Heterobifunctional Molecules Induce Dephosphorylation of Kinases—A Proof of Concept Study

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Supporting Information

ABSTRACT: Heterobifunctional molecules have proven powerful tools to induce ligase-dependent ubiquitination of target proteins. We describe here a chemical strategy for controlling a different post-translational modification (PTM): phosphorylation. Heterobifunctional molecules were designed to promote the proximity of a protein phosphatase (PP1) to protein targets. The synthesized molecules induced the PP1-dependent dephosphorylation of AKT and EGFR. To our knowledge, this work represents the first examples of small molecules recruiting non-native partners to induce removal of a PTM.

INTRODUCTION

Post-translational modification (PTM) effectively expands nature’s genetic code. It is estimated that nearly 5% of the human proteome consists of enzymes responsible for addition or removal of PTMs: chemical modifiers such as ubiquitin, phosphate, lipids, and glycans which can influence the localization, activity, and stability of their conjugated proteins. It is well recognized that the ability to selectively influence PTMs of a target protein may be an effective means to modulate its function. Recently, it has been shown that one class of post-translational enzymes, E3 ubiquitin ligases, can be co-opted in a novel approach to promote the degradation of target proteins of interest (POIs). In this strategy, heterobifunctional small molecules (sometimes referred to as “proteolysis targeting chimeras”) incorporating target-binding and ligase binding ends are hypothesized to promote the proximity of specific ligases to POIs promoting POI ubiquitination, subsequent recognition by the proteasome, and degradation (Figure 1a). These catalysts have been shown to be effective chemical tools to induce the degradation of members of several target classes including bromo-domains, kinases, and nuclear hormone receptors. Inspired by this groundbreaking work, we envisioned that heterobifunctional molecule approaches may be useful to influence some of the other >200 types of PTMs (beyond ubiquitination). Considering diverse PTMs can drive a broad variety of cellular processes, we envision such strategies could

Figure 1. (a) Heterobifunctional molecule containing an E3 ubiquitin ligase and a POI binder (sometimes referred to as a “proteolysis targeting chimera”). (b) Heterobifunctional molecule containing a protein phosphatase and POI binder. (c) Design to provide proximity between POI and protein phosphatase, promoting POI dephosphorylation. POI = protein of interest; PP = protein phosphatase; P = phosphate.
provide flexible and versatile tools to fine-tune cellular features of POIs as well as lead to therapeutically relevant agents.

Phosphorylation/dephosphorylation of serine, threonine, and tyrosine residues of proteins, elicited by kinases and phosphatases, respectively, serves to regulate many cellular processes. Inclusion of this small and polar functionality can lead to large conformational changes that alter protein function. We became interested in the design of compounds that could promote the selective dephosphorylation of POIs. We hypothesized heterobifunctional molecules comprised of a POI-binding moiety linked to a protein phosphatase (PP) binding moiety would recruit a phosphatase to, and promote dephosphorylation of, target proteins (Figure 1b,c). Herein, we report our studies directed at promoting protein–phosphatase 1 (PP1) recruitment to dephosphorylate oncogenic kinases AKT (protein kinase B) and EGFR (epidermal growth factor receptor).

RESULTS AND DISCUSSION

Promoting dephosphorylation of kinases is an attractive strategy for pharmacological intervention as many kinases have activity that is regulated by phosphorylation state. In classic examples, phosphorylation of the A-loop (and other motifs) increases enzymatic activity by conformational changes that lead to improved substrate binding, cellular localization, or copartner recruitment. Extracellular growth signals are propagated by kinases phosphorylating, and thus in many cases activating, downstream kinases. Inducing dephosphorylation of a pathway kinase would thus simultaneously inhibit the kinase’s ability to receive and transmit an upstream signal. The PI3K/AKT pathway is often overactivated in cancer cells, having a central role in cell proliferation and survival. AKT (protein kinase B) and EGFR (epidermal growth factor receptor) have activity that is regulated by phosphorylation state. In

Figure 2. 1 decreases pAKT in the cells expressing HaloTag-PP1-FLAG only. HaloTag-PP1-FLAG was transfected in LNCaP over 24 h. (a) Structure of HaloTag-reactive AKT inhibitor. (b) Immunoblot analysis of AKT phosphorylation after 8 h treatment of LNCaP cells with 10 μM 1. (c) Quantification of pAKT levels after normalization over total AKT in LNCaP, MCF7/neo HER2, and PC-3 cells after treatment with 10 μM 1 for 8 h. NS, P > 0.05; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

Figure 3. Allosteric AKT inhibitor-PDP1 decreased pAKT levels in the presence of PP1. (a) Structures of allosteric AKT inhibitor (2, AKTI) and AKTI-PDP1 conjugate (3). (b) pAKT levels after 1 h incubation of PP1, pAKT, and 2 or 3 in the presence of I2. (c) Competition assay with unconjugated PDP1 (10 μM).
inhibition through competition by the PP1-binding peptide (PDP1).\textsuperscript{20} We next observed that moderately decreased pAKT\textsuperscript{T308} levels in a concentration-dependent manner, which is consistent with the finding that the AKT inhibitor used in this experiment stabilizes the conformation susceptible to phosphatase-mediated dephosphorylation (Figure 3b).\textsuperscript{22} 3 induced greater reduction in pAKT\textsuperscript{T308} levels, compared to free AKT inhibitor 2 treatment. Additionally, 3 induced the lowest level of pAKT at the concentration of 2.5 μM and higher concentration of 3 had slightly less effects on pAKT. This modest trend toward a bell-shaped dose response is consistent with the expected hook/Prozone effect in a mechanism where ternary complex formation is necessary (Figure 3b, SI, Figure S2).\textsuperscript{23} We next demonstrated rescue of the dephosphorylation phenotype by competition with an excess amount of free PP1-binding peptide (Figure 3c). Taken together, these data indicate proximity of pAKT and PP1, promoted by a small molecule, is sufficient to promote dephosphorylation.

Molecule 3 was tested for influence on pAKT levels in LNCaP cells. Somewhat unsurprisingly, there was no obvious change in pAKT levels at concentrations up to 10 μM (SI, Figure S3). We hypothesize this lack of phenotype is a result of insufficient cellular permeability or potential proteolytic instability. We therefore decided to explore the shortest reported PP1 peptide to facilitate drug permeabilization and mitigate metabolic instability. Chamberlin and Tappan identified tetrapeptide sequence (RVSF) to be a minimum requirement for PP1 binding and activation in vitro.\textsuperscript{16} We synthesized heterobifunctional molecules with both allosteric\textsuperscript{16,21} and ATP-competitive\textsuperscript{24} AKT inhibitors that included either active PP1-binding RVSF or inactive RVSA (4a/b, 5a/b, N-Ac-Cys included to enable synthesis, Figure 4). Previous work demonstrated mutation of RVSE to RVSA completely abolishes the binding to PP1,\textsuperscript{18} so we incorporated the latter as a properties-matched negative control. Solvent exposed vectors for the PP1 peptides and AKT inhibitors were clear from analysis of published X-ray structures and SAR, and linkers were designed to be sufficient in length as to allow simultaneous binding of both partners.\textsuperscript{16,21,24}

4a and 4b include an allosteric AKT binder and RVSF or RVSA sequences, respectively. At a concentration of 10 μM over 8 h, 4a decreased the level of pAKT\textsuperscript{T308} and pAKT\textsuperscript{T473} in LNCaP cells whereas 4b (containing the PP1-inactive RVSA peptide) did not (Figure 5a,b). Importantly, cotreatment of 4b with free CRVSF 6 did not result in significant decreases in pAKT, suggesting dual binding of the heterobifunctional molecule is required for enhanced dephosphorylation. The difference in phenotype between 4a and 4b for pAKT\textsuperscript{T308} was consistent at higher concentration (LNCaP, Figure 5b, pAKT\textsuperscript{T473} displayed in SI, Figure S4) as well as in additional cell lines (MCF7/neu-HER2, PC-3, Figure 5c,d). We monitored the time course of dephosphorylation, and maximal effect was observed at 6–8 h (SI, Figure S5). The dephosphorylation phenotype of 4a was substantially rescued by depletion of PP1 by transfected siRNA (Figure 5e,f). Consistent with the mechanism of action of 4a, levels of downstream pS6\textsuperscript{2813} were substantially decreased relative to 4b (Figure 5g).

5a and 5b include an ATP competitive inhibitor. ATP-competitive AKT inhibitors are known to promote an increase
pAKT<sub>T308</sub> and pAKT<sub>S473</sub> by a mechanism that protects from dephosphorylation by protein phosphatase 2A (PP2A). At 10 μM test concentrations, 5a demonstrated a modest reduction in pAKT<sub>T308</sub> and pAKT<sub>S473</sub> compared to 5b alone or 5b in combination with RVSF 6 (Figure 6a,b). This result reached significance (P ≤ 0.01) at 50 μM test concentrations. Notably, relative to DMSO controls, 5a protected against the typical increase in pAKT observed for ATP-competitive AKT inhibitors (SI, Figure S6).

We next turned to demonstrate applicability to a second kinase, EGFR. EGFR has five autophosphorylation sites in its C-terminal tail, and phosphorylation is necessary for its maximal activity. EGFR inhibitor AZD-9291 (Tagrisso) was modified to include a HaloTag reactive chloroalkane (7, Figure 7) and dosed to HaloTag-PP1-FLAG transfected HCC827 cells at 10 μM for 8 h. The pEGFR levels were compared between HaloTag-PP1-FLAG transfected and nontransfected experiments. In both cases, pEGFR<sub>Y1068</sub> was shown to be substantially decreased relative to the DMSO control. This is expected as a major mechanism of EGFR inhibition is inhibition of trans autophosphorylation. The reduction in pEGFR<sub>Y1068</sub> was significantly more pronounced in the transfected experiment consistent with our designed mechanism of action. This case is notable as it suggests dephosphorylation of a tyrosine by a serine/threonine phosphatase, proximity overcoming typical substrate selectivity.

**CONCLUSIONS**

Taken together, our findings validate for the first time the concept of using tool compounds to recruit a phosphatase to a POI to affect dephosphorylation. In particular, we have shown proof of concept against two oncogenic kinases, AKT and EGFR, utilizing either heterobifunctional small molecules (phosphatase recruiting chimeras, PhoRCs) or a HaloTag-based approach. For kinases, the ability to inhibit receptor and transmission of the phosphorylation signal by promoting dephosphorylation may prove a means to better dampen pathway signaling and potentially avoid paradoxical pathway reactivation.

Moreover, this work represents the first examples of small molecules recruiting non-native protein/enzyme partners to induce removal of a PTM, adding to the potential applications of heterobifunctional compounds. Incorporation of the RVXF motif into these chimeric molecules may not be optimal for a number of reasons. Use of the RVXF motif and associated binding site, for chimeric molecules might influence important PP1 regulatory protein interactions for this highly expressed and active phosphatase. Also, the dephosphorylation phenotype is only observed at high concentrations. Given their peptidic nature (high TPSA, low log P, high number of rotatable bonds, high H-bond donor count), we hypothesize this is likely due to a combination of poor intracellular penetration and instability toward cellular hydrolases. We anticipate discovery of a small molecule phosphatase binder, with improved properties, could overcome the limitations of the chimeric molecule, similar to advancements made in chimeric degrader field post initial discovery.

**EXPERIMENTAL SECTION**

**General Synthetic Procedures.** All solvents and reagents were used as obtained. 1H NMR spectra were recorded with Bruker spectrometers and referenced to tetramethyl silane. Nonpeptidic molecules were analyzed by HPLC (Waters Acquity UPLC column) with UV detection at 254 and 210 nm and purified by HPLC (Intersin, Phenomenex Luna-C18, Phenomenex Gemini-NX) or Teledyne ISCO CombiFlash (RediSep Rf silica gel column). Peptides and peptide–small molecule conjugates were analyzed by HPLC (Waters, Xevo QToF, UPLC column) with UV detection at 220 and 280 nm and purified by HPLC (Waters Autopurification System, Phenomenex Luna C18, 100 Å). Purity of final compounds was determined by HPLC to be >95% for all compounds. Spectral images are included in the SI. See the synthesis of 6 for a representative peptide synthesis procedure (standard Fmoc chemistry on Wang resin).

n-2 2-(2-(6-Chlorohexyloxy)ethoxy)ethyl)-N<sub>4</sub>-2-(2-oxo-2-(2-oxo-3-1(4-(1-5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)-benzyl)piperidin-4-yl)-2,3-dihydro-1H-benzo[di]imidazol-5-yl)-amino)ethoxy)ethoxy]ethyl)succinamide (1). Commercial 4-[2-(2-(6-chlorohexyloxy)ethoxy)ethylamino]-4-oxo-butanate (5 mg, 0.012 mmol) in 0.1 mL of acetonitrile was added to an ice-bath cooled solution of 2-(2-(2-aminooethyl)ethyloxy)ethyl)-N<sub>4</sub>-2-oxo-3-1(4-(1-5-oxo-3-phenyl-6H-1,6-naphthyridin-2-yl)phenyl)methyl]4-piperidyl]-1H-benzimidazol-5-yl]acetamide TFA salt (7, 9.0 mg, 0.012 mmol) in 0.1 mL of pH 8 borate buffer. The mixture was warmed to room temperature to stir for 2 h. Concentration and purification by reverse-phase chromatography on a Phenomenex Luna C18 (100 Å) column eluting with a linear 0–100% gradient of MeCN/water over 25 min at 0.5 mL/min yielded 28.5 mg of pure product 6.

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The compound was dissolved in 0.90 mL of pH 8 HEPES buffer. The solution was puriﬁed by reverse phase chromatography on a C18 column using acetonitrile/water buffers. Puriﬁed fractions were analyzed by liquid chromatography mass spectrometry, pooled, and lyophilized. LCMS (ES, m/z): [M + H]+ = 667.2. 

N(((2-(2-(2-(2-iodoacetamido)ethoxy)ethoxy)ethoxy)-N-pyrime
din-4-yl)piperazin-1-yl)-3-oxopropyl)(isopropyl)amino)ethyl)-2-(2-(2-(2-iodoacetamido)ethoxy)ethoxy)-N-((4-azido-5-oxo-3-phenyl-5,6-dihydro-1,6-naphthyridin-2-yl)benzyl)piperidin-4-yl)-2,3-dihydro-1H-benz[d][imidazol-5-yl]amine+ (DIPEA). Peptide was cleaved off the solid support with trifluoroacetic acid-trisopropylsilane:water (95:2.5:2.5) for 1 h at room temperature. Resin was ﬁltered, and ﬁltrate was evaporated and peptide was precipitated with ethyl ether, centrifuged, and ethyl ether decanted oﬀ. Addition of ethyl ether, centrifugation, and ether decantation was repeated twice, and peptide pellet was allowed to dry. Crude peptide pellet was solubilized in dimethyl sulfoxide and puriﬁed by reverse phase chromatography on a C18 column using acetonitrile/water buffers. Puriﬁed fractions were analyzed by liquid chromatography mass spectrometry, pooled, and lyophilized. LCMS (ES, m/z): [M + H]+ = 541.

The crude solid (40 mg, 0.069 mmol), 2-(2-(2-(2-(2-(6-chlorohexyl)oxy)ethoxy)ethoxy)acetyl)piperazin-1-yl)methyl)(methyl)amino)-4-methoxy-5-(4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)acrylamide (7). tert-Butyl 4-(2-(2-acrylamido-5-methoxy-4-(4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)ethyl)amine (DIPEA) (33.5 mg, 0.259 mmol) were diluted with DMA (2 mL) and the reaction mixture stirred for 30 min at room temperature. The solution was puriﬁed directly by reverse-phase HPLC (ACN:water 0.05% NH3) to give 7 (12.3 mg, 21%). LCMS (ESI): [M + H]+ = 805. 

H NMR (400 MHz, DMSO-δ6) 8.90 (s, 1H), 8.76 (d, J = 6.8 Hz, 1H), 8.27 (d, J = 7.9 Hz, 1H), 7.99 (s, 1H), 7.97 (d, J = 8.2 Hz, 1H), 7.10 (t, J = 7.1 Hz, 1H), 4.07 (s, 2H), 3.83 (m, 4H), 1.35 (m, 2H), 1.19 (m, 2H), 1.11 (m, 2H), 1.04 (s, 3H), 0.87 (t, J = 6.8 Hz, 2H), 1.33 (m, 2H), 1.23 (m, 2H), 0.82 (t, J = 6.8 Hz, 2H).

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**REFERENCES**


(4) Walsh, C. A-loop, activation-loop: AKT, protein kinase B; EGFRI, epidermal growth factor receptor; I2, PIP inhibitor 2; PDP1, PP1-disrupting peptide 1; PIP, phosphatase interacting protein; PI3K, phosphoinositol-3-kinase; PhoRC, phosphatase recruiting chimera; ProTaC, proteolysis targeting chimera; POI, protein of interest; PP1, protein phosphatase-1; PP2A, protein phosphatase 2a; PTM, post-translational modification; pS6, ribosomal protein S6


