ASGPR-Targeting Chimeras (ATACs): A New Class of Degraders for Targeting Extracellular Proteins

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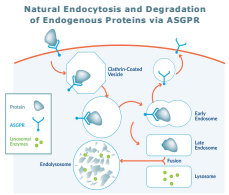
Abstract

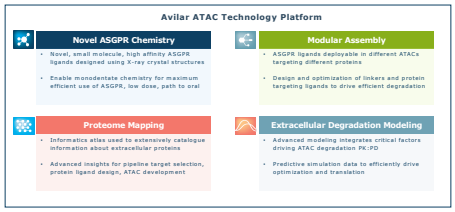
Targeted protein degradation is a promising new therapeutic modality that enables the removal of disease-causing proteins. First-generation protein degradation technologies have utilized the ubiquitin proteasome system to successfully degrade intracellular proteins. Recently, a new and attractive approach has emerged that enables the endolysosomal degradation of extracellular proteins using the asialoglycoprotein receptor (ASGPR), an endocytotic receptor expressed predominantly on the surface of hepatocytes. Various endogenous circulating extracellular glycoproteins are internalized via clathrin-mediated endocytosis and then degraded in the hepatocyte endolysosome. We describe herein the development of a novel ASGPR-targeting chimera (ATAC) platform using bifunctional compounds containing Avilar's novel, potent, small-molecule ASGPR-binding ligands. For initial proof-of-concept studies, ATACs were designed designed with Avilar's proprietary technology to target two extracellular proteins with different concentration and kinetic properties: one with high plasma concentration and a long half-life, the other with low plasma concentration and short half-life. In vitro characterization of the ATAC interactions with ASGPR and the target proteins, including binding, cellular uptake, and degradation via the endolysosomal pathway will be presented.

ASGPR Key Role in Body's Natural Cellular Degradation Machinery

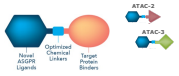
- ASGPR offers natural cellular machinery for extracellular degradation, analogous to E3 ligases in intracellular degradation
- Cell surface receptor mediates the endocytosis and degradation of various endogenous glycoproteins in endolysosome
- Highly expressed on hepatocytes (~1M receptors per cell in humans)
- Endocytosed and recycled from endosome back to plasma membrane every ~15 minutes

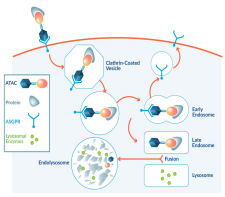


Proprietary Technology Platform to Design and Build ATACs



ATACs Harness ASGPR Pathway to Degrade Extracellular Proteins

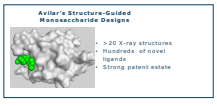




- Bi-functional molecules comprising ASGPR binder, optimized linker, and binder to a target protein
- Shuttle target protein from circulation to endolysosome for degradation
- Modular: optimized ASGPR binders and linkers deployed in synthesis of ATACs with diverse protein targeting binders
- New class of degrader and basis for transformative first-in-class medicines

Proprietary ASGPR Ligands with Significantly Improved Affinity

Novel high affinity monosaccharides superior to historical GalNAc analogs

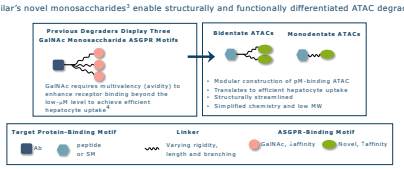


Compound ID	GalNAc	Bicyclic Bridged Ketal[2]	AVI-1	AVI-2	AVI-3
ASGPR SPR Binding: K_D (nM)	52,800	1,650	720	210	24
Increase in Affinity (X Fold)	1	32	73	251	2200

- Synthesized hundreds of monosaccharide ligands[3]
  - Many with $K_D \leq 1{,}000$ nM or even with $K_D \leq 100$ nM
- Determined >20 X-ray structures of ASGPR/ligand complexes
- Up to ~2,000-fold increase vs. GalNAc & >60-fold increase vs. bicyclic bridged ketal

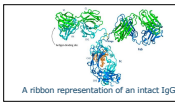
Novel ASGPR Ligand Chemistry Enables Modular Design of ATACs

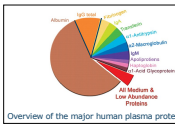
Avilar's novel monosaccharides[3] enable structurally and functionally differentiated ATAC degraders



ATAC Platform PoC Using Immunoglobulin G (IgG), a High Plasma Concentration and Long Half-Life Protein

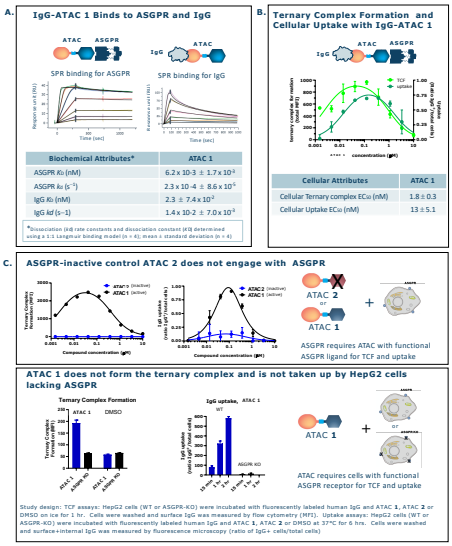
- To exemplify our ATAC platform, we designed ATAC molecules to target and degrade IgG, a high plasma concentration and long half-life extracellular protein
- IgG is a ~146 kDa antibody and it is the major class of immunoglobulins
- IgG binds to cell surface receptors on many types of cells to trigger phagocytosis or antibody-dependent cellular cytotoxicity[5a]
- IgG is the second most abundantly available and most common type of antibody found in blood
- IgG concentration[5b]
  - 1.06 g/kg total body IgG = 74.2 g total in 70 kg human = 508 μmol
- Resynthesis properties
  - $t_{1/2}$: 21 days
  - 32 mg/kg/day = 2.2 g/subject/day = 15 μmol/day
  - ~3% of total body IgG


A ribbon representation of an intact IgG[5a]



Overview of the major human plasma proteins[6b]

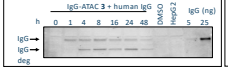
ATAC **1** Binds to IgG and ASGPR and Depends on ASGPR for Activity



IgG-ATACs Facilitate IgG Degradation in HepG2 Cells

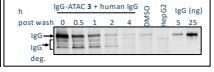
ATAC-dependent IgG uptake results in IgG degradation

- Continuous uptake of ATAC **3** + IgG for up to 48h
- Lyse cells and detect full length IgG and IgG degradation products by immunoblot



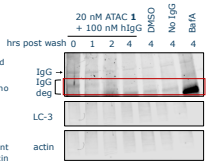
Degradation of endocytosed IgG-ATAC complexes is fast

- Uptake of ATAC **3** + IgG for 1h
- Wash cells, lyse after 30 min to 4h
- Detect full length IgG and IgG degradation products by immunoblot



IgG Degradation in Rat Hepatocytes Requires Lysosomal Function

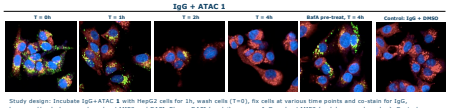
- IgG is taken up into hepatocytes for 1h (T-0) and then degradation is monitored over time
- Uptake into hepatocytes requires IgG-ATAC (no uptake with DMSO)
- IgG taken up in 1h (T=0) requires ~4h to be degraded. Both full length IgG and degradation product (red box) levels decrease over time
- As was observed in HepG2 cells, pre-treatment with the lysosomal function inhibitor Bafilomycin A blocks IgG degradation (no IgG fragment)
- IgG degradation kinetics consistent in HepG2 cells and rat hepatocytes.



Study design: ATAC **1** and hIgG are incubated with rat hepatocytes for 1h, cells are washed to remove excess IgG and further incubated for 0-4h, cells are then lysed, and IgG is detected by Western Blot with anti-IgG antibodies. Data is representative of 2 separate experiments in primary rat hepatocytes

IgG Intracellular Localization Following ATAC-Mediated Uptake

- IgG progresses through the endocytotic pathway to the endo-lysosome; colocalization of IgG (green) with endolysosome (red), starts at 1h (yellow)
- Degradation is complete by 4h (no yellow staining present) and depends on lysosomal function; in presence of BafA IgG (green stain) never enters the endolysosome (no yellow staining)



Study design: Incubate IgG+ATAC **1** with HepG2 cells for 1h, wash cells (T=0), fix cells at various time points and co-stain for IgG, lysosomal/endo-lysosomal marker LAMP2 and DAPI. Blue = DAPI (nuclei), green = IgG, red = LAMP2 (endolysosomal marker). Controls: HepG2 cells pre-treated with Bafilomycin A (lysosomal function inhibitor), HepG2 cells incubated with IgG and no compound (IgG+DMSO)

ATAC Platform PoC Using Tumor Necrosis Factor alpha (TNFα), a Low Plasma Concentration and Short Half-Life Protein

- To exemplify our ATAC platform, we designed ATAC molecules to target and degrade TNFα, a low plasma concentration and short half-life extracellular protein
- TNFα is a homotrimeric cytokine that binds two receptors, TNFR1 and TNFR2
- Engagement of TNFα with TNFR1 and TNFR2 initiates signaling cascades that result in inflammatory responses and control of apoptosis
- Soluble TNFα MW: 17 KDa, and assembles as a 51 KDa trimer
- Concentration: 2-20 pg/mL in healthy people and up to 5000 pg/mL in sepsis patients' serum[7a]

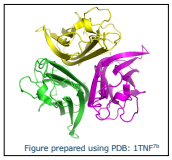
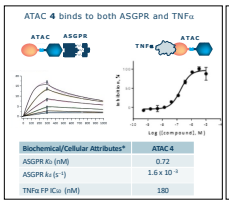
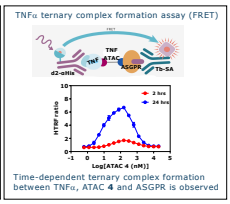


Figure prepared using PDB: 1TNF[7b]

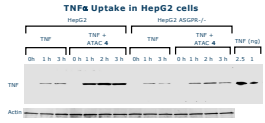
Confirmation of TNFα ATAC Binding and Characterization of Binary Complex





ATAC and ASGPR Mediated TNFα Uptake by ATAC **4** in HepG2 Cells

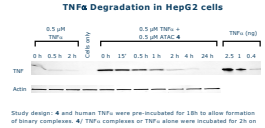
- Uptake of TNFα mediated by ATAC **4** is observed in HepG2 cells
- Low-level background TNFα uptake by cells presumably due to endogenous TNFR binding
- Significant uptake of TNFα requires the presence of an ATAC
- ASGPR is required to achieve significant ATAC-medicated TNFα uptake in HepG2 cells (no uptake in ASGPR knock-out cells)



Study design: 0.5 μM ATAC **4** and 0.5 μM human TNFα were pre-incubated for 18h to allow formation of stable complexes. ATAC **4**/ TNFα complexes or TNFα alone were incubated for 0-3h on HepG2 cells. Cells were washed and lysed for Western blot analysis. TNFα and actin were detected with anti-TNFα and anti-actin antibodies, respectively, and 800cw-conjugated secondary antibody.

ATAC-Mediated TNFα Degradation by ATAC **4** in HepG2 Cells

- Degradation of TNFα requires the presence of ATAC **4**
- Background TNFα internalization by cells presumably due to endogenous TNFR binding
- Half-life of TNFα degradation upon uptake by ASGPR is 15-30 min
- TNFα is fully degraded after 4h
- ATAC **4** and TNFα pre-incubated to allow formation of complex



Study design: **4** and human TNFα were pre-incubated for 18h to allow formation of binary complexes. **4**/ TNFα complexes or TNFα alone were incubated for 2h on HepG2 cells. Cells were washed, incubated with cell media without reagents for 0-24h and subsequently washed and lysed for Western blot analysis. TNFα and actin were detected with anti-TNFα and anti-actin antibodies, respectively, and 800cw-conjugated secondary antibody.

Summary

- Avilar created a library of proprietary, small molecule, high affinity ASGPR ligands
- We combined proprietary ASGPR ligands with IgG and TNFα ligands and linkers in modular manner to optimize ATAC functionality
- To exemplify our ATAC platform, we designed ATAC molecules to target and degrade two extracellular proteins with different concentration and kinetic properties: one with high plasma concentration and a long half-life, the other with low plasma concentration and short half-life
- As an ATAC platform proof of concept, we demonstrated in vitro ligand binding, ternary complex formation, cellular target uptake, and target degradation in liver cells for IgG and TNFα

Acknowledgements

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