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Parallel Multiplicative Target Screening Against Divergent Bacterial Replicases: Identification of Specific Inhibitors with Broad Spectrum Potential[†]

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Abstract

Typically, biochemical screens that employ pure macromolecular components focus on single targets or a small number of interacting components. Researches rely on whole cell screens for more complex systems. Bacterial DNA replicases contain multiple subunits that change interactions with each stage of a complex reaction. Thus, the actual number of targets is a multiple of the proteins involved. It is estimated that the overall replication reaction contains up to 100 essential targets, many suitable for discovery of antibacterial inhibitors. We have developed an assay, using purified protein components, where inhibitors of any of the essential targets can be detected through a common readout. Use of purified components allows each protein to be set within the linear range where the readout is proportional to the extent of inhibition of the target. By performing assays against replicases from model Gram-negative and Gram-positive bacteria in parallel, we show that it is possible to distinguish compounds that inhibit only a single bacterial replicase from those that exhibit broad spectrum potential.

Typically, *in vitro* high-throughput screening (HTS)[1] assays target single proteins or protein pairs. This approach has enabled significant success. To exploit all of the targets available in complex pathways or molecular machines, researchers often resort to cellular screens, to ensure the availability of all relevant targets. Using these approaches, novel targets have been revealed that have led to the discovery of new interactions, validating the power of forward chemical genetics (1). However, cellular screens have the drawback of missing compounds that cannot achieve suitable intracellular concentrations due to low permeability, unfavorable metabolism or efflux. These issues could be overcome if all of the machinery involved in a complex process could be reconstituted *in vitro*, enabling a biochemical screen. This approach would permit identification of inhibitors that could subsequently be optimized for potency and permeability in parallel with other favorable pharmacological properties.

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DNA replication is an essential process for the proliferation of all pathogens and offers a largely unexplored target for development of novel antibacterials. Therapeutically useful inhibitors have been developed that inhibit processes upstream (nucleotide precursor biosynthesis)(2) and downstream (DNA gyrase)(3) of DNA replication. Most of the subunits of the bacterial DNA replication apparatus are essential, suggesting that their inhibition should lead to blockage of cell proliferation or death (4). This has been validated by a class of compounds, 6-anilinouracils, targeted to the polymerase subunit of the Gram-positive replicase, Pol C. These compounds are not only potent biochemical inhibitors, but specifically block DNA replication in Gram-positive bacteria (5). While screens targeting individual replicase subunits have been described (6-9), complete bacterial replicases have not been explored by chemical genetic approaches.

Cellular replicases are tripartite multi-protein assemblies (for reviews, see (10-13)). They contain specific DNA polymerases that function as the catalytic elongation component and that derive enormous processivity from interaction with a bracelet-like ‘sliding clamp’ processivity factor that encircles DNA, tethering the replicative polymerase to it. The sliding clamp is loaded onto DNA by an ATP-powered ‘clamp loader.’ The ‘clamp loader’ serves additional roles, including communication with the replicative helicases and linking the leading and lagging strand polymerases, at least in *E. coli* (14,15).

The ten subunits of *E. coli* DNA Pol III holoenzyme interact to form a remarkably complex protein machine (10,14,16-18). Protein interactions change at the various steps of the replicative reaction. Counting all of the individual protein components and their interactions with other subunits and substrates, we estimate upwards of 100 essential targets that are potentially useful for development of antibacterial agents (see *Results and Discussion* for enumeration). Given the impracticality of running 100 specific screening assays and preferring to avoid the problems inherent in whole cell screens, we established a biochemical HTS where inhibition of any of the essential targets could be detected through a common endpoint. This would permit screening for all targets within the replicase in a single well of a microtiter plate, a goal never achieved for such a complex target in a biochemical screen using purified components. Using conversion of single-stranded DNA binding protein (SSB)-coated single-stranded DNA to a duplex, detected by binding of the fluorescent dye, PicoGreen, we developed robust screens for model Gram-negative and Gram-positive bacterial replicases. By screening a small trial 20,000-compound library against these related targets in parallel, we were able to distinguish compounds that inhibited the replicase of a single species from those compounds that exhibited broad spectrum potential. Counterscreens against non-orthologous enzymes with related activities revealed those compounds that are most likely to be target-specific.

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[1]Abbreviations:

HTS	high-throughput screening
SSB	single-stranded DNA binding protein
Pol	Polymerase
Pol III*	DNA polymerase III* (a subcomplex of all subunits of the *E. coli* Pol III holoenzyme except β_2)
MIC	minimal inhibitory concentration
MMS	macromolecular synthesis
IDD	intercalator dye displacement
SAR	structure activity relationship

MATERIALS AND METHODS

Chemicals and Reagents

Screening compounds were purchased from TimTec (10,000 compounds, Diversity Set collection, Newark, DE) and Chembridge (10,000 compounds, DIVERset collection, San Diego, CA). All nucleotide triphosphates, PicoGreen ds DNA detection reagent and fluorescein digalactoside were purchased from Invitrogen (Carlsbad, CA). T4 and T7 DNA polymerases and apyrase were purchased from New England Biolabs (Ipswich, MA). *E. coli* RNA polymerase core (αββ'ω) was purchased from Epicentre Biotechnologies (Madison, WI). β-Galactosidase was purchased from Worthington Biochemical Corp. (Lakewood, NJ).

Buffers

Buffer Q is 50 mM HEPES (pH 7.0), 20 mM NaCl, 10% glycerol, 5 mM DTT, 1mM EDTA. Buffer Q2 is 40 mM HEPES (pH 7.5), 50 mM NaCl, 10% glycerol, 0.5 mM DTT, 0.5mM EDTA. Buffer B is 50mM Tris-Cl (pH 7.5), 10% glycerol, 1.0 M ammonium sulfate, 0.5 mM EDTA, 5 mM DTT. Buffer QS is 50 mM Tris-Cl (pH 7.5), 10% glycerol, 5 mM DTT, 0.1M NaCl, 0.5 mM EDTA. Buffer HA is 25 mM Tris-Cl (pH 7.5), 10% glycerol, 5 mM DTT, 150 mM NaCl, 0.5 mM EDTA. Buffer S is 25 mM HEPES (pH 7.5), 10% glycerol, 0.2 M NaCl, 0.5 mM EDTA, 5 mM DTT. Buffer QE is 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 10% glycerol, 5 mM DTT, 0.5 mM EDTA. Buffer H is 50 mM imidazole (pH 7.0), 10% glycerol, 5 mM DTT. Buffer S is 50 mM Tris-Cl (pH 7.5), 10% glycerol, 10 mM NaCl, 0.1 mM EDTA, 5 mM DTT. Buffer I is 50 mM imidazole (pH 6.0), 20% glycerol, 20 mM NaCl and 5mM DTT. Buffer H2 is 50 mM Tris-Cl (pH 7.5), 400 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.25 mM DTT.

Cloning and Expression of B. subtilis DNA Replication Proteins

All *B. subtilis* DNA Pol III holoenzyme subunit genes were amplified by PCR of the relevant coding region from *B. subtilis* genomic DNA (ATCC) and the resulting amplified segments inserted into the expression plasmid pA1-CB (19,20) immediately behind the IPTG-inducible $P_{A1/04/03}$ promoter/operator using appropriate restriction enzymes. This resulted in the construction of expression plasmids pA1-BS-polC, pA1-BS-dnaX, pA1-BS-holBA, pA1-BS-holB, pA1-BS-dnaN and pA1-BS-ssb1, expressing *B. subtilis* DNA Pol III subunits PolC, τ, δ, δ', β, and SSB, respectively. All individual *B. subtilis* replicase subunits were heterologously expressed as native sequences, without affinity tags in the *E. coli* strain DH5-α. Due to extremely low levels of expression of the *B. subtilis* δ subunit in an initial expression construct containing only the *holA* gene, it was cloned into the polylinker region immediately downstream of the *holB* gene in pA1-BS-holB (resulting in expression plasmid pA1-BS-holBA). Expression of δ (*holA*) was significantly increased to levels above that of δ' (*holB*), which allowed purification of the single isolated δ subunit.

Cell lysates were prepared by procedures identical to those used for *E. coli* replicase component purifications. For all purifications, enzyme activity was monitored using DNA Pol III holoenzyme reconstitution assays measuring the incorporation of [^{3}H]-TTP into DNA synthesized on either $M13_{Gori}$ templates (dnaG-primase dependent *E. coli* system) or oligonucleotide-primed $M13_{Gori}$ templates (*B. subtilis* system), in a filter binding assay essentially as outlined in Figure 1 and (21).

Purification of Bacterial DNA Replication Proteins

E. coli Pol III*, β, SSB and DnaG were expressed and purified using published methods (22-24).

B. subtilis PolC was expressed in *E. coli* and was precipitated from a cell lysate with a 40-55% ammonium sulfate cut and purified by chromatography over Q Sepharose (buffer QE, gradient 0.05 to 0.6 M NaCl), hydroxylapatite (buffer HE, gradient 0.05 to 0.4 M potassium phosphate), BioRex70 (buffer I, gradient 0.075 to 0.4 M NaCl) and Sephacryl S-300 gel filtration in buffer S. A total of 25 mg purified PolC (specific activity 1.0×10^7 units/mg) was obtained from 600 g of cells.

B. subtilis τ was precipitated from cell lysate with a 35-45% saturated ammonium sulfate cut and purified by chromatography over Q Sepharose (buffer QE, gradient 0.1 to 0.4 M NaCl), hydroxylapatite (buffer HE, gradient 0 to 0.25 M potassium phosphate) and Sephacryl S-400 gel filtration in buffer S. A total of 160 mg were obtained from 280 g of cells (specific activity 3.3×10^5 units/mg).

B. subtilis δ was precipitated from extracts with a 35-50% saturated ammonium sulfate cut and purified by chromatography over Q Sepharose (buffer QS, gradient 0.1 to 0.35 M NaCl), hydroxylapatite (buffer H, gradient 0 to 0.2 M potassium phosphate) and Sephacryl S-100 gel filtration in buffer S. A total of 22 mg were obtained from 100 g of cells (specific activity 1.6×10^7 units/mg).

B. subtilis δ' was precipitated from extracts with a 40-55% saturated ammonium sulfate cut and purified by chromatography over Q Sepharose (buffer Q, gradient 0.02 to 0.18 M NaCl) and Butyl Sepharose (buffer B, gradient 0.8 M ammonium sulphate, 10% glycerol to 0 M ammonium sulphate, 30% glycerol). A total of 44 mg were obtained from 400 g of cells (specific activity 1.3×10^7 units/mg). Although two distinct bands are present in the purified preparation of δ' when analyzed by SDS-PAGE (Figure 2), both bands contain the δ' subunit. This doublet is also observed in purified *E. coli* δ' (25,26) and S. pyogenes δ' (27). The basis for this electrophoretic behavior of δ' is not known.

B. subtilis β was precipitated from extracts with a 65-75% saturated ammonium sulfate cut and purified by chromatography over Q Sepharose (buffer Q2, gradient 0.05 to 0.5 M NaCl) and heparin Sepharose (buffer Q2, gradient 0.05 to 0.25 M NaCl). A total of 25 mg were obtained from 400 g of cells (specific activity 8.0×10^7 units/mg).

B. subtilis SSB was precipitated from extracts with a 0-40% ammonium sulfate cut followed by backwashing the resulting pellet with 36% ammonium sulfate. The pellet was then resuspended in buffer QS and purified by chromatography over Blue Sepharose FF (24). This column was successively washed with 0.1, 0.4, 1 and 2 M NaCl in buffer QS before final elution in 4 M NaCl. SSB was further purified by hydroxylapatite (buffer H2, gradient 0 to 0.1 M potassium phosphate). A total of 110 mg were purified from 900 g of cells.

A polyacrylamide gel showing the final purified *B. subtilis* replicase subunits and SSB is shown in Figure 2.

High Throughput Screening Assays

HTS assays of the *E. coli* replicase were performed using final protein concentrations and conditions listed in the legend to Figure 3 using Biomek FX liquid handling robotics (Beckman Coulter Ltd.) equipped with a 96-well pipettor head and a plate stacker unit. An enzyme mixture containing all of the protein components for the assay in a buffer, optimized for maximal enzyme stability and activity (50 mM HEPES (pH 7.5), 5% glycerol, 0.02% Pluronic F-68, 80 μM TCEP, 150 mM potassium glutamate and 7 mM magnesium acetate). During HTS the enzyme mixture was kept on a chilled reservoir (4 °C), to ensure stability over a 6 hour screening period. Substrate mix (nucleotides and $M13_{Gori}$ ss circular DNA template) and test compounds were kept at room temperature. The HTS assay was performed by the addition of

1.5 μL of compound (500 μM in DMSO, 30 μM final concentration) into 18.5 μL of enzyme mix in black 384-well assay plates (Greiner Bio-One, Monroe, NC) and mixed by repeated aspiration. The compounds plus proteins were incubated at room temperature for 10 minutes after which 5 μL of substrate mix was added to initiate DNA synthesis (see Figure 3 legend for details). After incubation for 20 minutes at room temperature (22 ± 1 °C), 75 μL of PicoGreen dye reagent (containing 0.1 μL of reagent stock, as supplied by Invitrogen, in 10 mM Tris-Cl (pH 7.5), 10 mM EDTA) was added to stop the reaction. Assay plate fluorescence intensity was read using an excitation wavelength of 485 nm and an emission wavelength of 535 nm on an EnVision plate reader (Perkin Elmer, Waltham, MA). Compounds were not pre-screened for fluorescence at 535 nm and this could have caused a small number of false negative readings.

The *B. subtilis* HTS assay was performed essentially as described for *E. coli* except that the *B. subtilis* DnaG primase was not included in the HTS. Instead, a synthetic DNA primer (5′-AGGCTGGCTGACCTTCATCAAGAG TAATCT) was annealed to ss circular $M13_{Gori}$ DNA in an equimolar ratio in a quantity sufficient for all assays and kept frozen until needed. The assay buffer for the *B. subtilis* screen was optimized for maximal enzyme stability and activity and contained 40 mM HEPES (pH 7.5), 0.5% polyethylene glycol, 0.02% Pluronic F-68, 20 μM TCEP, 200 mM potassium glutamate, 3 μM zinc sulfate, 12.5 mM manganese chloride and 12.5 mM magnesium acetate. The *B. subtilis* HTS assay used 0.5 μL of compound (500 μM in DMSO, 10 μM final concentration) added into 19.5 μL of enzyme mix. All subsequent steps were identical to the *E. coli* HTS assay described above. We also included the known inhibitor of *B. subtilis* PolC, HB-EMAU (provided as a kind gift from Drs. Neal Brown and George Wright, University of Massachusetts Medical Center, (5)) as a positive control inhibitor in all *B. subtilis* replicase screening and IC_{50} assays (compound 13, Table S1 of *Supporting Information*).

Primary Screening – Bacterial Replicases

Screening compounds from TimTec and Chembridge were tested in each screening system in duplicate (Figure 4). Each 384-well screening plate contained DMSO (volume equivalent to compound added in each screen as a negative control) and EDTA (final concentration 20 mM as a positive control) in the two outer columns of every assay plate. Data from these controls were used to normalize compound activity calculations and determine assay signal-to-background ratios and Z- and Z'-factors (28). Compounds displaying activity ≤50% of the normalized DMSO control activity in both screens were scored as primary hits and were re-ordered from suppliers or sampled from internal stores for retesting to confirm inhibitory activity and potency.

IC_{50} Measurements

IC_{50} values were determined using protocols identical to the HTS and enzyme specificity assays described above and under *Supplemental Information*, except that assays were carried out in 96-well plates using 10, 2-fold serial dilutions of test compounds dissolved in DMSO at starting concentrations of 5 mM. This resulted in dilution series ranging from 300-0.29 μM for all assays. IC_{50} values were calculated by non-linear least squares curve fitting of the fluorescence data to the equation: $Y = Ymin + ((Ymax-Ymin)/(1+(IC_{50})/x)^h)$, where Y is the normalized percent activity of the reaction, x is the inhibitor concentration and h is the Hill coefficient.

Secondary Screening – Specificity Assays

Test compound-intercalator dye displacement (IDD) assays (29) were performed by modifications of published procedures (30). Double stranded calf thymus DNA (5 ng) was mixed with 0.1 μL of PicoGreen reagent in 23.5 μL of the *E. coli* HTS assay buffer and allowed

to bind for 10 minutes. Two-fold dilutions of test compounds (1.5 μL) were added in a final volume of 25 μL. Determination of fluorescence intensity and IC_{50} values were done as described above. *E. coli* β-galactosidase assays were performed by published procedures (31) using fluorescein digalactoside as a fluorogenic substrate (fluorescence intensity was read using an excitation wavelength of 485 nm and an emission wavelength of 535 nm). Apyrase ATPase activity was assayed using 0.5 mM ATP at 22 °C for 10 minutes using a colorimetric assay employing ammonium molybdate and malachite green dye to detect inorganic phosphate as described in (32).

E. coli RNA polymerase core transcription was assayed essentially as described in (33). T4 DNA polymerase was assayed in a gap-filling DNA polymerase assay essentially as described in (34). Both of these assays used DNaseI-activated calf thymus DNA as templates and measured the incorporation of either [^{3}H]-UTP (*E. coli* RNA polymerase core) or [^{3}H]-TTP (T4 DNA polymerase) into their respective RNA or DNA products. RNA and DNA synthesis was measured via scintillation counting after the addition of poly-L-lysine coated polyvinyl acetate scintillation proximity assay beads (Invitrogen) in 0.3 M sodium citrate, pH 3.0 (35).

Saccharomyces cerevisiae Pol δ holoenzyme (36) and human mitochondrial DNA polymerase (37) were assayed as described. These assays and the T7 DNA polymerase assay used the oligonucleotide primed $M13_{Gori}$ template described for the *B. subtilis* replicase assay and PicoGreen as described in *Materials and Methods*. The yeast Pol δ holoenzyme assay contained 25 ng Pol δ, 2 ng PCNA, 1 ng RFC and 0.6 μg RPA in a total volume of 25 μL. Assays were performed at 22 °C for 40 minutes. The human mitochondrial polymerase assay contained 4 ng Pol γ – α subunit, 10 ng Pol γ – β subunit and 1 μg human mitochondrial SSB in a total volume of 25 μL.

Antimicrobial Susceptibility Testing

The reference strains for antimicrobial susceptibility testing, *E. coli* ATCC 25922 and *B. subtilis* ATCC 6633 were used for all experiments and were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Antimicrobial susceptibility testing was done using Mueller-Hinton broth according to the Clinical Laboratory Standards Institute (38). Microtiter plates containing serial dilutions of each test compound (beginning at 512 μg/mL final concentration) were inoculated with each organism (10^5 CFU/ml) in a 100-μl final volume. Assay plates were incubated for 20 h at 35°C in ambient air. Minimum inhibitory concentrations (MICs) were scored as the lowest compound concentration at which no bacterial growth was visible. The antibiotics tetracycline, rifampicin and novobiocin were used as reference standards (39).

Macromolecular Synthesis Assays

Macromolecular synthesis (MMS) assays were adapted from Wilson *et al.* (40). *E. coli* strain ATCC 25922 and *B. subtilis* strain ATCC 6633 were grown overnight in L Broth at 37°C before inoculation into M9 minimal medium (10 mL) and grown to an absorbance (600nm) of 0.15. Radiolabelled precursors of three MMS pathways (DNA synthesis, 10 μCi/ml of [methyl-^{3}H] thydmidine; RNA synthesis, 3 μCi/ml of [5-^{3}H]uridine; Protein synthesis, 1 μCi/ml of L-[2,5-^{3}H]histidine) were then added and incubated at 37°C to allow incorporation of radiolabeled precursors in the presence of test compounds or control inhibitors. Compounds were added to 25 μL of cells at concentrations of 4, 2, 1, 0.5 and 0.25 times the MIC in a final volume of 50 μL. Novobiocin (and HB-EMAU for *B. subtilis*), rifampicin and tetracycline were used as controls for inhibition of DNA, RNA and protein synthesis, respectively. For *B. subtilis* MMS assays, radiolabeled cells were incubated with compounds for 40 minutes before lysis. For *E. coli* MMS assays, cells were incubated 30 minutes for DNA and RNA synthesis MMS assays and 10 minutes for the protein synthesis MMS assay before lysis. Labeled cells

were lysed using the detergent lysis reagent, Y-PER (Pierce Chemical), which lyses both Gram-negative and –positive bacteria. After addition of 100 μL of Y-PER reagent and mixing by repeated pipetting, cells were lysed by incubation at 37°C for 20 minutes with shaking. Macromolecules were precipitated by the addition of TCA to 10% (v/v) and incubation on ice for 1 h prior to filtration on 96-well Whatman GF/C filter plates. Filter plates were washed 8 times with cold 10% TCA (200 μL/well) and once with 100% ethanol. Incorporation of radioactivity into DNA, RNA and protein was detected by scintillation counting. Data was expressed as the percentage of macromolecule precursor incorporated, normalized to DMSO controls.

RESULTS

DNA replication is essential for the propagation of living cells. In spite of the widespread use of drugs that target processes upstream and downstream of DNA replication, no clinically useful compounds have been developed that target the central DNA replication apparatus. We describe an approach whereby purified, reconstituted bacterial replicases are assayed *by a screen that* simultaneously detects inhibitors of the numerous component targets. Parallel screens of replication systems from Gram-negative and Gram-positive organisms and comprehensive counterscreening including eukaryotic replicases allows biochemical assessment of the spectrum of potential antibacterial agents as well as potential toxins. The strategies outlined here should prove general and useful in initiating reverse chemical genetic approaches to any complex biological process.

Development of HTS Assays to Detect Inhibitors of Bacterial DNA Replicases

The origin of the single-stranded (ss) circular DNA of bacteriophage G4 cloned into M13 provides a convenient template for the assay of the *E. coli* Pol III holoenzyme, SSB and DnaG primase (41,42). The G4 origin possesses an ss DNA element that allows primase to bypass normal DnaB helicase-dependent primase recruitment during chromosomal replication, allowing direct primer synthesis (43).

We adapted a fluorescent PicoGreen assay (44) to monitor conversion of ss templates to a duplex and optimized the assay in a format suitable for HTS. PicoGreen binds preferentially to double stranded (ds) DNA and becomes fluorescent (Figure 1), providing a homogenous and sensitive indicator of compounds interfering with the synthesis of ds DNA. Given the complexity of this multicomponent system, the *E. coli* replicase HTS assay was extensively optimized and was dependent upon all of the purified protein components (Figure 3A,B). Each protein component was assayed at limiting concentrations that would respond linearly to binding of an inhibitor. Nucleotide substrate concentrations were also near-limiting (data not shown), permitting the detection of inhibitors of dNTP binding to the polymerase active site, rNTP binding to the primase active site or dATP/ATP binding to the ATPase site within the DnaX complex.

We next extended our capability to screen for inhibitors of a Gram-positive replicase. This would permit identification of inhibitors of both Gram-negative and Gram-positive DNA replication and, thus, would have the potential of being developed into a detection system for broad spectrum antibacterials or chemical biological tools. A minimal five subunit replicase has been demonstrated from *S. pyogenes* (27). For the development of basic chemical biological approaches, we chose to use a replicase from a related model organism, *B. subtilis*, so that we could exploit biochemical and genetic tools and knowledge available for this organism in downstream studies. We expressed the *B. subtilis* PolC, the β_2 processivity factor, the three subunits of the DnaX complex (τ, δ and δ') and SSB and purified them in sufficient quantity for HTS (Figure 2).

The *B. subtilis* replicase HTS assay was formulated using a strategy similar to that used for *E. coli*. The identical DNA template was used in both assays. Since the *B. subtilis* DnaG primase cannot synthesize primers directly on the G4 origin (unlike the *E. coli* primase) without the participation of additional proteins (45,46), we annealed an oligonucleotide primer complementary to the $M13_{Gori}$ ss DNA template. Titrations of all protein components and HTS assay optimization studies were performed as described for the *E. coli* replicase (Figure 3C,D).

Reaction kinetics were measured to determine the longest time for which a linear response was observed, about 20 minutes for both *E. coli* and *B. subtilis* (Figure 3E). As our screening compounds are dissolved in DMSO, we tested the DMSO tolerance of the assay in order to determine compound screening concentration limits (Figure 3F). Greater than 80% activity was retained at 8% and 15% DMSO for the Gram-negative and – positive screens, respectively. Since screening would be conducted over the course of a day, we tested the stability of the enzyme in assay buffer and determined that approximately 20% the activity was lost after 4 hours at room temperature (Figure 3G). Consequently, the *E. coli* protein component mixes used during screening were kept in a chilled reservoir (at 4°C) for no longer than 6 hours to ensure maximum enzyme activity.

HTS of both Gram-negative and –positive Replicases against a Compound Library

Having established robust, reliable replicase-targeted biochemical HTS systems, we tested a trial library comprised of 20,000 commercially obtained compounds in duplicate screens against both the Gram-negative and –positive replicases (Figure 4). Compounds that limited enzyme activities to ≤50% of uninhibited DMSO controls in both duplicates (red circles in Figure 4) were considered primary hits and selected for follow-up studies. Statistics from the screens yielded Z' factor averages >0.7, which was extremely encouraging given the complexity of both systems. Typically, Z-factors between 0.5 and 1 indicate a very robust assay (28). A total of 831 and 290 compounds were identified as primary hits in the Gram-negative and –positive screens, respectively (Figure 4).

Initial hit confirmation was done using a combination of compound titrations against the primary screen and a DNA intercalator dye displacement (IDD) assay (29). Preliminary studies indicated that the most common source of artifactual hits were compounds that affected the PicoGreen assay readout either by binding to the DNA template or product, subsequently blocking polymerase activity, by blocking PicoGreen dye binding to ds DNA, or more trivially, by interfering with the fluorescence of the PicoGreen-ds DNA complex due to absorption. The IDD assay measures the ability of a test compound to interfere with binding of the ds DNA-selective dye PicoGreen to ds calf thymus DNA. For example, compounds such as 133 (Figure 5 and Table 1) yielded comparable IC_{50}s in both bacterial replicase assays and the IDD assay. 251 and 143 of the primary hits resulting from the *E. coli* and *B. subtilis* screens, respectively, titrated with IC_{50} values ≤100 μM in the primary screen and were inactive (with IC_{50}s >5-fold of the replicase IC_{50}) in the IDD assay, strongly suggesting that these were specific inhibitors. These compounds were chosen for further analysis against a panel of specificity assays (Table S1 of *Supporting Information*).

Development of Assays to Eliminate Non-specific Inhibitors

Since our screening systems comprised a large range of potential enzymatic, protein-protein and protein-nucleic acid targets against which hits could act, we counterscreened active compounds against a diverse panel of specificity assays in order to distinguish specific inhibitors of the bacterial Pol III holoenzymes from non-specific inhibitors of unrelated DNA polymerases (bacteriophage T7 and T4 DNA polymerases, *Saccharomyces cerevisiae* DNA Pol δ holoenzyme and human mitochondrial DNA polymerase), an unrelated RNA polymerase

(*E. coli* RNA polymerase core), an unrelated ATPase (Apyrase), and a mechanistically unrelated enzyme *(E. coli* β-galactosidase).

The IC_{50} values of confirmed inhibitors were determined and compared with IC_{50} values determined in specificity assays. A threshold of 5-fold was used to score the specificity of a given compound for a particular assay or bacterial replicase target. A compound such as 133 (Figure 5 and Table 1) was scored as non-specific with respect to most specificity assays, since the IC_{50} values all fell within 5-fold of one of the bacterial holoenzyme target IC_{50} values. Compound 26 (Figure 5 and Table1) was considered specific for the *B. subtilis* replicase, because the IC_{50} (20.5 μM) was more than 5-fold more potent compared to the *E. coli* replicase assay IC_{50} (>300 μM), or that of any specificity assay. A complete listing of the annotated activities of all 394 compounds tested is provided in Table S1. Figure 5 and Table 1 illustrate the structures and activity profiles of selected compounds that will be presented as examples of the classes of inhibitors we have identified.

The panel of specificity assays was selected not only to identify problematic compounds such as protein reactive and aggregation-based inhibitors (47), but also to detect mechanistically non-specific compounds (i.e., general polymerase, ATPase or RNA polymerase inhibitors) and, as such, proved critical to a thorough evaluation of the replicase inhibitors identified here. Out of all compounds tested, relatively few were found to be active against β-galactosidase, *E. coli* RNA polymerase or human mitochondrial DNA polymerase (16, 19 and 18 compounds, respectively). Most of these compounds were generally non-specific, inhibited many other targets and were eliminated from further consideration.

DNA Pol IIIs, involved in chromosomal replication, are a special subclass of type X polymerases and have a unique fold in their active site that distinguishes them from type B polymerases, which replicate eukaryotic chromosomes, or type A polymerases, which are responsible for mitochondrial replication (48-51). To eliminate compounds that generally inhibited DNA polymerases, we performed counterscreens against two type A polymerases (bacteriophage T7 and human mitochondrial DNA polymerase) and two type B polymerases (bacteriophage T4 and *S. cerevisiae* DNA Pol δ holoenzyme). The bacteriophage T7 DNA polymerase assay was by far the most efficient specificity filter, identifying a total of 96 compounds that inhibited T7 DNA polymerase (plus an additional 50 that inhibited T7 and the 3 other polymerase specificity targets). Compounds such as 395 exhibited comparable levels of inhibition in the T7 assay and the bacterial replicase screens. T7 DNA polymerase is a highly processive polymerase (52) and may be particularly sensitive to compounds that interact with the ss DNA template, blocking processive replication by blocking progression of the polymerase. In contrast to T7 DNA polymerase, the human mitochondrial DNA polymerase (53) was inhibited by only 18 compounds, all of which were also active against T7. This enzyme was included because it is a known predictor of mitochondrial toxicity. DNA replication inhibitors (notably anti-reverse transcriptase inhibitors) have displayed toxicity directed at mitochondrial DNA polymerase (53,54) and have even led to the clinical failure of drugs such as the anti-hepatitis B agent Fialuridine (55). The majority of the compounds identified here have proven inactive against this specificity filter.

Only 5 compounds (e.g., compound 162) were found to significantly inhibit T4 DNA polymerase. The *S. cerevisiae* DNA Pol δ holoenzyme (56) specificity filter was included to permit detection of compounds that result from inhibition of a eukaryotic enzyme highly similar to the human replicase. Such compounds, of course, would have toxic potential from the perspective of antibacterial therapeutic agents, but could also be among the most useful as chemical biological tools, as they may target central mechanisms common to diverse life forms. We identified 15 compounds (e.g., compound 152) that fall into this class of agents that do not

inhibit the simpler DNA polymerases or other specificity targets tested, but do inhibit both a bacterial and the eukaryotic replicase.

Because a major enzymatic target in both replicase screens is the processivity clamp-loader ATPase, a eukaryotic E-type ATPase, apyrase (57), was included to identify compounds that could target ATPases nonspecifically. A total of 14 compounds inhibited apyrase (e.g., compound 100) at comparable IC_{50}s to either bacterial replicase, as well as 48 others that inhibit both apyrase and T7 DNA polymerase. While the majority of these compounds do not appear to inhibit any other specificity target (notably the *S. cerevisiae* DNA pol δ, which contains an AAA^+ ATPase clamp loader, as in the bacterial replicases), it will be of interest to determine if selected compounds in this set do inhibit bacterial clamp loader ATPases in future work and if structural analogs of these compounds could be identified that confer greater specificity towards the bacterial replicases.

Identification of Specific Bacterial Replicase Inhibitors

Most encouragingly, a total of 74 compounds were determined to exhibit a significant degree of specificity towards one or both bacterial replicases. According to our specificity threshold, a 5-fold difference in IC_{50}s between replicases and specificity assays, 18 compounds were specific for the *E. coli* replicase (examples are compounds 56, 39, 45 and 48), 28 compounds were specific for the *B. subtilis* replicase (e.g., compounds 19, 67, 33 and 26), and 28 compounds were found to inhibit both replicases (e.g., compounds 51, 70, 69, 65, 29 and 61), revealing a potential to be developed into broad spectrum antibacterial agents. Our specificity threshold is based on relative numbers. We acknowledge that some compounds, such as 3 (Table S1), were designated Gram-positive specific, but still yield a credible 25 μM IC_{50} against the *E. coli* replicase.

We anticipate that these highly specific replicase inhibitors will act by a variety of mechanisms against numerous protein sub-targets within the replicase assays. Although we have identified compounds that appear to be specific to Gram-negative replicases, this conclusion cannot be firmly established until the actual targets are identified. The primase activity in the Gram-positive replicase systems was not included, and templates were primed by annealing an exogenous primer. Thus, a broad spectrum primase inhibitor could appear to be Gram-negative specific in our assays, as constituted.

Identification of Compound Structural Series

We examined all 394 compounds identified from the bacterial replicase screens using a chemical structure clustering algorithm (ChemTK, Sage Informatics LLC, Santa Fe, NM) to identify structurally similar inhibitors. We identified 7 distinct chemical series (A-G, Table 2), all with representatives among the specific bacterial replicase inhibitors listed in Table 1 (and further described in Table S1).

The largest series identified (Series A, 46 compounds) contained a purine-2,6-dione core and included 9 compounds predominantly displaying broad spectrum replicase-specific inhibition and also *B. subtilis* replicase specificity, as shown with compounds 51 and 19. Other members of this series showed nonspecific activities primarily against apyrase, T7 DNA polymerase and *S. cerevisiae* DNA Pol δ, possibly reflecting the nucleoside-like core structure.

The next-largest series (Series C, 28 compounds) contained 5-arylmethylenethiazolidine-2,4-diones or -2-thioxo-4-ones. Many of these compounds showed broad spectrum activity (e.g., compounds 65 and 69) and a few were specific for the *E. coli* targets (e.g., compound 45). Other members of the series displayed non-specific activities predominantly towards other DNA polymerases.

An additional series (Series F, 20 compounds) contained variously substituted 2-quinazolinyl-guanidines. The 8 bacterial replicase-specific members of this series (e.g., compounds 67 and 71) predominantly targeted the *B. subtilis* replicase. Other non-specific members of the series predominantly inhibited T7 DNA polymerase (compound 395) and the other DNA polymerases in the specificity panel. This series has been previously identified as possessing antimicrobial activity (58), and later, as a polymerase inhibitor (59).

The remaining series identified (Series B, compounds 29 and 48; Series D, compound 56; Series E, compounds 33 and 61; Series G, compounds 26 and 162) all contain compounds displaying broad spectrum and organism-specific replicase inhibition. The data obtained from the above series of compounds provides information that can be used to derive preliminary structure-activity relationships (SAR) that will prove useful during the pursuit of more defined SAR and the design of library expansion strategies. Likewise, the remaining 44 bacterial replicase-specific compounds that do not fall into obvious inhibitor series can also provide starting points for testing structurally related compounds to establish whether or not a common structural series can be identified.

Identification of Compounds with Target-Specific Biological Activity

To determine which compounds, initially identified using our multiplicative target screen, exhibited antimicrobial activity and retained target specificity in a whole cell context, we took a two-pronged approach. First, we screened all compounds with promising biochemical profiles for antimicrobial activity. Minimal inhibitory concentrations (MICs) were determined. Then, all compounds that exhibited a MIC of 256 μg/mL or less were screened using macromolecular synthesis assays where incorporation of precursors for proteins, RNA and DNA were quantified. Among 196 compounds with biochemical replicase specificity (or activity against few other specificity targets) screened, 33 compounds exhibited MICs of 256 μg/mL or less. Among these, seven preferentially inhibited DNA replication and provide candidates for the development of more potent and specific probes that can be used to block bacterial DNA replication (Table 3). All of the compounds identified show biochemical activity against both Gram-positive and Gram-negative replicases (Table S1). We were unable to explore the specificity for the *E. coli* replicase for five compounds because of high MICs (Table 3). Two compounds (#309 and #345) that exhibited low MICs against both *E. coli* and *B. subtilis* only showed replicase specificity against *E. coli*, but not *B subtilis.*. In all cases, increasing the potency and specificity with attention to cell permeability issues promise to yield specific biological probes.

DISCUSSION

Typically, screens of compound libraries are performed on single targets. While this has led to the efficient identification of inhibitors of an enzymatic activity or a targeted interaction, it misses inhibitors of interactions absent from the relatively simple screening assay. To target more complex interactions, whole cell screens are generally performed. In a particularly elegant early example, Mitchison and colleagues, using a screen for mitotic arrest in tissue culture cells, identified monastrol, an inhibitor of the kinesin Eg5 that binds to a site remote from the ATP or microtubule binding sites (60).

An alternative approach to the whole cell screens would be to include all of the components of a multi-protein machine in a fully reconstituted biochemical system. Indeed, progress has been reported in the development of more complex biochemical assays; for example, a fatty acid biosynthesis pathway screen has been described where a precursor is converted to product through several intermediates and independent enzyme-catalyzed reactions (61). Screens have also been performed against multi-subunit enzymes, permitting identification of individual subunits and their interactions (62,63). In this report, we exploited the most complex

biochemical system reassembled from purified components for screening purposes to date. We reconstituted and screened the entire apparatus for both Gram-positive and Gram-negative bacterial replicases. For the *E. coli* assay, this includes the 10 subunits of the Pol III holoenzyme plus SSB and DnaG primase.

We estimate that this assay contains upwards of 100 targets, including all of the essential subunits plus their interactions and essential conformers. These include an α-ε interaction, required for shuttling of the primer terminus between these two essential subunits (64). Polymerization itself contains at least six kinetic steps and essential changing enzyme conformations (65). Additionally, the replicative elongation reaction contains additional steps involved in partitioning of the primer terminus between the polymerase and exonucleolytic proofreading active sites (66). Each stage of the replication pathway provides a target that can be trapped by a small molecule, arresting the overall reaction. Indeed, the Benkovic lab has demonstrated that gp59 arrests the bacteriophage T4 replicase by trapping a conformation that blocks both polymerase and exonuclease activity (67).

In addition, α interacts with the ss DNA template, the duplex immediately behind the template, the primer of the preceding Okazaki fragment, and the essential β and τ subunits (16,19,68, 69). The subunit interactions changes during initiation complex formation and also in the dissociative reactions requisite for cycling during Okazaki fragment synthesis on the lagging strand of the replication fork (18,69). The DnaX complex contains essential intra-complex interactions: τ-τ, τ-γ, γ-δ', δ'-δ, δ-τ, γ-ψ and ψ-χ. This is just the static picture. Assembly of a processivity factor onto DNA requires at least six kinetic steps in which the intersubunit interactions change during a tightly regulated process that involves ATP binding and turnover, accompanied by ordered β and DNA binding and release (70). Blockage of any of these discrete kinetic steps by trapping the complex in a specific conformation should result in inhibition of the whole reaction.

SSB interacts with DNA and DnaG primase and regulates the transfer of nascent primers to the polymerase, a process that likely involves DnaG-χ-ψ-α or DnaG-α interactions (71). An additional SSB-χ interaction is important for initiation complex formation and stabilization of the polymerase on DNA in the presence of physiological salt (72,73). Blockage of any of these targets by a small molecule inhibitor would prevent conversion of the ss DNA template to the ds DNA product detected in our HTS assay. We refer to the exploitation of complex dynamic protein machines for screening purposes as *multiplicative target screening* to reflect the combinatorial nature of interacting components providing a quantity of targets that is a multiple of the number of individual components required.

We have extended multiplicative target screening to replication systems from two diverse organisms. Parallel analysis of a compound library in replication systems from diverse organisms provided direct determination of which compounds have broad spectrum potential and which compounds are specific to one or more related organisms. Compounds that inhibit replicases from distinct Gram-negative and Gram-positive organisms would be expected to have broad spectrum potential. Compounds that inhibit replicases from only closely related organisms would be expected to have a narrower spectrum (Figure 6). This approach, which we refer to as *parallel target screening*, provides information useful for both reverse chemical genetic approaches and the development of therapeutics. For chemical genetics, knowing which small molecules are inhibitors of a specific enzyme in a variety of systems will improve their utility as a tool, both in serving to block a common process in multiple organisms and to serve as a probe to study conserved mechanistic features. For therapeutics, broad spectrum agents are most often pursued, but a need for targeted therapeutics exists (74), such as antimicrobial agents used to treat chronic diseases without interfering with normal flora.

Inclusion of counterscreens against targets that, if inhibited, could result in toxicity permitted early discrimination of compounds with an undesirable lack of specificity. In our studies, we used the *S. cerevisiae* pol δ replicase as a surrogate for the closely related human replicase and the recombinant human mitochondrial replicase. Screening for compounds that interact with ds DNA and interfere with dye binding provided a preliminary indication that a compound might be acting by interacting with the substrate of the replicase rather than the protein itself. Inclusion of unrelated phage polymerases provided an additional means of detecting compounds that can block DNA synthesis by general or non-specific mechanisms. The *E. coli* replication system contains a specialized RNA polymerase that makes primers for DNA replication. Inclusion of the unrelated *E. coli* RNA polymerase provided a means of eliminating general RNA polymerase inhibitors. In both the *E. coli* and *B. subtilis* systems, the β processivity factor is placed on DNA by the action of a AAA+ ATPase, the DnaX complex. We included an unrelated ATPase, apyrase, to eliminate simple ATP analogs or nonspecific ATPase inhibitors.

We have also demonstrated that our approach yields a subset of interesting compounds with biological activity, retaining the specificity indicated by the biochemical screen. The good yield (seven compounds with biological target specificity) from a small 20,000-compound test library, is an indication of the richness of the target explored by the biochemical multiplicative target screening approach. We expect that, by selecting a range of compounds showing high biochemical specificity, further optimization to increase potency will reveal additional useful biological probes as well as useful compounds to study the structure and mechanism of replicases. However, a strength of the biochemical approach resides in the ability to identify compounds and their accompanying targets that would have been missed in whole cell screens because the compounds fail to achieve the high cellular concentrations required for the relatively weak inhibitors expected from first-stage screening efforts. Those additional compounds that demonstrate high biochemical specificity can be further optimized to increase both their potency and cellular permeability.

A key advantage of our multiplicative target screening strategy is that a very large number of targets can be screened simultaneously. These targets include subtle conformational changes and interactions that may not yet be understood. However, this powerful approach leaves the challenge of identifying the exact target, both in terms of the binding sites and the specific interactions and mechanistic/kinetic steps affected. We are developing an efficient deconvolution process whereby the target can be identified without testing all possibilities. We anticipate this will involve broad classification of the general reaction stage (priming, initiation complex formation and elongation) and iteratively narrowing our search until the specific target is identified. For example, inhibitors shown to block initiation complex formation specifically, will be screened to determine whether ATP, DNA or β_2 binding to the DnaX complex is blocked or if the ATPase activity of DnaX is repressed. Once these details are understood, the specific kinetic step affected and specific binding site can be pursued, exploiting the extant wealth of functional and structural information. This will not only permit direct identification of known interactions and kinetic steps but also reveal unknown features of our complex system, by discovering compounds that interfere with weak, essential interactions or block fast non-rate limiting kinetic steps that have eluded detection by other techniques. As our studies progress, we hope to gain a better understanding of the structure of target-inhibitor complexes and exploit this information to gain mechanistic insight and to rationally progress promising inhibitors toward useful therapeutics and valuable biological and mechanistic probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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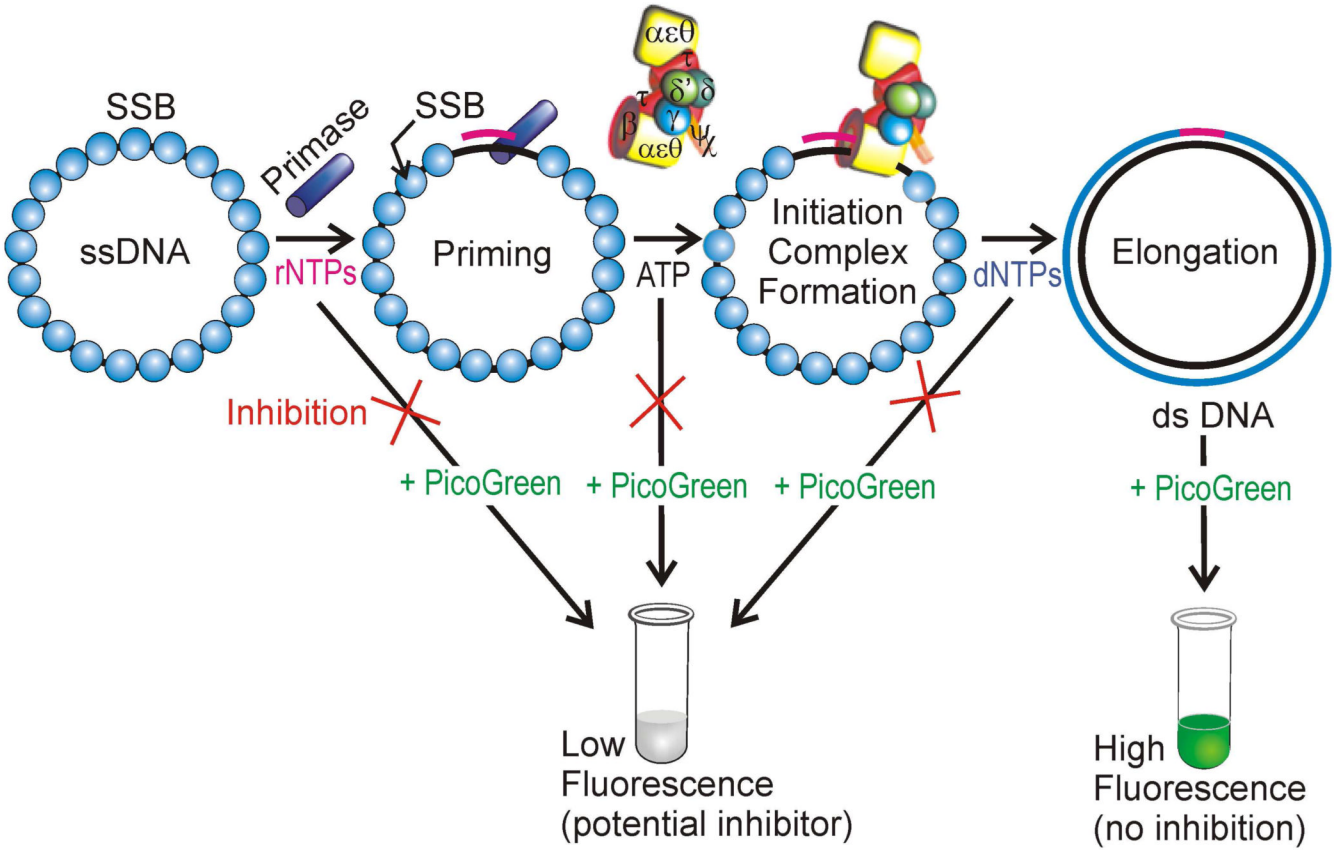


Figure 1.

Homogenous, PicoGreen Fluorescence-based Assay for High-Throughput Screening of Bacterial Replicases. In all replicase screening assays, the conversion of ss phage DNA to the duplex form was monitored by an increase in fluorescence upon binding the ds DNA-specific dye, PicoGreen. This permitted detection of inhibitors that act at any of the reaction steps, including priming on the SSB-ss DNA nucleoprotein filament, ATP-dependent initiation complex formation by Pol III holoenzyme, and subsequent elongation in the presence of dNTPs. Assays of the *B. subtilis* replicase were similar, except a primer was provided by annealing of a synthetic oligonucleotide.

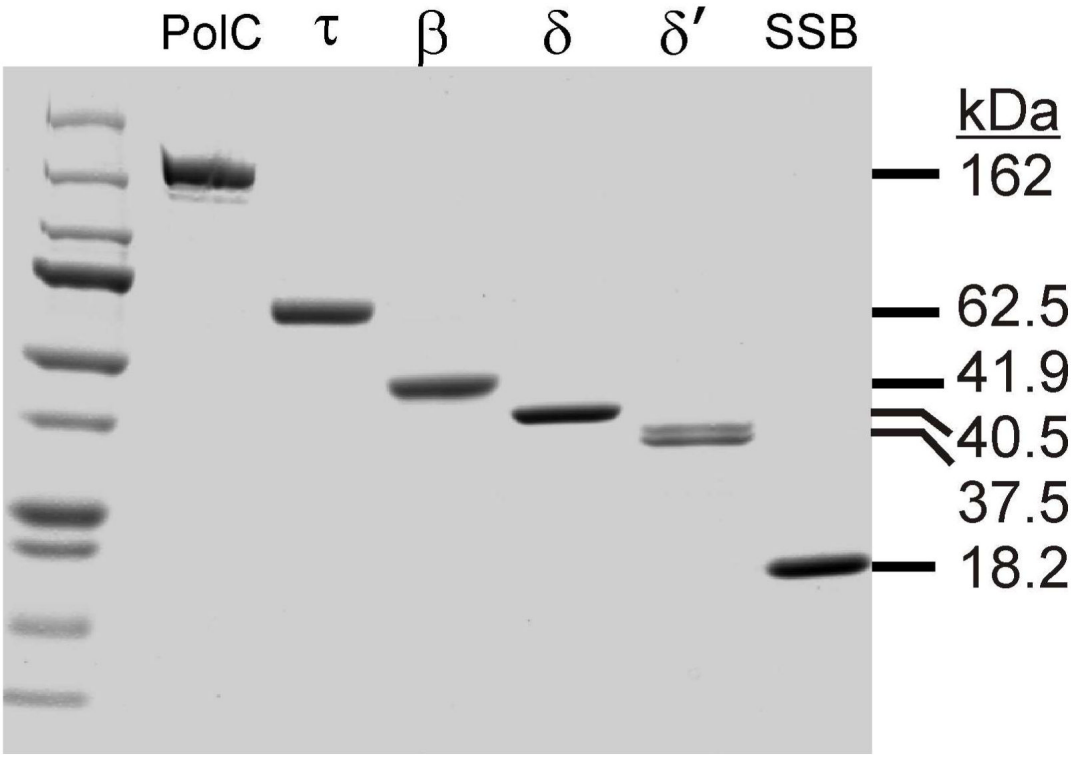


Figure 2.
Purified *B. subtilis* DNA Replication Proteins. Purified *B. subtilis* minimal replicase components PolC, τ, β_2, δ, δ' and SSB were analyzed on a 4-20% gradient SDS polyacrylamide gel. The first lane contained protein molecular weight standards. Other lanes contained 2 μg samples of each B. subtilis replicase protein as indicated. Molecular weights of each replicase protein are listed on the right.

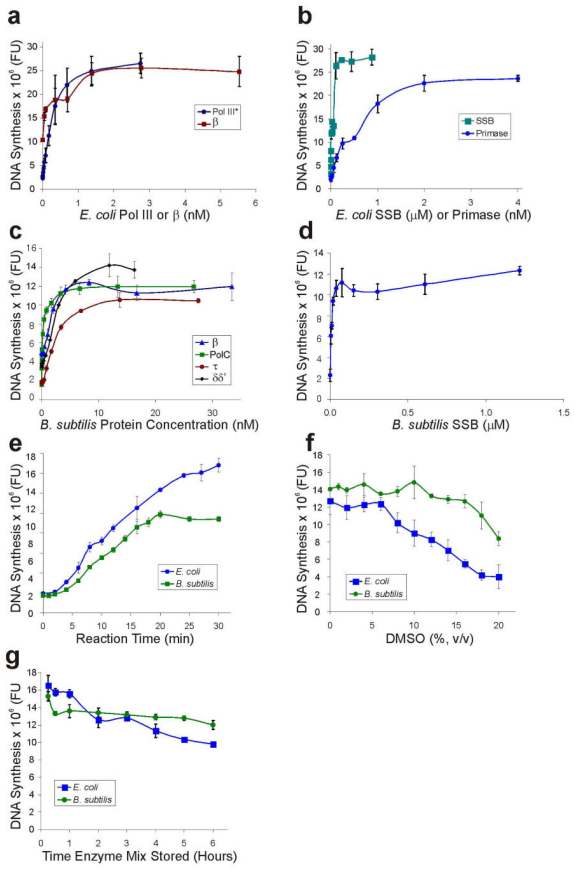


Figure 3.

Optimization of *E. coli* and *B. subtilis* Replicase High-Throughput Screening Assays. (A) Titration of *E. coli* Pol III holoenzyme components in the presence of saturating levels of the other assay components. Screening assays (25 μL volume) were conducted using 0.35 nM Pol III* and 1.4 nM β_2. Substrate concentrations used in all *E. coli* HTS assays were 5 μM dATP, dGTP, dCTP and dTTP, 200 μM CTP, GTP, UTP and 50 μM ATP, and 40 pmol of ss $M13_{Gori}$ (as nucleotide). (B) Titration of *E. coli* SSB and DnaG primase in the presence of saturating levels of the other *E. coli* assay components. Screening assays were conducted using 154 nM SSB_4 and 2 nM DnaG. (C) Titration of *B. subtilis* replicase components in the presence of saturating levels of the other assay components. Screening assays (25 μL volume) were conducted using 1.7 nM PolC, 2.8 nM β_2, 12 nM τ and 6 nM δδ'. Substrate concentrations used in all *B. subtilis* HTS assays were 7 μM dATP, dGTP, dCTP and dTTP, 15 μM ATP, and 40 pmol of oligonucleotide-primed ss $M13_{Gori}$ (as nucleotide). (D) Titration of *B. subtilis* SSB in the presence of saturating levels of the other assay components. Screening assays were conducted using 156 nM SSB_4. (E) Determination of time course of optimized, reconstituted replicase reactions. All assay components for each experiment were used at the concentrations specified in Figure 3A-D and contained 5% DMSO. All screening assays were performed for the indicated times before quenching with a PicoGreen dye/EDTA mixture as described under *Materials and Methods*. (F) Determination of the sensitivity of the HTS assay to DMSO. All assays were performed as specified in panels A-D. *E. coli* screening assays were conducted at a maximum of 8% DMSO (v/v). *B. subtilis* screening assays were conducted at a maximum of 15% DMSO (v/v). (G) Determination of stability of assay components at room temperature (22 °C). An enzyme premix containing protein components (at concentrations specified in Figure 3A-D) diluted in the optimized HTS assay buffer was mixed with 8% (*E. coli*) and 15% (*B. subtilis*) DMSO and incubated for the indicated times at room temperature before the

addition of substrates. Reactions were carried out for 20 minutes before the addition of PicoGreen dye reagent and EDTA as described under *Materials and Methods*.

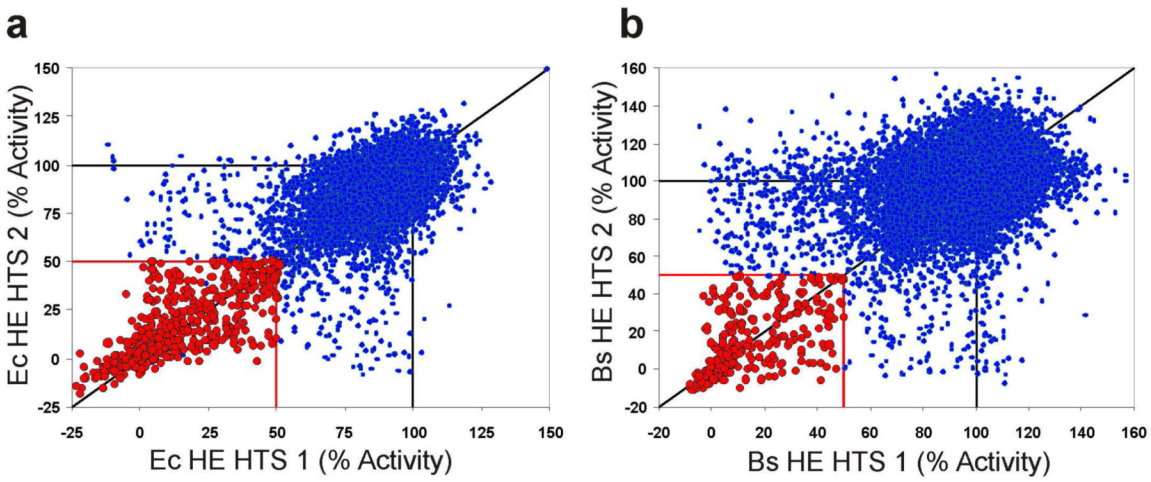


Figure 4.

Duplicate Screening Plots of the Bacterial DNA Pol III holoenzyme HTS. Compound activity (expressed as % of normalized DMSO control activity) for two screening runs is plotted. The black diagonal line represents the theoretical position of equivalent activity for the 20,000 compounds in each duplicate screen. The red lines outline the hit definition zone in which compounds scoring ≤50% of the activity of the uninhibited DMSO controls are considered primary hits (red points). (A**)** *E. coli* DNA Pol III HTS. Average assay signal-to-background ratios were 9.4 ± 2.2 and 9.8 ± 1.8 for screens 1 and 2. Average Z' factors were 0.73 ± 0.14 and 0.76 ± 0.16. (B**)** *B subtilis* DNA Pol III HTS. Average assay signal-to-background ratios were 11.7 ± 1.0 and 10.3 ± 0.7 for screens 1 and 2. Average Z' factors were 0.71 ± 0.10 and 0.74 ± 0.10.

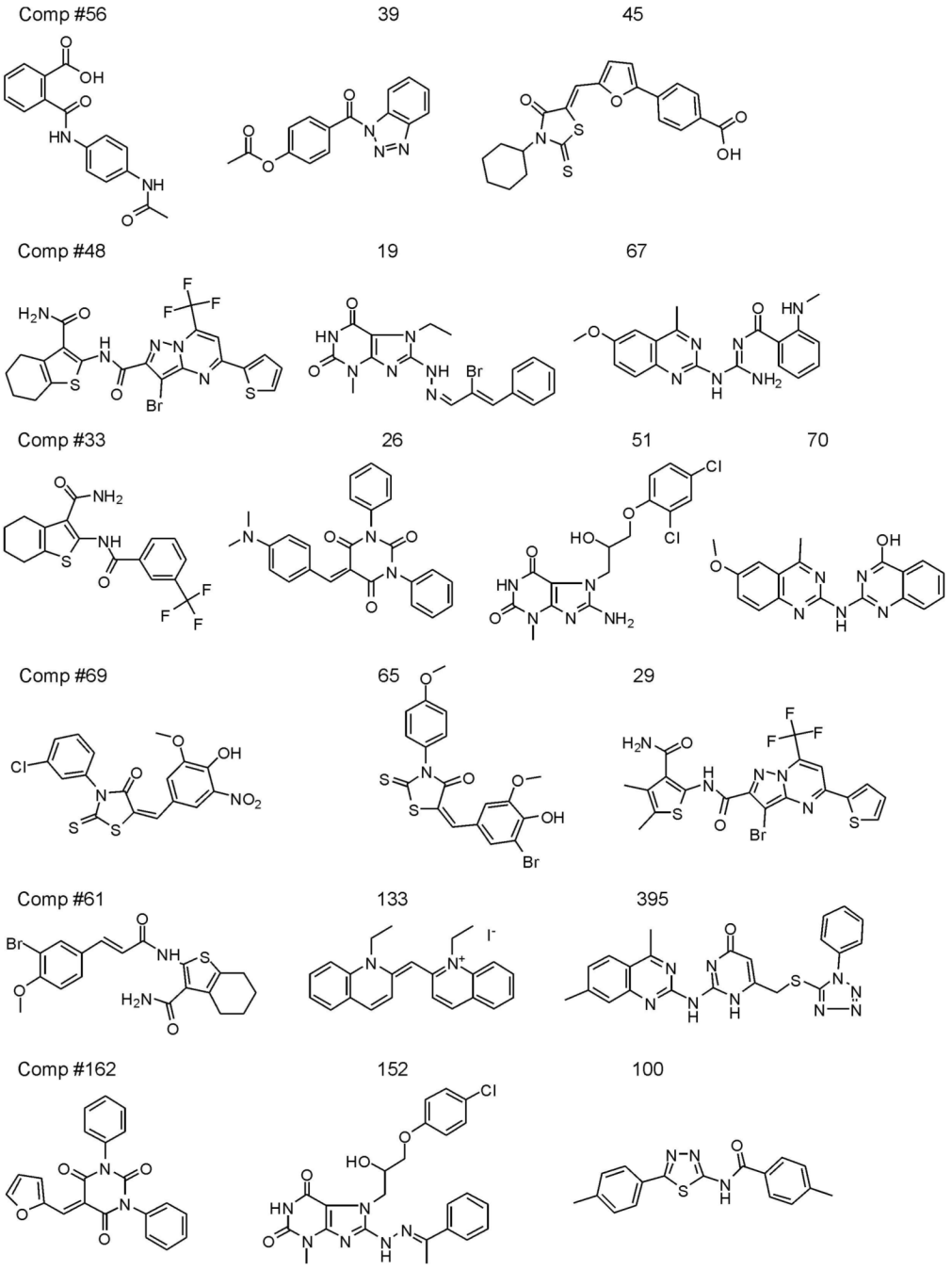


Figure 5.

Representative Inhibitors Identified through HTS against Bacterial Replicases. Structures of selected inhibitors of the *B. subtilis* and *E. coli* replicases are shown. Activity profiles of each compound in all replicase screens and specificity assays are shown in Table 1 and in Table S1.

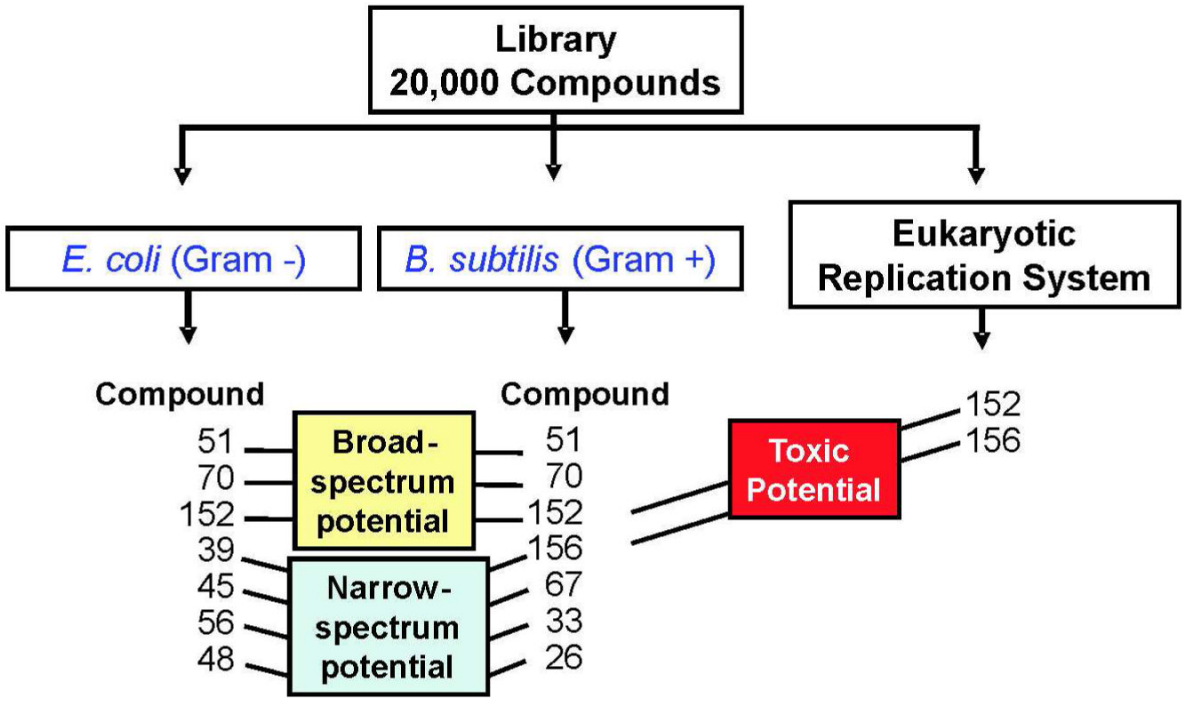


Figure 6.

Use of Parallel Multiplicative Target Screening Assays to Identify Specific Bacterial DNA Replication Inhibitors with Broad Spectrum Potential. The use of the *E. coli* replicase permits identification of compounds that have potential for general Gram-negative antibacterial activity. The same approach, utilizing the *B. subtilis* replicase, permits distinguishing compounds with general Gram-positive activity. Compounds that inhibit both Gram-positive and -negative replicases have the highest potential for being developed into broad spectrum agents. For therapeutic purposes, having a spectrum that is so broad that it includes the analogous human target is, of course, undesirable. Inclusion of a human target or a homologous eukaryotic target, permits elimination of compounds inhibiting the eukaryotic target. Compound numbers correspond to those in Tables 1 and S1.

Table 1

Representative Inhibitors Identified through HTS against Bacterial Replicases

	DNA Replication IC50 (μM)[b]		Specificity Assay IC50 (μM)								
Comp #[a]	***Eco***	***Bsu***	**DNA binding**	**T7**	**T4**	**Pol δ**	**Pol γ**	**Apy**	**RNAP**	**β-Gal**	**Specificity[c]**
56	5.6	86.0	>300	110	300	>300	>300	>300	>300	>300	*E. coli*
39	2.7	62.9	>300	>300	>300	>300	>300	>300	>300	>300	*E. coli*
45	13.4	192	300	>300	>300	>300	>300	>300	>300	>300	*E. coli*
48	60.6	>300	>300	>300	>300	>300	>300	>300	>300	>300	*E. coli*
19	>300	8.7	>300	>300	>300	300	>300	>300	>300	>300	*B. subtilis*
67	>300	1.3	>300	28.0	>300	>300	>300	>300	>300	>300	*B. subtilis*
33	260	35.0	>300	>300	>300	180	>300	>300	>300	>300	*B. subtilis*
26	>300	20.5	>300	>300	>300	>300	>300	>300	>300	>300	*B. subtilis*
51	28.6	22.3	>300	250	>300	300	>300	250	>300	>300	Broad
70	1.3	3.2	>300	10.2	>300	>300	>300	>300	>300	>300	Broad
69	0.9	0.9	55.4	11.3	14.6	4.5	>300	8.1	129	>300	Broad
65	6.0	3.0	33.0	31.8	57.2	36.6	>300	300	>300	>300	Broad
29	25.2	25.2	175	>300	>300	147	>300	>300	>300	>300	Broad
61	46.2	9.4	>300	68.1	127	>300	>300	>300	>300	>300	Broad
133	8.2	22.3	19.5	14.2	99.4	12.0	>300	198	94.5	15.8	Non-specific
395	36.9	2.8	>600	12.6	>600	>600	>600	>600	>600	>600	T7 Pol/ *B. subtilis*
162	20.4	34.0	>300	200	2.8	>300	>300	>300	>300	>300	T4
152	12.4	10.5	>300	>300	>300	25.0	>300	>300	>300	>300	Polδ/ Broad
100	22.7	18.8	>300	>300	>300	>300	>300	0.8	>300	>300	Apyrase

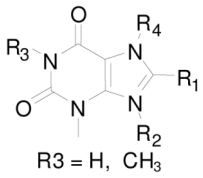
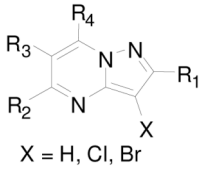
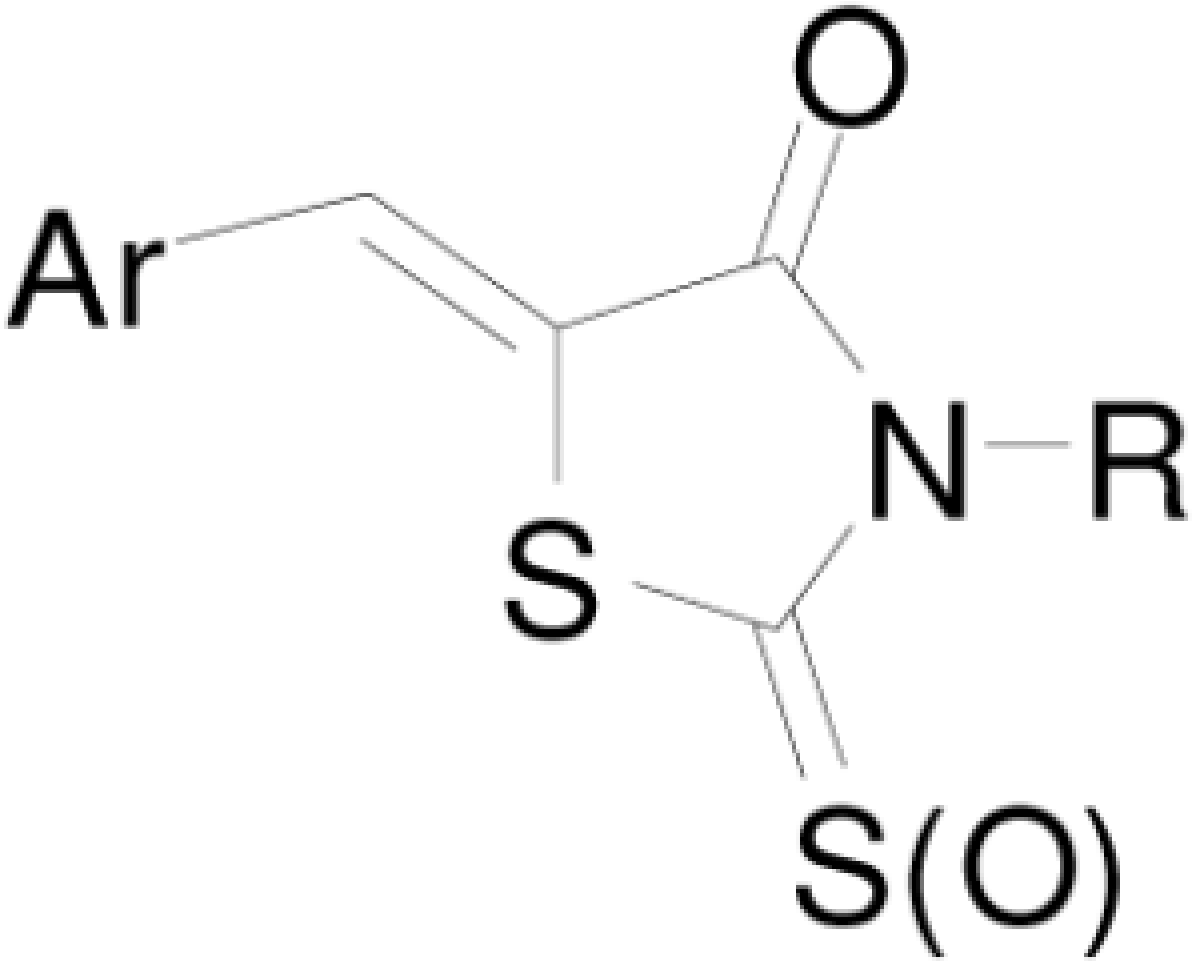
[a] Comp # refers to compounds shown in Figure 5 and Table S1.

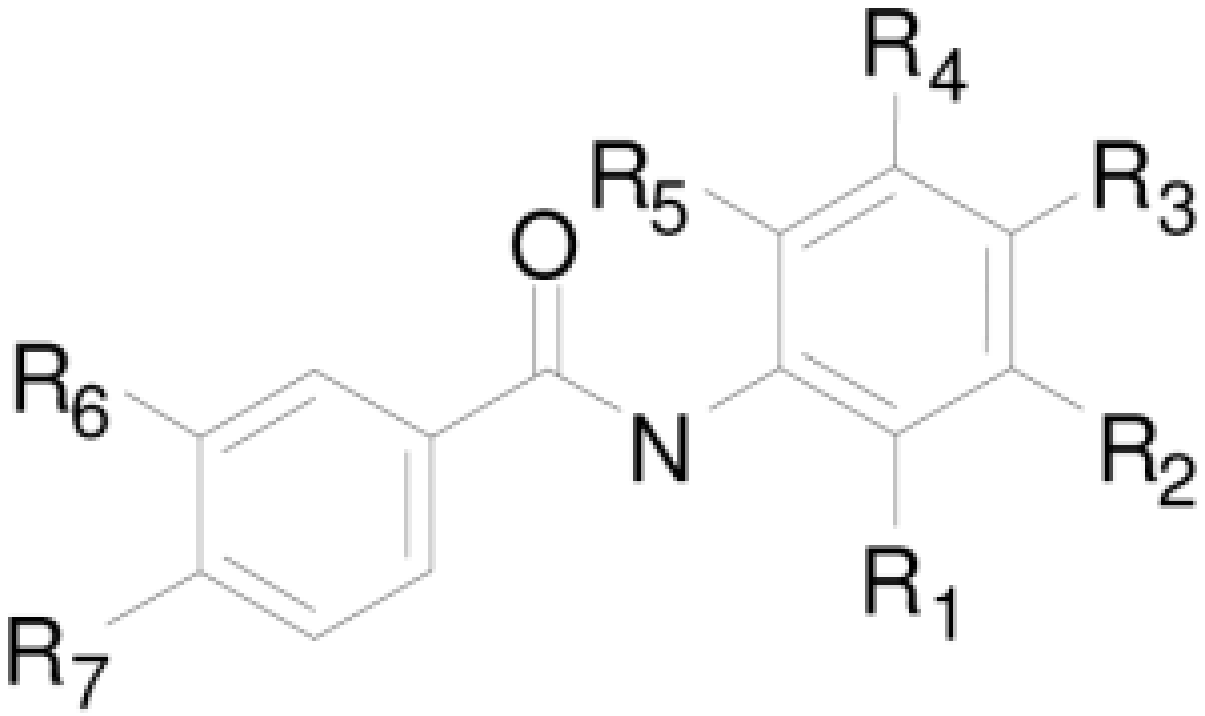
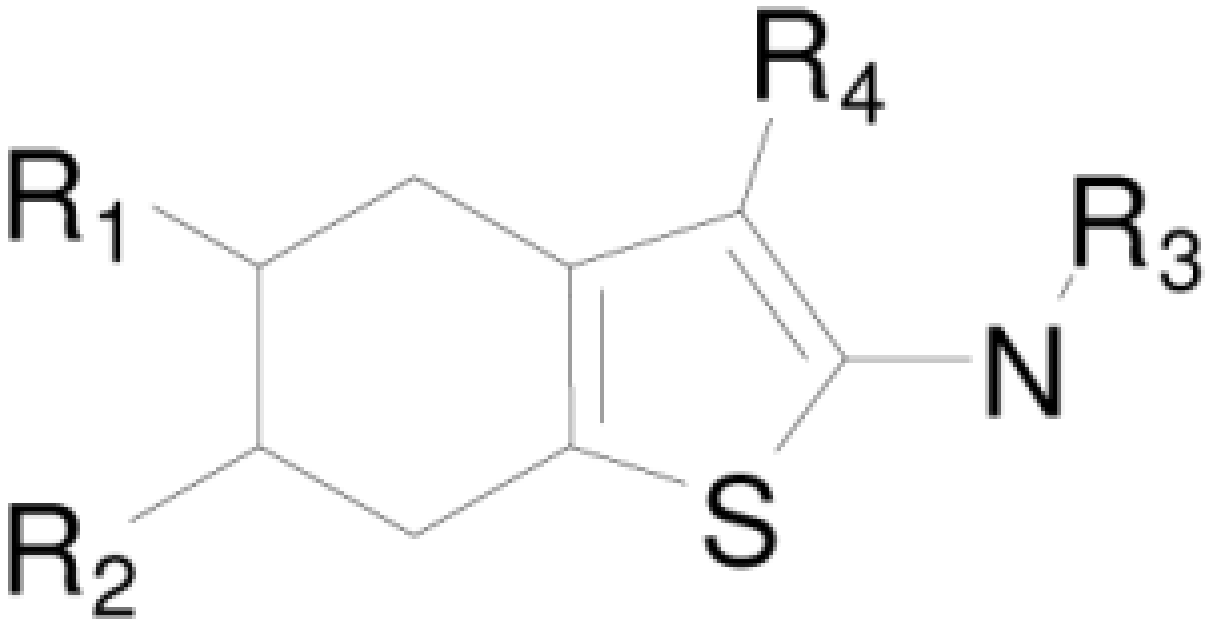
[b] IC_{50} values for both bacterial replicase assays (Eco, *E. coli* replicase; Bsu, *B. subtilis* replicase) and all specificity assays (DNA binding, IDD assay; T7, T7 DNA polymerase; T4,T4 DNA polymerase; Pol δ, *S. cerevisiae* DNA Pol δ; Pol γ, Human Mitochondrial DNA Polymerase; Apy, Apyrase ATPase; RNAP, *E. coli* RNA polymerase core; β-Gal, β-galactosidase) are shown. Bacterial replicase assays were conducted as described in *Materials and Methods.* IC_{50} values containing a ">" sign indicate that the compound did not inhibit the assay lower than the 50% activity level at the highest compound concentration tested.

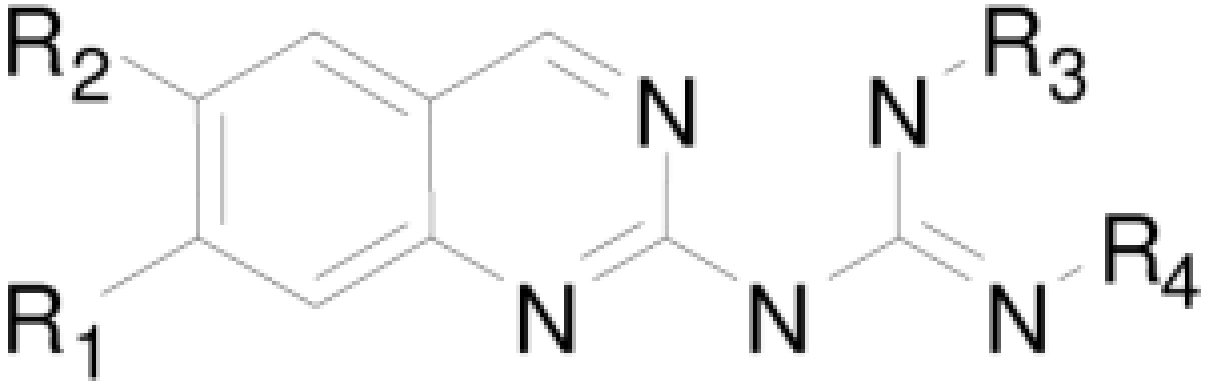
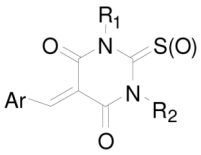
[c] A threshold of 5-fold was used to score the specificity of a given compound for a particular assay or bacterial replicase target. Specificity assays were conducted as described under *Materials and Methods.*

Table 2

Inhibitor Series Identified in Replicase Screens

Series	Core Structure [a]	Comp # [b]
A		19,51 152
B		29,48
C		45,65 69

Series	Core Structure [a]	Comp # [b]
D		56
E		33,61

Series	Core Structure [a]	Comp # [b]
F		67,70 395
G		26,162

[a] Ar = Aromatic Substitution

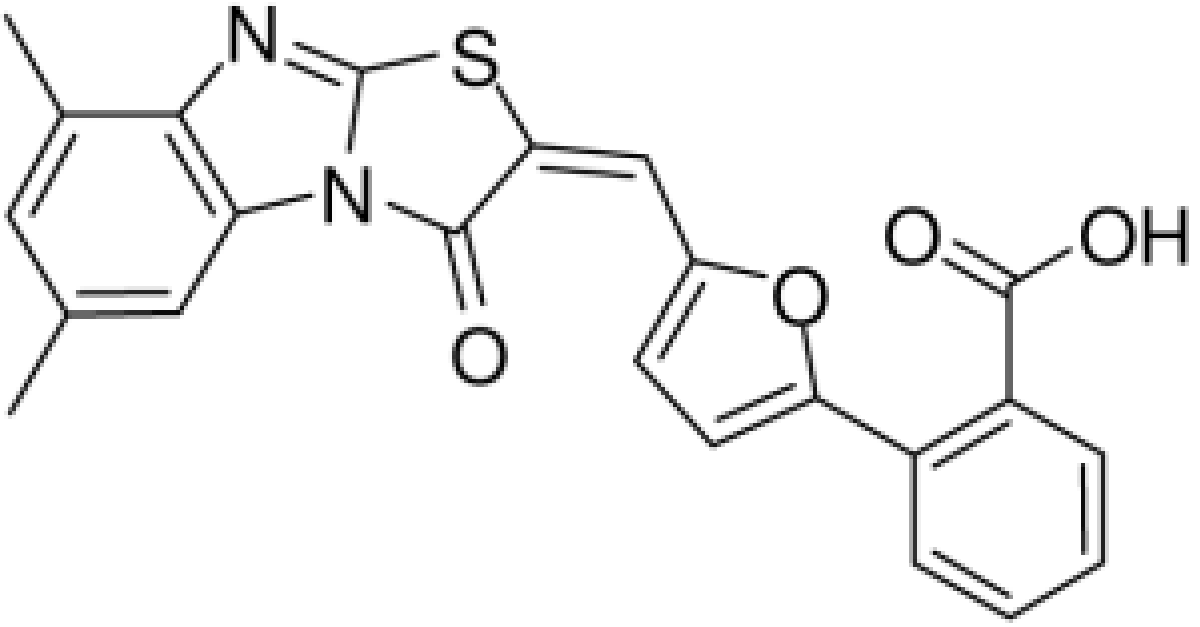
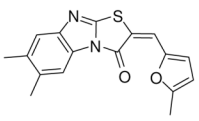
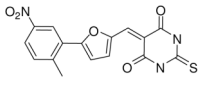
[b] Comp # refers to all compounds shown in Figure 5 and in Table 1.

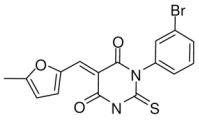
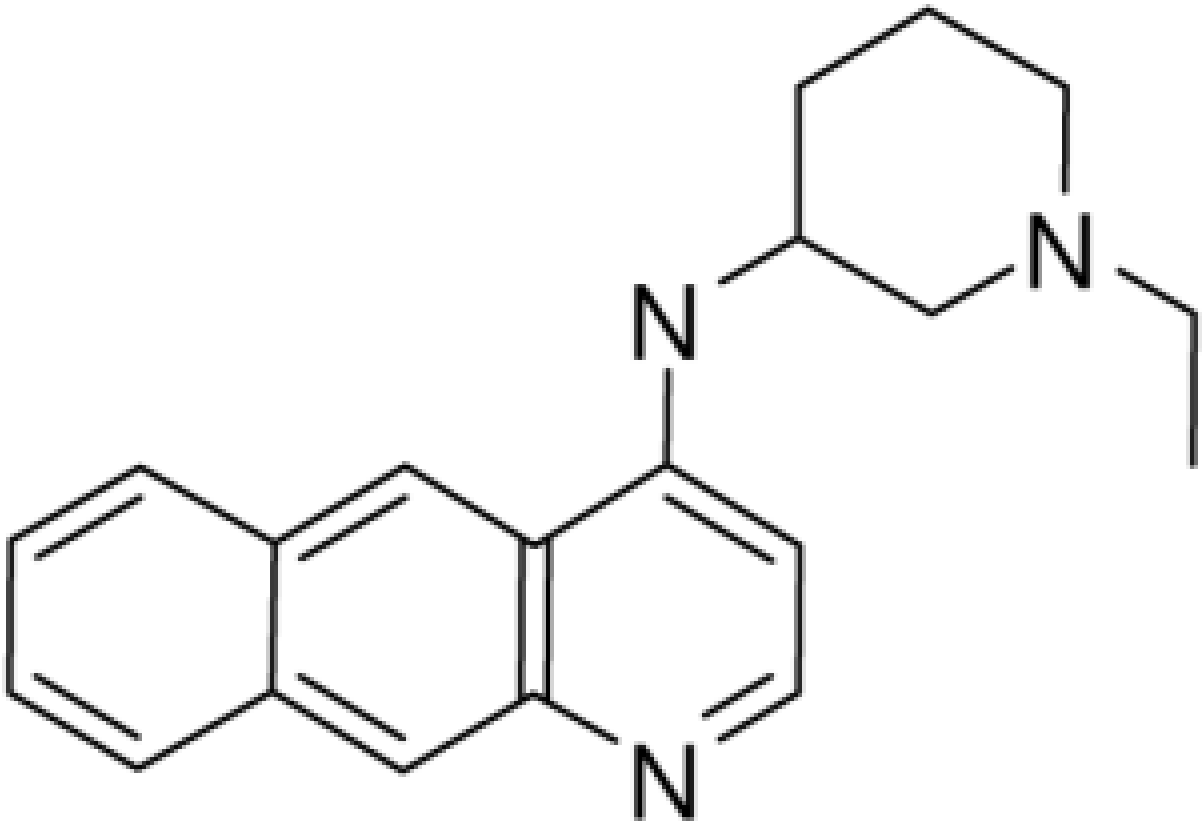
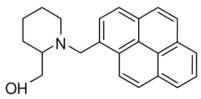
[c] Compounds that were hits in the HTS and titrated with an IC_{50} of 100 μM or less

[d] Compounds that titrated with and IC_{50} of 5-fold or less than the most inhibited specificity assay.

Table 3

Inhibitors of Cellular DNA Replication

Comp #	Structure	Antimicrob MIC (μ *E. coli*
72		>512
4		512
153		512
161		>512

Comp #	Structure	Antimicrob MIC (μ *E. coli*
285		>512
309		16
345		128

[a] MIC values of >512 μg/mL indicate no growth inhibition at the highest compound concentration tested.

[b] Data for MMS assay of these compounds is shown in Figure S1 in *Supplemental Information*.