DIFFERENTIAL INTERACTIONS BETWEEN THE HOLLIDAY JUNCTION-BINDING ANTIMICROBIAL PEPTIDE WRWYCR AND TWO DNA GYRASE INHIBITORS: WRWYCR’S EFFECTS ON MEMBRANE INTEGRITY AND DRUG EFFLUX

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The Undersigned Faculty Committee Approves the

Thesis of Ilham Naili:

Differential Interactions between the Holliday Junction Binding Antimicrobial Peptide wrwyr and Two DNA Gyrase Inhibitors: wrwycr’s Effects on Membrane Integrity and Drug Efflux

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DEDICATION

To my parents, Mohamed and Elisabeth Naili, for their unconditional love, understanding, and support for all these years.
The synthetic peptide wrwycr binds Holliday junctions and other branched DNA repair intermediates, and interferes in vitro with enzymes that participate in DNA repair. Peptide wrwycr is bactericidal for both Gram- and Gram+ bacteria. Because peptide wrwycr is synergistically lethal with DNA-damaging agents such as UV and mitomycin C, we hypothesized that the peptide traps DNA repair intermediates that arise during the repair of DNA damage. Therefore, we investigated the potential synergy between peptide wrwycr and two distinct topoisomerase II inhibitors: norfloxacin, a fluoroquinolone antibiotic that stabilizes open DNA complexes, and novobiocin, a coumarin that inhibits their ATPase activity. Repair of breaks caused by topoisomerases II requires recombination-dependent DNA repair. Co-treatments with sublethal concentrations of peptide wrwycr and a bacteriostatic concentration of norfloxacin indicated synergy only at high sublethal concentrations of the peptide. Surprisingly, low sublethal concentrations of wrwycr protected cells from the effects of norfloxacin: co-treated cells showed increased cell viability and reduced DNA damage compared to treatment with norfloxacin alone. E. coli lacking tolC lost this “protection”, and wrwycr co-treatment with norfloxacin induces the expression of TolC and its regulator MarA. In contrast, wrwycr demonstrated synergistic lethality with novobiocin even at low peptide concentration. We propose that this synergy is due not only to inhibition of DNA damage repair but also to the peptide-mediated increased membrane permeability, thereby permitting novobiocin to be more efficient.
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CHAPTER 1
INTRODUCTION

Antibiotic resistance in bacteria is a major health issue, and grows at an alarming rate. Although several new and very effective antibiotics, such as levofloxacin (Fu et al., 1992), clarithromycin (Fernandes et al., 1986) and doripenem (Tsuji et al., 1998), have been released on the market over the past decades, the utility of many has already been diminished as pathogens have developed resistance to these drugs (Davies and Davies, 2010; French, 2010; Silver, 2011). A major area of bacterial physiology that has not been targeted by antibiotics is DNA repair, a crucial component for bacterial survival to endogenous and exogenous damage. DNA repair via homologous recombination (HR) constitutes the only means of repairing double strand DNA breaks (DSB) in bacteria (McGlynn and Guy, 2008; Persky and Lovett, 2008). Holliday Junctions (HJ), consisting of two double-stranded helices linked by two DNA strands, are central intermediates in HR and DNA repair. DSB are often formed when a replication fork reaches a nick in double stranded (ds) DNA, causing replication fork collapse (McGlynn and Lloyd, 2002; Michel et al., 2004). The reassembly and restart of functional replication forks after collapse frequently require the successful resolution of HJ (Kuzminov, 1999; Michel et al., 2007).

Although DNA repair may initially appear to be a poor choice of antibiotic target given similarities between repair processes in prokaryotes and eukaryotes, several reasons mitigate against this. Eukaryotic cells have a considerably slower division rate and may thus have fewer replication-associated errors at any particular time, which renders formation of HJ a rarer event. Second, eukaryotes possess nuclear membranes, which impose an added selective barrier against environmental and cytoplasmic compounds. Third, DNA repair enzymes often differ sufficiently from those of bacteria to permit differential inhibition of eukaryotic versus prokaryotic homologs, such as is seen in the case of topoisomerase II inhibitors (Drlica et al., 2008; Pommier et al., 2010). These factors may sufficiently protect eukaryotic cells from an exogenous DNA repair inhibitor, and support the idea that DNA repair would be an attractive and effective new target.
Synthetic hexapeptides such as WRWYCR and KWWCRW, active as homodimers, have been isolated in our lab and shown to trap HJ in their open conformation, preventing their resolution into recombinant products (Boldt et al., 2004; Gunderson et al., 2009). To a lesser extent, these peptides bind to replication fork mimics (with up to 10X lower affinity and lesser stability; (Kepple et al., 2008)). Several of these, including the d-amino acid peptide wrwycr, were shown to inhibit the activity of the branched DNA-dependent helicase RecG and the HJ resolvase complex RuvABC (Kepple et al., 2005). The RuvABC resolvasome resolves HJ during DNA repair, while RecG prevents “pathologic replication” initiating at DNA repair intermediates (Rudolph et al., 2009; Rudolph et al., 2010). When present in biochemical reactions in vitro, peptide wrwycr has no intrinsic DNA damaging activity (Boldt et al., 2004; Kepple et al., 2008). Peptide wrwycr is bactericidal for both Gram+ (MIC 8-32 µg/ml) and Gram- (MIC 32-64 µg/ml) bacteria (Gunderson and Segall, 2006). Treatment of E. coli cells with wrwycr leads to the generation of DNA breaks, chromosome segregation defects, accumulation of anucleate cells, and cell death (Gunderson and Segall, 2006). Preliminary studies in our lab using two-dimensional gel electrophoresis showed an accumulation of HJ in wrwycr-treated E. coli cells (Marcusson et al., unpublished results). Moreover, this peptide stabilizes HJ of phage lambda site-specific recombination inside E. coli, and interferes with the excision of several prophages (Gunderson et al., 2009). Peptides with related sequence that have low affinity for HJ and bind HJ less stably have little or no antimicrobial activity (Gunderson and Segall, 2006; Kepple et al., 2008; Gunderson et al., 2009). Peptide wrwycr also inhibits the growth of Salmonella inside murine macrophages at concentrations that have no effect on the host cells (Su et al., 2010). A recent study by collaborators (Lino et al., 2011) pretreated a number of clinical E. coli strains with peptide wrwycr and found a large increase in acid-induced killing of these pathogenic strains, without concomitant increase in Shiga toxin production or increased cytotoxicity to host cells. Lastly, we have discovered that peptide wrwycr also has significant effects on E. coli membrane integrity. Indeed, treating cells with high concentrations of the peptide leads to induction of the membrane stress response, as well as rapid intracellular potassium leakage and significant membrane depolarization (Rostron, 2011). Further studies in our lab, as well as others (Kohanski et al., 2008), have showed a link between membrane damage, and
subsequent envelope stress, and DNA damage. Therefore, we believe part of wrwycr-mediated DNA damage may be due to its effects on the membrane of *E. coli* cells.

We showed previously that peptide wrwycr was synergistic with DNA damage-causing agents such as mitomycin C and UV, as expected if repair of the DNA damage creates branched DNA intermediates that become targets for the peptide, and results in a greater-than-additive drop in viability (Gunderson and Segall, 2006). Based on these observations, we speculated that peptide wrwycr could be used effectively in combination with antibiotics that induce DNA damage, such as the fluoroquinolones.

Stalled or collapsed replication forks are thought to occur at least once during each bacterial replication cycle (Cox *et al.*, 2000; Cox, 2001). Forks often stall or collapse at DNA lesions such as abasic sites (created by low pH (Tamm *et al.*, 1952), such as found in the stomach), DNA strand breaks, and proteins that are stably bound ahead of the replication fork (Mirkin and Mirkin, 2005; Payne *et al.*, 2006; Mirkin and Mirkin, 2007; Rudolph *et al.*, 2007). Topoisomerases offer a particular case in the sense that, although they ensure a successful proceeding of DNA replication, they also constitute an endogenous source of fork collapse and DNA breakage (Champoux and Dulbecco, 1972; Champoux, 1977a; Champoux, 1977b; Kirkegaard and Wang, 1978; Liu and Wang, 1979; Champoux, 2001). By catalyzing a transient single strand (type I topoisomerases) or double strand (type II topoisomerases) DNA break, these enzymes help maintain a proper DNA topology during both replication and transcription. During each catalytic cycle, topoisomerases perform a transresterification reaction that generates a transient protein-DNA covalent intermediate. Normally, upon strand passage or rotation and subsequent change of the DNA supercoiling level, the DNA ends are quickly religated by the enzymes. However, prolonging the time where topoisomerases are bound to DNA, or increasing the concentration of these covalent complexes on DNA, could have detrimental consequences on the fulfillment of replication, and thus cell viability; this property makes topoisomerases a powerful target for antimicrobials (Kreuzer and Cozzarelli, 1979; Froelich-Ammon and Osheroff, 1995; Pohlhaus and Kreuzer, 2005; Pommier *et al.*, 2010).

Both type II topoisomerases, DNA gyrase and topoisomerase IV, are able to relax positive supercoils during replication and transcription. DNA gyrase, in addition, has the unique ability to introduce negative supercoils, a required step to ensure the start of a
replication or transcription cycle (for review, see (Schvartzman and Stasiak, 2004)). The two GyrA subunits are responsible for covalently binding to a G segment (or Gate segment) of DNA and catalyzing a DSB, while the two GyrB subunits perform ATP hydrolysis to trigger a conformational change, allowing for strand passage of a T segment (or Transport segment) through the cleaved G segment. The broken DNA ends are then religated, and a second molecule of ATP is hydrolyzed to allow DNA gyrase to reset for the next catalytic cycle (Champoux, 2001; Nollmann, Crisona, et al., 2007; Schoeffler and Berger, 2008). Both DNA gyrase and topoisomerase IV are the targets of two distinct classes of antibiotics. Quinolones and the related fluoroquinolones (Ferrero et al., 1994; Khodursky et al., 1995) target the cleavage subunits of type II topoisomerases (GyrA in the case of DNA gyrase) and stabilize the covalently-bound enzymes on the cleaved DNA. The resulting ternary complexes stall the replication fork, leading to its collapse and to the generation of DSB (Goss et al., 1965; Chen et al., 1996; Drlica and Zhao, 1997; Khodursky and Cozzarelli, 1998; Grompone et al., 2003; Malik et al., 2006; Drlica et al., 2008). On the other hand, the coumarins bind to the ATP-binding subunits (GyrB in the case of DNA gyrase) and act as competitive inhibitors of ATP hydrolysis (Gilbert and Maxwell, 1994; Lewis et al., 1996).

Norfloxacin, a potent fluoroquinolone, has been used extensively, although its mechanism of action is still not completely elucidated (Malik et al., 2007; Drlica et al., 2008; Wang et al., 2010). The formation of reversible norfloxacin-gyrase complexes quickly arrests ongoing replication and induces the SOS response. Accumulation of the drug inside the cells renders these complexes irreversible and lethal by leading to the accumulation of DSB that fragment the chromosome and can overwhelm repair (Pohlhaus and Kreuzer, 2005; Hsu et al., 2006; Dwyer et al., 2007; Drlica et al., 2008). In the case of novobiocin, a well-studied coumarin, inhibition of ATP hydrolysis would prevent DNA gyrase and topoisomerase IV from introducing negative supercoils and relaxing positive supercoils, and would therefore stall active replication (Smith and Davis, 1967; Sugino et al., 1978; Maxwell, 1993; Hardy and Cozzarelli, 2003; Maxwell and Lawson, 2003). It is important to note, however, that recent studies demonstrated the ability of gyrase to relax negative supercoils in an ATP-independent manner (Nollmann, Stone, et al., 2007); this observation potentially indicates that the enzyme may still function in the presence of novobiocin, although this hypothesis has not been confirmed.
We predicted that, if peptide wrwycr interferes with repair of DSB, co-treating *E. coli* with norfloxacin and wrwycr will result in synergistic loss of viability. We have tested this hypothesis by monitoring their combined effects on the viability of *E. coli*, on the induction of the SOS response and the accumulation of DNA damage. At some concentrations of the peptide (and norfloxacin), our results supported our hypothesis. However, we found that low concentrations of wrwycr protected *E. coli* against norfloxacin’s effects; we discovered that this “protection” is at least partly due to a peptide-mediated stimulation of drug efflux. We also tested the effects of peptide wrwycr in co-treatment with novobiocin, in order to evaluate and compare any potential synergistic effect with those seen with wrwycr and norfloxacin. In contrast to norfloxacin, we showed that the MIC of novobiocin decreased 64-fold when used in co-treatment with the peptide (the MIC of wrwycr itself is reduced 4-fold in the combination treatment). Because we know that peptide wrwycr affects *E. coli*’s membrane integrity, we believe this result may be due not only to greater permeability of the antibiotic due to the peptide’s effect on the membrane, but also to a synergistic action between novobiocin and wrwycr with respect to the generation of DSB. Taken together, our results give new insights into peptide wrwycr’s effects on cell physiology and its potential utility in antibiotic combination therapies.
CHAPTER 2

MATERIALS AND METHODS

**Strains and bacterial culture methods:** Bacterial strains used in this work and their sources are listed in Table 1. Strains were grown on LB agar plates, with antibiotics when needed (tetracycline, 15 µg/ml final concentration; kanamycin, 50 µg/ml final concentration; ampicillin, 100 µg/ml final concentration), and cultures were generally grown in Luria-Bertani (LB) broth. For experiments involving peptide, we used Mueller-Hinton Broth (MHB, Becton-Dickinson). All chemicals were purchased from Sigma-Aldrich (St Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise indicated.

**Table 1. *E.coli* Strains Used in this Study**

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<th>Strain designation</th>
<th>Genotype/description</th>
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<tr>
<td>G 582/MG1655</td>
<td>F’ λ’ rph1</td>
<td>Stanley R. Maloy</td>
</tr>
<tr>
<td>G 775</td>
<td>sulB103 lacMS286 φ80 dllac BK1 argE3 his-4 thi-1 xyl-5 mtl-1 rpsL31 tsx sulAp-mCherry gal76::Tn10</td>
<td>Steven J. Sandler</td>
</tr>
<tr>
<td>EDT 1754</td>
<td>MG1655 sulAp-mCherry gal76::Tn10</td>
<td>Lab collection</td>
</tr>
<tr>
<td>EDT 1350</td>
<td>MG1655 ΔtolC::f rt</td>
<td>Lab collection</td>
</tr>
<tr>
<td>EDT 2117</td>
<td>MG1655 ΔtolC sulAp-mCherry</td>
<td>This study</td>
</tr>
<tr>
<td>EDT 2224</td>
<td>gal76::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>EDT 2227</td>
<td>N99 (ΔlacZYA galK rpsL) tolC::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>G 1004</td>
<td>N99 (ΔlacZYA galK rpsL) marA::lacZ</td>
<td>James M. Slauch</td>
</tr>
<tr>
<td></td>
<td>DH5α λ pir pKG137 (FRT-lacZY kan)</td>
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To obtain strains EDT 2224 and EDT 2227, we used the *tolC* FRT-Kan-FRT and *marA* FRT-kan-FRT constructs from the Keio collection strains (Datsenko and Wanner, 2000) and moved the deletion constructs into N99 by generalized transduction using phage P1. We then removed the kanamycin resistance cassette by transforming the strains with
pCP20 (AmpR), which encodes the Flp protein. We transformed these strains with pKG137 (FRT-lacZY KanR). After selection of transformants and insertion of lacZ under the tolC or marA promoters, both pCP20 and pKG137 plasmids were removed from the transformants by placing the cultures at 42°C and subsequent plating on LB with appropriate antibiotics.

**Peptides:** All peptides were synthesized by Biosynthesis (Lewisville, TX) or by Sigma Genesis (Sigma-Aldrich, St Louis, MO) using d amino acids with an amidated C terminus and purified to >95% purity by HPLC. Peptide stock solutions (10 mM) were maintained in 100% dimethyl sulfoxide (DMSO). The extent of dimerization of the peptide wrwycr was monitored by reverse phase HPLC on a C8 column as previously described (Orchard et al, unpublished results), and stocks used contained at least 86% dimers via a disulfide bridge (data not shown). Final DMSO concentrations in experimental procedures were, at most, 0.64%, and DMSO at the appropriate concentration was used in the absence of peptide as a control for solvent effects.

**Minimum Inhibitory Concentration (MIC) determinations and Viability assays:** MIC determinations were performed according to the NCCLS protocol for broth microdilutions (National Committee for Clinical Laboratory Standards, 1997), with the following adaptations. Briefly, overnight cultures to be tested were subcultured 1:100 and grown to an OD$_{600}$ of 0.1 in MHB. These subcultures were then mixed in a 1:1 ratio with 100 µl aliquots of MHB containing twice (2X) the desired final peptide wrwycr, norfloxacin, or novobiocin concentration (or 4X the desired final concentration of peptide d8 and norfloxacin or novobiocin combined) in a microtiter plate, as well as 2X the amount of EDTA desired when needed. Microtiter plates were sealed with parafilm to prevent evaporation and incubated for 20 h in a 37°C shaker incubator (Precision Scientific, Chennai, India). The OD$_{600}$ was read at t= 0 h and t= 20 h using a SpectraMax 384 plate reader (Molecular Devices, Inc; Sunnyvale, CA, USA), and the lowest concentrations of peptide and/or norfloxacin/novobiocin that completely inhibited growth were identified as the MIC. The MIC values were determined from a minimum of 3 independent colonies in each experiment, with most experiments performed at least three times.

For cell viability determinations, aliquots of each treated culture were taken at the time points indicated and serial dilutions were performed in TM buffer (1 mM TRIS-HCl, pH 7.4, 10 mM MgCl$_2$) in microtiter plates. Five µl of each dilution, from $10^0$ to $10^-6$, were
plated on LB agar using a multichannel pipettor. Plates were incubated overnight in a 37°C incubator before colonies were counted.

**Microscopy**: Bacterial cultures were grown in MHB until they reached an OD$_{600}$ of 0.1, then treated with wrwyr, norfloxacin, or a combination in a microtiter plate for 3 h or 12 h in the 37°C shaker. Cells were pelleted and resuspended in 1X phosphate-buffered saline (PBS), then mixed 1:1 on the microscopy slide with a PBS solution containing Slowfade antifade reagent in 50 % glycerol (at a final concentration of 0.04X from the stock solution) (Molecular Probes, Eugene, OR), the DNA-specific fluorophore DAPI (Molecular Probes, Eugene, OR), at a final concentration of 50µg/ml, and FM4-64, a membrane-specific fluorophore (Molecular Probes, Eugene, OR) at a final concentration of 20 µg/ml. Epifluorescence images were taken using a Carl Zeiss Axio Observer.Z1 inverted microscope with a 100X objective. Cell size was measured using the Axio Observer.Z1 software, and a minimum 50 different cells were analyzed for each treatment to obtain an average cell length.

**LacZ assays using 4 methyl umbelliferyl β-D-galactoside (MUG)**: Bacterial cultures of the LacZ reporter strains EDT2224 and EDT2227 were subcultured 1:100 in MHB at 37°C until an OD$_{600}$ of ~0.1, after which 150 µl of cells were transferred to a microtiter plate (Costar 3370) and treated for 1 h with varying concentrations of wrwyr, norfloxacin, novobiocin versus DMSO. The OD$_{600}$ was read using a SpectraMax 384 plate reader (Molecular Devices, Inc; Sunnyvale, CA, USA) and 10 µl aliquots were transferred to a black microtiter plate (Costar 3915). Freeze-thaw steps of 15 minutes at -80°C were repeated three times to ensure cell lysis, and aliquots were allowed to thaw at room temperature for 20 min before being resuspended in 100 µl of MUG buffer (60 mM K$_2$HPO$_4$, 40 mM KH$_2$PO$_4$, 100 mM NaCl). The β-galactosidase substrate 4-methyl umbelliferyl β-D-galactoside (MUG) was added (10 µl at 0.4 mg/ml dissolved in DMSO), and the plate was placed in the dark for 45 min at room temperature. We then added 100 µl of stop buffer (200 mM Glycine pH 10.3). Fluorescence was measured with an excitation of 350 nm, and an emission of 450 nm, using a SpectraMax Gemini XS fluorimeter (Molecular Devices, Inc; Sunnyvale, CA, USA). Units were calculated similarly to the calculation of Miller units, by dividing the fluorescence values by the OD$_{600}$.

**Flow cytometry**: All flow cytometry experiments were performed using a BD FACSARia desktop cell sorter with a 70 µm nozzle (Becton-Dickinson, San Jose, CA) 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, San Jose, CA) and/or FlowJo (Tree Star Inc., Ashland, OR.). In most cases, bacterial cells were identified using the forward scatter (FSC) and side scatter (SSC) parameters compared to a 1X filtered PBS control. Forward scatter due to light scattering denotes the size of the particles analyzed. The side scatter parameter was originally defined as denoting granularity of immune cells, and is not easily “translated” to bacterial cells. We do not use SSC except in distinguishing cells from debris.

**TUNEL assay:** We measured DNA strand breaks by labeling free 3’OH ends in DNA using a TUNEL assay (Rohwer and Azam, 2000; Gunderson and Segall, 2006). Overnight cultures were subcultured 1:100 in MHB and grown until they reached an OD\textsubscript{600} of 0.08-0.1, and then treated with 2X concentrations of peptide wrwycr, norfloxacin, or both dissolved in media, as described earlier, in a microtiter plate for 3 h or 12 h in a 37°C shaker (IKA®-Schuttler MTS 2, Wilmington, NC). 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, Germany) according to the manufacturer’s protocol, except that we resuspended our samples in a final volume of 25 µl of the detection solution. Cells were counterstained with DAPI (final concentration of 25 µg/ml) for 10 min. 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 violet laser (405 nm; the DAPI channel) for DAPI. The percentage of TUNEL\textsuperscript{+} cells was calculated as a fraction of the DAPI\textsuperscript{+} cells.

**SOS response:** To measure the induction of the SOS regulon, we used strains EDT1754 or EDT2117 encoding the mCherry fluorescent protein gene fused to the promoter of the SOS-regulated sulA gene. These strains were subcultured 1:100 to an OD\textsubscript{600} of 0.08-0.1, and then treated with DMSO, peptide wrwycr, norfloxacin, or both compounds, for 3 h or 6 h before being pelleted and resuspended in 1X PBS. Cells that were mCherry\textsuperscript{+} were quantified by flow cytometry, using the blue laser (488 nm) and the PE Texas Red channel.

**Anucleate cells:** To measure the formation of anucleate cells, we used strains MG1655 and EDT1350 (MG1655\textsuperscript{ΔtolC}) and employed the same methods as used for the TUNEL assay for subcultures, treatment, fixation and permeabilization of each sample. This
time however, in order to look for cells lacking DNA, we first stained our samples with DAPI (final concentration of 25 µg/ml in 1X PBS) for 10 min, then pelleted our samples, and resuspended them in a solution of FM4-64 (final concentration of 10 µg/ml in 1X PBS) for 10 min. Cells were pelleted and resuspended in 1X PBS for quantification by flow cytometry. We used the violet laser (405 nm; the DAPI channel) and the blue laser (488nm; the X channel) for FM4-64. Anucleate cells were identified as the FM4-64+/DAPI− population.

**Pulsed Field Gel Electrophoresis (PFGE):** The PFGE protocol used was adapted from Liu and Sanderson (Gunderson and Segall, unpublished results; Liu and Sanderson, 1995). Briefly, overnight cultures were subcultured 1:100 and grown until they reached an OD600 of 0.08-0.1, then treated with peptide wrwycr and/or norfloxacin at the indicated concentrations for 3 h. Cells were pelleted and resuspended in resuspension solution (10 mM Tris HCl pH7.2, 20 mM NaCl, 10 mM EDTA), to a final OD of 1.3. Bacteria were embedded in agarose plugs by mixing cells 1:1 with 1.5% PFGE agarose (Bio-Rad, Hercules, CA), and poured into a gel 10x5mm plugs. Solidified plugs were incubated with cell lysis solution (10 mM Tris HCl pH7.2, 50 mM NaCl, 100 mM EDTA, 0.2% sodium sodecyl sulfate, 0.5% N-lauryl sarcosine) for 1 h at 65°C. Plugs were then decanted and incubated with proteinase K solution (1 mg/ml−1 in 4ml of buffer containing 100 mM EDTA, 0.2% SDS, 1% N-lauryl sarcosine) for 48-72 h at 42°C. Proteinase K was inactivated by washing the plugs in phenyl methyl sulfonyl fluoride (PMSF) solution for 1 h at room temperature. Plugs were then washed several times with a wash solution (20 mM Tris HCl pH 8.0, 50 mM EDTA) and kept in storage solution (10X diluted wash solution) at 4°C. To make the gel, plugs were cut into 5 mm squares, washed with sterile nanowater, and placed on a gel comb. The plugs were embedded into a 1% PFGE agarose gel, and electrophoresis was carried out in 0.5X TBE at 14°C for 22 h, in a Bio-Rad CHEF III Mapper XA system (Bio-Rad, Hercules, CA) at 6.0V/cm, with 120° included angle, linear ramp, 50-90 second switch time. Each gel was stained with 1X SYBRGreen I (Molecular Probes, Eugene, OR) for 30 minutes in the dark and scanned using a GE Healthcare Storm 8600 or a Typhoon 9400 scanner (GE Healthcare, United Kingdom). Image J software version 1.41 (NIH) was used to quantify the DNA present in the gel and was performed by Rena Hiedo and Sarah Rafo.
CHAPTER 3

RESULTS

Based on our predictions of peptide wrwyryr’s interactions with norfloxacin and novobiocin, we hypothesized that co-treatments with wrwyryr and norfloxacin would be synergistic, and co-treatments with wrwyryr and novobiocin would be additive. We wanted to test our hypotheses by performing viability assays using both treatment combinations, and comparing the effects of co-treatments to those of single treatments.

VIABILITY OF E. COLI CO-TREATED WITH WRWYCR AND NORFLOXACIN OR NOVOBIOCIN

Peptide wrwyryr can lead to protection from norfloxacin. We first determined the MIC values of both compounds, individually and in combination, using a checkerboard MIC assay performed in microtiter plates (Table 2). Overnight cultures of E. coli MG1655 were subcultured in MHB and treated with either wrwyryr alone, norfloxacin alone, or both compounds at increasing concentrations. The OD$_{600}$ was measured at t=0 h and t=20 h of growth at 37°C (Table 2). The MIC value of peptide wrwyryr for MG1655 was 64 µM, as before (Gunderson and Segall, 2006). The MIC value of norfloxacin was 0.25 µg/ml. When treatments were combined, growth was inhibited with 16 µM of wrwyryr and 0.06 µg/ml norfloxacin. This MIC value represents a 4-fold decrease of both compounds, compared to the MIC value of each compound used alone. Similar MICs were obtained when using Salmonella enterica serovar Typhimurium LT2 (data not shown).

To test the effect on viability, MG1655 cells were treated in a similar fashion as for MIC assays; aliquots were taken at the indicated time points and cells were serially diluted and plated on LB agar (Figure 1). Bacterial viability dropped in a dose-dependent manner, with hardly any loss in viability at 8 µM wrwyryr and a 4-log drop in viability at 32 µM wrwyryr compared to DMSO during the first 6 h, but by 24 h the cultures recovered even from this higher dose. This recovery may be due both to repair of the damage created by the peptide and to the cells’ ability to efflux this peptide (Orchard et al., unpublished results). Norfloxacin at a concentration of 0.06 µg/ml is bacteriostatic, as seen by unchanged CFU/ml
Table 2. Growth of *E. coli* MG1655 Cells Treated with wrwycr and/or Norfloxacin

<table>
<thead>
<tr>
<th>wrwycr NFX</th>
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<th>8</th>
<th>16</th>
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Growth (+) or growth inhibition (-) was recorded by calculating the OD$_{600}$ change of each treatment between $t=0$ h and $t=20$ h. An OD$_{600}$ increase equal to or lower than 0.1 was considered evidence of growth inhibition.

values during the entire experiment. However, after 1.5 h of treatment, cells treated with both norfloxacin and either 8 µM or 16 µM wrwycr showed greater viability than when treated with norfloxacin alone after 1.5 h of treatment (Figure 1A and 1B). The results seen at low (8-16 µM) peptide concentrations were contrary to our hypothesis that the two compounds would have synergistically detrimental effects on growth and viability. In contrast, the combination of norfloxacin and 32 µM wrwycr showed a greater-than-additive effect on cell viability: after 6 h of treatment, the number of viable cells continued to decrease and by 24 h essentially no viable cells were recovered (Figure 1C). We also tested the combination of peptide doses with 0.125 µg/ml norfloxacin. However, this norfloxacin concentration was epistatic to all doses of wrwycr with respect to their effect on cell viability (Appendix A). In order to further investigate the combined effects of peptide and norfloxacin, and the basis of the peptide-dependent protective effect, we focused on 0.06 µg/ml norfloxacin in subsequent experiments.

**Peptide wrwycr is synergistic with novobiocin.** We also wanted to evaluate the effects of wrwycr in co-treatment with novobiocin, and used the same methods as employed above to establish a checkerboard MIC table with single treatments and co-treatments with the two compounds.

The MIC value of novobiocin in MG1655 was 512 µg/ml (Table 3). When we combined novobiocin with wrwycr, bacterial growth was inhibited at 16 µg/ml novobiocin and 8 µM peptide, representing a 32-fold drop in the MIC of novobiocin and a 8-fold drop in
Figure 1. Viability analysis of *E. coli* MG1655 treated with single and co-treatments. Cells were treated with DMSO, wwywr at 8 µM (A), 16 µM (B), or 32 µM (C), with or without norfloxacin at 0.06 µg/ml, for a total period of 24 h. Aliquots were taken at t= 0 h, 1.5 h, 3 h, 6 h, 12 h and 24 h, serial diluted and spot plated onto LB plates for colony counting. Error bars represent the standard error of the mean (n=9).
Table 3. Growth of *E. coli* MG1655 Cells Treated with wrwycr and/or Novobiocin

<table>
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<tr>
<th>NVB (µg/ml)</th>
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<th>8</th>
<th>16</th>
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the MIC of peptide wrwycr. The same inhibition was attained with 8 µg/ml novobiocin and 16 µM wrwycr. Both results show a synergistic effect of peptide wrwycr and novobiocin when combined in *E. coli* cells.

When we determined viability counts using the same technique as mentioned above (Figure 2), we were able to confirm our results obtained from the MIC assays. Indeed, even at 64 µg/ml novobiocin, MG1655 cells were as viable as untreated cells after 24 h of treatment, even though their viability decreased slightly for the first 3 h. Peptide wrwycr alone at 16 µM, as observed in Figure 1 (p. 13), affected viability only during the first 6 h, while at 8 µM, it did not decrease viable counts overall. When cells are co-treated with 8 µM wrwycr and 8 µg/ml or 16 µg/ml novobiocin, we observed a significant decrease in viability, although cells recovered from these treatments by 24 h (Figure 2A). However, co-treatment with 8 µM wrwycr and 32 µg/ml or 64 µg/ml novobiocin led to a severe viability defect, with a ~ 5 log drop in viable counts after 6 h of treatment. Importantly, after 24 h, viability was still 3 log lower than untreated cells when co-treated with 32 µg/ml novobiocin, while essentially no cells were recovered with co-treatment using 64 µg/ml novobiocin. Co-treatments using 16 µM wrwycr were synergistic for all concentrations of novobiocin used (Figure 2B). With 16 µg/ml, 32 µg/ml and 64 µg/ml novobiocin, we saw almost complete killing of the co-treated cultures by 24 h.
Figure 2. Viability analysis of *E. coli* MG1655 treated with single and co-treatments of wrwyrcr and novobiocin (NVB). Cells were treated with wrwyrcr at 8 µM (A) or 16 µM (B) with or without novobiocin for 24 h. Aliquots were taken at t= 0 h, 1.5 h, 3 h, 6 h, 12 h and 24 h, serial diluted and spot plated onto LB plates for colony counting. Error bars represent the standard error of the mean (n=6).

Both MICs and viability results showed a clear synergistic effect between wrwyrcr and novobiocin, thus distinct when compared to peptide in co-treatments with norfloxacin. With the goal of understanding further the mechanism behind such difference, we started by identifying further the “protective” effect seen during co-treatments with norfloxacin.
PEPTIDE WRWYCR AT LOW CONCENTRATIONS PROTECTS *E. coli* FROM NORFLOXACIN-INDUCED DNA DAMAGE

Less filamentation is observed in co-treatments with wrwycr and norfloxacin. Independently, both wrwycr and norfloxacin cause accumulation of DNA damage in bacteria (Pohlhaus and Kreuzer, 2005; Gunderson and Segall, 2006; Dwyer *et al.*, 2007). If *E. coli* cells treated with both norfloxacin and 8 µM or 16 µM wrwycr recover more than in the presence of norfloxacin alone, they may have suffered less DNA damage (or repaired the DNA damage more efficiently) than with norfloxacin alone. Similarly, cells treated with both norfloxacin and 32 µM peptide wrwycr may have been overwhelmed by the amount of DNA breaks. Therefore we tested the extent of DNA damage present in *E. coli* after each treatment.

Unrepaired DNA damage frequently gives rise to single-stranded (ss) DNA, which is a substrate for RecA binding and a precursor for the SOS response (Radman, 1975). The binding of RecA to ssDNA allows RecA to induce the autocleavage of the SOS regulon repressor LexA, and to subsequently launch the SOS response (Little and Mount, 1982; Walker, 1996). A common feature of a prolonged SOS response is the filamentation of bacterial cells due to the inhibition of the FtsZ ring formation by the SOS-induced SulA protein (Huisman *et al.*, 1984; Hill *et al.*, 1997). We treated MG1655 cells for 3 h with norfloxacin at 0.06 µg/ml and/or wrwycr at 8 µM, 16 µM or 32 µM, and stained them with FM4-64, a lipophilic dye for membranes, and the DNA stain DAPI. Aliquots of each sample were analyzed by epifluorescence microscopy (Figure 3). An end point of 3 h was initially chosen for this experiment as it is the earliest time point, based on the viability assay results, at which we observed the different phenotypes of the co-treatments. Compared to the DMSO-treated sample, most cells appeared to be ~50% longer when treated with 8 µM wrwycr (3.21 ± 0.33 µm versus 2.13 ± 0.05 µm for untreated cells, Figure 3B). Cells treated with norfloxacin at 0.06 µg/ml typically filamented to lengths over 10X their normal size (33.35 ± 1.92 µm) compared to untreated cells, consistent with inhibition of cell division by SulA. Co-treatment with 8 µM peptide and norfloxacin resulted both in fewer filamenting cells, and in filaments that were shorter (7.93 ± 1.1 µm) when compared to cells treated with norfloxacin alone. A similar effect was seen when cells were treated with 16 µM wrwycr, with or without norfloxacin: in the co-treated sample, fewer filaments were observed than
Figure 3. Microscopy of *E. coli* MG1655 cultures treated for 3 h with wrwycr at 8 µM, 16 µM or 32 µM, norfloxacin at 0.06 µg/ml, or both treatments, compared to DMSO. Cells were stained with FM4-64 (red) and DAPI (blue). (A) Representative fields from each treatment at 100X. Samples treated with wrwycr at 32 µM were concentrated to get a significant number of cells in each field. All panels have the same scale. (B) Average cell length measured for each treatment, with error bars representing the standard error of the mean (n=50).

with norfloxacin alone (average length of 5.08 ± 0.38 µm with wrwycr alone and 10.44 ± 0.96 µm in the co-treatment).

Cells treated with 32 µM wrwycr were mostly not filamentous, and when cells were treated with both norfloxacin and 32 µM wrwycr the filamentation caused by norfloxacin alone was almost completely inhibited (average length decreased to only 3.94 ± 0.08 µm). This absence of filamentation with at 32 µM peptide might be due to its effects on the membrane, a topic we will discuss later in the results’ section.
To measure the SOS response, we used a derivative of MG1655 containing a sulA-p-mCherry fusion, a construct in which the mCherry gene is transcribed by the promoter of the sulA gene. Cells were then treated with the same concentrations of peptide and/or norfloxacin as above, and the mCherry activity was quantified using flow cytometry (Figure 4). After 3 h, treatment with 8 µM or 16 µM peptide resulted in only 2 or 4-fold induction of mCherry, respectively, in fewer than ~10% cells on average. Compared to treatment with norfloxacin alone, which induced the SOS reporter 10 fold in ~26% of cells, co-treatments with 8 µM or 16 µM wrwycr and norfloxacin led to a modest decrease both in the number of mCherry+ cells (19% and 15%, respectively) and in the mean fluorescence per cell (Figure 4A). This trend was even more pronounced when the fluorescence was measured after 6 h of treatment (Figure 4B) where 10% of cells were positive if co-treated with wrwycr at 8 µM or 16 µM, respectively. The mean fluorescence values corroborated the “protective effect” of low peptide doses (Figure 4B). Treatment with 32 µM wrwycr on its own caused greater than a 20-fold SOS induction in ~50% cells, and 45% cells on average were SOS+ when treated for either 3 h or 6 h with norfloxacin and the peptide together (Figure 4). As seen earlier, 32 µM peptide conferred the dominant effect at either 3 h or 6 h (Figure 1C, p. 13; Figure 3A, p. 17).

Figure 4. Quantification of the SOS response of cells treated with peptide wrwycr and/or norfloxacin. The SOS induction was measured by the expression of mCherry after 3 h (A) and 6 h (B) of treatment of E. coli EDT1754 (MG1655 sulA-p-mCherry) with DMSO, wrwycr and/or norfloxacin at indicated concentrations. mCherry fluorescence was quantified by flow cytometry. Error bars represent the standard error of the mean (n=6).
Fewer DNA breaks are formed in co-treatments with wrwycr and norfloxacin. In light of these results, we quantified the DNA damage generated by these single and co-treatments. We first evaluated the extent of DNA breaks using a TUNEL assay, which fluorescently labels free 3′ hydroxyl DNA ends with Terminal Deoxynucleotidyl Transferase (TdT). We treated MG1655 cells for 3 h, and samples were processed with the TUNEL reagents and counterstained with DAPI for total DNA content, then analyzed by flow cytometry. Figure 5A shows dot plots obtained from one representative experiment, while the results of 3 experiments and 9 independent samples were graphed in Figure 5B. The TUNEL+ cells suffered DNA damage, either ss or ds DNA breaks. Treatment of MG1655 cells with increasing concentrations of wrwycr revealed a dose-dependent but non-linear increase of TUNEL+ cells (Figure 5A, 5B). Either treatment with 8 µM wrwycr was not enough to induce DNA breaks in a significant fraction of cells, or the DNA breaks had already been repaired in the majority of cells. The mean TUNEL fluorescence per cell clearly showed a dose-dependent increase of free 3′OH ends in peptide-treated cells (Figure 5A-C). At 3 h, 30.8% of cells on average with norfloxacin had DNA breaks (Figure 5B). The combination of norfloxacin with 8 µM wrwycr showed a greater than 3-fold decrease in the fraction of TUNEL+ cells compared to norfloxacin alone (Figure 5A, 5B). Treating cells with the combination of norfloxacin and 16 µM or 32 µM wrwycr at 3 h did not significantly increase the population of TUNEL+ cells compared to treatment with peptide alone (Figure 5B and 5C). Thus peptide-dependent damage at the higher concentrations was epistatic to norfloxacin-dependent damage.

To further investigate the trend towards fewer SOS-induced cells at longer time points, we performed the TUNEL experiment after 12 h of treatment. The results confirmed that, after 12 h, cells completely recovered from treatments with 8 µM or 16 µM peptide, while still experiencing DNA damage with 32 µM wrwycr treatment (Figure 5B and 5C). In contrast, treatment with norfloxacin alone resulted in ~50% increase in cells with DNA damage at 12 h compared to 3 h. Treatment with both norfloxacin and 8 µM or 16 µM wrwycr showed a reduced number of TUNEL+ cells compared to norfloxacin alone, while treatment with norfloxacin and 32 µM wrwycr resulted in about the same number of damaged cells as with the peptide alone. These results agreed with those of the viability assays (Figure 1, p. 13).
Figure 5. TUNEL analysis of E. coli MG1655 treated with single and co-treatments. Cells were treated for 3 h with DMSO, wrwycr at 8 µM, 16 µM, or 32 µM, with or without norfloxacin at 0.06 µg/ml, before being fixed and permeabilized. ss and ds DNA breaks were labeled with fluorescein-dUTP using TdT and cells were counterstained using DAPI for DNA content. Samples were resuspended in PBS and quantified by flow cytometry. (A) Example of dot plots. The X-axis displays the amount of DAPI fluorescence (Pacific-blue channel), and the Y-axis displays the amount of TUNEL fluorescence (FITC channel); each dot represents a cell. In all plots, populations are colored as follows: purple for Q1 (TUNEL single positive events), green for Q2 (double positive events), red for Q3 (unstained events), and blue for Q4 (DAPI single positive events). For each plot, the average % TUNEL and DAPI double positive events are shown, as well as the average mean fluorescence. (B) Graph of the percentage of TUNEL and DAPI double positive events for each treatment after 3 h. (C) Graph of the mean TUNEL fluorescence for each treatment after 12 h. For both (B) and (C), error bars represent the standard error of the mean (n=9). (D) Histogram of Forward Scatter (FSC) values obtained from representative samples. The X-axis displays the FSC of each sample, and the Y-axis represents the number of events. Overlay of samples was done using the FlowJo software.
The forward scatter (FSC) of light reflects cell size and was used to monitor filamentation due to wrwycr and/or norfloxacin treatment in the flow cytometry experiments. In agreement with the microscopy results (Figure 3, p. 17), we saw a large shift in the size of cells treated with norfloxacin at 0.06 µg/ml (Figure 5D, pp. 20-21). The three histograms also showed an increase in the size of cells treated with wrwycr at 16 µM and 32 µM, respectively. At 32 µM wrwycr the size increase reflected by FSC was higher than that observed by microscopy; this could be explained by the fact that flow cytometry allowed us to analyze many more cells, and to specifically discard any “events” that are debris rather than cells based on the gating criteria. For all peptide concentrations, co-treated cells were shorter than cells treated with norfloxacin alone, as previously shown by microscopy. In the case of cells co-treated with either 16 µM or 32 µM wrwycr and norfloxacin, FSC was the same as that of cells treated only with peptide. As was the case for accumulation of DNA damage, the peptide appeared in some way to antagonize or reverse norfloxacin’s effect on cell filamentation (Figure 3A, 3B, p. 17).

We tested whether the DNA damage occurring with peptide and/or norfloxacin treatment included DSB, using Pulsed Field Gel Electrophoresis (PFGE). PFGE separates intact circular DNA and large branched DNA repair intermediates, which stay in the wells of the agarose gels, from linear DNA fragments, which migrate into the gel and appear as smears. We treated MG1655 cells for 3 h with increasing concentrations of peptide or norfloxacin, as well as with combinations of the two. As seen from a representative gel (Figure 6A), treatment with either wrwycr or norfloxacin led to a dose-dependent loss of intact DNA from the wells and the accumulation of smaller fragments that migrated into the gel. At high concentrations of the peptide, little or no DNA was seen either in the wells or in the gel, suggesting that the treatments resulted in degradation of the DNA into fragments too small to remain in the gel. To test this hypothesis, we embedded agarose plugs containing cells from the same experiment into a 1% agarose gel, to observe short DNA fragments. Fragments of around 500-1000 bp could be seen for some treatments, such as the DMSO-treated cells and cells treated with 0.06 µg/ml norfloxacin (Figure 6B). However, we recovered few if any visible fragments from samples treated with 32 µM or 64 µM peptide. This observation suggested that fragments generated by DNA damage due to peptide and/or
Figure 6. PFGE analysis of *E. coli* MG1655 treated with peptide wrwycr and/or norfloxacin. Cells were treated for 3 h with DMSO, wrwycr at 8 µM, 16 µM, or 32 µM, with or without norfloxacin at 0.06 µg/ml. (A) Example of a pulsed field gel scanned after a 22 h run at 14°C. The gel was stained with SYBR Green I. Treatments are indicated at the top of the gel: MW, molecular weight; NFX, norfloxacin; Y, yeast chromosome ladder; λ, lambda concatemer ladder (the lowest band is the 48.5kb unit-sized lambda genome); D, DMSO-treated sample. (B) Same experiment as above, and run in 1% agarose gel using unidirectional electrophoresis for 90 minutes at 100V. The ladder used is Hyperladder I. The gel was stained using ethyldium bromide. (C) Histogram of the values of each defined DNA pools recovered from the gel, normalized to the total DNA present in each plug before the electrophoresis run. Quantification was done using the ImageJ software.
norfloxacin treatment were degraded to fragments too short to be retained even on a 1% agarose gel.

To determine the extent of fragmentation and degradation generated by each treatment (a work done by Rena T. Hiedo with the help of Dr. Peter Salamon at SDSU), we quantified the total amount of DNA present in each plug before performing the electrophoresis. We divided the DNA visible in the pulsed field gels into two pools: the DNA in the wells, and the fragmented DNA seen as smears. The DNA “missing” from the total DNA was considered to be “degraded DNA” and calculated as the remaining third pool. The volumes of each pool were normalized to the total DNA and graphed (Figure 6C, pp. 23-24). Note, however, that this protocol is only approximate and undercounts the DNA in the wells because in the samples with relatively low DNA fragmentation, the well signal is saturated. As indicated by the PFGE scan, more of the DNA is fragmented and degraded with increasing concentrations of either wrwycr or norfloxacin alone. When cells were co-treated with norfloxacin and wrwycr, no significant difference was seen in the extent of DNA fragmentation compared with norfloxacin treatment alone, whether at 0.06 µg/ml or 0.125 µg/ml of the fluoroquinolone. At 32 µM wrwycr, the very high levels of fragmentation and degradation of the DNA depleted almost all the DNA from both the wells and the lanes. Taken together, these results confirmed that treatment of MG1655 cells with wrwycr, like with norfloxacin, significantly damaged DNA and created DSB. Co-treatment of cells at 8 µM or 16 µM of wrwycr and norfloxacin resulted in approximately the same degree of DNA fragmentation as treatment with norfloxacin alone, while at 32 µM the peptide had the dominant effect.

Degradation of the chromosome should lead to an increased number of anucleate cells. Cells treated as above were stained with both FM4-64 and DAPI. We quantified the fluorescence by flow cytometry and counted events positive for FM4-64 but negative for DAPI as anucleate cells. As expected from previously published results (Gunderson and Segall, 2006), we observed a dose-dependent increase in the number of anucleate cells in populations treated with wrwycr (Figure 7). The low dose of norfloxacin alone (0.06 µg/ml) did not greatly increase the fraction of anucleate cells (0.22% ± 0.1%). Co-treatment of cells with norfloxacin and wrwycr led to similar numbers of anucleate cells as with the peptide alone. The high levels of anucleate cell formation seen at 32 µM wrwycr, whether in single
Figure 7. Percentage of anucleate cells in MG1655 after treatment with wrwycr and/or norfloxacin. Cells were treated for 3 h with DMSO, wrwycr at 8 µM or 16 µM, with or without norfloxacin at 0.06 µg/ml, and stained with DAPI (25 µg/ml) and FM4-64 (10 µg/ml). Quantification was done through fluorescence analysis of FM4-64⁺-DAPI⁻ cells by flow cytometry. Error bars represent the standard error of the mean (n = 9).

or co-treatments, agreed with the results obtained with the PFGE: cells experiencing a high level of DNA degradation indeed probably become anucleate cells.

**PULSED FIELD GEL ELECTROPHORESIS REVEALS SYNERGISTIC DNA FRAGMENTATION IN CO-TREATMENTS WITH WRWYCR AND NOVOBIOCIN**

Our results with co-treatments between wrwycr and norfloxacin led us to investigate whether those with wrwycr and novobiocin would yield greater than additive DNA damage, based on the viability counts obtained for these particular treatment combinations (Figure 2, p. 15). Moreover, we were interested in looking at the effect of novobiocin, as a single treatment, on the formation of DNA breaks in *E. coli*. Indeed, as mentioned earlier, it was recently discovered that gyrase could perform an ATP-independent negative supercoiling relaxation (Nollmann, Stone, *et al.*, 2007). This finding raises the interesting question of whether gyrase would still be able to function in the presence of novobiocin, and thus whether a treatment with novobiocin would lead to the formation of DSB. To answer these questions, we looked directly at the effect of co-treatments via PFGE. MG1655 cells were
treated for 3 h with increasing concentrations of peptide and/or novobiocin versus DMSO, samples were processed and the plugs were run for 22 h. We indeed observed the generation of DSB upon novobiocin treatment (Figure 8A and 8B); these DSB only appeared at high concentrations of novobiocin, from 128 µg/ml to 512 µg/ml, the latter being the MIC of novobiocin (Table 1, p. 6). Because the DNA signal in the wells does not vary linearly, we quantified only the extent of DNA fragmentation by calculating the DNA signal intensity throughout each lane (based on the average of 5 different “readouts” spread along the width of the lane), and reported our results relative to the length of DNA fragments obtained from the two DNA ladders used (a work done by Sarah Rafo with the help of Dr. Peter Salamon and Victor Seguritan at SDSU; Figure 8B). Peptide wrwycr at 8 µM and 16 µM did not lead to any DNA fragmentation, nor did novobiocin at 8 µg/ml, 16 µg/ml and 32 µg/ml. However, these latter doses of novobiocin in co-treatments with 8 µM peptide did cause DNA fragmentation, particularly visible at 8 µM wrwycr and 8 µg/ml novobiocin with a peak of DNA of high intensity towards the bottom of the lane (Figure 8A and 8B). With 16 µM wrwycr, co-treatment with low concentrations of novobiocin did not result in any visible fragmentation (Figure 8A, Appendix B) which, based on results from the viability assay (Figure 2, p. 15), can probably be attributed to cell death, rather than a lack of DNA damage.

In summary, performing this PFGE was successful in showing not only a novobiocin-dependent fragmentation of the DNA, but also an accumulation of small fragments in the presence of concentrations of peptide and novobiocin that do not generate DSB by themselves.

Thus, these results corroborate those from our viability assay (Figure 2, p. 15), and provide further evidence of a synergistic effect between wrwycr and novobiocin.

EFFECT OF WRWYCR CO-TREATMENTS ON EFFLUX

Sensitivity of MG1655 ΔtolC with wrwycr and norfloxacin. In general, our results showed that co-treating cells with 8 µM or 16 µM wrwycr and 0.06 µg/ml norfloxacin resulted in greater recovery than treatment with norfloxacin alone (Figure 1A, 1B). The cells treated with both norfloxacin and wrwycr experienced less DNA damage and formed fewer filamenting cells as well as shorter filaments. Hence, we investigated the basis of wrwycr’s observed protection of cells’ viability from the effects of norfloxacin. One possibility we
Figure 8. PFGE analysis of *E. coli* MG1655 treated with peptide wrwyrc and/or novobiocin. Cells were treated for 3 h with DMSO, wrwyrc at 8 µM or 16 µM, with or without several concentrations of novobiocin. (A) Scan of the PFGE after a 22 h run at 14°C. The gel was stained with SYBR Green I. Treatments are indicated at the top of the gel: MW, molecular weight; NVB, novobiocin; Y, yeast chromosome ladder; λ, lambda concatemer ladder (the lowest band is the 48.5kb unit-sized lambda genome); D, DMSO-treated sample. Two plugs from the same treatment with 16 µM wrwyrc were run in this gel. (B) Quantification of the DNA signal intensity from representative single and co-treatments relative to the fragment size in kilobases. The quantification was done using the ImageJ software.
considered was that the peptide induced greater expression of an efflux pump that in co-
treatments also reduced the intracellular concentration of norfloxacin, thereby conferring the
observed protective effect. In Enterobacteria, the AcrAB-TolC tripartite efflux system is a
key component in the emergence of efflux-mediated multi-drug resistant (MDR) bacteria to a
vast array of antibiotics, including fluoroquinolones (Piddock, 1999; Hooper, 2001). To
address the possibility that TolC may be involved in the observed phenotypes, we performed
viability assays with a MG1655 ΔtolC mutant (EDT 1350).

We tested the effect of single and combination treatments with various concentrations
of peptide and norfloxacin, by performing both an MIC assay and a time course viability
assay on EDT1350. We observed a striking decrease in the MICs for wrwycr and norfloxacin
with EDT 1350 after comparison with wild type MG1655 (Table 4). We found that 16 µM
wrwycr completely inhibited growth in TolC− cells. The MIC of norfloxacin decreased 4-fold,
coming down to 0.03 µg/ml in the tolC deletion mutant; such decrease was previously
reported by other researchers (Sulavik et al., 2001). As expected from these results, the MIC
in the case of co-treatments also decreased significantly, and was attained with 2 µM wrwycr
and 0.03 µg/ml norfloxacin, compared to 16 µM wrwycr and 0.06 µg/ml norfloxacin in
MG1655.

Table 4. MIC Results Recorded for MG1655 and MG1655 ΔtolC After 20 h with
Peptide, Norfloxacin and Novobiocin

<table>
<thead>
<tr>
<th></th>
<th>wrwycr (µM)</th>
<th>NVB (µg/ml)</th>
<th>wrwycr + NVB (µM + µg/ml)</th>
<th>NFX (µg/ml)</th>
<th>wrwycr + NFX (µM + µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>G652</td>
<td>32-64</td>
<td>512</td>
<td>8 + 16</td>
<td>0.25</td>
<td>16 + 0.06</td>
</tr>
<tr>
<td>EDT 1350</td>
<td>16</td>
<td>16</td>
<td>2 + 4</td>
<td>0.06</td>
<td>2 + 0.03</td>
</tr>
</tbody>
</table>

Values are means obtained from *4 independent experiments with 12 independent replicates, or *2 independent
experiments with 6 independent replicates. NT, not tested in this study.

For our viability assay, EDT1350 cells were treated with peptide and/or norfloxacin
as before, and viability counts were determined at several time points. By comparing the
effect of wrwycr alone in TolC− (Figure 9) and TolC+ (Figure 1, p. 13) cells, we could see a
more pronounced dose-dependent viability defect in TolC− cells. At 3 h of treatment, there
Figure 9. Viability analysis of *E. coli* MG1655 Δ*tolC* treated with wrwyrcr and/or norfloxacin. Cells were treated with various concentrations of wrwyrcr with or without norfloxacin, or DMSO as a control. Aliquots were taken at t= 0 h, 1.5 h, 3 h, 6 h, 12 h and 24 h, serial diluted and spot plated onto LB plates for colony counting. Error bars represent the standard error of the mean (n=3).

already was a greater than 3-fold difference between TolC− cells and TolC+ cells treated with 16 µM wrwyrcr, and the former did not recover from this treatment after 24 h of treatment.

When we looked at co-treatment with norfloxacin at 0.03 µg/ml and several concentrations of peptide, the results agreed with those of the MICs (Figure 9). MG1655 Δ*tolC* cells treated with 0.03 µg/ml norfloxacin and 2 µM peptide did suffer from a viability defect, although we did still recover few viable cells by 24 h. However, co-treatment with 0.03 µg/ml and 4 µM or 8 µM or 16 µM led to complete killing of the culture, unlike in MG1655, evidencing the sensibility of the mutant compared to the wild type.

These results suggested that TolC indeed may contribute to the observed protection from norfloxacin in co-treated cells; we next determined how the *tolC* deletion affected the accumulation of DNA damage. In principle, if our hypothesis holds true, co-treatments with low concentrations of wrwyrcr and norfloxacin should provoke a greater accumulation of
DNA damage compared to a single treatment of norfloxacin, rather than a reduction, as seen with wild type MG1655.

**Analysis of DNA damage in MG1655 ΔtolC.** We tested whether the TolC- cells experienced greater DNA damage by comparing SOS inductions in the TolC+ and TolC- cells using the *sulA::mCherry* fusion (Table 5). Indeed, about 5 times more TolC- cells induced the SOS response than TolC+ cells after treatment with wrwycr at 8 µM and 16 µM. Surprisingly, the TolC- mutant treated with norfloxacin at 0.06 µg/ml had fewer mCherry+ cells, with a lower mean fluorescence, than the wild type; this might be due to more DNA repair. Both TolC+ and TolC- cultures co-treated with norfloxacin and wrwycr at 8 µM or 16 µM had fewer mCherry+ cells than those treated with norfloxacin alone. The viability assay results at both 6 h and 12 h (Figure 9, p. 30; Appendix C) suggested that the reduced mCherry activity did not reflect the same protective effect seen with TolC+ cells but was rather due to rapid death of TolC- cells due to their inability to efflux norfloxacin and peptide wrwycr.

**Table 5. Induction of the SOS Response by wrwycr and/or Norfloxacin After 6 h Treatment of EDT1754 (MG1655) or EDT2117 (MG1655 ΔtolC) Cells**

<table>
<thead>
<tr>
<th>wrwycr (µM)</th>
<th>0</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>wt</td>
<td>ΔtolC</td>
</tr>
<tr>
<td>NFX (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.15±0.0a (100±8)</td>
<td>0.1±0.0 (60±1)</td>
</tr>
<tr>
<td>0.06</td>
<td>40.4±12.2 (1825±542)</td>
<td>24.3±1.3 (845±24)</td>
</tr>
<tr>
<td>16</td>
<td>17.4±2.8 (871±134)</td>
<td>19.0±1.4 (890±87)</td>
</tr>
<tr>
<td>32</td>
<td>49.7±4.6 (2300±228)</td>
<td>44.9±4.0 (2133±166)</td>
</tr>
</tbody>
</table>

*The top values are averages of mCherry+ cells. The values in parentheses are the mean mCherry fluorescence (n=6).*

Because we expected the TolC- cells to experience more DNA breaks than the TolC+ cells, we performed a TUNEL assay on EDT1350 cells treated for 3 h with wrwycr and/or...
norfloxacin. For comparison, we also included the TUNEL results obtained with wild type MG1655 cells (Figure 10). Similarly to what we observed in the SOS induction assay (Table 5, p. 31), MG1655 ΔtolC cells treated with 8 µM wrwyrcr experienced a greater than 3-fold increase in the number of TUNEL+ cells compared to MG1655 (23.4 % ± 6.9 % compared to 5.83% ± 1.57%), suggesting again that the absence of TolC might lead to a higher accumulation of peptide inside the cells. Treatment with norfloxacin at 0.06 µg/ml did not change significantly the number TUNEL+ cells in the mutant compared to the wild type (23.9 % ± 5.2% compared to 30.3% ± 1.5%), while the co-treatments with wrwyrcr at 8 µM and 16 µM gave on average a similar number of cells positive for DNA breaks.

Figure 10. Quantification of TUNEL+ cells in *E. coli* MG1655 ΔtolC compared to MG1655 cells. Cells were treated with wrwyrcr and/or norfloxacin for 3 h. Each sample was fixed and permeabilized before being treated with TUNEL reagents. Cells were counterstained with DAPI and resuspended in PBS before analysis by flow cytometry. Results are presented as the percentage of FITC+ cells from the DAPI+ population.

The fraction of anucleate cells after 3 h of treatment increased about 6-fold when TolC− cells versus TolC+ cells were treated with 8 µM wrwyrcr (Figure 11, data from MG1655 taken from Figure 7, p. 26, for comparison). Presumably, greater accumulation of the peptide in TolC− cells causes significant DNA damage and degradation while not disrupting cell division, thereby generating anucleate cells. At 16 µM wrwyrcr, on the other hand, both TolC+ and TolC− cells accumulated a roughly equivalent fraction of anucleate cells after 3 h (2.59% ± 0.35% vs. 3.17% ± 0.5%, respectively). The higher fractions of
Figure 11. Percentage of anucleate cells in MG1655 and MG1655 ΔtolC after treatment with wrwycr and/or norfloxacin. Cells were treated for 3 h with DMSO, wrwycr at 8 µM or 16 µM, with or without norfloxacin at 0.06 µg/ml, and stained with DAPI (25 µg/ml) and FM4-64 (10 µg/ml). Quantification was done through fluorescence analysis of FM4-64⁺-DAPI⁻ cells by flow cytometry. Error bars represent the standard error of the mean (n = 3).

Anucleate cells observed with 32 µM wrwycr presumably reflect significant DNA degradation in both mutant and wildtype strain. The TolC⁻ strain treated only with 0.06 µg/ml norfloxacin accumulated only about twice as many anucleate cells as the TolC⁺ strain. Co-treatment with norfloxacin and wrwycr led to a similar fraction of anucleate cells as treatment with wrwycr alone in both TolC⁻ and TolC⁺ cells.

Although we did observe some effects of the tolC deletion strain treated with the peptide alone, the results obtained here for the co-treatments were hard to interpret. Because MG1655 ΔtolC cells are much more sensitive than their wild type counterpart with the same treatments (Figure 9, p. 30), the reason a co-treatment with 8 µM wrwycr and 0.06 µg/ml led to a decreased amount of DNA damage may be that cells are dying from the overwhelming damage of both drugs. It would be necessary to investigate the effects of peptide and norfloxacin on DNA damage with co-treatments corresponding to the MIC of MG1655 ΔtolC, that is 2 µM wrwycr and 0.03 µg/ml norfloxacin, rather than the MIC of co-treatments in wild type MG1655.
In a further attempt to determine whether wrwycr, alone or in co-treatments, influences the efflux system of *E. coli* cells, rather than using indirect assays we sought to look for a more practical, direct way to establish a link between drug treatments and TolC.

**Effect of wrwycr co-treatments on induction of tolC.** In view of our viability results on MG1655 Δ*tolC* cells (Figure 9, p. 30), we hypothesized that the norfloxacin protection seen in co-treatments with wrwycr at 8 µM or 16 µM and 0.06 µg/ml norfloxacin was due to a peptide-mediated higher induction of *tolC*. To look for potential *tolC* induction, we constructed a strain of N99 (LacZ') in which a *lacZ* operon was fused to the *tolC* promoter, at the same time deleting *tolC* and providing a direct reporter assay of the expression level of *tolC*. We assayed LacZ activity, based on the conversion of the substrate 4-methyl umbelliferyl β-D-galactoside (MUG) into the fluorophore 4-methylumbelliferone by β-galactosidase; the more fluorescence is seen, the higher the induction of *tolC*. Cells were treated for a period of 24 h; at various time points, the OD$_{600}$ was read and aliquots were taken for measurements of β-galactosidase activity. We analyzed the results relative to the OD$_{600}$ recorded at each time point.

We first tested N99 *tolC::lacZ* cells with peptide wrwyrcr and/or norfloxacin (Figure 11A, p. 33). Peptide wrwyrcr at 8 µM led to a slight *tolC* induction compared to DMSO-treated cells; both transiently induced *tolC* during log phase growth. At 16 µM, we observed a somewhat low induction of *tolC* after 6 h of treatment but higher at later time points, to reach close to a 4-fold higher induction than DMSO-treated cells after 24 h. Norfloxacin at 0.06 µg/ml increased β-galactosidase activity after 24 h similarly to 16 µM wrwyrcr, although over a longer time. This was expected, as fluoroquinolones such as norfloxacin were previously reported to induce the expression of *tolC* (Coldham *et al.*, 2006). When cells were co-treated with 8 µM wrwyrcr and 0.06 µg/ml norfloxacin, *tolC* induction was higher than either of the two treatments at 12 h and 24 h, and it was higher than the addition of each drug independently at 12 h. Co-treatment with 16 µM wrwyrcr and norfloxacin led to the same induction pattern as with wrwyrcr alone. These results showed that co-treatments with 8 µM wrwyrcr and norfloxacin significantly induce the expression of *tolC*. The rather late induction is surprising, as protection from norfloxacin in co-treatments was seen as early as 3 h post treatment (Figure 1, p. 13). This may reflect the sensitivity limit of the lacZ reporter assay. Also, we might not observe a higher induction with co-treatment
with 16 µM as TolC− cells at these concentrations were not viable. Assaying the induction of tolC should be repeated in TolC+ cells to avoid these consequences.

We wanted to investigate whether this finding could explain the difference of phenotypes seen between the norfloxacin and novobiocin co-treatments. Presumably, co-treated cells are “protected” from norfloxacin because co-treatment with peptide mediates induction of efflux via TolC; we thus considered the possibility that co-treatment with novobiocin does not induce tolC, therefore explaining the synergistic effect seen with novobiocin and peptide. We treated N99 tolC::lacZ cells with wrwycr and novobiocin using the same method. We used 8 µg/ml, 16 µg/ml and 32 µg/ml novobiocin, and are only presenting in this figure the results for the two latter concentrations (Figure 12, Appendix D for other treatment combinations).

Figure 12. Results of the MUG assay of strains (A) EDT 2224 (N99 tolC::lacZ), and (B) EDT 2227 (N99 marA::lacZ) with wrwycr and norfloxacin. Cells were treated with wrwycr at 8 µM or 16 µM, with or without norfloxacin at 0.06 µg/ml for 24 h. At each time point, 10 µl aliquots were taken for β-galactosidase activity determination, and the OD600 of each sample was taken. The MUG activity was normalized to the OD600 at each time point. Error bars represent the standard error of the mean (n=6).
Novobiocin at 16 µg/ml induced tolC expression in a time-dependent manner, but reached only a 2-fold induction after 24 h; at 32 µg/ml, β-galactosidase activity was even lower (Figure 13A). Results from an MIC performed on MG1655 ΔtolC cells showed that the MIC for novobiocin dropped to 16 µg/ml in the tolC mutant (Table 4, p. 29); thus, the low activity recovered with 32 µg/ml is most likely due to cell death. When cells were co-treated with wrwycr, at 8 µM or 16 µM, and novobiocin, at 16 µg/ml or 32 µg/ml, induction of tolC was lower than for DMSO-treated cells, during the entire length of the experiment. Considering the very low OD₆₀₀ recorded under any of these co-treatments (data not shown), these results probably reflect cell killing.

Because these results render a comparison between norfloxacin and novobiocin not achievable, we decided to use the same technique, placing this time the lacZ operon under the marA promoter.

**Effect of wrwycr co-treatments on induction of marA.** MarA is known to be a key transcription regulator in the marA/soxS/rob operon. This operon comprises more than 40 genes, including tolC, and is involved in multi-drug resistance to antibiotics and xenobiotics in Gram- bacteria. Induction of marA, often seen during treatment with antibiotics, is most likely accompanied by an induction of TolC via the binding of MarA to its promoters (Zhang et al., 2008). Using such a reporter strain would give us a good indication of an induction of tolC (though in the absence of MarA) while still being in a TolC+ background. We used co-treatments with both norfloxacin and novobiocin and recorded the β-galactosidase activity.

The results did not entirely match our hypothesis (Figure 12B, p. 35). After 24 h of treatment, 8 µM wrwycr and 0.06 µg/ml norfloxacin induced marA 8-fold more than DMSO treatment (52335 ± 641 compared to 6460 ± 354), this induction is also nearly 2-fold higher than the combined induction due to wrwycr and norfloxacin alone (7641 ± 155 and 22160 ± 1034, respectively). A more modest but still significant marA induction was recorded in co-treated cells with 16µM wrwycr and 0.06 µg/ml norfloxacin. Co-treatments with novobiocin and peptide also induced marA, especially at 8 µM wrwycr (Figure 13B). After 24 h of treatment, with 8 µM wrwycr and 16 µg/ml novobiocin, the overall induction was slightly higher than the addition of β-galactosidase activities from both separate treatments.

As co-treatments with peptide and novobiocin significantly induced marA, it is very likely that AcrAB or TolC were also induced with these co-treatments; this should be
Figure 13. Results of the MUG assay of strains (A) EDT 2224 (N99 tolC::lacZ), and (B) EDT 2227 (N99 marA::lacZ) with wrwycr and norfloxacin. Cells were treated with wrwycr at 8 µM or 16 µM, with or without novobiocin at 16 µg/ml or 32 µg/ml for 24 h. At each time point, 10 µl aliquots were taken for β-galactosidase activity determination, and the OD_{600} of each sample was taken. The MUG activity was normalized to the OD_{600} at each time point. Error bars represent the standard error of the mean (n=6).
confirmed by repeating these MUG assays and looking directly for tolC induction in a TolC+ background. Therefore, despite a clear induction of marA and tolC with wrwycr and norfloxacin that might explain the observed “protection” from norfloxacin, this induction is not sufficient to account for the difference between co-treatments with norfloxacin and those with novobiocin. We thus wished to further investigate the mechanism behind such difference.

**Effect of Peptide wrwycr on Membrane and Cell Permeabilization: Consequence on Novobiocin Co-Treatment**

**Peptide wrwycr’s affects E. coli’s membrane.** From various experiments in our lab, we learned that peptide wrwycr was not only targeting HJ but also affecting the membrane integrity of E. coli cells. Indeed, as mentioned earlier, we found the peptide to induce, even at lower concentrations like 8 µM, several components of the envelope stress response (ESR) in treated E. coli, with 32 µM inducing the σE, which regulates the major envelope stress response, more than 7-fold. Additionally, wrwycr at high concentrations (32 µM) led to an immediate loss of E. coli intracellular potassium, which is tightly linked to the loss of turgor pressure (Rostron, 2011; Naili *et al.*, unpublished results).

We analyzed peptide-treated bacteria by microscopy. We treated MG1655 cells for 3 h with increasing concentrations of wrwycr or DMSO, and looked at cells using Differential Interference Contrast (DIC) (Figure 14). Compared to DMSO-treated cells, cells treated with 32 µM wrwycr had a striking phenotype: they appeared corrugated, uneven and “deflated”, and many cells formed aggregates. Although such obvious features were not as visible at lower concentrations of the peptide, they were also noticeable in some cells. It is however at 32 µM that we observed the most significant changes in membrane integrity, whether it was potassium leakage, or induction of the envelope stress response.

These findings prompted us to investigate whether this peptide-mediated disruption of E. coli membrane integrity participated in the observed synergy with novobiocin.

**Peptide wrwycr may increase novobiocin intracellular accumulation and efficacy.** We hypothesized that the peptide affected, directly or indirectly, the selective permeability of E. coli cells to molecules, hence increasing novobiocin’s availability inside cells and decreasing its MIC. Indeed, novobiocin and other coumarins have a high MIC for
Gram-negative bacteria due to their very hydrophobic nature, high molecular weight and consequent low diffusion rate (Nikaido and Vaara, 1985). To test our hypothesis, we performed an MIC assays with MG1655 treated with novobiocin added to media (MHB) with either DMSO, or with 0.2 mM or 1 mM EDTA. EDTA is known to increase the permeability of E. coli cells by chelating Mg\(^{2+}\) and reducing the effectiveness of the LPS barrier (Leive, 1965; Nikaido, 2003); moreover, it was shown to enhance the efficiency of novobiocin in E. coli (Cleeland et al., 1970). In our experiment, EDTA decreased the MIC of novobiocin as much as 16-fold, from 512 µg/ml to 32 µg/ml (Table 6). This result might suggest that the presence of wrwycr in co-treatments decreased the MIC of novobiocin via two different paths; first, by permeabilizing the membrane and allowing novobiocin to easily traverse the membrane, second, by acting synergistically in the context of generation of DNA damage. Importantly, no decrease in the MIC of norfloxacin was observed when the same experiment was performed, indicating that norfloxacin would not benefit from wrwycr-mediated membrane permeabilization, and fits with the lack of synergy observed between norfloxacin and 8 µM or 16 µM wrwycr.
Table 6. Effect of wrwycr Compared to EDTA on the MIC Values of Novobiocin and Norfloxacin in *E. coli* MG1655

<table>
<thead>
<tr>
<th>Condition</th>
<th>wrwycr&lt;sup&gt;a&lt;/sup&gt; (µM)</th>
<th>NVB&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>wrwycr + NVB&lt;sup&gt;b&lt;/sup&gt; (µM + µg/ml)</th>
<th>NFX&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>wrwycr + NFX&lt;sup&gt;a&lt;/sup&gt; (µM + µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHB + DMSO, 0.64%</td>
<td>32</td>
<td>512</td>
<td>8 + 16</td>
<td>0.5</td>
<td>16 + 0.06</td>
</tr>
<tr>
<td>MHB + EDTA, 0.2 mM</td>
<td>NT</td>
<td>256</td>
<td>NT</td>
<td>0.5</td>
<td>NT</td>
</tr>
<tr>
<td>MHB + EDTA, 1 mM</td>
<td>NT</td>
<td>32</td>
<td>NT</td>
<td>0.5</td>
<td>NT</td>
</tr>
</tbody>
</table>

Values are means obtained from <sup>a</sup>4 independent experiments with 12 independent replicates, or <sup>b</sup>2 independent experiments with 6 independent replicates. NT, not tested in this study.
CHAPTER 4

DISCUSSION

Based on previous studies showing that peptide wrwycr is synergistic with several DNA damaging agents (Gunderson and Segall, 2006), we sought to investigate and compare, in this project, the potential synergy of wrwycr in co-treatments with two distinct topoisomerase II inhibitors, norfloxacin and novobiocin. Norfloxacin forms ternary complexes between DNA, gyrase, and itself, causing the collapse of replication forks, generating DNA damage and initiating DNA repair (Chen et al., 1996; Hsu et al., 2006). Our hypothesis was that the peptide would prevent the resolution and completion of DNA repair intermediates initiated at such complexes. Novobiocin acts by blocking gyrase’s ATPase activity, which should prevent DNA gyrase from functioning and halt replication and transcription. In this case, we were expecting wrwycr and novobiocin not to be synergistic, as the lack of ATPase activity would block the resetting of DNA gyrase and subsequent catalytic cycles (Sugino et al., 1978). To verify both our predictions, our strategy was to determine whether combining sublethal doses of norfloxacin or novobiocin with sublethal doses of wrwycr would result in lethality. The answers turned out to be more complex than were predicted: data presented herein revealed not only dose-dependent distinct effects of the peptide with norfloxacin, but also a surprising synergistic effect between wrwycr and novobiocin.

Our results showed differences between the combination of the low concentration of norfloxacin (0.06 µg/ml) and low levels of peptide wrwycr (8 and 16 µM) versus a higher level of peptide (32 µM).

At the lower doses, the peptide conferred a protective effect on cells, reflected in greater viability, fewer filamentous cells and shorter cell length, lower levels of induction of the SOS regulon and, at the 8 µM concentration of peptide, fewer free 3’OH ends that were labeled in the TUNEL assay in co-treated cells compared to cells treated with norfloxacin alone.
At the higher peptide dose, 32 µM, we did observe strong synergy between norfloxacin and peptide. Interestingly, the TUNEL assay revealed that at 3 h, the presence of norfloxacin did not affect the fraction of cells positive for DNA breaks nor the average DNA breakage per cell when treating with 32 µM wrwycr (Figure 5, pp. 20-21). Similarly, at both 3 h and 6 h, wrwycr-only treatment and co-treatment with norfloxacin led to approximately the same number of SOS-induced cells and mCherry mean fluorescence per cell (Figure 4, p. 18). The same observations were made when looking at the formation of anucleate cells with 32 µM wrwycr and/or norfloxacin (Figure 7, p. 26). These results could suggest that the addition of norfloxacin in a treatment with 32 µM wrwycr does not lead to additional DNA breaks. It is possible that at this concentration, the peptide alone has generated so many sites of damage on the chromosome that it would lead to a complete block of replication. We also observed an inhibition, although mild (2 to 3-fold), of transcription of *E. coli* cells with 32 µM wrwycr (data not shown). In any case, norfloxacin would therefore have significantly fewer targets to bind to, as gyrase mainly functions during active replication and transcription. This could also explain why, although peptide at 32 µM induces expression of sulA (Figure 4, p. 18), we recovered very few if any filaments when we treated cells with both peptide at 32 µM and norfloxacin (Figure 3, p. 17); filamentation due to blockage of the FtsZ ring would suggest that treated cells can replicate, which we think is not the case.

The data obtained from our PFGE experiment was harder to fit into our prediction, yet helpful to understand the peptide’s mechanism of action. By itself, wrwycr led to a dose-dependent appearance and shortening of DNA fragments. At 32 µM, we could not see or quantify any DNA in the well or in the lane, nor did we recover small fragments when separating our samples on a 1% agarose gel. Thus high concentrations of peptide treatment result in significant degradation of DNA fragments, perhaps as short as nucleotides. This idea was supported by the fact that we recorded a high percentage of anucleate cells upon treatment of cells with 32 µM wrwycr (Figure 7, p. 26). However, the high percentage of TUNEL+ cells, coupled with the recovery of viable cells, after 3 h of treatment with 32 µM wrwycr is somewhat contradictory to the little, even absent, DNA seen from the PFGE scan. First, it should be noted that the TUNEL assay records individual events (representing individual cells) while the PFGE shows the result of a population of cells. Moreover, the sensitivity of the PFGE may be a limiting factor. It is possible that some of the DNA, intact
or fragmented, is below the threshold of detection of PFGE; thus, we may have only looked at the severe DNA degradation of a subpopulation of cells, in which the peptide may have accumulated in large amounts and resulted in extensive DNA fragmentation. The nearly complete degradation of fragments at high concentrations of the peptide suggests that the RecBCD proteins might be involved in the processing of peptide’s targets. Additionally, because DNA left in the well may be not only intact DNA but also branched DNA repair or replication intermediates that cannot leave the plugs (Nakayama et al., 1994; Garcia and Moss, 2001), the interpretation of the gel becomes difficult, as we could be missing an important class of treatment-induced fragments. This could explain more of the discrepancies between the PFGE and the TUNEL assay results. For example, the assumed intact DNA recovered from cells treated with wrwycr seems to be higher than what we would expect given the significant level of DNA breakage observed with the TUNEL assay. For similar reasons, it is difficult for us to draw conclusions from the co-treatment results. However, as we expected, the co-treatment between wrwycr at 8 µM and norfloxacin at 0.06 µg/ml does generate fewer DNA fragments and larger fragment sizes compared to each individual treatment, corroborating the evidence that co-treatment at these specific concentrations has a protective effect.

To address the question of the role of RecBCD in DNA degradation with our treatments, we have analyzed the effect of increasing concentrations of wrwycr in MG1655 ΔrecB (Appendix E); preliminary results indicate a higher fraction of fragments recovered in the mutant compared to the wild type. Notably, when we ran a shorter version of the gel in a regular 1% agarose, we noticed that treatment of RecB− cells with higher concentrations of the peptide led to a decrease of intact DNA present in the well, but a higher accumulation of large fragments towards the top of the lane, compared to RecB+ cells. This would indicate that RecB− do not experience as much DNA degradation due to the lack of a functional RecBCD, but that these cells have a lower survival rate than RecB+ cells as RecBCD-mediated HR is absent, therefore impairing DNA repair. Further analysis, including through quantification of these results, is needed to validate our hypothesis.

As a mean of comparison to co-treatments with norfloxacin, we also assessed the effect of co-treatments between wrwycr and novobiocin. Contrary to our hypothesis, we saw a synergistic effect in *E. coli* cells treated with wrwycr and novobiocin. The MIC and
viability assays show a severe growth defect in cells co-treated with concentrations of peptide and novobiocin (8 µM and 32 µg/ml, respectively) otherwise ineffective on their own, and results from the PFGE indicate an accumulation of small fragments with these same co-treatments, and even lower. Note that novobiocin alone generated a high amount of DNA fragmentation at high concentrations, a phenomenon that, in our knowledge, has not been reported before in E. coli (although it was seen in the context of eukaryotic cells (Jaxel et al., 1988)). Thus, peptide wrwyrc has very distinct effects with these two topoisomerase inhibitors.

The protective effect of the peptide from norfloxacin is lost in TolC⁻ bacteria, which become more sensitive to all treatments, including to the peptide by itself. This indicates that the peptide is likely to get extruded, at least partly, through an efflux system involving TolC, as was previously suggested in our lab from other experiments (Orchard et al., unpublished results). However, it should be noted that TolC serves a much wider purpose than drug efflux. Indeed, TolC has been shown to be critical to efflux of the siderophore enterobactin and subsequent iron scavenging (Bleuel et al., 2005). Also, it was found that tolC deletion mutants in E. coli experience membrane stress and metabolites depletion (Dhamdhere and Zgurskaya, 2010, Corbalan et al., 2010). Given that wrwyrc causes membrane stress in E. coli as well (Rostron, 2011), the results obtained here may also reflect a synergistic damage by the peptide on the membrane, causing MG1655 ΔtolC cells to be even more sensitive in the presence of wrwyrc.

Low concentrations of peptide wrwyrc, whether in co-treatments with norfloxacin or novobiocin, led to the induction of tolC and marA of higher levels than single treatments. TolC is under the control of the marA/soxS/rob regulon, and induction of the transcriptional activator gene marA, along with marA-induced genes, has been reported upon treatment with various antibiotics and antimicrobial agents, such as tetracycline (Viveiros et al., 2007) and cationic antimicrobial peptides (CAMPs) (Warner and Levy, 2010). Mar mutants recovered after incubation with tetracycline or chloramphenicol were also shown to be much more resistant to fluoroquinolones (Cohen et al., 1989). It is thus plausible that wrwyrc, a cationic hydrophobic peptide, may also affect the expression of MarA-regulated genes by increasing the expression of the AcrAB-tolC pump and thus the active efflux of both norfloxacin and peptide. In agreement with our hypothesis, results from a proteomics analysis obtained from
MG1655 cells treated by wrwycr showed increased protein levels of DamX (Rostron, 2011). The protein DamX has been characterized as a cell division protein in *E. coli* (Gerding *et al.*, 2009; Arends *et al.*, 2010), and *E. coli* damX mutants were recently reported to be bile-sensitive, suggesting a role in membrane integrity (Lopez-Garrido and Casadesus, 2010). Although little is known about its specific function, a recent study showed that damX mutants in *E. coli* had reduced MarA-induced MDR, due to a specific decrease in MarA-induced expression of the *acrA* gene, and therefore reduced activity of the AcrAB-TolC efflux pump system (Ruiz and Levy, 2010). We thus suggest that by increasing the expression of DamX, through a yet unknown mechanism, wrwycr indirectly leads to an increased expression of the AcrAB-TolC pump. Preliminary results show that co-treated MG1655 Δ*damX* cells lose protection from norfloxacin otherwise seen in the wild type (data not shown), corroborating our hypothesis.

Another possibility could account for the peptide’s effect. Because efflux pumps can often be induced by the ESR (Baranova and Nikaido, 2002; Nishino *et al.*, 2010), and wrwycr has been shown to induce the three major branches of the ESR, two questions are raised. First, it is possible that induction of *tolC* could be due to wrwycr-mediated induction of the ESR. Additionally, growth curves performed on MG1655 Δ*acrAB* showed no significant difference compared to MG1655 when treated with peptide or norfloxacin (data not shown), suggesting that these compounds may also get effluxed by means other than the AcrAB-TolC tripartite system, and could potentially participate in the protective effect. We are currently investigating this aspect further, by looking both at other TolC-dependent pumps and at pumps that do not require TolC. Preliminary data with NorE (MdtK) and AcrD in an MG1655 Δ*acrAB* background do not show hypersensitivity of these mutants in the context of co-treatments, while we are still working on other potential pumps involved in the efflux of fluoroquinolones.

Nevertheless, these hypotheses are not sufficient to explain the phenotypic differences seen between peptide co-treatment with norfloxacin and novobiocin. We also observed an induction of *marA* upon co-treatment with novobiocin (although not of *tolC*, which might be explained by a severe viability defect in the mutant), yet these co-treatments are synergistic. We believe that at least part of the answer behind such synergy is explained by the peptide’s effect on the membrane of *E. coli*. We found that novobiocin had an
increased efficiency with peptide treatment similar to one with an EDTA treatment. This suggests that the peptide may permeabilize the cell membrane to such extent that novobiocin would accumulate to higher amounts inside the cells. In another study, novobiocin was previously shown to gain significant efficacy in co-treatments with lactoferrin (Sanchez and Watts, 1999); like EDTA, lactoferrin destabilizes the lipopolysaccharide chains on the surface of Gram negative bacteria (Ellison and Giehl, 1991), therefore increasing cell permeability and facilitating the entry of novobiocin. We would like to confirm a similar ability of the peptide, for example by monitoring the accumulation of novobiocin in the presence of wrwycr, by using High Purification Liquid Chromatography (HPLC), or a fluorescently labeled version of novobiocin.

Other potential causes of the synergistic effect between wrwycr and novobiocin might come from novobiocin’s potential broader effects in the cell. Indeed, although it has not really been investigated in bacteria, work on novobiocin in eukaryotic mitotic cells showed that novobiocin could be a more generalized ATPase inhibitor, as it led to a decrease of the ratio ATP/ADP in treated cells (Downes et al., 1985). We found that wrwycr causes a rapid intracellular potassium loss and interferes with membrane potential (Rostron, 2011). Interestingly, to re-establish their membrane potential cells would need to pump out the accumulated protons from their cytoplasm, which can be done either via ATP hydrolysis or cellular respiration. While others in our lab are working to identify which of these holds true, in the case of compensation by ATP hydrolysis, ATP levels could be reduced by novobiocin. It would be interesting to know whether synergy is also indirectly attained through a depletion of the ATP pool and permanent loss of membrane potential in treated cells. A simple assay would consist in looking at the total ATP levels in the case of single and co-treatments.

It is clear, from our microscopy results, that peptide wrwycr is perturbing the membrane of *E. coli* cells. At 32 µM wrwycr, most cells were aggregating, and their membranes showed a corrugated, rough surface with deep craters (Figure 14, p. 39). Also, at low concentrations of the peptide (8 µM and 16 µM), the uneven FM4-64 staining pattern may reveal some membrane perturbation (data not shown), although the surface of the cells still appeared fairly smooth (Figure 14, p. 39). On occasion, we also noticed the accumulation of the dye in polar regions of treated cells, potentially indicating an opening in
the periplasmic space, and the budding of empty vesicles from membranes (data not shown). Many of these phenotypic observations are commonly found with bacteria treated with Cationic AntiMicrobial Peptides (CAMPs). The mechanism of killing by many CAMPs involves in part the accumulation of the amphipathic peptides in the membrane and, at high concentration, the rupture of the outer and/or inner membrane and leakage of periplasmic and/or cytoplasmic content (da Silva and Teschke, 2003; Yeaman and Yount, 2003; Brogden, 2005; Wu et al., 2010). Regardless of the models proposed for CAMP-induced membrane perturbations, their effect is not linear: CAMPs appear to need a minimum threshold to efficiently perturb the bacterial membrane (Huang, 2006; Melo et al., 2009). Recently, a group studying the effect of two CAMPs in E. coli, PGLa and Gramicidin S, proposed a model suggesting that, at sub-MIC concentration, the peptides barely destabilize the outer membrane and mostly interfere with osmoregulation, but above a threshold concentration severe inner membrane disruption leads to the formation of blisters, and the outer membrane eventually succumbs to turgor pressure and lyses (Hartmann et al., 2010). In line with our results, showing peptide-induced internal potassium leakage, increased membrane permeability, and activation of the envelope stress response, it appears that at high concentration the cationic hydrophobic peptide wrwycr may function similarly to other well-described CAMPs, by accumulating in the lipid bilayer of treated cells, leading to membrane perturbation, osmoregulation defects, and ultimately cell lysis.

In summary, we documented that, at low to moderate concentrations, the synthetic peptide wrwycr protects the cell from norfloxacin, most likely due to increased efflux via a TolC-dependent efflux pump. On the other hand, almost certainly through its effect on both membranes and DNA repair, peptide wrwycr could be used effectively in co-treatments with antimicrobials, like novobiocin, whose efficacy is compromised by their low permeability. The damage of E.coli’s membrane from the peptide brings even more similarities with other well defined membrane-perturbing antimicrobial peptides (Nguyen et al., 2011). Further understanding the mode of action of peptide wrwycr and similar molecules, as well as bacterial responses to these compounds, will be useful for future development of antimicrobial molecules or designing combination therapies between current antimicrobials.
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APPENDIX A

VIABILITY IN MG1655 TREATED WITH WRWYCR AND/OR 0.125 µG/ML NORFLOXACIN
Figure A.1. Viability analysis of *E. coli* MG1655 treated with single and co-treatments. Cells were treated with wrwycr at 8 µM (A), 16 µM (B) or 32 µM (C) with or without norfloxacin at 0.125 µg/ml, versus DMSO for a total period of 24 h. Aliquots were taken at various time points, serial diluted and spot plated onto LB plates for colony counting. Error bars represent the standard error of the mean (n=9).
APPENDIX B

PFGE ANALYSIS FROM MG1655 TREATED WITH WRWYCR AND/OR NOVOBIOCIN
Figure B.1. Quantification of the DNA signal intensity from *E. coli* cells treated with wrwycr at 16 µM and various concentrations of novobiocin, relative to the fragment size in kilobases. The quantification was done using the ImageJ software, from the scanned gel represented in Figure 8, p. 28.
APPENDIX C

VIABILITY OF MG1655 ΔTOLC TREATED WITH WRWYCR AND/OR 0.06 µG/ML NORFLOXACIN
Figure C.1. Viability analysis of *E. coli* MG1655 ΔtolC treated with single and co-treatments. Cells were treated with various concentrations of wrwyrcr with or without norfloxacin at 0.06 µg/ml, or DMSO as a control. Aliquots were taken at t= 0 h, 1.5 h, 3 h, 6 h, 12 h and 24 h, serial diluted and spot plated onto LB plates for colony counting. Error bars represent the standard error of the mean (n=3).
APPENDIX D

MUG ASSAY FROM EDT 2224 AND EDT 2227
WITH WRWYCR AND/OR NORFLOXACIN AND
NOVOBIOCIN
Figure D.1. Results of the MUG assay of strains EDT 2224 (N99 tolC::lacZ) and EDT 2227 (N99 marA::lacZ) with wrwycr and/or novobiocin. Cells were treated with wrwycr at 8 µM or 16 µM, with or without novobiocin at 8 µg/ml for 24 h. At each time point, 10 µl aliquots were taken for β-galactosidase activity determination, and the OD600 of each sample was taken. Colors represent different treatments as stated in the graphs’ legends. The MUG activity is displayed relative to the OD600 at each time point (n=6).
APPENDIX E

PFGE SCANS FROM MG1655 ΔRECB TREATED WITH WRWYCR
Figure E.1. Results of PFGE experiment with *E. coli* MG1655 Δ*recB* treated with peptide wrwycr. (A) Scan of the pulsed field gel after a 22 h run at 14°C. The gel was stained with SYBR Green I. The lambda concatemer ladder (the lowest band is the 48.5kb unit-sized lambda genome) was loaded on both sides of the gel. (B) Same experiment as above, and run in 1% agarose gel using unidirectional electrophoresis for 90 minutes at 100V. The ladder used is Hyperladder I and was loaded on both sides of the gel. The gel was stained using ethydium bromide. Some treatments are missing due to the lack of additional plugs at high concentrations of the peptide. D, DMSO at 0.64%; 8, 16, 32 and 64 represent peptide treatments at 8 µM, 16 µM, 32 µM, and 64 µM, respectively.