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A complementary pair of rapid molecular screening assays for RecA activities

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Abstract

The bacterial RecA protein has been implicated in the evolution of antibiotic resistance in pathogens, which is an escalating problem worldwide. The discovery of small molecules that can selectively modulate RecA's activities can be exploited to tease apart its roles in the de novo development and transmission of antibiotic resistance genes. Toward the goal of discovering small-molecule ligands that can prevent either the assembly of an active RecA-DNA filament or its subsequent ATP-dependent motor activities, we report the design and initial validation of a pair of rapid and robust screening assays suitable for the identification of inhibitors of RecA activities. One assay is based on established methods for monitoring ATPase enzyme activity and the second is a novel assay for RecA-DNA filament assembly using fluorescence polarization. Taken together, the assay results reveal complementary sets of agents that can either suppress selectively only the ATP-driven motor activities of the RecA-DNA filament or prevent assembly of active RecA-DNA filaments altogether. The screening assays can be readily configured for use in future automated high-throughput screening projects to discover potent inhibitors that may be developed into novel adjuvants for antibiotic chemotherapy that moderate the development and transmission of antibiotic resistance genes and increase the antibiotic therapeutic index.

Keywords: RecA; Antibiotic resistance; DNA repair; Recombination; High-throughput screen

Bacteria maintain a dynamic balance between the contrasting needs to preserve genomic information and to generate genetic diversity. The repair of damaged DNA is essential to the maintenance of heritable genetic information, while the variation of that information drives evolutionary adaptation [1]. In *Escherichia coli*, the RecA protein helps balance these needs by detecting the influence of environmental stress on DNA replication and initiating a programmed response to the resulting DNA damage [2–6]. Recently, RecA functions have been linked to various aspects of bacterial pathogenicity, including the induction of toxin biosynthesis [7], antigenic variation [8], and survival responses to antibacterial agents [9,10]. Of particular interest is the identification of RecA as a likely player in the mechanisms leading to the de novo development and transmission of antibiotic resistance genes. In these respective phenomena, RecA facilitates the development of antibiotic resistance via its roles in stress-induced DNA repair [1,11,12] and in horizontal transfer of genes between organisms [13,14]. The importance of these processes in bacterial pathogenecity continues to make RecA an attractive target for mechanistic and pharmacologic study [15–17].

Although RecA is highly conserved and may play similar roles in other bacteria [6], RecA-dependent processes have not been elucidated in many pathogens of interest. To delineate its roles in pathogenicity, including the development of antibiotic resistance, potent and selective modulators of RecA function are needed. To the best of our knowledge, however, no small-molecule natural product inhibitor of RecA activities has been reported [15,16]. The present paper describes the development of a pair of

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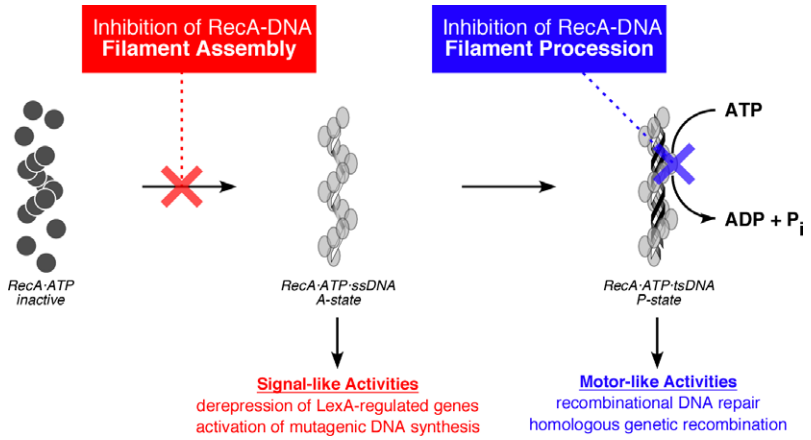

Fig. 1. Cartoons depicting the conformational states of RecA in the absence and presence of single-stranded DNA (ssDNA) and the two classes of activities relevant to the de novo development and transmission of antibiotic resistance genes. In the absence of DNA, RecA adopts an "inactive" conformation and a quaternary state favoring monomers and low aggregates (e.g., dimers and hexamers). In the presence of DNA and ATP, RecA adopts one of two "active" conformational states in which the protein self-assembles into a homopolymeric filament that coats the DNA strands (one RecA monomer per three DNA nucleotides). The A-state RecA-DNA filament, which requires ATP binding but not its hydrolysis, activates SOS by derepression of LexA-regulated genes. An important component of SOS is the overexpression and activation of low-fidelity DNA polymerases whose activity leads to heritable genetic changes in the bacterium. The P-state RecA-DNA filament, comprising RecA, ATP, and three DNA strands (tsDNA), uses ATP hydrolysis to carry out processive activities such as DNA recombinational repair and homologous recombination. These recombinational activities promote the horizontal transfer of antibiotic resistance genes. As described in the text, inhibitors that selectively bind the inactive conformation of RecA (red) would prevent nucleoprotein filament assembly, simultaneously precluding RecA's signaling and motor activities. Inhibitors that prevent the assembled RecA-DNA filament from hydrolyzing ATP (blue) would prevent only motor-dependent processive activities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

rapid, microvolume molecular screening assays to facilitate the discovery of potent RecA inhibitors from libraries of small molecules.

All RecA functions require formation of an active RecA-DNA filament comprising multiple RecA monomers, ATP, and DNA (i.e., states A and P in Fig. 1). This activated filament is responsible for two sets of biological functions: (1) induction of the SOS response to genomic damage by stimulation of LexA repressor autoproteolysis (state A in Fig. 1) [18] and (2) upon further DNA binding, direct participation in recombination and DNA repair (state P in Fig. 1) [6,19]. We posit that the discovery of small molecules that interfere with the assembly or subsequent processive activities of RecA-DNA filaments would be an important step in the development of inhibitors for the suppression of the development and transmission of antibiotic resistance. Moreover, we expect such agents to be useful as tools for dissecting resistance gene development and transmission pathways in bacterial pathogens. To tease apart the roles of RecA in these pathways, we envisaged two complementary sets of agents: one that can selectively suppress only the processive activities of the P-state RecA-DNA filament and a second that can prevent assembly of active RecA-DNA filaments altogether (blue and red text, respectively, in Fig. 1).

One strategy for developing RecA inhibitors is to exploit the structural differences between the active and the inactive conformers of the protein [20,21]. To carry out its biological functions, RecA must be bound to DNA in an active conformation (states A and P in Fig. 1); however, in the absence of DNA, RecA adopts an inactive conformation. Importantly, ADP and other select nucleotides stabilize the inactive conformer and inhibit the assembly of active RecA-DNA filaments [15,17,22–26]. Inhibitors of this class would abrogate all activities of the RecA-DNA filament, including both signaling and processive recombinational activities. In contrast, because the ATP-hydrolysis-dependent motor activities associated with recombination require the P state, inhibitors that are selective for the conformational P state may allow separation of the motor-like and signaling functions of RecA [27].

Toward the goal of discovering small-molecule ligands that can prevent either assembly of active RecA-DNA filaments or subsequent ATP-dependent motor activities (Fig. 1), we report the design and initial validation of a complementary pair of high-throughput-compatible screening assays suitable for the identification of inhibitors of RecA activities. One assay is based on established methods for monitoring ATPase enzyme activity and the second is a novel assay for RecA-DNA filament assembly using fluorescence polarization (FP)[1]. Our laboratory has recently reported the characterization of nucleotide analogs that are capable of differentiating between the active and the inactive conformations of RecA as a means of modulating the protein's activity [15,17]. Based on these results, we have used select nucleotide analogs as control compounds for assay validation. Importantly, this pair of screening assays allows the ready identification and segregation into

[1] *Abbreviations used:* FP, flurorescence polarization; HTS, high-throughput screening; DTT, dithiothreitol; SA-PMP, streptavidin paramagnetic particles.