Engineering an anti-Neuropilin-2 (NRP2) antibody that selectively blocks NRP2 interactions with Semaphorin and Plexin

Kaitlyn Rauch[1], Luke Burman[1], Yanyan Geng[2], Liting Zhai[2], Yeeting E. Chong[1], Ann Menefee[1], Kristina Hamel[1], Zhiwen Xu[1], Nathaniel Bloom[1], Lauren Guy[1], Matt Seikkula[1], Christoph Burkart[1], Leslie A. Nangle[1]*

1. aTyr Pharma, 2. Pangu Biopharma, *Contact: lnangle@atyrpharma.com

Abstract

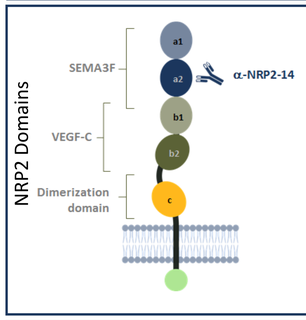
Neuropilin-2 (NRP2) is a multifunctional receptor that exerts its cellular effects by dimerizing with co-receptors upon ligand binding to modulate signaling across various biological processes including vascularization and inflammation. The most well studied of these ligands and co-receptors are vascular endothelial growth factors (VEGFs) and VEGF receptors (VEGFRs), and Class 3 Semaphorins (Sema3s) and Plexins. aTyr Pharma has generated a panel of high quality anti-human NRP2 monoclonal antibodies to study NRP2 signaling.

One of these antibodies, aNRP2-14, binds primarily to the a2 domain of NRP2 and selectively blocks the NRP2/Sema3 axis while not affecting the NRP2/VEGF axis. aNRP2-14 was isolated from a mouse immunized with human NRP2 using traditional hybridoma methods. The initial antibody was reactive towards human NRP2, with no cross-reactivity towards rodent NRP2. In order to further understand the binding interaction with NRP2, we solved the aNRP2-14/NRP2 co-crystal structure by X-ray diffraction. Using this structural information, we successfully engineered aNRP2-14 to recognize rodent NRP2 instead of human NRP2. This created a functionally equivalent surrogate antibody targeting the rodent NRP2 ortholog which could then be used to conduct proof-of-concept studies in pre-clinical rodent models. Both human- and rodent-reactive versions of aNRP2-14 were subsequently affinity matured *in vitro* to achieve high-affinity binding.

Through multiple *in vitro* assays, we confirmed that aNRP2-14 blocked binding of Sema3F to NRP2. aNRP2-14 blocked Sema3F-induced NRP2/PlexinA1 dimerization in NanoBiT receptor dimerization assays, while having no effect on NRP2/VEGFR dimerization. Sema3F signaling through NRP2/PlexinA1 has been reported to affect multiple pathways, with a common feature being the inhibition of Akt phosphorylation. *In vitro* cell based assays showed that aNRP2-14 treatment did indeed reverse the effects of Sema3F, relieving this inhibition and leading to higher levels of phosphorylated Akt. In an *in vivo* proof-of-concept study, aNRP2-14 was shown to enhance tumor growth in mice in a 4T1 breast tumor syngeneic mouse model. This is consistent with previous literature, in which Sema3F plays a role as a tumor suppressor. Interestingly, this data also suggests the potential utility of a bispecific aNRP2/aPLXNA1 antibody that could serve as a Sema3F mimetic in targeting tumors that express these co-receptors, an approach that we are actively pursuing.

aTyr has developed and characterized a domain specific antibody against human NRP2 that selectively blocks binding of Sema3 ligands and blocks dimerization and subsequent signaling through NRP2/PlexinA1. We successfully engineered a rodent surrogate of this antibody to be used in pre-clinical rodent models. The effect of aNRP2-14 in both *in vitro* and *in vivo* assays are consistent with blocking of Sema3F/NRP2/PlexinA1 signaling. Recent data linking Sema3F signaling to persistent inflammation in disease states like chronic obstructive pulmonary disease, specifically through retention of neutrophils at sites of inflammation, indicate blocking the NRP2/Sema3F axis could have valuable therapeutic utility.

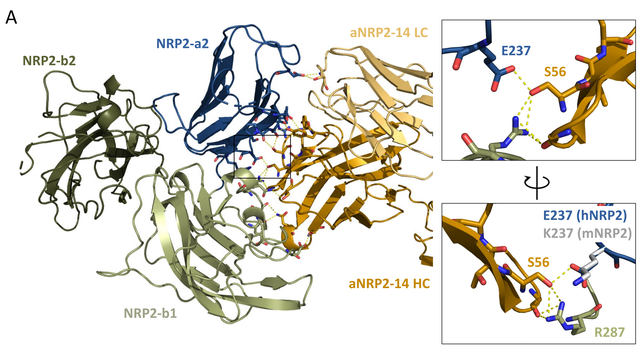
Introduction

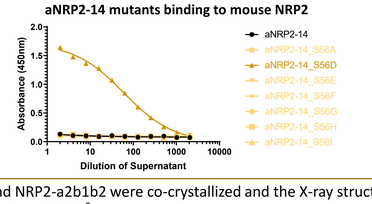


- NRP2 is a multi-functional receptor that exerts its cellular effects by dimerizing with co-receptors, the most well characterized of which are VEGFRs and Plexins, upon binding of ligands such as VEGF and Sema3s.
- Sema3F interacts with NRP2 at two distinct sites. One binding site includes the N-terminal region of semaphorin and the a1a2 domains of NRP2. The second binding site is formed between the carboxyl tail of Sema3F and the b1 domain of NRP2, however the specificity of binding Sema3F is dictated by the binding to the a1a2 domain of NRP2 [Ref 1].
- Recent data has shown that inflammatory human neutrophils highly express Sema3F and NRP2 and have shown a link between loss of Sema3F and accelerated inflammation resolution. This could indicate that blocking the Sema3F/NRP2 pathway could be useful in therapeutic regulation of chronic immune conditions [Ref 2].
- Sema3F/NRP2 pathway has also been shown to play a role in tumor suppression in various types of cancers. Part of this role is related to its regulation of phospho-Akt and mTOR signaling [Ref 3-4].
- aTyr has developed a diverse panel of monoclonal antibodies that target NRP2, including aNRP2-14 that has epitopic sites overlapped with the Sema3F binding region. aNRP2-14 was previously shown to specifically bind A549 cells that express endogenous NRP2, but not to NRP2-knockout A549 clonal cells [Ref 5].

Results

Figure 1. Co-crystal structure of aNRP2-14 bound to human NRP2 & structure-based engineering of aNRP2-14 to gain mouse NRP2 reactivity



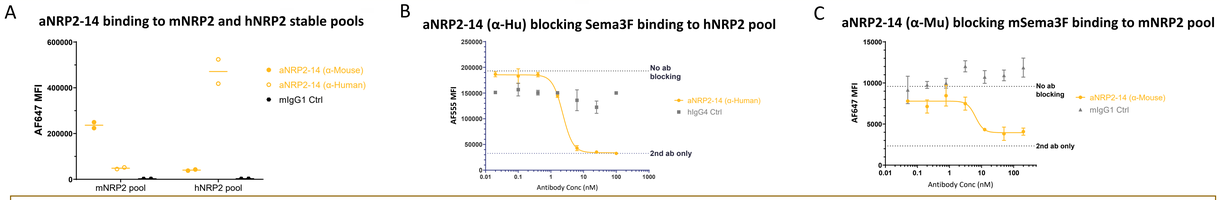


- aNRP2-14 Fab and NRP2-a2b1b2 were co-crystallized and the X-ray structure was solved to a resolution of 1.90Å.
- aNRP2-14/NRP2 co-crystal structure revealed that the a2 domain is primarily responsible for binding with a few contacts to the b1 domain.
- Residue 237 of NRP2 makes contact with residue S56 of the antibody heavy chain. This residue is E237 in human NRP2 and is K237 in mouse NRP2 and is the sole antigen-antibody contact that is not conserved between hNRP2 and mNRP2.
- NNK mutagenesis was performed on S56 of aNRP-14. When S56 was mutated to D, the aNRP2-14 antibody gained binding to both mouse & rat NRP2 (data not shown). However, decreased binding to human NRP2 was observed.

References

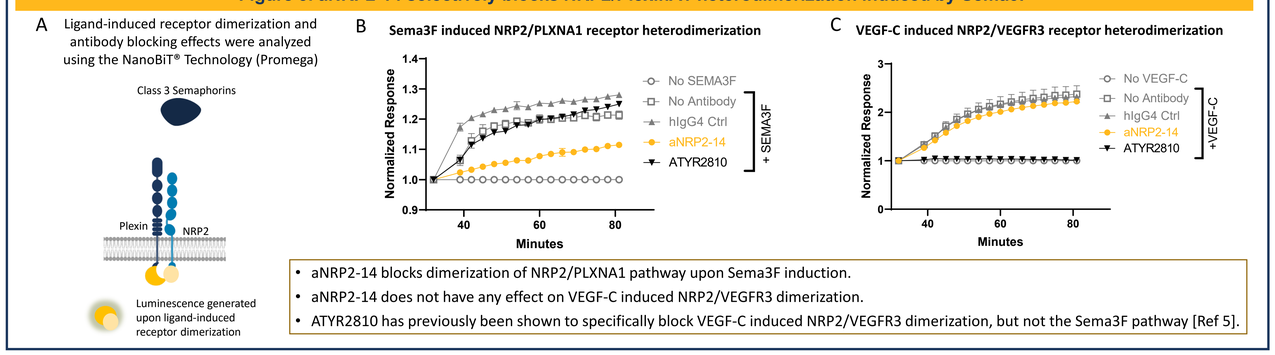
1. Appleton, Brent A et al. "Structural studies of neuropilin/antibody complexes provide insights into semaphorin and VEGF binding." The EMBO journal vol. 26,23 (2007): 4902-12. doi:10.1038/sj.emboj.7601906
2. Plant, Tracie et al. "Semaphorin 3F signaling actively retains neutrophils at sites of inflammation." The Journal of clinical investigation vol. 130,6 (2020): 3221-3237. doi:10.1172/JCI130834
3. Nakayama, Hironao et al. "Semaphorin 3F and Netrin-1: The Novel Function as a Regulator of Tumor Microenvironment." Frontiers in physiology vol. 9 1662. 23 Nov. 2018, doi:10.3389/fphys.2018.01662
4. Nakayama, Hironao et al. "Regulation of mTOR Signaling by Semaphorin 3F-Neuropilin 2 Interactions In Vitro and In Vivo." Scientific reports vol. 5 11789. 9 Jul. 2015, doi:10.1038/srep11789
5. Nangle, Leslie A. et al. "Domain-specific Antibodies to Neuropilin-2 Implicate VEGF-C and Not Semaphorin 3F in Breast Cancer Stem Cell Function", 2020 AACR Virtual Annual Meeting II (Abstract #7308, Poster #1785)

Figure 2. aNRP2-14 blocks Sema3F binding to NRP2



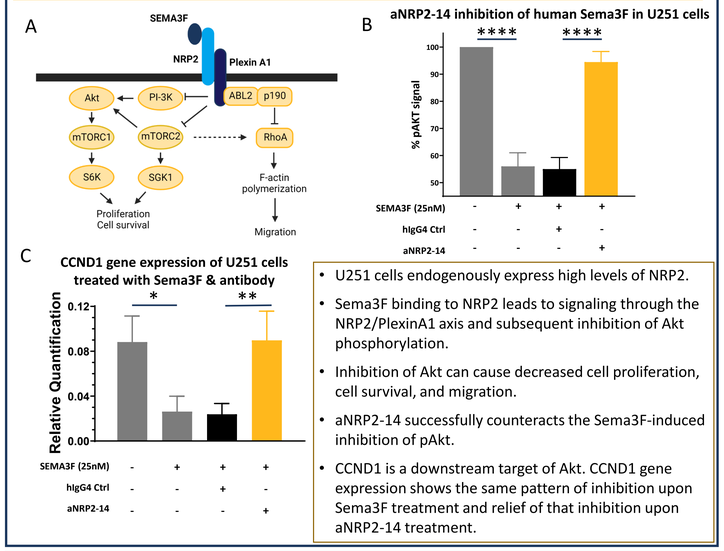
- Mutating the S56D residue of aNRP2-14 created an antibody that is functionally equivalent to the original aNRP2-14 (anti-human) while allowing the antibody bind to mouse NRP2. The S56D mutant was able to bind the 293F cells stable expressing mNRP2, but not hNRP2. The original anti-human aNRP2-14 binds strongly to the human NRP2-293F stable pool but not much to the mouse NRP2-293F pool.
- Both aNRP2-14 antibodies were also able to demonstrate their ability to block Sema3F binding to species specific NRP2 expression cells. Since the S56D mutant does not retain the ability to bind hNRP2, it only blocks the interaction of mSema3F to mNRP2 and vice versa for the anti-human aNRP2-14 antibody.

Figure 3. aNRP2-14 selectively blocks NRP2/PlexinA1 heterodimerization induced by Sema3F



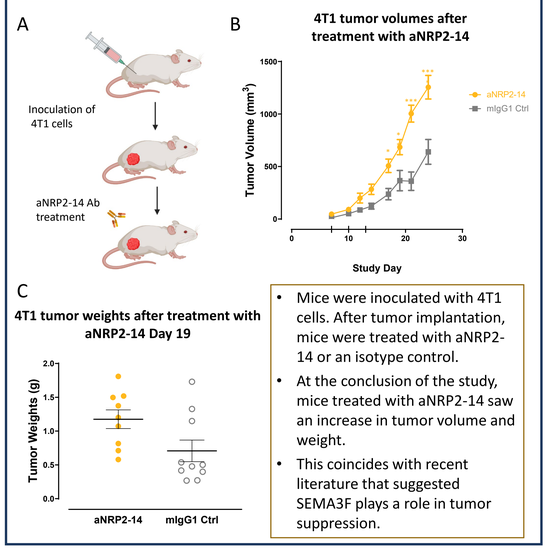
- aNRP2-14 blocks dimerization of NRP2/PLXNA1 pathway upon Sema3F induction.
- aNRP2-14 does not have any effect on VEGF-C induced NRP2/VEGFR3 dimerization.
- ATYR2810 has previously been shown to specifically block VEGF-C induced NRP2/VEGFR3 dimerization, but not the Sema3F pathway [Ref 5].

Figure 4. Sema3F inhibition of phospho-Akt is reversed by aNRP2-14



- U251 cells endogenously express high levels of NRP2.
- Sema3F binding to NRP2 leads to signaling through the NRP2/PlexinA1 axis and subsequent inhibition of Akt phosphorylation.
- Inhibition of Akt can cause decreased cell proliferation, cell survival, and migration.
- aNRP2-14 successfully counteracts the Sema3F-induced inhibition of pAkt.
- CCND1 is a downstream target of Akt. CCND1 gene expression shows the same pattern of inhibition upon Sema3F treatment and relief of that inhibition upon aNRP2-14 treatment.

Figure 5. 4T1 *in vivo* model treated with aNRP2-14



- Mice were inoculated with 4T1 cells. After tumor implantation, mice were treated with aNRP2-14 or an isotype control.
- At the conclusion of the study, mice treated with aNRP2-14 saw an increase in tumor volume and weight.
- This coincides with recent literature that suggested SEMA3F plays a role in tumor suppression.

Conclusions

- aNRP2-14 is a human NRP2 specific antibody. The co-crystal structure of aNRP2-14 and NRP2 confirmed that the main antibody contacts were with the a2 domain of NRP2.
- Based on this structure we were able to engineer a single amino acid mutation in aNRP2-14 that conferred the ability to bind mouse NRP2.
- aNRP2-14 blocks Sema3F binding to human NRP2, and similarly the engineered mouse NRP2-reactive aNRP2-14 (S56D) blocks mSema3F binding to mouse NRP2.
- aNRP2-14 selectively blocks Sema3F-induced NRP2/PlexinA1 heterodimerization, while not affecting VEGF-induced NRP2/VEGFR heterodimerization.
- aNRP2-14 reversed inhibition of Sema3F-induced Akt phosphorylation as well as gene expression of the downstream CCND1 gene.
- aNRP2-14 was tested in a 4T1 *in vivo* model which showed an increase in tumor volume and tumor weight upon antibody treatment. This was consistent with previous literature that suggested a role of Sema3F in tumor suppression and could suggest potential utility of a NRP2/PlexinA1 bi-specific antibody [Ref 3].
- The blocking ability of aNRP2-14 could have therapeutic potential as a novel treatment based on recent literature implicating Sema3F activity in potentiating persistent inflammation [Ref 2].