

1 **Rapid evolution of reduced susceptibility against a balanced dual-**
2 **targeting antibiotic through stepping-stone mutations**

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26 **Abstract**

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28 Multi-targeting antibiotics, i.e., single compounds capable of inhibiting two or more bacterial targets
29 are generally considered as a promising therapeutic strategy against resistance evolution. The
30 rationale for this theory is that multi-targeting antibiotics demand the simultaneous acquisition of
31 multiple mutations at their respective target genes to achieve significant resistance. The theory
32 presumes that individual mutations provide little or no benefit to the bacterial host. Here we propose
33 that such individual, stepping-stone mutations can be prevalent in clinical bacterial isolates, as they
34 provide significant resistance to other antimicrobial agents. To test this possibility, we focused on
35 gepotidacin, an antibiotic candidate that selectively inhibits both bacterial DNA gyrase and
36 topoisomerase IV. In a susceptible organism, *Klebsiella pneumoniae*, a combination of two specific
37 mutations in these target proteins provide an over 2000-fold reduction in susceptibility, while
38 individually none of these mutations affect resistance significantly. Alarming, strains with decreased
39 susceptibility against gepotidacin are found to be as virulent as the wild-type *Klebsiella pneumoniae*
40 strain in a murine model. Moreover, numerous pathogenic isolates carry mutations which could
41 promote the evolution of clinically significant reduction of susceptibility against gepotidacin in the
42 future. As might be expected, prolonged exposure to ciprofloxacin, a clinically widely employed
43 gyrase inhibitor, co-selected for reduced susceptibility against gepotidacin. We conclude that
44 extensive antibiotic usage could select for mutations that serve as stepping-stones towards resistance
45 against antimicrobial compounds still under development. Our research indicates that even balanced
46 multi-targeting antibiotics are prone to resistance evolution.

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