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Targeting VEGF signalling via the neuropilin co-receptor

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The blockade of tumour vascularisation and angiogenesis continues to be a focus for drug development in oncology and other pathologies. Historically, targeting vascular endothelial growth factor (VEGF) activity and its association with VEGF receptors (VEGFRs) has represented the most promising line of attack. More recently, the recognition that VEGFR co-receptors, neuropilin-1 and -2 (NRP1 and NRP2), are also engaged by specific VEGF isoforms in tandem with the VEGFRs has expanded the landscape for the development of modulators of VEGF-dependent signalling. Here, we review the recent structural characterisation of VEGF interactions with NRP subdomains and the impact this has had on drug development activity in this area.

Introduction

Angiogenesis, the physiological process of new blood vessel formation, is an essential component of tumour progression. Tumour growth relies on the development of new vasculature to provide oxygen and nutrients to the proliferating cells while removing carbon dioxide and metabolic waste. Targeting angiogenesis has emerged as a prominent strategy to complement chemotherapeutic approaches to treat cancer [1–3]. A pivotal pro-angiogenic signalling molecule is vascular endothelial growth factor A (VEGF-A) which promotes proliferation, survival, migration and permeability of endothelial cells lining the inner layer of blood vessels [4]. The creation and maintenance of the vasculature not only supports rapid growth of malignant tumours but also increases metastatic potential of cancer by providing a route for the escape and travel of malignant cells to remote sites in the body. An increased level of circulating VEGF is directly correlated to a poor patient outcome. In other contexts angiogenesis is an instrumental physiological response to inflammation, and it has a central role in eye diseases such as age-related macular degeneration and diabetic retinopathy. Here again, blood vessel formation, maintenance and permeability are regulated via VEGF signalling, providing a potential target for therapeutic intervention.

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VEGFs and VEGFRs

The VEGF family of secreted glycoproteins comprises VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PGF) (Fig. 1). The best-characterised variant, VEGF-A is linked to important physiological processes such as wound healing, pregnancy and maintenance of blood pressure, as well as various pathologies dependent upon angiogenesis including cancer [4–6]. Alternative splicing of the VEGF-A gene produces several isoforms of the mature protein containing between 121 and 206 amino acid residues, with VEGF-A_{165} being the dominant isoform responsible for pathological angiogenesis [7,8]. VEGFs exert their activity through interaction with a family of three transmembrane tyrosine kinase receptors, the VEGF receptors (VEGFRs) [9,10]. The individual VEGF isoforms bind to the VEGFRs with differing affinity and the combinatorial nature of the growth-factor-receptor signalling complexes mediates a range of cellular responses (Fig. 1). In the endothelium, the pro-angiogenic function of VEGF-A is mediated primarily via interaction with VEGFR2, also known as kinase insert domain-containing receptor (KDR) [11]. VEGF binding to VEGFR2 is associated with receptor dimerisation and activation that triggers downstream signalling pathways including phosphatidylinositol 3-kinase (PI3-K)/Akt and phospholipase C gamma/extracellular signal-regulated kinase (PLCγ/ERK) cascades that, together, support cell survival

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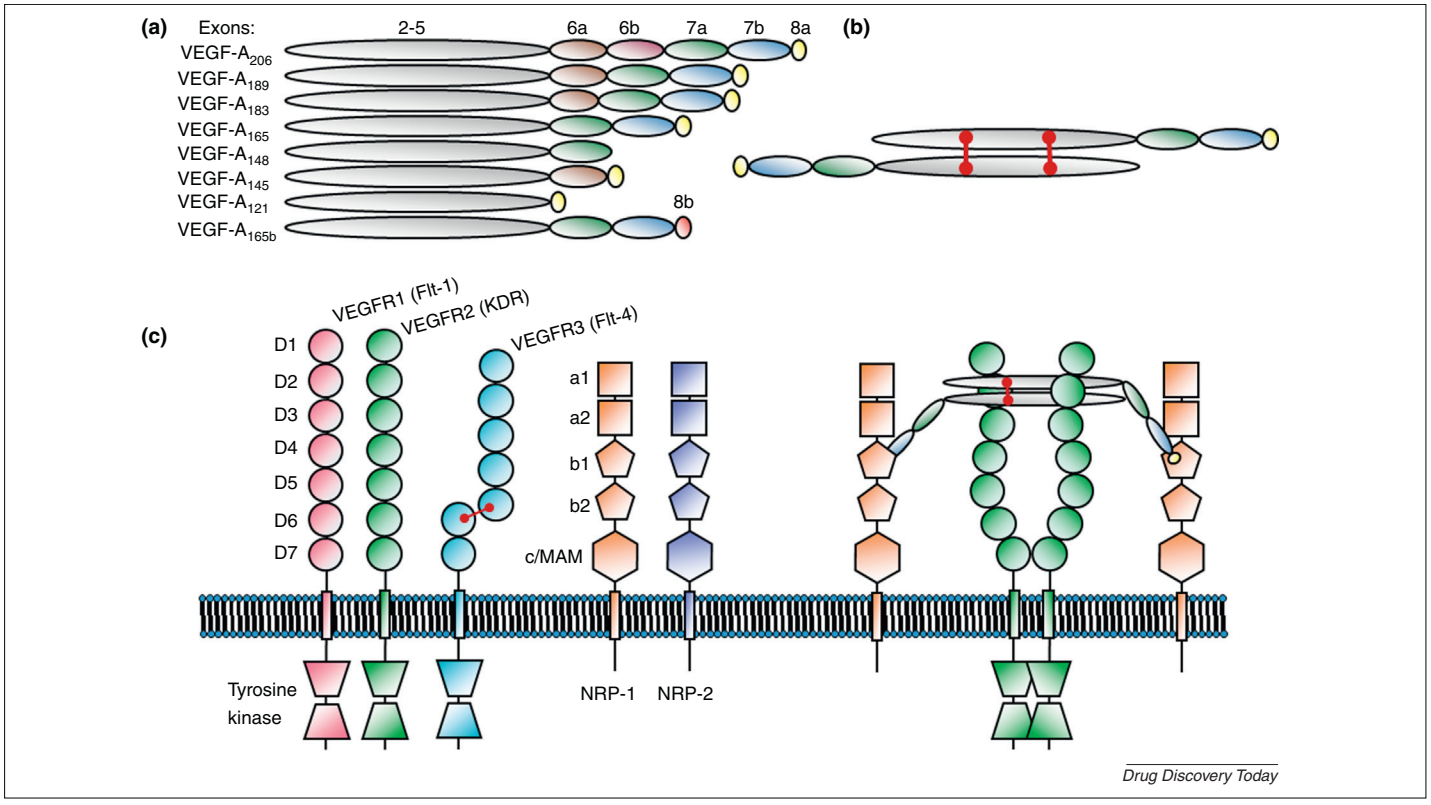

FIGURE 1

(a) Schematic representation of the family of VEGF-A splice isoforms indicating the exon-based origin of the domain organisation. The VEGFR binding domain derives from exons 2–5, the two-module heparin binding domain from exons 6 and 7 and the extreme C-terminal tail region (six amino acid residues) from exon 8. **(b)** VEGF proteins are disulphide-crosslinked (red) antiparallel homodimers, indicated here for VEGF-A_{165}. **(c)** Outline domain structure of VEGFR and NRP isoforms, drawn as transmembrane monomers (left). Cartoon representation (right) illustrating how VEGF-A_{165} might crosslink VEGFR2 and NRP1 to effect signalling, principally through *trans*-autophosphorylation of the VEGFR2 cytoplasmic domain. The position of glycosaminoglycans, implicated in complex formation, is not shown, as is the engagement of the NRP1 C-terminal region with putative binding partners, or the potential for direct contacts between the ectodomains of VEGFR2 and NRP1.

and the stimulation of proliferation. Other manifestations of VEGF signalling such as cell migration, blood vessel guidance and branching involve the formation of a more complex receptor assembly containing the VEGFR2 co-receptor neuropilin (NRP) [12,13].

In principle, interference of VEGF signalling, arguably the most direct and practical way to inhibit angiogenesis, can be carried out either through suppression of the activity of VEGF itself or through the blockade of VEGFR function. Both of these strategies have been explored experimentally. For example, removal of VEGF from the circulation is the mechanism of action of the first commercially available angiogenesis inhibitor bevacizumab, a specific humanised monoclonal antibody [14,15]. Bevacizumab (Avastin®) is approved for the treatment of certain metastatic cancers and, despite some reservations regarding safety and effectiveness [16,17], further clinical trials are underway to investigate other potential uses of this antibody in cancer and in eye disease.

Removal of VEGFs from the circulation has also been achieved with so-called 'VEGF traps' comprising engineered soluble VEGFR fragments. One of these, aflibercept, recently gained FDA approval for the treatment of age-related macular degeneration [18,19]. Similarly, it was reported that a mutated soluble NRP2 can reduce VEGF bioactivity [20]. In addition, some therapeutic success has been achieved by targeting VEGF receptor function through the development of antibodies that block ligand binding or VEGFR dimerisation, and with small molecule inhibitors of the intracellular receptor tyrosine kinase activity [21–27]. For example, sorafenib [23,24] and sunitinib [25–27] have been approved for use against advanced stage renal cell carcinoma. However, these molecules exhibit limited specificity: they not only interact with VEGFR2 but also with the platelet-derived growth factor (PDGF) receptor kinase and Raf kinase [28].

Ever since Folkman's hypothesis that inhibition of angiogenesis in solid tumours could be used to treat cancer [2], many potential inhibitors have been tested and currently there are numerous antiangiogenic therapeutics in development or undergoing clinical trials [1,15,29]. Unfortunately, many of the VEGF and/or VEGFR inhibitors tested so far have been reported to exhibit limited efficacy and high toxicity with problems including hypertension, protein in urine and arterial blood clots that can lead to stroke or heart attack, and result in only a mild improvement in patient survival [3,16,17,30–32]. New approaches to confront cancer-associated angiogenesis are being explored [33,34]. In particular, it would be desirable to achieve higher selectivity of VEGF-signalling inhibitors to eliminate off-target activity and thereby reduce toxicity. In the search for relevant new approaches, NRP1 has emerged as an attractive target owing to its role as a co-receptor for VEGF-A alongside VEGFR2. Here, we describe the molecular

biology of NRP1, its 3D structure and potential for therapeutic exploitation.

NRP: history and target validation

The NRPs, single-pass transmembrane receptors, originally discovered in the Xenopus nervous system [35], are highly conserved among vertebrates. In humans, the two related proteins NRP1 and NRP2 exhibit 44% sequence identity. NRPs are differentially expressed with NRP1 found primarily in arterial endothelial cells, whereas NRP2 expression is localised to venous and lymphatic endothelium. In addition, the two proteins exhibit differences in the subset of ligands that they recognise; for example, aside from VEGFs, NRP1 is a receptor for semaphorin-3A, -3C and -3F, whereas NRP2 preferentially binds semaphorin-3B, -3C, -3D and -3F [36–39]. Class 3 semaphorins are members of a family of axon guidance molecules that signal by interaction with transmembrane receptor complexes that incorporate NRPs as co-receptors to plexins – major receptors for all semaphorin family members. NRPs also recognise various VEGFs that, compared to semaphorins, represent a rather distinct set of ligands. Similar to the situation with semaphorins, there is distinct preference between the NRPs for different subsets of VEGFs. NRP1 interacts with heparin-binding isoforms of VEGF-A, -B, -E and PGF, whereas NRP2 interacts with VEGF-A, -C and -D [40–42]. VEGF–NRP binding is manifested primarily in the context of the NRP having a co-receptor role alongside the VEGF receptor.

The difference in expression patterns along with the distinct agonist specificity are reflected in the separate physiological roles that NRP1 and NRP2 have in development and disease. NRP1 gene deletion in mice results in embryonic lethality with embryos exhibiting abnormalities in heart, vasculature and neuronal guidance [43–45]. By contrast, NRP2-deficient mice are viable, although smaller in size than wild-type, and they display minor abnormalities in the lymphatic system [46]. Double knockout mice show an even more severe phenotype and die *in utero* at day E8.5 [47]. Most of the effects from the mutant mouse models point toward interaction of the VEGF-A_{165} isoform with NRP1 in endothelial cells and semaphorin-3A and/or semaphorin-3F with NRP1 and/or NRP2 in the nervous system [43–48].

The presence of NRPs has also been demonstrated in cancer cell lines as well as in various primary tumours. NRP1 participates in an autocrine $VEGF_{165}$-dependent signalling mechanism that promotes breast cancer [49,50]. Preclinical studies support a role for tumour cell NRP1 in lung and renal cancer cell migration, proliferation and invasion [51,52]. Recently, it was shown that NRP1 is essential in skin tumourigenesis, because NRP1 deletion abrogated the response of cancer stem cells to autocrine VEGF [53]. NRP1 also appears to support proliferation of human glioma stem-like cells in glioblastoma multiforme [54].

Many research groups have investigated NRP expression patterns and generated evidence for NRPs in endothelial and tumour cells [39,49,55–60]. Recently, a comprehensive evaluation of NRP1 expression was carried out for breast, colorectal and lung cancer [61]. In this study, a validated, highly specific monoclonal antibody was used for *in situ* analysis of NRP1 expression in cancerous tissue and during normal developmental angiogenesis. NRP1 was detected in more than 98% of blood vessels associated with primary and metastatic lung, colorectal and breast tumours. By contrast, the pattern of NRP1 expression on tumour cells themselves is much more varied: NRP1 was detected on 6% of primary breast carcinomas, 14% of secondary breast carcinomas, 36% of primary non-small-cell lung cancers (NSCLCs) and 50% of secondary NSCLCs; there was no NRP1 detectable on colorectal cancer cells. When the same antibody was used for the analysis of NRP1 expression during mouse development, NRP1 was found throughout the endocardial endothelium, whereas expression in the vasculature of other tissues was only detected in localised areas [61]. In addition, NRP1 was detected in the nervous system, smooth muscle cells and pericytes. Furthermore, blockade of NRP1 signalling resulted in defective VEGF-dependent angiogenesis in the postnatal mouse trachea providing further support for the hypothesis that NRP1 is a valid antiangiogenic target and potential antitumour target in at least a subset of cancers.

Additional NRP1 functions

There is a growing amount of evidence available that suggests NRP1 might display separate functions through mechanisms that might not involve VEGFR2. For example, suppression of NRP1 protein levels via siRNA results in changes in endothelial cell adhesion properties, whereas the same effect is not reproduced by siRNA knockdown of VEGFR2. It appears that NRP1 supports endothelial cell matrix adhesion through interaction with integrins in a VEGF-dependent manner [62–64]. Additionally, several studies have shown that NRP1 might signal through other receptor tyrosine kinases in response to ligands such as hepatocyte growth factor (HGF) and PDGF. It was reported that NRP1 and NRP2 mediate HGF-activated endothelial cell migration and proliferation [65] and that NRP1 interaction with PDGF-BB (the dimeric B-form of PDGF) stimulates migration of smooth muscle cells [66]. An increase in tyrosine phosphorylation of a scaffolding protein p130Cas, downstream of the kinase Pyk2, appears to be the major output of NRP1 signalling in response to HGF and PDGF in U87 glioma cells [67,68] and in human coronary artery vascular smooth muscle cells [69]. p130Cas tyrosine phosphorylation is linked to control of cell migration and is reduced upon NRP1 knockdown by siRNA. The activation of p130Cas seems to be dependent upon the cytoplasmic region of NRP1 because inhibition of VEGF-induced p130Cas tyrosine phosphorylation was also observed in experiments involving overexpression of NRP1 lacking its entire cytoplasmic region [67]. In addition, the cytoplasmic domain was implicated in a VEGF-dependent regulation of spatial separation of arteries and veins [70]. A knockin mouse model expressing NRP1 lacking the cytoplasmic domain showed atypically frequent occurrence of crossover of veins and arteries with no abnormalities in vasculogenesis and angiogenesis, suggesting that the cytoplasmic domain of NRP1 is required for normal arteriovenous patterning [70]. Furthermore, it was suggested that the NRP1 has $VEGF_{165}$- and/or semaphorin-3A-independent activity in regulating $\alpha5\beta1$-integrin traffic and downstream signalling [63]. NRP1 has also been implicated in signalling that leads to Pyk2-dependent phosphorylation at the Tyr407 site of focal adhesion kinase (FAK) [67] and activation of p38 mitogen-activated protein kinase (MAPK) which is involved in formation of pericyte-associated vessels [71]. These studies are however at an early stage; further investigation of the signalling pathways that operate downstream of NRP1 and the precise co-receptor context for these events is required to resolve the apparent complexity.

NRP structure

NRPs are type 1, single-pass, transmembrane proteins with a large (>920 amino acid residues) extracellular region comprising five modular domains named *a1, a2, b1, b2* and *c*, joined to a transmembrane helical region and a short (~44 residue) cytoplasmic domain [37] (Fig. 1). The different extracellular domains each share similarity to functionally diverse structural modules commonly found in cell-surface receptors and proteins involved in mediating cellular interactions (Figs 1 and 2a). The tandem *a* domains (*a1a2*) belong to the structural family of C1r/C1s-Uegf-BMp1 (CUB) domains homologous to complement binding factors C1r and C1s. The *b1* and *b2* domains are homologous to the C1 and C2 (discoidin) domains of coagulation factors FV and FVIII. The membrane-proximal *c* domain of NRP belongs to the family of MAM domains (meprin, A-5 protein and receptor protein tyrosine phosphatase μ) that have been implicated in protein homodimerisation. A single transmembrane helix that is reported to mediate NRP1 dimerisation [72] links the modular ectodomain to the intracellular region, the structure of which is unknown. It has been shown that the extreme C-terminal tripeptide (-Ser-Glu-Ala-COOH) is required for NRP1 interaction with the PDZ domain of synectin (also known as NIP or GIPC), a protein that is reported to play a part in the regulation of arterial branching [73–75]. Several reports confirm the important contribution of the NRP1 cytoplasmic region in various contexts, although it is not clear whether synectin is always involved.

There are several X-ray crystal structures available that describe either the isolated NRP1 *b1* domain or various combinations of tandem NRP1 (and NRP2) domains (i.e. *b1b2*, *a1a2* and *a1a2b1*) [76–78] (Fig. 2a). These structures have provided valuable information that has directed the development of NRP-targeted therapeutics. Several structures describe complexes of NRP domains with anti-NRP Fab fragments that interfere with either semaphorin or VEGF binding. Although biochemical evidence had already indicated that $VEGF_{165}$ binds to the *b1* domain of NRP1 via its C-terminal tail region, the first atomic model for this ligand–receptor interaction was derived from the crystal structure of a complex between NRP1 tandem *b1b2* domains and Tuftsin – an immunoreactive tetrapeptide that is produced by proteolysis of IgG heavy chain Fc fragments [77,79]. The Tuftsin amino acid sequence, Thr-Lys-Pro-Arg, is similar to the C-terminal tail of $VEGF_{165}$, Lys-Pro-Arg-Arg. The NRP1–Tuftsin structure enables the prediction of the mode of VEGF binding via the conserved carboxy-terminal Arg residue which, with specific electrostatic, H-bonding and van der Waals contacts, nestles in a shallow binding site on the *b1* domain surface (Fig. 3a). The rest of the peptide extends away from the groove formed by the β-strand-connecting loops of the *b1* domain β-sandwich fold. The location of the interaction site is analogous to the ligand-binding region for other examples of discoidin domains including those found in coagulation factors [77,80,81]. An interesting feature of the NRP1–Tuftsin structure is an extensive interface between the *b1* and *b2* domains that suggests a 'stable' relative orientation of these modules in the NRP extracellular region. In all subsequently determined crystal structures of NRP1 and NRP2 constructs the organization of this interface is conserved (Figs 2a and 3). The presence of an extensive

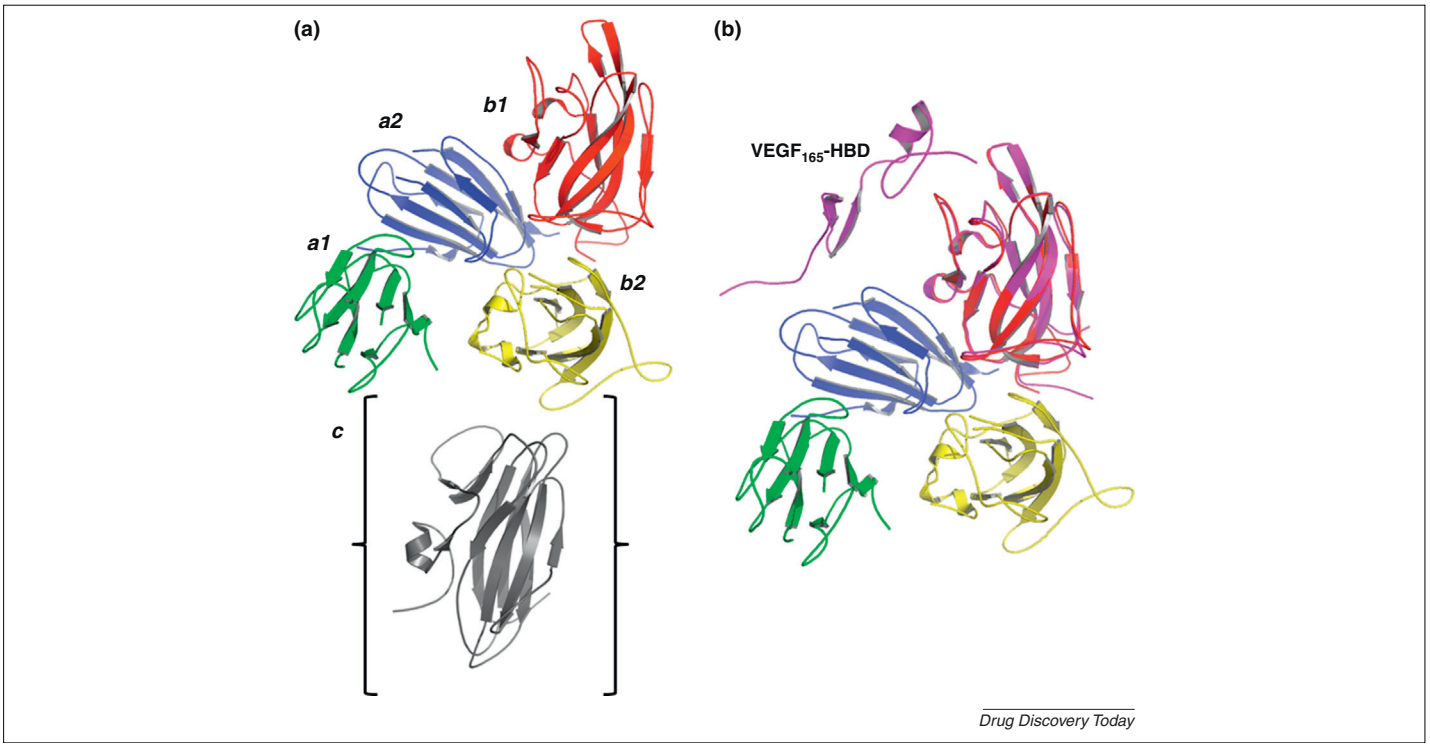

FIGURE 2

Topology and domain organisation of the NRP ectodomain. **(a)** A ribbon diagram of domains *a1a2b1b2* is shown with each domain coloured separately. The figure is based on the NRP2 chain from the crystal structure of NRP2 in complex with a semaphorin-blocking Fab (not shown; PDB 2QQL). A similar structure for NRP1 has not yet been obtained. No crystal structure of the c domain is available and a putative topology of this domain is shown in parentheses based on the crystal structure of the MAM domain from protein phosphatase μ (PDB 2C9A). **(b)** $VEGF_{165}$-HBD (magenta) and the *b1* domain from the fusion protein structure are superimposed over the *b1* domain from the *a1a2b1b2* structure shown in (**a**).

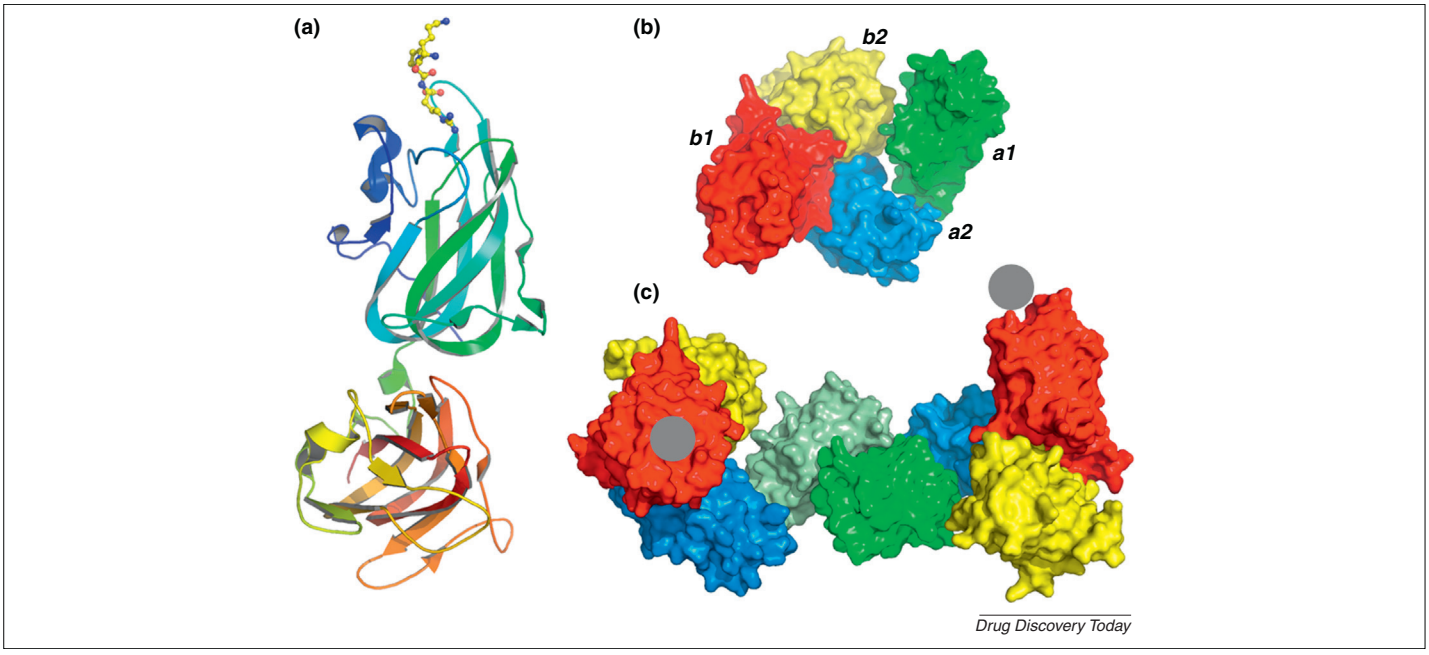

FIGURE 3

(a) Binding of Tuftsin to NRP1 *b1b2* domains (PDB 2ORZ). The peptide (ball-and-stick representation) sits in a groove on the top of the *b1* domain. The ribbon diagram of NRP1 is coloured as rainbow from the N (blue) to the C (red) terminus of the polypeptide. **(b)** Surface representation of the NRP2 *a1a2b1b2* monomer. **(c)** Putative dimer of the NRP2 *a1a2b1b2* structure based on the crystallographic symmetry contacts. The crystallographic interface that results in a dimer formation was observed in two different crystal forms of NRP2 suggesting that this type of interaction might exist in a solution before crystallisation; however, there is no direct confirmation that this dimerisation, mediated by reciprocal contacts between *a1* domains, is the biologically relevant form of NRP. Grey circles indicate areas engaged in binding to the C-terminus of the $VEGF_{165}$-HBD.

interdomain interface was also observed for the tandem *a2b1* domain combination [76]. In general, the packing of all domains within the context of longer polypeptide NRP constructs (i.e. *a1a2b1* or *a2b1b2*) is conserved irrespective of crystal symmetry, implying that even though the NRPs possess, in sequence terms, a modular organisation the tertiary domain architecture is well defined and probably presents defined orientations of outer faces for interaction with protein partners (Fig. 3). With exception of the relatively small domain interface between domains *a1* and *a2*, every conformational change involving disruption of the other interdomain contacts would probably incur a large energetic penalty.

Binding of $VEGF_{165}$ to NRPs is dependent upon the presence of the VEGF heparin-binding domain (HBD) encoded by exons 7 and 8 of the VEGF gene [77,82,83]. By contrast, the VEGFR2 binds $VEGF_{165}$ at a site that spans a region encoded by exons 2 to 5 [84–86]. A prevalent model for the tertiary complex formed between $VEGF_{165}$, VEGFR2 and NRP1 unites the two binding modes by suggesting that $VEGF_{165}$ acts as a bridge between the two receptor ectodomains (Fig. 1). However, the model does not differentiate between scenarios where NRP and VEGFR2 are present on the same or on neighbouring cells (Fig. 1). Furthermore, it appears that binding of $VEGF_{165}$ to NRP1 is required for the formation of detectable levels of complexes between VEGFR2 and NRP1 [87–90] and that the PDZ-binding domain of NRP1 is indispensable for NRP1–VEGFR2 complex formation [74].

Recently, in an attempt to gain an insight into the NRP–VEGF interaction the crystal structure of a fusion protein was determined, where the polypeptide sequence corresponding to $VEGF_{165}$–HBD was appended to the C-terminus of the NRP1 *b1* domain [91]. The fusion protein forms a homodimer such that the C-terminus of $VEGF_{165}$–HBD, comprising two Cys-bridged modules, binds to the ligand-binding groove of the *b1* domain of the neighbouring molecule in the asymmetric unit. The structure of this chimeric protein provides some additional information with respect to the mode of $VEGF_{165}$–HBD binding to NRP1 *b1* domain. However, it is possible that the orientation of the HBD with respect to the *b1* domain might be restricted by the covalent link of HBD to the C-terminus of the neighbouring *b1* domain, and influenced by crystal packing constraints (Fig. 2b). Based on this structure, and in the absence of definitive information about the segmental flexibility of $VEGF_{165}$, it is difficult to extrapolate from this result exactly how the full-length $VEGF_{165}$ homodimer interacts with intact NRP1.

So far, very little is known about the structure of the NRP *c* (MAM) and intracellular domains. Presently, there are only two MAM domain structures deposited in the Protein Data Bank (PDB), both of which are common to the receptor protein tyrosine phosphatase μ [92]. Although in the latter case the all-β domain is heavily glycosylated, there are no recognisable sequence motifs within the NRP1 and NRP2 MAM domains that suggest the presence of glycosylation sites. However, it has been demonstrated that NRP1 has an appended glycosaminoglycan – dominated by either heparan sulphate or chondroitin sulphate – attached at Ser612 which resides between the *b2* and *c* domains [68,93]. Interestingly, this NRP1 glycosylation, that as much as doubles the molecular mass of the polypeptide, has a profound effect on signalling. Although the effect of glycosylation on VEGF-dependent signalling remains to be investigated *in vivo*, it has been shown that the presence of chondroitin sulphate on NRP1

modulates p130Cas tyrosine phosphorylation in U87MG human glioma cells [68] and that the different composition of the NRP1 glycosaminoglycan in endothelial and smooth muscle cells leads to opposing responses to VEGF [93]. Furthermore, it has also been observed that the presence of heparin in NRP1 binding assays results in increased affinity for $VEGF_{165}$ [94,95]. The mechanistic basis for this enhancement and, in particular, whether this is different for covalently versus exogenous non-covalently attached GAGs is currently unclear.

A similarly unresolved issue is the structural nature of the NRP cytoplasmic domain. Standard secondary structure prediction algorithms suggest an absence of a significant quotient of regular structure. It is therefore tempting to assign this region as an intrinsically disordered polypeptide that perhaps adopts a specific conformation only in a context of a complex with an interaction partner. To date, the only protein identified to interact with the cytoplasmic NRP domain is synectin which, as described above, binds to the NRP C-terminal Ser-Glu-Ala tail through its PDZ domain [73,74]. Although the structure of the NRP intracellular domain is not known, the C-terminal Ser-Glu-Ala sequence does have the typical sequence characteristics of a PDZ domain-binding motif. In other cases PDZ domains bind to their partners by extension of a β-sheet through the addition of an antiparallel β-strand constituting the C-terminal tail of the protein ligand [96]. Whether synectin is constitutively bound to NRPs in the cell or whether additional proteins contribute to NRP-dependent membrane-associated signalling complexes is not currently known.

NRP-targeting strategies

The demonstrated involvement of NRP in the pathogenesis of cancer has catalysed interest in targeting this molecule to combat the disease. The modular organisation of the protein offers several avenues for attack, such as the blocking of agonist–NRP interaction, interference of NRP association with partner receptors (e.g. VEGFR2) independent of ligand binding and inhibition of the function of the intracellular NRP-signalling domain. In addition to experimental approaches based upon these concepts, computer simulations have also been employed to predict the most efficient method for targeting VEGF–receptor interactions [97,98]. Using an *in silico* model of VEGF interactions with endothelial cell receptors that includes experimental estimates of the rate of VEGFR2–NRP coupling by $VEGF_{165}$ the authors concluded that blockade of NRP–VEGFR interaction might provide the most effective decrease in VEGF–VEGFR2 signalling [98].

Current efforts to target NRPs, and NRP1 in particular, have focused on the specific interaction with the exon 7-8-encoded region of $VEGF_{165}$. Researchers at Genentech (http://www.gene.com/gene/index.jsp) have developed a range of anti-NRP1 and anti-NRP2 monoclonal antibodies that block interaction with $VEGF_{165}$ and semaphorins [12,76,99], and are undertaking clinical trials to test the effectiveness of these monoclonal antibodies in cancer treatment either as single agents or combination therapies. An antibody to NRP2 that blocks VEGF-C binding has also been reported [100]. Others have identified specific peptides and peptidomimetic inhibitors of $VEGF_{165}$–NRP binding with antiangiogenic activity [101–105]. All of these molecules are competitive inhibitors of $VEGF_{165}$ binding and were able to reduce downstream VEGF signalling as demonstrated by reduced VEGFR2 tyrosine phosphorylation. Although peptides are not considered to be viable drug candidates they provide a starting point for structure-based design of peptidomimetics and small molecule inhibitors. Giordano *et al.* employed a method of amino acid retroinversion that involves substitution of D- for L-amino acids and sequence reversal, to generate the potent peptidomimetic D-(Leu-Pro-Arg) [105]. In initial studies this D-tripeptide was resistant to proteolysis and exhibited antiangiogenic activity mediated through NRP1 (and VEGFR1) based on *in vivo* assays in three animal models of cancer and retinopathy. In addition, a synthetic peptide targeting the transmembrane domain of NRP1 showed antitumour activity [106]. A synthetic oligonucleotide (G_{18}) has also been reported to bind NRP1 resulting in receptor internalisation and inhibition of angiogenesis [107].

The first non-peptidic small molecule antagonist of NRP1 function has recently been reported by researchers at Ark Therapeutics (http://www.arktherapeutics.com/main/index.php) [108] (Fig. 4). Starting from the previously characterised bicyclic peptide EG3287

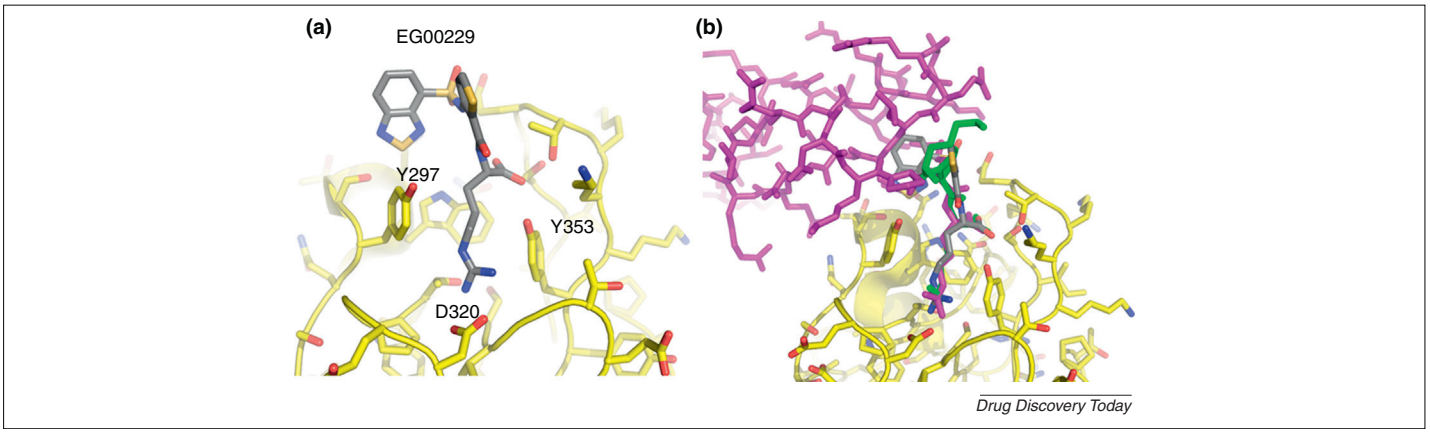

FIGURE 4

Ligand-binding pocket of the NRP1 *b1* domain. **(a)** Stick representation of small molecule VEGF antagonist EG00229 (grey). Key residues in the binding pocket are labelled. **(b)** Superposition of Tuftsin molecule (sticks, green) and $VEGF_{165}$-HBD (magenta) on the structure of the NRP1 *b1*/EG00229 (PDB 3I97) complex. The *b1* domains from the corresponding structures were not included in the figure for clarity. The differences in the side-chain conformations of the *b1* domain residues among three structures are negligible.

[103] that corresponds to the C-terminal 28-residue segment of VEGF-A_{165}, the small molecule inhibitor EG00229 was developed. Mutagenesis, X-ray crystallography and NMR spectroscopy were used to show that EG00229 binds to the targeted pocket on the *b1* domain of NRP1 (Fig. 4). The compound exhibits activity consistent with inhibition of VEGF-A_{165} binding to NRP1 and decreases VEGFR2 phosphorylation and cell migration *in vitro*. EG00229 is also reported to demonstrate activity against tumour cells by enhancing the cytotoxic effect of the chemotherapeutic drugs paclitaxel and 5-fluorouracil. Currently, EG00229 is a valuable tool for probing the molecular biology of NRP1 function; other more potent compounds with a superior pharmacokinetic profile are being developed by the same team.

Although co-immunoprecipitation experiments have demonstrated association between NRP and VEGFRs [87,88], the lack of biophysical and structural data describing the molecular basis of putative protein–protein interaction surfaces between NRP and VEGFR2, or other receptor tyrosine kinases, creates an obstacle to the formulation of strategies to target NRP function. For example, it would be helpful to understand better the role of the NRP MAM domain and its putative interaction with the membrane-proximal region of the VEGFR2 ectodomain. Analysis of the dimeric structure of the membrane-proximal domain D7 of VEGFR2 [109] suggests that this domain is crucial for receptor signalling. It would be reasonable to suppose that the (agonist-dependent) proximal location of the MAM domain of associated NRP – in addition to the potential role of the latter in NRP dimerisation on the cell surface [83,110] – could promote interaction with VEGFR2 D7 or other domains and thereby bring the NRP cytoplasmic region within the orbit of the VEGFR kinase domain. Finally, a better understanding of the contribution of NRP to intracellular signal transduction, through complex formation with proteins that associate with the intracellular domain, could provide additional scope for the development of therapeutic approaches to address the angiogenesis-related and -independent functions of NRPs.

Additional application of NRP1-targeting peptides

An important property of NRP1-binding peptides has emerged that could have significant implications for cancer drug targeting. A series of investigations using phage particle libraries that were initiated to search for tissue-penetrating peptides led to the observation that short peptides with basic (Arg or Lys) residues at the C-terminus are readily internalised. To emphasise the requirement for the basic residues being located at the C-terminus this activity was named the 'C-end rule' [111,112]. It was shown that internalisation of these peptides is mediated by NRP1 and, because NRP1 is upregulated in many cancer cell lines, this activity is being explored as a mechanism for the targeted delivery of therapeutic and diagnostic agents to tumours [113,114]. For example, when pro-apoptopic amino acid sequences were fused to NRP1-binding peptides they were not only internalised but also exhibited a potent antileukaemia cell effect [114]. Furthermore, a recent report showed that gold nanoparticles could be functionalised with NRP1-targeting peptides to effect internalisation [115]. Double-decorated gold nanoparticles, carrying a therapeutic p53-stabilising peptide alongside the NRP-targeting peptide, showed promising *in vitro* anticancer activity. Although the development of this NRP1-dependent drug delivery system is at an early stage, this offers significant promise for the targeted application of multifunctional agents with potential in areas as diverse as imaging, diagnosis and combination therapy.

Concluding remarks

This account introduced the role of VEGFs in signalling via the principal class of receptor (the VEGFRs) – signalling events that provide the most direct opportunity for the therapeutic blockade of angiogenesis. During recent years, drug discovery efforts in this area have been substantial, with marketed drugs available for oncology and ophthalmology indications, and are continuing as evidenced by a number of ongoing clinical trials. Experience has shown that these agents are associated with variable efficacy and various side-effects. We have described the emergence of the co-receptor role of the NRPs that also bind a subset of VEGF isoforms. The contribution that NRPs make to VEGF signalling, whether synergistic or otherwise, adds complexity to the VEGF interactome yet, at the same time, provides a secondary means to target VEGF signalling in disease. The acknowledged role of NRPs in cell migration, along with the role in VEGF signalling, suggests that a different spectrum of responses could emerge by targeting this axis. Early efforts have demonstrated the potential for drug development targeted to VEGF–NRP interactions, and this is likely to expand as further understanding of the structural and biochemical aspects of these interactions becomes available. Moreover, new discoveries concerning the function of VEGFs and NRPs, such as the determination of the 'stemness' of skin cancer cells [53] and endothelial lipid transport [40,116] relevant to type II diabetes, will probably provide additional avenues to exploit drug discovery efforts in this niche.

Disclosure statement

S.D. has been involved as consultant to Ark Therapeutics on a project involving small molecule inhibitors of NRP1. The work was described in part in Jarvis *et al.* [108].

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