Targeted Protein Internalization and Degradation by ENDoosome TArgeting Chimeras (ENDTACs)

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Supporting Information

ABSTRACT: Targeted protein degradation has generated excitement in chemical biology and drug discovery throughout academia and industry. By hijacking the machinery responsible for protein degradation via the ubiquitin proteasome system (UPS), various cellular targets have been selectively degraded. However, since the tools used, often termed PROteolysis TArgeting Chimeras (PROTACs), hijack the intracellular quality control machinery, this technology can only access targets within the cell. Extracellular targets such as growth factors, cytokines, and chemokines bind to cell surface receptors, often initiating aberrant signaling in multiple diseases such as cancer and inflammation. However, efforts to develop small molecule inhibitors for these extracellular target proteins have been challenging. Herein, we developed a proof-of-concept approach to evaluate if extracellular proteins can be internalized and degraded via the receptor-mediated endolysosomal pathway. Using a heterodimeric molecule, termed “ENDoosome TArgeting Chimera” (ENDTAC), internalization and degradation of an extracellular recombinant eGFP-HT7 fusion protein was achieved by hijacking the decoy GPCR receptor, CXCR7. This proof-of-concept study suggests that using ENDTACs to co-opt the endosomal–lysosomal degradation pathway, in contrast to PROTACs using the UPS, may provide an avenue for degrading extracellular targets such as cytokines. Overall, the technology described herein provides a novel expansion to the field of targeted protein degradation.

INTRODUCTION

Traditional drug development efforts are focused mainly on small molecules that target druggable protein classes such as enzymes and receptors. The majority of drugs operate as inhibitors of protein function; however, because this mode of action utilizes a target occupancy paradigm requiring high drug concentrations to sustain the biological response, it also can lead to undesirable off-target effects. As an alternative, PROteolysis TArgeting Chimeras (PROTACs) hold great promise as a therapeutic modality since they require only a transient interaction with the target protein to promote its degradation. However, despite promoting the degradation of various proteins, PROTACs are limited to target engagement within the intracellular space for ubiquitination. PROTACs are therefore unable to act on secreted proteins, such as cytokines and chemokines, which exert their biological activity from the extracellular space. These proteins bind to cell surface receptors and can initiate the aberrant signaling implicated in multiple diseases (Figure 1A). While monoclonal antibodies can target secreted proteins or their cognate receptors to block signaling, efforts to develop small molecule inhibitors for secreted proteins have so far been less successful. Given the functional importance of secreted proteins in diseases and the current limitations on inhibiting their activities, alternative technologies to efficiently target them are needed. Receptor–ligand-mediated delivery systems have gained significant attention in the past few years. Several studies have reported that cell surface receptors such as transferrin receptor, asialoglycoprotein (ASGPr), and folate receptor can be used to selectively deliver a wide range of therapeutic agents into cancer cells via receptor-mediated endocytosis. In addition, a recent study has shown that Cas9 could be selectively delivered into cells by harnessing the receptor–ligand interactions followed by endosomal escape. Here we report a new approach to potentially target extracellular proteins for receptor-facilitated lysosomal degradation using chimeric molecules termed ENDoosome TArgeting Chimeras (ENDTACs) (Figure 1B). An ENDTAC is a heterodimeric molecule consisting of a small molecule (agonist) that binds to a plasma membrane-localized receptor of interest (e.g., a GPCR) while the other end, connected via a linker, of the ENDTAC binds and
recruits the extracellular protein of interest (POI) (e.g., cytokine) (Figure 1C). Upon binding to the GPCR, the tethered extracellular protein can undergo receptor-mediated endocytosis and subsequently degradation by the lysosome (Figure 1B).

**RESULTS AND DISCUSSION**

To validate the feasibility of the ENDTAC approach, we performed a proof-of-concept study using the decoy receptor, CXCR7 (ACKR3), as the cell surface receptor and an engineered HA-eGFP-HaloTag7 (eGFP-HT7) fusion protein.
as the extracellular target protein. The GPCR CXCR7 is constitutively endocytosed at a low level to degrade its cognate chemokines via transport to the lysosome. A previous report has shown that two potent small molecule agonists VUF11207 and VUF11403 induce CXCR7 internalization (Figure S1). Since both these 3,4-dimethoxy and 3,4,5-trimethoxy styrene amides (VUF11403 and VUF11207, respectively) exhibit similar agonist activity in various pairwise comparisons, we incorporated the HT7-recruiting chloroalkane at their 5-position (Figure 2A). Using a facile synthetic method (Scheme S1, in the Supporting Information), linkers ranging from a diethylene glycol to pentaethylene glycol were incorporated in both series (R1: H or OMe) to afford ENDTACs-1−8 (Figure 2A). Agonist activity for ENDTACs-1−8 was evaluated by the Tango GPCR assay (Table 1). Interestingly, the dimethoxy-containing ENDTAC series (ENDTACs-1−4; R1, OMe; R2, H) displayed only slightly reduced potency compared to the parent warhead VUF11207 (Figure 2B; Table 1; left EC50 column), while the monomethoxy-containing ENDTACs (ENDTACs-5−8; R1, H; R2, H) exhibited greater than 10- to 40-fold reduction in activity compared to the respective warhead, VUF11403 (Figure S1 and Table 1). Accordingly, we focused on the dimethoxy-containing ENDTAC series and measured the ability of the prereacted ENDTAC-eGFP-HT7 complex to activate the CXCR7 receptor. Purified eGFP-HT7 was used as the reporter POI (Figures S2 and S3) to evaluate ENDTACs-1−4. The reaction between purified eGFP-HT7 and ENDTACs was confirmed by LC-MS analysis (Figures S4 and S5 and Table S1).

Among these four ENDTACs, ENDTAC-1 retained a similar potency compared to VUF11207 after reacting with eGFP-HT7 (Table 1). On the basis of this initial activity profile, we selected ENDTAC-1 for use in subsequent experiments. To ensure that eGFP-HT7 internalization is mediated via CXCR7 activity, we synthesized a negative control molecule, ENDTAC-neg, using a similar synthetic approach (Figure 2A; Scheme S2). The para-methoxy group on the styrene ring (R1, H; and R2, OMe) of ENDTAC-neg was previously identified to abrogate CXCR7 agonist activity. Therefore, ENDTAC-neg was evaluated for activity in the absence or presence of eGFP-HT7. According to Tango assay data, prereacted ENDTAC-neg-eGFP-HT7 did not show any CXCR7 activation compared to ENDTAC-1-eGFP-HT7, supporting the use of ENDTAC-neg as a negative control. (Figure 2C and Figure S6).

### Table 1. EC50 Values of VUF11207 and CXCR7-Recruiting ENDTACs As Determined in the Tango Assay

| entry | compound ID | n | EC50 (nM) compound alone | EC50 (nM) compound + eGFP-HT7 | EC50 (nM) compound + Nanoluc-HT7 |
|-------|-------------|---|-------------------------|-------------------------------|---------------------------------|
| 1     | VUF11207    | N/A | 68 ± 1.2                | 169 ± 1.5                     | 146 ± 1.3                      |
| 2     | ENDTAC-1    | 2  | 113 ± 1.3               | 251 ± 1.5                     |                                |
| 3     | ENDTAC-2    | 3  | 111 ± 1.2               |                                |                                |
| 4     | ENDTAC-3    | 4  | 145 ± 1.1               | 373 ± 2.0                     |                                |
| 5     | ENDTAC-4    | 5  | 158 ± 1.2               | 287 ± 3.0                     |                                |
| 6     | VUF11403    | N/A | 76 ± 1.3                |                                |                                |
| 7     | ENDTAC-5    | 2  | 1360 ± 3.8              | ND                            |                                |
| 8     | ENDTAC-6    | 3  | 2453 ± 1.1              | ND                            |                                |
| 9     | ENDTAC-7    | 4  | 4705 ± 5.2              | ND                            |                                |
| 10    | ENDTAC-8    | 5  | 1815 ± 1.4              | ND                            |                                |
| 11    | ENDTAC-neg  | 2  | ND                      | ND                            |                                |

"ENDTACs (1−4) contain OMe at the R1 position and H at the R2 position whereas ENDTACs (5−8) contain H at both R1 and R2 positions. These ENDTACs are generated by adding a chloroalkane to VUF11207 or VUF11403 via varying linker lengths. The ENDTAC-neg structure is slightly different than ENDTACs-1−4 where R1 is OMe and R2 is OMe. VUF11207 and VUF11403 are known small molecule agonists of CXCR7. eGFP-HT7 is HA-eGFP-Halotag7 protein, and Nanoluc-HT7 is a HA-nanoluciferase-Halotag7 fusion protein. ND: not determined. All data represent mean ± SEM. **N/A: not applicable."

Furthermore, we also analyzed the saturation kinetics of the prereacted ENDTAC-1-eGFP-HT7 complex using the Tango assay. The warhead VUF11207 and ENDTAC-1 show fast saturation kinetics, whereas ENDTAC-neg-eGFP-HT7 reaches saturation within 4 h (Figure 2D). Given the similar agonistic activities of the warhead and corresponding ENDTAC, as well as favorable binding kinetics, we next proceeded to study ENDTAC-induced internalization of eGFP-HT7 protein.

CXCR7-expressing MCF7 cells were treated for 4 h with either ENDTAC-1 or ENDTAC-neg conjugated to purified eGFP-HT7 (10 μM), and internalization was monitored by confocal microscopy. Internalized eGFP-HT7 was visualized as GFP-positive puncta (green), and we observed a greater uptake of eGFP-HT7 in the presence of 10 μM ENDTAC-1 after 4 h (Figure 3A). The quantitation of GFP-positive cells suggests ENDTAC-1 induced internalization of eGFP-HT7, as compared to ENDTAC-neg (Figure 3B). We also analyzed eGFP-HT7 internalization by immunostaining with HA antibody and observed an overlap between the GFP and HA puncta in the presence of ENDTAC-1 (Figure S7). eGFP-HT7 uptake was confirmed as being ENDTAC-dependent in CXCR7-expressing MCF7/7 and MIA PaCa-2 cells, where greater uptake is observed in the presence of 500 nM ENDTAC-1, compared to ENDTAC-neg, via immunoblotting (Figure 3C). To corroborate this finding, we used purified Nanoluc-HT7 (Figures S2 and S3) as the extracellular POI and evaluated Nanoluc-HT7 uptake by measuring luciferase activity in MCF7 and HTLA cells (Figure 3D and Figure S8). As was observed with the ENDTAC-1-eGFP-HT7 adduct, ENDTAC-1 retained CXCR7 agonistic activity after reacting with Nanoluc-HT7 (Table 1). We first incubated prereacted Nanoluc-HT7 + ENDTAC-1 (1 μM) with MCF7 cells for 2 h and then changed to ENDTAC-free media and assayed for Nanoluc-HT7 uptake over 24 h. Consistent with previous data, we observed a 2.5-fold uptake of Nanoluc-HT7 in the presence of ENDTAC-1 compared to ENDTAC-neg in the first 2−6 h (Figure 3D, Figure S8). In both MCF7 and HTLA cells, there is an observed reduction in Nanoluc-HT7 activity at 24 h, suggesting that internalized protein has been degraded in the lysosome.

Given the possibility that bulk endocytosis could lead to nonspecific uptake of proteins, we performed a ligand competition assay with excess CXCR7 agonist VUF11207 to...
probe that the ENDTAC-1-mediated HT7 uptake is CXCR7-dependent. We incubated MCF7 cells with DMSO:eGFP-HT7, ENDTAC-neg:eGFP-HT7, and ENDTAC-1:eGFP-HT7 in the absence or presence of excess warhead (VUF11207). The ENDTAC-1-mediated HT7 uptake was completely inhibited in the presence of the excess warhead (Figure 3E), suggesting that the ternary complex formation between eGFP-HT7, CXCR7, and EEA1 is required to facilitate selective internalization of eGFP-HT7.

We next sought to determine whether the level of CXCR7 expression could play a key role in the efficiency of the ENDTAC system. To probe the hypothesis, we first compared the uptake of eGFP-HT7 in CXCR7-overexpressing 293T cells. Upon incubation of ENDTAC-1:eGFP-HT7 with 293T cells, we observed an ENDTAC-1-dependent uptake of eGFP-HT7 compared to nontransfected cells (Figure S9A), suggesting that increased expression of CXCR7 enhances the ENDTAC-1-mediated internalization of HT7. To further support this result, we compared the ENDTAC-1-dependent cellular uptake in MDA-MB-231 cells (CXCR7-negative) and MCF7 cells (CXCR7-positive). Compared to MDA-MB-231 cells, we observed an increased eGFP-HT7 uptake in ENDTAC-1-treated MCF7 cells (Figure S9B), suggesting that the level of CXCR7 surface expression is a key parameter that dictates ENDTAC efficiency.

Upon receptor-mediated endocytosis, we propose that the eGFP-HT7 protein traffics sequentially to the early endosome and the late endosome and subsequently is degraded in the lysosome. To evaluate this hypothesis, we treated MCF7 and MIA PaCa-2 cells with ENDTAC-1:eGFP-HT7 or ENDTAC-neg:eGFP-HT7 for 4 h, washed the cells, replaced the medium with fresh medium, and cultured the cells for another 3 h, to monitor the fate of internalized eGFP-HT7. We observed eGFP-HT7 uptake by the cells after a continuous 4 h ENDTAC-1:eGFP-HT7 incubation, followed by the disappearance of eGFP-HT7 after a 3 h chase (Figure 4A,B). Therefore, it is possible that internalized eGFP-HT7 protein traffics to the lysosome and is therein degraded, given the mechanism of CXCR7 internalization following agonist treatment. To evaluate if lysosomal-mediated eGFP-HT7 degradation occurs, we incubated cells with ENDTAC-neg:eGFP-HT7 or ENDTAC-1:eGFP-HT7 for 4 h, washed the cells, and cultured them in the absence or presence of the lysosome inhibitor, bafilomycin A1 (baA1). Interestingly, eGFP-HT7 is degraded only in the absence of baA1, suggesting that eGFP-HT7 degradation indeed occurs via the lysosomal pathway (Figure 4C,D).

To further confirm endolysosomal accumulation of eGFP-HT7 in the presence of ENDTAC, we examined the uptake and degradation of eGFP-HT7 in MCF7 cells by confocal microscopy. MCF7 cells were treated with either Alexa Fluor 488-conjugated transferrin (TFN488), a positive control for endocytosis, or the ENDTAC-1/ENDTAC-neg:eGFP-HT7 conjugate (10 μM) for 4 h, fixed, and analyzed by confocal microscopy. Interestingly, as similarly observed with TFN488 treatment, eGFP-HT7-positive cells were observed in the presence of ENDTAC-1:eGFP-HT7 (Figure 5A–C). These eGFP-HT7 foci colocalize with the early endosome marker (EEA1) suggesting that internalized eGFP-HT7 traffics to the endosome (Figure 5, panel C). Furthermore, we also observed that eGFP-HT7-positive foci partially colocalize with the lysosome marker (LysoTracker), suggesting that ENDTAC-1 promotes receptor-mediated uptake and lysosomal degradation of eGFP-HT7 protein (Figure 5D,E).

While PROTACs are widely used in the field to target many intracellular proteins, a key limitation is their inability to target extracellular proteins. Here we show for the first time that chimeric molecules, which we designate as "ENDTACs", are capable of recruiting an extracellular protein (eGFP-HT7), for internalization and lysosomal degradation by hijacking a GPCR-mediated endocytosis pathway. Although we present a proof-of-concept approach using a covalent HaloTag-based system, we anticipate that the ENDTAC technology could be further optimized and modified for a noncovalent setting. In summary, the ENDTAC technology represents a new chemical biology tool to study extracellular proteins and has the potential for depleting disease-causing extracellular proteins in the future.
Figure 4. Cellular uptake and lysosomal degradation of eGFP-HT7 in the presence of ENDTAC-1. (A) Immunoblot analysis of uptake and fate of internalized eGFP-HT7 after 4 h of incubation followed by a 3 h chase in ENDTAC-neg/ENDTAC-1:eGFP-HT7-free medium in MCF7 (n = 4) and (B) MIA PaCa-2 cells (n = 3). (C) MCF7 or (D) MIA PaCa-2 cells were incubated with ENDTAC-neg/ENDTAC-1:eGFP-HT7 (500 nM) for 4 h, released to ENDTAC-free medium, and chased for an additional 3 h in the absence or presence of bafilomycin A1 (bafA1) (100 nM) (n = 3).

Figure 5. Endolysosomal trafficking of eGFP-HT7 in the presence of ENDTAC-1. Confocal microscopy analysis of (A) AF488-conjugated transferrin (TFN488) was used as a positive control for endocytosis. (B–E) Trafficking of internalized eGFP-HT7 (10 μM) to the endolysosome compartment after 4 h. (B, C) EEA1, early endosome marker, and (D, E) LysoTracker, lysosome marker. Red, EEA1 and LysoTracker; green, eGFP-HT7; yellow, merged images; blue, Hoechst stain for nuclei. Scale bar: 5 μm.

ASSOCIATED CONTENT

Supporting Information
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Experimental procedures and synthesis and characterization of ENDTACs (PDF)

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