Activity-Based Protein Probes

# Propargyl Amides as Irreversible Inhibitors of Cysteine Proteases—A Lesson on the Biological Reactivity of Alkynes**

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bioorthogonality · irreversible inhibitors · proteases · protein-templated reactions · ubiquitin

Oligomers of the small protein ubiquitin and of ubiquitin-like proteins such as SUMO attached to cellular proteins are the main signal in the regulation of protein turnover and lifetime in cells.[1,2] Therefore, the opposing mechanisms of ubiquitinylation and of de-ubiquitinylation are carefully regulated within cells. While ligating enzymes build up ubiquitin oligomers on lysine residues of intracellular proteins, the de-ubiquitinating enzymes (DUBs) hydrolyze these protein tags (Scheme 1). The latter enzymes represent
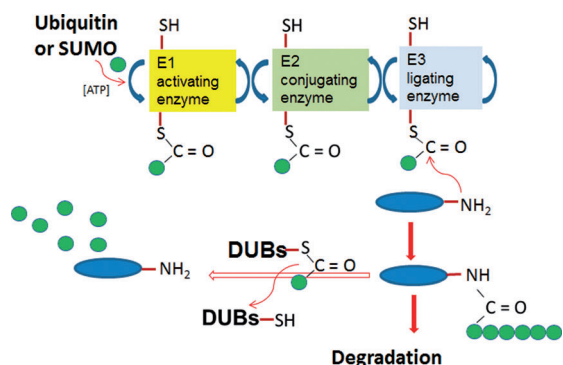
Scheme 1. Ubiquitin-like proteins are transferred to target proteins and bound by means of isopeptide bonds in an ATP-driven enzymatic transfer reaction with thioester intermediates. Modified target proteins are often driven towards degradation. DUBs are capable of hydrolyzing the isopeptide bonds thereby reliberating ubiquitin-like proteins and target proteins.

cysteine-dependent isopeptidases and can be divided into different subclasses according to their protein modifier substrate. By their action on modified cell proteins they are thought to influence diverse key cellular processes such as gene expression, DNA replication, DNA repair, cell cycle control, protein sorting, and protein halflife times. The

understanding of how DUBs function in health and disease is growing rapidly.[3]

Chemically modified ubiquitin derivatives have had a major impact on the progress in the field of DUBs and therefore chemical methods have been devised to prepare these DUB-specific probes, which include fluorogenic substrates and covalent reversible inhibitors as well as irreversible inhibitors.[4] The latter, for example, ubiquitin vinyl sulfone, have been proven as useful as active-site-directed probes for pull-down, identification, and possibly quantification of interacting proteins. Most of these ubiquitin derivatives modified at the carboxy terminus have been prepared by combinations of biochemical and chemical methods, but chemical total synthesis has been reported as a means to produce ubiquitin derivatives as well.[5] Recently, the preparation of ubiquitin derivatives by dipolar cycloadditions of azides and alkynes has been reported.[6]

Now, in two independent reports from the groups of Ovaa[7] and Mootz[8] C-terminal propargyl amides have been prepared as alkyne components for dipolar cycloadditions—with completely unexpected results. When the C-terminal propargylamide of ubiquitin or of SUMO, an ubiquitin-like protein modifier, came into contact with DUB isopeptidases specifically recognizing them, they were found to be potent irreversible inhibitors of the respective cysteine proteases at stoichiometric amounts. While considering the alkyne functionality as biochemically and chemically inert under the conditions applied, both groups found the addition of the thiol group of the enzyme's active-site cysteine to the carbon triple bond of the propargyl amide. A vinyl thioether product could be identified in the crystal structure of the protein complex, and the involvement of the active-site cysteine residue was further confirmed by site-directed mutagenesis.

As alkynes have never been reported as active-site-directed inhibitors of cysteine proteases before, the authors investigated the generality of propargyl amides as cysteine protease inhibitors. When propagyl amide was attached C-terminally to ubiquitin, members of all four families of cysteine DUBs were observed to react. The situation changed when caspase-1 served as a cysteine protease target not related to ubiquitin-derived substrates. For this enzyme the tetrapeptidyl aldehyde was known as a low-nanomolar inhibitor;[9,10] however, the propargyl amide of this short

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peptide was inactive in the inhibition experiments. Elongation of the progargyl amide to 16 amino acids was required to regain potent inhibition of the enzyme.

The results by Ovaa et al. and Mootz et al. raise interesting questions regarding the claimed bioorthogonality, or biological inertness of alkynes in biological systems.[11] While the Markovnikov-selective hydrothiolation of terminal alkynes requires temperatures of 170–180°C with base[12a] and still 120°C with a Lewis acid catalyst,[12b] obviously the addition of a protein thiol can proceed smoothly, if the protease positions the thiolate and alkyne in close proximity and thereby enforces the reaction. In this context, the observed reactivity of propargyl amides could be attributed to a template effect: recognition of the ubiquitin-derived protein by the DUB isopeptidase favors the formation of the transition state and of the vinyl thioether product. Considering the structure of the protein complex, the terminal alkyne of the modified ubiquitin substrate indeed seems to be able to interact with the oxoanion hole of the enzyme's active site, which is lined with positive partial charges of the H-bond donor groups (Scheme 2).
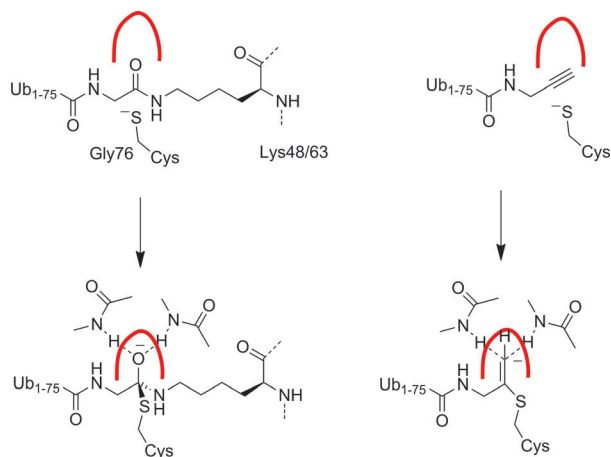
Scheme 2. Cleavage of the ubiquitin isopeptide bond by a de-ubiquitinating enzyme (DUB). Left: The thiolate anion attacks the amide carbon being activated by binding to the acidic, positively charged oxoanion hole (red). Right: The same H-donor-lined protein pocket might activate the propargyl amide for nucleophilic attack by using its capability to stabilize the intermediate carbanion and/or to raise the electrophilicity at C2 of the alkyne.

These partial charges might stabilize the negative polarization of the terminal alkyne carbon formed by attack of the enzyme's thiolate at C2 of the alkyne. In this way, the DUB isopeptidase favors formation of a thermodynamically rather unfavored product through a templated reaction. In principle, these results highlight the general significance of spatial substrate recognition for enzymatic activity—with the only difference that here the irreversible addition of the enzyme to the modified substrate derivative is the net effect. In the case of the propargyl amide of ubiquitin, recognition of the entire protein suffices to generate an isopeptidase–ubiquitin complex stable enough to initiate the thiol addition to the alkyne. In the case of caspase-1, the tetrapeptide alkyne apparently

does not bind tight enough to effect the reaction. Here the 16-mer peptide was required to raise the binding affinity up to a level that enabled the templated thiol addition. As a result, the findings by the groups of Ovaa and Mootz do not generally question the biological inertness of alkynes. Only when binding is strong enough to induce templated reactivity is the carbon triple bond activated.

In a broader perspective, the reported findings on propargyl amides emphasize the potential of protein-templated reactions for the discovery of novel protein ligands. Noncovalent interactions with the protein template were demonstrated earlier to shift dynamic covalent equilibria on the protein surfaces and this effect has been exploited for the identification of novel, protein-binding ligands.[11] In contrast, in the examples discussed here, the protein template induces an irreversible reaction, which is not likely to proceed in solution. These examples possibly can be extended to other rather unreactive functional groups and future research will have to show how generally this principle can be applied to convert tight-binding protein ligands into relatively specific irreversible inhibitors.

The major practical relevance of the protein propargyl amides presumably will be in the area of active-site-directed probes. Already in the initial research the authors have demonstrated the reactivity of the propargyl amide toward the four major classes of DUBs. What is missing so far, however, are probes with selectivity for single DUBs or for defined subgroups of DUBs. Such further advanced probes would allow us to study the chemical biology of single enzymes or defined groups and thereby validate the hypotheses about the physiological and pathological relevance of these proteins. So far, this central challenge for the experimental use of active-site-directed probes in DUB research still remains: how can improved specificity of the probes be attained? In other words, how can probes be designed that do not react with *any* DUB, but with a defined subgroup of DUBs? Recent results from our own research show that this goal may already be reached by carefully selecting the chemical character of the reactive group attached to ubiquitin. Another approach for improved specificity may be to include into the protein-modifying substrates (ubiquitin, SUMO, Nedd 8) at least one isopeptide bond that mimics one of the naturally occurring branching points. In any case, even after more than 30 years of research on the ubiquitin pathway the field is still full of fundamental questions awaiting creative ideas.

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