

Small-Molecule Inhibitors of Bacterial AddAB and RecBCD Helicase-Nuclease DNA Repair Enzymes

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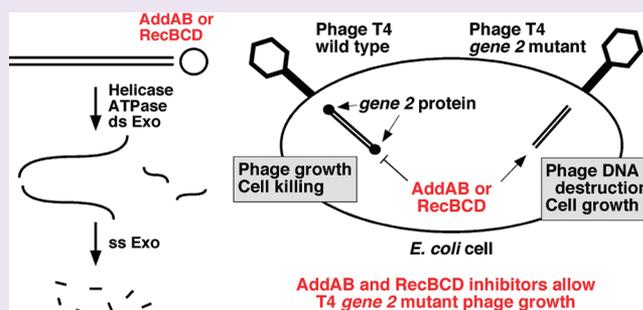
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S Supporting Information

ABSTRACT: The AddAB and RecBCD helicase-nucleases are related enzymes prevalent among bacteria but not eukaryotes and are instrumental in the repair of DNA double-strand breaks and in genetic recombination. Although these enzymes have been extensively studied both genetically and biochemically, inhibitors specific for this class of enzymes have not been reported. We developed a high-throughput screen based on the ability of phage T4 *gene 2* mutants to grow in *Escherichia coli* only if the host RecBCD enzyme, or a related helicase-nuclease, is inhibited or genetically inactivated. We optimized this screen for use in 1536-well plates and screened 326,100 small molecules in the NIH molecular libraries sample

collection for inhibitors of the *Helicobacter pylori* AddAB enzyme expressed in an *E. coli* *recBCD* deletion strain. Secondary screening used assays with cells expressing AddAB or RecBCD and a viability assay that measured the effect of compounds on cell growth without phage infection. From this screening campaign, 12 compounds exhibiting efficacy and selectivity were tested for inhibition of purified AddAB and RecBCD helicase and nuclease activities and in cell-based assays for recombination; seven were active in the 0.1–50 μM range in one or another assay. Compounds structurally related to two of these were similarly tested, and three were active in the 0.1–50 μM range. These compounds should be useful in further enzymatic, genetic, and physiological studies of these enzymes, both purified and in cells. They may also lead to useful antibacterial agents, since this class of enzymes is needed for successful bacterial infection of mammals.



Small-molecule inhibitors are exceptionally useful in enzymology and physiology, for they can allow immediate inactivation of an enzyme, either in the purified state or in cells. With multifunctional enzymes, such as those studied here, they can also halt an enzymatic reaction at an intermediate step of the reaction and permit, for example, determining the structure of reaction intermediates. Notable examples include the aminocoumarin and quinolone classes of DNA gyrase inhibitors and the camptothecin class of topoisomerase I inhibitors. In these cases, inhibitors were instrumental in showing that the proteins make covalent links with their DNA substrate.^{1,2} The quinolone inhibitors, such as ciprofloxacin, are also useful antibacterial agents, since DNA gyrase is widely distributed in bacteria but closely related enzymes appear to be absent in eukaryotes.

The RecBCD class of enzymes and the closely related AddAB enzymes are bacterial helicase-nuclease complexes important for repair of broken DNA and for genetic

recombination.^{3–5} Starting at a double-strand (ds) DNA end, these enzymes unwind DNA rapidly and highly processively while hydrolyzing ATP or another nucleoside triphosphate (Figure 1a). During unwinding, they also hydrolyze DNA by making endonucleolytic scissions at a rate dependent on the ratio of [ATP] to [Mg²⁺], both of which are required for the helicase and nuclease activities. The RecBCD enzyme of *Escherichia coli* makes endonucleolytic scissions at especially high frequency at Chi sites (5' GCTGGTGG 3'), which as a consequence are hotspots of recombination.⁶ The RecBCD and AddAB enzymes from other species similarly act at other short nucleotide sequences.⁷ The single-stranded (ss) DNA resulting from unwinding is a potent substrate for the enzymes' ATP-dependent ss nuclease, which at least for the RecBCD enzyme

Received: January 18, 2012

Accepted: February 21, 2012

Published: March 23, 2012

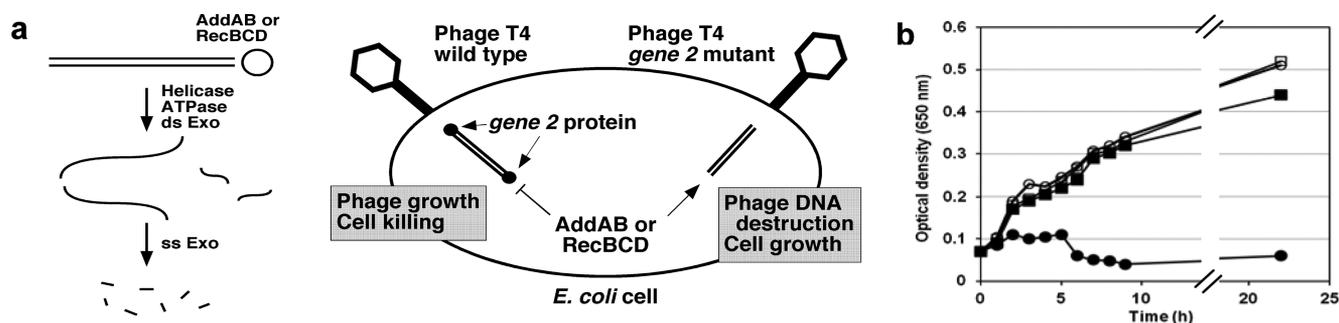


Figure 1. (a) Principle of the cell-based screen for AddAB and RecBCD inhibitors. (Left) Activities of RecBCD and AddAB helicase-nucleases. Both enzymes (open circle) are active on linear duplex DNA (double lines). ds exonuclease activity involves a combination of ATP-dependent DNA unwinding and endonucleolytic cuts. ss DNA intermediates are digested to short TCA-soluble oligonucleotides by the ss exonuclease activity. (Right) RecBCD or AddAB nuclease activity blocks the growth of phage T4 *gene 2* mutants. Upon injection into *E. coli* cells, wild-type T4 DNA is protected from AddAB and RecBCD nucleases by the *gene 2* protein bound to the linear duplex DNA ends in the virion; phages grow and the cells are killed. Unprotected T4 *gene 2* mutant DNA is digested by the nucleases; cells grow. Inhibition of AddAB or RecBCD is detected by lack of cell growth after T4 *gene 2* mutant infection. (b) T4 *gene 2* mutant phage prevent the growth of *E. coli* lacking RecBCD enzyme but not growth of wild type. *E. coli* strain V66 (*recBCD*⁺; open symbols) and strain V67 (*recB21*; closed symbols) were infected with T4 *gene 2* triple non-nonsense mutant phage (MOI = 0.01; circles) or not (squares). See Supplementary Figure 2 for details.

of *E. coli* produces a limit digest of primarily tetra- to hexanucleotides.⁸ Because this class of enzymes is important for the repair of DNA double-strand (ds) breaks, mutants lacking them are deficient in infecting mammals, likely because mammalian cells produce reactive oxygen species, such as hydrogen peroxide, that break the bacterial DNA upon infection.^{9,10} Inhibitors of these enzymes may therefore be effective antibacterial drugs.

Although these complex enzymes have been studied by both biochemistry and genetics for over 50 years,^{5,11} no small molecule inhibitors specific for them have, to our knowledge, been reported. Dziegielewska et al.¹² reported inhibition of RecBCD helicase activity by three compounds, but these compounds bind and alkylate DNA and likely would inhibit other helicases as well. Ca²⁺ and other divalent ions inhibit *E. coli* RecBCD and *Bacillus subtilis* AddAB nuclease,¹³ though not the helicase,^{14,15} but Ca²⁺ inhibits many other enzymes. The Gam protein of phage λ also partially inhibits RecBCD,^{16,17} perhaps by binding to the site at which DNA binds.^{18,19} Nonspecific inhibitors, such as EDTA and SDS, have also been used to stop the enzyme but have limited utility in studying reaction mechanisms.

To aid studies of RecBCD and AddAB and to find potential novel antibacterial agents, we have sought inhibitors of these enzymes by screening large libraries of small organic molecules. We developed a cell-based assay, so that compounds that do not enter *E. coli* would be immediately eliminated. The assay used here is based on the ability of phage T4 *gene 2* mutants to grow in *E. coli* only if the RecBCD nuclease is inactivated by mutation²⁰ (Figure 1a). The nuclease, which resides in the RecB polypeptide,²¹ is active only if the RecD subunit is present and only if the RecB helicase is active.^{22,23} The RecB helicase in turn is highly active only in the presence of the RecC subunit.²⁴ In AddAB, each subunit contains a nuclease domain; only AddA appears to have an active helicase domain, but its inactivation blocks all detectable nuclease activity.^{13,25–27} Consequently, small molecules that bind to any subunit of either enzyme might inhibit the nuclease, either directly or indirectly, and allow T4 *gene 2* mutant phage to grow and thereby block the growth of *E. coli*. We report here five structural classes of inhibitors of the three-subunit *E. coli*

RecBCD enzyme and the related two-subunit *Helicobacter pylori* AddAB enzyme.

RESULTS AND DISCUSSION

Rationale and Development of the Screen. Because phage T4 *gene 2* mutants grow in *E. coli* mutants lacking RecBCD nuclease activity but not in *E. coli* wild-type or other nuclease-deficient mutants,²⁰ RecBCD evidently is the only nuclease that blocks this mutant phage's growth. Wild-type T4 phage are able to grow in wild-type *E. coli* presumably because the *gene 2* protein binds to the ends of the ds DNA in the phage virions and, upon injection of the DNA into the host cell, blocks the action of RecBCD²⁰ (Figure 1). On the basis of these observations, we developed conditions that allow *E. coli recB21* null mutants but not *recBCD*⁺ cells to be lysed by T4 *gene 2* mutant phage. After infection at a multiplicity of infection (MOI) of 0.01 in liquid culture, *E. coli recB21* cells increase in OD for about 2 h and then cease growth, presumably when the phage have multiplied sufficiently to infect and begin to lyse most or all of the cells (Figure 1b and Supplementary Figure S1). Under these conditions, *recBCD*⁺ cells grow about the same with or without phage infection. *E. coli* cells bearing a deletion of the *recBCD* genes and harboring a plasmid expressing the *H. pylori addAB*⁺ genes also grow about the same with or without phage infection.¹⁰ We reasoned that an inhibitor of RecBCD or AddAB nuclease would allow T4 *gene 2* mutant phage to block the growth of *recBCD*⁺ (or *addAB*⁺) cells; an inhibitor specific for RecBCD or AddAB would not block the growth of uninfected cells. We used these criteria to screen for specific inhibitors of these two enzymes.

To screen large numbers of compounds in 0.1 mL cultures in 96-well plates, we found reproducible results by diluting freshly grown cells about 1:100 into LB broth containing compound and adding phage after 1 h of incubation. In every well *recB21* cells were lysed or failed to grow, and in nearly all of the wells *recBCD*⁺ cells grew to at least as high OD as without infection (e.g., Figure 1b and Supplementary Figure S1); however, in about 2% of the wells *recBCD*⁺ cells were also lysed. Similar results were found with *E. coli* expressing *H. pylori* AddAB (Amundsen and Londoño, unpublished data). We found that these wells with lysed cells contained revertants or pseudorevertants of the *gene 2* mutation, a nonsense mutation at codon

Table 1. Summary of Properties of 18 Most Active Compounds Found in the Screens for AddAB and RecBCD Inhibitors

CID [scaffold type] ^a	inhibition (approximate IC ₅₀ , μM)					relative recombinant frequency ^b		Chi hotspot ^c activity at 200 μM compound
	<i>E. coli</i> (<i>paddAB</i>) growth with T4 2 ⁻ phage	AddAB nuclease	<i>E. coli</i> <i>recBCD</i> ⁺ growth with T4 2 ⁻ phage	RecBCD nuclease	inhibition of RecBCD Chi cutting	<i>E. coli</i> Hfr recombination at 200 μM compound	phage λ recombination at 200 μM compound	
1045135 [A]	2.5	34	>40	13	10	16, 15	9, 14	4.1, 4.6
4831 [A]	ND ^e	>100	ND	>100	ND	0.2, 0.4	9, 11	3.1, 3.5
1517823 [A]	ND	26	ND	5.1	ND	20, 10	18, 23	2.5, 2.1
2295461 [B]	18	96	>120	51	20	37, 21	ND	ND
697851 [B]	ND	13	ND	33	>500	118, 121 ^d	ND	ND
771978 [C]	8.2	>100	>40	>100	>100	7, 5	0.4, 0.3	5.3, 5.2
693996 [C]	9.7	>100	86	>100	>500	6, 7	5, 6	4.9, 3.8
845583 [C]	10	>100	>120	>100	100	34, 42	ND	ND
5341736 [C]	4.9	>100	>120	>100	50	37, 43	ND	ND
3157809 [C]	>13	>100	>120	>100	>500	47, 51	ND	ND
2473636 [C]	4.8	>100	>120	>100	>500	65, 73	ND	ND
3577588 [C]	17	>100	>120	>100	20	47, 55	ND	ND
2345741 [C]	21	>100	>120	>100	>500	49, 63	ND	ND
774021 [C]	ND	>100	ND	>100	200	49, 54	ND	ND
2743870 [D]	12	130	>120	>100	>500	0.6, 0.3	90, 50	3.1, 2.9
4189453 [E]	ND	ND	>100	10	50	64, 72 ^d	ND	ND
4426551 [E]	ND	ND	>100	80	50	78, 67 ^d	ND	ND
43945278 [E]	ND	ND	ND	3	50	57, 47 ^d	ND	ND

^aSee Figure 2 and Supplementary Figures S4 and S6. A, pyrimidopyridone; B, cyanothiophene; C, nitrofurans; D, nitrothiazole; E, iminobenzothiazole. ^bData, from two experiments for each compound, are the recombinant frequencies as a percentage of that without compound. For Hfr recombination these were 8.7% and 8.9% His⁺ Str^R per viable Hfr cell in the two experiments, respectively; for λ recombination these were 11.2% and 3.4% J⁺ R⁺, respectively. ^cChi hotspot activity, measured in crosses between λ phages 1081 × 1082 and 1083 × 1084 as in ref 34, is the frequency of recombinants in an interval with Chi to that in the same interval without Chi. In the absence of compounds, values were 5.1 and 4.9 in the two experiments, respectively. ^dCompound was 100 μM; recombinant frequencies in the absence of compound were 7.6% and 8.4%. ^eND, not determined.

247 of 275 codons in the gene (Amundsen unpublished data) (NCBI NP_049754).²⁸ To circumvent this problem, we constructed a phage with three nonsense mutations, at codons 247, 248, and 249. [We were unable to construct a deletion of *gene 2*, presumably because part of the *gene 2* protein is also required for packaging phage DNA.²⁹] We have not observed revertants of this triple nonsense phage, designated *gene 2 am149*, in >10³ assays without a compound added.

Screen of 326,100 Compounds. To screen larger libraries, we converted the assay for use in 1536-well plates as detailed in Methods and summarized in Supplementary Table S1. The AddAB (strain V3065) phage assay was selected as the primary screen to test a total of 326,100 distinct chemical entities from the National Institutes of Health's Molecular Libraries Small Molecules Repository (MLSMR). All compounds were tested at 12 μM in singlicate. Primary screen results were reviewed, and 937 compounds that appeared nominally active ("hits") were advanced to secondary assays; 52 of these compounds were unavailable from the MLSMR. In

secondary assays, the 885 available compounds were first retested in triplicate in the primary screening assay to confirm activity. The same compounds were also tested in triplicate for their effect on the viability of strain V3065 (i.e., in the absence of phage) and also screened in triplicate in strain V66 in the presence of phage to determine RecBCD inhibition. From these efforts, 225 hits that appeared active in either the RecBCD or AddAB inhibition assays were advanced to titration assays. In titration assays, compounds were tested in triplicate as 10-point titrations using the same protocols used for secondary assays; IC₅₀ values were then determined. All HTS assays were determined to be robust, as each assay demonstrated Z'-scores greater than 0.8. A summary of the entire HTS effort, including summary statistics for all screening assays, is presented in Supplementary Table S2.

Direct Enzyme Assays of 12 Compounds. From the screens above, we chose the seven most potent compounds and five related compounds for direct tests with purified enzymes. These compounds, listed in Table 1 and diagrammed in Figure

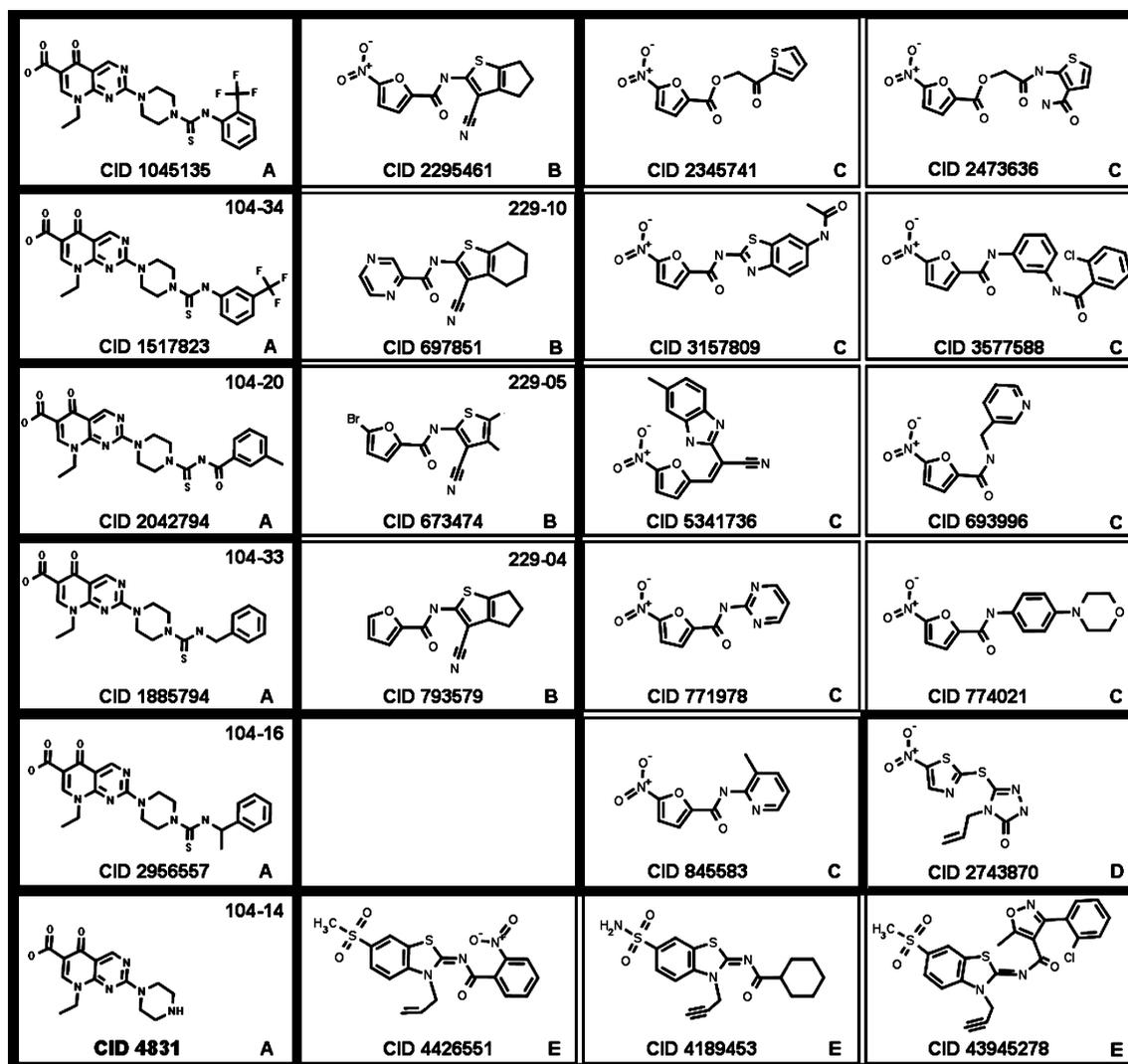


Figure 2. Structures of AddAB and RecBCD inhibitors. Compounds are grouped into “scaffolds” (A, B, C, D, and E in lower right corner of each panel) reflecting related structures. Trivial names, such as 104–34 and 229–10 in the upper right corner of some panels, refer to derivatives of the parent compound (see Supplementary Figures S4 and S6, which show additional compounds related to CID 1045135 [A] and CID 2295461 [B]). Heavy outlines separate compounds of different scaffold types.

2, form four structural classes, designated here “pyrimidopyridones” (group A), “cyanothiophenes” (group B), “nitrofurans” (group C), and “nitrothiazole” (group D with one member). For ease of identification, CID numbers are followed by the group designation in square brackets.

We first assayed the ability of the compounds to inhibit the ds exonuclease activity of purified RecBCD and AddAB enzymes. Compound concentrations from 0.2 to 500 μM were tested. IC_{50} values ranged from ~ 15 to >100 μM (Figure 3 and Table 1). For both enzymes, CID 1045135 [A] (a pyrimidopyridone) and CID 2295461 [B] (a cyanothiophene) were the most potent. In helicase assays, these compounds did not significantly inhibit AddAB (Supplementary Figure S2), but several inhibited RecBCD’s helicase and Chi-cutting activities. CID 2295461 [B] inhibited both of these activities with an IC_{50} of ~ 20 μM (Figure 4), and three nitrofurans (CID 3577588 [C], CID 774021 [C], and CID 845583 [C]) inhibited with IC_{50} ’s of <500 μM . CID 1045135 [A] appeared to inhibit in a biphasic way; it inhibited both helicase and Chi-cutting activities at 50 μM , but at 500 μM it appeared to stimulate the helicase and to change the position of specific cuts. Further

investigations are required to determine the nature of the effect of CID 1045135 [A] on RecBCD.

Further Tests of Structurally Related Compounds. To find more potent compounds, we tested 22 compounds related to CID 1045135 [A] and 15 compounds related to CID 2295461 [B]. In the T4 *gene 2* mutant-sensitization assay, used for the initial screen, derivative CID 1517823 [A] appeared RecBCD-specific and derivatives 104–29, 30, 31, and 34 appeared AddAB-specific, i.e., growth of cells with each enzyme was inhibited only if cells were infected with T4 *gene 2*⁻ phage (Supplementary Figure S3). (See Supplementary Figure S4 for the structures and CID numbers corresponding to these trivial designations of group A compounds.) Derivative CID 4831 [A] inhibited growth of cells with either enzyme even without phage infection; thus, this compound appears to inhibit *E. coli* growth independently of RecBCD or AddAB. Derivatives 104–20, 21, 32, 33, and 35, and to a lesser extent 104–15 and 16, also inhibited growth without phage infection, but only of cells with AddAB. This result may reflect the poorer growth of *E. coli* with AddAB than with RecBCD, perhaps because AddAB does not effectively utilize *E. coli* RecA but RecBCD does.²⁵ With

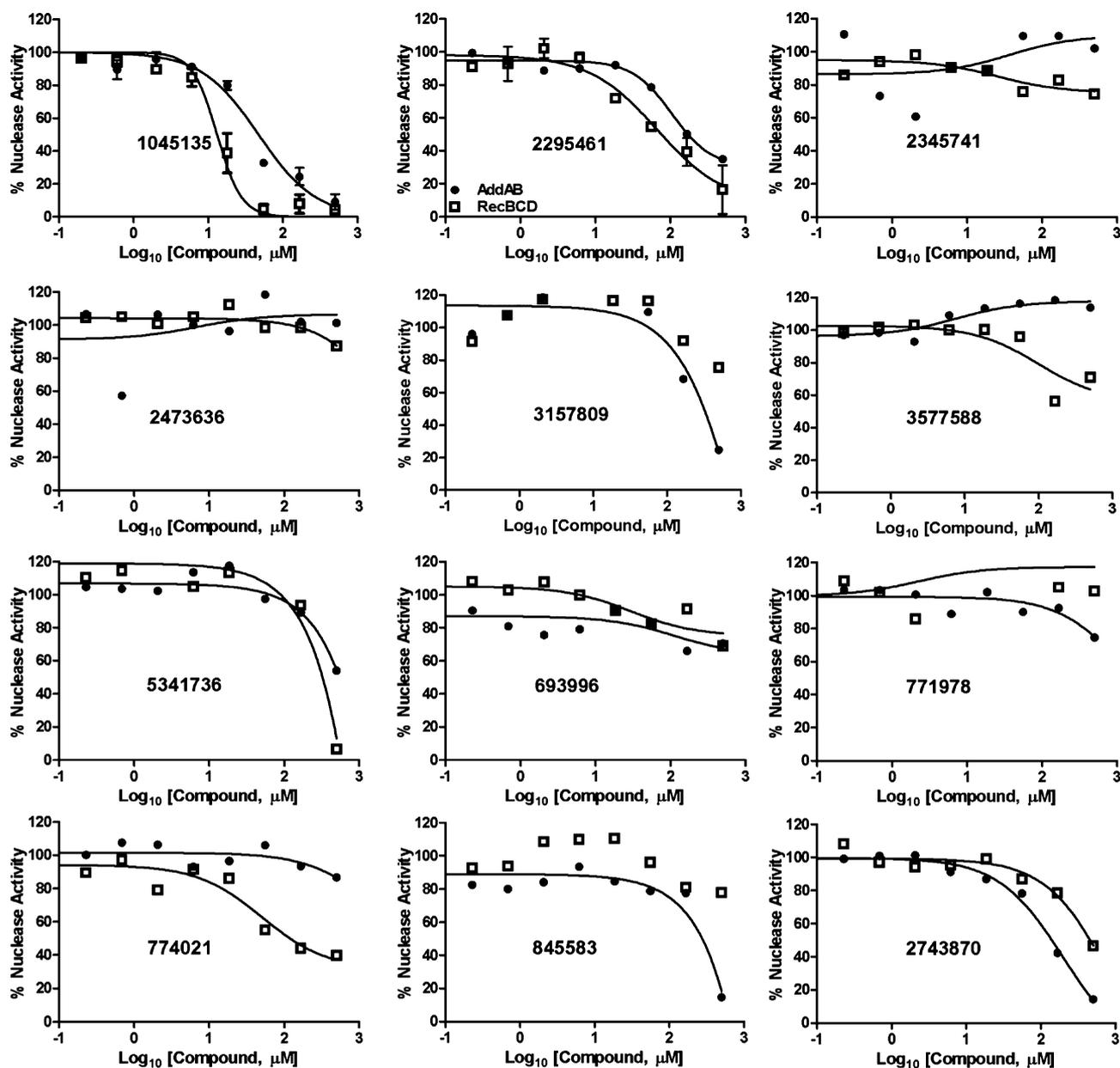


Figure 3. Inhibition of AddAB and RecBCD nuclease activities by the 12 most active compounds identified in the initial screen. ds exonuclease activity of AddAB (●) and RecBCD (□) was measured in the presence of the indicated concentration of each compound (identified by CID number) and expressed as a percent of the activity in the absence of compound. Curves were fit by GraphPad software using the four-parameter logistic nonlinear regression model. For CID 1045135 [A] and CID 2295461 [B] data are means \pm SEM; $n = 3$ or 4; for other compounds data are from one experiment. Structures are in Figure 2.

one exception, derivatives of CID 2295461 [B] did not greatly inhibit either enzyme in this cell-based assay; the exception was the parent compound, CID 2295461 [B], which slightly inhibited AddAB, though not as much as in the original screen at this concentration (50 μ M).

In direct tests of inhibition of the purified enzymes, we found that most of the derivatives of CID 1045135 [A] inhibited RecBCD nuclease activity and several inhibited AddAB nuclease activity (Supplementary Figure S5). More derivatives may inhibit in these assays than in the cell-based assay because we were able to test a higher concentration with purified enzymes (100 μ M) or because some compounds may not enter cells. Notably, however, derivative CID 1517823 [A] inhibited both enzymes in both cell-based and enzyme-based assays.

Derivative CID 4831 [A] did not inhibit either purified enzyme, a result consistent with its inhibition of cell growth independent of RecBCD or AddAB (Supplementary Figure S3). Thus, these results validate the cell-based screen.

Three derivatives of CID 2295461 [B] (229-04, 05, and CID 697851 [B]) inhibited the nuclease activity of both of the purified enzymes with IC_{50} 's of <100 μ M (Figure 5 and Supplementary Figure S5), although they did not significantly inhibit in either cell-based assay using T4 *gene 2* mutant infection. (See Supplementary Figure S6 for the structures and CID numbers corresponding to these trivial designations of group B compounds.) Thus, these compounds may not effectively enter *E. coli* cells. From these nuclease assays, compounds CID 1517823 [A] and CID 697851 [B] appear to

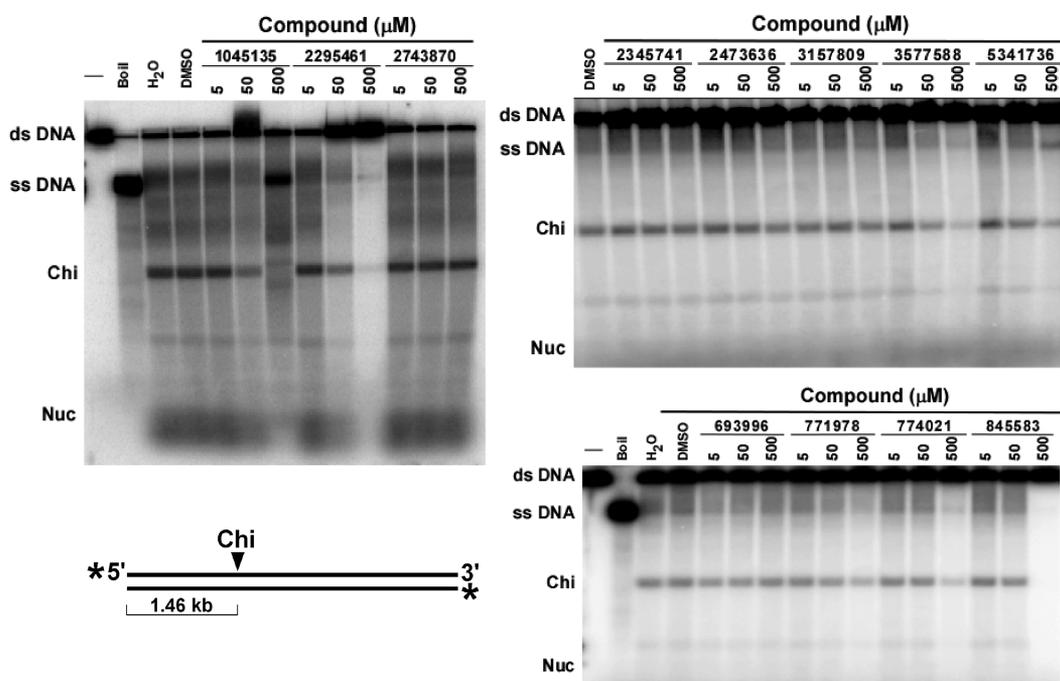


Figure 4. Inhibition of RecBCD unwinding and Chi-cutting activities by the 12 most active compounds identified in the initial screen. DNA unwinding and cutting at Chi hotspots by RecBCD enzyme was assayed in the presence of the indicated concentration of each compound (CID number shown). Unwinding is indicated by the amount of ss DNA, and Chi cutting by the amount of Chi-dependent 1.46 kb ss DNA fragment (“Chi”) produced from the 4.36 kb ds DNA substrate. “Nuc”, oligonucleotides. Structures are in Figure 2.

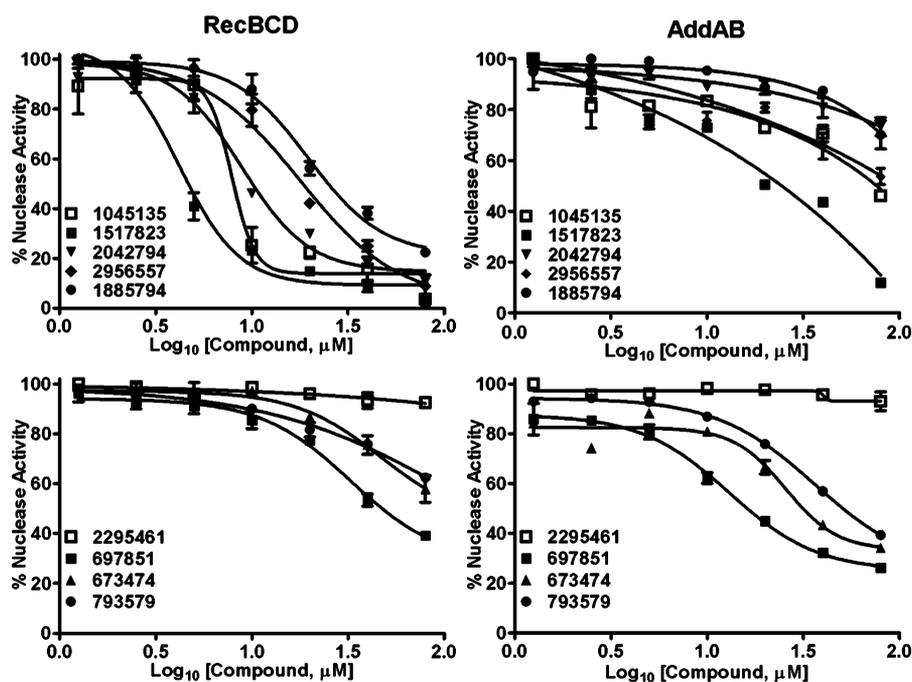


Figure 5. Inhibition of AddAB and RecBCD nuclease activities by derivatives of CID 1045135 [A] and CID 2295461 [B] (dose responses). ds exonuclease activity was measured in the presence of the indicated concentration of each compound and expressed as a percent of the activity in the absence of compound. Structures are in Figure 2 and Supplementary Figures S4 and S6.

be the most potent inhibitors identified in our screen. The IC_{50} of CID 1517823 [A] is $\sim 5 \mu M$ for RecBCD and $\sim 25 \mu M$ for AddAB and that of CID 697851 [B] is $\sim 30 \mu M$ for RecBCD and $\sim 15 \mu M$ for AddAB (Figure 5).

Assays of the RecBCD helicase and Chi-cutting activities showed that these two compounds inhibited these activities much like the parent compounds: CID 1517823 [A] inhibited

in a biphasic way and CID 697851 [B] in a monophasic way (IC_{50} 's of ~ 5 and $\sim 30 \mu M$, respectively); neither compound inhibited AddAB unwinding activity (Karabulut, unpublished data).

Inhibition of Intracellular Recombination. To explore further the ability of these compounds to inhibit RecBCD or AddAB in cells, we assayed the ability of the compounds to

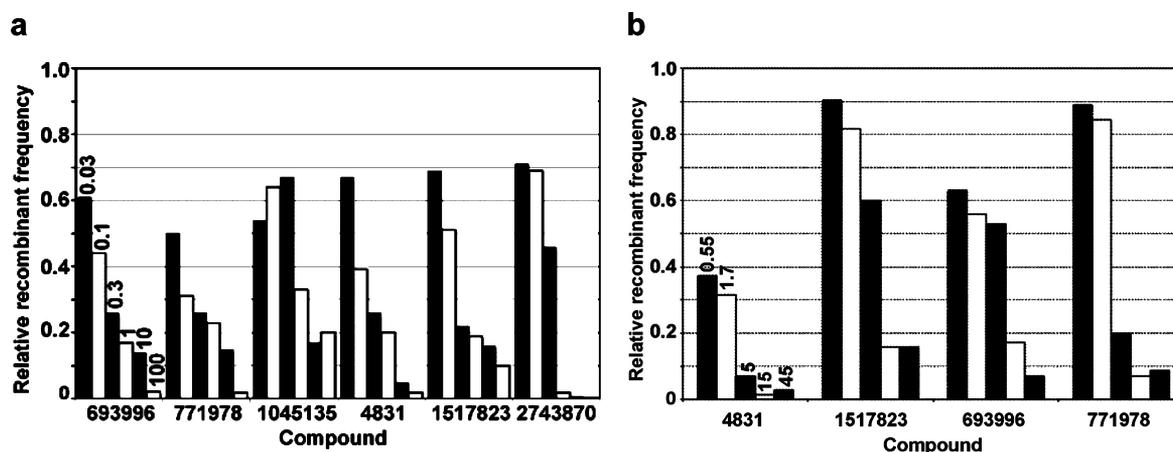


Figure 6. (a) Inhibition of *E. coli* Hfr recombination by selected compounds. The frequency of His⁺ Str^R recombinants in matings between strains V66 (F⁻ *recBCD*⁺ *hisG4* *rpsL31*) and V1306 (Hfr PO44 *hisG*⁺ *rpsL*⁺) in the presence of compound (concentration in μM as indicated for CID 693996 [C] applies to all compounds) is expressed as a fraction of that in the absence of compound (9.3% per viable Hfr cell). Structures are in Figure 2 and Supplementary Figures S4 and S6. Data are from one experiment; similar results were obtained in two others. (b) Inhibition of phage λ recombination by selected compounds. The mean frequency of J⁺ R⁺ recombinants in λ crosses (1081 × 1082 and 1083 × 1084) in strain V66 in the presence of the indicated compound (concentration in μM as indicated for CID 4831 [A] applies to all compounds) is expressed as a fraction of that without compound (6.9 ± 0.25%; *n* = 4). Structures are in Figure 2 and Supplementary Figures S4 and S6.

Table 2. Pathway Specificity of Hfr Recombination Inhibitors

pathway	genotype	fold-reduction in recombinant frequency ^a						
		null mutant ^b	2743870 [D]	1045135 [A]	4831 [A]	1517823 [A]	693996 [C]	771978 [C]
RecBCD	<i>recBCD</i> ⁺	1000	450	6.9	300	9	14	13
RecF	<i>recB21 recC22 sbcB14, C(D)</i>	1000	13	1.4	13	1.8	1.0	1.2
RecE	<i>recB21 recC22 sbcA23</i>	100	11	2.0	5.6	1.8	2.7	3.1

^aRecipient strains were grown and mated with donor strain V1306 (Hfr PO44) in LB plus the indicated compound (100 μM). Structures are in Figure 2 and Supplementary Figure S4. Data are the mean factor of reduction (*n* = 3) in recombinant frequencies compared to those of the untreated control, which were for V66 (*recBCD*⁺) 4.5%, 10.2%, and 11.1%; for JC9387 (*recB21 sbcB14 sbcC* or *D*) 1.3%, 2.2%, and 1.9%; and for JC8679 (*recB21 sbcA23*) 2.2%, 2.1%, and 1.8%. SEM or range was <20% of the mean (*n* = 2 or 3). Strain JC9387 (*recB21 sbcB14*) presumably also carries an *sbcC* or *sbcD* mutation.⁵² ^bThe factor of reduction in the frequency of recombinants observed in crosses between an Hfr donor strain and a recombination-deficient null mutant compared to that in the corresponding *rec*⁺ parent: for the RecBCD pathway V67 (*recB21*) compared to V66;³⁷ for the RecF pathway JC8111 (*recF143*) compared to JC9387;⁵³ for the RecE pathway N2510 (*recN262*) compared to JC8679.⁵⁴

inhibit *E. coli* Hfr-based recombination and phage λ recombination. When tested at 100 or 200 μM, seven of the initial 12 compounds reduced Hfr recombinant frequencies by less than a factor of 2, but the remaining five compounds and four of the derivatives inhibited more, by factors up to ~200 (Table 1, Figure 6a and Supplementary Figure S7). For six of these latter nine, dose–response assays showed that CID 693996 [C], CID 771978 [C], CID 1045135 [A], CID 4831 [A], CID 1517823 [A], and CID 2743870 [D] inhibited with IC₅₀'s of <1 μM (Figure 6a).

For CID 693996 [C], CID 771978 [C], and CID 4831 [A] this outcome was surprising, since these compounds did not significantly affect RecBCD nuclease, unwinding or Chi-cutting activities (Figures 3 and 4 and Supplementary Figure S5). These compounds at ≥40 μM inhibit cell growth without T4 *gene 2* mutant infection (Supplementary Figure S3; Table 1) and may inhibit some enzyme other than RecBCD required for recombination. For example, DNA gyrase, which is required for RecBCD pathway recombination³⁰ is inhibited by pyrimidopyridones such as CID 4831 [A] (pipemidic acid).^{31,32} In contrast CID 1045135 [A], CID 1517823 [A], and CID 2743870 [D] inhibited Hfr recombination with IC₅₀'s of <1 μM (Figure 6a), as well as RecBCD nuclease, unwinding, or Chi-cutting activities, albeit at higher IC₅₀ (Figures 3, 4, and 5 and Supplementary Figure S5). CID 1045135 [A] also inhibited Hfr

recombination when AddAB replaced RecBCD in *E. coli* cells: 20 μM CID 1045135 [A] reduced the recombinant frequency to that of cells lacking AddAB or RecBCD (0.02% His⁺ Str^R recombinants per Hfr donor cell), although this reduction was by a factor of only ~5 since AddAB does not confer as high recombination proficiency as RecBCD.²⁵ Inhibition by additional compounds of AddAB-dependent Hfr recombination was not tested because of instability of strains expressing both *H. pylori addAB* and *recA* from plasmids.

To determine if inhibition of Hfr recombination was specific to RecBCD, we tested the six compounds used in Figure 6a for inhibition of Hfr recombination by two alternative pathways, called RecE and RecF, that are activated by mutations (*sbc*) that suppress the recombination deficiency of *recB recC* null mutants.³³ Each compound inhibited recombination by the RecBCD (wild-type) pathway to a greater extent than recombination by the RecE or RecF pathway (Table 2). Two compounds, CID 2743870 [D] and CID 4831 [A], significantly inhibited recombination by the latter pathways by factors of 6–13, but the other four compounds inhibited recombination by these pathways only marginally if at all (by factors of ≤3). Thus, these data suggest that these six compounds are specific to RecBCD enzyme. They may, however, inhibit instead an enzyme required more stringently by the RecBCD pathway than by the other pathways, or both that enzyme and RecBCD.

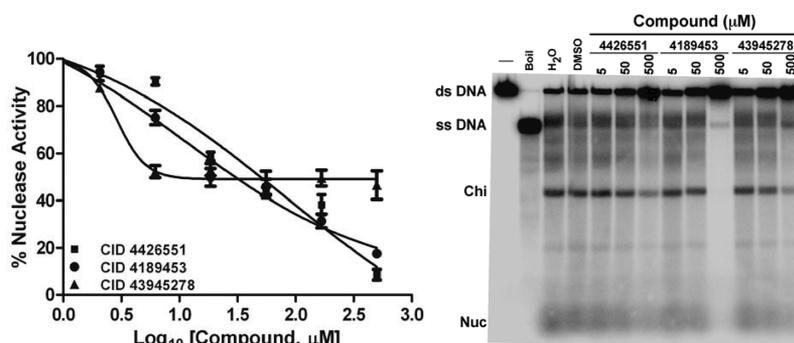


Figure 7. Inhibition of RecBCD nuclease, unwinding, and Chi cutting activities by “iminobenzothiazoles” [group E]. ds exonuclease activity (left panel) and unwinding and Chi cutting activities (right panel) were measured in the presence of the indicated concentration of each compound. ds exonuclease activity is expressed as a percent of the activity in the absence of compound. Unwinding is indicated by the amount of ss DNA, and Chi cutting by the amount of Chi-dependent ss DNA fragment (“Chi”). “Nuc”, oligonucleotides. Structures are in Figure 2.

For example, DNA gyrase, which is inhibited by CID 4831 [A],^{31,32} may be the target of CID 4831 [A], as suggested above. Inhibition of another component, such as RecA protein, required differentially, at least quantitatively, may also explain the differential inhibitions seen by these compounds. Nevertheless, the effects of CID1045135 [A], CID 1517823 [A], CID 693996 [C], and CID 771978 [C] on purified RecBCD (Figures 3, 4, and 5 and Supplementary Figure S5) and on intracellular recombination (Tables 1 and 2, Figures 6a, and Supplementary Figure S7) suggest that at least these four compounds inhibit recombination by RecBCD in cells.

To extend the intracellular assays, we tested the effects of the compounds on phage λ recombination dependent on RecBCD (the phages are *red gam* mutants). The most interesting results were obtained with CID 693996 [C], CID 771978 [C], CID 4831 [A], and CID 1517823 [A], which inhibited λ recombination with IC_{50} of <15, 5, 0.6, and 5 μ M, respectively (Figure 6b). These compounds also inhibited RecBCD-dependent Hfr recombination, as noted above. In λ crosses, the activity of the Chi hotspot, which regulates RecBCD activity,^{3–5} is measured as the ratio of the recombinant frequency in a genetic interval with Chi to that in the same interval without Chi; this ratio is \sim 5 in wild-type *E. coli* and 1 in *recBCD* null mutants, meaning that Chi is inactive in the absence of RecBCD.³⁴ Three compounds, CID 4831 [A], CID 1517823 [A], and CID 2743870 [D], significantly lowered Chi hotspot activity to a value of \sim 3 (Table 1). Since RecBCD directly interacts with Chi,⁶ this result suggests that the compounds directly inhibit RecBCD, but indirect effects on a step of recombination after that stimulated by Chi cannot be ruled out.

Another Class of Inhibitors Identified by Similarity to Other Helicase Inhibitors. Large-scale screens for three DNA helicases have been reported on PubChem: AID 485395 for *Staphylococcus aureus* replicative helicase,³⁵ AID 1945 for hepatitis C virus helicase NS3 helicase, and AID 2549 for human RecQ-like helicase 1. We noted that one class of compound, here called “iminobenzothiazoles”, appeared to be common among the reported inhibitors. We therefore tested 60 such compounds in a library from Life Chemicals. We tested these for inhibition of RecBCD nuclease activity and found three with IC_{50} of <100 μ M (Figure 7 and Supplementary Figure S8), but their dose responses were unexpected. CID 43945278 [E] detectably inhibited at concentrations as low as \sim 2 μ M; inhibition was \sim 50% at \sim 5 μ M and remained at that level at concentrations as high as 500 μ M. As their

concentrations were raised, CID 4426551 [E] and CID 4189453 [E] inhibited more gradually than expected for single-site inhibition: activity decreased from \sim 90% to \sim 10% over a range of \sim 2.5 \log_{10} rather than over 1 \log_{10} as expected for single-site inhibition.

These curious results may reflect differential inhibition of the two helicases in RecBCD, since DNA unwinding by both helicases is essential for full nuclease activity.^{22,36} CID 43945278 [E] may inhibit only one of the helicases, with IC_{50} of \sim 2 μ M, and this helicase may be responsible for only half of the nuclease activity measured. The other two compounds may inhibit this helicase with IC_{50} of \sim 10 μ M and the other helicase with IC_{50} of \sim 100 μ M, so that nuclease activity is inhibited only gradually as the concentration is raised. Further experiments are required to test this hypothesis.

DNA unwinding and Chi-cutting activities were also inhibited by these three compounds (Figure 7). The IC_{50} values, most readily quantified for Chi cutting, were \sim 50 μ M. In cell-based assays (sensitization to T4 *gene 2* mutant infection and Hfr-dependent recombination) these compounds were inactive at the concentration tested (100 μ M; Supplementary Table S7), which may explain why this class of compounds was not identified in the initial screens.

Advantages of a Cell-Based Screen. By using a cell-based assay, we would obviously study only compounds that entered *E. coli* cells sufficiently readily to inhibit the target enzyme, either the native RecBCD enzyme or the AddAB enzyme expressed from the *H. pylori* genes. In addition, these compounds must inhibit the enzyme in its natural environment, which might be markedly different from that of conditions normally employed to study the purified enzymes. Using these stringent criteria, we identified only about a dozen effective inhibitors from the 326,100 compounds initially tested, and four structurally different molecules (“scaffolds”) were identified. For two of these, we studied structurally related compounds and found six more potent inhibitors. Further structure–activity relationships (SAR) may reveal additional, highly potent inhibitors.

The assay used here is simple and inexpensive, since it employs only bacteria and phage, which are readily grown in large quantities, and reliable: Z' factors of \sim 0.9 were routinely observed (Supplementary Table S2). In principle this assay is specific for RecBCD or related nucleases, such as AddAB, since activity of the critical reagent used, phage T4 *gene 2* mutants' lysis of *E. coli* cells (Figure 1), is detected only in *recBCD* mutants.²⁰ As anticipated, we found compounds that inhibit

AddAB or RecBCD or both. The most potent compounds, CID 1045135 [A] (a “pyrimidopyridone”) and CID 2295461 [B] (a “cyanothiophene”), inhibited the ds exonuclease, DNA unwinding, and Chi-cutting activities of RecBCD and the ds exonuclease activity of AddAB (Figures 3, 4, and 5 and Supplementary Figure S5). Under the conditions used, neither compound inhibited the unwinding activity of AddAB (Supplementary Figure S2), but more sensitive assays under other conditions may reveal inhibition of AddAB unwinding activity. Thus, this screen indeed revealed compounds of the desired type.

An unexpected result of our screen, however, was the identification of CID 2743870 [D], which strongly inhibits Hfr recombination but appears to have little effect on purified RecBCD enzyme. Although Hfr recombination was inhibited at IC_{50} of $\sim 0.3 \mu\text{M}$ (Figure 6a), the nuclease was inhibited only at about 1000-fold higher concentration (IC_{50} of $\sim 200 \mu\text{M}$; Figure 3), and unwinding and Chi-cutting were not detectably inhibited even at $500 \mu\text{M}$ (Figure 4). Although differences in the cellular conditions versus those used with the purified enzyme may account for this marked disparity, CID 2743870 [D] may affect another component required for Hfr recombination, since it also significantly inhibits Hfr recombination by the RecE and RecF pathways (Table 2). For example, it may affect RecA protein loading onto DNA or its DNA strand exchange activity, since RecA is required for all three pathways of recombination.³³ The partial inhibition of Chi hotspot activity (Table 1) might also reflect inhibition of RecA, because RecA is required for the Chi-stimulated RecBCD pathway.³³ If indeed CID 2743870 [D] does not inhibit RecBCD, its identification in our screen must be fortuitous, because RecA is not needed to block the growth of phage T4 *gene 2* mutants,³⁷ the basis of our screen. We note that CID 2743870 [D] has been identified as an active compound in many screens listed on PubChem and may inhibit many cellular components.

Identification of Potent Inhibitors of AddAB and RecBCD Enzymes. By direct nuclease assays in the presence of ~ 20 compounds structurally related to each of the two initial compounds CID 1045135 [A] and CID 2295461 [B], we found more potent inhibitors. In our view, the most interesting are CID 697851 [B] (most active against AddAB) and CID 1517823 [A] (most active against RecBCD); their structures are in Figure 2. CID 697851 [B] inhibits AddAB nuclease with an IC_{50} of $\sim 15 \mu\text{M}$, but like its parent compound CID 2295461 [B] it does not detectably inhibit AddAB unwinding activity under the conditions used (Figure 5 and Supplementary Figures S2, and S5; Karabulut, unpublished data).

Like its parent compound CID 1045135 [A], compound CID 1517823 [A] inhibited all of the activities of RecBCD tested, both with purified enzyme and with cell-based recombination assays (Figures 3–6, Supplementary Figures S5, and S7; Tables 1 and 2). IC_{50} values were $\sim 3 \mu\text{M}$ for nuclease and Chi-cutting with purified enzyme (Karabulut, unpublished data) and $\sim 0.3 \mu\text{M}$ for Hfr recombination and $\sim 5 \mu\text{M}$ for phage λ recombination promoted by RecBCD; it also significantly reduced Chi hotspot activity in λ crosses (Table 1). It only marginally inhibits recombination by the *E. coli* RecE and RecF pathways, which do not employ RecBCD (Table 2). In the T4 *gene 2* mutant screen, it only slightly inhibits *E. coli* growth in the absence of phage but strongly inhibits in the presence of phage (Supplementary Figure S3). These observations indicate that CID 1517823 [A] specifically inhibits

RecBCD in cells. We expect this compound would have been detected in our initial screen of 326,100 compounds had it been in that library. Thus, compound CID 1517823 [A] has the properties that we expected for a specific inhibitor of RecBCD enzyme and is a strong validation of our screen.

Unexpected Properties of RecBCD Inhibitors CID 1045135 and 1517823. We were surprised that the IC_{50} values of these compounds were ~ 10 times lower in the intracellular assays for Hfr recombination than in assays with purified enzyme. This result may reflect differences in the enzyme's environment during the assays, or it may reflect some activity of RecBCD not yet assayed, such as the loading of RecA protein after action at Chi,³⁸ that is even more sensitive to inhibition than the nuclease, DNA unwinding, and Chi-cutting. In any case, this result encourages the possibility that these or related compounds may be effective antibacterial drugs, as discussed below.

The biphasic dose–response curve for inhibition of DNA unwinding and Chi-cutting by CID 1045135 [A] suggests a complex interaction between this compound and RecBCD enzyme. At $\sim 50 \mu\text{M}$ compound, unwinding and Chi-cutting were partially inhibited, but at $\sim 500 \mu\text{M}$ unwinding appeared to be stimulated and DNA was cut at novel positions (Figure 4). Since RecBCD has two helicases and a nuclease involved in these activities, the compound may have differential effects on two or more primary activities. For example, one helicase may be simply inhibited at low concentrations, and the other stimulated or altered at high concentrations. The cutting of DNA at novel positions is reminiscent of the behavior of RecBCD enzyme from two mutants altered in the RecB helicase domain, *recB2732* (Y803H) and *recB2734* (V804E). These mutant enzymes cut not at Chi but at a position that depends on the length of the DNA substrate.³⁹ We hypothesize that the RecB nuclease cuts wherever it is on the DNA when the faster helicase, RecD, reaches the end of the substrate and that the *recB* mutations sensitize the enzyme to a signal from Chi through RecC to stop RecD when RecBCD encounters Chi. CID 1045135 [A] may similarly sensitize the enzyme to signaling between RecD and RecB. In any case, further studies of the effects of CID 1045135 [A] and related compounds, such as CID 1517823 [A], may help elucidate how this complex enzyme works.

Searching for more effective inhibitors related to CID 1045135 [A], we tested CID 4831 [A], which has the pyrimidopyridone part but not the benzene ring part of CID 1045135 [A] (Figure 2). CID 4831 [A] did not detectably inhibit the nuclease activity of purified AddAB or RecBCD enzyme (Supplementary Figure S5), but it strongly inhibited Hfr and phage λ recombination (Figure 6 and Supplementary Figure S7). In the T4 *gene 2* mutant assay it inhibited *E. coli* growth with or without addition of phage (Supplementary Figure S3). These results indicate that CID 4831 [A] inhibits some cellular component other than RecBCD, such as DNA gyrase, which is inhibited by CID 4831 [A] (pipemidic acid)^{31,32} and is required for cell growth⁴⁰ and for recombination.³⁰ These facts suggest, in turn, that the benzene ring part of CID 1045135 [A] is critical for inhibition of RecBCD. Indeed, CID 1517823 [A], the *m*-trifluoromethyl isomer of CID 1045135 [A], is more potent than the *o*-trifluoromethyl parent compound in assays of both RecBCD and AddAB nuclease (Figure 5). The results of this limited SAR survey suggest that potent inhibitors of both RecBCD and

AddAB nuclease can be identified, an outcome important for preparing broad-spectrum antibiotics (see below).

Mode of Action of Inhibitors. Our primary screens were based on inhibition of the nuclease activity of AddAB or RecBCD, but because the nuclease activity depends on the helicase and ATPase activities, the inhibitors could act on any of these activities. None of the inhibitors significantly affected the AddAB helicase activity under the conditions used (Supplementary Figure S2). Although further experiments, including ATPase assays, are required to test more rigorously an effect on AddAB helicase, the inhibitors of AddAB nuclease activity, such as CID 2295461 [B] and its derivatives (Figure 3 and 5 and Supplementary Figure S5) may directly inhibit the nuclease. For RecBCD, the most potent inhibitors of the nuclease activity also inhibited the unwinding activity (Table 1), suggesting that these inhibitors (CID 1045135 [A], CID 1517823 [A], CID 2295461 [B], and CID 697851 [B]) may act primarily on the helicase or ATPase. In contrast, three compounds (CID 771978 [C], CID 5341736 [C], and CID 3577588 [C]) appear to inhibit the helicase but not the nuclease activity of RecBCD. This result may reflect the 200-fold higher ATP concentration used for the helicase assay than for the nuclease assay. Determining the primary target of the inhibitors will require more investigation, including the isolation of mutant enzymes resistant to the inhibitors. The requirement for RecBCD for growth of certain *E. coli* mutants, such as *ligA*, *polA*, and *rnh*,^{41,42} may enable isolation of resistant mutants and thereby determine, with the aid of the crystal structure of RecBCD,⁴³ the site of action of the inhibitors.

Potential for Identification of Novel Antibacterial Drugs. Inhibitors of AddAB and RecBCD are potentially useful antibacterial drugs for two reasons. First, these enzymes are required for repair of DNA damage inflicted upon bacteria by their host cells upon infection. *Salmonella recB* mutants are unable to kill a mouse,⁴⁴ and *H. pylori addAB* mutants less effectively colonize the mouse stomach than wild type.¹⁰ Second, RecBCD, and perhaps AddAB, is required for the induction of the SOS response to DNA damage, which includes the induction of mutagenic polymerases responsible for most induced mutations.^{45,46} Inhibition of RecBCD should thus lessen the evolution of bacteria resistant to the inhibitor, an important goal in current antibacterial drug therapy. The AddAB and RecBCD class of enzymes is found in ~90% of all bacterial species whose genomes have been sequenced and reported.⁴⁷ Thus, their inhibitors may be effective broad-spectrum antibiotics. CID 1045135 [A] and its derivatives may be especially effective, since they contain the pyrimidopyridone moiety that inhibits DNA gyrase and leads to dsDNA breaks whose repair requires RecBCD enzyme.⁴⁵ A derivative that inhibits both gyrase and RecBCD would thus lead to both DNA damage and the failure to repair it. Such a compound might be a single-molecule combination drug more effective than two drugs. The screen described here may be useful in finding such a drug.

METHODS

Bacterial and Phage Strains. The *E. coli* strains used are listed in Supplementary Table S3, and phage λ strains are in Supplementary Table S4. Phage T4 wild type and *gene 2 amNS1* mutant are from our collection.³⁷ A derivative bearing three adjacent nonsense mutations is described below. Stocks of T4 phage were grown in strain V67, which lacks RecBCD and does not suppress the nonsense mutation(s) so that the phage particles contain DNA not protected by *gene 2* protein.

Plasmids and Oligonucleotides. Plasmids are listed in Supplementary Table S5, and oligonucleotides are in Supplementary Table S6.

Bacterial Growth Media. Luria–Bertani (LB) broth contains 1.0% Tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. (All percentages in this paragraph are w/v.) LB agar is LB broth with 1.5% agar (Difco). Cation-adjusted Mueller–Hinton broth was purchased from Becton-Dickinson. TB contains 1.0% Tryptone and 0.5% NaCl; for phage λ crosses, 0.1% maltose was added. BBL top and bottom agar contain TB with 0.75% and 1% agars, respectively. BBL-YE is BBL bottom agar supplemented with 0.2% yeast extract. For growth of strains with plasmids, media were supplemented with ampicillin (100 $\mu\text{g}/\text{mL}$) or chloramphenicol (15 $\mu\text{g}/\text{mL}$). Cultures were grown at 37 °C.

Compound Libraries and Additional Compounds. For 1536-well screening, the Molecular Libraries Small Molecule Repository (MLSMR) library was provided by the NIH's Roadmap: Molecular Libraries Initiative. Details regarding compound selection for this library can be found online at <http://mli.nih.gov/mli/compound-repository/mlsmr-compounds/>. The MLSMR library is a highly diversified collection of small molecules, with more than 50% of compounds having molecular weights between 350 and 410 g/mol. These compounds comprise both synthetic and natural products, from either commercial or academic sources, that can be grouped into three categories: (1) specialty sets of known bioactive compounds, such as drugs and toxins, (2) focused libraries aimed at specific target classes, and (3) diversity sets covering a large area of the chemical space. Powders of compounds in Table 1 were obtained from ChemBridge, except for Chemical Identifier (CID) 693996, 4831, and 1517823 (Vitas-M lab), 2473636 and 2345741 (Enamine), 2743870 (Maybridge), 3577588 (ChemDiv), and 4189453, 4426551, and 43945278 (Life Chemicals). An additional library of ~18,400 compounds from Life Chemicals (Burlington, Canada) was a generous gift of Kineta, Inc. (Seattle, WA).

RecBCD and AddAB Enzyme Assays. Nuclease assays measured the formation of TCA-soluble radioactive material from phage T7 [³H] DNA (2 $\mu\text{g}/\text{mL}$; 6 μM nucleotides) substrate in a 20 min incubation at 37 °C.⁴⁸ AddAB assays were in 50 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, polyvinylpyrrolidone (1 mg mL⁻¹), 1 mM DTT, and 50 μM ATP. RecBCD assays used the same condition but with 25 μM ATP. Compounds were diluted in DMSO and added to enzyme in assay buffer on ice; final DMSO concentration was 5.0% (v/v) in each assay. DNA substrate was added, and after <5 min the reactions were started by transferring the samples to 37 °C. Reactions were stopped by addition of calf thymus DNA to 0.2 mg mL⁻¹ and TCA to 5% (w/v). After 10 min on ice, the mixtures were centrifuged for 5 min at 16,100 \times g, and the soluble radioactive material was determined in a scintillation counter.

Helicase assays measured the formation of ss DNA from 5' [³²P] pBR322 χ^+ F (or χ^0 control) DNA (0.1 nM molecules) linearized by digestion with *Hind*III enzyme. AddAB assays were in 25 mM Tris-acetate (pH 7.5), 2.0 mM Mg(OAc)₂, 5 mM ATP, 1.5 μM SSB and used 1 nM enzyme. RecBCD assays used the same condition but with 0.15 nM enzyme and without SSB. Compounds were added to the reaction mixture containing all the reagents except ATP; final DMSO concentration was 2.5% (v/v) for each assay. (All other percentages in this paragraph are w/v.) Reactions were started by addition of ATP and were at 37 °C for 1 min (RecBCD) or 2 min (AddAB). Reactions were stopped by addition of 1/3 vol of stop buffer (2.5% SDS, 100 mM EDTA, 0.125% bromophenol blue, 0.125% xylene cyanol FF, and 10% Ficoll), and the products were subjected to electrophoresis in a 1.25% agarose gel at 5 V/cm for 2.5 h in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA). Gels were dried under vacuum, and the products were detected by autoradiography or analyzed with a Typhoon Trio PhosphorImager (GE Lifesciences) and ImageQuant TL software (Amersham). With RecBCD, this assay also detects cutting of DNA at the Chi site χ^+ F, which produces a 1.46 kb fragment.⁴⁹

Genetic Assays. *E. coli* Hfr recombination, phage λ recombination, and Chi hotspot activity assays were conducted as described.³⁷

To test for inhibition of Hfr recombination, recipient cells were grown in LB to an optical density (OD) of 0.25 at 650 nm. Compound was added, and incubation was continued until the OD reached 0.5 (typically 30–45 min later). An aliquot was mixed with donor strain V1306 (Hfr PO44) in the ratio of one donor cell per 10 recipient cells. After 8 min, the mixture was diluted 1:50 into LB with compound. After 20 additional min, cells were vortexed to separate mating pairs, diluted, and plated to select recombinants. Viability of the cells was not significantly affected by compounds during this 1.5 h incubation.

To test for inhibition of phage λ recombination and Chi hotspot activity, *E. coli* strain V66 (*recBCD*⁺) was grown as above except in TB plus 0.1% (w/v) maltose. Cells were infected with λ phages at an MOI of 5 each; cross 1 was λ 1081 \times λ 1082, and cross 2 was λ 1083 \times λ 1084. After 15 min, cells were diluted 1:100 into TB with compound at the same concentration, incubated at 37 °C for 90 min, and treated with chloroform. Phage were titered on strain 594 (*sup*⁺) for J⁺ R⁺ recombinants and on strain C600 (*supE44*) for total phage. Chi hotspot activity was measured as $\sqrt{(T1/C1)/(T2/C2)}$, where T1/C1 is the ratio of turbid (*c*⁺) to clear (*cl857*) J⁺ R⁺ recombinants in cross 1 and T2/C2 is the same for cross 2.³⁴

Construction of T4 gene 2 am149 Triple Nonsense Mutant Phage. Phage T4 gene 2, including 851 bp 5' and 842 bp 3' of the ORF, was amplified from a lysate of phage T4 gene 2 *amN51* in a DNA polymerase chain reaction using oligonucleotides OL2636 and OL2637, Platinum Taq Polymerase (Invitrogen), and the manufacturer's suggested conditions. The product was purified on a QIAquick column (Qiagen), digested with *Hind*III (New England Biolabs), and ligated to *Hind*III-cleaved pBR322 to yield plasmid pSA520. The sequence of gene 2 in this plasmid was that of wild type except for 5' TAG 3' at codon 247 (5' TGG 3' in wild type). Two additional nonsense mutations were introduced into this gene at codons 248 (5' GCG 3' \rightarrow 5' TAG 3') and 249 (5' AAC 3' \rightarrow 5' TAA 3') using a QuikChange reaction (Statagene-Agilent Technologies) and oligonucleotides OL2652 and OL2653 to yield plasmid pSA524. Strain V67 (*recB21*) transformed with this plasmid was grown in TB; $\sim 1 \times 10^6$ cells were embedded in BBL top agar on an LB agar plate, and $\sim 1 \times 10^5$ T4 wild-type phage were spotted on this lawn. After overnight incubation at 37 °C, phage were harvested, diluted, and plated on strain V67. About 100 small plaques, from a total of about 600 plaques, were transferred with toothpicks to a lawn of V67 and to a lawn of strain V66 (*recB*⁺). Phage that grew on V67 but not on V66, about 10% of the total tested, were plaque-purified, grown in V67, and confirmed to contain the expected triple nonsense mutations. This complex mutation is designated gene 2 *am149*.

Compound Screen in 96-Well Format. A fresh overnight culture of strain V66 in LB was diluted 1:100 into LB broth with 0.1% (v/v) DMSO and grown with aeration at 37 °C to an OD₆₅₀ of 0.05. Each well of a 96-well plate (Costar, Corning Inc.) was prepared by adding 10 μ L of compound in 20% (v/v) DMSO or the appropriate control (LB with 20% (v/v) DMSO), and then 100 μ L of the bacterial culture (containing about 2.5×10^6 cells) or uninoculated medium was added. The OD₆₅₀ of each well was read on a VERSAmax microplate reader (Molecular Devices), and the plates were incubated without shaking at 37 °C. After about 1 h the OD₆₅₀ of the cultures was approximately 0.1, and 10 μ L of a phage suspension containing 5×10^4 phage or 2.5 μ g of chloramphenicol was added to the appropriate wells. The plates were incubated for approximately 20 h, and the OD₆₅₀ was determined.

Compound Screen in 1536-Well Format. These assays, including assay statistics, are summarized in Supplementary Table S2. Detailed assay protocols and data generated for the high-throughput screen (HTS) and probe development are found at PubChem AID 449731: pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=449731.

AddAB (Strain V3065) HTS assay. All reagents were purchased from Sigma unless noted otherwise below. Prior to assay, strains V3065 (*addAB*⁺) and V3069 (vector control) were grown at 37 °C to an OD₆₀₀ of 0.05 ($\sim 2.5 \times 10^7$ cfu/mL). Three microliters of assay buffer containing glycerol (0.1% v/v) and ampicillin (100 μ g/mL) (Fisher) in Cation-adjusted Mueller Hinton Broth (Becton-Dick-

inson) were added to each well of a 1,536-well clear-bottom plate (Aurora, Nexus Biosystems). Sixty nanoliters of test compound (12 μ M final concentration), ciprofloxacin (0.95 μ g/mL final concentration, as a control for complete inhibition), or DMSO alone (1.2% v/v final concentration) were added to the appropriate wells; compounds and ciprofloxacin were in DMSO. One microliter of strain V3065 (*addAB*⁺) or strain V3069 (vector control) was dispensed into the appropriate wells, and plates were incubated for 60 min at 37 °C. One microliter of phage T4 gene 2 *am149* mutant was dispensed to the appropriate wells at a multiplicity of infection (MOI) of 0.02. Plates were centrifuged, and after 18 h of incubation at 37 °C the absorbance, as OD₆₀₀, was read on an Envision microplate reader (PerkinElmer). All data were normalized to that of the positive control (wells containing strain V3065, ciprofloxacin, and phage) and negative control (wells containing strain V3065, DMSO, and phage). This protocol was used for primary, secondary, and titration screening assays (PubChem AID 435030, 488942, and 492957, respectively). The hit-cutoff used to qualify active compounds in the primary assay was calculated as the average percentage activity of all compounds tested plus three times their standard deviation.⁵⁰ The secondary assay used the same conditions as the primary screening assay, except that plates were assessed in triplicate and results for each compound were reported as the average percentage activity of the three measurements, plus or minus the associated standard deviation. For titration experiments, assay protocols were identical to those described above, except that compounds were prepared in 10 point, 1:3 serial dilutions starting at a nominal test concentration of 120 μ M and assessed in triplicate.⁵¹

Bacterial Viability HTS Assay. This assay was identical to the AddAB (strain V3065) screening assay, except that no phage was added to the wells. All data were normalized to that of the positive control (wells containing strain V3065 and ciprofloxacin) and negative control (wells containing strain V3065 and DMSO). This protocol was used for primary, secondary, and titration screening assays (PubChem AID 449728, 488956, and 492958, respectively).

RecBCD (Strain V66) HTS Assay. This assay was identical to the primary AddAB screening assay except that strains V66 (*recBCD*⁺) and V67 (*recB21*) replaced strains V3065 and V3069, respectively. All data were normalized to that of the positive control (wells containing strain V66, ciprofloxacin, and phage) and negative control (wells containing strain V66, DMSO, and phage). This protocol was used for secondary and titration screening assays (PubChem AID 488955 and 492957, respectively).

■ ASSOCIATED CONTENT

📄 Supporting Information

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are especially grateful to Julian Simon and Antonio Bedalov (Fred Hutchinson Cancer Research Center; FHRC) for discussions of inhibitor screens and, with Gemma Park, for aiding our preliminary screens for RecBCD and AddAB inhibitors, which were supported by grants from the FHRC

Synergy Fund and the Bill and Melinda Gates Foundation Grand Challenges Explorations. We thank Pierre Baillargeon and Lina DeLuca (Lead Identification Division, Scripps Florida) for compound management, Peter Chase (Lead Identification, Scripps Florida) for assistance in screening activities, Nina Salama (FHCRC) for discussions of *H. pylori*, Julian Simon for comments on the manuscript, Micah Ferrel (Seattle Structural Genomics Center for Infectious Disease) for purified *H. pylori* AddAB enzyme, Andrew Taylor (FHCRC) for purified *E. coli* RecBCD enzyme, and Kineta, Inc. for a compound library from Life Chemicals. This work was supported, in part, by grants from the FHCRC Synergy Fund (G.R.S.), Bill and Melinda Gates Foundation Grand Challenges Explorations grant 53290 (G.R.S.), and National Institutes of Health grants R01 GM031693-S1 (G.R.S.), R03 AI083736 (G.R.S.), and U54 MH084512 (P.H.).

REFERENCES

- (1) Tse, Y. C., Kirkegaard, K., and Wang, J. C. (1980) Covalent bonds between protein and DNA. Formation of phosphotyrosine linkage between certain DNA topoisomerases and DNA. *J. Biol. Chem.* 255, 5560–5565.
- (2) Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., and Tomizawa, J. (1977) Nalidixic acid resistance: A second genetic character involved in DNA gyrase activity. *Proc. Natl. Acad. Sci. U.S.A.* 74, 4772–4776.
- (3) Smith, G. R. (2001) Homologous recombination near and far from DNA breaks: Alternative roles and contrasting views. *Annu. Rev. Genet.* 35, 243–274.
- (4) Dillingham, M. S., and Kowalczykowski, S. C. (2008) RecBCD enzyme and the repair of double-stranded DNA breaks. *Microbiol. Mol. Biol. Rev.* 72, 642–671.
- (5) Smith, G. R. How RecBCD enzyme and Chi promote DNA break repair and recombination. — A molecular biologist's view. *Microbiol. Mol. Biol. Rev.*, in press.
- (6) Ponticelli, A. S., Schultz, D. W., Taylor, A. F., and Smith, G. R. (1985) Chi-dependent DNA strand cleavage by RecBC enzyme. *Cell* 41, 145–151.
- (7) Touzain, F., Petit, M. A., Schbath, S., and El Karoui, M. (2011) DNA motifs that sculpt the bacterial chromosome. *Nat. Rev. Microbiol.* 9, 15–26.
- (8) Goldmark, P. J., and Linn, S. (1972) Purification and properties of the RecBC DNase of *Escherichia coli* K-12. *J. Biol. Chem.* 247, 1849–1860.
- (9) Buchmeier, N. A., Libby, S. J., Xu, Y., Loewen, P. C., Switala, J., Guiney, D. G., and Fang, F. C. (1995) DNA repair is more important than catalase for *Salmonella* virulence in mice. *J. Clin. Invest.* 95, 1047–1053.
- (10) Amundsen, S. K., Fero, J., Hansen, L. M., Cromie, G. A., Solnick, J. V., Smith, G. R., and Salama, N. R. (2008) *Helicobacter pylori* AddAB helicase-nuclease and RecA promote recombination-related DNA repair and survival during stomach colonization. *Mol. Microbiol.* 69, 994–1007.
- (11) Taylor, A. F. (1988) RecBCD enzyme of *Escherichia coli*. In *Genetic Recombination*, Kucherlapati, R., Smith, G. R., Eds. American Society for Microbiology, Washington, DC, pp 231–263.
- (12) Dziegielewska, B., Beerman, T. A., and Bianco, P. R. (2006) Inhibition of RecBCD enzyme by antineoplastic DNA alkylating agents. *J. Mol. Biol.* 361, 898–919.
- (13) Kooistra, J., Hajjema, B. J., Hesselting-Meinders, A., and Venema, G. (1997) A conserved helicase motif of the AddA subunit of the *Bacillus subtilis* ATP-dependent nuclease (AddAB) is essential for DNA repair and recombination. *Mol. Microbiol.* 23, 137–149.
- (14) Taylor, A., and Smith, G. R. (1980) Unwinding and rewinding of DNA by the RecBC enzyme. *Cell* 22, 447–457.
- (15) Rosamond, J., Telander, K. M., and Linn, S. (1979) Modulation of the RecBC enzyme of *Escherichia coli* K12 by Ca²⁺. *J. Biol. Chem.* 254, 8646–8652.
- (16) Karu, A. E., Sakaki, Y., Echols, H., and Linn, S. (1975) The gamma protein specified by bacteriophage lambda. Structure and inhibitory activity for the RecBC enzyme of *Escherichia coli*. *J. Biol. Chem.* 250, 7377–7387.
- (17) Murphy, K. C. (2007) The lambda Gam protein inhibits RecBCD binding to dsDNA ends. *J. Mol. Biol.* 371, 19–24.
- (18) Court, R., Cook, N., Saikrishnan, K., and Wigley, D. (2007) The crystal structure of lambda-Gam protein suggests a model for RecBCD inhibition. *J. Mol. Biol.* 371, 25–33.
- (19) Murphy, K. C. (1991) Lambda Gam protein inhibits the helicase and chi-stimulated recombination activities of *Escherichia coli* RecBCD enzyme. *J. Bacteriol.* 173, 5808–5821.
- (20) Oliver, D. B., and Goldberg, E. B. (1977) Protection of parental T4 DNA from a restriction exonuclease by the product of gene 2. *J. Mol. Biol.* 116, 877–881.
- (21) Yu, M., Souaya, J., and Julin, D. A. (1998) Identification of the nuclease active site in the multifunctional RecBCD enzyme by creation of a chimeric enzyme. *J. Mol. Biol.* 283, 797–808.
- (22) Hsieh, S., and Julin, D. A. (1992) Alteration by site-directed mutagenesis of the conserved lysine residue in the consensus ATP-binding sequence of the RecB protein of *Escherichia coli*. *Nucleic Acids Res.* 20, 5647–5653.
- (23) Amundsen, S. K., Taylor, A. F., Chaudhuri, A. M., and Smith, G. R. (1986) *recD*: The gene for an essential third subunit of exonuclease V. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5558–5562.
- (24) Masterson, C., Boehmer, P. E., McDonald, F., Chaudhuri, S., Hickson, I. D., and Emmerson, P. T. (1992) Reconstitution of the activities of the RecBCD holoenzyme of *Escherichia coli* from the purified subunits. *J. Biol. Chem.* 267, 13564–13572.
- (25) Amundsen, S. K., Fero, J., Salama, N. R., and Smith, G. R. (2009) Dual nuclease and helicase activities of *Helicobacter pylori* AddAB are required for DNA repair, recombination, and mouse infectivity. *J. Biol. Chem.* 284, 16759–16766.
- (26) Hajjema, B. J., Meima, R., Kooistra, J., and Venema, G. (1996) Effects of lysine-to-glycine mutations in the ATP-binding consensus sequences in the AddA and AddB subunits on the *Bacillus subtilis* AddAB enzyme activities. *J. Bacteriol.* 178, 5130–5137.
- (27) Sinha, K. M., Unciuleac, M. C., Glickman, M. S., and Shuman, S. (2009) AdnAB: a new DSB-resecting motor-nuclease from mycobacteria. *Genes Dev.* 23, 1423–1437.
- (28) Miller, E. S., Kutter, E., Mosig, G., Arisaka, F., Kunisawa, T., and Ruger, W. (2003) Bacteriophage T4 genome. *Microbiol. Molec. Biol. Rev.* 67, 86–156.
- (29) Wang, G. R., Vianelli, A., and Goldberg, E. B. (2000) Bacteriophage T4 self-assembly: in vitro reconstitution of recombinant gp2 into infectious phage. *J. Bacteriol.* 182, 672–679.
- (30) Ennis, D. G., Amundsen, S. K., and Smith, G. R. (1987) Genetic functions promoting homologous recombination in *Escherichia coli*: A study of inversions in phage λ . *Genetics* 115, 11–24.
- (31) Zweerink, M. M., and Edison, A. (1986) Inhibition of *Micrococcus luteus* DNA gyrase by norfloxacin and 10 other quinolone carboxylic acids. *Antimicrob. Agents Chemother.* 29, 598–601.
- (32) Shen, L. L., and Pernet, A. G. (1985) Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. *Proc. Natl. Acad. Sci. U.S.A.* 82, 307–311.
- (33) Clark, A. J. (1973) Recombination deficient mutants of *E. coli* and other bacteria. *Annu. Rev. Genet.* 7, 67–86.
- (34) Stahl, F. W., and Stahl, M. M. (1977) Recombination pathway specificity of Chi. *Genetics* 86, 715–725.
- (35) Aiello, D., Barnes, M. H., Biswas, E. E., Biswas, S. B., Gu, S., Williams, J. D., Bowlin, T. L., and Moir, D. T. (2009) Discovery, characterization and comparison of inhibitors of *Bacillus anthracis* and *Staphylococcus aureus* replicative DNA helicases. *Bioorg. Med. Chem.* 17, 4466–4476.
- (36) Taylor, A. F., and Smith, G. R. (2003) RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity. *Nature* 423, 889–893.

- (37) Schultz, D. W., Taylor, A. F., and Smith, G. R. (1983) *Escherichia coli* RecBC pseudorevertants lacking Chi recombinational hotspot activity. *J. Bacteriol.* 155, 664–680.
- (38) Anderson, D. G., and Kowalczykowski, S. C. (1997) The translocating RecBCD enzyme stimulates recombination by directing RecA protein onto ssDNA in a χ regulated manner. *Cell* 90, 77–86.
- (39) Amundsen, S. K., Taylor, A. F., Reddy, M., and Smith, G. R. (2007) Intersubunit signaling in RecBCD enzyme, a complex protein machine regulated by Chi hot spots. *Genes Dev.* 21, 3296–3307.
- (40) Gottesman, M. M., Hicks, M. L., and Gellert, M. (1974) Genetics and function of DNA ligase in *Escherichia coli*. *J. Mol. Biol.* 77, 531–547.
- (41) Kushner, S. R. (1974) *In vivo* studies of temperature-sensitive *recB* and *recC* mutants. *J. Bacteriol.* 120, 1213–1218.
- (42) Kogoma, T., Hong, X., Cadwell, G. W., Barnard, K. G., and Asai, T. (1993) Requirement of homologous recombination functions for viability of the *Escherichia coli* cell that lacks RNase HI and exonuclease V activities. *Biochimie* 5, 89–99.
- (43) Singleton, M. R., Dillingham, M. S., Gaudier, M., Kowalczykowski, S. C., and Wigley, D. B. (2004) Crystal structure of RecBCD enzyme reveals a machine for processing DNA breaks. *Nature* 432, 187–193.
- (44) Buchmeier, N. A., Lipps, C. J., So, M. Y., and Heffron, F. (1993) Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. *Mol. Microbiol.* 7, 933–936.
- (45) Cirz, R. T., Chin, J. K., Andes, D. R., de Crecy-Lagard, V., Craig, W. A., and Romesberg, F. E. (2005) Inhibition of mutation and combating the evolution of antibiotic resistance. *PLoS Biol.* 3, e176.
- (46) McPartland, A., Green, L., and Echols, H. (1980) Control of *recA* gene RNA in *E. coli*: regulatory and signal genes. *Cell* 20, 731–737.
- (47) Cromie, G. A. (2009) Phylogenetic ubiquity and shuffling of the bacterial RecBCD and AddAB recombination complexes. *J. Bacteriol.* 191, 5076–5084.
- (48) Eichler, D. C., and Lehman, I. R. (1977) On the role of ATP in phosphodiester bond hydrolysis catalyzed by the RecBC deoxyribonuclease of *Escherichia coli*. *J. Biol. Chem.* 252, 499–503.
- (49) Smith, G. R., Kunes, S. M., Schultz, D. W., Taylor, A., and Triman, K. L. (1981) Structure of Chi hotspots of generalized recombination. *Cell* 24, 429–436.
- (50) Hodder, P., Cassaday, J., Peltier, R., Berry, K., Inglese, J., Feuston, B., Culberson, C., Bleicher, L., Cosford, N. D., Bayly, C., Suto, C., Varney, M., and Strulovici, B. (2003) Identification of metabotropic glutamate receptor antagonists using an automated high-throughput screening system. *Anal. Biochem.* 313, 246–54.
- (51) Madoux, F., Li, X., Chase, P., Zastrow, G., Cameron, M. D., Conkright, J. J., Griffin, P. R., Thacher, S., and Hodder, P. (2008) Potent, selective and cell penetrant inhibitors of SF-1 by functional ultra-high-throughput screening. *Mol. Pharmacol.* 73, 1776–84.
- (52) Lloyd, R. G., and Buckman, C. (1985) Identification and genetic analysis of *sbcC* mutations in commonly used *recBC sbcB* strains of *Escherichia coli* K12. *J. Bacteriol.* 164, 836–844.
- (53) Horii, Z.-I., and Clark, A. J. (1973) Genetic analysis of the RecF pathway to genetic recombination in *Escherichia coli* K12: Isolation and characterization of mutants. *J. Mol. Biol.* 80, 327–344.
- (54) Lloyd, R. G., Buckman, C., and Benson, F. E. (1987) Genetic analysis of conjugational recombination in *Escherichia coli* K12 strains deficient in RecBCD enzyme. *J. Gen. Microbiol.* 133, 2531–2538.

■ NOTE ADDED AFTER ASAP PUBLICATION

This article was published ASAP on March 23, 2012. Co-author, Christina Eberhart's name was misspelled. The corrected version was re-posted on April 20, 2012.