Novel Inhibitors of *E. coli* RecA ATPase Activity

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Abstract: The bacterial RecA protein has been implicated as a bacterial drug target not as an antimicrobial target, but as an adjuvant target with the potential to suppress the mechanism by which bacteria gain drug resistance. In order to identify small molecules that inhibit RecA/ssDNA nucleoprotein filament formation, we have adapted the phosphomolybdate-blue ATPase assay for high throughput screening to determine RecA ATPase activity against a library of 33,600 compounds, which is a selected representation of diverse structure of 350,000. Four distinct chemotypes were represented among the 40 validated hits. SAR and further chemical synthesis is underway to optimize this set of inhibitors to be used as antimicrobial adjuvant agents.

INTRODUCTION

Antibiotic resistance is an innate problem involved in the treatment of bacterial infectious diseases and the incidence of these infections is escalating at an alarming rate [1-3]. Resistance not only renders current antibiotics useless against dangerous pathogens, but it is also a financial deterrent to efforts towards the discovery of novel antibiotics [4]. As a result, many pharmaceutical companies have reduced or eliminated their antibacterial drug discovery efforts [5], and since 1962 only three new classes of antibacterial agents have been introduced [6]. Genomics-driven discovery against targets considered essential has not yielded any clinically approved drugs [5], and one way to avert this looming public health crisis could be to replenish antibiotic drug pipelines with drugs against targets not considered essential for survival. In fact, two-thirds of *Escherichia coli* genes fall into this population [7], and targeting this previously ignored segment of the bacterial genome may offer possibilities for the discovery of non-traditional pharmaceutical agents that attenuate pathogenicity [8] or potentiate the pharmacologic effects of known antibacterial agents [9]. In this context, we have focused on the bacterial RecA protein as a prospective target in the treatment of bacterial infectious diseases. RecA's activities are central to the repair of DNA damage and stalled replication, but are also important for adaptive mutagenesis and horizontal gene transfer. We hypothesize that small molecule inhibitors of RecA may sensitize bacteria to established antibacterial agents and prevent the development and acquisition of genes conferring drug resistance.

In *Escherichia coli*, a model system to study DNA damage, RecA's expression is upregulated by events that challenge the integrity of the organism's genome, and lead to the formation of single-stranded DNA (ssDNA), a hallmark of DNA damage [10]. In this event, RecA monomers respond by forming a homopolymer on the exposed ssDNA, initiating a series of programmed events that can be fundamentally associated with two major functions, de-repression of the SOS regulon and recombinational DNA strand exchange [11, 12]. In the first function, the SOS regulon is initiated when the RecA nucleoprotein filament comes into contact with the LexA repressor, which sits on promoters of up to 40 genes that are involved in repairing DNA damage [13]. Gene products expressed in the early stages of SOS repair the DNA damage by nucleotide excision (e.g., UvrABCD) or recombination (e.g., RecA), while gene products of the late stages of SOS are involved in error-prone DNA synthesis (e.g., PolIV and PolV) that systematically introduces mutations into the bacterial genome and facilitates adaptive evolution [14-17]. In the second function, RecA pairs ssDNA with homologous duplex DNA, and hydrolyzes ATP to induce the conformational rearrangements necessary to catalyze a strand exchange reaction that produces two new DNA molecules [18, 19]. This ATPase-dependent activity is necessary for recombinational DNA repair [20, 21] and horizontal gene transfer during bacterial transformation, transduction and conjugation [22].

Recently, the SOS and recombination processes mediated by RecA have been shown to be activated in response to antibiotic treatment [23-25]. The biological activities controlled by RecA likely enhance a bacterium's tolerance to antibiotics and can result in the de novo development and horizontal transfer of antibiotic resistance genes. Because RecA and its functions in overcoming antibacterial chemo-

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therapy are highly conserved among bacteria, small molecule modulators of RecA would be valuable for further exploration of its roles in bacterial pathogens and its potential exploitation as a therapeutic target. While ATP-competitive nucleotide analogs [26, 27], polysulfated naphthyl compounds [28], short alpha-helical peptides [29] and select transition metal cations [30] are effective inhibitors of RecA *in vitro*, none of these molecular classes have demonstrated RecA-specific biological activities in bacterial cell cultures.

Towards the goal of discovering potent cell-permeable inhibitors for the validation of RecA as an important and novel target for the chemotherapeutic treatment of bacterial infections, we carried out a two-tiered HTS campaign against RecA. As ssDNA-dependent ATP hydrolysis is a useful indicator that the RecA protein is active, we elected to screen the compounds using an inexpensive, yet robust and reliable phosphomolybdate blue ATPase assay to identify compounds that inhibit ATP hydrolysis. We initially screened the BRITE diversity set, which is comprised of approximately 10% of our proprietary collection of nearly 350K compounds selected to maximize chemical diversity. From this collection, we identified 40 hits that clustered into four chemotype classes which were then carried into dose-response confirmatory testing. We have also performed Tanimoto-based similarity searching in the entire BRITE library to identify related compounds that will be screened to provide initial insights into structure-activity relationships.

MATERIALS AND METHODOLOGY

RecA and ATPase Reagents

For the single-concentration screening, RecA protein was purified and stored as previously described by Singleton, *et al.* [31] and for additional screen of 8640 compounds, the enzyme was purchased from Epicentre Biotechnologies for the dose/response study. The commercially available enzyme has similar purity and specific activity as purified in-house, which is greater than 90% pure as judged by SDS-PAGE. Polydeoxythymidylic Acid (poly(dT)) single-strand DNA (average length = 300 nucleotides) was purchased from Midland Certified Reagent Company (Cat #P-2004, Lot 110607-403). Crystalline L-ascorbic acid and sulfuric acid were purchased from Fisher Scientific. Unless otherwise stated all other reagents were purchased from Sigma-Aldrich at the highest level of purity possible.

Compound Libraries and Handling

The general strategy for RecA screening was to test a small diverse library and then elaborate on the confirmed active compounds by similarity searching in the entire BRITE compound library. The BRITE diversity library screened in this HTS effort consists of 33,600 compounds selected for chemical diversity from the entire BRITE library, which was generated by combinatorial chemistry synthetic routes and was a gift from Biogen-Idec in 2005. All of the compound plates were stored in polypropylene deep-well blocks at 4 °C without humidity controls. The BRITE compound library is plated in 384-well Greiner V-bottom polypropylene plates in 100% DMSO in 10 mM and 1 mM aliquots. Plates are stored at 4 °C. Using the Biomek® NX and P20 tips, 0.5 µl of 1 mM compound in DMSO was spotted into columns 3 - 22 of Costar clear flat bottom 384-well as-

say plates (Corning # 3702) for single shot analysis. Columns 1, 2, 23, and 24 were spotted with 0.5 µl of DMSO with the Biomek® NX and P20 tips and used as positive and negative controls. This resulted in a final concentration of 10 µM for single-point screening.

For dose-response/IC$_{50}$ curve analysis, 1 µl of the 10 mM stock of selected compounds was added to 4 µl of DMSO and mixed in an Axygen 384-well PCR plate for a 5-fold dilution and beginning concentration of 2 mM. This was performed with the Biomek® 3000 and P20 tips. A 10-point 2-fold serial dilution was then performed across the plate with the Biomek® 3000. The serially diluted compounds were then spotted to columns 3 – 22 of Costar clear flat bottom 384-well assay plates with the Biomek® NX and P20 tips. Columns 1, 2, 23, and 24 were spotted with 0.5 µl of DMSO with the Biomek® NX and P20 tips and used as positive and negative controls.

Phosphomolybdate-Blue ATPase Assay

A modified phosphomolbydate-blue ATPase assay (Unpublished data; Yeh L.A.) was used to quantify the amount of free phosphate as a measure of ATP hydrolysis, as an indirect measure of RecA nucleoprotein filament assembly [32]. The ATPase reactions were carried out in Costar clear flat-bottom 384-well assay plates (Corning, Lowell, MA) that the compounds were spotted in, and the final volume in each well was 30.5 µl, giving final concentrations of 17 µM for the library compound and 1.6% for DSMO. A 2.25 µM stock of ATP was prepared in H$_2$O and 10 µl of this was added to all wells of the assay plates using a Thermo Mutidrop 384-well dispenser (Thermo Fisher, Waltham, MA), yielding a final ATP concentration of 0.75 µM. The reaction rate is linear at this low ATP concentration. The assay development, optimization and validation studies of known compounds were described in a recent publication by Wigle [33]. After assay assembly, the plates were then incubated at room temperature for 15 min. To columns 3-24 of the assay plates, using a Thermo Mutidrop 384-well dispenser, was added 20 µl of a cocktail of containing 0.5 µM RecA, 5 µM poly(dT) ssDNA, 10 mM Mg(OAc)$_2$, 25 µM Tris·HOAc, and 5% v/v glycerol. For the negative control (minimum signal control), 20 µl of an identical solution omitting poly(dT)-DNA was added to columns 1 and 2 using a Thermo Mutidrop 384-well dispenser. The positive control (maximum signal control) in lanes 23 and 24 contained the full ATPase reaction reagents and 0.5 µl DMSO representing the full/uninhibited RecA activity. Compounds that exhibited inhibition greater than 50% in the primary screen were carried into the dose-response confirmation step.

For the IC$_{50}$ determinations, the PMB ATPase assay was improved by the addition of 10 µg /mL BSA to reduce the immobilization of RecA in the assay plates, thereby increasing the amount of RecA in the well that can participate in nucleation and filament formation. It was also determined that the assay window was wider when the optical absorption was tabulated at 825 nm. Serial dilutions of compounds were performed in 100% DMSO with a two-fold dilution factor resulting in 10 final concentrations spanning 33 µM to 65 nM. The serially diluted compounds (0.5 µL) were dry spotted into assay plates and were assembled as described for the single-concentration screen. Four-parameter sigmoidal re-

gressions were performed and IC_{50} values were determined using the XLfit software (XLFit software, IDBS, Guildford UK).

The assay plates were sealed by a Thermo Scientific microplate heat sealer and centrifuged at 1000 RPM for 1 min. The plates were then transferred to a 37 °C air incubator and the ATPase reaction was allowed to proceed for 35 min. The plates were subsequently removed from incubator and 30 µl of freshly made phosphomolybdate blue dye (10% ascorbic acid and 2.5% ammonium molybdate at the ratio of 1:1 and adding 12 M sulfuric acid at 6.25% v/v) was added to all wells using a Thermo Mutidrop 384-well dispenser. After 30 min of incubation at room temperature, the plates were scanned for absorbance at 825 nm using a Spectramax-384 Plus (Molecular Devices, Sunnyvale, CA).

HTS Results and RecA Inhibitor Identification

In lieu of direct measurement of RecA nucleoprotein filament formation, we chose a more HTS-amenable endpoint of ATP hydrolysis in the presence of single-stranded DNA to identify RecA inhibitors. Here we report confirmed molecular structures for the most potent RecA inhibitors in each distinct class discovered in this screening effort. This screening approach successfully identified several chemotype classes and gave initial insight into the structure-activity relationship of small molecule inhibition of bacterial RecA.

RESULTS

The PMB ATPase screen of the BRITE diversity library resulted in 73 active compounds exhibiting RecA inhibition above a 50% threshold. RecA inhibitors were triaged by selecting the most potent single-point hits and were confirmed with dose-response. The average Z′ for this screen was 0.83, with an overall hit rate of 0.22%. Of the 73 hits obtained, 71 of 73 compounds could be classified into one of four chemotype classes based on structural similarity (Fig. **1**). The label for chemotype classes D and C are reversed in the lower portion of Fig. (**1**). "C - 9 compounds" should appear on the lower left, and "D – 2 compounds" should appear on the lower right.
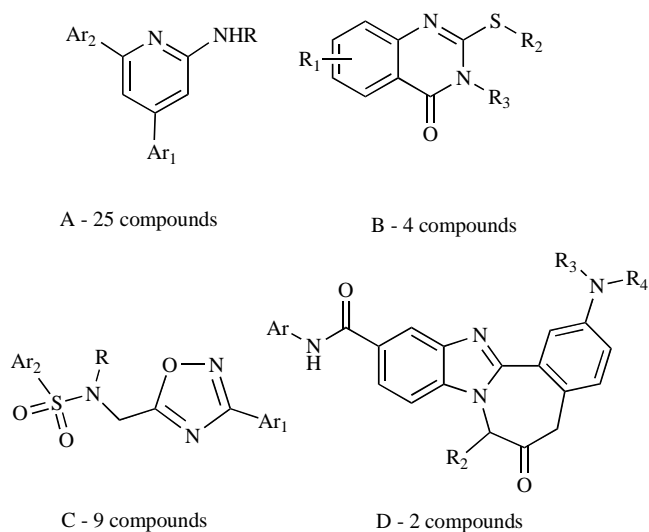
Fig. (1). Four chemotype classes of RecA inhibitors showing the number of compounds discovered for each classes in this PMB ATPase high thoughput screening effort.

The 73 hits identified in the single-point 10 µM screen were carried into dose-response confirmation with 40 compounds exhibiting dose-response behavior, yielding a 55% confirmation rate. A typical dose-response curve for RecA inhibitors is shown in Fig. (**2**), with characteristic steep dose-response curve likely due to multi-site inhibitor binding and well known positive cooperative binding kinetics [34, 35]. The structure and dose-response curve for the most potent RecA inhibitor identified in the BRITE diversity set screen, BRITE-338733, is shown in Fig. (**2**) and has been analyzed and exhibits a 4.7µM IC_{50} and a Hill slope of 7.6. The compound BRITE-345133 (chosen as representative of the 2-amino-4,6-diarylpyridine scaffold) has been analyzed by LC/MS and has a confirmed mass of 524.1 m/z (m+1 peak) and is 89% pure. The range of IC_{50} values for the 40 confirmed compounds is 4.7 µM to 39 µM and the average Hill slope is 7.4. The molecular structures and corresponding IC_{50} values are shown in Table **1**. The 2-amino-4,6-diarylpyridine scaffold is the most potent as shown by BRITE-338733 having an IC_{50} of 4.7 µM. The 1,2,4-oxadiazole scaffold of BRITE-212537 followed by the quinaxolinone scaffold of BRITE-379089 and the benzimidazole diazepinone scaffold of BRITE-082776 produced IC_{50} values of 6.2, 11 and 32 µM respectively, and represent the most potent members of their respective classes.
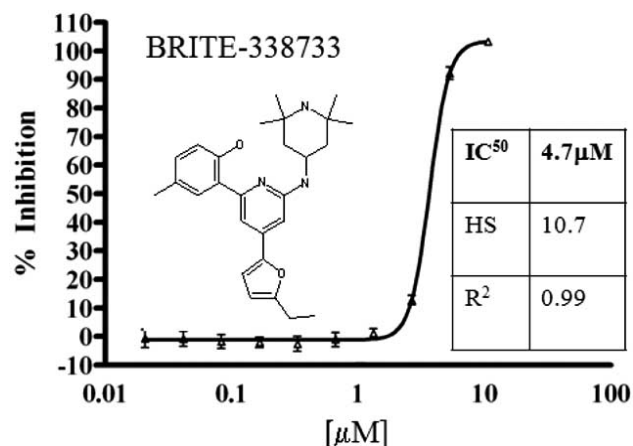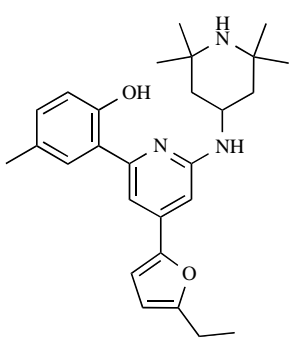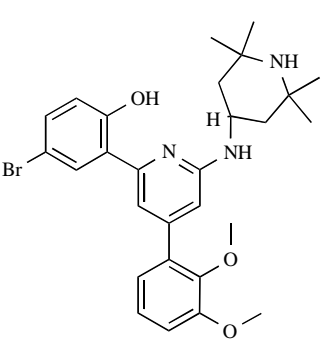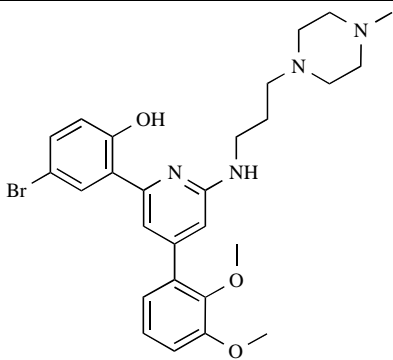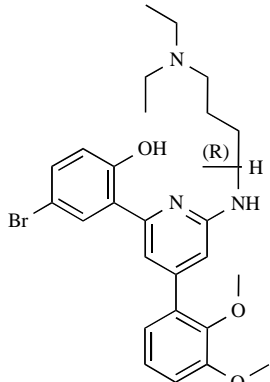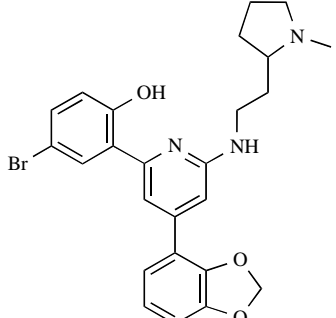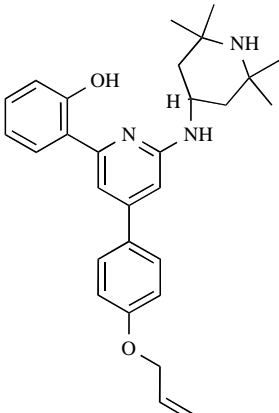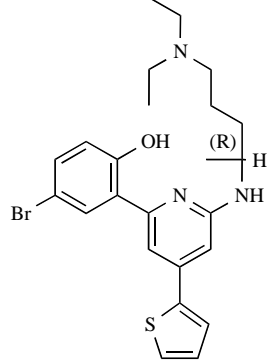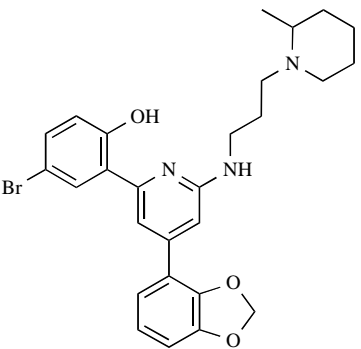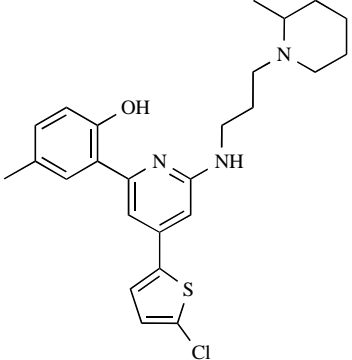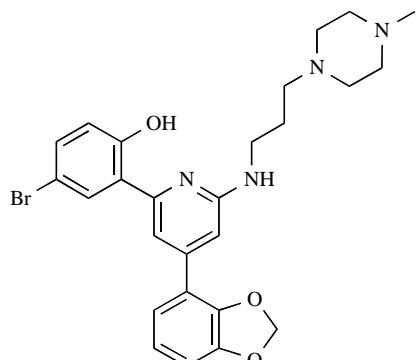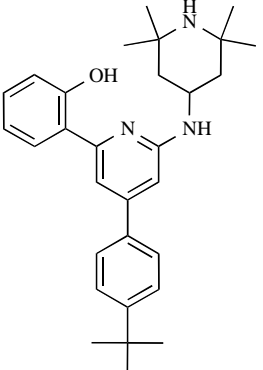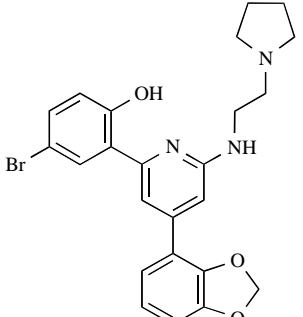

Fig. (2). Dose-response curve for the RecA inhibitor BRITE-338733, exhibiting an IC_{50} value of 4.7 ± 0.5 µM and characteristic steep Hill-slope (> 5).
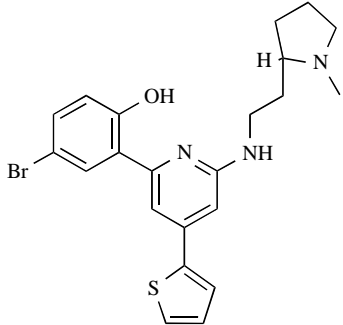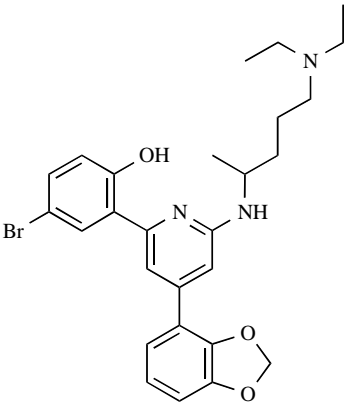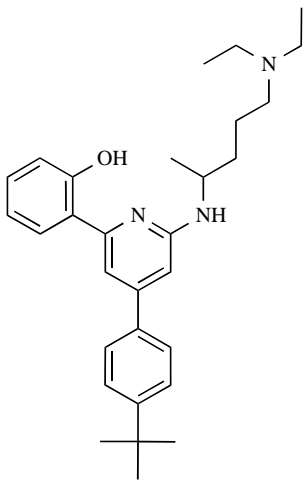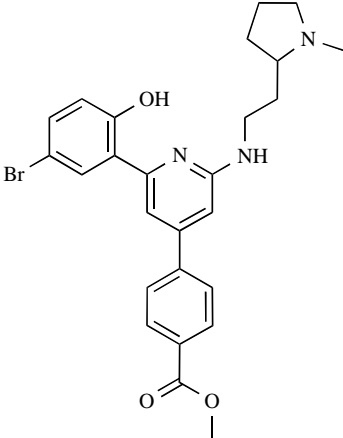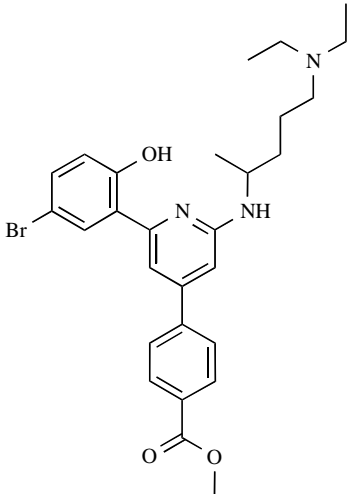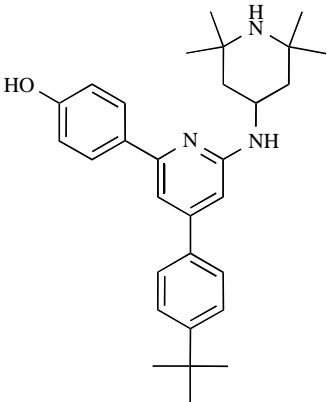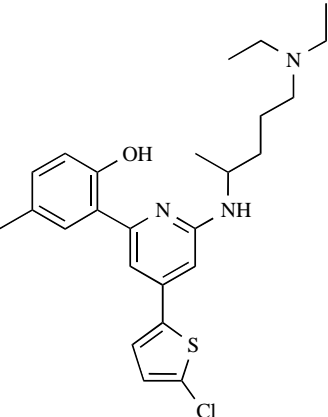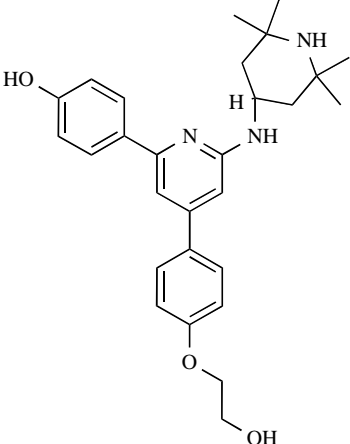
DISCUSSION
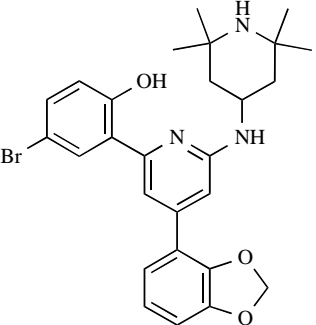
Here we present a focused high throughput screening effort that effectively identified several novel RecA inhibitors for molecular probes to investigate the impact of RecA inhibition on antibacterial drug resistance. Multiple chemical scaffolds were identified but the 2-amino-4,6-diarylpyridine scaffold was the most potent class of inhibitors. We have independently verified biological activity of this chemotype as described in Wigle, 2009 [33].

The PMB ATPase assay, while not a direct measure of nucleoprotein filament formation, provides a valuable means for the identification of RecA inhibitors with excellent screening statistics and normal confirmation rates (see Wigle, 2009) [33]. We found the assay to be very robust;
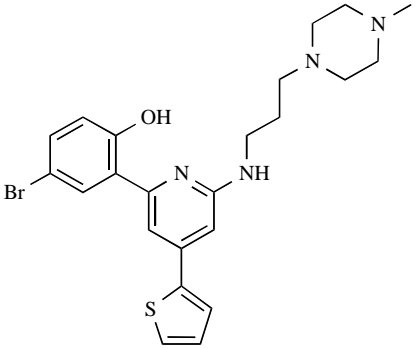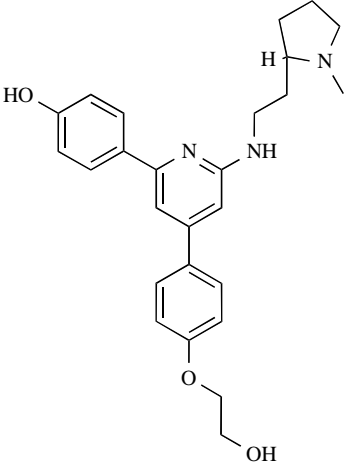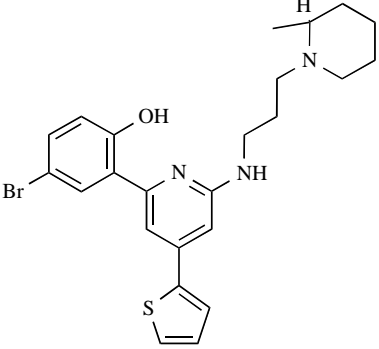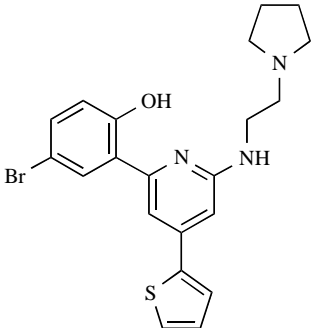
Table 1.   **Chemical Structures of Confirmed RecA Inhibitors**

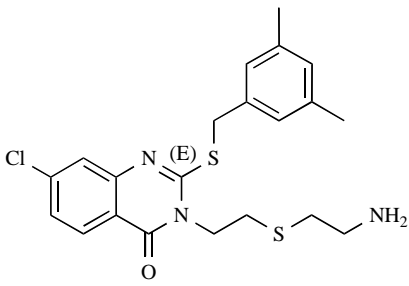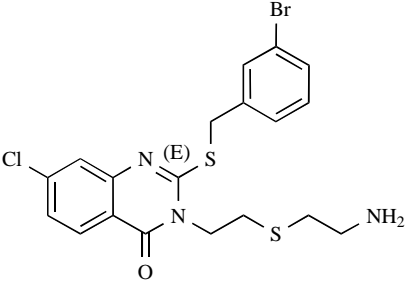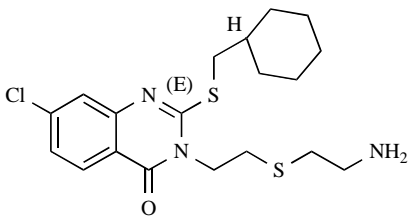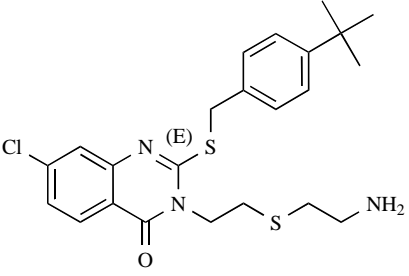| A. Chemotype-A Structures, Sorted by Potency		
Compound ID: BRITE-338733 IC50:4.73	Compound ID: BRITE-150984 IC50: 8.33	Compound ID: BRITE-150981 IC50:10.24
Compound ID: BRITE-150980 IC50:10.55	Compound ID: BRITE-345125 IC50:10.59	Compound ID: BRITE-151007 IC50:10.73
Compound ID: BRITE-149666 IC50:10.85	Compound ID: BRITE-345132 IC50:11.7	Compound ID: BRITE-340894 IC50:13.49
Compound ID: BRITE-345130 IC50:14.77	Compound ID: BRITE-340613 IC50:15.55	Compound ID: BRITE-345138 IC5016.19

A. Chemotype-A Structures, Sorted by Potency

Compound ID: BRITE-149655 IC50:20.54

Compound ID: BRITE-345129 IC50:20.64

Compound ID: BRITE-340617 IC50:23.02

Compound ID: BRITE-344741 IC50:24.02

Compound ID: BRITE-345133 IC50:24.23

Compound ID: BRITE-344730 IC50:25.72

Compound ID: BRITE-345173 IC50:26.66

Compound ID: BRITE-340897 IC50:28.55

Compound ID: BRITE-150422 IC50:29.92

(Table 1). Contd…..

A. Chemotype-A Structures, Sorted by Potency

Compound ID: BRITE-149665 IC50:34.09

Compound ID: BRITE-150415 IC50:35.55

Compound ID: BRITE-149663 IC50:36.11

Compound ID: BRITE-149649 IC50:39.33

B. Chemotype-B Structures, Sorted by Potency

Compound ID: BRITE-379089 IC50:10.73

Compound ID: BRITE-379081 IC50:10.89

Compound ID: BRITE-379078 IC50:11.6

Compound ID: BRITE-379099 IC50:11.66
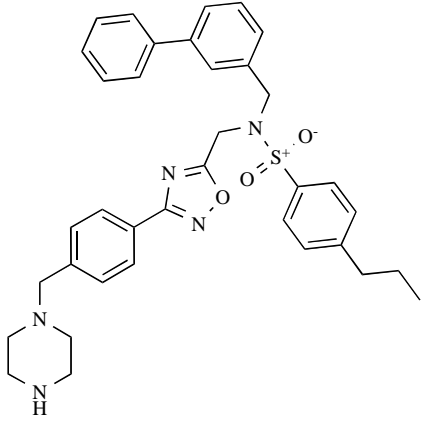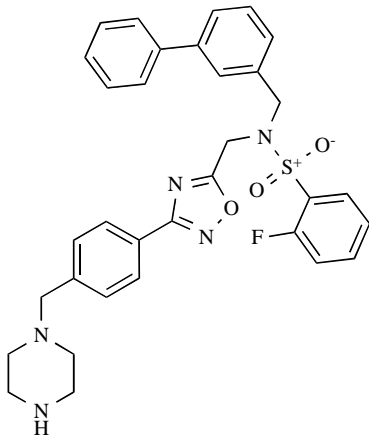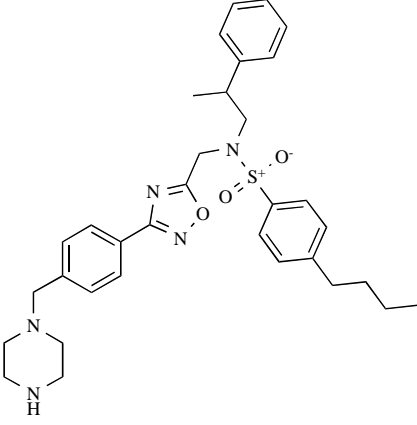
C. Chemotype-C Structures, Sorted by Potency

Compound ID: BRITE- 082776 IC50:31.87

Compound ID: BRITE-148538 IC50:36.44

D. Chemotype-D Structures, Sorted by Potency

Compound ID: BRITE-212537 IC50:6.19

Compound ID: BRITE-212534 IC50:8.42

Compound ID: BRITE-212535 IC50:9.02

Compound ID: BRITE-212536 IC50:10.75

Compound ID: BRITE-212540 IC50:12.21

Compound ID: BRITE-212533 IC50:12.23
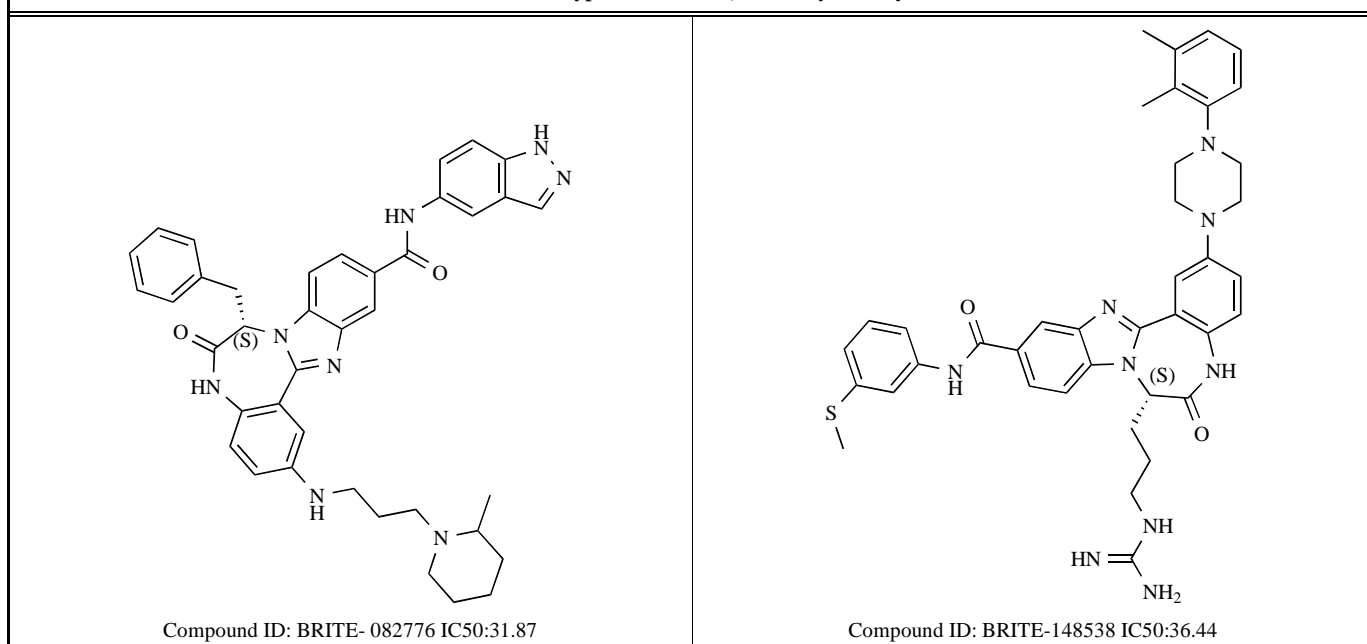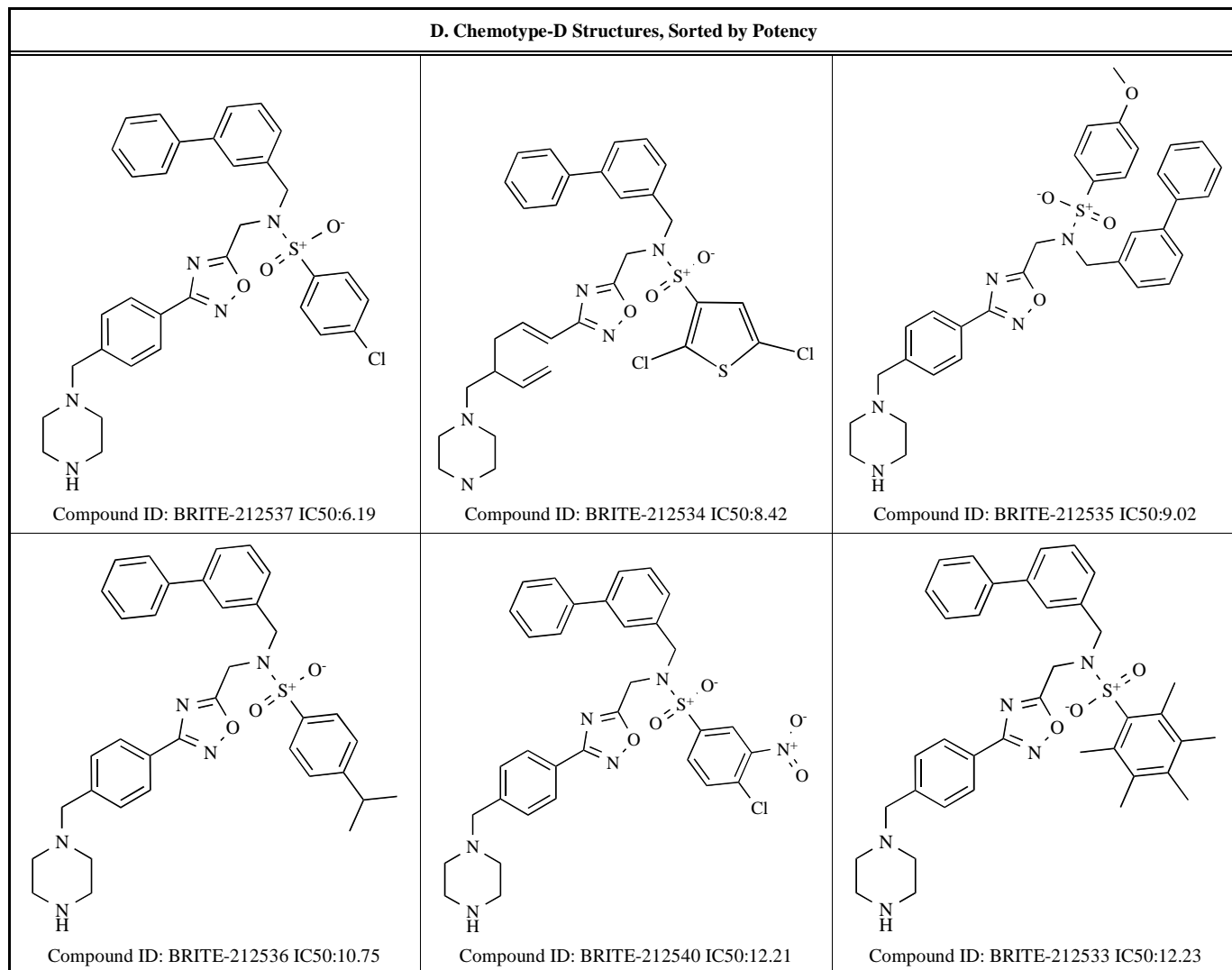
(Table 1). Contd…..

D. Chemotype-D Structures, Sorted by Potency

Compound ID: BRITE-101417 IC50:34.15	Compound ID: BRITE-101419 IC50:34.97	Compound ID: BRITE-094936 IC50:35.59

however, the assay window and statistics could vary significantly from RecA protein batch to batch. The cause of this variation was determined to be residual free phosphate from the protein purification process involving phosphate-based buffers. We routinely performed min-max experiments in one 384-well plate (half-min, half-max) to investigate the assay window and signal-to-noise ratio for batch validation and would dialyze the RecA protein in the assay buffer to reduce the amount of free phosphate. Excellent assay statistics were achieved when the UV/Vis absorption assay window (max-minus-min) was above 0.2 for A_{650}, and above 1.0 for A_{825}. Two changes were made upon reflection on the initial BRITE-diversity set screen prior to the confirmation step; 1) shifting absorption wavelength to 825 nm and 2) with the addition of 10 µg/mL BSA, which resulted in an increase in Z′ from 0.83 to 0.90.

Typical IC_{50} curves for RecA inhibition are steep, with fit Hill slopes typically above five. It is expected that positive cooperative binding (long known in the experimental RecA literature) would result in steep inhibitory Hill slopes. We have performed dynamic light scattering (DLS) experiments (Dynapro-384, Wyatt Tech. Santa Barbara, CA) to independently assess if the 2-amino-4,6-diarylpyridine scaffold (class A, most potent overall) was aggregating RecA protein in the absence of DNA. We found no indication of RecA aggregation in the presence of up to 10 µM BRITE-345133 (chosen as representative of the 2-amino-4,6-diarylpyridine scaffold) with DLS, which is approaching the solubility limit for the compound (data not shown). These DLS experiments confirm that chemotype-A compounds are genuine RecA inhibitors and the kinetics of inhibition adhere to a multi-site kinetic model. The DLS data will be published in a separate manuscript.

The preliminary structure-activity-relationship of the additional 8640 compounds selected from the rest of the 350,000 compound library for the active scaffolds shows a relatively flat profile, with most IC_{50} values hovering in the low micromolar range (data not shown). We believe that this PMB ATPase assay is not suitable for profiling the SAR because low micromolar concentrations are at the lower edge of the assay dynamic range. We are developing more sensitive assays to adequately profile the SAR for these compounds in the nano- to micromolar range.

CONCLUSION

We have screened 33,600 compounds selected from a library containing 350,000 compounds. Here we present 40 confirmed inhibitors of RecA ATPase activity. The 40 confirmed hits represented four novel scaffolds for RecA inhibition. These scaffolds will be used for future structure activity relation (SAR) analysis. Among the four scaffolds, the 2-amino-4,6-diarylpyridine compounds are the most potent with IC_{50} values in the single-digit micromolar range. We are in the process of developing new sensitive assays to further the SAR exploration. This high-throughput screening effort effectively identified a diverse set of active chemical scaffolds to serve as molecular probes for studying the effects of RecA in the development of antimicrobial resistance.

ACKNOWLEDGEMENTS

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