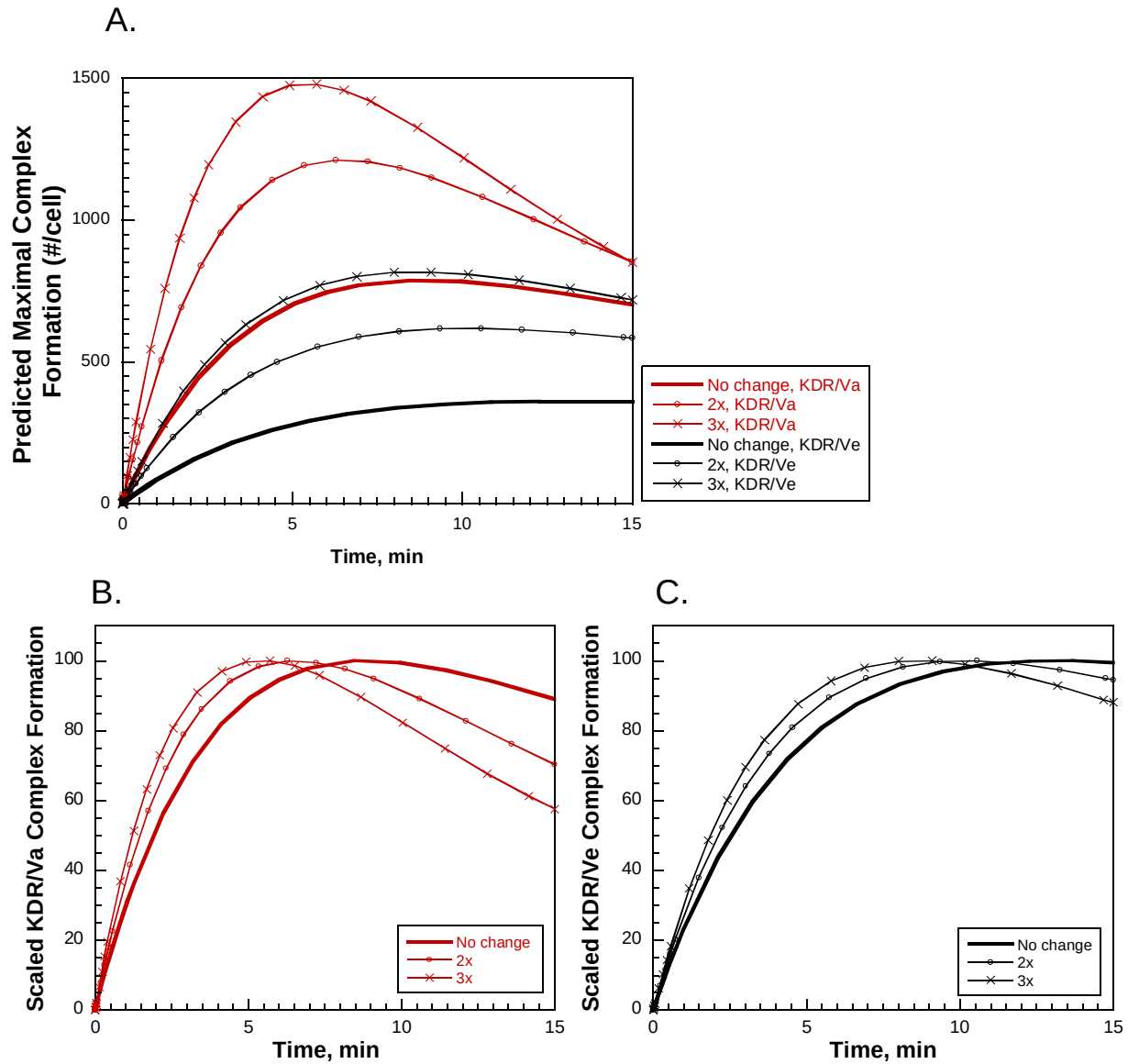


Figure 35. Sensitivity analysis for alterations in “on” rates. Original model predictions for KDR/Va & KDR/Ve complex formation are indicated by the thick red and black lines, respectively. **(A)** On rates are increased by 2 and 3 times and the corresponding time course predictions are indicated by (o) and (x), respectively. A gradual decrease in time to peak complex formation is coupled to an increase in maximal complex formation, overall. When scaled to the maximal complex formation, the effects of increasing on rates on time-to-peak complex formation are shown for **(B)** KDR/Va complexes and **(C)** KDR/Ve complexes.

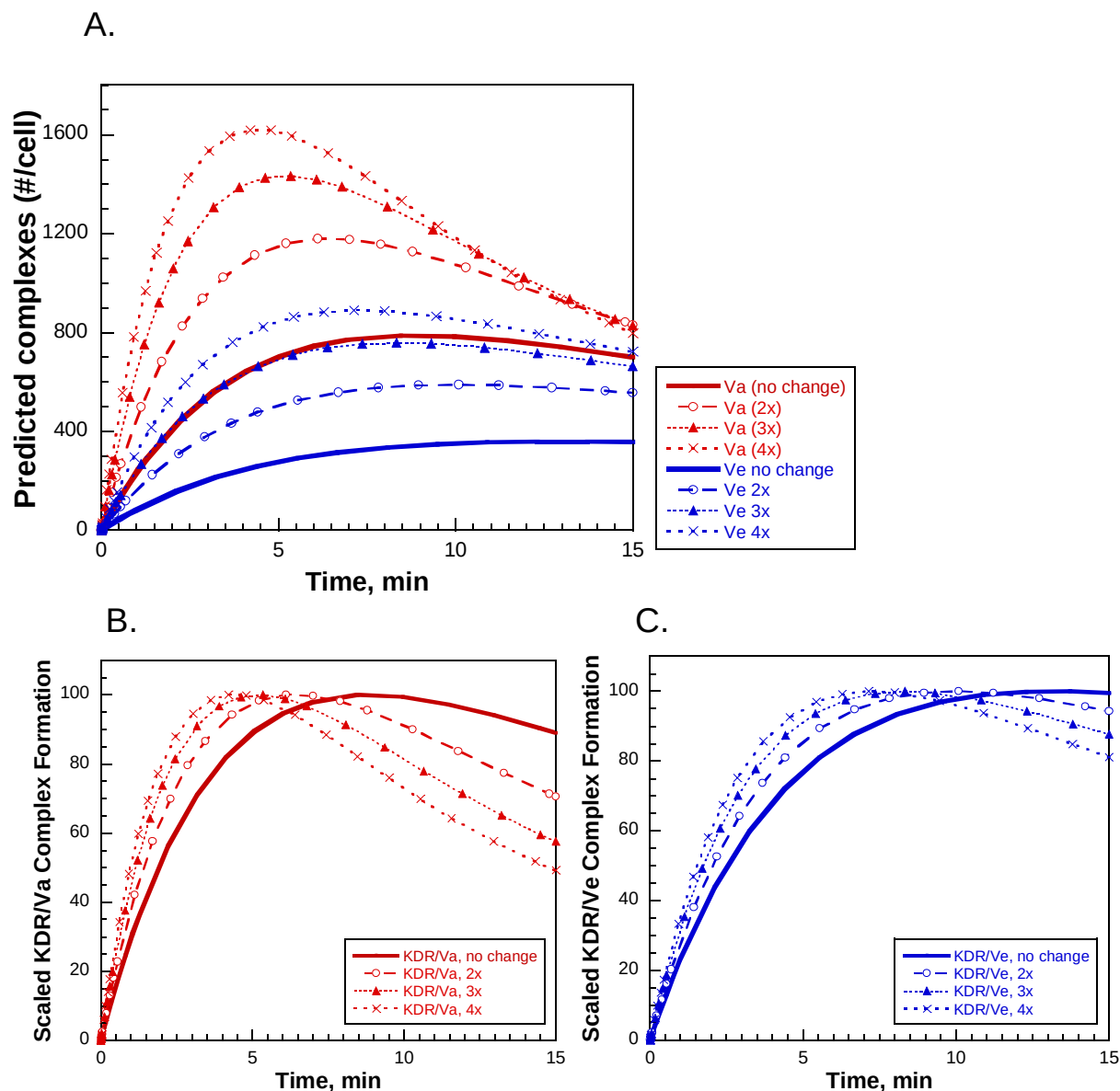


Figure 36. Sensitivity analysis for alterations in “off” rates. Original model predictions for KDR/Va & KDR/Ve complex formation are indicated by the thick red and blue lines, respectively. **(A)** Off rates are increased by 2, 3, and 4 times and the corresponding time course predictions are indicated by (o), ($\diamond$), and (x), respectively. In each case, the on rate was adjusted in concert to the off rate. These new rates were calculated according to the K_D values for each ligand binding to KDR. A gradual decrease in time to peak complex formation is coupled to an increase in maximal complex formation, overall. When scaled to the maximal complex formation, the effects of increasing off rates—with simultaneous on rate adjustment—on time-to-peak complex formation are shown for **(B)** KDR/Va complexes and **(C)** KDR/Ve complexes.

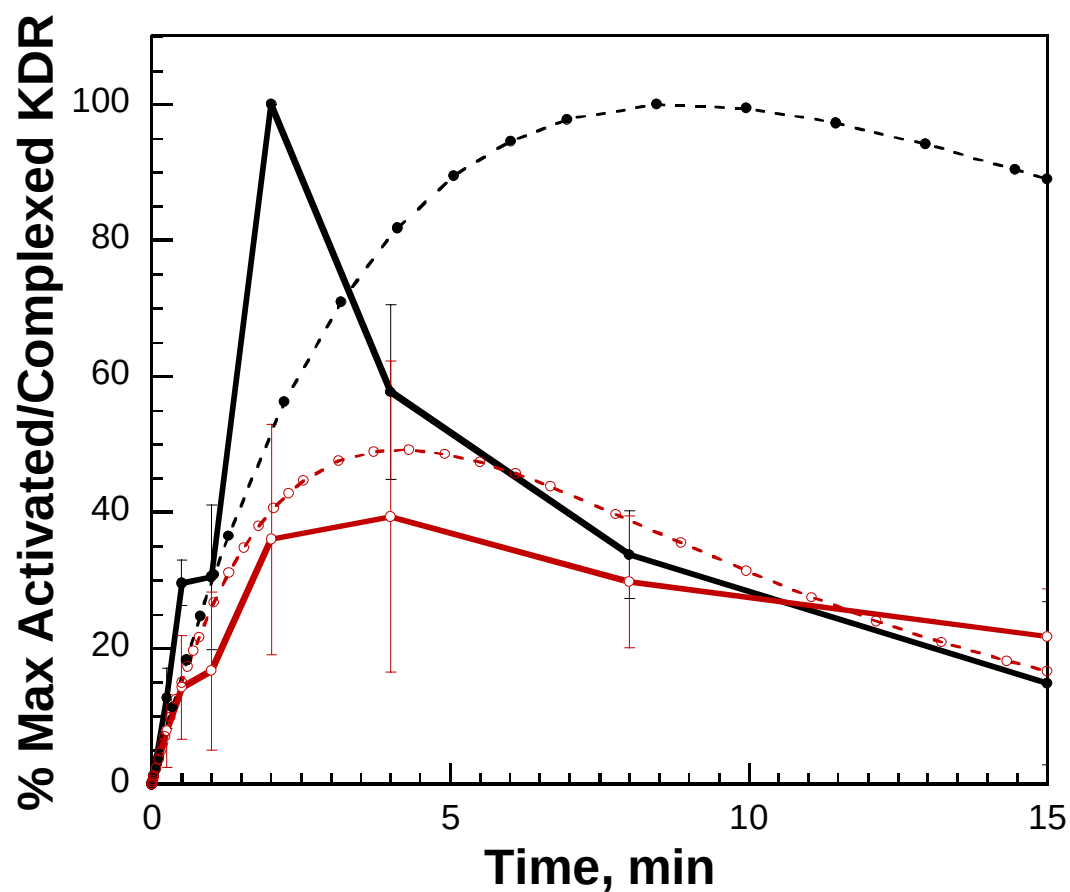


Figure 37. Comparison of scaled time-course activation data for (●) VEGF-A (25 ng/ml) and (○) VEGF-A & sFlt-1 at 25 ng/ml and 50 ng/ml, respectively. Results are scaled to peak activation/complex formation for exogenous VEGF-A addition (V_{a0}) without sFlt-1 present in the systems. Normalized, averaged values for activated KDR, as determined by phospho-tyrosine residues on precipitated KDR from western blotting, are depicted with solid lines (—), and model results of simulated KDR complex formation over time is depicted with dashed lines (---).

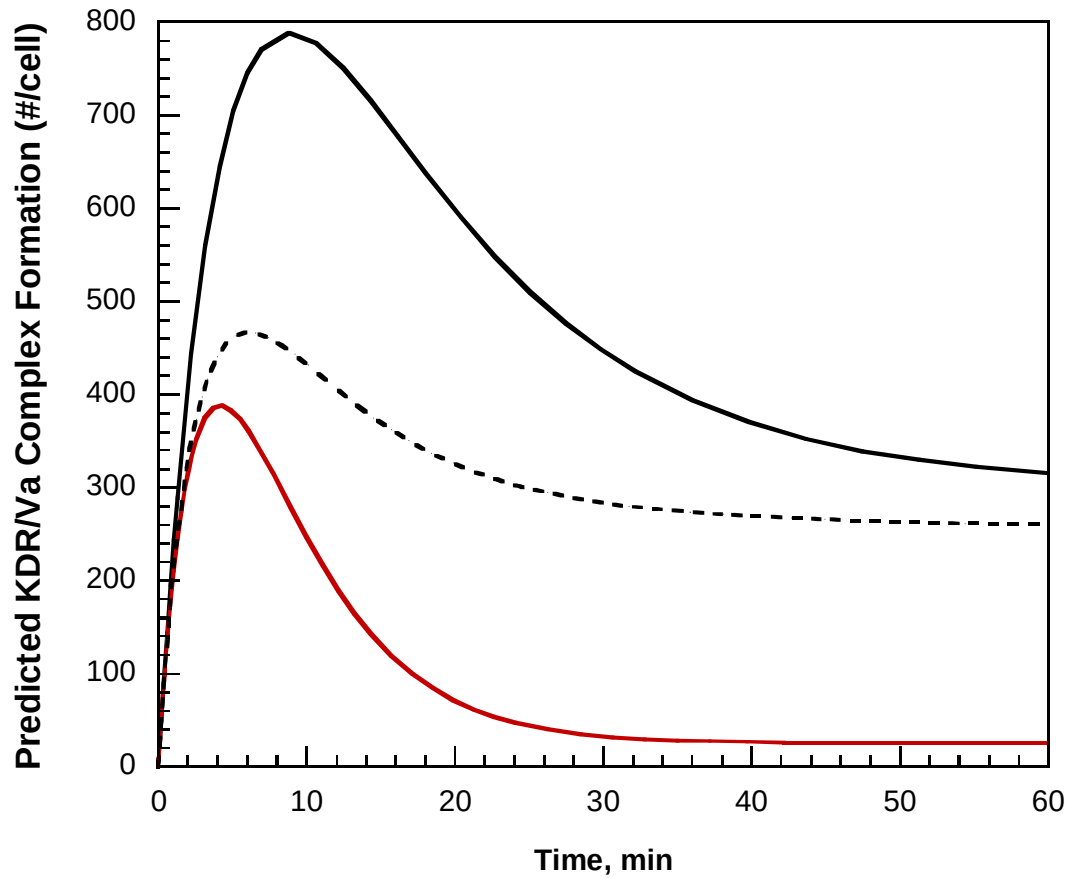


Figure 38. Predicted ligand-shifting effect of PIGF on simulated sFlt-1 inhibition of the formation of KDR/Va complexes over 60 minute timespan. The number of KDR/Va complexes/cell in the absence of either sFlt-1 or PIGF is depicted by the thick black line. With the addition of sFlt-1 at 50 ng/ml, the predicted decrease in complex formation is depicted with the thick red line. These effects are reversed when PI_0 is added at 2.38 nM (---). The Va effectively shifts to KDR and ~50% of the original KDR/Va complex predictions are restored.

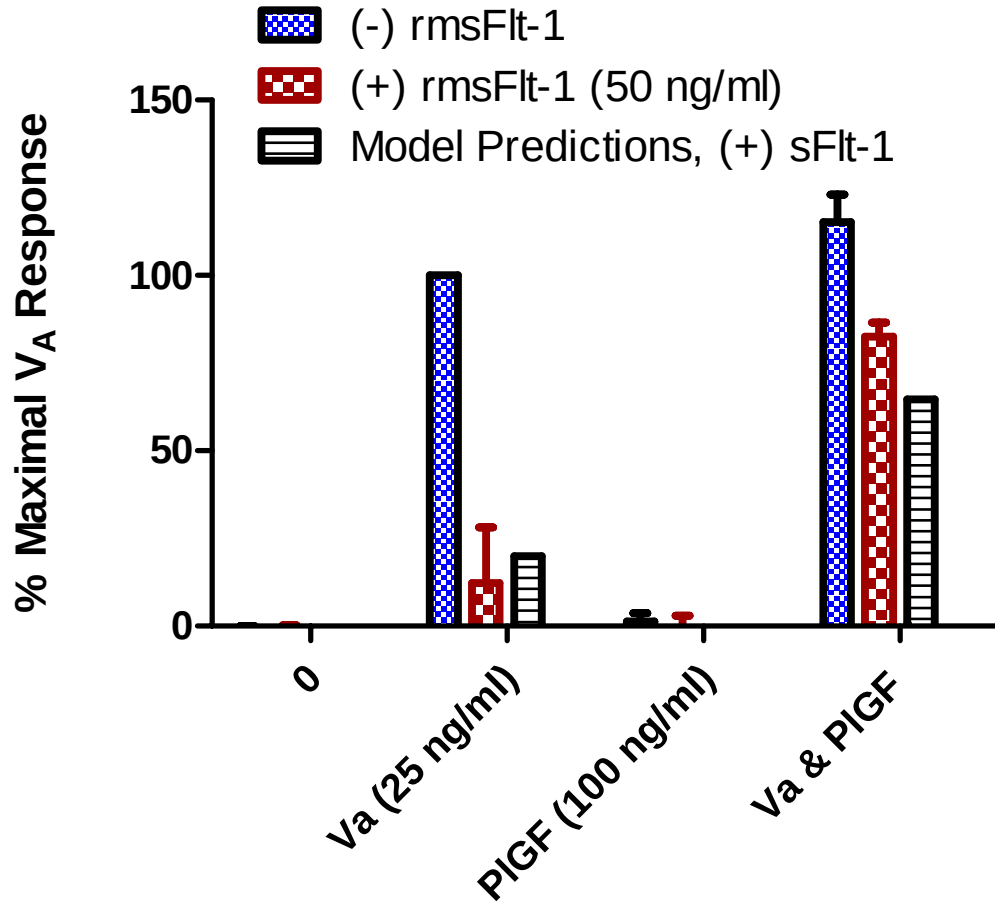


Figure 39. Ligand-shifting effects of PlGF on sFlt-1 inhibition of VEGF-A signaling and simulated complex formation. For inositol phosphate accumulation experiments, average values are shown after subtraction of basal inositol phosphate accumulation and normalized to the maximal stimulation by VEGF-A within each experiment. Plotted data represent the averaged normalized values from three, 60 minute experiments. Model predictions represent “net signaling effects” which were scaled to the percentage of maximal VEGF-A binding and complex formation. The signaling effects were derived by integrating and calculating the area under complex simulation curves—representing the sum of signaling complexes formed over time.

CHAPTER 5: DIMERIZATION MODEL

INTRODUCTION

There is a generally held belief that sFlt-1 inhibits VEGF action by simply binding and sequestering free VEGF from the overall pool of available growth factor, but there has not been a consensus reached on whether sFlt-1 may also act in an inhibitory fashion by forming of inactive heterodimers with KDR and Flt-1 [84]. Kendall *et al.* (1996) were able to demonstrate a dominant negative inhibitory role for sFlt-1 when they showed that the receptor, produced by HUVE cells in culture, formed VEGF-stabilized ternary complexes with KDR. Of note was the observation that the heterodimeric sFlt-1/KDR complexes were stabilized by VEGF and only a small amount of these receptor complexes were observed when VEGF was not present in the incubation mixture [84].

In another unrelated study, Ruch *et al.* (2007) demonstrated, via electron microscopy, that uncoupled KDR monomers normally have low affinity for each other and the receptors remain predominantly monomeric in the cell membrane [164]. The authors concluded that the likely mechanism of dimerization begins with the binding of VEGF to the Ig-like domains of one receptor subunit, which in turn increases the probability that a second receptor can bind the second monomer unit in already tethered ligand. After that, the authors suggest that the two receptors become essentially cross-linked to each other through interactions with ligand, whereby the Ig domains closest to the cell membrane are held in close proximity forming a low-affinity homotypic interaction further stabilizing receptor dimers [164].

These sorts of observations are important when determining the extent to which sFlt-1 may act in a dominant-negative role. If the formation of heterodimers between KDR and sFlt-1 is restricted to when VEGF is present, this would dramatically alter its heterodimeric, inhibitory role. It would be limited to forming these heterodimers when it can diffuse to the cell surface and bind to the receptors with VEGF. At the same time, even if dependent on the presence of VEGF for receptor coupling, the “pool” of available receptor homodimers could still be significantly reduced. This observation is of particular importance when trying to model the interactions of VEGF with the Flt-1 or KDR. Though the *in vitro* results presented here lead to no strong conclusions about dimerization, an ensuing model of sFlt-1 inhibition of KDR/Va complex formation is

presented with the possibility of receptor dimerization included. In light of recent observations about ligand-promoted receptor coupling, receptor dimerization is included in this model only after monomeric receptors are complexed with VEGF-A. Ultimately, the simulations from this model are compared to those obtained from the original base model presented earlier in this document.

METHODS

Model description

This modified model consists of a system of 9 ordinary differential equations, based on mass action kinetics, which describe secretion, transport, binding, and internalization of KDR and VEGF in with sFlt-1 present in the system. The equations formulated, describing the change in species concentration with time, are conceptually similar to those formulated earlier in this document and are extended to the VEGF binding kinetics model illustrated in Figure 40. The empty and complexed receptors are internalized with characteristic rate constants and this act of internalization consumes the receptors and ligands. The main volumetric species represented in the model is VEGF-A, whose concentration is denoted as V (M). I assume that the only surface species, monomeric KDR denoted $R1$, is the only species capable of binding V at the cell surface. Further, it is assumed that, within the simulated experimental “wells” there is symmetry, such that the concentrations of V and receptors are spatially uniform throughout the plane of the cell surface. In addition to KDR, monomeric sFlt-1 denoted $R2$, is the only moiety interacting with V in the extracellular space. Just as it is assumed that receptor concentration is uniform over the cell surface, it is also assumed that $R2$ is uniformly distributed throughout the cell medium. It is assumed that all receptors initially exist in their monomeric form—with homodimerization and heterodimerization occurring only after binding VEGF. In this amended model, V binds to monomeric sFlt-1 or KDR in a single step, and dissociates from those receptors in a single step with no further process modifying this interaction. After that, the monomeric ligand complexes are able to couple to unbound receptor monomers to form additional complexes. This includes the heterodimerization of KDR with sFlt-1 at the cell surface. With these constraints in mind, six complexes are included in the model: $C1$, formed from KDR monomers and

VEGF-A; **C2**, formed from the dimerization of **C1** and a KDR monomer; **C3**, an sFlt-1 monomer and VEGF-A; **C4**, an sFlt-1 monomer coupling with **C3**; **C5**, the dimerization of sFlt-1 monomers with **C1**; and **C6**, the coupling of KDR monomers with **C3**. Aside from the inclusion of dimerization, all of the previous assumptions that were made during the formulation of the previous model are maintained here. Though this amended model includes the possibility of heterodimerization, it remains that only KDR homodimers can become activated and produce intracellular signaling cascades. Their simulated formation over time will be the focus of model predictions here.

The time evolutions of the receptors and complexes are represented by equations 5.1-5.8. The effective on rate and off rate constants for *V* and *R1* interactions ($M^{-1} \cdot \text{min}^{-1}$ and min^{-1}) are represented as k_1 and k_{-1} , respectively. The effective association and dissociation rate constants for *V* and *R2* interactions are represented as k_2 and k_{-2} , respectively. The coupling and uncoupling rates for are represented as k_c and k_{uc} . As before, it is assumed that synthesis rates (V_{SR1} & V_{SR2}) are essentially equivalent to internalization/degradation of unbound receptors. The internalization rates (min^{-1}) for receptors and ligand-bound complexes are represented as the k_e values.

$$\frac{dR1}{dt} = V_{SR1} - k_{eR1}R1 + k_{-1}C1 - k_1VR1 + k_{uc,C2}C2 - k_{c,R1C1}R1C1 + k_{uc,C6}C6 - k_{c,R1C3}R1C3 \quad (5.1)$$

$$\frac{dR2}{dt} = V_{SR2} + k_{-2}C3 - k_2VR2 + k_{uc,C4}C4 - k_{c,R2C3}R2C3 + k_{uc,C5}C5 - k_{c,R2C1}R2C1 \quad (5.2)$$

$$\frac{dC1}{dt} = -k_{eC1}C1 + k_1VR1 - k_{-1}C1 - k_{c,R1C1}R1C1 + k_{uc,C2}C2 - k_{c,R1C3}R2C1 + k_{uc,C5}C5 \quad (5.3)$$

$$\frac{dC2}{dt} = -k_{eC2}C2 + k_{c,R1C1}R1C1 - k_{uc,C2}C2 \quad (5.4)$$

$$\frac{dC3}{dt} = k_2VR2 - k_{-2}C3 + k_{uc,C4}C4 - k_{c,R2C3}R2C3 - k_{c,R1C3}R1C3 + k_{uc,C5}C6 \quad (5.5)$$

$$\frac{dC4}{dt} = k_{c,R2C3}R2C3 - k_{uc,C4}C4 \quad (5.6)$$

$$\frac{dC5}{dt} = -k_{eC5}C5 + k_{c,R2C1}R2C1 - k_{uc,C5}C5 \quad (5.7)$$

$$\frac{dC6}{dt} = -k_{eC6}C6 + k_{c,R2C1}R1C3 - k_{uc,C5}C6 \quad (5.8)$$

The evolution over time of the volumetric species concentrations (e.g., dV/dt) is represented by equation 5.9. As previously stated, one of the inherent assumptions in this model is a well-mixed assumption; therefore, the fluid phase diffusivities of ligand/soluble receptor are neglected.

$$VNa \times \frac{dV}{dt} = [-k_2VR2 + k_{-2}C3 - k_1VR1 + k_{-1}C1] \quad (5.9)$$

The initial conditions for the simulations are listed in equation 5.10. The model is intended to help in understanding the inhibitory effects of sFlt-1 on KDR binding of VEGF, with the inclusion of receptor coupling dynamics. For this reason only a few simulated conditions are included: (1) where only V is added to the system with KDR; (2) where both V and $R2$ are added as volumetric species. The differences in predicted complex formation were compared between these two cases, but also compared to those results generated earlier in this document with the base model of sFlt-1 inhibition.

$$\begin{aligned} V(t=0) &= V_0 \\ R1(t=0) &= R1_0 \\ R2(t=0) &= R2_0 \\ C1(t=0) &= C2(t=0) = C3(t=0) = C4(t=0) = C5(t=0) = C6(t=0) = 0 \end{aligned} \quad (5.10)$$

Initially, there were no preformed complexes $C1$ - $C6$ at time = 0, and V is added as a step change at this point. The equations were solved on a PC in Matlab version 7.5.0.342 R2007b (The Mathworks, Inc.) using ODE15S, a stiff differential equation solver, with a relative error tolerance of 1×10^{-3} and an absolute tolerance of 1×10^{-6} . While many of the parameter values used in the earlier model will remain the same, some unique values were used here and are listed in Table 3.

In the existing experimental literature, there are no available coupling and uncoupling rate constants for the process of KDR or sFlt-1 dimerization; therefore, other growth factor systems that have been more extensively studied served as a bench-mark

for the determination of these optimized parameters. In a recent modeling paper by Mac Gabhann and Popel (2007) the authors selected specific coupling and uncoupling rates for a ligand-induced dimerization study with VEGF [109]. These rates for ligand-induced coupling agreed with estimates of the theoretical diffusion-limited rate constants derived in previous studies [165, 166] and are, therefore, used in the current model. Though the coupling rates for heterodimerization of sFlt-1 to membrane-bound KDR may very well be different from those for homodimerization, this initial model will only include one effective rate constant for all coupling events. Differences in the coupling and uncoupling rates of homo- and heterodimeric VEGF receptors are areas that should be explored in future studies. These respective rates are used in this initial model—keeping in mind that they may be altered or optimized in later expansions of this dimerization model. This is only an initial attempt to investigate the effects of receptor dimerization; therefore, these rates are not optimized here.

In addition to this difference to the base model, it is apparent from the new binding schematics (Figure 37), that multiple receptor internalization rates need to be included for different complexes as well as unbound KDR monomers. It has previously been reported that maximal rates of receptor kinase activity are required for VEGF-induced receptor internalization [78]; therefore, the original internalization rate of KDR complexes (used in the base model) is used for homodimeric KDR complexes (C2). All other complexes and KDR monomers are assumed to be internalized at a rate 10-fold slower than the C2 complexes.

Cross-linking experiments for determination of sFlt-1 and KDR dimerization

In order to investigate if dimerization was occurring to a measurable level between rmsFlt-1 and KDR *in vitro*, I attempted a series of cross-linking experiments. KDR cells were grown to a level of ~80% confluence on BD BioCoat™ 60-mm poly-L-lysine dishes, and growth medium was replaced with 2.0 ml/dish assay medium (Hank's balanced salt solution containing 0.1% BSA, 25 mM Hepes pH 7.5). There were several treatment groups in this assay: (1) a no treatment, non-cross-linked control; (2) a dish with VEGF-A added at 50 ng/ml; (3) a dish with only rmsFlt-1 added at 100 ng/ml; (4) a dish with both VEGF-A and rmsFlt-1 added. Because sFlt-1 dimerization with cell-

surface KDR was of primary importance, rmsFlt-1 was added to the appropriate dishes and incubated at 37°C for approximately 30 minutes. Those dishes not receiving rmsFlt-1 were also heated in this step. This part of the protocol, ideally, allows the rmsFlt-1 to associate with the cell surfaces, whether through association with the extracellular matrix or receptor dimerization. The dishes were then cooled and VEGF-A was added to the appropriate dishes as a 10X solution to a final concentration of 50 ng/ml. These dishes were incubated, on ice, for approximately 3 hours. Following the incubation the dishes were washed three times with ~5 mls PBS (ice-cold). Immediately before its use, the crosslinker, Bis(Sulfosuccinimidyl)suberate (BS³, Pierce, Rockford, IL), was prepared by dissolving it in water to a final concentration of 30 mM. Following the washes, ~0.2 mls of BS³ was added to 1.8 mls of PBS in the dishes resulting in a final concentration of 3 mM BS³. The cross-linker was incubated on the cells, on ice, for 1 hour. The cross-linking was quenched for 20 minutes with the addition of 22 mM Tris-Glycine. Following quenching, the cells were lysed with buffer containing: 20 mM HEPES, 50 mM NaF, 1.0 mM Na₃VO₄, 10% glycerol, and 1% Triton X-100, and a protease inhibitor cocktail (final dilution 1:200, Sigma[®] P8340). After cell lysis, and removal of cellular debris by centrifugation, 1 µg (5 µl) of anti-KDR antibody (sc-504, Santa Cruz Biotechnology, Inc.) was added, and the samples were placed on a Labquake[®] overnight for end-over-end mixing at 4°C. After this incubation step, 20 µl (10µl packed beads) of protein A/G agarose bead slurry (sc-2003, Santa Cruz Biotechnology, Inc) was added to each sample and placed back on the Labquake[®] for end-over-end mixing at 4°C for 1 hour. The samples were spun at 8,000 x g in a microcentrifuge for 5 minutes and washed twice with 1 ml of cell lysis buffer. Samples were eluted from beads with the addition of 40 µl of 2x Laemmli Sample Buffer (BioRad[®]) and 30 µl of H₂O. Samples were heated for 5 minutes at 95°C which allowed the receptor to be separated on a polyacrylamide gel and probed by western blotting. SDS-PAGE was performed (as previously described for rmsFlt-1 and KDR) using precast 3-8 % XT Criterion gels loaded with 30 µl of each sample. Western blotting was performed for KDR and Flt-1 proteins as previously described.

Measurement of phospholipid metabolites for cell-association of rmsFlt-1

In order to determine if sFlt-1 was associating, in any way, with the cell surface, the generation of phospholipid metabolites were measured with or without the soluble receptor present in solution. These experiments were carried out as previously described in this document with one major difference. The order of sFlt-1 addition was different in two separate experiments. In one experiment, chimeric mouse sFlt-1 (R&D Systems, Minneapolis, MN) was added to the wells with KDR cells prior to the addition of VEGF-A. In a separate experiment, recombinant sFlt-1 was not preincubated with the KDR-expressing cells, but it was instead added along with VEGF-A at the time of cellular activation. Both activations were carried out for 60 minutes. Chimeric sFlt-1 was added at a final concentration of 100 ng/ml, and when added for the purpose of preincubating the cells, sFlt-1 was added to the activation medium with the cells for ~30 minutes at 37°C. In both cases, VEGF-A was added at a final concentration of 50 ng/ml and cellular activation was carried out as previously described.

The [³H]inositol phosphate data were normalized by subtracting off basal (non-activated) values, and within each experiment the values were expressed as a percentage of the maximal elicited response. In this way the data could be directly compared between experiments. The averaged values were analyzed and a two-way repeated measures ANOVA with Bonferroni post-tests was performed using GraphPad Prism.

RESULTS

No sFlt-1/KDR dimerization apparent through crosslinking

There was no evidence of sFlt-1 dimerizing with KDR when crosslinked samples were probed with antibodies directed against KDR and Flt-1 (Figure 41). The control cell sample, whole cell lysates from KDR cells activated with VEGF-A (lane 1) was the standard used to determine the presence of KDR in all other samples. This sample displayed the characteristic doublet which migrated to ~190-200 kDa when probed for KDR. This indicated that the receptor was being expressed on the cells. In all subsequent samples, with the exception of Lane 2 of Figure 38 which contained only rmsFlt-1, KDR was present as evidenced by the intense bands. If rmsFlt-1 had

dimerized with KDR at the cell surface, and been successfully crosslinked to the membrane-bound receptor, there should be bands that migrate slightly above the KDR bands. These higher molecular weight bands would represent the combined M.W. of both monomeric receptor subtypes coupled together. In all cases, when the cells were incubated with sFlt-1 with and without VEGF-A present, there were no bands apparent that would indicate dimerization had occurred—or at least that the event had been captured with the crosslinking protocol described above. A darker “smear” appears at the top of the lane representing VEGF-A treatment only (lane 5, Fig. 41) which may indicate ligand induced cross-linking of the KDR monomers that was unable to be resolved due to the high molecular weight of these cross-linked receptor dimers. This is not seen in the lane where VEGF-A and sFlt-1 were incubated together on these cells.

Cell association of sFlt-1 may significantly increase its inhibitory effect

In order to further investigate the possibility of dimerization of sFlt-1 with KDR, VEGF-A promoted inositol phosphates accumulation was measured with chimeric sFlt-1 introduced at different times relative to the cellular activation. In one case, the chimera was added at the same time that VEGF-A was added to the cells. In the second experiment, the sFlt-1 was preincubated for a period of time with the KDR cells before VEGF-A was added. In principle, this preincubation provides a period of time whereby the soluble receptor can associate with the cell surface—either by binding to HSPGs or by dimerizing with KDR. The order of addition mattered greatly to the amount of inhibition observed for VEGF-A mediated cellular activation. When the cells were not pre-incubated with sFlt-1, significant inhibition of VEGF-A activation ($P < 0.05$) was observed, as cellular activation was inhibited by ~36% of the non-inhibited value (Figure 42). In contrast, when the cells were pre-incubated with sFlt-1, the inhibition of the original VEGF-A signaling was decreased by 90%—a value not only significantly different ($P < 0.05$) from the uninhibited cell sets, but also significantly less ($P < 0.001$) than the value obtained without preincubation of sFlt-1. These initial results suggest that sFlt-1 is associating with the cells in a way that caused a significant increase in the soluble receptor’s ability to inhibit VEGF-A binding and activation.

Inclusion of dimerization drastically affects sFlt-1 inhibitory function

The challenges in comparing *in vitro* western blot results to *in silico* model simulations have already been discussed. It was not concluded whether the differences noted between the two data sets were due to the limitations in comparing the two methods or an inherent weakness in the model development. Every attempt was made to reconcile the differences by altering the parameters that governed model solutions but this did not resolve the issues in a satisfactory manner. In this case, an initial attempt was made to alter the model itself and include dimerization of receptors—cell associated and soluble—to determine if this could improve the overall model predictions.

Before attempting to model sFlt-1 inhibition the base model, including dimerization, was tested to determine if it effectively predicted VEGF complexing with KDR. As before, the predictions in the maximal complex formation numbers match quite well to the *in vitro* western blotting results, but there is an obvious delay in the peak formation of predicted KDR/Va from both models (Figure 43). The time to peak formation for KDR/Va in the original model occurs at roughly 8.5 minutes, while the same value for KDR/Va falls at approximately 7.2 minutes. On initial inspection, it seems that the inclusion of a coupling rate actually improved the time-to-peak formation of predicted complexes, when compared to *in vitro* results. This needs to be qualified by the fact that the maximal number of complexes formed with the inclusion of dimerization in the model dropped by 34% compared to the base model (data not shown). While improvements were noted in the timing of complex formation, there was a similar drop in maximal complexes formed over time.

When the same approach was taken for modeling sFlt-1 inhibition, it seems that dimerization dramatically increased the ability of sFlt-1 to inhibit VEGF-A promoted complex formation. As noted in chapter 4, when starting conditions for Va_0 and $[sFlt-1]_0$ are the same, the percentage of maximal VEGF-A-dependent activation/complex formation for *in vitro* experiments and the original computation modeling experiments compare reasonably well, with values of 39% and 49%, respectively. With all ligand and receptors concentrations kept the same, the inclusion of sFlt-1 in a computational modeling system that includes heterodimerization leads to complete inhibition of the

formation of KDR/Va complexes (Figure 44). The addition of coupling rates for both receptor types increases the potency of sFlt-1's inhibitory role in such a way that only a 1:1 ratio of sFlt-1 to KDR (#/cell) inhibits maximal KDR/Va complex formation by 35% (Figure 45). As suggested by Kendall *et al.* [84], when sFlt-1 is able to heterodimerize with KDR, its inhibitory value increases in dramatic fashion.

DISCUSSION

One of the challenges in developing this model was predetermining the level of complexity to use in the steps of binding and complex formation. There have been few studies that have actually investigated the dimerization process for VEGF receptors—fewer still that have attempted to determine the extent to which heterodimerization occurs between the primary cell-surface receptors. Some of the more convincing evidence, to date, was a study by Kendall *et al.* (1996) which demonstrated that sFlt-1, produced by HUVE cells in culture, formed VEGF-stabilized ternary complexes with KDR. With similar methods to those described above, the authors preserved dimerized complexes by covalently crosslinking the receptors in complex with VEGF. One difference between this study and the experiments I described in this chapter is that the Kendall *et al.* (1996) used radiolabeled VEGF and cation-exchange resin to determine that the receptors had heterodimerized. This is a much more sensitive method and the presence of even very small amounts of radioactivity can be detected. One observation of note was that the heterodimeric sFlt-1/KDR complexes were stabilized by VEGF and only a small amount of these receptor complexes were observed when VEGF was not present in the incubation mixture [84]. Studies about VEGF receptor dimerization since then have been few in number, but one of the more recent examples, a study by Ruch *et al.* (2007), also demonstrated that uncoupled KDR monomers normally have low affinity for each other and the receptors remain predominantly monomeric in the cell membrane [164]. Like Kendall *et al.* (1996) they concluded that the likely mechanism of dimerization begins with the binding of VEGF to the Ig-like domains of one receptor subunit, which in turn increases the probability that a second receptor can bind to the monomeric ligand-receptor complex. In light of these observations, one unique aspect

of this subsequent model is that the dimerization events do not occur appreciably in the absence of VEGF.

Results of attempts presented here to determine the extent to which sFlt-1 associates with KDR were conflicting, at best. The cross-linking experiments presented earlier did not resolve any sFlt-1/KDR heterodimers. Even when VEGF was presented—in theory to stabilize dimer formation—still no receptor dimers appeared. Curiously, there was no evidence of even KDR homodimers that were covalently cross-linked, but this may be in part because of the difficulty in resolving proteins of that size. The gel system, the XT Criterion™ gel system was specially selected for its potential ability to resolve proteins of high molecular weight, but the faint “smear” appearing in the sample lane from which VEGF-induced KDR dimerization would occur was inconclusive. Ultimately, it cannot be concluded from these results that heterodimerization occurred with sFlt-1 and KDR. In the same way, it cannot be concluded that this is an optimal method to resolve very small amounts of heterodimerization. Perhaps the best method for resolving dimer formation would be a very sensitive method like that presented by Kendall *et al.* (1996).

An indirect method for determining sFlt-1 cell association was presented with the assay of VEGF-promoted inositol phosphate accumulation. When pre-incubated with KDR cells, the level of inhibition of cell activation lent by sFlt-1 was significantly greater than that which occurred with simultaneous introduction of both of the volumetric species together. In fact, generation of phospholipid metabolites was almost completely ablated. It is true that this method of introducing sFlt-1 into the system does not directly delineate between simple cell association of sFlt-1 or true heterodimerization of sFlt-1 and KDR, but the location of its association can be inferred. It seems unlikely that sFlt-1 would simply be binding to HSPGs or some other component of the extracellular matrix because in these cases it would not lead to more inhibition of cellular activation. In truth, the only way that this level of inhibition could be achieved is by association, in some way, with KDR. Whether it is direct heterodimerization, or some sort of HSPG-potentiated dimer formation, sFlt-1 is very likely acting in a dominant-negative fashion. This, coupled to its inherent ability to simply bind VEGF in the assay medium, is the best explanation of the inhibitor role of sFlt-1.

While these *in vitro* observations were far from conclusive for determining how sFlt-1 associates with KDR cells, a subsequent model was developed that included receptor coupling. This model effectively demonstrated that sFlt-1, when acting in a dominant-negative fashion, is able to inhibit KDR/Va complex formation to a much greater extent. In fact, the level of inhibition was so great that it may represent an unrealistic level of inhibition. Even at very low ratios of sFlt-1 to KDR, predicted complex formation was much less than the uninhibited values (Figure 45). If the *in vitro* conditions are replicated *in silico*, with VEGF-A at a concentration of 25 ng/ml and sFlt-1 at a concentration of 50 ng/ml, no KDR/Va complexes are predicted to form. It is likely that the problem here lies in the coupling rates. Because of the lack of literature values for coupling rates of VEGF receptors, a theoretical rate was used that was previously gleaned from another ligand-receptor system. This will already introduce some variability when comparing the predictions to *in vitro* results. Further, this same value was used for all coupling events in the model—even though it is likely that a different coupling rate would govern the formation of heterodimers between KDR and sFlt-1. Using the same coupling rate for heterodimerization of a soluble and membrane-associated receptor will almost assuredly amplify the dominant-negative inhibitory role of sFlt-1. The different coupling rates should be determined through future experimentation if an accurate model is to be developed that includes dimerization.

This supplementary model represents the first attempt to model sFlt-1 inhibition of VEGF binding with the inclusion of its dimerization to KDR. The model demonstrates that when that sFlt-1 is allowed to form inactive heterodimers with KDR, its ability to inhibit complex formation is greatly increased. The model is much simpler than the original base model presented earlier in this document, but the results here indicate that the base model could safely be extended to include these dimerization events—in order to produce a novel model of VEGF binding that is the most relevant to the physiologic system.

Parameter	Value	Source
Initial ligand concentrations, pM		
V_0	600, varied accordingly	Based on M.W. of V_A
Initial receptor populations, #/cell		
$R1_0$	3900	Experimental
$R2_0$	varied	
Equilibrium dissociation constants (K_D), pM		
$K_D, V, R1$	124	Experimental
$K_D, V, R2$	120	Experimental
Association “on” rates, $M^{-1} \cdot min^{-1} \times 10^7$		
$k_1, V, R1$	11.4	“off”/ K_D
$k_2, V, R2$	4.68	“off”/ K_D
Dissociation “off” rates, min^{-1}		
$k_{-1}, V, R1$	0.0142	Experimental
$k_{-2}, V, R2$	0.0056	Experimental
Coupling rates, $10^{-3}, (\#/cell \cdot min)^{-1}$		
$K_c, R1, R2, C1, C3$	2.1	Literature, [166]
Uncoupling rates, min^{-1}		
$K_{uc}, C2, C4, C5$	1.6	Literature, [166]
Internalization rates, min^{-1}		
k_{eC2}	0.186	Experimental
$k_{eR1}, k_{eC1}, k_{eC5}$	0.0186	literature
Insertion rate of receptors, $receptors \cdot cell^{-1} \cdot sec^{-1}$		
$V_{SR1}, V_{SR2}, V_{SR3}$	$k_{eR} \cdot R(t)$	Based on k_e
Translation of ligand conc, L/cell, $\times 10^{-9}$		inversely related to cell density and volume of well,
V	0.5	0.5 ml and 1×10^6 cells per well
Avagadro’s number, sites/mol		
N_A	6.02×10^{23}	

Table 3. Parameter values used in simulating VEGF-induced binding and dimerization of KDR and sFlt-1.

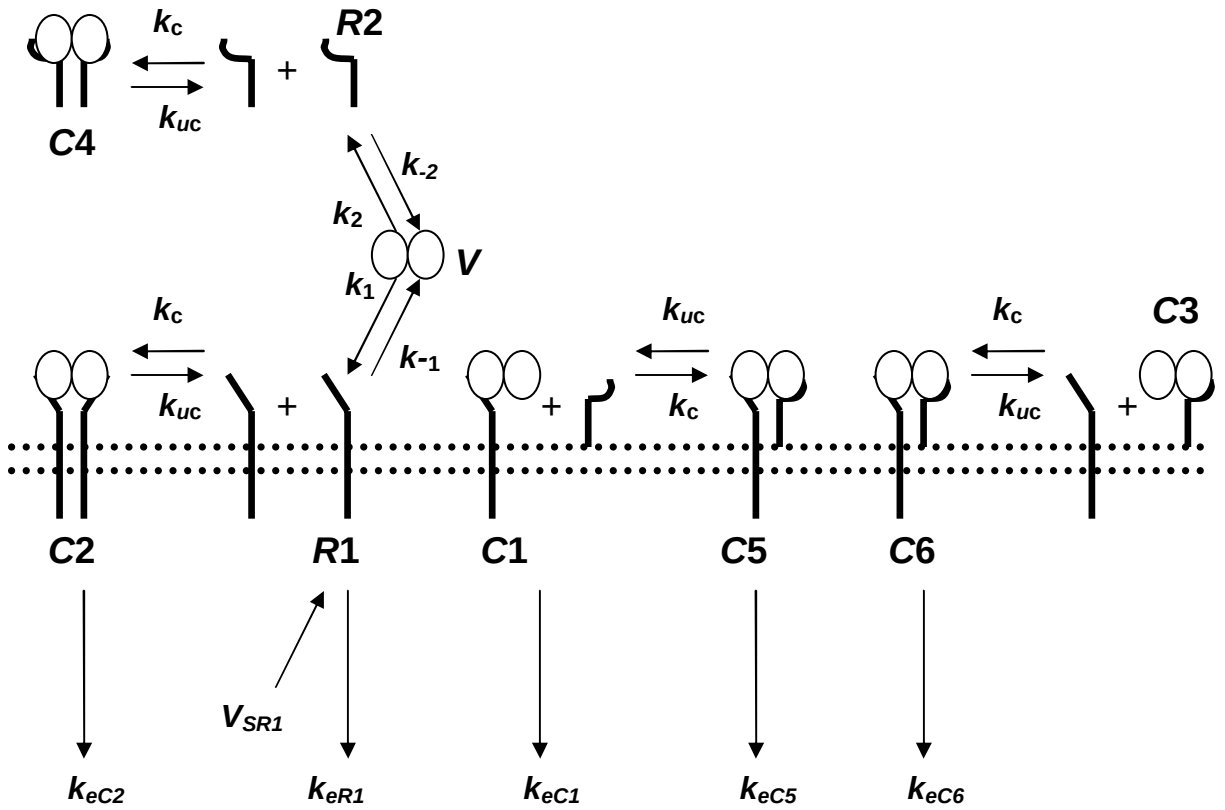


Figure 40. Schematic diagram of receptor-ligand system used to model VEGF-A-promoted KDR binding and dimerization with sFlt-1 present in the model system.

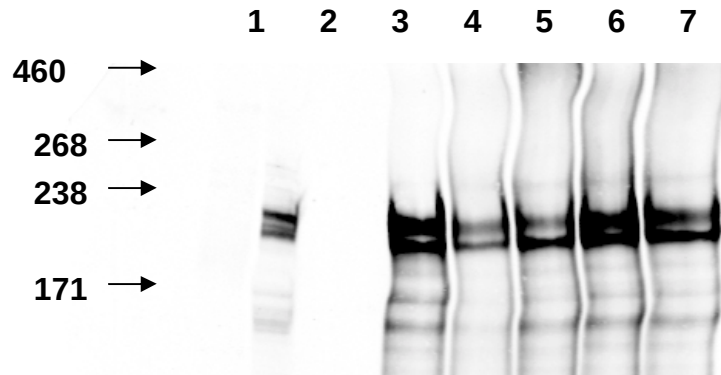


Figure 41. Western blotting results for cross-linking of rmsFlt-1 with KDR on C57 cells. When probed with an α -KDR antibody (sc-6251, Cell Signaling Technology®) the western blot demonstrated the presence of KDR in the cellular samples with characteristic duplicate bands for KDR, migrating to approximately 190-200 kDa. Lanes 1-3 contain control KDR, sFlt-1, and non-crosslinked cell lysate sample, respectively. After that, lane 4 contains cross-linked KDR cells with no treatment. Lane 5 shows cross-linked KDR cells with the addition of VEGF-A (50 ng/ml). Lane 6 contains crosslinked KDR cells that were pretreated with sFlt-1 (100 ng/ml). Finally, lane 7 contains crosslinked KDR cells that were treated with VEGF-A and sFlt-1.

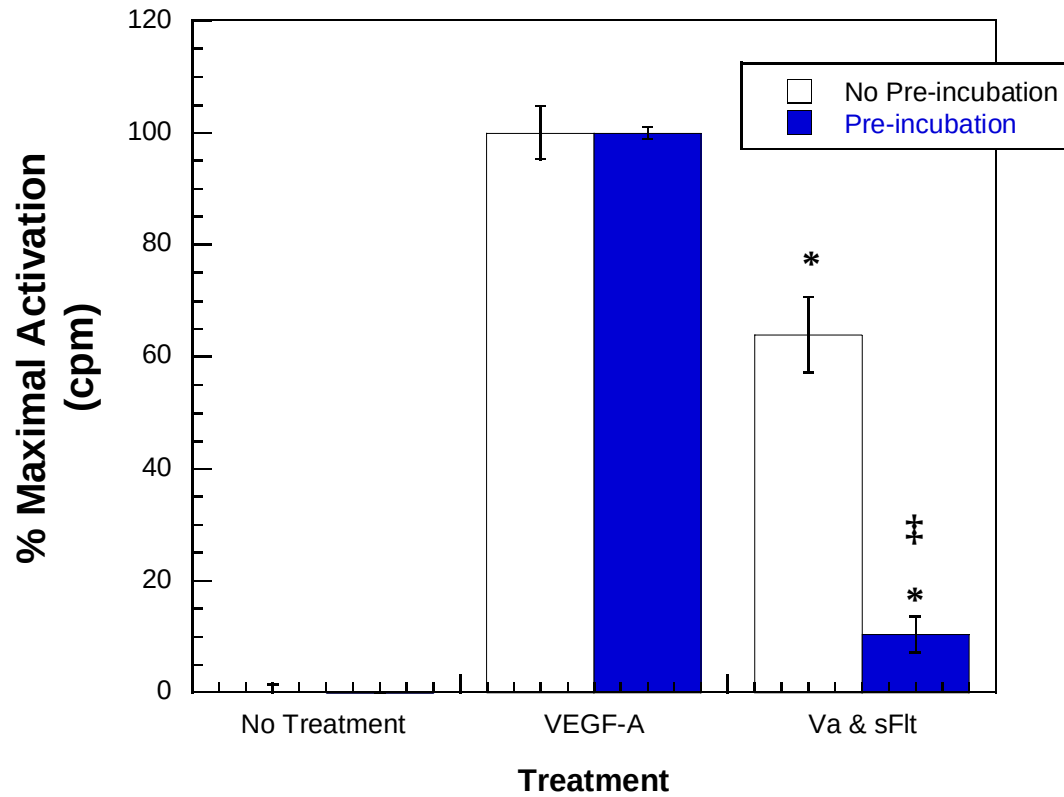


Figure 42. Effects of pre-incubation of sFlt-1 on its ability to inhibit VEGF-A signaling. Average values are shown after subtraction of basal inositol phosphate accumulation and normalized to the maximal stimulation by VEGF-A within each experiment. The plotted data represent the averaged normalized values for one 60-minute incubation experiment (performed in triplicate). *Indicates statistically significant difference from VEGF-A stimulation ($P < 0.05$), and ‡ indicates significant difference from “No Pre-incubation” ($P < 0.001$). Two-way repeated measures ANOVA with Bonferroni post-tests was performed using GraphPad Prism.

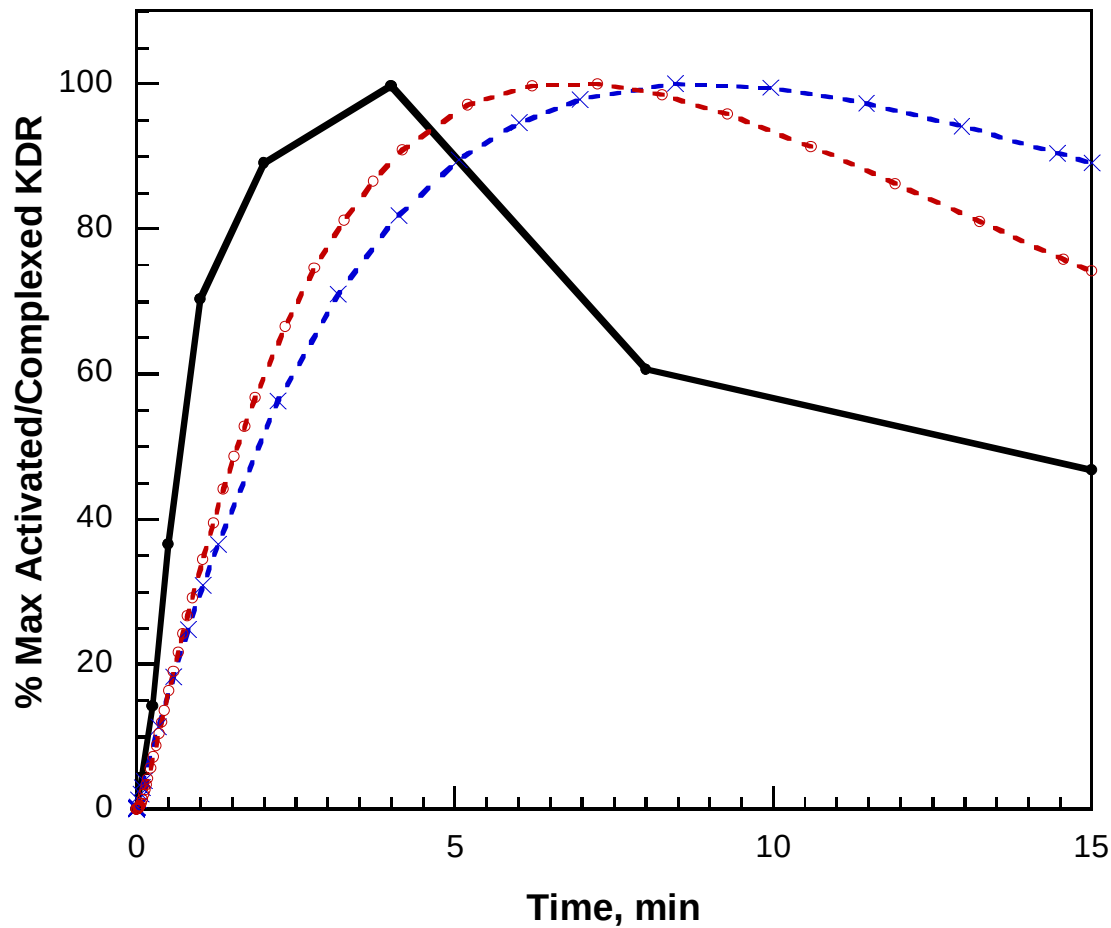


Figure 43. Comparison of scaled time-course activation data and model predictions for VEGF-A activation with and without dimerization included in model equations. Normalized, averaged values for activated KDR (C2) from *in vitro* activation experiments, represented by the thick black line (—) are scaled to peak activation/complex formation for exogenous VEGF-A (25 ng/ml). Model predictions, without dimerization (x), of simulated KDR complex formation over time is depicted with a dashed blue line (---). Model predictions, with dimerization (o), of simulated KDR complex formation over time is depicted with a dashed red line (---).

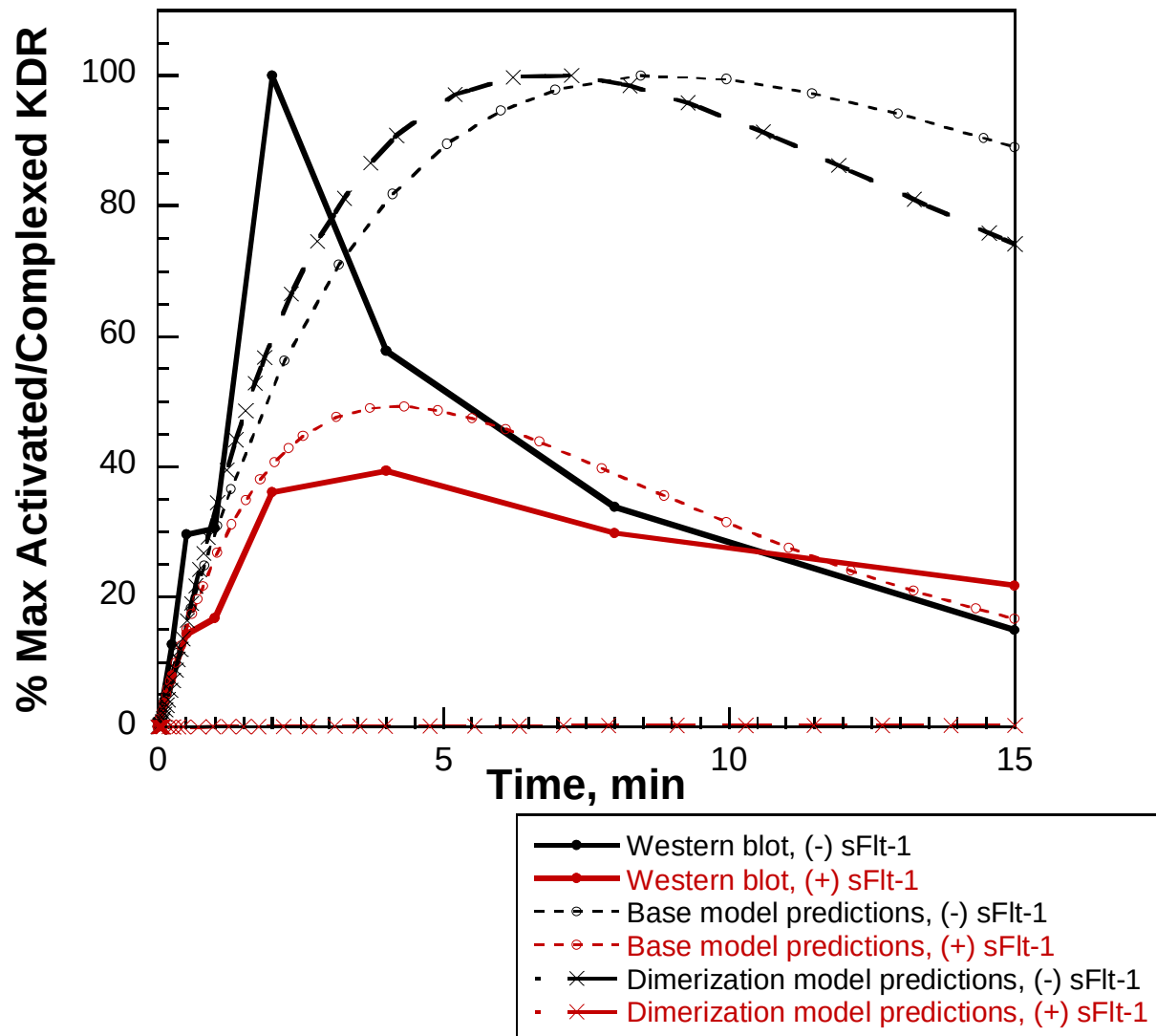


Figure 44. Comparison of scaled time-course activation data for VEGF-A (25 ng/ml) and VEGF-A & sFlt-1 at 25 ng/ml and 50 ng/ml, respectively. Results are scaled to peak activation/complex formation for exogenous VEGF-A addition (V_{a0}) without sFlt-1 present in the systems. Normalized, averaged values for activated KDR, as determined by phospho-tyrosine residues on precipitated KDR from western blotting (●), are depicted with solid lines (—). Model predictions of simulated KDR complex formation over time is depicted with dashed lines (---).

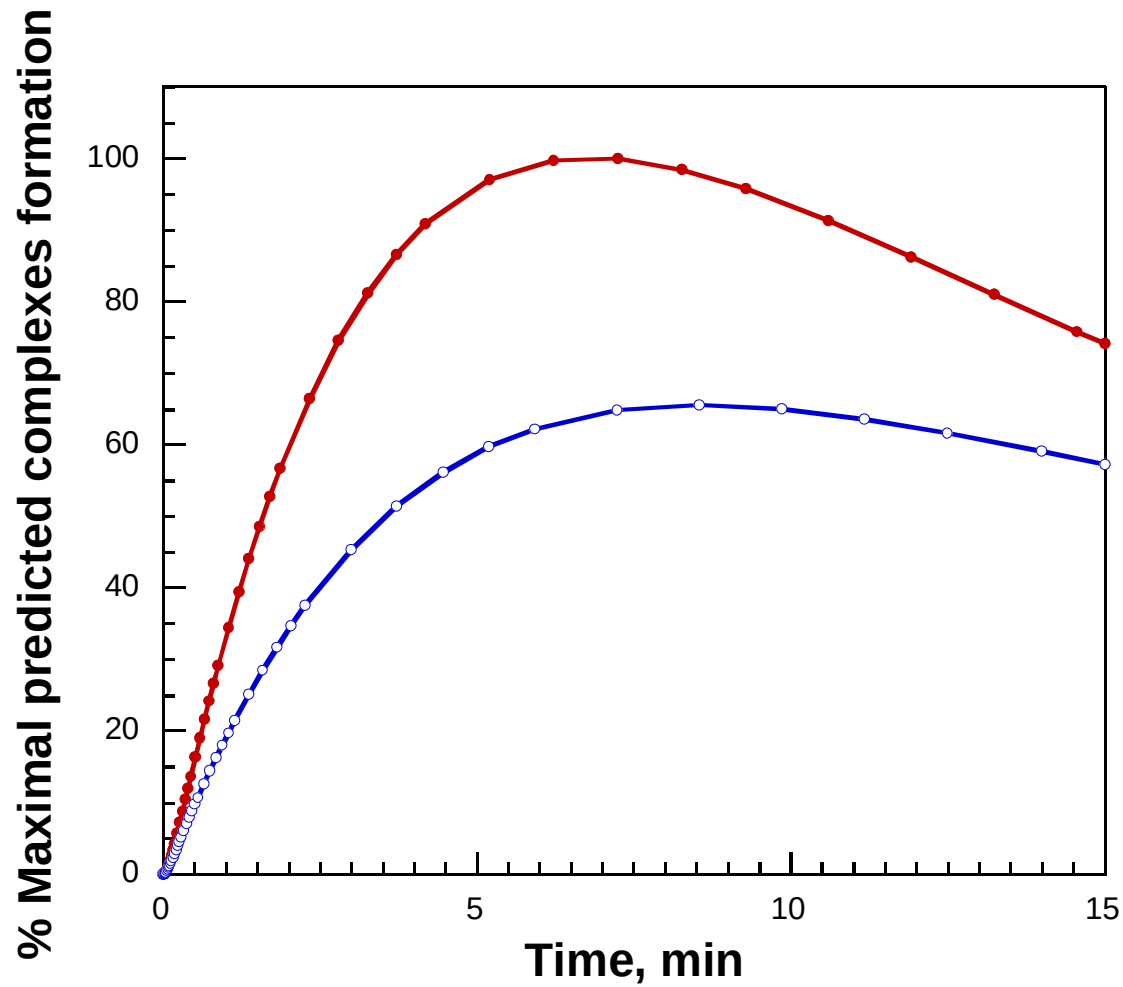


Figure 45. Comparison of scaled model predictions for sFlt-1 inhibition with heterodimerization. Model predictions for the formation of KDR-Va without sFlt-1 present (●) are compared to model predictions with the inclusion of sFlt-1 (○) at in a 1:1 ratio with KDR (#/cell).

SUMMARY & CONCLUSIONS

The system of ligands and receptors that potentiate the angiogenic process is large and complex. Though studying the binding and signaling of ligands in this system has been a growing area of research over the last few decades, there is still a need to better understand the interplay that exists between the ligands and receptors. Furthermore, there is a growing interest in identifying potential new inhibitors of VEGF-mediated actions in the microenvironment for angiogenic processes like the vascularization of tumors and events such as wound healing and preeclampsia. Soluble Flt-1 is a naturally-occurring inhibitor that can specifically and effectively block VEGF-A binding and signaling, but its mechanism of inhibition needs to be further studied. It has been proposed that sFlt-1 acts as a decoy receptor by binding VEGF-A and thereby regulating the availability of VEGF-related ligands for the activation of KDR [75, 136]. One very useful tool for understanding the consequences of sFlt-1 binding on the status of ligand binding and KDR activation would be a computational model that is able to replicate, *in silico*, the processes that potentially occur *in vitro* or *in vivo*. Until now, there have been no effective modeling attempts made to classify the inhibitory process of sFlt-1 binding.

The results presented in this document support the hypothesis that a mathematical model simulating VEGF binding in a multiple ligand, multiple receptor system could be developed which mimics experimental behavior of this system *in vitro*. One of the primary aims in developing this model was its use in investigating the competitive inhibitory effects that sFlt-1 may have on the ability of Flt-1 and KDR to bind ligands. It was designed with an eye toward *in vitro* experiments so as to provide a way of testing potentially interesting physical mechanisms of regulation in the VEGF system. Specifically addressed were the mechanisms leading to the inhibitory effects of sFlt-1 on VEGF-related ligands binding to cells expressing receptor populations that would normally be present. The model simulated a series of experiments in which the ratio of sFlt-1 to KDR and sFlt-1 to VEGF-A was varied. Not only was inhibition modeled, but the reversal of sFlt-1 inhibition was demonstrated in a series of simulations where ligands were competitively shifted from one receptor population to another in the presence of sFlt-1. The power of the model was further demonstrated by simulating

experiments not yet proposed for *in vitro* experimentation. The potentiation of VEGF-E binding was modeled, and a potentially new corollary of the VEGF 'sink' theory was proposed. The ability to mathematically model interactions prior to *in vitro* or *in vivo* experimentation is beneficial, and the potential uses of a model like this are far-reaching. It may be used in angiogenic drug design and development or even for determining *in vitro* conditions for tissue engineering applications. The possible applications are quite varied, but the model must be accurate in its predictions for it to be truly useful.

Not only was the development of the model a novel attempt for the VEGF system, but the testing of model predictions via *in vitro* experiments was an additional step not previously taken for this complex system of ligands and receptors. The results from western blotting experiments provided information about the concentration of sFlt-1 that is necessary for inhibiting the maximal VEGF-A response. A direct comparison of the scaled predictions of KDR complex formation over time and *in vitro* cellular activation assays showed a good qualitative match for the predictions in maximal complex formation numbers and activated KDR receptors. These *in vitro* observations could not discount the possibility that sFlt-1 associates with KDR at the cell surface, and this is an area that should be further investigated in studies subsequent to this. In light of these possibilities, an additional model was developed that included receptor coupling. This model predicted that sFlt-1, when acting in a dominant-negative fashion by forming inactive heterodimers with KDR, is able to inhibit KDR/Va complex formation to a much greater extent. The model is much simpler than the original base model presented earlier in this document, and in future studies the base model should be extended to include these dimerization events for all receptors present. This should result in a model of VEGF binding that is the most relevant to the physiologic system. Overall, the results from both models presented in this document represent a successful attempt at modeling the interplay of ligands and receptors in the VEGF system.

APPENDICES

Appendix A. Sequencing and PCR Primer Sequences

BH-261, GTGTCCAAGGTCAAGGTGCTGCAG

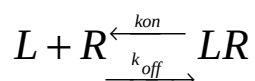
BH-436, ATTAAAATGATAACCATCTCGC

BH-437, GTCCAAGTTTCCCTGTAG

Appendix B. Equation derivations

Theory and derivation of homologous competition binding equation

To derive the equation used for non-linear regression to derive K_D and B_{max} values from the homologous competition binding experiments (Chapter 2), you must first derive the model that describes equilibrium binding. This model starts with the binding of a ligand with a receptor, where the binding follows the law of mass action:



For this equation, L is the concentration of free ligand and R is the concentration of free receptor. The association rate constant, k_{on} , is expressed in units of $M^{-1}min^{-1}$, while the dissociation rate constant, k_{off} , is expressed in units of min^{-1} . The formation of reaction products is similar to what is seen for enzyme kinetics, where the rate of formation of LR is equal to $L \cdot R \cdot k_{on}$ and the rate of the dissociation of LR is equal to $LR \cdot k_{off}$. When equilibrium is reached, this essentially means that the rate at which new ligand/receptor complexes are formed equals the rate at which they dissociate; therefore, at equilibrium:

$$L \cdot R \cdot k_{on} = LR \cdot k_{off}$$

In the case of radioligand binding studies, you measure specific binding, which is a measure of LR, so you can rearrange the equation to obtain on the left side of the equation:

$$LR = L \cdot R \cdot \frac{k_{on}}{k_{off}}$$

Previously, it was defined that the K_D , the equilibrium dissociation constant, is equal to k_{off}/k_{on} which has the units, M. Substituting this term into the above equation yields:

$$LR = \frac{L \cdot R}{K_D}$$

Because, in radioligand binding, is normally a measure of LR instead of free R, it is beneficial to remove the term for free receptor concentration and express it, instead, as the total number of receptors less the number bound, $R = R_{tot} - LR$. Substituting this into the previous equation leads to:

$$LR = \frac{L \cdot (R_{tot} - LR)}{K_D} = \frac{L \cdot R_{tot} - L \cdot LR}{K_D}$$

Again, because radioligand binding provides a measure of LR, rearranging the equation leads to the following derivation:

$$\begin{aligned} LR \cdot K_D &= L \cdot R_{tot} - L \cdot LR \\ LR(K_D + L) &= L \cdot R_{tot} \\ LR &= \frac{L \cdot R_{tot}}{K_D + L} \end{aligned}$$

Assuming that the amount of ligand, L, is varied and specific binding, LR, is measured in these assays, you can redefine these terms as X and Y, respectively. The total number of binding sites, also called B_{max} , can also be substituted into the equation for a new equation for equilibrium binding to a single class of receptors:

$$\text{Specific Binding} = Y = \frac{B_{max} \cdot X}{K_D + X}$$

For homologous competition experiments, like the ones used for the determination of K_D values for VEGF binding to KDR and Flt-1, the ligand concentration is equal to the sum of the labeled (hot) radioligand and the unlabeled (cold) radioligand. For specific binding, in this instance, you are measuring specific binding of all ligand times a fraction of the ligand that is labeled, $[hot]/([hot]+[cold])$; therefore, the new equation for specific binding is:

$$\text{Specific Binding} = \frac{B_{\max} \cdot ([hot] + [cold])}{K_D + [hot] + [cold]} \cdot \frac{[hot]}{[hot] + [cold]}$$

In the case of the homologous competition experiments performed here, total binding equaled specific binding plus nonspecific binding. Since nonspecific binding was the same for all wells in which it was included, it only depends only on the concentration of hot ligand, which is constant. The equation for specific binding, therefore, should have a nonspecific binding term added to define total binding:

$$\text{Specific binding} = Y = \frac{B_{\max} \cdot [hot]}{K_D + [hot] + [cold]} \cdot NSB$$

Because the concentration of the hot ligand is fixed during each experiment, it is the concentration of cold competitor that varies from well to well—normally equally spaced along a log scale. Therefore, in order to find the most accurate confidence intervals, the K_D values are fit as the $\log(K_D)$:

$$\text{Specific binding} = Y = \frac{B_{\max} \cdot [hot]}{10^{\log K_D} + [hot] + [cold]} \cdot NSB$$

Theoretical derivation of Cheng & Prusoff equation and its relationship with K_i

For competition binding studies, the concentration of competing ligand determined to effectively decrease specific binding of the radioligand by 50% (IC_{50} value) is not equivalent to the K_D for the competing ligand, but the K_D value for the competitor can be calculated from the IC_{50} [131]. This can be attained using the method

first introduced by Cheng and Prusoff (1973) and Chou (1974), whereby the authors demonstrated how to calculate the K_i (dissociation constant of an inhibitor) from the IC_{50} value attained from competition experiments [129, 130]. This method describes the kinetic situation of enzyme-substrate complex formation in the presence of an inhibitor. Velocity of the reaction, involving one substrate, can be described in a similar manner as for specific binding:

$$V_0 = \frac{V_{\max} \cdot S}{K_m + S}$$

where, V_0 = velocity of the reaction in the absence of an inhibitor; $V_{\max}$ = maximum velocity of the reaction; K_m = Michaelis constant of the substrate (S); S = substrate concentration [129]. Now when an inhibitor is present to compete for enzymatic activity, the velocity of the reaction is altered and a new term for inhibition is introduced that directly affects the Michaelis constant:

$$V_I = \frac{V_{\max} \cdot S}{K_m \left(1 + \frac{I}{K_i} \right) + S}$$

where, V_I = velocity of the reaction in the presence of the inhibitor; I = the concentration of the inhibitor; K_i = dissociation constant of the enzyme-inhibitor complex. Cheng & Prusoff (1973) went on to describe the situation where the concentration of inhibitor is roughly equal to the IC_{50} value [129]. When $I = IC_{50}$, $V_0 = 2V_I$, then

$$\frac{2 \cdot V_{\max} \cdot S}{K_m \left(1 + \frac{IC_{50}}{K_i} \right) + S} = \frac{V_{\max} \cdot S}{K_m + S}$$

When you rearrange this equation, you are left with the following equation:

$$IC_{50} = K_i \left(1 + \frac{S}{K_m} \right)$$

This equation can be related to competitive radioligand binding, whereby IC_{50} is the concentration of competitor that effectively competes for 50% of the specific radioligand binding; K_i , is the equilibrium dissociation constant for the competitor, K_i ; S represents the concentration of the radioligand, $[^*L]$ with which the competitor is competing; and K_m , the Michaelis constant for the substrate, would represent the K_{DL} for the radioligand. In this case, the equation can be rewritten as:

$$IC_{50} = K_i \left(1 + \frac{^*L}{K_{DL}} \right)$$

This equation can also be rewritten in such a way to solve for K_{DL} :

$$K_i = \frac{IC_{50}}{\left(1 + \frac{^*L}{K_{DL}} \right)}$$

Using this equation allows you to calculate the K_i of a competing, unlabeled ligand if you can determine the IC_{50} value of the competition. This was the approach taken earlier in Chapter 2, where the IC_{50} values for PIGF and VEGF-E were determined from their competition of VEGF-A binding. Of course, to determine these values, you must also know the concentration of radioligand (a constant throughout the experiment) and the K_D of this radioligand. Having already calculated the K_D values for VEGF-A binding to each respective receptor type, I used the aforementioned equation for determination of K_i .

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