SIMULTANEOUS RANDOMISATION OF EIGHT KEY ACTIVE SITE RESIDUES IN *E. COLI* NFSA TO GENERATE SUPERIOR NITROREDUCTASES FOR PRODRUG ACTIVATION.

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There is a substantial gap between the levels of enzyme activity Nature can evolve and those that scientists can engineer in the lab. This suggests that conventional directed evolution techniques involving incremental improvements in enzyme activity may frequently fail to ascend even local fitness maxima. This is most likely due to an inability of step-wise evolutionary approaches to effectively retain mutations that are beneficial in combination with one another, but on an individual basis are neutral or even slightly deleterious (i.e., exhibit positive epistasis). To overcome this limitation, we are seeking to “jump” straight to an enzyme with peak activity by conducting simultaneous mass randomisation of eight key active site residues in *Escherichia coli* NfsA, a nitroreductase enzyme that has several diverse applications in biotechnology. Using degenerate codons, we generated a diverse library containing 425 million unique variants. We then applied a powerful selection system using either or both of two recently identified positive selection compounds, which has enabled us to recover a diverse range of highly active nitroreductase variants. These have been screened against a panel of prodrug substrates to identify variants that are improved with specific prodrug substrates of interest. A primary focus has been developing nitroreductases as tools for targeted cell ablation in zebrafish. The basic system involves co-expression of a nitroreductase and fluorescent reporter under the control of a cell type specific promoter in a transgenic fish. Expression of the nitroreductase selectively sensitises target cells to a prodrug which, following nitroreduction, yields a cytotoxic compound that causes precise targeted cell ablation. We have identified several nil-bystander prodrugs that are able to selectively ablate nitroreductase expressing cells with no harm to nearby cells, and have paired these with highly specialised NfsA variants to improve the efficacy and accuracy of cell ablation. We have also screened our mass-randomisation libraries to recover nitroreductases that have non-overlapping prodrug specificities, to be used in a multiplex cell ablation system. This expands upon the previous system, by using pairs of selective nitroreductases and two different prodrugs to facilitate independent ablation of multiple cell types. For example, we have identified a specialist NfsA variant that has activity for tinidazole and not for metronidazole, achieved by including metronidazole as a simultaneous counter-selection during the initial positive selection process. This elegant positive/negative selection eliminated activity with metronidazole, while still ensuring that some level of nitroreductase activity was retained overall.