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Combinatorial active-site variants confer sustained clavulanate resistance in BlaC β-lactamase from *Mycobacterium tuberculosis*

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Abstract: Bacterial resistance to β-lactam antibiotics is a global issue threatening the success of infectious disease treatments worldwide. *Mycobacterium tuberculosis* has been particularly resilient to β-lactam treatment, primarily due to the chromosomally encoded BlaC β-lactamase, a broad-spectrum hydrolase that renders ineffective the vast majority of relevant β-lactam compounds currently in use. Recent laboratory and clinical studies have nevertheless shown that specific β-lactam–BlaC inhibitor combinations can be used to inhibit the growth of extensively drug-resistant strains of *M. tuberculosis*, effectively offering new tools for combined treatment regimens against resistant strains. In the present work, we performed combinatorial active-site replacements in BlaC to demonstrate that specific inhibitor-resistant (IRT) substitutions at positions 69, 130, 220, and/or 234 can act synergistically to yield active-site variants with several thousand fold greater *in vitro* resistance to clavulanate, the most common clinical β-lactamase inhibitor. While most single and double variants remain sensitive to clavulanate, double mutants R220S-K234R and S130G-K234R are substantially less affected by time-dependent clavulanate inactivation, showing residual β-lactam hydrolytic activities of 46% and 83% after 24 h incubation with a clinically relevant inhibitor concentration (5 μg/ml, 25 μ*M*). These results demonstrate that active-site alterations in BlaC yield resistant variants that remain active and stable over prolonged bacterial generation times compatible with mycobacterial proliferation. These results also emphasize the formidable adaptive potential of inhibitor-resistant substitutions in β-lactamases, potentially casting a shadow on specific β-lactam–BlaC inhibitor combination treatments against *M. tuberculosis*.

Keywords: Class A β-lactamases; β-lactam; antibiotic resistance; *Mycobacterium tuberculosis*; site-directed mutagenesis; clavulanic acid; combinatorial mutagenesis