Evaluation of two engineered antibody fragments, derived from the human anti-CD20 Ofatumumab, as tracers for immunoPET

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Abstract

Background: CD20 is a cell surface antigen that is expressed on the majority of B-cell lymphomas and B-cell leukemias. Several monoclonal antibodies (mAbs), including the fully human IgG1, Ofatumumab, have been used successfully to target human CD20. Ofatumumab is FDA-approved for the treatment of patients with chronic lymphocytic leukemia (CLL) and is under ongoing clinical development for other hematological malignancies and autoimmune diseases. In this study Ofatumumab was engineered into smaller fragments including a minibody (scFv-C_H3; 80kDa) and a cys-diabody (scFv dimer; 50kDa) to enhance pharmacokinetic parameters and optimize possible application for immunoPET imaging.

Methods: The variable (V) genes of Ofatumumab were reformatted into two minibodies (Mb) and four cys-diabodies (Cys-Db), differing in orientation of the V genes and linker lengths. Following *in vitro* characterization of each variant, a lead candidate from each format was produced at larger scale for *in vivo* evaluation. Ofatumumab, Mb and Cys-Db were radioiodinated with I-131 ($t_{1/2}$ 8d) for biodistribution and I-124 ($t_{1/2}$ 4.2d) for PET imaging studies using the Iodogen method. Female SCID mice bearing Ramos ($CD20^+$) and BC-1 ($CD20^-$) tumors were used. Blocking studies were performed by pre-injecting 0.2-1mg of unlabeled Ofatumumab 24h prior to imaging. The percent injected dose per gram (%ID/g) was determined in harvested tumor, blood and organs.

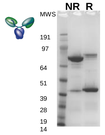
Results: The lead Cys-Db and Mb were shown to have similar binding affinity (0.8 nM) to that of the parental Ofatumumab. Receptor specific binding was confirmed by competitive cell binding assays using both fluorophore-conjugated and radiolabeled antibody fragments. Blood clearances resulted in MRTs of 3.8h, 8.3h and 24.9h for Cys-Db, Mb and Ofatumumab, respectively. Due to its slower initial distribution, the Mb had a higher blood AUC (306%ID/g*h) than that of Ofatumumab (234% ID/g*h) and Cys-Db (102% ID/g*h). Biodistribution studies of Ofatumumab revealed significant uptake in spleen, bone and liver, which was attributed to Fc-mediated clearance as this was not observed with the antibody fragments. Imaging studies with Mb and Cys-Db showed clear delineation of Ramos xenografts; 1.3% ID/g Cys-Db at 8h and 2.7% ID/g Mb at 24h. This resulted in a tumor to blood ratio of 1.7 for the Cys-Db at 8h and 1.1 for the Mb at 24h. Prior administration of excess unlabeled Ofatumumab reduced tumor uptake of the Cys-Db by 50% at 8h and decreased uptake of the Mb by 17% at 24h.

Conclusion: Our preliminary results show that antibody fragments derived from Ofatumumab can be used to target CD20 positive cells *in vitro* and *in vivo*. The favorable pharmacokinetics of the Cys-Db resulted in higher tumor to blood ratios at 8h and 24h compared to the Mb or Ofatumumab and support the further development of this fragment as a CD20-targeted immunoPET agent.

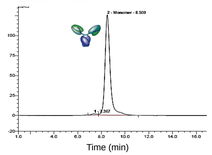
In Vitro Characterization

Supernatants from large scale production of the Mb and Cys-Db in mammalian cell culture were purified by ion exchange chromatography followed by ceramic hydroxyapatite. Purity and aggregation of the Mb and Cys-Db were evaluated by SDS-PAGE and size exclusion chromatography (SE-HPLC). Binding was evaluated by flow cytometry. Receptor-specific binding was evaluated by competition studies.

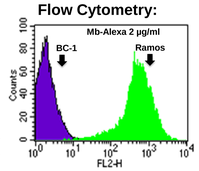
SDS-PAGE:



SEC-HPLC:

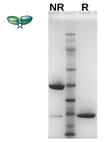


Flow Cytometry:

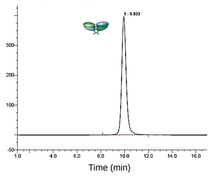


SDS-PAGE showed that the Mb and Cys-Db migrated as covalent dimers of ~80 kDa and 50 kDa, respectively. In the presence of a reducing agent (DTT) they migrated as monomers of ~40 kDa and 25 kDa. The purity by SDS-PAGE was >90%.

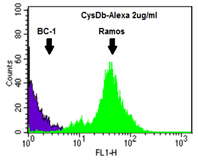
SDS-PAGE:



SEC-HPLC:



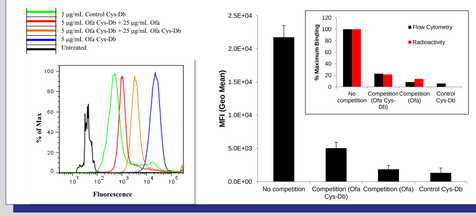
Flow Cytometry:



SE-HPLC analysis showed that both proteins eluted as a single peak corresponding to dimers of ~80 kDa and 50 kDa in size. The purity by SEC was estimated to be >98% for both fragments.

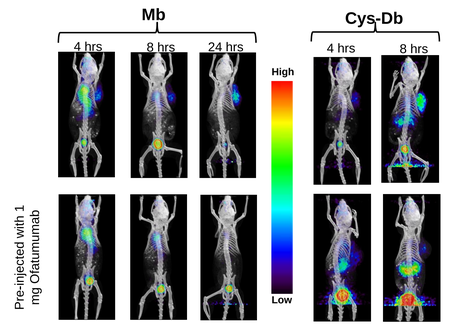
Flow cytometry confirmed binding to CD20 expressing Ramos cells, and not to the negative BC-1 cells.

Competition Studies:



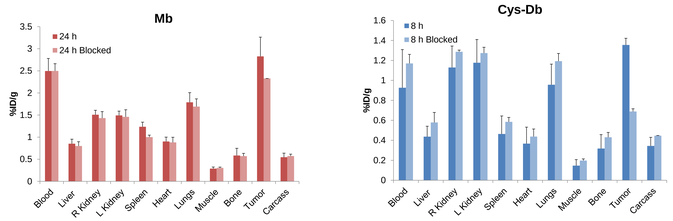
Receptor-specific binding of Cys-Db to CD20(+ve) Ramos cells was confirmed by flow cytometry after fluorophore conjugation and radioiodination. Binding of Cys-Db to Ramos cells could be inhibited by addition of excess unlabeled Cys-Db, Mb, or Ofatumumab. Similar displacement was obtained with the Mb (not shown)

In Vivo PET Imaging and Blocking Studies

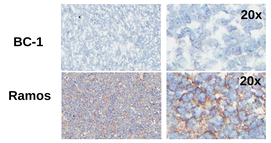


The Mb and Cys-Db were radioiodinated with I-124 ($t_{1/2}$ 4.2 days) using the Iodogen method. Groups of 4-5 nude, female mice harboring Ramos xenografts were injected with ~133 µCi (SA 3.4 µCi/µg) of Cys-Db or ~135 µCi (SA 3.9 µCi/µg) of Mb and serially imaged by PET/CT. Two mice injected with the Cys-Db and three mice injected with the Mb were pre-injected with 1 mg of unlabeled Ofatumumab. Rendered Images of one mouse from each group are shown. Blocking resulted in 50% and 17% reduction in tumor uptake of the Cys-Db at 8 hrs and Mb at 24 hrs, respectively.

Biodistribution



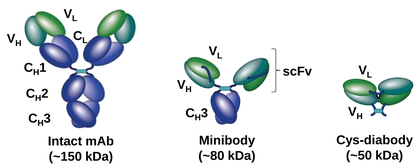
After the last scan time point, mice were sacrificed and tumor, blood and organs were harvested, weighed and counted. The radioactivity was decay corrected and the %ID/g calculated.



Ramos and BC-1 tumors were evaluated following immunohistochemistry staining. The panels shows CD20 staining on frozen specimens. The Ramos express CD20 whereas the BC-1 is negative for CD20.

Background and Rationale

- Ofatumumab, a human monoclonal antibody directed to CD20, was approved in 2009 for the treatment of refractory chronic lymphocytic leukemia.
- CD20 is a B-cell–specific differentiation antigen that is expressed on mature B cells and in most B-cell non-Hodgkin's lymphomas but not on early B-cell progenitors or later mature plasma cells.
- The CD20 antigen is a member of the membrane-spanning 4A gene family and Ofatumumab binds both the large and small extracellular loops.
- Type I antibodies (Rituximab and Ofatumumab) induce redistribution of CD20 into large lipid rafts in the plasma membrane and confer strong complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity but have minimal direct antitumor effects.
- In this study a diabody with terminal cysteines (Cys-Db) and a Minibody (Mb) were generated from ofatumumab and evaluated for their suitability to image CD20 positive cancers.



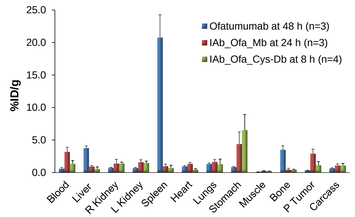
Schematics of an intact monoclonal antibody (mAb), a minibody and a Cys-Db. The various domains are indicated. Molecular weights are shown below. V = variable, C = constant, L = light, H = heavy

In Vivo Biodistribution and Pharmacokinetics

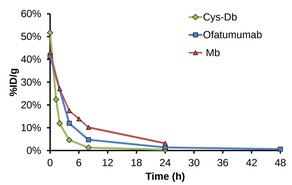
The fragments and Ofatumumb were radioiodinated with I-131using the Iodogen method. High immunoreactivity to Ramos cell were maintained after the labeling

`	Cys-Db x2	Mb	Ofatumumab
Radiochemical Purity	99.3%	96.8%	96.4%
Specific Activity (µCi/µg)	3.33/3.33	3.57	3.56
Immunoreactivity	66.3 vs.4.0%	72.7 vs. 5.7%	83.5 vs. 2.7%

Biodistribution



Blood clearance



Groups of 3-4 mice were injected i.v. with 10-14 µCi of I-131-labeled Cys-Db, Mb or Ofatumumb. Biodistributions were performed at 8, 25 and 48 hrs for the Cys-Db, Mb and Ofatumumab, respectively. At specific time-points tails were nicked with a scalpel and 10 µL blood collected and counted in a gamma counter. The activity was decay corrected and the %ID/g was calculated. The MRTs from the blood clearance profiles shown above, were determined to be 2.9h, 6.2h and 13.2h for the Cys-Db, Mb and Ofatumumab, respectively. Due to its' slower initial distribution, the Mb had a higher blood AUC (277%ID/g*h) than that of Ofatumumab (212% ID/g*h) and Cys-Db (100% ID/g*h) in this experiment. The iodistribution shows that Ofatumumab has high uptake in spleen, liver and bone which is probably due to Fc-mediated clearance.

Summary of Results

- Protein production of the anti-CD20 Mb and Cys-Db yielded highly pure (>95%), homogeneous antibody fragments that bound to CD20
- *In vitro*, binding to CD20 was inhibited by competition with Ofatumumab
- *In vivo*, the Mb cleared more slowly from the blood than the Cys-Db and Ofatumumab
- The tumor to blood ratios were 1.7 for the Cys-Db at 8 hrs and 1.1 for the Mb at 24 hrs
- PET imaging revealed specific localization of the fragments in Ramos xenografts that could be inhibited by pre-administration of excess Ofatumumab
- Blocking reduced the tumor uptake from 1.4 to 0.7 %ID/g for the Cys-Db at 8 hrs and from 2.8 to 2.3% ID/g for Mb at 24 hrs indicating that tumor localization was driven by receptor-specific interactions
- The favorable pharmacokinetics of the Cys-Db resulted in higher tumor to blood ratios at 8h and 24h compared to the Mb or Ofatumumab and support the further development of this fragment as a CD20-targeted immuno PET agent

Acknowledgments
The authors would like to acknowledge the UCLA Crump Institute for Molecular Imaging and UCLA Pathology core for assistance with imaging and IHC.