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1,3,5-Triazine as a Modular Scaffold for Covalent Inhibitors with Streamlined Target Identification

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Supporting Information

ABSTRACT: Small-molecule inhibitors can accelerate the functional annotation and validate the therapeutic potential of proteins implicated in disease. Phenotypic screens provide an effective platform to identify such pharmacological agents but are often hindered by challenges associated with target identification. For many protein targets, these bottlenecks can be overcome by incorporating electrophiles into small molecules to covalently trap interactions in vivo and by employing bioorthogonal handles to enrich the protein targets directly from a complex proteome. Here we present the trifunctionalized 1,3,5-triazine as an ideal modular scaffold for generating libraries of irreversible inhibitors with diverse target specificities. A divergent synthetic scheme was developed to derivatize the triazine with an electrophile for covalent modification of target proteins, an alkyne as a click-chemistry handle for target identification, and a diversity element to direct the compounds toward distinct subsets of the proteome. We specifically targeted our initial library toward cysteine-mediated protein activities through incorporation of thiol-specific electrophiles. From this initial screen we identified two compounds, RB-2-cb and RB-11-ca, which are cell permeable and highly selective covalent modifiers for Cys239 of β-tubulin (TUBB) and Cys53 of protein disulfide isomerase (PDI) respectively. These compounds demonstrate in vitro and cellular potencies that are comparable to currently available modulators of tubulin polymerization and PDI activity. Our studies demonstrate the versatility of the triazine as a modular scaffold to generate potent and selective covalent modifiers of diverse protein families for chemical genetics applications.

Small-molecule modulators of protein activity can serve as tools to annotate biochemical pathways and validate therapeutic targets relevant to disease. Despite the emergence of high-throughput screening platforms and cellular assays to identify bioactive compounds, a vast subsection of the proteome remains functionally unannotated with no available pharmacological tools.[1] Proteins from diverse functional families contain reactive amino acids that can be exploited in the development of covalent modulators. This subset of potential protein targets is not restricted to enzymes that utilize a catalytic nucleophile, but encompasses the myriad of proteins that contain reactive amino acids within either the active site or distal, yet functionally relevant, regions of the protein. This is exemplified by the kinase family, where nonconserved, yet functionally relevant, cysteine and lysine residues have proved invaluable in the development of selective inhibitors and activity-based probes, respectively.[2,3] Developing and screening libraries of small molecules targeted toward these reactive amino acids has potential to reveal pharmacological modulators for diverse protein classes.

One such reactive amino acid found within protein frameworks is cysteine. Cysteine residues are vital for redox and nucleophilic catalysis and allosteric regulation in proteins from diverse classes.[4] These functional cysteine residues display significantly enhanced reactivity with thiol-targeted electrophiles, such as haloacetamides and acrylamides.[5,6] Here we exploit this elevated reactivity to develop a small-molecule library to covalently modify functionally relevant cysteines. The covalent mode of action of these small molecules helps to overcome one of the largest bottlenecks that currently exist in high-throughput screening efforts: target identification. Incorporation of protein-reactive electrophiles into small-molecule scaffolds serve to trap the protein target within a physiologically relevant system. Here, we further expedite this target identification step by incorporating a bioorthogonal alkyne handle into our small molecules, thereby enabling the use of click-chemistry[7] to enrich, visualize and identify protein targets. The potency and selectivity of our small molecules can be directly assessed within the context of an entire cellular proteome, and target identification is streamlined.

Our platform is centered upon a small-molecule library that possesses the following elements: (1) a rigid triazine scaffold, (2) chloroacetamide (CA), chloronitrobenzyl (CNB) or acrylamide (Acr) reactive groups (R_2) known to covalently modify reactive cysteine residues,[5,8,9] (3) an alkyne handle for downstream conjugation to reporter tags (R_1), and (4) a variable diversity element (R_3) to direct reactivity to distinct subsets of proteins (Figure 1A). The heterocyclic triazine scaffold has recently been utilized in multiple large-scale combinatorial efforts, due to the ease of functionalization and structural similarity to bioactive endogenous heterocycles such as purines and pyrimidines.[10−14] The triazine has three-fold symmetry, allowing positional modification with the required R_1, R_2 and R_3 groups. Here we report the synthesis and evaluation of 20 compounds incorporating the CA, CNB and Acr electrophiles, together with a variety of diversity elements (R_3 groups) (Figure 1B). The R_3 group was varied across the library and encompasses a multitude of physicochemical

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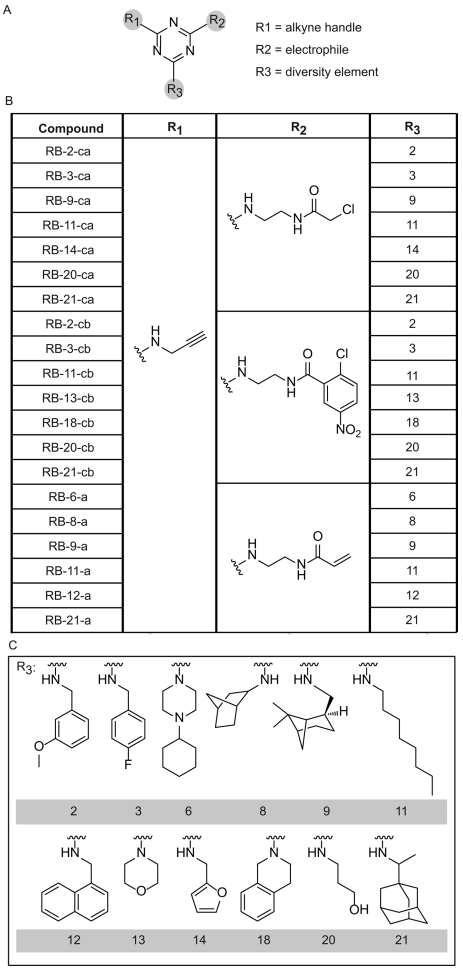

Compound	R_1	R_2	R_3
RB-2-ca			2
RB-3-ca			3
RB-9-ca			9
RB-11-ca			11
RB-14-ca			14
RB-20-ca			20
RB-21-ca			21
RB-2-cb			2
RB-3-cb			3
RB-11-cb			11
RB-13-cb			13
RB-18-cb			18
RB-20-cb			20
RB-21-cb			21
RB-6-a			6
RB-8-a			8
RB-9-a			9
RB-11-a			11
RB-12-a			12
RB-21-a			21

Figure 1. 1,3,5-Triazines provide a modular scaffold for covalent inhibitor design. (A) Trifunctionalization of the triazine scaffold to present an alkyne (R_1), cysteine-reactive electrophile (R_2) and a diversity element (R_3). (B) R_1, R_2 and R_3 groups for the 20 compounds evaluated in this study. (C). Structures of the diversity elements (R_3) incorporated in this study.

properties to direct the reactivity of these small molecules toward distinct subsets of proteins (Figure 1C).

The synthesis of the trifunctionalized triazines was performed using a solution-phase scheme adapted from previously reported solid-phase synthetic routes.[11] This synthetic scheme allows for the large-scale divergent synthesis of the final compounds from common intermediates (Scheme S1, Supporting Information). Our first goal was to assess the cell permeability of these trifunctionalized triazines and furthermore to determine if each compound targets a unique protein or subset of protein targets. To achieve this, we incubated HeLa cells with the triazines for 1 h and evaluated protein labeling after cell lysis, click chemistry to incorporate a fluorescent rhodamine moiety, and in-gel fluorescence.[15,16] These studies confirmed the ability of these small molecules to permeate cell membranes and covalently modify a disparate set of protein targets (Figure 2A and B, and Figures S1, S2, and S3 in Supporting Information). As demonstrated for the CA electrophile (Figure 2A), varying the R3 group on CA-containing triazine molecules results in varied protein-labeling patterns. Subtle changes such as a *m*-methoxybenzyl versus a *p*-fluorobenzyl (RB-2-ca vs RB-3-ca; Figure 2A) resulted in a noticeable change in the protein targets, serving to exemplify the target diversity we can access through minor changes in R_3 structure. In addition to changes in the diversity element, we can also modulate the protein targets by varying the electrophile. For three R_3 groups, we illustrate that varying the electrophile results in significant diversification of the protein targets (Figure 2B). These results highlight the modularity of the trifunctionalized triazine scaffold, which allows for varied targeting of proteins driven by modifications to either diversity element or electrophile structure.

We followed up on target identification and mechanistic characterization for two of the compounds in our initial library, RB-2-cb and RB-11-ca, which demonstrated high selectivity toward a single protein target (Figure 2C). We proceeded to identify the targeted proteins using mass spectrometry. A biotin group was incorporated onto labeled proteins using click chemistry, followed by avidin enrichment and subsequent identification using LC/LC−MS/MS.[17] Spectral counts for proteins identified in RB-2-cb- and RB-11-ca-treated proteomes were compared to a DMSO-treated control to identify the direct targets of these compounds (Table S2 and S2, Supporting Information). These mass-spectrometry studies identified the ∼50 kD target of RB-2-cb as a tubulin β chain

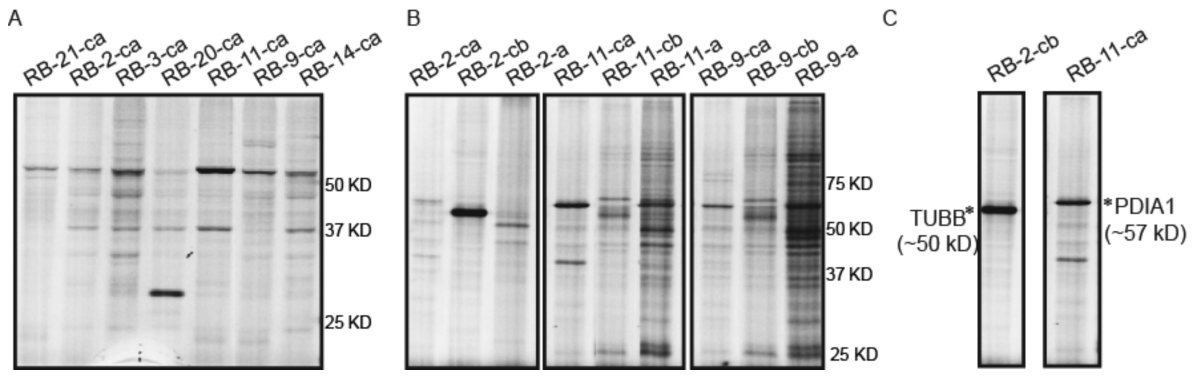

Figure 2. Protein targets of the triazine-based compounds. Library members were incubated with HeLa cells, and protein labeling by each compound was evaluated after cell lysis, click-chemistry-based incorporation of rhodamine, SDS-PAGE and in-gel fluorescence. (A). CA-containing probes with seven different R_3 groups demonstrate that modifications to the diversity element results in variations of the protein targets. (B) CA, CNB and Acr electrophiles demonstrated diverse labeling patterns even though the same R_3 group was employed. (C) In-gel fluorescence studies of RB-2-cb and RB-11-ca show labeling of one-major protein identified using LC/LC−MS/MS as TUBB and PDIA1 respectively.

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and the ~57 kD target of RB-11-ca as protein disulfide isomerase (PDI).

To verify the protein targets identified by mass spectrometry and to determine the specific cysteine on each of these proteins that is covalently modified by these compounds, we overexpressed isoforms of tubulin and PDI (TUBB and PDIA1) with a C-terminal Myc/His tag using transient transfection in HEK293T cells. We confirmed labeling of the wild-type proteins by RB-2-cb and RB-11-ca (Figure 3A and B). The

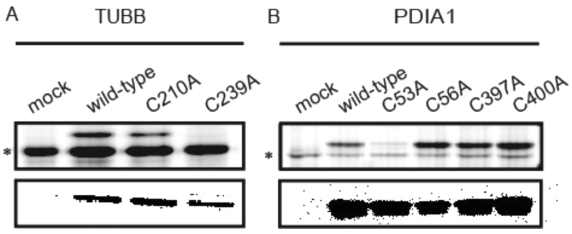

Figure 3. Target verification and identification of site of labeling. Wild-type and mutant TUBB (A) and PDIA1 (B) were overexpressed and labeled with RB-2-cb and RB-11-ca, respectively, to identify C239 and C53 as the sites of modification (top panel). The lower band * is the endogenous protein, and the upper band represents the overexpressed Myc/His-tagged variant. Western blotting with an α-Myc antibody verified equal protein expression for wild-type and mutant proteins (bottom panel).

lower band in both gels (*) represents the endogenous protein, whereas the upper band denotes the overexpressed variant. In addition to the wild-type proteins, we also overexpressed cysteine to alanine mutants of several known reactive cysteines on these proteins.[6] This gel-based analysis of wild-type and mutant proteins confirmed that RB-2-cb targets Cys239 on TUBB and RB-11-ca targets Cys53 of PDIA1.

Microtubule disruptors that function through inhibition of tubulin polymerization hold great promise as chemotherapeutic agents.[18] These microtubule-targeting agents include tubulin stabilizers (e.g., taxanes and epothilones) as well as tubulin depolymerizers (e.g., colchicine).[18] β-Tubulin is a major component of microtubules and Cys239 in β-tubulin has been shown to be targeted by electrophilic small molecules and nitrosylating agents, leading to inhibition of tubulin polymerization.[19−22] To assess the effect of Cys239 modification by RB-2-cb, we used an in vitro assay to monitor tubulin polymerization in the presence of our compound.[23] We compared the effect of RB-2-cb to colchicine,[24] a natural product known to disrupt microtubule formation by reversible binding to β-tubulin (Figure 4A). Similar to colchicine, RB-2-cb is a potent inhibitor of tubulin polymerization in vitro, with a >60% inhibitory effect at 2 μM. Furthermore, given that colchicine is a potent inhibitor of mitotic growth in cancer cells, we examined the ability of RB-2-cb to inhibit the growth of HeLa cells using an MTT cell viability assay. As expected, RB-2-cb is a potent inhibitor of cell growth with an EC_{50} value of 2.7 μM (Figure 4B).

PDIs are primarily localized to the endoplasmic reticulum, and act as chaperone proteins that catalyze the isomerization and rearrangement of disulfide bonds.[25] Increased PDI levels have been documented in various cancers, including ovarian,[26] lung[27] and prostate[28] cancers. PDIs contain two active sites for redox catalysis, denoted as α and α' domains.[29] Each active site contains a pair of cysteine residues within a CGHC motif (Cys53/Cys56 and Cys397/Cys400 in PDIA1).[29] Recently,

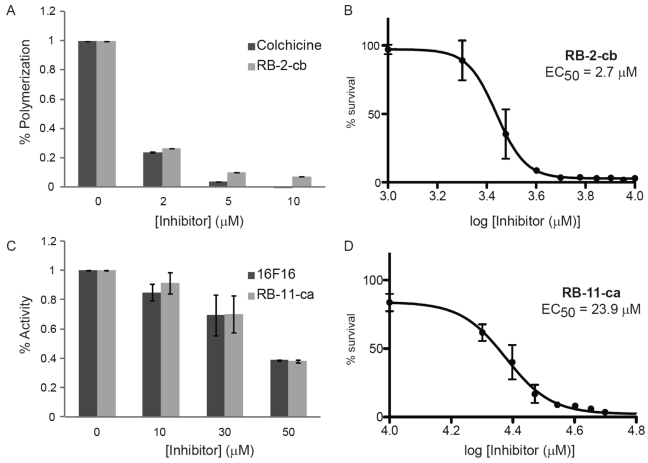

Figure 4. Effects of RB-2-cb and RB-11-ca on protein activity and cell proliferation. (A) Tubulin polymerization assay comparing the effects of RB-2-cb with colchicine. (B) Effects of RB-2-cb on cell proliferation of HeLa cells. (C) PDI activity assay comparing RB-11-ca to the commercially available PDI inhibitor, 16F16. (D) Effects of RB-11-ca on HeLa cell proliferation.

two irreversible inhibitors of PDI, 16F16 and PACMA 31, have been shown to suppress apoptosis in a model of Huntington disease,[30] and decrease ovarian tumor growth,[31] respectively. These previous studies underscore the importance of developing selective small-molecule inhibitors for PDI. To study the effect of RB-11-ca on PDI activity, we performed an in vitro fluorescence-based activity assay and compared RB-11-ca to the commercially available PDI inhibitor 16F16 (Figure 4c).[30] RB-11-ca demonstrated comparable inhibition to 16F16, adding to the arsenal of small molecules available to perturb PDI activity in vivo. Given the intriguing selectivity of RB-11-ca for one of the PDI active sites, this small molecule could provide insight into the regulation and activity of each of the two PDI active sites. Furthermore, given the report that targeted PDI therapy was effective in ovarian cancer cells,[31] we sought to evaluate the effect of RB-11-ca on HeLa cell proliferation and demonstrate that our compound shows micromolar inhibition (23.9 μM) of cell proliferation (Figure 4D).

In summary, we explore the trifunctionalized 1,3,5-triazine as an ideal modular scaffold for development of libraries of covalent inhibitors with streamlined target identification. By modifying either the diversity element or the electrophile in the triazine, we can modulate the protein targets of our small-molecule library. Using a preliminary set of 20 compounds, we demonstrate the modularity, cell permeability and target diversity of these triazine-based compounds. Furthermore, we identified highly selective covalent modifiers for β-tubulin and protein disulfide isomerase, with comparable efficacy to existing inhibitors of tubulin polymerization and PDI activity, respectively. These compounds add to the armory of small-molecule probes available for these protein activities, and furthermore exemplify the potential of similar libraries directed toward cysteines or other reactive amino acids to yield selective pharmacological agents. Such agents will hold great promise in chemical genetic screens to identify novel bioactive agents. This triazine-based platform is an attractive alternative to traditional small-molecule libraries due to the ease of target identification that is built into the library. Our preliminary studies lay the groundwork to support the future generation of larger triazine-

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based covalent libraries for cell-lysate or phenotypic screens with the goal of identifying selective covalent inhibitors for diverse proteins.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures, experimental protocol, synthetic scheme and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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