Targeting VEGF–neuropilin interactions: a promising antitumor strategy

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Inhibition of vascular endothelial growth factor (VEGF) or its corresponding receptor (VEGFR) has been validated as an efficacious antiangiogenetic approach for cancer treatment. More recently, neuropilins (NRPs), the essential coreceptors for VEGF, have also been shown to have a significant role in VEGF signaling. Given the multifaceted effects of VEGF–NRP interactions on tumor initiation and progression, the exploration of new chemical entities that selectively block these interactions has recently attracted considerable interest as a novel antitumor strategy. Here, we summarize the biological functions of VEGF–NRP interactions in tumor biology, analyze the structural basis for these interactions, and present a detailed discussion of the development of the NRP antagonists reported so far.

Introduction

Therapeutic approaches targeting VEGF and its receptors (VEGFRs) have witnessed remarkable clinical success since the US Food and Drug Administration (FDA)'s approval of the first VEGF inhibitor, bevacizumab, in 2004. The initial rationale for inhibiting VEGF as an antitumor strategy was based on the fact that angiogenesis, which is mediated by VEGF–VEGFR signaling, is closely related to tumor initiation and progression. Tumor cells secrete VEGF, which binds to surrounding endothelial cells (ECs) and results in the sprouting of new blood vessels in tumor tissues. These newly formed blood vessels not only participate in the efficient transportation of oxygen and nutrients to tumor cells for quick proliferation, but also provide an excellent route for these tumor cells to migrate along. Thus, drugs that directly sequester VEGF (anti-VEGF antibodies) or disrupt its downstream signaling (VEGFR inhibitors or other kinase inhibitors) could attenuate tumor angiogenesis, and have proven to be effective therapeutics against a range of tumor types [1].

The underlying molecular mechanism of VEGF signaling is well characterized and has been extensively reviewed in the literature [2]. The VEGFs (primarily VEGFA and VEGFC) stimulate cellular responses by binding to their cognate receptors (VEGFR2 and VEGFR3, respectively) on the cell surface and triggering receptor homo- or heterodimerization. The VEGFRs then become activated through autophosphorylation and subsequently trigger downstream intracellular signaling pathways that result in vascular permeability, and EC survival, migration, and proliferation. Meanwhile, other research has shown that the participation of other auxiliary proteins or VEGF coreceptors is important for optimal VEGF–VEGFR signaling. The most well-known VEGF coreceptors are NRPs, and their key roles in mediating tumor-associated VEGF functions are well elaborated [3].

NRPs (NRP1 and NRP2) are a family of single-pass transmembrane glycoproteins that are highly conserved in all vertebrates. They were originally identified on neurons as receptors for semaphorins with roles in neural development. Later, it was found that NRPs were also expressed or upregulated in a variety of cells, including ECs, immune cells, and tumor cells, and were involved in a series of signaling cascades [4]. For instance, in ECs where both NRP and VEGFR are highly expressed, NRP receptors bind to VEGF and coordinate with VEGFR, which results in enhanced VEGF signaling. Therefore, the interruption of VEGF–NRP associations in this context is deemed an attractive antiangiogenic approach [5–7]. More recently, there is growing evidence suggesting that VEGF–NRP interactions mediate multiple cellular functions other than angiogenesis and promote tumorigenesis in a

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VEGFR-independent manner, which further justifies potential antitumor strategies targeting these interactions [3,8].

Herein, we review the multifaceted roles of the VEGF–NRP interaction in angiogenesis, cancer stem cell (CSCs) and immune tolerance. We analyze the structural basis and characteristics of these interactions, highlighting recent findings. Finally, we present a detailed discussion of the development of NRP antagonists that target this type of interaction to shed light on the future development of this potential drug class.

Roles of VEGF–NRP interactions in tumor biology

Angiogenesis

VEGF and its receptors (VEGFRs) are pivotal in the development of the vascular system and the maintenance of vascular integrity. When VEGF is overexpressed, it can also contribute to related pathological processes, such as uncontrolled angiogenesis and cellular metastasis in many types of cancer. It is well established that endothelial NRP binds to VEGF and works as coreceptor facilitating VEGF-mediated signaling [9]. Several studies have correlated this particular role of NRP with VEGFA-mediated angiogenesis and VEGFC-mediated lymphangiogenesis. For example, it was reported that the blockade of VEGFA–NRP1 and VEGFC–NRP2 binding via site-specific anti-NRP antibodies [anti-NRP1b monoclonal antibodies (mAb) and anti-NRP2b mAb, respectively] were effective in reducing tumor-associated angiogenesis and lymphangiogenesis, respectively. In addition, synergistic antiangiogenic effects were also observed when anti-NRP1 and anti-VEGF antibodies were used in combination. However, these anti-NRP antibodies only led to a mild reduction in VEGFR phosphorylation and demonstrated few effects on downstream ERK1/2 or Akt activation, suggesting that NRP mediates VEGF functions partly through an unknown mechanism that might be independent of VEGFR phosphorylation [10,11].

Although NRPs have been identified as important potential targets for antiangiogenic therapies, the underlying mechanism of how NRP regulates endothelial VEGF signaling remains controversial and is yet to be fully understood. Nonetheless, it is generally accepted that, in ECs, VEGF binds to both NRP and VEGFR simultaneously, forming a 'bridged' tertiary VEGF–VEGFR–NRP complex (Fig. 1b). Formation of this complex is suggested to promote receptor internalization and intracellular trafficking, which are crucial for activating downstream intracellular signaling [12,13].

However, recent findings suggest that NRP1 instead suppresses angiogenesis when NRP1 and VEGFR2 are presented *in trans* (NRP1 and VEGFR2 are expressed on different cells, Fig. 1c). It was reported that, by forming a *trans* complex, endothelial VEGFR internalization is arrested and the subsequent intracellular signaling is inhibited [14]. Given the opposing effects of NRP1 expressed *in cis* and *in trans*, the expression status of NRP1 in different tumor types could be a prognostic predictor of antiangiogenic therapy [15], and the *cis*/*trans* ratio of a certain tumor type should be taken into consideration in the future development of potential NRP1 antagonists targeting angiogenesis.

Cancer stem cells

CSCs are a subpopulation of cancer cells with stem-cell-like properties, including indefinite self-renewal, asymmetric division, and strong vitality, and are responsible for cancer recurrence and therapeutic resistance. Several modulators, such as Wnt, FAK, and Hedgehog, are involved in the regulation of cancer stemness, and several related therapies are currently in clinical trials [16].

Recent studies suggest a role for NRP1 in CSCs, based on the observation that NRP1 expression or upregulation correlates with CSC properties and NRP1-knockdown mice demonstrate diminished CSC function [17,18]. In addition, many tumor types do not express VEGFR essentially, but respond to autocrine VEGF; thus, it is reasonable to speculate that NRPs act as important alternative modulators in these tumor cells because they are the only VEGF-binding receptors expressed by these cells. Recently, many reports have demonstrated that VEGFA–NRP1 signaling has an essential role in promoting stemness of several types of cancer, including skin cancer [19], breast cancer [20], squamous cell carcinoma [21], and glioma [22]. In a recent report, NRP2 was also shown to promote stem-like traits upon binding to VEGF in breast cancer [23]. Therefore, blocking VEGF–NRP interactions might represent a novel approach for future CSC-targeted therapies.

Although disruption of VEGF–NRP signaling has been demonstrated to reduce cancer stemness, the exact molecular mechanism remains largely unknown. Given that NRPs have no known enzymatic function, it is puzzling how the VEGF–NRP complex participates in the regulation of various biological functions, such as promotion of stemness, in tumor cells. In breast cancer, Wnt and the hippo factor TAZ have been proposed to facilitate NRP1- and NRP2-mediated cancer stemness, respectively, although more conclusive evidence is still needed [20,23]. Although even fewer details have been determined in other types of cancer, further mechanistic studies could provide more insights into this emergent regulator of cancer stemness.

Immune tolerance

NRPs are found in a variety of immune cells, including macrophages, regulatory T cells (Tregs), and dendritic cells (DCs), and have been identified as a potential target for immunotherapy [24]. Tregs are key modulators in the immune tolerance of tumors because they are strongly immunosuppressive and have tumor-infiltrating properties that correlate with poor prognosis [25]. Tumor-derived VEGF might serve as a chemotactic factor that guides NRP1-expressing Tregs into tumors, and the chemotactic interaction between VEGF and NRP1 is crucial for the recruitment and subsequent immunosuppressive functions of Tregs [26,27]. Tuftsin, a natural immunostimulatory tetrapeptide that competes with VEGF in NRP binding [28], was shown to avert the negative immunoregulation of $NRP1^{high}$ Tregs in septic mice [29], which supports the potential role of NRP1 antagonists in immunotherapy.

Inhibition of DC maturation also contributes to tumor immunotolerance in that only matured DCs are able to stimulate a productive T cell response. In a related study, VEGF was reported to robustly inhibit lipopolysaccharide (LPS)-induced DC maturation, and it was demonstrated that NRP1 was indispensable to this inhibitory process because NRP1-deficient DCs were insensitive to VEGF-dependent inhibition. Therefore, NRP1 may be a promising target to optimize DC maturation within VEGF-rich environments, such as tumors [30].

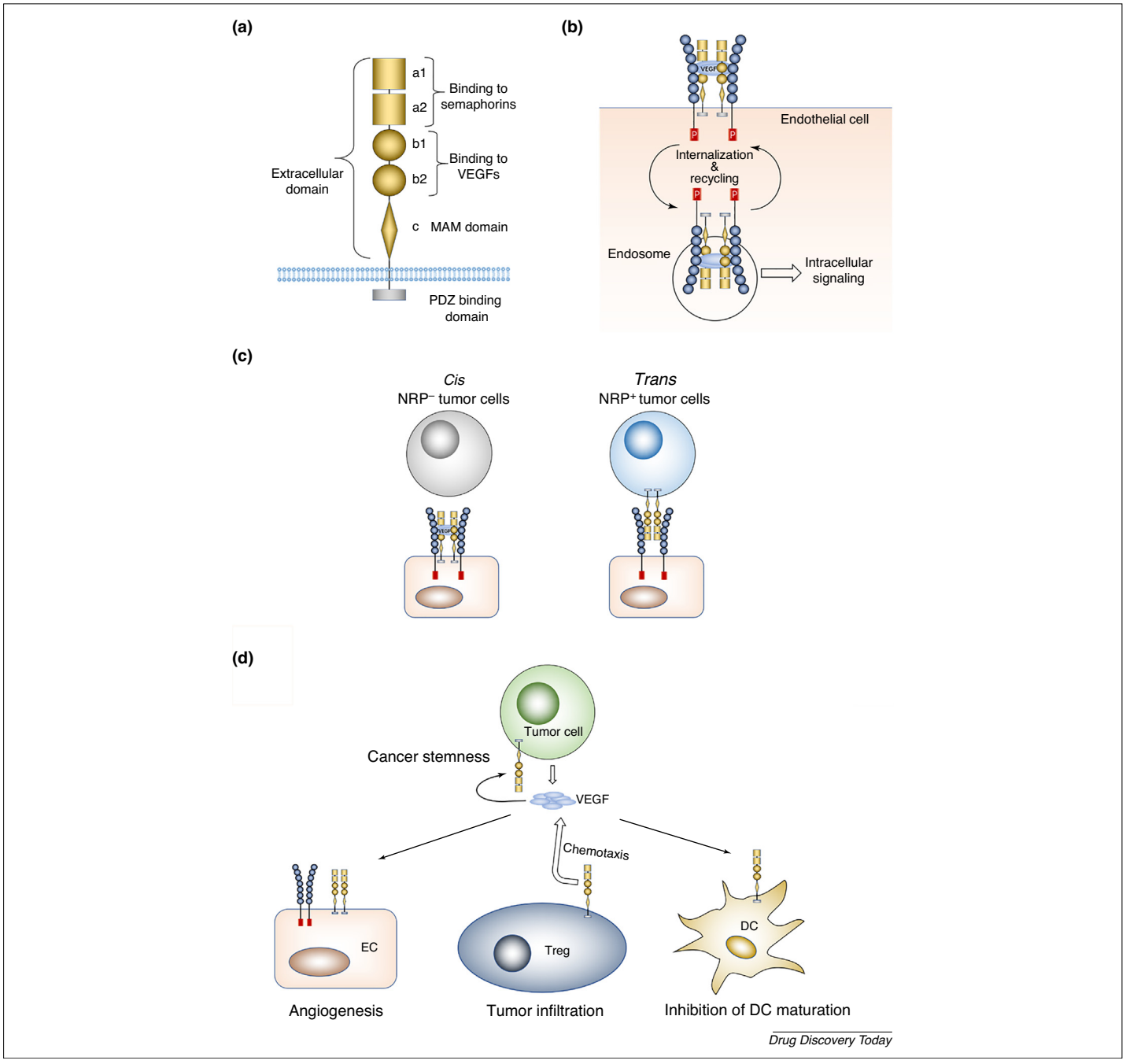

FIGURE 1

Roles of VEGF-NRP interactions in tumor biology. **(a)** General domain structure of neuropilins (NRPs). **(b)** Possible vascular endothelial growth factor (VEGF)–VEGR receptor (VEGFR)–NRP complex promoting receptor internalization and subsequent intracellular signaling. **(c)** *Cis* and *trans* presentation of NRP and VEGFR in tumor cells and endothelial cells. **(d)** Summative cartoon representation of the multifaceted roles of the VEGF–NRP interaction in tumor biology. Abbreviations: DC, dendritic cell; EC, endothelial cell; Treg, regulatory T cell.

Given that the VEGF–NRP complex participates in multiple pathological processes in tumors (Fig. 1d), the development of novel NRP antagonists provides an attractive route to realize the favorable antitumor effects, especially when used in combination with other targeted therapies synergistically.

Structural basis for VEGF–NRP interactions

To design new chemical molecules that are able to interfere with the interactions between NRP and VEGF, we must first understand their binding mode. A series of protein structures of both NRPs and VEGFs have been extensively characterized and their binding patterns have been largely uncovered. NRP1 and NRP2 share 44% sequence homology and both comprise two extracellular ligand-binding domains (primarily a1a2 for semaphorin binding and b1b2 for VEGF binding), a MAM domain (c) responsible for multimerization, a transmembrane helix and a intracellular PDZ-binding tail, which is suggested to mediate signaling (Fig. 1a) [31]. The VEGF family comprises five members, VEGFA, -B, -C, -D, and placental growth factor (PlGF), among which VEGFA and VEGFC execute the major biological functions of

this family by binding to the corresponding VEGFR2–NRP1 and VEGFR3–NRP2 complex, respectively.

It is now widely accepted that the exon 7- and 8-encoded domain of VEGF is responsible for NRP binding, and that different VEGF members or isoforms exhibit different affinities for NRP1 and NRP2. For instance, VEGFA exhibits a 50-fold stronger binding affinity towards NRP1 compared with binding to NRP2. This selectivity is unexpected given that the VEGF-binding domains of NRP1 and NRP2 are similar. It is suggested that the electronegative nature of VEGFA exon-7-encoded residues, which could lead to repulsion against the NRP2 b2 domain, accounts for the selective VEGFA–NRP1 binding [32,33]. In addition, Tsai *et al.* discovered a Zn^{2+}-binding pocket in NRP2, remote from VEGF-binding pocket, which might also affect the ligand selectivity [34].

Despite their different binding preferences, one common feature of all VEGFs that are able to bind NRP is a C-terminal arginine residue. In fact, any peptide with a C-end arginine shows affinity to NRP to some extent. This pattern is termed as 'C-end rule' or 'CendR' [35], and results from a highly conserved arginine-binding pocket located in the b1 domain of NRP (Fig. 2a). Occlusion of this pocket abolished ligand-binding affinity, as observed for both NRP1 and NRP2 [32,33]. Therefore, this arginine pocket constitutes a 'hot spot' that drives the protein–protein interactions (PPIs) between NRP and VEGF [36], and small molecules that are able to occupy this pocket are most likely to disrupt VEGF–NRP interactions.

Alanine scanning of the arginine pocket revealed that Y297, W301, T316, D320, S346, T349, Y353, and W411 are crucial for VEGFA–NRP1 binding [37]. All of the above key residues are also conserved in NRP2, which renders it challenging to develop selective NRP1 or NRP2 antagonists. The only significant structural divergence between NRP1 and NRP2 b1 domains lies in the L1 loop region (Fig. 2b). The L1 T299 of NRP1 is replaced by D301 in NRP2, which might lead to electronic repulsion between the exon-7 residues of

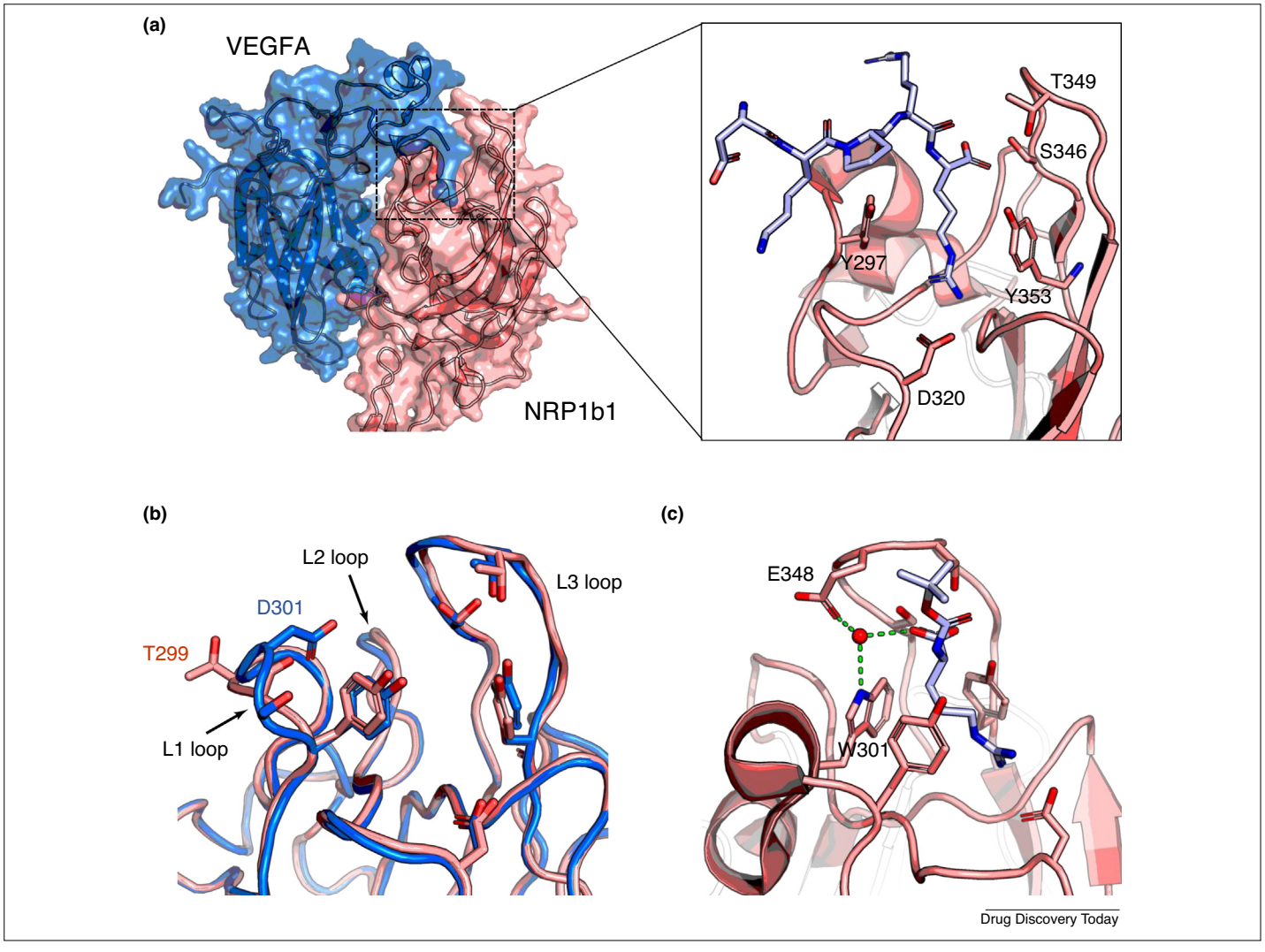

FIGURE 2

Structural basis for VEGF-NRP interactions. **(a)** Structure of neuropilin (NRP)-1 b1 domain (pink) in complex with VEGFA (blue) and a close-up view of the vascular endothelial growth factor A (VEGFA) C terminus binding to the arginine pocket [Protein Data Bank (PDB) ID: 4DEQ]. Carbon atoms in VEGFA C terminus are in light blue. Key interacting residues are shown as sticks. **(b)** Superposition of NRP1 (salmon) and NRP2 (marine) arginine pockets. Key residues are shown as sticks and protein backbones are shown as tubes. **(c)** Binding of an arginine derivative to the NRP1 b1 domain (PDB ID: 5J1X). A conserved water molecule (red spheres) forms hydrogen bonds (green dashes) with E348, W301, and the ligand.

VEGFA and NRP2, thus partially explaining the binding preference of VEGFA for NRP1 over NRP2 [33]. The C-terminal arginine of VEGFA fully extends and reaches to the bottom of the arginine pocket, with the guanidine group forming a salt bridge with D320. The long alkane chain of the arginine is stacked between Y297 and Y353, and the free terminal carboxyl group engages in hydrogen bond interactions with S346, T349, and Y353. By analyzing the X-ray structures of a series of arginine derivatives binding to NRP b1 domain, a recent report identified a highly conserved water molecule in this pocket, which bridges a hydrogen bond network among W301, E348, and the ligand (Fig. 2c). It is suggested that this water molecule be retained during structure-based drug design, because its displacement resulted in significantly decreased ligand affinity [38]. Furthermore, this binding pocket appears to be tailor-made for C-end L-arginine, and any modifications to this arginine are usually poorly tolerated. Switching this arginine to D-arginine, capping the free carboxyl group via amidation, or lengthening the alkane chain abolished the binding capabilities [35,38].

Although a C-end arginine is required for NRP binding, structural features other than CendR also have significant roles in contributing to an optimized affinity, which poses challenges to the development of potent NRP antagonists. For instance, a tuftsin-like pentapeptide with a TKPPR motif similar to VEGFA exon-8 only weakly inhibits bt-VEGFA binding to NRP1, with an IC_{50} of 47 μM, whereas the IC_{50} for VEGFA is <1 nM [28]. Indeed, PPIs are usually considered as complicated processes that occur in a rather vast interface possibly involving numerous bridging waters, and to disrupt PPI with a small-molecule inhibitor is never an easy task because of the multiple interactions sites [36]. Nevertheless, significant progress has been made recently to block VEGF–NRP interactions with peptide-based, peptidomimetic, or nonpeptide antagonists. Here, we summarize representative efforts in the design and discovery of novel NRP antagonists.

Antagonists of the VEGF–NRP interaction

Peptide-based antagonists

Given that the NRP1 isoform is more ubiquitous and a better characterized protein receptor compared with NRP2 in terms of its cell biology, studies have focused on the design and discovery of NRP1 antagonists that mediate the regulation of the NRP1-related biological functions, while giving less attention to NRP2 antagonists. However, given the structural similarity between NRP1 and NRP2 in the VEGF-binding pockets, a NRP1 antagonist usually displays inhibition of NRP2 function to some extent, although this is rarely reported.

Based on the exon 7- and 8-encoded domain of VEGFA, Jia and coworkers identified the first specific NRP antagonist EG3287 (Table 1) that inhibits VEGF-induced tyrosine phosphorylation and its downstream signaling in ECs. They found that the C-terminal 7-residue peptide RXDKPRR (X: aminobutyric acid), which contains the six residues encoded by VEGFA exon-8, retained a significant degree of activity, indicating the crucial role of the exon 8-encoded C terminus [39]. N-terminal octanoylation of compound EG3287 led to the new peptide EG00087 with an enhanced IC_{50}. Modeling studies indicated that new intramolecular hydrogen bonds formed by the newly introduced acyl group accounted for the increased activity [40]. Starzec *et al.* reported another novel type of peptide-based NRP antagonist with the sequence of ATWLPPR (named A7R) through phage library screening. A7R was shown to inhibit VEGFA binding to NRP1 and to decrease tumor angiogenesis and growth *in vivo* [41]. Subsequent structural–function analyses revealed that the -LPPR minimal fragment in A7R is essential for maintaining such activity [42]. Branching the position 1 lysing in LPPR with additional homoarginine (Har) residue and increasing the flexibility of the middle part of LPPR, accompanied by the introduction of additional interacting elements, led to Lys(Har)-Dab-Pro-Arg (Dab: L-2,4-diaminobutyric acid), which exhibited 30-fold stronger potency compared with A7R as well as significantly improved proteolytic stability [43]. Starting with the LPPR sequence, the Grabowska research group successfully designed a cyclic peptide H-c[Lys-Pro-Glu]-Arg-OH to evade the drawbacks of linear peptides, such as fast elimination. In this cyclic peptide, a lactam bond is formed between the side chains of lysine in position 1 and glutamine in position 3, while the crucial C-terminal arginine remains exocyclic. Significantly increased *in vitro* activity was observed with H-c[Lys-Pro-Glu]-Arg-OH compared with A7R [44]. Structure–activity relationship studies indicated that the

TABLE 1

Peptide-based NRP antagonists and their biological activities[a]

Sequence	IC_{50} (μM)	% Inhibition at 100 μM	Refs
H-SCKNTDSRCKARQLELNERTCRCDKPRR-OH (EG3287)	2.8[b]	97[b]	[39]
 (EG00086)	1.2[b]	–	[40]
H-ATWLPPR-OH (A7R)	5.9	82	[41,44]
H-LPPR-OH	–	75	[42]
H-Lys(Har)-Dab-Pro-Arg-OH	0.2	–	[43]
H-c[Lys-Pro-Glu]-Arg-OH	0.18	100	[44]
[b]H-Har-Dab-Pro-Arg-OH	0.8	–	[46]
H-KPPR-OH	14[b]	97.8	[37,47]
$[(H\text{-}TKPRKHG)_2K]_2KG$-OH (MY1340)	7.5 ng/ml	–	[48]

[a] Values of IC_{50} and inhibition rate are based on cell-free VEGFA–NRP1 binding assays in accordance with original references.
[b] These values were obtained from cell-based VEGFA binding assays.

ring size, rather than the position of the lactam bond, was more important for maintaining the strong inhibitory effect of H-c[Lys-Pro-Glu]-Arg-OH [45]. Tymecka *et al.* designed a series of tetrapeptides, such as Har-Dab-Pro-Arg (Har: L-Homoarginine; Dab: L-2,4-diaminobutyric acid), that also exhibited potent activity. They found that simultaneous interactions of the basic amino acids in position 1 and position 4 (Arg) with NRP1 are crucial [46,47]. Mo *et al.* recently reported a new peptide, MY1340, that inhibited the formation of the VEGF–NRP1 complex with nanomolar activity and reversed the inhibitory effects of VEGF on DC differentiation and maturation *in vitro* [48]. However, it remains unclear how MY1340 disrupts the VEGF–NRP interaction, because it lacks a C-end arginine residue, which occurs widely in traditional peptide antagonists.

Peptidomimetics

Although many peptide-based NRP antagonists have been reported, they are essentially 'undruggable' because of metabolic liability and low bioavailability. Therefore, the design and development of small molecules that mimic their parent peptides presents another rational approach to inhibit PPIs in biological systems [36].

Jarvis *et al.* described the first small-molecule NRP antagonist EG00229 (Fig. 3a) [37]. The structure of EG00229 was derived from KPAR, the minimal sequence that could retain the activity of EG3287. The crucial C-terminal arginine was entirely conserved in EG00229, and the central sulfonylaminothiophene core linked arginine with benzothiadiazole, which supposedly mimicked the important position 1 lysine in KPAR. It was demon-

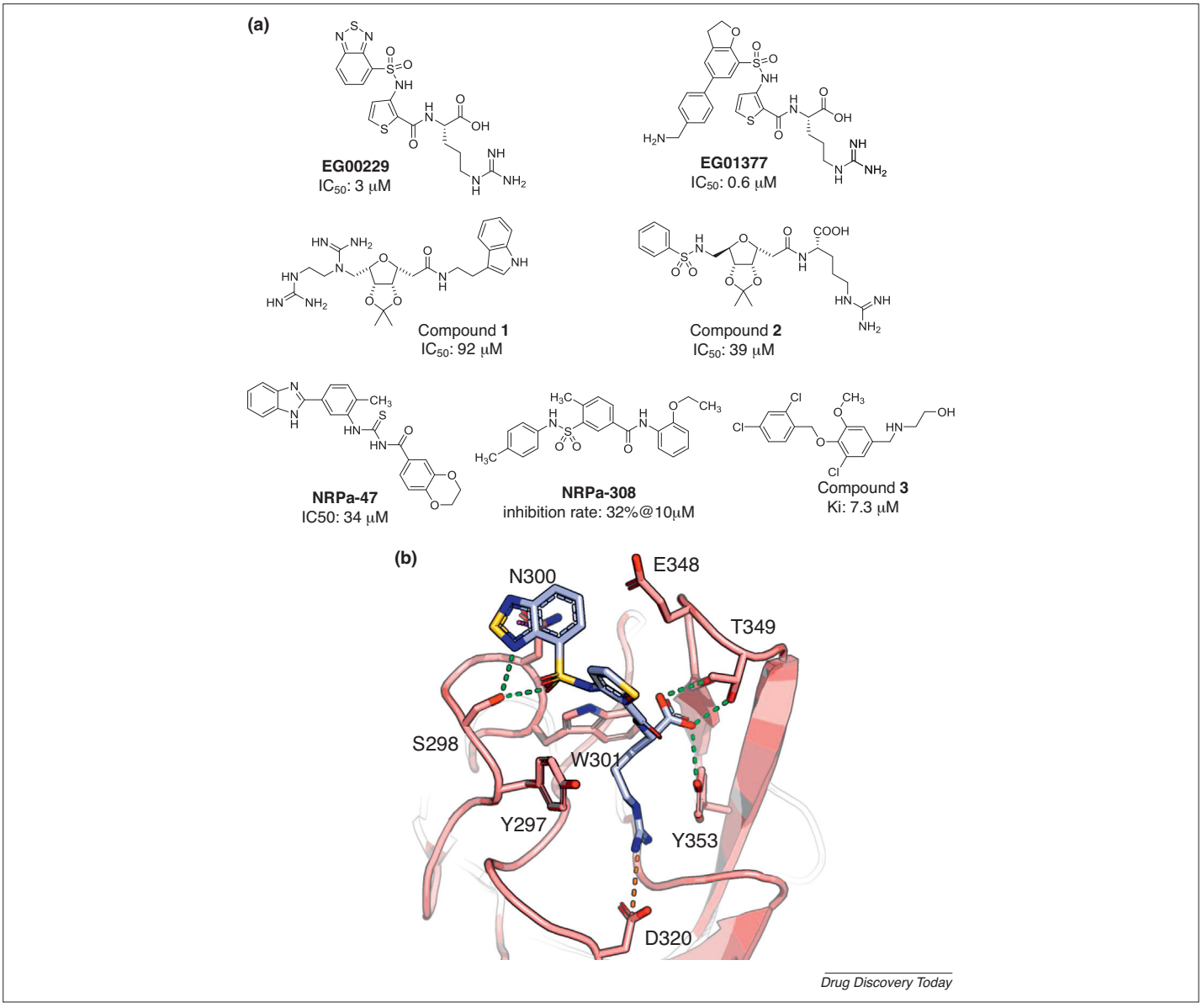

FIGURE 3

Antagonists of NRP-VEGF interactions. **(a)** Chemical structures and biological activities of small-molecule neuropilin (NRP) antagonists so far reported. Values of IC_{50}, K_i and inhibition rate are based on vascular endothelial growth factor A (VEGFA)–NRP1 binding assay in accordance with their original references. **(b)** Cocrystal structure of EG00229 (carbons in light blue) bound to NRP1 b1 [Protein Data Bank (PDB) ID: 3I97]. EG00229 and key residues are shown as sticks. Hydrogen bonds, stacking interactions, and salt bridges are shown as green, magenta and orange dashes, respectively.

strated that EG00229 moderately inhibited VEGFA binding to NRP1-expressing porcine aortic endothelial (PAE) cells with an IC_{50} of 3 μM and displayed enhanced cytotoxicity of paclitaxel and 5-fluorouracil *in vitro*. X-ray analysis of the EG00229-NRP1 complex (Fig. 3b) indicated that all the interactions within the arginine pocket were conserved compared with the VEGFA arginine-binding pattern and there were additional interactions, including two hydrogen bonds with S298 and a stacking interaction with N300. An intramolecular hydrogen bond between the carbonyl amide –NH– and the sulfonamide –NH– was also observed, and this feature might be crucial for the biological activity of this compound. The same group recently reported the further structural modification of EG00229, which yielded a new compound EG01377 (Fig. 3a) with significantly improved inhibitory activity (IC_{50} = 0.6 μM) [49]. In EG01377, a phenethylamine moiety was introduced to the benzene ring *meta* to the sulfonylamine. The newly incorporated phenethylamine was thought to form an extra hydrogen bond with E348 that might contribute to the significant increase in biological activity. EG01377 was shown to demonstrate antiangiogenic, antimigratory, and antitumor effects, and to block the protumorigenic polarization of $NRP1^+$ Tregs. It was intriguing that compound EG01377 was reported to specifically target the NRP1 receptor and showed almost no affinity to NRP2 in binding assays.

Inspired by A7R (ATWLPPR), Novoa and coworkers designed a series of sugar-based peptidomimetics [50]. The authors used a sugar ring as a scaffold to mimic the rigid LPP sequence in A7R. Decoration of the sugar scaffold with various functionalities mimicking arginine and tryptophan in A7R on different sides of the sugar ring generated compound **1** (Fig. 3a) with relatively weak affinity for NRP1 (IC_{50} = 92 μM). A second round of sugar-based antagonist optimization studies that investigated the structural composition as well as the spatial arrangement of the two residue-mimicking side chains led to the discovery of compound **2** (Fig. 3a) with moderately increased affinity (IC_{50} = 39 μM) [51].

Nonpeptide antagonists

Borriello *et al.* identified a series of nonpeptide VEGF–NRP antagonists, including NPRa-47 and NRPa-308 (Fig. 3a), via tandem virtual screening and cell-based screening [52–54]. These molecules displayed antitumor effects both *in vitro* and *in vivo*. Molecular docking studies of these target compounds indicated that their binding patterns were different from previously mentioned arginine-based peptides or peptidomimetics, featuring a sandwiched π–π stacking interaction with Y297 and Y353 that anchored the antagonists deeply in the arginine pocket. Indeed, scrutiny of this pocket revealed a π-box comprising the benzene rings of Y297 and Y353 almost in parallel on two sides and the indole ring of W301 in the back of the box. The π–π stacking interactions within this box might provide an extra clue for the future design of small-molecule NRP antagonists with better activity. Although both NRPa-47 and NRPa-308 inhibited VEGFA binding to NRP1 only moderately (IC_{50} > 10 μM), they displayed considerably potent antitumor activities in a range of cell-based evaluations (IC_{50} <1 μM), suggesting that these molecules also interact with tumor targets other than NRP1.

Through virtual screening and similarity searches, the Starzec group also discovered several NRP antagonists that share a common benzyloxybenzylamine scaffold [55]. Among these molecules, compound **3** (Fig. 3a) displayed the strongest inhibitory activity against VEFGFA–NRP1 interactions, with a K_i value of 7.3 μM, similar to that of A7R. Docking models revealed stacking and hydrophobic interactions between compound **3**, and amino acid residues W301 and Y353 had significant roles in the observed inhibitory activities of the VEFGFA–NRP1 complex, which further supports the hypothesis that the π-box structural motif is essential in the design of nonpeptide NRP antagonists. Additionally, loss of the crucial salt bridge interaction with D320 in the arginine pocket, as observed in NRPa-308 as well as in compound **3**, did not eliminate NRP affinity. Therefore, structural features of C-end arginine should not be regarded as the 'golden rule' in the future design of small-molecule NRP antagonists.

Concluding remarks

VEGF–NRP interactions have been observed in many pathological processes associated with tumor biology, including angiogenesis, CSCs, and immune tolerance, although more in-depth mechanistic studies are still required. Nonetheless, the blockade of VEGF–NRP interactions through the development of novel NRP antagonists represents a viable antitumor approach. In addition, transforming growth factor-beta (TGFβ) has recently been shown to interact with NRP1 to mediate tumorigenesis in a KRAS-dependent manner [56,57]. This could provide additional incentives for the development of VEGF–NRP antagonists, because TGFβ (or its latent form) was shown to bind to the NRP1 arginine pocket similarly to VEGF [58].

Furthermore, there is a growing body of evidence suggesting that NRP1 is upregulated in various tumor cells in adaptive response to targeted antitumor therapies. Upregulation of NRP1 correlates with drug resistance and tumor recurrence, which provides a rationale for combination therapies with NRP antagonist [59,60]. Although VEGF inhibitors appear to be perfect partners for NRP antagonists given their promising synergistic antiangiogenic effects [11], this combination is dangerous because of the severe adverse effect of proteinuria observed in a Phase 1b study that evaluated the combination therapy of MNRP1685A (an anti-NRP1 antibody) with bevacizumab [61]. This nephrotoxicity was not observed with MNRP1685A administered alone [62]. It was later suggested that dual blockade of NRP1 and VEGF leads to full inhibition of VEGFR activity, thereby incurring endothelial damage in glomeruli that could cause proteinuria [63]. Therefore, combination therapy involving NRPs should be designed with caution in light of the promiscuous and ubiquitous nature of these multifunctional coreceptors. Synergistic effects of NRP antagonists with other targeted therapies would only be safely exploitable once investigations of the functions of NRPs with regard to different tumor types have been conducted and their respective molecular mechanisms clarified and verified.

Many significant medicinal chemistry efforts have been made in the development of VEGF–NRP antagonists, The binding mode of VEGF and NRP has been largely uncovered, and many peptide-based antagonists featuring C-end arginine have been reported. To obtain better bioavailability, several hit or lead structures of small-molecule NRP antagonists have also emerged (e.g., NRPa-308 and EG01377). We expect that future medicinal chemistry efforts will focus on hit-to-lead or lead optimization processes, hopefully

resulting in compounds with significantly enhanced potency and improved druggability. Moreover, structural exploration of new subpockets in the VEGF–NRP interface amenable to small-molecule occupation will undoubtedly aid in the design and discovery of NRP antagonists with novel chemotypes and, hopefully, better efficacy.

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