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Investigating the fluoroquinolone-topoisomerase interaction by use of novel fluoroquinolone and quinazoline analogs

Kevin Randall Marks
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INVESTIGATING THE FLUOROQUINOLONONE-TOPOISOMERASE INTERACTION
BY USE OF NOVEL FLUOROQUINOLONONE AND QUINAZOLINE ANALOGS

by

Kevin Randall Marks

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacy (Medicinal and Natural Products Chemistry)
in the Graduate College of
The University of Iowa

May 2011

Thesis Supervisor:  Associate Professor Robert J. Kerns
Fluoroquinolones are broad-spectrum antibacterial agents based on the structure of nalidixic acid. For nearly five decades it has been known that fluoroquinolones inhibit bacterial growth by blocking the enzymatic action of type II topoisomerases such as DNA gyrase and topoisomerase IV. Only recently has it been discovered that some fluoroquinolones are capable of a mechanism that results in fragmented DNA and leads to rapid bacterial cell death. This mechanism is not well understood. Presented here are studies towards understanding the structure activity relationship (SAR) of fluoroquinolones, specifically to determine what leads to the novel mechanism termed “rapid lethality.” This work is based on the hypothesis that structurally unique fluoroquinolones interact with the DNA-topoisomerase complex in a unique manner that ultimately leads to rapid cell death.

The first approach to understand SAR for killing was to evaluate the effect of a ring fusion between N-1 and C-8 of the fluoroquinolone core. Known lethal fluoroquinolones are substituted by N-1 cyclopropyl and C-8 methoxy, but some clinically important fluoroquinolones contain a 2-methylmopholino moiety between these two positions. Novel fluoroquinolones were synthesized and clinically available agents were obtained to create a panel of drug molecules with one of six C-7 substituents and either the morpholine ring system or N-1 cyclopropyl and C-8 methoxy. Bacteriostatic and bactericidal activities of these compounds were determined. Bactericidal studies were conducted both in the presence and absence of chloramphenicol, a protein synthesis inhibitor used to simulate non-growing bacteria. Lethality in the presence of chloramphenicol is also important when considering co-administration of fluoroquinolones with other antibiotic classes.

In a second study, fluoroquinolones were synthesized with a C-2 thioalkyl substitution. Substitutions at the C-2 position are severely lacking in clinical fluoroquinolones, with only prulifloxacin, a newly developed antibiotic, being substituted by an N-1 to C-2 thiazetidine ring structure. Analogs of ciprofloxacin and moxifloxacin
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In a third study, quinazoline-2,4-diones, a new antibiotic class structurally and mechanistically similar to fluoroquinolones, were modified at the C-4 position in an effort to understand the binding interaction between these compounds and the target enzyme. Importantly, the quinazoline-2,4-diones typically retain activity against bacterial cells known to be resistant to fluoroquinolones and are less likely to select for resistant mutants. In this study, the C-4 carbonyl was replaced with either a thiocarbonyl or a hydroxylimine and the new compounds, bearing C-7 substituents common to potent antibiotic fluoroquinolones and quinazolines, were evaluated for activity against bacterial cells.

Despite the findings of recently published X-ray crystallography, it was determined that one of the greatest determinants in antibiotic activity of fluoroquinolones is the C-7 substituent. Additionally, there is increasing evidence that the C-2 carbonyl of quinazoline-2,4-diones affords the increase in activity against resistant mutants by creating a unique binding interaction. Collectively, the conclusions reached here add to our understanding of the structure activity relationship of the fluoroquinolone antibiotic class for rapidly killing bacterial cells and overcoming resistant mutants.

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Thesis Supervisor: Associate Professor Robert J. Kerns
CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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ABSTRACT

Fluoroquinolones are broad-spectrum antibacterial agents based on the structure of nalidixic acid. For nearly five decades it has been known that fluoroquinolones inhibit bacterial growth by blocking the enzymatic action of type II topoisomerases such as DNA gyrase and topoisomerase IV. Only recently has it been discovered that some fluoroquinolones are capable of a mechanism that results in fragmented DNA and leads to rapid bacterial cell death. This mechanism is not well understood. Presented here are studies towards understanding the structure activity relationship (SAR) of fluoroquinolones, specifically to determine what leads to the novel mechanism termed “rapid lethality.” This work is based on the hypothesis that structurally unique fluoroquinolones interact with the DNA-topoisomerase complex in a unique manner that ultimately leads to rapid cell death.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>Deuterated Chloroform</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
</tr>
<tr>
<td>DCE</td>
<td>1,2-Dichloethane</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DME</td>
<td>1,2-Dimethoxyethane</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DMSO-d$_6$</td>
<td>Deuterated Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Correlation</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure/Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>KHMDS</td>
<td>Potassium Hexamethyldisilazane [Potassium bis(trimethylsilylamide)]</td>
</tr>
<tr>
<td>LRMS</td>
<td>Low Resolution Mass Spectrometry</td>
</tr>
<tr>
<td>LD$_{(x)}$</td>
<td>Lethal Dose (for X% of the population)</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple Drug Resistant</td>
</tr>
<tr>
<td>MIC$_{(x)}$</td>
<td>Minimum Inhibitory Concentration (for X% of the population)</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance spectroscopy</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trfluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
</tbody>
</table>
SAR – Structure-Activity Relationship
XDR – Extensively Drug Resistant
CHAPTER 1 INTRODUCTION

1.1 Antibiotics

Antibiotic agents are a class of chemical compounds, originally specifically of bacterial origin [3], possessing the ability to “inhibit the growth of and even to destroy bacteria and other micro-organisms.” This definition encompasses traditional antibiotic agents such as aminoglycosides, tetracyclines, macrolides, and β-lactams. The contemporary definition of antibiotic has been further updated to include chemically modified substances and those of fully synthetic chemical origins, such as fluoroquinolones (a class that includes isothiazoloquinolones and quinazoline diones), sulfonamides, and oxazolidinones [4]. Optimum antibiotic agents exploit the unique differences between infectious bacteria and the host, such as bacterial cell wall synthesis or folate coenzyme synthesis, to reduce off-target effects and damage to host cells. Additionally, two other general mechanisms of antibiotic action are known: protein biosynthesis inhibition and inhibition of DNA replication/repair. Though the latter two processes are not unique to bacterial cells in comparison to mammalian cells, there are differences that allow selective inhibition [5].

The history of naturally occurring antibiotics began in 1928 with the discovery by Alexander Fleming that the bacterium *Penicillium notatum* inhibited growth of other bacteria [6]. The penicillins were first used clinically during World War II and became widely available to the civilian market shortly after the war with the increase in mass production capability [7]. Over the next twenty years, booming antibiotic research produced several natural product derived classes of antibiotics including tetracyclines [8], glycopeptides [9], macrolides, and phenylpropanoids [10]. After a 41 year lag, the most recent naturally derived antibiotic class, lipopeptides, was introduced with daptomycin in 2003 [11].
Prontosil, a synthetically prepared sulfonamide and the first clinical antibiotic, was released by Bayer in 1935 [12]. It would be almost 30 years before the next fully synthetic antibiotics, the quinolones, would hit the market in 1962, when nalidixic acid was found to be effective for the treatment of urinary tract infections [13]. Nearly four more decades would pass before the next synthetic antibiotic class, the oxazolidinones, would enter clinical use in 2000 [14].

The long periods of relative inactivity in development of antibiotic agents can be attributed to the incorrect belief that, by the 1960s, bacterial infections would soon no longer be a threat in most countries [15]. However, bacterial resistance was already being recognized in the early 1960s [16] and even with vigilant and responsible use of clinical antibiotics, developing resistance is becoming increasingly problematic [17], with even antibiotics of last resort, such as vancomycin, becoming ineffective against mutated pathogens [18, 19]. The recent increase in the threat of community-acquired- and healthcare-acquired-methicillin resistant staphylococcus aureus (CA-MRSA and HA-MRSA, respectively) has brought the threat of resistant bacteria into the public consciousness [20, 21].

1.2 Fluoroquinolones

The fluoroquinolone antibiotic class is structurally based on the fully synthetic napthyridone, nalidixic acid (Figure 1). Nalidixic acid, first reported in 1962, was the first active agent in the class and the cornerstone of the first generation of the fluoroquinolone class of antibiotics. The fluoroquinolone antibiotic class has evolved through four subsequent generations, each loosely defined by either structural modifications, ability to overcome resistant mutant bacteria, or type of bacterial infection targeted [22, 23]. The only characterization that is universally applied is that the non-fluorinated compounds are grouped into the first generation and fluorinated compounds are grouped into the latter
Table 1. Examples of antibiotic class by origin.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Class</th>
<th>Example</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biosynthetic</td>
<td>Penicillins</td>
<td>Penicillin G</td>
<td>Inhibition of cell wall biosynthesis</td>
</tr>
<tr>
<td></td>
<td>Tetracyclines</td>
<td>Tetracycline</td>
<td>Protein synthesis inhibition by inhibiting 30S ribosomal subunit</td>
</tr>
<tr>
<td></td>
<td>Glycopeptides</td>
<td>Vancomycin (see Fig A41)</td>
<td>Inhibition of cell wall biosynthesis</td>
</tr>
<tr>
<td></td>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>Protein synthesis inhibition by blocking 50S rRNA complex</td>
</tr>
<tr>
<td></td>
<td>Phenylpropanoids</td>
<td>Chloramphenicol</td>
<td>Protein synthesis inhibition</td>
</tr>
<tr>
<td></td>
<td>Lipopeptides</td>
<td>Daptomycin (see Figure A42)</td>
<td>Cell membrane disruption/depolarization</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Sulfonamides</td>
<td>Prontosil</td>
<td>Folate synthesis inhibition (by direct inhibition of dihydropterate synthetase)</td>
</tr>
<tr>
<td></td>
<td>Fluoroquinolones</td>
<td>Ciprofloxacin</td>
<td>Inhibition of DNA replication</td>
</tr>
<tr>
<td></td>
<td>Oxazolidinones</td>
<td>Linezolid</td>
<td>Protein synthesis inhibition</td>
</tr>
</tbody>
</table>
generations. However, some recently described broad spectrum antibacterial agents, such as garenoxacin, do not contain a fluorine, despite being derived from the later generations of the fluoroquinolone class [24].

1.2.1 Fluoroquinolones by generation

First generation quinolone antibiotics have been used as drugs of choice for the treatment of uncomplicated urinary tract infections since their arrival to the market. The first generation agents are typically active against Gram-negative bacteria, with the exception of *Pseudomonas* species. Few first generation quinolones remain on the market due to genotoxic and carcinogenic side effects [25].

Second generation quinolones were the first in class to contain a C-6 position fluorine atom, hence addition of the fluoro- prefix (Figure 1). Targets of the second generation tend to be limited to Gram-negative pathogens, now including *Pseudomonas* species, but they also include activity against some Gram-positive species, such as *Staphylococcus aureus*. Clinical infections that have been treated using second generation fluoroquinolones include urinary tract infections (both complicated and uncomplicated), pyelonephritis, sexually transmitted diseases, prostatitis, and localized skin and soft tissue infections [25]. The second generation is often separated into class IIa and class IIb. IIb agents tend to have greater broad-spectrum applications, particularly enhanced activity against Gram-positive species and respiratory infections, and a pharmacokinetic profile allowing once daily dosing [25]. In perhaps the most well publicized use of a second generation fluoroquinolone, ciprofloxacin (Cipro) was administered to several government workers as a prophylactic treatment during the anthrax scare in the fall of 2001 [26].

Third generation fluoroquinolones are used almost exclusively against respiratory infections [25]. Broad-spectrum activity is further increased over second generation agents, and treatment targets include expanded Gram-positive bacteria and some
penicillin resistant bacteria. Like the second generation, the third generation agents are occasionally split into IIIa and IIIb, however, IIIb agents are commonly classified as fourth generation [23].

The line between what is a third generation or fourth generation fluoroquinolone is poorly distinguished, with some agents, such as moxifloxacin, being reported as in either third or fourth generation, depending on the source. In general, fourth generation
fluoroquinolones are used to treat similar infections as third generation drugs, but also have activity against anaerobic targets as well as infections of the pelvis, abdomen, and nosocomial pneumonia. Overall activity against targets of second and third generation quinolones is retained, but further increased. Of key importance, the fourth generation fluoroquinolones are active against ciprofloxacin-resistant pneumonococci.

1.2.2 Mechanism of action by fluoroquinolones

The general mechanism of action employed by the fluoroquinolone class is inhibition of type II topoisomerases DNA gyrase or topoisomerase IV (topoIV). TopoIV and DNA gyrase are the enzymes responsible for the relaxing of supercoiled DNA and inducing supercoiling after replication. When functioning properly, topoIV and gyrase work to relax negatively supercoiled DNA by creating a nicked site in one strand of double stranded DNA, passing the complementary strand through the break, and then ligating the nicked ends [27] (Figure 2). When a fluoroquinolone binds to the DNA-topoisomerase complex, the resulting ternary complex inhibits cell growth and DNA replication [28, 29]. Most fluoroquinolones preferentially bind either topoIV or DNA gyrase; dual target agents are nearly equipotent against both [30]. Agents that exhibit dual targeting are likely to be more active against resistant mutants.

Ternary complexes involving first and second generation quinolones exclusively result in bacteriostatic conditions and, upon lysis of the cell and addition of a detergent such as sodium dodecyl sulfate (SDS) [31, 32], DNA fragments of varying lengths can be visualized by polyacrylamide gel electrophoresis (PAGE). By a unique mechanism, only recently discovered and not well understood, some third and fourth generation fluoroquinolones will induce destabilization of the ternary complex, resulting in fragmented DNA, a process that has been termed “rapid lethality” [33]. Furthermore, there appear to be two different pathways in this new rapid lethality mechanism, one route which requires ongoing protein synthesis and one which remains active when
protein synthesis is halted by addition of chloramphenicol. The particular pathway is determined by the specific fluoroquinolone in the ternary complex, but currently it is not understood what properties of the fluoroquinolones lead to one mechanism over the other.

1.3 Bacterial resistance to antibiotics

Emerging bacterial resistance to antibiotics is increasingly becoming a problem throughout the world [34]. In many cases, in fact, the prevalence of resistance is so great that patients are treated as having a resistant infection upon admission into the clinic and only tested to determine a specific strain after treatment has begun [35]. Generally, bacterial resistance can be defined as mutations in bacteria leading to future generations displaying lower or no susceptibility to antibiotic drugs. The rapid reproduction rate of most infectious bacteria, coupled with the slow rate of bacterial killing by most antibiotics is attributed to the rapidly increasing resistance to antibiotics.

Rapidly increasing antibiotic resistance has led to restrictions on the use of antibiotics in order to reduce exposure of bacteria to “drugs of last resort,” decreasing the chances of selecting for strains resistant to these drugs [36]. Other areas of focus include

Figure 2. DNA passes between the GyrA (parC) dimer and GyrB (parE) dimer subunits. Fluoroquinolone binds in the GyrA (parC) dimer interface near the active site. (Figure adapted from http://www.triusrx.com/trius-therapeutics-gyrb-parc.php)
preventing the spread of infection by isolating patients known to have a resistant infection, and increased hygienic practices by healthcare workers to prevent cross-infection between patients.

1.3.1 Mechanisms of bacterial resistance to antibiotics

Mechanisms of antibiotic resistance in bacteria have been studied extensively [37, 38]. The two overarching categories of resistance mechanisms are phenotypic, reversible resistance and heritable, permanent genetic resistance. The most common example of phenotypic resistance is bacteria that can exist in a latent phase; latent bacteria, or persister cells, are tolerant to most antibiotic drugs until they exit latency and begin replicating. The concept of persister cells was first observed and described by Joseph Bigger in 1944 [39]. Bigger found that when a population of Staphylococcus spp. was treated with penicillin, a small portion of the cells survived, but were not strictly antibiotic resistant mutants. He proposed that these cells were in fact dormant, having temporarily traded growth for antibiotic tolerance. Further research in E. coli found that cells having a mutation to the hipA gene were approximately 1000 fold more likely to be tolerant to ampicillin treatment, though not truly resistant [40]. Persister cells, due to the long-term infection that is essentially untreated, can increase the production of truly resistant mutant bacterial cells [41].

Prevalence of genetic resistance is greatly increased in correlation with infection load. That is, the larger the infectious bacteria population, the more likely it is that there exists genetic variation within the population that results in cells that are inherently resistant to one or more antibiotic agents. The most common treatment plan for overcoming this form of resistance is combination therapy, in which multiple drugs are administered at once, decreasing the chances that a mutant will survive because it is statistically extremely unlikely that a viable cell has developed preexisting mutations resulting in resistance to multiple mechanisms of action from different drugs [38].
However, a hazard of combination therapy is that some drug-drug interactions may result in decreased effectiveness or increased toxicity [25].

Resistance of bacteria to antibacterial treatments is ever increasing, and for a number of reasons. In general, two events must occur to result in clinically significant resistant strains: (1) resistant bacteria must be generated, a process that occurs randomly in nature, and (2) resistant bacteria must be selected for [42]. Unfortunately, overuse of antibiotics, both clinically and agriculturally, increases the risk of generating resistant mutations, resulting in what could be untreatable infectious diseases [43-46]. It has also become evident that some antibiotics, including fluoroquinolones, can even promote resistance by inducing mutations in bacterial cells [47].

1.3.2 Bacterial resistance to fluoroquinolone antibiotics

Bacterial resistance to fluoroquinolones is commonly a result of mutations to amino acids in the area of the active site of gyrA (DNA gyrase) or ParC (TopoIV), from positions 67-106 [48], with specific amino acid positions 83 and 87 (gyrA) often associated with clinical resistance [49]. This region, known as the quinolone resistance determining region (QRDR) [50]. This region is within the DNA binding interface of gyrase [27]. Fluoroquinolone antibiotics bind to both DNA and the topoisomerase with amino acids in the QRDR form a binding pocket; mutations in this region result in lower affinity for fluoroquinolone binding and decreased activity [51].

1.4 DNA Gyrase/Topoisomerase IV

1.4.1 Structure

The general structure of all type II topoisomerases, including DNA gyrase and topoisomerase IV, is an $A_2B_2$ heterotetramer. The structure of gyrase consists of two GyrA and two GyrB subunits, and topoIV consists of two parC and two parE subunits. GyrB (parE) is the subunit associated with ATP binding and energy transfer. GyrA
10

(parC) contains the DNA cleavage domain and the quinolone binding site (Figure 3).

1.4.2 Mechanism of Gyrase/TopoIV

As briefly mentioned previously, type II topoisomerases (e.g. gyrase and topoIV) are responsible for controlling the level of supercoiling in DNA strands (Figure 4). DNA in most organisms exists predominantly in a negatively supercoiled, tightly wound state. Supercoiling can be defined as an abstract mathematical concept, linking number (L), as the sum of two principles, twist (T) and writhe (W), illustrated by the equation:

\[ L = T + W \]

Twist is simply defined by the number of twists in a length of double stranded DNA. In fully relaxed DNA, one full twist occurs approximately every 10.4 base pairs [52]. In the case of circular or long linear DNA, increasing twist results in a torsional strain on the
DNA strand known as writhe, which keeps the linking number static. During replication, DNA is untwisted and strands are separated. This untwisting, however, leads to increased writhe. The increased strain can only be relieved by changing the linking number, which requires a strand break. Topoisomerases are able to break the DNA strand and change linking number by changing twist before religating. DNA gyrase is the only enzyme that is capable of introducing negative supercoils into DNA [53].
1.4.3 Crystallography of the fluoroquinolone-topoIV-DNA ternary complex

Due to the flexibility of the enzyme, the transient nature of the ternary complex, and the constant motion involved in the activity of topoIV/gyrase, crystallography of the ternary complex was not resolved until recently. Three articles published between June of 2009 and August of 2010 have shown, with increasing resolution, various fluoroquinolone drug molecules bound to topoisomerase IV complexed with DNA (Figure 5) [54-56]. Illustrated most clearly in Figure 5C, a key binding interaction occurs
between the C-3 carboxylic acid and the C-4 carbonyl of the quinolone, DNA, and the topoisomerase, mediated by a divalent magnesium cation. Interestingly, none of the three crystal structures show interaction between the fluoroquinolone C-7 substituent and topoIV. Vast differences in activity are observed between quinolones that differ only in the C-7 substituent, such as gatifloxacin and moxifloxacin[57]. Also, different mutations may be induced or overcome by different C-7 groups [42, 58, 59]. Thus, the x-ray structures do not fully explain the structure activity relationship (SAR) of fluoroquinolones.

In as yet unpublished data, Arkady Mustaev used an earlier crystal structure of the DNA-topoisomerase IV complex (lacking bound fluoroquinolone) as a basis to investigate the putative fluoroquinolone binding site on topoisomerase IV. The binding interaction between quinolone and topoisomerase IV is known to be a two step mechanism [60-62], and I propose that the x-ray crystal structures have all isolated only one of the two steps, a step in which the fluoroquinolone makes contact with the QRDR via a magnesium bridge. However, it is not known whether this is the first or second step. The two step binding mechanism might also explain the discrepancy between the lack of indication of any interaction with the C-7 group in each of the crystal structures and the wide variation in activity between quinolones differing only at the C-7 group. A second binding step, possibly a different orientation, must rely on a contact made between the C-7 substituent and the topoisomerase.

As shown in Figure 6, modeling strongly suggests points of contact between quinolone and enzyme not only through the magnesium bridge between Asp87 and the carboxylic acid at C-3, but also in the area of Gly81, Asp82, Ser83, and Ala119. All three binding interactions are further supported by mutations at these locations, induced by treatment with fluoroquinolone drugs, and the ability of some fluoroquinolone drug molecules to overcome resistance caused by mutations at the positions by varying substitutions to the core, particularly at N-1 (Ala119), C-3/4 (Ser83, Asp87, Tyr86), and
Thus, while there is increasing evidence explaining some SAR of fluoroquinolone action, substitution to nearly every available position of the fluoroquinolone core results in effects on activity that cannot be explained by a simple model of drug binding.

Figure 6. Modeling images of ciprofloxacin in the putative binding site of topoisomerase IV. Panels A and B: Space filling render of ciprofloxacin in the binding site viewed from two different angles. Panels C and D: Ribbon model of the enzyme with key amino acids highlighted as ball and stick models. Panels E and F: Close up view of the interaction between fluoroquinolone and key amino acid residues. (Images courtesy Arkady Mustaev)
CHAPTER 2 STATEMENT OF PURPOSE

The increasing rate of bacterial resistance to clinical antimicrobial agents and its impact on treatment of infectious diseases has begun to present a unique problem throughout the world. Drug resistant, multiple drug resistant (MDR), and extensively drug resistant (XDR) infectious bacterial pathogens put a greater risk on the population at large due to the risk of pandemic illness. Increasing complication is that the fact that many antibacterial agents can induce mutations and resistance, often by different mechanisms.

Many compounds considered to be drugs of last resort are becoming increasingly ineffective against emerging mutations. Future treatment of drug resistant bacterial infections relies on development of new agents that are able to overcome current resistance mechanisms while not increasing the rate of emerging resistance. Overcoming resistance by targeting a unique pathway avoids current resistance mechanisms [63], but identifying new targets and drug classes is becoming increasingly difficult, and may only lead to new resistant mechanisms [64]. Drugs developed by structural changes to existing drugs, and having the same mechanism of action, may be ineffective against current resistance mechanisms.

The most effective method of overcoming bacterial resistance is the development of antimicrobial agents that reduce the emergence of resistance. The recently discovered, but poorly understood, mechanism of rapid lethality asserts that this approach is achievable. As the name implies, rapid lethality is a mechanism by which some fluoroquinolones, when dosed at levels approximately ten fold higher than the minimum inhibitory concentration (MIC) of drug for blocking growth in 50 percent of a bacterial population, will initiate chromosomal fragmentation within bacterial cells which leads to very rapid cell death. The clinical benefit of this mechanism is patients’ infections can be treated more quickly, increasing patient compliance and, perhaps more importantly, the
bacterial population is eliminated quickly enough that occurrence of mutations is restricted. In addition, some later generation fluoroquinolones are active against mutants resistant to earlier fluoroquinolone generations. Therefore, new fluoroquinolones that are active against wild-type and current fluoroquinolone resistant cells, particularly those agents that rapidly kill, are expected to have decreased ability to select for or induce resistant mutations.

This study focused on understanding the structure activity relationship of fluoroquinolone class antibiotics as it applies to the rapid lethality mechanism and activity against resistant mutants. The widest variation in fluoroquinolone substitution is at positions N-1, C-7, and C-8. The specific combination of substituents that results in rapid lethality is unknown, but a change at only one position can lead to a large variation in activity.

The first portion of this study investigated the effect of ring fusion between N-1 and C-8, a common structural motif represented by the second and third generation quinolones ofloxacin and levofloxacin, on rapid lethality. The approach used here was to compare fluoroquinolones substituted by cyclopropyl at N-1 and methoxy at C-8 to structurally similar counterparts in which N-1 and C-8 positions were fused as part of a 3-methylmorpholino moiety. The working hypothesis was that the N-1 cyclopropyl and C-8 methoxy groups, which are common on all known rapidly lethal fluoroquinolones, must be free to arrange in a relative trans configuration when interacting with the enzyme. It was believed that the ring fusion between N-1 and C-8 would limit the flexibility of these substituents, resulting not in higher MIC, but reduced rapid lethality potential of the drug.

Functionalization of the fluoroquinolone core at the C-2 position has been limited. Prulifloxacin (Figure 1), the prodrug form of ulifloxacin, a compound developed in the 1990s [65] that has recently undergone clinical approval in several countries, introduced a 2-methyl thiazetidine ring between N-1 and C-2. Ulifloxacin displays a lower than average MIC against a broad range of bacterial targets [66-68] and the C-2 thioether is
believed to be important. A modeling study suggested a potential binding interaction between the sulfur and topoisomerase. In order to understand the contribution of the C-2 thioether and its possible impact on rapid lethality, N-1 cyclopropyl, C-8 methoxy fluoroquinolone molecules were synthesized in which the C-2 position was substituted with a thioethyl or thioisopropyl group. It was anticipated that this study would reveal the contribution of the thioether functional group and possibly result in development of novel fluoroquinolones that exploit the interaction between the C-2 thioalkyl group and the enzyme.

Quinazoline-2,4-diones, a class of compounds structurally similar to fluoroquinolones have been shown to exhibit antibacterial activity analogous to that of fluoroquinolones. Interestingly, quinazoline-2,4-diones, compared to structurally similar fluoroquinolones, do not select for resistant mutants. One key structural difference between fluoroquinolones and quinazoline-2,4-diones is that the diones lack a C-3 carboxylate, a structural feature that is known to be important for magnesium mediated binding between fluoroquinolones and the DNA-topoisomerase complex. In order to investigate the structural requirements for the quinazoline-2,4-diones to interact with the enzyme, a structure activity relationship study was undertaken in which specific modifications to the N-3 and C-4 positions of the quinazoline core were synthesized. The expected outcome for this portion of the project was a better understanding of the pharmacophore and specific interactions between quinazoline-2,4-diones and topoisomerase IV that lead to antibacterial activity and reduced mutant selection.

The overarching goal of this work was to better understand the structure activity relationship of various substitutions to the fluoroquinolone antibiotic class, specifically as it applies to the recently discovered, but poorly understood mechanism of rapid lethality. This study was accomplished by the design and synthesis of fluoroquinolones and quinazoline-2,4-diones with unique ring structures and substitution or modification at the largely neglected positions C-2, C-3, and C-4. The synthesized compounds were
tested with bacterial cells alongside known fluoroquinolones to compare for various activities.
CHAPTER 3 EFFECT OF N-1 TO C-8 RING FUSION ON RAPID LETHALITY OF FLUOROQUINOLONES

The work presented in this chapter, in part, has been published in *Antimicrobial Agents and Chemotherapy* [69].

The general mechanism of fluoroquinolone mediated cell death has long been known. Formation of a ternary drug-enzyme-DNA complex, which is reversed by removal of quinolone (5, 23), blocks DNA replication, RNA synthesis, and bacterial growth (3, 5, 21). Events subsequent to complex formation are irreversible and kill bacteria. The mechanism of rapid cell death, which correlates with chromosome fragmentation, is illustrated in Figure 7.

DNA + Gyrase

\[ \text{DNA-Gyrase Complex} \]

Quinolone

Quinolone-DNA-Gyrase Ternary Complex

\[ \text{Cm} \]

\[ X \text{ Cm} \]

Chromosome Fragmentation (Synthesis Dependent) \hspace{2cm} Chromosome Fragmentation (Synthesis Independent)

Figure 7. The quinolone-DNA-gyrase complex may lead to chromosome fragmentation and cell death by either of two pathways. In one pathway (left) ongoing protein synthesis is required. Fluoroquinolones that act by this pathway can be inhibited by addition of chloramphenicol. In the other pathway (right) protein synthesis is not required and cell death is not inhibited by addition of chloramphenicol.
fragmentation, is much less well understood. However, it is known that rapid cell death occurs by two pathways, one that requires protein synthesis and one that does not, as illustrated in Figure 7.

The former pathway is known to involve production of the toxic hydroxyl radical, since agents that inhibit hydroxyl radical accumulation block the lethal action of quinolones, such as oxolinic acid [70]. The latter pathway does not require hydroxyl radical accumulation for cell death. It has been suggested that this pathway involves drug-induced destabilization of ternary complexes that leads to chromosome fragmentation, because fragmentation occurs when gyrase and fluoroquinolone are added to isolated nucleoids [33]. What specific characteristics of fluoroquinolone structure lead to this pathway of chromosomal fragmentation is not known.

Using chloramphenicol to inhibit protein synthesis, several key features have been identified that contribute to rapid cell death. Comparison between fluoroquinolones differing only in N-1 substituent, such as norfloxacin and ciprofloxacin (Figure 1) has determined that one key feature contributing to the rapid lethality mechanism in the absence of protein synthesis is the N-1 cyclopropyl group: ciprofloxacin is lethal against E. coli in the presence of chloramphenicol, but norfloxacin is not [71, 72]. Also determined to be of key importance is the C-8 substituent. The inclusion of a methoxy group at the C-8 position has been shown to improve the lethal activity of fluoroquinolones against S. aureus [73, 74], E. coli [71, 75-80], M. smegmatis [57] and M. tuberculosi [81]. Furthermore, inclusion of specific C-7 ring systems also plays a part in the lethal activity of fluoroquinolones. With E. coli, a C-7 N-ethyl piperazine renders the action of an N-1 cyclopropyl, C-8 methoxy compound (PD161144) insensitive to chloramphenicol, while the C-7 diazabicyclo ring of moxifloxacin affords this insensitivity with mycobacteria [33, 81]. Thus, structure-activity relationships for killing in the absence of ongoing protein synthesis are beginning to emerge.
3.1 Effect of the orientation of the N-1 and C-8 substituents

The N-1 cyclopropyl and C-8 methoxy substituents, when modeled in an energy minimized state, arrange in a relative trans configuration to one another. However, due to the lack of chiral bonds, there is no absolute stereo chemistry in the fluoroquinolone core, and no absolute configuration of the N-1 and C-8 substituents can be inferred and modeling outcome is based on the initial configuration (Figure 8).

The absolute configuration of the N-1 substituent is implied in the difference in activity seen between ofloxacin and levofloxacin (Figure 1). Ofloxacin, the racemic form of levofloxacin, displays approximately twice the MIC (one half the antibacterial activity) of levofloxacin across all susceptible species. Incidentally, the inactive R isomer has been blamed for human toxicity following clinical administration of ofloxacin [75].

Figure 8. Energy minimization of moxifloxacin results in the N-1 cyclopropyl and C-8 methoxy groups aligning trans to each other. Absolute conformation depends on starting orientation. Shown: Two dimensional drawing of moxifloxacin (left) and MM2 energy minimization of moxifloxacin from different starting conformations, viewed along the C-4 carbonyl bond (center) and along the plane of the aromatic ring (right).
3.2 Effect of ring fusion

The particular importance of the N-1 cyclopropyl and C-8 methoxy groups’ contribution to killing in the absence of protein synthesis raises interesting questions about the lethality of clinically important fluoroquinolones such as levofloxacin. As shown in the overlay in Figure 9, levofloxacin can be envisioned as having a fusion between its N-1 cyclopropyl and C-8 methoxy substituents. The result is a rigid ring structure in which the two substituents are not free to rotate or reposition relative to the quinolone core. This loss of flexibility could affect the formation of ternary complexes, either positively or negatively. Comparison between fluoroquinolones differing only in the structural motif of either N-1 cyclopropyl, C-8 methoxy or N-1/C-8 fusion gives the best insight into the effect of ring fusion and whether it promotes or impedes the killing of bacterial cells in the presence of chloramphenicol.

In order to determine the effect of ring fusion on killing in the presence of chloramphenicol, levofloxacin-like fluoroquinolones, that is, those with N-1 to C-8 ring fusions were compared to similar fluoroquinolones substituted by N-1 cyclopropyl and C-8 methoxy. Bactericidal studies show that in all cases, the N-1 cyclopropyl, C-8 methoxy fluoroquinolones displayed greater lethal activity against bacterial cells when

![Figure 9](image)

Figure 9. Comparison between 1-cyclopropyl-6,7-difluoro-8-methoxy fluoroquinolone core (top left) and 6,7-difluoro-levofloxacin core (top right). Overlay of 1-cyclopropyl-6,7-difluoro-8-methoxy core (blue) on 6,7-difluoro levofloxacin core (red) illustrates the structural similarity between the two (bottom).
protein synthesis was inhibited by treatment with chloramphenicol. This result is best explained by a necessary interaction between the N-1 and C-8 substituents. However, X-ray structures of ternary complexes, shown previously, indicate that the N-1 and C-8 positions are not in contact with the enzyme. Combined, these data support the hypothesis of a two step binding process between fluoroquinolone and gyrase/topoIV, resulting in the ternary complex.

3.3 Goals of this study

With the discovery that some fluoroquinolones are capable of initiating a rapid lethality mechanism even in the presence of a protein synthesis inhibitor such as chloramphenicol new research is required. Some fluoroquinolones exhibiting comparatively higher MICs show greater potential for lethality in the presence of chloramphenicol than more potent inhibitors of bacterial growth.

The first goal of this study was to use N-1 to C-8 ring fused analogs to confirm that the orientation of the N-1 and C-8 substituents that leads to greater inhibitory activity is also responsible for greater lethality. This hypothesis was tested by synthetically preparing levofloxacin derivatives, differing from cognate levofloxacin by substitution at the C-7 position, and similar ofloxacin derivatives (Scheme 1). Assuming the hypothesis is correct, racemic ofloxacin compounds were expected to display half the inhibitory potential (twice the MIC) and be half as effective in the presence of chloramphenicol as the levofloxacin derivatives, if at all.

In the second half of this study, a broader panel of levofloxacin analogs, again differing from cognate levofloxacin by C-7 substitution, was compared to N-1 cyclopropyl, C-8 methoxy fluoroquinolones with similar C-7 substituents (Figure 10). Comparisons were made strictly on the ability to kill bacteria in the presence and absence of chloramphenicol. The use of chloramphenicol, a protein synthesis inhibitor, serves two purposes: (1) inhibiting protein synthesis creates a model for studying fluoroquinolone
Scheme 1. Synthesis of ofloxacin and levofloxacin derivatives used for this study.

action against non-growing cells, and (2) fluoroquinolones that remain active in the presence of chloramphenicol can be co-administered with chloramphenicol to achieve a synergistic effect.

Data was normalized to the factor of the MIC needed to affect lethality in bacterial cells to account for differences in MIC between fluoroquinolones. Working under the hypothesis that N-1 cyclopropyl and C-8 methoxy must be able to arrange in a relative trans configuration, it was expected that the fused ring derivatives would not only have a higher MIC (lower inhibitory potency) but also that a greater dose as a factor of the MIC would be required to induce the rapid lethality mechanism. Additionally,
differences in both the MIC and lethality were expected, dependent upon the C-7 cyclic amine substituent.

3.4 Synthesis of ofloxacin and levofloxacin derivatives

As shown in Scheme 1, derivatives of ofloxacin and levofloxacin were prepared by combining three different cyclic amine compounds with purchased 6,7-difluoro levofloxacin and ofloxacin core compounds by nucleophilic aromatic substitution. The choice of 2-methyl piperazine, 2-ethyl piperazine, and cis-octahydropyrrolo[3,4-b]pyridine as cyclic amine compounds was based on their inclusion at the C-7 position of commercially available fluoroquinolones gatifloxacin, PD161144, and moxifloxacin, respectively.

In short, commercially available core structures were stirred in DMSO along with triethylamine and the desired C-7 substituent at 100° to 110° C overnight. Purification was performed by semi-preparative HPLC, followed by concentration of fractions containing product by lyophilizer. Other compounds used in this study were either purchased or synthesized by other group members for previous studies.

3.5 Bacteriostatic and bactericidal studies

Lethality and bacteriostatic studies were carried out on E. coli K-12 strain DM4100. MIC was determined following serial two-fold dilutions of quinolone, dissolve in 1N sodium hydroxide solution. Lethality data was determined from incubating cells for two hours in the presence of quinolone. Cells were removed from drug and plated on drug free agar to determine the number of colony forming units. To determine the necessity of protein synthesis and compatibility with chloramphenicol, cells were pretreated in a chloramphenicol solution for 10 minutes before adding drug.
3.5.1 Bacteriostatic activity comparison

Structures of the fluoroquinolone agents used in this study are shown in Figure 10. Table 2 shows the MIC for each compound. With the exception of only one C-7 substitution, the cis-octahydropyrrolo[3,4-b]pyridine seen in moxifloxacin and KRM-I-033, ring fusion has a decidedly negative impact on MIC. In this particular case, there is no difference seen between moxifloxacin, the cognate N-1 cyclopropyl, C-8 methoxy fluoroquinolone, and KRM-I-033, the fused ring derivative. In general, it was determined that N-1 to C-8 ring fusion does not promote inhibitory activity of fluoroquinolones and in most cases is disruptive to growth inhibition.

3.5.2 Bactericidal activity comparison

The effects of N-1 cyclopropyl and C-8 methoxy groups on killing in the presence and absence of chloramphenicol is shown on in Figure 11. Most dramatic is the difference between PD161144 and NG4-257, its fused ring counterpart. Addition of
chloramphenicol had no effect on the lethal potential of PD161144. However, the bacterial survival rate of cells treated with NG4-257 was increased by approximately three orders of magnitude. While other compound pairs did not show quite as large of a difference, there was nonetheless telling evidence that the N-1 to C-8 fusion reduced the lethal potential for each. For example, although gatifloxacin’s lethal activity is severely impaired, by as much as two orders of magnitude, when cells are treated with chloramphenicol, its ring fused counterpart, KRM-I-025 is further inhibited by as much as three orders of magnitude. Also important to note, HS-IIa-93 retains far more lethal activity in the presence of chloramphenicol than its clinically important ring fused
Figure 11. Lethal activity of N-1 cyclopropyl, C-8 methoxy fluoroquinolones and N-1 to C-8 fused ring analogs in the presence and absence of chloramphenicol. Survival of *E. coli* was measured as a function of the fluoroquinolone concentration expressed as a multiple of the MIC in the presence (filled circles) or absence (open cycles) for chloramphenicol. N-1 cyclopropyl, C-8 methoxy compounds are in the left column, fused ring analogs are in the right. The names and C-7 substituent for each panel are as indicated.
counterpart, levofloxacin. One compound, PD135042, was strongly affected by addition of chloramphenicol until a concentration approximately ten times the MIC, at which point cell survivability dropped to levels similar to that seen when no chloramphenicol was present. The lethality of compounds with a 3-ethyl piperazinyl substitution, present on PD161148 and KRM-I-025, was negatively impacted about equally, regardless of ring fusion. Interestingly, in comparison between the two compounds in the series with the cis-octahydropyrrolo[3,4-b]pyridine substituent, despite being much more active than its ring fused counterpart, moxifloxacin shows about the same lethality in the presence of chloramphenicol as KRM-I-033. It is important to note that none of the fused ring compounds retains greater activity than its N-1 cyclopropyl, C-8 methoxy counterpart.

Investigating the differences between C-7 ring structures revealed the impact of the N-ethyl piperazinyl group. PD161144, the only N-1 cyclopropyl, C-8 methoxy fluoroquinolone with the N-ethyl piperazinyl substituent at C-7, is completely insensitive to chloramphenicol. In the N-1 cyclopropyl, C-8 methoxy series, the C-7 substituent had a significant impact on the lethal activity of the fluoroquinolone in the presence of chloramphenicol. However, in the fused ring series, varying the C-7 substituent had little impact on the lethality of the compound in the presence of chloramphenicol. Modeling data (Section 3.6) supports a connection between the N-1/C-8 configuration and the effect of the C-7 substituent on lethal activity: the steric clash between the N-1 cyclopropyl group and the C-8 methoxy group leads to the C-8 methoxy being limited to configurations that impede the free rotation of the C-7 ring structure.

To further investigate the effect of ring fusion between N-1 and C-8, we purchased commercially available clinical and veterinary medicine fluoroquinolones pazufloxacin, marbofloxacin, and rufloxacin, which were then evaluated for lethal action in the presence and absence of chloramphenicol. Pazufloxacin has the same core structure as levofloxacin, differing only in C-7 substituent, an aminocyclopropyl structure (Figure 10). Marbofloxacin and rufloxacin are substituted at C-7 with the same N-methyl
substituent as levofloxacin, but differ from each other and levofloxacin in the fusion between N-1 and C-8. None of these three clinically important fluoroquinolone agents exhibited rapid lethality potential in the presence of chloramphenicol. The unique C-7 substituent of pazufloxacin failed to impart lethal activity to the core structure. Lethal activity of rufloxacin, the only fused ring derivative without an exocyclic methyl group, was almost completely reversed upon treatment with chloramphenicol, indicating the importance of the exocyclic methyl functionality despite its inability to impart the level of lethality conveyed by the N-1 cyclopropyl and free C-8 methoxy (Figure 12). This is consistent with modeling studies (Section 3.6), which show that the exocyclic methyl group occupies the same relative space as the N-1 cyclopropyl group in N-1 cyclopropyl, C-8 methoxy derivatives (Figure 12).

Figure 12. Lethality data for pazufloxacin, marbofloxacin, and rufloxacin. Survival of E. coli was measured as a function of the fluoroquinolone concentration expressed as a multiple of the MIC in the presence (filled circles) or absence (open circles) of chloramphenicol. Compound names are listed in each panel.
3.6 Modeling comparisons

To better understand why fluoroquinolones bearing a ring fusion between N-1 and C-8 display lower lethal potential in the presence of chloramphenicol as compared to cognate N-1 cyclopropyl, C-8 methoxy derivatives, energy minimized structural models of levofloxacin and its N-1 cyclopropyl, C-8 methoxy counterpart, HS-IIa-93, were created (Figure 13). For best comparison, HS-IIa-93 was minimized from an initial configuration in which the N-1 cyclopropyl group most closely mimicked the enantiomerically pure S configuration of levofloxacin’s exocyclic methyl group. Doing so maintains consistent three dimensional orientation of these two groups. Shown in Figure 13, the N-1 cyclopropyl and C-8 methoxy groups extend in a relative trans configuration from the core of HS-IIa-93. In comparison between the two fluoroquinolone models, it quickly becomes clear that the exocyclic methyl of levofloxacin occupies the same relative space as the N-1 cyclopropyl group of HS-IIa-93. Shown in Figure 13b, viewed from the carboxyl end of the fluoroquinolone, the aromatic core of HS-IIa-93 appears to be supported on the “legs” of the N-1 cyclopropyl and the C-8 methoxy. Similarly, the levofloxacin core appears to be supported by the exocyclic methyl group and a pucker in the ring away from the methyl group. However, viewed along the axis of the C-4 carbonyl bond (Figure 13a) there is a vacant space in the levofloxacin model that is filled by the C-8 methoxy of HS-IIa-93.

Due to the limited structural flexibility of the fused ring of levofloxacin, the fluoroquinolone core ring system is nearly coplanar with the N-1, C-3, C-4, and C-8 substituents. In contrast, the flexibility of the N-1 cyclopropyl and C-8 methoxy groups of HS-IIa-93 and the resulting steric clash between the two results in a skewing of the fluoroquinolone core structure, which breaks the coplanarity of the substituents and the ring (Figure 13b). Similar skewing of the ring, indicated by dashed lines of Figure 13b has been shown to correlate with increased bacteriostatic activity [82] when caused by...
steric repulsion between N-1 5-amino-2,4-difluorophenyl substituent and C-8 halide. This result indicates that the induced geometry of the core ring structure, caused by the specific N-1 and C-8 substituents, likely affects fluoroquinolone activity, similar to binding interactions between these two substituents and topoisomerase or DNA. Because steric effects of the trans orientation of the N-1 cyclopropyl and C-8 methoxy groups is expected to restrict movement of the C-8 methoxy group and, thereby, the free rotation of C-7 substituent, the rotational freedom of the bond between C-7 and the substituent was evaluated for three fluoroquinolone cores: PD161144, UING4-257, and ciprofloxacin. As shown in Figure 10, PD161144 and UING4-257 vary by N-1 cyclopropyl, C-8 methoxy, or N-1 to C-8 ring fusion, respectively. The C-8 H of ciprofloxacin would not be expected to affect the free rotation of the C-7 group.

Rotational energy data were generated by two different methods: (1) the fluoroquinolone core and substituents other than C-7 were held rigid and a steric energy profile was approximated by calculating the conformational energy at predetermined angles of the dihedral indicated in Figure 14a; (2) energy was minimized for the entire fluoroquinolone structure when the C-7 substituent bond was held rigid at various predetermined dihedral angles. The former method approximates a situation in which the fluoroquinolone structure is held rigid by binding to DNA or protein and the latter approximates rotational freedom of the C-7 substituent prior to interaction with DNA and/or protein and represents a measure of the ability of the fluoroquinolone to undergo structural changes that alleviate steric conflict.

Shown in Figure 14b, the steric energy model indicates that the energy of rotation of the C-7 piperazine ring of ciprofloxacin, unimpeded by a C-8 methoxy substituent, is uniform, displaying high and low energies 180° apart, correlating to steric interactions between the two ring structures. However, both PD161144 and UING4-257 have additional energy barriers to rotation, indicated by arrows in Figure 14b, with the C-8 methoxy group of PD161144 imparting a slightly higher energy barrier than the fused
Figure 13. Molecular modeling indicates several differences between the N-1 cyclopropyl, C-8 methoxy and fused ring fluoroquinolones. (A) At the minimum energy conformation, the C-8 methoxy group extends into what is unoccupied space in the fused ring system. (B) Coplanarity of the two rings is disrupted by the N-1 cyclopropyl, C-8 methoxy system. (C) Freedom of rotation of the C-8 methoxy results in steric clash with the C-7 substituent.
ring of UING4-257 at dihedral angles of approximately 0° and 180°.

Figure 14c shows the relative minimum energy calculations for PD161144 and UING4-257 at specific values of the dihedral angle indicated in Figure 14a. This simulation compares the ability of each core structure to undergo structural changes that alleviate steric interactions. The total energies of the minimized structures were compared at 18 different dihedral angles and normalized to the relative energy to the minimum energy conformation. The highest energy rotational barriers for the C-7 ring are similar between the two fluoroquinolones. As expected, the highest energy conformations shown in Figure 14c are slightly lower than those shown in Figure 14b in which the structures were held rigid. The allowed conformational change of the core structure nearly eliminated the steric conflict indicated by the arrows in Figure 14b for UING4-257. However, the high steric conflict caused by the C-8 methoxy substituent of PD161144 is still evident in the lowest energy conformations at approximately 0° and 180°. This indicates that the lowest energy conformation of the C-8 methoxy containing PD161144 is constricted to a narrower range of rotation than that of UING4-257.

The C-8 methoxy substituent restricts free rotation of the C-7 substituent to a greater degree than the N-1, C-8 ring fusion as indicated in Figure 14c. Additionally, when the fluoroquinolone is bound to DNA or protein, further restricting flexibility of the core, the C-8 methoxy substituent creates in an even greater restriction on the free rotation of the C-7 ring system (Figure 14b). This indicates that the N-1 and C-8 substituents of fluoroquinolones likely impact the activity or lethality of the specific compound by affecting the rotation of the C-7 ring as well as the direct effects caused by these two groups upon binding to protein or DNA. For this reason, the identification of optimal N-1, C-7 and C-8 substituents for quinolone structures that display improved killing in the absence of protein synthesis cannot be considered independently of each other or the core structure.
Figure 14. Minimum energy calculation for different values of the dihedral angle between the C-7 substituent and fluoroquinolone core. (A) Two dimensional representation of a dihedral angle of 0° (left) and 180° (right) measured by the three bold faced bonds. (B) Plot of steric energies for 360° rotation about the specified bond for PD161144 (solid line), NG4-257 (dashed line), and ciprofloxacin (dotted line). (C) Plot of energies of energy minimized structures, calculated at 20° increments of the indicated dihedral bond for PD161144 (solid line) and NG4-257 (dashed line).
CHAPTER 4 FLUOROQUINOLONES WITH A C-2 THIOETHER SUBSTITUTION

Literature examples of 2-thioquinolone derivatives lacking an additional ring structure are limited [83], and only ring-fused structures such as the isothiazoloquinolones and thiazetoquinolones (Figure 15) and are reported to have potent antibacterial activity [84-86]. Furthermore, despite extensive modification of the fluoroquinolone core, little precedent exists for removing the 3-carboxylate due to its assumed requirement for activity [87]. However, quinazoline-2,4-diones and 1,3-diones have recently been shown to possess potent, broad-spectrum antibacterial properties despite the lack of a 3-carboxylate substituent [30, 64, 88-93], thus suggesting the possibility that further modification to the C-2 and C-3 positions of quinolones will provide novel structures that maintain antibacterial activity.

![Figure 15](image)

Figure 15. Structures of known active C-2 substituted fluoroquinolones ulifloxacin and isothiazoloquinolone core, with commonly substituted positions labeled.

4.1 C-2 Sulfur containing fluoroquinolones

First developed in the early 1990s and having recently begun testing for clinical safety and usability in a number of countries, prulifloxacin (Figure 1) is the prodrug form or ulifloxacin. The dimethyl cyclic carbonate moiety, used to increase lipophilicity, is cleaved as prulifloxacin is rapidly and extensively metabolized to the active agent upon
absorption in the gastrointestinal tract [1]. Comparison of inhibitory and bactericidal activities of ulifloxacin and ciprofloxacin is shown in Table 3. Ulifloxacin does show lethal activity against many Gram-positive and Gram-negative bacteria, but is relatively ineffective against anaerobic bacteria [1].

Table 3. Activity of ulifloxacin and ciprofloxacin against selected species of Gram negative and Gram positive bacteria [1].

<table>
<thead>
<tr>
<th>Species</th>
<th>Ulifloxacin</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (nalidixic acid resistant)</td>
<td>&lt; 0.015</td>
<td>&lt; 0.015</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (ciprofloxacin sensitive)</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. aureus</em> (methicillin resistant)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>1-2</td>
<td>1-2</td>
</tr>
</tbody>
</table>

Data presented as MIC₅₀ in concentration of mg/L

In addition to ulifloxacin, other fluoroquinolones having a C-2 sulfur functionality have been shown to possess potent antibacterial activity, specifically the isothiazoloquinolones. As with fluoroquinolones, addition of a C-8 methoxy substituent has been shown to increase activity against bacterial cells, including methicillin resistant and vancomycin resistant *S. aureus* [94]. However, many isothiazoloquinolones are active against topoisomerase II, equating to increased cytotoxic potential [95, 96].

4.2 Modeling studies of protein binding

Prior to the recent publication of crystal structures showing a fluoroquinolone-topoIV-DNA ternary complex, Arkady Mustaev undertook a modeling investigation to determine the possible binding site(s) and orientation of the fluoroquinolone onto protein. Taking into account the known sites of mutation induced by fluoroquinolones on gyrase and topoIV, and how these mutations may be overcome or induced depending on substituents of the fluoroquinolone core, the possible binding orientation shown in Figure
6 was determined. This orientation and location is further supported by cross linking studies in which fluoroquinolones were synthesized with an N-haloacetyl-piperazinyl substituent at C-7 (Figure 16). In combination with a DNA gyrase G81C mutation, these haloacetyl compounds resulted in irreversible inhibition of gyrase activity, likely by forming a covalent bond between cysteine-81 and fluoroquinolone (Figures 16 and 17). Our collaborators at UMDNJ continue to work towards proving that the irreversible inhibition is a result of crosslinking.

Inserting ulifloxacin into the purported binding site of the model suggested some unique binding interactions that may explain the activity of ulifloxacin (Figure 18). In the model showing binding of ciprofloxacin and C-8 methoxy ulifloxacin on opposite sides of the dimer interface it becomes evident that the C-2 sulfur of ulifloxacin is likely
making a binding contact with the enzyme. Additionally, it appears that there is a potential hydrophobic pocket in the area of aspartate-87 (square, Figure 18) that could be exploited by thioalkyl substitution at C-2 of the fluoroquinolone core.

4.3 Goals of this study

Initially, this study began as an investigation into the effect on lethality in the presence of chloramphenicol of adding a C-8 methoxy substituent to ulifloxacin.
Although ulifloxacin is administered clinically as the racemate, it is known that the exocyclic methyl group of the N-1 to C-2 thiazetidine ring assumes an S stereochemistry in the active isomer [97, 98]. Although ulifloxacin is ineffective against anaerobic bacteria and has not been shown to be lethal in the presence of chloramphenicol, it is possible that the C-8 methoxy substitution may impart lethal activity on the core structure.

Also of interest was the effect of the C-2 thioether moiety. Ulifloxacin differs from ciprofloxacin only by the N-1 to C-2 thiazetidine ring, yet is equipotent or more potent against both Gram-negative and Gram-positive bacteria. The potential binding contacts suggested by modeling shown in Figure 18 required investigation into the

Figure 18. Model of C-8 methoxy ulifloxacin and ciprofloxacin on opposite sides of the dimer interface of E. coli gyrase. The area indicated by the black square was determined to be a potential binding pocket for C-2 thioalkyl substituents.
potential activity of fluoroquinolones substituted at the C-2 position by thioalkyl substituents.

4.4 Synthesis of C-2 thioether substituted fluoroquinolones

Synthetic preparation of C-8 methoxy ulifloxacin was attempted by two different routes, each reported in literature [65, 94]. The first route attempted is shown in Scheme 2. The hydroxyl of 2,3-difluoro-5-nitrophenol (3) was methylated by addition of methyl iodide. The nitro group of resulting compound 4 was then reduced by treatment with acetic acid in the presence of iron powder [99]. From this point, synthesis followed published procedure [68] as compound 5 was converted in two steps via carbon disulfide formation of a dithiocarbamate intermediate (6), followed by reduction of the intermediate to aryl isothiocyanate 7. The isothiocyanate was then added to deprotonated

Scheme 2. First attempted synthesis of C-8 methoxy ulifloxacin.
diethyl malonate and the free thiolate of the resulting intermediate (8) was then protected by methoxymethyl (9). At this point, however, likely due to the meta methoxy substituent, attempts at Friedel-Crafts acylation of the aromatic ring, forming the fluoroquinolone core structure, resulted in extremely low yields.

In an attempt to avoid addition to the aromatic ring meta to the methoxy group, a new synthetic approach was developed. Again, following literature procedure [94], shown in Scheme 3, commercially available 2,4,5-trifluoro-3-methoxy benzoic acid (10) was converted via acid chloride 11 to propionic acid ethyl ester 12. Following treatment of 12 with potassium hydroxide and benzyl isothiocyanate, the free thiolate was capped with ethyl iodide, resulting in compound 13. The second ring was then closed by intramolecular nucleophilic aromatic substitution to yield 14. Deprotection of the benzyl group by polyphosphoric acid mediated hydrolysis resulted in 2-ethylsulfanyl quinoline 15. However, attempts at forming the thiazetidine ring via functionalizing the ethyl moiety to 2-chloroethyl by reaction with sulfuryl chloride were ineffective. Additionally,
protecting groups such as methoxy methyl (MOM), benzyl (Bn), benzoyl (Bz), carbonylbenzyloxy (CBz), and trichloroethoxycarbonyl (troc) were attempted in place of the ethyl iodide. These groups did not successfully add to the free thiolate. As a result, efforts to synthesize C-8 methoxy ulifloxacin were abandoned in favor of exploiting this synthetic route as a way of making C-2 thioalkyl substituted fluoroquinolones.

Encouraged by the effectiveness of forming the 2-thioethyl fluoroquinolone core, and wanting to explore the potential effects on antibacterial activity imparted by a C-2 thioalkyl group on an N-1 cyclopropyl, C-8 methoxy core, the knowledge gained from

![Chemical structures and reactions](image)

the efforts shown in Scheme 3 was applied to generating C-2 thioethyl and C-2 thioisopropyl derivatives, as shown in Scheme 4. Beginning with propionic acid ethyl ester 12, synthesized in Scheme 3, N-1 cyclopropyl, C-2 thioalkyl, C-8 methoxy fluoroquinolone core was synthesized in two steps: first, cyclopropyl isothiocyanate was added to propionic acid ethyl ester 12, previously deprotonated by potassium hydroxide, followed by reaction of the free thiolate with either ethyl iodide (16a) or isopropyl iodide (16b). The two different quinolone cores (17a, 17b) were then generated by intramolecular nucleophilic aromatic substitution. Penultimate fluoroquinolone products 18a-d were achieved by nucleophilic aromatic substitution at the C-7 position by either cis-octahydropyrrolo[3,4-b]pyridine (18a, 18c) or piperazine (18b, 18d).

Surprisingly, what was assumed to be a simple ester hydrolysis proved not to be so (Scheme 5). Application of different known methods for ester hydrolysis each resulted in different products. For example, the first attempts at hydrolysis were performed in

Scheme 5. Hydrolysis of the C-3 ethyl ester.
aqueous basic conditions. While no reaction occurred in a 10% aqueous solution of potassium hydroxide at room temperature, upon heating the solution to 110°C, the fluoroquinolones quickly underwent a surprising reaction. Ester starting material converted to one major product, in which the C-3 carboxyl group was lost and the C-2 thioalkyl, whether thioethyl or thioisopropyl, was replaced by hydroxyl. An explanation for this unexpected product can be inferred from the cyanide mediated decarboxylation of fluoroquinolones proposed by Reuman, et al. [100], and shown in Figure 19. In the cyanide mediated decarboxylation mechanism, cyanide anion adds to the C-2 position as in a 1,4-Michael addition to an alpha, beta unsaturated ketone. The resulting intermediate quickly decomposes to the C-3 H fluoroquinolone following decarboxylation and subsequent loss of the cyanide as a leaving group. Here, I propose that decarboxylation and resulting C-2 hydroxyl substitution follows a similar mechanism. In this case, however, the thioalkyl substituent serves as a more stabilized leaving group and is ejected in favor of the hydroxyl.

Most acid based attempts to hydrolyze the ethyl ester under aqueous acidic conditions were also ineffective (Scheme 5). Although still leading to decarboxylation, under aqueous acidic conditions, products were recovered in which the C-2 thioalkyl substituent was retained. Despite the recovered products (KRM-II-183,185,197,195) not being the intended result, the compounds obtained were still useful in this study. The C-3 carboxyl group is known to be important in mediating the binding interaction between fluoroquinolone and protein by participating in a magnesium chelation-mediated binding interaction with aspartate 87 (gyrase). Removal of magnesium from cell media after treatment with fluoroquinolone is known to reverse fluoroquinolone mediated growth inhibition [101]. Since these compounds would now lack the C-3 carboxyl chelation-mediated binding interaction, the importance of increased interaction of the C-2 thioalkyl group was determined to be greater.
Finally, desired C-2 thioalkyl fluoroquinolones **KRM-II-241** and **KRM-II-045** were obtained by ester hydrolysis in fuming sulfuric acid. These conditions were extremely water sensitive, however, and led to low yields (Scheme 5). Interestingly, when applied to C-2 thioisopropyl derivatives (**18c, 18d**), these conditions resulted in an unexpected tetracyclic compound that was not used for further analysis.

In order to fully investigate the contribution of the C-2 thioalkyl derivatives, ciprofloxacin and moxifloxacin were subjected to cyanide mediated decarboxylation conditions, similar to that reported by Reuman, et al. [100], (Scheme 6). Additionally, ulifloxacin, which was found to be resistant to cyanide mediated decarboxylation, was
treated with 10% aqueous sulfuric acid at elevated temperature to yield to cognate descarboxy compound.

4.5 Bacteriostatic activity of C-2 thioalkyl fluoroquinolones

Inhibitory activity of the synthesized compounds was determined against three different cell types. Wild type E. coli (KD65) and an efflux pump deficient E. coli strain (KD1397) represented Gram-negative bacteria and M. smegmatis (KD1163), a relatively safe, fast growing Gram-positive species, was evaluated as a surrogate for M. tuberculosis. The efflux pump (tolC) deficient E. coli strain was used to better determine protein inhibition caused by the drug without bacterial cell efflux complications.

As expected, the decarboxylated analogs of compounds ciprofloxacin, moxifloxacin, and ulifloxacin were significantly less active against all three cell lines than respective parent compounds (Table 4). Relative activity between parent and decarboxylated compounds was reduced by a factor of between 60 and 25,000 against wild type *E. coli* cells. Of note in this series, however, both parent and decarboxylated compounds display a marked increase in activity against efflux pump deficient *E. coli* cells as compared to wild type, indicating that the C-3 carboxyl is not required for recognition and transport by the *tolC* efflux pump.

### Table 4. Antibacterial data for C-2 thioalkyl and decarboxylated fluoroquinolones.

<table>
<thead>
<tr>
<th>Compound</th>
<th>C-2</th>
<th>C-3</th>
<th>C-7</th>
<th>Bacterial Strain (MIC µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>E. coli</em> (KD65)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>H</td>
<td>CO₂H</td>
<td>Piperazinyl</td>
<td>0.04</td>
</tr>
<tr>
<td>KRM-III-145</td>
<td>H</td>
<td>H</td>
<td>Piperazinyl</td>
<td>50</td>
</tr>
<tr>
<td>Ulifloxacin</td>
<td>*</td>
<td>CO₂H</td>
<td>Piperazinyl</td>
<td>0.062</td>
</tr>
<tr>
<td>KRM-III-163</td>
<td>*</td>
<td>H</td>
<td>Piperazinyl</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>H</td>
<td>CO₂H</td>
<td>Octahydropyrrolopyridinyl</td>
<td>0.156</td>
</tr>
<tr>
<td>KRM-III-157</td>
<td>H</td>
<td>H</td>
<td>Octahydropyrrolopyridinyl</td>
<td>&gt;200</td>
</tr>
<tr>
<td>KRM-II-241</td>
<td>S-Et</td>
<td>CO₂H</td>
<td>Octahydropyrrolopyridinyl</td>
<td>&gt;200</td>
</tr>
<tr>
<td>KRM-III-045</td>
<td>S-Et</td>
<td>CO₂H</td>
<td>Piperazinyl</td>
<td>200</td>
</tr>
<tr>
<td>KRM-II-183</td>
<td>S-Et</td>
<td>H</td>
<td>Octahydropyrrolopyridinyl</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-II-197</td>
<td>S-Et</td>
<td>H</td>
<td>Piperazinyl</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-II-185</td>
<td>S-iPr</td>
<td>H</td>
<td>Octahydropyrrolopyridinyl</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-II-199</td>
<td>S-iPr</td>
<td>H</td>
<td>Piperazinyl</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

* N-1 to C-2 thiazetidine ring
MIC values for all C-2 thioalkyl derivatives are higher in comparison to parent fluoroquinolones. Interestingly, in the C-7 cis-octahydroprrolopyrroldinyl series, comparison between decarboxylated moxifloxacin (KRM-III-157) and decarboxylated thioalkyl compounds KRM-II-183 and KRM-II-185, compounds bearing C-2 thioalkyl substitution are distinctly more active than the C-2 H compound against all three strains tested. This result indicates that either a C-2 thioalkyl or C-3 carboxylate can result in increased activity when compared to corresponding compounds not substituted at either C-2 or C-3.

The increased MIC for the C-2 thioalkyl, C-3 carboxyl compounds KRM-II-241 and KRM-III-045 is in contrast to typically reported low values of MIC for ulifloxacin and isothiazoloquinolone compounds. Structurally, these compounds differ in that the C-2 thioalkyl compounds lack an additional ring structure, either the thiazetidine of ulifloxacin, or the isothiazolidinone of the isothiazoloquinolones. An explanation for the increased activity of the fused ring congeners is the interaction between the C-2 sulfur atom and gyrase/topoIV. However, substitution at C-2 of the fluoroquinolone core by thioalkyl groups, while leading to a marginal increase in activity in moxifloxacin derived compounds lacking a C-3 carboxyl, did not enhance activity over parent compounds. Due to their poor inhibitory activities, these novel compounds were not carried forward into bacterial lethality studies.

4.6 Molecular modeling explanation of reduced activity

Molecular modeling shows a possible explanation for the reduced activity upon C-2 thioalkyl substitution. Shown in Figure 20, ulifloxacin, an isothiazoloquinolone, and KRM-III-045 are compared side by side after energy minimization. In the cases of the isothiazoloquinolone and ulifloxacin, the two fused ring congeners, the core structure and C-3 carboxyl maintain planarity. However, lacking the fused ring moiety, the C-2 thioalkyl substituent results in disrupted planarity of the fluoroquinolone core and C-3
carboxyl as a result of steric conflict between the C-2 thioalkyl substituent and the C-3 carboxyl group.

Figure 20. Side by side comparisons of ulifloxacin (left), an isothiazoloquinolone (center), and KRM-III-045. Energy minimized three dimensional structures indicate increasing disruption in planarity of the core ring from left to right. KRM-III-045 also displays a rotation of the C-3 carboxyl group due to steric conflict with the C-2 thioethyl.
CHAPTER 5 EFFECT OF MODIFICATION AT C-4 OF 2,4-QUINAZOLINE DIONES

Quinazoline-2,4-diones have recently been investigated for antibacterial activity [2, 30, 64, 88, 89, 91-93, 102-106]. In 2006, a new class of drug molecules active as topoisomerase inhibitors was reported [64]. The 3-aminoquinazoline diones represent a new class of topoisomerase inhibitor that is similar to fluoroquinolones in both structure and antibacterial mechanism of action. Of key importance in structural comparison to fluoroquinolones, the 3-aminoquinazoline diones lack the C-3 carboxylic acid group (Figure 21). The carboxylic acid group has been shown to be of critical importance in quinolone binding and inhibition; however, it has recently been shown that in some cases, molecules retain activity, even in the absence of this group [90, 92].

5.1 Antimutant activity of quinazoline-2,4-diones

The fluoroquinolones are among the groups of antibacterial agents that are susceptible to bacterial resistance to antibiotics. While it has been shown that the

![Figure 21. Examples of fluoroquinolones and quinazoline-2,4-diones studied previously.](image)
inclusion of the C-8 methoxy increases activity of fluoroquinolones against bacterial strains shown to be resistant to traditional fluoroquinolones unsubstituted at the C-8 position, this is becoming less effective as new resistant mutants continue to emerge [107-109]. Recently, however, it has been shown that changing the core structure to that of the quinazoline-2,4-diones also increases activity over resistant mutants. In concert with the C-8 methoxy substitution, the quinazoline-2,4-dione core results in little increase in MIC against gyrA and gyrB mutants of E. coli normally resulting in resistance to fluoroquinolone antibiotics [30].

Hiroshi Hiasa, recently compared the activity of a C-8 methoxy quinazoline-2,4-dione to that of cognate C-8 methoxy fluoroquinolone, ciprofloxacin, and a C-8 methyl quinazoline-2,4-dione as shown in Table 5 [2]. Inhibitory activity is shown against E. coli gyrase and topoIV, as well as S. aureus gyrase and topoIV. The best replacement for a fluoroquinolone would be a drug that is active as a dual targeting agent, retaining antibiotic potential if a mutation to either DNA gyrase or topoIV results in decreased inhibition. As shown, the addition of C-8 methoxy to the fluoroquinolone, decreases selectivity for E. coli gyrase over topoIV by nearly threefold. Conversion from

Table 5. Selectivity of fluoroquinolone and quinazoline-2,4-dione compounds for DNA gyrase over topoIV in E. coli and S. aureus [2]. Structures are shown in Figure 21.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ E. coli (µM)</th>
<th>Selectivity</th>
<th>IC₅₀ S. aureus (µM)</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gyrase</td>
<td>Topo IV</td>
<td>Gyrase</td>
<td>Topo IV</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.45</td>
<td>15.9</td>
<td>35.3</td>
<td>31.5</td>
</tr>
<tr>
<td>NG5-249</td>
<td>0.16</td>
<td>1.7</td>
<td>10.6</td>
<td>1.1</td>
</tr>
<tr>
<td>NG5-207</td>
<td>2.8</td>
<td>16</td>
<td>5.7</td>
<td>2.3</td>
</tr>
<tr>
<td>JR1-048</td>
<td>0.95</td>
<td>6.0</td>
<td>6.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Selectivity is defined by the ratio of the IC₅₀ for the secondary target over the IC₅₀ for the primary target.
the fluoroquinolone core to the quinazoline-2,4-dione core decreases selectivity even further, approaching a true dual target value of one.

The quinazoline-2,4-diones are also less susceptible to resistance as a result of mutation to DNA gyrase. Shown in Table 6, the IC$_{50}$ of both C-8 methoxy and C-8 methyl quinazoline-2,4-diones are less affected by mutations to *E. coli* gyrase at positions 67, 81, and 83 than are either C-8 methoxy fluoroquinolone or ciprofloxacin [2]. Although the C-8 methoxy fluoroquinolone is the most active, in absolute terms, against wild type *E. coli* gyrase and against all three mutants, a much greater increase in drug concentration is needed to reach the same inhibitory levels against G81C and S83W mutants than that necessary for either of the quinazoline-2,4-dione derivatives. Against the A67S mutant, the two C-8 methoxy derivatives have improved inhibitory activity, indicating the importance of this functional group against the particular mutation.

Table 6. Activity of selected fluoroquinolone and quinazoline-2,4-dione compounds against wild-type and mutant *E. coli* purified DNA gyrase [2].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Median IC$_{50}$ (µM)</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>GyrA S83W</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.45</td>
<td>101 (224)</td>
</tr>
<tr>
<td>NG5-249</td>
<td>0.16</td>
<td>1.4 (8.8)</td>
</tr>
<tr>
<td>NG5-207</td>
<td>2.8</td>
<td>5.9 (2.1)</td>
</tr>
<tr>
<td>JR1-048</td>
<td>0.95</td>
<td>3.8 (4.0)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the ratio of IC$_{50}$ for the mutant over wild-type.

5.2 Modeling of quinazoline-2,4-diones on topoisomerase IV

Shown in Figure 22, C-8 methoxy quinazoline-2,4-dione was modeled and overlaid onto an x-ray crystal structure of C-8 methoxy fluoroquinolone interacting with
topoIV at the putative fluoroquinolone binding pocket of topoIV. The model indicates the importance of the C-3 carboxylic acid and C-4 carbonyl groups’ interaction through magnesium in binding with topoIV. However, this model indicates a binding interaction between the C-2 carbonyl and arginine 129, which may account for dione binding despite lacking the C-3 carboxylic acid. Fluoroquinolone inhibitory activity against gyrase can be reversed by chelation and removal of magnesium [110] due to the interaction between the C-3 carboxylic acid functionality and the carboxylate of aspartate 95, mediated by a magnesium bridge. The quinazoline-2,4-diones have been shown to retain activity even in the presence of a magnesium chelator [103], indicating a difference in the binding interaction. Shown in the overlay of dione in Figure 22A, the interaction between C-2 carbonyl and arginine 129 may account for this difference. Additionally, there is likely a hydrogen bonding interaction with serine 79 when an amino substituent is present at N-3.
of the dione core. The N-3 amino substitution is also known to increase inhibitory activity over resistant mutant strains [30].

5.3 Goals of this study

Due to the novelty of the quinazoline-2,4-diones as an antibiotic class, only limited information is available in the primary literature concerning structure activity relationship. It is known that the N-3 amino substitution, C-8 methyl or C-8 methoxy, and particular cyclic amine substituents at C-7 lead to lower ratios between MIC for wild-type and MIC for resistant mutants in *E. coli* [30]. However, for antibiotic purposes, alteration of the quinazoline-2,4-dione core structure has not been investigated. With modeling indicating the importance of the C-2 carbonyl and the relative lack of binding interaction between the C-4 carbonyl and target proteins, investigating the effect of changes made at these two positions may lead to further understanding of the binding interaction between quinazoline-2,4-diones and target enzymes. Furthermore, this may create the ability to make modifications to the quinazoline core, specifically at the C-4 position, that alter the solubility, membrane permeability, and recognition by bacterial efflux pumps.

5.3.1 Investigating the effect of thiocarbonyl substitution

Knowing that the C-2 thioether functionality of ulifloxacin and isothiazoloquinolones results in increased antibacterial activity, the effect of substituting the C-2 position of a quinazoline-2,4-dione with a thiocarbonyl became of interest. Shown in Figure 23, the target C-2 thioquinazoline compound is expected to have increased antibacterial potential over both ulifloxacin and the similar quinazoline-2,4-dione, NG5-207.

Also of interest is altering the C-4 position of the quinazoline core. Although the C-4 carbonyl is important for magnesium mediated binding to protein for fluoroquinolones, molecular modeling of the interaction between protein and
quinazoline-2,4-diones does not indicate an important hydrogen bond occurring at this position (Figure 22). Thus, a change in the C-4 substitution will either increase the evidence that this position is not involved in binding, or potentially lead to a better understanding of how the quinazoline-2,4-diones interact with the target enzyme. An increase or decrease in the inhibitory activity of these compounds over cognate quinazoline-2,4-dione, especially when tested against resistant mutants suggests an important interaction that is not explicit from modeling.

5.3.2 Investigating the effect of imine substitution at the C-4 position

Further changes to the C-4 substituent may also lead to changes in the interaction with target enzyme. As shown in Figure 24, substituting the C-4 position with a group
capable of making a hydrogen bond directly to asp95 (topoIV), such as oxime, while retaining the C-2 carbonyl may result in increased binding and inhibitory activity. This interaction would be reversed if the oxime is replaced by a similar group, incapable of the hydrogen bond donation, such as methoxylimine (Figure 24). In side by side comparison between C-4 oxime and C-4 methoxylimine quinazoline compounds, modeling suggests that the C-2 carbonyl and C-3 amino contributions to hydrogen bonding are retained. However, the free hydroxyl moiety of the hydroxylamine substituent is able to participate in hydrogen bond donation either directly to the carboxylate of asp95 or through solvent, effectively serving as a surrogate for the C-3 carboxylic acid of fluoroquinolones.

5.3.3 Target synthetic compounds

The synthetic approach to this section focuses on the preparation of quinazoline core structures with alternative substitutions at the C-2 and C-4 positions. Interest in synthesizing and testing a C-2 thiocarbonyl derivative stems from earlier work in attempting to elucidate the impact of the C-2 thioether substituent of ulifloxacin and isothiazoloquinolones. Alternate substituents at the C-4 position, expected to have less impact on inhibition, are of interest as a possible way of functionalizing quinazoline compounds to increase desirable properties, such as solubility, targeting, and membrane crossing.

Synthetic goals include the following core structures:

- C-2 thiocarbonyl, C-4 carbonyl quinazoline derivative
- C-2 carbonyl, C-4 thiocarbonyl quinazoline derivative
- C-2, C-4 dithiocarbonyl quinazoline derivative
- C-2 carbonyl, C-4 oxime quinazoline derivative
- C-2 carbonyl, C-4 methoxylimine quinazoline derivative
5.4 Synthesis of quinazoline derivatives

5.4.1 Synthesis of C-2 carbonyl, C-4 thiocarbonyl derivatives

Synthetic preparation of C-4 thiocarbonyl derivatives were completed first due to easy access to advanced intermediates by following published literature procedure [88]. As illustrated in Scheme 7, beginning with the same commercially available 2,4,5-trifluoro-3-methoxy benzoic acid used in the C-2 thioalkyl fluoroquinolone study (10), synthesis proceeds through the quinazoline-2,4-dione core as an intermediate. The

Figure 24. Modeling of the proposed C-4 oxime (A) and C-4 methoxylimine (B) quinazoline compounds indicates a possible hydrogen bonding interaction between hydroxylimine and asp95. The increased steric bulk and lipophilicity of the C-4 methoxylimine substitution may also impact binding and inhibition to the target enzyme. Figure adapted from unpublished data provided by Arkady Mustaev.
benzoic acid was converted to amide 22 in two steps, first by forming the unpurified acid chloride, which was then stirred at room temperature with ammonium hydroxide solution. Stirring amide 22 in the presence of oxalyl chloride at reflux afforded the reactive isocyanate intermediate 23. The isocyanate was then added dropwise to a solution of cyclopropyl amine in anhydrous dichloromethane. After recrystallization, stable $N$-cyclopropyl-$N'$-(2,4,5-trifluoro-3-methoxybenzoyl) urea 24 was recovered in high yield as off-white crystals. Attempts at the intramolecular nucleophilic aromatic substitution yielding the quinazoline-2,4-dione core were initially problematic. Different published literature procedures propose the use of different bases, including potassium carbonate [93], KHMDS [93], and sodium hydride [88]. After several attempts, it was determined that the best yield was obtained by portioned addition of fresh 60% sodium hydride as a mineral oil dispersion.

With quinazoline-2,4-dione core (25) in hand, two routes were possible, both leading to the target quinazoline compound (28). In route one, the N-3 amino substitution was made first by nucleophilic attack by the N-3 nitrogen on synthetically prepared $O$-(2,4-dinitrophenyl)-hydroxylamine [111]. The resulting N-3 amino quinazoline-2,4-dione core structure (26) was previously used to synthesize the controls for this study. By this route, the final step in preparing the C-4 thiocarbonyl quinazoline compound (28) was accomplished in high yield by conversion of the C-4 carbonyl using Lawesson’s reagent [112]. Literature precedent indicates that the C-4 position is more susceptible to conversion by Lawesson’s reagent due to the electronic impact of the benzylic position as compared to the less reactive urea carbonyl. C-4 thiocarbonyl substitution was later confirmed by heteronuclear multiple bond correlation (HMBC) NMR (Figure A23).

The second route from 25 to 28 utilized the same two reactions, but in opposite order. The benefit to this route is that by obtaining the Lawesson’s reagent mediated C-4 thiocarbonyl intermediate (27) a comparison can be made between compounds with and without the N-3 amino substitution, a functionality known to increase activity against
resistant mutants. N-3 amino quinazoline derivative was then achieved by nucleophilic substitution addition of the amine, following the same procedure as with compound 26.

Substitution of the C-7 positions of compounds 27 and 28 was then carried out as presented previously with fluoroquinolone derivatives, shown in Scheme 8. **KRM-III-211** and **KRM-IV-251** were synthesized in two steps by nucleophilic aromatic substitution of the C-7 position with 3-(R)-Boc-aminomethyl pyrrolidine, followed by deprotection of the tert-butoxycarbonyl via 10% TFA solution in DCM. C-7 N-ethyl piperazinyl derivatives **KRM-IV-053** and **KRM-IV-249** were prepared similarly by nucleophilic aromatic substitution of N-ethyl piperazinyl at the C-7 position.

![Scheme 7. Synthesis of the C-4 thiocarbonyl quinazoline core (28). Intermediates 26 and 27 were also functionalized with C-7 cyclic amines and used for final antibacterial studies.](image-url)
5.4.2 Synthesis of C-2 thiocarbonyl derivatives

Due to the stability of the C-2 carbonyl and resistance to treatment with Lawesson’s reagent, a de novo synthesis of quinazoline compounds with a thiocarbonyl substitution at the C-2 position would be required. Retrosynthetic analysis, shown in Scheme 9, proceeds through a thiourea intermediate. Upon intramolecular cyclization via aromatic nucleophilic substitution, as with the quinazoline-2,4-dione synthesis, the C-2 thiocarbonyl core is completed. Moving forward, the C-4 carbonyl could then be subjected to the Lawesson’s reagent directed thiocarbonyl substitution, as before,
yielding a quinazoline-2,4-dithione.

Although literature precedents exist for similar compounds, the formation of the
tetrasubstituted benzoyl thiourea has not been reported [102, 106, 113, 114]. Literature
examples of similar syntheses of N-aroyl-N'-aryl thioureas result in stable products that
can be induced to undergo rearrangement to aryl amides by addition of copper iodide
[102, 106]. However, as shown in Scheme 10, in attempts to synthesize N-cyclopropyl-
N'-benzoyl thiourea 30, it appears that the aryl substitution is important for stability and
non-aromatic substituents, such as cyclohexyl [106] or the cyclopropyl used here, result
in instability and spontaneous conversion to amide 31.

The most successful attempt at synthesizing compound 30 is the first shown in
Scheme 10. 2,4,5-Trifluoro-3-methoxy benzoic acid was converted to the acid chloride
following the previously reported method using oxalyl chloride and DMF in
dichloromethane. The acid chloride was then stirred with ammonium isothiocyanate to
generate the aroyl isothiocyanate (29) analogous to aroyl isocyanate 23 in the
quinazoline-2,4-dione synthesis. The reactive intermediate was quickly subjected to
cyclopropyl amine, generating thiourea 30 in low yields of 20% or less, accompanied by
cyclopropyl amide 31 as the major component. Although purification of 30 was possible
by silica column, the product quickly underwent rearrangement to 31, even when stored
at 0° C away from light. Attempts to quickly perform the intramolecular cyclization did
not result in significant yields of desired quinazoline analog.
Scheme 10. Attempts to synthesize thiourea 30. Literature examples of N-benzoyl-N'-aryl thioureas are stable. However, compound 30 quickly and spontaneously converts to cyclopropylamide 31.
Having determined that current methods would not be capable of synthesizing the C-2 thiocarbonyl derivatives, this portion of the project was abandoned and the focus turned to functionalizing the C-4 position alone.

5.4.3 Synthesis of C-4 imino quinazoline derivatives

Synthesis of the C-4 imino substituted quinazoline compounds proceeds from an advanced intermediate in the synthesis of the C-4 thiocarbonyl compounds. Scheme 11 illustrates the methylation of compound 27 at the C-4 thiocarbonyl to yield the C-4 thiomethyl quinazoline core structure 32 in excellent yield. Subsequent treatment of 32 with the hydrochloride salt of either hydroxylamine or methoxylamine resulted in C-4 imino quinazoline compounds 33 or 34, respectively, in quantitative yield. Interestingly, the attempted addition of C-7 cyclic amine substituents by the previously reported nucleophilic aromatic substitution was ineffective. Attempts at using DMSO as solvent...
led to a single major product that was not able to be identified by NMR or MS after purification. Additionally, multiple MS injections over the course of one hour using DMSO as a solvent indicated instability of compound 33 in DMSO at room temperature. No progress was observed in attempting to add the C-7 cyclic amine substituents using either DMF or acetonitrile as solvent.

The difficulty in completing the C-4 imino compounds was not unforeseen. As such, plans for reversing the order of addition of C-4 and C-7 substituents were already made and quickly proved effective. Shown in Scheme 12, methylation of the C-4 thiocarbonyl of KRM-IV-249 and compound 36, the protected precursor of KRM-IV-251, was accomplished following the method above. Due to extensive loss of product during HPLC purification, the intermediates were not purified before being used in the
next reaction. C-4 thiomethyl compounds were then treated with the hydrochloride salts of hydroxylamine and methoxylamine, and warmed to 50° C in ethanol. Hydroxylamine reactions proceeded quickly and were recovered as TFA salts in good yield following purification by semi-preparative HPLC, but methoxylamine addition was not completed to any degree, even after continuous stirring in warmed ethanol for as long as three weeks. This outcome is not surprising, as addition of hydroxylamine to the 6,7-difluoro core was accomplished within thirty minutes while methoxylamine addition required several days.

5.5 Activity of C-4 modified quinazoline compounds

Inhibitory activity of the novel C-4 substituted quinazoline compounds was determined against the same cell types used in the C-2 thioalkyl study. Again, wild type *E. coli* (KD65) and an efflux pump deficient *E. coli* strain (KD1397) represented Gram-negative bacteria and *M. smegmatis* (KD1163) was evaluated as a mycobacteria representative. Because quinazoline-2,4-diones are known to be very susceptible to bacterial efflux, the efflux pump (*tolC*) deficient *E. coli* strain is important to develop an

Table 7. MIC₅₀ for the novel quinazoline analogs. All six compounds were strongly affected by efflux, but displayed similar activity against efflux pump deficient bacterial strain as cognate quinazoline-2,4-dione NG5-207.

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>E. Coli</em> (KD65)</th>
<th><em>E. coli (tolC)</em> (KD1397)</th>
<th><em>M. Smegmatis</em> (KD1163)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRM-III-211</td>
<td>25</td>
<td>2.5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-IV-053</td>
<td>&gt;50</td>
<td>5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-IV-249</td>
<td>50</td>
<td>5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-IV-251</td>
<td>50</td>
<td>2.5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-IV-287</td>
<td>&gt;50</td>
<td>5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>KRM-IV-295</td>
<td>&gt;50</td>
<td>2.5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>NG5-207</td>
<td>5</td>
<td>2.5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>NG5-249</td>
<td>0.062</td>
<td>0.004</td>
<td>0.062</td>
</tr>
</tbody>
</table>
understanding of the direct impact on inhibition of the target proteins.

Minimum inhibitory concentration determined for the six synthesized compounds is shown in Table 7. As a control, all of the novel compounds were compared to quinazoline-2,4-dione NG5-207.

Cognate 1-cyclopropyl-7-[3-(R)-aminomethylpyrrolidinyl]-8-methoxy quinazoline-2,4-dione NG5-207 has been found to display similar activity against both strains of *E. coli* [2]. Compounds substituted at the C-7 position by the 3-(R)-aminomethylpyrrolidinyl moiety, including NG5-207, KRM-III-211, KRM-IV-251, and KRM-IV-295, were all found to have an MIC$_{50}$ against efflux pump deficient *E. coli* (KD1397) of 2.5 µg/mL. None of the new compounds or NG5-207 displayed activity against *M. smegmatis*. Structurally, these four compounds vary only at the N-3 and C-4 positions: NG5-207 and KRM-III-211 are both N-3 amino compounds while KRM-IV-251 and KRM-IV-295 are both N-3 H compounds; the C-4 substituent of NG5-207 is carbonyl, that of KRM-III-211 and KRM-IV-251 is thiocarbonyl, and KRM-IV-295 bears a hydroxylimino substitution at C-4.

Interestingly, despite the importance of the N-3 amino group in reducing the ratio of the MIC for wild-type *E. coli* versus resistant strains [30], there is no difference seen here against wild-type *E. coli* and efflux pump deficient strains (gyrase enzyme remains wild-type) between N-3 amino and N-3 H compounds. This indicates that the N-3 substituent might not play a direct role in a binding contact between the quinazoline compounds and DNA gyrase, as was suggested by modeling.

Varying the substituent present at C-4 also had no impact on the inhibitory potential of the quinazoline derivatives. Modeling suggests that the quinazoline C-4 carbonyl is not involved in binding to target protein in the same way as the C-4 carbonyl of fluoroquinolones. In fluoroquinolone binding, the C-4 carbonyl and C-3 carboxylic acid together chelate a magnesium ion to form a bridge with asp87 in gyrase or asp95 in topoIV. Unlike fluoroquinolones, chelation of magnesium does not reverse inhibition by
quinazoline-2,4-dione compounds. The environment surrounding the C-4 carbonyl of bound quinazoline-2,4-dione compounds is open enough to accommodate the added bulk and electronic differences of either a thiocarbonyl or oxime substituent at C-4, illustrated by electron density and potential maps in Figure 25, without affecting inhibitory activity. The increase in electropositive potential at C-4 would be expected to have a major impact on binding if this substituent is involved. The C-2 carbonyl is not affected by changes made at the C-4 position. This property could be taken advantage of to functionalize the quinazoline core structure to increase desirable properties or decrease susceptibility to

Figure 25. (A) Overlay of energy minimized structures of 1-cyclopropyl-2-oxo-6,7-difluoro structures on calculated electron density maps. Blue represents areas of most positive density, red represents more negative density. (B) minimized structures with potential map overlaid. C-4 substituents, from left to right: oxime, thiocarbonyl, carbonyl.
efflux mechanisms.

As expected, the C-7 substituent had a major impact on inhibition of cell growth. C-7 3-(R)-aminomethylpyrrolidinyl substituted derivatives were twice as active against efflux deficient *E. coli*. It has been previously reported that a partial list of C-7 substituents for decreasing inhibitory activity against wild type and resistant mutants of 1-cyclopropyl-8-methoxy quinazoline-2,4-dione is 3-aminomethyl pyrrolidinyl > 3-aminopyrrolidinyl > octahydropyrrolopyridinyl > 2-ethyl piperazinyl [30].
CHAPTER 6 EXPERIMENTAL SECTION

6.1 General Methods and Equipment

NMR spectra were obtained for $^1$H, $^{13}$C, $^{19}$F, COSY, HMBC, and HMQC using a Bruker Ultrashield 300 MHz instrument. Chemical shifts are given in ppm relative to tetramethylsilane and solvent. Solvent chemical shifts are as follows: DMSO-$\delta_6$ – 2.50; Methanol-d$_4$ – 3.31; CDCl$_3$ – 7.26.

Mass spectrometry was determined using a Thermo LCQ Deca mass spectrometer with ESI ionization and quadrupole ion trap mass analyzer.

Analytical HPLC analysis was determined using a Shimadzu system equipped with LC-20AT pump, DGU-14A degasser, CBM-20A system controller, and SPD-M10A vp photodiode array detector. The system was connected to a Dell Optiplex GX400 PC and controlled by Shimadzu Client/Server Version 7.4. Either Phenomenex Luna C18 or Restek Allure PFP Propyl was used as stationary phase. Mobile phase consisted of solvent A (water, buffered 0.1% TFA) and solvent B (acetonitrile, buffered 0.1% TFA). Gradient elution followed one of two programs: (Program A) from $t = 0$ min [solvent A (0.95 mL/min), solvent B (0.05 mL/min)] to $t = 30$ min [solvent A (0.05 mL/min), solvent B (0.95 mL/min)], to $t = 35$ min [solvent A (0.05 mL/min), solvent B (0.95 mL/min)], to $t = 40$ min [solvent A (0.95 mL/min), solvent B (0.05 mL/min)]; (Program B) from $t = 0$ min [solvent A (0.95 mL/min), solvent B (0.05 mL/min)] to $t = 15$ min [solvent A (0.05 mL/min), solvent B (0.95 mL/min)] to $t = 18$ min [solvent A (0.05 mL/min), solvent B (0.95 mL/min)], to $t = 20$ min [solvent A (0.95 mL/min), solvent B (0.05 mL/min)].

Specified final products that were purified by semi-preparative HPLC were separated using a Shimadzu system. The system was equipped with two LC-10AT pumps, one for each solvent A and B, SPD-M10A vp photodiode array detector, and SCL-10A vp system controller. The system was connected to a Dell Optiplex 755 running
Shimadzu EZStart Version 7.4 control software. Either Phenomenex Luna C18 or Phenomenex Luna PFP(2) was used as stationary phase. Mobile phase consisted of solvent A (water, buffered 0.1% TFA) and solvent B (acetonitrile, buffered 0.1% TFA). Mobile phase solutions were degassed once daily by bubbling helium through the solution for 20 minutes prior to injections. Gradient elution from $t = 0$ min [solvent A (0.35 mL/min), solvent B (6.65 mL/min)] to $t = 40$ min [solvent A (6.65 mL/min), solvent B (0.35 mL/min)] to $t = 50$ min [solvent A (6.65 mL/min), solvent B (0.35 mL/min)] to $t = 60$ min [solvent A (0.35 mL/min), solvent B (6.65 mL/min)].

Reactions were monitored by analytical HPLC (methods described previously) or by thin layer chromatography (TLC) carried out on 0.25 mm TLC Silica Gel 60 plates with fluorescence indicator $F_{254}$ (EMD Sciences). Typical visualization methods were: (1) by UV absorption/fluorescence (254 nm and 366 nm); (2) staining by ninhydrin solution (0.3% ninhydrin in 3% TFA solution of n-butanol), followed by heating; (3) molydate stain (5.0 g cerium ammonium nitrate, 25.0 g ammonium molybdate tetrahydrate, 450 mL water, 50 mL concentrated sulfuric acid) followed by heating.

Silica gel flash chromatography was performed using Silica Gel 60 (particle size 0.040 – 0.063 mm; 230 – 400 mesh ASTM). Solvent ratios given are volume/volume.

Organic solvents were dried over anhydrous magnesium sulfate or anhydrous sodium sulfate, and then concentrated by rotary evaporator and reduced pressure (high vacuum). Aqueous solutions were concentrated by lyophilization.

Dry DCM, toluene, and THF were collected from laboratory solvent distillation system. Other dry solvents were prepared ad hoc by distillation as noted. When necessary, dry glassware was prepared by oven drying followed by flame drying under reduced pressure. Anhydrous reactions were carried out under argon in a closed system.

Molecular modeling studies were performed using Spartan '06 Essentials (Wavefunction). Energy minimization for Figures 13, 20, and 25 was determined using Hartree-Fock 3-21G parameters. Electron density and potential maps were calculated
according to minimized structures. Data in Figure 14B was determined by calculating static energy for the molecule at specific dihedral angles of the C-7 bond, as illustrated in Figure 14A, following minimization of the molecule. Data in Figure 14C was determined by 6-31G* minimization for the molecule while locking the dihedral angle of the C-7 bond.

Bacterial cells used in inhibition and lethality tests were grown on LB agar or in LB liquid medium. The MIC was measured by incubation of $10^4$ to $10^5$ cells/mL in LB liquid medium containing serial 2-fold dilutions of quinolone at 37°C. To measure lethal action, cells were grown aerobically at 37°C in liquid medium to midlog phase. Solutions of quinolone were added, and incubation was continued for 2 h. The cells were diluted in liquid growth medium, applied to agar plates lacking the drug, and incubated overnight at 37°C to determine the number of colony forming units. Percent survival was determined relative to number of colony forming units at the time of quinolone addition.

Chloramphenicol (MIC = 2 µg/ml), when used, was added to 20 µg/ml 10 min prior to the addition of quinolone for measurement of killing in the absence of protein synthesis.

6.2 Synthesis of ofloxacin and levofloxacin derivatives for fused ring studies (Chapter 3)

6.2.1 Preparation of 8-fluoro-3-methyl-9-(3-methylpiperazin-1-yl)-6-oxo-2,3,5,6-tetrahydro-4H-1-oxa-3a-aza-phenalene-5-carboxylic acid (KRM-I-017)

9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (1) (Acros, 15.8 mg, 0.06 mmol) was stirred in 2 mL DMSO with 2-methyl piperazine (Acros, 9.3 mg, 0.09 mmol) and 50 µL TEA (Fisher). The solution was stirred for 15 hours at 105°C. The reaction was cooled to room temperature and diluted in 10 mL water (0.1% TFA). The solution was filtered through cotton and product was purified by semi-preparative HPLC. Fractions containing product were concentrated by
rotary evaporator to remove acetonitrile and TFA, followed by lyophilization to yield pure KRM-I-017 (14.9 mg, 60%). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ = 15.11 (s, 1H), 9.00 (s, 1H), 7.64 (d, $J_{HF}$ = 11.2 Hz, 1H), 5.07 (m, 1H), 4.64 (m, 1H), 4.42 (m, 1H), 3.50 (m, 7H), 1.47 (t, $J = 7.1$ Hz, 3H), 1.25 (d, $J = 6.4$ Hz, 3H). HRMS (ESI) calcd for (M+H$^+$) 362.1511, found 362.1517

6.2.2 Preparation of 8-fluoro-3-methyl-9-(3-ethyl-piperazin-1-yl)-6-oxo-2,3,5,6-tetrahydro-4H-1-oxa-3a-aza-phenalene-5-carboxylic acid (KRM-I-021)

9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (1) (Acros, 19.7 mg, 0.07 mmol) was stirred in 2 mL DMSO with 2-ethyl piperazine (Atlantic Scitech Group, 16.1 mg, 0.14 mmol) and 50 µL TEA (Fisher). The solution was stirred for 15 hours while heating to 105° C. The reaction was cooled to room temperature and diluted in 15 mL water (0.1% TFA). The solution was filtered through cotton and the product was purified by semi-preparative HPLC. Fractions containing product were concentrated by rotary evaporator to remove acetonitrile and TFA, followed by lyophilization to yield pure KRM-I-021 (12.2 mg, 45%). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ = 15.11 (s, 1H), 9.00 (s, 1H), 7.64 (d, $J_{HF}$ = 11.2 Hz, 1H), 4.96 (m, 1H), 4.60 (m, 1H), 4.40 (m, 1H), 3.55 (m, 4H), 3.22 (m, 3H), 1.63 (m, 2H), 1.46 (d, $J = 7$ Hz, 3H), 0.97 (t, $J = 7.4$ Hz, 3H). HRMS (ESI) calcd for (M+H$^+$) 376.1667, found 376.1669.

6.2.3 Preparation of 8-fluoro-3-methyl-9-(octahydropyrrolo[3,4-b]pyridin-6-yl)-6-oxo-2,3,5,6-tetrahydro-4H-1-oxa-3a-aza-phenalene-5-carboxylic acid (KRM-I-023)

9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (1) (Acros, 15.7 mg, 0.06 mmol) was stirred in 2 mL DMSO with cis-octahydropyrrolo[3,4-b]pyridine (3B Medical Systems, 40.6 mg, 0.32 mmol) and 50 µL
TEA (Fisher). The solution was stirred for 15 hours while heating to 105°C. The reaction was cooled to room temperature and diluted in 15 mL water (0.1% TFA). The solution was filtered through cotton and the product was purified by semi-preparative HPLC. Fractions containing product were concentrated by rotary evaporator to remove acetonitrile and TFA, followed by lyophilization to yield pure KRM-I-023 (7.4 mg, 32%). ¹H NMR: (300 MHz, DMSO-d₆) δ = 15.35 (s, 1H), 8.93 (s, 1H), 7.58 (d, J_H-F = 14.4 Hz, 1H), 4.88 (m, 1H), 4.55 (m, 1H), 4.29 (m, 1H), 4.20 (m, 1H), 3.74 (m, 5H), 2.95 (m, 2H), 1.74 (m, 5H), 1.46 (m, 3H). HRMS (ESI) calcd for (M+H⁺) 388.1667, found 388.1687.

6.2.4 Preparation of S-(-)-8-fluoro-3-methyl-9-(3-ethylpiperazin-1-yl)-6-oxo-2,3-dihydro-6H-1-oxa-3a-aza-phenalene-5-carboxylic acid (KRM-I-025)

S-(-)-9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (2) (Aldrich, 24.8 mg, 0.09 mmol) was stirred in 2 ml DMSO with 50 µl triethylamine (Fisher). 2-Ethyl piperazine (Atlantic Scitech Group, 19.0 mg, 0.17 mmol) was added and the mixture heated to 110°C overnight. The solution was cooled to room temperature, diluted in 30 ml water (0.1% TFA), filtered through cotton, and purified by semi-preparative reverse phase HPLC. Fractions containing product were concentrated by rotary evaporation to remove acetonitrile and TFA, and then lyophilized, yielding KRM-I-025 (7.9 mg, 24%). ¹H NMR (300 MHz, Methanol-d₄) δ = 8.87 (s, 1H), 7.68 (d, J_H-F = 13.1 Hz, 1H), 4.59 (d, J = 13.1 Hz, 1H), 4.44 (d, J = 13.1 Hz, 1H), 3.61 (m, 3H), 3.40 (m, 4H), 1.74 (m, 2H), 1.57 (d, J = 6.4 Hz, 3H), 1.09 (t, J = 7.3 Hz, 3H). HRMS (ESI) calcd for (M+H⁺) 376.1667, found 376.1675.
6.2.5 Preparation of S-(-)-8-fluoro-3-methyl-9-(3-methyl-piperazin-1-yl)-6-oxo-2,3-dihydro-6H-1-oxa-3a-aza-phenalene-5-carboxylic acid (KRM-I-029)

S-(-)-9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (2) (Aldrich, 28.5 mg, 0.10 mmol) was stirred in 2 ml DMSO with 50 µl triethylamine (Fisher). 2-Methyl piperazine (Acros, 17.0 mg, 0.17 mmol) was added and the mixture heated to 110°C overnight. The solution was cooled to room temperature, diluted in 30 ml water (0.1% TFA), filtered through cotton, and purified by semi-preparative reverse phase HPLC. Fractions containing product were concentrated by rotary evaporation to remove acetonitrile and TFA, and then lyophilized to yield pure KRM-I-029 (15.0 mg, 41%). 1H NMR (300 MHz, DMSO-d6) δ = 15.13 (s, 1H), 9.02 (s, 1H), 7.64 (d, J=11.7 Hz, 1H), 4.94 (q, J = 5.8 Hz, 1H), 4.61 (d, J = 11.7 Hz, 1H), 4.40 (d, J = 11.3 Hz, 1H), 3.42 (m, 7H), 1.46 (d, J = 6.4 Hz, 3H), 1.24 (d, J = 6.4 Hz, 3H). HRMS (ESI) calcd for (M+H+) 362.1511, found 362.1468.

6.2.6 Preparation of S-(-)-8-fluoro-3-methyl-9-(octahydro-pyrrolo[3,4-b]pyridin-6-yl)-6-oxo-2,3,5,6-tetrahydro-4H-1-oxa-3a-aza-phenalene-5-carboxylic acid (KRM-I-033)

S-(-)-9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (2) (Aldrich, 27.7 mg, 0.10 mmol) was stirred in 2 ml DMSO with 50 µl triethylamine (Fisher). Octahydro-pyrrolo[3,4-b]pyridine (3B Medical Systems, 20.4 mg, 0.16 mmol) was added and the mixture heated to reflux overnight. The solution was cooled to room temperature, diluted in 20 mL water (0.1% TFA), filtered through cotton, and purified by semi-preparative reverse phase HPLC. Fractions containing product were concentrated by rotary evaporator to remove acetonitrile and TFA, and then water was removed by lyophilization, yielding pure KRM-I-033 (8.8 mg, 23%). 1H NMR (300 MHz, DMSO-d6) δ = 15.41 (s, 1H), 8.93 (s,
6.3 Synthesis of crosslinking agents (Chapter 4)

6.3.1 Preparation of 7-(4-acetyl-piprazin-1-y1)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (KRM-II-273)

Ciprofloxacin hydrochloride (IGN Biomed, 175.1 mg, 0.5 mmol) was stirred with sodium hydroxide (Fisher, 21.4 mg, 0.5 mmol), tetrabutylammonium bromide (Aldrich, 4.2 mg, 0.01 mmol), and acetic anhydride (Fisher, 199.6 mg, 2.0 mmol) in 5 mL DMF while heating to 70° C for one hour. The reaction was diluted in water and KRM-II-273 precipitated as a white solid. The solid was collected by filtration, washed with ethyl acetate and DCM, then dried under reduced pressure (97.0 mg, 52%).$^{1}$H NMR (300 MHz, Methanol-d$_4$) δ = 8.85 (s, 1H), 7.97 (d, $J_{H,F}$ = 13.7 Hz, 1H), 7.46 (d, J = 7.4 Hz, 1H), 3.82 (m, 5H), 3.42 (m, 4H), 2.21 (s, 3H), 1.45 (d, 2H), 1.23 (m, 2H). $^{19}$F NMR (282 MHz, Methanol-d$_4$) δ = -122.52. LRMS (ESI) calcd for (M+H$^+$) 374.15, found 374.09.

6.3.2 Preparation of 7-[4-(2-bromo-acetyl)-piprazin-1-y1]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (KRM-III-043)

Ciprofloxacin hydrochloride (IGN Biomed, 198.8 mg, 0.6 mmol) was stirred in 15 mL water with sodium hydroxide (Fisher, 49.1 mg, 1.23 mmol) until clear. The solution was concentrated by lyophilizer and the white residue was suspended in 1.5 mL anhydrous DMF and filtered through cotton to yield ciprofloxacin.
Ciprofloxacin (29.2 mg, 0.1 mmol) was stirred in 1.5 mL anhydrous DMF with \( p \)-nitrophenyl bromoacetate for four hours at 110° C. The solution was cooled to room temperature and diluted in 10 mL water (0.1% TFA). **KRM-III-043** was purified by semi-preparative HPLC as the trifluoroacetate salt (13.58 mg, 23%). \(^1\)H NMR (300 MHz, DMSO-\( d_6 \)) \( \delta = 15.20 \) (s, 1H), 8.68 (s, 1H), 7.95 (d, \( J_{H-F} = 12.1 \) Hz, 1H), 6.55 (s, 1H), 4.23 (s, 2H), 3.71 (m, 5H), 3.37 (m, 4H), 1.25 (m 4H). \(^{19}\)F NMR (282 MHz, DMSO-\( d_6 \)) \( \delta = -121.60 \). LRMS (ESI) calcd for (M+H\(^+\)) 452.06, found 452.05.

6.4 Synthetic routes towards C-8 methoxy ulifloxacin

(Chapter 4)

6.4.1 Preparation of 1,2-difluoro-3-methoxy-4-nitrobenzene

(4)

2,3-Difluoro-6-nitro phenol (3) (Matrix Scientific, 1.05 g, 6.0 mmol) was combined with oven dried potassium carbonate (Fisher, 1.05 g, 7.6 mmol) and iodomethane (Acros, 20.52 g, 144.5 mmol). The whole was dissolved in 30 mL acetone and heated to reflux for 2.5 hours under argon. Acetone was diluted in 100 mL water and extracted twice with 150 mL ethyl acetate. Organic fractions were combined, washed with water and brine, dried over magnesium sulfate and concentrated by rotary evaporator to yield 4 as a dark yellow oil (878 mg, 77%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta = 7.68 \) (m, 1H), 7.02 (dd, \( J_{H-F} = 15.4, 9.5 \) Hz, 1H), 4.15 (d, \( J = 2.0, 3H \)).

6.4.2 Preparation of 3,4-difluoro-2-methoxy phenylamine

(5)

1,2-difluoro-3-methoxy-4-nitrobenzene 4 (878 mg, 4.64 mmol) was suspended in 25 mL water. Acetic acid (Fisher, 0.7 mL glacial) and reduced iron powder (Alfa Aesar 1.91 g, 34.2 mmol) were added and the suspension was stirred while heating to reflux, protected from light, overnight. The suspension was cooled to room temperature,
neutralized to pH 7 with aqueous sodium bicarbonate solution and filtered to remove solids. The filtrate was extracted twice with 175 mL ethyl acetate. Organic fractions were combined, dried over sodium sulfate, and concentrated by rotary evaporator to yield 5 as a dark red oil (719 mg, 97%). Spectral data were consistent with published literature values [99].

6.4.3 Preparation of (3,4-difluoro-2-methoxyphenyl)-dithiocarbamic acid (6)

3,4-Difluoro-2-methoxy phenylamine 5 (7.8 g, 49.0 mmol) was diluted in 37 mL TEA (Fisher) and stirred over an ice bath. Carbon disulfide (Fisher, 8.8 g, 115.9 mmol) was added dropwise and the solution warmed gradually to room temperature and stirred for six days. Pure 6 was collected as a pale yellow precipitate by filtration. The precipitate was washed with water and dried under reduced pressure (9.4 g, 81%). Spectral data was consistent with published literature values [68].

6.4.4 Preparation of 1,2-difluoro-4-isothiocyanato-3-methoxy benzene (7)

(3,4-Difluoro-2-methoxyphenyl)-dithiocarbamic acid (6) (9.4 g, 39.9 mmol) was suspended in 31 mL chloroform and 3.5 mL TEA and stirred over ice. Ethyl chloroformate (Acros, 2.9 g, 26.3 mmol) was dissolved in 8.5 mL chloroform and added dropwise. The mixture stirred at 0° C for two hours, and was then poured over ice water and neutralized to pH 7 with 6N hydrochloric acid. The chloroform layer was recovered and the aqueous layer was extracted with 50 mL chloroform. Organic layers were combined, dried over sodium sulfate, and concentrated by rotary evaporator. 7 was purified by silica gel flash chromatography eluted with 50:1 hexanes:ethyl acetate mobile phase (3.0 g, 38%). Spectral data were consistent with published literature values [68].
6.4.5 Preparation of 2-[(3,4-difluoro-2-methoxy-phenylamino)-methoxymethylsulfanyl-methylene]-malonic acid diethyl ester (9)

Potassium hydroxide (Fisher, 4.5 g, 80.2 mmol) was stirred in 80 mL anhydrous toluene while diethyl malonate (Acros, 8.0 g, 49.8 mmol) was added dropwise. The suspension stirred at room temperature for 2.5 hours. Previously prepared 1,2-difluoro-4-isothiacyanato-3-methoxy benzene (7) (5 g, 24.9 mmol) was diluted in 20 mL anhydrous toluene and added to the stirred mixture. The whole was stirred at room temperature for seven hours, and then stored at 0° for 20 hours. The precipitated potassium salt (8) (2 g, 5 mmol) was collected by filtration.

Chloromethyl methyl ether (Aldrich, 530 mg, 6.6 mmol) was stirred in a solution of 300 µL TEA in 30 mL toluene over an ice bath. After 30 minutes, potassium salt (8) (2 g, 5 mmol) was added and the whole stirred for 44 hours, gradually warming to room temperature, and then poured over ice. The toluene layer was recovered and washed, in sequence, with 1% hydrochloric acid solution (w/v), saturated sodium bicarbonate solution, and brine. The toluene layer was recovered and concentrated by rotary evaporator, yielding 9 as a yellow oil (161.5 mg, 8%). Spectral data were consistent with published literature values [68].

6.4.6 Preparation of 3-oxo-3-(2,4,5-trifluoro-3-methoxy-phenyl)-propionic acid ethyl ester (12)

Thionyl chloride (Aldrich, 4.5 g, 37.8 mmol) was added to a stirring solution of 2,4,5-trifluoro-3-methoxy benzoic acid (10) (Aldrich, 2.0 g, 9.8 mmol) and sodium chloride (Aldrich, 90 mg, 1.5 mmol) in 50 mL ethyl acetate. The solution was heated to reflux for two hours, then cooled to room temperature and concentrated by rotary evaporator. The resulting acid chloride (11) was twice dissolved in 50 mL anhydrous toluene and concentrated by rotary evaporator.
Ethyl potassium malonate (Aldrich, 2.0 g, 11.9 mmol) and anhydrous magnesium chloride (Amresco, 2.7 g, 28.3 mmol) were stirred at room temperature in 30 mL ethyl acetate for 30 minutes, followed by addition of triethyl amine (Fisher, 2.9 g, 28.5 mmol) and continued stirring at room temperature for 30 minutes. Acid chloride 11 was dissolved in 20 mL ethyl acetate and added to the stirring malonate solution. The reaction was then heated to reflux for 2.5 hours, and then cooled to room temperature. The solution was diluted with 50 mL water, and then acidified to pH 1-2 by dropwise addition of 6N HCl. The biphasic solution was stirred vigorously. The ethyl acetate layer was collected, dried over sodium sulfate, and concentrated to an oil by rotary evaporator. The oil was recrystallized in 50 ml ethanol, 20 ml water. Crystals were collected by filtration, washed with cold 70% ethanol, and dried under reduced pressure to yield 12 (1.4 g, 53%). Spectral data were consistent with published literature values [115].

6.4.7 Preparation of 1-benzyl-2-ethylsulfanyl-6,7-difluoro-8-methoxy-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid ethyl ester (14)

Previously prepared 3-oxo-3-(2,4,5-trifluoro-3-methoxy-phenyl)-propionic acid ethyl ester (12) (165.2 mg, 0.60 mmol), potassium hydroxide (Fisher, 87.2 mg, 1.55 mmol), and tetrabutylammonium bromide (Aldrich, 5 mg, 0.02 mmol) were stirred in 8 mL DMF at room temperature for 30 minutes. Benzyl isothiocyanate (Acros, 107.9 mg, 0.72 mmol) was added and the solution stirred at room temperature for 16 hours. Iodoethane (Acros, 300 mg, 1.92 mmol) was added and the solution continued to stir at room temperature for 90 minutes. Ethyl acetate was distilled off by rotary evaporator leaving crude 2-(benzylimino-ethylsulfanyl-methyl)-3-hydroxy-3-(2,4,5-trifluoro-3-methoxy-phenyl)-acrylic acid ethyl ester 13 as a green residue. The residue was twice dissolved in 10 mL anhydrous toluene and concentrated by rotary evaporator.

Potassium tert-butoxide (Acros, 70.5 mg, 0.63 mmol) was added to the crude
reside, dissolved in 10 mL anhydrous toluene. The solution was heated to reflux for four hours, and then quenched with 30 mL water. The toluene layer was recovered and the aqueous layer was extracted twice with 20 mL ethyl acetate. Organic fractions were combined and concentrated by rotary evaporator. The resulting residue was purified by silica gel flash chromatography eluted with 4:1 hexanes:ethyl acetate to yield **14** (107.8 mg, 41%).

**1H NMR** (300 MHz, CDCl$_3$) $\delta$ = 7.86 (dd, $J_{HF}$ = 9.8, 8.7 Hz, 1H), 7.21 (m, 3H), 6.93 (dd, $J = 7.9$, 2.1 Hz, 2H), 6.04 (s, 2H), 4.42 (q, $J = 7.1$ Hz, 2H), 3.80 (d, $J = 1.9$, 3H), 2.97 (q, $J = 7.5$ Hz, 2H), 1.39 (t, $J = 7.1$ Hz, 3H), 1.28 (t, $J = 7.5$ Hz, 3H).

**19F NMR** (282 MHz, CDCl$_3$) $\delta$ = -136.01, -143.91.

6.4.8 Preparation of 2-ethylsulfanyl-6,7-difluoro-4-hydroxy-8-methoxyquinoline-3-carboxylic acid ethyl ester

**15**

1-Benzyl-2-ethylsulfanyl-6,7-difluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (**14**) (77.4 mg, 0.17 mmol) was stirred in acetonitrile and polyphosphoric acid at room temperature for three days. Acetonitrile was distilled off by rotary evaporator and the residue was taken up in a minimum of 3:1 hexanes:ethyl acetate. The product was purified by silica gel flash chromatography, eluted with 3:1 hexanes:ethyl acetate to afford **15** (22.6 mg, 37%).

**1H NMR** (300 MHz, CDCl$_3$) $\delta$ = 7.90 (dd, $J_{HF}$ = 9.4, 9.4, Hz, 1H), 4.44 (q, $J = 7.0$ Hz, 2H), 3.82 (s, 3H), 2.98 (q, $J = 7.5$ Hz, 2H), 1.415 (t, $J = 6.9$ Hz, 3H), 1.30 (t, $J = 7.4$ Hz, 3H).

**19F NMR** (282 MHz, CDCl$_3$) $\delta$ = -135.92, -143.88.
6.5 Synthesis of C-2 thioalkyl fluoroquinolone derivatives

(Chapter 4)

6.5.1 Preparation of 2-(cyclopropylimino-ethanesulfanyl-methyl)-3-hydroxy-3-(2,4,5-trifluoro-3-methoxyphenyl)-acrylic acid ethyl ester (16a)

Previously prepared 3-oxo-3-(2,4,5-trifluoro-3-methoxyphenyl)-propionic acid ethyl ester (12) (168.7 mg, 0.6 mmol) was stirred with potassium hydroxide (Fisher, 36.1 mg, 0.6 mmol) and tetrabutylammonium bromide (Aldrich, 11.3 mg, 0.03 mmol) at room temperature in 7 mL anhydrous DMF for 90 minutes. Cyclopropyl isocyanate (Acros, 111.2 mg, 1.1 mmol) was added and the mixture continued to stir at room temperature for five hours, followed by addition of iodoethane (Acros, 116.5 mg, 0.8 mmol). The reaction was stirred overnight at room temperature, quenched with saturated ammonium chloride, and extracted with ethyl acetate. Organic fractions were recovered, dried over sodium sulfate, and concentrated by rotary evaporator. Residue was purified by silica flash chromatography, eluted with 2:1 hexanes:ethyl acetate to yield 16a (182.7 mg, 74%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta = 11.82\) (s, 1H), 6.97 (m, 1H), 4.03 (s, 3H), 3.97 (q, \(J = 7.8\), 2H), 3.08 (m, 1H), 3.05 (q, \(J = 7.8\) Hz, 2H), 1.34 (t, \(J = 7.8\) Hz, 3H), 0.99 (t, \(J = 7.8\) Hz, 3H), 0.98 (m, 2H), 0.87 (m, 2H). \(^19\)F NMR (282 MHz, CDCl\(_3\)) \(\delta = -135.21, -140.86, -149.39\).

6.5.2 Preparation of 2-(cyclopropylimino-isopropylsulfanyl-methyl)-3-hydroxy-3-(2,4,5-trifluoro-3-methoxyphenyl)-acrylic acid ethyl ester (16b)

Previously prepared 3-oxo-3-(2,4,5-trifluoro-3-methoxyphenyl)-propionic acid ethyl ester (12) (505.6 mg, 1.8 mmol) was stirred with potassium hydroxide (Fisher, 106.1 mg, 1.9 mmol) and tetrabutylammonium bromide (Aldrich, 32.6 mg, 0.1 mmol) in 10 ml DMF at room temperature under argon for two and one half hours. Cyclopropyl
isothiocyanate (Acros, 222.5 mg, 2.2 mmol) was added and the solution stirred at room
temperature overnight. Isopropyl iodide (Acros, 654.9 mg, 3.9 mmol) was added and the
solution continued to stir at room temperature for seven hours. The reaction was
quenched by dilution with saturated ammonium chloride and product was extracted by
ethyl acetate. Organic fractions were combined, dried over sodium sulfate, and
concentrated by rotary evaporator. Residue was diluted in anhydrous toluene and
concentrated by rotary evaporator a total of three times. The orange oil was purified by
silica flash chromatography, eluted with 3:1 hexanes:ethyl acetate to yield 16b (581.3
mg, 76%). ¹H NMR (300 MHz, CDCl₃) δ = 11.84 (s, 1H), 6.96 (m, 1H), 3.99 (s, 3H),
3.96 (q, J = 6.9 Hz, 2H), 3.74 (m, 1H), 3.15 (m, 1H), 1.32 (d, J = 6.9 Hz, 6H), 0.99 (d, J =
6.9 Hz, 3H), 0.97 (m, 2H), 0.83 (m, 2H). ¹⁹F NMR (282 MHz, CDCl₃) δ = -135.17, -
140.89, -149.26.

6.5.3 Preparation of 1-cyclopropyl-2-ethylsulfanyl-6,7-
difluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-
carboxylic acid ethyl ester (17a)

2-(Cyclopropylimino-ethylsulfanyl-methyl)-3-hydroxy-3-(2,4,5-trifluoro-3-
methoxy-phenyl)-acrylic acid ethyl ester (16a) (182.7 mg, 0.45 mmol) was stirred at
reflux in 15 mL toluene with potassium tert-butoxide (Acros, 53.0 mg, 0.47 mmol) for 19
hours. The reaction was diluted in water and the toluene layer recovered. The aqueous
layer was extracted with toluene and the organic fractions were combined and
concentrated by rotary evaporation. The residue was purified by silica gel flash
chromatography eluted with 3:1 hexanes:ethyl acetate to yield 17a (66.7 mg, 15%). ¹H
NMR (300 MHz, CDCl₃) δ = 7.74 (m, 1H), 4.37 (q, J = 7.1 Hz, 2H), 4.06 (d, J = 2.3 Hz,
3H), 3.70 (m, 1H), 3.06 (q, J = 7.4 Hz, 2H), 1.36 (t, J = 7.2 Hz, 3H), 1.33 (t, J = 7.4 Hz,
3H), 1.18 (bd, J = 5.8 Hz, 2H), 0.70 (bs, 2H). ¹⁹F NMR (282 MHz, CDCl₃) δ = -137.15, -
146.06. LRMS (ESI) calcd for (M+H⁺) 384.11, found 384.02. 17b was prepared similarly
from 16b and taken to the next step without purification. LRMS (ESI) calcd for (M+H$^+$) 398.12, found 398.02.

6.5.4 Preparation of 1-cyclopropyl-2-ethylsulfanyl-6-fluoro-8-methoxy-7-(octahydropyrrolo[3,4-b]pyridin-6-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester

(18a)

1-Cyclopropyl-2-ethylsulfanyl-6,7-difluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (17a) (99.6 mg, 0.26 mmol) was dissolved in 1 mL anhydrous DMF and octahydropyrrolo[3,4-b]pyridine (3B Medical Systems, 65.9 mg, 0.52 mmol) and stirred while heating to 60°C for 26 hours. The reaction was followed by analytical HPLC, and then the DMF was distilled off by rotary evaporator. The oily residue was dissolved in 10 mL ethyl acetate and washed twice with 10 mL water. The organic layer was dried over sodium sulfate and concentrated by rotary evaporator. The resulting oil was recrystallized from ethanol to yield 18a (46.4 mg, 36%). 18b was prepared similarly from 17a and piperazine and taken to the next step without purification. 18c and 18d were prepared similarly from 17b and taken to the next step without purification. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 9.72 (bs, 1H), 7.33 (d, $J_{H-F}$ = 12.8 Hz, 1H), 4.30 (dq, $J = 7.1, 1.6$ Hz, 2H), 4.04 (t, $J = 1.1$ Hz, 1H), 3.89 (bs, 1H), 3.74 (m, 2H), 3.55 (s, 3H), 3.35 (m, 1H), 3.13 (q, $J = 7.4$ Hz, 2H), 3.05 (m, 1H), 2.73 (bs, 1H), 1.96 (m, 3H), 1.87 (m, 2H), 1.80 (bs, 1H), 1.33 (t, $J = 7.1$ Hz, 3H), 1.29 (t, $J = 7.4$ Hz, 3H), 0.97 (bs, 2H), 0.63 (bs, 2H). $^{19}$F NMR (282 MHz, CDCl$_3$) $\delta$ = -124.20. LRMS (ESI) calcd for (M+H$^+$) 490.22, found 490.18.
6.5.5 Preparation of 1-cyclopropyl-2-ethylsulfanyl-6-fluoro-8-methoxy-7-(octahydropyrrolo[3,4-b]pyridin-6-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (KRM-II-241)

1-Cyclopropyl-2-ethylsulfanyl-6-fluoro-8-methoxy-7-(octahydropyrrolo[3,4-b]pyridin-6-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (18a) (6.4 mg, 0.013 mmol) was stirred in 0.5 mL fuming sulfuric acid for two hours. The reaction was monitored by analytical HPLC, using Program B. The reaction was diluted in 10 mL acetonitrile (0.1% TFA), filtered through cotton, and purified by semi-preparative HPLC. Fractions containing product were concentrated by rotary evaporator to remove acetonitrile and TFA, then by lyophilization to yield KRM-II-241 as a brown-yellow solid. Product was confirmed by mass spectrometry as loss of ethyl (1.94 mg, 32%). LRMS (ESI) calcd for (M+H⁺) 462.19, found 462.11. KRM-III-045 was prepared similarly. LRMS (ESI) calcd for (M+H⁺) 422.15, found 422.03.

6.5.6 Preparation of 1-cyclopropyl-2-ethylsulfanyl-6-fluoro-8-methoxy-7-(octahydropyrrolo[3,4-b]pyridin-6-yl)-1$H$-quinolin-4-one (KRM-II-183)

1-Cyclopropyl-2-ethylsulfanyl-6-fluoro-8-methoxy-7-(octahydropyrrolo[3,4-b]pyridin-6-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid ethyl ester (18a) (46.4 mg, 0.09 mmol) was stirred in aqueous sulfuric acid (1.0% v/v) and heated to reflux for 36 hours. Reaction was monitored by analytical HPLC, using Program B. Upon complete conversion of starting material to a single, more polar product, purification was accomplished by semi-preparative HPLC. Fractions containing product were combined and concentrated by rotary evaporator to remove acetonitrile and TFA, followed by lyophilization to yield KRM-II-183 (23.4 mg, 60%). KRM-II-197, KRM-II-185, and KRM-II-199 were prepared similarly from 18b-d, respectively. $^1$H NMR (300 MHz,
DMSO-d$_6$) $\delta = 9.26$ (bd, $J = 9.4$ Hz, 1H), 8.58 (bd, $J = 8.5$ Hz, 1H), 7.40 (d, $J_{H-F} = 13.9$ Hz, 1H), 6.08 (s, 1H), 4.02, (m, 1H), 3.88 (bs, 1H), 3.72 (m, 2H), 3.59 (m, 1H), 3.49 (s, 3H), 3.22 (bd, $J = 11.1$ Hz, 1H), 3.10 (m, 2H), 2.97 (m, 1H), 2.64 (bs, 1H), 1.74 (m, 4H), 1.34 (t, $J = 7.4$ Hz, 3H), 1.19 (m, 1H), 1.07 (m, 1H), 0.68 (m, 2H). LRMS (ESI) calcd for (M+H$^+$) 418.20, found 418.22.

**KRM-II-197** $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta = 7.40$ (d, $J_{H-F} = 12.6$ Hz, 1H), 6.11 (s, 1H), 3.84 (m, 1H), 3.54 (s, 3H), 3.40 (m, 4H), 3.28 (m, 4H), 3.04 (q, $J = 6.9$ Hz, 2H), 1.38 (t, $J = 6.9$ Hz, 2H), 1.18 (m, 2H), 0.85 (m, 2H). LRMS (ESI) calcd for (M+H$^+$) 378.16, found 378.17.

**KRM-II-185** $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta = 9.35$ (bd, $J = 9.3$ Hz, 1H), 8.66 (bs, 1H), 7.39 (d, $J_{H-F} = 14.4$ Hz, 1H), 6.19 (s, 1H), 4.02, (m, 1H), 3.88 (bs, 1H), 3.68 (m, 1H), 3.58 (m, 1H), 3.48 (s, 3H), 3.22 (bd, $J = 10.1$ Hz, 1H), 3.10 (m, 2H), 2.99 (m, 1H), 2.64 (bs, 1H), 1.77 (m, 4H), 1.38 (d, $J = 6.7$ Hz, 3H), 1.36 (d, $J = 6.7$ Hz, 3H), 1.18 (bs, 1H), 1.05 (bs, 1H), 0.66 (m, 2H). LRMS (ESI) calcd for (M+H$^+$) 432.21, found 432.24.

**KRM-II-199** $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta = 7.40$ (d, $J_{H-F} = 12.4$ Hz, 1H), 6.19 (s, 1H), 3.69 (m, 1H), 3.58 (s, 3H), 3.39 (m, 4H), 3.27 (m, 4H), 3.15 (m, 1H), 1.26 (d, $J = 6.3$ Hz, 6H) 1.09 (d, $J = 7.1$ Hz, 2H), 0.84 (bs, 2H). LRMS (ESI) calcd for (M+H$^+$) 392.18, found 392.20.

**6.5.7 Preparation of 1-cyclopropyl-6-fluoro-7-piperazin-1-yl-1H-quinolin-4-one (KRM-III-145)**

Ciprofloxacin hydrochloride (19) (ICN, 41.6 mg, 0.13 mmol) and sodium cyanide (Fisher, 26.7 mg, 0.54 mmol) were combined and stirred in 1 mL DMSO at 150° C for one hour. Temperature was lowered to 100° C for four hours. The solution was cooled to room temperature, diluted in 10 mL water, and extracted twice with 20 mL ethyl acetate. Organic fractions were combined, dried over sodium sulfate, and concentrated by rotary evaporator to yield KRM-III-145 as a yellow solid (11.4 mg, 31%). KRM-III-157 was
prepared similarly from moxifloxacin (20) (ChemPacific). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 7.98 (d, J = 8.2 Hz, 1H), 7.76 (d, J$_{H-F}$ = 13.9 Hz, 1H), 7.45 (d, J = 8.2 Hz, 1H), 6.00 (d, J = 7.4 Hz, 1H), 3.57 (m, 1H), 3.42 (m, 4H), 3.34 (bs, 4H), 1.22 (q, J = 6.6, 2H), 1.04 (m, 2H). $^{19}$F NMR (282 MHz, CDCl$_3$) $\delta$ = -126.30. LRMS (ESI) calcd for (M+H$^+$) 288.15, found 288.24.

**KRM-III-157** $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 7.72 (d, J$_{H-F}$ = 15.1 Hz, 1H), 7.61 (d, J = 7.5 Hz, 1H), 6.10 (d, J = 7.5 Hz, 1H), 3.96 (m, 2H), 3.82 (m, 2H), 3.54 (s, 3H), 3.47 (m, 2H), 3.35 (d, J = 10 Hz, 1H), 3.10 (d, J = 10 Hz, 1H), 2.74 (t, J = 10 Hz, 1H), 2.35 (m, 1H), 1.86 (m, 8H), 0.89 (m, 5H), 0.74 (m, 2H). $^{19}$F NMR (282 MHz, CDCl$_3$) $\delta$ = -124.44. LRMS (ESI) calcd for (M+H$^+$) 358.19, found 358.23.

6.5.8 Preparation of 6-fluoro-1-methyl-7-piperazin-1-yl-2-thia-8b-aza-cyclobuta[a]naphthalen-4-one (KRM-III-163)

6-Fluoro-1-methyl-4-oxo-7-piperazin-1-yl-4H-2-thia-8b-aza-cyclobuta[a]naphthalene-3-carboxylic acid (21) (ulifloxacin, Bosche Scientific, 16.7 mg, 0.05 mmol) was stirred in 2 mL aqueous sulfuric acid (1% v/v) at 100° C for 17 hours. Reaction progress was followed by analytical HPLC, Program B. Product was purified by semi-preparative HPLC. Fractions containing product were combined, and concentrated by rotary evaporator to remove acetonitrile and TFA followed by lyophilization, to yield **KRM-III-163** (6.1 mg, 40%). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ = 7.72 (d, J$_{H-F}$ = 13.6 Hz, 1H), 6.90 (d, J = 6.4 Hz, 1H), 6.22 (q, J = 6.2 Hz, 1H), 6.13 (s, 1H), 3.40 (m, 4H), 3.31 (m, 4H), 2.05 (d, J = 6.2 Hz, 3H). Note: due to small amounts of TFA, the water peak is shifted to 9.03 ppm [116]. $^{19}$F NMR (282 MHz, DMSO-d$_6$) $\delta$ = -125.56. LRMS (ESI) calcd for (M+H$^+$) 306.11, found 306.23.
6.6 Synthesis of quinazoline derivatives (Chapter 5)

6.6.1 Preparation of 2,4,5-trifluoro-3-methoxybenzamide

(22)

2,4,5-Trifluoro-3-methoxybenzoic acid (10) (Oakwood, 5.0 g, 24.4 mmol) was dissolved in 36 mL anhydrous DCM and stirred at room temperature under argon for 45 minutes. Oxalyl chloride (Alfa Aesar, 4.3 g, 33.9 mmol) was added slowly, followed by addition of anhydrous DMF (Aldrich, 377.6 mg, 5.2 mmol). After bubbling had subsided, the solution was stirred for two hours at room temperature, and then concentrated by rotary evaporator. The acid chloride residue was twice dissolved in 50 mL anhydrous DCM and concentrated by rotary evaporator.

Acid chloride intermediate was dissolved in 40 mL anhydrous DCM and stirred vigorously over an ice bath during dropwise addition of ammonium hydroxide solution (Fisher, 70% NH₃, 22 mL). The solution was stirred while gradually warming to room temperature over two hours, and was then poured into 50 mL water. The DCM layer was recovered and the aqueous layer was extracted with 30 mL 1:1 DCM:ethyl acetate solution. The combined organic layers were concentrated by rotary evaporator to yield 22 as a white, crystalline solid (4.3 g, 87%). Spectral data were consistent with published literature values [88].

6.6.2 Preparation of 1-cyclopropyl-3-(2,4,5-trifluoro-3-methoxybenzoyl)-urea (24)

2,4,5-trifluoro-3-methoxybenzamide (22) (1.1 g, 5.1 mmol) was dissolved in 50 mL anhydrous DCM and stirred at room temperature while oxalyl chloride (Alfa Aesar, 945.8 mg, 7.5 mmol) was added dropwise. The solution was warmed to 60° C and stirred for one hour, then heated to reflux for 20 hours. DCM was distilled off by rotary evaporator to leave the reactive isocyanate intermediate (23) as a yellow oil.

Cyclopropyl amine (Alfa Aesar, 0.4 g, 7 mmol) was diluted in 50 mL anhydrous
DCM and stirred over a dry ice/acetone bath at -78°C. 2,4,5-Trifluoro-3-methoxybenzoyl isocyanate (23) was dissolved in 10 mL anhydrous DCM and added dropwise. The mixture was slowly warmed to room temperature and continued stirring for 17 hours. The reaction was concentrated by rotary evaporator and recrystallized from 1:1 isopropanol:heptane to yield 24 as an off white solid (1.4 g, 97%). Spectral data were consistent with published literature values [88].

6.6.3 Preparation of 1-cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione (25)

1-Cyclopropyl-3-(2,4,5-trifluoro-3-methoxybenzoyl)-urea (24) (1.4 g, 4.7 mmol) was stirred in 20 mL of a 1:1 mixture of anhydrous toluene and dimethoxyethane under argon at 0°C. Sodium hydride, 60% mineral oil dispersion (Acros, 418.9 mg, 17.4 mmol) was added in four portions, 15 minutes apart. The reaction mixture was then heated to reflux under argon for 18 hours. The reaction was concentrated by rotary evaporator and the resulting residue was dissolved in a minimum of 1:1 hexanes:ethyl acetate and purified by silica gel flash chromatography, eluted with 1:1 hexanes:ethyl acetate to yield 25 as a white solid (491.2 mg, 40%). Spectral data were consistent with published literature values [88].

6.6.4 Preparation of O-(2,4-dinitrophenyl)-hydroxylamine

Ethyl N-hydroxyacetimidate (Alfa Aesar, 4.0 g, 19.7 mmol) and potassium hydroxide (Fisher, 1.3 g, 24.3 mmol) were stirred in a solution of 40 mL water, 2 mL ethanol at -5°C for 10 minutes. 2,4-Dinitrochlorobenzene was dissolved in 70 mL ethanol and added dropwise such that the temperature of the reaction mixture remained below 0°C. The solution stirred at -1°C for three hours, and then the solid was collected by filtration and recrystallized from ethanol to yield N-(2,4-dinitrophenox)-acetimidic acid ethyl ester as a light cream colored solid (4.9 g, 94%).

N-(2,4-Dinitrophenoxy)-acetimidic acid ethyl ester (508.2 mg, 1.89 mmol) was
stirred at room temperature for 30 minutes in a solution of 2.5 mL 1,4-dioxane, 1 mL 70% perchloric acid. The reaction mixture was poured over 15 g ice and stirred vigorously until the ice had melted. The solid was collected by filtration, washed with ethanol, then suspended in a 1:1 mixture of anhydrous THF and anhydrous toluene. The mixture was concentrated by rotary evaporator and the process repeated twice with DCM. The residue was dried under high vacuum to yield an off-white solid (180.8 mg, 48%). Spectral data were consistent with published literature values [111].

6.6.5 Preparation of 3-amino-1-cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione (26)

1-Cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione (25) (601.4 mg, 2.2 mmol) was suspended with oven dried potassium carbonate (Fisher, 774.5 mg, 5.4 mmol) in a solution of 26 mL anhydrous DMF and 6.5 mL anhydrous THF. Previously prepared O-(2,4-dinitrophenyl)-hydroxylamine (1.2 g, 6.1 mmol) was added resulting in an immediate color change from white to dark orange. The reaction was heated to 90° C for 18 hours, and then additional O-(2,4-dinitrophenyl)-hydroxylamine (320 mg, 1.6 mmol) was added. The reaction mixture continued to stir at 90° C for one hour before cooling to room temperature and being concentrated by rotary evaporator. The resulting residue was suspended in water and extracted with ethyl acetate. Organic fractions were combined, dried over sodium sulfate and concentrated by rotary evaporator. The residue was purified by silica gel flash chromatography eluted with a gradient of 1:1 → 0:1 hexanes:ethyl acetate. Fractions containing product were combined and concentrated to yield 26 (235.2 mg, 38%). Spectral data were consistent with published literature values [88].
6.6.6 Preparation of 1-cyclopropyl-6,7-difluoro-8-methoxy-4-thioxo-3,4-dihydro-1H-quinazolin-2-one (27)

1-Cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione (25) (109.3 mg, 0.4 mmol) was stirred with Lawesson’s reagent (Alfa Aesar, 165.7 mg, 0.4 mmol) in 5 mL anhydrous toluene and heated to reflux for two hours. Reaction was cooled to room temperature and concentrated by rotary evaporator. The residue was dissolved in a minimum of 1:1 hexanes:ethyl acetate and purified by silica gel flash chromatography eluted with 2:1 hexanes:ethyl acetate to yield 27 as a yellow crystalline solid (110.5 mg, 95%). \( \text{H NMR (300 MHz, CDCl}_3) \delta = 8.13 \text{ (dd, J}_{H-F} = 10.5, 8.5, 1H), 4.00 \text{ (d, J = 1.8, 3H), 3.53 \text{ (m, 1H), 1.21 \text{ (m, 2H), 0.71 \text{ (m, 2H).}} \}
\text{C NMR (75.5 MHz, CDCl}_3) \delta = 188.60, 149.70, 148.66, 146.32, 139.13, 131.6, 119.53, 113.41, 62.88, 31.79, 10.92. \text{F NMR (282 MHz, CDCl}_3) \delta = -137.86, -140.73. \text{LRMS (ESI) calcd for (M+H\(^+\)) 285.05, found 285.03.} \)

6.6.7 Preparation of 3-amino-1-cyclopropyl-6,7-difluoro-8-methoxy-4-thioxo-3,4-dihydro-1H-quinazolin-2-one (28)

3-Amino-1-cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione (26) (82.3 mg, 0.3 mmol) was stirred with Lawesson’s reagent (Alfa Aesar, 121.8 mg, 0.3 mmol) in 5 mL anhydrous toluene and heated to reflux for four hours. The reaction mixture was cooled to room temperature and concentrated by rotary evaporator. The residue was dissolved in a minimum of 1:1 hexanes:ethyl acetate and purified by silica gel flash chromatography, eluted with 1:1 hexanes:ethyl acetate, to yield 28 as a yellow solid (60.4 mg, 70%). \( \text{H NMR (300 MHz, CDCl}_3) \delta = 8.25 \text{ (dd, J}_{H-F} = 10.9, 8.5, 1H), 6.63 \text{ (bs, 2H), 4.03 \text{ (d, J = 2.3 Hz, 3H), 3.50 \text{ (m, 1H), 1.22 \text{ (m, 2H), 0.72 \text{ (m, 2H).}} \}
\text{C NMR (75.5 MHz, CDCl}_3) \delta = 177.95, 150.09, 146.31, 138.60, 134.09, 127.79, 118.62, 113.29, 62.81, 32.64, 10.77. \text{F NMR (282 MHz, CDCl}_3) \delta = -137.63, -143.12. \)
6.6.8 Preparation of 7-(3-aminomethyl-pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-8-methoxy-4-thioxo-3,4-dihydro-1H-quinazolin-2-one (KRM-IV-251)

1-Cyclopropyl-6,7-difluoro-8-methoxy-4-thioxo-3,4-dihydro-1H-quinazolin-2-one (27) (26.3 mg, 0.09 mmol) was combined with (R)-3-N-boc-aminomethyl prrolidine (Astatech, 28.3 mg, 0.14 mmol) and TEA (Fisher, 15.7 mg, 0.15 mmol) in 2 mL acetonitrile and heated to 100° C. After 1 hour, acetonitrile, TEA, and excess cyclic amine were distilled off by rotary evaporator and the bright orange residue (36) was taken up in 3 mL anhydrous DCM and treated with 300 µL TFA. Deprotection progress was followed by analytical HPLC, Program A. The reaction was concentrated by rotary evaporator and the residue was dissolved in 5 mL acetonitrile (0.1% TFA) and purified by semi-preparative HPLC. Fractions containing product were combined and concentrated by rotary evaporator to remove acetonitrile and TFA, followed by lyophilization to yield KRM-IV-251 as the trifluoroacetate salt (6.3 mg, 19%). \(^1\text{H}\) NMR (300 MHz, DMSO-\(d_6\)) \(\delta = 12.54\) (s, 1H), 7.92 (bs, 3H), 7.76 (d, J_{HF} = 15.5 Hz, 1H), 3.66 (m, 3H), 3.46 (s, 3H), 3.20 (m, 1H), 2.96 (m, 2H), 2.47 (m, 1H) 2.10 (m, 1H), 1.71 (m, 1H), 0.96 (m, 2H), 0.59 (m, 2H). \(^{19}\text{F}\) NMR (282 MHz, DMSO-\(d_6\)) \(\delta = -127.12\). LRMS (ESI) calcd for (M+H\(^+\)) 365.14, found 365.18.

KRM-III-211 was prepared similarly from 28 and isolated as the di-trifluoroacetate salt. \(^1\text{H}\) NMR (300 MHz, DMSO-\(d_6\)) \(\delta = 7.95\) (bs, 2H), 7.80 (d, J_{HF} = 12.4 Hz, 1H), 6.61 (bs, 2H), 3.65 (bs, 3H), 3.49 (s, 3H), 3.39 (m, 1H), 3.20 (bs, 1H), 2.97 (bs, 2H), 2.12 (bs, 1H), 1.73 (bs, 1H), 0.99 (m, 2H), 0.58 (m, 2H). \(^{19}\text{F}\) NMR (282 MHz, DMSO-\(d_6\)) \(\delta = -126.38\). LRMS (ESI) calcd for (M+H\(^+\)) 380.15, found 380.20.
6.6.9 Preparation of 1-cyclopropyl-7-(4-ethyl-piperazin-1-yl)-6-fluoro-8-methoxy-4-thioxo-3,4-dihydro-1H-quinazolin-2-one (KRM-IV-249)

1-Cyclopropyl-6,7-difluoro-8-methoxy-4-thioxo-3,4-dihydro-1H-quinazolin-2-one (27) (20.5 mg, 0.07 mmol) was combined with N-ethyl piperazine (TCI, 13.5 mg, 0.12 mmol) and TEA (Fisher, 7.8 mg, 0.08 mmol) in 2 mL acetonitrile and heated to 100° C for 19 hours. Reaction progress was followed by analytical HPLC, Program A. Upon completion, the crude mixture containing KRM-IV-249 was cooled to room temperature and purified directly by semi-preparative HPLC as the trifluoroacetate salt. Fractions containing product were combined and concentrated by rotary evaporator to remove acetonitrile and TFA, followed by lyophilization (12.5 mg, 47%). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta = 12.85 \text{ (s, 1H), } 9.94 \text{ (bs, 1H), } 7.86 \text{ (d, } J_{H,F} = 13.0 \text{ Hz, 1H), } 3.67 \text{ (s, 3H), } 3.53 \text{ (m, 6H), } 3.23 \text{ (m, 3H), } 3.12 \text{ (q, } J = 8.0 \text{ Hz, 2H), } 1.28 \text{ (t, } J = 8.0 \text{ Hz, 3H), } 0.96 \text{ (m, 2H), } 0.60 \text{ (m, 2H).} \(^1\)F NMR (282 MHz, DMSO-\(d_6\)) \(\delta = -126.20.\) LRMS (ESI) calcd for (M+H\(^+\)) 379.16, found 379.23.

KRM-IV-053 was prepared similarly from 28, substituting DMSO as solvent for acetonitrile. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta = 8.04 \text{ (d, } J_{H,F} = 12.4 \text{, 1H), } 6.61 \text{ (s, 2H), } 3.66 \text{ (s, 3H), } 3.64 \text{ (m, 1H), } 3.46 \text{ (m, 4H), } 2.63 \text{ (bs, 2H), } 2.54 \text{ (q, } J = 7.2 \text{ Hz, 2H), } 1.45 \text{ (m, 4H), } 1.18 \text{ (t, } J = 7.2 \text{ Hz, 3H), } 1.16 \text{ (m, 2H).} \(^1\)F NMR (282 MHz, CDCl\(_3\)) \(\delta = -123.81.\) LRMS (ESI) calcd for (M+H\(^+\)) 394.16, found 394.24.

6.6.10 Preparation of 1-Cyclopropyl-3-(2,4,5-trifluoro-3-methoxy-benzoyl)-thiourea (30)

2,4,5-Trifluoro-3-methoxy benzoyl chloride (11) (223 mg, 0.99 mmol) was stirred at 0° C with ammonium isothiocyanate (83.7 mg, 1.10 mmol) in acetone for fifteen minutes. Upon TLC indicating consumption of starting acid chloride, cyclopropyl amine (156 mg, 2.73 mmol) was added dropwise and the solution warmed to 60° C for one
hour. The reaction was quenched by addition of water. The solution was partially concentrated by rotary evaporator, followed by extraction of the aqueous solution by ethyl acetate. Ethyl acetate was concentrated by rotary evaporator and the residue was dissolved in a minimum of 8:1 ethyl acetate:hexanes and purified by silica flash chromatography, eluted with 8:1 ethyl acetate:hexanes. Fractions 6-10 contained desired product \(30\) (60.2 mg, 20%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta = 10.37\) (bs, 1H), 9.47 (bd, \(J = 11.6\) Hz, 1H), 7.41 (m, 1H), 4.03 (s, 3H), 3.11 (m, 1H), 0.90 (m, 2H), 0.69 (m, 2H). \(^1^3\)C NMR (75.5 MHz, CDCl\(_3\)) \(\delta = 180.60, 161.07, 150.23\) (d, \(J_{C\text{-F}} = 249.5\) Hz), 147.75 (d, \(J_{C\text{-F}} = 250.0\) Hz), 147.62 (d, \(J_{C\text{-F}} = 250.6\) Hz), 138.38, 115.48, 111.35, 62.39, 28.15, 7.20. \(^1^9\)F NMR (282 MHz, CDCl\(_3\)) \(\delta = -131.02, -137.39, -141.27\).

Fractions 15-22 contained N-Cyclopropyl-2,4,5-trifluoro-3-methoxy-benzamide (31). \(30\) quickly underwent rearrangement to \(31\) following purification. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta = 7.57\) (m, 1H), 6.77 (bd, \(J = 10.6\) Hz, 1H), 4.01 (s, 3H), 2.90 (m, 1H), 0.86 (m, 2H), 0.61 (m, 2H). \(^1^3\)C NMR (75.5 MHz, CDCl\(_3\)) \(\delta = 162.68, 150.22\) (d, \(J_{C\text{-F}} = 246.2\) Hz), 147.50 (d, \(J_{C\text{-F}} = 249.5\) Hz), 146.50 (d, \(J_{C\text{-F}} = 249.5\) Hz), 137.79, 117.13, 111.65, 62.24, 23.29, 6.83.

### 6.6.11 Preparation of 1-cyclopropyl-6,7-difluoro-8-methoxy-4-methylsulfonyl-1H-quinazolin-2-one (32)

1-Cyclopropyl-6,7-difluoro-8-methoxy-4-thioxo-3,4-dihydro-1H-quinazolin-2-one (27) (103.9 mg, 0.37 mmol) was refluxed with oven dried potassium carbonate (Fisher, 67.1 mg, 0.49 mmol) and iodomethane (Aldrich, 672 mg, 4.73 mmol) in 5 mL acetone for one hour. The reaction was concentrated by rotary evaporator and the resulting yellow residue was dissolved in a 1:1 mixture of hexanes:ethyl acetate. \(32\) was obtained as a white crystalline solid following purification by silica gel flash chromatography, eluted with 1:1 hexanes:ethyl acetate (94.4 mg, 87%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta = 7.45\) (dd, \(J_{H\text{-F}} = 9.5, 7.8\) Hz, 1H), 4.00 (d, \(J = 1.9\) Hz, 3H), 3.42 (m,
6.6.12 Preparation of 1-cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione 4-oxide (33)

1-Cyclopropyl-6,7-difluoro-8-methoxy-4-methylsulfanyl-1H-quinazolin-2-one (32) (91.6 mg, 0.31 mmol) was combined with hydroxylamine hydrochloride (Baker, 39.7 mg, 0.62 mmol) and TEA (Fisher, 64.8 mg, 0.64 mmol) in 5 mL absolute ethanol and warmed to 40° C for 80 minutes. The reaction was concentrated by rotary evaporator and the residue was taken up in a suspension of ethyl acetate and saturated aqueous sodium bicarbonate solution. The ethyl acetate layer was recovered, washed with brine, dried over sodium sulfate, and concentrated to yield 33 as a white solid, which was further dried under reduced pressure (87.8 mg, 100%). ^1H NMR (300 MHz, DMSO-d_6) δ = 10.12 (bs, 1H), 7.31 (dd, J_H-F = 10.4, 8.4 Hz, 1H), 3.89 (d, J = 1.7, 3H), 3.09 (m, 1H), 0.91 (q, J = 6.8 Hz, 2H), 0.48 (m, 2H). ^19F NMR (282 MHz, DMSO-d_6) δ = -142.93, -151.25. The compound was unstable to ESI MS and DMSO.

6.6.13 Preparation of 1-cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione 4-(O-methyl-oxime) (34)

1-Cyclopropyl-6,7-difluoro-8-methoxy-4-methylsulfanyl-1H-quinazolin-2-one (32) (95.6 mg, 0.32 mmol) was combined with methoxylamine hydrochloride (Alfa Aesar, 89.3 mg, 1.07 mmol) and TEA (Fisher, 117.8 mg, 1.16 mmol) in 5 mL absolute ethanol and warmed to 40° C for 48 hours. The reaction was concentrated by rotary evaporator and the residue was taken up in a mixture of ethyl acetate and water. The ethyl acetate layer was recovered, washed with brine, dried over sodium sulfate, and concentrated to yield 34 as a light yellow crystalline solid (96.9 mg, 100%). ^1H NMR (300 MHz, CDCl_3) δ = 8.16 (bs, 1H), 7.39 (dd, J_H-F = 10.0, 8.0 Hz, 1H), 3.95 (d, J = 1.6
Hz, 3H), 3.89 (s, 3H), 3.23 (m, 1H), 1.06 (m, 2H), 0.60 (m, 2H). $^{19}$F NMR (282 MHz, CDCl$_3$) $\delta = -140.52, -148.34$. LRMS (ESI) calcd for (M+H$^+$) 298.10, found 298.08.

6.6.14 Preparation of 1-cyclopropyl-7-(4-ethyl-piperazin-1-yl)-6-fluoro-8-methoxy-4-methylsulfanyl-$^1$H-quinazolin-2-one (35)

1-Cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-8-methoxy-4-thioxo-3,4-dihydro-$^1$H-quinazolin-2-one (KRM-IV-249) (79.5 mg, 0.21 mmol) was stirred with oven dried potassium carbonate (Fisher, 30.4 mg, 0.22 mmol) and iodomethane (Aldrich, 228 mg, 1.6 mmol) in acetone heated to 70$^\circ$ C for 5 hours. Reaction progress was monitored by analytical HPLC, Program A. Upon completion, the reaction was concentrated by rotary evaporator and taken to the next step without purification or characterization.

6.6.15 Preparation of 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-8-methoxy-$^1$H-quinazoline-2,4-dione 4-oxime (KRM-IV-287)

1-Cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-8-methoxy-4-methylsulfanyl-$^1$H-quinazolin-2-one (35) was dissolved in 4 mL ethanol and divided in two 2 mL portions. To one portion was added hydroxylamine hydrochloride (Baker, 25.5 mg, 0.37 mmol) and TEA (Fisher, 36 mg, 0.35 mmol). The solution was stirred while warming to 40$^\circ$ C for 8 hours and followed by analytical HPLC, Program A. Upon completion, the solution was concentrated by rotary evaporator and the residue dissolved in 1:1 water:acetonitrile (0.1% TFA). KRM-IV-287 was purified as the di-trifluoroacetate salt by semi-preparative HPLC. Fractions containing product were combined and concentrated by rotary evaporator to remove acetonitrile and TFA, followed by lyophilization (11.9 mg, 30%). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta = 9.64$ (s, 1H), 7.19 (d, $J_{HF} = 12.3$, 1H), 3.73 (d, $J = 7.0$ Hz, 3H), 3.50 (m, 8H), 3.23 (m, 1H), 3.10 (m, 3H), 1.27
(m, 3H), 0.89 (q, J = 7 Hz, 2H), 0.46 (m, 2H). $^{19}$F NMR (282 MHz, DMSO-d$_6$) δ = -126.57. LRMS (ESI) calcd for (M+H$^+$) 378.19, found 378.24.

6.6.16 Preparation of [1-(1-cyclopropyl-6-fluoro-8-methoxy-4-methylsulfanyl-2-oxo-1,2-dihydro-quinazolin-7-yl)-pyrrolidin-3-ylmethyl]-carbamic acid tert-butyl ester

(37)

Protected KRM-IV-251 precursor [1-(1-cyclopropyl-6-fluoro-8-methoxy-2-oxo-4-thioxo-1,2,3,4-tetrahydroquinazolin-7-yl)-pyrrolidin-3-ylmethyl]-carbamic acid tert-butyl ester (36) (120.8 mg, 0.26 mmol) was stirred with oven dried potassium carbonate (Fisher, 38.7 mg, 0.28 mmol) and iodomethane (Aldrich, 456 mg, 3.2 mmol) in acetone heated to 62°C for 10 hours. Reaction progress was monitored by analytical HPLC, Program A. Upon completion, the reaction was concentrated by rotary evaporator and taken to the next step without purification or characterization.

6.6.17 Preparation of 7-(3-aminomethyl-pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione 4-oxime (KRM-IV-295)

[1-(1-Cyclopropyl-6-fluoro-8-methoxy-4-methylsulfanyl-2-oxo-1,2-dihydro-quinazolin-7-yl)-pyrrolidin-3-ylmethyl]-carbamic acid tert-butyl ester (37) was dissolved in 4 mL absolute ethanol and divided into two 2 mL portions. To one portion was added hydroxylamine hydrochloride (Baker, 18.8 mg, 0.27 mmol) and TEA (Fisher, 36 mg, 0.35 mmol). The solution was stirred while warming to 40°C for 48 hours and followed by analytical HPLC, Program A. Upon completion, ethanol was distilled off by rotary evaporator and the residue was dissolved in 2 mL DCM and 200 µL TFA. Deprotection was monitored by analytical HPLC, Program A. Upon completion, the DCM was distilled off by rotary evaporator and the residue was taken up in 1:1 water:acetonitrile (0.1% TFA). KRM-IV-295 was purified as the tri-trifluoroacetate salt by semi-
preparative HPLC. Fractions containing product were combined and concentrated by rotary evaporator to remove acetonitrile and TFA, followed by lyophilization (11.8 mg, 25%). \( ^1H \) NMR (300 MHz, DMSO-\( d_6 \)) \( \delta = 9.47 \) (s, 1H), 7.91 (bs, 3H), 7.09 (d, \( J_{H-F} = 12.4 \) Hz, 1H), 3.53 (m, 3H), 3.51 (s, 3H), 3.31 (m, 1H), 3.10 (m, 1H), 2.94 (m, 2H), 2.47 (m, 1H), 2.08 (m, 1H), 1.69 (m, 1H), 0.88 (m, 2H), 0.65 (m, 2H). \( ^19F \) NMR (282 MHz, DMSO-\( d_6 \)) \( \delta = -127.75 \). LRMS (ESI) calcd for (M+H\(^+\)) 364.18, found 364.25.
CHAPTER 7 CONCLUSIONS AND FUTURE DIRECTIONS

Being one of the newest classes of antibiotics, the mechanism and potential of fluoroquinolones are only just beginning to be fully understood. With the recent discovery of the phenomenon of rapid lethality, many fluoroquinolones, having been considered of limited clinical usefulness because of high MIC, must be reevaluated. It is also important to consider substitution of the fluoroquinolone core at non-traditional positions, such as C-2, or to evaluate the effect of substitutions to the quinazoline-2,4-dione core at the C-2 and C-4 positions.

Rapid killing of bacterial pathogens by fluoroquinolones is important in patient treatment and in restricting the emergence of resistance [80, 117, 118]. Killing non-growing bacteria is particularly important with pathogens that enter dormant states, such as tuberculosis and persister cells. The knowledge that quinolones kill by one of two mechanisms, either requiring ongoing protein synthesis or not, and that the particular mechanism followed is a direct result of the structure provides an opportunity for further study of the two pathways. The pathway not requiring protein synthesis is restricted to only a small subset of fluoroquinolones. This pathway is expected to result from destabilization of the quinolone-topoisomerase-DNA complex, leading to chromosome fragmentation and cell death. In the work presented here, E. coli was treated with chloramphenicol to induce protein synthesis arrest, simulating the dormant state of some infectious agents. The effect of two different sets of fluoroquinolones, either N-1 cyclopropyl, C-8 methoxy or fused ring derivatives, otherwise differing by the C-7 substitution, was then evaluated for the ability to destabilize the topoisomerase-DNA complex, leading to chromosome fragmentation and cell death. The ability of quinolones to kill E. coli in the absence of protein synthesis was impaired by fusion between N-1 and C-8. Levofloxacin, a clinically useful fluoroquinolone antibiotic contains the N-1 to C-8 fusion, and additional experiments should be completed to determine if levofloxacin is
suboptimal against pathogens that enter a dormant state. Additional experiments will also reveal whether killing by PD161144, the compound found to be least affected by chloramphenicol, is also unaffected by other protein synthesis inhibitors, thereby exploring the possibility of co-administration of protein synthesis inhibitors with quinolone-based gyrase inhibitors.

Energy minimization and molecular modeling suggest that the N-1 cyclopropyl and C-8 methoxy substituents arrange in a trans orientation, forming a platform which restricts free rotation of the C-7 substituent. The resulting constraint may lead to a restriction in the free movement of the quinolone while bound to the topoisomerase-DNA complex, causing destabilization of the ternary complex and ultimately resulting in chromosome fragmentation and cell death. The comparison PD161144 and the other fluoroquinolones used in this study provides a striking example of this phenomenon in which the specific C-7 substituent combines with N-1 cyclopropyl and C-8 methoxy to lead to rapid lethality that is unaffected by chloramphenicol (Figure 11). Changing the C-7 group, even as small a change as the size or position of the alkyl group attached to the piperazinyl ring, resulted in reversal of lethal activity when protein synthesis was inhibited by chloramphenicol. Enzyme structure is also likely to be important, since moxifloxacin, rather than PD161144, is currently the compound that is least sensitive to chloramphenicol with mycobacteria [57, 81].

As suggested by modeling studies, the greater rotational freedom of the bond between the fluoroquinolone core and C-7 substituent for the fused ring derivatives than for C-8 methoxy compounds, especially in simulations in which all other points of the molecule are held rigid to replicate a protein-bound situation, the rotational and conformational freedom of the C-7 substituent is important in explaining why different C-7 groups affect the lethal ability of the N-1 cyclopropyl, C-8 methoxy fluoroquinolones in the presence of chloramphenicol though the C-7 substituent had only minimal impact on synthesis independent killing by fused ring derivatives. It is proposed here that the
unique ability of N-1 cyclopropyl, C-8 methoxy fluoroquinolones with specific C-7 substituents to kill *E. coli* in the absence of protein synthesis is a direct result of the effect of the *trans* orientation of the N-1 and C-8 substituents on binding to gyrase and the impeded or altered rotation of the C-7 substituent, particularly when the quinolone is bound to the topoisomerase-DNA complex.

In order to further understand the mechanism of rapid lethality, further studies are necessary. Variation of the N-1 and C-8 substituents, as well as deeper study into the C-7 substituents may lead to a better understanding of the structural requirements for killing non-replicating cells. An interesting structural feature to investigate, though it would eliminate the free C-8 methoxy, would be a fusion between C-7 and C-8, more effectively locking the rotation of the C-7 substituent than the limited rotation of C-7 caused by the C-8 methoxy. Further study must also be completed in the area of X-ray analysis of the binding state. X-ray crystallography of the quinolone-topoisomerase-DNA ternary complex has only just begun to be developed and, as such, there is only very limited information. With further elucidation of the environment immediately surrounding the fluoroquinolone and the interactions occurring during binding, especially in the case of compounds shown to cause chromosome fragmentation in the absence of protein synthesis, it will be possible to better design fluoroquinolones with this goal in mind.

In the work presented in Chapter 4, substitution of the C-2 position of the fluoroquinolone core was investigated for ability to increase the growth inhibition of fluoroquinolone class antibiotics against bacterial cells. This investigation was based on the knowledge that some of the most potent fluoroquinolone class agents to date are ulifloxacin and the isothiazoloquinolones, each bearing a C-2 thioether moiety as part of a cyclic system.

Molecular modeling, based on low resolution crystal structures, suggests that the C-2 sulfur plays a part in the interaction between quinolone and protein, possibly making a binding contact in a previously unexploited area. Also discovered during molecular
modeling studies was a hydrophobic area that could be utilized as an additional binding pocket by substituting an alkyl chain at the C-2 position. In an effort to determine the existence and impact of this potential interaction, a series of fluoroquinolone analogs were synthesized in which the C-2 position was substituted by either a thioethyl or thioisopropyl moiety. To determine the effect of the C-2 thioalkyl substitution alone, the C-3 carboxylic acid group was removed and comparison between the activities of compounds unsubstituted at both C-2 and C-3 and compounds bearing only the C-2 thioalkyl substituents were made.

Structural features of C-2 thioalkyl fluoroquinolone derivatives were identified that contribute to both increased and decreased antibacterial activity. It was determined that if thioalkyl substitution at the C-2 position was present along with C-3 carboxylic acid substitution, resulting quinolones were far less active than parent fluoroquinolones not substituted at the C-2 position. However, it was also determined that in some cases, the addition of the C-2 thioalkyl group to fluoroquinolone compounds lacking the C-3 carboxylic acid, a group known to be important for binding of fluoroquinolone to enzyme, increased inhibitory activity over compounds lacking both C-2 and C-3 substitution. Again, as determined in earlier work, the C-7 substituent plays a major role in the overall activity of the fluoroquinolones.

Molecular modeling then suggested that the additional ring structure found in ulifloxacin and the isothiazoloquinolones restrains the C-2 substituent in such a way that co-planarity of the core and C-3 carboxylic acid is not disrupted as it is in C-2 thioethyl derivatives. Interestingly, restraint of the C-2 thioether was extremely detrimental in the lack of C-3 carboxylic acid as KRM-III-163, the descarboxy ulifloxacin derivative, was the least active compound in the panel.

Further work in understanding the importance and effect of C-2 substitution needs to be addressed. No clinically important C-2 substituted quinolone-class agents currently exist, with prulifloxacin on track to be the first, following approval in several countries.
Further studies into the effect of C-2 functionalization should be focused on linkages through heteroatoms other than sulfur, such as oxygen or nitrogen, alkyl chains, aromatic substituents, and additional ring structures that both do and do not include a carbonyl analogous to the C-3 carboxylic acid or the carbonyl of the isothiazolidinone ring of isothiazoloquinolones. Also, understanding the importance of the C-7 substituent on both inhibitory activity and lethal activity, it is imperative that a panel of compounds with a wider range of C-7 substituents be studied.

In the final chapter, the effect of modification of the C-4 position of quinazoline-2,4-diones was determined. Thought not fluoroquinolones, the quinazoline-2,4-diones are a recent addition to the fluoroquinolone antibiotic class. The unique binding between quinazoline-2,4-diones and gyrase has been theorized to occur by a hydrogen bond interaction between the C-2 carbonyl and arg121 (arg129 in topoIV). It was hypothesized, as suggested by modeling studies, that alternate substitution of the C-4 position would have relatively little impact on binding between the quinazoline derivative and target enzyme, though adding a hydrogen bond donor, such as oxime at this position may result in a direct interaction with asp87 of gyrase (asp95 of topoIV). Thiocarbonyl substitution of the C-2 position, however, was expected to have a major impact on the binding interaction. Thiocarbonyl was chosen as a target because it forms a logical link between the quinazoline-2,4-dione core and the ulifloxacin core.

Synthesis of C-2 thiocarbonyl quinazoline compounds proved more problematic than expected and was not completed during this study. However, a panel of six quinazoline analogs was synthesized in which the C-4 position was modified from carbonyl to thiocarbonyl or hydroxylimine. The N-3 position was either left unsubstituted or amino substituted as this group is known to decrease the ratio of MIC against resistant mutant strains versus MIC against wild type cells.

As predicted by modeling, the changes made at the C-4 position had no impact on the binding of the novel quinazoline analogs as compared to parent quinazoline-2,4-
dione. Again, the only difference in activity observed was as a result of differing C-7 substitution, with the C-7 N-ethylpiperazinyl derivatives approximately half as active as 3-(R)-aminomethylpyrrolidinyl derivatives.

Experiments involving changes to the C-2 substituents must be the focus for further investigation of the quinazoline antibiotic class. New synthetic developments have opened the possibility of obtaining alternate C-2 substituents by proceeding through a novel 2-(aminoalkyl)benzamide intermediate (Figure 26). The unique opportunity presented here is that the pyrimidine portion of the quinazoline core can be completed by a number of carbonyl analogs by reaction with reagents such as thiophosgene or thionyldiimidazole.

![Chemical structure](image)

Figure 26. Synthetic approaches towards 2-(aminocyclopropyl)benzamide intermediates have been developed by Kerns group member Luis Hernandez that may be useful in developing novel quinazoline compounds uniquely substituted at the C-2 position. Possibilities exist such that X represents carbon, phosphorous or sulfur, and Y represents sulfur, oxygen, or nitrogen. 2-sulfuryl has also been proposed.

Additionally, having unequivocally determined that modification to the C-4 position did not affect the inhibitory activity of the quinazoline class compounds, this position is now open to modification. Size and electronic difference between carbonyl, thiocarbonyl, and hydroxylimino, as illustrated in Figure 25, are broad enough to assert that this position could be utilized for modifications that increase properties such as solubility, membrane crossing, absorption, and metabolism as well as functionalization that will inhibit efflux pumps or reduce recognition by efflux pumps. Future substitution at this position should focus on determining the limits to both steric bulk and electronic
differences of substituents that can be put at C-4 without negatively affecting inhibition of bacterial growth. Coupling C-4 modifications to increase desired PK/PD properties with alternate C-2 substituents that yield greater inhibitory activity may lead to active agents with fewer side effects.
APPENDIX

A.1 Selected Spectral Data

Figure A 1. $^1$H NMR of KRM-I-017

Figure A 2. $^1$H NMR of KRM-I-021
Figure A 3. $^1$H NMR for **KRM-I-023**.

Figure A 4. $^1$H NMR for **KRM-I-025**.
Figure A 5. $^1$H NMR for **KRM-I-029**.

Figure A 6. $^1$H NMR for **KRM-I-033**.
Figure A 7. $^1$H NMR for KRM-II-273.

Figure A 8. $^1$H NMR for KRM-III-043.
Figure A 9. $^1$H NMR for 14.

Figure A 10. $^1$H NMR for 15.
Figure A 11. $^1$H NMR for 16a.

Figure A 12. $^1$H NMR for 16b.
Figure A 13. $^1$H NMR for 17a.

Figure A 14. $^1$H NMR for 18a.
Figure A 15. $^1$H NMR for **KRM-II-183**.

Figure A 16. $^1$H NMR for **KRM-II-197**.
Figure A 17. $^1$H NMR for KRM-II-185.

Figure A 18. $^1$H NMR for KRM-II-199.
Figure A 19. $^1$H NMR for **KRM-III-145**.

Figure A 20. $^1$H NMR for **KRM-III-157**.
Figure A 21. $^1$H NMR for **KRM-III-163**.

Figure A 22. $^1$H NMR for **27**.
Figure A 23. HMBC NMR spectra for 25 (top) and 27 (bottom). The aromatic proton at C-5 correlates with the C-4 carbon, but not C-2. The shift, indicated by green boxes, of both the aromatic proton and one correlated carbon (C-4) confirms C-4 thiocarbonyl substitution over C-2.
Figure A 24. $^1$H NMR for 28.

Figure A 25. $^1$H NMR for KRM-III-211.
Figure A 26. $^1$H NMR for **KRM-IV-053**.

Figure A 27. $^1$H NMR for **KRM-IV-251**.
Figure A 28. $^1$H NMR for **KRM-IV-249**.

Figure A 29. $^1$H NMR for **30**.
Figure A 30. $^1$H NMR for 31.

Figure A 31. $^1$H NMR for 32.
Figure A 32. $^1$H NMR for 33.

Figure A 33. $^1$H NMR for 34.
Figure A 34. $^1$H NMR for **KRM-IV-287**.

Figure A 35. $^1$H NMR for **KRM-IV-295**.
Figure A 36. Analytical HPLC (Program B) was used to follow the addition of the C-7 side chain forming 18a (10.7 min) from 17a (13.3 min).
Figure A 37. Analytical HPLC (Program B) was used to follow the hydrolysis and decarboxylation of 18a (10.7 min) to KRM-II-183 (9.9 min).
Figure A 38. Analytical HPLC (Program B) was used to follow the ester hydrolysis forming KRM-II-241. From top to bottom, the starting material (18a) elutes at 10.8 min and converts to KRM-II-241, eluting at 10.6 min.
Figure A 39. Analytical HPLC (Program B) indicates that 18b (10.0 min) proceeds cleanly to KRM-II-195 (8.9 min). The peak at 9.6 min represents a small amount of KRM-III-045 that forms during the reaction.
Figure A 40. Analytical HPLC of the ester hydrolysis forming **KRM-III-045** indicates that the hydrolysis does not proceed cleanly. Starting material (18b) elutes at 10.0 min and is converted to C-3 carboxyl product, eluting at 9.6 min. The product was stable to semi-preparative HPLC.
A.2 Structures of antibiotics from Table 1

Figure A 41. Structure of vancomycin.

Figure A 42. Structure of daptomycin.
REFERENCES


