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Rational Targeting of Active-Site Tyrosine Residues Using Sulfonyl Fluoride Probes

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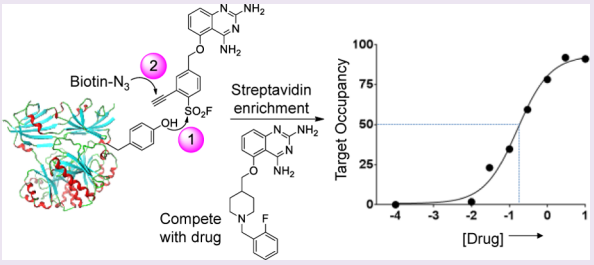
Supporting Information

ABSTRACT: This work describes the first rational targeting of tyrosine residues in a protein binding site by small-molecule covalent probes. Specific tyrosine residues in the active site of the mRNA-decapping scavenger enzyme DcpS were modified using reactive sulfonyl fluoride covalent inhibitors. Structure-based molecular design was used to create an alkyne-tagged probe bearing the sulfonyl fluoride warhead, thus enabling the efficient capture of the protein from a complex proteome. Use of the probe in competition experiments with a diaminoquinazoline DcpS inhibitor permitted the quantification of intracellular target occupancy. As a result, diaminoquinazoline upregulators of survival motor neuron protein that are used for the treatment of spinal muscular atrophy were confirmed as inhibitors of DcpS in human primary cells. This work illustrates the utility of sulfonyl fluoride probes designed to react with specific tyrosine residues of a protein and augments the chemical biology toolkit by these probes uses in target validation and molecular pharmacology.



Technologies that measure target engagement are important in the development of clinical candidates and for the validation of new therapeutic targets using chemical probes.[1,2] A quantitative assessment of target coverage by a drug or probe provides a direct measure of the interactions with its target, which is independent of downstream functional consequences. Moreover, target-occupancy values give a more accurate determination of predicted efficacious drug concentrations in the clinic, allowing therapeutic indices to be better defined.[3]

We[4,5] and others[6−12] have illustrated the importance of developing probes that assess intracellular target engagement to provide a more physiologically accurate picture of molecular interactions that includes the influence of factors such as post-translational modification, autoinhibition, scaffolding, substrate competition, and subcellular distribution. Of particular relevance in this area has been the use of covalent inhibitors with embedded click handles that allow subsequent attachment of a reporter, which is useful for profiling protein activity and drug occupancy in intact cells.[13]

Spinal muscular atrophy (SMA) is an autosomal−recessive disease caused by mutations in the survival motor neuron gene (*SMN1*).[15] As the name suggests, the SMN protein is essential to the survival of neurons, and its diminished abundance in SMA results in degeneration of motor neurons and muscle wasting (atrophy). Humans have two genes that encode the SMN protein (*SMN1* and *SMN2*), but *SMN2* contains a single nucleotide difference that reduces its splicing efficiency. This results in the production of full-length SMN protein in small amounts, which is sufficient for the survival of some neurons. One approach to modulate the severity of this devastating disease is to find ways to upregulate the amount of SMN protein.

A high-throughput phenotypic assay was previously developed to unearth molecules that upregulate the expression of SMN protein by activating *SMN2*.[16] This work led to the discovery of a series of diaminoquinazolines (DAQs) that efficiently upregulated SMN protein, [17] and a protein microarray (>5000 proteins printed onto glass slides) that used a radiolabeled DAQ probe identified the decapping scavenger enzyme DcpS as a target of these molecules.[14] DcpS is involved in mRNA metabolism, mediating the catalytic hydrolysis of the 5′ cap structure (m^7GpppN) in mRNA fragments.[18] Although there is currently no known biochemical link between DcpS inhibition and SMN modulation, DAQ

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inhibitors have shown considerable promise for the treatment of SMA.[19]

Here we describe the rational design and development of sulfonyl fluoride (SF) covalent inhibitors of DcpS to deliberately target specific binding-site tyrosine residues for the first time, relying on the nucleophilicity of phenol in a context-specific environment (see below).This led to the creation of an occupancy biomarker that confirmed intracellular DcpS−target engagement. This work validates DcpS as the *bona fide* target of DAQ SMN modulators in human primary cells and illustrates the value of chemoproteomics to studies in translational pharmacology.

To expedite the design of covalent inhibitors of DcpS, we used the X-ray structures already reported with the DAQ series.[14] In the structure of compound D153249 with DcpS (Figure 1a), the basic diaminoquinazoline makes key electro-

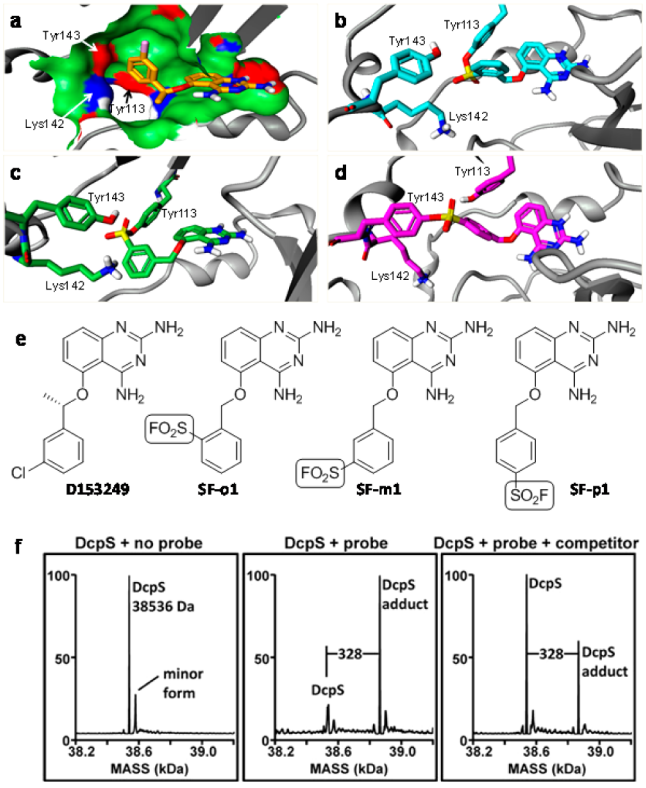


Figure 1. X-ray crystal structures of DcpS with (a) D153249 (Protein Database (PDB) 3BLA),[14] (b) **SF-o1** (PDB 4QDE), (c) **SF-m1** (PDB 4QEB), and (d) **SF-p1** (PDB 4QDV). (e) Structures of DcpS inhibitor D153249 and SF probes **SF-o1**, **SF-m1**, and **SF-p1**. (f) LC-MS showing adduct formation of DcpS with probe **SF-p1** and competition with inhibitor (center and right panels show spectra taken 30 min after the start of incubation).

static and bidentate hydrogen-bonding interactions with Glu185 through protonation of position 1 of the quinazoline ring and amino group 2. The DAQ ring also forms $\pi-\pi$ stacking interactions with Trp175 and additional hydrogen bonding through the interaction of amino group 4 with Asp205. The ligand conformation is further stabilized through an intramolecular hydrogen bond between amino group 4 and the ether oxygen. While there are no nucleophilic cysteine residues available for electrophilic attack in the binding site, the structure shows that Tyr113, Lys142, and Tyr143 are proximal to the benzylic group of the ligand and may serve as targets for templated covalent inhibition.[20]

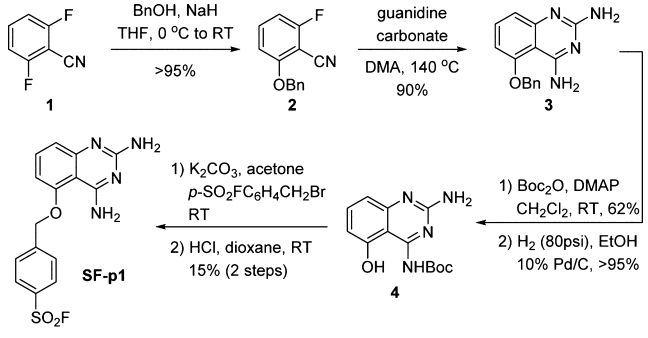
SF probes have found considerable utility in chemical biology and medicinal chemistry.[21] The reactive adenosine derivative 5′-fluorosulfonylbenzoyl 5′-adenosine (FSBA) was originally developed 40 years ago by the Colman group to explore the binding sites of glutamate dehydrogenase.[22] The probe was subsequently found to react with the conserved lysine residue in the ATP site of kinases, and a related probe bearing a click handle was recently used to selectively target the active site of Src kinases in intact cells.[11] Another recent example used a series of SF inhibitors of transthyretin that covalently reacted with a pK_a-perturbed lysine in the thyroxine binding site.[23]

Recently, SF reactive probes were developed that were based on 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, also called Pefabloc), a reagent that is normally used to inactivate serine proteases.[24] In this work, a clickable SF probe was found to label different glutathione S-transferases (GSTs) in complex proteomes.[25] Interestingly, MS peptide-mapping experiments determined that the probe had fortuitously labeled the GSTs on the tyrosine residues in the xenobiotic binding site. The context-dependence of SF reactivity was illustrated by the reaction of FSBA with tyrosine or lysine residues at a number of nucleotide binding sites (including those of kinases) in a complex proteome.[26] These previous observations suggested that the SF functionality may be used to deliberately target reactive tyrosines in protein active sites.

As a result, three regioisomeric SF probes were rationally designed on the basis of the structure of D153249 to explore structure−function relationships, with the aim of creating covalent inhibitors of DcpS (Figure 1e). In the DcpS inhibitor pocket, it appeared that the para isomer **SF-p1** was positioned to react preferentially with Tyr143 and the ortho isomer **SF-o1** was positioned to react with Tyr113. However, the residue that would react with the meta isomer **SF-m1** was unclear, and it was also possible that given the opportunity all of the probes may react with the more flexible Lys142 regardless of the predicted proximity of the tyrosine residues in the static X-ray structure. Nevertheless, the basic residues His139 and Lys142 (neighboring Tyr113 and Tyr143, respectively) may facilitate phenolate reaction with SF electrophiles.[25]

Scheme 1 outlines the synthesis of fluoride inhibitor **SF-p1**, which relies on guanidine cyclization onto cyano-fluorobenzene derivative **2** and the alkylation of phenol **4** with *p*-$SO_2FC_6H_4CH_2Br$. SF inhibitors **SF-o1** and **SF-m1** were prepared in an analogous manner (Supporting Information).

Scheme 1. Preparation of Sulfonyl Fluoride Inhibitor PF-06746716



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A biochemical assay was developed to measure the inhibition of DcpS activity, following the modifications of a previously reported protocol.[27] Briefly, hydrolysis of a biotinylated cap substrate (m^7GpppA-biotin) by recombinant DcpS resulted in the formation of ADP-biotin, and subsequent streptavidin capture and use of an antibody to ADP enabled measurement of DcpS activity *via* an ELISA format (Supporting Information). In this assay, the SF probes were found to be subnanomolar inhibitors of DcpS activity *in vitro* (Table 1).

Table 1. Potency of DcpS Inhibitors D153249, SF-o1, SF-m1, SF-p1, SF-p1-yne, and D156844

compound	D153249	SF-o1	SF-m1	SF-p1	SF-p1-yne	D156844
DcpS IC_{50} (nM)	1.9	0.066	<0.02	<0.02	1.1	0.10

To confirm the covalent nature of inhibition, the probes were incubated with recombinant DcpS and analyzed using LC-MS (Figure 1f). On the basis of the presence of M+328 in the mass spectra (i.e., addition to protein with loss of fluoride), the probes appeared to be covalent inhibitors; competition with the inhibitor reduced labeling, thus indicating the specificity of the chemistry. The rate of adduct formation was also investigated using LC-MS as a readout (Figure S2).

X-ray crystal structures of SF probes with DcpS unambiguously confirmed the modified residues (Figure 1b,c,d) and the maintenance of the key diaminoquinazoline interactions. **SF-o1** and **SF-m1** reacted with Tyr113, whereas **SF-p1** reacted with Tyr143 as expected. Additionally, LC-MS peptide-mapping experiments that used trypsin and chymotrypsin were in line with the X-ray data (Supporting Information). These results show that subtle changes in the position of the SF functionality will determine which of the tyrosine residues react with the warhead. The preference for tyrosine labeling over lysine labeling is an interesting observation in this case and could impact the future design of SF inhibitors. As a result, to assess the residues proximal to tyrosines that were labeled by SF reagents, we carried out a review of the literature and the PDB and analyzed the proteomics experiments in Gu *et al.*[25] and Hanoulle *et al.*[26] (see protocol and data in Supporting Information). Labeled tyrosine residues proximal to basic residues account for the majority of cases (i.e., Arg = 23%, Lys = 23%, and His = 9%), whereas neutral residues/backbone interactions (i.e., Gln = 8%, Asn = 7%) and acidic residues (i.e., Asp = 12%, Glu = 4%) are also represented. Therefore, in nearly every case, a proximal residue facilitates deprotonation of the tyrosine −OH. This analysis may help delineate the targetable "tyrosinome" that is ongoing in our group.[20]

To illustrate the utility of the tyrosine-targeted probes, an intracellular-occupancy biomarker technology was developed. An alkyne-tagged SF probe was prepared to enable click-chemistry attachment of biotin azide, using a copper-mediated azide−alkyne cycloaddition (CuAAC)[28,29] to mediate protein enrichment and subsequent Western blot analysis. SF probe **SF-p1-yne** (Figure 2b) was designed on the basis of a balance among its likely reactivity with DcpS, its toleration of the terminal alkyne within the binding site, and its expedience of synthesis (Scheme 2 and Supporting Information). Treatment of commercially available sulfonyl chloride **6** with KF and borane reduction furnished SF intermediate **7**. Subsequent conversion to benzyl bromide **8** and coupling with phenol **4** provided **9**. Stille coupling installed the terminal alkyne, and Boc-deprotection yielded the desired SF probe, **SF-p1-yne**.

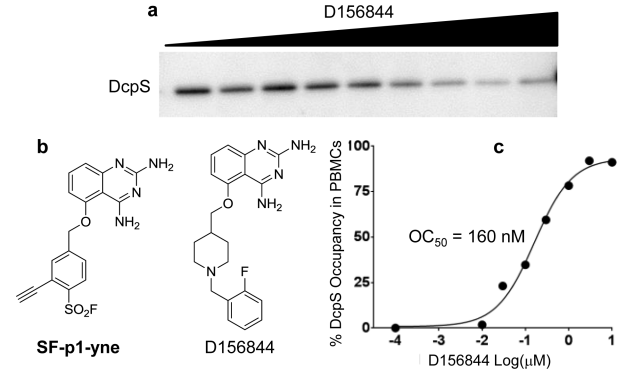
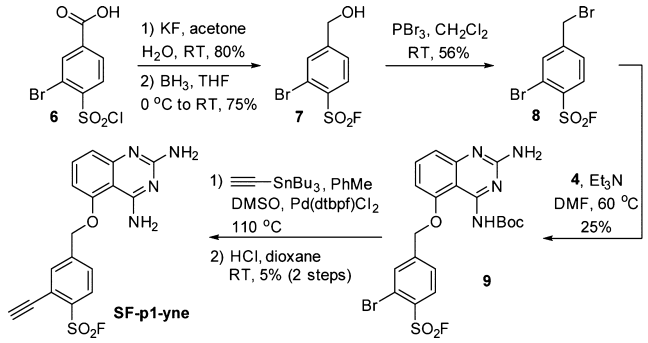


Figure 2. (a) Western blot showing DcpS that was isolated from PBMCs by using probe **SF-p1-yne** and competed with by inhibitor D156844. (b) Structures of clickable SF probe **SF-p1-yne** and DcpS inhibitor D156844. (c) Densitometry of WB shown in panel a.

Scheme 2. Preparation of Sulfonyl Fluoride Probe SF-p1-yne



As expected, **SF-p1-yne** was found to be a potent inhibitor of DcpS (Table 1) and was used in further cell-based experiments. However, it is difficult to compare the potencies of covalent inhibitors because the described assay does not take into consideration the time-dependence of inhibition. To assess DcpS target engagement in intact human primary cells, protein isolated using **SF-p1-yne** was competed in a dose-related manner with varying concentrations of the known inhibitor D156844 (Figure 2). Peripheral blood mononucleated cells (PBMCs) were incubated with D156844 for 2 h, then treated with 1 μM **SF-p1-yne** for 20 min (see Figure S6 for optimization of labeling conditions), followed by cell lysis and click attachment of azido biotin, which enabled streptavidin capture of DcpS, visualization using Western blot analysis, and quantification by densitometry (Figure 2). This experiment allowed us to determine the inhibitor concentration in cells that provides 50% occupancy of the DcpS enzyme (i.e., OC_{50}). Inhibitor D156844 had an OC_{50} of 160 nM under these conditions. There is a notable difference between the OC_{50} and the biochemical IC_{50} value for this compound, which may reflect an intrinsic difference in the target DcpS in a cellular context, the influence of the cellular milieu (e.g., substrate concentration), or the methodological aspects of the technique. Other potential targets of SF probe **SF-p1-yne** are currently under investigation (see Figure S7 for in-gel fluorescence of TAMRA-labeled proteins).

Future work in our group is taking into account the potential shift in OC_{50} that results from covalent-labeling kinetics, as was recently explored when ATP covalent probes were used to

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profile reversible kinase inhibitors.[30] Nevertheless, we are able to use the technology described here to provide quantitative validation of DcpS as the biological target of DAQ SMN upregulators and to measure intracellular DcpS coverage. Importantly, the DcpS-occupancy values reported here are more in-line with the reported phenotypic efficacy of D156844 (100 nM increased the number of intranuclear gem bodies that are rich in SMN protein in type-I-SMA patient fibroblasts by 5.5-fold).[17]

In conclusion, we have rationally designed SF probes that deliberately target specific tyrosine residues in a protein (i.e., the mRNA-decapping scavenger enzyme) for the first time. An SF probe bearing a silent click reporter (i.e., an alkyne tag) was used to measure intracellular target engagement of the DcpS enzyme in human primary cells. The quantitative correlation of target occupancy with pharmacological and phenotypic modulation is an essential component of successful target validation and clinical progression.[2] For example, the biochemical potency of inhibitor D156844 significantly underestimates the efficacious concentrations required for this molecule to achieve the requisite *in vivo* effects. As a result, we have provided further confidence that DcpS inhibition is the mode of action of the DAQ molecules.

More broadly, chemical biology tools are required to further elucidate the therapeutic potential of RNA binding proteins, and the work herein advances these efforts. This study illustrates the utility of tyrosine-targeted probes, thus enhancing the chemical biology toolbox. This is particularly important when reactive cysteine or lysine residues are not available for template covalent modification. Also noteworthy is our ability to develop an SF probe for applications in cells rather than those in cell lysate, the latter of which do not represent the biology of an intact cell. With regard to the use of lysate in chemoproteomic experiments, another complicating factor could be the millimolar concentrations of AEBSF that are used to inhibit proteolysis because these reagents are known to modify several other proteins.[31−35]

Future work in our group will include exploring the selectivity of sulfonyl fluorides for amino acids to provide methods of predicting reactive residues in other protein targets. In particular, further biochemical/physical and mutational studies are required to elucidate the origins of tyrosine reactivity (e.g., possibly through pK_a perturbation) in this and other cases.

■ ASSOCIATED CONTENT

Supporting Information

Preparation and analytical characterization (including NMR spectra) of all new molecules, design of probe **SF-p1-yne**, X-ray crystallography protocols and diffraction data, DcpS enzyme assay protocol, peptide mapping for DcpS−probe adducts, and protocol for determining DcpS occupancy in PBMCs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors are employees and shareholders at Pfizer.

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