Feasibility of PNA-mediated
Affibody-based radionuclide pretargeting

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Outline

- Difference between conventional radioimmunotherapy (RIT) and pretargeting RIT
- Affibody Molecules, HER2 receptor and HER2 binding Affibody molecules
- Design, production and *in vitro* characterization of PNA and Affibody-PNA chimeras
- Biodistribution of PNA and in vivo pretargeting
- Summary
- Future Work
Conventional radioimmunotherapy

Injection of radiolabeled Ab

Tumor cell

Accumulation in tumor, but slow clearance from blood

Time
Pretargeting for radioimmunotherapy

First injection: Ab

Accumulation in tumor and slow clearance from blood

Second injection: Radionuclide (after waiting time)

Covalent or non-covalent interaction linking the primary and secondary agents
Affibody molecules – Alternative scaffold affinity proteins

- Affibody molecules are 58 amino acid three-helix-bundle affinity alternative scaffold proteins
- Derived from the B domain in staphylococcal protein A
- Small MW (6.5 kDa) – allows for fast biodistribution and high tissue penetration
- Using phage display, 13 solvent-exposed sites in helix one and two are mutated randomly to generate binder libraries
- High affinity binders have been generated to cancer associated targets such as HER2, EGFR, HER3, IGF-1R, PDGFRβ and CAIX
- High solubility, high thermal stability
- Chemical synthesis or low-cost recombinant expression in E. coli
HER2 - Human epidermal growth receptor 2

- Also known as receptor tyrosine-protein kinase 2, ERBB2
- Over-expressed in numerous cancers, including ovarian and bladder cancers, and about 20% (15-20%) of all breast cancer cases
- HER2-positive breast cancers are associated with poor survival
- Since the introduction of HER2-targeted treatments, survival of patients with HER2-positive breast cancer has improved significantly

Structural basis for high-affinity HER2 receptor binding by an engineered protein, Eigenbrot C. et al. PNAS (2010)
PET/CT imaging of HER2+ metastatic breast cancer using [\(^{68}\text{Ga}\)]-ABY-025

- ABY-025 is a HER2-binding Affibody molecule conjugated with a DOTA chelator
- Labeled with \(^{68}\text{Ga}\) for PET imaging
- High contrast HER2 images were acquired 2-4 h after injection of \(^{68}\text{Ga}\)-ABY-025
- Non-invasive way of determining HER2 status in metastatic breast cancer
- HER2-targeted treatment was changed in 3/16 patients as a consequence of \(^{68}\text{Ga}\)-ABY-025 PET
- Affibody molecules labeled with residulizing radiometals are suitable for imaging studies but not for therapy due to a high kidney uptake

Two patients (A and B) with wide-spread metastatic breast cancer
- Patient A has a HER2-negative primary tumor
- Patient B has a HER2-positive primary tumor

A1 and B1 \([\(^{18}\text{F}\)]\)-FDG PET
A2 and B2 \([^{68}\text{Ga}]\)-ABY-025 PET

Measuring HER2-Receptor Expression in Metastatic Breast Cancer Using [\(^{68}\text{Ga}\)]ABY-025 Affibody PET/CT
Design of complementary PNA-based hybridization probes

Hybridization probe 1 (HP1):
Conjugated to Affibody molecule

Hybridization probe 2 (HP2):
Complementary PNA sequence
**Peptide nucleic acid (PNA)**

- **PNA (Peptide nucleic acid)** is a synthetic DNA mimetic in which the phospho-sugar backbone has been replaced by a more peptide-like charge neutral backbone.
- PNA has four different bases just as DNA and complementary PNA sequences are able to base-pair with each other, obeying Watson-Crick complementarity rules.
- PNA:PNA duplexes show thermal stabilities that exceed that of natural nucleotides.
- Being neither a peptide nor a nucleotide makes PNA resistant to nucleases and proteases and PNA molecules have excellent serum stability.
- PNA molecules have low toxicity, are nonimmunogenic and show low cellular uptake in vivo.
Solid phase synthesis of PNA-based hybridization probes

*HP1* and *HP2* were synthesized in-house using solid phase synthesis with commercially available monomers.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Teor. Mass</th>
<th>Exp. Mass</th>
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<tr>
<td>G-G-G-S-S-agtctggatgtagtc-E-K(DOTA)-AEEA-E-CONH₂</td>
<td>5396.4 Da</td>
<td>5396.5 Da</td>
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<tr>
<td>DOTA-AEEA-S-S-gactacatccagact-E-Y-CONH₂</td>
<td>5157.8 Da</td>
<td>5158.3 Da</td>
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Production of $Z_{HER2:342}$-SR-$HP1$

I.
- Cloning
- Expression
- IMAC purification

II.
- Solid phase synthesis
- HPLC purification

$Z_{HER2:342}$-SR-$H_6$ + $HP1$

III.
- Sortase A*/eSortase A mediated ligation
- HPLC purification
- IMAC purification

$Z_{HER2:342}$-SR-$HP1$

DOTA: macrocyclic metal chelator
[AEEA]: PEG-based spacer

GGGSS-agt ctg gat gta gtc-EK-[AEEA]-E-NH$_2$

DOTA
Biophysical characterization - Biacore affinity measurement

[Graph showing SPR sensorgrams with time (minutes) on the x-axis and resonance units (RU) on the y-axis.]

- HER2 on chip, pre-hybridized Z\textsubscript{HER2:342}-SR-\textit{HP1:HP2} is flown over the surface.

- Biacore SPR sensorgrams of binding pre-hybridized Z\textsubscript{HER2:342}-SR-\textit{HP1:HP2} complex to immobilized HER2 receptor.

K\textsubscript{D}~212 pM

- Resonance units (RU)

<table>
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<th>Concentration (nM)</th>
<th>Time (minutes)</th>
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<td>23.5 nM</td>
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<tr>
<td>11.8 nM</td>
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<td>5.9 nM</td>
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<tr>
<td>1.5 nM</td>
<td>20</td>
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<tr>
<td>0.7 nM</td>
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**Biophysical characterization**

- **Biacore affinity measurement**

- Biacore SPR sensorgrams of HP2 binding to immobilized $Z_{HER2:342}$-SR-HP1.

- The $Z_{HER2:342}$-SR-HP1:HP2 complex has a very slow dissociation rate, 17 hours after injection with 725 nM HP2 the dissociation was still <5% of the initial response.
Biodistribution of $^{125}$I-HP2 and $^{111}$In-HP2 in normal (non-tumor) NMRI mice

Groups of five NMRI mice were injected with $1 \ \mu g$ radiolabeled HP2, either $^{125}$I-HP2 or $^{111}$In-HP2, and the biodistribution was measured 1 and 4 h.p.i.

As a comparison, renal uptake of $1 \ \mu g$ $^{111}$In-labeled DOTA-Z$_{HER2:342}$, 4 h.p.i. was (180–200 %ID/g), i.e., 45–50-fold higher.
Specific HER2 targeting by the $Z_{HER2:342}$-**HP1** Affibody-PNA chimera

- **Specific HER2 targeting** demonstrated by injection of 5 µg $^{111}$In-$Z_{HER2:342}$-SR-**HP1** in mice with HER2-positive SKOV-3 xenografts.

- Control 1: a blocked group co-injected with an excess amount (500 µg) of non-labeled $Z_{HER2:342}$ Affibody molecule

- Control 2: a group with HER2-negative Ramos cell xenografts.
Pretargeting: BALB/c Nude mice with HER2-expressing SKOV-3 xenografts

All mice were injected with 1 μg radiolabeled HP2, $^{125}$I-HP2 (left) or $^{111}$In-HP2 (right), and the biodistribution was measured at 1 h p.i. 

**Pretargeted mice** had been injected with 100 ug $Z_{HER2:342}$-SR-HP1 4 hour prior to HP2 injection, the **control mice** received no such injection
Imaging of HER2-expressing SKOV-3 xenografts in mice

A- Direct targeted mouse, 2 μg $^{111}$In-DOTA-Z$_{HER2:K58}^	ext{}$
B- Pretargeted mouse, preinjected with 100 μg Z$_{HER2:342}$-SR-HP1 4 hours prior to injection with 1 μg $^{111}$In-HP2
C- Control mouse, Injected with 1 μg $^{111}$In-HP2

All three mice were injected with 650 kBq of radiolabeled compound 1 h before image acquisition

Gamma-camera imaging of HER2-expression in SKOV-3 xenografts
Summary:

- Binding of $^{111}$In-$Z_{HER2:342}$-SR-$HP1$ to HER2-expressing SKOV-3 xenografts was HER2-specific.

- Accumulation of $^{125}$I- and $^{111}$In-labeled $HP2$ in HER2-expressing tumors is dependent on pretargeting.

- Without $Z_{HER2:342}$-SR-$HP1$ pre-injection the tumor uptake of HP2 was very low.

- Affibody-based PNA-mediated pretargeting can thus provide higher accumulation of radiometals in tumors in comparison with kidneys.
Future work

- Optimize labeling of HP2 with a therapeutic radionuclide, i.e. $^{177}$Lu (Ongoing)

$^{177}$Lu is a commercially available medium-energy $\beta$-emitter, suitable for treatment of small and medium-size tumors

- Experimental therapy on mice with SKOV-3 xenografts

$Z_{\text{HER2:342}}$-SR-HP1 and $^{177}$Lu-HP2 (Planned autumn 2016)
Publications:


Feasibility of Affibody Molecule-Based PNA-Mediated Radionuclide Pretargeting of Malignant Tumors (2016)
Hadis Honarvar, Kristina Westerlund et al. Theranostics 6, 93–103.

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QUESTIONS?