Rapid identification of the pharmacophore in a peptoid inhibitor of the proteasome regulatory particle†

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Here we report a simple and effective method to identify the minimal pharmacophore in the first peptoid inhibitor of the 19S proteasome regulatory particle, which has led to the development of a derivative that exhibits improved cellular activity, presumably due to a reduction in mass of about two-fold and the elimination of positively charged lysine-like residues.

The 26S proteasome is a 2.5 MDa multi-catalytic protease complex that degrades polyubiquitinylated and damaged proteins.[1] It is composed of a barrel-like catalytic 20S core particle (CP) that contains four stacked hetero-heptameric rings, two α and two β (Fig. 1).[2] The openings at the top and bottom of the barrel are small enough that folded proteins are precluded sterically from accessing the internal proteolytic active sites. Substrate introduction into the catalytic maw of the 20S CP is achieved through the activity of a 19S regulatory particle (RP) that caps each end of the 20S CP. The 19S RP contains a putative hetero-hexameric ring of ATPases (Rpt1-6) as well as several other proteins (Fig. 1). It binds polyubiquitinylated proteins, unfolds them, and feeds the resultant chain into the interior of the barrel where the proteins are degraded into small peptides.[3]

There is considerable interest in pharmacological inhibitors of proteasome function both as research tools[4] and as therapeutic agents.[5] However, while several compounds are known that target the proteolytic active sites in the 20S core, there were no chemical inhibitors of the 19S RP until our recent report[6] of the isolation of such a compound from a combinatorial library of peptoids (*N*-substituted oligoglycines).[7] This compound, which we called Regulatory Particle-Inhibiting Peptoid-1 (RIP-1) (Fig. 1) was shown to target one of the six ATPases in the 19S RP, Rpt4,[8] and blocks 19S RP-mediated protein unfolding *in vitro* with an IC_{50} of $\approx$3 μM.[6] RIP-1 also inhibits proteasome-mediated turnover of proteins in living cells, but with an IC_{50} of $\approx$50 μM. This differential between the *in vitro* and *in vivo* activity of the compound may reflect modest cell permeability. In this report, we take advantage of the modular structure of peptoids to develop a facile and rapid 'glycine scanning' method to identify the minimal pharmacophore in the molecule.

The data derived from this approach facilitated the development of a 'minimal' RIP-1 derivative with a molecular mass that is less than half that of RIP-1 and which exhibits $\approx$5-fold greater potency as a proteasome inhibitor in cultured cells, presumably due to enhanced cell permeability.

To identify residues in RIP-1 critical for activity, a series of derivatives was synthesized in which various parts of the molecule were deleted and each derivative was tested for activity. The assay employed in these experiments was to monitor the effect of the peptoid on 26S proteasome-mediated peptidolysis. Specifically, hydrolysis of a fluorogenic peptide, *N*-succinyl-Leu-Leu-Val-Tyr 7-amino-4-methylcoumarin (Suc-LLVY-amc), can be monitored conveniently *via* a spectroscopic assay[9] and is thus well-suited to evaluate the activity of significant numbers of compounds. In order for this reaction to occur, a 'flap', comprised of the N-terminal tails of the 20S α proteins, that otherwise blocks substrate access to the internal cavity of the proteasome (Fig. 2A), must be held open by the ATPases of the 19S RP.[10] This activity of the ATPases does not require ATP hydrolysis, in contrast to their protein unfolding activity.

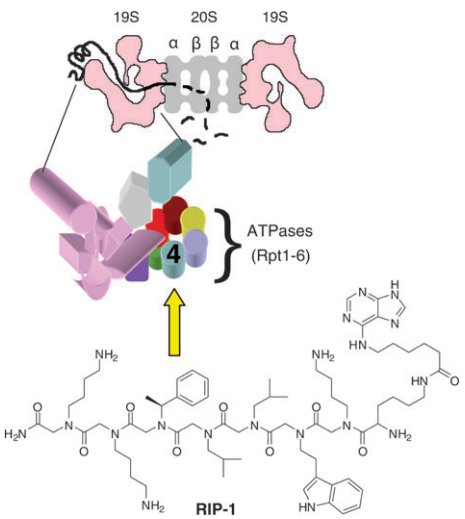

Fig. 1 RIP-1 is an inhibitor of the 26S proteasome that targets Rpt4. The ultrastructure of the 26S proteasome is shown at the top of the figure. It is comprised of a barrel-shaped central 20S core particle (CP) (gray) capped on each end with a 19S regulatory particle (RP) (pink). The 19S RP is, in turn, comprised of a lid sub-unit and a base subcomplex that includes six AAA class ATPases,[14] including Rpt4, the target of RIP-1.[8] The ATPases are responsible for ATP-dependent unfolding of substrate proteins.

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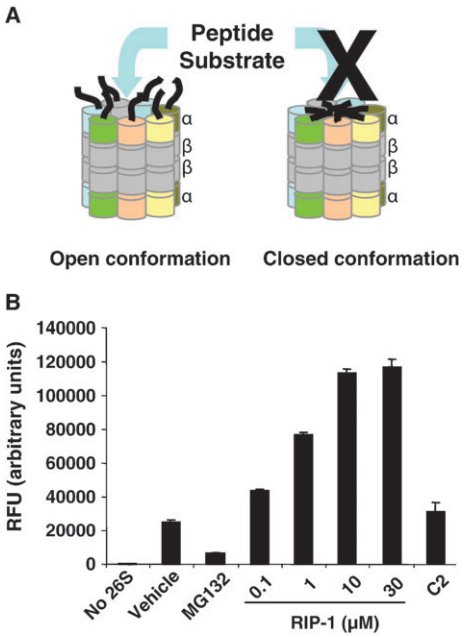

Fig. 2 RIP-1 stimulates 26S proteasome-mediated peptidolysis. (A) Hydrolysis of an unfolded peptide substrate by the 26S proteasome is inhibited by a 'flap' structure comprised of the N-terminal tails of the 20S CP α sub-units that occlude the opening into the catalytic interior of the complex (right). The ATPases of the 19S CP (not shown here) must hold these peptides in an 'open' conformation in order for the substrate to diffuse into the barrel. (B) Effect of RIP-1, MG132 and a control peptoid, C2, on this activity. Vehicle denotes the solvent in which the peptoids were dissolved.

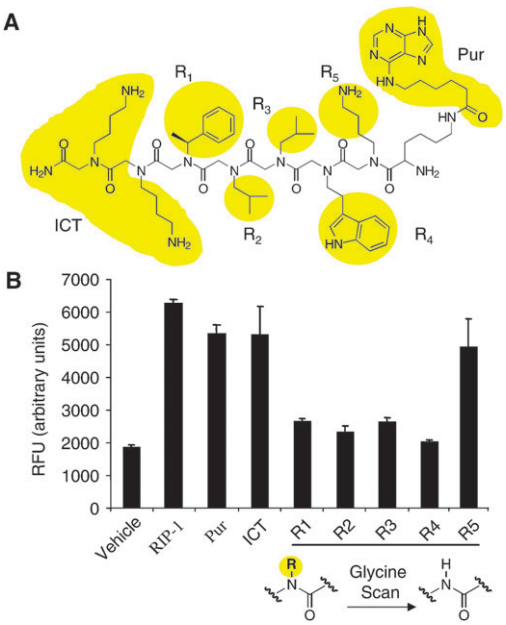

Fig. 3 Identification of the pharmacophore of RIP-1. (A) Structure of RIP-1 indicating the pieces that were eliminated in the various derivatives analyzed. ICT = invariant C-terminus; Pur = purine. In each derivative, the atoms included in the yellow region were replaced with hydrogen. (B) Activities of the various derivatives in the peptidolysis assay.

As expected, MG132, a peptide aldehyde inhibitor of the chymotrypsin-like activity of the proteasome, inhibits peptidolysis. In contrast, RIP-1 stimulates the peptidase activity of the 26S proteasome with an EC_{50} of $\approx$ 1 μM, whereas a control peptoid, C2, not selected to bind the proteasome had no effect (Fig. 2B). As will be discussed in a full paper on the mechanism of action of RIP-1 (H.-S.L. and T.K., in preparation), we believe that this stimulation is a result of the peptoid 'freezing' Rpt4 in a conformation suitable for flap opening. The EC_{50} of RIP-1 in this peptidolysis assay was essentially identical to the IC_{50} of the peptoid in blocking 19S RP-mediated protein unfolding,[6] arguing that the same peptoid–Rpt4 binding event is responsible for both activities.

Fig. 3A shows schematically the deletion strategy employed to identify the minimal pharmacophore of RIP-1. First, a derivative lacking the two lysine-like C-terminal residues (Nlys) was synthesized since these residues were not randomized in the library from which RIP-1 was isolated.[6] As shown in Fig. 3B, this had little effect on the peptidolysis stimulation activity. We then created a derivative lacking the purine-containing appendage. Surprisingly, this compound was only slightly less potent than RIP-1, showing that the nucleoside contributes only minimally to the activity of the molecule. Finally, in the context of the molecule lacking the two C-terminal Nlys residues, five additional derivatives were synthesized in which each side chain was, in turn, replaced with a hydrogen atom. This 'glycine scanning' approach is similar to the alanine scanning technology often used in biochemistry to identify amino acids important for protein–protein interactions or other functions, except that the 'point mutants' are made synthetically rather than being encoded at the DNA level. Only the Nlys residue near the N-terminal end of the molecule (R_5 in Fig. 3) was found to be dispensable for activity. Substitution of any of the other four groups with hydrogen essentially abolished the activity of the molecule.

The data shown in Fig. 3 led us to hypothesize that the minimal pharmacophore in RIP-1 is the tetrapeptoid shown in Fig. 4A. To test this idea, this molecule, called RIP-1.4, was synthesized and its activity in several biochemical assays was analyzed. As shown in Fig. 4B, RIP-1.4 stimulated 26S proteasome-mediated peptidolysis (EC_{50} $\approx$5 μM), though about 5-fold less potently than RIP-1. We then carried out an *in vitro* assay for proteasome-mediated protein unfolding developed in our laboratory.[11] This involves exposure of an immobilized Gal4-VP16·DNA complex to the proteasome. Binding of the 19S ATPases to the VP16 domain[12] of this chimeric protein allows the ATPases to engage Gal4-VP16 as a substrate for ATP-dependent unfolding, which is scored *via* monitoring the time-dependent loss of the protein from the DNA.[11] As shown in Fig. 4C, RIP-1.4 inhibited this unfolding process in a dose-dependent fashion with an IC_{50} of $\approx$5–10 μM, a potency that is again 30–50% that exhibited by RIP-1.[6] We conclude from these data that RIP-1.4 indeed represents the minimal pharmacophore of RIP-1, with *in vitro* activities no worse than 2- to 3-fold off those of the parent compound.

Since RIP-1.4 is less than half the mass of RIP-1 (604 *vs.* 1347 Da) and lacks the positively charged Nlys residues present in RIP-1, we speculated that it might be significantly more cell permeable than the parent compound. To test this point, the ability of RIP-1.4 to inhibit both p27 and p53

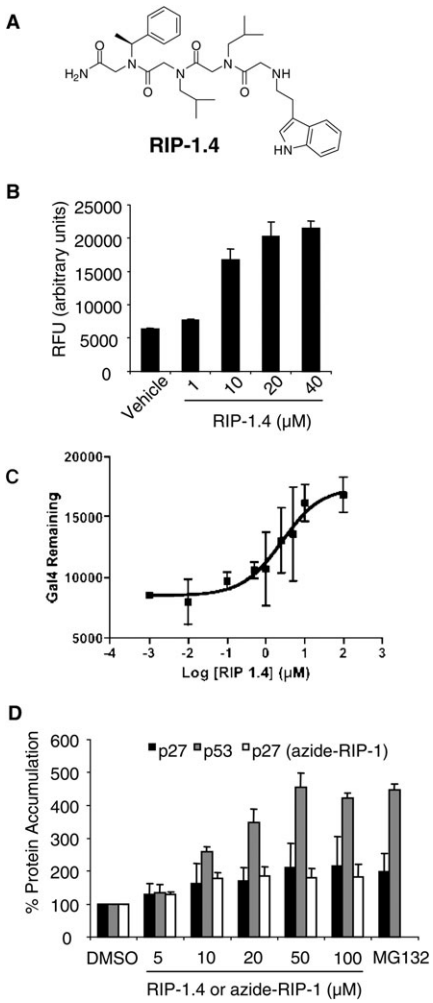

Fig. 4 A biologically active 'minimal' RIP-1 derivative. (A) Chemical structure of RIP-1.4, the proposed minimal pharmacophore of RIP-1. (B) Dose-dependent stimulation of 26S proteasome-mediated peptidolysis by RIP-1.4. (C) Dose-dependent inhibition of ATP-dependent unfolding of Gal4-VP16 by RIP-1.4.[11] (D) Dose-dependent inhibition of p27 (black bars) and p53 (gray bars) in C2C12 myoblasts by RIP-1.4. White bars indicate dose-dependent inhibition of p27 by azide-RIP-1.

turnover in cultured C2C12 muscle cells was measured. As shown in Fig. 4D, a build-up of both proteins was observed as increasing amounts of the peptoid were incubated with the cells. The results were identical for both proteins, as would be expected for a compound that targets the proteasomal ATPases and thus should affect the turnover of all proteins equally. The IC_{50} was approximately 10–15 μM, a value about 3- to 5-fold more potent than that measured for RIP-1 in the same assay.[6] Given the fact that RIP-1.4 is slightly more potent than RIP-1 *in vivo*, but somewhat less potent *in vitro*, we conclude that RIP-1.4 indeed exhibits enhanced permeability, as expected.

To assess the relative importance of mass reduction and elimination of the charged residues in the increased cell permeability of RIP-1.4, we synthesized a derivative of RIP-1 that substitutes neutral azide moieties for the three primary amines in RIP-1 (for the structure and synthesis, see ESI†) but has a similar molecular mass. We tested the effect of azide-RIP-1 on proteasome-mediated p27 turnover. As shown in Fig. 4D (white bars), azide-RIP-1 is almost as potent as RIP-1.4,[13] indicating that the elimination of charge is the dominant contributor to increased permeability,[14] though the reduced mass probably contributes somewhat as well.

This study highlights the simplicity with which structure–activity relationships (SARs) can be probed using peptoid lead compounds. The modular structure and simple 'sub-monomer' synthesis of peptoids[15] lend themselves to the rapid synthesis of a modest number of derivatives of a lead compound that allow for the rapid identification of the pharmacophore. In this case, this exercise suggested that the core pharmacophore of RIP-1 is a simple tetrapeptoid lacking the two C-terminal Nlys residues, the N-terminal Nlys residue and, surprisingly, the purine moiety that was employed to cap all of the molecules in the library.[6] Resynthesis of this much smaller and uncharged compound, called RIP-1.4, revealed that, indeed, it is nearly as potent as RIP-1 *in vitro* in the stimulation of 26S proteasome-mediated peptidolysis and the inhibition of protein unfolding (Fig. 3B and C) and is slightly more potent than RIP-1 in blocking proteasome-mediated protein turnover in living cells.

The next step in this work will be to create large numbers of derivatives of the minimal pharmacophore RIP-1.4 and to assay this new library for compounds with significantly increased potency. The discovery that this compound stimulates the peptidolysis activity of the 26S proteasome, an assay that is readily adaptable to high-throughput screening, should facilitate these efforts.

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Notes and references

1 G. N. DeMartino and T. G. Gillette, *Cell*, 2007, **129**, 659.
2 J. Lowe, D. Stock, B. Jap, P. Zwickle, W. Baumeister and R. Huber, *Science*, 1995, **28**, 533.
3 G. N. DeMartino and C. A. Slaughter, *J. Biol. Chem.*, 1999, **274**, 22123.
4 A. F. Kisselev and A. L. Goldberg, *Chem. Biol.*, 2001, **8**, 739.
5 J. Adams, *Trends Mol. Med.*, 2002, **8**, S49.
6 H. S. Lim, C. T. Archer and T. Kodadek, *J. Am. Chem. Soc.*, 2007, **129**, 7750.
7 R. J. Simon, R. S. Kania, R. N. Zuckermann, V. D. Huebner, D. A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C. K. Marlowe, D. C. Spellmeyer, R. Tan, A. D. Frankel, D. V. Santi, F. E. Cohen and P. A. Bartlett, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 9367.
8 H. S. Lim, D. Cai, C. T. Archer and T. Kodadek, *J. Am. Chem. Soc.*, 2007, **129**, 12936.
9 A. F. Kisselev and A. L. Goldberg, *Methods Enzymol.*, 2005, **398**, 364.
10 A. F. Kisselev, D. Kaganovich and A. L. Goldberg, *J. Biol. Chem.*, 2002, **277**, 22260.
11 A. Ferdous, D. Sikder, T. G. Gillette, K. Nalley, T. Kodadek and S. A. Johnston, *Genes Dev.*, 2007, **20**, 112.
12 C. T. Archer, L. Burdine and T. Kodadek, *Mol. BioSyst.*, 2005, **1**, 366.
13 At 50 and 100 μM of azide-RIP-1 in culture media, some precipitation was observed.
14 P. Yu, B. Liu and T. Kodadek, *Nat. Biotechnol.*, 2005, **23**, 746.
15 R. N. Zuckermann, J. M. Kerr, S. B. Kent and W. H. Moos, *J. Am. Chem. Soc.*, 1992, **114**, 10646.

