Bioorganic & Medicinal Chemistry Letters 20 (2010) 4531–4534

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Small molecule functional analogs of peptides that inhibit λ site-specific recombination and bind Holliday junctions

Dev K. Ranjit [a,†], Marc C. Rideout [a], Adel Nefzi [b], John M. Ostresh [c], Clemencia Pinilla [c], Anca M. Segall [a,*]

[a] Department of Biology and Center for Microbial Sciences, San Diego State University, San Diego, CA 92182-4614, USA
[b] Torrey Pines Institute for Molecular Studies, 11350 SW Village Parkway, Port St. Lucie, FL 34987-2352, USA
[c] Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, 2-129, San Diego, CA 92121-1120, USA

ARTICLE INFO

Article history:
Received 5 April 2010
Revised 3 June 2010
Accepted 4 June 2010
Available online 8 June 2010

Keywords:
Site-specific recombination
λ-Integrase
Holliday junction
Combinatorial libraries
RecG helicase

ABSTRACT

Our lab has isolated hexameric peptides that are structure-selective ligands of Holliday junctions (HJ), central intermediates of several DNA recombination reactions. One of the most potent of these inhibitors, WRWYCR, has shown antibacterial activity in part due to its inhibition of DNA repair proteins. To increase the therapeutic potential of these inhibitors, we searched for small molecule inhibitors with similar activities. We screened 11 small molecule libraries comprising over nine million individual compounds and identified a potent *N*-methyl aminocyclic thiourea inhibitor that also traps HJs formed during site-specific recombination reactions in vitro. This inhibitor binds specifically to protein-free HJs and can inhibit HJ resolution by RecG helicase, but only showed modest growth inhibition of bacterial with a hyperpermeable outer membrane; nonetheless, this is an important step in developing a functional analog of the peptide inhibitors.

Site-specific recombination (SSR) catalyzed by bacteriophage λ Integrase (Int) requires the formation and subsequent resolution of a 4-armed DNA intermediate known as a Holliday Junction (HJ) (Supplementary data Fig. 1A, and reviewed in Ref. 1). We have previously identified hexapeptide inhibitors from combinatorial peptide libraries that stabilized the HJ intermediate, or blocked recombination completely.[2,3] The activity of many of these peptides, such as WRWYCR (Supplementary data Fig. 1B), is not dependent upon interactions with any of the proteins required for recombination. In fact, these peptides inhibit several mechanistically distinct tyrosine recombinases,[3,4] DNA cleavage and HJ resolution by vaccinia virus topoisomerase,[5,6] and structurally unrelated HJ-processing enzymes like RecG helicase and RuvABC resolvase.[7] All of these activities are based on the ability of the peptides to bind to protein-free HJs, and with somewhat lesser affinity to other branched DNAs and replication forks.[7] Because HJs are central intermediates in several DNA repair pathways,[8] we reasoned that these peptides may interfere with DNA repair and thus have antibacterial activity. Indeed, minimal inhibitory concentration (MIC) values range from 32 to 64 μg/ml in Gram negative bacteria and 4 to 32 μg/ml in Gram positive bacteria, including methicillin resistant *Staphylococcus aureus* (MRSA).[4] Evidence from genetic and biochemical studies supports a model where the need for DNA repair causes the formation of Holliday junctions that are trapped by the peptides; in this way repair is blocked and the cells die.[4,7,9,10]

DNA repair is a novel antibacterial target. Moreover, target-site resistance is unlikely to develop because HJs are generated in multiple independent ways.[8] Consistent with this hypothesis, we have been unable to isolate stable peptide resistant mutants. Due to the increasing problem of multiple drug resistance in bacteria, we wanted to identify small molecules with similar activities as the peptides. We performed screens of 11 small molecule libraries comprising over nine million individual compounds to identify functional analogs of the HJ trapping peptides. Here we present the identification and characterization of an *N*-methyl aminocyclic thiourea that is an active inhibitor of our in vitro recombination assays, binds specifically to protein-free HJs, and inhibits a HJ-processing helicase.

Compound identification. The mixture-based libraries we screened were synthesized at Torrey Pines Institute for Molecular Studies (reviewed in Ref. 11). In general the compounds in the libraries we tested had low molecular weights and three diversity positions, or R groups, built on unique scaffolds (Fig. 2 in Supplementary data). These scaffolds were first evaluated for inhibition of site-specific recombination without regard to individual R groups in the libraries by making a 'mixture of mixtures.' For example, the 40 R^1, 37 R^2, and 80 R^3 mixtures of the *N*-methyl aminocyclic thiourea library (DCR 528, Supplementary data Table 1) were pooled together to make one mixture comprising the total diversity of that library (40 $R^1 \times 37$ $R^2 \times 80$ $R^3 = 118{,}400$ compounds). This 'mixture of

* Corresponding author. Tel.: +1 619 594 6528; fax: +1 619 594 5676.
E-mail address: asegall@sciences.sdsu.edu (A.M. Segall).
† Present address: Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198-5900, USA.

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.06.029

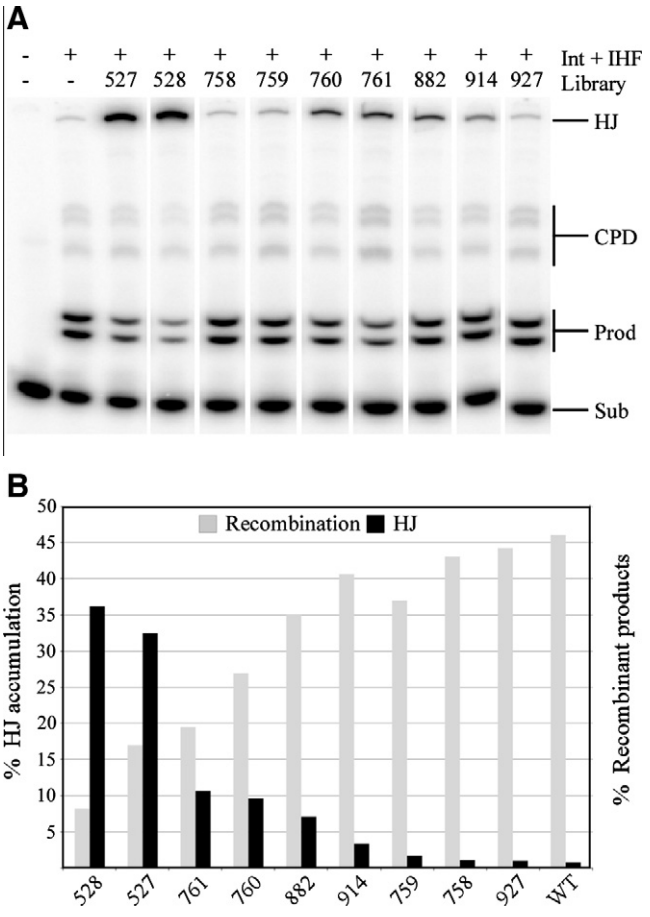

Figure 1. *Scaffold ranking.* (A) Lanes from Tris–Tricine–SDS gels showing recombination reactions in the presence of the small molecule mixtures at 10 μg/ml in bent-L recombination. 'Sub' refers to the double radioactive end-labeled DNA substrate which recombines with an unlabeled partner to generate products, 'Prod'. 'CPD' refers to covalent protein–DNA complexes and 'HJ' is the Holliday junction. (B) Quantitation of the reactions shown in Figure 2A for HJ accumulation (left *Y*-axis) and concurrent reduction in the formation of recombinant products (right *Y*-axis). Int and IHF are the proteins performing the recombination reaction.

mixtures' was tested in Int-mediated recombination between two *attL* sites known as the bent-L[2,12] pathway at 10 μg/ml and quantitated with respect to accumulation of HJs (Fig. 1). Individual compounds from the mixture are present in the recombination reactions at ~84 pg/ml (~225 pM for DCR 528 given an average molecular weight of 374 g/mol for compounds in that library).[‡] This concentration is well below the IC_{50} of even our most potent peptide inhibitor. However, as discussed previously,[2,11] the inhibitory activity seen in the libraries is likely the result of a number of chemically similar compounds with characteristics and potency close to the most active inhibitors and this group increases the 'effective concentration' of the inhibitors in the mixtures. As seen in Figure 1, the accumulation of HJs in treated reactions coincides with a reduction in recombinant products as catalysis in the context of the HJ is blocked (refer to Supplementary data, Fig. 1A). Of the libraries screened, the *N*-benzyl aminocyclic thiourea scaffold (DCR 527) and the *N*-methyl aminocyclic thiourea scaffold (DCR 528) accumulated the most HJs and inhibited recombination to the greatest extent (Fig. 1B). Therefore, we pursued both libraries.

Following scaffold selection, defined R group mixtures were screened to identify individual compounds with activity (reviewed in Refs. 11,13). Each mixture has a 'defined position' and individual compounds within that mixture all have the same functionality at that R group position; the other two R group positions contain equimolar mixtures of the other functionalities. For example, mixture 126 in the *N*-methyl aminocyclic thiourea library (Supplementary data Table 1) contains the defined functionality *N′N*-dimethyl-*S*-propylamine at position R^1, and a mixture of the 37 R^2 and 80 R^3 functionalities at the other two positions. Thus, this single mixture contains a total of 2960 ($1 \times 37 \times 80$) possible compounds, each present at ~337 pg/ml (~900 pM) when tested in reactions at 1 μg/ml, and this allows evaluation of the functionality *N′N*-dimethyl-*S*-propylamine at position R^1 with respect to inhibition of recombination. Functionalities at R^2 and R^3 were screened in the same fashion. The mixtures from DCR 528 (Fig. 3 in Supplementary data) averaged twofold more HJ accumulation than DCR 527 (data not shown) and thus we chose to identify compounds from DCR 528. These top ranking mixtures are designated with an asterisk in Supplementary data, Figure 3, and each mixture was titrated more extensively in the bent-L pathway and ranked based on potency. The top defined mixtures were also shown to inhibit *attL* × *attR* (excisive[12]) recombination, albeit with lower potencies.[2,14] In order to test the specificity of these mixtures, they were assayed in several other reactions involving DNA transactions and were found not to inhibit restriction digests by NdeI and HindIII, or plasmid relaxation by *E. coli* topoisomerase I at 10 μg/ml.[14] The mixtures were also tested (as described in Ref. 4) at 25 μg/ml, and shown to inhibit the growth of *S. aureus*.[14] Based on these data, three R^1, three R^2, and one R^3 functionalities were selected and the combinations resulted in nine individual compounds (Table 1) that were synthesized as described.[15,16]

The crude compounds were characterized in several in vivo and in vitro assays similar to those used to assess the defined mixtures.[14] Based on these data TPI1530-1 (Fig. 2) was selected for further study.

Compound characterization. Compound 1530-1 was purified to >95% and verified by LC-MS. We titrated 1530-1 into excision recombination reactions (Fig. 3A) and determined IC_{50} values of 3 μg/ml for accumulation of HJs, and 5 μg/ml for inhibition of recombination (compared with IC_{50} values for peptide WRWYCR of 0.04 and 0.12 μg/ml, respectively; Table 2). We tested for whether 1530-1 inhibits several restriction enzymes as well as whether it binds non-specifically to double stranded DNA (an *attL* site[12] in the absence of recombination enzymes). 1530-1 inhibited restriction digests at 100 μg/ml by 22% compared to untreated reactions (Table 2); however, no evidence of binding to double stranded DNA alone was detected at this concentration. This suggested specificity for the common HJ intermediate in the recombination assays. To test this further, 1530-1 was examined for its ability to bind to protein-free HJ substrates and shown to form a specific complex similar to that seen with peptide WRWYCR (Fig. 3B). We then tested the effects of the compound on RecG-mediated unwinding of HJs. RecG is a monomeric SFX family helicase that recognizes HJs and unwinds these substrates in the presence of ATP and magnesium.[17] This helicase bears no structural or mechanistic similarity to the proteins in the recombination assays and has been used previously to examine the specificity of our peptides for the HJ.[7,10] As seen in Figure 3C, 1530-1 does inhibit RecG activity in a dose dependent manner with an IC_{50} of 0.85 μg/ml (compared with 0.12 μg/ml for WRWYCR). These data further corroborate a model where the inhibitor recognizes the structure of the HJ intermediate.

The ability of 1530-1 to inhibit bacterial growth was examined using several Gram (+) and Gram (−) bacteria. In contrast to the activity of the DCR 528 library, compound 1530-1 did not inhibit bacterial growth, even at the highest concentration tested

[‡] The concentration of individual compounds (in μg/ml) that are present in the library or in the defined mixtures are calculated from the final concentration of the mixture in the treated reaction divided by the diversity of the mixture. For instance, treatments with library DCR 528 at 10 μg/ml gives a final compound concentration of 84 pg/ml (10 μg/ml/118,400 compounds), and treatments with a defined DCR 528 mixture (such as mixture 126) at 1 μg/ml gives a final compound concentration of 337 pg/ml (1 μg/ml/2960 compounds).

Table 1
Deconvolution of positional scanning data for the *N*-methyl aminocyclic thiourea library

Compound	R^1	R^2	R^3
1530-1	*N*′*N*-Dimethyl-*S*-propylamine	*R*-Hydroxy-methyl	2-(4-Nitro-phenyl)-ethyl
1530-2	*R*-Hydroxy-methyl		
1530-3	*S*-3-Methyl-1-methyl-indole		
1530-4	*N*′*N*-Dimethyl-*S*-propylamine	*S*-3-Methyl-1H-indole	
1530-5	*R*-Hydroxy-methyl		
1530-6	*S*-3-Methyl-1-methyl-indole		
1530-7	*N*′*N*-Dimethyl-*S*-propylamine	*S*-Hydroxy-methyl	
1530-8	*R*-Hydroxy-methyl		
1530-9	*S*-3-Methyl-1-methyl-indole		

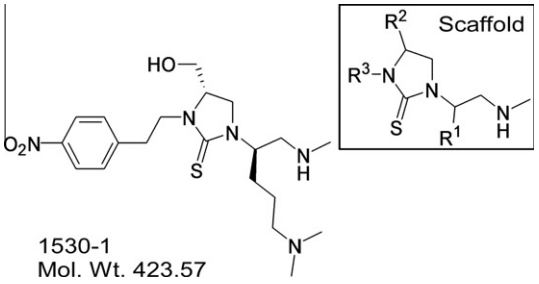

Figure 2. 1530-1 is built on a *N*-methyl aminocyclic thiourea scaffold with functionalities *N*′*N*-dimethyl-*S*-propylamine, *R*-hydroxy-methyl, and 2-(4-nitro-phenyl)-ethyl (R^1, R^2, and R^3, respectively).

(100 μg/ml, data not shown). Lack of inhibitory activity may be due poor to permeability of 1530-1 into bacterial cells. To address this possibility we tested a hyperpermeable *Salmonella enterica* serovar Typhimurium (Stm) strain with mutations in the *galE* and *rfa* genes; this strain has shorter lipopolysaccharide (LPS) chains in the outer membrane and increased permeability to many compounds, including our peptides.[4,18–20] At 100 μg/ml, 1530-1 inhibited the growth of this mutant by 50% after 22 h of incubation (data not shown). Alternatively, 1530-1 may be rapidly effluxed from the cell. To address this we tested an *E. coli* strain with a deletion of the *acrAB* genes, which inactivates the major drug efflux pump in the bacterium and increases susceptibility to many antibiotics.[21]

At 100 μg/ml of 1530-1 the growth of the efflux mutant was not inhibited (data not shown). These data suggest that 1530-1 may have antibiotic activity but that poor membrane permeability may prevent the compound from entering cells and finding its target.[14]

In summary, we have identified and purified an *N*-methyl aminocyclic thiourea that recognizes HJs and inhibits enzymes that catalyze the resolution of these intermediates of DNA recombination and repair. This compound is composed of a similar combination of R-group functionalities as the side chains of the peptide inhibitors:[3,6] aromatic and basic functionalities are present in both the most potent peptides and in the other *N*-methyl aminocyclic thioureas that were identified (Table 1). Molecular modeling corroborated by fluorescence quenching studies on peptide WRWYCR suggested that aromatic functionalities may be involved in base stacking interactions with the HJ,[10] while functionalities that are positively charged could stabilize binding to the negatively charged DNA. Together, these functionalities may represent the core characteristics necessary for interactions with HJs, although not necessarily for entry into bacteria. One attribute which 1530-1 does not share with the peptides is an efficient mechanism for forming dimers, which the most effective hexapeptides do via disulfide linkage between two monomers. Future screening of other chemical libraries combining a primary screen for HJ binding activity, followed by a

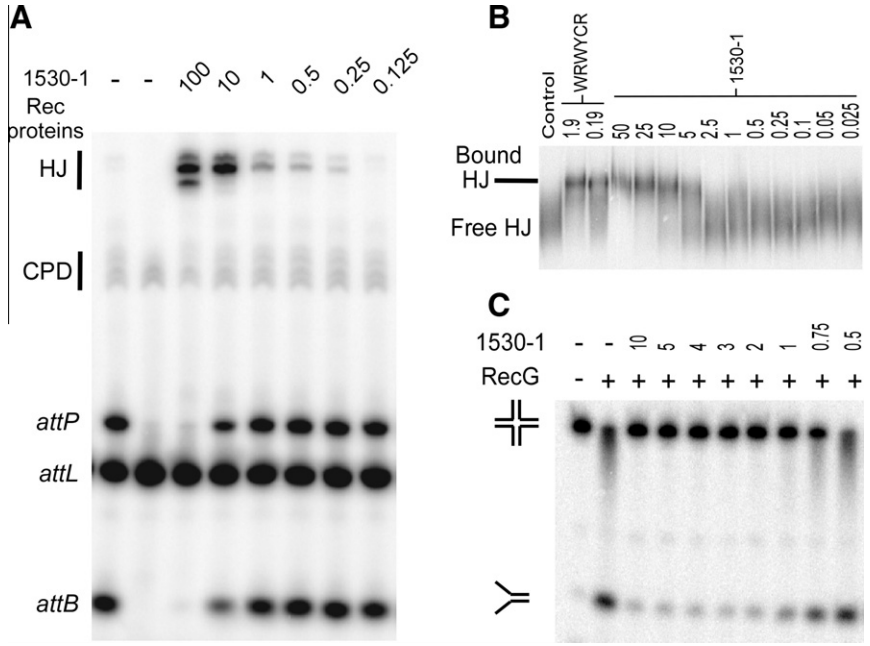

Figure 3. 1530-1 interacts with Holliday junctions. All concentrations in μg/ml. (A) Dose dependent inhibition of excision SSR. Rec protein refers to inclusion of Int and Xis proteins and Integration Host Factor (IHF). (B) 1530-1 causes a shift in the migration rate of HJ DNA. The specific shift seen with 1530-1 is comparable to the shift seen in control reactions with peptide WRWYCR. (C) 1530-1 inhibits RecG-mediated unwinding of HJ DNA. RecG binds to HJs and, in the presence of ATP and Mg^{+2} will unwind the DNA to a replication fork. This activity is inhibited by 1530-1 in a dose dependent manner.

Table 2
Comparison of 1530-1 and WRWYCR for selected activities

Compound	Mol. weight[a]	Excision		RecG	NdeI	Growth inhibition[c]
		IC_{50} (Rec)	IC_{50} (HJ)	IC_{50}	% cleavage[b]	Stm (*galE rfaA*)
1530-1	423.5	5	3	0.85	78	100
WRWYCR[a]	1926.2	0.04	0.02	0.12	100	12.5

All concentrations in μg/ml.
[a] Molecular weights given are for active species, which is a homodimer in the case of WRWYCR.
[b] Restriction digest data are given as a percentage of activity at 100 μg/ml compared to untreated reactions.
[c] The concentration at which 50% growth inhibition is seen.

secondary screen for growth inhibition, may yield compounds with greater antimicrobial activity.

Acknowledgements

This work was supported by Public Health Service Grant RO1 GM052847 from the National Institute of General Medical Sciences to A.M.S. M.C.R. is the recipient of an Achievement Rewards for College Scientists scholarship. We thank Matt Parker for critical reading of the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.029.

References and notes

1. *λ Integrase and the λ Int family*; Azaro, M. A., Landy, A., Eds.; ASM Press: Washington, DC, 2002.
2. Cassell, G.; Klemm, M.; Pinilla, C.; Segall, A. *J. Mol. Biol.* **2000**, *299*, 1193.
3. Boldt, J. L.; Pinilla, C.; Segall, A. M. *J. Biol. Chem.* **2004**, *279*, 3472.
4. Gunderson, C. W.; Segall, A. M. *Mol. Microbiol.* **2006**, *59*, 1129.
5. Klemm, M.; Cheng, C.; Cassell, G.; Shuman, S.; Segall, A. M. *J. Mol. Biol.* **2000**, *299*, 1203.
6. Fujimoto, D. F.; Pinilla, C.; Segall, A. M. *J. Mol. Biol.* **2006**, *363*, 891.
7. Kepple, K. V.; Boldt, J. L.; Segall, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 6867.
8. Michel, B.; Boubakri, H.; Baharoglu, Z.; LeMasson, M.; Lestini, R. *DNA Repair (Amst)* 2007, *6*, 967.
9. Gunderson, C. W.; Boldt, J. L.; Authement, R. N.; Segall, A. M. *J. Bacteriol.* **2009**, *191*, 2169.
10. Kepple, K. V.; Patel, N.; Salamon, P.; Segall, A. M. *Nucleic Acids Res.* **2008**, *36*, 5319.
11. Houghten, R. A.; Pinilla, C.; Appel, J. R.; Blondelle, S. E.; Dooley, C. T.; Eichler, J.; Nefzi, A.; Ostresh, J. M. *J. Med. Chem.* **1999**, *42*, 3743.
12. Segall, A. M.; Nash, H. A. *Genes Cells* **1996**, *1*, 453.
13. Pinilla, C.; Appel, J. R.; Borras, E.; Houghten, R. A. *Nat. Med.* **2003**, *9*, 118.
14. Ranjit, D. K., San Diego State University, 2004.
15. Nefzi, A.; Ostresh, J. M.; Giulianotti, M.; Houghten, R. A. *J. Comb. Chem.* **1999**, *1*, 195.
16. Nefzi, A.; Ostresh, J. M.; Meyer, J.-P.; Houghten, R. A. *Tetrahedron Lett.* **1997**, *38*, 931.
17. Whitby, M. C.; Lloyd, R. G. *J. Biol. Chem.* **1998**, *273*, 19729.
18. Ames, B. N.; Lee, F. D.; Durston, W. E. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 782.
19. Kadam, S. K.; Rehemtulla, A.; Sanderson, K. E. *J. Bacteriol.* **1985**, *161*, 277.
20. Nikaido, H. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 593.
21. Murakami, S.; Tamura, N.; Saito, A.; Hirata, T.; Yamaguchi, A. *J. Biol. Chem.* **2004**, *279*, 3743.