Preclinical Antimicrobial Drug Discovery:

Development and Evaluation of a Platform for High-Throughput Screening *in vitro* and an Immunocompromised Animal Model

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Abstract

The incidence of infections caused by antibiotic-resistant bacteria and fungi is rising rapidly. Once considered as little more than a nuisance, antibiotic resistance has become a serious threat. The mortality rate for some infections is approaching that of the pre-antibiotic era. New antimicrobials are needed urgently. Prior to the introduction of any new antimicrobial, comprehensive toxicity and efficacy profiles are assessed in preclinical studies. This thesis focuses on two key stages of preclinical antimicrobial drug development, specifically compound screening *in vitro* and animal efficacy testing *in vivo*. We developed a sensitive colorimetric platform with high-throughput capacity for the rapid screening of candidate antimicrobials. This platform could be adapted to assess compounds targeting a range of bacteria, fungi (such as *Candida albicans*), and protozoan parasites (such as *Leishmania major*). When this assay was modified to measure minimum inhibitory concentrations (MICs) for bacteria, 100% agreement within one dilution was achieved compared to the gold-standard method. A novel antifungal compound was taken forward to animal testing in an immunocompromised mouse model. We demonstrated herein that a histone deacetylase inhibitor in combination with an imidazole can synergise to produce a potent antifungal effect. A dose-dependent response, defined as a lower fungal burden and a higher survival rate, was achieved with increasing concentrations of the novel inhibitor.

Résumé

L'incidence des infections provoquées par les bactéries et les mycètes résistants aux antibiotiques ne cesse d'accroître. Une fois considéré en tant qu'un petit ennui, la résistance aux antibiotiques est devenue une menace sérieuse. Le taux de mortalité pour certaines infections est comparable à l'ère précédant l'avènement de l'antibiotique. Le besoin urgent de nouveaux antimicrobiens est devenu réel. Avant l'introduction d'un nouvel antimicrobien, la compréhension de la toxicité et des profils complets de l'efficacité sont évalués dans des études précliniques. Cette thèse se focalise sur deux étapes principales de développement de médicaments antimicrobiens précliniques, le criblage spécifique de composés *in vitro* dans un premier temps et l'efficacité du produit chez l'animal dans un deuxième temps. Nous avons développé une plate-forme colorimétrique sensible de haute capacité pour le criblage rapide des candidats antimicrobiens. Cette plate-forme pourrait être adaptée pour évaluer des composés visant une gamme de bactéries, de mycètes (tel que *Candida albicans*), et des parasites protozoaires (tel que *Leishmania major*). Quand cette analyse a été modifiée pour mesurer des concentrations inhibitrices minimales (CIMs) pour des bactéries, l'accord à 100% à moins d'une dilution a été réalisé comparé à la méthode de référence. D'ailleurs, un nouveau composé antifongique a été testé chez des souris immunodéprimées. Nous avons démontré ci-dessus qu'un inhibiteur de déacétylase d'histone en combinaison avec un imidazole peut avoir un effet synergique afin de produire un effet antifongique efficace. Une réponse dépendante de la dose, définie comme une charge fongique inférieure et un taux de survie plus élevé, a été réalisée avec l'augmentation des concentrations du nouveau inhibiteur.

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Contribution of Authors

Manuscript I

- Bill Lee:
  - conceptualized and executed all experiments
  - collected and analyzed data from all experiments
  - wrote the manuscript
- Momar Ndao provided guidance
- François Sanschagrin provided *Escherichia coli* JM101/pNU359 and assistance in data analysis
- Roger C. Lévesque was a co-investigator for this work:
  - provided sulbactam and clavulanic acid
  - provided advice
- Jeffrey Besterman provided the experimental compounds for screening
- Brian J. Ward was the Principal Investigator:
  - provided guidance
  - edited the manuscript

Manuscript II

- Bill Lee:
  - conceptualized and executed all experiments
  - collected and analyzed data from all experiments
  - wrote the manuscript

- Momar Ndao assisted during experimental design, IV drug administrations in mice and data analysis
- Nafsika Georgopapadakou and Jeffrey Besterman provided MG 3290 and input for experimental design
- Brian Ward was the Principal Investigator,
    - provided guidance
    - edited the manuscript

Abbreviations

ABC	ATP (adenosine tri-phosphate)-binding-cassette
ADME	absorption, distribution, metabolism, and elimination
ADMET	administration, distribution, metabolism, elimination and toxicity
ATCC	American Type Culture Collection
AUC	area under the curve
CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DMSO	dimethyl sulfoxide
DNA	deoxy-ribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ESBL	extended-spectrum β–lactamase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACI	histone deacetylase inhibitor
HIV	human immunodeficiency virus
HTS	high-throughput screening
IND	investigational new drug
INT	4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride
LC/MS	liquid chromatography/mass spectrometry
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium inner salt
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NBT	nitrobluetetrazolium
NCCLS	National Commmittee for Clinical Laboratory Standards
OD	optical density
OD_{450}	optical density at 450 nm
$OD_{biomass}$	optical density values from the susceptibility assay using MHB alone
OD_{XTT}	optical density from the XTT assay

PBP	penicillin binding protein
PD	pharmacodynamics
PK	pharmacokinetics
PMS	phenazine methosulfate
QSAR	quantitative structure-activity relationship
RODU	relative optical density unit
RPM	Revolutions Per Minute
SAR	structure-activity relationship
SDA	Sabouraud dextrose agar
SEM	standard error of the mean
STC	2,3-Diphenyl-5-thienyl-(2)-tetrazolium chloride
TCA cycle	The tricarboxylic acid cycle
TNBT	tetranitrobluetetrazolium
TSA	trichostatin A
TTC	2,3,5-triphenyltetrazolium chloride
VRE	vancomycin-resistant enterococci
VRSA	vancomycin-resistant *Staphylococcus aureus*
WST-1	4-(3-4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)- 1,3-benzenedisulfonate
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

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Section I: LITERATURE REVIEW

Chapter 1: Antibiotic resistance among bacteria and fungi

Antibiotic resistance may be defined as the absolute or relative insensitivity of a microorganism to a natural or synthetic substance that normally kills or restricts growth. With Alexander Fleming's serendipitous discovery of penicillin as a so-called "contaminant" on plates inoculated with *Staphylococcus aureus* in 1928, a process was started that would have a profound impact on humankind (54). When penicillins were introduced for clinical use in the 1940s, life expectancy steadily rose while leading causes of death including pneumonia and bacterial meningitis fell proportionately (5). Following the introduction of nystatin, the first major antifungal agent, in 1949 (22) and subsequently amphotericin B, azoles, and other antimycotics, similar improvement in outcomes were achieved against candidemia, aspergillosis and other fungal infections. The tremendous success of antibiotic therapy created a sense of victory. In 1969, the US Surgeon General of the time, William Stewart, declared that it was time to "close the books on infectious diseases" (110). Although this statement was certainly premature, pharmaceutical companies nonetheless heeded the call. Antibiotic-related investigational new drug (IND) applications rapidly fell to a trickle through the last one-third of the 20th century. Meanwhile, in true Darwinian fashion, many pathogenic microorganisms evolved and adapted to the selective pressure exerted by antibiotics. As a consequence, many "old" pathogens are now once more serious threats. Indiscriminate and excessive use of antibiotics in human care and agricultural settings has catalyzed this phenomenon (119,130). If novel therapies are not introduced in the coming years, the continued emergence and dissemination of resistance mechanisms between pathogenic

microorganisms may thrust mankind back to the frightening conditions of the pre-antibiotic era.

The following describes the clinical impact of antibiotic-resistance and the mechanisms of resistance employed by bacteria and yeasts of the *Candida* genus. Two strategies for overcoming resistance – one well-established; the other with great potential – are also discussed.

1.1 Clinical significance

Antibiotic resistance has created a unique set of challenges in the clinical setting. Complications arising from infections by drug-resistant pathogens result in extended hospital stays, increased morbidity and mortality, and greater treatment costs. Some infections, such as multi-drug resistant tuberculosis, require supervised treatment (175). Though a comprehensive discussion of the clinical importance of antibiotic resistance is beyond the scope of this thesis, the following examples illustrate the impact of resistance, primarily from a Canadian perspective.

Methicillin-resistant *S. aureus* (MRSA) is commonly associated with pneumonia, abscesses, skin and wound infections, bronchitis, furuncles, and osteomyelitis (1). The incidence of MRSA jumped from <3% to nearly 40% during the 1980s in hospitals in the United States and Europe (131,176). First appearing in Canada in 1981 (99), the number of patients infected by MRSA increased 8-fold between 1995 and 1999 (159). One particular strain of MRSA contributed significantly to the spread. Although vancomycin is typically used to treat MRSA infections, vancomycin-resistant *S. aureus* (VRSA) has been reported (12). Treatment options are extremely limited for VRSA infections.

Vancomycin is a potent glycopeptide antibiotic that was once used as a last line of defence against resistant bacteria. Until 1989 in the United Sates, there were no reports of vancomycin-resistant enterococci (VRE). However by 1994, reports of patients infected by VRE in intensive care units reached almost 14% (28). Treatment of VRE infections with newer antibiotics is difficult as a result of emerging resistance and toxicity (179).

Diseases resulting from pneumococcal infections include sinusitis, meningitis, otitis media, bacteremia, and pneumonia (1). In Canada, pneumococcal isolates that were insensitive to penicillin increased dramatically from the late 1980s until 1994 (34), but stabilized between 1994 and 2000, at approximately 12.7% (100). However, an increase in macrolide resistance from 2.8% to 11.6% occurred during the latter period. A combination of antibacterials commonly is prescribed for pneumococcal infections, though the efficacy of this strategy to reduce mortality has been called into question (66).

Candida spp. represents the fourth most commonly isolated pathogen from blood cultures (132). Azole antifungals such as fluconazole and itraconazole are effective inhibitors of fungal growth, including that of *Candida* spp. A recent Canadian study showed that 30% of isolates tested had increased resistance to fluconazole (89). Antifungals available for the treatment of azole-resistant infections are exceedingly limited. Moreover, the 40% mortality rate for those infected by *Candida* species underscores the severity of this infection. Of particular concern was one isolate identified in this survey that was resistant to amphotericin B, a drug widely considered as the "gold standard" of antifungal treatments. Amphotericin B is equally considered a drug of last resort because of the severe adverse effects it may induce.

1.2 Antibiotic resistance among bacteria

1.2.1 Mechanisms of resistance

Resistance mechanisms can be grouped into four broad categories. These include: 1) mutation of the drug target, 2) target protection, 3) drug exclusion and evacuation via porins and efflux pumps, and 4) enzymatic degradation (Fig. 1). A drug-resistant microorganism may express any combination of these mechanisms.

Mutation of the drug target

Quinolone resistance is an example of resistance arising from drug target mutation. Quinolones, such as ciprofloxacin and nalidixic acid, target bacterial DNA gyrase and topoisomerase IV. DNA gyrase produces negative supercoils to offset the positive supercoils produced during DNA transcription or replication. Topoisomerase IV decatenates daughter chromosomes following replication. Studies have shown that the primary targets of quinolones are topoisomerase IV in Gram-positive bacteria and DNA gyrase in Gram-negative bacteria (74,83). Quinolone antibiotics act by binding to the DNA gyrase - topoisomerase IV complex, thus preventing DNA re-ligation. Drug resistance occurs following multiple mutations in the genes encoding the two target enzymes. In Gram-positive bacteria, a single point mutation in *parC* confers low-level resistance; an additional point-mutation occurring in *gyrA* results in high-level resistance. In Gram-negative bacteria, two mutations in *gyrA* followed by two mutations in *parC* induce resistance to quinolones. Differences in the primary drug target in the various bacterial species are thought to be the result of the higher-resistance of DNA gyrase in Gram-positive bacteria than the same enzyme in Gram-negatives (67).

Drug target protection

Tetracycline resistance is a good example of drug target protection. In this case, the bacterium produces an enzyme that physically removes the drug from its target, the ribosome. Tetracyclines act by binding to the A-site of ribosomes, thus inhibiting translation and protein synthesis. There are two possible mechanisms by which tetracyclines may arrest translation: either through steric hindrance (21,138) or by inducing conformational changes (35,36,123,166) within the ribosome A-site. Various bacteria have evolved at least two enzymes that can remove tetracycline from ribosomes to restore protein translation, namely tet(O) (an elongation-factor-like protein) and tet(M) (25,165).

Drug exclusion and efflux

This mechanism involves the modification of the bacterial membrane to reduce drug permeability and uptake or the upregulation of genes encoding efflux pumps. The multiple antibiotic resistance (*mar*) regulon, found in drug–resistant *Escherichia coli* and other members of the Enterobacteriaceae family, controls a series of genes that code for regulatory proteins. Located on the bacterial chromosome, the *mar* regulon can be activated by a diverse set of triggers including sodium salicylate, tetracycline and chloramphenicol (13,31,65,157). Once triggered, MarR (a repressor) becomes inactivated. This induces the overexpression of MarA (an activator) which downregulates porin OmpF synthesis (8), causing a reduction in drug up-take, and upregulates the AcrAB-TolC multidrug efflux system (8), which rapidly pumps out "toxins" such as

antibiotics (55,126). This mode of resistance not only confers insensitivity to antibiotics, but to disinfectants (107,112), organic solvents (8,182), and oxidative stress agents (13).

Enzymatic degradation

Enzymatic degradation involves the chemical alteration of an antibiotic such that it is rendered inactive. ß-lactamase hydrolysis of penicillins is the classic example of this mechanism of resistance. Composed of cross-linked peptidoglycan chains, the cell wall is essential in maintaining cell integrity and shape. The transpeptidases and carboxypeptidases involved in cross-linking the peptidoglycan chains are the targets of ß-lactam antibiotics. The peptidases are therefore collectively referred to as the penicillin binding proteins (PBPs). By binding these enzymes, ß-lactams disrupt cell wall synthesis. This ultimately destabilises the cell wall and triggers autolysins (specifically, peptidoglycan hydrolases) to induce autolysis (92). β-lactamases, which may have evolved from a common ancestor with the peptidases (62,105), confer resistance by binding ß-lactam antibiotics and hydrolysing the amide bond of the ß-lactam ring, the core structure characterising all penicillins (Fig. 2). The inactivated drug is then released, thus permitting catalysis of additional ß-lactam molecules.

In Gram-positive bacteria, β-lactamases are secreted into the extracellular environment while in Gram-negatives, the enzymes are located in the periplasmic space. Genes that encode β–lactamases may be found on the chromosome (84), on plasmids (101), and on mobile genetic elements such as transposons (75) and integrons (173). The dissemination of β-lactamase resistance within and between species is mediated by plasmids and mobile genetic elements.

Extended-spectrum β–lactamases (ESBLs) are of particular clinical concern. Over the past 25 years, different classes of β–lactam antibiotics have been developed to be more resilient to β–lactamase hydrolysis in order to circumvent resistance. However, bacterial ESBLs adapted in parallel and are capable collectively of hydrolysing virtually all prescribed β–lactams. As a consequence, the choice of effective drugs can be exceedingly limited when confronted by infection by ESBL-harbouring pathogens.

β–lactamases will be discussed in greater detail as these molecules were the principal targets in our first manuscript.

1.2.2 Classes of ß-lactamases

As a consequence of the disparate substrate profiles of different β–lactamases, simple identification of a pathogen infecting a patient may not be sufficient for effective treatment. It is frequently necessary to determine the type or types of β–lactamase(s) harboured within the isolate. This can be achieved by antibiotic susceptibility profiling using a range of technologies including both manual (such as the E-test) and automated systems (such as VITEK and Phoenix) (45,51).

At the time of writing, 532 varieties of β–lactamase enzymes have been identified from clinical isolates (71). ß-lactamases are categorized according to the Ambler (4) and Bush-Jacoby-Medeiros schemes (26). In the former system, enzymes are organized by sequence homology whereas the Bush-Jacoby-Medeiros system focuses on enzyme substrate profiles (Table 1). This thesis will discuss ß-lactamases according to the Ambler classification system.

Class A ß-lactamases

Also known as the penicillinases, class A enzymes are the most frequently encountered in the clinic and are certainly the best studied. Class A ß-lactamases have an active-site serine residue that mediates ß-lactam hydrolysis, a feature shared with class C and D enzymes. The spread of this diverse class of hydrolytic enzymes has been greatly facilitated by their location on plasmids and transposons. Several classes of β–lactam antibiotics (such as the isoxazolyl penicillins and the second and third-generation cephalosporins) were designed to evade hydrolysis by class A enzymes. In Gram-negative bacteria, most class A variants are derived from the TEM and SHV proteins (147).

The first plasmid-mediated β–lactamase isolated from a clinical isolate of *E. coli* in 1960 (38). The patient's name was Temoniera, hence this first β-lactamase was called "TEM" (108). TEM family enzymes are constitutively expressed, thus their impact varies primarily according to the plasmid copy number within the bacterium. SHV (sulphydryl variable) hydrolases can be both plasmid-mediated (in *E. coli*) and chromosomally encoded (in *Klebsiella pneumoniae*). Although most class A enzymes are plasmid-mediated, all of the SHV variants are classified within class A enzymes since they were first discovered on plasmids in *E. coli*. SHV enzymes typically do not hydrolyze third generation cephalosprins sufficiently to protect bacteria, but when highly expressed, resistance can occur. Many variants of TEM and SHV proteins have been identified as ESBLs (147).

Class B ß-lactamases

First discovered as chromosomally-encoded enzymes in *Bacillus cereus* (152), the class B ß-lactamases attracted little research attention initially since no other bacteria were found to harbour them. However, their subsequent transfer on to plasmids and mobile genetic elements has resulted in their rapid dissemination and greater scientific respect. These metallo-enzymes are now found in many bacteria including *Pseudomonas aeruginosa* (178), *Bacteriodes fragilis* (144), *Serratia marcescens* (127), and *K. pneumoniae* (124). This class is further divided into 3 subgroups based on sequence dissimilarities (56). The most commonly known class B enzymes belong to subgroup B1, namely the ß-lactamase II (BcII) enzymes from *Bacillus* spp. (76,79) and the VIM and IMP proteins from *P. aeruginosa* (88,90). Subgroup B2 includes enzymes identified from *Aeromonas* spp. while subgroup B3 includes FEZ-1, THIN-B and the L1 and GOB proteins. Subgroups B1 and B3 demonstrate potent hydrolytic activity against almost all β-lactams including cephamycins and carbapenems. In contrast, B2 enzymes are effective only against imipinem and ampicillin (147).

Unlike the serine β–lactamases, class B enzymes contain 2 Zn^{2+} ions positioned in the active site, hence metallo-enzyme (32). These enzymes remain active when the Zn^{2+} ions are substituted with Co^{2+} or Cd^{2+} (39) and are inactivated in the presence of EDTA, a chelating agent. Unlike the β–lactamases of other classes that hydrolyse β–lactams using a water molecule associated with a residue in the active site (the general base), it has been proposed that an OH^- group associated with either one or both metal ions acts as the general base in class B enzymes (33).

Class C ß-lactamases

Currently, class C hydrolases are second only to the class A enzymes in clinical importance. These cephalosporinases are capable of inactivating all three generations of cephalosporins. Class C ß-lactamases are exclusively produced in Gram-negative bacteria and were found initially in *Morganella morganii*, *Enterobacter cloacae*, *Citrobacter freundii, S. marcescens* and *P. aeruginosa* (147). However, it has been reported that virtually all Gram-negatives express class C enzymes to some extent. In the past, these enzymes were predominantly chromosomally-encoded, which limited their spread. However, the incidence of infections caused by class C ß-lactamase–expressing bacteria sharply increased following their migration to plasmids. Plasmid-based class C expression has been detected in *K. pneumoniae*, *Klebsiella oxytoca E. coli*, *Proteus mirabilis*, and *Salmonella* spp. (147).

Class D ß-lactamases

Of the four classes of ß-lactamases, the class D enzymes (the oxacillinases) are the least understood at the molecular level. This lack of understanding is not a reflection of their importance, as class D enzymes are capable of hydrolysing the latest generation ß-lactams including oxacillin, cloxacillin, and carbapenems. Moreover, as the genes encoding oxacillinases are found on plasmids and integrons of Gram-negative bacteria, class D-mediated resistance is spreading rapidly. Fortunately, oxacillinases are not as prevalent as the other three classes at this time. OXA-1 is the most common variant isolated from members of the Enterobacteriacae family and *P. aeruginosa* (23,120).

Interestingly, studies have shown that these enzymes function more efficiently as homodimers rather than in their monomeric form (37).

1.2.3 Inhibitors of ß-lactamase enzymes

Two strategies have been employed to circumvent ß-lactamase activity. One of these, mentioned above, involves the development of ß-lactams poorly recognized by ß-lactamases, such as methicillin and imipenem. The other strategy involves developing compounds that directly inhibit the bacterial enzymes by irreversible covalent binding (also known as suicide inhibition). Clavulanic acid, sulbactam, and tazobactam are licensed ß-lactamase inhibitors acting via this latter strategy.

Current ß-lactamase inhibitors have relatively weak intrinsic antibacterial activity and are consequently paired with a ß-lactam. The first ß-lactam - ß-lactamase inhibitor formulation consisted of amoxicillin and clavulanic acid (145). Discovered in 1977, clavulanic acid is naturally produced by the Gram-negative bacterium *Streptomyces clavuligerus*. Together, the compounds act synergistically to inhibit the growth of many ß-lactamase-expressing bacteria including: *Neisseria gonorrhoeae*, *Proteus mirabilis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Bacillus fragilis*, and *K. pneumoniae*. Shortly after the development of clavulanic acid, sulbactam and tazobactam were introduced by Pfizer and Wyeth Pharmaceuticals respectively. The three inhibitors are structural analogues of the ß-lactam antibiotics (and hence contain a ß-lactam ring). Additionally, all three compounds target class A enzymes, though tazobactam is known to have moderate activity against class C and D ß-lactamases (59). None is effective against the class B metallo-enzymes (20).

Following the same fate as virtually all other antibiotics, resistance has developed against the ß-lactamase inhibitors. For clavulanate, evidence of resistance was documented even prior to its approval for clinical use (146): ß-lactam – ß-lactamase inhibitor-resistant mutants can easily be generated in the laboratory (44). Through the early 1990s, resistance to clavulanate (and the whole class of inhibitors by association) arrived in the clinic and the frequency of reports of resistant isolates increased (172). Resistance is conferred as a result of specific mutations of active site residues of TEM and SHV ß-lactamases (29,128). For example, the mutation of Asn-276 to Asp in SHV-5 results in a ten-fold increase in the IC_{50} of clavulanate and tazobactam and a 5.5-fold increase for sulbactam (61). These mutations increase the activity against the vulnerable ß-lactam ring of the inhibitor itself (53). Another resistance mechanism involves the induction of ß-lactamase hyperexpression by clavulanic acid (114). An increase in ß-lactamase expression not only causes more efficient ß-lactam hydrolysis, but also proportionately increases the inhibitor concentration needed to inactivate enzyme activity. Sufficiently high doses to inhibit the growth of these isolates often cannot be achieved safely for the various ß-lactam - ß-lactamase inhibitor combinations (20).

1.2.4 New molecular entities for inhibiting ß-lactamase activity

Several new classes of compounds that target the ß-lactamases are currently in preclinical development. Among the most promising are the phosphonate-based compounds (80-82) that can bind all serine ß-lactamases, specifically classes A, C, and D (2). As the core structure of phosphonates does not feature a ß-lactam ring, these compounds are less susceptible to the hydrolytic action of ß-lactamases. Molecular

modeling data have shown that the cyclic phosphonate derivatives inhibit ß-lactamases most effectively. Once a cyclic derivative binds a ß-lactamase enzyme through a phosphonylation reaction, conformational changes are induced that impede the hydrolysis of the substrate. The phosphonate-enzyme complex remains stable and the enzyme is therefore inhibited. Phosphonates are not significantly hydrolysed by mammalian hydrolases, specifically acetylcholinesterase and phosphodiesterase I (80). These compounds may therefore exhibit good pharmacokinetic activity *in vivo*. The development of a high-throughput screen to identify novel ß-lactamase inhibitors is the subject of the first manuscript (chapter 5).

1.3 Antibiotic resistance among fungi

1.3.1 Mechanisms of resistance

Drug resistance by fungi may be classified as primary or secondary. Primary resistance means that the pathogen is intrinsically resistant to the antifungal irrespective of prior exposure. For example, *Candida krusei* isolates are naturally resistant to fluconazole (170). Secondary resistance is acquired and develops following exposure to antifungals. The proposed mechanisms for both primary and secondary resistance are identical, occurring either through reduced drug accumulation via multi-drug transporters and efflux pumps or through mutations of genes encoding the target enzymes involved in ergosterol biosynthesis.

Drug efflux

Similar to bacteria, fungi can also become resistant to antimicrobials by upregulation of drug transporters and efflux pumps. This is the principal mechanism by which antibiotic resistance is achieved in fungi (135). In *C. albicans*, the *CDR1* (141) and *CDR2* (155) genes code for ATP-binding-cassette (ABC) transporters (Fig. 3). The upregulation of *CDR1* and *CDR2* results in an increased density of transporters on the cell membrane. Resistance to many azole antifungals is conferred via this mechanism. Resistance may also be mediated by the *MDR1* gene product. *MDR1* is a member of the major facilitator (MF) superfamily of transporters. Interestingly, the upregulation of *MDR1* causes resistance only to fluconazole, suggesting that this transporter exhibits substrate specificity (154). *Candida* spp. possess a sensory system to detect the presence of antifungals. Triggering this system results in *CDR* and *MDR* upregulation (85).

Gene mutations

Mutations in genes encoding enzymes involved in ergosterol biosynthesis can also contribute to antifungal resistance, specifically *ERG11* (coding for 14α-lanosterol-demethylase) and *ERG3* (coding for $\Delta^{5,6}$-desaturase) (Fig. 4). Analogous to cholesterol in mammalian cells, ergosterol is essential in fungi for stabilising the cell membrane. As human cells do not produce this sterol, targeting ergosterol itself and the enzymes involved in its synthesis are effective strategies for inhibiting fungal growth without inducing severe side effects (136).

The specific target of all azole-based antifungals is the cytochrome P-450 enzyme 14α-lanosterol-demethylase. When this enzyme is inhibited, lanosterol and other sterol

intermediates accumulate on the cell membrane. The ratio of ergosterol to sterol intermediates decreases and ultimately compromises membrane stability. The membrane instability in turn may affect membrane-associated proteins involved in nutrient transport and chitin synthesis (60,137,170). Resistance conferred by mutations in *ERG11* produce catalytically functional enzymes that demonstrate a reduced affinity for azoles. From studies of clinical isolates of azole-resistant *C. albicans*, it has been proposed that the mutations do not occur on residues within the active site (139). Instead, they occur in other regions of the enzyme that mediate conformational changes that take place during drug binding and release.

Potent azole resistance also may result from mutations that render $\Delta^{5,6}$-desaturase non-functional (135). It has been suggested that this allows the accumulation of less toxic sterol intermediates (78). Though fungal cells expressing defective *ERG3* are viable, virulence is attenuated (30). This likely accounts for the rare occurrence of this mutation in the clinical setting.

Amphotericin B is a potent polyene antifungal that acts by binding ergosterol and disrupting cell membrane integrity. Pores are created in the cell membrane that cause leakage of cations and proteins into the extracellular space, ultimately leading to cell death (15). Resistance to this drug can be conferred by mutations in *ERG* genes that divert ergosterol synthesis towards the production of ergosterol-like molecules. Cell integrity therefore is maintained while pore formation is avoided. Though amphotericin B resistance has been documented in strains of *C. lusitaniae*, *C. albicans*, *C. tropicalis* and *C. parapsilosis*, their clinical occurrence is rare (47).

1.3.2 Potential role of histone deacetylase inhibitors in azole resistance

Within the nucleus of eukaryotic cells, DNA strands are compacted into assemblies of chromatin. The basic assembly unit of chromatin is the nucleosome, comprising a segment of DNA wrapped around a histone. By virtue of this compression, gene expression and repression may be elegantly controlled. To induce gene expression, DNA must first be decompressed. Histones in the vicinity of the gene promoter therefore are "tagged" via a methylation, phosphorylation, or acetylation reaction (86). Histone acetyltransferases (HATs) activate gene transcription by acetylating the ϵ-amino group of histone lysine residues. Inversely, histone deacetylases (HDACs) repress gene expression by hydrolysing the acetyl moiety of tagged histones (115).

The role of histone deacetylases in cancer is an active field of research. In cancerous cells, HDACs are aberrantly recruited to promoters that mediate transcriptional repression (3). Expression of these regulatory enzymes therefore can be maintained by small-molecule inhibitors of HDAC activity. A particular advantage of histone deacetylase inhibitors (HDACIs) is their high specificity: Spurious histone deacetylation, which can cause toxicity in normal cells, is minimised (115). This high specificity also applies in the context of fungal versus human HDACs.

A recent study by Smith and Edlind (160) demonstrated a correlation between HDAC inhibition and an enhanced sensitivity to antifungals that deregulate ergosterol synthesis in *C. albicans*. Sensitivity was mediated through a reduction in the expected upregulation of *CDR* (porins) and *ERG* (ergosterol synthesis enzymes) in the presence of azoles and terbinafine. In this study, the HDACI trichostatin A (TSA) acted synergistically only with the antifungals that inhibit *ERG* enzyme activity. A minimal

effect was observed when TSA was combined with antifungals acting through other mechanisms. The authors then analysed *ERG*, *CDR* and *MDR* expression in the presence of fluconazole or terbinafine, with and without TSA. When *C. albicans* was incubated with the antifungal only, an upregulation of the *ERG* and *CDR* genes was detected, indicating that the drugs were triggering enhanced efflux. When the antifungal and HDACI were combined, gene upregulation was reduced by 50 - 100%. These data set a precedent for further studies in azole-HDACI combination therapy.

MethylGene Inc. has evaluated six HDACIs in combination with ketoconazole against *C. albicans*, *C. glabrata* and *A. fumigatus in vitro*[*]. Two of the HDACIs tested were found to synergize with ketoconazole against all isolates. Synergy was defined as a reduction in the minimum inhibitory concentration (MIC) by at least 4-fold compared to the ketoconazole-only control. One of the HDACIs was TSA (as used by Smith and Edlind, thus confirming their results). The other was MG-1, a proprietary compound synthesized by MethylGene Inc. A related compound, specifically MG 3290, is the compound evaluated in our animal model development studies described in manuscript #2 (chapter 6).

[*] Campeol, N., J. Bedard, and N.H. Georgopapadakou. 2005. Synergism of histone deacetylase (HDAC) inhibitors with ketoconazole in *Candida albicans* and *Candida glabrata*. Relationship to their effects on HDAC Activity in protoplasts. 45th Intersci. Conf. Antimicrob. Agents Chemother., **abstr. M-2154**; Hu, W., N. Campeol and N.H. Georgopapadakou. 2005. Synergism of histone deacetylase (HDAC) inhibitors with ketoconazole in *Aspergillus fumigatus*. Relationship to inhibitory effects on HDAC activity in protoplasts. 45th Intersci. Conf. Antimicrob. Agents Chemother., **abstr. M-2165**.

Chapter 2: Preclinical drug development

For a "compound" to be considered a "drug," a series of rigorous tests for efficacy and safety must be passed. Efficacy and toxicity are emphasized during preclinical studies while both safety and efficacy are evaluated in clinical trials. The following describes essential principles in preclinical drug development in the context of antibacterials and antifungals (Fig. 5).

2.1 Target selection

In the selection of an appropriate target for antimicrobial drug development, the desired effect of the drug-target interaction is a loss of pathogen viability. Ideally, the target should be pathogen-specific to reduce the risk of cross-reactions causing toxicity. If there is no homolog of the target in human cells, the likelihood of causing side-effects is reduced. The penicillin-binding proteins, the target of ß-lactam antibiotics, are a good example of targets that have no human equivalents. This absence of homology accounts for the low toxicity of ß-lactam antibiotics. Conversely, target similarity also accounts for the relatively high toxicity of amphotericin B. Since cholesterol found on mammalian cells is structurally very similar to the drug target ergosterol, considerable side effects are commonly experienced when this drug is taken.

Drug development can proceed using established or new targets. Established targets include the peptidases in bacterial cell wall synthesis, bacterial ribosomes, fungal ergosterol synthesis enzymes, among others. Though such targets are well-understood, resistance mechanisms to evade inhibition are equally well-established. Drug discovery

against new targets became viable with the advent of functional genomics through the 1990s. After mapping all genes within the genome of a pathogen, functional genomics attempts to identify genes that are essential for viability or virulence. The products of these genes may then be targeted in drug discovery. Developing therapies against new targets has inherent advantages. For example, the target is likely to be very pathogen specific, thus reducing potential toxicity. Moreover, intrinsic resistance mechanisms may not exist as selective pressure may never have been exerted. This genomic approach can also be applied in the discovery of new targets in problem pathogens such as *Mycobacterium tuberculosis* or *P. aeruginosa*. Despite a very optimistic outlook and intensive efforts to find essential genes amenable for drug discovery, relatively few potential targets have actually been identified to date by this approach (174).

2.2 Primary screening

Primary screening involves searching for molecules that can inhibit target function or activity. Compounds for primary screening have been developed using 3 strategies, largely depending on resources available. Big pharmaceutical companies, having the greatest financial resources, often use a brute, empiric approach. Compounds in individual corporate libraries, which may number in the hundreds of millions to over a billion, have accumulated through decades of drug research as well as mergers and acquisitions of competing firms. Screening campaigns in such "big pharma" settings typically test a million compounds or more. Compounds selected for screening are molecularly very diverse. The empiric approach therefore represents a completely random, non-hypothesis driven method of screening. Having fewer resources, smaller

pharmaceutical and biotechnology companies, and academia usually take a rational or lead-based approach. In rational drug design, compounds are designed based on known structures of the target, which are commonly determined from X-ray crystal structure analyses. A good visual representation of the active site of the target of interest may reveal clues for optimal compound design. If compound libraries are available, this permits more-stringent filtering for compounds that may bind (at least on a theoretical level). If designing compounds *de novo*, a limited number of small molecules that bind or interact optimally with residues in the target active site can be synthesized. If these compounds show inhibitory activity, analogues may be synthesized quickly through combinatorial chemistry and then screened. This strategy was employed in synthesizing phosphonate-based compounds as potential inhibitors of ß-lactamase (82). Lead-based compound design involves synthesizing analogues of drugs with known activity against the target. For example, many penicillins were designed using this approach. As >90% of all prescribed drugs have a molecular weight between 200 - 800 daltons, the typical size of molecules in screening libraries ranges from 300 to 600 daltons (174).

As considerable resources are invested in drug development based on primary screening data, great care must be taken during assay design and implementation. The assay should be simple, robust, generate reproducible results (including intra-assay and inter-assay reproducibility), and accurately measure the desired effect. In a typical primary screen, between 1 000 to 10 0000 compounds are tested daily. Big pharmaceutical companies often can test over 100 000 compounds per day. In order to test this number of compounds on a daily basis, high-throughput screening (HTS) assays are necessary. To reduce the intense labour involved in manually processing such large

numbers of compounds and to consider the typically small volumes involved, any HTS assay must be automated. Innovations in automation technology have greatly assisted the implementation of high-throughput assays (174).

A HTS assay may be cell-based (to detect the impact on pathogen viability) or cell-free (biochemical assay focusing on compound – target binding). Target biology dictates the type of assay developed. For example, receptor-binding assays cannot differentiate between agonists and antagonists (177). If a receptor agonist is sought, a cell-based primary screen is most appropriate. When such differentiation is unnecessary, the primary screen is preferably cell-free, involving only the purified target and the test compound. Cell-free assays are preferred as they are much shorter in duration and less subject to data scatter than cell-based screens. However, these assays typically require target purification, which can be a limiting step in the screening process (174).

Assay quality can be measured using various statistical formulas. One of the most commonly used is the Z' factor (or Z' score) (189). This formula considers the signal-to-background ratio as well as data precision. Generally, a minimum Z' score of 0.5 is necessary to consider an assay appropriate for HTS applications.

A good example of such an HTS assay is the previously described enzymatic assay for ß-lactamase inhibitors (142). This assay relies on the fluorescence of enhanced green fluorescent protein (EGFP) to indicate binding. A gene construct of an EGFP - ß-lactamase fusion protein was incorporated into an expression vector, transformed into *E. coli*, and expressed. The fusion protein was then purified. Upon ß-lactam hydrolysis by the fusion protein, a decrease in the local pH also decreases fluorescence emission of EGFP. In the presence of a ß-lactam, effective inhibitors of the enzyme would therefore

sustain fluorescence. In a screening assay, compounds producing sustained fluorescence would be identified as "hits."

2.3 Post screening analysis

In primary screening assays using "sets" of structurally-related compounds, results typically show a spectrum of activity. By correlating chemical structures with binding activities, the structure-activity relationship (SAR) of the hits may be characterised. The activity of the hits is then summarized according to one of the following three descriptions: A "flat SAR" suggests all structurally-related compounds have similar activity. "Singletons" are an anomaly; the hit represents the only compound in the set to be active. Hits showing an "evolvable SAR" show a range of activity that may be correlated with structural properties. No further research is normally conducted on singletons. Compounds showing a flat SAR may proceed to secondary screening, though data from cell-culture toxicity assays (as described below) must be carefully considered. Hits showing an evolvable SAR are generally selected for further development. Knowledge gained from these analyses are used for synthesizing improved analogues of the original hits through multiple rounds of SAR-guided development (174).

2.4 Secondary screening

In contrast to primary screening where random compounds may be tested, secondary screening is usually more focused. The secondary screen confirms target

specificity and compound efficacy by testing compounds against the pathogen. The assays implemented for screening are therefore cell-based, either involving one pathogen or a variety of pathogens. Hits identified from the primary screen may be filtered out at this stage as a result of poor permeability or stability. Compounds identified as hits in secondary screening are usually sufficiently permeable to reach their target and potent enough to either inhibit growth or induce cell lysis. Secondary screening may also explore compound toxicity to human cell cultures. This is commonly tested using cell viability and proliferation assays. Compounds that show toxicity in these assays are usually abandoned. However, SAR analysis may reveal analogues with lower toxicity profiles (174). For reasons similar to those discussed in primary assay design, meticulous attention to detail is also important when designing a secondary screening approach. If the assay is designed such that a linear relationship exists between the response signal and the biological effect, quantitative structure-activity relationship (QSAR) analyses can be performed.

For both primary and secondary screens, controls are of the utmost importance and should be included in every assay run. Any mechanical failures (such as a blocked pipette tip) or biological failures (such as a failure of the pathogen to grow in the absence of a suppressive agent) may be detected by verifying the control wells. Statistical software is available to detect such failures and should be used prior to data analysis. Controls should include:

1) blank controls – containing medium only, indicates the baseline signal

2) neutral controls – lacks test compound but contains the pathogen, signal correlates with microbial growth

3) maximum effect controls – contains a reference drug that completely inhibits growth

4) sensitivity controls – contains a reference compound producing 50% growth inhibition (73).

2.5 Animal studies

Animal studies are used to assess compound efficacy and provide an understanding of compound behaviour within a mammalian organism. Compound dynamics are analysed first in animal models as their physiological environment closely resembles that of humans. Toxicity is evaluated in preliminary studies. Typically, small rodents receive doses of the compound that range from well-below therapeutic to doses that are far above the expected human dose. Water and food consumption, behaviour, urine and stool excretion, blood chemistries and body weight are all monitored closely for changes that suggest deteriorating health. If results from short-term toxicity studies indicate a wide dose range without toxicity (also known as a wide therapeutic window), the compound advances to pharmacologic activity studies. Long-term or repeated-dosing toxicity studies may also be initiated. Compounds having a narrow therapeutic range are frequently abandoned at this stage (174).

Pharmacologic studies seek to define the pharmacokinetic (PK) and pharmacodynamic (PD) properties of a compound in animal systems. PK studies assess the absorption, distribution, metabolism, and elimination (ADME) of a compound. In

initial PK studies, rodents (usually rats) are injected intravenously with a fixed dose of compound. Blood samples are taken at various time points and analyzed by liquid chromatography/mass spectrometry (LC/MS) or other quantitative analytic techniques (148). LC/MS data is then used to determine distribution and elimination times (from a graph of blood/serum concentration versus time) and total compound exposure (area under the curve (AUC) from the same graph). Organ homogenates also are analyzed by LC/MS to determine compound distribution and metabolism in various tissues. Other important pharmacokinetic parameters such as bioavailability, drug-drug interactions, and mutagenic potential are also assessed (174).

PD studies, which consider the *in vivo* efficacy of the compound with respect to compound concentration, may be conducted simultaneously with PK studies. Despite high potency and low toxicity *in vitro*, factors such as poor tissue penetration, high protein binding, or rapid metabolism and elimination may interfere with similar results *in vivo*. For compounds demonstrating only moderate efficacy at sub-toxic doses, it is possible that additional rounds of QSAR analysis and secondary screening can lead to compounds with better pharmacodynamic properties. However, further drug development usually ceases for compounds that are bioavailable but penetrate tissues poorly or are hepatotoxic (174).

For infectious diseases, animal modeling involves infecting animals to directly assess compound efficacy *in vivo*. To establish the model, the course of infection in the animal ideally should mimic as closely as possible the course followed in humans. For some opportunistic infections, such as systemic candidemia, infections are established normally in immunocompromised hosts only. The ideal animal model for systemic

candidemia would therefore involve animals with deficient immune function. Mice are commonly used to study systemic candidemia. Though genetically-modified immunodeficient mouse strains are available, cost of such mice in large numbers is prohibitive. Normal mice therefore are commonly used and are immunosuppressed through irradiation or the use of drugs such as prednisone (or other glucocorticosteroids) or cyclophosphamide.

Antimicrobial compounds having good PK and PD profiles and low toxicity proceed to clinical testing. Drug candidates first are tested in increasing doses in small groups of healthy humans to assess safety and PK (phase I), then in a small number of infected individuals (phase II). Treatments with candidate compounds can proceed in parallel with existing therapeutics (for a direct comparison of efficacy with existing antibiotics) or following treatment failure (often arises as a consequence of antibiotic resistance). Candidates that continue to show good efficacy and low toxicity are then tested on considerably larger groups of infected patients (phase III). Compounds that continue to demonstrate high potency while producing tolerable side effects may be approved for licensure and clinical use.

Chapter 3: Tetrazolium salts

High-throughput cell-based assays utilising a variety of strategies to measure cell viability and growth have been developed for screening antimicrobial compounds. One cell-based assay involving luminescence was recently described (6). To create this assay, an inducible plasmid constructed of luciferase genes was transformed into an *E. coli* strain to create luminescent bacteria. Replication of the plasmid and gene expression depended on the bacterial cell machinery. Luminescence should be low for compounds that inhibit growth. Light emission was found to correlate poorly with growth inhibition.

Another strategy detects dissolved oxygen in the assay medium through fluorescence (187). Fluorescence of the indicator, a ruthenium dye, is quenched in the presence of oxygen. As oxygen is depleted by respiring cells, signal intensity increases. Since the relationship between the signal and cell number was linear, data collected from this assay can be analysed quantitatively. Cost of using this (proprietary) technology is relatively high.

A third and increasingly-popular strategy for the detection of cell viability and proliferation involves the use of tetrazolium salts. These compounds are small-molecule colorimetric indicators of overall metabolic activity or cell viability. First described over a hundred years ago (133,134), the structure of the original compound has been modified to produce a variety of biologically-functional tetrazolium salts. The range of currently-available compounds allows for qualitative and quantitative assessments of many different biological systems.

3.1 The biochemistry of tetrazolium salts

When dissolved, tetrazolium salts may range from colorless to faintly-colored. In the presence of metabolically active cells, the salt is reduced to a purple (MTT), blue (NBT), red (TTC), orange (XTT, MTS) or yellow (WST-1) formazan product. CTC is unique in that its reduction produces a fluorescent formazan.

At the heart of each tetrazolium compound is a positively charged quaternary tetrazole ring composed of 4 nitrogen atoms and 1 carbon atom (Fig. 6). Differences in formazan colors result from modifying any combination of the three aromatic groups attached to the tetrazole ring (17).

NBT is highly impermeable to cells and its formazan is highly water insoluble. As seen in Fig. 6, the bulky, largely hydrophobic nature of this ditetrazolium salt accounts for its lack of penetration and its insolubility. The success of MTT, the first widely-used monotetrazolium dye, in cellular assays may be accounted for by its smaller size compared to the ditetrazolium salts and its net positive charge, which permits cellular uptake via the plasma membrane potential. Since MTT is cell-permeable, it was believed originally that the dye was reduced by succinate. Later studies showed that reduction largely depended on NADH and NADPH, and not on succinate (18). When multiple negatively-charged sulfonate groups are added at the aromatic moieties of a tetrazolium core (for example: MTS, WST-1, and XTT; Fig. 6), the molecule acquires a net negative charge. These dyes cannot penetrate cells and thus are reduced extracellularly (Fig. 7) (19). Direct reduction of water-soluble tetrazolium salts by cell-membrane NADH oxidases (156) occurs at a low-to-moderate rate. An electron coupling agent such as

phenazine methosulfate (PMS) or menadione is therefore required to enhance redox rates (Fig. 7) (17).

3.2 Applications, advantages and limitations

Over the past 20 years, tetrazolium salts have been extensively used for *in vitro* assays. The development of tetrazolium dyes producing increasingly water-soluble formazans contributed significantly to their widespread use. First generation tetrazolium salts such as neotetrazolium (NT), nitrobluetetrazolium (NBT) and tetranitrobluetetrazolium (TNBT) formed water-insoluble formazans and were primarily used in histological applications for their high affinity to tissue proteins (162). NBT was also used in cellular assays (14,72), though its associated formazan was highly water-insoluble. MTT, INT and CTC were designed specifically to produce less insoluble formazans and penetrate cells more readily. This led to the development of an MTT-based cell proliferation and cytotoxicity assay by Mosmann (118), which greatly expanded the popular use of tetrazolium dyes. Despite improvements generated by MTT, INT and CTC, these dyes still produced water-insoluble formazans, and thus were not appropriate for use in experiments requiring multiple real-time measurements. This issue was resolved following the development of fully water-soluble tetrazolium salts including XTT, MTS, WST-1 and WST-8 (17).

As tetrazolium salts may be reduced by both prokaryotic and eukaryotic cells, they are frequently used for histochemical preparations (24,43), cell proliferation and cytotoxicity assays (161,190), cell viability testing (140) and drug susceptibility screening for anti-cancer (181), anti-bacterial (116), anti-viral (48) and anti-parasitic (57)

compounds. Several susceptibility tests using tetrazolium dyes have been developed for pathogenic bacteria including *Helicobacter pylori* (168,171), *M. tuberculosis* (16,40-42,77,103), *S. aureus* (113), and *P. aeruginosa* (167).

Research has favoured the use of tetrazolium compounds for their inherent advantages, the most important one being their ability to indicate cellular activity through an amplified signal, specifically a colored-formazan product. These colorimetric indicators are particularly effective in assays in which assessment of growth is unclear or difficult. For example, accurate determination of the MIC for *Candida* species according to the standard CLSI method M27-A2 (122) can be difficult as a result of a trailing effect (no complete inhibition observed). An STC-based susceptibility assay has been developed in which the MIC is clear without evidence of a trailing effect (158). In antimicrobial susceptibility testing, a more-relevant measure (total metabolic activity of viable cells only) is achieved compared to standard methods which measure biomass including dead, damaged and live cells. Unlike ^{3}H-thymidine, tetrazolium compounds do not involve radioactive materials or high waste-management costs. The simplicity of the redox reaction of these colorimetric compounds makes them amenable for high-throughput applications (17).

However, there are limitations to the use of tetrazolium salts. The dyes are not recommended when the percent of viable cells versus non-viable cells must be assessed. The crystalline structure of water-insoluble formazans tends to damage cell membranes while soluble formazans tend to diffuse out of the cell. As the rate of formazan production is proportional to metabolic activity, tetrazolium salts may not be appropriate for detecting the growth of cells if growth arrest is induced. Moreover, as the metabolic

rate of different cell lines or infectious agents varies, direct comparisons of results may not be meaningful unless data are normalised (17).

Chapter 4: Hypotheses and justification

4.1 Hypotheses

In tandem with the drug discovery programs at MethylGene Inc., our interest focused on the two last stages of preclinical antimicrobial drug development, specifically HT assay design and animal efficacy studies:

Research Question 1:

Is it possible to design a rapid cell-based assay with high-throughput capacity in which potent class A and class C ß-lactamase inhibitors can be identified?

We postulate that by using the colorimetric tetrazolium salt XTT, a rapid and high-throughput screening program for aerobic Gram-negative bacteria expressing defined ß-lactamase enzymes can be created.

Research Question 2:

Can the inhibition of yeast histone deacetylation increase the sensitivity of *Candida* species to commercially-available azole antifungals *in vivo*?

We postulate that histone deacetylase inhibitors are capable of potentiating the activity of azole antifungals in an animal model of systemic infection with *C. albicans*.

4.2 Justification

One method that could be employed in the screening for ß-lactamase inhibitors is the conventional broth microdilution assay described by the Clinical and Laboratory Standards Institute (CLSI) (121). Including variations based on similar principles of this method (such as the E-test or VITEK system (51)), CLSI procedure M7-A5 is the standard method used in hospitals across North America, primarily for its high reliability and clinical relevancy. In the context of a rapid test, a disadvantage of this method is the long incubation period required (16 - 20 hours) before results can be interpreted. When designing a rapid cell-based assay with high-throughput capacity, it is preferable that results are obtained within at most a few hours. To use an assay that requires long incubation times to screen large compound libraries is impractical. Additionally, if data are collected spectrophotometrically, it is likely that the Z' score of the assay will be relatively low. This may be the reason that section 7.3.4 of the CLSI protocol emphasizes a visual assessment of drug susceptibility (121).

As described earlier, Puckett *et al.* (142) reported the development of an EGFP-ß-lactamase fusion protein that can be applied in high-throughput screening applications. Though likely useful in primary screening, this method does not account for compound permeability or efficacy against pathogenic ß-lactamase-expressing bacteria. Moreover, the signal produced by EGFP may be subject to non-specific effects such as environmental changes and sample composition (111). Although a reporter gene could be used to account for such factors, this would increase the complexity of the assay. Änko *et al.* (6) have previously described an *E. coli* strain expressing luciferase that also may be adapted for use as a cell-based screening system for ß-lactamase inhibitors. Though drug

sensitivity could be assessed within 150 minutes, quantitative analysis of the data is not possible as the fluorescence signal is not in linear proportion with growth inhibition. Results obtained using this system are not suitable for QSAR analysis and therefore may hinder an efficient drug development process.

Currently, the literature contains little information regarding rapid, cell-based screens for ß-lactamase inhibitors for high-throughput use. Such an assay would be of tremendous value by accelerating the research and development of novel therapies targeting multiple classes of ß-lactamase enzymes, currently an unmet medical need.

The use of histone deacetylase inhibitors as an adjunct to conventional antifungal therapies is a promising strategy for controlling fungal infections. Published literature on HDACIs with anticancer properties is substantial. In contrast, very limited data are available on potential activity with antimycotics. Even less is known of their physiological activity with azoles and efficacy in mammalian systems. Animal studies lead to a better understanding of the interplay between the regulation of genes encoding drug transporters and the effect of such regulation on azole sensitivity. This in turn may lead to the validation of this strategy for treating azole-resistant fungal infections, a serious and growing clinical problem.

Figures and tables

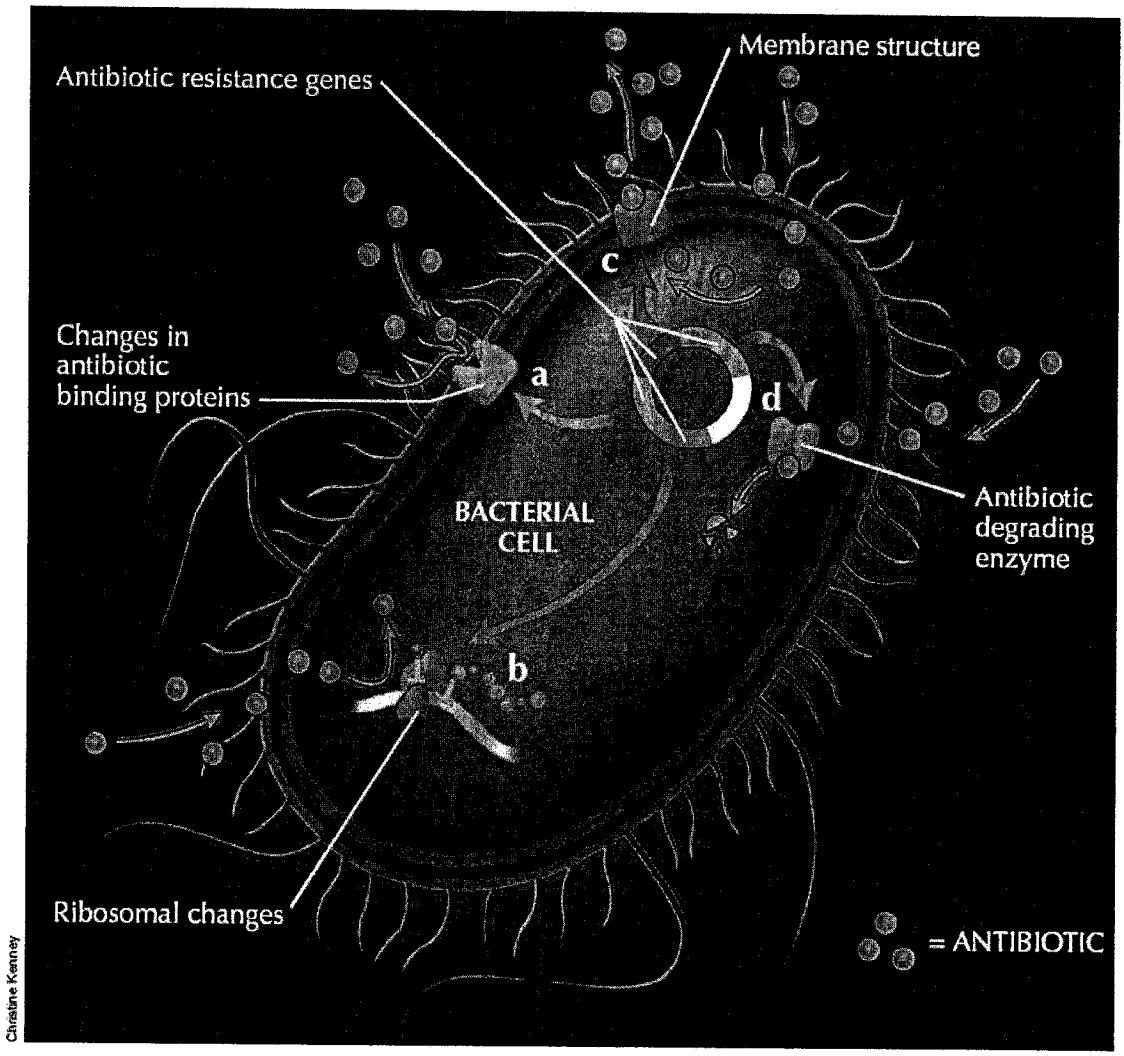

FIG. 1: Principal genetic mechanisms of antibiotic resistance in bacteria. Development into a resistant phenotype may be innate (via spontaneous mutation) or acquired. When acquired, it may occur through horizontal (transfer from one bacterium to another) or vertical transmission (from mother cell). The resistant phenotype may be expressed as (a) drug target mutations, (b) target protection (c) modification of cell membrane composition, including the addition of efflux pumps, and (d) enzymatic degradation. This figure has been reproduced from reference (34).

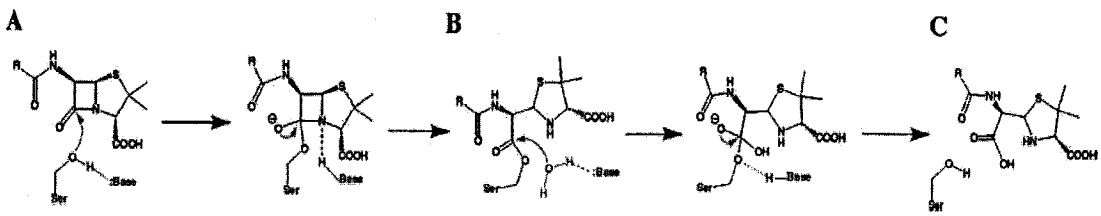

FIG. 2: Proposed mechanism of inactivating ß-lactams by serine ß-lactamases. The ß-lactam initially forms a Michaelis-Menten complex with the enzyme (A). The active site serine attacks the carbonyl group of the ß-lactam and ejects a nitrogen atom and a proton, resulting in an acyl-enzyme intermediate (B). An activated water molecule then attacks the acyl moiety of the complex and releases the inactivated antibiotic (C). This figure has been reproduced from reference (63).

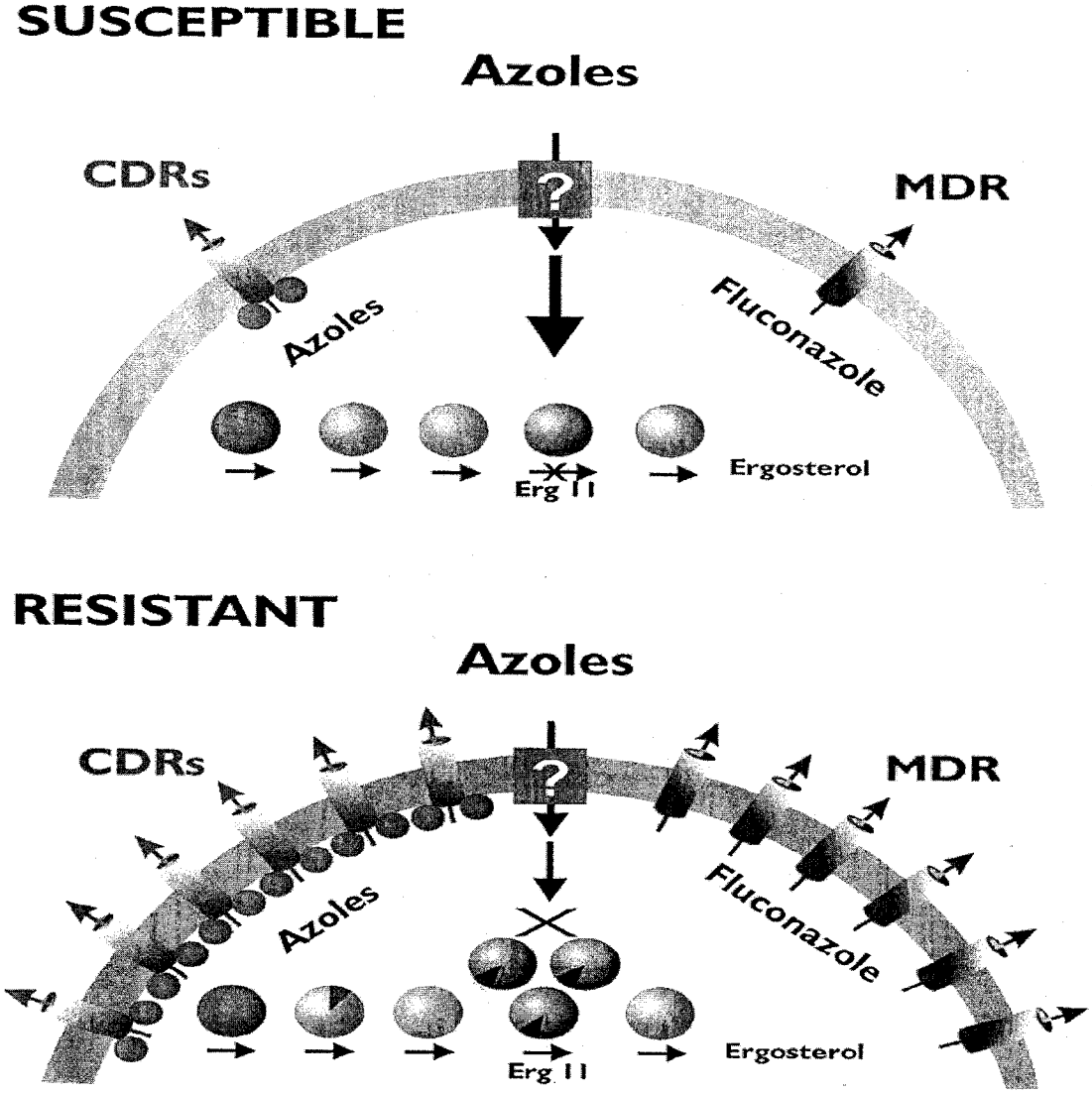

FIG. 3: Molecular mechanisms of azole resistance. In a susceptible cell, azole drugs enter the cell through an unknown mechanism, perhaps by passive diffusion. The azoles then inhibit Erg11, blocking the formation of ergosterol. Two types of efflux pumps are expressed at low levels. The *CDR* proteins are ABC transporters. The *MDR* protein is a major facilitator transporter. In a "model" resistant cell, the azoles also enter the cell through an unknown mechanism. The azole drugs are less effective against Erg11 for two reasons; the enzyme has been modified by specific point mutations and the enzyme is overexpressed. Modifications in other enzymes in the ergosterol biosynthetic pathway contribute to azole resistance. The sterol components of the plasma membrane are modified. Finally, the azoles are removed from the cell by overexpression of the *CDR* genes and *MDR*. The *CDR* genes are effective against many azole drugs, while *MDR* appears to be specific for fluconazole. This text was adapted from reference (184). This figure has been reproduced from reference (183).

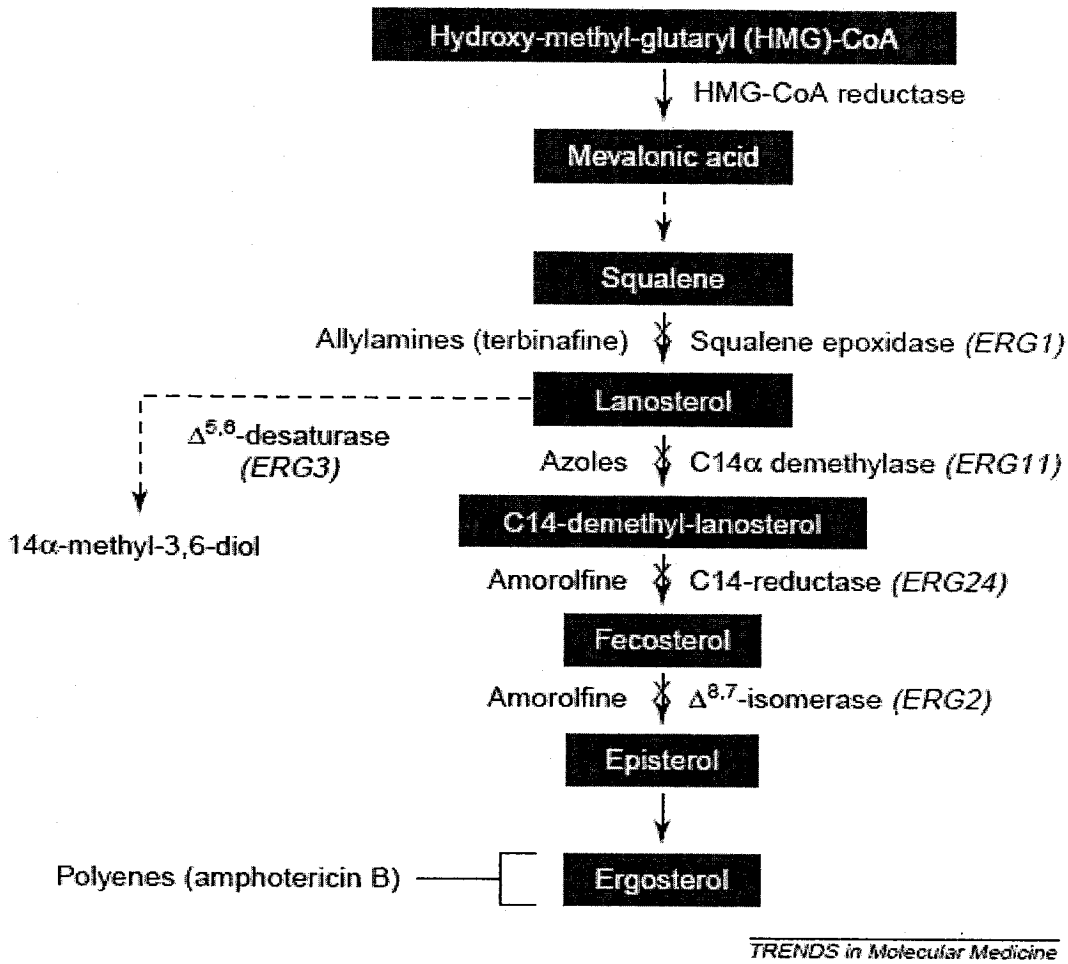

FIG 4: Mechanism of action of antifungal drugs affecting the ergosterol biosynthetic pathway. The target enzymes are reported on the right with encoding genes in parentheses, whereas the antifungal drugs are reported on the left of the arrows indicating the sequential steps of sterol biosynthesis. Text and figure have been reproduced from reference (102).

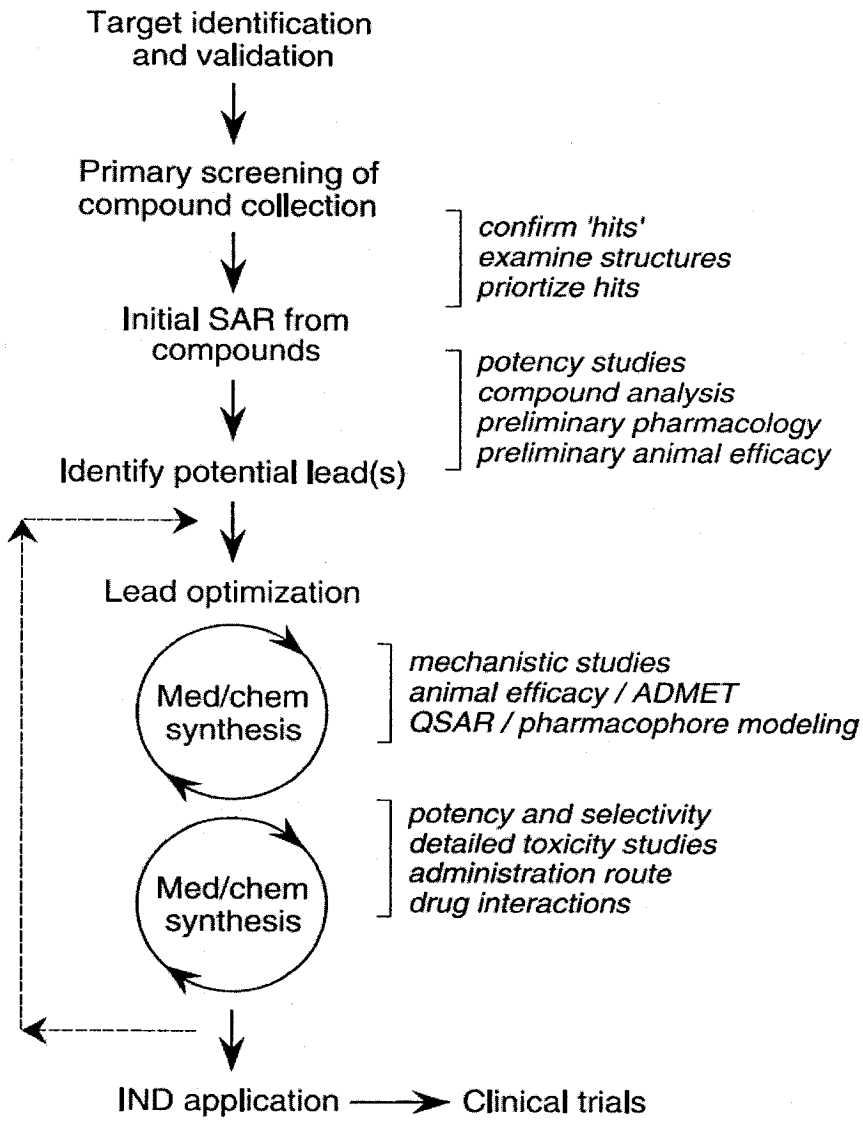

FIG 5: Strategy for preclinical drug development. After selecting a target for drug development, compound libraries may be used for primary screening. Data generated from this screen is then analyzed for structure activity relationships (SAR). This information may then be used for lead optimization and secondary screening. Hits identified during secondary screening then are tested in animals to determine their administration, distribution, metabolism, elimination and toxicity (ADMET) profiles. Lead optimization continues via quantitative SAR (QSAR) or pharmacophore modeling. If a compound is effective, bioavailable and demonstrates low toxicity, an investigational new drug (IND) application is filed (in United States). Clinical trials then commence. This figure has been adapted from reference (174).

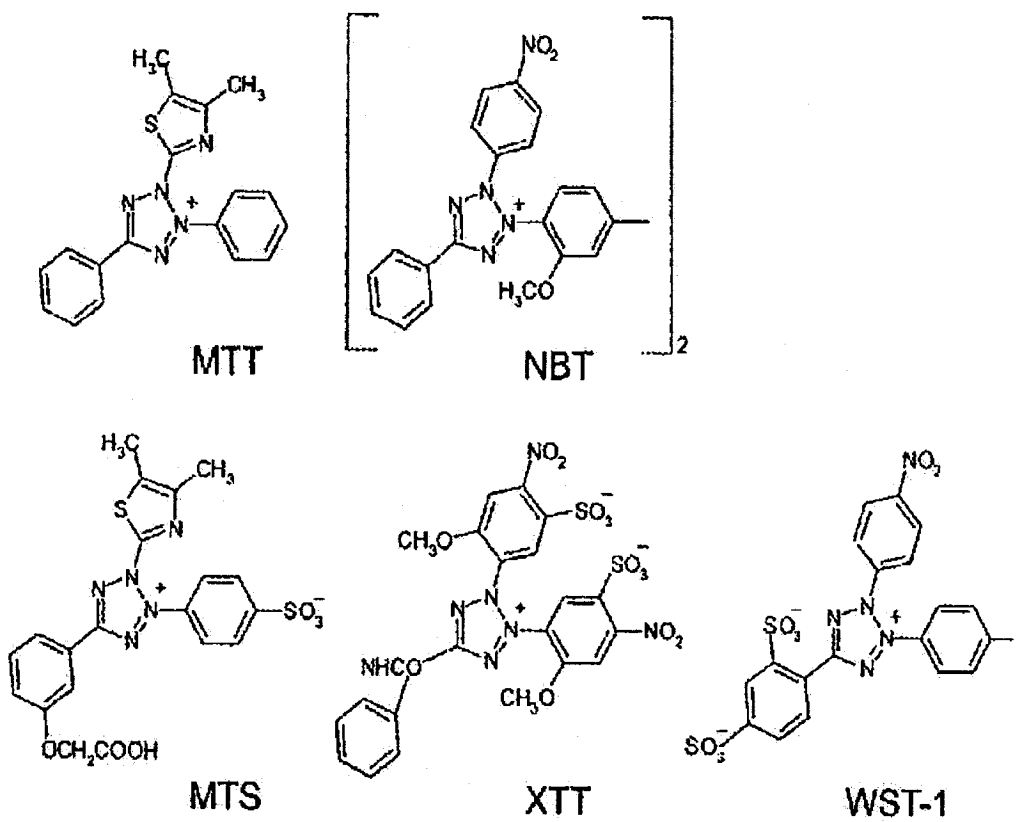

FIG. 6: Chemical structures of select tetrazolium salts. This figure has been adapted from reference (17).

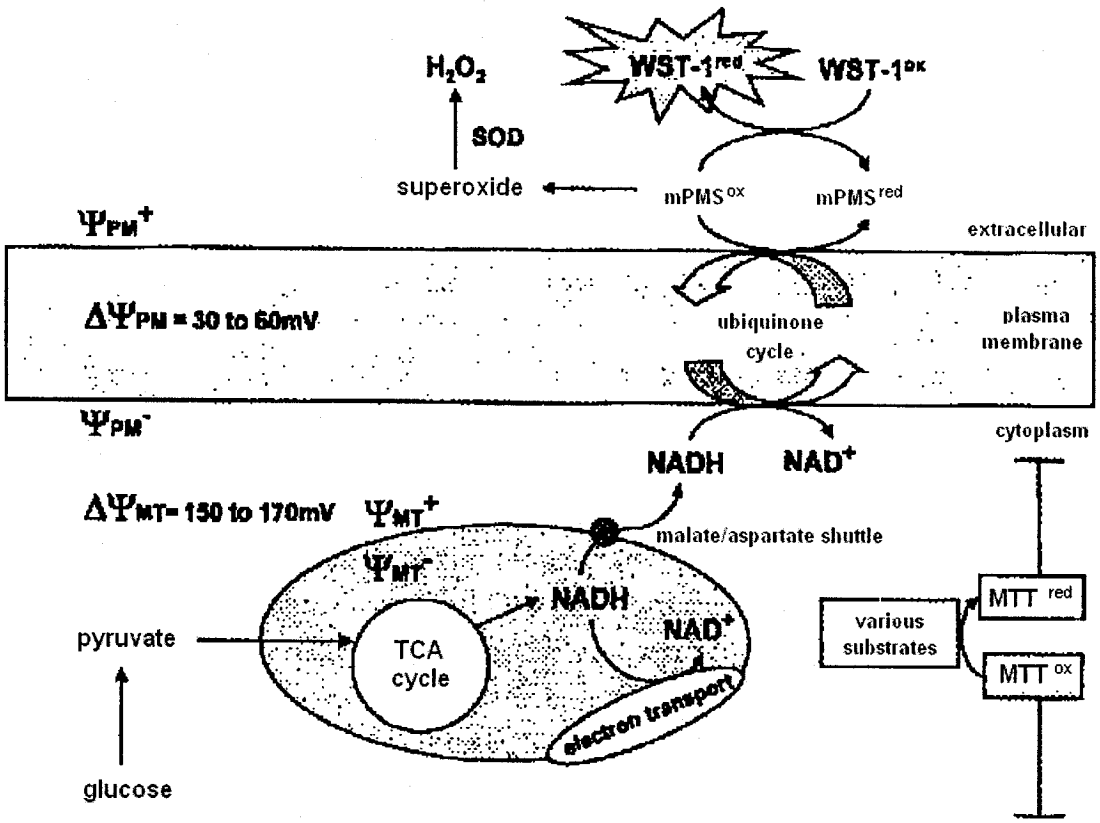

FIG. 7: Schematic representation of the proposed mechanisms of cellular reduction of monotetrazolium salts. MTT represents dyes producing water-insoluble formazans (such as INT and CTC) while WST-1 represents those producing water-soluble formazans (such as MTS and XTT). Whereas MTT is reduced by a variety of intracellular reductants, most notably NADH, WST-1 is reduced by trans-plasma membrane electron transport via the electron carrier, mPMS, in which case the cellular reductant is NADH derived mainly from the mitochondrial TCA cycle. The plasma membrane potential, which is proposed to be the major cellular determinant of tetrazolium dye uptake is also depicted. This text and figure has been adapted and reproduced respectively from reference (17).

TABLE 1: Classification schemes for ß-lactamases[a]

Bush–Jacoby–Medeiros	Ambler	Comment
Functional group (groups 1–4)	Molecular classification (class A–D)	
Group 1 cephalosporinases	Class C-cephalosporinase	Examples include AmpC of *E. coli* and P99 of *Enterobacter* spp. These enzymes are usually clavulanic acid-resistant and are usually chromosomally encoded. There are several of these cephalosporinases that are plasmid encoded (e.g. CMY-2, ACT-1).
Group 2 penicillinases (+clavulanic acid) 2a – staphylococcal penicillinase 2β – TEM-1 and SHV-1 β-lactamases 2be – ESBLs 2br – inhibitor-resistant β-lactamases 2c – carbenicillin-hydrolyzing 2e – cephalosporinases inhibited by clavulanic acid 2f – carbapenem-hydrolyzing enzymes inhibited by clavulanate	Class A-penicillinases	Examples are penicillinases of *E. coli*, *K. pneumoniae* and *Staphylococcus aureus*. These enzymes are usually clavulanic acid-susceptible, with notable exceptions (inhibitor-resistant TEMs and SHV enzymes) from the 2br group. It is noteworthy that the extended-spectrum β-lactamases and carbapenemases found in this group are all clavulanic acid-susceptible.
Group 2d	Class D – cloxacillin-hydrolyzing enzymes (OXA)	These enzymes are less clavulanic acid-susceptible than 2a, 2b or 2be. These represent a growing group of β-lactamases, in which are found many carbapenemases.
Group 3 (3a, 3b, 3c)	Class B – metallo-β – lactamases (zinc)	The metal ion is necessary for hydrolysis. These enzymes confer resistance to carbapenems and are not inhibited by clavulanic acid. It is noteworthy that some are inhibited by aztreonam.
Group 4	Unknown	Miscellaneous or unsequenced/uncharacterized enzymes that do not fit into any function or molecular grouping.

[a]This table has been reproduced from reference (71).

Section II: MANUSCRIPT I, MANUSCRIPT II

Connecting statement I

The assay described in this chapter was conceived to evaluate a family of novel ß-lactamase inhibitors generated by MethylGene Inc. Beginning with a rational drug design approach and multiple cycles of QSAR, the company was developing these compounds to inhibit both class A and C ß-lactamases. At the time my graduate work began, the company was poised to use a combinatorial chemistry approach to develop potential lead candidates. A sensitive, high-throughput, cell-based assay was needed. The first manuscript describes the development of a high-throughput assay to screen large numbers of compounds for ß-lactamase inhibitory activity. By optimizing the concentration of XTT (the colorimetric indicator), menadione (the electron-coupling agent assisting XTT reduction), the antibiotic concentration, and the bacterial inoculum, hits could be identified within 5 hours.

Chapter 5: Manuscript I

A colorimetric cell-based assay for the rapid and high-throughput screening of beta-lactamase inhibitors

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Key words: tetrazolium, XTT, beta-lactamase inhibitors, antibiotic resistance, colorimetric methods, high-throughput, HTS, susceptibility testing

5.1 Abstract

A high-throughput colorimetric screen for small-molecule compounds having ß-lactamase inhibitory activity is described. The screen is based on the reduction of the tetrazolium salt 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenyl-amino)Carbonyl]-2H-Tetrazolium-5-carboxanilide (XTT). Using 200 μg/mL XTT, 25 μM menadione (an electron-intermediate carrier), a ß-lactamase-producing bacterial inoculum of 5×10^5 CFU/mL and a sub-inhibitory dose of piperacillin (16 μg/mL), compounds inhibiting ß-lactamases and thus re-establishing antibiotic activity were identified within 5 hours. The assay was validated by using it to measure minimum inhibitory concentrations (MICs) and comparing these results to MICs determined according to the CLSI broth microdilution method. The XTT-based assay achieved 100% essential agreement with the CLSI reference method. The colorimetric assay described herein shows potential for screening compounds that inhibit ß-lactamases.

5.2 Introduction

Antibiotic resistance in pathogenic bacteria is an increasing global phenomenon with serious implications for the treatment of infections. Current strategies to circumvent resistance mechanisms focus on both the search for new targets and the inhibition of established targets. Among the many targets for which drugs are under development are peptide deformylase (9-11), DNA gyrase (58) and ß-lactamase. The last target is well-established: clavulanic acid, sulbactam and tazobactam are all used in medical practice in combination with various ß-lactams (95). The three inhibitors primarily target plasmid-mediated Ambler class A ß-lactamases (91), though tazobactam is thought to have limited activity against class C and D enzymes (129). Nonetheless, the efficacy of all currently-available ß-lactamase inhibitors cannot address the spread of class B, C, and D ß-lactamases or inhibitor-resistant class A enzymes. New inhibitors that target multiple classes of ß-lactamases and are impervious to enzyme hydrolysis are urgently needed. Phosphonate-based compounds have shown such potential (2,80,82).

To screen large combinatorial chemistry libraries for potent ß-lactamase inhibitors, a simple, sensitive and rapid assay is needed. To ensure the compounds can access ß-lactamases within the periplasmic space of Gram-negative bacteria, bind irreversibly and render bacteria susceptible to ß-lactams, a cell-based assay is most appropriate. Standard methods such as the broth microdilution assay proposed by the Clinical and Laboratory Standards Institute (CLSI, formerly the NCCLS) are highly reliable but require long incubation times (121). If the process of compound screening is not to become a bottleneck, an assay based on the CLSI method is not practical. Few cell-

based assays have been described specifically for the high-thoughput screening of ß-lactamase inhibitors.

XTT is a member of a family of tetrazolium salt compounds extensively used in cell proliferation and viability assays (17). This tetrazolium salt has been used in susceptibility testing of bacteria including *Mycobacterium tuberculosis* (16,40-42,77) and *Pseudomonas aeruginosa* (167), as well as other pathogens including *Aspergillus* species (109), *Candida* spp. (68-70,87), *Leishmania* spp. (185), *Madurella mycetomatis* (169) and HIV (46,48,125). The popularity of XTT is based laregly in its ability to indicate growth with much shorter incubation times. XTT is reduced by metabolically active cells, producing a deep orange-colored formazan. In microplate reaction wells, changes in optical density therefore depend on cellular activity rather than on changes in biomass. Unlike other tetrazolium salts such as NBT or MTT (17), the formazan product of XTT is water-soluble. Solubilization steps required in earlier tetrazolium salt-based assays therefore are not required for spectrophotometric reading. By simplifying the assay as much as possible, the complexities of implementing the assay in a high-throughput format therefore are minimised.

In the studies described here, a quantitative high-throughput assay for screening small-molecule ß-lactamase inhibitors was created. The assay was applied in the screening of a small set of compounds. Two hits were identified from the screen. Using the parameters established for the screening assay, a susceptibility assay was designed and used to determine the MICs of piperacillin combined with each "hit" for three strains of bacteria. These results were compared to MICs assessed by the reference CLSI broth microdilution method.

5.3 Materials and methods

Bacterial strains

Four strains were used in this study, including three *Escherichia coli* strains (ATCC 25922, ATCC 35218, JM101/pNU359) and one *Enterobacter cloacae* strain (P99). All strains except *E. coli* ATCC 25922 were resistant to ß-lactam antibiotics. Resistance in *E. coli* ATCC 35218 was mediated by TEM-1 (class A) (163). ß-lactam resistance in *E. cloacae* P99 was mediated by AmpC (class C) (94). *E. coli* JM101 was transformed with plasmid pNU359 (94). The plasmid contains an inducible class C ß-lactamase from *E. cloacae* P99. All strains were stored at -80°C in Mueller Hinton broth (MHB, Becton Dickinson, Cockeysville, MD, USA) supplemented with 10% glycerol.

Medium

Mueller Hinton broth (Becton Dickinson) was used in all studies.

Compounds

Ampicillin, ticarcillin, and tazobactam were obtained from Sigma (St. Louis, MO, USA). Piperacillin was obtained from Mayne Pharma (Montreal, Canada). Sulbactam and clavulanic acid were kindly provided by Pfizer Laboratories (Montreal, Quebec, Canada) and GlaxoSmithKline (Mississauga, Ontario, Canada) respectively. Eight test compounds were provided by MethylGene Inc. (Montreal, Canada). All test compounds were selected from a larger library of compounds targeting Ambler class A and/or C ß-lactamases.

XTT

XTT (Sigma) was dissolved in MHB at a concentration of 222 μg/mL. The solution was filter-sterilized using a 0.22 μm-pore-size Steritop filter (Millipore, Billerica, MA, USA). Further dilutions were prepared in the medium.

Electron coupling agents

The efficacies of two electron coupling agents were evaluated. Phenazine methosulfate (PMS) (Sigma) was dissolved in saline at a concentration of 111 μM. Menadione (Sigma) was dissolved in acetone at a concentration of 1.11 mM and then diluted 1:10 in saline. Additional dilutions of each agent were performed in MHB containing XTT.

Parameter optimization assay

The parameters assessed in this assay include XTT concentration, electron coupling species and concentration, bacterial inoculum, and incubation time. To optimize assay parameters, a series of checker board tests were performed based initially on a previously reported assay with some modifications (109). Briefly, a 3.0 McFarland suspension of the ß-lactam sensitive *E. coli* ATCC 25922 was prepared and serially diluted tenfold in MHB to yield an inoculum range of 10^3 – 10^9 CFU/mL. Aliquots from each dilution were plated on Mueller Hinton agar (Becton Dickinson, Sparks, MD, USA) in duplicate for colony counting. 20 μL of the suspension and dilutions were inoculated in 96-well flat-bottom microplates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). 20 μL of sterile medium were also added to microplates as a control. 180 μL of

the XTT-PMS or XTT-menadione solutions prepared at various concentrations were aliquoted to the wells. Final concentrations of XTT were 50, 100, and 200 μg/mL. For each XTT concentration, the final concentrations of PMS or menadione were 0.39, 1.56, 6.25, 25, and 100 μM respectively. The final inoculum ranged between 10^2 – 10^8 CFU/mL. Microplates were incubated at 37°C for 6 hours. At 1 h intervals, the optical density (OD) at 450 nm was assessed using a universal microplate reader (EL800, Bio-Tek Instruments, Winooski, VT, USA). Each experimental condition was tested in triplicate. Spectrophotometric data were normalized by subtracting the OD of background wells from the OD of experimental condition wells. After 5 hours incubation, the correlation between the OD and inoculum was analyzed by linear regression for the various concentrations of menadione tested at 200 μg/mL XTT. Regression lines and the 95% confidence intervals were plotted. The slope and r^2 values were reported to indicate the rate of XTT reduction and the "goodness of fit" respectively. Statistical analyses were performed with Graphpad Prism Software version 3.02 (San Diego, CA, USA).

Bacterial cryoprotection assay

To reduce the potential of inter-assay variability resulting from the frequent preparation of fresh bacterial suspensions, the feasibility of cryoprotecting microplates pre-inoculated with a representative strain, specifically *E. coli*, was evaluated. *E. coli* ATCC 25922 was grown overnight in MHB and centrifuged at 3000 RPM for 10 minutes. A 0.5 McFarland suspension was prepared in MHB containing 5% glycerol. 20 μL were inoculated into two 96-well flat-bottom microplates. An aliquot was serially diluted and plated for colony counting. After 30 minutes equilibration at ambient

temperature, one of the plates was placed in a -80°C freezer for 24 hours. 180 µL of MHB with XTT (222 µg/mL) and menadione (27.7 µM) were added to a set of wells of the other microplate. The final concentrations of XTT and menadione were 200 µg/mL and 25 µM respectively. To quantify the OD change as a consequence of the use of XTT, MHB alone was added to another set of wells. After incubating the microplate for 5 hours at 37°C, the OD at 450 nm was assessed as above. After 24 hours, the microplate containing cryopreserved bacteria was placed in a 37°C incubator for rapid thawing and was assayed according to the procedure described for the first plate. OD readings from the XTT assay (OD_{XTT}) indicated changes in colour (i.e. the metabolic activity of viable bacteria). OD values from the inoculated wells containing MHB alone reflected changes in turbidity (or biomass) and are referred to as $OD_{biomass}$.

Determination of the optimal bacterial inoculum

To standardize the inoculum, a range between 5×10^4 CFU/mL and 5×10^7 CFU/mL was evaluated using a ß-lactam - ß-lactamase inhibitor combination. The TEM-bearing ß-lactamase positive *E. coli* ATCC 35218 was grown overnight in MHB and centrifuged at 3000 RPM for 10 minutes. A 0.5 McFarland suspension was prepared and serially diluted tenfold. Aliquots from each dilution were plated in duplicate for colony counting. 20 µL of each dilution were inoculated in a 96-well microplate. Ampicillin and sulbactam (2:1 ratio) were dissolved and serially diluted twofold in an XTT – menadione solution as described in the cryoprotection assay. 180 µL of the ampicillin – sulbactam dilutions were loaded in the microplate wells. Final concentrations ranged from 64 to 512

μg/mL for ampicillin and from 32 to 256 μg/mL for sulbactam. After incubation for 5 hours at 37°C, the OD_{450} was assessed.

Antimicrobial susceptibility assay

After optimizing each of the parameters described above, the accuracy of the optimized XTT assay was evaluated. Using the optimized assay, minimum inhibitory concentration (MIC) values for two ß-lactam - ß-lactamase inhibitor combinations were compared to expected MICs described in CLSI protocol M7-A5 (121). *E. coli* ATCC 35218 was grown overnight in MHB and centrifuged at 3000 RPM for 10 minutes. A 0.5 McFarland suspension was prepared and diluted to 5×10^6 CFU/mL in MHB. 20 μL aliquots of the suspension and the sterile MHB were transferred to a 96-well microplate. Ampicillin and sulbactam (2:1 ratio) were dissolved together at initial concentrations of 142 μg/mL and 71.1 μg/mL respectively and serially diluted twofold in an XTT – menadione solution as described in the cryoprotection assay. Ticarcillin was dissolved at 142 μg/mL and serially diluted twofold in an XTT – menadione solution containing clavulanic acid (2.22 μg/mL). 180 μL of the ß-lactam – ß-lactamase inhibitor combinations were loaded in the microplate wells. Plates were incubated for 5 hours at 37°C, and the OD_{450} was determined. Results were expressed in relative optical density units (RODU) calculated according to the following formula: [(OD of drug-containing well – background OD)/(OD of drug-free well – background OD of drug-free well)] × 100% (109).

Compound screening

To assess the performance of the colorimetric assay as a screen, three ß-lactamase-expressing bacterial strains were used to evaluate the inhibitory activity of eight compounds synthesized by MethylGene Inc. *E. coli* ATCC 35218, *E. coli* JM101/pNU359 and *E. cloacae* P99 were cultured overnight and centrifuged at 3000 RPM for 10 minutes. A 0.5 McFarland suspension was prepared and diluted to 5×10^6 CFU/mL in MHB containing 5% glycerol. 20 µL of each suspension was aliquoted to a 96-well microplate. Aliquots also were taken from each suspension, serially diluted and plated for colony counting. After equilibration, the microplate was stored as described in the cryoprotection assay. After thawing the plate, 180 µL of XTT – menadione solution containing piperacillin (17.8 µg/mL) were loaded into the microplate wells. The final concentration of piperacillin was 16 µg/mL. Stock solutions of test compounds MG506H1, MG514X1, MG867, MG2102, MG2914, MG4545, MG4581 and MG4684 were dissolved in DMSO at 400 µg/mL. Tazobactam was dissolved in sterile saline at 160 µg/mL. 5.0 µL of each test compound and tazobactam were loaded into the wells yielding final concentrations of 10 µg/mL and 4 µg/mL respectively. The OD_{450} was read after incubation for 5 hours at 37°C.

MIC of "hit" compounds according to the colorimetric susceptibility assay

For compounds identified as hits, the MIC of piperacillin combined with the test compound was determined. Inoculated microplates were prepared, stored and thawed as described under compound screening. Piperacillin was dissolved at an initial concentration of 71.1 µg/mL and diluted twofold in an XTT – menadione solution. 180

μL of each piperacillin dilution was loaded into microplate wells. 5.0 μL of each test compound and tazobactam solutions were loaded into the wells yielding final concentrations of 10 μg/mL and 4 μg/mL respectively. The OD_{450} was determined after incubation for 5 hours at 37°C.

CLSI broth microdilution assay

The colorimetric assay as described above was validated against the CLSI M7-A5 reference method (121). The 2 hits identified from the screen were tested with piperacillin against the three ß-lactamase positive organisms used in the colorimetric susceptibility assay. Tazobactam was also tested as a control. Briefly, MG2102 (10 μg/mL), MG2914 (10 μg/mL) and tazobactam (4 μg/mL) were dissolved and dispensed into tubes. Piperacillin was dissolved and serially diluted twofold in these tubes to yield a concentration range between 1 μg/mL and 64 μg/mL. 100 μL of each drug combination and drug-free controls were dispensed into microplates. 0.5 MacFarland suspensions of *E. coli* ATCC 35218, *E. coli* JM101/pNU359 and *E. cloacae* P99 were prepared and diluted tenfold. 5.0 μL of the suspensions were inoculated into the wells, yielding a final inoculum of 5×10^5 CFU/mL. The microplate was incubated at 35°C for 18 hours and then read spectrophotometrically at 630 nm. A visual assessment of MICs was also made.

5.3 Results

Optimization of the high-throughput assay

In order to select the most appropriate electron-coupling agent and determine the optimal XTT concentration indicative of metabolic activity and produces a strong signal,

E. coli ATCC 25922 was exposed to a range of concentrations of XTT and menadione or PMS over a 6 hour period. Results shown in Figure 1 indicate that the isolate is capable of reducing XTT without the presence of an electron coupler. However, after a minimum of 3 hours of incubation, XTT reduction efficiency is increased at coupling agent concentrations of 6.25 μM and above. After exposure for 5 hours or longer, a 100 μM concentration slightly reduced formazan production, suggesting possible toxicity. Of the two electron couplers, menadione was more potent than PMS at equivalent concentrations in our hands. Throughout the assay, menadione produced lower background ODs than PMS (data not shown). A maximal signal was achieved when bacteria were incubated with XTT at 200 μg/mL; two-times and three-times higher than with XTT at 100 and 50 μg/mL respectively.

After 6 hours of incubation, XTT reduction could not be detected with initial inocula of 10^2 and 10^3 CFU/mL (Figure 2). At 100 μM menadione, absorbance exceeded OD 3.0 (the upper limit of detection of the spectrophotometer) at an inoculum range between 10^5 and 10^8 CFU/mL. However with a 10^4 CFU/mL inoculum, little formazan production was detected. Because the absorbance rose from a barely measurable signal to exceeding the limit of detection within a brief one-hour interval, the use of 100 μM menadione was abandoned. At menadione concentrations of 6.25 and 25 μM, linear XTT reduction occurred over a wider range of inocula between 10^5 and 10^8 CFU/mL within 5 hours of incubation. There was no significant difference in XTT reduction rates at menadione concentrations of 1.56 μM or below for respective inocula.

The correlation between XTT reduction and the bacterial inoculum is shown in Figure 3. Bacteria were exposed to 200 μg/mL XTT and menadione at concentrations of

0.39, 1.56, 6.25, 25 and 100 μM for 5 hours. At all concentrations of menadione, a linear relationship existed between absorbance and log CFU ($P < 0.0001$). Results from assays in which linearity exists between the signal and the biological effect are amenable to quantitative analysis. As described earlier, the data confirm that formazan production occurs at a low rate with 0.39 and 1.56 μM menadione (slopes were 0.35 and 0.37 respectively) (Table 1). A higher XTT reduction rate was achieved with 6.25 μM menadione, though the highest OD recorded was only 1.5 (at an inoculum of 10^7 CFU/mL). An optimal correlation coefficient and rate of XTT conversion were observed at 25 μM menadione. As the number of metabolically-active bacteria may be assessed over a wide signal range, good assay sensitivity therefore is achieved at this menadione concentration. In addition, high OD readings can be achieved from growing bacteria within 5 hours of incubation with low background noise.

Effect of cryoprotection on XTT reduction

Microplates were inoculated with 10^7 CFU/mL *E. coli* ATCC 25922 and incubated with 200 μg/mL XTT and 25 μM menadione for 5 hours. MHB alone was also added to a set of inoculated wells to assess changes in turbidity (or the number of bacteria per well). Following cryopreservation, $OD_{biomass}$ values indicated no decrease in growth compared to freshly-prepared bacteria (Table 2). These data indicated that any loss of viable bacteria due to cryopreservation was insignificant. As expected, OD_{XTT} values were considerably higher than respective $OD_{biomass}$ values. Prior to and following cryopreservation, OD_{XTT} were 3.9–fold and 4.5–fold higher respectively. Colorimetric assessments of growth through the use of XTT therefore have a superior dynamic range

compared to turbimetric assessments. When evaluated colorimetrically, cryopreserved *E. coli* reduced XTT more than non-cryopreserved *E. coli*, suggesting possible enhanced metabolic activity. A similar increase in OD was observed when the $OD_{biomass}$ of cryopreserved and non-cryopreserved *E. coli* were compared. The increase in metabolic activity observed in the XTT assay therefore was correlated with increased bacterial growth. The metabolic activity of reconstituted *E. coli* was consistent when stored at -80°C for up to six months (data not shown).

Determination of the optimal bacterial inoculum

In order to standardize the inoculum, the sensitivity of inocula ranging from 5×10^4 to 5×10^7 CFU/mL of *E. coli* ATCC 35218 was tested against various concentrations of ampicillin:sulbactam (64:32, 128:64, 256:128, and 512:256 µg/mL). At inocula of 5×10^6 and 5×10^7 CFU/mL, increasing metabolic activity (i.e. growth) was detected despite relatively high drug concentrations (Table 3). Since *E. coli* ATCC 35218 is a CLSI quality control strain (121), the CLSI report that the MIC for ampicillin – sulbactam should have been between 8:4 µg/mL and 32:16 µg/mL. Prior testing of this strain according to the CLSI method revealed compliance with the recommended limits. The results we observed in the XTT assay may be accounted for by the inoculum effect (52,93,96) commonly associated with ß-lactam antibiotics (52). At an inoculum of 5×10^4 CFU/mL, little formazan production was detected in the antibiotic-free control, indicating that this inoculum was too low to test susceptibility within 5 hours. When an inoculum of 5×10^5 CFU/mL was used, complete inhibition was noted at all drug concentrations tested. An OD of 2.546 in the antibiotic-free control wells showed that

growth inhibition had occurred rather than a lack of growth. At this inoculum, growth inhibition at the drug doses tested was consistent with expected results based on CLSI methods.

XTT-based antimicrobial susceptibility assay

To evaluate whether the sensitivity and performance of the XTT assay are satisfactory at clinically-relevant antibiotic concentrations, susceptibility of *E. coli* ATCC 35218 to ampicillin:sulbactam and ticarcillin:clavulanic acid was assessed. Concentrations of drug combinations encompassed the interpretive breakpoints as described in CLSI method M7-A5 (121). The MIC was interpreted according to Tunney *et al.* (167) with some modifications. The MIC was defined as the concentration at which the blanked OD is reduced by 90% compared to the blanked control growth. For ampicillin:sulbactam and ticarcillin:clavulanic acid, the observed MICs in the XTT assay were 2:1 μg/mL and 8:2 μg/mL respectively (Figure 4). Compared to CLSI-recommended limits for the *E. coli* isolate used, the MIC of ampicillin:sulbactam was two dilutions below the reference range while the MIC of ticarcillin:clavulanic acid was within the expected range. These data suggest that the XTT assay may be more sensitive than the CLSI method.

Test compound screening

XTT assay performance was evaluated using eight test compounds provided by MethylGene Inc. Tazobactam was included as a control. The activity of the compounds was not known to the operator (B.L.) prior to screening. Compounds producing at least a

50% reduction in absorbance compared to the antibiotic-free control for the three ß-lactamase producing bacterial strains tested were considered hits. Hits therefore were active against both class A and C ß-lactamases. The piperacillin concentration was fixed at a sub-inhibitory concentration of 16 μg/mL. After screening, 3 compounds were identified as hits, including tazobactam (Figure 5). Assay quality was assessed by calculating Z' scores (189). Z' scores varied between 0.52 and 0.98, with a median of 0.75.

XTT assay validation

The inhibitory activity of hits combined with piperacillin was quantitatively assessed using the XTT assay and the CLSI broth microdilution method. Tazobactam was included as a reference. Two MICs from the XTT assay exactly matched the MICs from the CLSI method (Table 4). The remaining seven XTT-derived MICs were within one dilution above or below their respective reference MIC. Exact agreement therefore was 22% and essential agreement was 100%. In addition, all except one MIC from the XTT assay were below their corresponding reference MIC. This observation again suggests that the colorimetric method was more sensitive than the CLSI method.

5.4 Discussion

The expression of ß-lactamase enzymes is one of the principal mechanisms by which pathogenic bacteria resist the bactericidal action of ß-lactam antibiotics. Though small-molecule inhibitors of ß-lactamases have been discovered, the available products

target only class A enzymes. The spread of inhibitor-resistant class A ß-lactamases and the emergence of class B, C, and D enzymes have spurred a search for novel drugs that can target multiple enzyme classes and resist the hydrolytic action of ß-lactamases.

In the current study, we have developed a tetrazolium-based high-throughput screen for the identification of ß-lactamase inhibitors. When dissolved, XTT is a colorless tetrazolium compound that becomes vibrant orange upon its reduction into a formazan product via bacterial metabolic activity. Though it has been reported that tetrazolium salts inhibit bacterial growth (180), no inhibition was observed at the concentrations tested in this study. Without an electron coupling agent, bacteria reduced XTT only moderately over a 5 h incubation. The dynamic range of an assay using XTT alone in this context would therefore have been small. To improve the dynamic range (i.e. increasing the rate of formazan production per unit of time), an electron coupling agent was found to be necessary. In our hands, menadione proved to be the superior compound. The superiority of menadione over PMS has been previously reported for detecting growth of *Aspergillus* spp. (109). In addition, menadione produced generally lower background ODs.

Metabolic activity as indicated by tetrazolium salt reduction can be linearly proportional to the number of exponentially-growing bacteria (41,167). In our studies, the relationship between XTT reduction and increasing inocula was linear ($r^2 \geq 0.86$) at an inoculum range between 10^4 and 10^7 CFU/mL. With 200 µg/mL XTT, 25 µM menadione, XTT reduction could linearly gauge the bacterial biomass over a 5 hour incubation. Variation in inoculum size can be a source of considerable experimental variability (7). As the OD signal from formazan production represents an amplification of metabolic activity, small differences in the inoculum at the start of the assay may

considerably influence subsequent OD readings. In order to reduce the inter-experimental variability as well as the labour involved in the frequent preparation of fresh bacterial suspensions, the feasibility of cryoprotecting microplates pre-inoculated with a stock *E. coli* concentration was assessed. When bacteria were assayed before and after cryopreservation, metabolic activity was found to have increased slightly following the freezing process. It is possible that this increase is the result of the penetration of glycerol into the cryopreserved bacteria. Glycerol can be metabolized and thus used as an energy source for growth (191). This observation is consistent with previous reports in which bacterial metabolic activity increased in the presence of an energy source (106,143,149). Although it is likely that a small number of bacteria were rendered non-viable due to cryopreservation, our results indicate that this loss does not significantly influence antibiotic susceptibility or test compound efficacy. The OD of colorimetric and turbimetric measurements were compared. OD_{XTT} values were approximately fourfold higher than respective $OD_{biomass}$ values. The former method therefore has a considerably wider dynamic range and is thus more appropriate for high-throughput applications than the latter.

The XTT assay was applied to eight compounds with known activity as ß-lactamase inhibitors supplied by MethylGene Inc. Tazobactam was also tested as a reference. In order to select for compounds demonstrating inhibitory activity against class A and C ß-lactamases, screening was performed using *E. coli* ATCC 35218 (class A), *E. coli* JM101/pNU359 (inducible class C) and *E. cloacae* P99 (constitutive class C). Piperacillin was fixed at 16 μg/mL since this antibiotic concentration was sub-inhibitory for the ß-lactamase-expressing bacterial strains used. However, in the presence of an

effective ß-lactamase inhibitor, sensitivity to the ß-lactam should have been re-established. Growth consequently would have been inhibited and interpreted as a weak signal in the screen. Using a hit threshold of ≥50% inhibition for each strain, two compounds, specifically MG 2102 and MG 2914 were found to be active. For both compounds, the growth of the two *E. coli* strains was strongly inhibited (nearly 100% inhibition) though *E. cloacae* was only marginally above the 50% threshold. Identical results were observed for the reference inhibitor, tazobactam. Although *E. coli* JM101/pNU359 and *E. cloacae* P99 express the same ß-lactamase variant (P99), we observed clearly different efficacy profiles. A potential contributing factor could be the lower permeability of piperacillin and/or the ß-lactamase inhibitor test compounds into the periplasm of *E. cloacae* (50). If the passive diffusion of either the antibiotic or the inhibitor through porins in the outer membranes of *E. cloacae* is lower compared to *E. coli*, then it would be expected that growth inhibition also would be lower. Another possibility accounting for the decreased sensitivity of *E. cloacae* could be the result of higher ß-lactamase activity. It has been reported that the periplasmic concentration of ß-lactamase enzymes in *E. cloacae* P99 is 300 μM (49). Although the ß-lactamase concentration of the *E. coli* strain expressing P99 used in the current study was not calculated, it is reasonable to assume based on the high sensitivity of *E. coli* JM101/pNU359 to piperacillin in the presence of either test compound that the concentration was lower. The median Z' score was 0.75, thus quantitatively confirming that the XTT assay has a wide dynamic range and is amenable to high-throughput screening. After screening the compounds, it was revealed to the operator (B.L.) that only MG 2102 and MG 2914 were designed to be ß-lactamase inhibitors. The other

compounds were developed for a related project. The screen therefore accurately identified all potential "hits."

The colorimetric screen was validated by comparing the MICs of the two hits using an XTT-based susceptibility assay and the CLSI method. Tazobactam was again used in both assays as a reference. The MIC results using the XTT method correlated well with the screening data. Between the screening and the XTT-based susceptibility assays, it is important to note the difference in RODU at which growth inhibition is defined for "hits" and MICs respectively. In the former, a "hit" produced at least 50% inhibition ($\geq$50% RODU) for each strain. In the latter, the MIC is defined as the lowest concentration of ß-lactam - ß-lactamase inhibitor combination that resulted in at least 90% inhibition ($\leq$10% RODU). Since almost 100% inhibition was observed for MG 2102, MG 2914, and tazobactam for both *E. coli* strains in the screening assay, the piperacillin MICs in combination with these inhibitors therefore should have been 16 µg/mL or below. All piperacillin MICs determined according to the colorimetric method were 16 µg/mL or below. Since approximately 50% inhibition was observed for *E. cloacae* for all inhibitors tested in the screen, it was expected that the piperacillin MICs should have been above 16 µg/mL. This assumption was confirmed by the results obtained from the XTT susceptibility assay for this bacterial strain. Though exact agreement was 22%, essential agreement (MIC within 1 twofold dilution) was 100%. The MIC difference largely favoured the lower concentration, therefore indicating a slight sensitivity advantage over the CLSI method. Both MG 2102 and MG 2914 sufficiently inhibited ß-lactamase activity to render both *E. coli* strains sensitive to piperacillin. *E. cloacae* remained resistant, possibly for similar reasons described above. The potency of

tazobactam was higher than both test compounds against both *E. coli* strains, but was equally ineffective at inhibiting class C ß-lactamase activity in *E. cloacae*. Though neither MG 2102 nor MG 2914 were as potent as tazobactam, designing structural analogues of these compounds may produce a more effective ß-lactamase inhibitor.

Some elements of the assay may be modified as appropriate for various applications. For instance, screening may be performed using any ß-lactam, providing that it does not have any direct effect on the colorimetric reaction. No interaction was noted between the ß-lactams used in this study and either XTT or menadione. Additionally, bacteria other than *E. coli* or *E. cloacae* may be used for screening. When bacteria of other species are selected, the menadione concentration may require adjustment to account for differences in metabolic activity. The ß-lactam concentration may also require adjustment to ensure it is sub-inhibitory unless a compound impeding ß-lactamase function is present. To test for inhibitor efficacy against different classes of ß-lactamases or variants of ß-lactamases within the same class, a ß-lactam sensitive *E. coli* strain (such as ATCC 25922) may be transformed with a plasmid expressing the desired variant or variants.

The colorimetric assay described here is best suited as a secondary screen in drug discovery programs. As the assay is cell-based, only compounds that permeate the bacterial cell wall and inhibit ß-lactamase function (thus allowing the bactericidal action of ß-lactams) are identified.

In summary, our report describes for the first time a complete colorimetric, cell-based system for the high-throughput screening of ß-lactamase inhibitors. Our results show that bacteria can be pre-inoculated in microplates and cryopreserved without

detrimentally affecting viability and drug susceptibility results. Moreover, quantitative analyses can be performed on screening data because of the linear correlation between optical densities and the number of metabolically-active bacteria. The screen is simple to perform, adaptable to screening objectives, amenable for automation and does not require costly investments for implementation.

Acknowledgments

We gratefully acknowledge Momar Ndao for helpful discussions.

MethylGene Inc. had no involvement in the design of this study, the interpretation of the data, or the preparation of this report.

5.5 Figures and tables

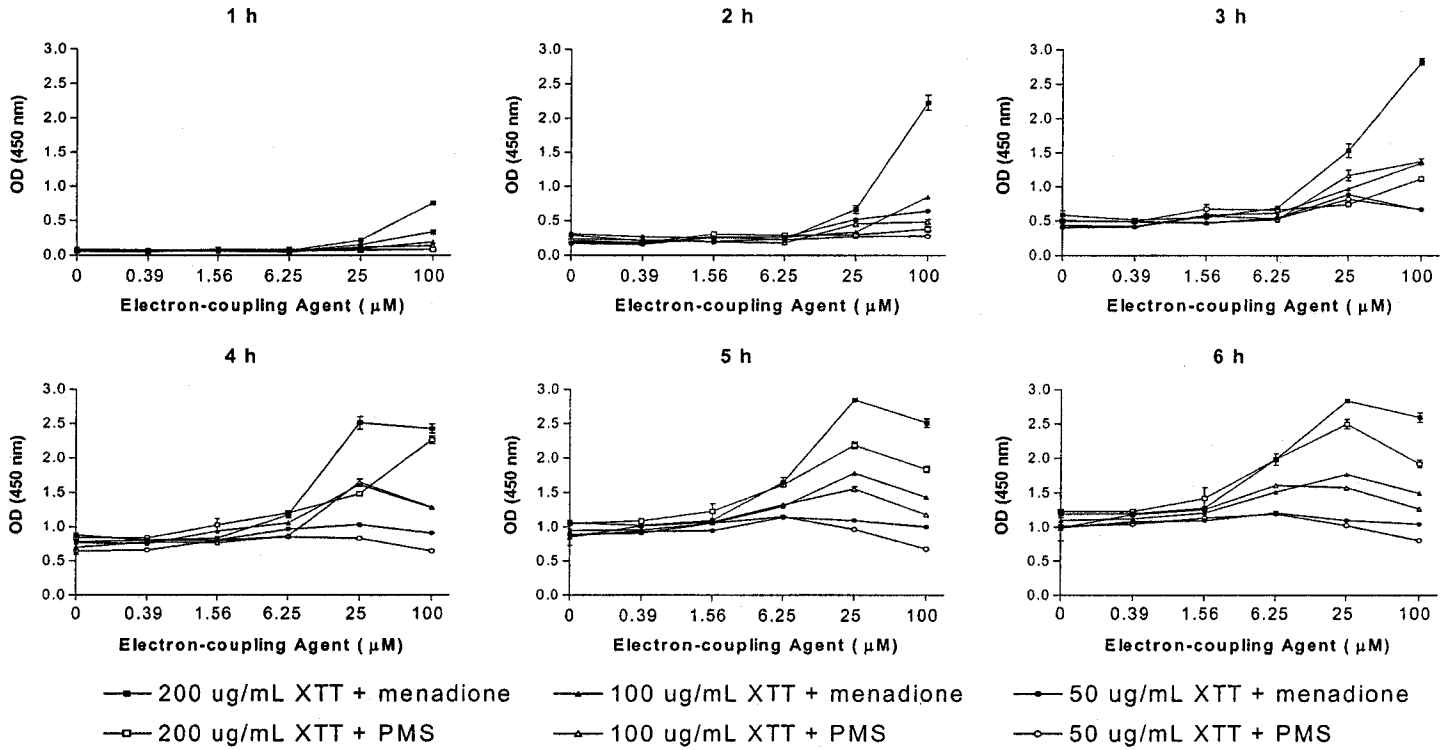

FIG 1. Reduction of XTT by *E. coli* at an initial inoculum of 1×10^7 CFU/mL. *E. coli* was exposed to various concentrations of XTT, 200 (squares), 100 (triangles) and 50 (diamonds) μg/mL, and menadione (solid symbols) and PMS (hollow symbols) at various concentrations (0.39, 1.56, 6.25, 25, and 100 μM). Data points represent the mean of each experimental condition tested in triplicate. Error bars represent the SEM.

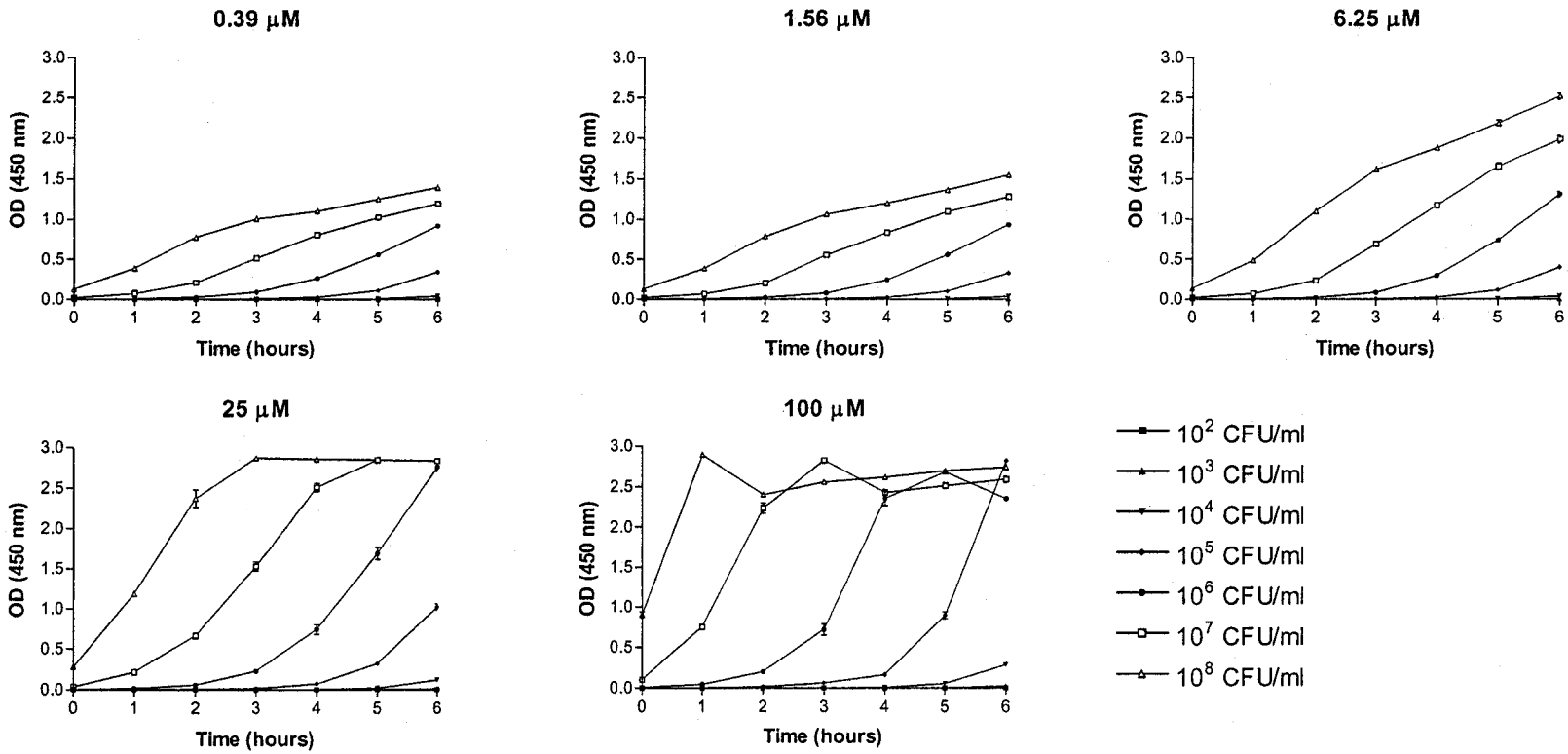

FIG 2. XTT reduction by various inocula of *E. coli* ATCC 25922. Various inocula of *E. coli* were exposed to XTT at 200 µg/mL and various concentrations of menadione over a 6 hour period. Each datum point represents the mean of each experimental condition tested in triplicate. Error bars indicate standard errors.

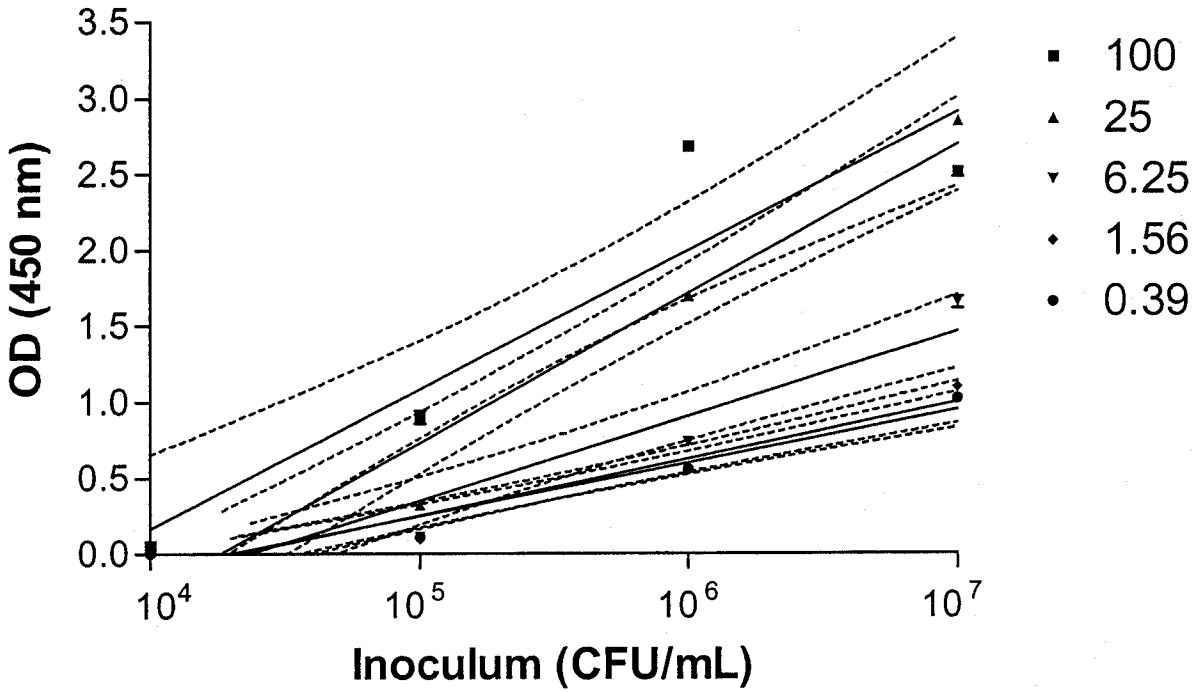

FIG 3. Correlation between XTT reduction and increasing inocula. 200 μg/mL XTT was reduced in the presence of various concentrations of menadione (0.39, 1.56, 6.25, 25, and 100 μM). Each experimental condition was tested in triplicate. The symbols indicate the means of the triplicates. Lines were produced by linear regression analysis. Dotted lines show the 95% confidence intervals of the regression lines.

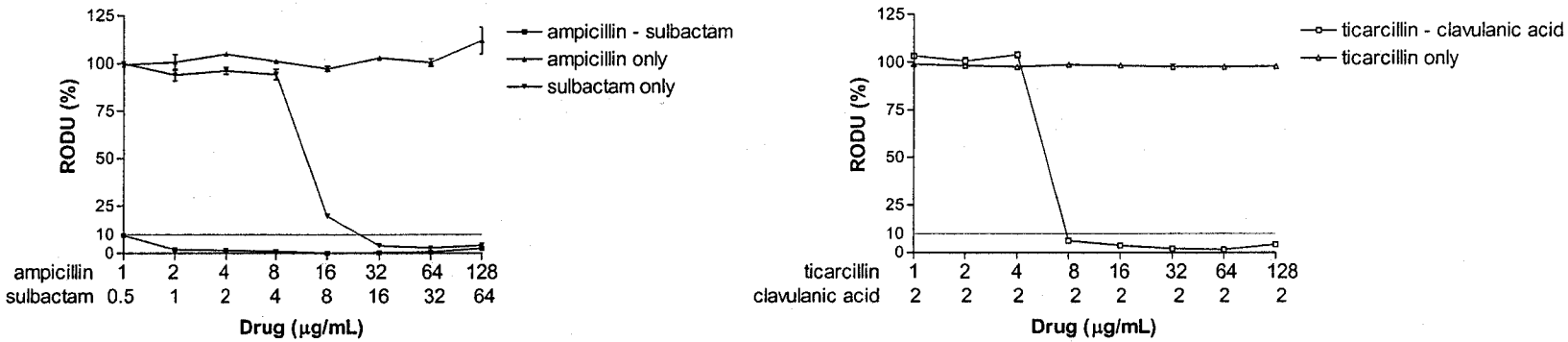

FIG 4. Concentration effect curves for ampicillin - sulbactam and ticarcillin - clavulanic acid. An inoculum of 5×10^5 CFU/mL of *E. coli* ATCC 35218 was incubated for 5 hours with 200 µg/mL XTT and 25 µM menadione. ODs were measured at 450 nm. RODUs represent the quantity of formazan produced with respect to the growth control. 10% RODU indicated on the graphs show the threshold at which the MIC was determined. Data points indicate the mean of triplicates. Error bars show the standard error.

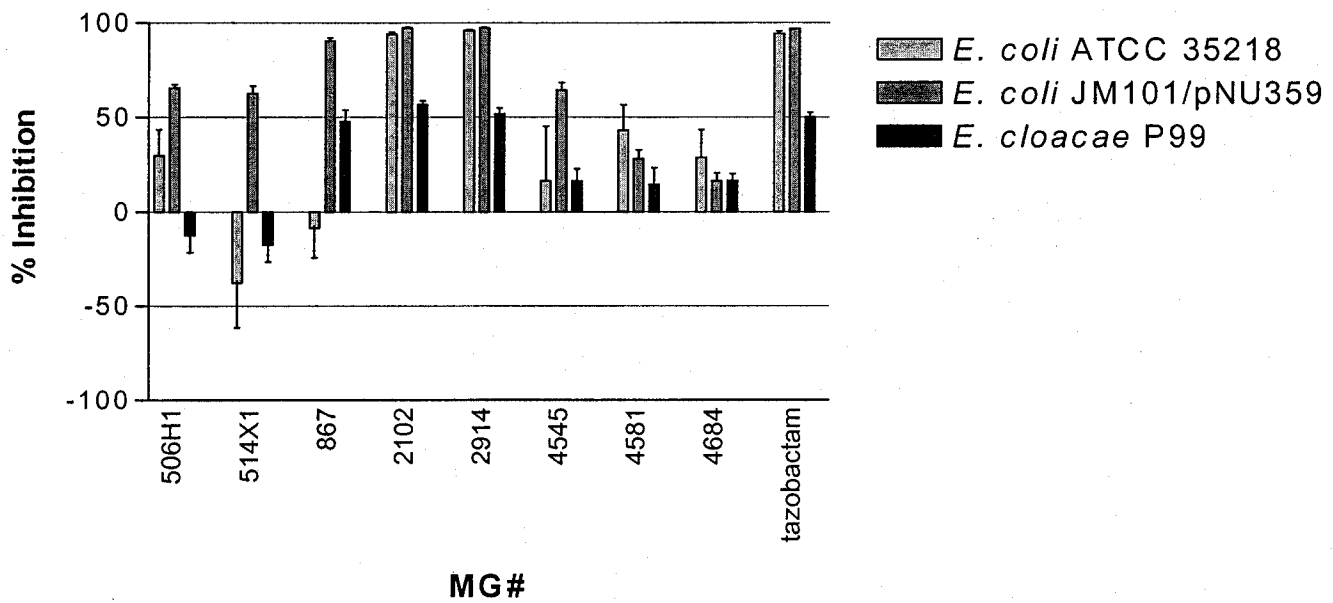

FIG 5. Inhibitory activities of test compounds and tazobactam against two strains of *E. coli* and *E. cloacae*. Test compounds were evaluated at 10 μM. Tazobactam was evaluated at 4 μM. Piperacillin concentration was 16 μg/mL. Data represent the mean of triplicates ± SEM of two independent experiments.

TABLE 1: Linear regression analysis of the correlation between the rate of XTT reduction at various concentrations of menadione and increasing inocula of *E. coli* ATCC 25922

	Correlation between menadione concentration and inoculum (log CFU/mL)				
	0.39 μM	1.56 μM	6.25 μM	25 μM	100 μM
Slope	0.35	0.37	0.56	0.98	0.92
r^2	0.941	0.928	0.902	0.946	0.860

TABLE 2: Effect of cryopreservation on metabolic activity of *E. coli* ATCC 25922

	Mean absorbance values			
	Pre-cryopreservation		Post-cryopreservation	
	OD (450 nm)	± SD	OD (450 nm)	± SD
XTT - menadione	2.288	0.012	2.772	0.028
MHB only (control medium)	0.594	0.001	0.614	0.014

TABLE 3. XTT reduction by *E. coli* ATCC 35218 in the presence of ampicillin - sulbactam

Inoculum (CFU/mL)	Mean absorbance at various concentrations of ampicillin - sulbactam (μg/mL)									
	512-256		256-128		128-64		64-32		0-0	
	OD (450 nm)	± SD	OD (450 nm)	± SD	OD (450 nm)	± SD	OD (450 nm)	± SD	OD (450 nm)	± SD
10^4	- 0.003	0.001	- 0.002	0.002	0.006	0.003	0.031	0.005	0.285	0.006
10^5	0.028	0.003	0.041	0.005	0.140	0.001	0.364	0.034	2.546	0.470
10^6	0.403	0.007	0.640	0.018	1.352	0.009	2.686	0.122	2.103	0.460
10^7	2.811	0.000	2.817	0.000	2.797	0.031	2.032	0.053	2.280	0.427

TABLE 4. Piperacillin MICs as interpreted by the XTT reduction assay and by the CLSI method[a]

Strain	Piperacillin MIC (μg/mL)					
	MG 2102		MG 2914		Tazobactam	
	XTT	CLSI	XTT	CLSI	XTT	CLSI
E. coli ATCC 35218	16	32	8	16	1	2
E. coli JM101/pNU359	4	4	4	2	1	1
E. cloacae P99	64	>64	32	64	64	>64

[a]CLSI MICs were determined visually and spectrophotometrically in two independent experiments. Spectrophotometic MIC data corresponded to visual assessments.

Connecting statement II

After developing the assay described in the previous section, we were interested in testing candidate ß-lactamase inhibitors *in vivo*. However at the time, MethylGene Inc. was focusing on the *in vitro* stage of drug development for these inhibitors. In consequence, no candidate compounds with good safety and efficacy profiles were available for *in vivo* testing. Apart from antibacterial therapeutics, MethylGene Inc. also maintains an active interest in antifungal therapies. Initial *in vitro* tests of a novel histone deacetylase inhibitor, MG 3290, with a commercially-available antifungal demonstrated synergistic activity against the growth of *Candida albicans* and *Candida glabrata. In vitro* data was sufficiently compelling to warrant *in vivo* testing. Since fungal infections (such as systemic candidemia) are among the most common infections that cause morbidity and mortality in the clinical setting and because of the relative paucity of effective antimicrobials to combat antifungal resistance, we felt that it would be important to advance the preclinical development of this lead compound. First, we sought to develop an animal model of systemic candidemia. Based on a search of the literature, a mouse model of systemic *Candida* infection was designed. Immunocompromised mice were infected intravenously with *C. albicans* and treatment was initiated the following day. Drugs were administered intraperitoneally once per day for 7 days. On the 8^{th} day, mice were euthanized and kidneys were collected for fungal load assessment. The second manuscript describes the testing of MG 3290 with ketoconazole using this mouse model. Synergy between the compound and the antifungal was observed.

Chapter 6: Manuscript II

In vivo synergy between MG 3290, a histone deacetylase inhibitor, and ketoconazole in immunocompromised mice systemically infected with *Candida albicans*

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Key words: antifungal therapy, histone deacetylase inhibitor, HDAC, candidemia, murine model, systemic infection, ketoconazole

6.1 Abstract

As a result of the expanded use of immunosuppressive drugs and the increased number of individuals living with HIV infections, a greater segment of the population is immunocompromised and thus vulnerable to opportunistic infections. The incidence of fungal infections, in particular caused by azole-resistant strains, is increasing. Novel antifungals are needed urgently. Although antifungal drug development traditionally focuses on established targets, a new strategy involves the inhibition of fungal histone deacetylases to enhance the efficacy of existing azole antifungals. The *in vivo* efficacy of MG 3290, a novel histone deacetylase inhibitor, in enhancing activity of azole antifungals in a murine model of systemic *Candida* infection is reported. Neutropenic mice were infected with *Candida albicans* intravenously and treated once daily over 7 days with ketoconazole (10 – 40 mg/kg, intraperitoneally) and MG 3290 (1 – 10 mg/kg intraperitoneally). A dose-dependent response was observed with increasing doses of MG 3290 when 20 mg/kg ketoconazole was administered. In mice treated with 10 mg/kg MG 3290 and 20 mg/kg ketoconazole, kidney fungal burden decreased 3.2 log units and the survival rate doubled to 100% compared with ketoconazole-only treated control mice. Our findings suggest that histone deacetylase inhibitors may enhance *C. albicans* sensitivity to azole antifungals *in vivo*. This may have implications in the treatment of azole-resistant fungal infections.

6.2 Introduction

The incidence of systemic candidemia is increasing rapidly and systemic *Candida* infections have become the fourth leading cause of nosocomial disease (132). A growing segment of the population who are immunocompromised (i.e. those undergoing cancer chemotherapy, organ transplantation or living with HIV infection) is particularly vulnerable: *Candida* infection in hospitalised subjects considerably increases the risk of mortality (117). Amphotericin B is an effective treatment for a wide variety of fungal infections, but frequently causes side-effects including fever, rigors, and at high doses, nephrotoxicity (186). Azole antifungals such as itraconazole or fluconazole induce fewer side-effects and are used widely but their efficacy is increasingly challenged by the spread of azole-resistant fungi (153).

Histone deacetylases (HDACs) are essential in the regulation of DNA transcription. HDACs function by deacetylating N-terminal lysine residues of histone components H2A, H2B, H3 and H4 thereby inactivating gene expression (97,98). Though the inhibition of HDACs in *Candida* spp. is not necessarily lethal (150), it may increase susceptibility of these microbes to antifungal compounds. It has been proposed that this occurs by repressing the expression of genes encoding multi-drug transporters (*MDR1*, *CDR1*, *CDR2*) and enzymes involved in the biosynthesis of ergosterol (*ERG*), an essential stabilising component of the fungal cell wall (160).

MG 3290 is a small-molecule HDAC inhibitor developed and synthesized by MethylGene Inc. *In vitro* assays reveal substantial reductions in the growth of *Candida albicans* and *Candida glabrata* at increasing doses of MG 3290 in the presence of ketoconazole (Campeol, N., J. Bedard, and N.H. Georgopapadakou. 2005. Synergism of

histone deacetylase (HDAC) inhibitors with ketoconazole in *Candida albicans* and *Candida glabrata*. Relationship to their effects on HDAC Activity in protoplasts. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-2154). The present study describes the *in vivo* activity of MG 3290 with ketoconazole in a neutropenic mouse model.

6.3 Materials and methods

Organism

The *Candida albicans* strain used in this study is a clinical isolate obtained from the reference collection of the clinical mycology laboratory of the McGill University Health Centre (Montreal General Hospital). To prepare stock suspensions, an overnight growth on Sabouraud dextrose agar (SDA, Becton Dickinson, Sparks, MD, USA) plates was suspended in 0.9% saline supplemented with 10% glycerol and was cryopreserved in liquid nitrogen in cryogenic vials. 48 hours prior to infecting mice, organisms were streaked and grown on SDA plates at 37°C. On the day of infection, colonies were picked and suspended in 0.9% saline. Turbidity was adjusted to 0.5 McFarland (corresponding to an inoculum range between 1×10^6 and 5×10^6 CFU/mL) using a Vitek colorimeter (Hach Company, Loveland, CO, USA). 2.0 mL of this suspension was aliquoted to 18 mL of saline for inoculation. An aliquot was then serially diluted, plated on SDA plates, and incubated at 37°C overnight for colony counting.

Mice

6 – 8 week-old female CD-1 mice (Charles River Canada, Saint-Constant, Canada) weighing 20 to 25 g were used in this study. Animals were maintained according to guidelines established by the Canadian Council on Animal Care (188). Ethical approval was obtained from the McGill University Animal Care Committee prior to the commencement of the study. Mice had access to food and water *ad libitum*.

Antimicrobial Agents

MG 3290 was provided in powder form by MethylGene Inc. (Montreal, Canada). The compound was stored at -80°C. Ketoconazole was purchased from Sigma (St. Louis, MO, USA). Stock solutions of MG 3290 (200 mg/mL) and ketoconazole (200 mg/mL) were prepared in DMSO. Hydrochloric acid was added to the ketoconazole stock solution for complete dissolution (pH 3.0).

Drug combinations were prepared on the first day of drug treatment (Day +1 of infection). Combinations were prepared according to Table 1. DMSO was added to all tubes to a total volume 4.8 mL. Sterile water was then added to all tubes to a total volume 8.0 mL. Final solutions were stored at 4°C for up to 7 days.

To reduce secondary infection in the neutropenic animals, a solution comprising 1.0 mg/mL ampicillin (Wisent, Saint-Bruno, Canada) and 0.2 mg/mL kanamycin (Wisent, Saint-Bruno, Canada) was prepared as drinking water.

Immunosuppressive Agent

Cyclophosphamide (Sigma, St. Louis, MO, USA) was dissolved in 0.9% saline at concentrations of 24 mg/mL and 8.0 mg/mL. Solutions were filter-sterilized using a 0.22 μm-pore-size Millipore Express PES membrane (Bedford, MA, USA).

Model of Infection

To render mice neutropenic, cyclophosphamide was injected intraperitoneally on Day -3 (240 mg/kg), Day +1 (80 mg/kg), and Day +5 (80 mg/kg) of infection. Using the Unopette system (Becton Dickinson, Franklin Lakes, NJ, USA), white blood cell counts were performed on Day +1 and upon sacrificing mice. On the day of infection (Day 0), 0.2 mL of the yeast suspension (corresponding to an inoculum range from 2×10^4 to 1.22×10^5 CFU/mouse) was injected intravenously in the lateral tail vein. The control group was injected with an equal volume of sterile 0.9% saline. 24 h after infection (Day +1), kidneys from one group of mice were collected using aseptic techniques to assess the fungal load at the onset of treatment. Ketoconazole was administered at 10, 20, and 40 mg/kg of body weight with MG 3290 at 1, 2, 5, and 10 mg/kg. MG 3290 was not tested with 40 mg/kg ketoconazole in infected mice. However, ketoconazole and MG 3290 at 40 mg/kg and 10 mg/kg respectively were injected in the sham-infected group as a toxicity control. Drug combinations were injected intraperitoneally once per day starting on the day following infection (Day +1) and therapy continued for 7 days. Mice were weighed and monitored daily. Kidney pairs from mice succumbing to the infection were collected on the day of death or euthanasia. Surviving mice were sacrificed 24 hours following the final dose (Day 8) and their kidneys were harvested.

To assess the fungal load, kidney pairs from each mouse were homogenized in sterile saline, serially diluted, plated on Sabouraud dextrose agar in duplicate and incubated at 37°C for 24 hours. Colony counts were then performed. The study was initially conducted using 3 mice per group. The same study was repeated once, however using 5 mice per group. A total of 8 mice per group were used between both experiments. Statistical analyses were performed using Graphpad Prism Software version 3.02 (San Diego, CA, USA) and Microsoft Excel 2003 (Redmond, WA, USA). Fungal load and percent survival data (from Day 8) were paired and graphed.

6.3 Results

Effect of MG 3290 and ketoconazole combination therapy in systemic *Candida albicans* infection

Figure 1 shows the results of treating mice infected with *C. albicans* with various concentrations of MG 3290 and ketoconazole intraperitoneally. After treating sham-infected mice over 7 days with the 40 mg/kg ketoconazole and 10 mg/kg MG 3290, no signs of toxicity were observed (behaviour, food/water consumption, stool production, etc.). Treatment of mice with 10 mg/kg ketoconazole did not reduce the fungal burden in kidneys or increase survival times regardless of the dose of MG 3290. At a ketoconazole dose of 20 mg/kg however, a clear dose-dependent response was noted. The survival rate increased while the kidney fungal burden decreased with increasing doses of MG 3290. Treatment with 20 and 10 mg/kg ketoconazole and MG 3290 respectively resulted in a kidney fungal load of 2.1 log CFU/g and a 100% survival rate to the end of the experiment. In contrast, the fungal load of mice receiving 20 mg/kg ketoconazole alone

was 5.5 log CFU/g and the survival rate was only 50%. In the animals treated with 20 mg/kg ketoconazole, kidney lesion severity was reduced progressively with increasing doses of MG 3290 from 2 mg/kg. In mice receiving 10 mg/kg MG 3290, only very small lesions were observed with the unaided eye.

6.4 Discussion

HDAC inhibitors that target fungal HDACs have been previously reported to enhance the potency of azole-based antifungals against pathogens that may cause systemic mycosis. (Campeol, N., J. Bedard, and N.H. Georgopapadakou. 2005. Synergism of histone deacetylase (HDAC) inhibitors with ketoconazole in *Candida albicans* and *Candida glabrata*. Relationship to their effects on HDAC Activity in protoplasts. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-2154). In one study, trichostatin A, a HDAC inhibitor, potentiated the activity of fluconazole against *C. albicans*. (160).

In the present study, the potential for a novel HDAC inhibitor, MG 3290, to increase the sensitivity of *C. albicans* to ketoconazole in immunocompromised mice was evaluated. Neutropenic CD-1 mice were infected and treated over 7 days with varying doses of ketoconazole and MG 3290 intraperitoneally. Results show that MG 3290 can strongly enhance the potency of ketoconazole against disseminated candidemia. MG 3290 contributed to reduced fungal loads at doses of 2 mg/kg and above when 20 mg/kg ketoconazole was co-administered. In mice receiving combination therapy with 20 mg/kg ketoconazole and 10 mg/kg MG 3290, kidney fungal burden was reduced by 3.4 log CFU/g and the percentage of survivors doubled. In addition, the severity of kidney lesions (if any detected) was considerably reduced compared to the control group.

Results of this study suggest that the combination of MG 3290 (an HDAC inhibitor) and an azole antifungal is a promising strategy of treating systemic candidemia. Such drug combinations may also be effective in the treatment of azole-resistant infections. Further drug development is warranted. Whether MG 3290 is capable of synergizing with other azole antifungals remains to be evaluated. Further study is also necessary to determine the efficacy of HDAC inhibitor – azole combinations on fungi other than *C. albicans* (for instance, other members of the *Candida* genus and *Aspergillus* spp.). Routes of administration other than parenteral should also be considered.

Acknowledgments

We gratefully acknowledge Angela Brewer for technical assistance.

6.5 Figures and tables

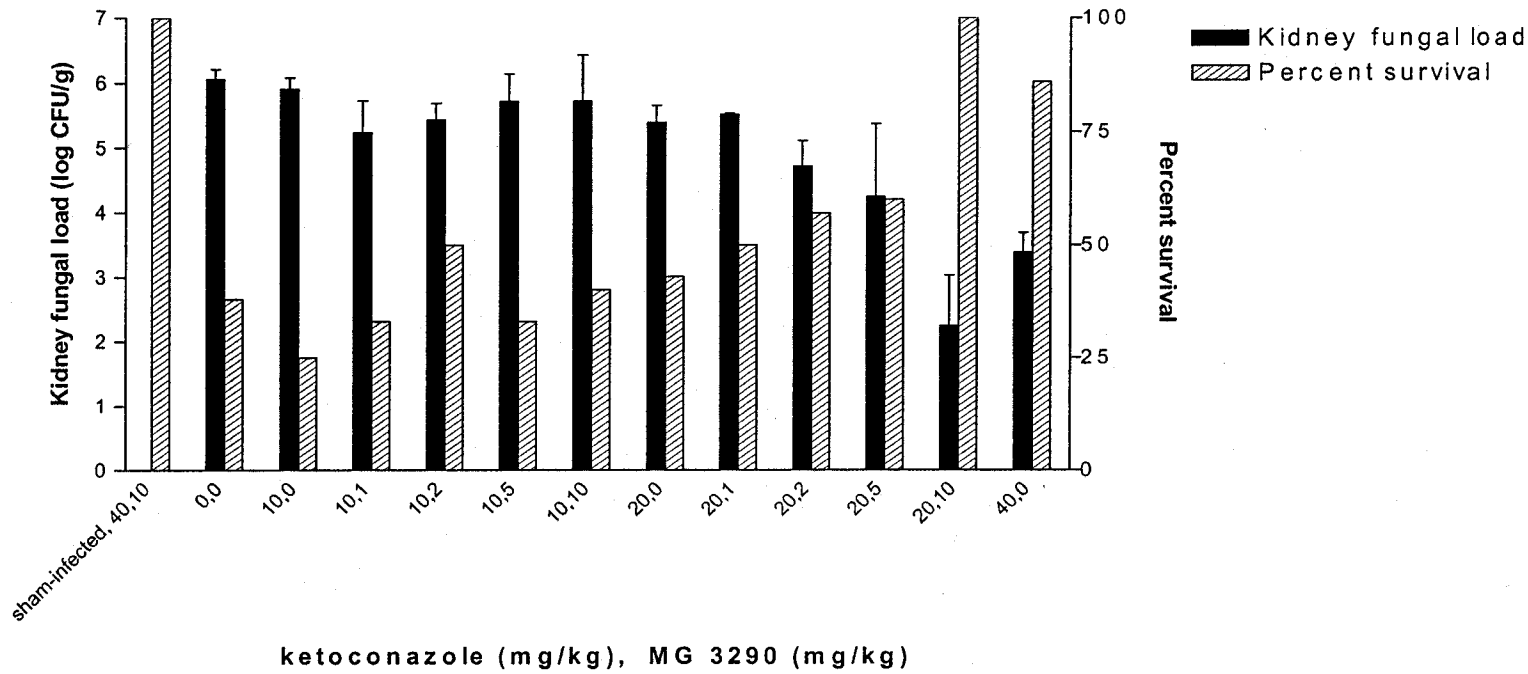

Fig 1. Effect of MG 3290 and ketoconazole combination therapy on systemic candidemia in immunocompromised mice. Starting on Day +1, mice were treated intraperitoneally once daily over a period of 7 days. Surviving mice were sacrificed the following day. Data from two independent studies were combined (N = 8) and are shown as the mean fungal load in kidneys (black bars) and percent survival (lined bars). Error bars show the standard error.

TABLE 1. Drug combination scheme for murine antifungal chemotherapy

Tube	Volume aliquoted from MG3290 stock solution (uL)	Volume aliquoted from ketoconazole stock solution (uL)
1	50	200
2	0	0
3	0	50
4	5	50
5	10	50
6	25	50
7	50	50
8	0	100
9	5	100
10	10	100
11	25	100
12	50	100
13	0	200

Section III: DISCUSSION, REFERENCES, APPENDIX

Chapter 7: Discussion

Over the past 60 years, the wide availability of antimicrobials has been a boon to human health. The severity of once lethal diseases such as pneumonia, meningitis, and invasive candidemia has been reduced dramatically. The initial impact of antibiotics against infections was so impressive that these drugs rapidly approached the status of a panacea. Accordingly, these "miracle drugs" were used for colds (viral infections, against which antibacterials have no effect) and non-specific reasons as simple as "not feeling very good;" thus leading to the emergence of resistant strains. Failure of patients to follow a strict antibiotic regimen also contributed to the emergence of resistance. Poorly-controlled agricultural use of antibiotics created an ideal environment in which bacteria could develop and spread resistance genes. Though antifungal resistance has not yet reached the epidemic proportions that antibacterial resistance has attained, all indications suggest a similar trend (89,151,164). It is not feasible for humans to rely on and wait for spontaneous mutations in the genome to confer protection against drug-resistant pathogens (as microorganisms have done to resist antibiotics). Active research and drug development are necessary to effectively address the problem.

ß-lactamases are among of the best-studied enzymes conferring antibacterial resistance. As validated targets for drug development, substantial research efforts on these enzymes have resulted in the elucidation of their mechanism of action, the resolution of their crystal structures with various substrates, and three clinically-relevant inhibitors (29,32,63,81,129,147). The three inhibitors, namely clavulanic acid, sulbactam, and tazobactam, have had good success in inactivating ß-lactamase activity and re-establishing the efficacy of once ineffective ß-lactam antibiotcs. However, all three

inhibitors are ß-lactams themselves, and thus are susceptible to hydrolysis. Current research efforts have concentrated on developing non-ß-lactam synthetic compounds that bind covalently while evading hydrolysis. Phosphonate-derived compounds have shown such activity against multiple classes of ß-lactamases (2,82).

Currently, the literature contains little information regarding high-throughput assays specifically designed to screen ß-lactamase inhibitors. We have successfully designed an XTT-based assay using Gram-negative bacteria expressing class A and C ß-lactamases that can identify "hits" within 5 hours. After optimizing each parameter, a linear correlation between metabolic activity and formazan production was achieved using the tetrazolium salt XTT. After screening a set of structurally related compounds, this linear correlation allows for QSAR analysis for lead optimization. Because of the water solubility of the colored formazan product, no additional solubilization steps were necessary to read the signal. The median Z' score of 0.75 suggests that this assay is characterised by an excellent dynamic range and low variability. The assay described in this thesis may be effectively adapted for high-throughput screening.

The differences between the assay systems described by Puckett *et al.* (142) involving EGFP, Änko *et al.* (6) involving luciferase, and the XTT assay described here highlight the importance of clearly establishing all objectives of the assay prior to its conceptualization. If the objective is to find molecules with good ß-lactamase specificity empirically, the EGFP assay would be the most appropriate of the three as the assay time is short and the target is highly purified (and therefore clearly defined). However, if the objective is to screen a smaller set of better-defined compounds for pathogen penetration and growth inhibition, the luciferase or XTT assays are better suited. If a quick screen is

desired and a quantitative assessment of the activity of compounds screening is not essential, then the luciferase screen would be superior because of the assay run time. However, if QSAR analysis is to be performed with the data generated, then the XTT assay is the best option.

As mentioned above, the phosphonate-derived compounds inactivate serine ß-lactamases and resist hydrolysis. The XTT assay could be used as a powerful tool for rapidly screening phosphonate analogues. QSAR analysis could be performed by comparing binding activity with respective chemical structures. Drug development of hits identified may proceed for toxicity testing *in vitro* and *in vivo*. Though phosphonate compounds have been emphasized, the XTT assay could be used to screen compounds from any source.

A number of experiments may be performed to confirm high-throughput applicability and increase assay efficiency. As described in section 5.2, the 8 compounds tested were screened using manual methods. In order to confirm high-throughput performance, the XTT assay would need to be adapted to a robotic platform and tested using a considerably greater number of test compounds. The original set of compounds described in section 5 could be included in the automated screen to verify assay reproducibility. To increase throughput capacity, the inoculum and reagent volumes can be proportionately reduced for testing in 384-well microplates. Though the XTT assay as described for 96-well microplates is in itself cost efficient, additional cost savings can be realised if the assay was adapted for 384-well plates.

The role of histone deacetylases in cancer therapeutics has been well studied over the last 35 years. HDAC inhibition was recently discovered to be a possible mechanism

of sensitizing some clinically relevant fungi to antifungals. Similar to the synergistic activity between ß-lactamase inhibitors and ß-lactams against ß-lactamase-expressing bacteria, HDAC inhibitors may synergize with azole drugs to inhibit fungal growth. In contrast to the former, the ß-lactamase inhibitors are effective exclusively in ß-lactamase-expressing bacteria whereas HDAC inhibitors may potentiate azole drug efficacy against both azole-resistant and -sensitive fungi. Irrespective of the resistance phenotype, the dose of the azole drug may be reduced and thus minimise the risk for adverse effects. The proposed mechanism of action of HDAC inhibitors in this context suggests that the upregulation of genes encoding drug efflux transporters and ergosterol biosynthesis enzymes is either reduced or inhibited (160). *In vitro* studies have shown growth inhibition at lower doses of ketoconazole when combined with an HDAC inhibitor for *Candida* spp. and *Aspergillus fumigatus*.

Our *in vivo* studies corroborate the HDAC inhibitor synergy observed *in vitro*. At a fixed dose of ketoconazole, immunocompromised and *C. albicans*-infected mice responded in a dose-dependent manner to increasing doses of the HDAC inhibitor MG 3290. All mice treated with 20 mg/kg of body weight ketoconazole and 10 mg/kg MG 3290 survived. No lethality or indications of deteriorating health were observed when normal, healthy mice were administered MG 3290 at doses up to 50 mg/kg (unpublished data). These data have important implications in terms of further drug development. First, further proof-of-principle is established: enzymes responsible for fungal histone deacetylation are viable drug targets when combined with an azole drug. Second, these studies may lead to the development of the first antifungal-HDAC inhibitor combination for clinical use.

The results obtained thus far are encouraging, though many questions remain unanswered. *In vivo* studies have focused on only one fungal species, therefore it can only be claimed that HDAC inhibition is effective for *C. albicans* infections. To test the wider applicability of this strategy, other species of *Candida* should be tested, including a variety of azole sensitive and resistant strains. In addition, other fungi should be incorporated in drug testing. Various strains of *A. fumigatus*, the second most common fungal isolate identified in clinical cultures, and *Cryptococcus neoformans* would be good candidates.

Smith and Edlind (160) have reported that some *Candida* strains did not respond to the HDAC inhibitor TSA. Though the authors did not address this issue, it is an important consideration for drug development as it may impact the utility of any drug combination conceived for clinical use. In these *Candida* strains that did not respond, it is possible that the *CDR*, *MDR*, or *ERG* genes were not upregulated, only weakly upregulated, or were already derepressed. If the azole does not trigger a significant increase in expression of these genes, then an HDACI would have little or no effect. Alternatively, enhanced compound efflux could have prevented adequate HDAC inhibition for drug synergy. Poor permeability of the HDAC inhibitor could have been another factor.

The issue of drug efflux is particularly important as this is a viable mechanism not only for antifungal resistance, but possibly for HDAC inhibitor resistance as well. Unlike bacteria, in which replication and protein synthesis function independently of ß-lactamase activity, HDAC activity is inextricably linked to all aspects of cellular function. Could

mutations in gene sequences encoding HDACs conferring resistance to HDACIs be lethal? If mutations are non-lethal, do they severely compromise virulence?

Drug resistance is well documented for anticancer therapeutics (27,64). Like fungi, the primary mechanism of resistance involves drug efflux (64), though it may also occur through other mechanisms (Fig. 8). HDAC inhibitor resistance in fungi can therefore theoretically develop. To explore this possibility, *Candida* cells may be grown in sub-inhibitory concentrations of an azole drug and an HDAC inhibitor and then aliquoted into fresh medium with the drug combination. Serial passages can be performed to observe if resistance can be acquired.

Although the discussion on HDAC inhibition has focused primarily on fungi, this strategy also may be effective against other eukaryotic organisms that activate otherwise quiescent genes to confer resistance. For example, the sensitivity of *Trypanosoma brucei*, the causative agent of African trypanosomiasis (also known as African sleeping sickness), to melarsoprol could be re-established through the inhibition of TbMRPA overexpression (104). TbMRPA encodes a trypanothione-conjugate efflux pump. Through the inhibition of specific trypanosomal histone deacetylases, the upregulation of TbMRPA could be abrogated. Similar to observations for *C. albicans*, HDAC inhibitors could render non-resistant strains of *T. brucei* more sensitive to antitrypanosomal drugs. Drug doses (and possible adverse effects from these drugs) therefore can be reduced.

Bacterial and fungal infections are serious threats to human health. Particularly for those with weakened immune systems and patients undergoing invasive surgical procedures, such infections not only impose undue stress and discomfort, but also greatly

increase the risk of morbidity and mortality. Despite the fact that antimicrobials have been discovered and designed to combat infections, pathogens have evolved and disseminated diverse mechanisms to resist the action of these drugs. The consequence of antibiotic resistance is clear: available therapeutics to treat drug-resistant infections are rapidly on the decline. Effective antibiotics to fight resistant pathogens such as VRSA and multi-drug resistant tuberculosis represent an urgent unmet medical need. MethylGene Inc. has compounds in preclinical development that address both bacterial and fungal antibiotic resistance. The company is developing compounds that target multiple classes of ß-lactamases (enzymes harboured by a large percentage of clinical isolates) and fungal histone deacetylases (increases sensitivity to azole-antifungals). The current thesis describes the advancement of preclinical drug development for each target. A sensitive colorimetric assay with high-throughput capacity has been developed to screen for potent ß-lactamase inhibitors. This screen is a powerful tool that can assist in the discovery of new ß-lactamase inhibitors for clinical use. Animal studies have confirmed the indirect anti-infective role of HDAC inhibition during antifungal therapy. This may be a novel strategy for treating infections caused by azole-resistant fungi and possibly parasitic protozoans.

Although rational drug design, pharmacophore modeling and QSAR analysis make it less likely that serendipity will play as major a role in the drug discovery process as it did for Fleming, the objective nevertheless remains the same. If humankind wishes to sustain the quality of life achieved thus far, it is imperative to stay one step ahead of the evasive mechanisms that pathogens evolve. Continuing research and drug development are essential to maintain humankind's supremacy over the microbe.

Figures

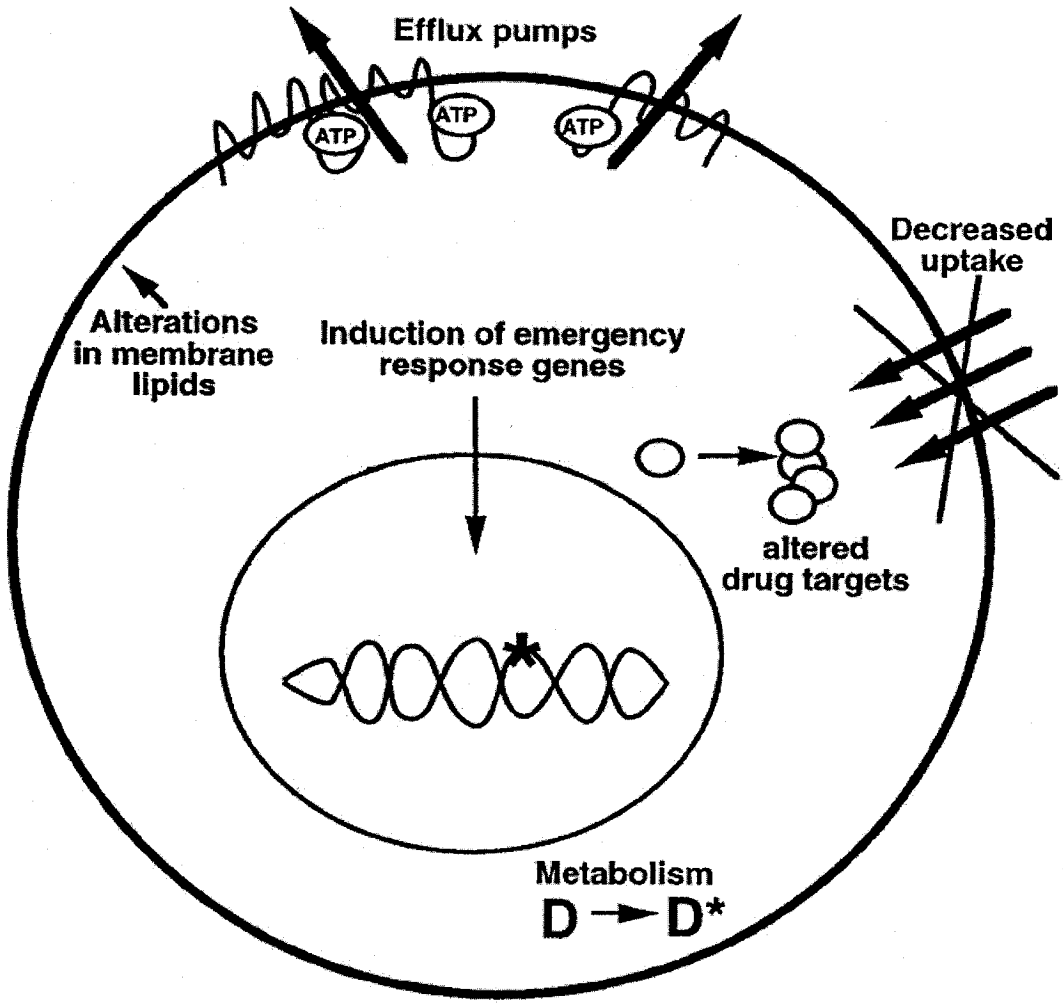

FIG 7: Potential mechanisms of histone deacetylase inhibitor resistance. Active efflux is the most likely method by which fungi will develop resistance as this is already an established mechanism of antifungal resistance. Changes in cell wall or membrane composition may reduce HDACI permeability. Mutations in HDACs may evade HDACI binding. Induction of HDAC gene expression increases the drug target density and therefore may render fungi resistant. The inhibitor may also metabolize the drug. Figure has been adapted from reference (64).

Chapter 8: References

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Chapter 9: Appendix