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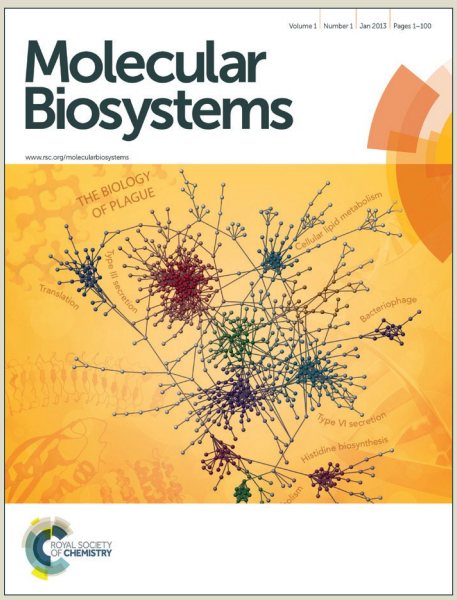

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A Library Approach to Rapidly Discover Photoaffinity Probes of the mRNA Decapping Enzyme DcpS

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Despite its diverse applications, such as identification of the protein binding partners of small molecules and investigation of intracellular drug-target engagement, photoaffinity labelling (PAL) is intrinsically challenging, primarily due to the difficulty in discovering functionally active photoaffinity probes. Here we describe the creation of a chemoproteomic library to discover a novel photoaffinity probe for DcpS, an mRNA decapping enzyme that is a putative target for Spinal Muscular Atrophy. This library approach expedites the discovery of photoaffinity probes and expands the chemical biology toolbox to include RNA cap-binding proteins.

Photoaffinity labelling has become a powerful tool in chemical biology and drug discovery, with uses including target identification, imaging and binding site mapping. A photoaffinity probe usually consists of three components: 1) a pharmacophoric element that provides the binding specificity; 2) a photoreactive group that converts into a reactive species upon irradiation (e.g. nitrene, carbene or radical) and then forms a covalent linkage with its target biomacromolecules through C-C, C-H and heteroatom-H insertions; and 3) a reporter tag (a radioactive, fluorescent, immunoreactive or biotin functionality) that allows for subsequent manipulations, such as imaging and affinity enrichment.[1, 2] A successful photoaffinity probe needs to possess a photoreactive group that enables reactivity with a proximal amino acid residue, while still maintaining the same biological activity as the parent. It is often challenging to design such a probe, due to the difficulty in satisfying both the structure-activity relationships (SARs) of the target protein and amino acid reactivity in the binding site. This process is usually sequential, that is, target protein binding is first optimised, and then photoaffinity handles are incorporated into the most potent template. However, in this approach, there is an assumption that the different SARs align, which may not always be the case, i.e. the most potent binding probe may not necessarily be the most efficient at labelling the target. Additionally, in our experience, efficiency levels are relatively low for protein labelling using PAL versus other techniques such as electrophilic labelling using sulfonyl fluorides.[3] However, photoaffinity probes benefit from being less reliant on specific amino acid targeting due to the promiscuity of carbene, radical and nitrene chemistry. Additionally, reporter groups such as fluorescent dyes, biotin or click handles are often incorporated into the photoaffinity probe to enable protein isolation, enrichment or visualisation, thus demanding further SAR optimisation. To expedite the discovery and utilisation of PAL, we describe here a library approach to discover photoaffinity probes. A previous approach applied library chemistry to the development of PAL probes for the matrix metalloproteases, although this work kept the benzophenone photoreactive group and click reporter moieties the same, whilst varying amino acid residues in the peptidic template.[4] By building a carefully designed photoprobe library using various photoreactive tags, we were able to explore a larger chemical-biology space in a more timely fashion. This strategy has the potential to improve efficiency in the discovery of photoaffinity probes, since chemical biology functionality is explored with protein binding/potency in a concurrent manner.

We used DcpS as a model protein to illustrate this approach. DcpS is an mRNA decapping enzyme involved in the degradation of mRNA fragments. It hydrolyzes short 5'-capped oligonucleotides (m^7GpppN) resulting from 3' to 5' mRNA decay.[5] DcpS is a putative target of 2,4-diaminoquinazolines (DAQs) that are under investigation for the treatment of spinal muscular atrophy (SMA),[6] an inherited neuromuscular disease caused by loss-of-function mutations in the SMN1 gene that encodes the survival motor neuron (SMN) protein.[7-9] By building an alternative technology to the sulfonyl fluoride probe work we performed previously,[10] we sought to confirm DcpS target engagement by the DAQ class, and provide further

confidence regarding target validation. This early report describes the discovery of a probe that labels recombinant DcpS, with a view to further development of a technology to label the protein in more physiologically relevant contexts (e.g. human primary cells – ongoing work). Additionally, photoaffinity probes in this class could augment the chemical biology toolkit for understanding RNA cap-binding proteins.

Photoaffinity probe design was explored using the DAQ inhibitor scaffold, which was enabled by the availability of the crystal structures of DAQ derivatives with the DcpS enzyme.[6] For example, the D156844-DcpS crystal structure highlights key interactions between the DAQ core and the protein binding site (Fig. 1A). The protonated scaffold engages the Glu-185 with two strong hydrogen bonds to N1 and C2-NH_2. The flat bicyclic quinazoline scaffold undergoes a productive π–π stacking interaction with Trp-175.

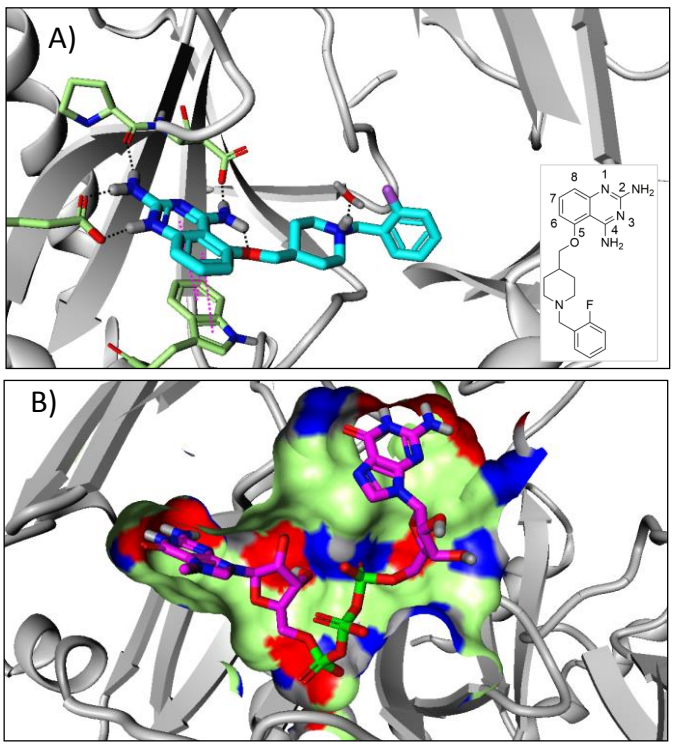


Fig.1 (A) Crystal structure of D156844 (cyan and inset) with DcpS (PDB code 3BL7) showing extensive hydrogen bonding to the protein and π-stacking to Trp175.[6] (B) Crystal structure of m^7GpppG (magenta) with DcpS (grey ribbon, PDB code 1ST0) showing the binding surface (red = oxygen, blue = nitrogen, green = hydrophobic surface).[11] The crystal structures provide confidence that larger DAQ photoaffinity probes could be accommodated in the binding site of the enzyme.

From the m^7GpppG-DcpS crystal structure (Fig. 1B)[11] it is also clear that the binding pocket is large enough to accommodate photoaffinity tags appended to the DAQ scaffold. This provided the basis for exploring the large binding site in a library fashion using a number of photoreactive functionalities attached via ether and amide linkers (Fig. 2). The length and the shape of the linkers that are attached to 2,4-diaminoquinazoline were varied in such a manner that the key interactions with the DAQ pharmacophore would likely be maintained. The photoreactive library monomers consisted of 22 benzophenones (different regioisomers), 6 aryl azides, and 2 diazirines that were obtained either commercially or from the Pfizer file. The lack of availability of diazirine monomers reflects the difficulty in preparing the strained ring system and more commercially available monomers bearing this group would likely advance PAL more broadly. The library was also populated with derivatives bearing an alkyne reporter handle, additional to a photoprobe (Fig. 2 and ESI), to facilitate subsequent copper(I)-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC 'click chemistry')[12, 13] attachment of various reporters depending on the functionality of the tag introduced through conjugation. However, only 6 reactive monomers contained both functionalities (click handle plus photoreactive group) and future work involves increasing the number of these useful monomers in our collection (see below).

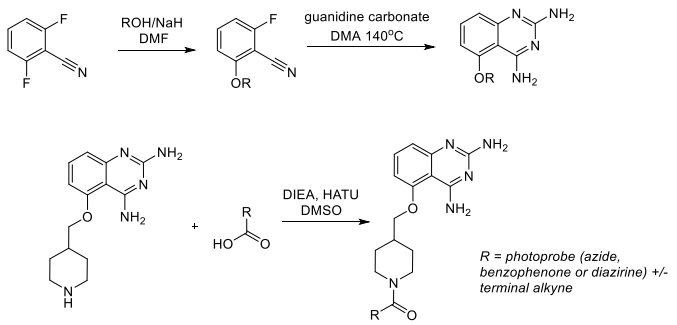


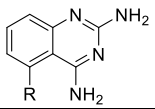
Fig. 2 Photoaffinity probes made in the library, using etherifications and amidations as the key steps. All members of the library are described in the ESI.

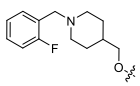
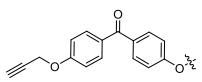
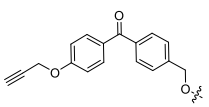
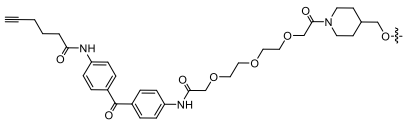
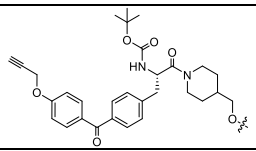
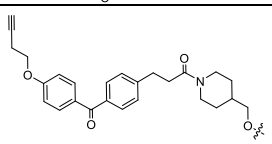
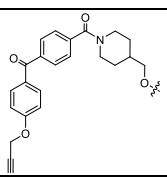
The 30 library compounds were first tested in an ELISA biochemical assay to determine their DcpS inhibitory activities.[10] The library displayed a range of DcpS potencies as shown in Table 1 and Table S1, with most possessing IC_{50} < 100 nM, and many < 1 nM (compare the potency of D156844) suggesting they are well accommodated in the DcpS active site, thus vindicating the original design strategy. Aryl ethers (linked directly into the benzophenone ring), are significantly less potent than benzyl ethers (e.g. compare compounds **1** and **2**, **7** and **9**) where the additional carbon atom likely provides the flexibility required to place the pendant benzophenone appropriately in the binding pocket. The difference in potency of compounds **9** and **10** also likely reflects this effect. Although less potent, compound **3**, which possesses a long linker to the photoreactive moiety, retains significant DcpS activity showing the somewhat promiscuous nature of this region of the binding site. The presence of the alkyne tag appears to improve potency in the piperidine amide benzophenone **6** (compared to **15**).

To further assess these probes in a proof-of-principle experiment, recombinant DcpS protein samples were subjected to intact protein mass spectrometric analysis following photoaffinity labelling. Unfortunately, we were unable to identify any probe that caused a predicted mass shift by LC-MS, likely due to the low PAL yield and/or the poor ionization of the protein-probe adduct (Figure S1 and S2). This observation highlighted the need to have an orthogonal

approach to assess probe performance, which was enabled in this case by the terminal alkyne handles present in six 'clickable' probes (DcpS activities are shown in Table 1). Therefore, in parallel with the intact protein mass spectrometric analysis, we quickly determined whether the six probes were capable of labelling DcpS protein by performing a click reaction with biotin azide after photoaffinity labelling, followed by affinity enrichment using streptavidin agarose and Western blot analysis (Figure 3).

Table 1. DcpS inhibition activities of the clickable members of the photoaffinity probe library.



Compound ID	R	DcpS IC_{50} (nM)
D156844		0.1
1		5560
2 (DcpS-BPyne)		6.0
3		27
4		7.6
5		12
6		0.29

In Figure 3A, significantly more DcpS was enriched by **1** and **2** after UV irradiation compared to the samples without irradiation treatment, suggesting these two probes successfully labelled DcpS. Interestingly, some probes enrich DcpS in the absence of light, which may reflect affinity isolation of the protein directly. Due to the potential non-specific reactivity of the photoactivated probes, PAL may cause nonspecific labelling.[14] It is therefore essential to further understand the specificity of the labelling. We performed a competition experiment with SF-p1 (Figure 3B), a covalent DcpS inhibitor that modifies Tyr-143 in the active site.[10] The labelling by compound **2** (which we named DcpS-BPyne) was efficiently competed by SF-p1, providing validation that the probe specifically labelled DcpS in the active site (Figure 3C). On the other hand, the labelling by compound **1** appeared to be non-specific since, unusually, SF-p1 appeared to increase the labelling by the probe (Figure 3D). These data also illustrate the divergence between DcpS potency and efficacious protein labelling. For example, compound **6** is one of the most potent DcpS inhibitors in the library, but a poor photoaffinity probe of the enzyme active site.

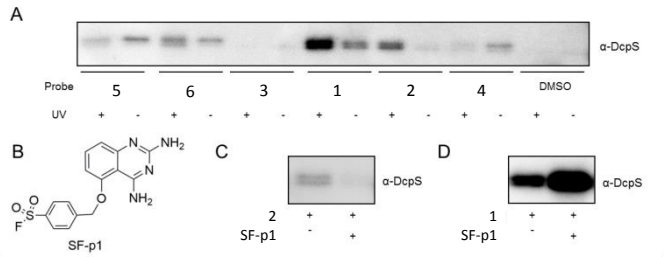


Fig. 3 Probe assessment by affinity enrichment. (A) Affinity enrichment of the six clickable probes with or without UV irradiation. DcpS was incubated with the probes. After irradiation or no irradiation, click chemistry with biotin azide was performed, followed by pulldown of DcpS with streptavidin agarose. (B) Chemical structure of a covalent DcpS inhibitor SF-p1 that modifies an active site tyrosine residue; (C) DcpS was pre-incubated with SF-p1, a covalent DcpS inhibitor, before PAL by compound 2. The enrichment was competed by SF-p1, suggesting the probe specifically labels the DcpS binding site. (D) Labelling by compound 2 enhanced in the presence of SF-p1, indicative of nonspecific labelling.

Our library approach led to the discovery of a novel DcpS photoaffinity probe, DcpS-BPyne (compound 2). This probe allowed us to use an orthogonal technology to validate DcpS as a target of DAQs currently being investigated for SMA. These preliminary results show potential for the use of DcpS-BPyne in labelling DcpS in a complex proteome (ongoing work). A key learning from this research is that it would be beneficial to build a future library in which all probes contain a clickable handle, and thus allow rapid probe assessment by orthogonal assays in parallel, e.g. both mass spectrometry *and* affinity enrichment. This is particularly useful given that photoaffinity labelling often has a low yield and the adduct may not be suitable for intact mass spectrometric analysis.

Conclusions

For the first time, we have illustrated the utilisation of a library approach to obtain a specific photoaffinity probe for DcpS, a putative drug target for SMA. This technique will no doubt facilitate the rapid and efficient discovery of useful PAL probes for many other proteins of therapeutic interests. Chemoproteomic libraries will likely find significant utility in target identification and molecular pharmacology, particularly

in the drug discovery setting, where new technologies are required to validate target protein engagement. The further utility of DcpS chemical probes will be described in due course.

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Notes and references

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† Electronic Supplementary Information (ESI) available: additional experimental details, including compound synthesis, analytical data and labelling procedures. See DOI: 10.1039/c000000x/

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