Extracellular Protein Degradation Using Novel M6PR-Targeting Chimeras (MTACs): In Vitro and In Vivo Proof-of-Concept Studies

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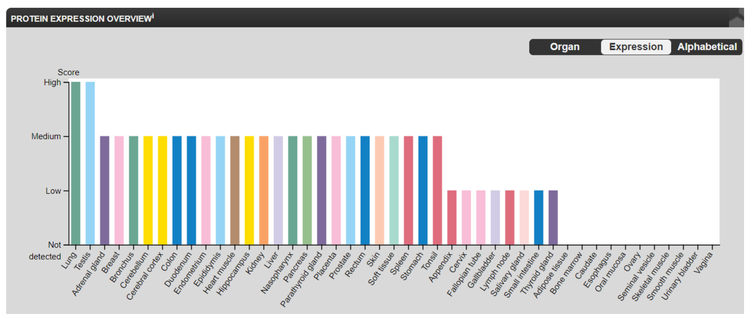
Abstract

- The mannose-6-phosphate-receptor (M6PR) traffics proteins from extracellular and intracellular locales to late endosomes/lysosomes for degradation and is ubiquitously expressed. This natural endogenous function of the mannose-6-phosphate-receptor (M6PR) may be harnessed for degradation of soluble circulating proteins and cell specific degradation of membrane proteins.
- Here we describe the design and development of novel M6PR targeting chimeras (MTACs). MTACs are heterobifunctional molecules containing Avilar's novel, potent, small molecule M6PR-binding ligands conjugated to ligands that bind a target protein of interest for degradation.
- For proof-of-concept studies, MTACs were designed to target the extracellular protein IgG, the second most abundant circulating protein with a long plasma half-life. Reduction of pathogenic IgG autoantibodies is a clinically validated strategy for treating multiple debilitating autoimmune diseases.
- In vitro characterization of tridentate and monodentate MTACs revealed potent biochemical binding to M6PR and IgG, and MTAC-mediated cellular uptake and degradation of IgG via the endolysosomal pathway in K562 and LNCaP cells.
- In vivo MTAC-mediated depletion of human IgG was demonstrated using a heterologous rat PK/PD model.

MTAC (M6PR-Targeting Chimera) Platform

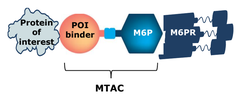
- ASGPR and M6PR are known endocytotic receptors that induce internalization and lead proteins to degradation in the endolysosomes
- ATAC (ASGPR-Targeting Chimera) degraders leveraging ASGPR have been previously described by Avilar and apply to circulating proteins and hepatocyte membrane proteins
- M6PR is more widely expressed compared with the nearly exclusive hepatocyte expression of ASGPR[1]

M6PR Protein Tissue Expression



- Avilar MTACs contain novel and proprietary M6PR ligands with improved affinity versus previous chemistries
- These higher affinity M6PR ligands enable efficient degradation with simplified and lower molecular weight monovalent designs
- Lower molecular weight translates to reduced dose and injection volume

Design of M6PR Ligands and MTACs



- Designed and synthesized (using structure based computational approaches) multiple M6PR-binding ligands with increasing affinity and varying avidity for in vitro characterization
- Designed and synthesized bifunctional molecules comprising various M6PR ligands in monodentate and tridentate presentation, various linkers, and a peptide binder to human IgG for in vitro and in vivo characterization
- Compared performance of monodentate MTACs containing a novel high affinity M6PR ligand (Ligand B) with tridentate and monodentate bifunctional molecules containing a lower affinity M6PR ligand (Ligand A)

Characterization of Cell Surface M6PR Across Cell Lines

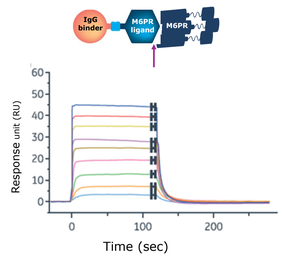
- Measured various rate-limiting factors for protein degradation: M6PR number, ability to form ternary complex, and ability to uptake MTACs in various commonly used cell lines
- Observed >10x difference in number of surface M6PR in cell lines with varying ability to form ternary complex and perform endocytosis of IgG MTACs
- Receptor number is not the sole determinant of IgG MTAC uptake and protein degradation
- HepG2 cells have as many receptors as LNCaP but are deficient in TCF, uptake, and degradation
- MCF7 cells are robust in TCF assay but we did not observe uptake and degradation
- Highest M6PR number, TCF, uptake, and degradation were observed in K562, a myeloid cell line

Cell Type	Cell Origin	M6P Receptors/ Cell	Ternary Complex Formation (TCF)	Uptake and Degradation
HepG2	Liver	16,073	+/-	-
K562	Myeloid	48,595	++++	++++
MCF7	Breast	4,715	++	-
LNCaP	Prostate	18,124	++	++
HeLa	Ovary	4,020	-	NA

- Methods: M6PR/cells determined by flow cytometry. Adherent cells were incubated with Accutase to obtain a single-cell suspension. Cells were incubated with an Fc blocker, followed by addition of a PE-conjugated anti-human M6PR monoclonal antibody (Novus Biological). Cells were washed two times and 7-aminoactinomycin D was added immediately prior to flow cytometry to eliminate dead cells. Number of receptors/cell was determined by comparison to a standard curve generated using Quantibrite (BD Biosciences).

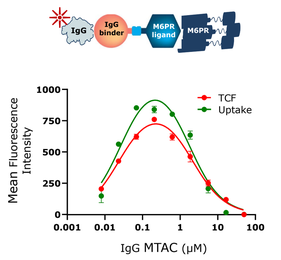
Tridentate Bifunctional Molecule – In Vitro M6PR Binding and TCF

Binding to M6PR



- Tridentate bifunctional molecule binding affinity and kinetics to M6PR determined by SPR
- K_D of IgG bif mol = 45 nM

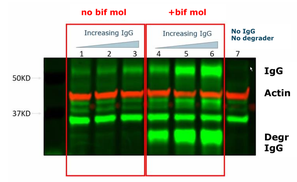
Ternary Complex Formation & Cellular Uptake



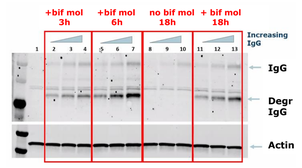
- Fluorescent measurement of labeled IgG on the surface (TCF) and inside (uptake) LNCaP cells
- TCF EC_{50} = 48 nM
- Uptake EC_{50} = 22 nM

Tridentate Bifunctional Molecule Mediates IgG Uptake and Degradation in Cells

Uptake and Degradation in K562 Cells



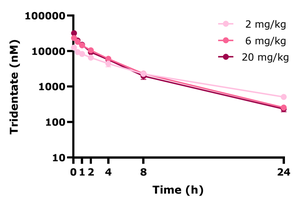
Uptake and Degradation in LNCaP Cells



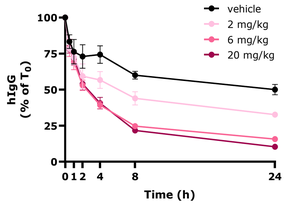
- K562 and LNCaP cell lines were selected for assessing in vitro uptake and degradation of IgG using a tridentate bifunctional molecule with M6PR ligand A
- Robust IgG uptake and degradation observed at 6 h in K562 cells and starting at 3 h in LNCaP cells
- In both cell lines, IgG uptake and degradation was mediated by tridentate bifunctional molecule
- Methods: Cells were incubated for 6 h (K562 cells) or 3-18 h (LNCaP cells) with IgG (100, 250, and 500 nM) in the presence or absence of a tridentate IgG bifunctional molecule (200, 500, and 1000 nM). Full-length IgG, IgG degradation products, and actin were detected by western blot (antibodies: rabbit anti-human IgG, mouse anti-β-actin, IRDye 800CW goat anti-rabbit IgG, IRDye 680RD goat anti-mouse IgG). Images were obtained using LiCor Odyssey Imager. Signal was quantified using Image Studio 5.0. Signals were normalized to an internal control (β-actin) and then to the 0 h time point.

Single Dose of Tridentate Bifunctional Molecule Depletes hIgG in Rat

Plasma PK



Rat % hIgG Degradation in Rat



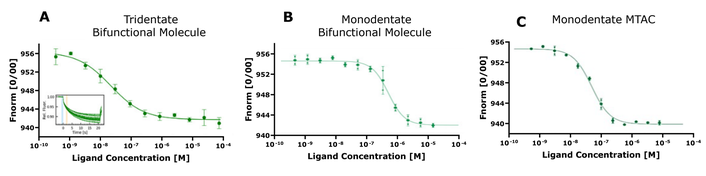
Study Design

- 1 h post IV administration of 200 mg/kg human IgG, 3 single IV doses of tridentate bifunctional molecule administered at 2, 6 or 20 mg/kg or vehicle control
- Plasma collected for 24 h for PK (by mass spectroscopy) and PD (plasma hIgG concentration by ELISA)
- Graphs represent the mean + SD of n=3 rats per group
- IgG degradation corrected by amount degraded in vehicle group at each timepoint

Study Results

- Dose dependent plasma exposure of the tridentate bifunctional molecule
- Time- and dose-dependent IgG degradation
- 2 mg/kg IV: 18% (1.4 μM) at 4 h and 16% (1.3 μM) at 8 h
- 6 mg/kg IV: 35% (2.6 μM) at 4 h and 35% (2.7 μM) at 8 h
- 20 mg/kg IV: 34% (2.1 μM) at 4 h and 38% (2.3 μM) at 8 h

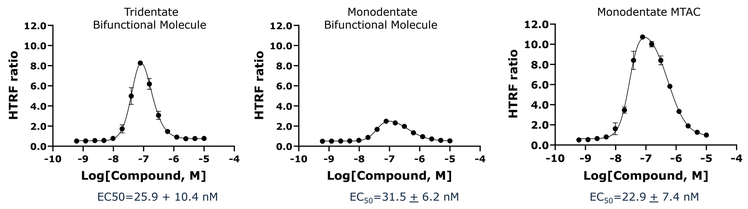
Progression from Tridentate Bifunctional Molecules to Monodentate MTACs – Binding Affinity



K_D=9.0 ± 1.4 nM | K_D=1,990 nM | K_D=106 ± 89 nM

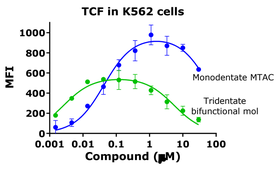
- Tridentate bifunctional molecules with M6PR ligand A potently bind M6PR (graph A) but lose affinity when synthesized as monodentates (graph B)
- Used M6PR structure-guided computational approaches to design novel M6PR ligands and monodentate IgG MTACs with increasing affinity for M6PR (graph C)
- Methods: Binding affinity to M6PR was assessed by MST (microscale thermophoresis). A His-tagged M6PR was labeled using RED-Tris-NTA. Labeled M6PR and bifunctional molecules/MTAC were incubated for 30 min at room temperature in the dark. A fluorescent change in MST signal was detected using a Monolith X. The fluorescent signal was normalized (Fnorm) and plotted as parts per thousand (0/00).

Progression from Tridentate to Novel Monodentate MTACs – TCF



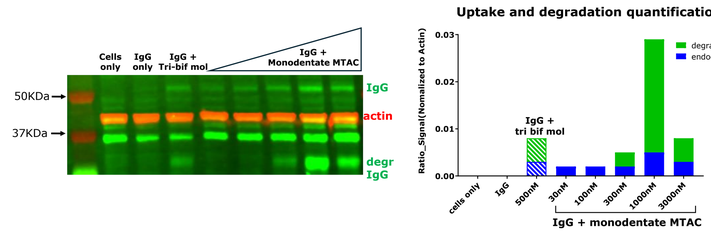
EC50=25.9 + 10.4 nM | EC_{50}=31.5 ± 6.2 nM | EC_{50}=22.9 ± 7.4 nM

- Monodentate IgG MTAC containing Avilar's novel M6PR ligand (ligand B) demonstrates robust ternary complex formation
- Monodentate IgG MTAC achieves higher and broader peak vs tridentate bifunctional molecule containing ligand A, indicating higher ternary complex stability in both HTRF and cellular TCF assays



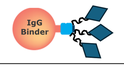
- Methods: TCF (ternary complex formation) assays identify the optimal concentration of bifunctional molecules/MTAC that bridges the formation of complex between a target (IgG) and the M6PR. TCF was assessed in HTRF biochemical assay and FACS cell-based assay. In HTRF assay, recombinant GST-tagged IgG (12.5 nM Fc) and His-tagged M6PR (150 nM Fc) were incubated with bifunctional molecules/MTAC at varying concentrations for 1 hr. Anti-His d2 (6.7nM Fc) and anti-GST-Tb (0.5 nM Fc) were added and the mixtures were incubated for 2 h. The mixtures were read on an EnVision and HTRF ratios (665 nm/615 nm) were calculated from the raw data. For cellular TCF assay, 100,000 K562 cells were dispensed to 96-well assay plates. Human IgG (Sigma) added to 1 μM final concentration to block Fc sites. After 30 min, cells were washed with ice cold PBS + 0.1% BSA. Bifunctional/MTAC compounds and AF488-labeled human IgG (100 nM Fc) were added and incubated for 1 h. AF-488 probe on the cell surface was detected by flow cytometry (iQue® Screener PLUS). Mean fluorescence intensity was graphed versus compound concentration and EC50 for TCF calculated from the inflection point of the curve (GraphPad).

Monodentate MTAC Achieves IgG Uptake & Degradation in K562 Cells



- Dose-dependent uptake and degradation observed with novel monodentate MTAC, consistent with its high-affinity M6PR binding and stable TCF properties
- Methods: K562 cells were incubated for 6 h with 300 nM IgG in presence or absence of novel mono IgG MTAC (30, 100, 300, 1,000, 3,000 nM). Tridentate bifunctional molecule was used at 500 nM as a positive control and comparison. Full length IgG and IgG degradation products were detected by western blot. K562 were incubated for 6 h with IgG (100, 250, 500 nM) in presence or absence of IgG MTAC. Full length IgG, IgG degradation products and actin were detected by western blot (antibodies: rabbit anti-human IgG, mouse anti-β-actin, IRDye 800CW goat anti-rabbit IgG, Dye 680RD goat anti-mouse IgG). Images were obtained using LiCor Odyssey Imager. Signal was quantified using Image studio 5.0. Signals were normalized to an internal control (β-actin) and then to the 0 h time point.

Avilar MTAC - Robust Degradation with Simplified Monodentate Design

	Tridentate Bifunctional	Monodentate Bifunctional	Monodentate MTAC
			
M6PR Ligand	**A**	**A**	**B**
M6PR Ligand Affinity to M6PR (nM)	**2,600**	**2,600**	**20**
Bifunctional Molecule Affinity to M6PR (nM)	**9**	**1,990**	**280**
Valency	**Tridentate**	**Monodentate**	**Monodentate**
Ternary Complex Formation	**Moderate**	**Weak**	**Strong**
Uptake/Degradation	**Moderate**	**N/A**	**Strong**

- Discovery of novel M6PR ligands enabled synthesis of monodentate MTACs with robust TCF and uptake/degradation with lower MW compared to tridentate bifunctional molecules

Summary

- Avilar invented novel M6PR ligands with higher affinity vs previously described ligands
- Synthesized monodentate MTACs containing these novel high affinity M6PR ligands
- Demonstrated efficient TCF, uptake, and in vitro degradation with Avilar's monodentate MTACs
- These studies provide PoC for MTACs, which offer the potential for effective M6PR-mediated protein degradation using streamlined lower molecular weight designs

References

1. Protein Atlas: https://www.proteinatlas.org/ENSG00000197081-IGF2R