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Two ways to skin a cat: ADEP antibiotics can kill bacteria through activation or inhibition of ClpP activity

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Infection by *Mycobacterium tuberculosis* (Mtb) has had a devastating effect on the world population. This problem is exacerbated by the fact that antibacterial resistant strains are emerging at an alarming rate, highlighting the need to develop new approaches. The discovery that acyldepsipeptides (ADEPs) can kill bacteria by over activating the bacterial ClpP peptidase speaks to the unexplored possibilities for new antibiotics (Brötz-Oesterhelt *et al.*, 2005; Gersch *et al.*, 2015). ADEP antibiotics exert activity against many bacteria, including Mtb (Brötz-Oesterhelt *et al.*, 2005; Sass *et al.*, 2011; Ollinger *et al.*, 2012; Carney *et al.*, 2014), with the assumption that uncontrolled proteolysis by ClpP is the common mode of killing. In a new paper, work from the Brötz-Oesterhelt lab shows that, although ADEPs can activate Mtb ClpP *in vitro*, cell death more likely comes from a failure to form active protease complexes that require ClpP.

Proteolysis in Clp systems is controlled by AAA+ unfoldases, such as ClpC or ClpX, that capture the energy from ATP hydrolysis to bind, unfold, and feed substrates into the catalytic ClpP chamber for degradation (Figure 1) (Baker and Sauer, 2012). ADEP binds ClpP in hydrophobic patches that encircle the normally closed pore to stabilize an ‘open’ conformation (Figure 1) (Lee *et al.*, 2010; Li *et al.*, 2010). This open form of ClpP is able to hydrolyze any polypeptide that gains access to its active sites, leading to trimming or destruction of proteins (Kirstein *et al.*, 2009). Prior to this work, the antibiotic activity of ADEP was thought to be due to the activation of uncontrolled ClpP proteolysis (Brötz-Oesterhelt *et al.*, 2005; Kirstein *et al.*, 2009; Sass *et al.*, 2011; Gersch *et al.*, 2015). For example, in *Bacillus subtilis*, ADEP activation of ClpP results in destruction of the essential cell division protein FtsZ (Sass *et al.*, 2011). Interestingly, the patches used by ADEP to bind ClpP are also used by the attendant AAA+ unfoldases, therefore ADEP could also block formation of protease complexes such as ClpCP and ClpXP (Figure 1). Because these proteases are essential in mycobacteria (Ollinger *et al.*, 2012; Raju *et al.*, 2012; Raju *et al.*, 2014), it is possible that the antibiotic activity of ADEP in these bacteria results from inhibiting these protease complexes rather than activation of ClpP. By using purified proteins and *Mycobacterium bovis* as a proxy for Mtb, Famulla, et al. now present support for this intriguing possibility.

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The active ClpP peptidase from most bacteria is composed of 14 ClpP monomers forming a two-ring chambered enzyme. By contrast, mycobacteria encode two types of ClpP proteins, ClpP1 and ClpP2, that form separate heptameric rings which come together to form the final ClpP1P2 peptidase (Benaroudj *et al.*, 2011; Akopian *et al.*, 2012). The asymmetry of the peptidase is important as the ClpP2 ring seems preferred for the binding AAA+ unfoldases or ADEP (Schmitz *et al.*, 2014; Leodolter *et al.*, 2015; Li *et al.*, 2016). For ADEP activation of ClpP1P2 *in vitro*, ligand mimics such as nonhydrolysable dipeptides are required (Akopian *et al.*, 2012; Schmitz *et al.*, 2014; Li *et al.*, 2016; Famulla *et al.*, 2016) (Figure 1). These unique properties of ClpP1P2 lead the authors of this current work to speculate that ADEP activation of ClpP1P2 might not be sufficient to kill mycobacteria.

As FtsZ had been previously shown to be a particularly sensitive substrate of ADEP activated ClpP in *Bacillus* (Sass *et al.*, 2011) (Figure 1), the authors first determined if FtsZ could be a fragile ClpP1P2 substrate in mycobacteria as well. Using purified Mtb ClpP1P2 and FtsZ, they found that activated Mtb ClpP1P2 was unable to degrade Mtb FtsZ and failed to degrade *Bacillus* FtsZ (Figure 1). Levels of FtsZ in *M. bovis* do not fall during ADEP induced cell death, suggesting that uncontrolled turnover of FtsZ does not underlie the toxic effects of ADEP in this system. Finally, the authors validated that ADEPs inhibit the activity of ClpP1P2 dependent proteases, specifically ClpC1P1P2 (Raju *et al.*, 2014; Leodolter *et al.*, 2015; Famulla *et al.*, 2016).

Based on these *in vitro* observations, it remained possible that ADEP kills mycobacteria by activating ClpP1P2 destruction of proteins other than FtsZ. Rather than pursuing a fishing expedition for this potential substrate Famulla, et al. determined the mode of ADEP killing in a more general fashion. They reasoned that if ClpP1P2 activation was required for ADEP killing, then depletion of ClpP1P2 should protect cells from ADEP. By contrast, if ADEP blocked ClpP1P2-dependent protease activity, then depletion of ClpP1P2 should sensitize cells to ADEP. Because ClpP1P2 is essential, it was important that the authors picked conditions where these lower peptidase levels had minimal basal effects. After identifying these conditions, the authors showed that lowering ClpP1 levels resulted in sensitizing cells to ADEP (Famulla *et al.*, 2016). They showed that this was in sharp contrast to the case in *Bacillus*, where a similar depletion of ClpP results in protection against ADEPs (Sass *et al.*, 2011; Famulla *et al.*, 2016). The clear synergistic effect of ADEP activity and depletion of ClpP1P2 in mycobacteria provides satisfying evidence that ADEP kills *Mycobacteria* not by promoting unrestrained ClpP1P2 activity, but by blocking formation of ClpP1P2 dependent protease complexes.

In a more general sense, this work highlights the fact that antibiotics may impact multiple pathways so that the major mode of antibiotic action in different bacteria may differ among susceptible species. With particular regard to combating resistance, it is urgent that we understand how different bacteria respond to similar antibiotics in order for us to either reduce or predict resistance mechanisms. Finally, this work specifically showcases how highly conserved proteolytic systems in bacteria are excellent targets for new antibiotics as either inhibiting or activating these systems seem to be effective treatments.

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Figure 1.

How ADEPs kill bacteria is not common to all bacteria (A) During normal energy dependent degradation folded substrates (green) are translocated into the ClpP peptidase chamber by AAA+ unfoldases. (B) Activation of mycobacterial ClpP1P2 by ADEP antibiotics requires additional dipeptide ligand mimics that together result in pore opening and unconstrained proteolysis. In *Bacillus*, ADEP activation of ClpP leads to uncontrolled degradation of essential protein (such as FtsZ). By contrast, Famulla et al, now show that in *Mycobacterium*, cell death is due to a loss of active AAA+ protease complexes. In these cases, the principal toxicity of ADEP is the inhibition of active complexes of AAA+ unfoldases and ClpP1P2 chambered peptidases.