Design of new Angiopep-2-anti-EGFR and Angiopep-2-anti-HER2 derivatives with increased blood-brain barrier permeability for treatment of brain tumors.

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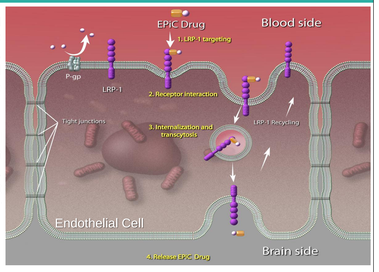
ABSTRACT

The therapeutic potential of mABs to treat a variety of tumor types is well established. However, this class of agents is generally excluded from the brain due to poor blood-brain barrier (BBB) permeability. While preventing entry of xenobiotics into the brain, BBB endothelial cells do express multiple transporters and receptors to permit entry of essential molecules, such as nutrients and hormones. One such receptor, the low density lipoprotein-related receptor 1 (LRP1), enables numerous ligands to access the brain via receptor-mediated transcytosis. We have previously demonstrated that therapeutic brain levels of non-brain penetrant peptides or small molecules can be achieved by conjugation with Angiopep-2 (An2), a proprietary 19 amino acid peptide which binds LRP1. For example, An2-paclitaxel (GRN 1005, licensed to Geron, Inc.) is currently in Phase II studies for brain metastases. Applying this Engineered Peptide Conjugate (EPiC) technology to mAbs, we have now been able to expedite transport of anti-Her2 and anti-EGFR mAbs across the BBB. Several conjugates were created using various chemical methods and conditions, which were optimized for brain penetration and pharmaceutical properties. By measuring *in vivo* brain uptake in mice, we observe that incorporation of An2 increases the rate of entry into brain parenchyma by 5-10-fold over unconjugated mAbs, depending on the number of An2 molecules per mAb. EPiC-mAb conjugation does not impact target binding affinity or plasma stability. In mouse, the plasma half-life of Epic-anti-Her2 is the same as that of the unconjugated molecule, while brain:plasma ratio is higher. Overall, these data extend the validation of An2 conjugation beyond small molecules and peptides to include larger molecules such as therapeutic mAbs for development of new brain-penetrant antitumor therapeutics.

INTRODUCTION

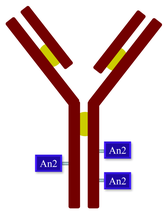
Angiochem is a clinical-stage biotechnology company discovering and developing new breakthrough drugs that are uniquely capable of crossing the blood brain barrier (BBB) to treat brain diseases. These new drugs have the potential to address significant medical needs, many of which cannot be effectively addressed due to the fundamental physiological challenge the BBB presents.



The BBB is a selective barrier formed by tightly packed endothelial cells that line the cerebral capillaries. The BBB is important as it provides an insulated environment for stable neuronal function. Endothelial cells forming the BBB are not only able to form tight junctions, but also possess the following characteristics that further protect the brain, they:

- ✓Lack fenestra;
- ✓Lack transendothelial channels;
- ✓Lack pinocytic vesicles; and
- ✓Express high levels of the active efflux pump (P-gp).

Angiochem's proprietary peptide-drug conjugate platform targets low-density lipoprotein receptor-related protein-1 (LRP1). This endogenous transcytosis system has a number of inherent biochemical advantages for drug transport across the BBB, including high capacity, rapid turnover, recognition of numerous ligands, and limited down-regulation. We have created peptides (Angiopeps), including Angiopep-2 (An2) using a library based on LPR-1 binding sequences of known LRP-1 ligands. These peptides can be introduced, by chemical or fusion, to small molecules and biologics, thus forming NCEs that are brain-penetrant Peptide-Drug Conjugates.



HER2 (ErbB2, human epidermal growth factor receptor 2) and EGFR (HER1, ErbB1, epidermal growth factor receptor) belong to a family of receptor tyrosine kinases whose effects on intracellular signaling pathways lead to cell proliferation, differentiation and survival. Humanized monoclonal antibodies to these receptors (trastuzumab, Genentech and cetuximab, Imclone) are currently in clinical use for treating tumors expressing HER2 and EGFR receptors. While success has been achieved in treating such cancers in peripheral tissues, these mAbs do not readily cross the BBB, thus limiting their utility for metastatic brain tumors. Targeting lysine residues on the full-length mAbs for conjugation with stable linkers to An2, we have created peptide-mAb conjugates that are recognized by LRP1 and cross from plasma to brain by receptor-mediated transcytosis. These NCEs retain target binding characteristics and cytotoxicity potency of the native molecule. Here we report increased brain entry by these peptide mAb conjugates.

METHODS

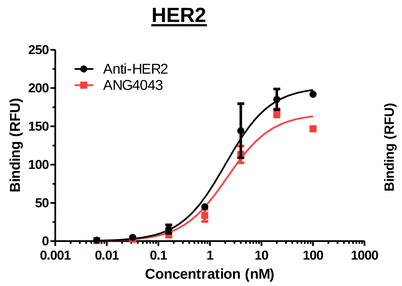
1. Evaluation of in vivo brain uptake. Test drug (^{125}I-labeled) was administered via carotid artery of mice for up to 4 minutes, followed by a 30-second saline wash. Brains were removed followed by capillary depletion, radioactivity quantification, and calculation of rate constant, Kin.

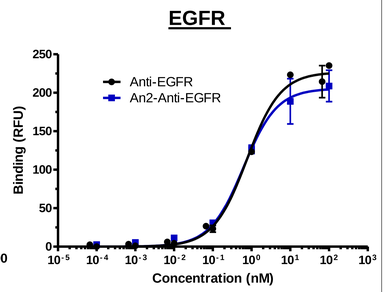
2. Evaluation of Cytotoxicity. BT-474 cells were incubated with increasing concentration of anti-Her2 antibody or ANG4043 for 5 days. After incubation, the medium was aspirated and cells were pulse labeled for 4 hrs at 37°C with medium containing 2.5 µCi/mL [methyl-^{3}H]-thymidine. Cells were fixed, liquid scintillation cocktail was added and ^{3}H-thymidine uptake was quantified in a radioactivity counter. Results show the amount of incorporated ^{3}H-thymidine as a function of mAb concentrations.

3. Binding of anti-HER2 and ANG4043 to BT-474 cells. Confluent BT-474 cells were first detached from flaks with PBS-citrate non-enzymatic dissociating buffer. Cells in suspension were washed in ice-cold binding buffer (BB: Hepes 10mM, NaCl 150mM, $CaCl_2$ 2.5mM, pH: 7.3), counted and dispensed to a count of 10^6 BT-474 cells per tube. Binding of test articles was performed with increasing concentrations in ice-cold BB for 30 minutes at 4°C. Cells were then washed and incubated with an α-Human-AlexaFluor488 secondary antibody in ice-cold BB for 30 minutes at 4°C. Cells were extensively washed with ice-cold BB and analyzed by flow cytometry (10,000 gated events per condition). Binding of anti-EGFR and An2-anti-EGFR to U87 cells overexpressing the wild-type EGFR was measured using the same experimental conditions.

4. Imaging of mice in tumor cells For near infrared (NiR) in vivo imaging studies, ANG4043 and anti-Her2 antibody were labeled with a NiR reactive dye Cyto750-NHS ester (Cytodiagnostics, Burlington, ON) according manufacturer's protocol. Briefly, cyto750-NHS ester dissolved in DMSO was incubated with mAb at pH=9 with a dye to protein molar ratio of 1 :2. After 1 hour incubation at room temperature, the Cyto750 labeled proteins were separated from the unreacted dye by gel filtration (Pierce Dextran desalting column), the first running coloured band being the Cyto750 labeled proteins. Mice were injected with 5 mg/kg of test mAb. Animal imaging was performed 24 hrs after injections using the in vivo Xtreme imaging system from Carestream.

BINDING TO TARGET RECEPTOR



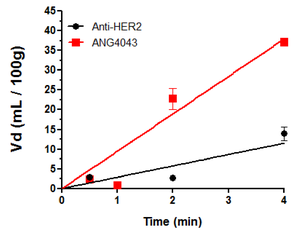


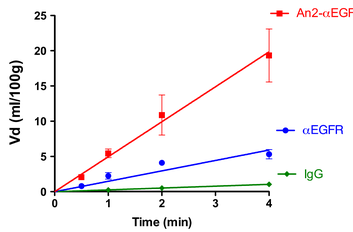
	(IC_{50} nM)
Anti-HER2	0. 2 +/- 0.13
ANG 4043	0.3 +/- 0.09

	(IC_{50} nM)
Anti-EGFR	0. 2 +/- 0.13
An2-Anti-EGFR	0.3 +/- 0.09

An2 conjugation does not affect target binding affinity

BRAIN UPTAKE of mABs in MOUSE



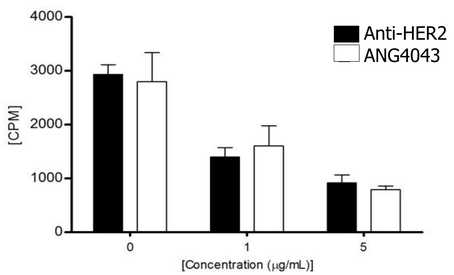


	K_{in} (mL/s/g)
Anti-HER2	4.8 x 10^{-4}
ANG 4043	1.6 x 10^{-3}

	K_{in} (mL/s/g)
Anti-EGFR	2.5 x 10^{-4}
An2-Anti-EGFR	8.3 x 10^{-4}

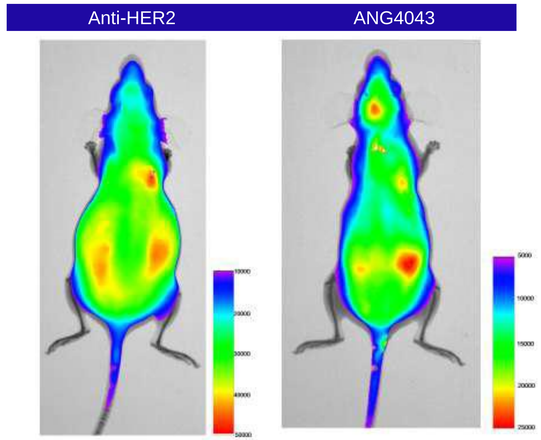
Initial rate of transport into brain (Kin) is higher for An2-antibody conjugates.

ANG4043 CYTOTOXICITY in BT-474 CELLS



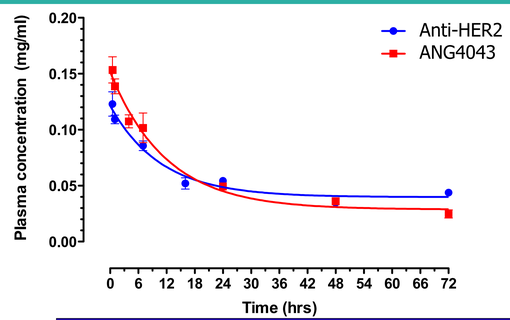
Effect on cell proliferation is similar between H4043 and unconjugated anti-HER2

IMAGING of ANG4043 in INTRACRANIAL BT-474 MICE



Mice bearing intracranial BT-474 tumors were dosed with Cyto-750-labeled Anti-HER2 or ANG4043 24 hours prior to NiR imaging. The signal in the brain observed following ANG4043 treatment indicates that this mAb is able to penetrate the blood-brain barrier and access the intracranial tumor.

ANG4043 PLASMA PHARMACOKINETICS IN MOUSE



	$T_{1/2}$ (hr)
Anti-HER2	5.8
ANG4043	6.0

Plasma half-life in mouse is similar for ANG4043 and anti-HER2.

CONCLUSIONS

- New brain-penetrant Peptide-Antibody Conjugates are described here for the first time.
- The An2-mAbs retain target receptor binding characteristics of the unconjugated mAbs.
- ANG4043, the An2-anti-HER2 conjugate selected for further evaluation, displays similar in vitro cytotoxicity potency to that of unconjugated anti-HER2.
- Plasma half-life values for ANG4043 and anti-HER2 are similar.
- In mice implanted intracranially with BT-474 cells, higher accumulation of Cyto750-ANG4043 is observed in brain tumor tissue compared to Cyto750-anti-HER2.
- **These results indicate that ANG 4043 crosses the blood-brain barrier, enters the CNS, and targets tumor tissue within the brain.**

Angiochem BEYOND BARRIERS