Published in final edited form as:

Peptides. 2013 February ; 40: 112–122. doi:10.1016/j.peptides.2012.12.025.

wrwrggrywrw is a single-chain functional analog of the Holliday junction-binding homodimer, (wrwycr)₂

Marc C. Rideout^a, Ilham Naili^a, Jeffrey L. Boldt^{a,1}, America Flores-Fujimoto^{a,2}, Sukanya Patra^{a,3}, Jason E. Rostron^a, and Anca M. Segall^{a,*}

^aDepartment of Biology and Center for Microbial Sciences, San Diego State University, San Diego, California 92182

Abstract

DNA repair pathways in bacteria that use homologous recombination involve the formation and subsequent resolution of Holliday junction (HJ) intermediates. We have previously identified several hexameric peptides that bind to HJs and interfere with HJ processing enzymes *in vitro*. The peptide WRWYCR and its D-amino acid stereoisomer wrwycr, are potent antibacterial agents. These hexapeptides must form homodimers in order to interact stably with HJs, and inhibit bacterial growth, and this represents a potential limitation. Herein we describe a disulfide bond-independent inhibitor, WRWYRGGRYWRW and its D-stereoisomer wrwrggrywrw. We have characterized these single-chain, linear analogs of the hexapeptides, and show that in addition to effectively binding to HJs, and inhibiting the activity of DNA repair enzymes that process HJs, they have equal or greater potency against Gram-positive and Gram-negative bacterial growth. The analogs were also shown to cause DNA damage in bacteria, and disrupt the integrity of the bacterial cytoplasmic membrane. Finally, we found that they have little toxicity toward several eukaryotic cell types at concentrations needed to inhibit bacterial growth.

Keywords

Antibacterial peptides; Homodimer; Holliday junction-binding peptides; RecG helicase; RuvABC resolvase; DNA repair inhibitors

© 2012 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed: anca.segall@gmail.com, Phone: (619) 594-6528, Fax: (619) 594-5676.

¹Current address: Genomatica, Inc., 10520 Wateridge Circle, San Diego, CA 92121

²Current address: Monserate Biotechnology Group, 8395 Camino Sante Fe, Suite E. San Diego, CA 92121

³Current address: In Vitro Pharmacology Department, Lupin Ltd., Nande Village, Mulshi Taluka, Pune-42

6. Conflict of Interest

The authors declare that they have no conflicts of interest.

Contributors:

MCR: Performed excision assays, RecG unwinding assays, MIC assays. Assembled data and wrote the manuscript.

IN: Performed MIC assays, hemolysis assay, TUNEL assay as well as MTT and Live/Dead assays on PMs

JLB: Performed band shifts, excision reactions, and RuvABC assays

AAF: Screened single chain peptides, performed band shifts

SP: Performed MTT and Live/Dead assays on HCT116 cells.

JR: Performed DiBac experiment

AMS: Principle Investigator

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Holliday junctions (HJ) are 4-way branched DNA intermediates that form *in vivo* from the union of two duplex strands of DNA. These junctions arise as a result of two distinct recombination reactions, homologous recombination (HR) or site-specific recombination mediated by tyrosine recombinases (Y-SSR). HJs formed by HR are required for the non-mutagenic repair of double-stranded DNA breaks [26, 35]. They can also be intermediates in repair pathways that process single stranded gaps [36] or collapsed replication forks [34, 36]. Y-SSR reactions (reviewed in [3]) are critical for the segregation of newly replicated chromosome dimers [34] as well as antigenic variation in pilins and fimbriae [27]. The HJs formed during both types of recombination must be resolved in order to separate the duplex DNA molecules and maintain genome integrity. In the case of the tyrosine recombinases, resolution is accomplished by the recombinases themselves (reviewed in [22]), while in the case of HR, it is accomplished by structure selective nucleases such as the RuvABC resolvase in bacteria [14], or Yen1 or Mus81-Mms4 in yeast [13, 16]. We have previously identified hexapeptide inhibitors, screened from combinatorial libraries, that are inhibitors of Y-SSR reactions catalyzed *in vitro* by phage γ -Integrase (Int) [6, 9], Cre [6] and XerC/D [24], among others [20, 40]). *In vitro*, the peptides have been shown to bind stably and specifically to synthetic protein-free HJs, and to a lesser extent branched DNAs such as replication forks [28]; it is through these interactions that they inhibit the activity of DNA repair enzymes such as the RuvABC resolvase [28, 29]. The peptides stabilize HJs formed *in vivo* by Int [23] as well as those formed by RecA (Marcusson, Agrawal, Medina-Cleghorn, Segall, unpublished data). They are potent antibacterials and, while they affect membrane potential (Rostron, Naili, and Segall, unpublished results) and may induce DNA damage indirectly through effects on iron-sulfur cluster proteins [39], their activities are also consistent with the possibility that HJs and DNA repair are a direct target of these compounds [24]. WRWYCR or KWWCRW are among the most potent peptides identified to date and the formation of a disulfide-bridged homodimer between two hexapeptides is a critical component for activity *in vitro* [6, 30]. Additionally, dimer formation is necessary for potent antibacterial activity, as analogs such as wrwyar are up to 100-fold less potent [51]. We hypothesized that single-chain linear analogs of WRWYCR or KWWCRW could retain characteristics of the homodimers while eliminating the need for dimer formation and stable maintenance of the disulfide bridge in the reducing environment of the bacterial cytoplasm [50, 58] or in reaction conditions for crystallography, for example [20]. Here we describe the activities of peptide WRWYRGGRYWRW and its D-stereoisomer wrwyrgrgrywrw and show that they trap HJs formed in Y-SSR reactions, bind to protein-free HJ DNA, and inhibit the activity of two classes of DNA repair enzymes that act on junctions *in vitro*. We found that treatment of bacteria with wrwyrgrgrywrw inhibits cell growth, causes DNA damage, and membrane depolarization, and is particularly potent against Gram-positive bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA). We also investigated the cytotoxicity of wrwyrgrgrywrw in several eukaryotic cell types, and found that the peptides are not toxic at the concentrations necessary to inhibit bacterial growth. These analogs, and the parental peptide wrwyar, fall into a class of cationic antimicrobial peptides that may have multiple cellular targets, including DNA repair and non-lytic disruption of the cell membrane.

2. Materials and Methods

2.1. General

All peptides were synthesized by Sigma Genosynthesis or Biosynthesis Inc., with C-terminal amidation. The peptides were of >95% purity by HPLC and the correct molecular weights were verified by mass spectroscopy (MS) by the manufacturers. Lyophilized peptides were resuspended as 10 mM stock solutions in 100% anhydrous DMSO and all peptide dilutions

were made in water. For each experiment, DMSO controls were performed using the residual DMSO concentration found in the highest peptide treatment. Oligonucleotides were obtained from Integrated DNA Technologies. Site-specific recombination substrates were made by PCR as described previously [6]. For the generation of HJ substrates, individual oligonucleotides were purified on 5% native polyacrylamide gels and the DNA was visualized by UV shadowing using a hand held mineral lamp, model UVGL-58 (366 nm). The major product was excised from the gel and eluted overnight in TE (pH 8.0) using a “crush/soak” method [46]. Eluted DNA was precipitated with three volumes of ethanol and 10% sodium acetate. The DNA was resuspended in TE and, if needed, individual strands were radiolabeled using [γ - 32 P]-ATP (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs) according to [46]. DNA strands were then directly annealed together to assemble HJ substrates by heating to 95°C and slow cooling to room temperature. The HJ was then purified as described above for the oligonucleotides except that the substrate was visualized by exposing the gel to photographic film (Kodak). Recombination and band shift gels were dried at 80°C under vacuum for 1–2 hours and were then exposed to a PhosphorImager (PI) screen (GE Lifesciences), visualized using a Typhoon 9000 (GE Lifesciences) and quantified with ImageQuant software from GE. All acrylamide gels were made with 29:1 polyacrylamide:bis-acrylamide solutions. The 12% HJ unwinding gels were exposed to PI screens without drying and scanned following overnight exposure.

2.2. Excision Recombination assays

Substrates, proteins and assay conditions are described in [6].

2.3. Non-competitive HJ band shifts

Non-competitive HJ band shifts were performed in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 7.8)), and 5% glycerol. Radiolabeled HJ DNA was the same as used for the RecG unwinding assay (below). The HJ DNA substrate was included in reactions at 2 nM final concentration. Peptide dilutions were added and reactions were allowed to incubate for 10 minutes on ice. The samples were loaded without loading-dye on 5% native Tris-borate-EDTA (TBE, pH 7.8) gels. Gels were electrophoresed at 240V for 3 hours at 4°C.

2.4. Competition band shifts

The HJ substrate was the same as used in the RecG assay (below). The linear duplex DNA substrate was a radiolabeled *attL* site. Competitive band shifts were performed as described above, except that sonicated salmon sperm DNA was added as a non-specific competitor prior to peptide addition.

2.5. RecG HJ unwinding assays

Purified RecG protein was the generous gift from Dr. Peter McGlynn (University of Aberdeen, UK). Unwinding assays were performed as described previously [28], using the same oligonucleotides as substrates.

2.6. RuvABC HJ cleavage assay

Purified RuvA, RuvB and RuvC proteins were the generous gift from Robert Lloyd (University of Nottingham, UK) or purified using the protocols of the Lloyd lab. HJ cleavage assays were performed as described previously [28], using the same oligonucleotides as substrates.

2.7. Minimal inhibitory concentrations (MICs)

The genotypes of the bacterial strains used to examine the inhibitory effects of the peptides are listed in Supplementary Table 1. Overnight cultures were diluted 1:100 in Mueller

Hinton Broth (MHB) in culture tubes and grown to an optical density 600 nm (OD₆₀₀) of 0.08 – 0.1. A 96-well microtiter plate was prepared with 2X peptide in 100 μ L of MHB and subcultured cells (100 μ L per well) were then mixed into each well and an initial OD₆₀₀ reading of the plate was taken. Final concentrations of peptide used for the MIC experiments were 1, 2, 4, 8, 16, 32, 64, and 128 μ g/mL, which are reported in Table 2 as 0.55, 1.1, 2.2, 4.4, 8.7, 17.5, 35, or 70 μ M respectively. Plates were incubated for 16 to 20 hours without shaking at 37° C. The final OD₆₀₀ reading was determined and the difference between the final and the initial reading was calculated to yield the increase in growth. The MIC values were defined as the lowest concentration of compound that inhibits growth [18] and are estimated from 3 independent replicates in each experiment.

2.8. Flow cytometry for bacterial cells

Experiments were performed using a BD FACSAria desktop cell sorter with a 70 μ m nozzle (Becton-Dickinson) at the SDSU Flow Cytometry Core Facility. For each sample, 50,000 events were recorded. Data acquisition and analysis was performed using FACSDiva software (Becton-Dickinson). In most cases, bacterial cells were identified by counterstaining the DNA as described below.

2.9. Bacterial Terminal dUTP transferase Nick-End Labeling (TUNEL) assay

Bacterial cells were grown as described above. Cells were treated with peptides at the concentrations indicated for this assay for 1.5 hours in a 37°C shaker. The cultures were then pelleted, fixed with 4% paraformaldehyde, permeabilized using a solution of 0.1% Triton X-100, 0.1% sodium citrate, and assayed using the *In Situ* Cell Death Detection Kit Fluorescein (Roche) according to the manufacturer's protocol, except that we resuspended our samples in a final volume of 25 μ L of the detection solution and counterstained cells with 0.5 μ M TOTO-3 (Life Technologies Inc.) for 10 minutes to monitor the presence of the chromosome. After treatment, cells were pelleted and resuspended in 1X PBS and quantified by flow cytometry using the blue laser (488 nm; FITC channel) for fluorescein, and the red laser (633 nm; APC channel) for TOTO-3. TUNEL-positive cells were calculated as a fraction of the TOTO-3-positive cells.

2.10. Disruption of membrane potential

Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3), Life Technologies Inc., (B-438)] can penetrate depolarized cells where it is reported to bind the cytoplasmic membrane or hydrophobic regions of proteins resulting in a red-shifted fluorescence. Bacterial cells were grown as described above and treated with wrwyrggrywrw at the indicated concentrations for 10, 90, or 180 minutes in a 37°C shaker. Immediately following incubation, 50 μ l of cells were pelleted, resuspended in 100 μ l of PBS + 10 μ g/ml DiBAC₄(3) in the dark at room temperature for 15 min. An additional 200 μ l of PBS was added and samples were immediately analyzed using flow cytometry using with an excitation wavelength of 493nm and monitoring emission at 516 nm.

2.11. Hemolytic activity assay

The assay and data collection were conducted as described in [42].

2.12. Collection of peritoneal macrophages and MTT reduction assays

Peritoneal murine macrophages were harvested as described in [51]. To gauge metabolic activity/viability, the MTT reduction assay was performed as described in [51] except that PMs were seeded in 96-well plates at 400,000 cells/mL.

2.13. MTT reduction assay in HCT 116 human colorectal cancer cells

The assay and data collection were conducted as described in [51] except that HCT116 cells were seeded at 100,000 cells/mL in 96-well plates, then incubated overnight at 37 °C with 5% CO₂ in complete growth media (DMEM with 10% FBS and 100 µg/mL of Penicillin/Streptomycin).

2.14. Live-Dead Assay on PMs

The Live-Dead assay on PMs was performed as described in [51] with the following exceptions. We used the green laser (488 nm) to detect intracellular calcein (FITC channel) and to detect intracellular ethidium homodimer (PE channel), and performed manual compensation to avoid overlap between the two colors used. We used cells permeabilized with ethanol at 70% as our positive single color control for ethidium homodimer (dead cells), and untreated cells in DMEM as our positive single color control for calcein (live cells). 30,000 events were recorded for each sample and analyzed using the FACSDiva software (Becton-Dickinson).

2.15. Live-dead assay on HCT116 cells

The assay and data collection were performed as described in [51] except that HCT116 cells were seeded at 200,000 cells/mL in 6-well tissue culture plates and incubated overnight at 37 °C with 5% CO₂ in complete growth media (DMEM supplemented with 10% FBS and 100 µg/mL of penicillin and streptomycin).

3. Results

3.1. Inhibition of site-specific recombination

We hypothesized that single-chain linear analogs of a WRWYCR dimer (expressed as (WRWYCR)₂ hereafter) could be designed that would retain the same characteristics (WRWYCR)₂ but without the need for disulfide bond formation or maintenance. To investigate this hypothesis, we designed several analogs of either 10 or 12 amino acids in length and screened them for the ability to inhibit the excisive site-specific recombination pathway (excision) mediated by Int. In this reaction, Int and the accessory proteins Integration Host Factor (IHF) and Excisionase (Xis) catalyze recombination between two DNA molecules encoding the *attL* and *attR* sequences (for review of pathway, see [3]). Initial testing indicated that a palindromic 12 amino acid analog, WRWYRGGRYWRW, had comparable activity to the parent peptide, (WRWYCR)₂ (Supplementary Table 2). We performed more extensive titrations of (WRWYCR)₂ and WRWYRGGRYWRW in excision reactions, in the presence (Figure 1A) or absence (gel not shown) of the reducing agent dithiothreitol (DTT). The peptides had equivalent IC₅₀ values in the reactions conducted in the absence of DTT; however, in contrast to reduced (WRWYCR)₂, WRWYRGGRYWRW maintained its activity in the presence of DTT, (Figure 1B and Table 1). The peptide concentrations necessary to trap the maximum amount of HJs in this reaction (40–50% of starting substrates) were approximately equivalent for the two peptides in the absence of DTT (data not shown). These data indicate a similar mode of action for (WRWYCR)₂ and WRWYRGGRYWRW, namely that they stabilize the HJ intermediate of recombination.

3.2. Peptide HJ interactions

All of the single chain linear analogs we designed were examined for the ability to interact with a HJ in the absence of recombination proteins using electrophoretic mobility shift assays (EMSA, Figure 2A). Several of the peptides including WRWYRGGRYWRW and KWWRGGRWWK caused a change in the mobility of protein-free HJ DNA similar to shifts seen with (WRWYCR)₂. This shift is likely due not only to the extra mass of the peptide but

also to the conformational change in the HJ from a faster migrating “closed” to a slower migrating “open” conformation (reviewed in [31]). We also observed supershifts in addition to the accumulation DNA in the wells of the gel with the highest amounts of several of the peptides. Both of these are probably due to different levels of nonspecific binding of the peptide to the HJ substrate. In the case of WRWYRGGRYWRW and KWWRGGRWWK these shifts occurred at concentrations at least 10-fold higher than the apparent binding constant (Table 1 and Supplementary Table 2), indicating that the shifts are indeed non-specific. Note that WRWYRGGRYWRW caused more supershifted DNA at lower concentrations than (WRWYCR)₂ suggesting that WRWYRGGRYWRW has greater non-specific binding for HJ DNA than (WRWYCR)₂. Neither WRWYGGYWRW nor WYCRGGRCYW caused specific shifts at low peptide concentrations, though WYCRGGRCYW caused both supershifts and accumulation of DNA in the wells at lower concentrations than the other peptides. These non-specific shifts are probably due to oligomerization of WYCRGGRCYW via disulfide bridge formation. We also compared band shifts with WRWYRGGRYWRW on radiolabeled HJ DNA or double stranded *attL* DNA using increasing amounts of unlabeled, non-specific dsDNA as a competitor. When the HJ was used as a substrate, the specific shifts remained the same or even increased, and the supershifts decreased with addition of competitor DNA (Figure 2B, compare lanes with 5 μM WRWYRGGRYWRW in the presence of increasing amounts of non-specific DNA). In contrast, increasing amounts of WRWYRGGRYWRW were only able to shift the *attL* dsDNA into the wells of the gel, demonstrating the peptides’ preference for HJ DNA over linear B-form DNA. Notably, the presence of competitor DNA increases the apparent K_D of WRWYRGGRYWRW binding to HJ DNA approximately 50-fold. This trend is also seen with (WRWYCR)₂, although to a lesser extent [28].

3.3. Interference with HJ-processing enzymes

(WRWYCR)₂ inhibits HJ resolution by several important DNA repair enzymes/complexes that process “open” HJ substrates, including RecG helicase and the RuvABC resolvase. RecG processes a variety of branched nucleic acid intermediates that form frequently inside of cells, such as R-loops and D-loops [32, 52]. Recently, Lloyd and colleagues have proposed that the primary function of RecG is to unwind these intermediates to prevent their use as templates for toxic, origin-independent replication (so-called “pathological replication,” see [44, 45]). RecG may also have a role in the replication fork repair (RFR) by interconverting forks and HJs [21, 33]. *In vitro*, we measured RecG’s activity of unwinding a HJ into a flayed duplex [55] in the presence of WRWYRGGRYWRW or the D-stereoisomer wrwyrggrywrw (Figure 3). Both peptides inhibit RecG-mediated HJ unwinding by more than 70% relative to DMSO treated controls, at 4–5 μM. The inhibition seen is dose-dependent, and while the D-stereoisomer was consistently more effective than the L-stereoisomer, neither was as effective as (WRWYCR)₂ or (wrwyrcr)₂ [28].

RuvABC is a multimeric complex of proteins that can branch migrate HJs and cleave them endonucleolytically [54]. This complex is the major HJ resolvase in bacteria, present in nearly all bacterial species, and required for recombination-dependent DNA repair [4, 53]. It resolves HJs formed during the repair of single stranded gaps and double-strand breaks [53], by making symmetrical cuts near the junction center and yielding two DNA duplexes with ligatable nicks [15]. RuvABC also plays a major role in RFR [4, 7, 36]. We tested whether WRWYRGGRYWRW or wrwyrggrywrw could inhibit the cleavage activity of the RuvABC resolvase [11] by a gel-based assay (Figure 3C). Both peptide stereoisomers were much more effective at inhibiting RuvABC cleavage than RecG unwinding, 5.6-fold and 13.5-fold lower IC₅₀ values for the L and D stereoisomers, respectively (Figure 3D and Table 1).

3.4. Antibacterial activity

The L- and D- stereoisomers of (WRWYCR)₂ are potent antibacterial compounds with minimal inhibitory concentrations (MIC) in the low μ M range [23]. Given the relatively similar *in vitro* characteristics of (WRWYCR)₂ and WRWYRGGRYWRW (Table 1), we tested if the peptides had similar *in vivo* activities. While both stereoisomers were tested, we focus our discussion on wrwyrggrywrw for the *in vivo* studies, since we expect it to resist degradation by proteolysis and thus confer an advantage over the L- stereoisomer. We assayed the antibacterial activity using standard MIC microtiter dilution assays in Mueller-Hinton broth (MHB) [17]. We found that wrwyrggrywrw was an effective inhibitor of bacterial growth, and in most cases, had 2–4 fold lower MICs than (wrwycr)₂ (Table 2). Several pathogenic clinical isolates were also susceptible to peptide treatment, including a methicillin resistant *Staphylococcus aureus* (MRSA, strain ATCC 33591), the *Staphylococcus aureus* Newman strain, and a *Pseudomonas aeruginosa* small colony variant (SCV) isolated from the lung of a deceased cystic fibrosis (CF) patient [56]. Both wrwyrggrywrw and (wrwycr)₂ were more effective against Gram-positive bacteria than Gram-negative bacteria, and we have previously shown that lipopolysaccharide (LPS) chains in the outer membrane of Gram-negative bacteria are a barrier to (wrwycr)₂ entry into the cells [24]. To test this possibility for wrwyrggrywrw, we compared the MICs of the wild-type *Salmonella enterica* serovar Typhimurium LT2 and *Salmonella* LT2 *gale rfa* carrying mutations that shorten LPS chains (AMES strain, see [1]). As seen in Table 2, the MIC was 2–4 fold lower in the AMES strain compared to LT2; however, L- and D- WRWYRGGRYWRW had more similar MICs between *Salmonella* AMES and LT2 than the L- or D- (WRWYCR)₂.

3.5. Analysis of the *in vivo* effects of peptide treatment

We next investigated the mechanisms of action that lead to the potent antibacterial activity seen. Unresolved HJs may lead to free DNA ends after subsequent DNA replication, a phenotype seen in both Gram-positive and Gram-negative bacteria treated with (wrwycr)₂ [24]. To investigate whether wrwyrggrywrw had similar effects, we measured DNA damage using a TUNEL assay, where 3'-OH ends are labeled by terminal deoxynucleotidyl transferase (TdT) with fluorescein-conjugated dUTP [24, 34, 43]. We treated MG1655 cells with wrwyrggrywrw or (wrwycr)₂ for 1.5 hours and analyzed the results from the TUNEL assay by flow cytometry. Both peptides caused a dose-dependent increase in TUNEL-positive cells (normalized to DMSO treated cells, Figure 4); however, treatment near the MIC for MG1655 led to only a modest increase in TUNEL-positive cells (7.9% for wrwyrggrywrw at 8 μ M). At the highest concentration tested, both peptides were effective at inducing DNA damage, though wrwycr led to a higher percentage of TUNEL-positive cells compared to wrwyrggrywrw ($75.6 \pm 2.2\%$ compared to $46.5 \pm 4.3\%$, respectively).

Peptides that are rich in arginine and tryptophan residues have been shown to affect bacterial cell membranes [10]; to investigate this possibility we analyzed the effect of wrwyrggrywrw treatment on the cytoplasmic membrane potential using bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)). Loss of membrane integrity allows DiBAC₄(3) to enter the cell and bind to intracellular proteins or to the membrane itself. Upon binding, DiBAC₄(3) displays enhanced, red-shifted fluorescence (Methods). We treated MG1655 cells with wrwyrggrywrw and DiBAC₄(3) for 10 minutes to 3 hours, and analyzed the results by flow cytometry. As seen in Figure 4B, there is a rapid and significant loss of cytoplasmic membrane potential with 16 μ M peptide treatment, $92.9 \pm 0.6\%$ DiBAC₄(3)-positive cells after 10 minutes. Similar levels of DiBAC₄(3)-positive cells are seen with 16 μ M wrwyrggrywrw treatment after 1.5 and 3 hours. When cells were treated with 8 μ M wrwyrggrywrw we saw that $55.1 \pm 8.7\%$ of the population was DiBAC₄(3) positive; after 3 hours, only $18.7 \pm 5.7\%$ cells were DiBAC₄(3)-positive. It is possible that the cells may

recover their membrane potential at lower peptide concentrations. At all time points examined with 4 μ M peptide treatment, only a small fraction of the population was DiBAC₄(3)-positive (fewer than 17% cells). The large fraction of DiBAC₄(3) positive cells at 16 μ M suggests that wrwyrggrywrw may be bactericidal; we tested the viability of treated cells and found that, while the peptide is not bactericidal at 16 μ M, it is bactericidal at 32 μ M (Figure 4C).

3.6. Cytotoxicity of wrwyrggrywrw in primary eukaryotic cells

As a preliminary measure of the toxicity toward eukaryotic cells, we examined the hemolytic activity of wrwyrggrywrw and compared it with that of (wrwycr)₂. Red blood cells were incubated with peptides or the indicated controls for one hour and the supernatant of each treatment was analyzed for evidence of hemoglobin release (Figure 5). For both peptides at all concentrations tested, the OD₄₁₄ readings, reflective of the extent of hemoglobin release, were approximately the same or lower than those observed with DMSO-treated or H₂O-treated red blood cells (OD₄₁₄ of 0.18 ± 0.1 and 0.14 ± 0.08 , respectively).

To further address the toxicity toward eukaryotic cells, we performed MTT assays on activated peritoneal macrophages (PMs) isolated from BALB/c mice and on a human colorectal cancer cell line (HCT116). This assay measures the activity of mitochondrial dehydrogenases in reducing the MTT dye (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) to a purple formazan derivative that precipitates intracellularly and is monitored optically [37]. Increasing amounts of wrwyrggrywrw led to a dose-dependent decrease in MTT reduction in the PMs (up to 31% inhibition at the highest concentration tested, 75 μ M; Figure 6A). At the lower peptide concentrations (16–32 μ M) we saw an overall increase in MTT reduction relative to DMSO treated cells (up to a 77% increase at 25 μ M), which may result from increased permeability of the MTT dye or from stimulation of redox metabolism as a result of low peptide concentrations; this was observed previously [51]. The HCT116 cells also showed a dose-dependent decrease in MTT reduction with increasing amounts of wrwyrggrywrw (up to 47% inhibition at the highest concentration tested, 150 μ M; Figure 6B).

As an independent measure of viability we used a Live/Dead assay on the PMs and the HCT 116 cells. This assay monitors the ability of active cellular esterases to convert calcein-AM (CAM, the “live” stain) to a fluorescent product that is retained in cells, in addition to monitoring the uptake of ethidium homodimer (EH), a membrane-impermeable DNA binding-dye (the “dead” stain). Thus, healthy active cells are EH-negative and CAM-positive, dead cells are EH-positive and CAM-negative, and a subpopulation of cells that are “dying” may be positive for both dyes if they have compromised membranes but retain enzymatic activity. Both cell types were treated with wrwyrggrywrw for 24 hours, stained, and fluorescence was measured by flow cytometry (Figure 6C and Figure 6D for the PMs and HCT116 cells, respectively). We saw a dose-dependent decrease in the number of live cells and a concomitant increase in dead cells with increasing peptide concentration for both cell types. The IC₅₀ of wrwyrggrywrw in this assay was approximately 40 μ M for the PMs and about two fold higher (85 μ M) for the HCT 116 cells.

4. Discussion

We previously identified Holliday junction-trapping, bactericidal hexapeptides, such as WRWYCR or KWWCRW, which require homodimer formation for maximum activity, both *in vitro* and *in vivo* [6, 30]. Despite the fact that they are dissolved in an oxidizer (100% DMSO), the rate of dimerization is slow. Moreover, we desired HJ-trapping peptides that would remain active in the presence of reducing agents such as β -mercaptoethanol or DTT,

which are often used during protein purification or in crystallization buffers. We therefore designed single-chain linear analogs and evaluated them in excision recombination reactions and binding reactions to protein-free HJs (Supplementary Table 2 and Figure 2). We found that single-chain decapeptides containing only two basic amino acid residues were not effective as inhibitors of excision recombination, nor did they bind protein-free HJs. This is likely the result of fewer electrostatic interactions between the peptides and DNA, resulting in weaker affinity for the HJ and/or less stable complex formation. We have seen a similar trend with two classes of small molecule inhibitors of Y-SSR that also bind HJs [41, 42]. On the other hand, peptides with multiple cysteine residues that may form oligomers were less specific and did not inhibit excision reactions as effectively as hexapeptides with a single disulfide bridge, such as (WRWYCR)₂ (Figure 2B). This characteristic was also seen with peptide inhibitors of HJ resolution by vaccinia virus topoisomerase that contain multiple cysteine residues [19]. Based on our initial evaluation of the single chain peptides, we selected WRWYRGGRYWRW for further study, and our analyses support that it is a functional analog of a dimer of the hexapeptide WRWYCR. First, WRWYRGGRYWRW and (WRWYCR)₂ inhibit excision reactions with similar potencies when corrected for molecular weight. Second, both peptides have similar affinity for protein-free HJs (Table 1). They do not bind specifically to linear B-form DNA, but do bind a variety of HJs regardless of DNA sequence, junction arm-length, or the ability to branch migrate (Kepple, Flores-Fujimoto, Boldt, Lai, and Segall, unpublished data). Third, the band shift pattern of WRWYRGGRYWRW suggests that it may bind HJs similarly to (WRWYCR)₂, which interacts with HJs that are in an “open” conformation [28]. The “open” conformation provides the maximum solvent-exposed area at the center of the junction for peptide binding, as exemplified by the crystal structure of Cre-recombinase trapped on a HJ by the WKHYN peptide identified in our original library screens [20]. In the Cre-*loxP*-WKHYN complex, the junction is held open by the recombinase tetramer and peptide binding leads to displacement of the scissile phosphate from the enzyme active site, thereby inhibiting junction resolution [20]. Tyrosine recombinases and RuvABC form multimeric complexes that hold the HJ in an “open” conformation ([2, 5, 12, 20], respectively). In contrast, RecG helicase recognizes “open” HJs [47, 48] and unwinds them as a monomer, but has low processivity and is easily out-competed for HJ binding. Competitive inhibition of RecG was seen previously with (WRWYCR)₂ [29], and is also likely for L- and D-WRWYRGGRYWRW. However, WRWYRGGRYWRW appears to have greater nonspecific binding to HJ DNA than (WRWYCR)₂ (compare the supershifts caused by WRWYRGGRYWRW and (WRWYCR)₂ in Figure 2A); this may explain the higher concentrations of WRWYRGGRYWRW necessary to inhibit RecG-dependent unwinding of junctions compared to (WRWYCR)₂, as well as the differences seen between wrwyrggrywrw and (wrwyrcr)₂ (Table 1).

Peptide wrwyrggrywrw has MIC values in the low μ M range (Table 2). This includes treatment for MRSA, small colony variants of *Pseudomonas*, and *Salmonella enterica*, SCV's are one of the many complications in the treatment of CF [25], and all are important bacteria in clinical settings. In general, Gram-positive bacteria were more susceptible to wrwyrggrywrw than Gram-negative bacteria, similar to (wrwyrcr)₂: the permeability of both peptides is impeded by the outer membrane [24]. When compared directly, the D-stereoisomers of either peptide sequence are generally more effective at inhibiting bacterial growth than the L-stereoisomers, and wrwyrggrywrw is generally more effective than (wrwyrcr)₂. If peptide uptake requires spanning the phospholipid bilayer, the single chain dodecapeptides would more efficiently accomplish this, whereas any reduction of the (wrwyrcr)₂ would diminish its uptake efficiency.

To compare the *in vivo* effects of wrwyrggrywrw and (wrwyrcr)₂ in bacteria we performed assays to measure the amount of DNA damage, membrane depolarization and cell viability

(Figure 4). The data from the TUNEL and DiBAC₄(3) assays suggest that the peptides initially perturb the membrane potential, and that DNA damage occurs subsequently. For instance, treatment with 16 μ M wrwyrggrywrw led to >90% DiBAC₄(3)-positive cells after 10 minutes and a 90% decrease in viability, but <30% TUNEL-positive cells after 1.5 hours. If DNA damage indeed results from inhibition of HJ resolution, this damage may take longer to accumulate than the 1.5-hour time point of the TUNEL assay. We do not see significant DNA damage at earlier time points ([24], Naili, Segall unpublished data). Interestingly, at lower concentrations of wrwyrggrywrw, cells appear to recover their membrane potential with time (compare 8 μ M treatment at 1.5 and 3 hours in Figure 4B). Either the affected cells recover, or at that concentration, two distinct cell populations exist – one in which the peptide causes early and irreparable damage and death, and another which incurs sublethal damage from which cells recover. The two populations may reflect high cooperativity among peptides as they cross the bacterial membrane. Our current experiments following bacterial growth (Figure 4C and growth curves, Rostron and Segall, unpublished data) do not distinguish between these possibilities.

The DNA damage and membrane depolarization seen may independently cause bacterial growth inhibition. In addition, we have recently shown that there is a strong correlation between membrane stress, derived endogenously or exogenously, and DNA damage ([57]). We do not know if the DNA damage is independent of and/or as a consequence of whatever membrane or other damage is associated with peptide treatment. We have demonstrated previously that wrwyrggrywrw can trap HJs formed during Y-SSR reactions *in vivo* [23] and these experiments show that the peptides can bind HJs and interfere with their resolution inside cells. To what extent this activity causes or contributes to inhibition of bacterial growth remains to be determined. It is also possible that additional, unidentified cellular targets contribute to the antibacterial activity of the peptides. Multiple independent targets are common for cationic antimicrobial peptides, which is highly desirable with respect to preventing antibiotic resistance [8, 38, 49]. In fact, we have been unsuccessful in finding *E. coli* or *Salmonella enterica* mutants resistant to (wrwycr)₂, either by spontaneous or by transposon mutagenesis (Gunderson, Rostron, Segall, unpublished data).

The toxicity of wrwyrggrywrw treatment was studied on three mammalian cell types: red blood cells, activated murine peritoneal macrophages, and human colorectal cancer cells. Peptide wrwyrggrywrw had little or no hemolytic activity even at 128 μ M (Figure 5). This concentration is 7X higher than the MIC for wrwyrggrywrw against the bacteria tested (Table 2). Peptide wrwyrggrywrw was more toxic to peritoneal macrophages than to HCT116 cells, in both the MTT and the Live/Dead assay (Figure 6). Direct comparison of these values is difficult as the two cell types are quite distinct. For instance, PMs are terminally differentiated and do not divide *in vitro*, in contrast to the transformed HCT116 cells, which divide, potentially “diluting” the peptide over time. Additionally, the activated PMs likely internalize more peptide due to their phagocytic properties than HCT116 cells, and low concentrations of peptide may further stimulate phagocytosis, leading to greater uptake of the MTT reagent and thus an increase in MTT reduction (Figure 6A). This increase is consistent with our previous studies on the effects of (wrwycr)₂ treatment on PMs; however, treatment of PMs with 75 μ M (wrwycr)₂ led to a 18% increase in MTT reduction [51] compared to a 31% decrease with wrwyrggrywrw at the same concentration. A second macrophage cell line, J774A.1 cells, showed a 17.3% decrease in MTT reduction with 50 μ M (wrwycr)₂ treatment [51], so the response will be cell-type specific. Peptides (wrwycr)₂ and wrwyrggrywrw may not be internalized by PMs equally and this may account for the differences between the MTT assays in this and the previous studies. In fact, the concentration of the single chain dodecapeptide, but not (wrwycr)₂, increases linearly in U2OS cells, showing that the former penetrates cells more efficiently (Patra, Segall, unpublished data). However, it is also possible that wrwyrggrywrw is more toxic than

(wrwycr)₂. Indeed, wrwyrggrywrw causes significantly more cell death than (wrwycr)₂ when measured with the Live/Dead assay (~10% dead PMs with 75 μM (wrwycr)₂ [51] versus >90% with 75 μM wrwyrggrywrw). Despite this modest specificity toward bacterial cells, the IC₅₀ values for wrwyrggrywrw in the Live/Dead assays (approximately 43 μM and 92 μM, for PM and HCT116 cells, respectively) exceed the MIC values for all of the bacterial species tested, especially of Gram positive bacteria (compare with values in Table 2). It may be possible to decrease the toxicity towards eukaryotic cells, for example by decreasing uptake into the cell and/or the nucleus, while improving antibacterial activity. Regardless, these peptides could become useful tools to follow the flux of DNA replication and repair intermediates in both prokaryotes and eukaryotes.

5. Conclusion

We have designed and characterized single-chain linear analogs of the HJ trapping, antibacterial peptides L- and D-(WRWYCR)₂. L- and D-WRWYRGGRYWRW are resistant to reducing conditions, and recognize branched DNAs such as HJs in the absence of junction-binding proteins. They are likely to have multiple cellular targets based on their inhibition of DNA repair enzymes, and induction of DNA damage, and loss of bacterial membrane potential. Finally, L- and D-WRWYRGGRYWRW are potent inhibitors of both Gram-positive and Gram-negative bacterial growth and their MICs are comparable, if not better, than L- or D-(WRWYCR)₂ yet remain below the range of toxicity that we see with eukaryotic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Brett Hilton and the SDSU Flow Cytometry Core for help and support. We also thank Sylvie Blondelle (Sanford Burnham Medical Research Institute), Lionello Bossi (CNRS, Gif-sur-Yvette), Doug Conrad (University of California San Diego), Susan Lovett (Brandeis University), Victor Nizet, and Forest Rohwer (SDSU) for their generosity in providing us with strains for this study. We thank Leo Su for training IN to collect peritoneal macrophages from mice. MCR would like to thank the Achievement Rewards for College Scientists (ARCS) organization for financial support. This work was supported by grants R01-AI058253 from the National Institute of Allergy and Infectious Diseases and R01-GM052847 from the National Institute of General Medical Services to AMS.

Glossary

Holliday junction (HJ) DNA	The central intermediate in recombination, a Holliday junction (also known as a 4-way junction) is formed by the union of two duplex strands of DNA to make a structure with four double-stranded arms (branches) and a branch point in the center
Branched DNA	Intermediates in DNA replication, DNA repair, or DNA recombination. Examples are a HJ, a D-loop, or a replication fork
Branch Migration	The process by which the branch point of the HJ migrates. Possible due to the complimentary nature of the DNA sequence in the arms
RuvABC	A multimeric complex of proteins that can branch migrate a HJ (RuvAB) and resolve it to duplex DNA by the nuclease activity of the RuvC proteins
RecG	An enzyme that unwinds a HJ into 2 flayed duplex molecules

Replication fork reversal (RFR)	When a replication fork encounters a blockage on the DNA template, the fork may be reversed in order to facilitate repair. Fork reversal leads to the formation of a HJ
Homodimer	Two molecules of peptide wrwycr that are connected by a disulfide bridge between the cysteine residues. Homodimers of wrwycr bind to HJs and inhibit their resolution
Single-chain functional analog	A sequence variant of peptide wrwycr that retains the same activity without the need for disulfide bridge formation. wrwyrgrgyrwrw is an example
DTT (dithiothreitol)	A chemical agent that reduces disulfide bonds to sulfhydryls
Open vs. Closed HJ	Conformational forms of the HJ that differ by the angle between the arms of the junction. In the open conformation, the arms are approximately 90° apart. In the closed conformation, two pairs of arms exist and each pair is subtended by an angle of less than 60 degrees. Both forms can be separated by electrophoresis
Specific shift versus supershift	Binding of a peptide to the center of the HJ (at the branch point) may change the conformation from closed to open and contribute to the retardation of the HJ DNA during electrophoresis in a gel. This is a specific shift. Binding of multiple peptides to the HJ, where at least some of the peptides interact in a non-specific manner with the dsDNA arms of the junction, may cause an even larger shift, also visible by gel electrophoresis

References

1. Ames BN, Lee FD, Durston WE. An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc Natl Acad Sci U S A*. 1973; 70:782–6. [PubMed: 4577135]
2. Ariyoshi M, Nishino T, Iwasaki H, Shinagawa H, Morikawa K. Crystal structure of the holliday junction DNA in complex with a single RuvA tetramer. *Proc Natl Acad Sci U S A*. 2000; 97:8257–62. [PubMed: 10890893]
3. Azaro, MAL. A. γ Integrase and the γ Int family. In: Craig, NL.; Craigie, R.; Gellert, R.; Lambowitz, AM., editors. *Mobile DNA II*. Washington D.C: ASM Press; 2002. p. 118–48.
4. Baharoglu Z, Bradley AS, Le Masson M, Tsaneva I, Michel B. *ruvA* Mutants that resolve Holliday junctions but do not reverse replication forks. *PLoS Genet*. 2008; 4:e1000012. [PubMed: 18369438]
5. Biswas T, Aihara H, Radman-Livaja M, Filman D, Landy A, Ellenberger T. A structural basis for allosteric control of DNA recombination by lambda integrase. *Nature*. 2005; 435:1059–66. [PubMed: 15973401]
6. Boldt JL, Pinilla C, Segall AM. Reversible inhibitors of lambda integrase-mediated recombination efficiently trap Holliday junction intermediates and form the basis of a novel assay for junction resolution. *J Biol Chem*. 2004; 279:3472–83. [PubMed: 14625310]
7. Bradley AS, Baharoglu Z, Niewiarowski A, Michel B, Tsaneva IR. Formation of a stable RuvA protein double tetramer is required for efficient branch migration in vitro and for replication fork reversal in vivo. *J Biol Chem*. 2011; 286:22372–83. [PubMed: 21531731]
8. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol*. 2005; 3:238–50. [PubMed: 15703760]
9. Cassell G, Klemm M, Pinilla C, Segall A. Dissection of bacteriophage lambda site-specific recombination using synthetic peptide combinatorial libraries. *J Mol Biol*. 2000; 299:1193–202. [PubMed: 10873445]

10. Chan DI, Prenner EJ, Vogel HJ. Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. *Biochim Biophys Acta*. 2006; 1758:1184–202. [PubMed: 16756942]
11. Connolly B, Parsons CA, Benson FE, Dunderdale HJ, Sharples GJ, Lloyd RG, et al. Resolution of Holliday junctions in vitro requires the *Escherichia coli* ruvC gene product. *Proc Natl Acad Sci U S A*. 1991; 88:6063–7. [PubMed: 1829835]
12. Conway AB, Chen Y, Rice PA. Structural plasticity of the Flp-Holliday junction complex. *J Mol Biol*. 2003; 326:425–34. [PubMed: 12559911]
13. De Muyt A, Jessop L, Kolar E, Sourirajan A, Chen J, Dayani Y, et al. BLM helicase ortholog Sgs1 is a central regulator of meiotic recombination intermediate metabolism. *Mol Cell*. 2012; 46:43–53. [PubMed: 22500736]
14. Declais AC, Lilley DM. New insight into the recognition of branched DNA structure by junction-resolving enzymes. *Curr Opin Struct Biol*. 2008; 18:86–95. [PubMed: 18160275]
15. Eggleston AK, West SC. Cleavage of holliday junctions by the *Escherichia coli* RuvABC complex. *J Biol Chem*. 2000; 275:26467–76. [PubMed: 10851230]
16. Ehmsen KT, Heyer WD. *Saccharomyces cerevisiae* Mus81-Mms4 is a catalytic, DNA structure-selective endonuclease. *Nucleic Acids Res*. 2008; 36:2182–95. [PubMed: 18281703]
17. Ferraro, MJ. National Committee for Clinical Laboratory Standards Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard; Wayne, PA. 2000. p. 36
18. Ferraro, MJ. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing: twelfth informational supplement. Wayne, PA: National Committee for Clinical Laboratory Standards; 2002.
19. Fujimoto DF, Pinilla C, Segall AM. New peptide inhibitors of type IB topoisomerases: similarities and differences vis-a-vis inhibitors of tyrosine recombinases. *J Mol Biol*. 2006; 363:891–907. [PubMed: 16996084]
20. Ghosh K, Lau CK, Guo F, Segall AM, Van Duyne GD. Peptide trapping of the Holliday junction intermediate in Cre-loxP site-specific recombination. *J Biol Chem*. 2005; 280:8290–9. [PubMed: 15591069]
21. Gregg AV, McGlynn P, Jaktaji RP, Lloyd RG. Direct rescue of stalled DNA replication forks via the combined action of PriA and RecG helicase activities. *Mol Cell*. 2002; 9:241–51. [PubMed: 11864599]
22. Grindley ND, Whiteson KL, Rice PA. Mechanisms of site-specific recombination. *Annu Rev Biochem*. 2006; 75:567–605. [PubMed: 16756503]
23. Gunderson CW, Boldt JL, Authement RN, Segall AM. Peptide wrwycr inhibits the excision of several prophages and traps holliday junctions inside bacteria. *J Bacteriol*. 2009; 191:2169–76. [PubMed: 19181810]
24. Gunderson CW, Segall AM. DNA repair, a novel antibacterial target: Holliday junction-trapping peptides induce DNA damage and chromosome segregation defects. *Mol Microbiol*. 2006; 59:1129–48. [PubMed: 16430689]
25. Haussler S. Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. *Environ Microbiol*. 2004; 6:546–51. [PubMed: 15142242]
26. Heitman J, Ivanenko T, Kiss A. DNA nicks inflicted by restriction endonucleases are repaired by a RecA- and RecB-dependent pathway in *Escherichia coli*. *Mol Microbiol*. 1999; 33:1141–51. [PubMed: 10510229]
27. Johnson, RC. Bacterial Site-specific DNA inversion systems. In: Craig, NLCR.; Gellert, R.; Lambowitz, AM., editors. *Mobile DNA II*. Washington D.C: ASM press; 2002. p. 230-71.
28. Kepple KV, Boldt JL, Segall AM. Holliday junction-binding peptides inhibit distinct junction-processing enzymes. *Proc Natl Acad Sci U S A*. 2005; 102:6867–72. [PubMed: 15867153]
29. Kepple KV, Patel N, Salamon P, Segall AM. Interactions between branched DNAs and peptide inhibitors of DNA repair. *Nucleic Acids Res*. 2008; 36:5319–34. [PubMed: 18689438]
30. Klemm M, Cheng C, Cassell G, Shuman S, Segall AM. Peptide inhibitors of DNA cleavage by tyrosine recombinases and topoisomerases. *J Mol Biol*. 2000; 299:1203–16. [PubMed: 10873446]
31. Lilley DM. Analysis of branched nucleic acid structure using comparative gel electrophoresis. *Q Rev Biophys*. 2008; 41:1–39. [PubMed: 18755052]

32. McGlynn P, Al-Deib AA, Liu J, Mariani KJ, Lloyd RG. The DNA replication protein PriA and the recombination protein RecG bind D-loops. *J Mol Biol.* 1997; 270:212–21. [PubMed: 9236123]
33. McGlynn P, Lloyd RG. Genome stability and the processing of damaged replication forks by RecG. *Trends Genet.* 2002; 18:413–9. [PubMed: 12142010]
34. McGlynn P, Lloyd RG. Recombinational repair and restart of damaged replication forks. *Nat Rev Mol Cell Biol.* 2002; 3:859–70. [PubMed: 12415303]
35. Meddows TR, Savory AP, Lloyd RG. RecG helicase promotes DNA double-strand break repair. *Mol Microbiol.* 2004; 52:119–32. [PubMed: 15049815]
36. Michel B, Boubakri H, Baharoglu Z, LeMasson M, Lestini R. Recombination proteins and rescue of arrested replication forks. *DNA Repair (Amst).* 2007; 6:967–80. [PubMed: 17395553]
37. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65:55–63. [PubMed: 6606682]
38. Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol.* 2011
39. Orchard SS, Rostron JE, Segall AM. Enterobactin synthesis and uptake mutants are hypersensitive to an antimicrobial peptide that limits the availability of iron in addition to blocking Holliday junction resolution. *Microbiology.* 2011
40. Rajeev L, Segall A, Gardner J. The bacteroides NBU1 integrase performs a homology-independent strand exchange to form a holliday junction intermediate. *J Biol Chem.* 2007; 282:31228–37. [PubMed: 17766246]
41. Ranjit DK, Rideout MC, Nefzi A, Ostresh JM, Pinilla C, Segall AM. Small molecule functional analogs of peptides that inhibit lambda site-specific recombination and bind Holliday junctions. *Bioorg Med Chem Lett.* 2010; 20:4531–4. [PubMed: 20598532]
42. Rideout MC, Boldt JL, Vahi-Ferguson G, Salamon P, Nefzi A, Ostresh JM, et al. Potent antimicrobial small molecules screened as inhibitors of tyrosine recombinases and Holliday junction-resolving enzymes. *Mol Divers.* 2011; 15:989–1005. [PubMed: 21938393]
43. Rohwer F, Azam F. Detection of DNA damage in prokaryotes by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling. *Appl Environ Microbiol.* 2000; 66:1001–6. [PubMed: 10698764]
44. Rudolph CJ, Upton AL, Briggs GS, Lloyd RG. Is RecG a general guardian of the bacterial genome? *DNA Repair (Amst).* 2010; 9:210–23. [PubMed: 20093100]
45. Rudolph CJ, Upton AL, Lloyd RG. Replication fork collisions cause pathological chromosomal amplification in cells lacking RecG DNA translocase. *Mol Microbiol.* 2009; 74:940–55. [PubMed: 19818016]
46. Sambrook, J.; Russell, DW. *Molecular cloning: a laboratory manual.* 3. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press; 2001.
47. Sharples GJ. The X philes: structure-specific endonucleases that resolve Holliday junctions. *Mol Microbiol.* 2001; 39:823–34. [PubMed: 11251805]
48. Sharples GJ, Ingleston SM, Lloyd RG. Holliday junction processing in bacteria: insights from the evolutionary conservation of RuvABC, RecG, and RusA. *J Bacteriol.* 1999; 181:5543–50. [PubMed: 10482492]
49. Silver LL. Challenges of antibacterial discovery. *Clin Microbiol Rev.* 2011; 24:71–109. [PubMed: 21233508]
50. Stewart EJ, Aslund F, Beckwith J. Disulfide bond formation in the Escherichia coli cytoplasm: an in vivo role reversal for the thioredoxins. *EMBO J.* 1998; 17:5543–50. [PubMed: 9755155]
51. Su LY, Willner DL, Segall AM. An antimicrobial peptide that targets DNA repair intermediates in vitro inhibits Salmonella growth within murine macrophages. *Antimicrob Agents Chemother.* 2010; 54:1888–99. [PubMed: 20176906]
52. Vincent SD, Mahdi AA, Lloyd RG. The RecG branch migration protein of Escherichia coli dissociates R-loops. *J Mol Biol.* 1996; 264:713–21. [PubMed: 8980680]
53. West SC. Processing of recombination intermediates by the RuvABC proteins. *Annu Rev Genet.* 1997; 31:213–44. [PubMed: 9442895]

54. Whitby MC, Bolt EL, Chan SN, Lloyd RG. Interactions between RuvA and RuvC at Holliday junctions: inhibition of junction cleavage and formation of a RuvA-RuvC-DNA complex. *J Mol Biol.* 1996; 264:878–90. [PubMed: 9000618]
55. Whitby MC, Lloyd RG. Targeting Holliday junctions by the RecG branch migration protein of *Escherichia coli*. *J Biol Chem.* 1998; 273:19729–39. [PubMed: 9677403]
56. Willner D, Haynes MR, Furlan M, Hanson N, Kirby B, Lim YW, et al. Case studies of the spatial heterogeneity of DNA viruses in the cystic fibrosis lung. *Am J Respir Cell Mol Biol.* 2012; 46:127–31. [PubMed: 21980056]
57. Yitzhaki S, Rostron JE, Xu Y, Rideout MC, Authement RN, Barlow SB, et al. Similarities between Exogenously- and Endogenously-Induced Envelope Stress: The Effects of a New Antibacterial Molecule, TPI1609-10. *PLoS One.* 2012; 7:e44896. [PubMed: 23071502]
58. Zheng M, Aslund F, Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science.* 1998; 279:1718–21. [PubMed: 9497290]

Highlights

wrwyrggrywrw inhibits RecG- and RuvABC- mediated processing of Holliday junctions in vitro.

wrwyrggrywrw causes DNA damage and loss of membrane potential in *E. coli* in vivo.

wrwyrggrywrw is a potent antimicrobial with minimum inhibitory concentrations in the low ug/ml range.

wrwyrggrywrw has minimal toxicity to eukaryotic cell types at the concentrations necessary to inhibit bacterial growth.

Figure 1.

Inhibition of excision under reducing conditions. (A) Selected lanes from a representative Tricine-SDS gel showing the effects of peptides (WRWYCR)₂ and WRWYRGGRYWRW on excision recombination in the presence of 25 mM DTT. Recombination proteins (Int, IHF and Xis) recombine radiolabeled “attL” DNA, with an unlabeled partner site, attR, to form the recombinant products “attP” and “attB”. The “HJ” is the intermediate of this reaction and is stabilized by the peptides, which blocks progression to products or regression to substrates, albeit at different potencies in the presence of DTT. “CPD,” covalent protein-DNA intermediate. (B) Comparison of the ability of peptides (WRWYCR)₂ (circles) and WRWYRGGRYWRW (squares) to accumulate HJs in excision recombination reactions in the presence (dashed lines) or absence (solid lines) of DTT. Treatment with DTT significantly reduces the % accumulation of HJ intermediates by (WRWYCR)₂, but not by WRWYRGGRYWRW. High concentrations of either peptide inhibit the initial cleavage step of the reaction, resulting in a reduced accumulation of HJs.

Figure 2.

(A) HJ band shifts by single-chain linear peptides. The band shift seen is a combination of both the greater mass of the HJ-peptide complex and the conformational change in the HJ from a faster migrating “free HJ” species to a slower migrating “bound HJ”. Supershifts and DNA in the wells of the gel are the result of non-specific interactions. (WRWYCR)₂ is shown here for comparison purposes. (B) Band shifts in the presence of competitor DNA of WRWYRGGRYWRW, with either “HJ DNA” (top) or “Linear DNA” (an *attL* site, bottom). The indicated amounts of non-specific DNA (nsDNA) were added prior to peptide treatment. DNA present in the supershift and in the wells are the result of weaker non-specific interactions as these bands disappear with increasing concentrations of competitor DNA (compare 0.55 μg nsDNA vs. 1 μg nsDNA). WRWYRGGRYWRW does not form a stable complex with double stranded linear *attL* DNA.

Figure 3.

Peptide-dependent inhibition of HJ processing. (A) *In vitro* in the presence of ATP and Mg^{+2} , RecG helicase unwinds a synthetic HJ into a flayed duplex with both double stranded and single stranded regions. Selected lanes from Tris-Tricine-SDS polyacrylamide gels are shown that demonstrate the dose-dependent inhibition of RecG unwinding activity due to treatment with peptides WRWYRGGRYWRW or wrwyrggrywrw. (B) Quantitation of gels in (A) normalized to the DMSO control. (C) *In vitro*, the RuvABC resolvase cleaves a synthetic HJ by introducing two staggered cuts (indicated by the solid or dashed arrows). The different arm lengths of the HJ result in two different size double-stranded products, which were separated on Tris-Tricine-SDS gels. Shown are selected lanes from titrations of WRWYRGGRYWRW or wrwyrggrywrw that demonstrate inhibition of the cleavage activity of RuvABC resolvase. (D) Quantitation of gels in (C), normalized to the DMSO control.

Figure 4.

In vivo effects of peptide treatment. (A) *E. coli* cells were treated for 90 minutes with peptide, before performing the TUNEL assay. Treatment with either peptide resulted in a dose dependent increase in TUNEL-positive cells. DMSO controls correspond to the highest peptide concentration and consistently resulted in less than 0.1% TUNEL-positive cells (not shown). (B) *E. coli* cells were treated with wrwyrggrywrw before performing the DiBAC₄(3) assay. Control treatments with DMSO, as described above, were subtracted from each treatment to give the corrected values seen. Loss of membrane potential occurs rapidly, and the extent is dependent upon the dose of wrwyrggrywrw used. (C) *E. coli*

colony forming units (cfu) were measured following treatment with wrwyrggrywrw at the indicated times.

Figure 5.

Hemolytic activity of peptides. A solution of red blood cells (0.5% final concentration) was incubated with peptides or controls at the indicated concentrations for 1 hour at 37°C in 5% CO₂. The optical density of the supernatant was read at OD₄₁₄ nm to monitor the release of hemoglobin. A isotonic solution of “1X PBS” was used as a negative control and provides an indication of “no hemolysis”. “Water” was used as a control for osmotic hemolysis. “1% Triton X-100” was used as a control for complete hemolysis.

Figure 6.

Effects of wrwyrggrywrw on eukaryotic cell metabolism and viability. (A) Peritoneal macrophages (PMs, 400,000 cells/mL) or (B) human colorectal cancer cells (HCT116, 100,000 cells/mL) were treated with wrwyrggrywrw or DMSO for 24 hours and then tested in the MTT assay. Results are shown as “% MTT reduction” and normalized to untreated cells. (C) Activated PMs (400,000 cells/mL) or (D) HCT116 cells (200,000 cells/mL) were treated with wrwyrggrywrw for 24 hours and then tested in the Live/Dead assay. Subpopulations of cells that stained stain positive for CAM (live), or for EH (dead), or both (dying) were quantified by flow cytometry and normalized to the DMSO control. The DMSO controls for all assays corresponds to the residual solvent content at the highest peptide treatments.

Table 1

Summary of *in vitro* characteristics^a

Assay	Site-specific recombination		Holliday junction interactions ^b		
	Excision (IC ₅₀ in μ M)	(+) DTT	HJ binding (K _d in μ M)	HJ processing ^c (IC ₅₀ in μ M)	
Peptide	(-) DTT		EMS A	RecG	RuvABC
(WRWYCR) ₂	0.010 ^d	0.25 ^d	0.014 ^e	0.06 ^e	0.032 ^e
WRWYRGGRYWRW	0.025	0.055	0.025	2.53	0.045
(wrwyct) ₂	0.022 ^d	NT	NT	0.25	0.2
wrwyrgrgywrw	NT	NT	NT	1.08	0.08

^a All data shown are averages of 2 or more independent titrations over 7 concentrations; all concentrations here are reported as dimer equivalents of L- or D-WRWYCR. By convention, D-stereoisomers are written in lower case.

^b All values reported for HJ interactions are (-) DTT.

^c Activity refers to HJ unwinding by RecG, and HJ cleavage by RuvABC

^{d,e} Published in [6] and [28], respectively, and shown here for comparison purposes.

Table 2

Summary of MICs (μM) for selected bacterial strains^a

Gram (–) bacteria				Gram (+) bacteria			
Species	<i>S. enterica</i> serovar Typhimurium	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	
Genotype	LT2	AMES	MG1655	PY79	MRSA	Newman	M49
Strain ^b	G255	G455	G582	G1018	G510	G748	G762
Peptide							
(WRWYCR) ₂	17.5 ^c	4.4 ^c	17.5 ^c	NT	2.2 ^c	8.7	NT
WRWYRGGRYWRW	17.5	8.7	17.5	NT	8.7	8.7	NT
(wrwycr) ₂	17.5 ^c	4.4 ^c	8.7–17.5 ^c	4.4	2.2 ^c	8.7	35
wrwyrgrgywrw	4.4	2.2	8.7	4.4	2.2	8.7	17.5
							2.2

^aSmall colony variant (SCV, [56])
^bRefers to Segall lab strain designations found in Supplementary table 1
^cPublished [24] and shown here for comparison purposes.