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Reversible protein inhibitors kept on target
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substrate-recognition domains or enabling binding to different proteins from the usual ones. It will be important to explore how the properties of individual proteins are altered by these substitutions and how it affects cancer cells.

This substitution phenomenon has not been found in non-cancerous cells, nor in some types of cancer cell. The authors suggest that differences in the immune-cell microenvironments surrounding tumours are responsible for this difference between cancer cells. However, the lack of identification of the key factors underlying the generation of the substitutants is a limitation of this study. Pinpointing the molecular components involved and the specific conditions required for substitutants to arise is a crucial task for the future.

Pataskar and colleagues' discovery adds to a growing appreciation of the diversity and dynamic nature of genetic decoding[4]. The genetic code (the 'rule book' that defines the relationship between codons and amino acids) is not universal; many variants are known, including those used by our own mitochondrial organelles, and new variants continue to be discovered[5–7]. The change of a codon assignment in the genetic code is thought to be a slow evolutionary process involving either the loss of a specific codon from the entire genome or its ambiguous decoding as either one of two possible amino acids over a long period of time. Once changed, such an alteration is generally thought to be hard-wired, and most proteins in a cell are synthesized according to the genetic code of that cell, with a small number of exceptions at specific regions of protein-coding sequences (such as during the incorporation of non-standard amino acids, or as a result of a process called ribosomal frameshifting)[8].

However, instances of low-efficiency incorporation of the 'wrong' amino acids into proteins have been observed in many microorganisms. This deviation is thought to allow the microbes to diversify their complement of proteins and thereby adapt to stressful conditions, and it occurs particularly often during infection of a host. For example, species of the disease-causing bacterium *Streptomyces* use partial switching of a specified amino acid to aid their invasion of plants[9]. From the host's side, in response to viral attack, mammalian cells increase the random misincorporation of a sulfur-containing amino acid into their proteins[10]. Such an amino acid is more likely to react with and neutralize reactive oxygen species. This process is thought to buffer proteins against the damage mediated by reactive oxygen species that are generated by the infection. However, the example that Pataskar *et al.* discovered is striking because it is a more efficient and more specific response to a cellular challenge.

Are other examples of conditional changes to the genetic code's rule book awaiting discovery? We might know the answer reasonably soon. Freely available data sets providing results generated by an approach called mass spectrometry could be explored to determine whether such proteomics information indicates that a rise in specific amino-acid substitutions is associated with a particular condition or disease. Given the large number of data sets already available, it is surprising that substitutants were not discovered earlier. One possible reason, as noted by the physicist Richard Feynman, is that the human imagination is limited compared with that of nature. However, once one example is discovered, it does not require a lot of imagination to search for others.

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Chemical biology

Reversible protein inhibitors kept on target

Stephan M. Hacker

Compounds that form reversible covalent bonds with lysine amino-acid residues in proteins have high potential for drug discovery. A chemical group has been reported that prolongs the time for which such compounds bind to their targets.

In the past few years, drug-discovery researchers have become increasingly interested in covalent inhibitors – compounds that bind and inhibit a target protein by forming a covalent bond to it[1,2], rather than binding through non-covalent interactions alone, as most conventional small-molecule drugs do. A subclass of compounds known as reversible covalent inhibitors has particular promise, but the chemical groups available for their design can pose the challenge that they do not bind to proteins for long enough to exert an effect. Writing in the *Journal of the American Chemical Society*, Reja *et al.*[3] report a chemically reactive group that targets commonly found lysine amino-acid residues in proteins, and that prolongs the time spent by reversible covalent inhibitors at their binding sites.

Covalent inhibitors have attracted interest for three main reasons. First, they can have a higher binding affinity for proteins than do conventional drugs, which allows them to target shallow binding sites. Second, they have potentially longer durations of action than conventional drugs, which can lead to less-frequent drug dosing. And third, they can be targeted specifically to just one out of a group of closely related proteins[1].

Inhibitors that form irreversible covalent bonds to their targets have had many successes in drug development, with some now approved for use in the clinic[2]. However, the reactivity and selectivity of irreversible covalent inhibitors need careful tailoring to prevent these compounds from becoming permanently attached to off-target proteins, because such attachment can cause toxicity or allergies[1]. Compounds that form reversible covalent bonds offer a solution to this problem, because they can potentially disengage from off-target proteins. Nevertheless, precise fine-tuning of the kinetics of binding to, and dissociation from, proteins is still needed to ensure that reversible covalent inhibitors reside at the target protein for long enough to exert an effect.

Groundbreaking work on reversible covalent inhibitors has produced compounds that target the thiol (SH) group of non-catalytic cysteine residues in kinase enzymes[4]. However, cysteine is relatively rarely found in the proteome[5] (the complete set of proteins encoded by a genome), so it would be useful to develop reversible covalent inhibitors that form bonds to other amino-acid residues, to broaden the applicability of these compounds.

The lysine residue is particularly interesting

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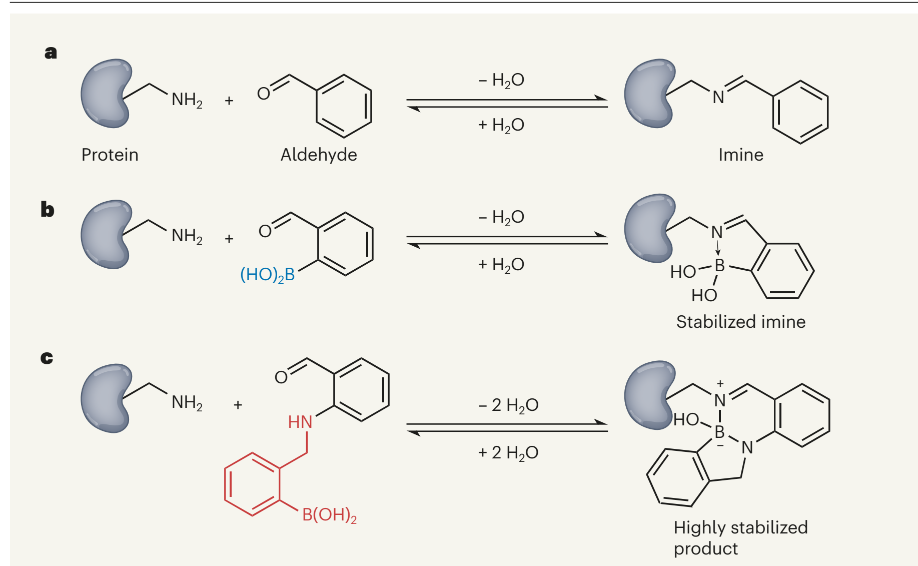

Figure 1 | Stabilization of products formed with lysine amino-acid residues. a, Aldehydes that react reversibly with the primary amine (NH_2) of lysine amino-acid residues of proteins are potentially useful as pharmaceutical protein inhibitors. However, simple aldehydes (such as the one shown) often produce imines that rapidly hydrolyse, re-forming the aldehyde and lowering the inhibitory activity. **b**, The attachment of a boronic acid group (blue) in proximity to the aldehyde allows the nitrogen atom in the imine to donate electrons (arrow) to the boron atom. This stabilizes the imine and slows hydrolysis. **c**, Reja *et al.*[3] report that the attachment of *o*-aminomethyl phenylboronic acid (AMPB; red), which contains both a boronic acid group and a nitrogen atom, stabilizes the product much more than does a boronic acid group alone. The authors used an AMPB group as the key component of an inhibitor for an enzyme of the disease-causing bacterium *Staphylococcus aureus*.

in this respect, partly because it is frequently found in the proteome (often in the active sites of proteins)[6], but also because it contains a primary amine group (NH_2) in its side chain – and many chemistries are available to form bonds to primary amines. Moreover, powerful methods for screening the proteome can be used to determine which proteins are bound by lysine-directed covalent inhibitors. This allows the reactivity and protein selectivity of these inhibitors to be tuned[6,7].

The reaction of primary amines with aldehydes to form imines (Fig. 1a) is particularly promising for the development of reversible covalent inhibitors. However, when simple aldehydes are used as reversible inhibitors, the rate of dissociation of the inhibitor from its target can be relatively high. For this reason, few target binding sites for simple aldehydes were identified in a proteome-wide study reported last year[7].

An attractive solution to this problem is to stabilize the imine product, which reduces the dissociation rate and thereby increases the length of time for which the inhibitors are bound to the target. One possibility is to use aldehydes known as salicylaldehydes, which produce an imine that is stabilized by an intramolecular hydrogen bond. This strategy was used in the development of voxelotor, a clinically approved drug that targets the primary amine at the amino terminus of haemoglobin to treat sickle-cell disease[8].

Another approach is to add a boronic acid group ($B(OH)_2$) in proximity to the aldehyde, which can stabilize the imine by accepting a pair of electrons from the nitrogen atom in the imine group[9] (Fig. 1b). This strategy has been used to develop reversible covalent inhibitors for difficult biological targets, including those involving protein–protein interactions[10]. Nevertheless, the half-lives of the resulting imines are still short (that is, the dissociation rates of the inhibitors are still relatively high) and few binding sites for boronic-acid-containing aldehydes have been identified in the proteome[7].

Reja *et al.* set out to stabilize the imine products even further by incorporating a strategically placed nitrogen atom close to the aldehyde, in addition to a boronic acid, producing a group called an *o*-aminomethyl phenylboronic acid (AMPB; Fig. 1c). The authors observed that the resulting aldehydes react with primary amines to produce a doubly stabilized product. By contrast, aldehydes that lack either the boronic acid or the nitrogen atom do not react substantially with primary amines under the same conditions. Excitingly, the AMPB-derived imines had half-lives of approximately 5–11 hours in experiments in which a solution of the compounds was diluted to induce imine hydrolysis (and therefore dissociation of the aldehyde); by comparison, imines that were stabilized solely by a boronic acid hydrolysed almost immediately.

Encouraged by the high stability of the AMPB-derived imines, Reja *et al.* went on to develop a reversible covalent inhibitor that contains the AMPB group. They identified a small molecule (a cyclic peptide) that binds non-covalently to the sortase A enzyme of *Staphylococcus aureus*, a disease-causing bacterium; sortase A is a potential target for drugs that combat *S. aureus* infections. The authors incorporated the AMPB group into the cyclic peptide and observed that the resulting molecule potently binds to isolated sortase A through covalent modification of a lysine residue in the enzyme. They also demonstrated that their compound inhibits sortase A activity in experiments with bacteria, thus establishing the AMPB group as a promising motif for use in other lysine-directed, reversible covalent inhibitors with long residence times.

Although the formation of imines that have long half-lives of many hours in biological systems could be beneficial for many applications, it could be undesirable in some cases. Such protein modification can be thought of as nearly irreversible, and therefore might cause some of the potential side effects of truly irreversible covalent inhibitors. Further research to precisely tune AMPB groups is warranted, and could yield a toolbox of reactive groups that produce imines with a range of stabilities. Indeed, such research is ongoing in Reja and colleagues' laboratory.

Another possible limitation of the AMPB group is its size: it might be too large to be grafted onto a wide range of conventional (non-covalent) small-molecule inhibitors to yield potent reversible covalent inhibitors. Even so, the AMPB group looks highly promising for specific applications. Moreover, Reja and colleagues' rational design of the AMPB group's imine-stabilization mechanism suggests that other reactive groups used in reversible covalent inhibitors could be similarly optimized, potentially enabling the development of many more groups suitable for clinical applications.

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