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THE EFFECT OF CHLORAMPHENICOL ON BB88
MURINE ERYTHROLEUKEMIA CELLS

by

Peter K. W. Harris

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
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Western Michigan University
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THE EFFECT OF CHLORAMPHENICOL ON BB88 MURINE ERYTHROLEUKEMIA CELLS

Peter K. W. Harris, Ph.D.

Western Michigan University, 2007

DNA microarrays can be used to measure genome-wide transcript levels. These measurements may be useful in understanding cellular changes induced by a chemical agent. In this study, Affymetrix microarray technology has been used to study the effects of chloramphenicol, an antibiotic that inhibits bacterial and mitochondrial protein synthesis, on the transcription profile in mammalian cells. Transcript levels in BB88 murine erythroleukemia cells treated with 50 micromolar (μM) chloramphenicol, a concentration shown to inhibit BB88 proliferation, are measured. Using total RNA from treated cells, biotin-labeled cRNA was prepared and hybridized to Affymetrix U74Av2 microarrays. From these hybridizations, expression levels were measured for approximately 12,000 transcripts. Methods to identify genes whose transcript levels increase or decrease during the treatments are described. In particular, chloramphenicol increases transcript levels for a subunit of cytochrome c oxidase (COX), a complex metalloprotein that plays a critical role in cellular respiration. Specifically, transcripts for cytochrome c oxidase VIa-H (COX VIa-H), a nuclear-encoded subunit of complex IV in the mitochondrial respiratory chain, increase greater than 20-fold after 24 hours of chloramphenicol treatment. While transcripts for COX VIa-H are increased in chloramphenicol-treated cultured erythroleukemia cells, transcripts for other nuclear-

encoded COX subunits appear unaffected. To follow-up on these findings, a reverse transcription-polymerase chain reaction (Taqman® PCR) method has been used to confirm that chloramphenicol causes specific increases in COX VIa-H expression in these cells not shown previously to contain COX VIa-H. Further, these results have been extended by showing that increases in the COX VIa-H transcript by chloramphenicol treatment are concentration- and time-dependent. Finally, in cells treated with other compounds that inhibit either mitochondrial protein synthesis or respiration, COX VIa-H transcripts are increased. While chloramphenicol's mechanism of mammalian cell toxicity remains uncertain, these findings support evidence that shows chloramphenicol has an affect on mitochondrial proteins and suggests the COX VIa-H transcript may be an anti-proliferation marker (biomarker) in these cells.

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LIST OF ABBREVIATIONS

ALAD – delta aminolevulinate dehydratase – enzyme involved in heme biosynthesis

ATP – adenosine triphosphate

COX – cytochrome c oxidase

COX VIa-H – nuclear-encoded cytochrome c oxidase subunit VIa-H

DD RT-PCR – differential display reverse transcription-polymerase chain reaction

Dec – decreased transcript based upon the change in p-value (closer to 1.0) computed for that transcript

EC – endothelial cells

EC₅₀ – effective concentration at which a 50% biological effect is seen

EtOH – ethanol

GAPDH – glyceraldehyde phosphate dehydrogenase

GO – gene ontology

Inc – increased transcript based upon the change in p-value (closer to 0.0) computed for that transcript

MD – marginally decreased transcript based upon the change in p-value (closer to 1.0) computed for that transcript

MI – marginally increased transcript based upon the change in p-value (closer to 0.0) computed for that transcript

MM – one nucleotide mismatch in Probe Set Pair

MPS – mitochondrial protein synthesis

ORF – DNA open reading frame

PCR – polymerase chain reaction

List of Abbreviations—Continued

PM – perfect nucleotide match to ORF in Probe Set Pair

RPA – ribonuclease protection assay

RT – reverse transcriptase or reverse transcription

SAGE – serial analysis of gene expression, technique for determining transcript levels

SAPE – streptavidin phycoerythrin stain solution used to enhance transcript level determination

SF – scaling factor is the amount that each Signal value on a microarray is multiplied by in order to normalize that microarray to other microarrays

SLR – signal log ratio is the computed mean of the log₂ ratios of probe pair intensities between any two arrays

SOS – sum of signals is the addition of the treated and control signal values for a particular Probe Set

TGT – target threshold is an arbitrary user-defined average value used to make average intensities from one microarray comparable

U – units of measuring enzyme activity

INTRODUCTION

Antibiotics: Mechanism of Action and Toxicity

Antibiotics are compounds that either stop microorganisms from growing or kill bacteria or fungi outright. In the 1940s, Selman Waksman, a 1952 Nobel laureate, used the term “antibiotic” to describe any antimicrobial compound that was derived from a living organism having therapeutic potential versus infectious diseases (Waksman & Geiger, 1944). Today, the term antibiotic is more inclusive and applies not only to natural, synthetic, or semi-synthetic agents that are used to treat infectious microbes, but also to natural products that can inhibit growth of human cancer cells. An antibiotic that inhibits the growth of bacteria is called bacteriostatic; chloramphenicol exemplifies this kind of antibacterial compound. Antibiotics that lower bacterial cell number by causing death are called bactericidal and penicillin exemplifies this kind of antibiotic. As mentioned above, while there are some man-made antibiotics, such as the sulfonamides, antibiotics are produced primarily by microorganisms. This production is probably an evolutionary adaptation so that the antibiotic producer has an advantage over its antibiotic-sensitive neighbors. Both bacteria and fungi synthesize antibiotics. However, since antibiotics often target cellular components or metabolic pathways unique to another group of organisms, it is rare for a specific antibiotic to be therapeutically useful as both an antibacterial and an antifungal. Antibacterial antibiotics will be discussed briefly, in part due to the current diversity of these therapeutic agents, and in particular,

due to the research described herein that focuses on toxicity of chloramphenicol, a broad-spectrum antibacterial antibiotic. The following paragraphs highlight mechanistic aspects and toxicity issues for some important antibacterial drugs, with only a brief mention of antibiotic selectivity and resistance.

There are several systems of classifying antibiotics. One classification system is based upon on the producing organisms. Of the approximately 12,000 known antibiotics, about 160 have been used in human therapy. *Streptomyces*, a genus of gram-positive (see below), filamentous bacteria found predominantly in soil and in decaying vegetation, is estimated to be responsible for 55% of the commercially available antibiotics (Champness, 2000). Another classification method is by spectrum of activity or the bacterial diseases they are used to treat (Levy, 1998). Some antibiotics are used in specific situations and against particular pathogens. For instance, bacitracin is used topically versus skin infections, while tetracyclines can be used internally versus infections of the digestive and urinary tracks. Historically, antibiotics were discovered based upon their effect on bacterial growth. It was not until later that an antibiotic's mode of action, selectivity, and toxicity, were made known. Through this information, perhaps the most common system of antibiotic classification is grouping compounds based upon their mode of action. There are four prime antibacterial metabolic targets: 1) cell wall synthesis, 2) protein synthesis, 3) DNA and RNA synthesis, and 4) folic acid metabolism (nucleotide precursor biosynthesis). Two of these targets, cell wall biosynthesis and folic acid metabolism do not have human counterparts. The other two targets, protein synthesis and DNA and RNA synthesis, are found in humans, but there

are enough structural differences that selective inhibition of disease causing bacteria has been achieved.

Antibiotics That Inhibit Cell Wall Synthesis

In the 1880s, Hans Gram (1884), a Danish bacteriologist, developed a stain to distinguish strains of bacteria. At the time Gram did not know how the stain worked, but later scientists learned that the Gram reaction is based on the structure of the bacterial cell wall (Popescu & Doyle, 1996). In gram-positive bacteria, an iodine-crystal violet complex is trapped in the outermost peptidoglycan (PG) layer of the cell wall and these cells appear purple under the microscope. This iodine-crystal violet complex also binds to the peptidoglycan layer that lies beneath the outer membrane in gram-negative bacteria. However, it is washed away along with the outer membrane upon exposure to ethanol. Subsequent counterstaining with safranin or basic fuchsin causes the decolorized gram-negative bacteria to appear pink. While other components of bacterial cell walls have been targets, it is this PG that has been successfully targeted by many antibiotics. These antibiotics have been involved in inhibiting enzymes or sequestering substrates involved in PG biosynthesis.

PG biosynthesis occurs in three phases (Figure 1). During the cytoplasmic phase, six enzymes, Mur A-F, are responsible for converting UDP-N-acetylglucosamine to UDP-muramyl-pentapeptide (El Zoeiby, Sanschagrín, & Levesque, 2003). While research into inhibitors for the cytoplasmic phase of PG synthesis is flourishing, fosfomycin, an antibiotic used to treat infections of the urinary tract by inhibiting MurA, is about the only antibiotic that has been used in humans (F. Kahan, J. Kahan, Cassidy, &

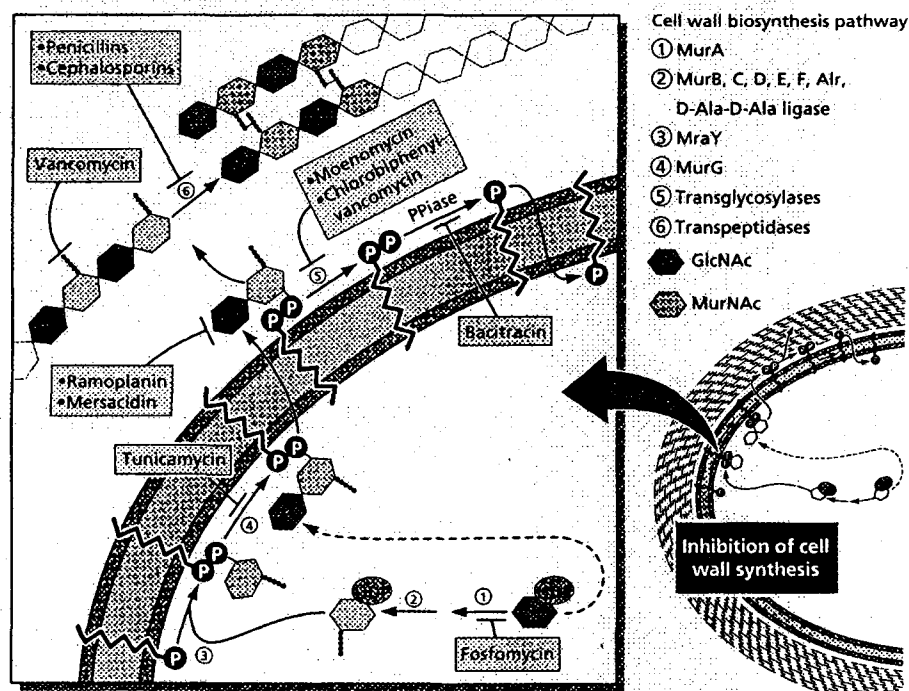


Figure 1. Antibiotics That Inhibit Cell Wall Biosynthesis. (courtesy of Walsh, 2003)

Kropp, 1974; Skarzynski et al., 1996). During the membrane-associated phase, MraY transfers the muramyl-pentapeptide to an unusual membrane lipid, bactoprenol, creating Lipid I (Bouhss, Crouvoisier, Blanot, & Mengin-Lecreux, 2004; Ikeda, Wachi, Jung, Ishino, & Matsushashi, 2001; Van Nieuwenhze, Mauldin, Zia-Ebrahimi, Aikens, & Blaszcak, 2001). Tunicamycin, a natural antibiotic commonly used in eukaryotic glycoprotein research, inhibits this reaction, whereas the mureidomycins selectively inhibit prokaryotic MraY (Lee & Hecker, 1999). Next, the essential MurG converts Lipid I to Lipid II by adding a UDP-N-acetyl glucosamine to the UDP-muramyl-pentapeptide (Ha, D. Walker, Shi, & S. Walker, 2000; Ha, Gross, & S. Walker, 2001). While not exactly understood, Lipid II translocates the disaccharyl-pentapeptide from the internal membrane to the external membrane face. Bacitracin, a decapeptide, inhibits

transglycosylation by interactions with Lipid II (Brotz, Bierbaum, Leopold, Reynolds, & Sahl, 1998). Originally, the lipoglycopeptide, ramoplanin, was thought to inhibit MurG, but recent research implies that this antibiotic inhibits the transglycosylation step of PG biosynthesis by binding to Lipid II (Fang et al., 2006).

The final phase of PG biosynthesis involves several separate reactions occurring in the external space outside of the bacterial internal membrane. First, enzymes called autolysins break both glycosidic bonds at the point of growth along the existing peptidoglycan and the peptide cross-bridges that link the rows of sugars together. Transglycosylases then insert the PG monomers from Lipid II into the breaks in the mature PG. Transpeptidases reform the peptide cross-bridges between the rows and layers of PG to make the cell wall strong osmotically. The glycopeptides, vancomycin and teicoplanin, complex with the pentapeptide portion of PG and Lipid II. This interaction effectively sequesters the PG from not only transpeptidases, but also transglycosidases. Since the outer membrane limits their access to PG in the periplasm, vancomycin and teicoplanin have little effect on most gram-negative bacteria. However, there has been some work on semi-synthetic glycopeptides that can cross the outer membrane of vancomycin insensitive bacteria (Ciabatti & Malabarba, 1997).

Moenomycin is one of the few antibiotics that interferes with transglycosylation. NMR structural studies have revealed that it binds to the target transglycosylase and blocks disaccharyl-pentapeptide addition to a growing PG layer (Kurz, Guba, & Vertesy, 1998).

β -lactams are a class of antibiotics that interfere with PG transpeptidation reaction.

Penicillins and cephalosporins are perhaps the most celebrated natural β -lactams. The

story of Alexander Fleming's accidental discovery of penicillin in 1928 is well known (Fleming, 1932, 1945, 1980); but what is not so well remembered is that it took more than ten years before scientists, Chain and Florey, had enough of the antibiotic purified for large-scale clinical use (Chain et al., 1940). While these three researchers shared equally a Nobel Prize in 1945, arguably it is Fleming who is best remembered. Some have suggested that the public adulation for Fleming was not only for his pioneering scientific work, but also because of his friendly and outgoing personality. Further, after one of his patients died on account of there not being enough antibiotic to complete the treatment, Howard Florey shied away from publicity (Bennett & Chung, 2001). Nevertheless, another quarter century passed before penicillin's mechanism of action was better understood. In Jack Strominger's lab at Harvard, it was proposed that the β -lactam portion of penicillin mimicked the acyl-D-alanyl-D-alanine structure on the PG pentapeptide chain (Tipper & Strominger, 1965). Experiments showed that when transpeptidases bind to β -lactams, the enzyme-antibiotic intermediates are extremely stable. This binding effectively inhibits the transpeptidation reaction, resulting in a weakly cross-linked cell wall and osmotic lysis of the bacterium (Strominger, Blumberg, Suganaka, Umbreit, & Wickus, 1971; Yocum, Rasmussen, & Strominger, 1980). Although the penicillins and cephalosporins are some of the most successful and widely used antibiotics, newer semi-synthetic β -lactams have been developed over the years (Scholar & Pratt, 2000). While the mechanism of action remains the same, these newer β -lactams are overcoming some of the issues experienced with the older antibiotics, such

as degradation by stomach acids, resistance to bacteria that produce penicillinases, and increased range of activity versus some Gram-negative bacteria.

Antibiotics That Inhibit Protein Synthesis

The second antibacterial metabolic target is protein synthesis (Figure 2). Protein synthesis is not unique to prokaryotes, as the process is an essential function for all cells. However, there exist enough differences between the eukaryotic and prokaryotic protein synthetic machinery to make this metabolic process an effective antibacterial target. Most protein synthesis antibiotics target the ribosome. The 70S bacterial ribosome consists of

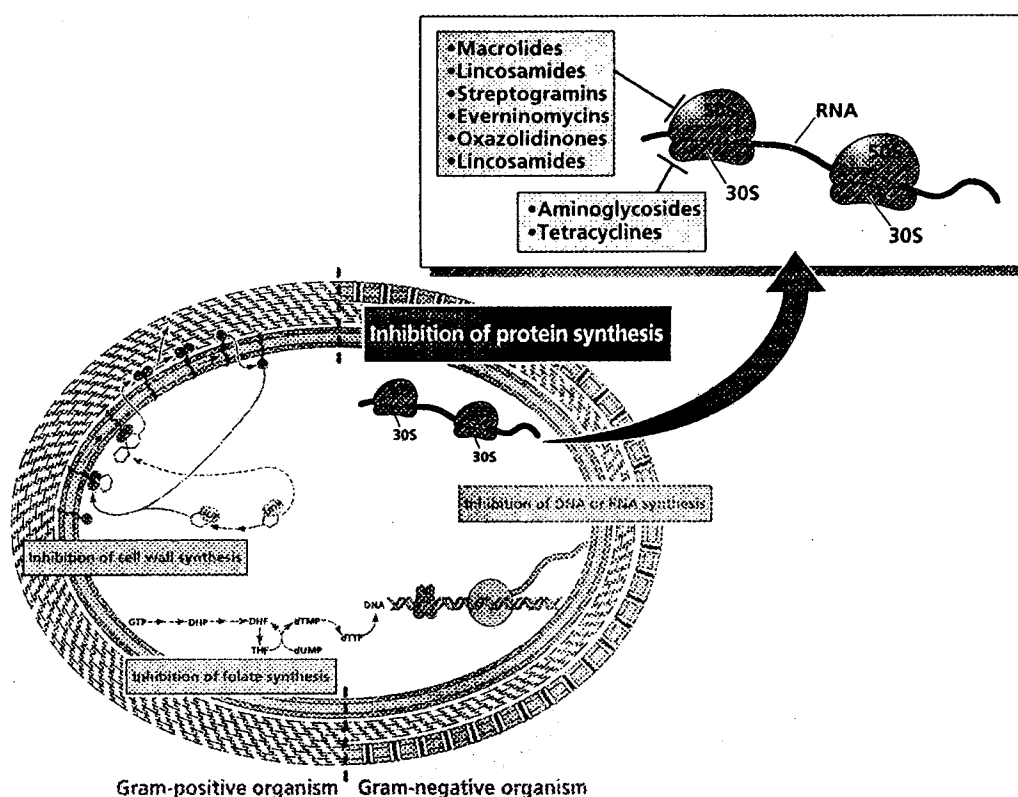


Figure 2. Antibiotics That Inhibit Bacterial Protein Synthesis. (courtesy of Walsh, 2003)

two nucleoprotein subunits. While the smaller 30S subunit contains a 16S ribosomal RNA (rRNA) and about 20 proteins, the larger 50S subunit is made up of a 23S rRNA, a 5S rRNA and about 30 proteins. The recent articles describing the crystal structures for the 30S, 50S, and the intact 70S ribosomal subunits have deepened our understanding of protein synthesis and the mechanisms involved in mRNA translation (Ban, Nissen, Hansen, Moore, & Steitz, 2000; Nissen, Hansen, Ban, Moore, & Steitz, 2000; Ramakrishnan, 2002; Wimberly et al., 2000). Each 30S and 50S subunit has three binding sites for transfer RNA (tRNA). The A-site accepts incoming aminoacylated-tRNA. The P-site holds the tRNA with the nascent polypeptide chain, while the E-site holds the de-acylated tRNA before it leaves the ribosome. The 30S ribosome binds mRNA and the anticodon subunits of tRNA. This subunit contributes to translation fidelity by monitoring base pairing between codon and anticodon. The 50S subunit catalyzes peptide bond formation between incoming amino acids on A-site tRNA and the nascent polypeptide chain attached at the neighboring P-site tRNA. Besides mRNA, tRNA, and the ribosome, bacterial protein synthesis requires additional proteins. Many of these proteins are GTP binding proteins that become activated upon ribosome binding and then aid in protein synthesis initiation, elongation, and termination (Nissen 2000).

Tetracycline and the aminoglycoside antibiotics affect protein synthesis by binding to the 16S rRNA of the 30S ribosomal subunit. Structural studies showed that tetracycline, a bacteriostatic antibiotic that has been around for more than 50 years, binds to the A-site on 16S ribosome (Carter et al., 2000). While tetracycline's binding does not appear to block the initial delivery of aminoacyl-tRNA to the ribosome and the accompanying hydrolysis of GTP by initiation factors, it does cause premature

aminoacyl-tRNA release without peptide bond formation (Brodersen et al., 2000, 2001). The aminoglycoside streptomycin was discovered in 1943 in the laboratory of Selman Waksman, who later won a Nobel Prize for its discovery. As an aside, this Nobel Prize has been accompanied by some controversy (Mistiaen, 2002). With some strong evidence to support his claims, Albert Schatz, a young scientist working for Waksman at the time, claimed not only to have discovered streptomycin, but first demonstrated that it was active against the tuberculosis bacterium, *Mycobacterium tuberculosis* (Schatz & Waksman, 1944; Schatz, Bugie, & Waksman, 1944). While some issues were resolved legally, this situation created some very hard feelings and tarnished reputations. As are most aminoglycosides, streptomycin is a hydrophobic sugar with multiple amino groups attached. The aminoglycosides are bactericidal versus most gram-negative bacteria and resistance mapping studies have shown that most of the mutations were found to map to a region close to the tetracycline binding site (Carter, 2000; Springer, 2001). However, since they are ineffective versus gram-positive bacteria, they have been used synergistically with some β -lactam antibiotics (Scholar & Pratt, 2000).

The macrolides are a small group of natural bacteriostatic antibiotics that inhibit protein synthesis by acting at the 50S ribosome. Structural, mutational, and footprinting studies have revealed that macrolide antibiotics like erythromycin interact with nucleotide residues between 2040-2609 on the 23S rRNA, a region at the entrance of the polypeptide export tunnel (Schlunzen et al., 2001; Vester & Douthwaite, 2001; Garza-Ramos, Xiong, Zhong, & Mankind, 2001). Binding of these antibiotics and certain semi-synthetic macrolides, such as azithromycin, disrupts the elongation step in protein

synthesis (Ramakrishnan, 2002). The antibiotic clindamycin, despite being structurally different, is a competitive inhibitor of erythromycin and inhibits protein synthesis by binding to the 23S rRNA at or near physically overlapping sites (Douthwaite, 1992a). Similarly, chloramphenicol, which will be discussed later, inhibits bacterial protein synthesis by interactions with 23S rRNA (Douthwaite, 1992b; Long & Porse, 2003).

The oxazolidinones are a class of compounds that inhibit bacterial protein synthesis. Linezolid, an oxazolidinone that was approved by the FDA in 2000, is the first structurally novel antibiotic introduced in the last three decades. Early mutational studies of linezolid-resistant bacteria strongly suggested that this synthetic antibiotic interferes with protein synthesis by binding to a site that is near to the peptidyl transferase center on the 50S ribosome (Kloss, Xiong, Shinabarger, & Mankin, 1999; Zhou, Swaney, Shinabarger, & Stockman, 2002). Using a photoactive probe, Colca et al. (2003) localized the site of oxazolidinone action in *Staphylococcus aureus*. In addition to cross-linking to the conserved adenine nucleotide at position 2602 on the 23S rRNA, the photoactive oxazolidinone cross-linked ribosomal protein L27, whose amino terminus may reach the peptidyltransferase center, and LepA, a protein homologous to translation factors.

Antibiotics That Inhibit DNA and/or RNA Synthesis

DNA and RNA synthesis are the third group of antibacterial metabolic targets. DNA gyrases are essential bacterial enzymes that introduce negative DNA supercoils into a double-stranded circular DNA (Figure 3). These negative supercoils release superhelical tension ahead of DNA polymerase so that replication can continue

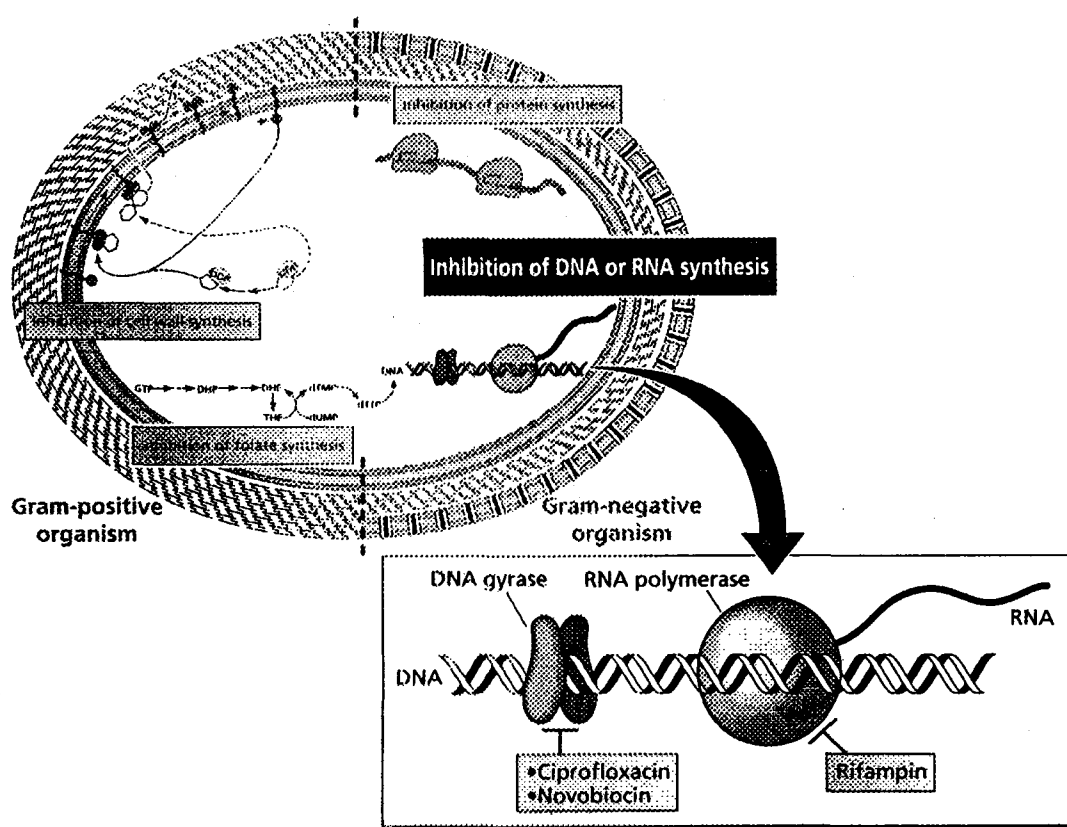


Figure 3. Antibiotics That Inhibit Bacterial RNA and DNA Synthesis. (courtesy of Walsh, 2003)

(Cozzarelli, 1980). The fluoroquinolones are a synthetic class of bactericidal antibiotics that block DNA synthesis by inhibiting DNA gyrase. DNA gyrase exists as a heterodimer and biochemical and genetic studies have identified the A subunit (GyrA) as a target of ciprofloxacin and other quinolones (Hooper, Wolfson, Ng, & Swartz, 1987). Quinolone resistance mutations frequently occur at residues Ser⁸³ and Asp⁸⁷ of the gyrase A subunit, suggesting that these residues are involved in drug binding (Barnard & Maxwell, 2001). However, recently the B subunit (GyrB) has been implicated in quinolone binding, as antibiotic resistance mutations are known to exist in GyrB. *In vitro*, two such mutant

proteins, GyrB(Asp⁴²⁶ → Asn) and GyrB(Lys⁴⁴⁷ → Glu), have been shown to behave similarly to GyrA quinolone-resistant proteins by decreasing the amount of radio-labeled ciprofloxacin bound to a gyrase-DNA complex (Hedde & Maxwell, 2002). Bacterial gyrases and the eukaryotic enzyme topoisomerase II are similar. However, enough differences exist between the two enzymes such that the quinolones selectively inhibit gyrases while topoisomerase II remains resistant. In terms of RNA synthesis, another antibacterial metabolic target that has a eukaryotic counterpart, rifampicin (rifampin) is the only antibiotic in clinical use. Rifampicin is a semisynthetic version of Rifamycin B, a natural product isolated from *Nocardia mediterranei* fermentations (Sensi, 1983). This antibiotic, which has been a key component of tuberculosis therapy, binds to the β subunit of RNA polymerase. X-ray structures of rifampicin bound to the core RNA polymerase of *Thermus aquaticus* revealed that this antibiotic blocks RNA elongation by binding in the DNA/RNA tunnel (Campbell et al., 2001).

Antibiotics That Inhibit Folic Acid Metabolism

The fourth primary antibacterial metabolic target is folic acid metabolism (Figure 4). Folic acid functions as a coenzyme in the pathway leading to purine nucleotide synthesis. Animals cannot synthesize folic acid and depend on exogenous sources. Therefore, animal cells are unaffected by inhibitors of the bacterial folic acid pathway. Sulfamethoxazole and trimethoprim are two drugs which block steps in bacterial folic acid metabolism. These two drugs are being used in combination to treat patients with urinary tract infections and also acquired immunodeficiency syndrome (AIDS) patients with *Pneumocystis carinii* infections (Scholar & Pratt, 2000). Sulfamethoxazole is a

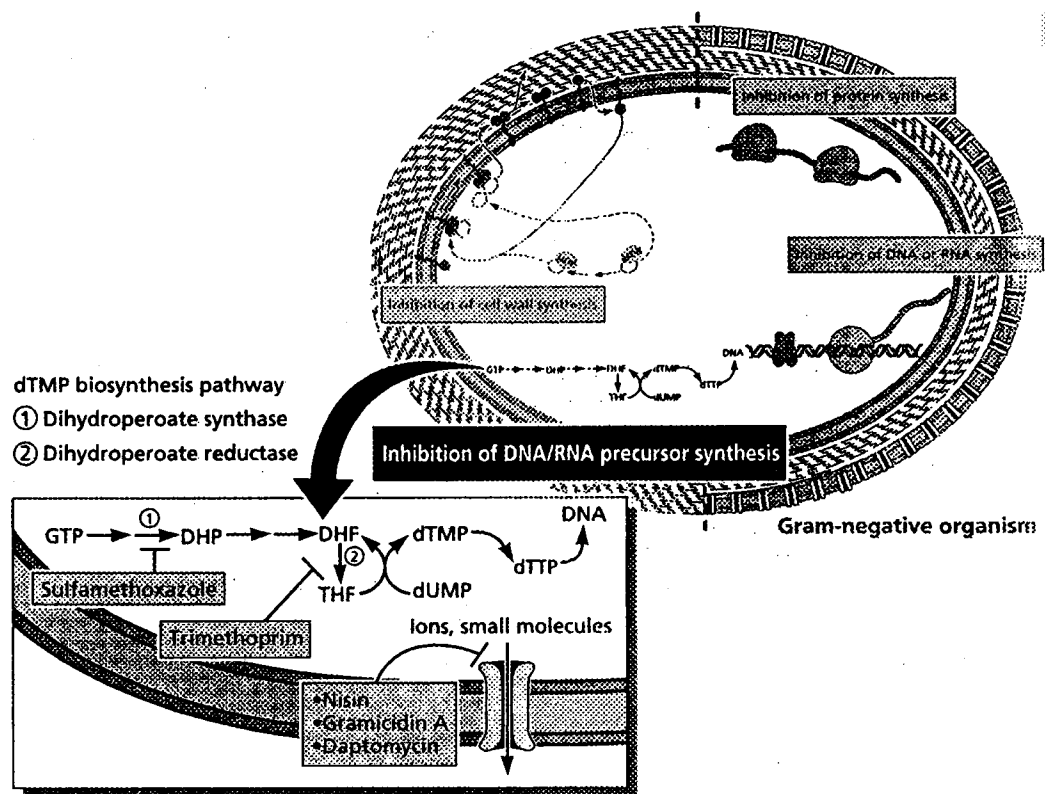


Figure 4. Antibiotics That Inhibit Folic Acid Metabolism. (courtesy of Walsh, 2003)

current generation sulfonamide. In his book, *The Demon Under the Microscope*, Thomas Hager (2006) argued that the key event in the history of antibacterials was not the emergence of penicillin in the 1940s, but Gerhard Domagk's discovery and the development of sulfonamides almost a decade earlier. In 1932, while working as a bacteriologist in a large German company, Domagk took chemicals originally introduced into the dye industry and tested them for their antibacterial activity. He found that a synthetic sulfonamide preparation, subsequently named Prontosil, cured mice infected with ten times the lethal dose of *Streptococcus* (Domagk 1935, 1937). An interesting point Hager makes is that, while Domagk was awarded a Nobel Prize in 1939 for this

work, two ironies existed to explain why his momentous work did not receive greater acclaim. First, Domagk worked in Nazi Germany and was not allowed to travel to receive his award. Second, Domagk's employer, IG Farben, became the major producer of poison gas that was used to kill Jews in the concentration camps. Hager argues that here is how politics and social situations can impact the importance or significance of a scientific discovery. Much later it was shown that the structure of sulfonamides resembles that of para-aminobenzoic acid (PABA), a precursor in folic acid synthesis, and sulfonamides compete for binding with PABA for the enzyme dihydropteroate synthase. In 1997, crystal structures of sulfonamide with the bacterial enzyme validated this mechanism of inhibition (Achari et al., 1997). In the case of the antibiotic trimethoprim, this compound inhibits bacterial dihydrofolic acid reductase, an enzyme that converts dihydrofolic acid to tetrahydrofolic acid, a step later in the bacterial folic acid metabolic pathway. While this enzyme exists in humans, the IC₅₀s for the bacterial and human enzymes are 10 nM and 300,000 nM, respectively (Scholar & Pratt, 2000). Thus, in combination with the sulfonamides, trimethoprim blocks the synthesis of nucleotides that will be used in bacterial DNA and RNA synthesis.

Antibiotic Toxicity

Antibiotics have been tremendous assets in the treatment of infectious diseases. However, even though many antibiotics follow the principle of selective toxicity, antibiotic therapy is not without some risk. In fact, even though the antimicrobial target may not be present in humans, most antibiotics have the potential to be toxic. Antibiotic adverse reactions can be classified as either dose-dependent or non dose-dependent. In

dose-dependent adverse reactions, the incidence and intensity of adverse effects increase proportionately to antibiotic concentration. Patient monitoring, considerations of patient pharmacokinetic differences, and dosage individualization can decrease these adverse reactions. Non dose-dependent adverse reactions are less common but are more severe. They are considered idiosyncratic, suggesting that the exact mechanism is unknown. These adverse reactions don't depend upon antibiotic pharmacodynamics, but appear related to a patient's immune system or the genetics of drug metabolism. These kinds of adverse reactions appear unexpectedly and often cause death (Gruchalla, 2001; Wawruch, Bozekova, Kremery, & Kriska, 2002).

Toxicity of Cell Wall Inhibitors

Despite penicillin and its many semi-synthetic derivatives being relatively nontoxic even at high doses, their use leads frequently to allergic reactions. These allergic reactions to β -lactams are mediated by an immune mechanism. Most antibiotics are chemically inert in their native state and need to be metabolized in order to covalently bond with other macromolecules. In the case of penicillin, and other β -lactams, degradation occurs spontaneously in the body and the intermediates bind to proteins. These protein bound intermediates are immunologically reactive (Solensky, 2003, 2006). Interestingly, while penicillin sensitivity has been estimated to be 10% of the general population, this estimate may be inaccurate. In a study where patients with a penicillin allergy were re-evaluated by a skin test, more than 90% were found not to be allergic and were able to tolerate the antibiotic (Gadde, Spence, Wheeler, & Adkinson, 1993). The discrepancy here may be explained by several factors. The initial allergic reaction

may have been unrelated to the antibiotic and caused by the illness or the loss of penicillin-specific IgE antibodies over time (Solensky, 2006). Vancomycin has been quite effective in the treatment of methicillin-resistant *Staphylococcus aureus* infections. Except for some allergic reactions, the major side effects of this antibiotic are nephrotoxicity and ototoxicity. While the mechanism of kidney toxicity is not completely understood, biochemical and histological analyses of rats treated seven days with vancomycin (200 mg/kg) revealed increased blood levels of urea/creatinine and destruction to glomeruli and necrosis of proximal tubules, respectively (Nishino et al., 2003). Thus, these researchers suggest oxidative stress might underlie the pathogenesis of vancomycin-induced nephrotoxicity because targeting the antioxidant superoxide dismutase to the kidneys reduces the damage caused by this antibiotic. In terms of vancomycin and ototoxicity, some studies suggest that this adverse effect may be a result of vancomycin-induced augmentation of aminoglycoside ototoxicity, see below. However, in animal models, vancomycin given alone at high doses causes no hearing damage (Brummett, 1993).

Toxicity of Protein Synthesis Inhibitors

The adverse effects of the aminoglycosides are dose-dependent and can be manifested by nephrotoxicity and ototoxicity. There seems to be two contributing factors here. Aminoglycosides have a narrow therapeutic concentration range and patients present with a wide variation in antibiotic tissue distribution and elimination characteristics (Garraffo, 1998). Large amounts of intravenous administered aminoglycoside accumulate in the kidney (about 10% of dose), whereas little distribution

of aminoglycosides to other tissues is observed (Nagai & Takano, 2004). In patients with normal serum creatinine levels, Zaske and coworkers (1982) observed half-lives of gentamicin between 0.4 and 32.7 hours (Zaske, 1982). It is felt that patients with longer gentamicin half-lives accumulate more of this antibiotic in the kidney and inner ear. Recently, there have been some links made between adverse antibiotic effects and mitochondrial protein synthesis (MPS). Since MPS machinery is similar to prokaryotic machinery, antibiotics that bind to prokaryotic ribosomes may also target mitochondrial ribosomes (Bottger, Springer, Prammananan, Kidan, & Sander, 2001). In a recent study on the oxazolidinones, results indicate that this class of antibiotic inhibits mammalian MPS in a variety of tissues (McKee, Ferguson, Bentley, & Marks, 2006). In addition to the oxazolidinones, of eight clinically approved antibiotics studied, only chloramphenicol and tetracycline were significant inhibitors of mammalian MPS. While chloramphenicol toxicity is a prime focus this work and will be discussed in detail later, the oxazolidinones and chloramphenicol have been linked to dose-dependent reversible suppression of bone marrow synthesis. Some have argued that severe clinical consequences result from the oxazolidinones inhibiting MPS (De Vriese et al., 2006). Interestingly, the macrolides, lincosamides, and aminoglycosides are relatively poor inhibitors of MPS; and of these three types of antibiotics, the macrolides and lincosamides are well tolerated and associated with few adverse effects.

Toxicity of DNA and/or RNA Synthesis Inhibitors

The most common adverse reactions of quinolones are effects to the gastrointestinal tract and the central nervous system. Although some gastrointestinal

problems could be related to antibiotic neurotoxicity, they arise probably from effects on the normal flora. Digestive bacteria may be sensitive to fluoroquinolones and other antibiotics, so that the symbiotic relationship that exists between the gastrointestinal system and bacteria is disrupted (Stahlmann, 2002). While the pathogenesis of neurotoxicity is still unknown, it may be related to inhibition of the γ -aminobutyric acid receptors or possibly to agonism of N-methyl-D-aspartate receptors (Ito, Miyasaka, Fukuda, Akahane, & Kimura, 1996; Lode, 1999). Using electrophysiological determinations on hippocampal slices, several fluoroquinolones increased the neuronal population spike amplitude in a concentration-dependent manner (Schmuck, Schurmann, & Schulter, 1998). An important point here is that antibiotic toxicity can be interpreted differently. For example, there have been several fluoroquinolones removed from the market due to severe toxicities. As an example, temafloxacin was withdrawn in 1992 after a uremic syndrome involving hemolysis, renal failure, and thrombocytopenia was associated with its use (Norrby & Lietman, 1993). The syndrome was estimated to occur in about one in 5000 patients treated with this antibiotic. This incidence was too low to detect during the clinical trials. From this perspective, antibiotic drug discovery is very difficult. Erythromycin and other newer fluoroquinolones have shown some cardiac effects. *In vitro* and *in vivo* studies have shown that erythromycin exerts electrophysiologic effects on the cardiac muscle. In a hospital study, doctors observed prolongation in QT intervals in EKGs of 15 patients who were being treated with intravenous erythromycin for simple pneumonia (Freeman & Platt, 1997). However, recent dog studies showed that the quinolone, moxifloxacin, and the erythromycin

analogue, azithromycin did not cause ventricular problems when compared with the control compound, dofetilide (Thomsen et al., 2006). Thus, there are some unresolved issues with the cardiac effects of these antibiotics. When used to treat tuberculosis, rifampicin has been associated with some dose-dependent adverse reactions. Most common were its gastrointestinal effects. However, this antibiotic has been shown to inhibit bilirubin transport and this hepatic effect may warrant monitoring liver chemistry (Yew & Leung, 2006).

Toxicity of Inhibitors of Folic Acid Metabolism

Antibiotics that target folic acid metabolism have some associated adverse reactions. The sulfonamides have been shown to cause hypersensitivity, nephrotoxicity, and hepatotoxicity. Hypersensitivity is the most common side effect of sulfonamide treatment and a possible mechanism by which antibiotics can invoke an immune response has been discussed already. Sulfonamides can cause renal insufficiency by precipitating in the kidney, especially in patients who already have an underlying kidney deficiency. Prevention and treatment includes drug dose monitoring, adequate hydration, and drinking alkaline mineral waters (Perazella, 1999). Idiosyncratic sulfonamide-induced hepatotoxicity may result from antibiotic metabolism. Sulfonamides are metabolized primarily by acetylation. Certain patients acetylate sulfonamides more slowly resulting in more of the antibiotic passing through the cytochrome P-450 pathway system. This system produces reactive toxic metabolites that are normally detoxified by scavenger compounds such as glutathione. In some patients known to have low levels of glutathione, these sulfonamide metabolites accumulate and are hepatotoxic (Larrey &

Pageaux, 1997). Also, patients who are slow acetylators have a higher incidence of sulfonamide hypersensitivity reactions (Carr, Gross, Hoskins, Penny, & Cooper, 1994; Rieder, Shear, Kanee, Tang, & Spielberg, 1991).

The essence of antimicrobial chemotherapy is selective toxicity. To be effective, antimicrobial agents must possess a selective action on microorganisms without being toxic to the host cells. Here some of the primary antibacterial mechanisms along with their potential toxicities have been described. As has been discussed, even though some antibacterial targets have no human counterpart, adverse reactions occur. Antibiotics can be recognized as foreign, eliciting an immune response, while some antibiotics become metabolized and it is these metabolites that are immunogenic or toxic. Accompanying the molecular biology and genomic revolutions is a deeper understanding of the similarities and differences between prokaryotic and eukaryotic cells (Fischer & Freiberg, 2007; Schnappinger, 2007). With the genomes of several pathogenic bacteria being sequenced completely, new essential and unique genes have been identified. Researchers are trying to see if they can synthesize chemicals that will not only affect these novel targets, but also not be toxic to the bacteria's host (humans). Hopefully, this work will delineate new antibiotic targets to counteract not only the growing problems of antibiotic resistance, but also issues with antibiotic toxicity.

Studies of Gene Expression

Gene expression is a process by which the hereditary information encoded in a gene's DNA sequence is converted into the structures and metabolic functions of a cell. This conversion is the result of transcription and translation, whereby DNA gets

transcribed into messenger RNA (mRNA) and the mRNA gets translated into protein. These two processes contribute to a dogma that forms the fundamental backbone of molecular biology. Since the middle of the 20th century, gene expression has been a major focus in molecular biology research. Experiments are designed to monitor changes in expression of an interesting gene in response to an internal or external stimulus and these changes have furthered our understanding of how a protein functions in particular biological pathways. As mentioned at the end of the previous section, genomic sequencing has been useful to identify open reading frames that may code for genes unique to a particular organism. However, measuring mRNA levels in cells can be highly reflective of a gene's influence on a cellular process such as proliferation, a major focus of this study. The methods used to study gene expression can be categorized as follows; those methods that measure the expression of specific genes and those that analyze the expression of hundreds or even thousands of genes simultaneously. While the assumption has been that transcriptional changes are being measured by these methods, few methods measure RNA synthesis directly. What is being measured are mRNA levels of particular genes. This level is the sum of RNA transcription and RNA degradation rates. Nuclear "run-on" transcription assays have been used to measure rates of transcription (Gatehouse & Thompson, 1995). However, because of its complexity, this technique is not often used. What follows is a description of some of the more popular procedures that are being used to measure eukaryotic mRNA transcript levels.

Northern and Dot Blotting

Because of its reliability and low cost, Northern blotting is one of the most common techniques used to measure specific gene expression. This technique was first described almost 30 years ago (Alwine, Kemp, and Stark, 1977). While some improvements have been made over the years, the basic methods remain unchanged (Sambrook, MacCallum, & Russell, 2001, Fernyhough, 2001). First, total RNA or mRNA is resolved on denaturing agarose gels. Next, the RNA is transferred to and immobilized on a solid matrix such as nitrocellulose or nylon membranes. Finally, the RNA is hybridized with a labeled DNA or labeled anti-sense RNA probe. Excess probe is removed by washing the membranes and the image of the hybridization product is captured and analyzed. Historically, the probe has been labeled by the incorporation of radioactive nucleotides and the membranes were exposed to x-ray film or scanned in a phosphor-imager. These exposed images can identify a transcript size and measure RNA expression qualitatively. For quantification, membranes can be re-hybridized to internal controls such as glyceraldehyde 3-phosphate dehydrogenase, beta actin and cyclophilin, whose expression supposedly does not fluctuate greatly (Suzuki, Higgins, & Crawford, 2000; Weisinger, Gavish, Mazurika, & Zinder, 1999). Bianchi et al. used Northern blotting in their studies on erythroid proliferation and differentiation. They showed that tallimustine treatment inhibited human leukemic K562 cell proliferation and induced erythroid differentiation as measured by increases in globin mRNAs (Bianchi et al., 2001). In another study, Dai, Price, Brunner, and Krantz (1998) looked at the effect of interferon gamma (IFN) on purified human erythroid colony-forming cells (ECFC). They

found that the addition of IFN gamma inhibited growth and differentiation of ECFCs in a time- and dose-dependent fashion. Accompanying these effects was a marked increase in the percentage of cells expressing Fas on the surface of the ECFCs as well as the intensity of Fas mRNA expression as measured by Northern blotting. Quite a few years ago, we used Northern blotting to observe qualitative differences in certain ribosomal protein mRNAs in wild type and mutant yeasts grown at the permissive and non-permissive temperatures. Based upon the size differences and the resulting migration patterns of these mRNAs, we determined a mutation existed that affected mRNA splicing (Rosbash, Harris, Woolford, & Teem, 1980). While very powerful, Northern blotting is limited in that it can be used for the identification of a small number of defined gene transcripts. Also, since constitutive expression of the internal controls has not been thoroughly studied, quantification results may be unreliable. Dot blotting is a similar, but simpler hybridization method that has been used to quantitate mRNA. Rather than being resolved on gels, RNA samples are "dotted" onto membranes directly (Sambrook et al., 2001). Hybridization of the labeled probes to the immobilized RNA occurs as in Northern blotting. Dot blotting can be used for quantification, but it cannot be used for mRNA size determination. Another potential problem here is false-positive signals resulting from DNA contamination. All DNA should be eliminated from the RNA sample to avoid hybridization of the probe with genomic DNA. Tonini et al. (1987) used Northern and dot blotting to study the modulation of c-myc expression in K562 cells treated with certain anti-neoplastic drugs. These drugs blocked proliferation and induced erythroid differentiation. They observed a short-term decrease in c-myc mRNA. At the

time, they speculated that this transient decrease in c-myc expression is an initial event in the differentiation of K562 cells.

Ribonuclease Protection Assay

The ribonuclease protection assay (RPA) is a sensitive and accurate method to measure gene expression (Bader et al., 1992; Gilman, 2001). While it can be used to measure the abundance of multiple mRNAs, in its simplest form, RPA is used to measure expression of a single target mRNA species in a complex mixture of total RNA purified from cells (Mitchell & Fidge, 1996; Rottman, 2002). Briefly, the method involves the hybridization of radio-labeled antisense RNA probes (riboprobes) to its complementary target mRNA. The double-stranded RNA (dsRNA) hybrids are digested with single-stranded specific ribonucleases, RNase A and T. The remaining protected dsRNA fragments are analyzed by electrophoresis on denaturing polyacrylamide gels and subsequent autoradiography (Sambrook et al., 2001). If the riboprobe is in molar excess, the intensity of the radioactive signal generated is directly proportional to the amount of specific target mRNA in the original total RNA sample. The major advantages of RPA are that multiple mRNA species can be measured simultaneously in a single total RNA sample and that the assay has relatively high throughput. The major disadvantage is that the assay requires moderate technical skill. Wang et al. (1997) studied the effects of alpha thrombin on proliferation and differentiation of endothelial cells (EC). They used RPA to measure changes in the expression of motility-related genes in human EC, and, depending upon the type of human EC used, alpha thrombin exerted its chemokinetic and mitogenic effects differentially.

Primer Extension

Primer extension is a technique that is used primarily to map the 5' end of mRNAs, but it also can be used to quantify particular transcripts (Boorstein & Craig, 1989). Historically, early cloning methods often produced complementary DNAs (cDNAs) missing the 5' end of the transcript. Primer extension was often used to identify sequences involved in transcription and translation initiation. As an example, Epstein et al. (1986) used reverse transcriptase and a 49 nucleotide-long primer to reverse transcribe rat RNA into cDNA. The size and sequence of this primer extension product were analyzed on denaturing polyacrylamide gels, resulting in the identification of the 5' end of rat parvalbumin mRNA. Gong, Alkhalif, Murphy, and Murphy (1992) used both RPA and primer extension in studies of mitogenic signals in human endometrial carcinoma cells. They showed that a phorbol ester enhanced transcription of the calcyclin gene from three identified transcription start sites (Gong 1992). While primer extension is considered less sensitive than RPA, this gene expression technique is relatively easy to implement, offers additional mRNA sequence information, and is more sensitive than Northern blotting (Spira, 2005).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

One of the most sensitive and widely used methods in gene expression studies is quantitative RT-PCR (Spira, 2005). This two-step method combines reverse transcription (RT) with the polymerase chain reaction (PCR), a powerful technique for which Kary Mullis (1990) won the 1993 Nobel Prize in chemistry. In the first RT step,

cDNA is synthesized from mRNA using an RNA-dependent DNA polymerase. In the second PCR step, using a special DNA polymerase (Taq polymerase) and cDNA-specific oligonucleotide primers, multiple copies of the cDNA are made. Based upon the thermostable properties of Taq polymerase, thermal annealing properties of nucleic acids, and the reaction cycling temperatures, the amount of cDNA can be amplified logarithmically.

The amplified cDNA can be viewed and analyzed on non-denaturing polyacrylamide or agarose gels. Because of the incredible power to amplify the DNA, problems such as contaminating DNA, may arise. However, with the inclusion of appropriate internal or external standards, these problems can be minimized and transcript levels quantified (Freeman, Walker, & Vrana, 1999). As with many other molecular biology techniques, commercial enterprises have made improvements to RT-PCR. With the discovery and implementation of molecular beacons (fluorescent probes), Taqman RT-PCR is a commercial method that has surpassed RT-PCR for transcription quantitation (Yin et al., 2001). This method, which is used in this study, relies on specific hybridization probes containing a fluorescent dye (fluorophore) at one end and a quenching dye at the other end. When free in solution, the probes' ends are in close proximity to one another, such that the fluorescent dye is quenched. At the beginning of the PCR reaction, the primers and probes anneal to the target cDNA. During DNA polymerization, Taq polymerase's exonuclease activity hydrolyzes the probe and the fluorophore is released. The detected fluorescence is proportional to the amount of PCR product that is being produced (Bustin, 2000). This cumulative fluorescence provides a kinetic measure of DNA amplification and is a more precise measure of mRNA levels

than RT-PCR. However, successful application of Taqman RT-PCR requires an understanding of the practical problems, such as DNA contamination, and a careful experimental design of primers and probes. As with the other methods, proper validation is essential for precise mRNA quantitation. Combined with microarray studies (see below), RT-PCR techniques have been used to study gene expression changes resulting from chemotherapeutic treatments. Using microarrays, Lehnhardt et al. (2005) analyzed the expression patterns displayed in HT1080 fibrosarcoma cells treated with several chemotherapeutic agents. Using RT-PCR, they validated the expression of 46 candidate genes. Closer analysis of these differentially regulated transcripts revealed that doxorubicin induced cell death by influencing the abundance of factors mediating the mitochondrial (intrinsic) apoptosis pathway. Despite being able to measure one transcript per reaction, Taqman RT-PCR can be automated and made somewhat high throughput. Nevertheless, because reagents and the equipment to measure fluorescence are rather expensive, Taqman RT-PCR is being used more in well-funded and commercial laboratories.

Differential Display Reverse Transcription-Polymerase Chain Reaction (DD RT-PCR)

Primarily, there are three methods that are used to measure expression changes in numerous genes simultaneously. DD RT-PCR, the first technique, was invented in 1992 and has been used to identify changes in large subsets of known and unknown mRNAs (Liang & Pardee, 1992; Liang, Averbouhk, & Pardee, 1994). Currently, DD RT-PCR uses oligo-dT primers in combination with a series of arbitrary 13-mer oligonucleotides to reverse transcribe (RT) and amplify (PCR) mRNAs from treated and control cells.

These amplified cDNA copies can then be separated and displayed on gels, providing a pattern of differentially expressed genes. GenHunter, a company in Nashville, Tennessee that markets DD RT-PCR products, advertizes that there have been more than 3500 citations using DD RT-PCR during the last 14 years (<http://www.genhunter.com/>). This method has been especially useful for studying the effect of drugs or xenobiotics on gene expression. While this method can be used to estimate differences in gene expression, the major advantage to DD RT-PCR is that it can be used to identify new and novel genes. In 1996, we used this technique to identify four transcripts whose expression is altered in dioxin-treated hepatoma cells (Wang, Harris, Ulrich, & Voorman, 1996). We showed and confirmed by Northern blotting that dioxin decreased fibrinogen gamma and plastin mRNA levels in a time-dependent manner; whereas, it increased two unidentified transcripts. Decreases in fibrinogen and plastin suggest that dioxin may play a role in hemostasis and tumorigenesis, respectively. Recently, from sequence similarity searches we have performed, one of the increased transcripts from this study is very similar to a human ribosomal protein L7-like 1 mRNA, accession # BC064519. This result suggests that dioxin may have effects on protein synthesis apparatus (Strausberg et al., 2002).

Serial Analysis of Gene Expression (SAGE)

SAGE is a second method for a comprehensive analysis of gene expression patterns. The method is broadly applicable for the quantitation and comparison of expressed genes in a variety of normal, developmental, and disease states (Porter, Yao, & Polyak, 2006; Velculescu, Zhang, Vogelstein, & Kinzler, 1995; Yamamoto, Wakatsuki, Hada, & Ryo, 2001). The SAGE procedure has three underlying principles:

1. Short DNA sequence tags (10-14 base pairs in length) contain enough information to uniquely identify a transcript. 2. Short sequence tags can be linked together. 3. The frequency of a particular tag correlates to the expression level of the corresponding transcript. In the SAGE protocol, the first step is the synthesis of cDNA using RT and a biotinylated oligo-dT primer. Next, the cDNA is digested with a four base pair cutter restriction endonuclease called the anchoring enzyme. The digestion products are immobilized on streptavidin-coated beads and then divided. Each half is ligated to a different DNA linker that binds to the cohesive ends previously created by the anchoring enzyme. Each linker contains a type IIS restriction site (asymmetric recognition sequences) and a unique priming sequence. Upon digestion with the type IIS restriction endonuclease, DNA fragments are released from the beads. These small DNAs are enzymatically blunt-ended and ligated together creating "ditags." Using PCR primers to the unique sites within the linkers, the ditags are amplified. Following the PCR amplification step, the ditags are digested with the anchoring enzyme to release the linkers. The ditags are then ligated in tandem and cloned into a plasmid vector used for sequencing. Because the tags are short, about 50 tags can be read per sequencing reaction. Finally, the repeat sequences are counted and searched for homology versus known DNA sequences. Menssen and Hermeking (2002) used SAGE in an attempt to identify target genes of the oncogenic transcription factor c-MYC. In primary human umbilical vein endothelial cells, in which cMYC was over-expressed, 216 unique mRNAs were induced, while 260 mRNAs were repressed. Transcript levels were confirmed on 53 genes using RT-PCR and microarray analysis. They concluded that the c-MYC-regulated genes/tags identified will help to define the set of bona fide c-MYC targets and

may have potential therapeutic value for inhibition of cancer cell proliferation, tumor-vascularization, and restenosis (Menssen & Hermeking, 2002). While the SAGE procedure is quite labor intensive, requiring a multitude of reactions, there are some advantages to this technique. One advantage is that SAGE is a counting-based method. Rather than relying on hybridization thermodynamics for transcript level determinations, the abundance of a transcript is assessed by counting the number of sequence tags representing that transcript relative to the total number of tags identified. Thus, the proportion of one transcript relative to another can be determined directly. Also, SAGE requires no a priori sequence information. As in DD RT-PCR, differential expression of yet unidentified genes can be detected.

DNA Microarrays

The third and newest technique for studying patterns of expression for numerous genes is DNA microarrays. Two recent advances, gene identification through massive DNA sequencing efforts, such as the human genome project, and the technical ability to immobilize orderly these gene sequences on solid silicon supports, have pushed this technique into the mainstream, or perhaps forefront, of molecular biology research (Venter et al., 2001; Pease et al., 1994). The basic principle of the technique involves hybridizing labeled cDNAs made from target RNAs to gene-specific DNAs that have been spotted onto glass slides (microarrays). Primarily, there are two types of microarray platforms in use today. The cDNA microarray, developed by Brown and colleagues in 1996, involves depositing PCR-amplified cDNAs onto a glass slide using a robotic spotting device (Shalon, Smith, & Brown, 1996). Sample RNAs are then converted into

cDNA into which fluorescent dyes Cy3 or Cy5 have been incorporated. Fluorescent sample (experimental) cDNAs and reference (control) cDNAs labeled with another fluor are mixed and hybridized to the same DNA microarrays. Measuring and comparing the sample fluorescent signal intensities relative to the reference signal intensities can determine the abundance of each transcript in each sample. In the case of oligonucleotide microarrays, either gene specific oligonucleotides are spotted onto or synthesized in situ on glass slides (Lockhart et al., 1996; Hughes et al., 2001). Today, perhaps the most widely used oligonucleotide microarray platform is the one commercially available from Affymetrix, Santa Clara, CA. The microarray (chip) contains gene-specific oligonucleotide probes that are photolithographically synthesized onto glass. In addition, probes containing a mismatch at the central position are included as controls for cross-hybridization. Sample RNAs are converted to cDNA, from which phycoerythrin-labeled cRNA is prepared. Next, the labeled test and control cRNAs are hybridized to separate, but identical, microarrays. Binding is detected by staining with a fluorescent dye coupled to streptavidin. Laser-captured signal intensities are used to measure the relative cRNA abundance for the genes represented on each microarray. To obtain differential expression results, sophisticated computer programs compare and analyze the signal intensities on both microarrays. With the ability within one experiment to measure changes in expression of complete genomes, whole new areas of molecular biology have been augmented. Pharmacogenomics examines how one's genetic makeup affects a person's response to drugs. Microarrays can be used to determine the effects of a drug on global transcription. In so doing, key genetic biomarkers may be identified and used in evaluating a drug's effect in the overall population. For an example of how microarrays

are furthering this area of research, researchers from Washington University School of Medicine in St. Louis, Missouri discussed “clinically relevant gene polymorphisms that influence the outcome of cancer therapy, and whole-genome expression studies using microarray technology that have shown tremendous potential for benefiting cancer pharmacogenomics” (Watters & MacLeod, 2003). More fundamentally, scientists are using microarrays to further classify tumors based upon the gene expression patterns. This classification may help in building a more focused treatment regimen. In the realm of toxicology and toxicogenomics, researchers are using microarrays to focus their analysis on the basic questions of how chemicals and drugs induce disease in normal cells or tissues (De Longueville, Bertholet, & Remacle, 2004). Bulera et al. (2001) used microarrays to analyze the expression pattern in liver from Wistar rats treated with six known hepatotoxicants. This analysis identified multiple genes and groups of genes that were affected by the six hepatotoxicants, indicating that high-density microarray expression data are useful to identify groups of genes involved in toxicity. Further, to support the use of microarrays to determine or predict toxic liver effects, the identity of an unknown sample was revealed by comparing its expression pattern profile with the compiled expression profiles from the known hepatotoxicants (Bulera et al., 2001). While the microarray technology is becoming more and more popular, it is not without problems. First, microarray technology is very expensive. Not only are the microarrays themselves quite expensive to produce, the laser detection system, microarray wash station, and accompanying computer equipment costs put this technology out of reach for most molecular biology labs. Further, there have been some basic concerns raised

over microarray reproducibility and standardization (Bammler et al., 2005, Waring et al., 2005).

We chose to use Affymetrix microarray technology to study the effects of chloramphenicol on gene expression in mammalian cells and confirmed these changes by Taqman RT-PCR. Because all mammalian cells contain mitochondria, we hypothesize that myeloid cells contain other factors, such as uniquely expressed genes, that make this tissue perhaps more sensitive to this antibiotic than other tissues. Leiter et al. (1999) have examined the effects of chloramphenicol on mitochondrial function in K562 cells, a human erythroleukemia cell line frequently used to study red blood cell precursors. In this work, it was shown that chloramphenicol inhibited oxidative metabolism, reduced the activity of cytochrome c oxidase (COX), and lowered the ATP content of the cells. In addition, this antibiotic reduced the production of the ferritin and the transferrin receptor. The reduction of these two proteins was somewhat surprising, since eukaryotic ribosomes in the cytosol are the sites of synthesis of these two iron-related proteins. Interestingly, using Northern blotting, they showed that chloramphenicol decreased only the mRNA levels for the transferrin receptor. Though it was unclear how the chloramphenicol-induced mitochondrial dysfunction caused a decline in these two important proteins in heme homeostasis, since total cellular protein synthesis was unaffected by this treatment in these cells, a link was established between the mitochondria and these two particular proteins. The goal of this study will be to see if global transcriptional changes in cells treated with chloramphenicol can identify genes whose expression correlates with effects on proliferation. It is proposed that we examine global transcriptional changes resulting from chloramphenicol treatment to see if these

events can be separated in any way from its known effects on mitochondria and proliferation.

Cell Model for Studying Effects of Chloramphenicol on Hematopoietic Cells

To study the effects of chloramphenicol on cells, we chose to study proliferation and gene expression changes in a murine erythroleukemia cell line. Since chloramphenicol has an effect on red blood cell number, a cell culture system that enables the study of erythroid progenitors is a useful and convenient tool for assessment of the effects of antibiotics on hematopoiesis. The cells used in this work are related to erythroleukemia cells first characterized by researchers back in the 1950s. Using electron microscopy, Charlotte Friend and Cecily Selby (1954), colleagues at Sloan-Kettering in New York, noticed that cells from Ehrlich carcinoma had cytoplasmic particles that were similar to those seen in thin-sections of virus-infected cells. Charlotte Friend decided to inoculate newborn mice with cell-free extracts from this cancer. While these mice appeared visibly healthy, upon being sacrificed and examined, several of the mice contained enlarged spleens. After Friend injected spleen cells from these mice into adult mice, several animals obtained palpable spleens. Soon Friend was able to show that the disease she was seeing was unique. This cancer was characterized by erythroblastosis and a profound anemia. Besides being transmissible in adult non-inbred mice, the short latent period before the first symptoms appeared was powerful evidence that the transmissible agent was directly involved in the induction of the leukemia (Friend, 1956). Later Friend demonstrated that in some instances, expression of the malignant phenotype could be reversed experimentally. Upon treatment of some of the erythroleukemia cells

with DMSO, she was able to inhibit cell proliferation and induce differentiation as measured by increases in cellular hemoglobin production (Friend, Scher, Holland, & Sato, 1971). For this work, the virus and leukemia now bear Friend's name. The Friend erythroleukemia cells became a widely used model for studying control of erythroid differentiation, as well as malignant transformation and disease (Ruscetti, 1995; Lee et al., 2003).

The cell line we used in this work is derived from a leukemia induced by a B-tropic Friend leukemia virus (ATCC TIB-55 BB88, Chesebro, Wehrly, & Housman, 1978). While there are several strains of erythroleukemia viruses, the B-tropism here refers to the ability of this particular retrovirus to grow preferentially in BALB strain mice cells. This growth property is regulated by a single genetic locus, Fv-1; such that B-tropic Friend retroviruses can integrate only into host DNA containing the permissive Fv-1^{b/b} genotype. The result of this integration is that the cells appear to be blocked at the colony-forming cell for erythropoiesis (CFU-e) stage of erythropoiesis. This block is caused primarily through the interaction of a unique viral envelope protein and the erythropoietin (Epo) receptor that results in receptor activation and subsequent erythroid signaling (Ruscetti, 1999). The aberrant receptor activation causes cell proliferation and differentiation in the absence of Epo, an essential regulator of erythroid cells (Richmond, Chohan, & Barber, 2005). These cells and other murine erythroleukemia (MEL) cells have been used in a variety of studies examining changes in gene expression as a result of differentiation induction (Gambari, Marks, & Rifkind, 1979; Karacay & Chang, 1999; Klinken, Holmes, Morse, & Thorgeirsson, 1988; Obinata, Kameji, Uchiyama, & Ikawa, 1978; Profous-Juchelka, Reuben, Marks, & Rifkind, 1983; Zaker, May, & Burnett,

2002). In addition, they have been used to show that compounds that inhibit MPS also hinder erythroid maturation as measured by hemoglobin accumulation in chemically-induced MEL cells (Kaneko, Watanabe, & Oishi, 1988). An objective of this study is to measure the effects of chloramphenicol in proliferating BB88 MEL cells.

Chloramphenicol and Its Toxicity

In 1947, Chloramphenicol (Chloromycetin) was first isolated from an actinomycete, *Streptomyces venezuelae*. This organism had been found in a soil sample obtained from a field near Caracas, Venezuela (Ehrlich, Bartz, Smith, Joslyn, & Burkholder, 1947). In sensitive bacteria, chloramphenicol is a potent and specific inhibitor of protein synthesis. The mechanism of this inhibition is attributed to its stereospecific and reversible binding to the 50S ribosomal subunit (Cundliffe 1967; Lorian, 1971). In particular, chloramphenicol has been shown to bind preferentially to bacterial protein L16 of the 50S subunit. This binding prevents the attachment of the amino acid containing end of the amino-acyl t-RNA complex to the ribosome and thus, inhibits peptide bond formation (Pongs, Bald, & Erdmann, 1973). Nucleotide footprinting studies identified that chloramphenicol interacts strongly with nucleotides Adenosine-2451 and Guanine-2505, sites within the peptidyl transferase region on 23S rRNA. This interaction bolstered chloramphenicol's influence in peptide translation inhibition (Moazed & Noller, 1987).

With great excitement and fanfare, chloramphenicol was approved in 1949 for distribution in the United States (Maeder, 1994). While this antibiotic showed great promise for treating a number of infections such as typhoid fever and meningitis,

chloramphenicol treatment was not without problems. Microbial resistance can and does occur (Calder 1972; Friedland & Klugman, 1992; Jorgensen, 1991; Linares, Tubau, & Dominguez, 1999; Scott et al., 2005). Primarily, this resistance is the result of a specific plasmid that causes the production of acetyltransferases. These transferases acetylate chloramphenicol so that it no longer binds to bacterial ribosomes (Gaffney, Foster, & Shaw, 1978; Shaw & Brodsky, 1967; Shaw, 1971). However, it was not chloramphenicol resistance that created the most controversy, as resistance occurs to most, if not all, antibiotics. It was the adverse effects chloramphenicol was having on patients' myeloid tissue that created the biggest problem for this medicine.

Two types of myelotoxicity have been linked to chloramphenicol usage. The most serious toxicity is idiosyncratic aplastic anemia. Death from aplastic anemia has been estimated to occur in one of 20,000-40,000 courses of chloramphenicol treatment. (Smick, Condit, Proctor, & Sutcher, 1964; Feder, Osier, & Maderazo, 1981) and while this type of anemia is quite rare, it is irreversible and most often fatal. To date, the mechanism by which chloramphenicol causes aplastic anemia remains elusive. Some researchers have suggested genetics may be involved in human chloramphenicol-induced aplastic anemia (Dameshek, 1969). Others have suggested that aplastic anemia is associated with the DNA damage caused by nitroso-chloramphenicol (Yunis, Miller, Salem, Corbett, & Arimura, 1980). Nitrosochloramphenicol is a product of the metabolic reduction of the para nitro group of chloramphenicol and the ability to perform this reduction may be genetically determined. Also, Skolimowski, Knight, and Edwards (1983) showed that thiamphenicol, a compound with similar antimicrobial properties to chloramphenicol, but whose structure lacks the para nitro group, did not cause DNA

damage in mammalian cells *in vitro*. While it does affect myeloid tissue, there have been no definitive cases of aplastic anemia attributed to thiamphenicol (Yunis, 1981).

Unfortunately, very little of this work can be corroborated by *in vivo* experiments (Turton et al., 2000). To date, there have been no successful animal models that link aplastic anemia to chloramphenicol treatment (Festing, Diamanti, & Turton, 2001; Chen, 2005). Nevertheless, the threat of aplastic anemia has caused a dramatic decline in chloramphenicol's usage in the United States and the pharmaceutical company Parke Davis (now Pfizer, Inc.) has not manufactured this antibiotic for over 25 years.

The second and most common type of myelotoxicity accompanying chloramphenicol therapy is dose-related and reversible bone marrow suppression (Erslev 1953; Yunis, 1988). Therapeutically relevant concentrations of chloramphenicol affect cell growth of a variety of mammalian cells (Firkin & Linnane, 1968; Holt, Ryder, Fairbairn, Hurley, & Harvey, 1998; Kang et al., 2005). This anti-proliferative effect appears to be as a result of MPS inhibition both *in vitro* and *in vivo* (Bread, Armentrout, & Weisberger, 1969; Fettes, Haldar, and Freeman, 1972; Kroon & Van Den Bogert, 1983; Martelo, Manyan, Smith, and Yunis, 1969; Summ, Draeger, and Von Wasielewski, 1976). In a related finding, Nagiec et al. (2005) have shown that the oxazolidinones, a new class of chemicals that inhibit bacterial and MPS by binding to a similar ribosomal site, also inhibit K562 human erythroleukemia cellular proliferation reversibly. Further, cells depleted of their mitochondrial DNA by ethidium bromide treatment were unaffected by the oxazolidinones (Lin, Murray, Vidamr, & Marotti, 1997; Nagiec et al., 2005). Reversible myelosuppression and thrombocytopenia was seen in patients receiving Linezolid, an oxazolidinone approved for marketing in 2000

(Attassi, Hershberger, Alam, & Zervos, 2002; Green, Maddox, & Huttenbach, 2001; Paladino, 2002; Waldrep & Skiest, 2002). While, the pathogenesis of this myelosuppression appears related to the antibiotic's effect on mitochondria, it is unclear why myeloid tissue is so vulnerable. Because mammalian cells depend upon mitochondrial function for energy and the toxic effects of chloramphenicol appear primarily in the bone marrow, there may be other factors involved. Some have argued that cellular sensitivity may be the result of a cell's respiratory "threshold capacity" (Rossignol et al., 2003). A high threshold capacity in a tissue means a greater loss in mitochondrial proteins can be sustained, without losing mitochondrial function. Perhaps erythroid tissue has a low threshold capacity whereby losses in mitochondrial protein brought on by chloramphenicol treatment, dramatically affect respiration.

To better understand chloramphenicol's anti-proliferative effects on erythroid tissue, experiments to examine transcriptional changes in a cell model of this tissue were initiated. This report describes the application of DNA microarray technology to measure changes in gene transcript levels in BB88 erythroleukemia cells. Because all cells contain mitochondria, we hypothesize that myeloid cells contain other factors, such as uniquely expressed genes, that make this tissue perhaps more sensitive to this antibiotic than other tissues. As has been mentioned earlier, Leiter et al. (1999) have examined the effects of chloramphenicol on mitochondrial function in K562 cells, a human erythroleukemia cell line frequently used to study red blood cell precursors. In this work, chloramphenicol treatment not only inhibited oxidative metabolism and ATP production, but also reduced the amount of certain proteins involved in iron homeostasis. Interestingly, Northern blotting showed that chloramphenicol decreased mRNA levels

for only one of the proteins, the ferritin receptor, suggesting that chloramphenicol affects genes differentially. From these results, the investigators concluded that the previously unsuspected link between mitochondrial function and cellular iron metabolism might contribute to the anemia that often develops even in the absence of sideroblastic changes in the bone marrow. In a more recent study using K562 cells, Kang et al. (2005) show that longer-term chloramphenicol treatment (6-9 days) induces apoptotic cell death through a caspase-dependent pathway presumably by suppressing the expression and protein levels of certain cell cycle regulatory molecules. Our effort in this study will be to see if global transcriptional changes in a murine erythroleukemia cells (BB88) treated with chloramphenicol can identify genes whose expression correlates with effects on proliferation. We propose to examine global transcriptional changes resulting from chloramphenicol treatment to see if these events can be separated in any way from its known effects on mitochondria and proliferation. By looking at early effects (24 hours) of chloramphenicol, we predict that transcription changes in these cells will be uniquely tied to the mitochondria, a primary toxic target for this and other similar antibiotics (Nagiec, 2005). Studying transcriptional changes may be useful in identifying not only cellular changes induced by chloramphenicol, but also a particular antibiotic marker of chloramphenicol toxicity.

MATERIALS AND METHODS

Chemicals, Reagents, and Compounds

Chemicals and reagents were purchased from Sigma (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), Invitrogen (Carlsbad, CA), and Applied Biosystems, Inc. (ABI, Foster City, CA), unless specified. Chloramphenicol (MW 323.1) was purchased from Boehringer Mannheim (#634-433). Chloramphenicol was dissolved in ethanol (EtOH) to 100 mM and used at 50 μ M final concentration unless specified. PNU-86983 and PNU-78714 were obtained from Pharmacia's (Pfizer) Research Compound Collection. 100 μ M stocks were prepared by dissolving these compounds in DMSO (Sigma D-2650). Similarly, a 100 μ M stock solution of Tetracycline (Sigma T- 8032) in water was prepared. Erythromycin (Sigma E-6376), oligomycin (Sigma O-4876), and succinylacetone (Sigma D-8645) stock solutions were prepared by dissolving compounds in DMSO. These stock solutions were stored at -20° C. The antibiotic Geneticin (50 mg/ml) (Invitrogen #10131035) was stored at 4° C. Oligonucleotide primers and probes were designed using Primer Express version 2.0 software (ABI, Applied Biosystems, Inc. Foster City, CA) and these oligonucleotides were synthesized by ABI or Sigma-Genosys (Woodland, TX).

Cell Culture

BB88 murine erythroleukemia cells (ATCC TIB 55) were maintained in RPMI 1640 medium (with L-glutamine) (Invitrogen # 11875-093), containing 10% heat-inactivated fetal bovine serum (Invitrogen #10082-139), 10 μ M 2-mercaptoethanol (β ME)(Aldrich M370-1), and Penstrep antibiotic (Invitrogen #15140-122) (5 ml per 500 ml of medium). Cells were seeded at approximately 2×10^4 cells per milliliter (cells/ml), maintained in 5% CO₂ at 37^o C, and split after 5-6 cell doublings (~12 hours per doubling). Cell counts were determined using a Coulter Counter ZM (Beckman-Coulter Inc.). Compounds were added to cells to a final diluent concentration of 0.1% v/v.

Total RNA Isolation

RNA was isolated from BB88 cells using Trizol (Invitrogen #15596-026). Briefly, cells were pelleted by centrifugation at 500 x g in a Sorvall RT6000B centrifuge. Cell pellets were resuspended in Trizol (1 ml per 10^6 cells). Nucleic acids were extracted with CHCl₃ and phenol and precipitated using EtOH following the procedures described in the Trizol package insert. RNA pellets were dissolved in DNase/RNase-free water (Invitrogen #10977-015) and quantitated using spectrophotometry (Genequant, Pharmacia Biotech). Residual DNA was removed by passing RNA aliquots (100 micrograms (μ g)) over RNeasy mini columns (Qiagen #74106). Briefly, immobilized RNA was treated for 15 minutes at room temperature with DNase 1 (Qiagen #79254), washed with the kit's RW1 buffer (Qiagen #74106), and eluted with water. Total RNA was quantitated spectrophotometrically (1 Optical Density unit at 260 nanometers for

RNA molecules equals 40 nanograms (ng) per microliter (μ l) of RNA) and analyzed for fidelity (DNA contamination and/or degradation) using an Agilent Bioanalyzer, see

Figure 5.

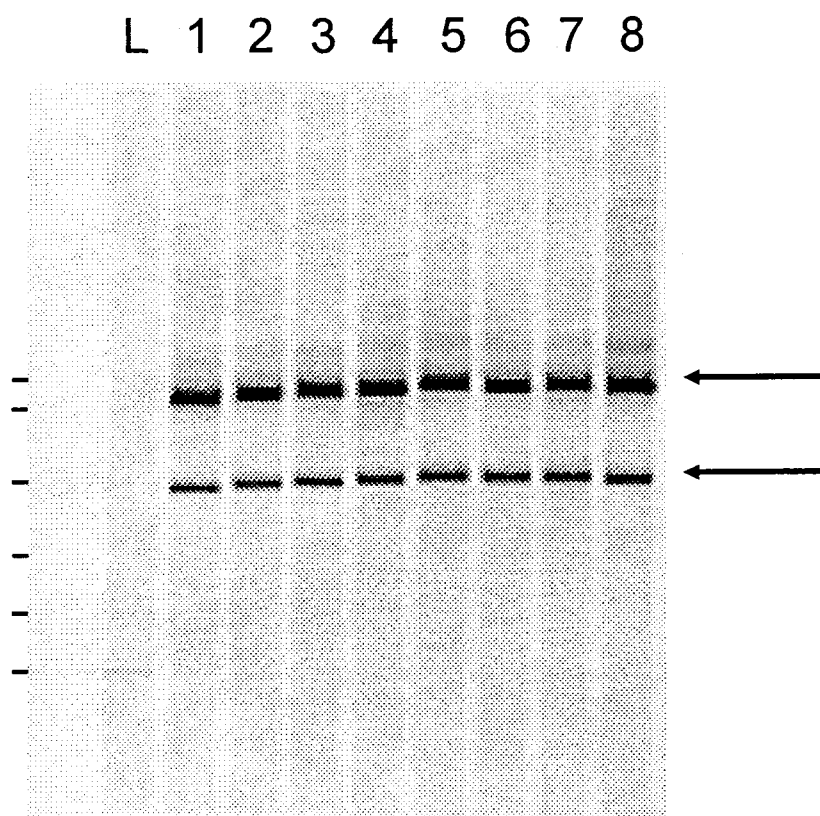


Figure 5. Agilent Bioanalyzer Analysis of BB88 RNA.

One microliter aliquots of DNAsed and Qiagen column-purified RNA (100 ng) were loaded into wells on the RNA LabChip (Agilent Technologies). Wells containing RNA and buffer, as well as RNA standards (L) and buffer, were mixed. The chips were loaded into and run through the bioanalyzer instrument. Lanes: L- RNA ladder (Agilent RNA 6000 Nano Ladder, 6000, 4000, 2000, 1000, 500, and 200 nucleotides), 1-2, Control RNAs 0 hours, 3-5 Control (0.1% ethanol) RNAs 24 hours, 6-8 Treated (50 μ M chloramphenicol) RNAs 24 hours. Ribosomal RNAs, 28S and 18S, are marked by arrows.

cDNA Synthesis and Labeled cRNA Preparation

To prepare double-stranded cDNA (ds-cDNA) and biotin-labeled cRNA for microarray chip hybridizations, the procedures described in the year 2000-GeneChip Expression Analysis Technical Manual were followed (Affymetrix, Santa Clara CA, Bammert 2000). Using components from a Custom Superscript Double-stranded cDNA Synthesis Kit (Invitrogen # 11917-010), ds-cDNA was prepared from a mix of 7.5 μ g total RNA and 100 picomoles of a T7-modified oligo-dT primer (GENSET Corporation, La Jolla, CA, cat. #GUAR10) in a final volume of 150 μ l. This two-step reaction was extracted with an equal volume of phenol:CHCl₃:isoamyl alcohol, and the organic and aqueous layers were separated using Phase Lock Gel I-Light matrix (5 Prime, Inc. #pl-188233). The aqueous layer was transferred to a DNase/RNase-free 1.5 ml microcentrifuge tube and precipitated using ethanol ammonium acetate in the presence of glycogen (5 μ g) (Ambion #9510). Next, a commercial *in vitro* transcription kit (Enzo #900182) was used for preparing biotin-labeled cRNA. Briefly, 10 μ l cDNA aliquots were combined with 30 μ l of a proprietary *in vitro* transcription reaction mix, containing hybridization buffer, biotin-labeled ribonucleotides, dithiothreitol (DTT), RNase inhibitor mix, and T7 polymerase. After incubation for 4.5 hours at 37°C (tubes were mixed gently every 30 minutes), each reaction was passed over an RNeasy mini column and labeled RNA was evaluated spectrophotometrically, see above.

Fragmentation of Labeled cRNA and Target Hybridization

In a total reaction volume of 30 μ l, 15 μ g cRNA aliquots were mixed with 6 μ l of 5x Fragmentation Buffer (200 mM Tris acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate). After heating the mixture for 35 minutes at 94° C, this cRNA Target was stored at -20° C until hybridization. Using the Affymetrix Gene Chip Protocol and the GeneChip Eukaryotic Hybridization Control Kit (Affymetrix # 900299), the cRNA Target Hybridization Mix was prepared as shown in Table 1.

Table 1. Reaction Components for Typical cRNA Target Hybridization Mix

Component	Volume (μ l)	Final concentration
cRNA Target	30	0.05 μ g/ μ l
3nMControlOligoB2*	5	50pM
20x Eukaryotic Hybridization Controls**	15	Varied**
Herring Sperm DNA 10 mg/ml (Invitrogen)	3	0.1 mg/ml
Acetylated BSA 50 mg/ml (Invitrogen)	3	0.5 mg/ml
2x Hybridization Buffer***	150	1X
water	94	
Final	300	

* Control Oligo B2 – Affymetrix synthetic control oligo used for alignment signals

** 20x Eukaryotic Hybridization Controls – Affymetrix mixture of 30 pM bioB(BIOB), 100 pM bioC(BIOC), 500 pM bioD (BIODN), and 2000 pM cre (CREX) cRNA Target controls. Heat separately at 65° C for 5 minutes before adding to mix.

*** 2x HybridizationBuffer – (final 1x concentration is: 100 mM MES, pH 6.6, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20).

Each cRNA target hybridization mix sample was heated to 99° C for five minutes, then incubated at 45° C for five minutes, followed by a five minute centrifugation at maximum speed (10,000 x g) to remove any insoluble material. During these incubations and centrifugation steps, each U74Av2 microarray (Affymetrix # 900343) was pre-wetted with 200 µl of 1x Hybridization Buffer (see above). Next, the 1x Hybridization Buffer was replaced in each microarray with 200 µl of cRNA Target Hybridization Mix. All microarrays were incubated for 16 hours at 45° C in a rotisserie oven (Affymetrix GeneChip Hybridization Oven 640).

Microarray Washing and Staining

After 16 hours, cRNA Target Hybridization mixes were removed from the individual microarrays and replaced with 200 µl of Non-Stringent Wash Buffer (6x SSPE, 0.01% Tween 20, 0.005% Antifoam (Sigma A-8082)). While the Affymetrix Genechip Fluidics Station was readied for the washing and staining steps, the microarrays were set aside and the Hybridization Mixes were stored at -20° C. The Fluidics Station reservoirs were filled with freshly-prepared Stringent Wash (100 mM MES pH 6.6, 100 mM NaCl, 0.01% Tween 20 (Pierce #28320) and Non-Stringent Wash Buffers (see above). To stain and amplify the hybridized cRNA Target on each microarray, two solutions, Antibody Amplification Solution (Ab) and Streptavidin Phycoerythrin Stain Solution (SAPE), were prepared as shown in Tables 2 and 3.

Next, the Fluidics station was primed and programmed to perform the EukGE-WS2v4 protocol (Affymetrix Microarray Suite User's Guide Version 5.0). This protocol included a series of steps that included a SAPE staining, an Ab amplification, and a

Table 2. Reaction Components for the Antibody Amplification Solution (Ab)

Reagent	Volume (μ l)	Final concentration
2 x Stain Buffer (200 mM MES pH 6.6, 2 M NaCl, 0.1% Tween 20, 0.01% Antifoam)	300	1 x
Acetylated BSA (50 mg/ml)	24	2 mg/ml
Goat IgG (10 mg/ml) (Sigma I5256)	6	0.1 mg/ml
Anti-streptavidin antibody (0.5 mg/ml) (Vector Laboratories BA-0500)	3.6	3 μ g/ml
water	266.4	
Final	600	

Table 3. Reaction Components for the Streptavidin Phycoerythrin Stain Solution (SAPE)

Reagents	Volume (μ l)	Final concentration
2x StainBuffer	600	1x
Acetylated BSA (50 mg/ml)	48	2 mg/ml
R-Phycoerythrin Streptavidin (1 mg/ml) (Molecular Probes # S-866)	12	10 μ g/ml
water	540	
Final	1200	

second SAPE staining. Stringent and non-stringent washes accompanied each of these staining and amplification steps, see the Affymetrix Microarray Suite User's Guide Version 5.0. Following washing and staining, each microarray was scanned on the

Affymetrix scanner. All of the scanned values were imported automatically into the Affymetrix Microarray Suite 5.0 gene expression analysis software.

Transcript Analysis Algorithm

Affymetrix microarrays consist of oligonucleotides synthesized and bound *in situ* to a 24 x 24 micron Probe Cell using photolithography (Pease et al., 1994). Each ORF (open reading frame) is represented by a Probe Set. Probe Sets consist of approximately twenty oligonucleotide pairs. Each oligonucleotide (20-mer) pair consists of an oligomer (PM-perfect match) that is designed to match the ORF sequence and an oligomer (MM-mismatch) that contains a homomeric mismatch in the middle of its sequence. A single intensity value for every Probe Cell, representative of the hybridization signal of its cRNA Target, is measured, and then corrected for background across the entire array, (see Appendix C in the Affymetrix Microarray Suite 5.0 User's Guide). Probe Cell intensities, both PM and MM, are used to determine the Signal. The Signal is a quantitative metric calculated from each Probe Set, which represents the transcript amount in solution (RNA sample). The Signal is calculated using the one-step Tukey's Biweight Estimate algorithm, yielding a robust weighted mean. To obtain this mean, each probe pair in a probe set potentially has a vote in the Signal value determination. The vote is an estimate of the real signal due to hybridization of the target cRNA. This real signal is estimated by taking the log (PM intensity minus the MM intensity). The Probe Pair vote is weighted more strongly if its real signal value is closer to the median value for an entire Probe Set. Next, a mean of the weighted intensities for a Probe Set is identified. This mean is corrected back to the linear scale and the Signal is its output. For

more details on Signals, (see Appendix C in the Affymetrix Microarray Suite 5.0 User's Guide and /or Affymetrix's GeneChip Expression Analysis – Data Analysis Fundamentals guide [available online at www.Affymetrix.com])).

Before comparing two microarrays, Signals were normalized by scaling. Scaling helps correct for variations between any two arrays. Biological and technical differences are two of the major sources of variation in any array experiment. Biological differences may include such things as time and treatment differences, while technical variation may be due to experimental variables such as the quality and quantity of target hybridized, staining, and handling error. In scaling, each Signal value on a particular array is multiplied by a scaling factor (SF) to make the average intensity of all of the signals on that microarray equal to an arbitrary user-defined average target threshold (TGT). Not only does this permit comparison of microarray data within an experiment, but also comparison of microarray data between experiments.

Two algorithms within Affymetrix Microarray Suite 5.0 GeneChip Comparison Analysis software are used to compare probes sets on one (experimental) array to their counterparts on another (baseline) array. To determine the magnitude and direction of a change in a transcript, the Signal Log Ratio (SLR) is computed. Similar to the Signal algorithm, the SLR is computed using a one-step Tukey's Biweight method by calculating the mean of the log₂ ratios of probe pair intensities between any two arrays. The Wilcoxon's Signed Rank test uses the differences between PM and MM intensities, as well as the differences between PM intensities and background to compute three, one-sided p-values. The most conservative value of the three is recorded and represents the Change p-value, Appendix B. Values close to 0.5 signify there has been no change in the

transcript level between experimental and baseline arrays, whereas values closer to 0 represent transcripts that have increased (Inc) or marginally increased (MI) while values close 1.0 represent transcripts that have marginally decreased (MD) or decreased (Dec). (Affymetrix's GeneChip Expression Analysis – Data Analysis Fundamentals Guide). See Appendix B for representative Signals and Change p-values for selected Probe Sets from this work. The results of these comparisons were exported into Excel and visualized by subsequent importation into Spotfire Decision Site 8.1 software (Spotfire, Inc., Somerville, MA).

Gene Ontology Analysis

For acquiring, analyzing and organizing the genetic information resulting from the microarray experiments, selected probe set names were entered into the Gene Ontology Mining Tool within NetAffx™ Analysis Center (<http://www.affymetrix.com/products/software/specific/netaffx.affx>).

RT-PCR

For measuring changes in transcript levels, Taqman® or SYBR Green two-step RT-PCR assay (ABI) was used. Briefly, the reverse transcriptase (RT) step was conducted by mixing 1-2 µg total RNA with 1X TaqMan® RT buffer, 5.5 mM MgCl₂, 500 µM dNTPs, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, ±1.25 U/µl reverse transcriptase [Taqman® RT reagents (ABI # N808-0234)]. Thermal cycling parameters for RT reactions included a primer incubation step at 25 °C for 10 min, RT at 48 °C for 30 min and an RT inactivation step at 95 °C for 5 min. In the subsequent PCR reactions,

we combined Taqman® Universal PCR Master Mix (ABI # 4304437) or SYBR Green PCR Master Mix (ABI #4309155), oligonucleotide primers, and oligonucleotide probes, see Table 4, with 1:10-1:200 dilution of RT reaction for unknowns or 1:50-1:50,000 dilution of murine genomic DNA (100 ng/μl stock, a gift from William Blake, Pharmacia Corp. Kalamazoo, MI) or human genomic DNA (Taqman Control Genomic DNA (human), # 4312660, ABI) for standards. These 25μl PCR reactions were placed in an ABI Prism 7700 Sequence Detection System (ABI). Thermal cycling parameters included an incubation step at 50 °C for 2 min, Amplitaq Gold (component of Universal PCR Master Mix) activation step at 95 °C for 10 min, and 40 cycles of PCR consisting of a denaturation step at 95 °C for 15 sec and an annealing/extension step at 60 °C for 1 minute. Using the ABI Prism quantitation software, relative transcript amounts were determined. We used glyceraldehyde phosphate dehydrogenase (GAPDH) transcript levels to normalize COX VIa-H and other transcript levels, as we found GAPDH to be constitutively expressed throughout the treatments. Fold increases were calculated as a ratio of transcript levels in treated versus untreated control samples. After a few RT-PCR experiments, we replaced genomic DNAs with pooled RT reactions in PCR reactions used for standard curve determinations. This replacement was necessary as we were unable to synthesize detectable PCR products using COX VIa-H primers with the mouse genomic DNA, (see Results section).

Data Analysis

We used Unistat (London, England, www.unistat.com) to perform t-test statistical determinations. For the time-course and concentration-response experiments,

data were analyzed in GraphPad Prism Software version 4.03 (GraphPad Software Incorporated, San Diego, CA).

Table 4. RT-PCR Oligonucleotide Primers

Gene Name	Organism	Primer Name	Accession Number (catalog number)	Sequence 5'→3'
COX VIa-H	Mouse	mCOX VIaH For	NM_009943	accacgagcgcccagag
		COX VIaH Rev	NM_009943	aggcgaagggttggttc
		COX VIaH Probe (FAM)	NM_009943	FAM-catccccgtatcaccacctccgcat-TAMRA
GAPDH	Rat	rodent GAPDH Forward	(ABI #4308313)	proprietary
		rodent GAPDH Reverse	(ABI #4308313)	proprietary
		rodent GAPDH Probe (VIC)	(ABI #4308313)	proprietary
COX VI	Mouse	mCOX IV For	M37831	catgaaggccaacccatt
		COX IV Rev	M37831	gcagcgggtctcacttct
		COX IV Probe (FAM)	M37831	FAM-actcattctgtcatagtccttgg-TAMRA
COX Vb	Mouse	mCOX Vb For	X52157	cagcttcaggcaccaagga
		COX Vb Rev	X52157	cacagatgcagcccactattctc
		COX Vb Probe (FAM)	X52157	FAM-ccctaattctagtcctccatcagcaa-TAMRA

RESULTS

Previous work has shown that chloramphenicol affects proliferation of a variety of cells (Morley, Furness, & Higgs, 1974; Ruben & Hooper, 1978; Madeira-Lopes & Van Uden, 1981; Leiter et al., 1999; Kang et al., 2005). In order to examine the effects of chloramphenicol on erythroid precursor proliferation to global transcription, we needed to set up an erythroid cell model that was sensitive to chloramphenicol. We chose BB88 murine erythroleukemia cells, a hematopoietic model described earlier in the Introduction section. These cells can be cultured easily and Figure 6 shows a representative growth curve for these cells. For the first 4 days in culture, BB88 cells diluted back to 20K per milliliter (20K/ml) grew exponentially and a doubling time of 12 hours was estimated. Thereafter, cells grew more slowly. In this experiment where the culture medium has not been replenished, by 7 days, BB88 cell growth was arrested. After determining these basic BB88 growth characteristics, we determined the effects of chloramphenicol on cell proliferation. BB88 cell cultures were set up as in Figure 6 and after 24 hours, various amounts (0-200 μ M) of chloramphenicol were added. After an additional 48 hours, cell counts of the various cultures were determined (Figure 7). The cell counts from this experiment fitted nicely a sigmoidal dose response curve such that the concentration at which chloramphenicol inhibited BB88 proliferation by 50% (EC_{50}) was 43 μ M.

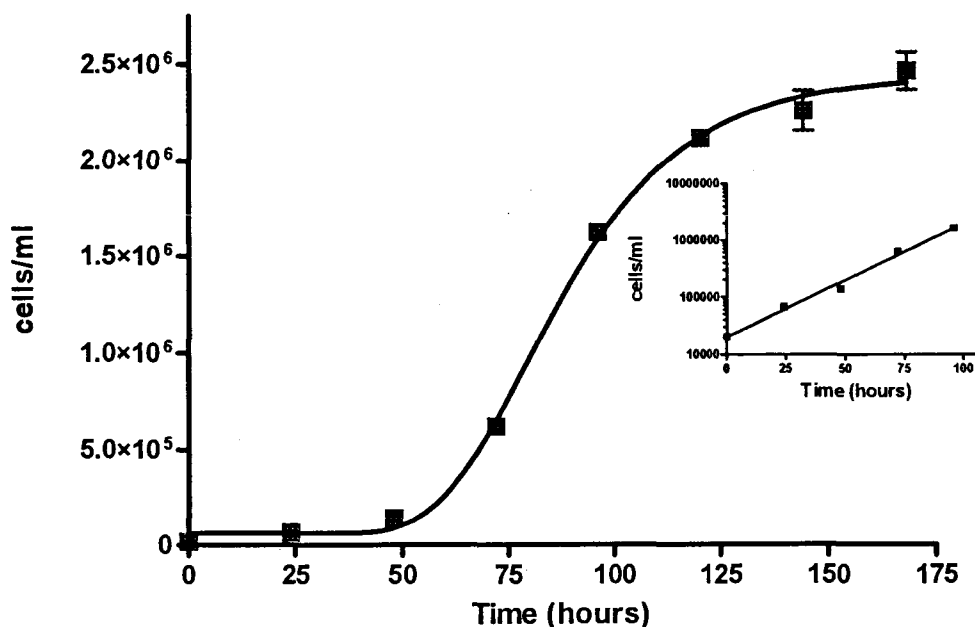


Figure 6. Proliferation of BB88 Murine Erythroleukemia Cells.

BB88 cells were maintained at 37°C under 5% CO₂ in motionless suspension culture in RPMI 1640 supplemented with 10% FBS (heat-inactivated), 10 μM β-mercaptoethanol, L-glutamine, and Penicillin-Streptomycin (100 Units/ml and 100 μg/ml, respectively). Cells were seeded at 2×10^4 /ml in 24-well Costar culture dishes, ~ 1 ml/well. Cell counts were determined using a Coulter Counter ZM (Beckman-Coulter Inc.) every 24 hours over 7 days (168 hours). Each square represents the mean of triplicate determinations. Using GraphPad Prism, the data was fitted using the Gompertz equation: $Y = N_0 + C \cdot \exp(-\exp((2.718 \cdot \mu_{\max}/C) \cdot (\text{Lag} - X) + 1))$. For fitting bacterial growth data (sigmoidal); N_0 = log initial number of cells; C = difference between initial and final cell numbers; Lag = delay before growth, same units as X ; μ_{\max} = maximum specific growth rate.; X = time, Y = log cell number. Inset graph shows logarithmic growth of BB88 cells over first 4 days. Using linear regression, doubling time of BB88 cells is 12 hours.

In the next experiment, we chose to follow BB88 proliferation after the addition of 50 μM chloramphenicol. As can be seen in Figure 8, chloramphenicol affects BB88 cell growth rate. After 5 days of antibiotic exposure, cell number in cultures containing

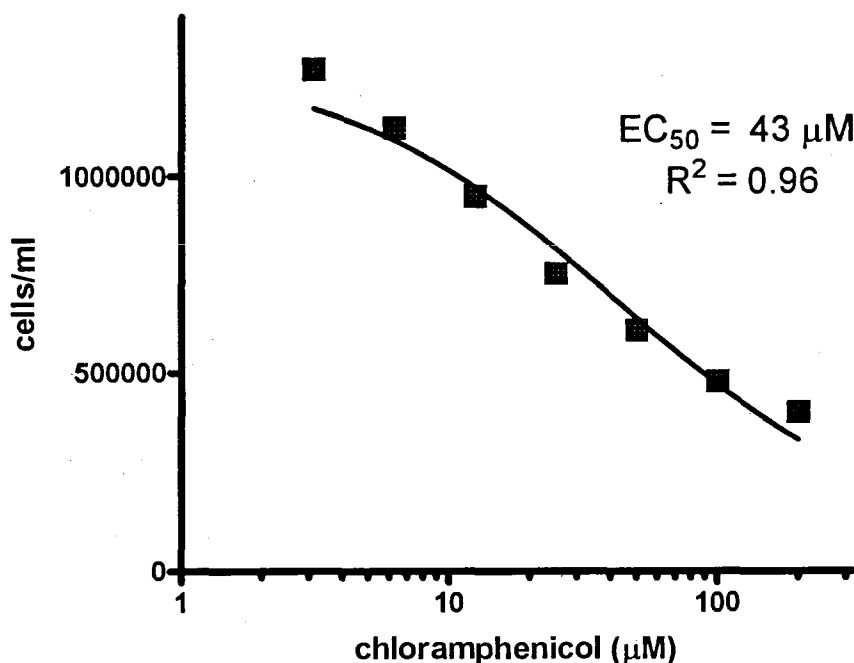


Figure 7. Effect of Chloramphenicol on Proliferation of BB88 Cells.

Cells were cultured as described in Figure 2. After 24 hours different amounts of chloramphenicol (0-200 μ M) were added to cells that were about 325K/ml wells. After an additional 48 hours, cells were harvested and counted. Cultures without chloramphenicol reached 1290K cells/ml. Each square represents the mean of triplicate proliferation determinations. Concentration response was generated using a Sigmoidal dose response equation within GraphPad Prism.

chloramphenicol are reduced by more than one half, declining from about 2660K cells/ml to about 1020K cells/ml, in 0.1% EtOH and 50 μ M chloramphenicol, respectively. In this experiment, a decline in cell number can be seen shortly after antibiotic addition. As depicted in the inset to Figure 8, after 24 hours in culture, proliferation decreased significantly ($P < 0.021$) from 226K cells/ml in 0.1% EtOH to 182K cells/ml in 50 μ M chloramphenicol. Despite there being a decline in the number of cells/ml after 6 hours in chloramphenicol, this decline was not significantly different from ethanol treated control

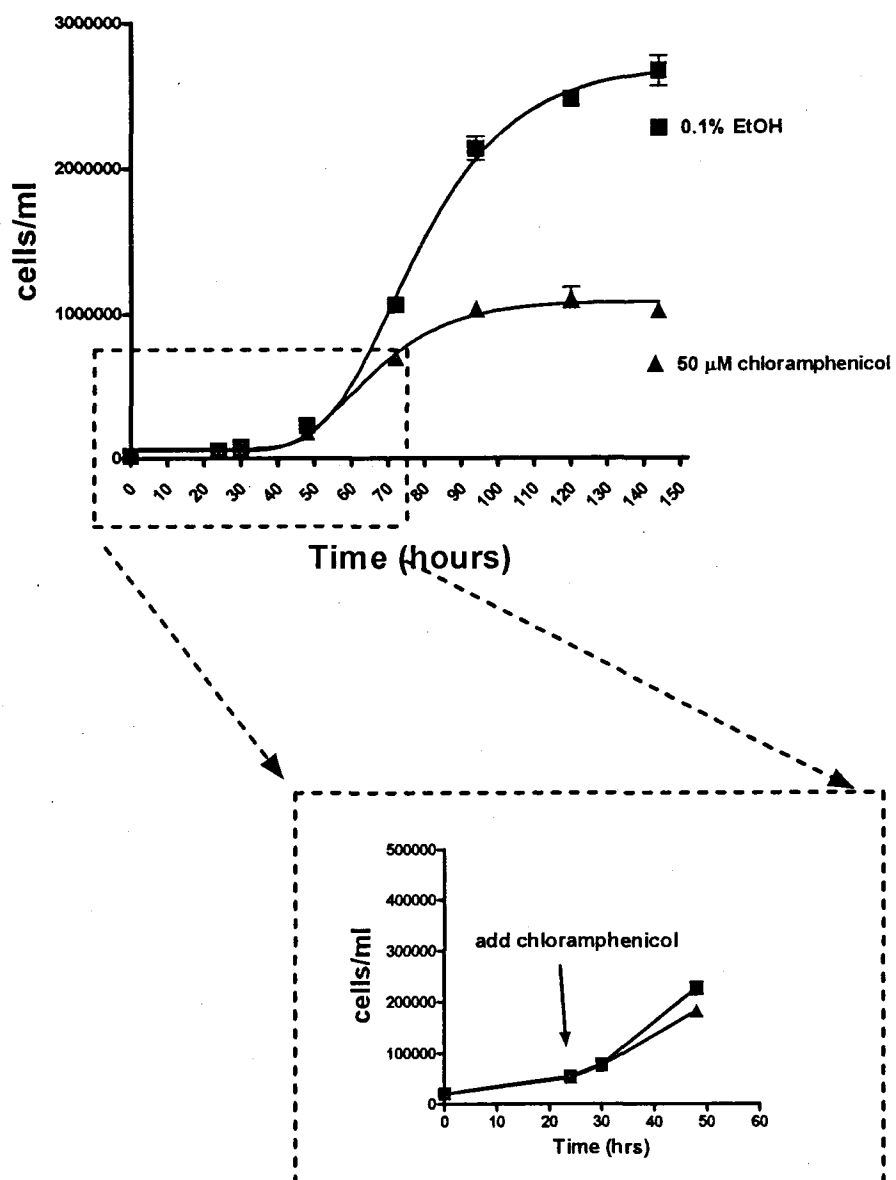


Figure 8. Effect of Chloramphenicol Treatment on BB88 Cell Proliferation Over Time.

BB88 cells cultures were set up and monitored as before, except after 24 hours, cultures received 50 µM chloramphenicol or ethanol (EtOH) (0.1% final concentration) and proliferation was monitored for an additional 5 days (144 hours). Each point represents the mean of triplicate determinations. Inset is an enlargement of the first 60 hours.

cultures ($P < 0.1$). Using linear regression, we examined the effect of the antibiotic treatment on proliferation over the first 48 hours. We calculated growth rates of 11.75 hours and 13 hours per doubling in the cultures containing 0.1% EtOH and 50 μ M chloramphenicol, respectively. We examined cell viability using trypan exclusion and saw no difference in the uptake of this stain in chloramphenicol and untreated cells (data not shown).

These effects of chloramphenicol on BB88 cell proliferation were useful in setting up an experiment to monitor global transcription changes, the primary focus of this work. We treated proliferating BB88 cells with and without 50 μ M chloramphenicol. After 0 and 24 hours treatment, we counted and harvested cells. While cell numbers for these cultures were not monitored beyond the last harvest time, as seen before, there is a significant decrease ($P < 0.005$) in cell number in the 24 hour antibiotic-treated cultures compared to the control cultures (0.1% EtOH), Figure 9A. In order to perform microarray hybridization experiments, we needed to prepare RNA from these treated cells. Although we used less Trizol per cell number than was suggested by the manufacturer, there was no significant difference in the RNA amounts in treated and control cells, Figure 9B.

To remove any contaminating DNA, we treated RNA that was immobilized on Qiagen RNeasy columns with DNase. To check for residual contaminating DNA, we analyzed DNase-treated RNAs using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Although we could not assess the mRNA component of each sample, we determined that the RNA samples were undegraded and intact based upon the

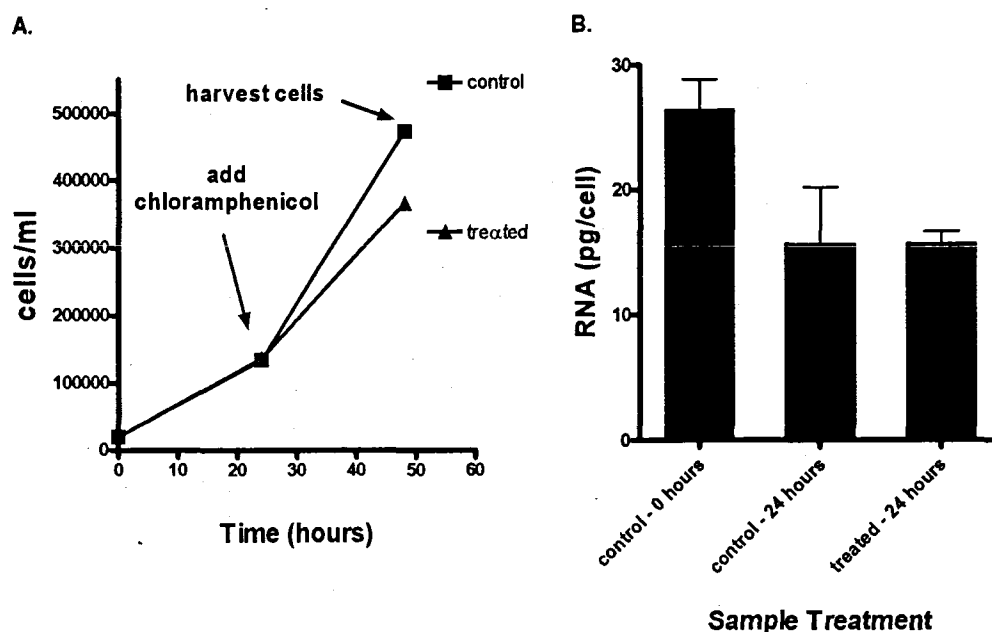


Figure 9. Effect of Chloramphenicol on Growth and RNA in BB88 Cells.

BB88 cells were diluted back to 20K/ml in 800 ml media and dispensed (100 ml) into 8 x 175 cm² culture flasks (Corning # 431080). After 24 hours, four cultures were treated with 50 μ M chloramphenicol (treated) and four cultures were treated with ethanol (0.1% final) (control). Immediately, one control and one treated culture were counted and harvested for RNA. After an additional 24 hours, the remaining cultures were counted and cells harvested for RNA. **A.** Cell counts in the various cultures during the experimental treatment, 8 cultures at 0 hours, 2 cultures at 24 hours (one control, one treated), and 6 cultures at 48 hours (3 control and 3 treated). **B.** RNA amounts (pg/cell) in cells harvested at 24 hours (2 x control 0 hours) or 48 hours (3 x control 24 hours and 3 x treated 24 hours). Bars represent the mean of either duplicate or triplicate determinations. There is no significant difference between the yields of RNA from the treated-24 hours versus the control-0 and 24 hours cells.

visualization of the 28S and 18S ribosomal RNA bands running at about 4000 base pairs (bp), respectively, see Figure 5. Since the RNA standards appeared very light in this particular electropherogram, they were marked by bars. At this point, we used these RNAs to synthesize cDNA and biotinylated cRNAs, see Methods. We prepared and

analyzed cDNA from equal RNA aliquots. These cDNAs were used to make biotinylated cRNA using a commercial *in vitro* transcription kit, see Methods. Figure 10 shows the results obtained from the *in vitro* transcription reactions done on the control and on treated RNA groups. The yields, ranging from 56.2 μg to 110.8 μg (average yield of 75.6 μg), were within the manufacturer's specifications (data not shown). There was no significant difference ($P < 0.8$) between any of average yields of these three groups of RNAs.

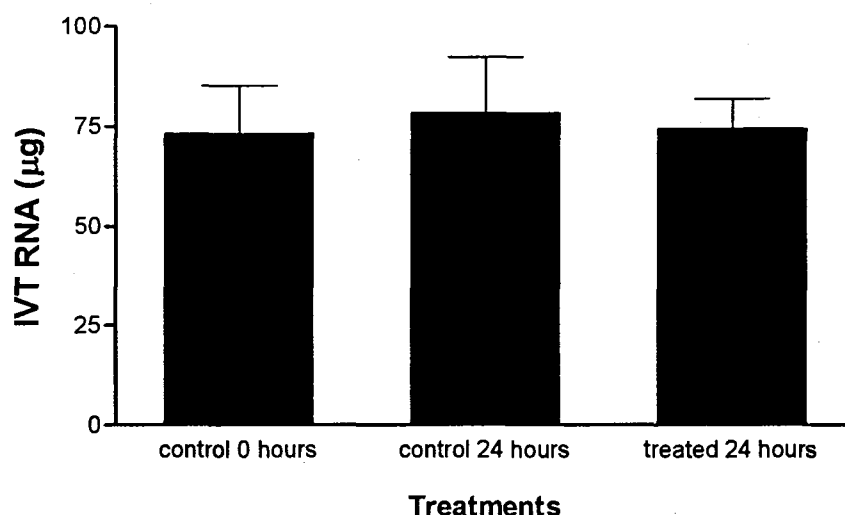


Figure 10. In Vitro Transcription Results – Yield of cRNA.

Double-stranded cDNA was synthesized from 7.5 μg total RNA aliquots. cDNAs were *in vitro* transcribed and then reaction products were passed over Qiagen RNeasy columns. Eluted RNA was quantified spectrophotometrically, see Methods. Bars represent the mean of either duplicate (Control 0 hours) or triplicate (control 24 hours and treated 24 hours) determinations. There is no significant difference between the average yields of these RNA ($P < 0.8$).

To obtain optimized cRNA for hybridization Targets, we fragmented cRNA by heating. RNA samples were compared on agarose gels, Figure 11. First, we compared

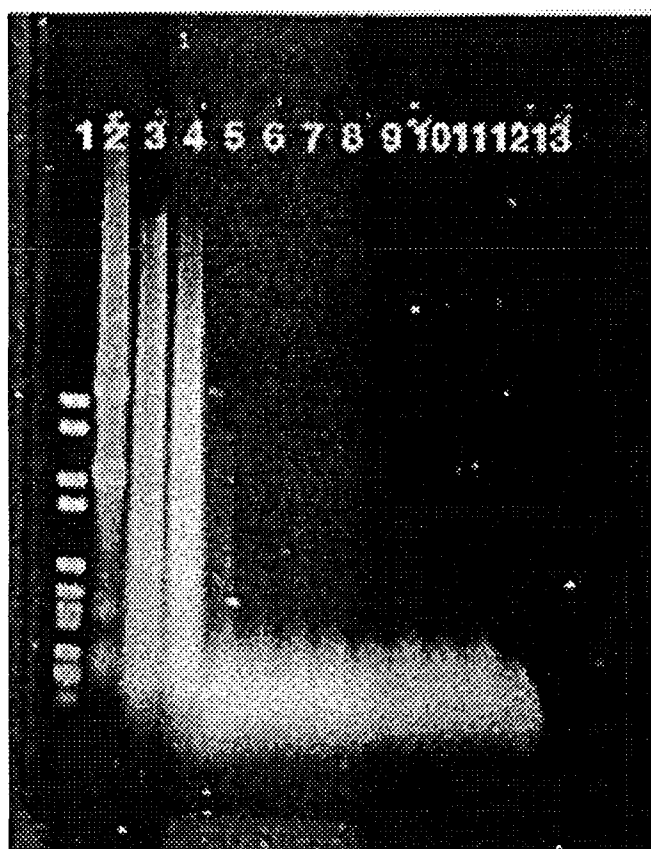


Figure 11. Gel Analysis of Fragmented cRNA Targets.

Fifteen micrograms cRNA aliquots were fragmented in thirty microliters buffer, see Methods. Five microgram aliquots were electrophoresed on a 1% Agarose gel, stained with Sybr Green (Molecular Probes), and photographed under UV light (Sambrook et al., 2001). Lanes: 1, Roche VI DNA molecular weight markers; 2, total RNA control 0 hours; 3, cRNA control 0 hours; 4, cRNA treated 24 hours; 5-6, fragmented cRNA control 0 hours; 7-9, fragmented cRNAs control 24 hours; 10-12, fragmented cRNAs treated 24 hours; 13, 2.5 μ g tRNA control.

total RNA with the unfragmented cRNA. Unfragmented cRNAs ran as smears between about 200 base pairs and on up above the highest DNA marker at 2176 base pairs. Also, these samples had no ribosomal bands, unlike the total RNA sample; compare lanes 3 and 4 with lane 2. All fragmented cRNAs from this experiment, lanes 5-12, migrated

with tRNA, lane 13, or near the lowest DNA marker at 154 base pairs (bp), lane 1. Because these particular cRNAs were of a particular fragment size as described in the Affymetrix technical manual (35-200 bp), we proceeded to hybridize these cRNA Targets to Affymetrix U74Av2 microarrays.

As part of our microarray technology evaluation, we had hybridized several cRNA Targets to Affymetrix Test 2 arrays to assess our cRNA Targets and the hybridization reaction and reagents (data not shown). These arrays contain far fewer Probes Sets than the U74Av2 microarrays that we used here; however, they contain Probe Sets from commonly expressed genes from the human, mouse, rat, and yeast genomes. For each of these genes, Probe Sets derived from the 5', middle, and 3' ends of the gene are represented on the microarray. Also, these arrays contained Probe Sets for genes that should be under-represented, such as murine 18S RNA (Affymetrix Probe Set name, 18SRNAMUR). Finally, these arrays also contain Probe Sets for control transcripts (BIOB, BIOC, BIODN, and CREX), such that the Signal intensity is generally proportional to the amount of the respective cRNA Target spiked into the hybridization mix (see Methods). By obtaining Signal measurements from these various Probe Sets, we can identify cRNA Targets that may be degraded or of poor hybridization quality.

Since the murine U74Av2 arrays contain most of these control Probe Sets, we decided to hybridize our fragmented cRNA Targets to the U74Av2 microarrays directly. After hybridizing, washing, and scanning the eight U74Av2 Arrays, we entered and analysed the various scanned output files within Affymetrix Microarray Suite version 5.0 software. After surveilling the hybridization signals to the various control Probe Sets

described above, we proceeded to perform some basic analyses. The first thing we wanted to know is whether there were differences between the transcription profiles on the control microarrays (RNA samples from two 0 hour [untreated] and three 24 hours [0.1% EtOH] cultures). While we did not expect to find many differences between transcription profiles from these control RNA samples, we felt that in order to alleviate large numbers of misinterpretations of the relative Signal intensities of the chloramphenicol-treated RNA samples, an analysis of control raw data needs to be evaluated. We started by performing six pairwise comparisons on the raw data (Affymetrix Signals) of three 24 hours control cultures (0.1% EtOH treated) versus the two 0 hour control cultures (untreated). Next, we did three pairwise comparisons of each 24 hours control cultures (Signal intensities from one 24 hours control microarray were compared to the Signal intensities of the other two replicate 24 hours control microarrays). The output from all these comparisons was exported into Excel, sorted, combined, and then imported into Spotfire 4.3. Within Spotfire, we were able to visualize and delineate those transcripts that had changed, as defined by the Affymetrix Change Call, see Transcript Analysis Algorithm section in Methods and the Affymetrix Gene Expression Analysis - Data Analysis Fundamentals.

Using GraphPad Prism, Figure 12 gives a visualization of the results of these control group comparisons. Unexpectedly, there was a larger number of transcripts changed within the 24 hour control group comparisons (average = 880 for the three comparisons), versus the 24 hour versus 0 hour control group comparisons (average = 635 for the six comparisons). However, overall number of changes between these two groups of pairwise comparisons (880 versus 635) is not significantly different ($p < 0.19$)

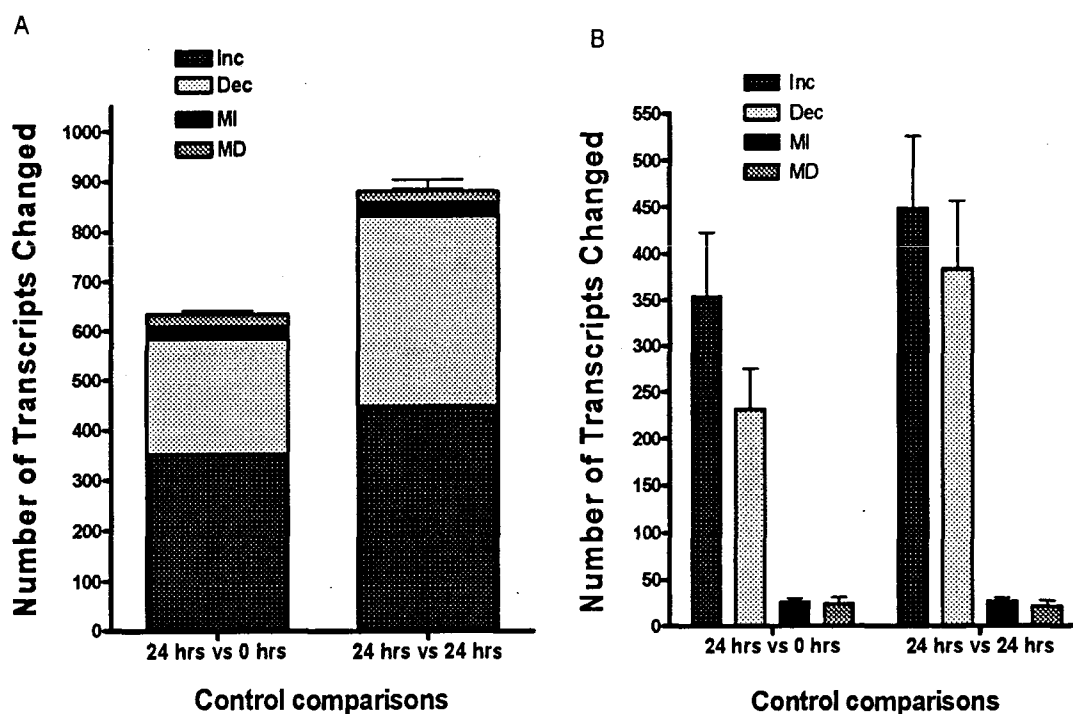


Figure 12. Number of Changed Transcripts in RNA Samples from Control BB88 Cultures.

Comparisons were performed using the Affymetrix hybridization output results from three 24 hour control (RNA from BB88 cultures grown in 0.1% EtOH for 24 hours) and the two 0 hour control samples (RNA from BB88 cultures grown in 0.1% EtOH for 0 hours). Six pairwise comparisons were done between the three 24 hours control and the two 0 hour control results (24 hrs versus (vs) 0 hrs) and three pairwise comparisons were done between the three 24 hour control results (24 hrs vs 24 hrs). **A.** Average total number of transcripts changed as a result of the comparisons (t-test, $P < 0.19$). **B.** Same data as in A, re-plotted to show the average number of transcripts increased (Inc), decreased (Dec), marginally increased (MI), and marginally decreased (MD), as a result of the comparisons. Each bar representing the 24 hrs vs 0 hrs comparisons is the average of six determinations, while each bar representing the 24 hrs vs 24 hrs comparisons is the average of three determinations.

(Figure 12A). In Figure 12B, the same data are plotted differently to visualize better the kinds of changes that have occurred. Here again, when looking at the average number of transcripts that have changed per comparison, while there are more increased (Inc),

decreased (Dec), marginally increased (MI), or marginally decreased (MD) transcripts in the within 24 hour control group, these changes were not significant. Also these graphs show that in all of these comparisons, the number of transcripts that were marginally increased (MI) or marginally decreased (MD), versus the control transcript levels, was less than 10% of the total number of changed transcripts. Taking the union of all the transcripts that changed in each of these pairwise comparisons and then sorting these transcripts within Spotfire, we were able to visualize what particular changes were common to all the comparisons within a group (data not shown, see Figure 14C for representative plot). In examining the three 24 hour control pairwise comparisons, we determined that there were a total of 2641 transcripts (represented by a unique probe set) that were changed out of a total of 37464 paired transcripts (12488 transcripts per microarray times three pairwise comparisons). From these changes, only 95 transcripts out of a total of 12488 transcripts (Probe Sets) on each U74Av2 microarray were changed in all three 24 hour pairwise comparisons. Since these three comparisons were between replicates from within the control group, transcripts that were either increased or decreased in two of the three pairwise comparisons were decreased or increased in the third comparison, respectively. Also, it should be noted that sometimes the micorarray contains multiple transcripts (Probe Sets) that are associated with the same gene. It turns out that three of the 95 changed transcripts were associated with the transferrin receptor and this transcript increased on two of three comparisons. Thus, there were annotations for 93 separate transcripts in BB88 cells that fluctuate randomly as a result of being cultured in 0.1% EtOH for 24 hours in different flasks. Further, about half of these transcripts changed less than two-fold.

In examining more closely the other six pairwise comparisons (three 24 hour 0.1% EtOH treated cultures versus the two 0 hour untreated control cultures), out of a total of 74928 paired transcripts (12488 transcripts per microarray times six pairwise comparisons), there were a total of 3809 changed. Of these transcripts, there were only 48 transcript comparisons that were commonly changed in all six pairwise comparisons. These changes represented only 8 transcripts (48 transcript changes/6 comparisons). Overall these eight transcripts were increased as a result of being cultured in 0.1% EtOH for 24 hours and only two transcripts were increased more than two-fold (data not shown).

To identify the transcripts that were changed in BB88 cells treated with chloramphenicol, we analysed the output from three U74Av2 microarrays hybridized to RNA from 50 μ M chloramphenicol-treated cultures. Similar to the pairwise comparisons described above, we performed two sets of comparisons (Figure 13). First, we compared transcript changes on the microarrays hybridized to RNAs from cells treated only with chloramphenicol. Out of a total of 37484 comparisons an average of 163 transcript changes per comparison (Figure 13A). From this total number of changes, 86 transcripts had changed (increased or decreased) more than two-fold. Further, only 9 transcripts were commonly changed in all three comparisons. These 9 transcripts, representing three unique genes, were all changed less than two-fold (data not shown). When the nine pairwise comparisons between the Affymetrix hybridization results from the three chloramphenicol-treated microarrays (Treated) and the three Control microarrays were tabulated, there was an average of 1310 transcript changes per

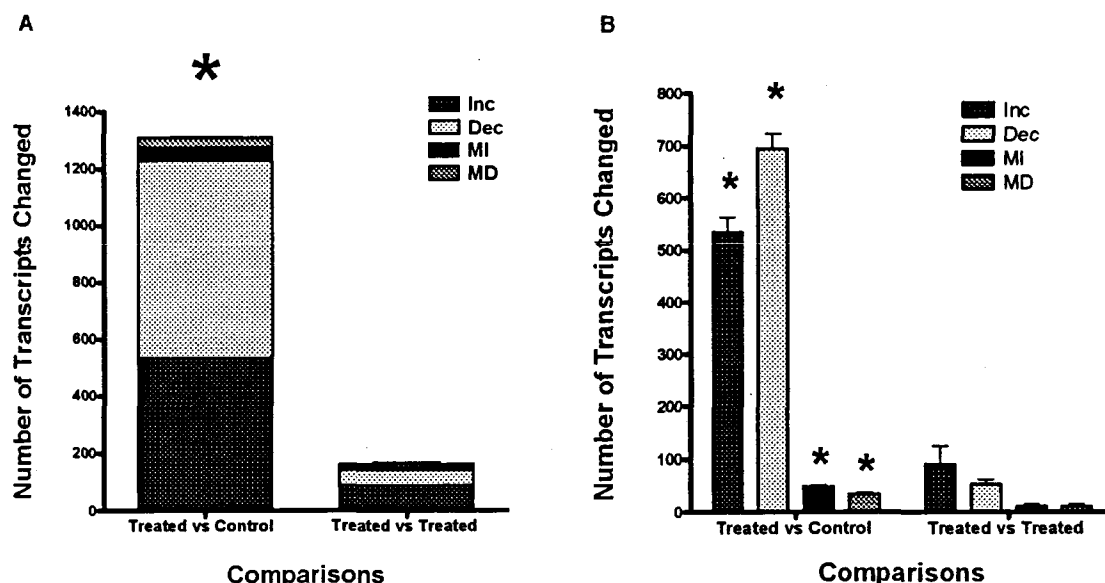


Figure 13. Number of Changed Transcripts in RNA Samples from Treated and Control BB88 Cultures.

Comparisons were performed using the Affymetrix hybridization output results from the three Treated (RNA from BB88 cultures grown for 24 in 50 μ M chloramphenicol) and the three Control samples (RNA from BB88 cultures grown in 0.1% EtOH for 24 hours). Nine pairwise comparisons were done between the three Treated and the three Control samples (Treated vs Control) and three pairwise comparisons were done between the three Treated samples (Treated vs Treated). **A.** Average total number of transcripts changed as a result of the comparisons (equal variance t-test, $P < 0.0001$). **B.** Same data as in A, re-plotted to show the average number of transcripts increased (Inc), decreased (Dec), marginally increased (MI), and marginally decreased (MD), as a result of the comparisons. Each colored bar representing the Treated vs Control 24 hrs comparisons is the average of nine determinations, while each bar representing the Treated vs Treated 24 hrs comparisons is the average of three determinations (equal variance t-test, $P < 0.0001$).

comparison (Figure 13). This number of transcripts was significantly larger (t-test, $P < 0.0001$) than the average computed for the within treatment comparisons (Figure 13A). When examining the kinds of changes that were made as a result of the chloramphenicol, there were significantly more ($P \leq 0.025$ for all comparisons) increased

(Inc), decreased (Dec), marginally increased (MI), and marginally decreased (MD) transcripts in comparisons of chloramphenicol-treated BB88 cells to control cells versus the comparisons of treated cells to themselves (Figure 13B).

Microsoft Excel files containing the increased or decreased transcript data from the pairwise comparisons described above were imported into Spotfire (Figure 14). Spotfire allows the visualization of complex datasets and we used this software to identify common increased or decreased transcripts from replicate samples. The bar graph in Figure 14A enumerates the number of changes in each pairwise comparison. By taking the union of these transcripts (11789) and plotting their fold change (Signal Log Ratio) versus their Affymetrix transcript identifier (Probe Set Name), a complex pattern appears (Figure 14B). While it appears that there may be two groups of transcripts whose transcript levels have increased greatly, we used Spotfire to eliminate transcripts whose expression did not change in all 9 pairwise comparisons. Each vertical line reaching the top of the graph in Figure 14C delineates a transcript that was increased in 9 of 9 Affymetrix comparisons. The fold changes for these remaining transcripts (1791 common to all nine pairwise comparisons) are shown in Figure 14D. From this visualization, one can easily identify three sets of transcripts that have changed dramatically, two that have increased, and one that has decreased. This set of changed transcripts, represents 199 genes, 106 genes whose transcripts have decreased and 93 genes whose transcripts have increased.

In Table 5 and Appendix B, we have reported not only the fold changes for those transcripts that have decreased (Log₂ ratio less than or equal to -1) or increased (Log₂ ratio greater than or equal to 1) two-fold or greater, but also have summarized gene

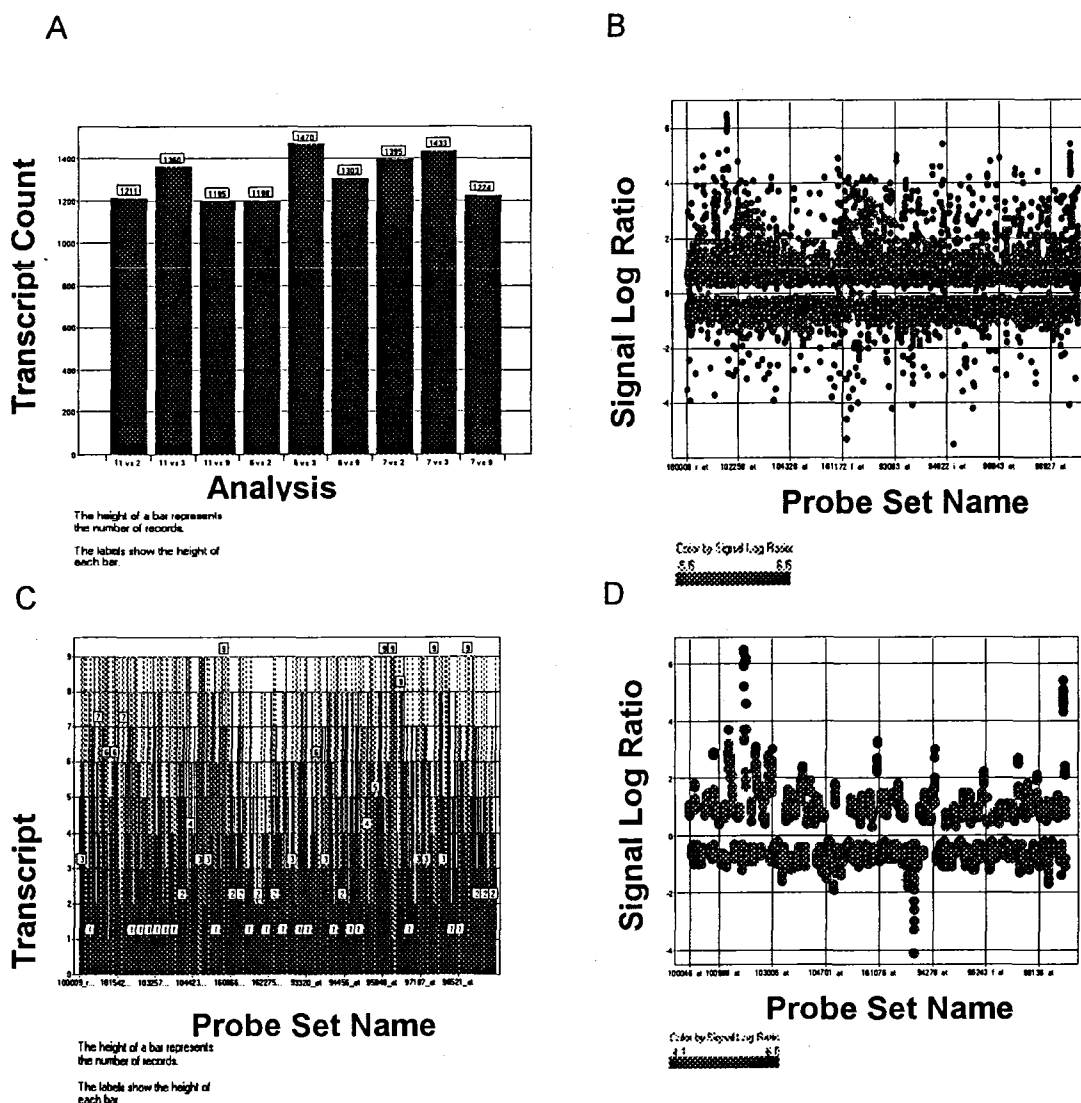


Figure 14. Transcripts Increased and Decreased as a Result of Chloramphenicol Treatment.

Microarrays hybridized to RNAs from three treated (50 μ M chloramphenicol) and three control (0.1% EtOH) BB88 cultures were analysed and compared within Microarray Suite 5.0. Comparison results were combined in Excel and visualized in Spotfire to obtain common transcripts changed, see Method. **A**. Number of transcripts changed in each pairwise comparison. **B**. Union of changed transcripts, fold change (Signal Log Ratio) versus Probe Set Name. **C**. Visualization of the common transcripts changed (199) in all nine pairwise comparisons. **D**. Visualization of fold change of the 199 transcripts.

ontology findings for these transcripts. Of the seven transcripts that decreased, two, Gpd2 and Pgam1, are involved in fatty acid and glucose metabolism, respectively and two of the transcripts code for integral membrane transporters (Table 5A). Slc2a1 is a glucose transporter, while Aqp1 is involved in water transport. Ptger4 is an integral membrane receptor that binds prostaglandins, while Bhlhb2 encodes a nuclear transcription factor. The seventh transcript, A030007L17Rik, remains unannotated. Of the 49 transcripts that increased more than two-fold after chloramphenicol treatment, two Probe Sets, 100306_at and 92268_at, code for the same unannotated transcript, 2700007P21Ri (Table 5B). There were additional redundancies with several other Probe Sets. Probe Sets, 103922_f_at and 103921_i_at, code for Cyb5r1, a nuclear-encoded mitochondrial protein involved in electron transport, Probe Sets, 100592_at and 160530_at, encode a growth hormone inducible integral membrane protein, and Probe Sets, 102879_s_at and 101793_at, encode an immunoglobulin receptor. These redundancies not only bring some confirmation to the identification of transcripts that have been affected by chloramphenicol, but also reduce the number of unique transcripts that have increased in BB88 cells treated with chloramphenicol to forty-five. Ten of the forty-nine total increased transcripts (20.4%) either encoded transcription factors or were involved in gene expression or cell cycle control (marked yellow in Table 5B). Seven transcripts (14.3%) were associated with amino acid or protein metabolism (marked red in Table 5B). As of this writing Affymetrix NetAffx Center annotated one Probe Set from this group, 161221_f_at, as asparagine synthetase (Table 5B). From our results and the Affymetrix nomenclature, this Probe Set's identity is suspicious. Affymetrix has another Probe Set, 95133_at, identified as asparagine synthetase and

Table 5. Decreased and Increased (≥ 2 -fold) Transcripts in Chloramphenicol-Treated BB88 Cells
(Transcripts have been color coded by biological process and molecular function based upon Affymetrix NetAffx gene ontology (GO) analysis.)

A. Decreased Transcripts

# Decreased	Probe Set Name	Gene Symbol	average signal log2 ratio	Change p-value	Gene Title	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description	Pathway
1	93738_at	Slc2a1	-2.4	1.00E+00	facilitated glucose transporter	glucose transport	glucose transporter activity	integral to membrane	---
2	160091_at	Pgam1	-1.3	1.00E+00	phosphoglycerate mutase 1	glycolysis	bisphosphoglycerate phosphatase activity	cytosol	Glycolysis_and_Gluconeogenesis
3	98984_f_at	Gpd2	-1.1	1.00E+00	glycerol phosphate dehydrogenase 2	glycerol-3-phosphate metabolism	glycerol-3-phosphate dehydrogenase activity	mitochondrial inner membrane	Fatty_Acid_Degradation
4	93330_at	Aqp1	-1.5	1.00E+00	aquaporin 1	water transport/excretion	water transporter activity	integral to membrane	---
5	103362_at	Ptger4	-1.2	1.00E+00	prostaglandin E receptor 4	G-protein coupled receptor protein signaling pathway	G-protein coupled receptor activity	integral to membrane	Prostaglandin_synthesis_regulation
6	104701_at	Bhlhb2	-1.0	1.00E+00	basic helix-loop-helix domain	regulation of transcription	transcription regulator activity	nucleus	---
7	94995_at	A030007L17Rik	-1.0	1.00E+00	RIKEN cDNA	---	---	---	---

Table 5—Continued

B. Increased Transcripts

#	Probe Set Name	Gene Symbol	average signal log2 ratio	Change p-value	Gene Title	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description	Pathway
1	161067_at	Trib3	2.7	1.00E-06	tribbles homolog 3 (Drosophila)	regulation of MAPK activity	transcription corepressor activity - kinase activity	nucleus	---
2	101429_at	Ddit3	2.4	0.00E+00	DNA-damage inducible transcript 3	regulation of transcription, DNA-dependent	transcription corepressor activity	nucleus	---
3	103006_at	Atf5	2.1	2.10E-05	activating transcription factor 5	regulation of progression through cell cycle -regulation of transcription, DNA-dependent	sequence-specific DNA binding	transcription factor complex	Smooth muscle contraction
4	102292_at	Gadd45a	2.1	1.00E-06	GADD 45 alpha	regulation of cyclin-dependent protein kinase activity	protein binding	nucleus	Cell Cycle
5	92925_at	Cebpb	1.5	0.00E+00	C/EBP beta	positive regulation of transcription	sequence-specific DNA binding	nucleus - cytoplasm	Ovarian Infertility Genes
6	98122_at	Lmo4	1.5	7.40E-05	LIM domain only 4	transcription from RNA polymerase II promoter	transcription factor binding	transcription factor complex	---
7	100599_at	Atf4	1.2	1.00E-06	activating transcription factor 4	gluconeogenesis - positive regulation of transcription from RNA polymerase II promoter	transcriptional activator activity	nucleus	Smooth muscle contraction

Table 5—Continued

#	Probe Set Name	Gene Symbol	average signal log2 ratio	Change p-value	Gene Title	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description	Pathway
8	94135_at	Tcfcp2	1.2	4.70E-05	transcription factor CP2	regulation of transcription from RNA polymerase II promoter	transcriptional activator activity	transcription factor complex	---
9	160535_at	Nfe2l1	1.1	0.00E+00	erythroid derived nuclear factor 2	transcription from RNA polymerase II promoter - heme biosynthesis	transcription factor activity	extracellular space /// nucleus	---
10	95502_at	Sirt2	1.0	1.00E-06	sirtuin 2	negative regulation of transcription	deacetylase activity	chromatin silencing complex	---
11	104144_at	Gtpbp2	1.7	2.00E-06	GTP binding protein 2			---	---
12	92589_at	Psph	1.3	0.00E+00	phosphoserine phosphatase			---	Glycine, serine and threonine metabolism
13	97960_at	Usp22	1.2	0.00E+00	ubiquitin specific peptidase 22			---	---
14	161221_f_at	Asns	1.2	1.33E-04	asparagine synthetase			soluble fraction	Nitrogen metabolism
15	98042_at	Scpep1	1.1	1.43E-04	serine carboxypeptidase 1			---	---

Table 5—Continued

#	Probe Set Name	Gene Symbol	average signal log2 ratio	Change p-value	Gene Title	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description	Pathway
16	160328_at	Lonp1	1.0	3.00E-06	lon peptidase 1			mitochondrion	---
17	97268_i_at	Krtcap2	1.0	1.00E-06	keratinocyte associated protein 2			integral to membrane	---
18	101441_i_at	Itpr2	1.4	1.30E-05	inositol 1,4,5-triphosphate receptor	calcium ion transport	inositol 1,4,5-triphosphate-sensitive calcium-release channel activity	integral to membrane	Smooth muscle contraction
19	96160_at	Slc6a9	1.3	4.67E-04	solute carrier family 6	amino acid transport	sodium:amino acid symporter activity	integral to membrane	---
20	100943_at	Slc1a4	1.1	2.90E-04	solute carrier family 1	neutral amino acid transport	neutral amino acid transporter activity	integral to membrane	---
21	103818_at	Slc7a7	1.1	5.90E-05	solute carrier family 7	amino acid transport	amino acid-polyamine transporter activity	integral to membrane	---
22	104328_at	Aqp9	1.0	3.40E-05	aquaporin 9	water transport	water channel activity	integral to membrane	---
23	101793_at	Fcgr1	5.2	3.10E-05	high affinity Fc receptor, IgG			integral to membrane	Inflammatory Response Pathway

Table 5—Continued

#	Probe Set Name	Gene Symbol	average signal log2 ratio	Change p-value	Gene Title	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description	Pathway
24	101845_s_at	Csprs /// Sp100-rs	4.1	2.38E-03	component of Sp100-rs			nucleus - integral to membrane	---
25	101209_at	Fcer1a	3.0	3.11E-04	high affinity Fc receptor, IgE			integral to plasma membrane	---
26	102879_s_at	Fcgr1	2.4	0.00E+00	high affinity Fc receptor, IgG			integral to membrane	Inflammatory Response Pathway
27	102337_s_at	Fcgr2b	1.1	2.30E-05	low affinity Fc receptor, IgG			integral to membrane	---
28	99330_at	Csf2ra	1.0	1.88E-03	CSF2 receptor			integral to membrane	---
29	99667_at	Cox6a2	4.8	0.00E+00	cytochrome c oxidase, subunit VIa-H			mitochondrion	Electron Transport Chain
30	103922_f_at	Cyb5r1	2.0	1.00E-06	cytochrome b5 reductase 1			---	---
31	97398_at	Pck2	1.9	0.00E+00	phosphoenolpyruvate carboxykinase 2			mitochondrion	Pyruvate metabolism

Table 5—Continued

#	Probe Set Name	Gene Symbol	average signal log2 ratio	Change p-value	Gene Title	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description	Pathway
32	103921_i_at	Cyb5r1	1.5	3.10E-05	cytochrome b5 reductase 1			---	---
33	97870_s_at	Ero1l	1.0	5.00E-06	ERO1-like (S. cerevisiae)			endoplasmic reticulum	---
34	160108_at	Nupr1	1.5	1.77E-04	nuclear protein 1	induction of transcription, cell growth		nucleus	---
35	103520_at	Vegfa	1.2	1.54E-04	VEGF A	positive regulation of angiogenesis, cell growth	growth factor receptor binding	extracellular matrix	---
36	95637_at	Flnb	1.2	1.00E-06	beta filamin	cell adhesion, cell growth	protein binding	cytoplasm - cytoskeleton	---
37	160463_at	Myd116	1.0	1.30E-03	myeloid differentiation primary response gene 116	cell growth, cell differentiation		---	---
38	94359_at	Plekha1	1.9	4.00E-06	pleckstrin homology domain containing			membrane	---
39	100306_at	2700007 P21Rik	1.3	6.30E-05	RIKEN cDNA 2700007P21 gene			---	---
40	100592_at	Ghitm	1.3	0.00E+00	growth hormone inducible transmembrane protein			integral to membrane	---

Table 5—Continued

#	Probe Set Name	Gene Symbol	average Signal Log2 ratio	Change p-value	Gene Title	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description	Pathway
41	100920_at	---	1.3	1.00E+00	---			---	---
42	92268_at	2700007 P21Rik	1.2	1.00E-06	RIKEN cDNA 2700007P21 gene			---	---
43	160530_at	Ghitm	1.1	3.70E-05	growth hormone inducible transmembrane protein			integral to membrane	---
44	102249_at	Avil	2.6	1.00E-06	advillin	actin filament organization/ barbed-end actin filament capping	actin binding - calcium ion binding - protein binding	actin cytoskeleton	---
45	94278_at	Lcp1	1.4	3.00E-06	lymphocyte cytosolic protein 1	response to wounding /// actin filament bundle formation	actin filament binding	cytosol - actin filament	---
46	96199_at	Mtm1	1.7	1.00E-06	X-linked myotubular myopathy gene 1	phospholipid dephosphorylation	inositol phosphatase activity - vesicular trafficking?	cytoplasm - membranes	---
47	102313_at	Gch1	1.6	1.00E-06	GTP cyclohydrolase 1	nitric oxide biosynthesis	GTP cyclohydrolase I activity	cytoplasm	Folate biosynthesis
48	102770_at	Gypa	1.6	3.00E-06	glycophorin A	regulation of response to osmotic stress	protein binding - protein homodimerization activity	integral to membrane	---
49	160602_at	Pde6d	1.2	1.20E-05	phosphodiesterase 6D, cGMP-specific, rod, delta	response to stimulus	3',5'-cyclic-nucleotide phosphodiesterase activity	---	---

we have shown that its transcripts were increased slightly by 24 hours ethanol treatment. However, transcripts for Probe Set 95133_at were unaffected by chloramphenicol treatment. This inconsistency suggests that gene identification and annotation are ongoing processes. In time, perhaps this discrepancy and the true identity of Probe Set 161221_f_at will be known. Five transcripts were transporters (10.2%), with three of the five transporters being involved in amino acid transport (marked green in Table 5B). Six increased transcripts (12.2%) appear to be receptors, with half of these receptors having immunoglobulins as their ligand (marked blue in Table 5B). Five transcripts (10.2%) were involved in either electron energetics or glucose metabolism (marked orange in Table 5B). There were four transcripts (8.2%) having something to do with cell proliferation, growth, development, and/or the cell cycle (marked light gray in Table 5B). Six transcripts (12.2%) were not readily grouped with any biological process or molecular function, although two of these transcripts appear to code for membraneous proteins (marked dark gray in Table 5B). Two increased transcripts (4.1%), Avil and Lcp1, coded for actin binding proteins (unmarked in Table 5B), while the four remaining transcripts (8.2%), Mtm1, Gch1, Gypa, and Pde6d, are more unique in their ontology, coding for a phosphatase, hydrolase, integral external membrane protein, and a nucleotide phosphodiesterase, respectively (marked tan in Table 5B).

The transcript that decreased most in this experiment was identified by Probe Set 93738-at (Table 5A and Appendix B). This Probe Set matched the gene for the facilitated glucose transporter. Similarly, two transcripts with some of the larger fold increases, 5.2 and 3.0 log₂ ratios, were Probe Sets, 101793_at and 101209_at, respectively (Table 5B and Appendix B). From the NetAffx Analysis Center, these

transcripts code for Fc receptor of immunoglobulins IgG and IgE, respectively. In chloramphenicol-treated BB88 cells, we saw large increases (4.8 signal log₂ ratio) in the transcript for Affymetrix Probe Set 99667_at (Table 5A and Appendix B). We decided to study further this phenomenon not only because of these dramatic increases in 99667_at mRNA, but also because Probe Set 99667_at codes for COX VIa-H, a subunit of mitochondrial cytochrome c oxidase, (Bread 1969; Wilkie, 1977) (Figure 15). Using RT-PCR, we decided to confirm the finding that chloramphenicol increases COX VIa-H RNA in RNA samples from chloramphenicol-treated BB88 cells. First, COX VIa-H oligonucleotide primers and probes were designed, see Methods and Table 4. These

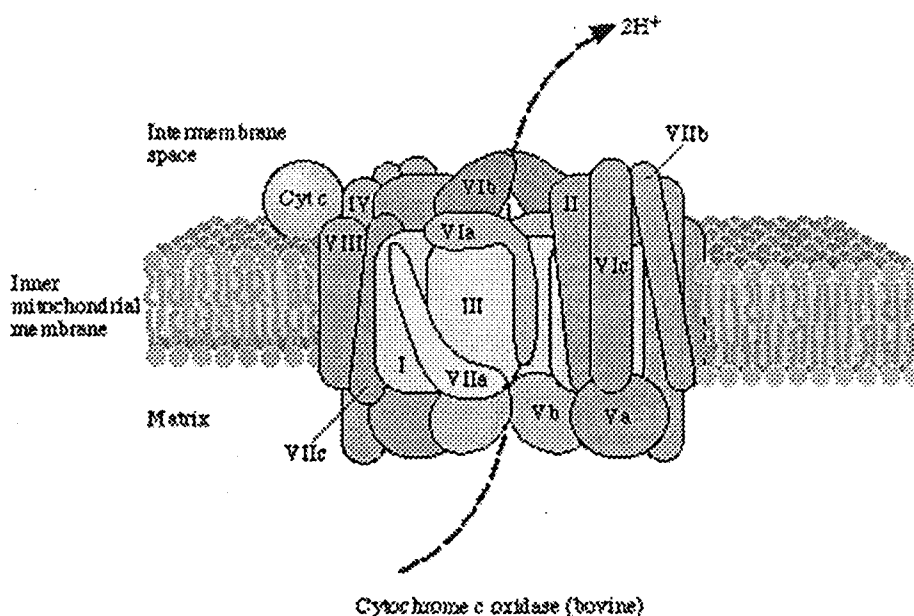


Figure 15. Model of Bovine Cytochrome C Oxidase.

Schematic diagram of cytochrome c oxidase (COX) based upon crystal structure of bovine COX (Tsukihara et al., 1996). This diagram shows the COX VIa-H subunit spanning the mitochondrial membrane and interacting with the COX I and COX III subunits. The diagram was downloaded from the Kyoto Encyclopedia of Genes and Genomes website (<http://www.genome.jp/kegg/pathway/map/map00190.html>).

oligonucleotides were used in matched RT-PCR reactions, along side of GAPDH primers and probe, see Methods. Despite a success in measuring GAPDH transcript levels in these RNA samples, initial reactions using COX VIa-H primers did not work properly (data not shown). Upon closer examination of the COX VIa-H genomic sequence (Genbank accession number, U34801), it became apparent that the COX VIa-H primers spanned an intron and, thus, they did not permit detectable amplification of genomic DNA in Taqman® PCR reactions. Since these reactions were used as standards to quantify mRNA levels of our unknown samples, an alternative approach was devised. Genomic DNA was replaced in the standard curve reactions with reaction dilutions from several pooled RT reactions. Using this strategy, it was determined that COX VIa-H transcripts increase greater than 20-fold after 24 hours in chloramphenicol-treated cells and this increase was significantly higher ($P < 0.00001$) than in EtOH-treated controls (Figure 16). Thus, the RT-PCR results confirm the microarray finding that chloramphenicol affects transcripts of this particular gene.

To extend these results, several additional experiments were performed. We added chloramphenicol to BB88 cultures to examine effects of chloramphenicol treatment times on relative COX VIa-H transcript levels. Figure 17 plots the fold increases from 3 separate time-courses. These results show that there is little change in COX VIa-H transcript levels until about 12 hours (one cell doubling). Thereafter, COX VIa-H transcript levels increase in chloramphenicol-treated cells relative to COX VIa-H transcript amounts in matched untreated control cells, reaching half-maximum increase in about 19 hours. Between 24 hours and our last time point at 50 hours, increases in COX

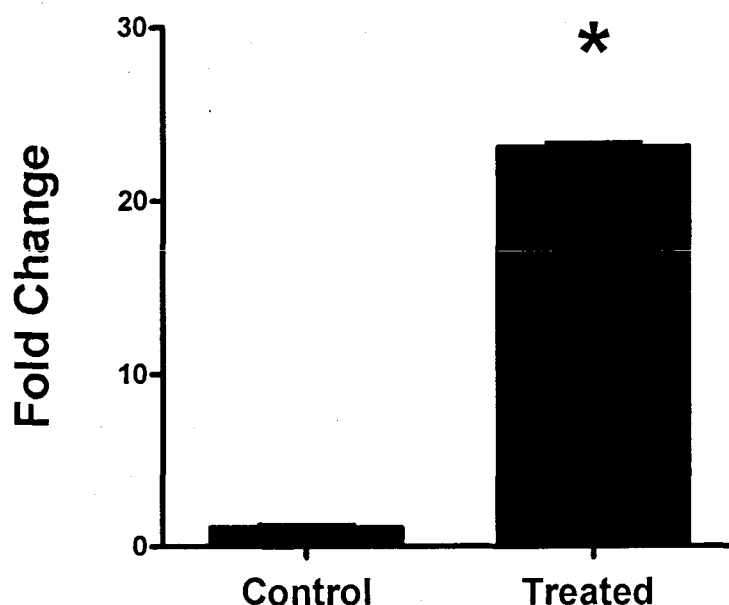


Figure 16. Treatment of BB88 Cells with Chloramphenicol Causes Increases in COX VIa-H Transcripts.

RT-PCR was performed on two Control (0.1% EtOH) and two Treated (50 μ M chloramphenicol) RNAs (RNAs were the same as in Figure 1) to determine the COX VIa-H transcript amounts in these RNAs, see Methods. Each RT-PCR reaction was done in triplicate. Plots show the fold increase of COX VIa-H transcripts in Treated (chloramphenicol) versus Control (EtOH) RNA samples normalized to GAPDH, see Methods. Control value represents the mean of six pairwise determinations, while the Treated value represents the mean from twelve determinations. COX VIa-H from treated cells is increased >20-fold over COX VIa-H RNA in control cells (*, $P < 0.00001$ equal variance t-test).

VIa-H transcripts appear to have slowed, with increases measuring approximately 20- and 30-fold, respectively (Figure 17). In a concentration-response experiment, we measured changes in COX VIa-H transcript levels in BB88 cells treated with various amount of chloramphenicol. Figure 18 shows that after 24 hours treatment, chloramphenicol causes a concentration-dependent increase in COX VIa-H transcripts. The data was analyzed using a four-parameter model of non-linear regression. The data

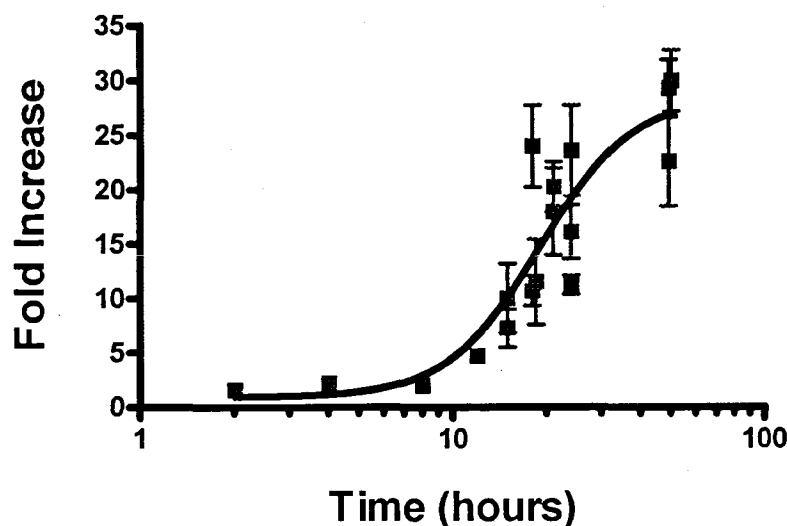


Figure 17. Effects of Chloramphenicol on COX VIa-H RNA in BB88 Cells Over Time.

Proliferating BB88 cells were treated with 50 μ M chloramphenicol and RNA was isolated from cells after various treatment times. Fold increases in COX VIa-H RNA levels were determined using RT-PCR reactions from three separate experiments, see Figure 12 and Methods. Squares represent the mean values of a minimum of duplicate determinations from RT-PCR reactions done in triplicate. Data were analyzed in GraphPad Prism and the curve fitted using non-linear regression-sigmoidal dose response. (EC_{50} = 19 hours and R^2 = 0.65).

fit the model quite well [R^2 = 0.95] with a half maximal increase in COX VIa-H transcript (EC_{50}) occurring at 33 μ M chloramphenicol.

Next, we measured the effect of other antibiotics on BB88 cell proliferation and COX VIa-H transcript levels. Figure 19A compares the effect of chloramphenicol, erythromycin, Geneticin, and tetracycline on BB88 cell growth to their respective diluents. At the concentrations selected, all the antibiotics inhibited BB88 cell proliferation after 24 hours. RNA was isolated from each of these single cultures and while yields of total RNA from the cultures were comparable (data not shown), levels of

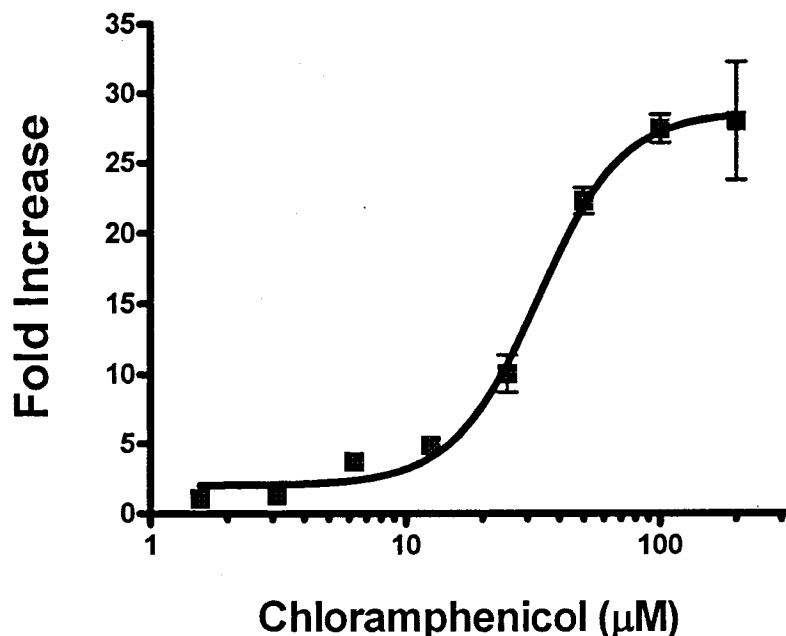


Figure 18. Fold Increases in COX VIa-H Transcripts in BB88 Cells Treated for 24 Hours with Various Amounts of Chloramphenicol.

BB88 cells were seeded at ~20K/ml. After 16 hours, cultures were counted and treated with various amounts of chloramphenicol (0-200 μ M). 24 hours later, RNA was prepared for Taqman RT-PCR studies, see Figure 3 and Methods. Squares represent the mean fold increase in COX VIa-H transcripts in RNA from three treated versus three untreated (0.1% EtOH) control BB88 cultures. Data were analyzed in Graph Pad Prism using a 4-parameter model of non-linear regression-sigmoidal dose response, EC_{50} = 33 μ M and R^2 = 0.95.

COX VIa-H transcript in each culture varied (Figure 19B). The amounts of COX VIa-H transcripts were significantly ($P < 0.007$) higher in all antibiotic-treated BB88 cells relative to the transcript levels in diluent-treated control cells. To determine whether this effect was specific for COX VIa-H transcripts, effects on two related nuclear transcripts were measured (Figure 20). Using nucleotide sequences with accession numbers M37831 and X53157, primers and probes were designed for measuring transcripts to COX IV and

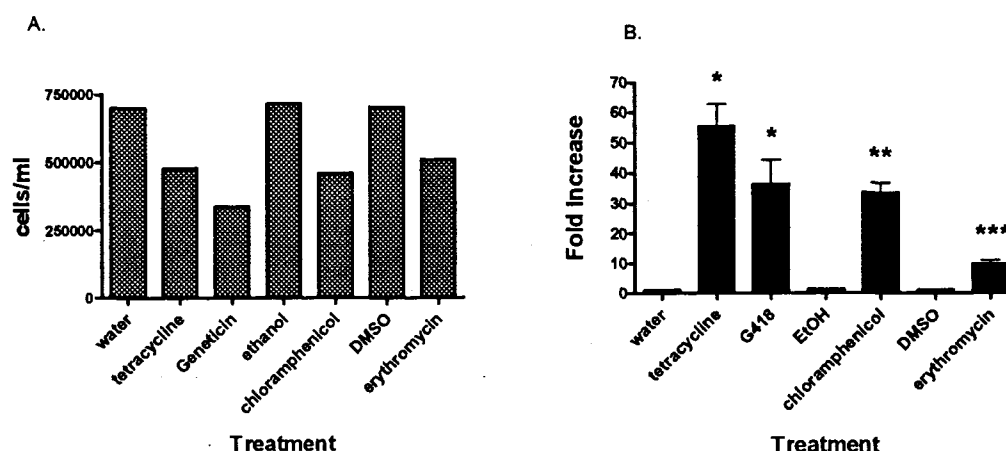


Figure 19. Effect of Antibiotics on BB88 Cell Proliferation and COX VIa-H Transcript Levels.

Cells were seeded as in Figure 18. After 24 hours, various antibiotics were added. **A.** After an additional 24 hours in culture, cells were counted and harvested. Bars represent the cell counts from individual cultures. **B.** RNA was isolated from harvested BB88 cells and then the COX VIa-H transcript levels were determined in each sample, see Figure 13 and Methods. Bars represent the mean fold increase of treated versus control from six RT-PCR determinations. Using a two-sample, unequal variance t-Test ; *, $P < 0.007$ for antibiotic versus water control, **, $P < 0.0001$ for antibiotic versus ethanol control, and ***, $P < 0.002$ for antibiotic versus DMSO control.

COX Vb subunits, respectively, see Methods and Table 4. Figure 20A and 20B show the fold increases in COX IV and COX Vb in transcripts the various RNA samples. While our RT-PCR assay easily detected COX IV and COX Vb transcripts, most of the RNAs for these COX subunits remained unchanged. The only change was a decrease in COX IV transcripts in RNA from chloramphenicol-treated BB88 cells ($P < 0.035$).

In an attempt to understand better the mechanism by which compounds may be affecting BB88 proliferation, we decided to experiment with several additional compounds (Figure 21). First, we examined the effects of compounds PNU-86983 and

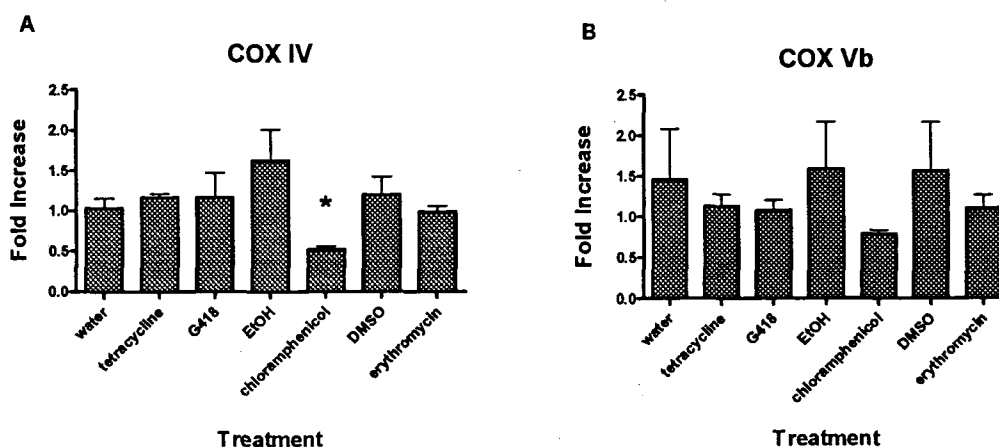


Figure 20. Effect of Antibiotics on Transcript Levels of COX IV and COX Vb Subunits.

Fold increases in COX IV transcript (A) and COX Vb transcript (B) were determined from the same RNAs as prepared in Figure 19. Bars represent the mean of six RT-PCR determinations. There is no difference between any of the antibiotic treatments on these RNAs, except for the RNA levels for COX IV in chloramphenicol-treated BB88 cells versus the ethanol (EtOH) control (*, $P < 0.035$).

PNU-78714. These two compounds have been shown to inhibit smooth muscle growth through an undefined mechanism (Bonin, Singh, Gammill, & Erickson, 1993; Erickson et al., 1994). We wanted to see whether the chloramphenicol effects in BB88 cells were somewhat selective or if these two compounds inhibited BB88 growth and increased COX VIa-H mRNA levels also. In the 24 hour-treated cultures done here, both 25 μ M PNU-86983 and PNU-78714 inhibited BB88 cell growth by 45% and 25%, respectively (Figure 21A). These effects on proliferation were accompanied by increases in COX VIa-H transcripts of about 5-fold (PNU-86983) and 8-fold (PNU-78714) over the transcript levels in DMSO-treated controls (Figure 21B). In the same experiment, we

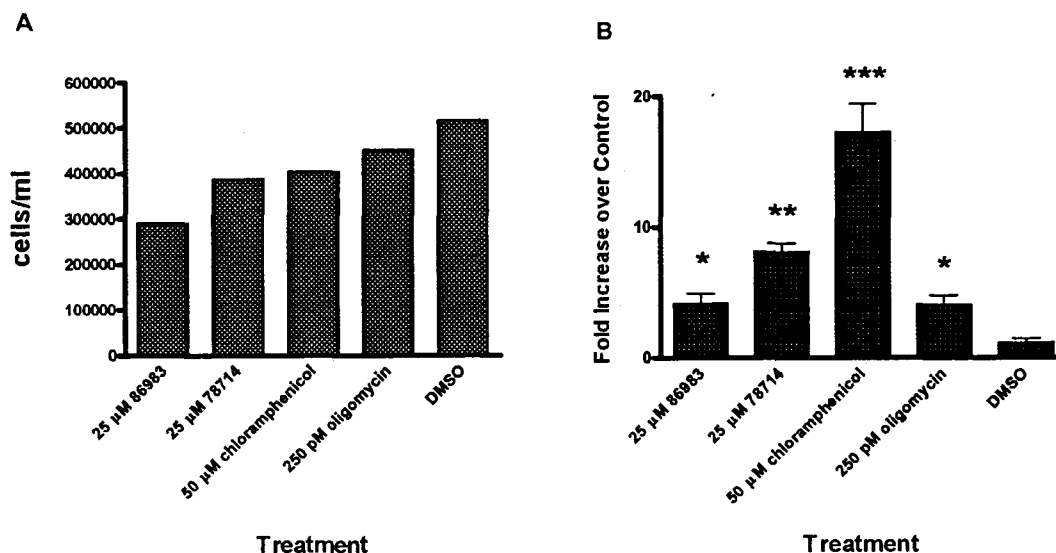


Figure 21. Effect of Compounds on BB88 Cell Growth and COX VIa-H Transcript Levels.

BB88 cells were seeded as in Figure 18 and various compounds were added after 24 hours. After an additional 24 hours, cells were counted and harvested (A). RNA was prepared from cells and fold increases of COX VIa-H transcript levels were determined as described in Figure 12 and Methods. Bars represent the mean of at least triplicate RT-PCR determinations from total RNA prepared from single cultures (B). (*, $P < 0.04$, **, $P < 0.0008$, ***, $P < 0.002$).

treated BB88 cells with oligomycin, a potent uncoupler of oxidative phosphorylation (Nagamune et al., 1994). In twenty-four hours, 250 pM oligomycin inhibited BB88 cell proliferation and enhanced COX VIa-H transcript levels, 13% and 4-fold, respectively (Figure 21A and B). In our last experiment, we observed the effects of succinylacetone on BB88 growth and COX VIa-H transcript levels (Figure 22). Succinylacetone is an inhibitor of delta aminolevulinate dehydratase (ALAD) (Woodard & Dailey, 2000). This enzyme plays a role in the synthesis of heme, an important component of hemoglobin. We wanted to know if this disruption in heme biosynthesis affected proliferating BB88

cells similar to chloramphenicol. While 50 μ M chloramphenicol inhibited BB88 cell proliferation and increased COX VIa-H transcript levels, 1 mM succinylacetone had no effect on growth or COX VIa-H transcripts (Figure 22A and B).

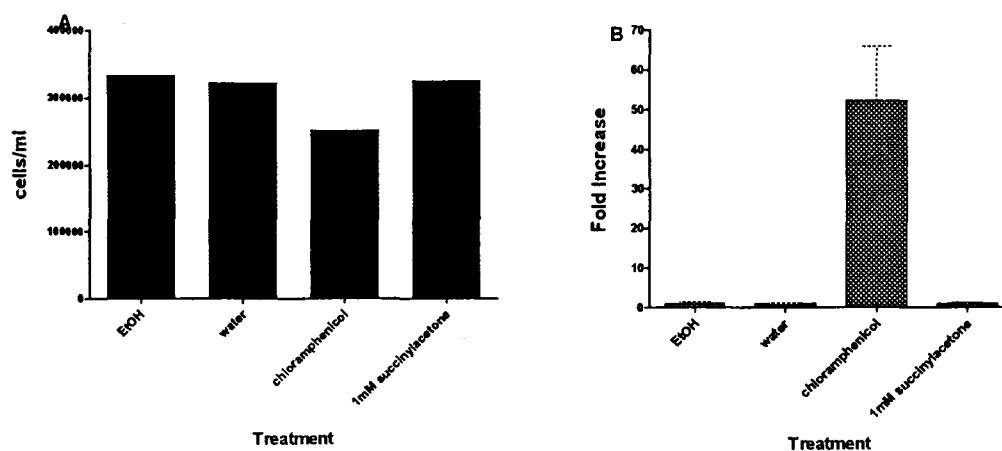


Figure 22. Effect of Succinylacetone on BB88 Cell Growth and COX VIa-H Transcript Levels.

A. Effect of Succinylacetone on BB88 proliferation. Cells were seeded as in Figure 14 and succinylacetone was added after 24 hours. Cells were counted and harvested after an additional 24 hours. Bars represent the cell counts from individual cultures. **B.** Effect of compounds on COX VIa-H transcript levels in proliferating BB88 cells. Bars represent the mean fold increase in COX VIa-H RNA determined by triplicate RT-PCR determinations using RNA isolated from cultures described in A.

DISCUSSION

As a consequence of chloramphenicol-induced myelosuppression, there may be gene expression changes that accompany and perhaps cause inhibition of erythroid cell proliferation. In order to see if we could identify antibiotic-induced changes in gene expression in erythroid cells, we decided to study the effects of chloramphenicol on cultured BB88 murine erythroleukemia cells. These murine erythroleukemia cells are easy to culture and under our culture conditions, have a doubling time of 12 hours (Figure 6). After 48 hours of treatment, chloramphenicol inhibits BB88 cell growth with an EC_{50} of 43 μ M (Figure 7), a concentration within this antibiotic's therapeutic serum concentration of 10-15 μ g/ml (~30-50 μ M) (Soldin, Golas, Rajchgot, Prober, & MacLeod, 1983; Wilson & Cockerill, 1987). Previous studies have examined changes occurring to cultured mammalian cells after longer chloramphenicol treatment (Holt, Andrews, Payne, Williams, & Turton, 1997; Kong, Holt, Ma, Lie, & Chan, 1999; Leiter et al., 1999; Kang 2005). In these studies, long-term (days) chloramphenicol treatment was causing apoptosis in erythroid cells. In this work, we focused on the early anti-proliferative effects of chloramphenicol where we observed proliferation changes within 24 hours of treatment (Figure 8). It should be added that chloramphenicol inhibits reversibly erythroleukemia cell proliferation (Kang et al., 2005). We treated BB88 cells with chloramphenicol for 24 hours and then removed the antibiotic by washing the cells. Treated , then washed BB88 cells returned to proliferating similar to untreated cells (data not shown). This reversible effect is what has been seen clinically for this antibiotic

and other antimicrobials (Kuter & Tillotson, 2001). Nagiec et al. (2005) determined that the oxazolidinones reversibly inhibit K562 human erythroleukemia cell proliferation *in vitro*. In addition, they showed that cells without mitochondria and treated with oxazolidinones proliferate, suggesting a link between proliferation and mitochondrial function. These results suggest that the BB88 cells are sensitive in a way that make them an appropriate model for examining the relationship between chloramphenicol's effects on proliferation and gene expression.

Microarray technology is a powerful tool in that global transcription changes can be studied in one experiment. This technology is being used to identify the effects of a wide variety of xenobiotics and has opened a whole new field of study named toxicogenomics, see Introduction (Rouse & Hardiman, 2003, Borlak, 2005). We attempted to use this technology to identify transcripts in BB88 cells that are sensitive to chloramphenicol. We first needed to validate Affymetrix microarray methods and procedures. There are two parts to this technology. There are experimental procedures involved in preparing cRNA Targets from experimental and control cells, hybridizing these Targets to Probe Sets on commercial murine microarrays, and then detecting each Target/Probe Set signal by scanning the microarray. Next, there are the analytical and bioinformatic procedures that are used to establish the significance of each data point, record, or hybridization signal. Both parts of the experimental exercise are labor intensive and developing. This technology and area of research is not without controversy. There have been recent reports with mixed results concerning the reproducibility and standardization of microarray experiments (Bammler, 2005; Waring et al., 2005). Nevertheless, the attractiveness of being able to examine large numbers of

transcriptional changes in a single experiment prompted us to use and validate this technology. We followed the Affymetrix protocols as closely as possible, see Methods and Affymetrix Microarray Suite User's Guide version 5.0, and although there are ways to ensure success of RNA preparation and cRNA Target synthesis, this technology is evolving. We have used a variety of analytical techniques, such as quantitation and DNase treatment of total RNA (Figures 5, 9, 10 and data not shown), Agilent Bioanalyzer gel analysis of fragmented cRNA Target samples (Figure 11), and hybridization to Test2 Arrays (data not shown) to increase our confidence to the results we received. In the case of Test2 arrays, these are more affordable micorarrays containing probe sets of spiked controls (eg BIOB) and housekeeping genes from a variety of organisms. While we did not use Test2 arrays in this particular experiment, we have used them to assess the quality of our cRNA Targets in other Affymetrix micorarray experiments. Since the U74Av2 microarrays contained the same spiked controls (see Methods) and many of the same murine control transcripts that are on the Test2 array, we decided to assess the cRNA Target and hybridization quality by performing one hybridization to these microarrays.

At any time in any cell, the number of genes being transcribed is limited. Also, those expressed genes are represented by differing amounts of RNA. A significant concern is whether this technology, or any current RNA quantitation technology for that matter, can be used to determine low copy transcripts. Even though the cRNA Target is an amplified representation of all the mRNAs in our total RNA, are we able to detect low-level transcripts or subtle changes in transcript levels using this technology? Put another way, are we to believe the changes we see in transcripts with very low Signals.

Affymetrix Analysis software has attempted to address this issue by attaching statistical significance (P-value) not only to each individual Signal, but also calculating a P-value for each computed fold change between any two microarray comparisons, see Methods and Table 5. We have included in our analysis a sum of singles (sos) calculation as an added tool to assess transcriptional changes, see Appendix B. Using the sos calculation, we may argue that one of the genes, 101845_s_at, whose transcripts increase significantly in our experiment (Affymetrix Change p-value equals 0.0024), but whose sos is 197, is not worthy of inclusion in the list of increased transcripts. Another issue with the microarray technology is dealing with the random fluctuations in cellular transcription (Figure 12). When comparing RNAs from the zero hour control cells to RNAs from cells grown in 0.1% EtOH for 24 hours, there were 3,809 increased or decreased transcripts out of a total of 74928 comparisons. This 5% represented a net change of only eight transcripts, all which increased slightly. From information obtained from NetAffx Analysis Center, four of the eight transcripts were annotated, data not shown. Probe Set Name 98603_s_at codes for RAN GTPase activating protein 1, a protein involved in mitosis and spindle assembly (Trieselman 2002). Another transcript, Probe Set Name 95133_at, codes for asparagine synthetase. This protein is involved in the control of growth or amino acid metabolism and is regulated by amino acid availability (Fafournoux, Bruhat, & Jousse, 2000). In this culture, perhaps amino acid pools declined prompting this increase. Probe Set Name 96596_at codes for TDD5, a phosphorylated stress-responsive protein whose phosphorylation may be related to cell growth (Agarwala, Kokame, Kato, and Miyata, 2000). Finally, Probe Set Name 160464_s_at codes for the cytoplasmic protein Ndr1 and is probably the same gene as Probe Set

Name 96596_at (Agarwala et al., 2000). These limited changes, now totaling 7 transcripts being changed in all 6 comparisons, suggest that 0.1% EtOH treatment causes few transcriptional changes in BB88 cells and correlate with the effects seen on proliferation. Unexpectedly, when we compared transcript changes within the 24 hour 0.1% EtOH control groups, the results are rather different. Here, for the three comparisons, out of a total of 37,464 comparisons (12,488 transcripts per U74Av2 microarray), 2,641 (880 average changed transcripts per comparison) (7%) transcripts are increased or decreased. In further examination of the 2641 changed transcripts, there were 95 transcripts common to all three 24 hours control comparisons. Since these RNAs were from the same treatment group, we would have expected fewer transcriptional changes amongst these RNAs. However, upon a closer examination of the within group comparisons, the number of transcript changes between U74Av2 control micorarray #3 vs #2, U74Av2 micorarray #9 vs #2, and U74Av2 micorarray #9 vs #3 were 633, 834, and 1174, respectively, data not shown. The number of changes in this first comparison, U74Av2 control micorarray #3 vs #2, matches closely the average number (634) of transcriptional changes we saw in the inter-control group comparisons, see Figure 12. Thus, there may be issues with the U74Av2 control micorarray # 9 and/or this particular sample RNA that surfaced only upon these analyses. It must be emphasized that the whole transcript profiling process involves a number of steps, such that any issue with a particular step may translate into downstream transcript changes. For example, RNA preparation, ds-cDNA synthesis, and cRNA Target amplification are manipulations where differences may occur. Obviously, while costly, the more replicate microarray hybridizations will eliminate some issues with random fluctuations in

transcript levels and enhance the sensitivity of any experiment. Also, using RT-PCR, could have been used to confirm these changes seen between control cultures.

As we expected to see with the intra-group control comparisons discussed above, there were much fewer changes when comparing chloramphenicol-treated RNAs with one another, see Figure 13. There was an average of 163 changes per comparison (%1.3). This represents only three transcripts that were commonly changed in the three comparisons, and none were changed more than two-fold. Despite the Affymetrix software assigning significance to these changes, we feel that these changes are random fluctuations accounting for the inherent noise in the datasets. When comparing chloramphenicol-treated RNAs with control (0.1% EtOH) RNAs, a total of 112,392 comparisons were done and there were 11,789 changes (1309 average changes per comparison, see Figure 13 and 14A). This number of changes suggests that on any treated microarray, 10% of the transcripts levels were different than on a control microarray. However, since random fluctuations occur, both within the treated and control groups respectively (see above), then the real changes may be substantially less, perhaps approaching 1.7% [$10\% - (7\% + 1.3\%)$]. Using Spotfire, we were able to capture 199 transcripts that were commonly changed in all nine comparisons. This number of changed transcripts is 1.5% of the total number transcripts (12488) per individual comparisons per chip (Figure 14C). These results were re-assuring as they were close to our theoretical calculations and they suggest that these changes are the result of chloramphenicol treatment.

Regardless of any problems concerning the identification of genes with subtle expression changes, our experiments have provided us with some noteworthy changes.

Of these 199 transcripts, 56, representing a total of 52 genes, were increased or decreased greater than or equal to two-fold (Figure 14D and Table 5). Table 5 summarizes what we found out about these transcripts and some gene ontology patterns have emerged. First, some of the largest changes occurred in transcripts that encode proteins involved in transcription. Even though the changes in transcript levels were not so dramatic like with COX VIa-H, even small changes in transcription factor levels can cause dramatic and amplified changes downstream. To see if there may be direct effects of chloramphenicol on transcription factor expression, a timecourse would help. Next, from the list of decreased and increased transcript lists, there were three and four genes that are involved in glucose metabolism, respectively. Of the genes sensitive to chloramphenicol, this number represented 13.5% of the total. By blocking mitochondrial protein synthesis, chloramphenicol appears to affect expression of nuclear genes involved in bioenergetics and metabolism. Down-regulation of such genes as Gpd2, a nuclear-encoded mitochondrial protein that is responsible for converting glycerol-3-phosphate to dihydroxyacetone phosphate, manifests this disruption. This process shuttles electrons at the level of Coenzyme Q, yielding to ATP production. While some mammalian cells use this energy shuttle, most cells prefer a more energy efficient system that uses malate and aspartate. In the case of transcript increases, the affected genes appear to be involved in mitochondrial electron transport (Table 5B). Here, chloramphenicol treatment may be stimulating gene expression to compensate for the reduction in mitochondrial proteins and the decline in ATP production. In terms of compensation, it was interesting to see that chloramphenicol caused increases in transcripts that code for proteins involved in amino acid transport and biosynthesis. Recently, Priquone and Cortopassi have been

studying mitochondrial deletions and showed that in osteosarcoma cells ATG12, a transcript involved in the autophagy pathway, is induced by chloramphenicol treatment (Priqione & Cortopassi, 2007). Further they showed that autophagy is accompanied by a decline in the concentration of intracellular amino acids in these cells. They argue this decline results from decreases in proteasomal activity. Here, while we did not measure amino acids levels in BB88 cells nor did we observe autophagy, perhaps all the increases we saw in transcripts involved in amino acid metabolism are compensating for effects of autophagy. Another compensatory mechanism may be the increased inositol phosphate receptor expression (Table 5B). Criollo et al. (2007) have been studying autophagy also and they have shown that declines in intracellular levels of 1,4,5-inositol phosphate and its receptor induce autophagy. Again, we did not measure inositol phosphate levels in chloramphenicol-treated BB88 cells, however we did see an increase in the transcripts for *Itpr2*, an inositol 1,4,5-triphosphate receptor. In relation to other's works on autophagy, our profiling results are intriguing.

Individually, there have some noteworthy changes in certain genes' transcript levels. From this group, three transcripts stood out. The first transcript, 101793_at, increased 37-fold ($\log_2=5.2$) and codes for the Fc portion, high affinity I, of IgG receptor. While the relationship between this cell surface immunoglobulin and chloramphenicol is not known, there is a report suggesting that this protein is related to stress and could act as an immunotoxin (Tur et al., 2003). Even though the relationship between proliferation and immunotoxin activity is unclear, perhaps the stress caused by chloramphenicol treatment is manifested by an increase in transcript levels for 101793_at. In the case of Probe Set Name 93738_at, transcript levels declined about 5-

fold ($\log_2 = -2.38$). This transcript codes for the facilitated glucose transporter. One can speculate that the effects of chloramphenicol on mitochondrial protein synthesis and hence respiration are causing a direct decline in the use of glucose. This decline may cause a decrease in the facilitated glucose transporter. Kansara and Berridge (2004) have shown that glucose transport tracks with the cell cycle. Expression of the facilitated glucose transporter in BB88 cells may be declining as a response to changes in the cell cycle. Kang et al. (2005) showed that long-term chloramphenicol treatment of K562 cells causes a decline in both translation and transcription of E2F-1 and cyclin D1, two cell cycle regulatory molecules. In the same cells, Leiter et al. (1999) reported that growth inhibition caused by 8 days of chloramphenicol treatment was associated with slight increases and decreases in the numbers of cells in G1 and S phase, respectively. While we saw no transcription changes in cell cycle genes, nor did we examine BB88 cell cycle profiles, we have shown that chloramphenicol inhibits BB88 cell growth by increasing its doubling time (Figure 8). Further, Nagiec et al. (2005) reported that short-term treatment of K562 human erythroleukemia cells with the oxazolidinones, antibiotics that inhibit MPS synthesis similar to chloramphenicol, also cause proliferation inhibition. Their findings suggest that the oxazolidinones do not cause cell cycle arrest, but affect growth by elongating the cell cycle. Therefore, perhaps the decline in the glucose transporter expression is tied to affects chloramphenicol has on the cell cycle. The third transcript, 99667_at, increased about 28-fold ($\log_2 = 4.8$) in chloramphenicol-treated cells. Since 99667_at transcripts code for subunit VIa of cytochrome c oxidase (COX) (see Table 5 and Figure 15), the terminal enzyme complex in the mitochondrial electron transport chain (Capaldi, 1990) and because there is a direct link between

chloramphenicol and mitochondrial function (Leiter et al., 1999), we chose to study this transcript change further. Interestingly, subunit VIa is not necessary for COX assembly, but it appears to modulate the enzyme's activity through interactions with ATP (Taanman & Capaldi, 1993). Also, in a COX assembly study, subunit VIa associated with S3 (COX subcomplex) suggesting that this subunit has a regulatory role (Nijtmans, Taanman, Muijsers, Speijer, & Van Den Bogert, 1998). Most likely, as a result of chloramphenicol's effect on the synthesis of COX, COX VIa-H expression increases. In mammals, subunit VIa exists as two isoforms, -H (heart) and -L (liver). Originally, these designations implied tissue-specific expression, although it appears now that these designations are interpreted as heart/muscle and constitutive forms, respectively (Ewart, Zhang, & Capaldi, 1991, Wan & Moreadith, 1995). From our experiments, it appears that subunit VIa-H is uniquely expressed in BB88 cells and its transcript is increased upon chloramphenicol treatment (Table 5 and Appendix B). Not only were we surprised by the magnitude of this increase, but that the COX VIa-H transcript was present at all. This finding may be the first report where this transcript has been found in erythroleukemic cells. Further, we observed that transcripts for the VIa-L isoform or other nuclear encoded COX subunits were not identified on the microarrays as having increased or decreased, data not shown.

We chose to use Taqman® PCR to confirm and expand on the microarray findings for COX VIa-H. RT-PCR confirms the finding that COX VIa-H transcripts are increased in proliferating BB88 cells. Initially, we were confounded by our measurements of COX VIa-H transcript in our RNA samples. There were fluorescent

signals coming from the experimental RNA samples, but the genomic standard control reactions were blank. Upon closer examination, it was discovered that the COX VIa-H primers were designed to a region of the mRNA spanning an intron. Thus, when using genomic DNA as a template, a PCR product was being synthesized that was too long and therefore was sub-optimal for Taqman® PCR. Therefore, the RT-PCR strategy was re-evaluated, and, rather than designing new oligonucleotide primers and probe, RT reactions made from RNA from several antibiotic-treated BB88 cells were pooled and used as a quantitative control, see Methods. Since the concern was to measure changes in COX VIa-H transcript levels between various RNAs from treated BB88 cells, these reactions were sufficient to use as standards for determining relative COX VIa-H transcript levels throughout this study. Another issue was the selection of a “housekeeping” control gene. Typically, a housekeeping gene is a constitutive gene that is transcribed independent of experimental conditions. While many genes have been designated housekeeping genes, the truth is no single housekeeping gene always manifests constant expression levels under all conditions (Bustin 2000; Doroudi 2002). While we selected glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as our housekeeping control gene to normalize the transcript levels for COX VIa-H, we measured some variation in GAPDH mRNA levels in our samples, data not shown. Nevertheless, Taqman® PCR confirmed that COX VIa-H transcripts increased greater than 20-fold in proliferating BB88 cells treated with 50 μ M chloramphenicol for 24 hours (Figure 16). This increase was slightly less than the increase we determined using Affymetrix MAS 5.0 software in the microarray experiment (Table 5). Taqman® PCR

has been determined to be very accurate; so even though the differences between the two methods of COX VIa-H measurement are less than two-fold, the results suggest that further experimental comparisons will be necessary to validate microarray methods (Bustin 2000). Also, it should be noted that there was variation in COX VIa-H transcripts levels within our Taqman® PCR experiments reported here; compare Figures 16, 18, 19, 21, and 22. We speculate that some of these differences may have resulted from real biological differences within the BB88 cultures or some of the variation may have resulted from the housekeeping gene normalization issue discussed above. Regardless, chloramphenicol causes time- and concentration-dependent changes in the relative amounts of COX VIa-H mRNA and these changes are probably due to transcriptional changes (Figure 17 and Figure 18).

Results from Taqman RT-PCR time-course experiments suggest that chloramphenicol affects COX VIa-H after about one cell doubling (Figures 6 and Figure 17). If chloramphenicol affected COX VIa-H transcription directly, we might have expected to see its transcript level change earlier. Rather, increases in COX VIa-H transcripts coincided with chloramphenicol's anti-proliferative effects, where effects were seen in growth and COX VIa-H transcript levels after 24 hours and 12 hours, respectively (Figure 8 and Figure 17). This result extended what we saw during our transcript profiling work. Results from our concentration-response experiment suggested that chloramphenicol has an $EC_{50} = 33 \mu M$ with respect to increases in COX VIa-H transcript after 24 hours. This concentration matched nicely with chloramphenicol's effect on cell proliferation. The proliferation inhibition EC_{50} measured

in BB88 cells was 43 μ M (Figure 7). These results suggest a link between changes in COX VIa-H transcripts and proliferation. Another piece of evidence that we had hoped would support this link was a finding that COX VIa-H transcript levels decline in differentiating BB88 cells treated with chloramphenicol. Marks and Rifkind (1989) showed that treatment of BB88 cells with polar compounds such as hexamethylene bisacetamide (HMBA) causes cell differentiation. Further this process is accompanied by a decline in cell proliferation. Kaneko et al. have shown that early addition of chloramphenicol to differentiating mouse erythroleukemia cells here inhibited this type of erythroid induction (Kaneko, 1988). In our hands however, preliminary experiments using chloramphenicol with HMBA-treated BB88 cells, there was no detectable affect on COX VIa-H mRNA (data not shown). This result may reflect the developmental pattern of regulation for this particular COX subunit, whereby COX VIa-H may not be expressed in differentiating BB88 cells (Ewart, 1991).

When BB88 cells were treated with other antibiotics, increases in COX VIa-H transcripts accompanied cell proliferation inhibition (Figure 19). Despite all the antibiotics inhibiting BB88 proliferation, the affect on COX VIa-H was somewhat surprising. Initially, we had expected there to be more selectivity of these antibiotics. However, since all these antibiotics are known bacterial protein synthesis inhibitors, perhaps this result is not so surprising. Others have shown a correlation between cell proliferation and mitochondrial function (Leiter et al., 1999; Kim et al., 2001; Nagiec et al., 2005). Perhaps as a result of these antibiotics affecting BB88 mitochondria similarly, there is a reduction in respiration and cellular ATP levels fall. Lower ATP levels in BB88

cells may have an effect on subunit COX VIa-H protein and the other nuclear encoded mitochondrial proteins whose transcripts levels were shown to increase upon chloramphenicol treatment (Table 5). Interestingly, the effect of chloramphenicol and other MPS inhibitors appeared to be specific for this particular COX subunit, as transcript levels of two additional nuclear-encoded COX subunits were unaffected by the antibiotics or respiratory inhibitors tested (Figure 20). These results extended the findings using Affymetrix microarrays. Gene ontology analysis revealed that despite chloramphenicol treatment affecting transcripts for only a few nuclear-encoded mitochondrial proteins, these affected proteins are involved in electron transport, gluconeogenesis, ion transport, and ATP-dependent protein hydrolysis (Table 5). These gene products appear sensitive to the effects these antibiotics have on mitochondrial bioenergetics and respiration.

PNU-86983 and PNU-78714 may be inhibiting BB88 cell proliferation and increasing COX VIa-H transcript levels similar to chloramphenicol (Figure 21). However, there may be some subtle differences here. In a personal communication with Ewa Nagiec, a scientist at Pfizer, Inc., we learned that when she treated rho zero cells with the aminochromone PNU-86983 and chloramphenicol, there were differences. Rho zero cells are human osteosarcoma 143B cells depleted of their mitochondria. This depletion occurs as a result of prolonged exposure to ethidium bromide, a chemical that binds to DNA and inhibits mitochondrial DNA replication. In Nagiec's experiments, PNU-86983 inhibited rho zero cell proliferation in a concentration-dependent fashion, whereas, while chloramphenicol inhibited parental 143B cell growth, it had no effect on rho zero proliferation (Nagiec et al., 2005). These results suggest that the

aminochromones do not affect proliferation via inhibiting MPS and more importantly point toward MPS as being a very important factor in control over cellular proliferation. These differences may be reflected in the extent to which COX VIa-H transcript levels are increased (Figure 21). In a personal communication with Paul Bonin, another Pfizer scientist, he explained that the aminochromones have been tested in a variety of assays, and, thus far, these compounds appear only to inhibit mammalian cell proliferation (Bonin, 1993; Erickson, 1994). Oligomycin is an antibiotic that is a potent uncoupler of oxidative phosphorylation by inhibiting ATP synthase (Nagamune, 1993). This antibiotic inhibited BB88 cell proliferation (Figure 21). These experiments were somewhat difficult to perform. Not only was oligomycin quite potent, but also its effect on BB88 growth occurred within a narrow concentration range. Nevertheless, in line with the relationship between mitochondrial function, cell proliferation, and COX VIa-H transcription, COX VIa-H mRNA increased 4-fold in cells treated 24 hours with 250 pM oligomycin (Figure 21). This compound may also have effects on MPS as oligomycin is a macrolide, a class of antibiotic that inhibits bacterial protein synthesis

Finally, we tested succinylacetone, a compound that affects heme production by inhibiting the enzyme delta-Aminolevulinate dehydratase (ALAD) in erythroid and non-erythroid cells (Woodard & Dailey, 2000). The results we obtained with succinylacetone were a little surprising since researchers have shown that 2 mM succinylacetone inhibited growth and decreased respiration in proliferating leukemia cells *in vitro* (Weinbach & Ebert, 1985). We found that 1 mM succinylacetone not only did not inhibit BB88 cell proliferation, but also had no effect on COX VIa-H transcript levels. It is possible that the differences here could be that Weinbach and Ebert used 2-4 mM succinylacetone.

Perhaps the 1 mM concentration that we used was insufficient to inhibit ALAD completely (Ebert, Hess, & Tschudy, 1985; Weinbach & Ebert, 1985). Further, in a study examining tissue variant effects of heme biosynthesis inhibitors, this compound has been shown to inhibit transcript levels for mitochondrial-encoded COX I and COX II subunits and a nuclear-encoded COX Vb subunits (Vijayasarathy, Damle, Lenka, & Avadhani, 1999). We speculated that, since chloramphenicol inhibits cell growth, affects MPS, and inhibits hemoglobin production in differentiating erythroleukemia cells (Kaneko, 1988), we might have found some common effects of chloramphenicol and succinylacetone. It turns out; 1 mM succinylacetone had no effect on BB88 cell proliferation, nor was there an effect of this ALAD inhibitor on COX VIa-H mRNAs (Figure 22). These results were somewhat unexpected, since chloramphenicol also decreased transcript levels for ALAD in proliferating BB88 cells and succinylacetone inhibited growth of HeLa cells after 5 days in culture (Ye & Zhang, 2004). Thus, it appears that chloramphenicol and succinylacetone affect BB88 cells differently.

CONCLUSION

Chloramphenicol affects mammalian cells and clinically this effect has manifested itself in myelosuppression. In an attempt to understand the myelosuppression that has accompanied chloramphenicol usage, we have examined the effects of chloramphenicol on murine erythroleukemia BB88 cells. These cells have proven to be an excellent model for studying the anti-proliferative effects of chloramphenicol. Chloramphenicol inhibits BB88 proliferation in a time- and concentration-dependent fashion. To begin to understand the cellular changes that accompany chloramphenicol's more immediate effects on proliferation, we implemented and validated the Affymetrix Microarray technology platform. This platform allowed us to observe changes occurring to approximately 6000 annotated gene transcripts and about 6,000 ESTs per U74Av2 Array in one experiment. By studying the early transcriptional changes resulting from chloramphenicol treatment, we have shown there is a correlation with the effects we see on proliferation. Under the experimental protocol we followed, we implemented and designed some relatively simple analytical methods to identify genes that may be increased or decreased in chloramphenicol-treated proliferating BB88 cells. In analyzing transcripts that decreased or increased two-fold or more, some patterns emerged. Notably, chloramphenicol affected nuclear-encoded transcripts involved in transcription control, mitochondrial electron transport and bioenergetics, and amino acid transport and metabolism.

Most interesting was the finding that in BB88 cells treated for 24 hours 50 μ M chloramphenicol, there is a dramatic increase in mRNA for COX VIa-H, a subunit of COX. While the function for COX VIa-H and most of the other nine nuclear-encoded mammalian COX subunits remains unclear, COX VIa-H may play an important role regulating mitochondrial energy metabolism (Kadenbach, Huttemann, Arnold, Lee, & Bender, 2000). Given that efficient cell proliferation depends on a functional electron transport system and mitochondrial energy production, we decided to study further this particular COX subunit. Using Taqman® PCR, we confirmed that proliferating BB88 cells contain COX VIa-H mRNA. To our knowledge, this finding is novel. Previous reports have not identified expression of COX VIa-H in erythroid cells (Grossman & Lomax, 1997). Unfortunately, an antibody to the murine COX VIa-H isoform does not exist; so we were unable to confirm whether protein levels matched mRNA levels. However, in murine BB88 erythroleukemia cells treated with chloramphenicol, we have shown that mRNA for the COX VIa-H subunit increases in a time- and concentration-dependent fashion. The effects on COX VIa-H transcripts appear to be specific; as other nuclear-encoded COX transcripts are unaffected by antibiotic treatment. At this time, it appears that COX VIa-H transcripts may be a biomarker for BB88 proliferation, as the changes we see in COX VIa-H transcript levels mimic chloramphenicol's effects on proliferation. Other compounds that are known to affect MPS or respiration inhibit BB88 proliferation and cause increases in COX VIa-H transcripts. These results match what we see with chloramphenicol treatment and suggest that chloramphenicol may be inhibiting cell proliferation and increasing COX VIa-H transcripts by affecting

mitochondria. Since we used undifferentiated cells, our experimental results may not translate perfectly to what happens clinically. However, compared with other tissues in the body, erythroid stem cells are proliferating continually, and differentiating cells are utilizing their mitochondria for energy and heme biosynthesis. Hence, despite the fact that other cells respond to chloramphenicol, this population of cells may be highly sensitive to the compounds that target mitochondria. In the short term, chloramphenicol inhibits cell proliferation through its effects on mitochondria and in our model of erythroid cell proliferation these inhibitory effects are manifested by changes in COX VIa-H expression. While further experiments are needed to definitively link COX VIa-H with proliferation, the evidence supports a role for this subunit in cell growth.

Appendix A

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> > As part of my scientific studies on chloramphenicol toxicity, I
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Appendix B

Transcripts Increased and Decreased as a Result of Chloramphenicol Treatment

# Decreased	Probe Set Name	average treated signal	average control signal	average signal log2 ratio	ave. signal ratio	Change p-value	average sos
1	94995_at	429	885	-1.0	-2.06	1.00E+00	1314
2	104701_at	403	858	-1.0	-2.13	1.00E+00	1261
3	98984_f_at	497	883	-1.1	-1.78	1.00E+00	1380
4	103362_at	342	978	-1.2	-2.86	1.00E+00	1320
5	160091_at	2654	8496	-1.3	-3.20	1.00E+00	11150
6	93330_at	554	1734	-1.5	-3.13	1.00E+00	2288
7	93738_at	101	449	-2.4	-4.45	1.00E+00	550
# Increased	Probe Set Name	average treated signal	average control signal	average signal log2 ratio	ave. signal ratio	Change p-value	average sos
1	101793_at	1500	67	5.2	22.4	3.10E-05	1567
2	99667_at	3095	50	4.8	61.8	0.00E+00	3145
3	101845_s_at	180	17	4.1	10.5	2.38E-03	197
4	101209_at	432	55	3.0	7.8	3.11E-04	487
5	161067_at	3835	707	2.7	5.4	1.00E-06	4542
6	102249_at	2967	470	2.6	6.3	1.00E-06	3437
7	102879_s_at	1843	206	2.4	8.9	0.00E+00	2049
8	101429_at	3200	652	2.4	4.9	0.00E+00	3852
9	103006_at	1019	199	2.1	5.1	2.10E-05	1218
10	102292_at	2323	568	2.1	4.1	1.00E-06	2891
11	103922_f_at	4890	974	2.0	5	1.00E-06	5864
12	94359_at	461	75	1.9	6.1	4.00E-06	536
13	97398_at	3758	799	1.9	4.7	0.00E+00	4557
14	96199_at	501	171	1.7	2.9	1.00E-06	672
15	104144_at	2247	765	1.7	2.9	2.00E-06	3012
16	102313_at	1321	345	1.6	3.8	1.00E-06	1666
17	102770_at	2711	883	1.6	3.1	3.00E-06	3594
18	92925_at	3021	1156	1.5	2.6	0.00E+00	4177
19	160108_at	595	202	1.5	2.9	1.77E-04	797
20	103921_i_at	1334	670	1.5	2	3.10E-05	2004
21	98122_at	496	186	1.5	2.7	7.40E-05	682
22	101441_i_at	1369	445	1.4	3.1	1.30E-05	1814
23	94278_at	2196	728	1.4	3	3.00E-06	2924
24	92589_at	5137	1833	1.3	2.8	0.00E+00	6970
25	96160_at	2506	794	1.3	3.2	4.67E-04	3300
26	100306_at	403	148	1.3	2.7	6.30E-05	551
27	100592_at	11164	4123	1.3	2.7	0.00E+00	15287
28	100920_at	291	174	1.3	1.7	1.00E+00	465
29	160602_at	1656	706	1.2	2.3	1.20E-05	2362
30	103520_at	232	121	1.2	1.9	1.54E-04	353
31	97960_at	460	186	1.2	2.5	0.00E+00	646

# Increased	Probe Set Name	average treated signal	average control signal	average signal log2 ratio	ave. signal ratio	Change p-value	average sos
32	161221_f_at	1145	520	1.2	2.2	1.33E-04	1665
33	95637_at	1045	426	1.2	2.5	1.00E-06	1471
34	100599_at	19818	8734	1.2	2.3	1.00E-06	28552
35	92268_at	2626	1152	1.2	2.3	1.00E-06	3778
36	94135_at	729	342	1.2	2.1	4.70E-05	1071
37	98042_at	302	140	1.1	2.2	1.43E-04	442
38	160530_at	1475	720	1.1	2	3.70E-05	2195
39	160535_at	2923	1438	1.1	2	0.00E+00	4361
40	100943_at	1735	848	1.1	2	2.90E-04	2583
41	102337_s_at	835	434	1.1	1.9	2.30E-05	1269
42	103818_at	1721	819	1.1	2.1	5.90E-05	2540
43	160328_at	6502	3070	1.0	2.1	3.00E-06	9572
44	104328_at	1318	750	1.0	1.8	3.40E-05	2068
45	97268_i_at	13077	5859	1.0	2.2	1.00E-06	18936
46	160463_at	962	504	1.0	1.9	1.30E-03	1466
47	99330_at	716	353	1.0	2	1.88E-03	1069
48	97870_s_at	3992	2139	1.0	1.9	5.00E-06	6131
49	95502_at	1278	826	1.0	1.5	1.00E-06	2104

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