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Discovery of New Antimicrobial Agents using Combinatorial Chemistry

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DISCOVERY OF NEW ANTIMICROBIAL AGENTS

USING COMBINATORIAL CHEMISTRY

A thesis submitted in partial fulfillment
Of the requirements for the degree of
Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPAIRED UNDER MY SUPERVISION BY William Isaac Northern ENTITLED Discovery of New Antimicrobial Agents Using Combinatorial Chemistry BE ACCEPTED IN PARTIAL FULFULLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

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Combinatorial chemistry has become an important aspect of medicinal research due to its flexibility and the ability to produce large numbers of potential therapeutic agents. Once compounds are made, they must be screened to determine if there is any biological activity. This research project focused on developing a screening method for chemical agents produced by a graduate student in the chemistry department at Wright State University. After an acceptable screening method was found, the goal of the project was to determine if compounds produced had either antibacterial activity, antifungal activity, or both. Seven compounds exhibited biological activity. Two of these compounds had activity against all organisms tested. Five compounds had activity against only *Staphylococcus aureus*. Also, initial toxicity studies were performed on the two compounds that had activity against both bacteria and fungi. The toxicity was detected by cytopathic effect (CPE) noted in human and monkey cell lines. One compound demonstrated severe toxicity while the other compound demonstrated slight toxicity. Additional research, including animal safety studies, will be required to determine if these compounds are viable prospects for development into antimicrobial agents. This research confirmed that it is possible to use combinatorial methods to produce agents. However, the ability to produce antimicrobial compounds is only a small part of producing a useful drug.

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INTRODUCTION

Importance of Finding New Antimicrobials

The use of antimicrobial agents is critical to successful treatment of infectious diseases. Although there are numerous classes of drugs that are routinely used to treat infections in humans, there are several reasons why the discovery and development of new antimicrobial agents are important. Over the past decade there has been an increased development of resistance in organisms that are typical pathogens in humans. These include methicillin/oxacillin-resistant *Staphylococcus aureus* (1,2), vancomycin-resistant and intermediate *Staphylococcus aureus* (3,4), vancomycin-resistant *Enterococcus* (2,5), gram-neagtive bacilli that produce extended spectrum beta-lactamases (6,7), carbapenem-resistant *Klebsiella pneumoniae* (8), and *Pseudomonas* and *Acinetobacter* (9) strains that are resistant to all antibiotics that are typically used for treatment. Figures 1 and 2 show a graphical representation of the rise in resistance for two common pathogenic organisms that are isolated in clinical laboratories (10). This increased resistance has limited the selection of antimicrobials that may be used to treat specific organisms.

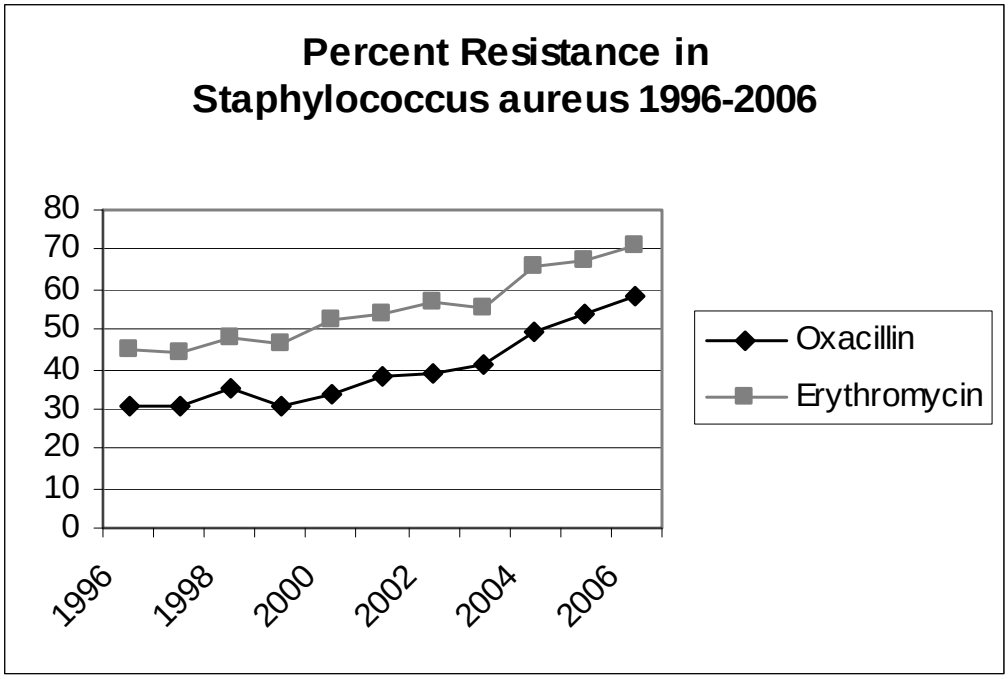


Figure 1: Percent resistance in selected antibiotics in *Staphylococcus aureus* at CompuNet Clinical Lab, Moraine, Ohio, 1996 to 2006 (10).

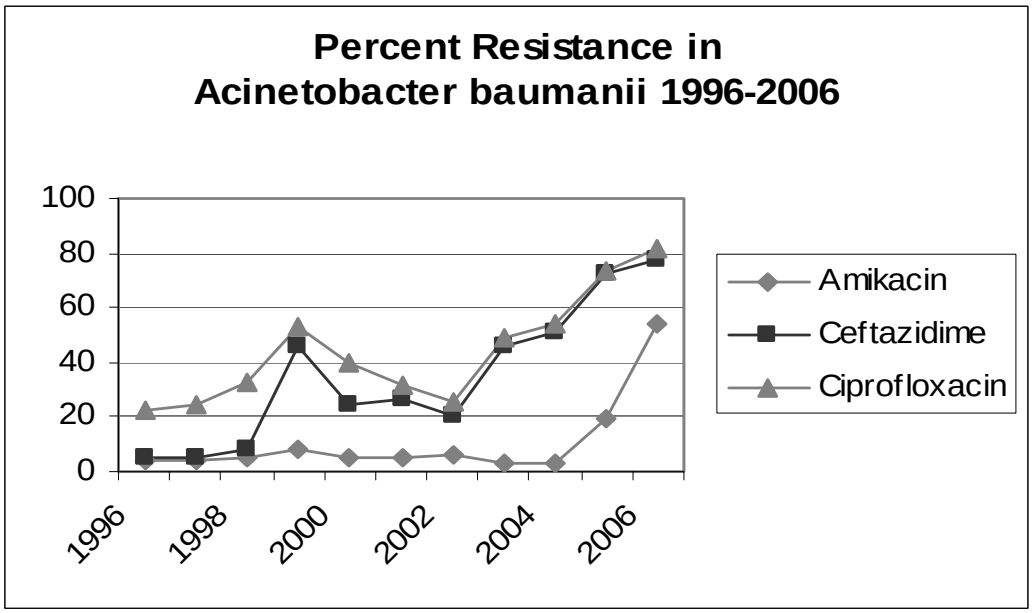


Figure 2: Percent resistance in selected antibiotics in *Acinetobacter baumanii* at CompuNet Clinical Lab, Moraine, Ohio, 1996 to 2006 (10).

New antimicrobials are also needed for certain groups of organisms. Very limited numbers of antimicrobials are available to treat infections caused by fungi and

mycobacteria. Infections with these organisms continue to be a major concern. Chemotherapy for cancer treatment, immunosuppressive drugs for treatment of autoimmune diseases and organ transplant recipients, and infections (such as AIDS) that alter the effectiveness of the host immune system render individuals at high risk for fungal infections and certain mycobacterial infections. Often these infections are caused by environmental organisms that would not typically cause disease in a normal host. There are increased numbers of reports of multi-drug resistant mycobacteria in the United States and throughout the world (11). Another complicating factor that reduces the effectiveness of treatment of these organisms is non-compliance in completing treatment regimens. Extended length of treatment (sometimes up to one year) and adverse side effects of the drugs used for treatment contribute to the lack of compliance.

Although many infectious diseases have been known for thousands of years, over the past 30 years a number of new infectious diseases have been discovered. Some examples include Lyme Disease caused by *Borrelia burgdorferi*, Legionnaires' Disease caused by *Legionella pneumophila*, peptic ulcers caused by *Helicobacter pylori*, antibiotic associated diarrhea caused by *Clostridium difficile*, and AIDS caused by Human Immunodifficiency Virus. In addition, microorganisms are constantly changing, finding new places to live and new ways to survive, and adapting to new situations. During this process, harmless organisms may turn deadly or deadly strains may move from their normal host to humans. With the continuing discovery of new infectious diseases and the development of new disease processes of existing pathogens (i.e., necrotizing fasciitis caused by *Streptococcus pyogenes*), it is important to continue to find anti-infective agents that can be used to treat these infections (12,13).

Development of novel classes of drugs, drugs with fewer side effects, and drugs with shorter lengths of treatment are key in continuing the fight against infectious disease.

Historical Perspective

In the early 1900's, a number of investigators were performing research on microorganisms. Paul Ehrlich was interested in distinguishing bacteria from tissue in histopathological slides. He noted that some dyes were taken up by human or animal cells but not by others. He also noted that there were dyes that were taken up by bacteria but not human cells and that there was a variation of up-take amongst bacteria. A Danish microbiologist, Hans Christian Gram, had already demonstrated that bacteria could be divided into two groups based upon their up-take of various dyes. (His research is the basis for the gram stain that is used in microbiology laboratories today.) Paul Ehrlich explained that the selective binding of the dyes was due to differences in the cell surfaces and felt that this property might be exploited. He postulated that if a dye could bind to a bacterial cell but not human cells and could kill the bacterial cell, an infection could be eradicated. He tested thousands of dyes to find one that met these criteria and in 1909 synthesized arsphenamine, a drug that could destroy the syphilis-causing organism *Treponema pallidum*. Unfortunately this drug compound had severe side effects and resulted in the death of numerous patients (14,15).

In 1915, Gerhard Domagk was an infantryman in the German military. He was wounded and sent to a hospital in Berlin. During his recuperation he trained as a medical assistant and was horrified when he recognized that many deaths in the military hospital did not come from direct war injuries but rather from the festering of wounds. This

experience convinced him that he should pursue a career in medicine. In 1932, he was working in an experimental pathological laboratory for a German chemical company I.G. Frabenindustrie that manufactured dyes for the textile industry. He was given the freedom to research whatever he thought was important and he chose to look for a compound that would selectively kill streptococci. He tested hundred of substances before finding a red dye called prontosil. He discovered that this compound protected mice from infections with streptococci. In 1936 it was discovered that a smaller, more effective compound known as sulfanilamide (a sulphonamide) was the active agent in prontosil. A demonstration for a group of German army surgeons proved to them that sulphonamides were effective in treating gangrenous wounds. Sulphonamide was soon packaged into small containers and became a standard issue for immediate disinfection of battle wounds during World War II (14).

In 1928, Alexander Fleming was working at St. Mary's Hospital in London researching agents that could be used to fight bacterial infections. Due to a serendipitous event, he discovered that staphylococcus would not grow around a mold that was growing on his culture plate. The mold was identified as *Penicillium notatum*. Fleming named the substance that was inhibiting the staphylococcus "penicillin". However, he was unable to purify it, nor did he realize the potential for use in treating infections. About ten years later two investigators, Ernest Chain and Howard Florey, tried to stabilize and purify the "penicillin". They were unable to produce enough of the purified substance to test its efficacy as an antimicrobial agent. Chain decided to inject an extract of the mold into healthy mice that were experimentally infected with streptococci. He noted that the infection was halted and the mice remained healthy after the experimental

treatment with the mold extract. In 1940, enough purified penicillin was produced to test it on mice. In these experiments, two groups of mice were infected with streptococci. One group was injected with penicillin while the other group was not treated. All of the mice that received penicillin survived while all the mice that were not treated died. Later studies showed that penicillin could safely be used to treat humans and was used to treat soldiers during World War II (12,14).

The discovery of penicillin led to the recognition that microbes produce substances that inhibit each other, setting off decades of intensive screening of bacterial cultures for new antibiotic classes. This resulted in the discovery and successful development of penicillins, cephalosporins, tetracyclines, aminoglycosides, rifamycins, glycopeptides and macrolide antibiotics (16).

Sources of New Antibiotics

Over the past 20 years, two general approaches have been taken in antimicrobial drug discovery. The first involves modification of existing antibiotics. The second is aimed at identifying new compounds that inhibit microorganisms that are not targeted by existing agents. Today the main sources for new drugs are natural products, synthetic products, existing drugs, and genomics (17). Each of these will be discussed in detail.

Natural products

The discovery of penicillin gave rise to the concept of seeking naturally occurring drugs (17). This was true for anti-infectives as well as other types of drugs. The discovery of vineristine and vinblastine in 1963 by R.L.Noble and its successful use by Eli Lilly launched the pharmaceutical industry into the search for natural products for

treatment of various cancers. Other drugs that started as natural products include avermectin (antihelmenthic), cyclosporine and FK-506 (immunosuppression), mevinolin and compactin (cholesterol-lowering), and taxol and comptothecin (anticancer) and have revolutionized therapeutic areas in medicine (18). Between one quarter and one third of all currently available drugs were derived from natural products (19).

In the anti-infective area, treatment is dominated by natural products and natural product analogs such as penicillins, cephalosporins, and vancomycin (20). These natural products can come from a number of different sources: bacteria, fungi, plants, and marine environments.

Bacteria and Fungi:

Natural products with antibiotic activity that come from bacteria or fungi are almost always products of secondary metabolic pathways. These pathways are not required for primary, life-sustaining functions of metabolism. When antibiotic-producing organisms face competition in their environment, they turn on genes that encode the antibiotic molecules and use them to wage chemical war on their neighbors. They then have a selective advantage for growth, including the nutrients from their dying neighbors (16). Organisms which cause human infections do not survive well in soil because they are destroyed by soil-inhabiting organisms. As a result, extensive soil-screening programs have resulted in the discovery of drugs such as streptomycin, chloramphenicol, chlortetracycline, erythromycin, neomycin, bacitracin, and polymixin (17).

Many of the drugs listed above were isolated from actinomycete species. Actinomycetes are filamentous gram-positive bacilli that make up a large portion of soil microbes. When these drugs were originally discovered, broth cultures of the

actinomycetes were grown and then tested for antimicrobial activity using whole-cell screening assays. Currently, many species of actinomycetes are non-cultivable and other means must be used to further discover new or novel antimicrobials. A company in Cambridge, Massachusetts, (NovoBiotic Pharmaceuticals) is making progress in learning how to grow actinomycetes that will not grow on standard laboratory media. Their progress, along with the use of genomic and combinatorial chemistry (to be discussed later) make actinomycetes a continuing potential source of clinically relevant antibiotics (21,22).

Marine Environments:

Chemical defenses employed by many organisms have proven to be available sources of novel molecules providing lead compounds for drug discovery. Among the least-explored of the planet's chemically defended organisms are the invertebrates, algae, fungi, and bacteria of the marine environment. There are greater than 2,000,000 marine animals and microorganism species available for investigation. The marine environment contains about half of the total global species and contains a biodiversity as extensive as all the world's rain forests combined. This biological diversity has resulted in a vast array of chemical diversity. Many marine organisms have a sedentary life style and require a chemical means of defense. As a result, they have the ability to produce or obtain toxic compounds to deter predators and keep competitors at bay. Bioprospecting in the marine environment has only started relatively recently but has already yielded thousands of novel compounds. One group studying the marine environment during the past 25 years has isolated over 10,000 compounds from marine invertebrates: algae, bacteria, fungi, and protozoa. Less than 0.5% of marine animals have been examined to

determine if they produce compounds that might be used as therapeutic agents against infectious diseases. Improved underwater life-support systems have provided marine scientists new mechanisms for collecting from unexplored regions and depths (23,24).

Marine actinomycetes are a group of organisms that have demonstrated great promise as a source of potential new drugs. These organisms account for 10% of marine snow (dead or dying animals and plants such as plankton, diatoms, fecal matter, sand, soot and other inorganic dust) that settles to the ocean floor. Marine actinomycetes exhibit very different 16S rRNA sequences compared to their terrestrial counterparts. As a result, marine actinomycetes produce novel metabolites that may be biologically active and a potential source of new anti-infective drugs (25).

One of the concerns of bioprospecting in the marine environment is the complexity of marine molecules. Because of this complexity, it is often difficult or impossible to manufacture the molecules in the large-scale production that is required when selling a drug. However, studies support the idea that most bioactive compounds isolated from marine invertebrates are the result of biosynthesis of the active compounds by microorganisms. Further studies allowing isolation of the particular microorganisms that are responsible for making the active compounds may reduce or eliminate this concern (26).

Plants:

Plants produce substances that help protect them from microorganisms, herbivores, competing plants, and aide in reproduction. They also produce chemicals for offensive chemical warfare targeting cell proliferation of pathogens. These chemicals may have general or specific activity against key target sites in bacteria, fungi, viruses, or

neoplastic diseases (18). It is estimated that more than 250,000 species of higher plants exist and that only a fraction have been investigated to characterize the chemical constituents for biological effects (19).

When plants are being examined, a successful drug discovery program requires several key components. It is necessary to have a continuing collection of new and preferably unique plants at a rate sufficient to generate several good leads per year. There must be a selective and effective set of bioassays to detect which plant extracts contain bioactive constituents. An effective mechanism for screening out compounds that have already been tested or otherwise uninteresting will allow drug companies to concentrate their work on compounds that have a high potential for becoming a marketable drug. An efficient fractionation and structure determination program will facilitate the isolation and identification of bioactive products (20).

When collecting samples, the goal is to obtain maximum chemodiversity and therapeutically useful biological activity within a minimum number of collected samples. Samples may be collected randomly or folklore information on medicinal plants may be used. Since many of the samples collected are found in remote areas of the world (i.e. rain forests), it is beneficial to collaborate with the endogenous people for collection. These people possess a vast knowledge of the plants in their part of the world. In particular, shamans or "witch doctors" use these plants in tribal medicine and have an understanding of how plants and other substances may be used to treat illnesses (20).

Some of the key decisions in sampling include: what quantity to collect, and how to store and process samples (19). Plant samples may be taken from roots, bark, twigs, and leaves. They are then dried and sent to the lab for extraction. If collaboration with

endogenous people is possible, the plants may be from "gardens" of tribal shamans or healers. Information should be collected about each plant. This includes: various medicinal uses, area where found, part of plant used for medicinal purposes, habitat, soil, visibility, abundance, how medicine is prepared by shaman, local name of plant, dosage, method of application, and side effects (20).

After the samples are tested, plants with bioactivity are recollected to confirm initial results. An extract from the plant is then fractionated in an effort to isolate and characterize the active entity (20). To fractionate samples, they are dried and reconstituted in solvents (27). Ethyl acetate and methanol may be used for this extraction (20). Initial fractionation of crude extracts can be done by countercurrent chromatography. This separation method is one of the least destructive of the compound of interest (27). Fractions are isolated and put in separate tubes and tested for biological activity. Fractions may be considerably pure and isolated in reasonable quantities after a single chromatographic step (27).

Testing Compounds

The goal of testing natural products is to identify compounds with selective and specific biological effects on contemporary and relevant disease targets. It is also important to ensure in vivo efficacy while at the same time preventing toxicity (19).

The most efficient primary assays used for screening compounds have a high through-put design to detect samples with the most promise for yielding interesting compounds (i.e. screening out the majority of low or moderate activity or non-selective activity). This is often based on identifying compounds that inhibited growth of target pathogens. In addition, the use of subcelluar assays and whole-cell assays make it less

likely to miss novel mechanisms on the primary screen (19). The subcellular and whole-cell assays are used to detect compounds that affect enzymes, cell receptors, ion channels, and nucleic acid replication in microorganisms. One of the advantages of subcellular assays is that they can yield a large number of potentially active compounds. However, many of the active compounds may not demonstrate activity when tested in whole-cell assays. Whole-cell assays simulate the natural milieu in which the targets reside and are often more indicative of the way molecular targets react in their natural state (28,29).

Samples found to have biological activity are subjected to "dereplication". This is the process of checking to see if a compound (or similar compounds) has been tested before. Samples that have not been tested before are subjected to additional studies to provide an early indication of novel chemotypes responsible for the activity.

Secondary assays are designed to corroborate and quantitate activity observed in the primary assay, to establish spectrum of activity, to provide insight into the mode of action, and to predict in vivo pharmaceutical properties. Criteria are necessary for determining the relative importance of lead compounds and if there is evidence to suggest a novel mechanism of action. This may be accomplished by testing to see if the compound is active against strains resistant to other known agents, if it inhibits known targets (i.e. cell wall biosynthesis), or how it influences general biochemical pathways (i.e. protein or nucleic acid synthesis). There are a number of assays that have been described that may be used to help make these determinations (30,31,32,33). Compounds with novel activity should be followed up with more extensive studies to determine molecular site of action (19).

A number or methods may be used to elucidate the structure. These include: liquid chromatography, mass spectrometry (MS), nuclear magnetic resonance (NMR), infared spectrometry (IR), and ultra-violet (UV) absorption.

Liquid chromatography and MS are used to study crude extracts and multi-component fractions for the presence of specific bioactive compounds or compound types. These methods are used as part of the dereplication process to help identify known classes of compounds when looking for novel bioactive compounds. MS is used in conjunction with NMR spectroscopy to allow determination of the molecular formula of a compound. Since nitrogen is found in many natural products, mass spectral data can be used to determine the position of nitrogen and allow elucidation and confirmation of structures (27). This data is then compared to a data base of compounds that have already been tested (34).

NMR is the best method to use for non-crytalline compounds. It allows fragments of compounds to be combined into complete molecules and may be used to definitively identify metabolites (23). Use of molecular weight and UV absorbance data with Berdy Bioactive Natural Products Database allows rapid identification of compounds. The identification is then confirmed by NMR (27). IR yields information about functional groups.

Results of bioassays and structure elucidation may indicate that an isolated product is not an effective drug. However, it may have a novel molecule. In such cases chemical modification of the natural product structure can often yield clinically useful drugs. (To be discussed later.) Appropriate and selective bioassays should be used to

screen potential drugs. "Hit rates" may be small, requiring thousands of compounds to be screened (20).

Existing Antibiotics

In the past, natural products have been the main source of new molecules that are developed into medically useful antimicrobials. However, after the first antimicrobial agents were discovered and routinely used to successfully treat infections, it became evident that these drugs had limitations. By studying the chemical properties of each drug and its limitations, modifications were made that resulted in new drugs that had improved characteristics and decreased limitations when compared to the original drugs.

Some drugs have toxic side effects. Amphotericin B causes renal toxicity while fluoroquinolones cause phototoxicity. A lipophilic formulation reduced the nephrotoxic effects of amphotericin B. Substitutions at a key position on the quinolone molecule eliminated this toxicity in newer fluoroquinolones (13,35).

Alterations in dosing regimens may show substantial improvement in patient treatment. Reducing the frequency of doses taken each day and the number of days that the drug is required to be taken greatly increases the chance that patients will be compliant in correctly taking their medicine. For example, the half-life of the macrolide erythromycin is 2-3 hours. However, the half-life of a newer macrolide, azithromycin, is 40-68 hours. This difference allows azithromycin to be taken once per day and is an improvement when compared to multiples doses per day required for erythromycin (36,37). Pharmacological changes in the antimicrobial molecule can affect the route of administration of the drug, such as a drug that is given intravenously or intramuscularly being altered to allow it to be administered orally. The route of administration can

ensure the antimicrobial reaches the site of infection. An example of a drug whose route of administration was modified to ensure that the drug reached the site of infection is the inhaled formulation of tobramycin. This is used by cystic fibrosis patients to get drugs directly to the lungs where infections typically occur (13,38).

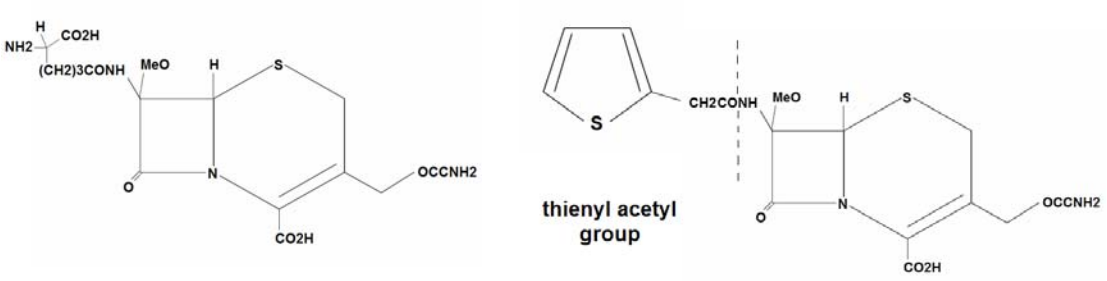
Development of resistance is a major concern with any antibiotic. Modifications of the molecule that protect the antimicrobial agent from resistance formation include combining complementary mechanisms of action in one drug, molecular hybrids or chimera, and chemical substitutions of functional chemical groups on the drug. Tigecycline is an example of a drug that has complementary mechanisms of action. It not only possesses the same action as tetracycline of inhibiting protein synthesis in the ribosomes but also evades a common resistance mechanism for tetracycline known as an efflux pump. Efflux pumps are transmembrane proteins that actively export the antimicrobial agent out of the microbial cell, often against a concentration gradient. Steric hindrance prevents extrusion of the tigecycline molecule from the cell due to a bulky substitution at a key position on the molecule that impedes its elimination from the bacterial cell. Combinations of a fluoroquinolone with a beta-lactam and a fluoroquinolone with an oxazolidinone molecule are examples of molecular hybrids (39,40). Although these combination drugs are not currently in use, they serve as models for chimeric drugs. Substitution of chemical groups on a known antibiotic has resulted in the development of numerous new drugs and will be discussed in detail (13,38,41).

Advances in understanding of bacterial physiology has led to the development of "structure-activity relationships" (SAR) that are used to direct the chemical modification of antibiotics to improve their activity. This led to discovery of new antibiotics that are

active against organisms that have acquired resistance. The SAR identifies important regions of the compounds that are necessary for biological activity and those regions that can be changed to improve the drug-like properties of the compounds (42). The approaches used to develop SAR usually rely on determining which substitution made on a particular molecule will translate to improved antimicrobial activity. An understanding of resistance mechanisms can play a major role in developing SAR. For example, if the compound under study is an aminoglycoside, knowledge of enzymes that can chemically modify aminoglycosides and make them inactive is important in designing new agents that might avoid such modifications (38).

A clear example of the importance of relationships between antibiotic structure and biological activity is in the development of cefoxitin from cephamycin C. Cephamycin C had an advantage over the classical cephalosporins because it contained a methoxy group in a key location on the beta-lactam ring that provided resistance to hydrolysis by beta-lactamases. In addition, on the same carbon atom that contained the methoxy group, a thienyl acetyl group was substituted for an alpha-amino-adipic acid moiety (See Figure 3). This substitution broadened the spectrum of cefoxitin compared to cephamycin C. This increased the activity of cefoxitin against gram-negative organisms and also gave it activity against gram-positive organisms (38). With the understanding that the methoxy group of cefoxitin conferred beta-lactam stability, efforts were taken to find other derivations with even greater stability. Using the unsubstituted compounds as the standard, it was found that any group at the seventh position (the carbon where the above substitutions were made) larger than the methyl group conferred enhanced stability to hydrolysis of the beta-lactam ring. It is hypothesized that

substitutions at this position sterically block the beta-lactam bond from attack by the enzyme. Substitution at this carbon decrease stability of some other cephalosporins (such as cefuroxime, cefamandole, and cephapirin) that have been shown to be less potent than the original molecule. This decrease is associated with a decreased affinity of the substituted analogs for penicillin binding proteins (PBP) (38). PBP's are natural proteins that are involved in the synthesis of the cell wall of bacteria, bind to the bottom of the β-lactam portion of β-lactam antibiotics, and are responsible for development of resistance when they are altered (43). The results of such structure-activity studies with cefoxitn illustrate how making changes in an active molecule can translate into major clinical benefits such as lower susceptibility to inactivation (38).



Cephamycin C Cefoxitin

Figure 3. Chemical structures of Cephamycin C and Cefoxitin. The methoxy group at the seventh carbon position is indicated by "MeO". The thienyl acetyl group is to the left of the line in the cefoxitin diagram (44).

Another example is naturally occurring monobactams which demonstrated antibacterial activity that was limited to gram-negative organisms. Competitive binding studies indicated that this limited spectrum resulted from poor affinity of the monobactam for the PBP of gram-positive organisms. Attempts were made to broaden the spectrum of naturally occurring compounds by making substitutions around the monobactem nucleus. Adding an aminothiazole oxime side chain conferred high affinity to PBP (See Figure 4).

This improved its activity against *Pseudomonas aeruginosa* (38). Aztreonam is the only example of a monobactam that is used as an antimicrobial agent (45).

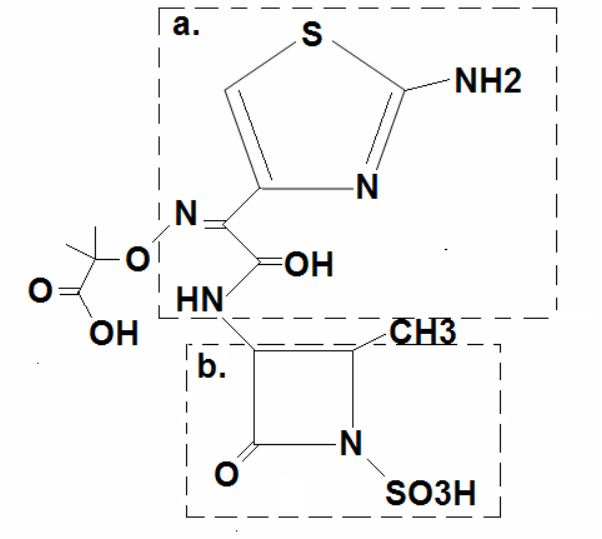


Figure 4. Structure of aztreonam. (a.) Aminothiazole oxime side chain. (b.) Monobactam nucleus (45).

SAR studies are also being used to determine which portions of molecules are toxic and to see if modifications of this portion will allow the production of an active non-toxic antimicrobial. An example of this is phototoxicity induced by fluoroquinolones (13). Studies by a team in Japan have shown that substitution of the carbon at position eight by a fluorine atom is one of the causes of toxicity (See Figure 5). If a methoxyl group (-OCH_3) is at that position, the phototoxicity is reduced (35).

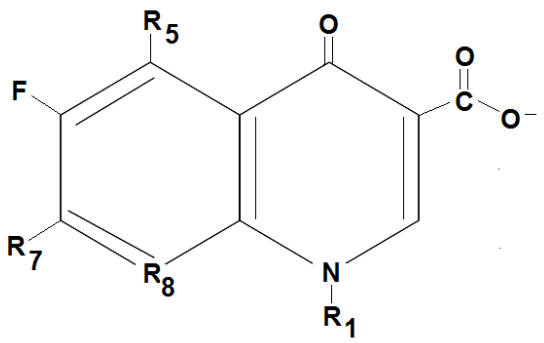


Figure 5. Basic structure of a quinolone. R_8 = Carbon 8. Substitutions at this site can determine the presence or absence of phototoxicity (46).

Routinely used antimicrobials are not the only source for lead compounds when developing new antimicrobials from existing drugs. Another avenue is to use veterinary and agricultural antimicrobial compounds as starting points. Linezolid was originally used to treat tomato infections due to *Agrobacterium tumefaciens*. Another example of other sources for new compounds is the modification of proton pump inhibitors (used to treat peptic ulcers) which allowed the synthesis of compounds active against *Helicobacter pylori*. Some drugs are rediscovered several decades after they are originally described. Some aminoglycosides that were active only against gram-positive organisms and not developed due to lack of medical need are being revived as potential treatment for methacillin-resistant *Staphylococcus aureus* (13).

Genomics

The first complete bacterial genome was published for *Haemophilus influenzae* in 1995. Since then many other bacterial genomes have been completed and are available for use in antimicrobial research (42). As a result of this increased knowledge, the drug discovery paradigm for some researchers has shifted away from finding compounds

against whole cells to identifying compounds that are active against selected protein targets (47).

Knowing the sequence of the microbial genome has allowed scientists to observe similarities and differences in the genetic makeup of various bacterial species. Many genes that have been sequenced are highly conserved (or shared among numerous bacterial species). It is postulated that these genes and the proteins for which they encode are most probably essential for life (48). Essential genes make up about 25% of the bacterial genome (47) and are responsible for DNA replication, transcription and protein synthesis, lipid biosynthesis, and cell wall assembly (48). Since these genes are responsible for basic life functions, substances that could block their activity could be developed into highly effective and broad spectrum antimicrobial agents.

With the completion of sequencing the human genome in 2003, it was possible to compare microbial genomes with the human genome to identify microbial genes that do not have comparable human genes (42,49). When eliminating genes found in the human genome and other eukaryotic cells, it is estimated that about 10% of a microbe's genes are unique and are prime targets for development of new and novel antimicrobial agents (47).

Once a gene is identified as a potential target for a new antimicrobial agent, assignment and confirmation of the gene function is crucial for genome-based drug discovery. The structure of proteins encoded by a particular gene can be determined by inserting the gene into an *Escherichia coli* strain. The *Escherichia coli* will then produce large quantities of the protein that may be used for screening for antimicrobial activity or allow characterization to occur (42). This characterization can be accomplished by

performing microarrays, protein-to-protein interaction studies, and mutagenesis (or "knock-out") studies. Microarrays can be used to monitor bacterial gene expression in response to environmental conditions (50). In a gene knockout study, the function of the gene is determined by altering the gene so it is non-functional. This may be accomplished by inserting a foreign sequence to disrupt the gene (51). If a particular cellular product or function is not found when the gene is "knocked-out", the gene's function is confirmed (48).

Comparing genomes of pathogens and non-pathogens might detect virulence factors and allow drugs to be developed that may not kill the organism but inactivate a virulence factor. Many differences between strains of a species are due to acquisition or deletion of DNA segments. Further sequencing of additional strains will allow a deeper understanding of the genomic source of virulence. In several organisms, acquired segments of DNA are associated with the virulence of that strain (52). For example, *Escherichia coli* 0157:H7 has 1300 genes that are not present in the *Escherichia coli* K12 laboratory strain. An antimicrobial agent that selectively inhibited the function of these genes would render the organism non-virulent but not kill it (53). This could avoid the inflammatory response in the host that is caused when endotoxin from the gram-negative bacterial cell wall is released due to lysis of the bacterial cell (50).

The use of genomics has many advantages. It may be used to find new and nontraditional protein targets which could be used as lead compounds for new drugs (42,52). Drugs with limited spectrum of activity that are selective for a particular organism may be developed to avoid the destruction of normal bacterial flora in the host (52). However, genomics does not consider cellular accumulation and efflux of the

compound being tested. As a result, whole cell assays for testing each potential lead compound is required to consider these issues when looking for new drugs (47).

Combinatorial Chemistry

In the past two decades a new sub-specialty of chemistry, combinatorial chemistry, has emerged with a great potential to revolutionize the discovery of new medicinal agents and diagnostic reagents. This special area of chemistry may increase the production of potential new drugs by using a set of innovative procedures to generate large libraries of compounds and identify the pharmacologically active ones (54). The idea of combinatorial chemistry is to make a large number of chemical variants all at one time. This may be accomplished by one of two methods: solid-phase synthesis and solution-phase synthesis. In solid-phase synthesis, the molecule of interest is attached to a polymeric carrier, such as a bead. Chemical reactions that modify the molecule of interest occur on the bead until the desired library of compounds is formed. Then the bead is separated from the compounds (See Figure 6). One of the advantages of this method is the ability to drive reactions to completion by using excess of reagents (55). The excess reagents are then removed by filtration and washing.

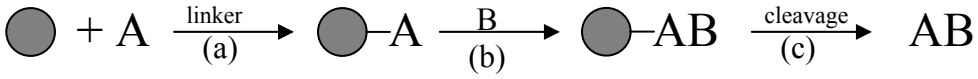


Figure 6. Solid-phase synthesis. (a) The molecule of interest "A" is attached to a polymeric bead via a linker. (b) Substance B reacts with and binds to molecule A. (c) The new molecule "AB" is cleaved from the bead (55).

In liquid-phase synthesis, excess reagents cannot be removed by filtration because reagents are in solution. However, scavenger molecules can remove excess reagents, allowing reactions to proceed to completion (See Figure 7) (55).

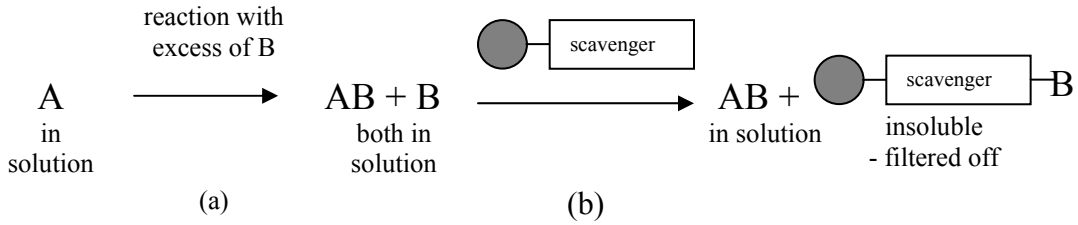


Figure 7. Liquid-phase synthesis. (a) Molecule A is reacted with an excess of molecule B. Both molecules are in solution. This results in a combination of AB and B molecules in solution. (b) A scavenger molecule that is attached to a bead reacts with the excess B and is filtered off, leaving molecule AB (55).

By using these methods a plethora of compounds can be produced in a short period of time (56). Biologically active compounds that are found (also known as "lead" agents) may be chemically modified in an attempt to find derivatives of the "lead" agents that have enhanced biological activity. The "lead" agent may be chosen from a library of diverse agents by testing for a specific biological activity. Combinatorial libraries of the "lead" agent may be made by one of two methods: split synthesis or parallel synthesis (57). In the split synthesis method, compounds are assembled on the surface of microparticles or beads. The "lead" compound is attached to the beads. Chemical components, or building blocks, are added to the "lead" compound. In subsequent steps, beads from the previous step are mixed and partitioned into several groups and a new building block is added. The process continues until the desired combinatorial library has been assembled. Each bead holds a single library member and may be separated from the others by mechanical means (See Figure 8) (55). In the parallel synthesis method, each compound is synthesized in a separate vessel. The "lead" compound may be attached to

a microwell tray. Each building block is added to the well and allowed to bind to the "lead" compound or previous building block. Both the split synthesis and parallel synthesis lend themselves to automation. Once a library of compounds is made, they can be tested for bioactivity by determining the ability to bind to a specific target or other desired properties. The most promising compounds must be isolated and identified for further development (57).

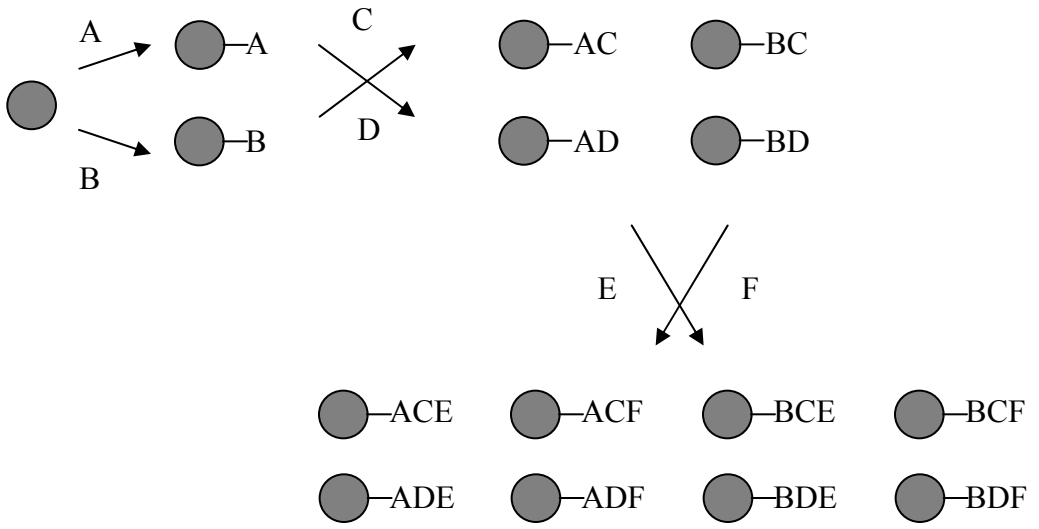


Figure 8. Split synthesis protocol (55).

There are several advantages to using combinatorial chemistry for discovery and development of new antimicrobial agents when compared to traditional methods. A collection of natural products is not necessary for combinatorial chemistry because all compounds are synthetically made. Combinatorial chemistry may be automated by robotic instrumentation that can make compounds and perform chemical and biological analysis with greater speed, accuracy, and precision than a laboratory scientist (58). Automation greatly decreases labor costs and allows huge numbers of compounds to be made and screened for biological activity in a short period of time.

Combinatorial chemistry may be used to produce compounds exhibiting both antibacterial and antifungal activity. An agent that has biological activity against either bacteria or fungi can be modified by adding building blocks to produce activity against the other group of microorganisms. Although little work has been done in this area, the methods used in combinatorial chemistry should allow production and screening of hundreds of agents that may have multiple antimicrobial indications.

Bringing a Drug To Market

Preclinical Trials

Once a compound has been identified as a potential new drug, it must go through a series of preclinical and clinical trials prior to being put on the market. During preclinical trials, the compound must be tested in animal models to assess safety, distribution in various tissues, and efficacy against experimental bacterial infections.

Early stages of testing ensure that the compound is not rapidly metabolized in vivo and that it is well tolerated by the animals in which it is tested. This is done by giving the drug by various routes of administration: intravenously, subcutaneously, and orally. The initial safety and metabolism is assessed for each form of administration. Typically 8-12 animals are used per administration route and dose. The doses that are used in these trials are selected based upon anticipated levels that will be used during the clinical trials. Blood samples are taken at strict time intervals after administration and analytical chemical methods, such as high-pressure liquid chromatography, are used to determine serum or plasma drug levels and to identify metabolites of the drug excreted in urine. The serum/plasma levels allow the determination of elimination half-life, peak drug concentrations achieved, and Area Under the Curve (AUC), which is a measure of

how much of a drug reaches the bloodstream in a set period of time (59). These parameters are determined for each species tested and can be used to predict how to use the drug in humans during the clinical trials. These early trials also evaluate how well experimental infections are cured by the drug.

Once a potentially new antibiotic has been tested, the data is reviewed and a decision is made whether the perceived advantages of the compound warrant the time and expense required for clinical development. Extensive safety evaluations must be done before proceeding (38).

Animal Safety Studies

The purpose of animal safety studies is to assess the drug for short-term (acute) and long-term (chronic) dosing, genetic or carcinogenic safety, and affects on reproductive capability. For acute studies, the drug is given over a 24 hour period at levels considerably above the anticipated level of use in humans. The animals are monitored for behavioral changes along with lethality. This allows the maximum tolerated dose to be determined.

Chronic studies use several different dosing schedules on multiple species of animals. The drug is given for 30 days of duration at a level several fold above the expected human-use level. Blood is drawn during these studies and tested for hematological and blood chemistry abnormalities, including liver and kidney function and central nervous system manifestations. At the end of dosing, animals are sacrificed and multiple tissues are examined for gross and histological abnormalities. If the drug is given perenterally, the injection site should be evaluated for irritation. These studies must be done before the drug can go into Phase I clinical trials. If the drug moves on to

Phase II clinical trials, a 6 month chronic study must be performed in at least 2 species of test animals.

Studies to assess reproductive capabilities are done to determine the effect on spermatogenesis in males and embryogenesis in females in animals receiving the drug. For mothers who received the drug during the gestational period, the effects of the drug on the fetus is evaluated by examination of the newborn for abnormalities. For antimicrobial agents that give an abnormal response in these studies, a 2-year carcinogenicity study is performed in rodents.

Once all safety studies are completed, the data must be compiled and submitted to the Food and Drug Administration (FDA) before clinical trials can begin. The FDA has 30 days to review and approve the drug for further testing (38).

In addition to preclinical trials and safety studies, the manufacturing process for the new drug must be scaled up from the small quantity that has been required up to this point, while ensuring reproducibility of each lot with the original samples. A determination must be made on the best way to produce large quantities of the drug to keep up with the demand for the new drug once approved. Before clinical trials can begin, susceptibility testing products (Kirby Bauer disks, E-test strips, or microbroth MIC panels) must be developed (60).

Clinical Trials

Clinical trials are divided into three phases and can take up to 5 years to complete. The FDA provides guidance on how these trials should be performed (61,62).

Phase I trials can take up to 2 years to complete and are performed to study the safety and pharmacokinetics profile in healthy volunteers. They confirm achievable

blood levels, elimination half-life, urinary recovery in humans, and help determine the extent of oral absorption. This data helps set up an appropriate dosing schedule for subsequent clinical trials.

Phase II trials can take up to 2 years to complete and are performed to assess the efficacy of the new antibiotic in treating patients with bacterial infections. This phase is usually done using patients who do not have life-threatening infections. The efficacy of the test drug is usually compared to a drug of known efficacy. The efficacy of the drug is monitored by both clinical and bacteriological cure. Clinical cure is determined by relief of symptoms and resolution of signs of infection. Bacteriological cure is determined by eradication of the etiologic agent on culture. During this phase, safety and toleration of the drug continue to be monitored (38).

Phase III trials can take several years and are similar to Phase II trials. However, these trials are done to show efficacy of the drug for multiple therapeutic indications. The efficacy should be demonstrated for each organism at each site of infection at which an indication for therapy is desired. The most meaningful data are obtained when the new drug is evaluated against a known standard therapy. This allows evaluation of improved side effect profiles, reduced dosing frequencies, and reduced cost (38).

After clinical trials are completed, all the data must be organized and submitted to the FDA for approval. The FDA takes about 21 months to review the data for approval. Once approved, the drug may be marketed and sold (38).

Future of Antimicrobial Development

While there is a need to produce new antimicrobial agents, some pharmaceutical companies have decided to discontinue the development of this group of therapeutic

agents. In the 1960's, the FDA approved 2.9 new antibacterial drugs per year. This decreased to 2.2 drugs per year in the 1990's and 1.6 drugs per year between 2000 and 2004. There are many reasons for this decrease. The profit for antimicrobial agents are a fraction of the profits for other types of drugs. For example, a company's best selling antibacterial agents made just over two billion dollars in 2003 compared to 9.3 billion dollars that was made on a lipid lowering agent sold by the same company. The lower profit margin may be attributed to the short term use of the drug when compared to other types of medicine. In addition, experts often recommend that newer antimicrobial agents be reserved for treating patients who have infections caused by resistant pathogens. Another contributing factor is the incredibly high cost of bringing a new antimicrobial to market. It is estimated that it costs about 800 million dollars to discover, develop, perform trials, and bring a drug to market. In the last several years, a number of large pharmaceutical companies have merged to form even larger companies. Larger companies require larger profits to sustain themselves and may be less willing to develop drugs that have a lower profit margin. Another deterrent to manufacturing antimicrobials is the length of time required to bring a drug to market and the regulatory requirements. The time from the discovery of a potential new drug to placing the drug on the market is up to ten years. The FDA is very involved in the developmental process and reviews the data for the new drugs at several time intervals during development. Even though the FDA is attempting to streamline this process, these changes cannot change a two billion dollar drug into a nine billion dollar drug (63).

Although the development of new antimicrobials has declined in recent decades, it is critical that research and development of antimicrobials continues. Cost effective

screening methods, such as genomics and combinatorial chemistry may make discovery of new agents more efficient. This coupled with a streamlined FDA process may reduce the cost and time required to bring a new drug to market. These factors are of key importance if we are to continue a successful fight against infectious diseases.

MATERIALS AND METHODS

Source of Compounds to be Tested

Combinatorial chemistry was used to produce sixty-one compounds in three general classes: hydantoins, sulfonamides, and amides. (See Table 1 for a list of compounds made.) These compounds were made by a fellow graduate student, Alen Cusak, in the Chemistry Department.

Table 1. Name and number of chemical compounds used.

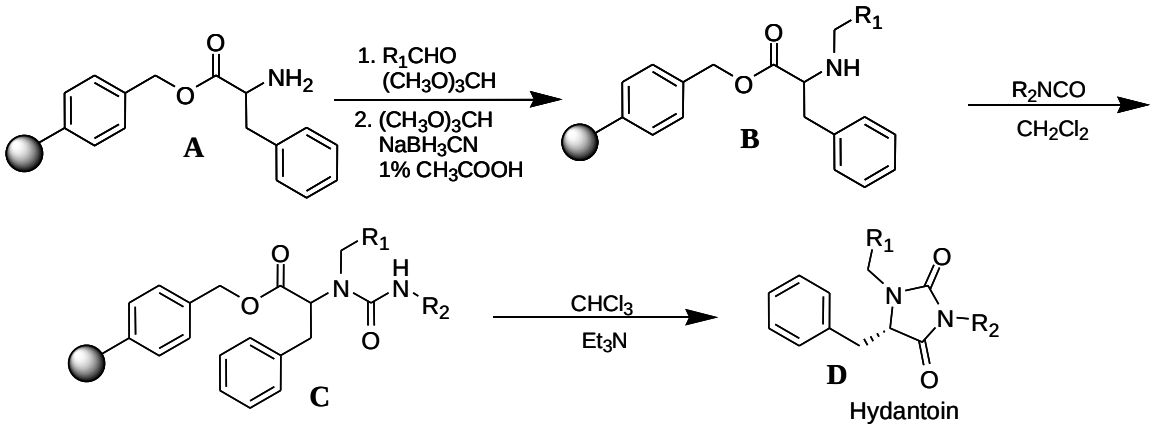
Sample Number	Chemical Name of Compound
1	N-butyl-4-chlorobenzamide
2	3,4-difluoro-N-(2-phenylethyl) benzenesulfonamide
3	N-benzylbenzenesulfonamide
4	N-(1-phenylethyl)benzenesulfonamide
5	3,5-dibenzyl-1-(4-nitrobenzyl)hydantoin
6	3,4-difluoro-N-(1-phenylethyl)benzenesulfonamide
7	N-benzyl-3,4-difluorobenzenesulfonamide
8	1,3,5-tribenzylhydantoin
9	N-[3,5-bis(trifluoromethyl)benzyl]-4-fluorobenzenesulfonamide
10	N-benzylbenzamide
11	4-chloro-N-(2-phenylethyl)benzamide
12	N-(1-phenylethyl)benzamide
13	N-(2-phenylethyl)benzenesulfonamide
14	3,5-dibenzyl-1-(4-methoxybenzyl)hydantoin
15	3,5-dibenzyl-1-isopentylhydantoin
16	N-butylbenzamide
17	N-butylbenzenesulfamide
18	4-chloro-N-(2-chlorobenzyl)benzamide
19	N-benzyl-4-chlorobenzamide
20	N-butyl-3,4-difluorobenzenesulfonamide
21	4-chloro-N-octylbenzamide
22	4-chloro-N-(1-phenylethyl)banzamide
23	N-isobutyl-3,5-bis(trifluoromethyl)benzamide
24	N-(2-chlorobenzyl)-4-fluorobenzenesulfonamide

25	3,5-dibenzyl-1-(3-nitrobenzyl)hydantoin
26	N-(2-phenyletheyl)benzamide
27	4-fluoro-N-(2-phenylethyl)benzenesulfonamide
28	3,5-dibenzyl-1-hexylhydatoin
29	N-(2-phenylethyl)-3,5-bis(trifluoromethyl)benzamide
30	3,5-dibenzyl-1-heptylhydatoin
31	3,5-dibenzyl-1-octylhydatoin
32	3,4-difluoro-N-(2-phenylethyl)benzene
33	4-chloro-N-(1-phenylethyl)benzene
34	N-(2-chlorobenzyl)-3,4-difluorobenzenesulfonamide
35	3,4-difluoro-N-octylbenzenesulfonamide
36	N-benzyl-4-fluorobenzenesulfonamide
37	3,5-dibenzyl-1-toluylhydatoin
38	N-(2,4-dichlorobenzyl)-4-fluorobenzenesulfonamide
39	3-benzyl-1-(2,4-difluorobenzyl)imidazolidine-2,4-dione
40	3-(2-chlorobenzyl)-1-(3-chloro-4-methoxybenzyl)imidazolidine-2,4-dione
41	3-benzyl-1-[4-(trifluoromethyl)benzyl]imidazolodine-2,4-dione
42	4-chloro-N-(4-fluorobenzyl)benzamide
43	3-benzyl-1-(4-fluorobenzyl)imidazolidine-2,4-dione
44	3-(2-chlorobenzyl)-1-(5-fluoro-2-methoxybenzyl)imidazolidine-2,4-dione
45	1,3-bis(2-chlorobenzyl)imidazolidine-2,4-dione
46	3-benzyl-1-(3-methylbutyl)imidazolidine-2,4-dione
47	3-(2-chlorobenzyl)-1-octylimidazolidine
48	1-benzyl-3-(2-chlorobenzyl)imidazolidine-2-4-dione
49	3-benzyl-1-(4-methoxybenzyl)imidazolodine-2,4-dione
50	N-hexyl-3,5-bis(trifluoromethyl)benzamide
51	1-[3,5-bis(trifluoromethyl)benzoyl]pyrrolidine
52	N-(4-fluorobenzyl)-3,5-bis(trifluoromethyl)benzamide
53	3,5-bis(trifluoromethyl)-N-[3-(trifluoromethyl)benzyl]benzamide
54	4-fluoro-N-(4-fluorobenzyl)benzenesulfonamide
55	1-[(4-fluorophenyl)sulfonyl]pyrrolidine
56	4-fluoro-N-[3-(trifluoromethyl)benzyl]benzenesulfonamide
57	N-(2-chlorobenzyl)-3,5-bis(trifluoromethyl)benzamide
58	4-fluoro-N-hexylbenzenesulfonamide
59	4-fluoro-N-isobutylbenzenesulfonamide
60	N-benzyl-3,5-bis(trifluoromethly)benzamide
61	N-[3,5-bis(trifluoromethyl)benzyl]-3,5-bis(trifluoromethyl)benzamide

The compounds were produced by attaching chemically reactive molecules to insoluble polymeric resin bead supports. Reactions were driven to completion by using excess reagents. After each step, purification of the resin-bound compounds was accomplished by filtrations and washing. The desired product was then cleaved from the

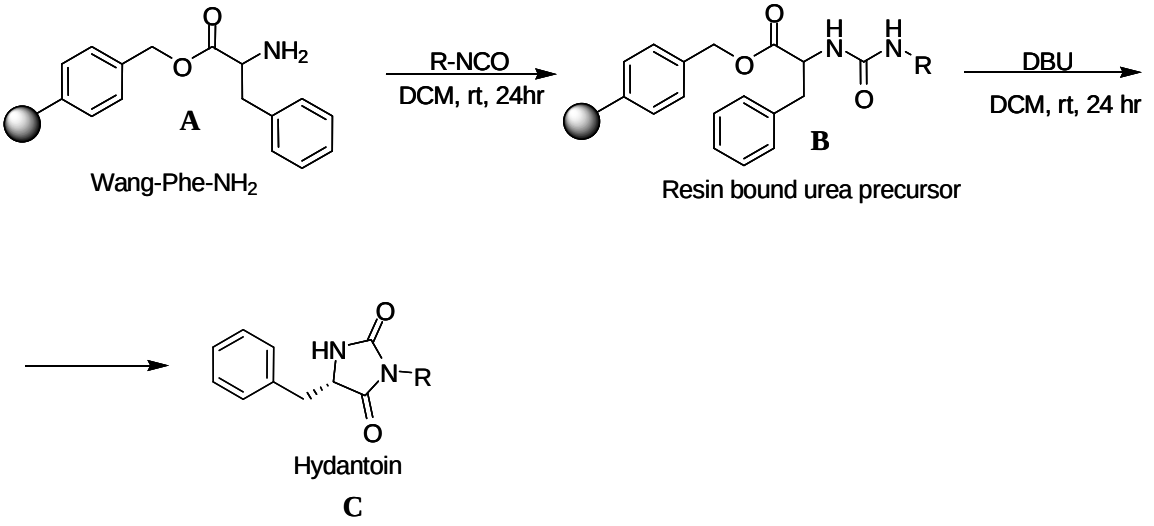
resin bead. (For a full description of how compounds were made, refer to Alen Cusak's thesis [64].)

The hydantoins that were synthesized fell into two categories: trisubstituted and disubstituted. The trisubstituted hydantoins were made using the following formula:



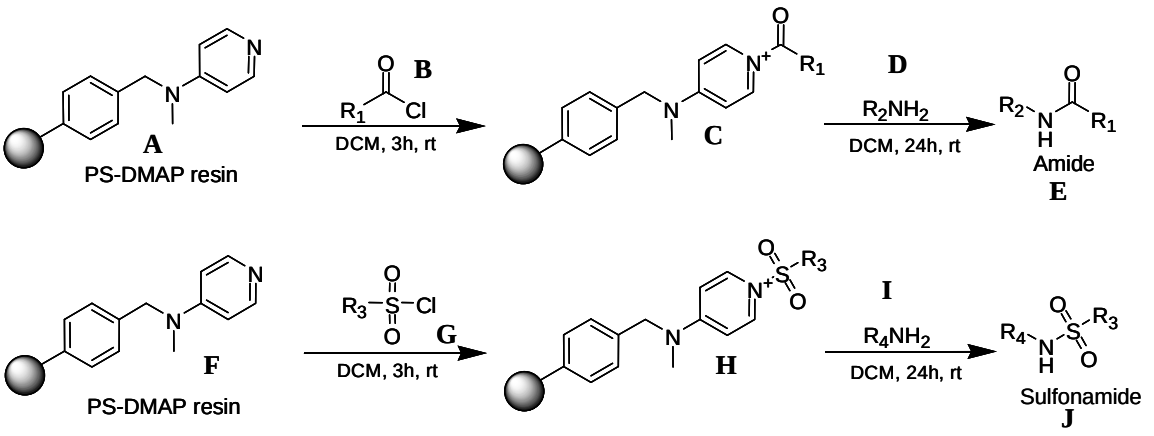
In this formula, a Wang resin bound amino acid (A) was reacted with various aldehydes (R_1CHO) in the presence of trimethylorthoformate ($(CH_3O)_3CH$). An imine intermediate was reacted with trimehylorthoformate and sodium cyanoborohydride ($NaBH_3CN$) under acidic conditions provided by acetic acid (1% CH_3COOH) and formed an N-alkylated resin (B). The resin was washed to remove excess aldehydes and excess $NaBH_3CN$ and was then reacted with isocyanate (R_2NCO). The resultant compound (C) was filtered and washed to remove excess isocyanate. A base induced cyclizative cleavage produced a relatively pure hydantoin (D) by causing the five member hydantoin ring to close and by cleaving the Wang resin from the hydantoin (64).

The disubstituted hydantoins were made using the following formula:



The Wang resin (A) was reacted directly with isocyanate to generate a resin bound urea precursor (B). It was washed with dichloromethane (DBM) to remove excess isocyanate. The resin was cleaved using a strong base (DBU) yielding the disubstitued hydantoins (C) (64).

The amides and sulfonamides were made by a "catch and release" application using the following formulas:



The polymer supported dimethylaminopyridines (PS-DMAP) resin (A) was placed in a reaction vessel and acid chlorides (B) or sulfonyl chlorides (G) were added. Upon reaction with acid chlorides (B) and sulfonyl chlorides (G) stable acyl-pyridinium

adducts (C) and sulfonyl-pyridinium adducts (H) were formed. This reaction describes "the catch" step in "catch and release" application. Acyl-pyridinium (C) and sulfonyl-pyridinium (H) adducts were cleaved from the resin by nucleophilic attack of pyridinium adduct with primary amine (D and I). The cleavage step is known as "release" (64).

The chemical properties of these polymers resulted in a product that had an oily consistency. In order to test these chemical compounds for antimicrobial activity, a test system that would accommodate the oily consistency was developed.

Bacterial and Fungal Isolates

The organisms used in this research were chosen to represent a wide range of bacteria and yeast. Strains of bacteria chosen were susceptible to most antibiotics that are tested in a clinical laboratory. The gram-positive organisms included *Staphylococcus aureus* (ATCC strain 25923) and a clinical isolate of Group B streptococci (or *Streptococcus agalactiae*). The gram-negative organisms included *Escherichia coli* (ATCC strain 35922) and *Klebsiella oxytoca* (ATCC strain 49131).

The yeasts used in this research were strains isolated from clinical specimens. The species chosen are generally susceptible to the most commonly used antifungal agents. The yeasts used were *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis*.

Inoculation of Media

In each of the experiments in this research, the media were inoculated by making a standardized suspension of the organism to be tested in sterile saline (0.85%) or Trypticase Soy Broth (TSB). This suspension was made by touching a swab to 2-3

colonies of the test organism grown on Trypticase Soy Agar (TSA) with 5% sheep blood. The swab was then plunged into a tube of sterile saline and twirled until a homogeneous suspension was made. A clean sterile swab was placed in the tube of the standardized inoculum. As the swab was removed from the tube, it was pressed against the side of the tube to express the excess liquid. Next, the swab was rubbed over the entire surface of the agar in one direction. The plate was then turned and the swab was rubbed over the entire surface in two additional directions. This technique ensured homogeneous growth of the organism on the plate (See Figure 9).

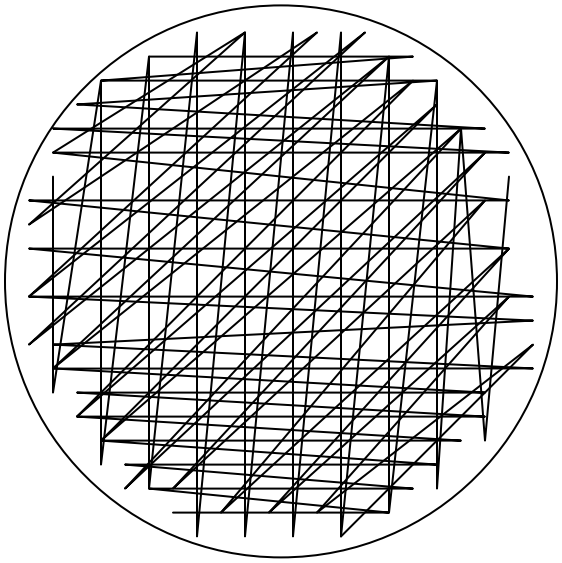


Figure 9. Diagrammatic representation of plate inoculation.

Inoculum Size

The available literature (68,87) is very specific about the proper inoculum size that should be used when performing susceptibility testing on bacteria. A suspension equaling a 0.5 McFarland [which is equivalent to approximately 1-2 X 10^8 Colony Forming Units/ml (CFU/ml)] was used in this research. The inoculum size was

confirmed by placing the inoculum tube in a turbidity meter (Figure 10) and adjusted to a specific turbidity range specified by the meter manufacturer.

Figure 10. Turbidity meter.

There are various published inoculum sizes that may be used for yeast. Some studies suggest the use of a 0.5 McFarland suspension while others suggest various dilutions of this 0.5 McFarland suspension (65,66,67). In order to determine the best inoculum size for this research, a 0.5 McFarland suspension was made (using a turbidity meter) in trypticase soy broth (TSB) for each yeast to be tested. These suspensions were then serially diluted in TSB to 1:100 and 1:2000. RPMI 1640 media (to be described later) was inoculated as described above. After the plates were incubated for 20-24 hours at 36^{o}C, the plates from the 0.5 McFarland suspension were the only plates to show confluent growth. As a result, it was determined that this was the best inoculum size. Since yeast grow more slowly than bacteria, the plates containing yeast were then reincubated and examined again after 36-48 hours to see if the results would be different after 48 hours of incubation. The 0.5 McFarland suspension was still the best inoculum size. As a result, a 0.5 McFarland suspension of yeast was used for the remainder of this research. This is equivalent to approximately 1-5 X 10^{6} cells per mL.

Incubation Time

The incubation time required for adequate growth of bacteria to detect inhibition by antimicrobial compounds is well defined (68). An incubation time of 16-24 hours at 36^{o}C is recommended. As with the inoculum size, the incubation time for yeast was not as well defined. During the experiment where the optimal inoculum size was determined, the plates were observed for growth at both 20-24 hours and 36-48 hours to determine the optimal incubation time. Adequate growth to allow detection of growth inhibition was seen at 20-24 hours. This incubation time was used for the remainder of the study.

Dimethyl Sulfoxide (DMSO) for Putting Compounds into Solution

The compounds that were tested were received in the microbiology laboratory as oily residues that were attached to the surface of glass vials. In order to test these compounds, they had to be suspended in solution. According the NCCLS guidelines for antibacterial susceptibility testing, many antimicrobial substances are soluble in water (69). However, a number of antimicrobial substances are not soluble in water and another diluent must be used. Some of the diluents suggested are phosphate buffer, sodium bicarbonate, dimethyl sulfoxide (DMSO), saline, acetic acid, ethanol, sodium hydroxide, methanol, and hydrochloric acid. In addition, the NCCLS guidelines for Antifungal Susceptibility Testing of Yeast (70) recommends the use of dimethyl sulfoxide (DMSO) to dissolve antifungal agents that are not water soluble. Since it was not known if the sixty-one substances that were tested were soluble in water, an "all-purpose" diluent was needed to suspend them in solution. Since many antibacterial agents are soluble in DMSO, it was used as the "all-purpose" diluent.

When using DMSO to dissolve antifungal powders as directed in the NCCLS guidelines, the DMSO is greatly diluted prior to inoculating organisms into the various concentrations of the antifungal agent. If the NCCLS guidelines are followed, the final concentration of DMSO would be only 1% of its original concentration. In addition, the antifungal agent would be diluted to 1% of its original concentration. Since the potency and purity of the compounds being tested were not known, there was a concern that high dilutions of the compounds may interfere with the ability to detect antimicrobial activity. Therefore, the smallest dilution possible of the compound should be used for testing. However, it was also necessary to ensure that the concentration of the DMSO used would not interfere with testing of the compounds.

To determine lowest concentration of DMSO that may be used to dissolve the compounds without inhibiting the test organisms, a 0.5 McFarland suspension of each test organism was made in 0.85% saline. Each organism was inoculated to either RPMI 1640 agar, Mueller Hinton agar, or Mueller Hinton agar with Sheep Blood. (Discussed later.)

Dilutions of the DMSO were made in sterile water. (1:1 [undiluted], 1:5, 1:10, 1:15, 1:20 dilutions were made.) Sterile filter paper disks were then dipped into the dilutions of the DMSO and placed on the surface of the Mueller Hinton, Mueller Hinton with Blood, and RMPI media. Each organism was tested with each dilution of DMSO.

The plates were incubated for 20-24 hours at 36^{o}C and then examined for inhibition of growth around the disk. Table 2 shows the results of this experiment.

Table 2. Zones of inhibition for DMSO dilutions.

	DMSO Dilutions (Zone sizes in mm)*				
	1:1	1:5	1:10	1:15	1:20
E. coli	0	0	0	0	0
S. aureus	0	0	0	0	0
K. oxytoca	0	0	0	0	0
Group B Strep	0	0	0	0	0
C. albicans	0	0	0	0	0
C. tropicalis	7	0	0	0	0
C. parapsilosis	0	0	0	0	0

* Note: The disk size was 6 mm in diameter. If no zone was noted, the result was recorded as "0

Only one organism was inhibited by undiluted (1:1) DMSO. None were inhibited by a 1:5 dilution of DMSO. As a result, a 1:5 dilution was used for further testing.

Volume of Liquid to Place on Filter Paper Disks

When testing the dilutions of DMSO, the filter paper disks were dipped into the various dilutions and placed onto the media. It was noted that the disks were very wet and did not stick well to the surface of the agar. This caused slight movement of the disks on the media and allowed accumulation of the DMSO in small puddles. This resulted in the liquid running over the surface of the plate away from the disks. Instead of dipping the disks in the solutions, it was hypothesized that placing a measured volume onto the disks would allow the disk to be saturated without being too wet. To determine this measured volume, filter paper disks were placed on an absorbent paper towel. Various volumes of a dark liquid (crystal violet stain) were pipeted onto the disks using an MLA pipet with disposable tips to determine the volume of liquid that was absorbed by the disks. After the crystal violet was pipeted, the disk was moved off of the paper towel to see if the disk was able to absorb the volume without excessive absorption by the paper towel. Table 3 shows the results of this experiment.

Table 3. Crystal violet volume required to saturate disk.

Crystal Violet Volume	Disk Description	Paper Towel Description
10 ul	Absorbed all crystal violet	No absorption of crystal violet
20 ul	Absorbed all crystal violet	Minimal absorption of crystal violet
30 ul	Over-saturated by crystal violet	Excessive absorption of crystal violet

Since the disks absorbed 20 µl of crystal violet with minimal absorption on the paper towel, this is the volume that was pipeted onto the disks in future experiments.

Evaluation of Testing Interference Due to Resin Beads

Resin beads were used in the production of each of the compounds to be tested. Although most beads were removed from the compounds during the final step of the combinatorial chemistry, a few beads remained in the samples. It was unknown whether these beads would interfere with the experiments of determining antibacterial or antifungal activity of the compounds. In order to determine if the resins would interfere, a 0.5 McFarland suspension was made of each organism and inoculated to appropriate plates. The resin was suspended by adding 100 µl of DMSO and 400 µl of water to a bottle that was coated with the resin beads. (It was the original bottle in which the resin beads were received and was left over from the combinatorial chemistry experiments performed by Alen Cusak.) Twenty microliters (µl) of this resin suspension was pipetted onto each of 7 filter paper disks. One disk was placed on each plate and inoculated for 20-24 hours. After this incubation, the plates were examined for zones of inhibition. Table 4 shows the results of this experiment.

Table 4. Zones of inhibition for resin beads.

	Zone size in mm*
E. coli	0
S. aureus	0
K. oxytoca	0
Group B Strep	0
C. albicans	0
C. tropicalis	0
C. parapsilosis	0

* Note: The disk size was 6 mm in diameter. If no zone was noted, the result was recorded as "0".

Since no inhibition was noted, the resin beads do not appear to affect the growth on the test organisms and was acceptable to use in this research.

Evaluation of the Newly Developed Test System

Once all of the variables in the test system were evaluated, it was necessary to test the entire system to confirm that antibacterial and antifungal activity could be detected. To do this a 0.5 McFarland suspension was made for each organism and inoculated to the appropriate media. *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella oxytoca* were plated to Mueller Hinton agar. Group B Strepotococcus was inoculated to Mueller Hinton agar with sheep blood. *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* were inoculated to RPMI medium. Antimicrobial powders for known antibacterial and antifungal agents (ciprofloxacin, amphotericin, and fluconazole) were put into solution for testing. This was done by placing a small amount of each powder (approximately 0.01 grams) into a vial and adding 100 µl of DMSO and 400 µl of water. The vial was gently swirled until the powder went into solution. Twenty µl of each solution was pipetted onto each of 7 filter paper disks. One disk for each antimicrobial agent was placed on each plate and incubated for 20-24 hours at 36^{o}C. After this incubation, the plates were examined for zones of inhibition. See Table 5 for results.

Table 5. Zones of inhibition for antimicrobial control compounds.

	Antimicrobial Solutions (Zone sizes in mm)*		
	Ciprofloxacin	Amphotericin-B	Fluconazole
E. coli	39	0	0
S. aureus	36	0	0
K. oxytoca	35	0	0
Group B Strep	31	0	0
C. albicans	0	18	0
C. tropicalis	0	18	0
C. parapsilosis	0	20	33

* Note: The disk size was 6 mm in diameter. If no zone was noted, the result was recorded as "0".

The test system worked well for detection of anti-bacterial activity and was acceptable for further testing. However, the zone sizes for the fungi were much smaller than the zone sizes for the bacteria and fluconazole demonstrated no activity against two of the fungi (*C. albicans* and *C. tropicalis*). Because of these results, there was a concern that the media formulation may not be optimal. Further investigation was done to see if the media formulation could be improved.

Media

The media used when screening for antimicrobial activity can have a dramatic effect on the results of an experiment. These media must fulfill two requirements: 1) support the growth of the organism being tested and, 2) allow inhibition of growth to be detected due to the presence of the compound being tested. Although this research was not done to perform susceptibility testing on clinical isolates of bacteria, Mueller Hinton agar and Mueller Hinton agar with sheep blood , the media used in clinical laboratories for bacterial susceptibilities as outlined in the NCCLS M2-A7 document (68), were suitable and effective for this research.

The medium used for the fungal isolates was less well defined. A number of different media have been described for testing fungal isolates. These include glucose-enriched Mueller Hinton agar (71), high resolution medium, high resolution medium with methylene blue (72), and RPMI medium. The NCCLS document M27-A describes media that may be used for fungal susceptibility testing and states that RPMI 1640 medium is "at least as satisfactory as several other synthetic media" (70). RPMI medium was chosen as the medium for this research. To make this medium 10.5 grams of RMPI 1640 powder was added to one liter of deionized water. In addition, 20 grams of Bacto agar was added to cause the medium to gel. The medium was then autoclaved at 121^{o}C for 20 minutes. After autoclaving, the medium was cooled to about 60^{o}C and poured into empty, sterile petri dishes. The dishes were left at room temperature for about 30 minutes to allow them to cool and gel. Once gelled, the plates were stored at 4^{o}C until used.

From the results of the quality control experiment that was performed to evaluate the test system that was developed, there was a concern that the fungal medium formulation may not be optimal. A new formulation of the RPMI 1640 medium and another readily available medium, Sabarouds Dextrose Agar (SDA), were considered for use in this study. The new formulation of the RPMI 1640 medium included MOPS buffer [3-(N-morpholino) propanesulfonic acid] to adjust the pH to 6.9-7.1. To make this medium 10.5 grams of RMPI powder, 20 grams of Bacto agar, and 34.53 grams of MOPS buffer powder were added to 900 ml of deionized water. The pH was adjusted by adding a 1 molar sodium hydroxide solution until the pH was between 6.9 and 7.1. The pH was determined by placing strips of pH paper in the liquid medium and then

comparing the color to a pH chart. After the proper pH was achieved, water was added to bring the volume to 1 liter. The medium was then autoclaved at 121^{o}C for 20 minutes. After autoclaving, the medium was cooled to about 60^{o}C and poured into empty, sterile petri dishes. The dishes were left at room temperature for about 30 minutes to allow them to cool and gel. Once gelled, the plates were stored at 4^{o}C until used.

To test the new formulation of RPMI 1640 medium and SDA, a 0.5 McFarland suspension was made for each of the yeast and inoculated to both the old and new formulations of RMPI media and the SDA. Antimicrobial powders for amphotericin, and fluconazole were put into solution (as described earlier) and placed on a filter paper disk for testing as described above. A 20 µl MLA pipettes was used to transfer the fluid onto the disks. One disk for each antimicrobial agent was placed on each plate and inoculated for 20-24 hours at 36^{o}C. After this incubation, the plates were examined for zones of inhibition. (See Table 6 for results.)

Table 6. Zones of inhibition for media formulations.

	RMPI – Original Formulation (Zone sizes in mm)*			
	Amphotericin-B	Fluconazole	DMSO	Resin
C. albicans	16	0	8	0
C. tropicalis	17	0	0	0
C. parapsilosis	20	42	0	0
	RMPI – With MOPS Buffer (Zone sizes in mm)*			
	Amphotericin-B	Fluconazole	DMSO	Resin
C. albicans	18	0	0	0
C. tropicalis	18	0	0	0
C. parapsilosis	22	42	0	0
	SDA (Zone sizes in mm)*			
	Amphotericin-B	Fluconazole	DMSO	Resin
C. albicans	13	0	0	0
C. tropicalis	10	0	0	0
C. parapsilosis	12	32	0	0

* Note: The disk size was 6 mm in diameter. If no zone was noted, the result was recorded as "0".

At the completion of this testing, it was determined that the RPMI 1640 with MOPS buffer was the best medium for detecting antifungal activity because it demonstrated the largest zone sizes but was not affected by DMSO. As a result, it was used for the remainder of fungal testing in this research.

Testing Compounds

Once the testing system was validated using known antimicrobial agents, it was possible to start testing the compounds provided by Alen Cusak, my colleague in the chemistry department. To do this, a 0.5 McFarland suspension was made for each of seven organisms and inoculated to the appropriate testing media. Chemical compounds produced by Alen were put into solution for testing by adding 100 µl of DMSO and 400 µl of water to the vial in which the compound was received. The vial was gently swirled

until the compound went into solution. Twenty µl of each solution was pipetted onto each of 7 filter paper disks. One disk for each compound was placed on each plate and inoculated for 20-24 hours at 36°C. After this incubation, the plates were examined for zones of inhibition. Results for each compound are found in the Table 11 in the "Results" section. Figures 11a and 11b demonstrate the zones of inhibition that were noted. The diameter of the zones were measured. The zone of inhibition was defined as the area where colonies failed to grow.

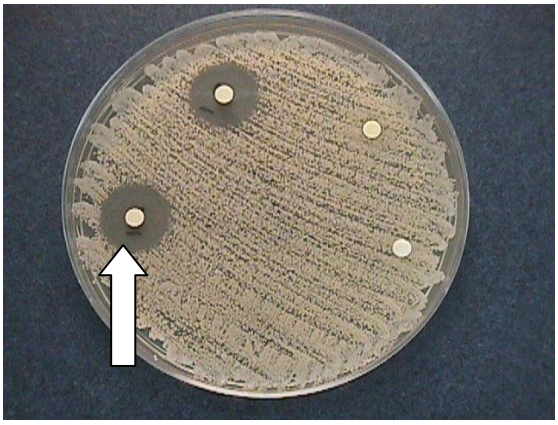


Figure 11a: RPMI 1640 Agar

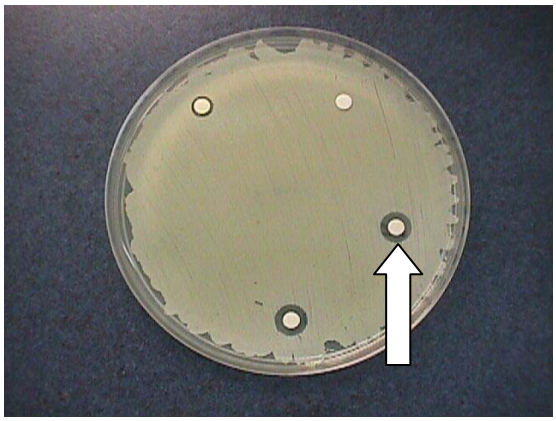


Figure 11b: Mueller Hinton Agar

Figure 11a and 11b. Photographs of RPMI 1640 and Mueller Hinton agars demonstrating zones of inhibition.

Effect of DMSO and Resin Beads on a Cell Culture System

Two compounds were found to have both antibacterial and antifungal activity. As mentioned earlier, all potential new antimicrobial agents must be tested for toxicity before clinical trials can begin. One way to perform cytotoxicity testing is to inoculate the compounds to live cells and observe for cytotoxic effects. Before that could be done, a test system had to be developed to ensure that results that would be obtained would be due to the compound being tested and not some other variable in the experiment.

The two compounds that were mentioned above were made on resin beads and DMSO was used to put the substances into solution. It was necessary to ensure that the resin beads and DMSO did not interfere with the cell culture test system. To do this, a bottle that was coated with resin beads was rinsed with 2 mL of sterile saline to suspend the resin beads in liquid. One mL of the resin bead solution was mixed with 1 mL of cell culture maintenance medium. (This medium contains proteins, buffer to control pH, and antibiotics to inhibit growth of bacteria and fungi (73) and is used to maintain the viability of the cells in a cell culture.) In addition, a 1:5 dilution of DMSO was made in the maintenance medium (200 µl of DMSO added to 800 µl of the maintenance medium).

The above solutions were inoculated into three living cell lines (human: MRC-5, A549; monkey: RMK). To do this, the maintenance medium that is routinely found in the cell culture tubes when purchased was discarded by inverting the tube and allowing the medium to run out into a waste container. Then 1.0 mL of the resin or DMSO solutions was inoculated to each tube. The tubes were placed into a rotating drum (Figure 12) and incubated up to 7 days at 36^{o}C. In the rotating drum, the tubes were lain on their sides and the medium periodically washed over the cell monolayer as the drum rotated and exposed the cells to the resin beads or DMSO. The tubes were observed for cytotoxicity at 24 hours, 48 hours, and 7 days. If cytotoxicity was observed in any tube, incubation was discontinued for that tube. Table 7 shows the results for this experiment.

Figure 12. Rotating drum for cell culture tubes.

Table 7. Cytotoxicity of DMSO and resin beads.

	MRC-5	A549	RMK
DMSO	Toxicity at 24 hours	Toxicity at 24 hours	Toxicity at 24 hours
Resin	No toxicity after 7 days	No toxicity after 7 days	No toxicity after 7 days

The resin beads did not effect the cell lines. However, the 1:5 dilution of DMSO that was used to put all compounds into solution caused cytotoxicity in all cell lines. As a result, this dilution of DMSO could not be used for testing in the cell lines and additional testing was required to find another diluent that may be used that would not cause cytotoxcity.

Determination of Best Diluent for Cytotoxicity Experiments

Since DMSO at a 1:5 dilution was not an acceptable diluent to use to put compounds into solution to test for toxicity in live cell lines, an alternate diluent had to be found to dilute compounds. NCCLS guidelines (68,70) indicate that some antimicrobial substances may be diluted with ethanol or water. To test the ethanol, 95% ethanol was diluted with cell culture maintenance media to make dilutions of 1:5, 1:10, and 1:20. Each of these dilutions, in addition to the neat 95% ethanol, was inoculated to the three

living human and monkey cell lines by discarding the cell culture maintenance medium that was in each tube and adding 1.0 mL of an ethanol solution to each tube. The tubes were placed into a rotating drum and incubated up to 7 days at 36°C. The tubes were observed for cytotoxicity at 24 hours, 48 hours, and 7 days. If cytotoxicity was observed in any tube, incubation was discontinued for that tube. Table 8 shows the results of this experiment.

Table 8. Cytotoxicity of DMSO dilutions and ethanol.

	MRC-5	A549	RMK
95% Ethanol	Little or no CPE, Questionaable viability of cells	Little or no CPE, Questionaable viability of cells	Little or no CPE, Questionaable viability of cells
1:5 Dilution	Toxicity at 24 hours	Toxicity at 24 hours	Toxicity at 24 hours
1:10 Dilution	Toxicity at 24 hours	Toxicity at 24 hours	Toxicity at 24 hours
1:20 Dilution	Toxicity at 24 hours	Toxicity at 24 hours	Toxicity at 24 hours

All tubes showed cytotoxicity except the 95% ethanol tubes. However, the cells in the tubes with 95% ethanol looked unusual. Since all other tubes showed toxicity, there was concern that cytoxicity was not demonstrated in the 95% because the cells were fixed or "pickled". Since most tubes demonstrated cytotoxicity, ethanol is not an acceptable diluent for putting compounds into solution. Further testing was required to find other possible diluents.

To determine if water was a suitable diluent for the two compounds, a small portion of the oily powder for each compound was placed in a test tube. A few drops of water were added to each tube. The tubes were then observed to see if the compounds would dissolve. Table 9 shows the results of this experiment and indicates that neither compound was soluble in water.

Table 9. Water as a possible diluent for two compounds.

Compound 21	Powdered crystals observed after water added.
Compound 35	Powdered crystals observed after water added

Since neither the ethanol nor water were acceptable diluents, another alternative had to be considered. A steroid diluent was considered as an option. However, since this diluent was not a known antimicrobial diluent, there was concern that this would introduce a potentially unnecessary variable into this research.

Although it was demonstrated that a 1:5 dilution of DMSO was an unacceptable diluent, higher dilutions of DMSO might work as a suitable diluent. To test this, DMSO was diluted 1:10, 1:20, 1:40, and 1:80 using cell culture maintenance medium. Each of these dilutions was inoculated into the three living human and monkey cell lines. To do this, the maintenance medium that was in each tube was discarded. Then 1.0 mL of each DMSO dilution was inoculated into each tube. The tubes were placed into a rotating drum and incubated up to 7 days at 36^{o}C. The tubes were observed for cytotoxicity at 24 hours, 48 hours, and 7 days. If cytotoxicity was observed in any tube, incubation was discontinued for that tube. See Table 10 for results.

Table 10. Toxicity of DMSO at high dilutions.

DMSO	MRC-5	A549	RMK
1:10 Dilution	Toxicity at 24 hours	Toxicity at 24 hours	Toxicity at 24 hours
1:20 Dilution	Toxicity at 24 hours	Toxicity at 48 hours	Dropped tube, Unable to assess toxicity
1:40 Dilution	No toxicity noted at 7 days	No toxicity noted at 7 days	No toxicity noted at 7 days
1:80 Dilution	No toxicity noted at 7 days	No toxicity noted at 7 days	No toxicity noted at 7 days

All cell lines demonstrated cytotoxicity in the 1:10 dilution of DMSO. At the 1:20 dilution, two of the cell lines demonstrated cytotoxicity. (It was not possible to examine the RMK cells at the 1:20 dilution because the tube was dropped.) At the 1:40 dilution, no cytotoxicity was noted in any cell line. As a result of this experiment, it was determined that a 1:40 dilution of the DMSO could be used to dissolve each compound for toxicity studies.

Testing Compounds for Toxicity

Each compound that had both antibacterial and antifungal activity was tested to determine if they produced cytotoxicity in human and monkey cell lines. To do this, 80 µl of DMSO was pipetted into the vials of the two compounds to be tested in order to put the powder into solution. 1.120 mL of water was added to each tube. Two mL of cell culture maintenance medium was used to bring each diluted sample to a 1:40 dilution of DMSO. The 1:40 dilution was inoculated to the three living human and monkey cell lines. To do this, the cell culture maintenance medium that was in each tube was discarded. Then 1.0 mL of each compound diluted in DMSO and maintenance medium was inoculated to each tube. The tubes were placed into a rotating drum and incubated up to 7 days at 36^{o}C. The tubes were observed for cytotoxicity at 24 hours and 7 days. If cytotoxicity was observed in any tube, incubation was discontinued for that tube. Results may be found in Table 15 in the "Results and Discussion" section.

RESULTS

Activity of Chemical Compounds

The test system that was developed to look for antibacterial and antifungal activity worked well on the sixty-one compounds that were tested. Table 11 shows the zones of inhibition for each compound that was tested against the seven test organisms.

Table 11. Zones of inhibition for each compound.

	Zone Sizes in mm*						
Compound	*C.tropicalis*	*C albicans*	*C.parapsilosis*	Group B Streptococcus	*S. aureus*	*K. oxytoca*	*E. coli*
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	0	7	0	0
4	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0
10	0	0	0	0	9	0	0
11	0	0	0	0	7	0	0
12	0	0	0	0	0	0	0
13	0	0	0	0	8	0	0
14	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0
21	16	15	14	11	11	15	14
22	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0

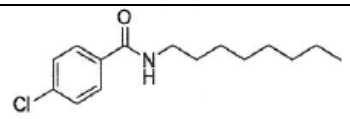
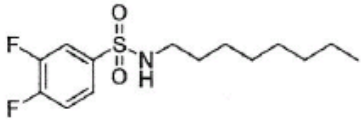
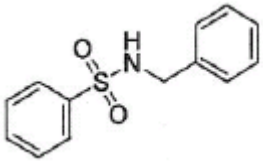
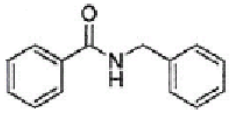
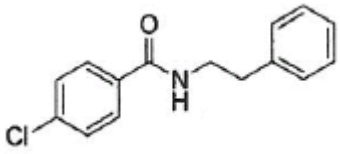
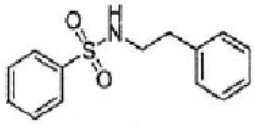
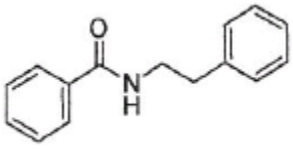
26	0	0	0	0	12	0	0
27	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0
35	18	10	10	11	11	11	13
36	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0
47	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0
54	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0
58	0	0	0	0	0	0	0
59	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0
61	0	0	0	0	0	0	0

* Note: The disk size was 6 mm in diameter. If no zone was noted, the result was recorded as "0".

Seven compounds exhibited biological activity. Two of these compounds (21 and 35) had activity against all organisms tested. Five compounds (3, 10, 11, 13, and 26) had

activity against just *Staphylococcus aureus*. The names of these compounds and their chemical structures are found in Table 12.

Table 12. Names and chemical structures of biologically active compounds.

Compounds With Both Antibacterial and Antifungal Activity		
Number	Chemical Name	Chemical Structure
21	4-chloro-N-octylbenzamide	
35	3,4-difluoro-N-octylbenzenesulfonamide	
Compounds With Only Antibacterial Activity		
3	N-benzylbenzenesulfonamide	
10	N-benzylbenzamide	
11	4-chloro-N-(2-phenylethyl)benzamide	
13	N-(2-phenylethyl)benzenesulfonamide	
26	N-(2-phenyletheyl)benzamide	

After using combinatorial chemistry to produce the chemical compounds, analysis of the compounds indicated that impurities were present. Both compounds 21 and 35 contained octylamine as an impurity (64). Additional testing was done to determine if octylamine could inhibit the growth of the bacteria and fungi in this study. Initial testing was performed using undiluted octylamine (99.9% pure). All organisms that demonstrated inhibition with the undiluted octylamine were tested using 1:5, 1:10, and 1:100 dilutions of the octylamine. Table 13 shows the results of this testing.

Table 13. Zones of inhibition caused by dilutions of octylamine.

	Octylamine Dilution (Zone Diameter*)			
Organism	Undiluted	1:5	1:10	1:100
E. coli	10	0	0	0
K. oxytoca	10	0	0	0
S. aureus	10	0	0	0
Group B Streptococcus	0	-	-	-
C. albicans	0	-	-	-
C. tropicalis	0	-	-	-
C. parapsilosis	0	-	-	-

* Note: The disk size was 6 mm in diameter. If no zone was noted, the result was recorded as "0".

Although three of the organisms tested were inhibited by 99.9% pure octylamine, a 1:5 dilution (representing a concentration of 20%) resulted in the lack of inhibition. Although the purity of compounds 21 and 35 was not indicated in Alen Cusak's thesis, all other amides and sulfonamides tested had purities of 59-99%. It is very unlikely that the octylamine impurity reached a concentration of 20% in the tested compounds. As a result, it is unlikely that the small amount of octylamine in the samples affected the results of this study.

Replicate Testing

To ensure that the results for this study could be replicated, the testing was repeated for all agents that exhibited antimicrobial activity. The two agents that demonstrated both antibacterial and antifungal activity were tested in duplicate. Due to limitations in the remaining amount of the compounds and supplies, the agents that demonstrated only bacterial activity were repeated in singlet. The results of the original testing and the repeat testing are shown in Table 14. (Note: Compound 3 was not retested due to insufficient quantity of the compound.)

Table 14. Replicate testing of biologically active compounds.

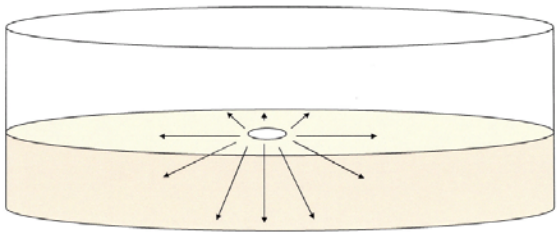
	Zone Sizes in mm*						
Compound **	*C.tropicalis*	*C albicans*	*C.parapsilosis*	Group B Streptococcus	*S. aureus*	*K. oxytoca*	*E. coli*
21 – O	16	15	14	11	11	15	14
21 – R	23	20	20	12	12	12	13
21 – R	22	20	18	12	12	12	13
35 – O	18	10	10	11	11	11	13
35 – R	23	21	18	13	11	12	13
35 – R	23	21	18	13	11	12	13
10 – O	0	0	0	0	9	0	0
10 – R	0	0	0	0	8	0	0
11 – O	0	0	0	0	7	0	0
11 – R	0	0	0	0	7	0	0
13 – O	0	0	0	0	8	0	0
13 – R	0	0	0	0	8	0	0
26 – O	0	0	0	0	12	0	0
26 – R	0	8	0	0	11	0	0

* Note: The disk size was 6 mm in diameter. If no zone was noted, the result was recorded as "0".
** O = Original Test R = Repeats

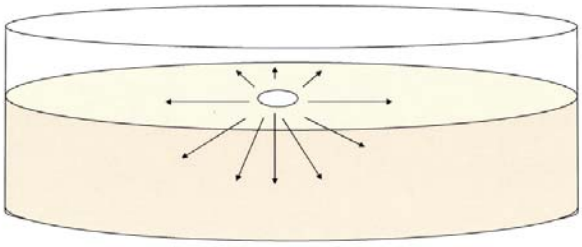
The repeat testing confirmed that each compound exhibited antimicrobial activity. It should be noted that the zones of inhibition for the bacteria when repeated were within 1 mm of the original results. There was only one exception to this finding. *Klebsiella oxytoca* when tested against compound 21 was originally 15 mm but was 12 mm upon

repeat testing. A less dense inoculum on the original test could have caused this variance from the original results for this organism. *Klebsiella oxytoca* produces a large capsule when it is grown on culture media. This causes the colonies to be mucoid and sticky and can make it difficult to separate the individual bacterial cells and obtain a homogeneous inoculum. If a larger inoculum size was used in the repeat experiment due to sticky clusters of the organism in the TSB, the zone sizes would have been smaller than the original test.

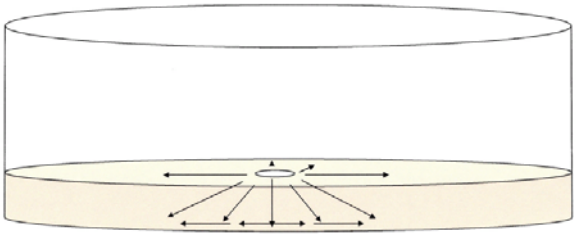
The yeast had a wider variation in the zones of inhibition than the bacteria on the repeats when compared to the original test. The most probable explanation for this difference is the thickness of the RPMI media that was used. It is known that when performing Kirby Bauer susceptibility testing, the thickness of the media can have an effect on the zone of inhibition. When the filter paper disk impregnated with a particular antimicrobial agent is placed on the media used for testing, the antimicrobial agent begins to diffuse out of the filter paper disk and into the agar in all directions away from the disk. In thin agar plates, the compound will reach the bottom of the media quickly and will start to diffuse laterally away form the disk, producing a high concentration of the agent relatively far away from the disk. In thicker agar, it takes longer for the agent to reach the bottom of the agar and causes a higher concentration of the agent near the disk (See Figure 13) (87). The difference in the concentration of the antimicrobial agent around the disk will result in a difference in the diameter of the zone of inhibition.



a. Appropriate thickness.



b. Too thick.



c. Too thin.

Figure 13. Effects of agar thickness when testing compounds.

Purchased media with strict quality control criteria was used when performing the bacterial testing. The RMPI media was made in the laboratory. When pouring the molten agar into the petri dishes, approximately 20-30 mL of agar was poured into each dish. However, this volume was estimated and not measured. This resulted in a variation of the media depth and a subsequent difference in zone sizes.

Toxicity Studies

Once it was determined which compounds had both antibacterial and antifungal activity, toxicity studies were performed. Some methods for performing toxicity testing that have been reported include the Sheep Erythrocyte Toxicity Assay and the Brine

Shrimp Toxicity Assay. In the Sheep Erythrocyte assay, toxicity is detected by hemolysis of the sheep red blood cells (86). In the Brine Shrimp assay, toxicity is detected and quantitated by the number of brine shrimp that die (76). For this research, compounds were tested to see if they were toxic to human and primate cells. The cells lines used for the toxicity studies included MRC-5, A549, and PMK. MRC-5 cells are derived from human lung fibroblasts and are typically used in a clinical laboratory for the cultivation of viruses from the Herpes group, such as Herpes Simplex Virus and Cytomegalovirus. A549 cells are derived from human epithelioid carcinoma and are used to detect respiratory viruses. PMK (or Primary Monkey Kidney) are cells taken directly from the kidneys of a sacrificed monkey. In the clinical laboratory, they are used to grow numerous types of viruses (88). In addition to observing cell lines that were inoculated with the test compounds, uninoculated control tubes for each cell line were observed to ensure the integrity of the cell line throughout the experiment.

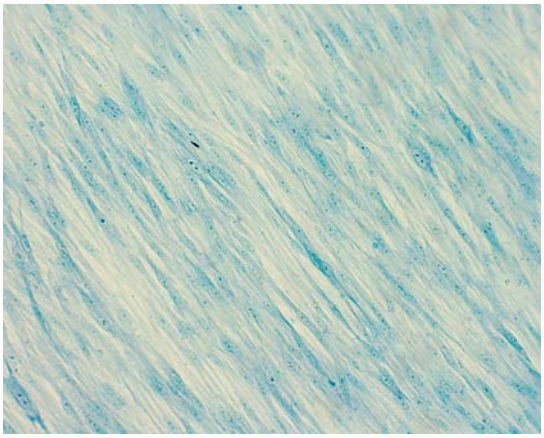
Compounds 21 and 35 were tested for toxicity. Table 15 shows the results of this testing.

Table 15. Toxicity of compounds that are biologically active against bacteria and yeast.

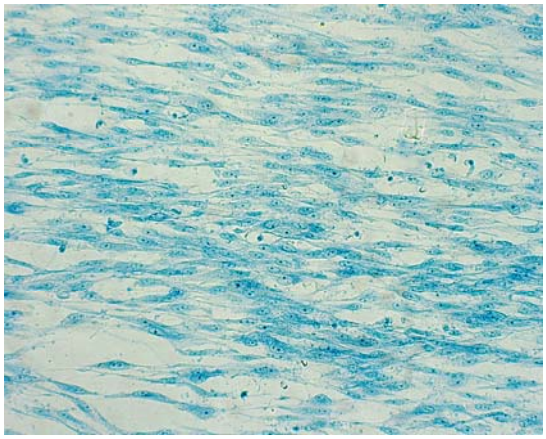
	MRC-5	A549	RMK
Compound 21	No toxicity after 24 hours but slight toxicity after 1 week.	No toxicity after 24 hours but slight toxicity after 1 week	No toxicity after 24 hours but slight toxicity after 1 week.
Compound 35	Toxicity at 24 hours	No toxicity after 24 hours but slight toxicity after 1 week	Toxicity at 24 hours
Control Tubes	No toxicity and normal appearance after 1 week	No toxicity and normal appearance after 1 week	No toxicity and normal appearance after 1 week

The toxicity was detected by cytopathic effect (CPE) noted in the cell lines. Compound 35 demonstrated severe toxicity in two cell lines after only 24 hours of

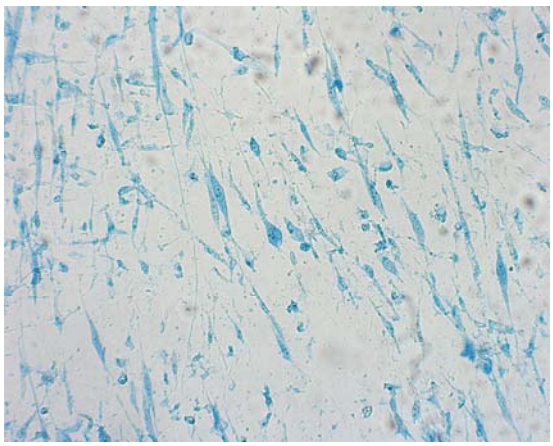
incubation. The CPE noted was total destruction of the cell monolayer with few remaining cells. Compound 21 demonstrated slight toxicity after one week of incubation. The CPE that was evident included rounding of some of the cells and some holes in the monolayer of cells. However, the cell monolayers were still intact. This indicated that though toxicity was present, it may not have been severe enough to kill the cells. Figure 14 shows the extent of cytopathic effect seen in the MRC-5 cells. Since the control tubes appeared normal throughout the length of incubation, the CPE was attributed to the toxicity of the compounds being testing and not degradation of the cell lines.



a. Normal – No Toxicity



b. Slight-Moderate Toxicity



c. Severe Toxicity

Figure 14. Toxicity seen for compound 21 on MRC-5 cells used in toxicity studies. a) Normal elongated fibroblasts. b) Slight toxicity demonstrating holes in the monolayer and rounding of some cells. c) Severe toxicity showing disintegration of the monolayer.

DISCUSSION

Chemical Compounds

The goal of this research was to use combinatorial chemistry to produce compounds that have both antibacterial and antifungal activity. The notion that agents may have both antibacterial and antifungal is not a new idea. Several agents have been described that exhibit activity against both groups of organisms. These include chandrananimycin, bonactin, and sulfonamides (74,75,76). However, none of these products were produced using combinatorial chemistry.

When making libraries of chemical compounds, molecules with structures similar to mefloquine (an antimalarial drug) were targeted. (Figure 15) The focus was on this drug because a study performed by Kunin, et al, tested a library of mefloquine-like compounds and found that some of the compounds had both antibacterial and antifungal activity (77).

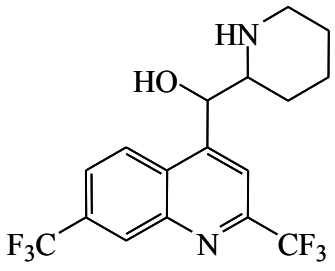
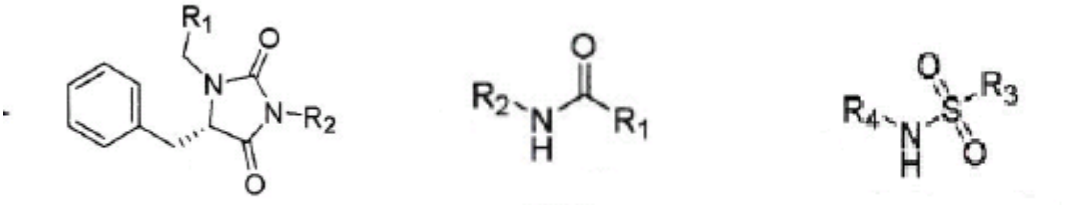


Figure 15. Structure of Mefloquine (77).

Various methodologies of solid-phase synthesis were considered when making the library. However, the "catch and release" method was the most attractive because it

required minimal purification of the final products. This method was used to make the amides and sulfonamides. The chemical constituents attached to the Wang resins were chosen based upon the cost of the constituents.

Libraries of hydantoins, amides, and sulfonamides were desired because of the biological activity of these groups of compounds. (See Figure 16 for molecular structure of hydantoins, amides, sulfonamides.) Hydantoins are a particularly important class of organic compounds because their five membered rings can be substituted to provide a great deal of variability. Derivatives of hydantoins have been identified as having antiviral, antibacterial, antifungal, herbicidal, anticonvulsant, antidiabetic, anti-inflammatory, and anti-ulcer activity (78). A number of amides and sulfonamides have been shown to exhibit antibacterial and/or antifungal activity. Some of these agents include cilofungin, novobiocin, nitazoxanide, sulfamethoxazole and a new drug for the treatment of *Helicobacter pylori* infections known as BAS-118 (79,80,81,82,83).



Hydantoin Amide Sulfonamide

Figure 16. Structures of hydantoins, amides, and sulfonamides (64).

Test System for Biological Activity

A number of methods have been described for detecting biological activity. Kleymann, et al, described a method in which the viability of Chinese hamster cells was

determined using fluorescein diacetate vital inclusion stain (84). In this method, cells were placed in a microwell tray and were infected with bacteria or fungi in the presence or absence of an antimicrobial agent. After incubation the viability of the cells was determined. The premise behind this approach is that infected cells will die and not take up the fluorescent dye. However, if the compound being tested is added to the cells and kills the infecting organism, the cells will remain viable and take up the dye. The amount of dye taken up is measured using spectrophotometry. The number of viable cells is proportional to the level of the fluorescent measurement.

Another type of bioassay was described by Ng, et al (85). In this assay, bacteria were inoculated to tubes of Mueller Hinton broth. Antimicrobial compounds were also added to the tubes and then they were incubated at 37^{o}C for 24 hours. The amount of growth or lack of growth was determined by measuring the optical density (OD) of the tube. If the compound added to the tube was active against the organism, the OD of the tube was lower than the OD of the bacterial growth control tube.

A method similar to the Kirby-Bauer method, known as the agar well diffusion method, was described by Aqil, et al (86). In this method, wells were punched in plates of Mueller Hinton agar. Test organisms were inoculated to the agar as described in this research. Test compounds were then added to the wells and incubated. The compounds diffused through the agar in a similar manner as described in the Kirby-Bauer method. Zones of growth inhibition were measured.

Although the methods described above would have been acceptable methods for determining bioactivity, in this research, a Kirby-Bauer-like method, employing a solid medium and looking for visible inhibition of growth, was used. A very small quantity of

each compound was available for testing. The Kirby-Bauer-like method required a very small amount of each compound while the other methods described would have required a larger amount. In addition, the supplies for the Kirby-Bauer-like method were readily available in the laboratory.

Toxiticity Testing

When performing toxicity testing, morphological changes occurred in the cells that indicated that toxicity was present. Compound 35 caused total destruction of the cells while compound 21 exhibited slight cellular changes. It was obvious that compound 35 caused cell death, but it was not known if the morphological changes caused by compound 21 resulted in cell death. Since only slight cytotoxic effects were seen, it was possible that the cells were still viable. Although viability of cells was not determined in this research, it would have been beneficial to make this determination to assess the potential for developing the compound into a marketable antimicrobial agent. The usefulness of an antimicrobial agent that causes cytotoxic activity while maintaining viability of the cells would be much greater than an antimicrobial agent that causes cell death. Vital stains, such as trypan blue, erythrosine B, and fluorescein diacetate may be used to establish viability of cells (103). Trypan blue and erythrosine B are vital exclusion stains while fluorescein diacetate is a vital inclusion stain. Exclusion stains mark only dead cells that have lost membrane integrity. Inclusion stains color vital cells that retain membrane integrity.

In this research, MRC-5, A549, and PMK cell lines were used for toxicity testing because they were readily assessable in the laboratory. However, it would have been prudent to broaden the extent of testing by using additional cell lines. In particular, renal

and hepatic cells might have allowed detection of toxicity to the kidneys and liver. Since nearly all antimicrobial agents are either excreted by the kidneys or metabolized by the liver, drugs tend to exist in high concentrations in these organs and are the sight of toxicity in some current antimicrobial agents. The use of other cells lines could also allow detection of organ-specific toxicity if it was noted that toxicity was seen in some types of cells and not in others.

Although using tissue culture cells in a laboratory setting may give an indication of whether toxicity is present or not, there are limitations to using this method. A number of factors may affect the ability to detect toxicity. These include age of the cell monolayer, testing temperature, and mechanical stress from the rotating drum used to incubate cells. In addition, some substances that are not generally considered toxic may cause toxicity in human and monkey cell lines. For example, human blood cells and urine may cause toxicity to these cells. Another complicating factor is that many cells that are available for testing from cell-line vendors are immortal cell lines (i.e., cancer cells). These cells are abnormal and may not react the same as normal cells in the human body. As a result, further testing is required to obtain a more definitive indication of the presence of toxicity. Toxicity testing in an animal model is superior to testing in cell culture because it allows the detection of toxicity in a living organism. This simulates the environment in which the compound would be used and allows long term toxicity testing to be performed.

Biological Activity of Compounds

The two compounds in this research with both antibacterial and antifungal activity were members of the amide and sulfonamide groups and had similar chemical structures

(See Table 12). Due to the presence of a benzene ring in each compound, they were more specifically identified as a benzamide and a benzenesulfonamide. Since the mechanism of action for these compounds was unknown, it was speculated that the molecular activity may be elucidated by evaluating the properties of similar compounds. A literature review identified the mechanism of action for a number of benzamides, sulfonamides, and benzenesulfonamides with antimicrobial activity. The results of this review are found in Table 16.

Table 16. Antimicrobial Activity of Benzamides, Sulfonamides, and Benzenesulfonamides

Compound Group	Biological Activity
Benzamides	• Inhibits DNA gyrase and topoisomerase activity (82) • Inactivates glucan synthesis, prohibiting the production of a fungal cell wall (81) • Interferes with anaerobic metabolism (89) • Prohibits cell division by inhibiting post-translational regulation of genes (90)
Sulfonamides	• Inhibits folic acid synthesis, interfering with production of nucleotides and amino acids (91)
Benzenesulfonamides	• Inhibits topoisomerase activity (92)

In addition, a literature search looking for the possible cytotoxic effects of benzamides, sulfonamides, and benzenesulfonamides in mammalian cells was performed. The results of this review are found in Table 17.

Table 17. Activity of Benzamides, Sulfonamides, and Benzenesulfonamides in Mammalian Cells

Compound Group	Biological Activity
Benzamides	• Inhibits DNA repair and synthesis (93) • Induces apoptosis (94,95) • Inhibits protein kinases (96)
Sulfonamides	• Effects acid-base balance in blood by inhibiting carbonic anhydrase (97) • Acts as an antagonist of folic acid (91)
Benzenesulfonamides	• Represses transcription and gene expression (98,99) • Interferes with prostaglandin production (100) • Antagonist of β-3-adrenergic receptor which is responsible for "fight-or-flight" response (101) • Antagonist to vasoconstrictor (102)

These results demonstrate that numerous types of biological activity are exhibited by benzamides, sulfonamides, and benzenesulfonamides. Another literature search was done in an attempt to further refine the results of the original search. Since each compound had an eight-carbon chain attached to the amide and sulfonamide, a search for the biological activity of octylbenzamide and octylbenzenesulfonamide was performed. This search yielded no information. As a result, it is impossible to speculate the exact mode of antimicrobial or cytotoxic activity. Further testing would be required to make this determination.

Limiting Factor

Two limiting factors in this study were the small amount of each of the compounds that was received and the lack of purity data. The small quantity received prevented the performance of an adequate number of repeat tests. It also prevented additional studies that would have allowed further characterization of the compounds. For example, if more sample of known purity had been available, additional testing would have been performed to quantify the level of antimicrobial activity and cytotoxic

effects. It would have been possible to determine the minimum concentration of the compounds that inhibited each organism and the minimum concentration that caused cytotoxicity. If the concentration required for microbial inhibition was greater than the concentration required for cytotoxicity, the compound would not have been a useful drug. Toxicity would have occurred before an adequate concentration was achieved to inhibit the organism. However, if the concentration required for microbial inhibition was less than the concentration required for cytotoxic effect, the compound would still have promise as a potential antimicrobial agent.

Conclusion

The original hypothesis for this research was that combinatorial chemistry may be used to produce agents that have both antibacterial and antifungal activity. This research confirmed that it is possible to use combinatorial methods to produce agents with these characteristics. However, the ability to produce antimicrobial compounds is only a small part of producing a useful drug. Other key components of drug discovery, such as toxicity studies and the ability to produce the compound in large quantities, are also necessary to bring a drug to market and continue the fight against infectious diseases.

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Appendix A

Table A1. Supplies

Item Name	Vendor	Catalog Number
Mueller Hinton agar	Becton Dickenson	B21800
Mueller Hinton agar with sheep blood	Becton Dickenson	B21801
RMPI 1640 powder	Sigma-Aldrich	R7755
Bacto agar	Beckton Dickenson	DF0140-15-4
Sterile petri dishes	Fisher Scientific	08-757-12
Sabarouds Dextrose Agar	Becton Dickenson	B21180
MOPS buffer	Sigma-Aldrich	M3183
Sodium hydroxide solution, 1 molar	Fisher Scientific	S318-500
pH paper	Cardinal Scientific	P1120-8
Amphotericin Powder	Bristol-Myers Squibb	NA
Fluconazole Powder	AstraZeneca	NA
Ciprofloxacin Powder	Bayer Corporation	NA
Filter paper disk	Becton Dickenson	231039
MLA pipette, 10 µl	Fisher	21-381-500
MLA pipette, 20 µl	Cardinal Scientific	P5065-20
MLA pipette, 30 µl	Cardinal Scientific	P5065-30
MLA pipette, 100 µl	Fisher	21381506
MLA pipette, 200 µl	Fisher	21381507A
Staphylococcus aureus (ATCC strain 25923)	Fisher Scientific	23-021104
Streptococcus agalactiae	Patient isolate	NA
Escherichia coli (ATCC strain 35922)	Fisher Scientific	4337015
Klebsiella oxytoca (ATCC strain 49131)	Fisher Scientific	23-021210
Sterile swab	Fisher Scientific	495996C
TSA agar with 5% sheep blood	Becton Dickenson	B21261X
Turbidity meter	Dade-Behring	B1018-66
DMSO	Sigma-Aldrich	D2650
Crystal violet stain	Becton Dickenson	B12526
Disposable pipette tips	Cardinal	P5048-40
Cell culture maintenance medium	Diagnostic Hybrids	RM-02
MRC-5 cells	Diagnostic Hybrids	51-0600
A549 cells	Diagnostic Hybrids	56-0600
RMK cells	Diagnostic Hybrids	49-0600
Rotating drum	Lab Line Instruments	Model 1645
Ethanol, 95%	Mallinckrodt	7018-4
Autoclave	Brinkman	Model 2540M