Dual targeting of angiogenesis pathways: combined blockade of VEGF and Ang2 signaling

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ABSTRACT

VEGF and Ang2 are important players in angiogenesis. VEGF is a well-known survival factor for endothelial cells, and several agents targeting VEGF signaling have been approved for use in cancer indications. Ang2 / Tie2 signaling regulates tumor vessel plasticity, allowing vessels to respond to other angiogenic factors. There is crosstalk between these angiogenic pathways, with Ang2 enhancing VEGF signaling, and VEGF upregulating Ang2 expression on endothelial cells. Thus, combined inhibition of VEGF and Ang2 might well result in improved clinical efficacy compared to VEGF pathway blockade alone.

We have generated a humanized trispecific Nanobody® comprising two single variable domains blocking VEGF and Ang2, and an additional module for half-life extension *in vivo*. This molecule was tested *in vitro* for pathway inhibition and effect on endothelial cells. In addition, efficacy of the Nanobody was tested in a series of patient-derived (PDX) xenograft models.

The VEGF/Ang2 blocking Nanobody® was found to inhibit signaling downstream of VEGF and Ang2, leading to a decrease of endothelial cell proliferation. Combined blockade of VEGF and Ang2 signaling pathways was found superior to inhibition of the individual pathways in patient-derived xenograft studies. The molecule was well tolerated in cynomolgus monkeys.

This novel VEGF/Ang2 blocking Nanobody® showed promising properties *in vitro* and *in vivo*, which strongly support the evaluation of this molecule in the clinic.

Nanobody® is an Ablynx trademark.

INTRODUCTION

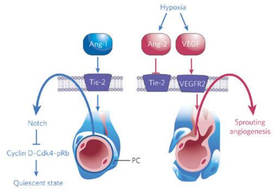
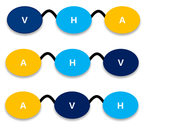


Figure 1. VEGF is a survival and growth factor for endothelial cells and induces proliferation of endothelial cells as well as sprouting and branching of new vessels. Ang2 promotes the detachment of pericytes (PC) and vessel destabilization. In addition, it sensitizes endothelial cells to VEGF signaling. Blockade of both VEGF and Ang2 signaling should thus be superior to inhibition of either pathway alone.

RESULTS

Dual angiogenic pathway inhibitors



V humanized VEGF blocker; cross-reactive to cynomolgus

A humanized Ang2 blocker; cross-reactive to cynomolgus, mouse and rat

humanized half-life extending moiety; cross-reactive to cynomolgus, mouse

Figure 2. Nanobodies® are antibody fragments consisting of a single variable antibody domain. Single-domain camelid antibodies are as specific as regular antibodies. Trispecific Nanobodies with variable domains blocking VEGF and Ang2 as well as an additional module for half-life extension *in vivo* were generated.

Binding to human, mouse and cynomolgus VEGF-A and Ang2

	K_D [M] (Biacore)		K_D [M] (Biacore)
hVEGF165	1.4E-09	hAng2-FLD	1.6E-11
mVEGF165	No binding	mAng2-FLD	5.1E-11
cVEGF165	1.4E-09	cAng2-FLD	8.1E-12

Table 1. Target binding was determined by Biacore. The VEGF/Ang2 Nanobody binds human and cynomolgus VEGF165 with similar affinities, and is human / mouse / cynomolgus crossreactive regarding Ang2 binding.

The VEGF/Ang2 Nanobody® is selective for human VEGF-A

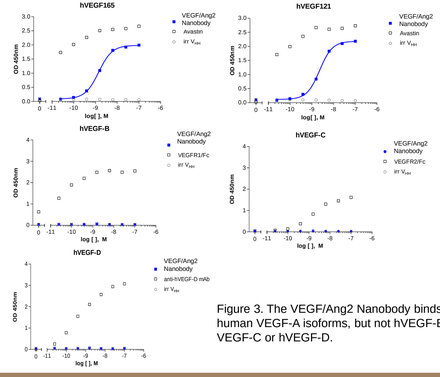


Figure 3. The VEGF/Ang2 Nanobody binds human VEGF-A isoforms, but not hVEGF-B, VEGF-C or hVEGF-D.

RESULTS

Inhibition of VEGF signaling

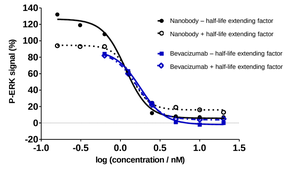


Figure 4. VEGF signals via the MAPK pathway. Phospho-ERK levels of VEGF-stimulated HUVECs were measured in the presence or absence of Nanobody or Bevacizumab by ELISA. The VEGF/Ang2 Nanobody efficiently blocked phosphorylation of Erk.

The VEGF/Ang2 Nanobody® impairs HUVEC proliferation

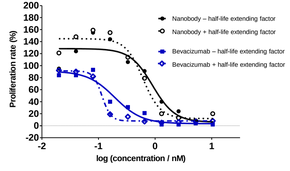


Figure 5. Proliferation of VEGF-stimulated HUVECs was measured in the presence or absence of Nanobody or Bevacizumab by [^{3}H]-Thymidine incorporation. The VEGF/Ang2 Nanobody inhibited HUVEC proliferation potently (Nanobody EC_{50} = 0.6 nM and Bevacizumab EC_{50} = 0.2 nM with half-life extending factor present).

The VEGF/Ang2 Nanobody® binds Ang2 selectively over Ang1

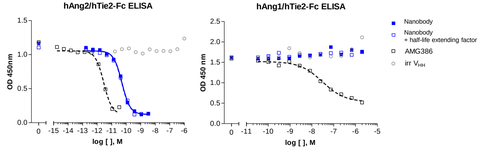


Figure 6. The VEGF/Ang2 Nanobody binds human Ang2, but not Ang1. Binding to Ang2 is comparable to AMG386, an Ang1/Ang2-neutralizing peptibody.

RESULTS

Inhibition of Tie-2 signaling

Ang1 stimulation:

	IC_{50} [nM]
Nanobody	No inhibition
AMG386	29.4 +/- 51.8

Ang2 stimulation:

	IC_{50} [nM]
Nanobody	3.0 +/- 2.6
AMG386	1.3 +/- 1.3

Table 2. HEK293-TIE2 cells were stimulated in the presence or absence of Nanobody or AMG386 with Ang1 or Ang2. Tie-2 phosphorylation levels were measured by ELISA.

Inhibition of HUVEC survival

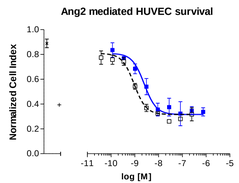


Figure 7. HUVECs were stimulated with Ang2 and cultured for 72h in the presence or absence of VEGF/Ang2 Nanobody or AMG386. HUVEC survival was determined by impedance measurement.

The VEGF/Ang2 Nanobody® shows superior *in vivo* efficacy

Indication	Model	Avastin %TGI	AMG 386 %TGI	Nanobody %TGI
Pancreas	PAXF 546	54	43	94
	PAXF 736	33	40	54
	PAXF 1872	13	65	46
Lung	LXFE 211	44	59	78
	LXFE 1422	67	58	86
Renal	RXF 631	43	39	54
	RXF 1220	20	4	58
Ovary	OVXF 1353	50	66	67
Colon	CXF 243	61	76	76

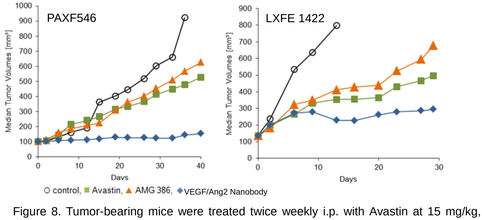


Figure 8. Tumor-bearing mice were treated twice weekly i.p. with Avastin at 15 mg/kg, AMG 386 at 15 mg/kg or VEGF/Ang2 Nanobody at 13.7 mg/kg. Tumor volumes were measured over time.

RESULTS

Angiogenesis is inhibited *in vivo*

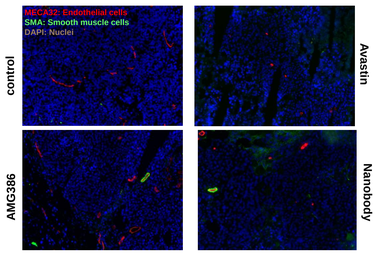


Figure 9A. SW620 tumor-bearing mice were treated twice weekly i.p. with 25 mg/kg Avastin, 15 mg/kg AMG386 or 15 mg/kg VEGF/Ang2 Nanobody. IHC stainings were performed using antibodies to MECA32 and SMA. B. Total levels of hVEGF and hAng2 were measured from tumor lysates by ELISA and showed a decrease upon VEGF/Ang2 Nanobody treatment.

CONCLUSIONS

- **The VEGF/Ang2 Nanobody® potently and selectively neutralizes VEGF-A and Ang2**
- **The molecule impairs HUVEC proliferation and survival**
- **The VEGF/Ang2 Nanobody® inhibits angiogenesis *in vivo* and shows superior efficacy to inhibition of the individual pathways**

These preclinical data strongly support the evaluation of the VEGF/Ang2 Nanobody® in the clinic.

DISCLOSURES

The authors IH, AB, FH, PGC, NK and KPK are employees of Boehringer Ingelheim. ED and JB were employees of Ablynx at the time of data generation.

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