Generation of SOS inhibitors as co-drugs to potentiate the activity of bactericidal antibiotics and to block the emergence of antibiotic resistance

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By

Areej Alhhazmi

ABSTRACT

The rapidly increasing emergence of antibiotic resistance amongst pathogenic bacteria is a major clinical and public health problem. The increase in resistant pathogens, accompanied with the small number of new antibiotics introduced in recent years, has limited the number of effective antimicrobials. The classical paradigm suggests that antibiotic resistance emerges by selection for pre-existing mutants in the bacterial population exposed to antibiotics. In contrast, recent data suggested that mutations evolve after cells encounter antibiotic therapy. This kind of mutation is known as adaptive mutation, which is activated by the SOS DNA repair and mutagenesis pathways. Accumulation of single-stranded DNA (ss-DNA) is the signal that induces the SOS response by promoting the formation of the RecA filament, which in turn activates the auto-cleavage activity of LexA and allows expression of SOS genes, including the SOS error-prone polymerases. In this project, phthalocyanine tetrasulfonic acid (PcTs)-based RecA inhibitors were characterized. PcTs molecules were found to potentiate the activity of bactericidal antibiotics and reduce the ability of bacteria to acquire antibiotic resistance mutations. This study highlights the ability of RecA inhibitors to potentiate the activity of antibiotics and provides a strategy for prolonging the life span of existing and newly developed antibiotics. We predicate that RecA inhibitors will be part of an antibiotic "cocktail" that enhances the activity of antibiotics and blocks resistance, which will ultimately prolong antibiotic lifespan.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	
PcTs	Phthalocyanine tetrasulfonic acid
NPF	Active helical nucleoprotein filament
ss-DNA	Single-stranded DNA
ds-DNA	Double-stranded DNA
DSEs	Double-stranded ends
DSBs	Double-stranded breaks
SBBs	ss-DNA binding proteins
DSBR	DNA double stranded break repair
QRDR	Quinolone resistance determining
	rejoin
polII	Polymerase II
polIII	Polymerase III
polIV	Polymerase IV
polV	Polymerase V
Fe-PcTs	Iron(III) phthalocyanine-4,4′,4″,4‴-tetrasulfonic acid
3,4′ Cu-PcTs	Copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid
MMR	Mismatch repair system
CFX	Ciprofloxacin
ROS	Reactive oxygen species
TSE	anti-transmissible spongiform
	encephalopathy
CJD	Creutzfeldt-Jakob disease
CWD	Chronic wasting disease (CWD)
(BSE)	Bovine spongiform encephalopathy
PrP-res	PrP-resistant
PrP-sen	PrP-sensitive

ADPT	Antimicrobial photodynamic therapy
DPT	Photodynamic therapy
Ps	Photosensitizer
ATCC	American Type Culture Collection
TSA	Tryptic soy agar
LBH	Lauria Bertani broth
MBH	Muller-Hinton broth
MIC	Minimum inhibitory concentration
SRBC	Sheep red blood cells
Apo-PcTs	Phthalocyanine tetrasulfonic acid
Al-PcTs	Aluminum(III) phthalocyanine tetrasulfonic acid
Zn-PcTs	Zinc(II) phthalocyanine tetrasulfonic acid
Ni-PcTs	Nickel(II) phthalocyanine tetrasulfonic acid
Cu-PcTs	Copper phthalocyanine tetrasulfonic acid

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

In recent years, there has been escalating concern over the growing number of antibiotic resistant bacteria, which reduces the efficacy of current antibacterial agents and discourages the incentive of developing new therapeutic agents. Despite contemporary efforts by some pharmaceutical companies, it is considered inadequate to continue introducing new and better antibiotics that are needed to forestall the threat of bacterial drug resistance to the market (Projan and Shlaes, 2004). Consequently, there is a clear and urgent need for developing and introducing novel strategies to overcome the antibiotic resistance crisis. Combination therapy is one approach for overcoming the problem of antibiotic resistance. Combination therapies can be roughly classified into four principal modes of action, which describe the mechanism by which a second compound enhances the activity of the main antibiotic. These modes of functions can act in the following ways: (i) when a second compound (an adjuvant) prevents the degradation or modification of the primary drug, (ii) when an adjuvant suppresses the accumulation and retention of the primary drug by inhibiting the efflux pumps, (iii) when an adjuvant is itself an antibiotic that targets the same or different pathway that is inhibited by the first antibiotic drug, or (iv) when an adjuvant inhibits the intrinsic repair pathway of cells exposed to the primary drug (Cottarel and Wierzbowski, 2007). The bacterial RecA protein represents an attractive target for treating bacterial infections, which fits into the last category of combination therapy. Bacterial RecA is a key player in regulating processes involved in repairing DNA damage or stalled replication forks. In addition, RecA and one of its downstream targets LexA control processes that lead to stress-induced mutations (Little *et al*., 1980; Luo *et al*., 2001; Thi *et al*., 2011) and horizontal gene transfer (Beaber *et al*., 2004). Because bacteria play a more proactive role in inducing stress-induced mutations or adaptive mutations in their genomes in response to certain antibiotics (Cirz and Romesberg, 2007; Riesenfeld *et al*., 1997), an emerging option to fight the growing number of resistant pathogens is to develop inhibitors for RecA, which would function as an adjuvant with the current or novel antibiotics. These adjuvants could potentiate the activity of antibacterial agents and prevent the acquisition of drug resistance.

The SOS pathway plays a critical role in the acquisition of mutations that lead to the emergence of antibiotic resistant bacteria. RecA and LexA are key elements in the SOS system, regulating DNA repair and mutagenesis mechanisms (Cirz *et al*., 2005). Certain antimicrobial agents exert stress that damages DNA structure or interferes with DNA replication (Cirz and Romesberg, 2007). These actions lead directly or indirectly to the production of free double-stranded breaks (DSBs) or ends (DSEs) (Cirz *et al*., 2005). DSBs or DSEs and stalled replication forks are consequently processed to ss-DNA, which is the signal that initiates SOS induction (Cirz *et al*., 2005). In this fashion, RecA is activated to form an active helical nucleoprotein filament (NPF), which coats the resulting ss-DNA. The NPF has both enzymatic and signaling activities. It mediates recombinational DNA repair, an enzymatic process that exchanges strands between homologous DNA strands. The NPF promotes the auto-cleavage of the LexA repressor (Cirz and Romesberg, 2007; Janion, 2001; Riesenfeld *et al*., 1997). Early expressed SOS gene products maintain the genetic integrity of the cell by high fidelity DNA repair mechanisms of the damaged DNA, while late SOS gene products induce stress-induced mutations (Cirz *et al*., 2005) and genome-wide hypermutation (Jolivet-Gougeon *et al*., 2011). Activation of the late stage of the SOS response includes expression of SOS error-prone polymerases IV (polIV) and V (polV). In this case, error-prone polymerases are active and produce mutations when the mismatch repair system (MMR) declines (Cirz *et al*., 2005). It has been demonstrated that RecA–dependent repair mechanisms result in acquired resistance to ciprofloxacin (CFX), and Δ*recA* strains are more susceptible to cell killing by CFX than wild type strains (Cirz *et a*l., 2005). Additionally, a great number of DNA damaging antibiotics (CFX, mitomycin C, and naldixic acid) and an RNA polymerase inhibitor (rifampcin) show a stronger effect on Δ*recA*$^-$ cells than on the wild type cells (Singleton and Hill., 2010). Therefore, we hypothesize that drugs that modulate RecA biological activity will provide an attractive pharmaceutical adjuvant by blocking the SOS pathway which will potentiate damaging effects of antimicrobial agents and inhibit mutagenesis mechanisms.

Acquisition of hypermutability reduces the fitness of bacterial mutators (Jayaraman, 2009). However, mutators can adapt to changing environments and stress more easily than non-mutators because of their mutagenic variation potential (Miller *et al*., 1999). Hypermutation is a property that leads to indiscriminate accumulation of unwanted mutations (Jayaraman, 2009). The cost of the increase in general mutability can be divided into two categories. First, lethal

mutations eliminate cells from the mutagenized population and reduce the potential yield of individuals with beneficial mutations. Second, beneficial mutations and deleterious nonlethal mutations can cause defects under most conditions, but others can cause major growth defects under specific conditions (Blazquez *et al*., 2002). The cost of most mutations in stable mutators that are deficient in the MMR is either neutral or lethal. Development of mutations in transient mutators costs less in terms of fitness since a mutation is produced only when needed (Macia *et al*., 2005). Environmental stressors, including antibiotics, can temporarily increase mutation frequency in a bacterial population. Bacterial cells transiently benefit from the elevated mutation frequency to overcome stressful conditions, while reducing the risk of accumulating deleterious mutations. A recent study suggests that the transient mutation state is turned on when cells are exposed to sub-lethal antibiotic selective pressure, and turned off when the stress disappears (Macia *et al*., 2005). The SOS system is activated in response to the formation of DSB or DSE in the DNA strand, eventually leading to the formation of ss-DNA. As a result, RecA is activated to form the NPF, coating the resulting ss-DNA. The NPF provokes the auto-cleavage of the LexA repressor, causing the activation of a series of about 40 different genes that are regulated by the product of the *lexA* gene and *recA*. Error-prone DNA polymerase IV and V are expressed and activated to overcome the blockage of DNA replication or the damage in DNA structure (Cirz and Romesberg, 2007; Janion, 2001). Moreover, induction of the SOS system by antibiotics increases the mutation rate in MMR-deficient cells (Jayaraman, 2009). Thus, the combined effects of both stable and transient hypermutations in a bacterial population, subjected to stress, increase the chance of acquiring antibiotic resistance. Transient hypermutations take place by using nonlethal conditions. In terms of antibiotics, the induction develops by using sub-lethal concentrations which are close to lethal since high concentrations will kill almost all the cells in the population and low concentrations will not activate the transient hypermutation system (Krasovec and Jerman, 2003).

To date, the number of natural or synthetic cell-permeable RecA inhibitors is extremely low. RecA and its structural and functional homologues are present in virtually all organisms, ranging from bacteria to humans (Cox, 2007). RecA belongs to recombinase proteins, which are a superfamily of strand exchange proteins, including archaeal RadA, and eukaryal Rad51 and DMC1. These proteins play a critical role in the DNA strand exchange process between a ss-DNA and a homologous ds-DNA in homologous recombination (Li *et al*., 2009). These proteins

exhibit noticeably similar filamentous assemblies from the electron microscopic and crystallographic data (Li *et al*., 2009). In principle, three common functionally important sites: (i) recruitment and polymerization, (ii) ATP binding, (iii) and DNA binding (Li *et al*., 2009) in RecA and its homologues filamentous structures could be targeted, which in turn inhibit these strand exchange proteins.

Candidate RecA inhibitors examined in this project were available from commercial small anionic aromatic libraries, specifically phthalocyanine molecules coordinated with different metal ions. The selection of these molecules was inspired by the ability of sodium tungstate (Na_2WO_4), a phosphate analogue, to be a potent inhibitor of ATPase and strand exchange activities of the RecA homologue MvRadA (Li *et al*., 2009). The metatungstate Na_2WO_4 cluster appears to be bound between the DNA-binding loops (L1 and L2), anchoring the protein in its inactive conformation (Li *et al*., 2009). The results suggest that small molecules could competitively inhibit DNA binding by RecA. A follow-up study showed that Na_2WO_4 was unable to abrogate RecA activity in living cells (Li *et al*., 2009). Therefore, in order to advance our goal of identifying molecules that are cell permeable and modulate RecA's biological activity, we screened commercially available anionic, aromatic molecules. Based on this screening, two phthalocyanine tetrasulfonic acid (PcTs) compounds coordinated with different metal ions, iron (III) phthalocyanine-4,4′,4″,4‴-tetrasulfonic acid (Fe-PcTs) and copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid (3,4′ Cu-PcTs), which inhibit RecA ATPase activity, were identified (Geyer and Luo personal communication, unpublished data). Abrogating ATPase activity is a useful tool to indicate the inhibition of the NPF and, consequently, the suppression of RecA activity. As a result, these RecA putative inhibitors represent attractive candidates to potentiate antibiotic efficacy and reduce the acquisition of resistance.

1.2 Antimicrobial Resistance

Since antibiotic discovery, antibiotics have been considered "miracle drugs", overwhelmingly used to fight infectious diseases. Antimicrobial drugs have saved many lives and contributed significantly to the control of infectious diseases (Bang *et al*., 1999; Zaidi *et al*., 2011). Looking back on the history of humans, infectious diseases were the leading cause of human morbidity and mortality (Aminov, 2010). The idea of a "magic bullet" that exclusively

steers its full action on disease-causing microbes, but not the host, was originated in 1904 by Paul Ehrlich (Abraham and Chain, 1988). This idea led him to start developing drugs that could cure diseases, such as syphilis. Salvarsan was the first synthetic antimicrobial drug and it had great success in treating syphilis (Projan and Shlaes, 2004). Following that, hundreds of drugs, including sulfonamides, were identified by Ehrlich for clinical settings. Sulfonamides were synthesized in 1935, yet they had limited clinical applications in terms of safety and efficacy (Projan and Shlaes, 2004). However, the true modern antibiotic era has been linked to Alexander Fleming, and his contribution to the clinical world. Fleming found that the growth of *Staphylococcus aureus* was inhibited in a zone surrounding a contaminated blue mold, named *Penicillium genus*, in culture plates (Aminov, 2010). This led to the hypothesis that a microorganism produces substances that could inhibit the growth of other microorganisms. These substances are weapons in the hands of antibiotic producer organisms to fight competitors, which might coexist with them. The antibiotic was named penicillin G, and it came into clinical use in the 1940s (Aminov, 2010). Following these discoveries, new classes of antimicrobial agents were developed, leading to the golden era of antimicrobial chemotherapy (Projan and Shlaes, 2004).

The major problem the world is encountering with antibiotic therapy is the ongoing race between the discovery and introduction of novel antibiotics and the remarkable ability of bacteria to evolve resistance against existing antibiotics. This scenario has been repeated on multiple occasions, demonstrating interesting facts about adaptive capabilities of bacteria to overcome the immense antibacterial drug abuses. Generation of resistant microbes or “superbugs” makes bacterial infections difficult to cure (Wright, 2000). Exposure to small doses of antibacterial agents introduces extreme selective pressure, causing bacteria to become resistant to these antibiotics instead of clearing up bacterial infections (Cirz and Romesberg, 2007). Lately, one of the greatest problems in medicine is bacterial resistance since diseases caused by microbes represent the second leading cause of death and is a primary cause of disability worldwide (Fauci, 2001). Twenty five thousands patients in the EU die from an infection by multidrug resistance bacteria per year while in the US more than 63.000 patients die every year by nosocomial infections (Aminov, 2010). The wide occurrence of antibiotic resistance suggests that, theoretically, any microbes could develop resistance to any antibacterial agents.

Additionally, the production of new agents for clinical use has dropped in the last 10-15 years (Alanis, 2005) (Figure 1.1), reflecting both the difficulty of discovering new drug classes and a declining interest in antibacterial drug discovery by the pharmaceutical industry (Aminov, 2010; Wright, 2000). As a result, the contemporary efforts by some pharmaceutical companies is considered inadequate to continue introducing new and better antibiotics needed to forestall the threat of bacterial drug resistance to the market (Projan and Shlaes, 2004). Since the same period has been accompanied with an escalating increase in bacterial resistance to existing antibacterial agents (Aminov, 2010) (Figure 1.1), it amplifies the emergence of a serious threat to global public health.

The accelerating evolution of antimicrobial resistant bacteria creates a considerable threat to public health and clearly is an unavoidable trend, demanding urgent solutions with either the discovered of new antibiotics or the revitalized of the pharmaceutical efficacy of current antibacterial agents. Improvement in existing antibacterial classes of drugs has been made to achieve better pharmacodynamic activity, including the absorption of oral drugs, concentration in the blood, distribution to the inflammatory tissue (Aminov, 2010), and overcoming the emergence of resistance (Hall, 2004). However, most of the current antibacterial agents in clinical use are already in their second or third generation of modification (Barbachyn and Ford, 2003). One approach to deal with antimicrobial resistance involves screening targets that are considered non-essential for bacterial viability (Cottarel and Wierzbowski, 2007). In principle, combination mutations in two non-essential genes can lead to a synthetic lethal phenotype, resulting in cell death; however, one mutation in any of these genes can slow down the cell growth. This principle could be employed chemically. An example would be the identification of two drugs, named drug A and drug B. Each drug inhibits a specific protein independently. Inhibition of any protein alone does not kill the cells, but slows down the growth. However, the combined effect of both drugs leads to lethal activity. This principle has been applied in *Mycobacterium tuberculosis*. *M. tuberculosis* utilize lipids as a source of energy when sugar is limited, which requires the activation of isocitrate lyase enzyme, encoded by *ICL1* and *ICL2* genes. Deletion of one of these genes alone in *M. tuberculosis* in an animal model has no lethal effect on the pathogen while deletion of both genes renders *M. tuberculosis* unable to replicate and the infection completely cleared. A dual ICL protein inhibitor suppressed bacterial

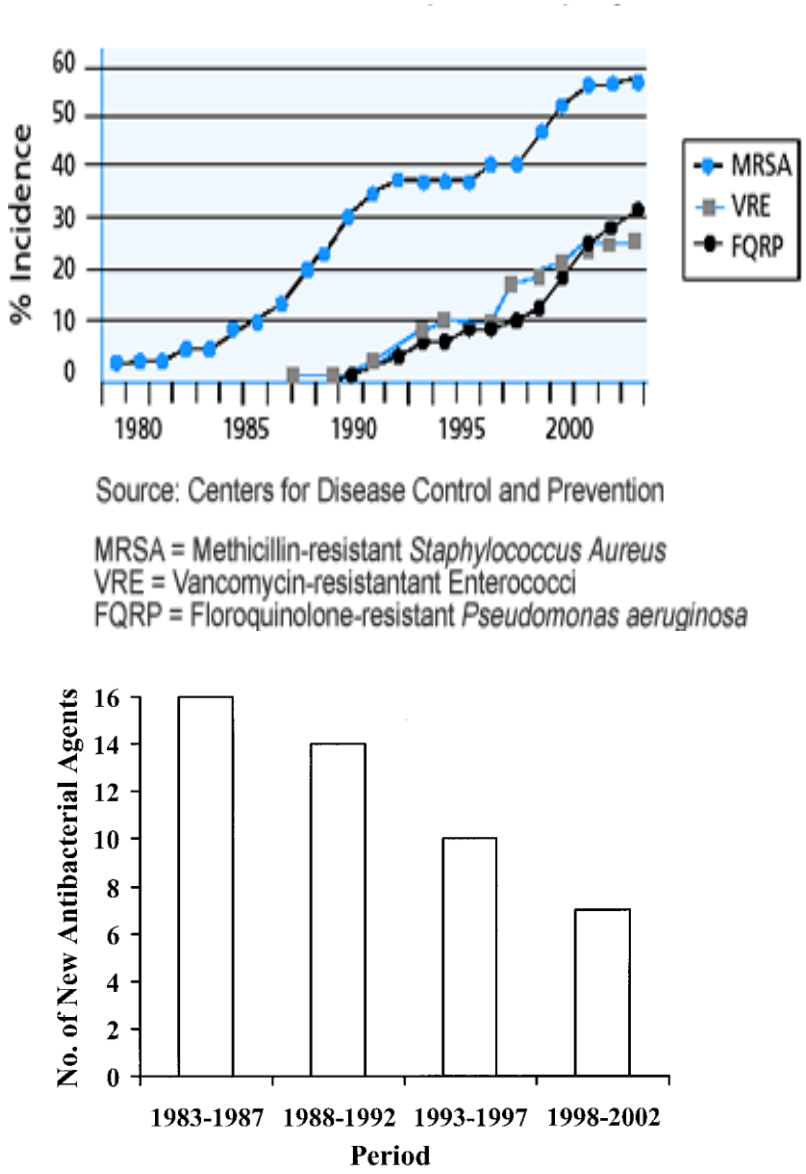

Figure 1.1. Trends associated with antibiotic resistance.

Upper graph*:* The percent of the increased incidence in nosocomial infections by resistant pathogens, including Methicillin-Resistant *S. aureus* (MRSA), Vancomycin-Resistant *Enterococci* (VRE) and Fluoroquinolone-Resistant *Pseudomonas aeruginosa* (FQRP) in the past 25 years. These data were collected from hospital intensive care units in the USA that participate in the National Nosocomial Infections Surveillance System, a component of the Centers for Disease Control and Prevention. The figure was obtained from the Infectious Diseases Society of America website, http://www.idsociety.org/. **Lower graph*:*** The declining number of licensed antibiotics in the past.20 years. This figure was reprinted: Spellberg *et al,* trends in antimicrobial drug development: implications for the future, clinical infectious diseases, 2004, 38, 9, 1279-1286, by permission of Oxford University Press.

replication in macrophage (Cottarel and Wierzbowski, 2007). This paradigm has shown that chemical inhibition of two non-essential proteins could produce a synthetic lethal phenotype, which can be applied to treat bacterial infections. Another way to resolve the antimicrobial resistance issue is the development of inhibitors of antibiotic resistance mechanisms, which can accompany the classical antimicrobials. In this manner, a current antibiotic is combined with an inhibitor that neutralizes the resistance mechanisms and, consequently, could potentiate the antibiotic's efficacy. This approach has the advantage of extending the lifetime of the current antimicrobials. The best well-known example is the combination of the β-lactam class antibiotic (amoxicillin) with a lactamase inhibitor (clavulanate). The β-lactamase inhibitor neutralizes the activity of β-lactamase to degrade amoxicillin and enhances amoxicillin ability to exert its action on bacterial cell wall (Cottarel and Wierzbowski, 2007; Wright, 2000). There are other examples of combination therapeutic drugs being introduced to the market, such as the semi-synthetic antibiotic, ampicillin, with the β-lactamase inhibitor, sulbactam (Wright, 2000). Another example of combination therapy is trimethoprim and sulfamethoxazole; these drugs inhibit different steps in the folic acid metabolism pathway at the transcription level. All combination therapy examples indicate that combination therapy could be a useful approach to combat antimicrobial resistance.

1.2.1 Factors Leading to Antimicrobial Resistance

There are two hypotheses regarding the emergence of resistance-conferring mutations. The classical theory links the evolution of antibiotic resistance to the selection of pre-existing mutants in a population of microbes exposed to an antibiotic therapy. This mutation is an unavoidable trend occurring as a result of replicating the bacterial chromosome with low fidelity polymerases (Cirz and Romesberg, 2007). This suggests that the emergence of mutations conferring resistance is a random event where intervention is not possible. Resistant microbes possess inherited resistant traits that allow them to survive killing or inhibitory effects of antimicrobial agents. Interestingly, these resistant genes can be transferred to other microbes by one of many genetic mechanisms, including transduction, transformation, and conjugation (Tenover, 2006). Nevertheless, current data suggests that the acquisition of mutations is a regulated process for which bacteria play a very proactive role by inducing particular proteins, at least when the DNA is exposed to certain antimicrobials and DNA-damaging agents (Cirz and

Romesberg, 2007). This kind of mutation belongs to adaptive or stress-induced mutations, occurring as a response to prolonged drug exposure or exposure to small doses of antibacterial agents for which these mutations relieve stress and allow microbes to grow (Cirz *et al*., 2005). This kind of mutation takes place only in non-dividing or slowly growing cells, and the stress that antibiotics create on bacteria is specific to a particular phenotype and produces a mutation in that phenotype that allows the bacteria to resume growth (Cirz *et al*., 2005; Riesenfeld *et al*., 1997). This paradigm supports the notion that resistance depends on particular biochemical pathways and intervention with these pathways would be a promising approach to combat the resistance issue.

The major antibacterial drug targets are bacterial cell-wall biosynthesis, protein synthesis, DNA replication and repair, metabolic pathway, and membrane structure (Tenover, 2006) (Figure 1.2). Numerous mechanisms have evolved in bacteria, which confer them with antibiotic resistance. Bacteria can have intrinsic or acquired resistance. In both cases, a heritable change in bacterial DNA is produced through either a mutation or acquisition of foreign resistant genes from other bacteria. The processes of horizontal gene transfer and stress inducible mutations have been shown to increase the chance of acquiring antibiotic resistance (Cirz *et al*., 2005; Kohanski *et al*., 2007; Thi *et al*., 2011), where RecA play a critical role in both processes (Beaber *et al*., 2004; Cirz *et al*., 2005).

Acquired mutations conferring resistance can render the ability of bacteria to: (i) chemically inhibit the active from of an antibiotic; (ii) physically remove an antibiotic from the cell through an efflux pump mechanism; or (iii) modify the target site of an antibiotic, so it is no longer recognized by the antibiotic (Tenover, 2006). The first effective strategy of antibiotic resistance is the destruction of the antibiotic (Figure 1.3). The most well-known example of this strategy is the hydrolytic deactivation of the β-lactam ring in penicillins and cephalosporins by production of the hydrolytic enzyme β-lactamase by resistant bacteria. The β-lactam ring is the functional component in these drugs, which irreversibly acylates and modifies the cell wall-crosslinking penicillin binding proteins (PBPs). When the ring is hydrolysed, it is deactivated and becomes nonfunctional as a PBP pseudosubstrate. The β-lactam resistant bacteria secrete this enzyme into the periplasm to destroy β-lactam antibiotics before they can reach the PBP

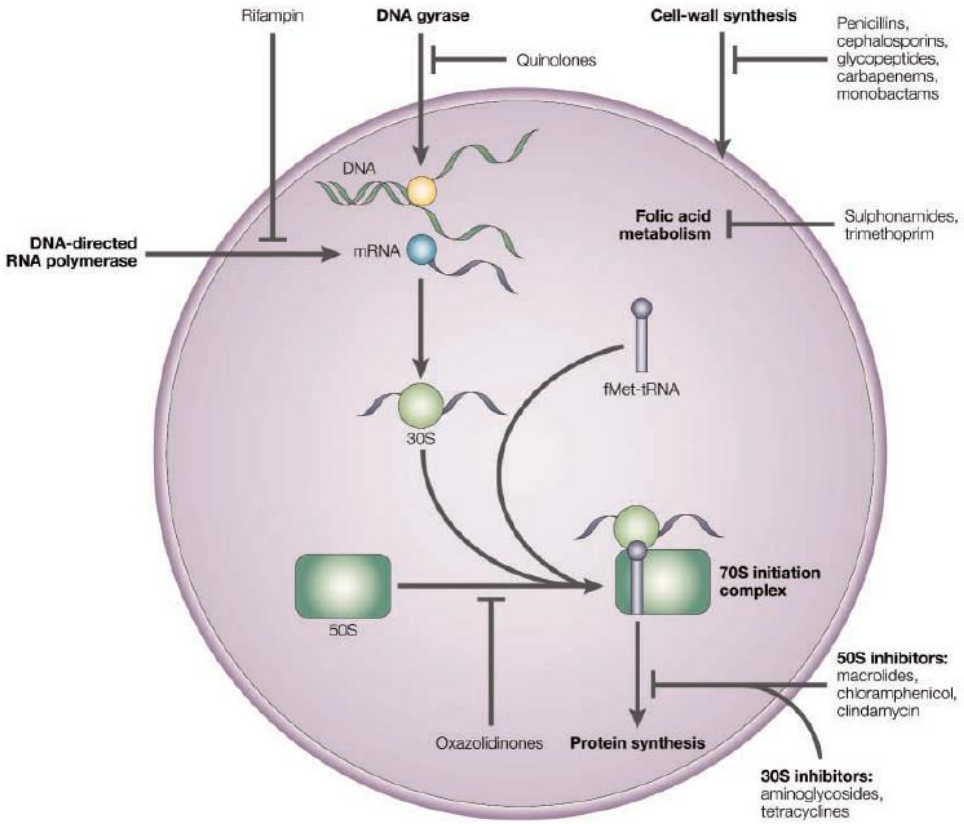

Figure 1.2. The major antibacterial agent targets.

There are five main antibacterial targets in bacteria: cell-wall biosynthesis, protein synthesis, DNA replication and repair, metabolic pathway, and membrane structure. This figure was reprinted by permission from Macmillan Publisher Ltd: [NATURE REVIEW DRUG DISCOVERY](Coates *et al*., 2002), copyright (2002).

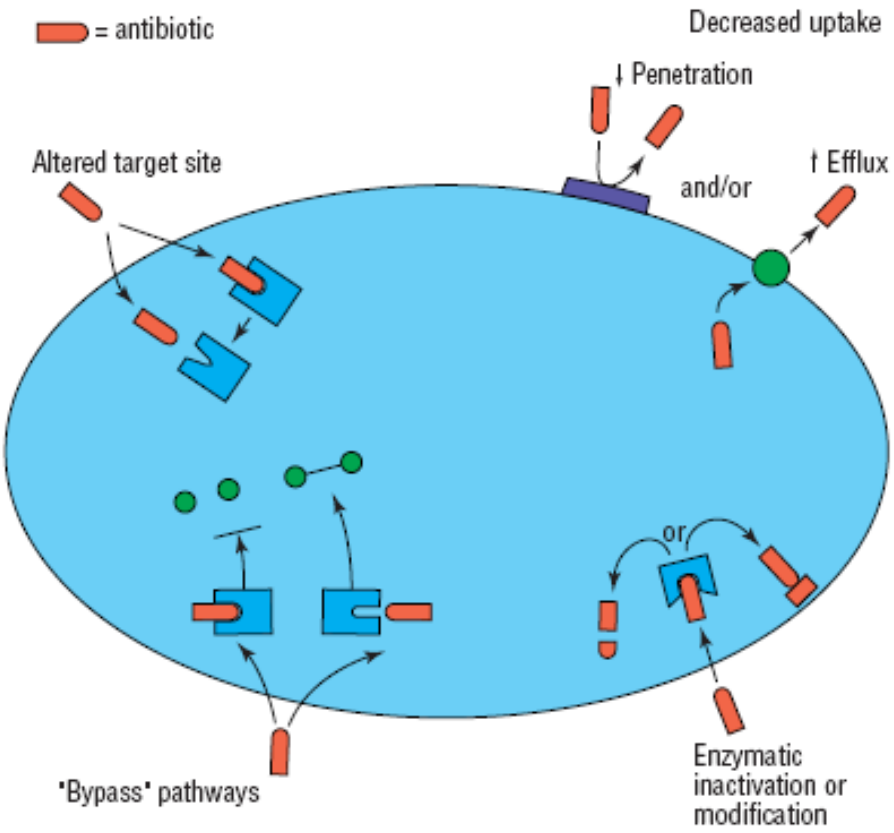

Figure 1.3. The molecular resistance mechanisms of antibacterial antibiotics.

The resistance mechanisms consist of chemical inhibition of the active from of an antibiotic, physical removal of an antibiotic from the cell through an efflux pump mechanism; or modification of the target site of an antibiotic. The figure was reproduced from [the origins and molecular basis of antibiotic resistance, Hawkey, 317, 657-660, copyright 1998] with permission from BMJ Publishing Group Ltd.

targets in the cytoplasmic membrane (Bradford, 2001). Other antibiotic classes, such as the aminoglycosides, use different mechanisms to neutralize antibiotic activity. These protein-synthesis inhibitors can be neutralized by antibiotic deactivating enzymes. The three classes of enzymes that confer aminoglycoside resistance are adenylyltransferases (ANTs), O-phosphoryltransferases (APHs), and N-acetyltransferases (AACs). These enzymes modify aminoglycoside structure, which causes the antibiotic to bind to their RNA targets in the ribosome (the 30S ribosome unit) with lower affinity (Smith and Baker, 2002). The second mechanism by which bacteria develop resistance to antibiotics is the physical removal of antibiotics from bacterial cells (Figure 1.3). All bacteria possess membrane proteins that organize the movement of lipophilic or amphipathic molecules in and out of cells. Gram-negative bacteria possess an outer membrane that acts as a front line defensive mechanism to stop toxic molecules, including antibacterial agents, from entering the cell. This mechanism has a non-specific property in protecting bacterial cells from toxic molecules. Although Gram-positive bacteria lack the presence of the outer membrane, they have the capability to restrict the movement of toxic drugs by physical means. The influx system only restricts antibiotics from entering bacterial cells, so it cannot take the entire responsibility of resistance (Nikaido, 1994). Resistance to antibacterial agents is linked in many cases to activation of the efflux system that prevents the intracellular accumulation of antibiotics inside the cell (Hawkey, 1998; Levy, 1992; Nikaido, 1994). Antibiotic-producing microorganisms use efflux pumps to transport antibiotics out of their cells (Nelson, 2002). In this fashion, they create a defensive mechanism for the bacteria to prevent being killed by their own toxic defense molecules. The same mechanism is used by resistant bacteria to pump antibiotics out of their system. Accordingly, antibiotics do not accumulate to lethal or inhibitory concentrations and reach their specific bacterial targets (Jarmula *et al*., 2011). Tetracycline-resistant bacteria (Gram-positive and Gram-negative bacteria) become resistant to tetracyclines by membrane proteins that act as efflux pumps for the antibiotic; therefore, the drug is pumped out faster than it can accumulate inside the cells, so the drug concentrations are maintained at a low level inside the cells and do not interrupt protein processes (Jarmula *et al*., 2011; Levy, 1992). Lastly, resistance can result from mutations that render the drug ineffective against the target; therefore, the drug cannot interact with the target, making the drug ineffective as an inhibitor (Jacoby, 2005) (Figure 1.3). In the case of quinolones, studies have shown that resistance is a result of *de nova* mutations in drug targets.

Mutations in *gyrA*, *gyrB*, *parC*, or *parE* result in quinolone resistance. Mutations in the *gyrA* gene are more common in quinolone-resistant clinical isolates of *E. coli* (Cirz *et al*., 2005), while mutations in *parC* are more dominant in Gram-positive bacteria (Martinez *et al*., 1998; Roca and Cox, 1997).

1.3 The SOS System

To maintain genomic functional and structural integrity, microorganisms react to stressful environmental conditions by producing or activating specific proteins that play roles in translesion DNA synthesis, recombination, repair, and cell division inhibition until DNA repair and replication are complete. This system is known as the SOS system, elaborately described in the *E. coli* by Walker in 1984 (Hilgers *et al*., 1989). This phenomenon was first proposed and named by Miroslav Radman in 1974 (Cirz and Romesberg, 2007). The SOS system is the bacterial DNA damage checkpoint response that is activated by DNA damage or stalled DNA replication. Activation of the SOS system results in the upregulation of at least 40 genes. The main proteins that regulate this system are LexA (transcriptional repressor) and RecA (SOS de-repressor) (Cirz and Romesberg, 2007).

1.3.1 The SOS Response

Activation of the SOS response induces a cascade of reactions associated with DNA repair pathways, cell cycle arrest, and mutagenesis (Janion, 2001). SOS genes are located at different sites on the *E. coli* chromosome and are normally activated by two proteins, LexA and RecA (Janion, 2001). SOS genes are negatively regulated when LexA binds to a specific set of related sequences in the operator site of SOS boxes, blocking the transcription of all SOS genes (Michel, 2005). A comparison of LexA regulons from several bacterial species revealed that the LexA regulon consists of approximately 42 genes and their expression is coordinated into three phases according to their functions. SOS genes are generally classified based on the order of their expression as early, middle, and late SOS genes (Figure 1.4). Their expression is dependent on the sequence of their SOS box and the position and strength of their promoter. *lexA* (a repressor of SOS genes), *uvrA* and *uvrB* (Uvr ABC-exonuclease-nucleotide exclusion repair), *uvrD* (HelicaseII), *polB* (DNA polyII), *ruv*A (RuvAB-helicase), *ruvB* (recombinational repair), and *dinI* (inhibitor of UmuD processing) are expressed in the first phase of the SOS response

and play a role in nucleotide excision repair mechanisms. If these genes do not repair the damaged DNA or the stalled replication fork, then *recA* (SOS de-repressor and recombinational repair) and *recN* (recombinational repair) are expressed and are responsible for recombination repair mechanisms. Lastly, if the SOS response is not successful in overcoming the blocked DNA replication, then the *sulA* and *umuDC* genes are expressed. *sulA* inhibits cell division and the *umuDC* operon encodes the error-prone DNA polV (Janion, 2001). The interaction between LexA and RecA regulates the expression and activation of PolV by stimulating UmuD to undergo auto-cleavage. Two cleaved UmuD units bind to one UmuC to create PolV. The lack of the proof reading function in PolV and PolIV facilitates their function in bacterial cells (Goodman, 2000). PolIV and V are crucial in translesion error-prone DNA synthesis, allowing for a gap across from the site of a lesion to be filled by any nucleotide, although they have distinctive preferences for particular lesions (Hastings and Rosenberg, 2002). When *sulA* is expressed in the late stage of the SOS gene expression, it arrests cell division by binding FtsZ (Trusca *et al*., 1998) and provides extra time for the mutagenic error-prone polymerases to acquire mutations that allow cells to escape from the metabolic and genomic stress. Induction of the SOS response is caused by the exposure of bacteria to stressful conditions, directly or indirectly interrupting DNA replication or damaging DNA structure. Both of these actions lead to accumulation of an ss-DNA (Cirz and Romesberg, 2007; Janion, 2001; Riesenfeld *et al*., 1997), which is the SOS signal that induces RecA to polymerize on the ss-DNA in the presence of dATP or ATP (Figure 1.5). When RecA forms the long helical NPF on the ss-DNA in the presence of ATP, it becomes active (RecA*) and induces the auto-proteolytic activity of LexA; promoting auto-cleavage of the LexA dimer repressor and CI repressor, and processing of the UmuD protein to the mutagenic UmuD' (Butala *et al*., 2009; Cirz and Romesberg, 2007; McKenzie *et al*., 2000). During this process, the concentration of ATP and dATP are increased several fold, while the level of LexA is decreased (Janion, 2001). This reduction in LexA concentration frees operator sites of SOS and allows the expression of SOS genes, which are responsible for DNA repair and mutagenesis mechanisms (Cirz and Romesberg, 2007; Janion, 2001; Riesenfeld *et al*., 1997). After cell revival from the DNA damage by DNA repair or mutations, the signal for activating RecA no longer exists, and as a result the de-repression of LexA is abolished. Thus, the SOS system returns to its repressed state (Riesenfeld *et al*., 1997).

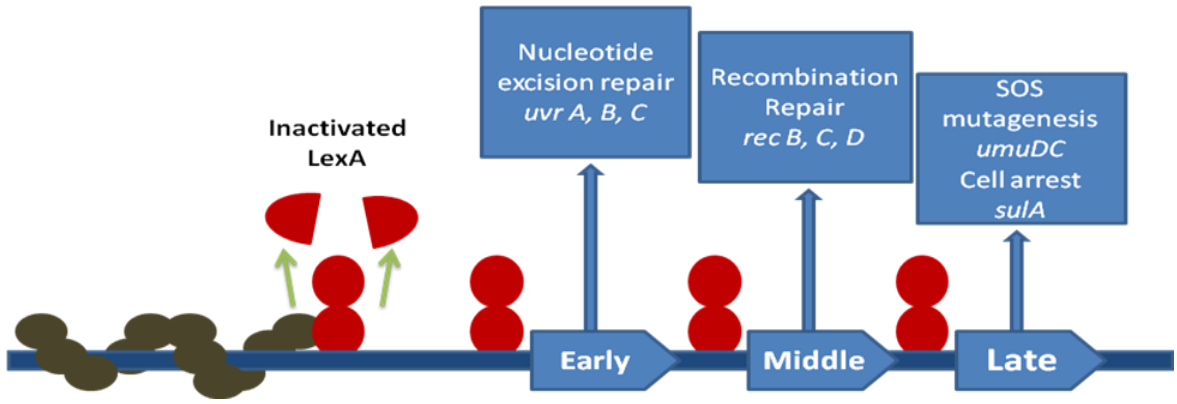

Figure 1.4. The SOS response to DNA damage or collapsed replication fork.

The SOS genes are divided into early, middle, and late genes based on their phase of expression. The SOS response in *E. coli* includes up to 40 genes, which are normally de-repressed based on the direct impact of interaction between two proteins, LexA and RecA. The early and middle expressed genes involve with repairing DNA damage while the late SOS genes activate the SOS mutagenesis in bacterial genome. The data used to drew this figure was obtained from (Janion, 2001)

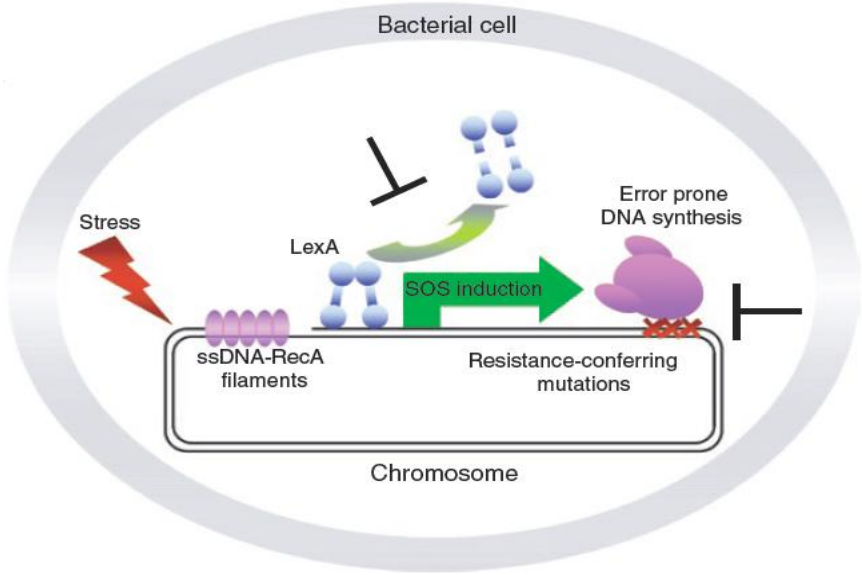

Figure1.5. The SOS response is activated when RecA protein forms the NPF on ss-DNA that accumulates in the presence of DNA damage or stalled DNA replication.

The active NPF assists repair of DSEs or restart collapsed replication forks. The NPF binds to the ss-DNA in the presence of ATP and promotes the auto-cleavage of the SOS transcriptional repressor protein, LexA. Cleavage of LexA leads to the dissociation from the SOS operator site and allows the transcription of the SOS genes. Bold Ts indicate possible ways of interventions to block the SOS response. This figure was reprinted by permission from Macmillan Publisher Ltd: [NATURE CHEMISTRY BIOLOGY], (Smith and Romesberg, 2007) copyright (2007).

1.3.2 Overview of RecA Protein and its Functions

RecA is a ubiquitous protein with structural and functional homologues present in all organisms, ranging from bacteria to humans (Cox, 2007). RecA is a recombinase protein, and is a member of the superfamily of strand exchange proteins, including archaeal RadA, and eukaryal Rad51 and DMC1 (Wu *et al*., 2005). These proteins play a critical role in the DNA strand exchange process between ss-DNA and homologous ds-DNA. Among bacteria, RecA is a highly conserved protein. Bacteria are constantly challenged by environmental stressors, such as DNA damaging agents. Bacteria have to maintain a dynamic conflict between the needs to reserve or vary their genetic information in the face of environmental stressors. DNA repair mechanisms are essential for the maintenance of bacterial genetic information while genetic variation provides evolutionary adaption to changing environments (Kowalczykowski and Eggleston, 1994; Roca and Cox, 1997). RecA plays a role in both pathways. It detects DNA damage or stalled replication forks and initiates the SOS response by stimulating LexA repressor auto-proteolysis (Cirz and Romesberg, 2007; McKenzie *et al*., 2000). The SOS response to DNA damage launches with up-regulating DNA repair activities, but if DNA damage is not successfully resolved, error-prone DNA synthesis is activated to promote mutagenesis (Cirz and Romesberg, 2007; McKenzie *et al*., 2000). RecA has an enzymatic activity in bacterial cells to promote the auto-cleavage and inactivation of the LexA and CI repressors and in promoting the processing of UmuD to mutagenic UmuD' (Cirz and Romesberg, 2007; McKenzie *et al*., 2000). RecA expression is also activated during the SOS response to perform recombinational activities which are the basis for DNA repair (Cox, 2007) and horizontal gene transfer processes (Beaber *et al*., 2004). Signaling and recombinational activities of RecA are capable of sustaining the genetic information of the bacterial genome or allowing the same genome to adapt to stressful conditions (Kowalczykowski and Eggleston, 1994; Roca and Cox, 1997).

Interestingly, RecA does not have any biological activities as a monomer. It functions as a helical filament with thousands of monomers polymerized on ss-DNA in the presence of ATP (Kowalczykowski and Eggleston, 1994). RecA protein is activated as a response to exposure to DNA damaging elements; however, it can be activated by a number of conditions such as antibiotic treatment, starvation, oxidative stress, heat shock, and pressure, all of which lead to metabolic and physiological stress that indirectly causes DNA damage and the ss-DNA

formation (VanBogelen *et al*., 1987). RecA binds to ss-DNA and causes a cascade of reactions, starting with nucleation. The active RecA NPF assembles and disassembles in one direction on the ss-DNA and ds-DNA. The RecA filament uniquely extends in the 5′ to 3′ direction. Its assembly is faster than its disassembly and its assembly on ss-DNA is faster than on ds-DNA. A gap in the ds-DNA is the most attractive site for RecA nucleation. If ss-DNA binding proteins (SSB) are bound to the DNA, the nucleation of RecA becomes significantly slower; therefore, RecO and RecR proteins form a complex that facilities RecA nucleation on SSB-bound ss-DNA. In addition, to prepare the ds-DNA for RecA binding, RecBCD cuts the 5′-end of DNA strands more than the 3′-ends of DNA strands, which generates a 3′ single-strand extension. This facilitates RecBCD function to load RecA on the processed DNA substrate (Cox, 2003). All types of DNA damage lead directly or indirectly to the formation of ss-DNA by processing the damage with recombinational accessory proteins to form the ss-DNA. The formation of DSB or DSE in the DNA leads to a stalled replication fork, where the RecBCD enzyme loads at DSEs and produces ss-DNA. In the case of DNA cross-link damage, the UvrABC exonuclease system nicks and removes small segments of DNA from one strand of the damaged duplex before and after the site of cross-linking, leaving a short ss-DNA gap (Sladek *et al*., 1989). Following that, RecBCD proteins apply exo- and endonuclease activities to unwind and degrade one strand of the damaged DNA duplex and produce ss-DNA (Smith *et al*., 1987). The active NPF starts to search for homologous DNA and mediates the ss-DNA invasion into the homologous DNA duplex, forming a D-loop structure or Holiday type structure. The RuvABC proteins carry on the branch migration, resulting in strand exchange (Kowalczykowski and Eggleston, 1994). This type of strand invasion is critical for resolving DSB in the DNA (Cox, 2003), stalled or collapsed replication forks, and cross-linked DNA strands (Roca and Cox, 1997).

The hydrolysis of ATP is necessary for some RecA functions. Upon RecA activation, RecA starts to polymerize on ss-DNA and forms the active ATP·RecA·ss-DNA nucleoprotein filament, promoting LexA auto-cleavage. The cleavage of LexA is one of RecA's signaling functions, which is dependent on the formation of the NPF, but does not require ATP hydrolysis (Courcelle and Hanawalt, 2003). However, the subsequent hydrolysis of ATP bound to the NPF is essential for recombinational activities, which are necessary for horizontal transfer and recombinational DNA repair. The recombinational DNA repair mechanism is necessary for restarting replication forks and repairing DNA damage even DNA damage that is bypassed by

the error–prone DNA synthesis (Cox, 2007). ATP hydrolysis contributes to unique functions of RecA, such as dissociating the RecA-filament at the disassembly end, rendering the strand-exchange reaction unidirectional (5′-3′ direction) in a three strand exchange reaction, and bypassing significant barriers in one of the DNA substrates since RecA-mediated bypass involves the unwinding of the entire DNA insertion. In addition, the four strand-exchange reaction must be initiated in a single-strand gap (as in the three-strand exchanges), yet the exchange readily grows beyond the gap. The transition from a three-strand reaction into a four-strand reaction is dependent on ATP hydrolysis. Lastly, upon RecA binding to a gap in the leading strand of the stalled replication fork, the fork regresses in a reaction that is dependent on ATP hydrolysis (Cox, 2007).

1.3.3 Responsibility of RecA in the Development of Acquired Resistance to Antibiotic Treatment

Some interesting features of RecA functions are its ability to facilitate the acquisition of antibiotic resistant genes by horizontal gene transfer and the development of stress-inducible mutations. The DNA recombinational repair mechanisms by RecA is a non-mutagenic process where problems in the DNA duplex are resolved. On the other hand, RecA-mediated recombination can incorporate a foreign DNA segment, which may contain resistant genes derived from exogenous homologous DNA, into the host genome through a process known as horizontal gene transfer (Beaber *et al*., 2004; McKenzie *et al*., 2000). Bacteria show the ability to incorporate a DNA fragment from bacteria of the same or different species (Davison, 1999). The significance that horizontal gene transfer has on bacterial evolution is emphasized by the fact that foreign DNA can represent up to one-fifth of a given bacterial genome (Rowe-Magnus et al., 2001).

The mechanism of stress-inducible mutagenesis involves switching the high fidelity DNA double strand-break repair (DSBR) mechanism via homologous recombination into a mutagenic version, controlled by SOS proteins. Fluoroquinolones have the ability to induce the SOS system. Bacterial RecA is known as a crucial element of the response to antibacterial quinolones that inhibit topoisomerase II. CFX is a member of the quinolones. The main target of CFX in *E. coli* is DNA gyrase, which is a tetrameric enzyme composed of two A subunits and two B subunits, encoded by *gyrA* and *gyrB*, respectively. The main function of this enzyme is to

supercoil and to uncoil the DNA helix by cleaving both strands of the helix, passing another piece of the helix through the resulting DSBs and rejoining these DSBs in the presence of ATP. These actions are necessary in DNA replication, transcription and recombination. There are additional targets for CFX in Gram-negative bacteria such as *ParC* and *ParE*, which encode subunits for topoisomerase IV (Jurado *et al*., 2008). CFX functions by reversibly binding to the topoisomerase II bridged-DSB intermediate and inhibiting rejoining of DNA ends. The proposed model for CFX resistance conferring acquired mutations begins with the formation of DSBs or DSEs, which can lead to DNA damage or a stalled replication fork (Fig. 1.6). The RecBCD enzyme loads at DSEs and produces ss-DNA where RecA can bind. The RecA-ss-DNA filament catalyzes strand invasion of the ss-DNA into a homologous sequence, resulting in the formation of the D-loop structure (Cirz *et al*., 2005). The persistence of the RecA-ss-DNA filament eventually leads to the degradation of LexA and activation of the SOS system, including the SOS error-prone polymerases. In this case, error-prone polymerases start to function and produce mutations when the MMR declines (Cirz and Romesberg, 2007). Criz *et al*., showed that *E. coli* cells with the uncleavable LexA transcriptional repressor do not develop resistance either in culture or in a murine infection model (Cirz *et al*., 2005).. Additionally, inhibiting LexA cleavage activity by mutating or deleting the *lexA* gene *in vitro*, considerably reduces the emergence of resistance to CFX (Cirz *et al*., 2005), and any defective mutations in one or more of the mutagenic polymerases are not able to develop post-exposure mutations (Cirz *et al*., 2005). DNA PolIV and PolV are Y-family error-prone DNA polymerases and mediate translesion DNA synthesis (Babynin, 2004). This indicates that the induction of error-prone polymerases (Pol II, PolIV and PolV) is required for CFX-resistant cell development. Defais *et al*., showed that deletion of the *recA* gene prevents mutations (Defais *et al*., 1971). RecA–dependent repair mechanisms resulted in acquired resistance to CFX, and Δ*recA* strains are more susceptible to cell death from CFX than wild type strains (Cirz *et al*., 2005). It has been revealed that a great number of DNA damaging antibiotics (CFX, mitomycin C, and naldixic acid) and an RNA polymerase inhibitor (rifampcin) have a stronger effect on Δ*recA*$^-$ cells than on wild type cells (Singleton and Hill., 2010). These studies indicated that the induced mutation process is regulated and related to regulation of the SOS response. Rifamycins function by inhibiting the initiation step of bacterial RNA synthesis by the binding to the β-subunit of RNA polymerase bound to DNA (Kohanski *et al*., 2010).

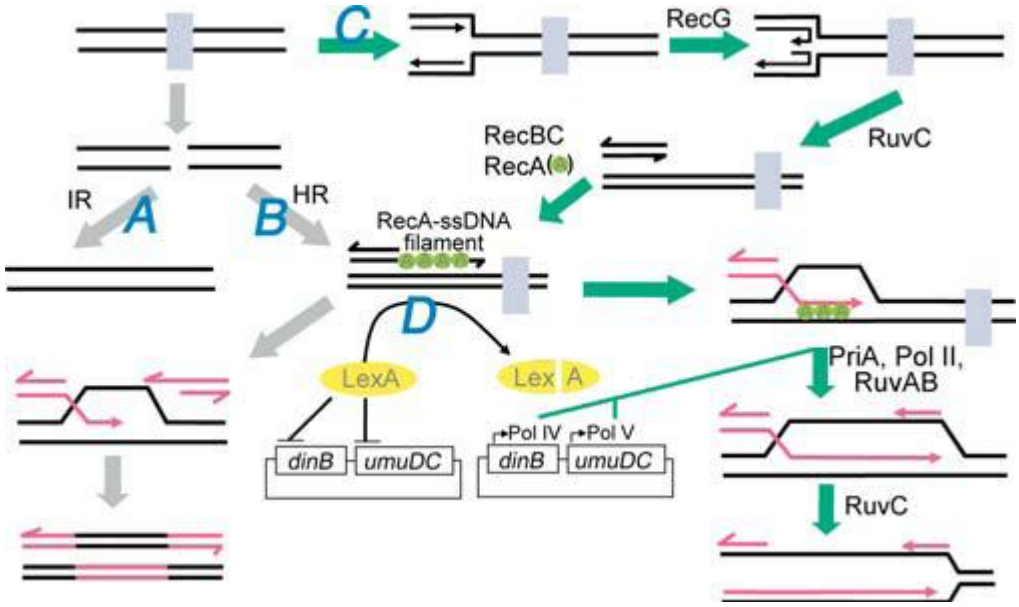

Figure1.6. Model of ciprofloxacin-acquired mutation conferring resistance.

Free DSBs are repaired by nuclease and polymerase-dependent illegitimate recombination, resulting in small deletions (Pathway A) in the absence of homologous sequences. Free CFX-induced DSBs can be repaired by recombination-dependent replication (Pathway B) in the presence of a homologous sequence. Lastly, replication forks that encounter topoisomerases that are covalently-bound to the DNA are repaired by recombination-dependent fork repair (Pathway C). This involves RecG-mediated fork regression and RuvC cleavage to free DSEs where RecBCD loads RecA on an ss-DNA to form the NPF. The NPF catalyzes strand invasion of a homologous sequence where PriA, and possibly Pol II, help to restart a processive replication fork. Sufficient accumulation of DSBs and blocked forks, persistent of the NPF induces LexA cleavage to levels sufficient to de-repress the error prone polymerases, Pol IV and Pol V, which cooperate to induce mutations (Pathway D). Once resistance-conferring mutations are made, DSBs, collapsed replication forks, and the NPF are no longer present and the cellular concentration of LexA increases, turning off expression of the error-prone mutagenic polymerases. This figure was obtained from (Cirz *et al*., 2005).

The inhibitory effect of rifamycins is attributed to two molecular actions: (i) binding of rifamycin molecule to the β-subunit (encoded by *rpoB*) of a DNA-bound RNA polymerase blocks transcription and (ii) oxidizing the hydroquinone moiety of rifamycin Sv-bound to RNA polymerase into rifamycin S leads to the production of free radicals (Kono, 1982). The ability of rifamycin to cycle between a radical and nonradical form (rifamycin Sv and rifamycin S, respectively) can damage DNA through a direct drug–DNA interaction (Kono, 1982). This can explain SOS induction following rifamycin Sv treatment. Later studies showed that during stress, a subpopulation of bacterial cells undergoes a transient state of hypermutation (Rosenberg *et al*., 1998). The cause of the temporary increase in mutations is linked to the SOS response since the SOS response regulates the expression of three nonessential DNA polymerases (Babynin, 2004). Error-prone polymerases PolIV, or PolV can bypass lesions that block replicative PolIII in a process known as translesion synthesis (Kang *et al*., 2006). These polymerases facilitate bacterial survival, but they have low fidelity and concurrently introduce mutations into the genome at high frequency (Kang *et al*., 2006). Activation of *E. coli* alternative sigma factor RpoS down-regulates the MMR genes during stressful conditions (Foster, 2007). The MMR remains active in stationary-phase cells, but two of the MMR proteins, MutS and MutH, are down-regulated in an RpoS-dependent manner. Consequently, decreases in the levels or functions of the MMR elevate mutation rate during stressful conditions (Foster, 2007). The MMR plays crucial roles in elevating the mutation rate and the emergence of antibiotic resistance (Blazquez *et al*., 2002; Macia *et al*., 2005). The main protein in the depletion of the MMR activity is *mutS*. The MMR mechanism could be an independent mechanism that allows spontaneous mutations to take place (Blazquez *et al*., 2002). However, in the case of LexA and RecA dependent acquired mutation, the MMR is dependent on the interaction between LexA and RecA since the complete inhibition of LexA cleavage and induction of the SOS system blocked the emergence of antibiotic resistant mutations in the *E. coli mutS* mutants (Cirz *et al*., 2005; Cirz and Romesberg, 2006). This indicates that evolution of antibiotic resistance is more likely to be inhibited by preventing the cleavage of LexA and induction of the SOS system, even in hypermutators. Isolating drugs that inhibit RecA; therefore, represents a fundamental new approach to combating the emergence of antibiotic resistance.

1.4 SOS Involvement in Biofilm Formation

Bacteria have been traditionally considered as individual organisms growing in homogenous planktonic populations. In most natural, clinical, and industrial settings, bacteria form biofilm communities of sessile cells embedded in an extracellular polymeric mixed matrix, consisting of proteins, polysaccharides, and nucleic acids (Wood, 2009). The functions of this structured community are dependent on a complex web of interdependent interactions. Biofilm formation is a dynamic process that passes through specific sequences (Fig. 1.7). Biofilm development is initiated with the attachment of sessile cells to an abiotic or biotic surface. Planktonic cells reversibly attach to the abiotic or biotic surface within a few minutes when microbes live in a liquid environment. In approximately two hours, the cells commit to irreversible attachment. Following the initial attachment to substratum, cells undergo programmed physiological changes (Wood, 2009). Therefore, early biofilm maturation starts to form a highly organized structure and then a more sophisticated structure is formed (Stoodley *et al*., 2002). Finally, some sessile cells disappear between the ninth and the twelve day. A remarkable feature of biofilm growing cells is the increased resistance to antimicrobial therapy and the eukaryotic immune system. The spatially extracellular matrix plays an important role in antimicrobial resistance and persistent infections (Costerton *et al*., 1999). It has been shown that biofilm formation was the cause of persistence infection by *P. aeruginosa* in cystic fibrosis (Costerton *et al*., 1999; Stewart and Costerton, 2001; Tart and Wozniak, 2008) and in immuno-compromised hosts (Costerton *et al*., 1999). The difficulty in eradicating bacterial infections is associated with biofilm development, and causes 65% of human infections in developed countries (Hall-Stoodley *et al*., 2004). This represents a serious threat to the health care system.

1.4.1 Role of Replication Inhibitory Drugs in Biofilm Formation

The concern of biofilm development is linked to multidrug resistance, persistent infections (Costerton *et al*., 1999) and increased resistance (Donlan and Costerton, 2002) to the host immune system (Donlan and Costerton, 2002). Interestingly, bacterial biofilm development can be induced by some antimicrobial and DNA damaging agents (Gotoh *et al*., 2008). Specifically, *E. coli* and *P. aeruginosa* biofilm formation is induced via treatment with subinhbitory concentrations of aminoglycosides (Hoffman *et al*., 2005). Quinolone

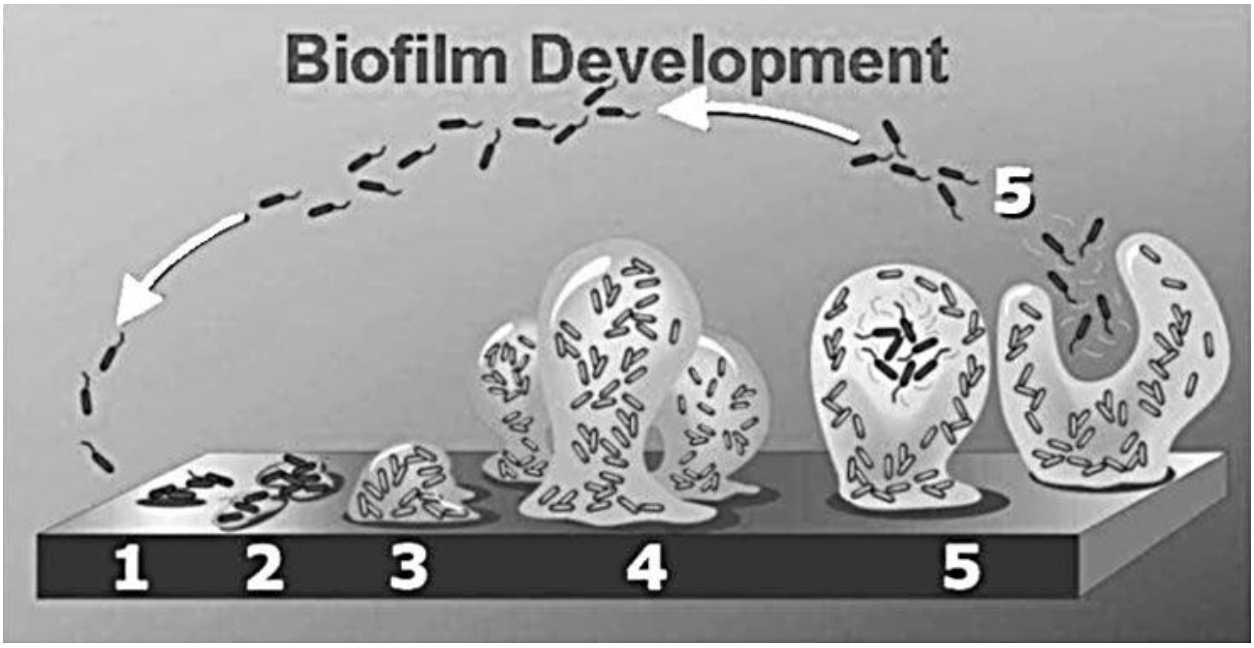

Figure1.7. The development of a biofilm in a five-stage process.

The first stage involves initial attachment of cells to the surface. In the second stage, production of extracellular polymeric substance matrix (EPS) results in irreversible attachment. The third Stage consists of early development of biofilm architecture while maturation of biofilm architecture takes place in the fourth stage. The fifth Stage contains dispersion of single cells from the biofilm. The figure was reprinted from Current Opinion in Biotechnology, 13, 3, 228-233, Hall- Stoodley and Stoodley, Developmental regulation of microbial films, copyright 2002, with permission from Elsevier.

antimicrobial drugs and hydroxyurea induce biofilm formation in *P. aeruginosa* (Gotoh *et al*., 2008; Takahashi *et al*., 1995). It has been proposed that, since such biofilm formation occurs under stressful conditions, it be named stress-inducible biofilm formation (Gotoh *et al*., 2010).

1.4.2 Role of LexA and RecA in Biofilm Formation

Regulatory mechanisms of stress-inducible biofilm formation response are not yet clear; however, it has been suggested that the SOS response may play a role (Walker, 1984). A study investigating the link between SOS induction and stress-inducible biofilm formation by DNA damaging agents suggested that stress-inducible biofilm formation is regulated by two SOS regulators, RecA and LexA. A mutant in *recA*, in which LexA is uncleavable, hindered biofilm formation in response to a DNA replication inhibitor hydroxyurea treatment (Beloin *et al*., 2004). These observation suggested that the positive role of RecA in biofilm formation. Another study showed that biofilm formation was repressed by the non-cleavable LexA of *P. aeruginosa*, while knocking out *lexA* resulted in a decrease in both normal and stress-inducible biofilm formation (Gotoh *et al*., 2010). These data suggest that the cleavable and non-cleavable LexA and RecA play a role in stress-inducible biofilm formation. In *E. coli*, SOS induction includes the expression of SOS genes *dinI, dinP, dinG, sbmC, recN*, and *sulA*. These genes were overexpressed in *E. coli* biofilms (Beloin *et al*., 2004). Additionally, the SOS genes including *recA, uvrD, uvrC, and recX* were induced in *M. smegmatis* biofilms (Gotoh *et al*., 2010). These SOS genes apparently play roles in biofilm formation since repression of those genes by LexA is expected to lead to biofilm reduction. Collectively, the data indicate that the SOS regulators, RecA and LexA play a role in biofilm formation since the repression of their role via mutation in *recA* or *lexA* and deletion of *lexA* prevent the formation of a biofilm. The link between the SOS system and stress inducible biofilm formation has a significant application in combating bacterial infections. It represents a novel approach to controlling biofilm formation by identifying drugs that modulate the SOS regulators, RecA or LexA.

1.5 The Oxidative Stress System and the SOS Response

The conventional model of how antimicrobials stimulate bacterial cell death is based on essential bacterial cell functions that are blocked by the primary drug-target interaction and whether this inhibition of cellular function is lethal or not (Kohanski *et al*., 2007).

Antimicrobials can be classified based on their systemic effects into bactericidal drugs that lead to bacterial cell death and bacteriostatic drugs that inhibit cell growth (Kohanski *et al*., 2010). Antimicrobial-mediated bacterial cell death is linked to: (i) the formation of DSBs following treatment with inhibitors of DNA gyrase, (ii) the inhibition of DNA-dependent RNA synthesis following treatment with rifamycins, (iii) the suppression of cell wall synthesis and loss of structural integrity following treatment with inhibitors of cell wall synthesis, and (iv) the inhibition of protein synthesis following treatment with inhibitors of protein synthesis (Kohanski *et al*., 2010).

1.5.1 Bactericidal Antibiotics Induce Hydroxyl Radical Formation

Antibiotic-induced cell death is a complicated process that involves specific sequences of events, beginning with the primary effect of the physical interaction between a drug molecule and its specific target in bacteria and terminating with bacterial cell death (Kohanski *et al*., 2010; Wright, 2007). In principle, inhibition of essential cellular function should lead to a sequence of consequences. Recent evidence has pointed out that all antibiotic-induced bacterial cell death involves the same killing mechanism (Kohanski *et al*., 2007). The major classes of bactericidal antibiotics, including β-lactams, aminoglycosides, and quinolones, stimulate the oxidative damage cellular death pathway (Kohanski *et al*., 2007). Despite the difference in their primary physical drug-target interactions, it has been found that all bactericides induce the generation of lethal hydroxyl radicals in both Gram-negative and Gram-positive bacteria (Kohanski *et al*., 2007), ultimately, contributing to cell death by interfering with the tricarboxylic acid (TCA) cycle and iron metabolism (Kohanski *et al*., 2007). However, the oxidative stress response following treatment with various bactericidal compounds has not been studied yet in anaerobic bacteria (Falconer *et al*., 2011). Oxygen (O^2) diffuses into bacterial cells and interacts with biomolecules, particularly, flavoenzymes, which have catalytic redox cofactors within their active sites and readily participate in electron transfer reactions with O_2, producing superoxide ($O^{2\bullet}$), hydrogen peroxide (H_2O_2), and highly destructive hydroxyl radicals ($OH^{\bullet}$) (Dwyer *et al*., 2009). Both $O^{2\bullet-}$ and H_2O_2 can be enzymatically eliminated from the cells by the actions of superoxide dismutases and catalases/peroxidases, respectively, as shown in equations (i) and (ii)

(i) $2O^{2\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$

(ii) $2H_2O_2 \rightarrow 2H_2O + O_2$

In contrast, there are no known enzymes that can eradicate and detoxify $OH^{\bullet}$. The hydroxyl radical is one of the highly deleterious molecules that can indiscriminately oxidize proteins, lipids, and DNA (Dwyer *et al*., 2009; Imlay, 2003) and exhibit cytotoxic or mutagenic effects on bacterial cells (Dwyer *et al*., 2009). Hydroxyl radical formation occurs via the Fenton reaction *in vivo*. The accessible solvent ferrous iron (Fe^{2+}) is oxidized into ferric iron (Fe^{3+}) by H_2O_2 to yield $OH^{\bullet}$ ($H_2O_2 + Fe^{2}+ \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$). To complete the cycle, $O^{2\bullet-}$ reduces unincorporated Fe^{3+}, or it interacts with iron-sulfur cluster-bearing enzymes, resulting in the destabilization of Fe^{2+} and/or release Fe^{2+} through a process known as the Haber-Weiss reaction (Dwyer *et al*., 2009) (Figure 1.8). It appears that Fenton reaction is the most significant contributor to cell death among the reactive oxygen species.

1.5.2 Bactericidal Antibiotics Induce the SOS System and Mutagenesis

Induction of the SOS response is associated with the phenomena of induced mutagenesis and the possibility of blocking the emergence of resistance by inhibiting SOS regulators: RecA or LexA. Considering that SOS induction is efficiently activated by DNA-damaging antimicrobials (Hassett and Imlay, 2007), it is not surprising that the majority of evidence connecting antimicrobial treatments to inducible mutagenesis and acquired resistance has been derived from quinolones studies. Induction of the SOS response by antimicrobials that do not cause direct DNA damage has been reported. One example is β-Lactam drugs that achieve their lethal action by disrupting cell wall integrity and inhibiting cell wall biosynthesis. It has been demonstrated that β-Lactam drugs trigger the SOS system via activation of the DpiAB two-component system (Kohanski *et al*., 2007; Miller *et al*., 2004). Additional studies have shown trimethoprim (a dihydrofolate reductase inhibitor), which is commonly formulated together with sulfamethoxazole (a sulfonamide) as co-trimoxazole and used to stem urinary tract

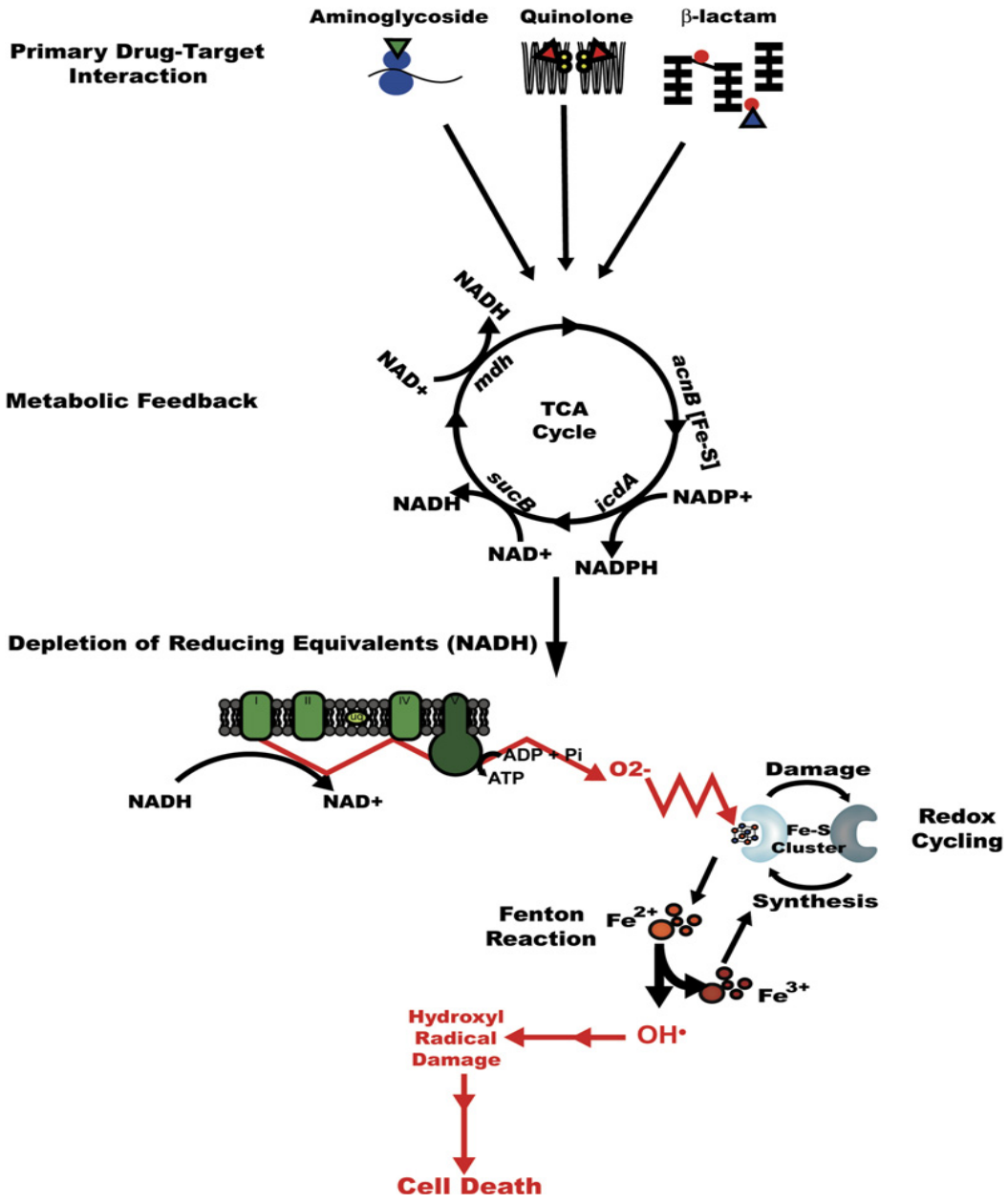

Figure 1.8. Proposed model for the oxidative cell death pathway by bactericidal antimicrobials.

The primary drug-target interactions with the RNA, DNA gyrase, and cell wall biosynthesis by aminoglycoside, quinolone, and β-lactam, respectively, induce oxidation of nicotinamide adenine dinucleotide (NADH) via the electron transport chain, which is dependent upon the TCA cycle. Hyperactivation of the electron transport chain decreases the reducing environment and increases superoxide formation. Superoxide damages iron-sulfur clusters, making ferrous iron available for oxidation by the Fenton reaction. The Fenton reaction leads to hydroxyl radical formation, and the hydroxyl radicals damage proteins, lipids, and DNA, resulting in cell death. The figures was reprinted from Cell, 130, 35, 797-810, Kohanski *et al*, A common mechanism of cellular death induced by bactericidal antibiotics, copyright 2007, with permission from Elsevier.

infections, induces the SOS system (Cirz and Romesberg, 2007). This drug combination applies stress on intracellular pools of deoxyribonucleotides by inhibition of ribonucleotide reductases. This signal may be perceived by the cell as a sign of overwhelming DNA stress (Cirz and Romesberg, 2007).

A recent study has demonstrated that all major classes of bactericidal drugs such as ampicillin, norfloxacin, and kanamycin, stimulate the production of highly deleterious ROS radicals in Gram-negative and Gram-positive bacteria, which ultimately contribute to cell death (Imlay, 2003; Kohanski *et al*., 2007). On the other hand, bacteriostatic drugs fail to stimulate the production of ROS molecules (Kohanski *et al*., 2007). Hydroxyl radicals are ROS molecules, which are extremely toxic and readily damage proteins, membrane lipids, and DNA (Imlay, 2003). Interestingly, it has been shown that following application of only bactericidal drugs, there is an increase in the DNA damage SOS response, where RecA is activated, promoting the autolysis of LexA. This would lead to the expression of the SOS genes (Kohanski *et al*., 2007). The majority of SOS genes expressed first function through the physical repair of damaged DNA. Based on the type and number of lesions, DNA repair may occur via nucleotide excision, base excision, or recombination pathways. The repair mechanisms involve the expression of the late SOS genes, error-prone polymerases (DNA polII, IV, and V) (Cirz and Romesberg, 2007). These polymerases catalyze error-prone DNA synthesis across lesions that cannot be achieved by the normal replicative DNA polymerase, DNA polIII. Expression of pol V is SOS-dependent and its activity is RecA-dependent, while expression of polII and IV is SOS-independent, yet they increase approximately 10-fold upon SOS induction (Babynin, 2004).

The discovery of a correlation between the activation of the SOS response and the common oxidative cellular damage pathway by a diverse set of bactericidal drugs has an important application in the development of more effective antibacterial therapies. More specifically, it indicates that all major classes of bactericidal drugs can be potentiated by inhibition of RecA and consequently the SOS system since the SOS response plays a key part in the repair of hydroxyl radical-induced DNA damage. This may be accomplished by identifying RecA inhibitors.

1.6 Previous Efforts to Develop RecA Inhibitors

Development of antimicrobial resistance is associated in large part with mutations that arise during stress-induced mutagenesis (Cirz *et al*., 2005) and the lateral transfer of genes between organisms (Beaber *et al*., 2004). In both cases, the bacterial RecA protein plays a crucial role as it is involved in DNA recombination and repair processes, including homologous recombination, SOS induction, and recombinational DNA repair (Cirz and Romesberg, 2007). The idea that RecA functions could be modulated by small molecules was previously investigated. All RecA-associated functions require the formation of the active NPF, encompassing multiple RecA monomers, ATP, and ss-DNA (Cox, 2007). Therefore, identifying small molecules that block RecA DNA-binding is a very attractive approach to develop inhibitors for the suppression of SOS-signaling and recombinational repairs, which, consequently, could potentiate the activity of bactericidal antibiotics, and inhibit the evolution and transmission of antibiotic resistance. The NPF formation normally causes ATP hydrolysis, which is an essential step for regulating SOS induction. ss-DNA-dependent ATP hydrolysis represents a valuable tool to detect the suppression of NPF formation (Sexton *et al*., 2010). The abrogation of ATPase activity would serve as a diagnostic indicator for RecA inhibition. To date, the number of cell-permeable natural or synthetic RecA inhibitors is extremely low. The only known inhibitors include native bacterial proteins, such as RecX (Lusetti *et al*., 2004a), DinI (Lusetti *et al*., 2004b), RdgC, and UvrD (Cox, 2007). These proteins play critical roles in regulating RecA functions. The RecX protein binds to the growing NPF ends and terminates filament growth (Lusetti *et al*., 2004a). DinI is one of the early expressed SOS genes, and functions by destabilizing the RecA filament in a concentration dependent manner; however, it would not be expected that an early SOS gene would terminate SOS induction. Moreover, this protein is encoded by a single copy on the bacterial chromosome, so it is not possible to reach a high concentration within the cells. At a low concentration, seen when it is expressed from a single copy in the bacterial chromosome, DinI's main activity is inhibition of the auto-cleavage of UmuD protein, delaying the activation of the mutagenic translesion synthesis, mediated by PolV. However, LexA cleavage is not affected, allowing the rest of SOS function to take place (Cox, 2007; Lusetti *et al*., 2004b). At the low level, the role of DinI in stabilizing the RecA filament requires further investigation. The RdgC protein competes with RecA to bind to DNA, specifically, to a ds-DNA. Additionally, if RdgC binds to the homologous DNA duplex bound

to the RecA-ss-DNA-ATP filament, it blocks stand exchange. When RecA functions come to the end and the RecA filament is no longer needed, UvrD, a member of the helicases, removes the filament from the DNA (Cox, 2007). In contrast, a variety of synthetic RecA inhibitors have been generated. Mechanisms by which these molecules inhibit RecA activities are not yet clear; however, some of the molecules, including, nucleotide analogs (Wigle and Singleton, 2007), α-helical peptides (Cline *et al*., 2007), and polysulfated naphthyl compounds (Lee *et al*., 2005) showed the ability to abrogate RecA ATPase activity, while others, such as metal cations, displayed the ability to aggregate RecA. All these small molecules have proven the principle that RecA functions can be selectively controlled by synthetic small molecules.

1.7 Phthalocyanine Drugs as Putative RecA Inhibitors

Phthalocyanine (PcTs) drugs are porphyrin-like molecules that possess characteristics similar to tetrapyrrole compounds (Caughey *et al*., 2007; Caughey *et al*., 1998; Priola *et al*., 2003; Priola *et al*., 2000). Many PcTs compounds are commercially used as pigments and dyes (Priola *et al*., 2003). Tetrapyrrole characteristics make PcTs compounds attractive as potential inhibitors since PcTs molecules contain hydrophobic aromatic rings and can be synthesized with sulfonate groups, making these molecules water soluble. More than 60 different kinds of metal atoms can be inserted into the central ring. Interestingly, tetrapyrroles can bind strongly and selectively to proteins and affect changes in protein conformation (Caughey *et al*., 2007; Caughey *et al*., 1998; Priola *et al*., 2003; Priola *et al*., 2000), potentially critical properties of an effective inhibitor. Recent studies have shown that certain PcTs molecules are useful as drugs against transmissible spongiform encephalopathy (TSE) diseases, such as scrapie in sheep, Creutzfeldt-Jakob disease (CJD) in humans, chronic wasting disease (CWD) in deer and elk, and bovine spongiform encephalopathy (BSE) (Caughey *et al*., 2007; Caughey *et al*., 1998; Priola *et al*., 2003; Priola *et al*., 2000). The pathological factor in these diseases is the accumulation of an abnormal form of the host prion protein, in the central nervous system. PrP-resistant (PrP-res) protein is derived from its normal protease-sensitive isoform, PrP-sensitive (PrP-sen). Normal PrP-sen, a glycoprotein that is expressed on the cell surface in a wide variety of tissues, is both soluble and sensitive to digestion with proteinase-K. The PrP-sen-to-PrP-res conversion involves changes in the conformation of PrP-sen. Significant amounts of data have demonstrated that PrP-res formation plays a major role in TSE disease pathogenesis. Recently, it has been

shown that several cyclic PcTs molecules inhibit PrP-res formation *in vitro* and significantly delay TSE disease *in vivo*. These PcTs molecules have several properties that work both prophylactically and therapeutically (Caughey *et al*., 2007; Caughey *et al*., 1998; Priola *et al*., 2003; Priola *et al*., 2000). The conversion of PrP-sen to PrP-res appears to be similar to the pathogenic processes of amyloid formation associated with a variety of other diseases including Alzheimer's disease and Type 2 diabetes. Thus, it is possible that these cyclic PcTs molecules might serve as inhibitors not only of PrP-res formation, but also of other types of amyloid formation (Lee *et al*., 2004). Another potential use of PcTs molecules is antimicrobial photodynamic therapy (APDT). It is a relatively novel therapeutic strategy, which is expected to be useful in the treatment of localized infections. APDT is a treatment that utilizes a combination of light, a chemical known as a photosensitizer (PS) that could be activated by light, and oxygen to achieve a cytotoxic effect. The process starts with delivering light of the appropriate wavelength to the PS molecule to bring it to its excited singlet state, which subsequently crosses to a more stable, lower-energy triplet state. The interaction between the PS excited states and the endogenous oxygen in the proximity of the target cells provides the cytotoxic effects through the production of ROS inside the microbial cells. APDT has high target specificity since the PS is localized in the microorganisms without affecting the surrounding tissues or cells (Giuliani *et al*., 2010). According to the principal of photodynamic therapy (PDT), PcTs molecules have shown the ability to cure cancer (Chan *et al*., 1989; Kessel, 1992). The broad spectrum of PcTs molecule's roles in biological system suggests that these molecules will play an important role in disease treatment.

RecA and its homologues play a critical role in DNA strand exchange process between an ss-DNA and a homologous ds-DNA. Recombinase proteins showed huge differences between the bacterial and nonbacterial (less than 30% sequence identity) in their primary structures. However, the electron microscopic and crystallographic data have revealed noticeably similar filamentous assemblies. Three functionally important sites are remarkably conserved in these filamentous structures. These sites are recruitment and polymerization, ATP binding, and DNA binding (Lee *et al*., 2005). In theory, targeting these functionally important sites can inhibit these strand exchange proteins.

Candidate RecA inhibitors examined in this project were available from commercial small anionic aromatic libraries, specifically phthalocyanine molecules coordinated with different metal ions (Figure 1.9). In an attempt to obtain a structure of the post-ATP hydrolysis conformation of the RecA homologue MvRadA, the MvRadA was co-crystallized in the presence of ADP and sodium tungstate (Na_2WO_4), a phosphate analogue (Lee *et al*., 2005; Li *et al*., 2009). A cluster of 12 tungsten atoms was unexpectedly located by outstanding anomalous scattering signals near DNA binding loops (L1 and L2). The metatungstate Na_2WO_4 was a potent inhibitor of ATPase and strand exchange activities of the MvRadA. The tungsten cluster appears to be bound between the DNA-binding loops, anchoring the protein in its inactive conformation (Lee *et al*., 2005; Li *et al*., 2009). The results suggest that small molecules could competitively inhibit DNA binding by RecA. A follow-up study showed that Na_2WO_4 was unable to abrogate RecA activity in living cells. In order to advance our goal of identifying molecules that are cell permeable and able to modulate RecA's biological activity, we screened commercially available anionic, aromatic molecules that inhibit RecA ATPase activity. Based on this screening, two drugs PcTs compounds, Fe-PcTs and 3,4′ Cu-PcTs, inhibiting RecA ATPase activity were identified (Geyer and Luo personal communication, unpublished data). Abrogating ATPase activity is a useful tool to indicate the inhibition of NPF and consequently the suppression of RecA activity. As a result, these putative RecA inhibitors may be good candidates to potentiate antibiotic efficacy and reduce the acquisition of resistance.

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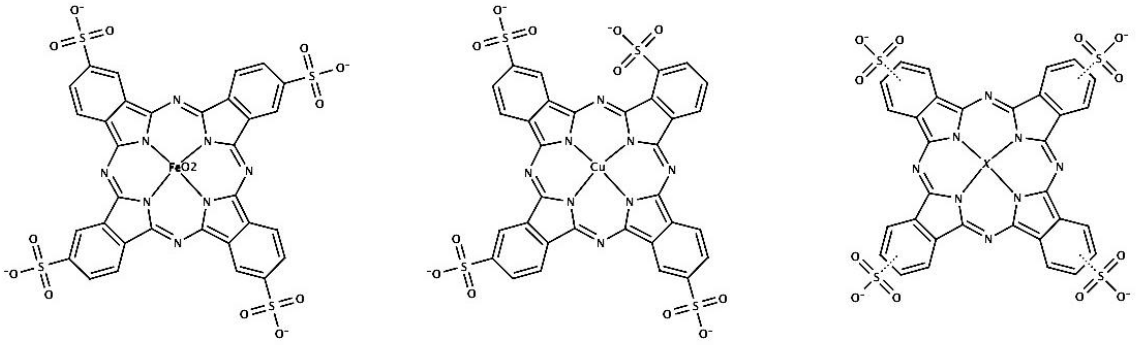


Fe-PcTs 3,4′ Cu-PcTs *X* = Al, Zn, Ni, Cu, H

Figure 1.9 Structures of phthalocyanine tetrasulfonic acid molecules coordinated with different metal ions.

Iron(III) phthalocyanine-4,4’,4’’,4’’’-tetrasulfonic acid (Fe-PcTs) (i), copper phthalocyanine-3,4’,4’’,4’’’-tetrasulfonic acid (3,4′Cu-PcTs) (ii), aluminum(III) phthalocyanine tetrasulfonic acid (Al-PcTs) (iii), zinc(II) phthalocyanine tetrasulfonic acid (Zn-PcTs) (iii), nickel(II) phthalocyanine tetrasulfonic acid (Ni-PcTs) (iii), copper phthalocyanine tetrasulfonic acid (Cu-PcTs) (iii), and phthalocyanine tetrasulfonic acid (Apo-PcTs) (iii).

CHAPTER 2

SPECFIC AIMS OF THIS PROJECT

RecA is a very attractive target for potentiating antibiotic chemotherapy since blocking RecA activity would prevent induction of the SOS response and block antibiotic-induced DNA repair and mutagenesis pathways. We hypothesized that aromatic anionic molecules may contain members that competitively bind to RecA in the DNA binding site. Blocking RecA DNA-binding should inhibit SOS-signaling and recombinational repair, emphasizing the ability of RecA inhibitors to potentiate the activity of bactericidal antibiotics and provide a mechanism to prolong the life span of existing and newly developed antibiotics. We envision that RecA inhibitors will be part of an antibiotic "cocktail" that enhances the activity of antibiotics and blocks of the emergence of resistance, which will ultimately prolong antibiotic lifespan.

The overall objective of this study was to identify molecules that could modulate RecA activity and eventually block the SOS induction. To achieve this goal, a group of experiments were performed to:

2.1. Compare the CFX potentiating activity of PcTs molecules coordinated with different metal ions.

2.2. Assay the ability of Fe-PcTs molecules to enhance the activity of bactericidal and bacteriostatic antibiotics.

2.3. Evaluate the ability of Fe-PcTs to abrogate RecA biological activities.

2.4. Determine whether Fe-PcTs blocks the development of CFX resistance in *in vitro* and *in vivo* assays.

CHAPTER 3
MATERIALS AND METHODS
3.1 Standard Laboratory Methods

3.1.1 Bacterial strains

The strains used in this study were *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *S. aureus* ATCC29213, *E. faecalis* ATCC29212, and *E. coli* SS996. All the clinical isolates were kindly provided by Dr. Joseph Blondeau. The SS996 strain is an *E. coli* K-12 with an SOS inducible GFP reporter gene and was obtained from Susan Rosenberg (McCool *et al*., 2004).

3.1.2 Storage of the Bacterial Isolates and Growth Conditions

S. aureus, *P. aeruginosa*, and *E. faecalis* isolates were streaked for colonies on tryptic soy agar (TSA) plates and incubated at 37°C for 18 to 24 hours. *E. coli* ATCC25922 and *E. coli* SS996 were cultured on Luria-Bertani (LB) plates and incubated at 37°C for 18 to 24 hours. All cultures were stored at -80°C in LB broth with 30% glycerol.

Five major biological aspects (growth and survival, SOS induction, hydroxyl radical production, cell elongation, and biofilm formation) were compared between untreated exponential-phase bacterial cultures and cultures treated with the examined antibiotics at given concentrations or combinations of each of antibiotics and PcTs molecules. Briefly, the *S. aureus*, *P. aeruginosa*, *E. faecalis* cultures were grown in 25 mL MHB while the *E. coli* ATCC25922 and the *E. coli* SS996 were grown in 25 mL LB broth in 250 mL flasks in a light insulated shaker, and then all were diluted 1:500 in 25 mL MHB or LB according to the strain type. Cultures were grown to an optical density (OD_{600}) of approximately 0.3, measured using the spectrophotometer (Thermo scientific, Genesys 20). PcTs molecules, antibiotics concentrations, length of treatment were determined as a result of optimization experiments (data not shown) unless it is stated otherwise. In all experiments, PcTs molecules were added to bacterial cultures at a concentration of 25 µM at early exponential phase and incubated for three hours. Following this, the antibiotic alone, Fe-PcTs, or both were added to the cultures and incubated under the appropriate conditions for a specific period of time according to the assay.

3.1.3 Susceptibility Study

3.1.3.1 Broth microdilution assay

Minimum inhibitory concentration (MIC) values were determined by the broth microdilution test for all organisms in accordance with the Clinical Laboratory and Standards Institute (CLSI) guidelines ((CLSI), 2004). Ninety-six well flat bottom microtitre plates (Falcon) were filled with 100 μL of MHB in each well of columns 2-to-12. An antimicrobial agent was serially diluted down the panel with the wells of column 1 containing the highest concentration and the wells of column 11 having the lowest concentration of the drug. The wells of column 12 were used as a growth control and, therefore, did not receive any drug.

All the clinical isolates were cultured onto TSA+5% sheep red blood cells (SRBC) plates and incubated under appropriate conditions for 18 to 24 hours at 37°C. Each isolate was standardized to a 0.5 McFarland (~1.0 X 10^8 cfus/mL) using a colorimeter (bioMérieux Vitek, Inc). The bacterial suspension was then diluted 1/100 with MHB (~1.0 X 10^5 to 10^6 cfus/mL). One hundred microlitres of diluted cells were added to each well on the plate, resulting in a final volume of 200 μL. Purity of the bacterial suspension was determined by plating a sample onto fresh TSA + 5% SRBC plates. The microtitre panels and purity plates were then incubated under the appropriate conditions in ambient air for 18 to 24 hours at 37°C. Following incubation, the lowest drug concentration at which there was no visible growth of the organism was recorded as the MIC. To ensure organism viability, the wells in column 12 (growth control) were examined. The appropriate ATCC strains were used as controls for susceptibility testing each time a susceptibility test was performed to validate the results based on the current CLSI breakpoints for each ATCC strain ((CLSI), 2004).

3.1.4 Synergy Testing

3.1.4.1 Synergy studies using the checkerboard assay

A two-dimensional checkerboard microdilution technique was used to characterize interactions between antibacterial agents and PcTs molecules. The two-dimensional microdilution checkerboard plates were prepared by dispensing a serially diluted antibacterial agent into the *x*-axis and a PcTs drug in the *y*-axis into a 96-well microtitreplate (Tateda *et al*.,

2006). Drugs were diluted in serial twofold dilutions, and concentrations ranged from those for several wells below the MIC to several wells above the MIC of each drug for each of the clinical isolates. Ninety-six well flat-bottom microtitre plates (Falcon) were filled with 50 μL of MHB in each well of columns 2-to-12. An antimicrobial agent was serially diluted down the panel with the wells of column 1 containing the highest concentration and the wells of column 11 having the lowest concentration of the drug. In the case of the PcTs molecule, it was serially diluted down the panel with the wells of row A containing the highest concentration and the wells of row G having the lowest concentration. The wells of column 12 received only serial concentrations of the PcTs molecule while the wells of row H solely received serial concentrations of the antimicrobial agent. Well H of column 12 was used as a growth control and, therefore, received no drug. Purity of the bacterial suspension was confirmed by plating each sample onto a fresh TSA plate containing 5% SRBC.

Inocula of the clinical isolates were prepared similarly to those for susceptibility testing with single drugs. Following inoculation, microplates and purity plates were incubated in ambient air for 18 to 24 hours at 35 to 37°C. At least three experiments were performed for each interaction. The MIC of the antibiotics tested in combination was defined as the lowest concentration of the tested antibiotic in the presence of PcTs that showed no growth.

3.1.4.2 Time-kill curve experiments

In order to demonstrate the lethal activity of antibacterial agents and PcTs molecules, the rate of killing by fixed concentrations of both drugs under controlled conditions was applied. This rate was determined by measuring the number of viable bacteria at various time intervals. The obtained data were used to draw the time-kill curve. The *P. aeruginosa* ATCC27853, *S. aureus* ATCC29213, and *E. faecalis* ATCC29212 were streaked onto TSA + 5% SRBC plates using a sterile wooden stick while the *E. coli* ATCC25922 cells were cultured onto LB plates. All plates were incubated under ambient conditions at 37°C for 18 to 24 hours. Following incubation, bacterial growth was standardized to a 0.5 McFarland (~1.0 X 10^8 cfus/mL) using a colorimeter. The bacterial suspension was then diluted 1/100 with MHB (~1.0 X 10^5 - 10^6 cfus/mL), exposed to either the antibiotic singly, Fe-PcTs, or both at the given concentrations in Table 3.1, and incubated for 48 or 72 hours at 37 °C with shaking (250 rpm). One hundred

microlitre aliquots of the cultures were taken at 0, 24, 48, and 72 hours. The aliquots were serially diluted in the appropriate 1x phosphate saline buffer (PSB), and applied to TSA + 5% SRBC or LB plates in triplicate and incubated at 37°C for 18 to 24 hours. Following incubation, the number of colonies on each plate was recorded. Aliquots were diluted 1/10 folds, so that viable counts had a countable number of colonies (20–200 cfus/plate). Positive controls were used which contained similar cell and solvent concentrations. Results were interpreted by the effect of the combination treatment in compared with the active single drug alone (Lin *et al*., 2005).

Table 3.1. Summary of the antimicrobial agent concentrations and PcTs molecules used in the time-kill curve assays

Bacterial strain	**Drug**	**Concentration (µM)**
E.coli ATCC25922	Ciprofloxacin (CFX)	0.04
	Kanimycin (KAN)	43
	Ampicillin (AMP)	40
	Chloroamphenicol (CAM)	46
	Tetracycline (TET)	21
	Spectinomycin (SPECT)	808
	Fe (III) phthalocyanine-4,4′,4″,4‴-tetrasulfonic acid (Fe- PcTs)	25
	Cu (II) phthalocyanine-3,4′,4″,4‴- tetrasulfonic acid (3,4′-Cu-PcTs)	25
	Cu (II) phthalocyanine tetrasulfonic acid (Cu PcTs)	25
	Ni (II) phthalocyanine tetrasulfonic acid (Ni-Pcts)	25
	Al (II) phthalocyanine tetrasulfonic acid (Al-PcTs)	25
	Zn (II) phthalocyanine tetrasulfonic acid (Zn-PcTs)	25
	Phthalocyanine tetrasulfonic acid (Apo-PcTs)	25
S. aureus ATCC29213 *E. feacilis* ATCC29212 *P. areuginosa* ATCC27853	CFX Fe-PcTs	6.5 25

3.1.5 Inhibition of RecA Activities

3.1.5.1 Inhibition of RecA-dependent SOS activation by Fe-PcTs

The ability of Fe-PcTs to inhibit the RecA-dependent activation of the SOS response was assessed using the *E. coli* SS996 having the *sulA* SOS promoter fused to the green fluorescent protein (*gfp*) reporter gene, which was inserted at *attλ* on the chromosome (McCool *et al*., 2004). *E. coli* cells were cultured on LB plates and incubated under appropriate conditions for 18 to 24 hours at 37°C. Two to three colonies were inoculated into 25 mL of LB broth and incubated for 18 to 24 hours at 37°C with shaking (250 rpm). Twenty five LB broth cultures were inoculated with saturated overnight cultures of bacterial cells to an OD_{600} of 0.3 in the presence of CFX, Fe-PcTs, or both at a concentration of 2.5 and 25 µM, respectively. Cultures were grown in a shaking incubator at 37°C for 180 minutes. Samples were taken immediately before the addition of the drugs (time zero) and then every hour for three hours. At each time point, approximately 10^6 cells were collected, washed once, and resuspended in filtered PSB (pH 7.2) prior to measurement. All data were collected using a flow cytometer (Beckman Coulter, Inc.) with a 488 nm argon laser and a 515–545 nm emission at low flow rate. Flow data were processed and analyzed with FLOWJO (Tree Star, Inc.).

3.1.5.1.1 Hydroxyl radical induction by combination therapy of a bactericidal agent and Fe-PcTs

We compared the production of hydroxyl radicals in cells treated with CFX to cells treated with both CFX and Fe-PcTs. *E. coli* ATCC25922 cells were cultured on LB plates and incubated under appropriate conditions for 18 to 24 hours at 37°C. Two to three colonies were inoculated into 25 mL of LB broth and incubated for 18-24 hours at 37°C with shaking (250 rpm). Twenty five millilitres of LB broth was inoculated with saturated overnight cultures of bacterial cells to an OD_{600} of 0.3. *E. coli* cells in their exponential-phase were treated with CFX or Fe-PcTs, or both, at 40 nM and 25 µM, respectively, and incubated for 180 minutes at 37°C with shaking (250 rpm). To detect hydroxyl radical formation, we used the fluorescent reporter dye 30-(p-hydroxyphenyl fluorescein (HPF, Invitrogen) at a concentration of 5 µM as described previously (Kohanski *et al*., 2007). To ensure that light-induced redox cycling of antibiotics was not a confounding factor, all experiments were performed in light-insulated shakers (Martin *et*

al., 1987; Umezawa *et al*., 1997). Samples were taken immediately before the addition of drugs (time zero) and then every hour for three hours. At each time point, approximately 10^6 cells were collected, washed once, and resuspended in filtered PSB (pH 7.2) prior to measurement. All data were collected using a flow cytometer (Beckman Coulter, Inc.) with a 488 nm argon laser and a 515–545 nm emission at low flow rate. Flow data were processed and analyzed with FLOWJO (Tree Star, Inc.).

3.1.5.2 Effect of CFX and Fe-PcTs on *sulA* induced-filamentation morphology

The effect of CFX and Fe-PcTs on cell morphology was studied by direct observation of treated cultures following staining with basic Gram stain. One hundred microliters of *E. coli* SS996 with the GFP reporter gene ($1x10^5$ cfus/mL), calculated from OD_{600} nm measurements, were added to 5 mL of LB broth. Bacterial cells were incubated with CFX or Fe-PcTs, or both (2.5 and 25 µM, respectively), for 180 minutes at 37°C with shaking (250 rpm). After three hours of treatment, 2 mL from each culture was used to prepare samples. These samples were stained with Gram stain and viewed by a Nikon Eclipse E400 compound microscope (Laboratory Optical Service, Inc.) at 100x power magnification. Photographs were taken 3 hours post treatment.

3.1.5.3 Biofilm formation suppression assay in 96 well Plates

To study CFX and Fe-PcTS effects on biofilm formation, a 96 well plate assay was used with lids (Falcon) as closures. One hundred microliter of *E. coli* ATCC25922 cells ($1x10^5$ cfus/mL) quantified from OD_{600} nm measurements were added to each well. Fixed concentrations of CFX and Fe-PcTs (40 nM and 25 µM, respectively) were applied. Plates were covered and incubated at 37°C for 18 to 24 hours under the appropriate conditions. To examine the biofilm formation by CFX alone, Fe-PcTs, or both, wells were washed 3 times with 150 µL of sterile filtered PSB, and then stained with 120 µL of 0.1% crystal violet (Fisher) for 20 minutes. The crystal violet stain was aspirated and wells were washed again 3 times with 150 µL of sterile PSB. Plates were allowed to air dry and the dye bound to adherent cells was resolubilized with 150 µL of 30% (v/v) glacial acetic acid (Fisher) per well. Plates were then placed on a rotational shaker for 20 minutes and the OD_{590} nm of each well was measured using a 96-well plate reader (Molecular Devices).

3.1.6 Inhibition of Antibiotic Resistance Induced by the SOS Response

3.1.6.1 *In vitro* CFX-resistance assay

The ability of Fe-PcTS to abrogate the emergence of CFX resistant mutations was assessed. The mutation rate of CFX was calculated *in vitro* in the presence and absence of Fe-PcTs and calculated by using the fluctuation test of Luria and Delbruck (DELBRUCK, 1943).

3.1.6.1.1 Inoculum preparation and resistance testing

E. coli ATCC25922 cells were cultured on LB plates using a sterile wooden applicator stick and incubated in ambient air at 37°C for 24 hours. After overnight incubation, a single colony was grown in 5 mL of LB broth and incubated for 18-24 hours at 37°C with shaking (250 rpm). The overnight diluted culture was inoculated into 25 flasks containing LB broth or LB with 25 µM Fe-PcTs. The twenty-five independent cultures were grown for 24 hours without selective pressure (CFX). Viable cell counts in these cultures were determined by plating serial dilutions onto LB agar plates. For determining resistance in the *E. coli* ATCC25922, 100 µL from each culture (approximately 10^8 cells) was plated in duplicate on LB plates, containing CFX (40 nM) in the presence or absence of Fe-PcTs (25 µM). Three additional 100 µL aliquots from three cultures were also plated on the same media to be used in the ‘‘survival’’ assay (see section 3.1.6.1.2). At 24 hour intervals over 10 days, visible colonies were marked, counted, and removed from the plates with a sterile wooden applicator. Isolated clones were grown in LB in a 96 well plates for 18-24 hours at 37°C with shaking (250 rpm). Each of these isolated clones was tagged with specific number and the day of isolation. All the isolated clones were stored at -80 °C in LB broth with 30% glycerol.

3.1.6.1.2 Survival assay

Every 24 hours, in parallel with the resistance assay, agar plugs among visible colonies were excised from plates used to assay cell viability. These plugs were removed and homogenized in M9 buffer. Dilutions were plated out in duplicate on LB plates to determine the total number of viable cells (CFX-sensitive cells present as a function of time) and on LB plates containing 40 nM CFX to determine if any CFX-resistant cells remained after excision (Cirz *et al*., 2005). The number of viable cells per plate was estimated for each plate and corrected for

CFX-resistant cells that remained after excision, and the counts of viable cells from the three survival plates were averaged.

To determine the number of viable cells present as a function of time in the presence of Fe-PcTs, a similar procedure was conducted with minor modifications. Three LB plates, containing CFX and Fe-PcTs (40 nM and 25 µM, respectively) were plated with 10^8 cells for use in the survival assay. Dilutions were plated out in duplicate on LB, LB, containing CFX (40 nM), or LB with CFX (40 nM) and Fe-PcTs (25 µM). The number of viable cells per plate was determined for each plate and corrected for CFX-resistant cells that remained after excision, and the counts of the viable cells from the three survival plates were averaged.

3.1.6.1.3 Reconstruction assay (low density)

To determine whether colonies isolated after plating on CFX were formed as a result of post-exposure mutation or as a result of mutation prior to the drug exposure, liquid cultures of LB media were inoculated with CFX-resistant clones isolated during the resistance assay and grown to saturation overnight. Overnight CFX-resistant cultures were diluted and inoculated with CFX-resistant clones stocked during the resistance assay in 96 well flat bottom plates. Cultures were replicated in duplicate using VP 408FH Replicator (V&P Scientific) on LB plates to confirm viability and LB plates containing CFX (40 nM) to confirm resistance. Resistant clones that were isolated in the presence of Fe-PcTs were plated out on LB, LB containing CFX and Fe-PcTs (40 nM and 25 µM, respectively), and LB containing CFX alone (40 nM). Clones that were resistant before exposure were defined as those that formed colonies on the CFX-containing media in the same number of days in the reconstruction assay as they had in the original resistance assay. On the other hand, clones that mutated after exposure to CFX were defined as those that formed colonies earlier than in the original resistance assay (Cirz *et al*., 2005). An additional reconstruction assay (high density) was employed to confirm results of pre- and post-exposure mutants.

3.1.6.1.4 Reconstruction assay (high density)

To recreate the conditions of the *in vitro* CFX-resistance assay and to determine if the presence of competing cells (CFX-sensitive cells) would affect colony regrowth time, the

reconstruction assay was performed in the presence of competing CFX-sensitive cells. Approximately 100 cfus of the CFX-mutant clones were plated on LB, containing CFX (40 nM) in the presence of ~10^8 CFX-sensitive cells. Five percent of randomly selected CFX-resistant clones stored during the resistance suppression assay in the absence of Fe-PcTs were analyzed, while all CFX-resistant clones isolated in the presence of Fe-PcTs were examined.

3.1.6.1.5 Calculation the mutation rate for CFX-acquired resistance

Mutation rate was defined as the number of CFX-resistant mutants per viable cell that evolved as a function of time. Those mutations represent only surviving cells that confer resistance to the drug. The mutation rate after exposure to ciprofloxacin was determined as the ratio of resistant colonies on a particular day to the number of viable cells present at the time the cells became resistant. Based on the assumption that a colony takes two days to form, the viable cell count was calculated two days prior to colony formation, accounting for both the actual time required for colony growth and the time required to turn over any residual CFX-sensitive protein, i.e., phenotypic lag (Cirz *et al*., 2005). The mutation rate was determined as the average of the mutation rate from the third day to the eighth day of incubation.

3.1.6.2 *In vivo* murine thigh infection model

A standard mouse thigh infection model described by Vogelman *et al*., was adapted (Vogelman *et al*., 1988) to examine the ability of Fe-PcTs to block CFX-resistance and to potentiate CFX activity *in vivo*. Female outbred Swiss white mice (CD1) were purchased from the Animal Resource Center of the University of Saskatchewan. These mice were six to eight week-old and specific-pathogen-free, weighing between 25 and 35 grams. Nine mice were used per group. Mice were rendered neutropenic (neutrophil counts less than 100/mm^3) by intraperitoneal injection with 150 mg/kg cylcophosphamide (Sigma) four days before infection and 100 mg/kg cyclophosphamide 24 hours before infection. This regimen has previously shown ability to induce neutropenia in this model for more than seven days (Andes and Craig, 2007).

3.1.6.2.1 Inoculum preparation and infection

The pathogenic *E. coli* ATCC25922 cells were cultured onto LB plates and incubated at 37°C for approximately 24 hours. After overnight incubation, a single colony was grown in five mL LB and incubated for 18-24 hours at 37 °C with shaking (250 rpm). LB broth cultures inoculated from freshly plated bacteria were grown to logarithmic phase (OD_{600} of approximately 0.3), and diluted 1:1000 in LB broth. Thigh infections were produced by injecting 0.05 mL volumes (approximately 10^6 cfus) of diluted broth cultures into halothane-anesthetized mice.

3.1.6.2.2 Administration of CFX and Fe-PcTs

A total of three treatment regimens were performed: (i) nine infected mice were given subcutaneous injections of 1 mg/kg CFX (CFX injectable USP, Hospira) alone every 24 hours for three days, (ii) nine infected mice received subcutaneous injections of 1 mg/kg CFX and interperitoneal injection of 10 mg/kg Fe-PcTs every 24 hours for three days, and (iii) nine mice received Fe-PcTs 24 hours pre-infection and then two hours after infection (defined as time zero) mice were given subcutaneous injections of 1 mg/kg CFX with interperitoneal injections of 10 mg/kg Fe-PcTs every 24 hours for three days.

3.1.6.2.3 Recovery of the infected thighs

After 48 and 72 hours, the skin was separated from the infected thigh by blunt dissection using sterilized forceps and the infected thigh was cut, washed with sterile saline, and collected in a tube with two mL fresh LB. Thighs were homogenized with conical tissue grinder (VWR international). Serial dilutions of the homogenates of infected thighs were plated onto LB plates and LB plates containing CFX (58 nM). MICs for CFX resistant clones were determined by standard microdilution methods as described previously (Andrews, 2006). The MICs of post-exposure to CFX mutants for *E.coli* were determined by examining ten clones isolated from LB agar plates at 48 and 72 hour time points.

3.1.6.3 DNA isolation, amplification and nucleotide sequence determination for recovered ciprofloxacin resistant mutatnts of the *E. coli* ATCC25922

Mutants of the *E. coli* ATCC25922 that were recovered and maintained from the resistance assay plates *in vitro* and *in vivo* were randomly selected. Ten CFX-resistant clones were cultured onto LB plates containing CFX (40 nM) and incubated at 37°C for approximately 24 hours. After overnight incubation, a single colony was grown in 5 mL LB and incubated for 18-24 hours at 37°C with shaking (250 rpm). Cells were centrifuged for 15 minutes and washed with PSB (pH 7.2) three times for 10 minutes. Following a third centrifugation step, the supernatant was used to create a bacterial suspension in TE buffer DNA isolation was done according to the manufacturer's recommendations (BioBasic).

3.1.6.3.1 Primer preparation and storage

The nucleotide sequences of the quinolone-resistance-determining regions (QRDR) of *gyrA* and *parC* were synthesized with an automated DNA sequencer at the Plant Biotechnology Institute, National Research Council of Canada. A DNA fragment of 648 bps from nucleotide 24 to 671 of the *gyrA* gene was obtained based on the procedure of (Oram and Fisher, 1991). *gyrA* is the most consistent location of ciprofloxacin resistance mutations in Gram-negative bacteria (Vila *et al*., 1994). To amplify *parC*, a DNA fragment of 395 bps from nucleotide 115 to 509 was obtained according to the procedure described previously (Vila et al., 1996). Primers were synthesized by Integrated DNA Technologies. Each primer set was made as a stock 100 μM concentration using TE buffer. Primers were stored in aliquots at –20^{o}C.

Table 3.2. Primers used to amplify and sequence *gyrA* and *parC* genes

Primer name	Sequence 5'-'3	Reference
gyrA-Forward	TACACCGGTCAACATTGAGG	(Oram and Fisher, 1991)
gyrA-Reverse	TTAATGATTGCCGCCGTCGG	(Oram and Fisher, 1991)
parC-Forward	AAACCTGTTCAGCGCCGCATT	(Vila *et al*., 1996)
parC-Reverse	GTGGTGCCGTTAAGCAAA	(Vila *et al*., 1996)

3.1.6.3.2 Analysis of PCR products

PCR reactions were performed in 50 µL volumes containing 0.4 µM of each primer, Buffer 1X, High Fidelity Platinum *Taq* Polymerase (Invitrogen), 1.5 mM MgSO4, 0.2 mM of each dNTP, 1 unit of Platinum *Taq* DNA Polymerase High Fidelity, and 4 µL of template DNA. All reactions were performed in a DNA thermocycler (BioRad); thirty cycles were used for each reaction, with the following temperature profiles: two minute denaturation step at 94^{o}C and each cycle consisted of a 94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute.

After the PCR was completed, products were visualized on an agarose gel at 1% (w/v) in order to verify amplification of the expected DNA segment of interest. For visualization of DNA, samples were mixed with loading buffer 6X (50% glycerol, 0.2M EDTA pH 8.3, and 0.05% bromophenol blue) to a final concentration of 1X, and 5 µL of the mixture were added per lane for PCR products. Six microlitres of tracking dye were added to each PCR reaction tube. For the marker, two µL of 1000 bp ladder (Sigma) were added to 12 µL of sterile distilled water and 4 µL of tracking dye. Samples were resolved on the agarose gel at 120 volts for 30 to 40 minutes in SB Buffer (5 mM disodium borate decahydrate, pH 8.0), then visualized and photographed using a UV light Transilluminator (BioRad). Agarose gels consisted of 0.5 to 1.5% (w/v) agarose (ultrapure agarose, Invitrogen), 1X SB Buffer, and 0.5 µg/mL ethidium

bromide. For purification of PCR products, a QIAquick kit from QIAgen was used according to the manufacturer's recommendations.

After PCR purification of DNA fragments, the same forward primers of *gyrA* and *parC* were used to sequence. DNA sequencing was performed using an automated gene sequencer at the Plant Biotechnology Institute, National Research Council of Canada DNA was diluted to 0.05 μg/μL, and primers were provided at a concentration of 3.2 pmol/μL.

3.1.7 Statistical Analysis

Unless otherwise noted, data was reported as the mean ± standard deviation. *P*-values were calculated using a two-tailed t-test with Prism 4.0c for Macintosh (Graphpad).

CHAPTER 4

RESULTS

4.1 Inhibition of RecA Activity

Bacterial RecA protein is an attractive target in the treatment of bacterial infections. Bacterial RecA is a main player in processes involved in repairing DNA damage or stalled replication forks (Thi *et al*., 2011). RecA and one of its downstream targets, LexA, control processes that lead to stress-induced mutations (Janion, 2008) and horizontal gene transfer (Beaber *et al*., 2004). In this manner, bacteria play a very active role in inducing stress-inducible mutations or adaptive mutations in their genomes in response to certain antibiotics (Cirz and Romesberg, 2007; Riesenfeld *et al*., 1997). Therefore, we hypothesized that an effective approach to combat the dramatically increasing number of resistant pathogens is to identify inhibitors for RecA, which could be utilized as an adjuvant to the current or novel antibiotics. These adjuvants could function as co-antibacterial agents that prevent the development and acquisition of genes conferring drug resistance. This hypothesis was based on previous studies that showed that deletion of the *recA* gene prevents mutations (Defais *et al.*, 1971), RecA–dependent repair mechanisms result in acquired resistance to CFX and Δ*recA* strains are more susceptible to cell killing by CFX than the wild type strains (Cirz *et al*., 2005). Furthermore, it has been shown that a great number of DNA damaging antibiotics, such as CFX, mitomycin C, and naldixic acids and an RNA polymerase inhibitor rifampcin have a stronger effect on Δ*recA* cells than on wild type cells (Singleton and Hill., 2010). It has been shown that bactericidal antibiotics, which induce hydroxyl radical production, also induce the SOS response simultaneously (Kohanski *et al*., 2007). In contrast, bacteriostatic antibiotics do not induce hydroxyl radical production or the SOS response (Kohanski *et al.*, 2007).

4.1.1 Inhibition of RecA-dependent SOS activation

The first goal of this project was identifying molecules that were cell permeable and limited SOS induction mediated by RecA *in viv*o. Fe-PcTs and 3,4′ Cu-PcTs molecules showed the ability to abrogate RecA ATPase activities *in vitro* (Geyer and Luo personal communication, unpublished data). Fe-PcTs molecule was subjected to further analysis to determine its ability to

effect RecA-dependent SOS induction by bactericidal or bacteriostatic antibiotics in bacteria. To examine the SOS response induced by various bactericidal or bacteriostatic drugs in the presence or absence of Fe-PcTs, we used an engineered promoter-reporter gene construct that expresses green fluorescent protein (GFP) upon LexA auto-cleavage. Following application of bactericides, we expected to observe the induction of the SOS response by RecA, which is activated by DNA damage and promotes the autolysis of the LexA repressor protein and induction of SOS response genes (Cirz and Romesberg, 2007; Riesenfeld *et al*., 1997). As expected, the *E. coli* exposed to CFX or AMP showed higher levels of SOS expression than untreated bacteria (Figs. 4.1, i & ii). KAN showed no significant increase in the SOS activity upon treatment (Fig. 4.1, iii). Three classes of ribosome inhibitors (bacteriostatic drugs), specifically, SPECT, CAM, and TET, were screened in this project. These bacteriostatic drugs specifically TET and CAM slightly induced the SOS response above the basal line (Fig. 4.1, iv). When the GFP strain was co-treated with Fe-PcTs and bactericidal antibiotics CFX, AMP, or KAN, the SOS induction was completely blocked for CFX and AMP (Figs. 4.1, i & ii). In the presence of Fe-PcTs, bacteria treated or untreated with CFX or AMP were characterized by a very similar SOS expression profile. Fe-PcTs completely blocked the GFP signals mediated by CAM and TET (Fig. 4.1, iv), which was consistent with the ability of these antibiotics to only induce a very low level of SOS response.

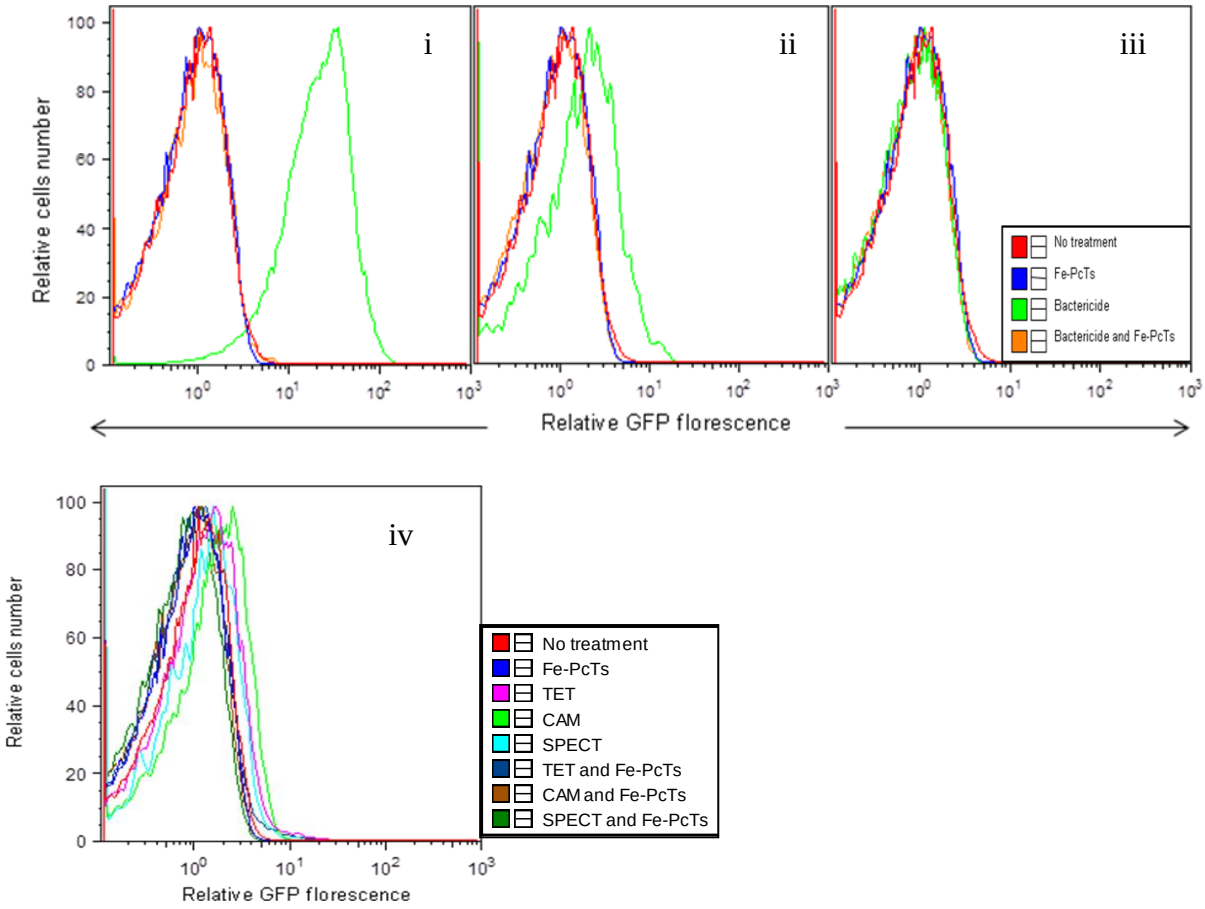


Figure 4.1. SOS suppression in *E. coli* by Fe-PcTs and bactericidal or bacteriostatic antibiotics.

Suppression of the induced of the SOS (DNA damage) response mediated by the addition of bactericidal antibiotics, 2.5 μM CFX (i), 40 μM AMP (ii), or 43 μM KAN (iii), or bacteriostatic antibiotics, 808 μM SPECT, 46 μM CAM, or 21 μM TET (iv), and 25 μM Fe-PcTs, or both Fe-PcTs and one of the bactericidal or bacteriostatic drugs, was monitored using an engineered sensor construct that employs the LexA repressor for control of green fluorescent protein (GFP) expression. Representative GFP histogram measurements were taken three hours after addition of the drugs using flowcytometery.

To examine whether or not Fe-PcTs interferes with the mechanism of hydroxyl radical production, we monitored the ability of Fe-PcTs to alter hydroxyl radical levels in the presence of bactericidal antibiotics in the pathogenic *E. coli* ATCC25922. Hydroxyphenyl fluorescein (HPF) was used to measure hydroxyl radical formation in bacteria, as described previously (Kohanski *et al*., 2007). Fe-PcTs did not alter the ability of CFX to induce hydroxyl radicals (Fig. 4.2), which is in agreement with the ability of Fe-PcTs to potentiate the activity of CFX by inhibiting RecA and blocking induction of the SOS response.

During growth of rod-shaped bacterial cells, two peptidoglycan synthesis mechanisms operate: (i) peptidoglycan insertion and (ii) septation (Uehara and Park, 2008; Varma *et al*., 2007). Inserting new peptidoglycan units into bacterial cell walls leads to cell elongation while septation causes cell division. Cell division is initiated by formation of an FtsZ ring at the centre of the cell, and this FtsZ ring recruits the other division proteins, including PBP3 (Uehara and Park, 2008; Varma *et al*., 2007). After activation of the SOS machinery, *sulA*, one of the SOS genes, is expressed. *sulA* is a key component in the process of filamentation, taking place in the last stage of SOS induction. The initiation of septation can be inhibited by SulA protein, a specific inhibitor of FtsZ functions causing cell elongation (Kohanski *et al*., 2010). Interestingly, inhibition of PBP3 by β-lactams results in the induction of filamentation, which has been shown to stimulate the DpiAB two-component system (Kohanski *et al*., 2010). Induction of this system can activate the SOS response. It has been shown that two actions can enhance β-lactam lethality: (i) disrupting DpiAB signaling and (ii) knocking out *sulA*. This suggests that *sulA* serves as a defense mechanism to protect bacterial cells from being killed by β-lactams, because SulA protects FtsZ and limits a ring interaction among PBPs and peptidoglycan hydrolases. Additionally, DNA-damaging antimicrobials, such as quinolones, which do not directly interrupt peptidoglycan synthesis, cause filamentation by activating the SOS response. Since RecA and LexA play critical roles in SOS induction, we hypothesize that Fe-PcTs is able to block RecA biological activity, and consequently, attenuate filamentation mediated by CFX. A subinhibitory concentration that induces expression of the *sulA*-GFP reporter gene in *E. coli* SS960 cells of CFX alone or combined with Fe-PcTs was added to the cultures. Three hours post treatment; Fe-PcTs was able to block filamentation mediated by CFX (Figure 4.3).

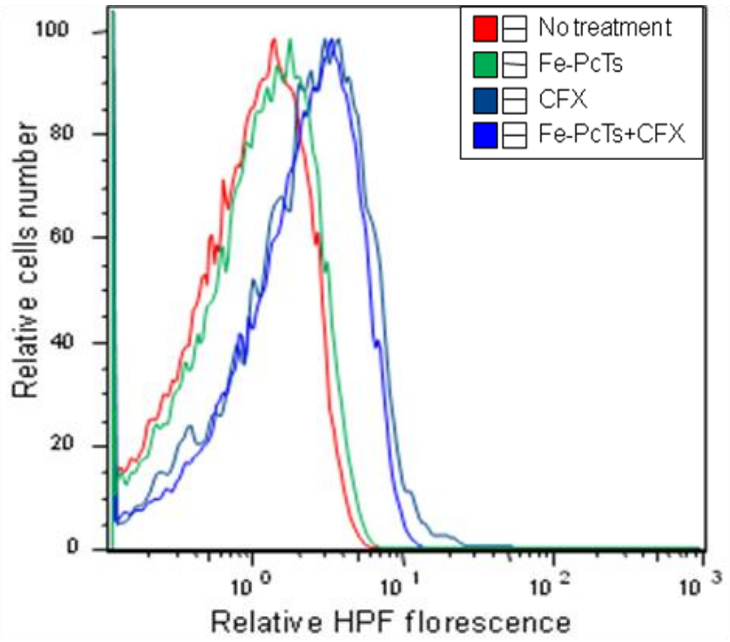


Figure 4.2. Hydroxyl radical production in *E. coli* by CFX and Fe-PcTs.

Generation of hydroxyl radicals following exposure to CFX and Fe-PcTs (40 nM and 25 µM, respectively), or both was determined. We used hydroxyphenyl fluorescein (HPF) to measure hydroxyl radical formation in bacteria. Representative measurements were taken three hours following addition of the drugs.

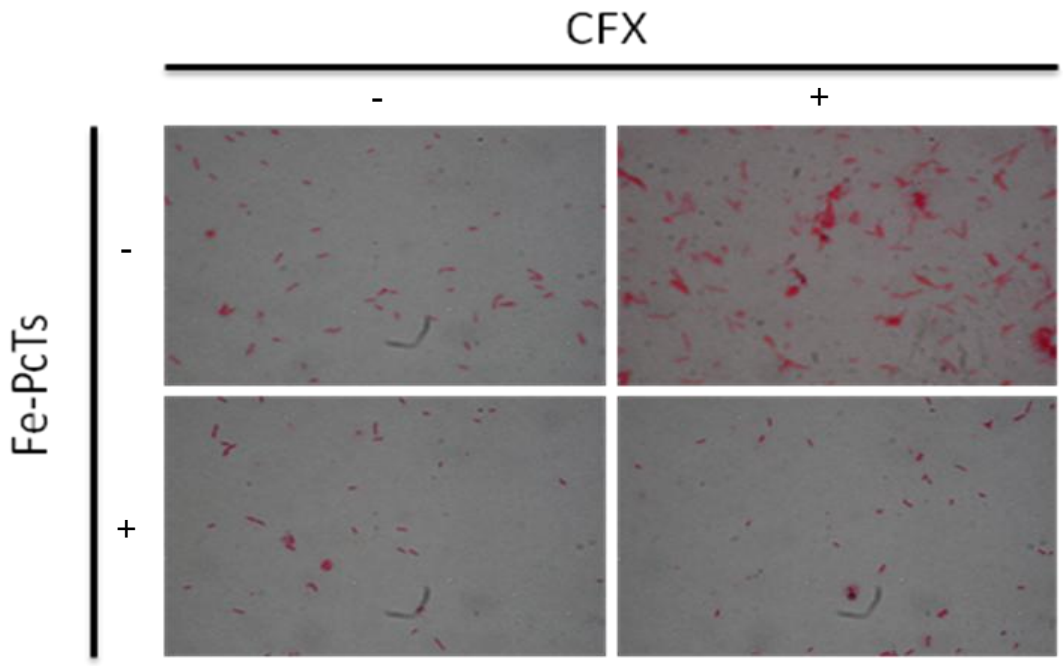


Figure 4.3. Suppression of filamentation mediated by the SOS response in *E. coli* by CFX and Fe-PcTs.

E. coli SS960 cells with GFP reporter gene were treated with a subinhibitory concentration of CFX (2.5 µM) alone or combined with Fe-PcTs (25 µM). The cells were stained using the basic Gram staining technique and viewed by a Nikon Eclipse E400 compound microscope at 100x power magnification. Photographs were taken three hours after addition of the drugs.

Recent evidence has suggested that the connection between stress inducible biofilm formation and the SOS machinery. RecA and LexA appeared to play a role in this response since biofilm formation was reduced in the presence of hydroxyurea in *recA* mutant cells compared with that of wild-type cells, and this defect was complemented by chromosomally inserted *recA*. This suggests that stress inducible biofilm formation is dependent on RecA. RecA is known to facilitate the auto-cleavage of LexA and, consequently, induces the SOS response. The biofilm repression in the *recA* mutant strain may have resulted from the fact that RecA was not able to promote the cleavage of LexA. Thus, the non-cleavable LexA led to non-inducible SOS machinery. This phenotype can be employed chemically which manifests the same repression activity (Gotoh *et al*., 2010). To test the hypothesis that Fe-PcTs was able to attenuate biofilm formation mediated by CFX, biofilm cell mass was determined in the presence and absence of Fe-PcTs. In this assay, ATCC2599 cells were treated with subinhibitory concentrations of CFX, Fe-PcTs, or both, and biofilm mass was determined spectrophotometrically by measuring the optical density at 600 nm (OD600). The results showed that Fe-PcTs was able to reduce the ability of CFX to induce *in vitro* biofilm formation (p-*value* ≤ 0.001) (Fig 4.4), which is mediated by SOS response (Gotoh *et al*., 2010).

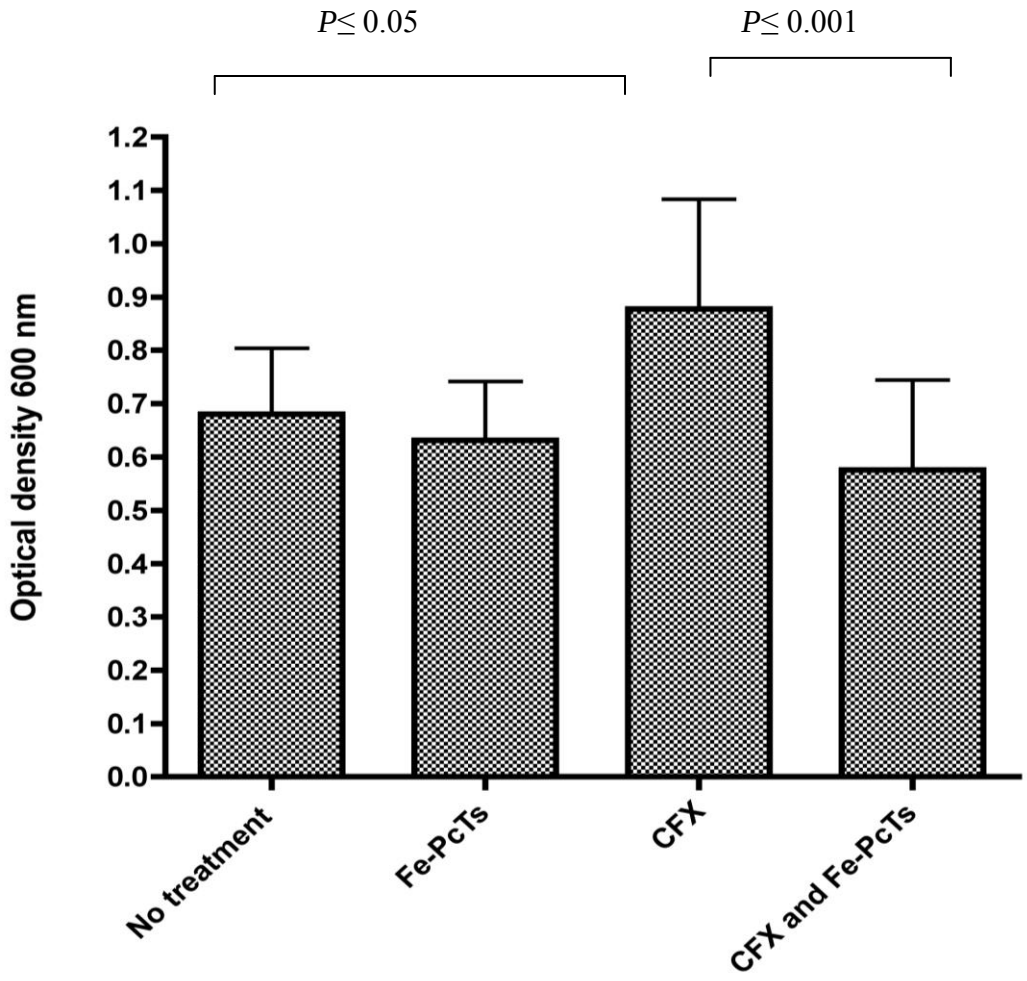


Figure 4.4. Suppression of biofilm formation mediated by the SOS response in the *E. coli* by CFX and Fe-PcTs.

E. coli ATCC25922 cells were treated with a subinhibitory concentration of CFX (40 nM) alone or combined with Fe-PcTs (25 µM). Biofilm cell mass on the wells of the 96-well microtiterplates after 24 hours of static conditions was determined spectrophotometrically by measuring the optical density at 600 nm (OD_{600}) after crystal violet staining. The results for the biofilm formation are from at least ten independent experiments and are plotted as the means and standard deviations.

4.2. Assaying Phthalocyanine (PcTs) Molecules' Ability to Enhance Bactericidal or Bacteriostatic Drugs Activities

4.2.1 PcTs molecules (metal derivatives) and CFX potentiation

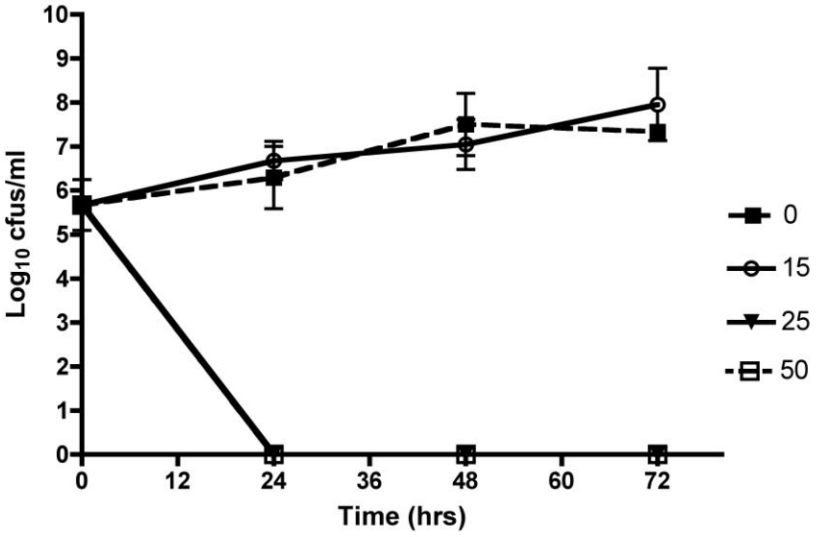
The ability of PcTs compounds to potentiate the activity of CFX in the *E. coli* ATCC25922 was evaluated. CFX produces free DSEs, which lead to a blocked replication fork. The formation of DSBs and a stalled replication fork are consequently processed to an ss-DNA, which is the signal that activates RecA to initiate SOS induction. The early expressed SOS gene products maintain genetic integrity of the cell by high fidelity DNA repair mechanisms to the damaged DNA, while late SOS gene products cause stress-induced mutations (Cirz *et al*., 2005) and genome-wide hypermutations (Jolivet-Gougeon *et al*., 2011). The potentiating activity of PcTs molecules in the *E. coli* ATTC25922 strain was characterized. The MIC of CFX in the ATTC25922 was 57 nM (±0.0153). Treatment of the ATCC25922 with Zn-PcTs, Al-PcTs, Ni-PcTs, or Apo-PcTs alone did not reduce the MIC of CFX at concentrations up to 100 μM. Co-treatment with CFX and Fe-PcTs or 3,4′ Cu-PcTs decreased the MIC of CFX, where Fe-PcTs showed greater activity than 3,4′ Cu-PcTs. MICs of CFX were 5.6 nM (±0.001) and 21 nM (±0.0043) in the presence of Fe-PcTs and 3,4′ Cu-PcTs, respectively (Table 4.1). Fe-PcTs and 3,4′ Cu-PcTs alone did not inhibit the growth of ATCC25922 cells at concentrations up to 100 μM. Fe-PcTs and 3, 4′ Cu-PcTs potentiated the activity of CFX and no colony forming units (cfus) were observed when ATCC25922 cells were co-treated with CFX and Fe-PcTs or 3,4′ Cu-PcTs at concentrations of 25 μM and above (Figs. 4.5, i & ii).

Table 4.1. MICs determination* in the *E. coli* ATCC25922 for PcTs and CFX treatment via the checkerboard assay.

Drug	**MIC (nM)**
No PcTs	57(±0.0153)
Fe-PcTs (100 μM)	5.6(±0.001)
Cu-PcTs (100 μM)	21(±0.0004)

*The results are means and standard deviations calculated from three independent experiments.

i



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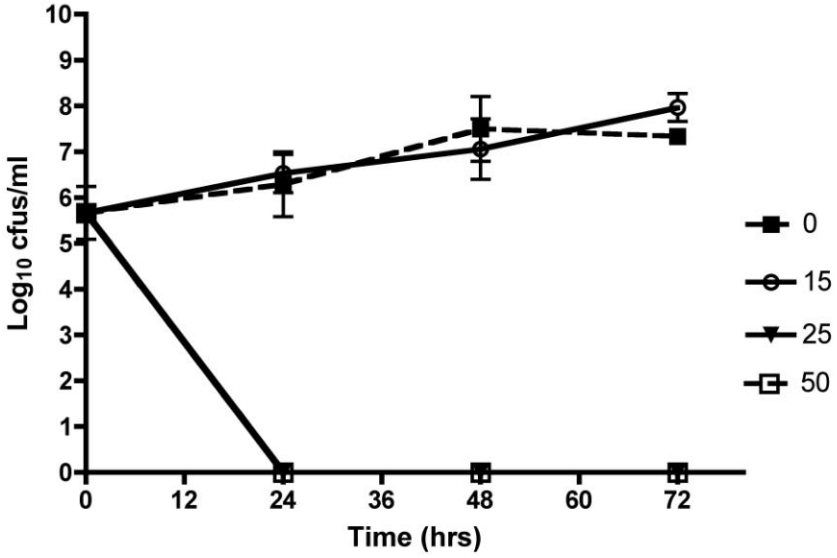

Figure 4.5. The *E. coli* survival assay for CFX and various concentrations of Fe-PcTs or 3,4′ Cu-PcTs treatments.

E. coli ATCC25922 cells were cultured in LB broth with various concentrations of Fe-PcTs (i) or 3, 4′ Cu-PcTs (ii) (0, 15, 25, or 50 μM) for three hours and then cultured in the same LB broth treatment with the addition of CFX 40 nM. Cfus were determined by plating 10-fold serial dilutions from each tube on LB plates at 0, 24, 48, and 72 hours. Error bars represent means and standard deviations calculated from three independent experiments.

The basic structure of the PcTs molecule is a flat aromatic macrocycle, four sulphonic groups, and a positively charged metal residue, attached in the middle of the PcTs molecules. The role of sulfonic acid position in the activity of Cu-PcTs was evaluated. The activity of 3,4′ Cu-PcTs with copper phthalocyanine-tetrasulfonic acid (Cu-PcTs), which contains mixture of sulfonic acid regioisomers was compared. Treatment of ATCC25922 cells with Cu-PcTs and CFX caused an ~30-fold decrease in cfus relative to CFX treatment alone, which was substantially less inhibitory than 3,4′ Cu-PcTs, where no cfus were detected (Fig**.** 4.6). This result suggests that sulfonic acid position appears essential for the activity of the PcTs and their potentiation activity.

To test whether the potency of PcTs molecules depends on the metal ion, the potentiation activity of the following molecules were evaluated: phthalocyanine tetrasulfonic acid (Apo-PcTs), aluminum(III) phthalocyanine tetrasulfonic acid (Al-PcTs), zinc(II) phthalocyanine tetrasulfonic acid (Zn-PcTs), nickel(II) phthalocyanine tetrasulfonic acid (Ni-PcTs), and copper phthalocyanine tetrasulfonic acid (Cu-PcTs). All these PcTs molecules consist of mixtures of different sulfonic acid regioisomers. All of these PcTs molecules slightly potentiated the activity of CFX with similar activity (~ 40-fold). We tentatively concluded that activities of PcTs molecules, which contain a mixture of sulfonic acid regioisomers, were not influenced by the identity of their chelated metal ion (Fig**.** 4.6).

In order to confirm the effect of Fe-PcTs was not specific to the Gram-negative strain ATCC25922, the ability of Fe-PcTs to potentiate CFX activity was determined in another Gram-negative strain (*P. aeruginosa* ATCC27853) and two Gram-positive strains (*S. aureus* ATCC29213 and *E. faecalis* ATCC29212). Co-treatment with Fe-PcTs and CFX eradicated bacterial growth 24 hours post treatment in the case of the ATCC 29213 and the ATCC 29212, while the combination therapy reduced the ATCC27853 growth at 48 hours (Figs. 4.7, i, ii, & iii).

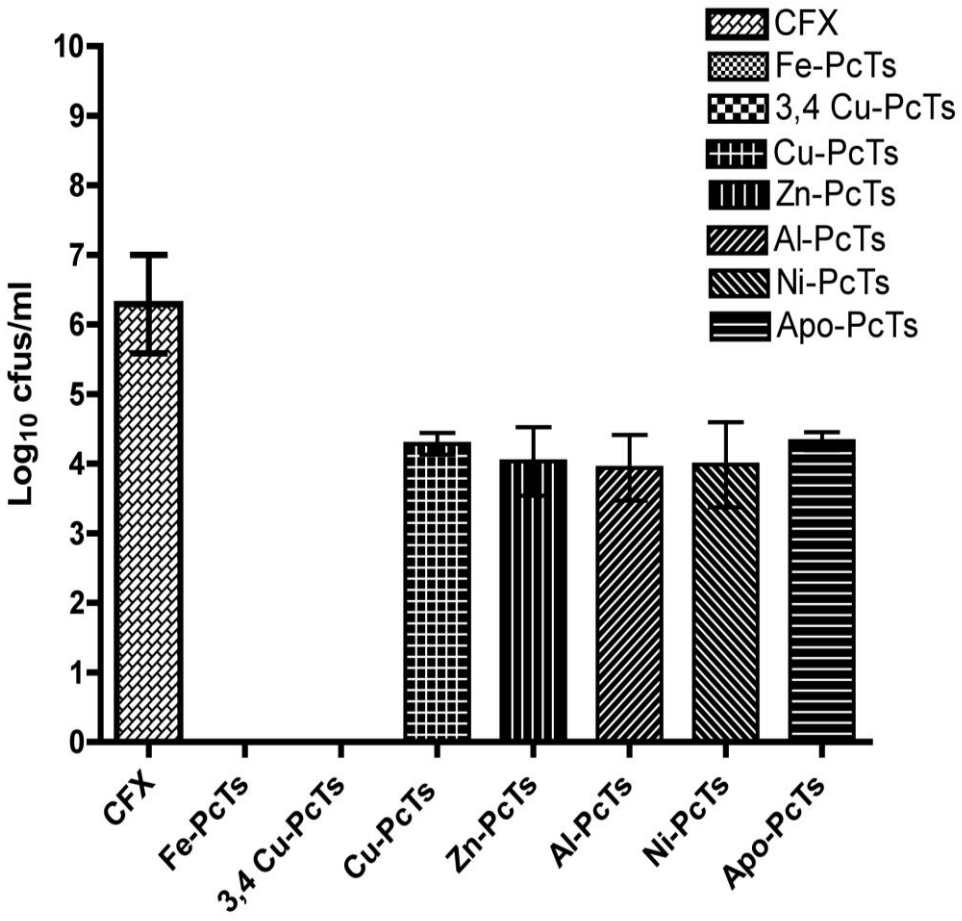

Figure 4.6. PcTs structure-potentiating activity relationships.

E. coli ATCC25922 cells were cultured in LB broth with two groups of PcTs moleculces:1) fixed sulfonic group site molecules (Fe-PcTs or 3,4′ Cu-PcTs) and 2) PcTs molecules consist of mixtures of different sulfonic acid regioisomers, at 25 µM for three hours. Then, *E. coli* cells were cultured in LB broth with the same treatment in addition to 40 nM CFX. Cfus were determined by plating 10-fold serial dilutions from each tube on LB agar plates at 24 hours. Error bars represent means and standard deviations calculated from three independent experiments.

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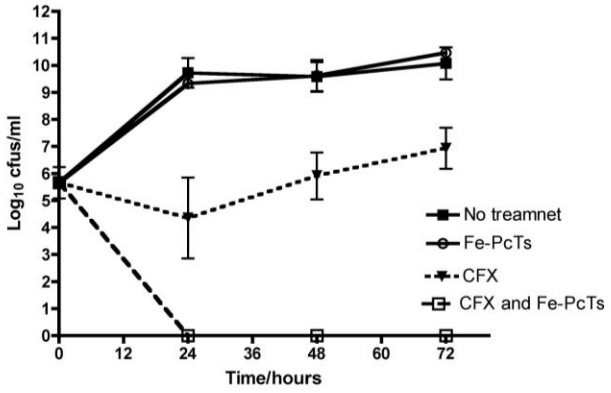

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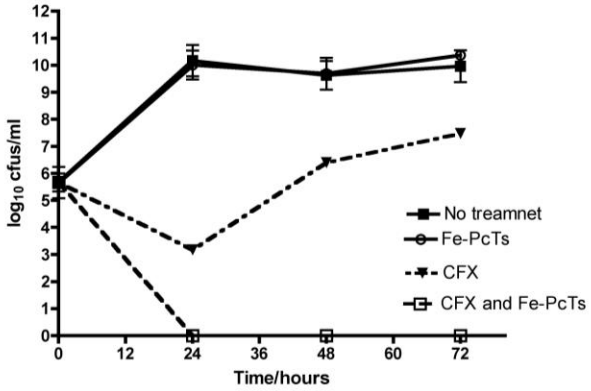

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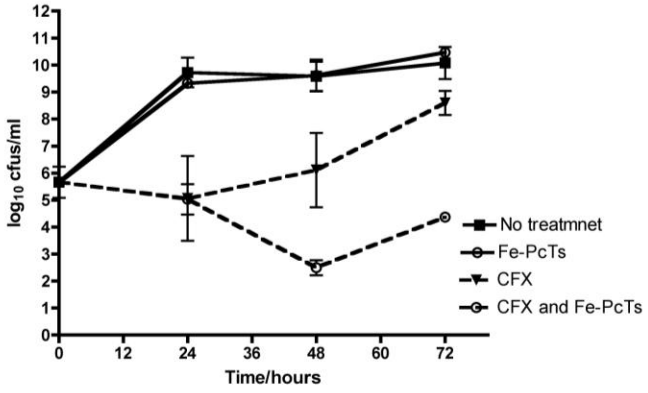

Figure 4.7. Survival assay for CFX and Fe-PcTs treatment for three different ATCC strains.

S. aureus ATCC29213 (i), *E. feacilis* ATCC29212 (ii), and *P. areuginosa* ATCC 27853 (iii) were cultured in MHB with Fe-PcTs 25 µM, CFX 6.5 µM , or both. Cfus were determined by plating 10-fold serial dilutions form each tube on Tryptic Soy Blood plates at 0, 24, 48, and 72 hours. Error bars represent means and standard deviations calculated from three independent experiments.

4.2.2 Effect of Fe-PcTs molecules on bactericidal or bacteriostatic antibiotic activity

A previous study demonstrated that all major classes of bactericidal drugs, such as ampicillin, norfloxacin, and kanamycin, stimulate the production of highly deleterious ROS radicals in Gram-negative and Gram-positive bacteria (Kohanski *et al*., 2007), leading to protein, lipid, and DNA damage (Imlay, 2003; Kohanski *et al*., 2007). On the other hand, bacteriostatic antibiotics do not induce hydroxyl radical production (Kohanski *et al*., 2007). In order to examine the ability of Fe-PcTs to potentiate the activity of a group of bactericidal or bacteriostatic antibiotics, we co-treated the ATTC25922 with Fe-PcTs and bactericidal antibiotics (CFX, AMP, or KAN), which are members of the quinolone, β-lactam, and aminoglycoside families, respectively, or bacteriostatic drugs (TET, CAM, or SPECT), which are ribosome inhibitors, and determined the MICs for both combination therapy and antibiotics alone. Fe-PcTs potentiated the activity of all bactericidal antibiotics tested by reducing their MIC. Fe-PcTs had no effect on the activity of bacteriostatic antibiotics (Table 4.2).

Following determination that Fe-PcTs limited the RecA-mediated SOS induction in bacteria exposed to bactericidal antibiotics, but not bacteriostatic drugs, we attempted to test the hypothesis that such a small molecule may act synergistically with a bactericidal or bacteriostatic antibiotic to kill bacteria. Experiments monitoring bacterial viability evaluated this hypothesis. Fe-PcTs increased the toxicity of CFX, and as the dose of Fe-PcTs was increased, the cell became more sensitive to CFX (Fig.4.5, i). Fe-PcTs weakened the ability of bacteria to withstand exposure to CFX, which was consistent with the ability of Fe-PcTs to block SOS response-mediated DNA repair pathways and SOS mutagenesis pathways. Although the killing effect of the β-lactam and the aminoglycoside drug alone were high, corresponding to the dose applied, Fe-PcTs increased these antibiotics' ability to eradicate bacterial growth rapidly (Figs 4.8, ii & iii). In contrast, co-treatment of Fe-PcTs with bacteriostatic antibiotics (SPECT, CAM, or TET) did not potentiate their ability to suppress bacterial growth (Fig 4.8, iv, v, & vi).

Table 4.2. MICs (µM) determination* in the *E. coli* ATCC25922 for Fe-PcTs and bactericidal or bacteriostatic antimicrobial treatment via the checkerboard assay.

	CFX	**AMP**	**KAN**	**TET**	**CAMP**	**SPECT**
Fe-PcTs free	0.057 (±0.0153)	6.7	6.4 (±1.25)	8.3	3.7	10
Fe-PcTs (100 µM)	0.0056 (±0.001)	3.4	1.6(±0.3125)	8.3	3.7	10

*The results are means and standard deviations calculated from three independent experiments.

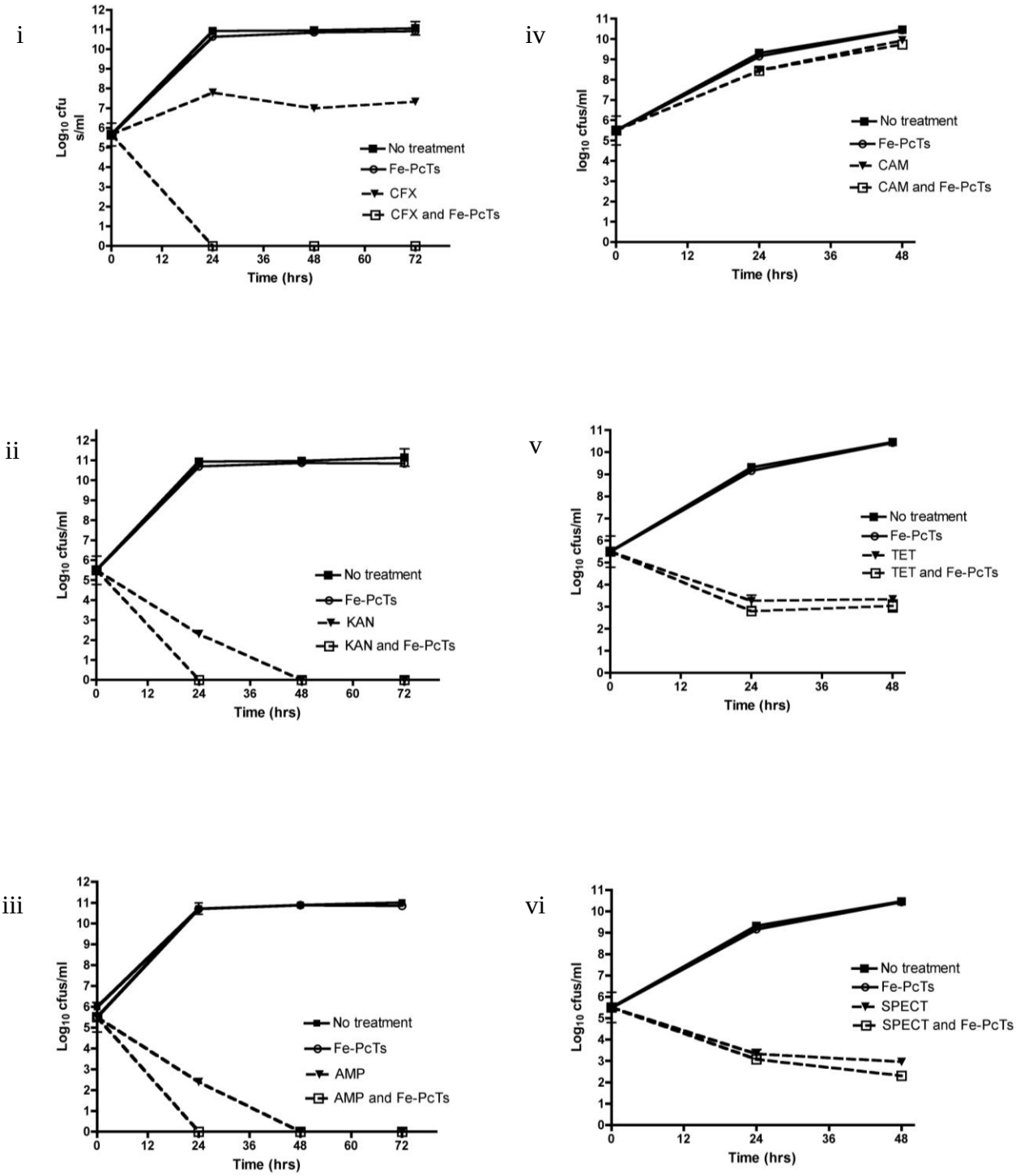

Figure 4.8. *E. coli* survival assays for Fe-PcTs and bactericidal or bacteriostatic treatment.

The *E. coli* ATCC 25922 was cultured in LB broth with 25 μM Fe-PcTs, or one of the bactericidal drugs, 40 nM CFX (i), AMP 40 μM (ii), or KAN 43 μM (iii), or one of the bacteriostatic drugs, CAM 46 μM (iv), TET 21 μM (v), or SPECT 808 μM (vi), or both Fe-PcTs and one of the bactericidal or bacteriostatic drugs. Cfus were determined by plating 10-fold serial dilutions form each tube on LB agar plates at 0, 24, 48, and/or 72 hours. Error bars represent means and standard deviations calculated from three or two independent experiments.

4.3 Determining whether Fe-PcTs Reduces Antibiotic Resistance

4.3.1 Effects of Fe-PcTs molecules on CFX-resistance *in vitro*

Antibiotic therapies that mediate DNA damage and/or lead to the formation of the RecA-ss-DNA filament are associated with antibiotic resistance through adaptive mutations. The blocked replication fork and the accumulation of RecA-ss-DNA filaments activate the SOS DNA repair and mutagenesis pathways (Cirz and Romesberg, 2007; McKenzie *et al*., 2000). The accumulation of the RecA filament activates the auto-cleavage activity of LexA. The inactivation of LexA frees the operator site of the SOS box and allows expression of SOS genes (Cirz and Romesberg, 2007; Janion, 2001; Riesenfeld *et al*., 1997). The early expressed SOS gene products maintain the genetic integrity of the cell by high fidelity DNA repair mechanisms of the damaged DNA. If these mechanisms are not successful in repairing the damage, this leads to the persistence of the RecA-ss-DNA filament and eventually results in the activation of the SOS error-prone polymerases (PolIV and PolV) (Cirz *et al*., 2005). In this case, error-prone polymerases start to function and produce direct mutations and promote a hypermutable state when the MMR declines (Cirz and Romesberg, 2007). Expression of these polymerases causes mutations in genes that enable the acquisition of resistance (Cirz *et al*., 2005).

An *in vitro* resistance assay was established to estimate whether or not the Fe-PcTs was able to attenuate or block the acquisition of CFX resistance. The *E. coli* ATCC25922 was plated onto two sets of plates containing CFX in the presence or absence of Fe-PcTs. Resistant colonies were counted as they arose, in 24 hour intervals over 10 days, as was previously described (Cirz *et al*., 2005). Colonies that formed immediately (at or before day 2) were attributed to cells that had acquired resistance prior to exposure to CFX, while colonies that formed on day three or later were attributed to cells that acquired resistance after exposure to CFX. Mutants that appeared late in the assay could also have occurred by mutations that effect normal growth rates that occurred after plating on LB with CFX, or they could have resulted from slow growing pre-existing mutant cells. To distinguish between pre- and post-exposure mutations, two different reconstruction assays were done to ascertain when mutations occurred. Clones that were resistant before exposure were defined as those that formed colonies on the CFX-containing media in the same number of days in the reconstruction assay as they did in the original resistance assay. Clones that mutated after exposure to CFX were defined as those that

formed colonies earlier than in the original resistance assay. Based on these criteria, we calculated that 29% of the CFX-resistant colonies were caused by pre-existing mutations in the presence of CFX (Table.4.3). For ATCC25922 cells treated with CFX and Fe-PcTs, 60% of CFX-resistant colonies were from pre-existing mutations (Table.4.3). To confirm that these observations were not due to the lower density of cell plating in the reconstruction assay, we repeated the reconstruction assay at a high cell density (10^8 cells/plate). The results of the high-density reconstruction assay were in agreement with the low-density assay. Treatment of ATCC2592 cells with CFX caused rapid emergence of CFX resistant clones (Fig.4.9). Cotreatment of ATCC25922 cells with Fe-PcTs and CFX dramatically reduced the number of CFX resistant clones (Fig. 4.9). Since Fe-PcTs potentiated the activity of CFX, it decreased the number of viable bacteria on the plates compared to CFX treatment alone. To determine the number of viable cells present as a function of time in CFX and CFX and Fe-PcTs treatments, a survival assay was conducted. In the absence of Fe-PcTs, the number of viable cells decreased from 3×10^8 to 10^4 cfus/plate on the seventh day of incubation. In the presence of Fe-PcTs, the number of viable cells decreased from 3×10^8 to 1×10^4 cfus/plate on the fourth day of incubation (Fig. 4.10). Since Fe-PcTs potentiates the activity of CFX, there were fewer cells present that could acquire resistance mutations. To account for the decreased viability in the presence of Fe-PcTs, we defined the CFX mutation rate as CFX resistant colonies per viable cell per day (Fig. 4.11), as described previously (Cirz *et al*., 2005). Fe-PcTs reduced CFX-induced resistance by a factor of 38-fold in day four. No mutations were observed in the Fe-PcTs cells after day four. These results are comparable to decreases in CFX-induced resistance observed in genetic mutations of SOS response and DNA repair genes. *E. coli* MG1655 was not able to produce pre- or post exposure mutants when *recA, recB, recG, ruvB,* or *ruvC* were deleted. (Cirz *et al*., 2005). These SOS gene products are essential for the acquisition of resistance-conferring mutations.

To confirm that cells isolated in the resistance assay developed CFX-resistance, we determined the MICs of randomly selected clones. The data showed that MICs of CFX for ten CFX-resistant mutants, isolated in the presence and absence of Fe-PcTs, were 189 or 377 nM, respectively. The CFX-MIC of these isolated mutants increased significantly from 57 nM to either 189 or 377 nM, which is considered a low range for quinolone resistance (Vila *et al*., 1994). In addition, the sequence of the quinolone resistance determining rejoin (QRDR) was analyzed in the previous selected resistant clones. The mutations in the *gyrA* gene involved in

the resistance are clustered in a region between nucleotides 199 (Ala-67) and 318 (Gln-106). All the examined mutants had a mutation in QRDR region, specifically at 87-Asp to Asn (Fig. 4.12).

Although changes in nucleotide 247 (Ser-83) are the most frequently changed in spontaneous *gyrA* mutations among clinical isolates or laboratory mutants of *E. coli*, and this mutation is responsible for the low-level resistance to fluoroquinolones, mutations in the Asp-87 for two strains, one of which is a spontaneous mutant of *E. coli* K-16 with a change to Asn (Oram and Fisher, 1991) and the other of which is a clinical isolate of *E. coli* with a change to Val has previously been reported (Oram and Fisher, 1991). In both cases, there was no accompanying mutation in Ser-83. Double mutations in Ser and Asp could account for a high level of quinolone resistance (Vila *et al*., 1994).

Table 4.3. *In vitro* CFX-resistance assay for the *E. coli* ATTCC25922 for CFX and Fe-PcTs therapy.

Day	CFX-mutants cfus/25 plates*	CFX-Fe-PcTs mutants cfus/25 plates*	Number of viable CFX-susceptible cell/plate*	Number of viable CFX-Fe-PcTs-susceptible cell/plate*
1	(± 62) 166	(± 2) 9	$(\pm 2\times10^{5})\ 1.92\times10^{7}$	$(\pm 3\times10^{4})\ 3.16\times10^{5}$
2	(± 97) 187	(±1) 3	$(\pm 9.9\times10^{5})\ 1.5\times10^{6}$	$(\pm 5\times10^{3})\ 1.5\times10^{4}$
3	(±70.5) 297	(±2) 4	$(\pm 1\times10^{4})\ 1.8\times10^{5}$	$(\pm 1\times10^{3})\ 1.3\times10^{4}$
4	(± 0) 276	(±3.5) 4	$(\pm 1.05\times10^{4})\ 1.8\times10^{4}$	$(\pm 0)\ 1\times10^{4}$
5	(± 156) 293	0	$(\pm 5\times10^{2})\ 1.29\times10^{4}$	-
6	(± 8.5) 113	0	$(\pm 1.6\times10^{3})\ 1.45\times10^{4}$	-
7	(± 1) 71	0	$(\pm 1.5\times10^{3})\ 1.5\times10^{4}$	-
8	(± 10.5) 11	0	$(\pm 0)\ 1\times10^{4}$	-
Total number mutants	1414	20		
Pre-existing mutants	**404**	**12**		
Early growing	353	12		
The late growing	51	None		
Post-existing mutants	**1010**	**8**		

*The results are means and standard deviations calculated from two independent experiments.

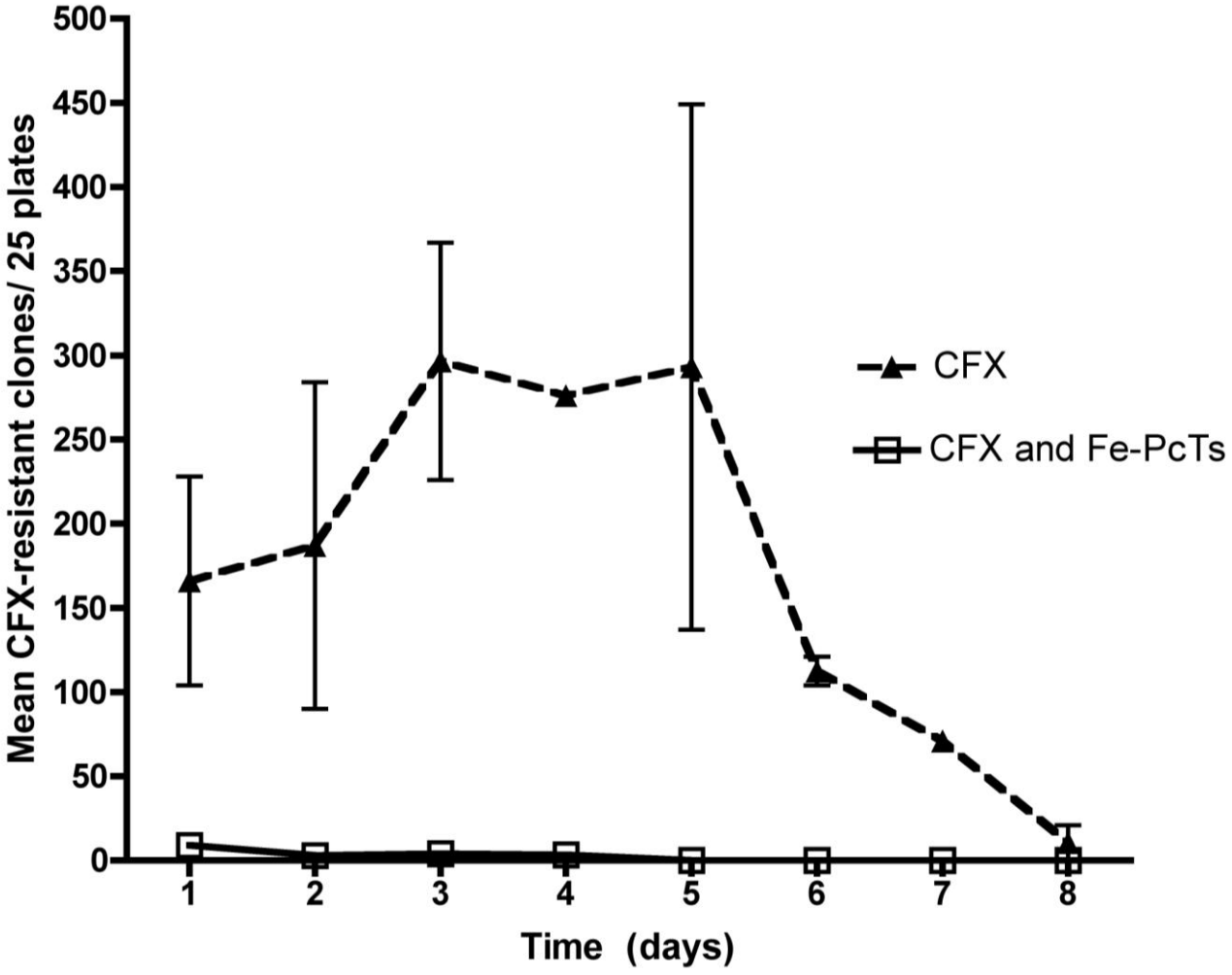

Figure 4.9. Resistance assay in *E. coli* for CFX and Fe-PcTs treatments.

E. coli ATCC2599 cells were plated out onto LB containing 40 nM CFX. In an independent experiment the *E. coli* ATCC2599 were pre-incubated with 25 µM Fe-PcTs followed by plating on CFX and Fe-PcTs 40 nM and 25 µM, respectively. The plates were incubated for 10 days. Every emerged mutant was isolated and stocked in -80 °C for use in the reconstruction. Error bars represent means and standard deviations, calculated from two independent experiments

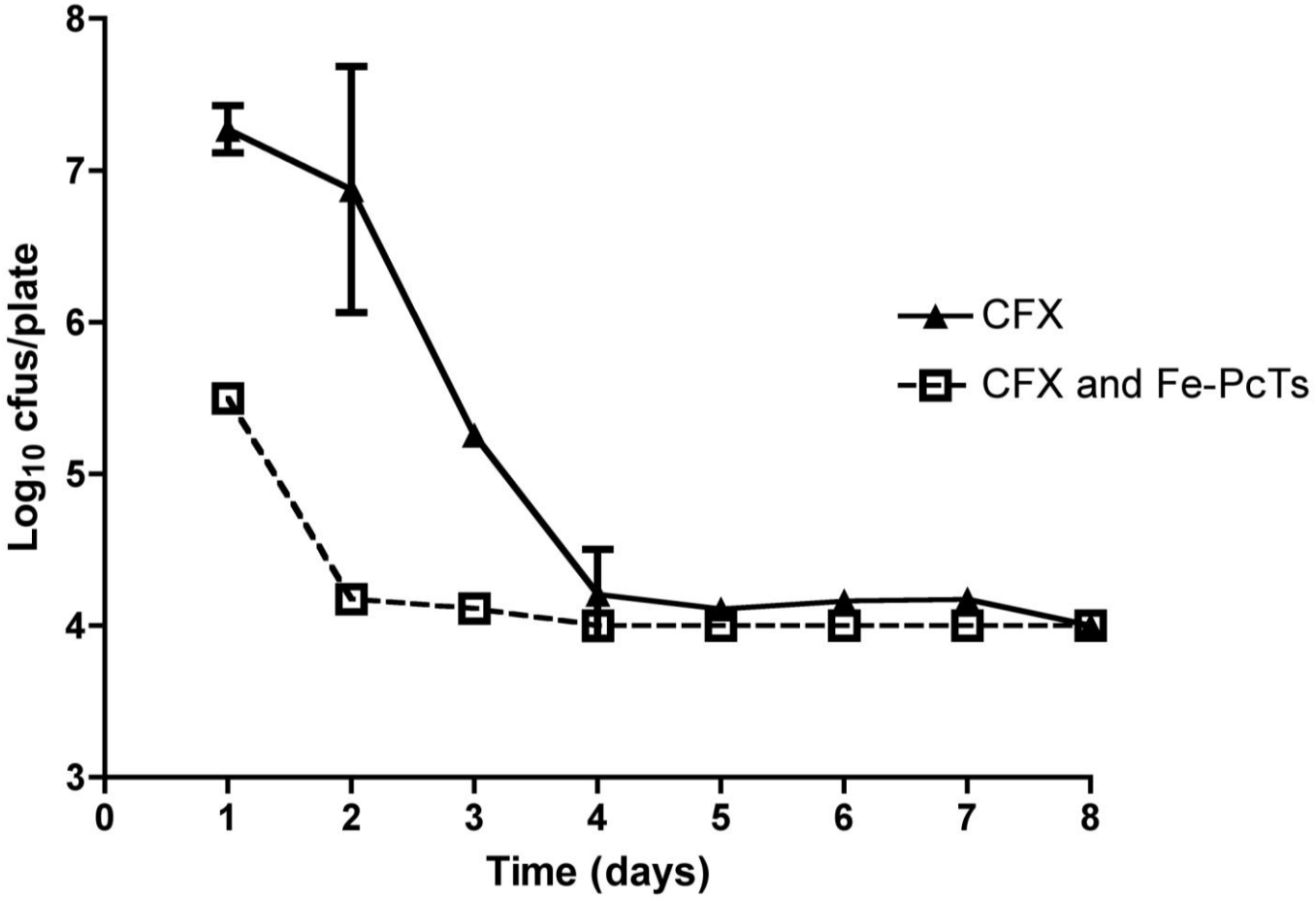

Figure 4.10. Survival assays in *E. coli* for CFX and Fe-PcTs treatments.

Three *E. coli* ATCC25922cultures, each of CFX or CFX and Fe-PcTs treatments were prepared exclusively for the survival assay. Agar plugs between visible colonies were excised from these cultures. These plugs were homogenized in M9 buffer. Dilutions were plated out in duplicate on LB plates, to determine the number of CFX-sensitive cells present as a function of time, and LB containing 40 nM CFX, to determine if any CFX-resistant colonies remained after excision. Error bars represent means and standard deviations, calculated from two independent experiments.

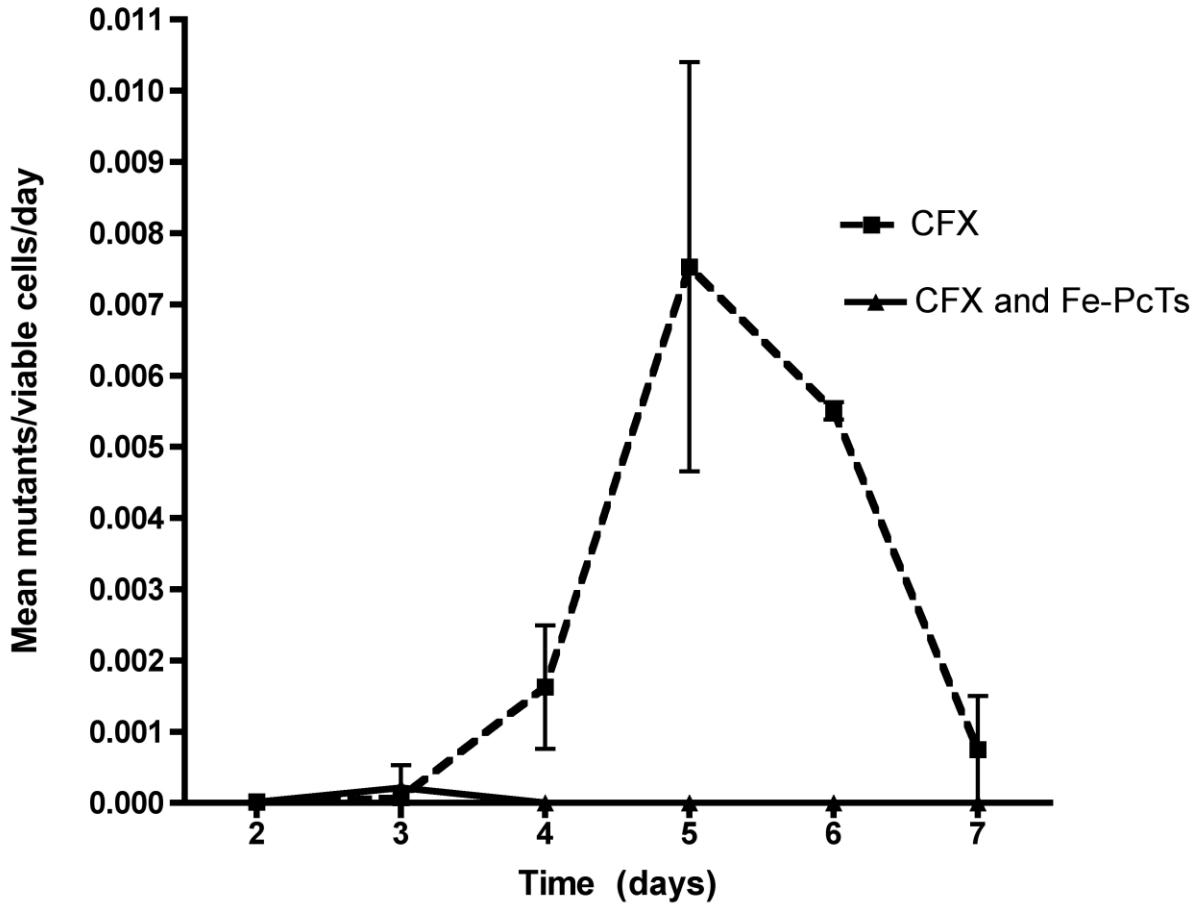

Figure 4.11. ***In vitro*** **post-exposure mutation rate of** ***E. coli*** **ATCC25922 for CFX or CFX and Fe-PcTs treatments fix legend.**

The post-exposure mutation rate at each day of incubation (starting from the third to the eight day of incubation) for the ATCC25922 treated with CFX or CFX and Fe-PcTs was determined as the ratio of resistant colonies on a particular day to the number of viable cells present at the time the cells became resistant. Since a colony takes two days to form, the viable cell count was calculated two days prior to colony formation. The post-exposure mutation rate was determined as the average of the mutation rate from the third to the eighth day of incubation. Error bars represent means and standard deviations, calculated from two independent experiments.

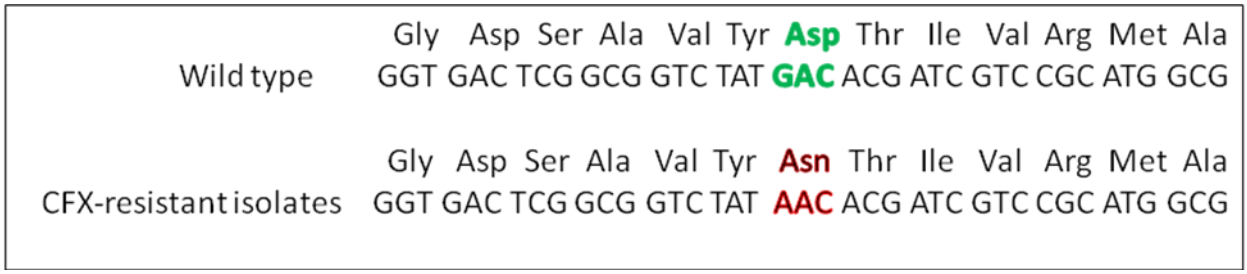

Figure 4.12. *gyrA* mutation determination in CFX-resistant isolates.

Colonies stored from the *in vitro* and *in vivo* resistance assays were streaked on LB agar containing 58 nM CFX. A single colony from each plate was used as a colony PCR template for *gyrA* fragment amplification. A substatution mutation from Asp into Asn at site 87 in *gyrA* was highlighted in red.

4.3.2 Effects of Fe-PcTs molecules on CFX-resistance *in vivo* murine thigh infection model

To examine whether Fe-PcTs can attenuate the acquisition of CFX resistance *in vivo*, we assayed the activity of Fe-PcTs in a neutrapenic murine thigh bacterial infection model (Cirz *et al*., 2005). Mice were rendered neutrapenic by intraperitoneal injection with cyclophosphamide. Mice were then injected with ATCC25922 cells. Two hours after infection, mice were administered subcutaneous injections of CFX or CFX and intraperitoneal injections with Fe-PcTs every 24 hours up to 72 hours. At 48 and 72 hours post infection, nine mice from each group were sacrificed and their thighs removed and homogenized to determine viable cells numbers of both CFX-sensitive and CFX-resistant *E. coli*. CFX-resistant cells were observed after 48 and 72 hours infection in the CFX-treatment group. Approximately, 50,000 CFX-resistant cells were observed after 72 hours infection when the mice were only treated with CFX. Remarkably, no CFX resistant cells were observed when mice were co-treated with CFX and Fe-PcTs (Fig. 4.13). Pretreatment of mice with Fe-PcTs prior to infection potentiated the activity of CFX more than when mice were only co-treated with CFX and Fe-PcTs shortly after infection (Fig. 4.13). No CFX-resistant colonies were observed in any mice treated with Fe-PcTs, which may reflect the time required for *E. coli* to develop resistance.

To confirm that cells isolated from a mouse model developed CFX resistant, we determined the MICs of randomly selected clones. The data showed that MICs of CFX for ten CFX-resistant mutants, isolated in the absence of Fe-PcTs, were 189 or 377 nM, respectively. The CFX-MIC of these isolated mutants increased significantly from 57 nM to either 189 or 377 nM, which is considered a low range for quinolone resistance (Vila *et al*., 1994). In addition, the sequence of the QRDR was analyzed in the previous selected resistant clones. The mutations in the *gyrA* gene involved in the resistance are clustered in a region between nucleotides 199 (Ala-67) and 318 (Gln-106). All the examined mutants had a mutation in QRDR region, specifically at 87-Asp to Asn (Fig. 4.12).

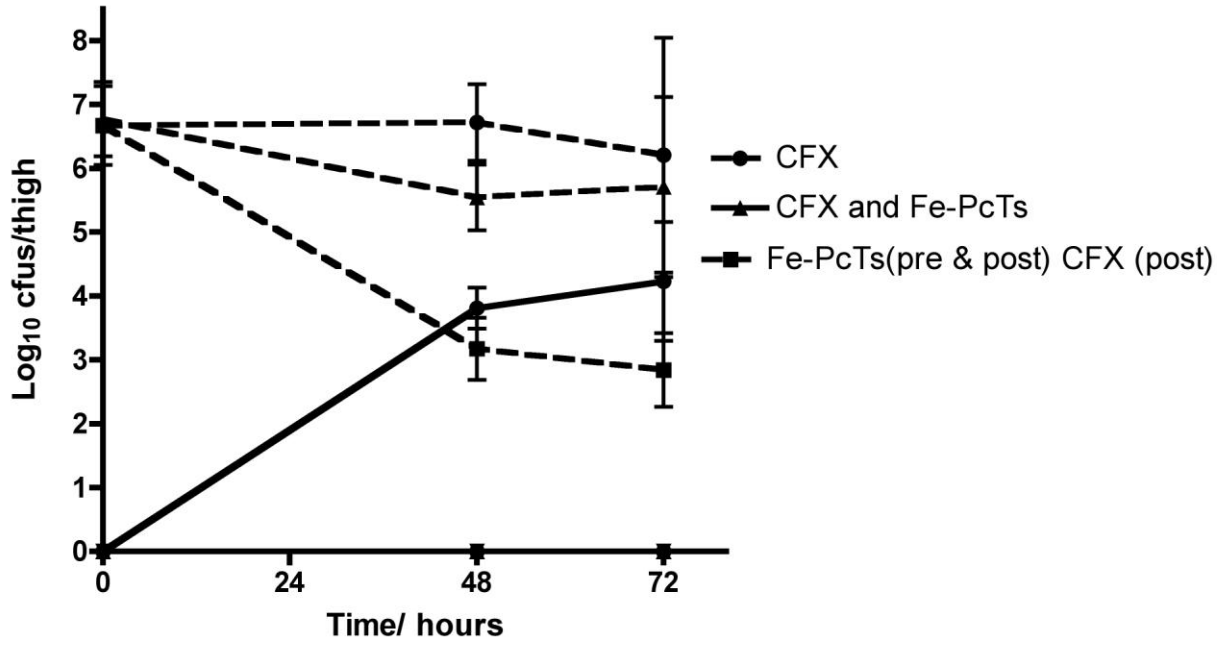

Figure 4.13. Survival and mutation of *E. coli in vivo* after starting CFX and Fe-PcTs therapy.

Survival and mutation of *E. coli* ATCC25922 in thighs of neutropenic mice at 48 and 72 hours after starting therapy with CFX, CFX and Fe-PcTs, or Fe-PcTs (pre and post treatment) and CFX. Disconnected lines represent the total cfus/thigh in the surviving *E.coli* and sold lines correspond to the total cfus/thigh of the CFX resistance. The results are means, calculated from nine sacrificed mice from each group and error bars are the standard deviations.

CHAPTER 5
DISCUSSION

Development and introduction of antimicrobial drugs have been considered a victory against the threat of infectious diseases. Unfortunately, numerous reports have demonstrated the ability of bacteria to evolve resistance to almost all current antibiotics (Aminov, 2010). In fact, the continual emergence of antibiotic resistance has amplified the global impact of infectious disease in this century. Antimicrobial resistance not only takes place rapidly, but it also spreads quickly. In addition, the antibiotic discovery industry has remarkably declined (Projan and Shlaes, 2004). This decline reflects both the difficulty of identifying novel classes of antibiotic and less commitment by the pharmaceutical industry towards discovering novel antibiotics. Some big pharmaceutical companies continue to invest billions of dollars in the development of new antibiotics; however, their production is considered insufficient to overcome the increased number of resistant pathogens (Projan and Shlaes, 2004).

There are several molecular mechanisms for drug resistance, but the most threatening ones are those that involve acquisition of antibiotic resistance genes via a processes known as horizontal gene transfer (Beaber *et al*., 2004) and stress-inducible mutation mediated by RecA (Cirz *et al*., 2005). The bacterial RecA protein is essential in both processes. Inactivation of RecA's biological activity in bacterial cells could abrogate DNA repair mechanisms, horizontal gene transfer, and SOS mutagenesis. It has been demonstrated that the mutagenic effect mediated by the sub-lethal concentrations of certain antibiotics was completely abolished when the biological activity of RecA was inactivated (Cirz *et al*., 2005; Cirz and Romesberg, 2007). In addition, bacterial cells became more susceptible to these antibiotics (Thi *et al*., 2011). Identification of molecules that inhibit RecA biological activities can potentiate the efficacy of current antibiotics and block the acquisition of resistant genes mediated by the SOS response.

RecA and other recombination proteins are essential for cell viability under normal growth conditions *in vivo*. The growth rate of Δ*recA*$^{-}$ bacterial is approximately 20% that of wild type strains (Courcelle and Hanawalt, 2003; Cox *et al.*, 2000). Reactivation of stalled replication forks under normal growth conditions, occurring regularly in bacterial cells, involves the activation of non-mutagenic homologous recombination pathways, where RecA plays a master

regulatory role. It has been previously shown that eliminating RecBCD leads to accumulation of unrepaired DNA-DSB, developing from fork inactivation and cleavage in 15-20% of cells under normal conditions (Courcelle and Hanawalt, 2003; Cox *et al*., 2000). Bacterial cells were inviable when null mutations in both *recA* and *pri* were combined with mutations in other recombination proteins, while cells that encompass a null mutation in *recA* alone were viable (Cox *et al*., 2000). However, the non-mutagenic recombination pathways can be switched to the mutagenic version when the damaged or stalled replication fork is not repaired successfully. Therefore, in order to maximize cell survival, the SOS response activates a number of downstream processes, such as cell-cycle arrest induced by SulA and the mutagenic repair, caused by DNA polIV (DinB) and V (UmuDC)-mediated replication fork bypass (Cirz *et al*., 2005; Cirz and Romesberg, 2007; Riesenfeld *et al*., 1997; Thi *et al*., 2011). As RecA functions rely upon active nucleoprotein filament formation stimulated by the simultaneous binding of ATP and ss-DNA, the NPF is an attractive target to block RecA functions. Isolating inhibitors that competitively block the DNA binding sites of RecA, freeze RecA in its ATPase inactive conformation, and prevent active nucleoprotein filament assembly is desirable. Inhibitors of this type would negate both the SOS response and processive recombinational activities of RecA by preventing the assembly of the active filament altogether. Such functionally selective inhibitors of the RecA-mediated stress response would permit a greater understanding of the antibiotic-induced bacterial stress response.

Recombinase proteins are ubiquitous proteins, which are present in all organisms, including bacterial RecA, archaeal RadA, and eukaryotic cells Rad51 and DMC1(Li *et al*., 2009). The similarity among RecA, Rad51, and RadA is mostly functional as the structural and biochemical approaches of these proteins are distinct (Yang *et al*., 2001a). The only common feature is the core ATP-binding domain, and even these domains are only modestly similar (Cox, 2007). RecA and its homologues can be inhibited by targeting three functionally important sites: recruitment and polymerization, ATP binding, and DNA binding (Li *et al*., 2009). The recruitment and polymerization site has a central hydrophobic residue that matches a hydrophobic pocket in an adjacent subunit (Li *et al*., 2009). Inhibition at the recruitment and polymerization site of Rad51 has been reported using a peptide mimicking the conserved polymerization motif in RecA orthologues (Tal *et al*., 2009). The ATP binding site, located at the inter-subunit interface, binds, hydrolyzes ATP, and regulates the conformation of the DNA

binding site (Li *et al*., 2009). The ATPase center of EcRecA has also been targeted (Sexton *et al*., 2010). The third site is the DNA binding center, consisting of L1 and L2 loops. The larger L2 has two universally conserved residues at each end while the smaller L1 is not highly conserved between bacterial RecA and nonbacterial recombinase proteins (RadA, Rad51, and DMC1). Despite the fact that the structure of the nonbacterial recombinase in complex with DNA is not yet determined, the structural similarities between the bacterial and nonbacterial recombinases indicate that L1 and L2 sites in the nonbacterial homologues are the most likely site of DNA interaction (Li *et al*., 2009). Developing a RecA inhibitor for therapeutic purposes involves identification of an inhibitor that is selective for a specific target since off target drugs can increase the toxicity by exposure to that particular drug. Therefore, when isolating a RecA inhibitor, this drug should not interfere with RecA homologues in eukaryotes, including Rad51 and DMC1. As bacterial RecA proteins are only functionally similar to their eukaryotic homologues, it should be possible to inhibit RecA specifically without blocking Rad51 and DMC1 functions. In agreement with the mentioned difference between RecA and Rad51, our research team's results have showed that the Fe-PcTs complexes were not toxic to mouse bone marrow at the maximum concentration tested (100 µM) or leukemia cells (Geyer and Luo personal communication, unpublished data). These results showed that Fe-PcTs has no toxicity in mice, which is in consistent with other studies showing that PcTs-based molecules are well tolerated by mice going under long-term treatment (Caughey *et al*., 2007). Additionally, PcTs-based molecules have been used to suppress infection mediated by prion such as scrapie infection in mouse models (Priola *et al.*, 2003; Priola *et al*., 2000).

Potential RecA inhibitors tested in this work were available from commercial small anionic aromatic libraries, specifically phthalocyanine molecules coordinated with different metal ions. The selection of these molecules was inspired by the ability of metatungstate Na_2WO_4 to inhibit the ATPase and strand exchange activities of the RecA homologue, MvRadA. Metatungstate ions appears to be bound between the DNA-binding loops, anchoring the MvRadA in its inactive conformation (Li *et al*., 2009), unfortunately, this molecule could not block RecA biological activity in living cells. Interestingly, when a copper phthalocyanine complex (3,4′ Cu-PcTs) was co-crystallized with MvRadA, the 1.9 Å resolution structure showed that a single 3,4′ Cu-PcTs molecule was sandwiched between two hexameric rings of MvRadA. 3,4′ Cu-PcTs binds near the L1 DNA-binding loop of MvRadA in its ring-shaped

oligomeric form (Geyer and Luo personal communication, unpublished data). Since such ring shaped oligomers are known to be the storage form for all studied RecA orthologues (Chen *et al*., 2007; Yang *et al*., 2001b), this mode of inhibitor/RadA interaction could be exploited for RecA. Phthalocyanine molecules (Fe-PcTs and 3,4′ Cu-PcTs) inhibited the ATPase, as well as DNA-binding, DNA stand-exchange, and LexA cleavage activities of EcRecA. The tested PcTs compounds have also been found to be micromolar inhibitors of ds-DNA-binding activity of EcRecA form (Geyer and Luo personal communication, unpublished data). These results suggest that anionic metatungstate molecules (Li *et al*., 2009) and PcTs inhibitors (Geyer and Luo personal communication, unpublished data), interacting with L1 DNA-binding loops of MvRad, could also effectively interact with the DNA binding loops of EcRecA.

RecA controls processes that are responsible for an increased tolerance to antibiotic chemotherapy and pathways, which ultimately lead to complete antibiotic resistance. Small molecules capable of abrogating RecA biological activities impact the following pathways: (i) DNA repair, (ii) SOS-response mutagenesis, and (iii) recombination-based horizontal gene transfer. It has been shown that bacteria impairing these functions by mutations in the *recA* gene are more susceptible to antibiotic treatments and are not able to develop resistance (Cirz *et al*., 2005; Cirz and Romesberg, 2007; Thi *et al*., 2011). A RecA inhibitor induces this phenotype to act synergistically with currently prescribed antibiotics, greatly enhancing their potency and preventing the accumulation of resistant cells. Therefore, identification of RecA inhibitors that are cell permeable, limit SOS induction, and increase the sensitivity of bacterial cells to the current antibiotics *in viv*o was investigated in this project. The Fe-PcTs, examined in *E. coli* cultures, abrogated the SOS response under stress conditions resulting from exposure to CFX or AMP, bactericidal agents known to upregulate SOS expression. CFX is considered a good inducer of the SOS response (Hassett and Imlay, 2007), since SOS induction is efficiently activated by DNA damaging antibiotics, leading to the expression of the DNA repair mechanisms and transcriptional induction of the error prone polymerases. Consequently, an increase in the acquisition of resistant genes, through either horizontal gene transfer or stress inducible mutation, takes place (Beaber *et al*., 2004; Riesenfeld *et al.*, 1997; Thi *et al*., 2011). In addition, β-lactams have recently been shown to induce the expression of the SOS response through the DpiBA two-component system. The effector of the DpiBA component system, Dpi, regulates all the following processes: DNA transcription, replication, and segregation by binding

to A+T rich sequence in the replication origin of the *E. coli* chromosome by unknown mechanisms. DpiA competes with the replication proteins DnaA and DnaB to bind to the replication origin. The overexpression of DpiA blocks replication and induces the SOS response (Miller *et al*., 2004). KAN induction of the SOS response could not be monitored by the GFP reporter gene since KAN is a translation inhibitor, and in order to monitor the GFP expression, active transcription and translation of the GFP reporter, regulated via LexA, are needed (Kohanski *et al*., 2007); therefore, the effect of Fe-PcTs could not be evaluated. However, it has been proven by Kohanski's group that abrogating the SOS response activity by knocking out *recA* made the cells more sensitive to KAN than the wild type, confirming the role of the SOS response in neutralizing the killing effects of the aminoglycoside family (Kohanski *et al*., 2007). Moreover, the compound Fe-PcTs potentiated the effect of a low dose of CFX, and this cocktail of compounds killed bacteria 24 hours post treatment with an efficiency equal to that of CFX by itself. It appears that the molecule Fe-PcTs weakens the ability of bacteria to withstand exposure to CFX based on the assumption that Fe-PcTs blocks the SOS response-mediated DNA repair pathways and SOS mutagenesis which repairs CFX-induced DNA DSB. The effect of Fe-PcTs carried over to other classes of bactericidal drugs, not the bacteriostatic drugs. Collectively, all the results related to induction of the DNA damage response by bactericidal or bacteriostatic microbial were consistent with the previous experiments by Kohanski's group. Fe-PcTs represented in the phthalocyanine class display a unique biological activity in antibacterial chemotherapeutics and may serve as lead candidates for the development of adjuvants for the treatment of bacterial infectious diseases. Additionally, using such cell-permeable small molecules, controlling RecA, proves the principle that RecA may be a novel target for antibacterial chemotherapy not belonging to the traditional classes of traditional antibiotics.

The SOS response is universally conserved in the entire bacterial kingdom, which repairs DNA spontaneously and environmentally stress-induced damage (Brendel *et al*., 1997; Roca and Cox, 1997). A small number of bacteria lack the SOS-inducible stress response. These bacteria are parasites, colonizing internal anaerobic environments in animals, such as the intestinal tract and reproductive system which are essentially free of DNA-damaging elements, like UV light and oxygen radicals (Black *et al*., 1998). These bacteria are member of *Neisseriaceae spp.* (Black *et al*., 1998; Kline *et al*., 2003), *Acinetobacter calcoaceticus* (Rauch *et al*., 1996), *Thiobacillus ferrooxidans* (Ramesar *et al*., 1989), and *Bacteroides fragilis* (Goodman *et al*.,

1987). However, RecA holds a highly conserved function in repairing DNA damage through its recombinational activity. Bacterial organisms that showed ability to express a defined SOS response were able to catalyze repair mechanisms and the SOS mutagenesis. Since, among bacterial species, RecA is a highly conserved protein in both structural and functional aspects, it is more likely that an inhibitor of RecA from one bacterial species will cross-inhibit the RecA from another bacterial species. The ability of Fe-PcTs to potentiate CFX activity in strains other than the ATCC25922 showed that the DNA damage by CFX treatment that produces DSB in the DNA and leads to the formation of a stalled replication fork that could not be repaired or to be tolerated in the presence of Fe-PcTs. The only exception was *P. aruginosa,* which showed a poor synergistic response to CFX and Fe-PcTs compared to the other strains. This strain's MIC exhibited moderate susceptibility to CFX (MIC $\geq$ 1.5-32 µg/ml), which is mediated by the efflux pumps that raises the MICs for penicillins, cephalosporins, quinolones, tetracyclines, and chloramphenicol (Pankey and Ashcraft, 2005). The poor sensitivity to the synergistic effect of CFX plus Fe-PcTs may be related in some part to the resistance property to CFX, exhibited by this strain. A study investigating the synergistic efficacy of synergistic antibiotic combinations in multidrug resistant *P. aeruginosa* strains showed no synergy has been detected in CFX and imipenem or tobramycin combinations (Dundar and Otkun, 2010). Resistance to CFX in *Pseudomonas* strains is due to a relative impermeability of the organism to CFX, which cannot be overcome by combination therapy.

The conventional paradigm links the evolution of antibiotic resistance to selection for pre-existing mutants in a population of microbes exposed to an antibiotic therapy. It suggests that resistance-conferring mutations are the inevitable consequence of polymerase errors and are random events where intervention is not possible. In contrast, the modern paradigm suggests a regulated process, for which bacteria play a very proactive role in the mutation of their own genomes by inducing particular proteins, at least when the DNA is exposed to certain antimicrobials and DNA-damaging agents (Cirz and Romesberg, 2007), including PolIV and PolV (Cirz *et al*., 2005). This is a paradigm supporting the notion that resistance depends on particular biochemical pathways, and intervention with these pathways would be a promising approach to combat the resistance issue. More specifically, inhibition of regulator proteins of these pathways, or the prevention of their de-repression by inhibition of LexA cleavage, with suitably designed drugs, might represent a fundamentally new approach to combating the

emerging threat of antibiotic-resistant bacteria. Based on the *in vitro* resistance assay, Fe-PcTs was able to suppress the acquisition of CFX resistance in the pathogenic *E. coli* ATCC25922 treated with CFX. These results are comparable to the decrease in CFX-induced resistance observed in genetic mutations of SOS response and DNA repair genes (Cirz *et al*., 2005). The *in vivo* resistance assay confirmed that Fe-PcTs attenuate the acquisition of CFX resistance in a neutrapenic murine thigh bacterial infection. Remarkably, no CFX resistant cells were observed when mice were co-treated with CFX and Fe-PcTs.

Drug-resistant pathogen infections are becoming more prevalent and one of the major issues in the health field. The increase in resistance has limited the number of effective antimicrobials, creating a problematic situation that has been amplified by the small number of new antibiotics introduced in recent years. The mechanism of killing by which bactericidal antibiotics kill bacteria, recently reported, provides new avenues for the development of new antibacterial compounds, as well as adjuvant molecules that could enhance the potency of current antibiotics. The roles that RecA plays in the SOS DNA repair, recombinational repair, mutagenic repair, and lateral gene transfer mechanisms are important to be effectively used in identifying inhibitors of RecA that potentiate the activity of bactericidal drugs and block resistance. Translation of this knowledge into new clinical treatments and approaches can effectively help to fight the growing threat from resistant pathogens. Most of the available combination therapies that are aimed to neutralize resistance mechanisms are specific to certain antibiotic classes. The best-well known example is the β-lactam class antibiotic (amoxicillin) with a lactamase inhibitor (clavulanate). In striking contrast, Fe-PcTs can be part of a wide-range of bactericidal antibiotic cocktails, providing a more general approach to counter the effects of resistance. Fe-PcTs can be combined to a wide array of antibiotic therapies, which will potentiate their activity and prolong their lifespan by reducing the acquisition of antibiotic resistance mutations.

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APPENDIX A

6.1 Solutions and Media

PSB

Dissolve the following ingredients: 8 gram of NaCl, 0.2 gram of KCl, 1.44 gram of Na_2HPO_4, and 0.24 gram of KH_2PO_4 in 800 mL of distilled water. Adjust pH to 7.4. Adjust volume to 1L with additional distilled H_2O. Sterilize by autoclaving

M9

Combine the following ingredients: 3 gram of KH_2PO_4, 6 gram of Na_2HPO_4, 5 gram of NaCl, 1 mL of $MgSO_4$ (1M) in 1 L of distilled water in a large beaker or a graduated cylinder using magnetic stir bar. Aliquot into appropriate sized bottles and then autoclave.

SB

Combine the following ingredients: 8 gram NaOH, and 45 gram boric acid in 1L of distilled water.

MHB

Add 21 gram MHB powder to 1 L of distilled water and then autoclave.

LBB

Add the following ingredients: 10 gram Bacto-tryptone, 5 gram yeast extract, 5 gram NaCl to 800 mL H_2O. Adjust pH to 7.5 with NaOH. Adjust volume to 1L with distilled water. Sterilize by autoclaving.

LBA

Prepare 1L of LBB and then add 15 gram agar. Sterilize by autoclaving.

TSA

Add 40 gram of TSA to 1 L of distilled water and then autoclave.

APPENDIX B

7.1 List of Reagents, Equipment and drugs

Table 7.1.1 List of reagents used in this project.

Item	**Supplier**
Agarose	Invitrogen
Acetic acid, glacial	Fisher
dNTP set	Fermentas
Ethidium bromide	BDH
Ethyl alcohol 95%	Commercial Alcohols
EDTA (ethylenediaminetraacetic acid)	EM Science
Glycerol	EMD
Magnesium sulfate	EMD
Sodium chloride	EMD
Sodium hydroxide	BDH
Sodium phosphate dibasic	EMD
Sodium phosphate	EMD
TRIS [Tris (hydroxymethyl) aminomethane]	EMD
10X Buffer *Taq* polymerase (platinum, high fidelity)	Invitrogen
Taq DNA polymerase (platinum, high fidelity)	NEB
30-(p-hydroxyphenyl) fluorescein (HPF)	Invitrogen
Crystal violet	Fisher

Table 7.1.2. List of reagents used in media preparation.

Item	Supplier
Agar	BD Biosciences
Trypton	BD Biosciences
Yeast extract	BD Biosciences
Muller Hinton broth (MHB)	Becton Dickinson
Tryptic soy agar (TSA)	Becton, Dickinson
Sheep red blood cells (SRBC)	Oxoid

Table 7.1.3. List of equipment used in this study.

Item	Supplier
96-1.2ml tube rack	Molecular Bioproducts
Centrifuge 5810	Eppendorf
Glass beads, 450-600μm in diameter	Sigma
MaxQ 4000 shaking incubator	Barnstead
Microfuge 18 centrifuge	Beckman Coulter
Micropipettors	Gilson
Spectramax M5 microplate reader	Molecular Devices
Ultraspec 3000 spectrophotometer	Pharmacia Biotech
Spectrophotometer	Thermo scientific, Genesys 20
Ultra-Tech WJ 301D incubator	Baxter
VP 408FH Replicator	V&P Scientific
Colorimeter	BioMérieux Vitek, Inc
Cytometer	Beckman coulter, Inc

Nikon Eclipse E400 compound microscope	Laboratory Optical Service, Inc
Tissue grinder	VWR international
UV light transilluminator	BioRad
Thermocycler	BioRad

Table 7.1.4. Drugs used in this study and their sources.

Drugs	**Types**	**Source**
Ampicillin (AMP)	Powder	Shelton Scientific
Ciprofloxacin hydrochloride (CFX)	Powder	Bayer Pharmaceutical
Kanamycin monosulfate (KAN)	Powder	Sigma-Aldrich Co.
Chloramphenicol (CAM)	Solution	Sigma-Aldrich Co.
Spectinomycin dihydrochloride (SPECT)	Powder	Sigma-Aldrich Co.
Tetracycline hydrochloride (TET)	Powder	Fluka
Iron (III) phthalocyanine-4,4′,4″,4‴-tetrasulfonic acid, compound with an oxygen monosodium salt hydrate (Fe-PcTs)	Powder	Sigma-Aldrich Co.
Copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt (Cu-PcTs)	Powder	Sigma-Aldrich Co.
Copper phthalocyanine-tetrasulfonic acid (Cu-Pcts isomer)	Powder	Sigma-Aldrich Co.
Nikel phthalocyanine tetrasulfonic acid (Ni-PcTs)	Powder	Sigma-Aldrich Co.
Phthalocyanine tetrasulfonic acid tetrasodium salt (Apo-PcTs)	Powder	Sigma-Aldrich Co.
Aluminum (II) phthalocyanine chloride tetrasulfonic acid (Al-PcTs)	Powder	Frontier Scientific

Zinc (II) phthalocyanine tetrasulfonic acid (Zn-PcTs)	Powder	Frontier Scientific