Insights into the Mechanism of Drug-Induced Agranulocytosis: A Study of the Immune Changes Induced by Clozapine and Amodiaquine

by

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A thesis submitted in conformity with the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Abstract

Drug-induced agranulocytosis is an idiosyncratic adverse drug reaction, and although its mechanism is not well understood, it is suspected to involve both metabolic (reactive metabolites) and immune components. Numerous drugs that cause agranulocytosis are metabolized to reactive species by neutrophils that, in turn, covalently bind to the cells; a process hypothesized to be dependent on neutrophil myeloperoxidase. However, despite extensive in vitro studies, in vivo evidence to support this is lacking. Therefore, the first goal of these studies was to characterize the involvement of myeloperoxidase in the covalent binding of amodiaquine and clozapine to neutrophils. In myeloperoxidase knockout mice treated with amodiaquine, covalent binding to neutrophils was found to decrease 2-fold compared to control. Interestingly, lack of the enzyme did not completely abolish binding, and lymphocytes were also found to extensively bind the drug. These studies confirm that myeloperoxidase is responsible for some of the covalent binding to neutrophils, but that other pathways not yet defined in neutrophils, as well as other cells, are also capable of oxidizing amodiaquine. The second goal of this work was to investigate the immune component associated with drug-induced agranulocytosis by studying
the response of neutrophils and the overall immune system to clozapine. Due to difficulties accessing patients, these studies were pursued by first designing a rat model of early clozapine treatment. Using this model, the response of neutrophils to clozapine was found to reflect that of G-CSF-mediated stimulation of the bone marrow. A proinflammatory response to clozapine was detected in the rats, characterized by neutrophilia and elevated levels of the acute phase protein α-1-acid glycoprotein, which led us to investigate the ability of clozapine to activate inflammasomes. These studies led to the proposal of a novel hypothesis, specifically, that the proinflammatory response to clozapine at the start of treatment is mediated by inflammasome-generated IL-1β.
Acknowledgments

Returning to the academic environment in pursuit of a Ph.D. has been one of the most challenging and rewarding experiences of my professional career. It feels like only a few years ago that I was completing my first undergraduate chemistry lab, and little did I know that it would eventually lead me to study with Dr. Jack Uetrecht, a passionate chemist at heart. The breadth of knowledge I have acquired throughout my studies I am certain will carry me through the next steps in my career, and I have many people to thank for this.

First and foremost, I extend a warm thank-you to my supervisor Dr. Jack Uetrecht, who throughout my time in his lab provided me with endless insight and guidance. Despite the fact that my studies on the myeloperoxidase hypothesis for neutrophil covalent binding ended up challenging his theory instead of supporting it, Jack continually encouraged me to pursue my studies, which I truly respect in him. Thank-you Jack for always having the door to your office open, as well as to your home and your cottage, ready to discuss research or more often the ways of the world. I have learned so much from you during my studies, and I thank-you for providing me with such a positive and rewarding experience.

Next I would like to thank my committee members, Drs. Michael Glogauer, Marciano Reis, Peter O’Brien, and Jean Wang, for putting in the extra effort to help me succeed. The insight and advice that you provided at my committee meetings, as well as outside the scheduled meeting times, helped shaped this thesis and allowed me to reach my goals.

No one can survive doctoral studies without a strong support network of colleagues, so I of course have to thank all of the past and present members of the Uetrecht lab, as well as the many visiting students and scientists that I had the pleasure of working with. Thank-you as well to the other Faculty members I was honored to work with, Drs. Christine Allen, Jillian Kohler, Ray Reilily, and Peter Wells.

Last but not least, I couldn’t have done this without my personal cheering squad. Thank-you to Dave, my editor and soon husband-to-be, for providing me with the endless love and support that I needed from beginning to end. To my parents, thank-you for encouraging me to pursue this path and for always supporting me wholeheartedly in all of my academic and personal endeavors.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-IMT</td>
<td>2-iminothiolane</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin</td>
</tr>
<tr>
<td>α-1-AGP</td>
<td>α-1-acid-glycoprotein</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-modified Eagle’s medium</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>AQ</td>
<td>amodiaquine</td>
</tr>
<tr>
<td>AQQI</td>
<td>amodiaquine quinone imine</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated specklike protein</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Tissue Culture</td>
</tr>
<tr>
<td>BCP</td>
<td>blue carrier protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase-associated recruitment domain</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>CLZ</td>
<td>clozapine</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine ligand</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>IDR</td>
<td>idiosyncratic drug reaction</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>INF</td>
<td>interferon</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAL</td>
<td>N-α-acetyl-L-lysine</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate-13-acetate</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin glycoprotein ligand</td>
</tr>
<tr>
<td>PYD</td>
<td>pyrin domain</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sc</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>
List of Thesis Publications and Abstracts

Journal Articles


Lobach, A.R. and Uetrecht, J. Clozapine promotes the proliferation of granulocyte progenitors in the bone marrow leading to increased granulopoiesis and neutrophilia in rats. Chemical Research in Toxicology. 2014 Jun 26. [Epub ahead of print].


Abstracts


CHAPTER 1

Introduction
Every year billions of dollars are spent globally on the development of new pharmaceutical products, encompassing the early stages of research and development to the testing of these agents in phase three clinical trials.¹ The drug approval process is governed by strict regulatory guidelines, and the reality is that only a small percentage of new discoveries possess the required efficacy and safety profiles to ever reach the market. For example, in 2012, the Food and Drug Administration (FDA) approved 39 new drugs for patient use, which was in fact the highest number of approvals seen since 1996; however, looking at the big picture, this is only a small percentage of the total number of drugs proposed during the research and development phase.² Although the regulatory process is designed to prevent drugs that are potentially harmful from obtaining approval, unfortunately, not all adverse drug reactions are detected during clinical trials. First off, the overall number of patients tested in clinical trials is often not high enough to detect reactions that are less common, and secondly, the exclusion criteria defined during recruitment tend to produce rather homogenous patient populations.³

1.1 Adverse drug reactions

Adverse drug reactions are ranked as the 4⁰ – 6⁰ leading cause of death in the US, generating approximately 100,000 fatalities per year.³, ⁴ They are a major clinical problem that increase the cost of the healthcare system, contribute to the high expenses associated with drug development, and most importantly, pose significant danger to patients.⁵ Adverse drug reactions have been classified into five categories, namely types A – E.⁶⁻⁸ Type A reactions are related to the pharmacological action of the drug, and therefore are largely predictable and dose-related. Type B reactions are not related to the pharmacological action of the drug, are unpredictable, and as a result, are described as bizarre. Reactions directly related to the chemical structure of a drug, type C, are the result of long term exposure and a resulting cumulative toxic effect. Type D reactions occur months to years following drug treatment, and lead to reactions such as carcinogenicity and teratogenicity. The type E reactions occur at the end of treatment, and arise due to the sudden withdrawal of the drug. Table 1-1 provides some common examples of each type of reaction.
Table 1-1. Types of adverse drug reactions

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A Augmented</td>
<td>• Excessive bleeding with warfarin administration</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Aminoglycoside-induced nephrotoxicity</td>
</tr>
<tr>
<td>Type B Bizarre</td>
<td>• Isoniazid-induced hepatotoxicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Clozapine-induced agranulocytosis</td>
</tr>
<tr>
<td>Type C Chronic</td>
<td>• Analgesic nephropathy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hypothalamic-pituitary-adrenal axis suppression by corticosteroids</td>
</tr>
<tr>
<td>Type D Delayed</td>
<td>• Phocomelia due to thalidomide</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Tardive dyskinesia induced by dopamine receptor antagonists</td>
</tr>
<tr>
<td>Type E End of use</td>
<td>• Seizures upon terminating anticonvulsant therapy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Opiate withdrawal syndrome</td>
</tr>
</tbody>
</table>

1.2 Idiosyncratic adverse drug reactions (IDRs)

In addition to being bizarre, type B reactions are also defined as idiosyncratic, meaning that they are peculiar to an individual. This patient-specific response is what makes them so rare and unpredictable, and therefore, they are extremely difficult to detect during clinical trials. Lasser et al. reported that over a 25 year period (1975 – 2000), 10.2% of newly approved drugs were labeled with black box warnings or completely withdrawn from the market due to adverse reactions that were not detected during clinical trials.9 As a result, IDRs present a major problem for drug development and health care industries. Although much effort has been put forth to try and predict the risk of such reactions before drug candidates reach the market, the overall mechanism of IDRs is not well understood, which makes it extremely difficult to develop effective predictive tools.10, 11 The more we unravel about the mechanism of these reactions, the more complex the overall picture gets; it is increasingly evident that not one, but multiple mechanisms are at play, and the combination of these factors appears to vary between drugs, as well as different types of reactions.12 IDRs most commonly target the skin, liver, or blood cells, and in some cases drugs can be responsible for causing more than just one type of IDR.13 For example, amodiaquine induces agranulocytosis in 1:2000 patients, but also causes hepatotoxicity at an incidence rate of 1:15 000.14
1.2.1 Clinical characteristics of IDRs

A feature common to most IDRs is the delay in onset of the reaction, which can vary from weeks to years, depending on the type of reaction; skin rashes tend to appear within weeks, whereas reactions targeting the liver and bone marrow generally develop 1 – 2 months into treatment.13 The delay in onset is highly suggestive of the involvement of an immune component in the mechanism of IDRs; however, evidence to support this has not been definitive, and it appears that the types of immune responses vary for different drugs. For example, in some cases of drug rechallenge, an IDR will reoccur more rapidly than the time to onset with first exposure, suggesting the involvement of memory T cells. In other cases, drug rechallenge does not shorten the time to onset of a second IDR, and it has been proposed that drugs displaying such characteristics may stimulate an autoimmune response that would lack immune memory.12

IDRs are often described as being dose independent, because the risk of reaction does not seem to increase in parallel with the dose.13 This is in fact incorrect. The probability that a drug will cause an IDR is related to the range of its therapeutic dose, and therefore drugs administered at very low doses (i.e. less than 10 mg/day) rarely induce IDRs.10 In most instances, the risk of an IDR does not increase within the therapeutic range due to the fact that the maximal incidence of induction occurs at a dose below this often narrow range.13

Another characteristic feature of most drugs that cause IDRs is the propensity of these molecules to be bioactivated into reactive metabolites.11 The formation of reactive, electrophilic derivatives of parent drugs is implicated in inducing toxicity, often the result of protein interactions in the form of direct covalent modification, or oxidation of redox-sensitive thiols or amino groups.15 This ultimately leads to immune activation, initiation of cellular apoptosis or necrosis, or DNA modification-induced carcinogenicity or teratogenicity.15, 16 It is suspected that the location of reactive metabolite formation is linked to the type of reaction induced, because it is unlikely that these unstable compounds would travel far from their site of production before encountering a protein, for instance, upon which to act. The majority of drug metabolism occurs in the liver, and most drugs that cause idiosyncratic liver injury are bioactivated to reactive species by cytochrome P450 enzymes present in the liver.13 However, in most cases, definitive evidence proving that a specific reactive species is responsible for causing the IDR is lacking, and therefore such findings must be interpreted with caution.17 Xenobiotics are also bioactivated in
the skin to reactive metabolites, and recently, using a rat model of nevirapine-induced skin rash, Sharma et al. demonstrated that a benzylic sulfate metabolite formed in the skin by sulfotransferases is responsible for the rash.\(^{18}\) The blood and bone marrow can also bioactivate drugs; specifically, myeloperoxidase-generated oxidants from neutrophils have been shown to produce reactive metabolites of numerous drugs known to cause agranulocytosis.\(^{19}\)

Several strong genetic associations between increased IDR risk, and specific human leukocyte antigen (HLA) genotypes have been recently identified, further suggesting that these reactions involve an immune component.\(^{12}\) Briefly, the major histocompatibility complex (MHC), in the case of humans HLA, is found on antigen presenting cells, and is responsible for presenting foreign antigens to T cells to initiate immune responses. Specific cases of drug-induced hypersensitivity, liver injury, as well as skin reactions have been linked to patients with particular HLA alleles. For example, one of the strongest associations reported is that of the HLA-B*15:02 haplotype and carbamazepine-induced skin reactions (approximate odds ratio of 895), which has led to the implementation of genetic testing of patients of relevant ethnicities prior to carbamazepine treatment.\(^{20}\) Weaker associations with genes involved in innate immune responses (i.e. interleukin (IL)-4 and IL-10 genes in diclofenac-induced hepatotoxicity), as well as polymorphisms in drug-metabolizing enzymes (i.e. N-acetyltransferase 2 polymorphisms in isoniazid-induced hepatotoxicity) have also been reported.\(^{20}\)

1.2.2 Proposed mechanisms of IDRs

Although the mechanisms of IDRs are still not well understood, the collection of clinical characteristics described above has provided a series of clues to guide our mechanistic understanding. The involvement of endogenously formed reactive metabolites of drugs, in combination with some form of immune activation, are thought to be of importance, and consequently are common components of a number of the proposed hypotheses.\(^{10}\)

1.2.2.1 Hapten hypothesis

The hapten hypothesis stems from the observations of Landsteiner and Jacobs in 1935, where they found that small molecules were not able to stimulate an immune response in guinea pigs unless the molecules were covalently bound to proteins.\(^{21}\) In the context of IDRs, this hypothesis postulates that drugs are bioactivated to chemically reactive species (or the parent drugs
themselves may be reactive) deemed haptens, which covalently bind to endogenous proteins. This results in the formation of drug-modified proteins that, when taken up by antigen presenting cells, are considered to be foreign or nonself. As a result, the drug-modified proteins are hydrolyzed by the antigen presenting cell into peptide fragments and presented in the context of MHC to T cells. The interaction between the T cell receptor and the drug-modified protein constitutes signal 1 and can lead to activation of T and B cells to stimulate an immune response (Figure 1-1). Penicillin-induced allergic reactions are a good example of a mechanism that can be explained by the hapten hypothesis. Penicillin is inherently reactive due to the strain present in the $\beta$-lactam ring, which leads to the interaction of penicillin with free amino and sulfhydryl groups on proteins and the formation of irreversible covalent bonds. In select patients, the presence of these drug-modified proteins leads to an immune response that generates IgE antibodies against penicillin, resulting in a severe allergic reaction. Another example of a drug that can act as a hapten to induce an antibody-mediated immune response is aminopyrine. In 1952, Moeschlin and Wagner transferred blood from a patient with aminopyrine-induced agranulocytosis into a recipient who took a dose of aminopyrine but had never previously been treated with aminopyrine, and within an hour the recipient’s granulocyte count dropped from 7100 to 1100 per mm$^3$. We now understand that this reaction is mediated by aminopyrine-dependent anti-neutrophil antibodies, that when introduced into the naïve recipient, were able to destroy mature neutrophils in the circulation. Furthermore, it is suspected that formation of an extremely reactive dication of aminopyrine by neutrophil-generated oxidants is responsible for acting as a hapten and covalently binding to neutrophil proteins.

1.2.2.2 Danger hypothesis

The danger hypothesis was proposed by Matzinger in 1994, which challenged the fundamental belief that the immune system’s primary goal is to discriminate between self and nonself. She put forth the idea that it is the threat of danger within the system that determines whether an immune response will be mounted; in the absence of danger, the result is immune tolerance. In this scenario, danger signals are released by injured tissue or stressed cells in the form of hydrophobic biological molecules, type 1 interferons, or stress proteins such as heat shock proteins. These factors upregulate costimulatory molecules found on antigen presenting cells, which promote the induction of an immune response through the interaction of B7 on antigen presenting cells with CD28 on T cells, for example, also known as signal 2 (Figure 1-1).
The importance of signal 2 is best illustrated by the use of adjuvant to upregulate costimulatory molecules on antigen presenting cells when administering vaccines. In general, foreign proteins do not induce significant immune responses, whereas in the presence of adjuvant, signal 2 is promoted, and the resulting immune responses are far more potent.\textsuperscript{10} In the context of IDRs, reactive metabolites of drugs could very likely be capable of causing cell damage leading to the release of danger signals, yet to date, clear evidence of such a response has not been reported. On the other hand, the presence of viral infections in patients (i.e. HIV, herpes virus) are known to increase the incidence of some drug hypersensitivity reactions, and the danger hypothesis provides a logical explanation for this observation.\textsuperscript{25}

Figure 1-1. The hapten and danger hypotheses
1.2.2.3 Pharmacological interaction hypothesis

For drugs that are inherently reactive or are biotransformed into chemically reactive intermediates, the induction of IDRs is best explained by the hapten and danger hypotheses. Drugs that are not chemically reactive, either as the parent compound or a reactive metabolite, but can still induce IDRs, are more appropriately described by a third hypothesis. The pharmacological interaction hypothesis states that a drug is able to stimulate T cells through direct interaction with the MHC-T cell receptor complex, either by directly binding to MHC molecules or interacting with the T cell receptor. In vitro studies have provided strong evidence supporting this mechanism, namely that T cell clones obtained from sensitized patients respond to the parent drug in the absence of metabolism via the T cell receptor in an MHC-dependent manner. However, in many cases, the metabolites of these drugs are also implicated in IDRs, which raises the issue that what T cells respond to is not necessarily what initially stimulated the immune response. For example, employing the animal model of nevirapine-induced skin rash, Chen et al. were able to show that lymphocytes obtained from sensitized animals responded in vitro to the parent drug, even though it is evident that a reactive metabolite initially induced the immune response in vivo.

The genetic association of various HLA alleles with certain IDRs is intriguing in the context of the pharmacological interaction hypothesis. Abacavir-induced hypersensitivity is associated with the HLA-B*57:01 haplotype, and recently it was discovered that the drug itself alters the specificity of HLA-B*57:01 by binding in the F pocket of the peptide-binding groove. Although related to the pharmacological interaction hypothesis, it appears that this binding leads to alteration of the repertoire of self-peptides, which is directly responsible for immune activation. As more drugs that cause IDRs are investigated in the context of this hypothesis, it appears that the hapten and pharmacological interaction mechanisms are not mutually exclusive.

1.2.3 Idiosyncratic drug-induced blood dyscrasias

Drugs cause a number of adverse reactions that affect the blood cells, including thrombocytopenia, aplastic anemia, hemolytic anemia, and agranulocytosis. These reactions can be predictable, for example the bone marrow toxicity associated with the administration of various anti-cancer agents, or idiosyncratic, which is the focus of this overview.
Thrombocytopenia is defined as a decrease in the number of circulating platelets, which leads to elevated risk of bleeding in the patient. Many cases of drug-induced thrombocytopenia, such as those caused by procainamide and quinine, are mediated by antibodies, providing ample evidence that such reactions are immune-mediated. The classic example of this reaction is heparin-induced thrombocytopenia, which involves the binding of heparin to platelet factor 4 to form an immunogenic complex. This leads to the generation of IgG antibodies against this complex that directly activate platelets, resulting in platelet destruction.\(^{30}\)

Hemolytic anemia involves the premature breakdown of the red cells, and many cases that are drug-induced appear to be mediated by drug-specific or autoimmune antibodies.\(^{13}\) A well-accepted example of hemolytic anemia is that of penicillin, which covalently binds to red cell membrane proteins to induce the production of anti-drug antibodies. These antibodies target the drug-bound red cells and flag them for premature clearance by macrophages.\(^{31}\) Other cases of this reaction, such as those induced by methyldopa and fludarabine, cause the formation of autoantibodies against epitopes on red cells. However, the mechanism of red cell autoantibody formation is still not well understood.\(^{31}\)

Aplastic anemia damages the hematopoietic stem cells residing in the bone marrow, and is characterized by a complete disappearance of all the major cell types produced in the marrow (red cells, granulocytes, and platelets). Bone marrow biopsy shows an almost complete absence of blood stem cells that have been primarily replaced by fat.\(^{29}\) Drugs such as chloramphenicol are associated with this reaction, and many drugs that cause agranulocytosis, such as propylthiouracil and carbamazepine, have also been reported to induce aplastic anemia.\(^{13}\) Nevertheless, due to the fact that aplastic anemia is usually idiopathic and can be caused by a variety of factors, it is often difficult to be certain that specific cases are indeed drug-induced.\(^{13}\)

1.2.3.1 Drug-induced agranulocytosis

Agranulocytosis is defined as a decrease in granulocyte count in the peripheral blood, which in humans is apparent when neutrophil counts drop below 500 cells/\(\mu\)L of blood. Agranulocytosis is the result of decreased production of neutrophils in the bone marrow, increased destruction of neutrophils in the peripheral blood, or sequestering of neutrophils in tissues.\(^{32}\) A systematic review by Andersohn et al. found that between 1966 and 2006, 125 drugs were reported to cause this adverse reaction, spanning eleven different drug categories.\(^{33}\) The time to onset of the
reaction is generally delayed (at least 1 month), represented clinically as septicemia, septic shock and/or severe infection. However, many patients remain relatively asymptomatic, and therefore routine monitoring of neutrophil counts remains important for high risk drugs.\textsuperscript{34} Ceasing treatment with the causative agent will most often allow the neutrophil count to return to baseline, and it is becoming more common to administer granulocyte-macrophage-colony stimulating factor (GM-CSF) or G-CSF to speed up recovery, thereby reducing the number of infectious and fatal complications.\textsuperscript{33} Bone marrow examination indicates that approximately 65\% of cases are due to decreased production of neutrophil precursors in the bone marrow, or impaired granulopoiesis.\textsuperscript{33} In other instances, a state of “maturation arrest” is evident in the bone marrow, where neutrophils less mature than myelocytes are preserved, but the effect may also reach to the precursor cells, which is more difficult to detect and depends on the timing of the bone marrow biopsy.\textsuperscript{13, 34} Overall, the underlying mechanism of drug-induced agranulocytosis is not well understood. Clinical observations combined with extensive basic and clinical research suggest that the reaction likely involves the formation of chemically reactive drug metabolites paired with an immune component.

1.2.3.1.1 Risk factors for drug-induced agranulocytosis

A) Metabolic factors

The fact that many drugs with high incidences of agranulocytosis are metabolized by neutrophils to reactive intermediates suggests that this reaction likely involves reactive metabolites of the parent drugs.\textsuperscript{35, 36} Neutrophils generate a variety of oxidative species mediated by the enzymes NADPH oxidase and myeloperoxidase; integral components of the oxidative burst pathway present in these cells (expanded upon in section 1.3.3.2.1). Hypochlorous acid, the strongest oxidant generated by neutrophils, has been shown to metabolize drugs such as clozapine, procainamide, carbamazepine, aminopyrine, and many others, to form reactive intermediates in vitro.\textsuperscript{35, 37} For example, clozapine is converted to a reactive nitrenium ion, amodiaquine to a quinone imine, aminopyrine to a reactive dication, and vesnarinone to an imidoiminium ion (Figure 1-2).\textsuperscript{38} Furthermore, many of these reactive metabolites are able to covalently bind to protein, particularly neutrophil proteins, and in some cases this has been demonstrated in patients.\textsuperscript{39-41} The formation of covalent adducts with either circulating mature neutrophils, or
those present in the bone marrow, could theoretically induce an immune response, leading to the
destruction of neutrophils in the bone marrow and/or the circulation.\textsuperscript{19}

B) Immune mechanisms

Antibodies against various epitopes, including neutrophils, drug-protein complexes, or other
endogenous antigens, have been described in some cases of drug-induced agranulocytosis.\textsuperscript{42}
Aminopyrine-induced agranulocytosis is clearly mediated by drug-dependent anti-neutrophil
antibodies, as demonstrated by the destruction of circulating neutrophils in control patients after
transferring blood from a patient with aminopyrine-induced agranulocytosis.\textsuperscript{12, 22} Anti-drug and
autoantibodies are associated with dipyrone and penicillin-induced agranulocytosis, and some
patients with propylthiouracil-induced agranulocytosis have antibodies that recognize
granulocytes, monocytes, hematopoietic precursor cells, or even neutrophil myeloperoxidase.\textsuperscript{42}
A few immune-related genetic associations have been identified as risk factors for drug-induced
agranulocytosis, such as the increased susceptibility of clozapine-induced agranulocytosis in
patients with certain HLA polymorphisms (i.e. HLA-B38 and DR4 in the Ashkenazi Jewish
population).\textsuperscript{19} Cell-mediated mechanisms have also been proposed for some drugs, suspected to
involve cytotoxic T cells or large granular T lymphocytes. For example, significant infiltration of
large granular T lymphocytes in the bone marrow and peripheral blood of patients with
rituximab-induced agranulocytosis has been reported, and it is thought that these cells may be
responsible for promoting the apoptosis of mature neutrophils.\textsuperscript{19}
1.2.4 Animal models of IDRs

The study of IDRs remains a challenge, as data from patients is often limited to case reports or studies of a retrospective nature, and in vitro experiments are not usually reflective of the complete scenario in vivo. Animal studies have provided insights into the metabolic aspects of these reactions, but the reality is that IDRs are just as idiosyncratic in animals as they are in humans, thereby making it a challenge to study the events leading up to the onset of the reaction. Despite extensive efforts, development of an animal model of drug-induced agranulocytosis has not been met with success; however, the Utrecht research group has reported two models that accurately represent drug-induced skin rash and autoimmunity, and more recently may have generated the first valid animal model of idiosyncratic drug-induced hepatotoxicity.

1.2.4.1 Nevirapine-induced skin rash

Nevirapine is associated with a significant incidence of skin rash, ranging from mild skin reactions to the more serious classification of Stevens-Johnson syndrome. Female Brown Norway rats treated with nevirapine develop a moderately severe skin rash following 2 – 3 weeks of treatment (150 mg/kg/day) that presents with very similar characteristics to the reaction.
in humans.\textsuperscript{43} Rechallenge with the drug leads to a faster onset of a second reaction, and depletion of CD4\textsuperscript{+} T cells in both patients and humans is protective. Furthermore, T cells from both patients and rats with nevirapine-induced skin rash proliferate in vitro in the presence of the parent drug and produce interferon-\(\gamma\) (INF-\(\gamma\)).\textsuperscript{43} Using this animal model, the mechanism of nevirapine-induced skin rash has been studied extensively, and to date numerous findings have been reported. Most importantly, Sharma et al. were able to demonstrate that a reactive benzylic sulfate metabolite formed in the skin is able to covalently bind to skin proteins and is responsible for inducing the skin rash.\textsuperscript{18}

1.2.4.2 D-Penicillamine-induced autoimmunity

D-Penicillamine leads to a variety of autoimmune reactions in patients, and treatment of Brown Norway rats with this drug leads to autoimmunity characterized by dermatitis, vasculitis, arthritis, and weight loss.\textsuperscript{43} The reaction appears after approximately 3 weeks of treatment (20 mg/day), and although there exists strong evidence that this reaction is immune-mediated, rechallenge does not shorten the time to onset. D-Penicillamine does not require metabolism to become chemically reactive, and studies with this animal model revealed that the drug preferentially binds to macrophages and activates them.\textsuperscript{43} The animals that developed autoimmunity had elevated levels of IL-6, IL-17, and IL-22 and increased numbers of T\(_h\)17 cells, a T cell subtype that has been implicated in mediating generalized autoimmune syndromes.\textsuperscript{43}

1.2.4.3 Amodiaquine-induced hepatotoxicity

Drug-induced liver injury has been particularly tricky to study in animals, likely because it is difficult to overcome the dominant response of immune tolerance. Amodiaquine is associated with hepatotoxicity in patients, and both Brown Norway rats and C57BL/6 mice develop liver injury when treated with this drug for 2 – 3 weeks (62.5 or 300 mg/kg/day, respectively). However, with continued treatment in both rodent models, the elevated levels of serum alanine transaminase return to baseline, and the injury resolves with tolerance.\textsuperscript{43} Attempts to overcome this immune tolerance in mice were initially not met with success, but more recently, by targeting two pathways of immune tolerance simultaneously, it appears that this liver injury can be sustained (I. Metushi, unpublished data).
Table 1-2. The mature blood cells

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype</th>
<th>Primary function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td></td>
<td>Bind oxygen in the lungs and deliver it to the tissues</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Mononuclear</td>
<td>T cells – involved in cell-mediated immune responses</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>B cells – involved in antibody-mediated immune responses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NK T cells – involved in innate immune responses</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Initiate innate immune responses and differentiate to tissue-specific macrophages</td>
</tr>
<tr>
<td></td>
<td>Polymorphonuclear</td>
<td>Phagocytose and destroy invading pathogens as part of the innate immune response</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>Combat parasites and some viral infections</td>
</tr>
<tr>
<td></td>
<td>Basophils</td>
<td>Participate in a number of inflammatory reactions, particularly those associated with allergy</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td></td>
<td>Form aggregates to promote clotting of the blood</td>
</tr>
</tbody>
</table>

1.3 Hematology overview

The blood is composed of three types of mature cells: red cells, white cells, and platelets. A brief description of each cell type is provided in Table 1-2.

1.3.1 Structure and function of the bone marrow

The bone marrow, functioning as the primary hematopoietic organ in humans, is located within the central cavities of the axial and long bones. It is responsible for generating the blood cells, including, erythrocytes, granulocytes (neutrophils, eosinophils, and basophils), monocytes, lymphocytes, and thrombocytes.\(^{44, 45}\) In general, the bone marrow can be described as a mixture of hematopoietic islands and adipose cells that are surrounded by an extensive vascular network (sinuses), all contained within a framework of trabecular bone.\(^{44}\) The wide variety of cell types that exist in the marrow are compartmentalized based on their structure and function, classified as either a hematopoietic or stromal subtype.\(^{46}\) The hematopoietic compartment consists of the hematopoietic stem and progenitors cells, which generate billions of blood cells per day that
remain in this compartment until they are mature enough to be released to the circulation. This process is supported by the surrounding bone marrow stroma, mainly comprised of fibroblasts, adipocytes, macrophages, nerves, the venous sinuses. Although the stroma is not directly involved in hematopoiesis, this tissue provides the necessary microenvironment to support the generation and growth of the hematopoietic cells.

1.3.2 Hematopoiesis

Pluripotent stem cells residing in the bone marrow are responsible for generating the blood cells of all lineages, a process of cellular division and differentiation known as hematopoiesis. The pluripotent stem cells are unique in that they are capable of self renewal, but they can also give rise to the multipotent myeloid and lymphoid stem cells, also known as colony forming units (CFUs). Mature progeny arising from the myeloid stem cell include granulocytes, erythrocytes, monocytes, and megakaryocytes, whereas only the T and B lymphocytes are a product of the lymphoid stem cell. These stem cells further differentiate into progenitor cells specific for megakaryocytes (CFU-Meg), erythrocytes (CFU-E), eosinophils (CFU-Eo), basophils (CFU-Ba), and lymphocytes (CFU-L); neutrophils and monocytes share a common progenitor cell (CFU-GM). Generation of the different cell lineages is a highly coordinated process that is strictly regulated by a number of local and peripheral signals, including cytokines, hormones, cell factors, and cellular interactions. Stem cell renewal is largely governed by interaction of stem cell factor, an early-acting hematopoietic growth factor, with the transmembrane KIT receptor (CD117) present on the pluripotent stem cells. The differentiation of stem cells into committed progenitor cells and eventually functional hematopoietic cells, requires different combinations of cell factors and cytokines, including G-CSF, GM-CSF, erythropoietin, thrombopoietin, and IL-11, to name a few. The specific requirements for each cell type are best described in Figure 1-3, and the details for neutrophils will be expanded on below.
1.3.3 Neutrophils

Neutrophils are essential contributors to innate immune responses, primarily as phagocytes that engulf and destroy invading pathogens. In humans, mature neutrophils are the most abundant type of white blood cell present in the circulation (circulating pool), but a large store of mature cells also exists in the bone marrow (marrow reserve), as well as transiently arrested within the narrow blood capillaries (marginated pool). In the presence of an inflammatory stimulus, the stored neutrophils are released rapidly into the circulation, and by the process of chemotaxis, circulating neutrophils enter the tissues and migrate to the site of infection.
Neutrophils are a type of granulocyte; they contain granules in their cytoplasm that are full of enzymes and toxic proteins that aid them in pathogen destruction. The contents of the granules are impermeable to the granule membrane, and therefore can only act on their target upon release into the phagocytic vesicle, or secretion from the cells to act extracellularly. The primary granules, or azurophilic granules, are the most potent type, and are characterized by the presence of myeloperoxidase. As expanded on below, myeloperoxidase is responsible for converting hydrogen peroxide to the strong oxidant hypochlorous acid in an oxygen-dependent manner. Primary granules also contain a variety of other proteins to help destroy pathogens, many which are not dependent on molecular oxygen, such as defensin, azurocidin, lysozyme, and bacterial permeability-increasing protein. The three other types of granules, secondary, tertiary, and secretory, all contain a variety of enzymes and proteins that either target bacterial processes necessary for survival, or alternatively aid the neutrophil in transiting through the tissues.

1.3.3.1 Neutrophil development

The production of neutrophils occurs in the bone marrow and begins with the differentiation of a hematopoietic stem cell into a common myeloid progenitor cell that becomes committed to the neutrophilic lineage under the influence of G-CSF. The earliest definable cell of the neutrophil lineage is the myeloblast, which is still capable of proliferating and has very few cytoplasmic granules. Promyelocytes are larger than myelocytes, and this is the stage at which the primary granules, containing large quantities of peroxidase, are formed in the cytoplasm. Promyelocytes are also capable of cell division, as are their more mature descendants, the myelocytes. The development of secondary granules characterizes the myelocytes, whereas primary granule formation ceases. Therefore, with each subsequent division of the promyelocytes and myelocytes, the number of primary granules decreases. Since the myeloblasts, promyelocytes, and myelocytes are all capable of proliferating, these cells are classified as the mitotic pool of neutrophil precursors within the bone marrow. In humans, it takes approximately 5 days for a neutrophil precursor to transit through the mitotic pool. The first cell of the non-mitotic pool, the metamyelocyte, is no longer capable of dividing, and at this stage the tertiary granules form. Metamyelocytes differentiate into band cells, which are characterized by an indented nucleus, followed by fully mature polymorphonuclear neutrophils, with nuclei containing two or more lobes connected by filamentous strands (Figure 1-4). The average time for a neutrophil precursor to transit through the post-mitotic pool in humans is 4 – 6 days.
1.3.3.2 Neutrophil recruitment and activation

Upon maturity, the majority of neutrophils are released to the peripheral blood, where it is generally accepted that they freely circulate for a very short time (less than 24 hours) before being cleared by the liver, spleen, or bone marrow. It is important to note that recent studies have reported much longer neutrophil life spans of 5.4 days in humans; however the mathematical modeling employed has been challenged by others and remains a topic of debate. Neutrophils generally circulate in an unactivated state, surveying the periphery diligently for signs of harmful invaders. Most microorganisms enter the body through the mucosa of the respiratory system or the gut, and therefore circulating neutrophils require notification that their services are required at the site of infection. Macrophages present in the submucosal tissues are most often the primary cells to encounter pathogens, and upon their activation they secrete a
variety of cytokines and chemokines to promote neutrophil recruitment. For instance, the chemokine CXCL8 induces neutrophils to exit the peripheral blood. In parallel, the cytokine tumour necrosis factor (TNF)-α activates the vascular endothelium and increases vascular permeability, thereby making it easier for neutrophils to exit the bloodstream. The act of neutrophils crossing the vascular endothelium to enter the tissues is called extravasation, and begins with the TNF-α-induced endothelial upregulation of P-selectin and E-selectin. These selectins interact reversibly with P-selectin glycoprotein ligand (PSGL)-1 and L-selectin (CD62L) on the circulating neutrophils, causing them to slow down and begin rolling along the vessel wall. This tethering allows the adhesion molecules ICAM-1 and ICAM-2 on the endothelium to firmly bind the neutrophil β2 integrins (i.e. CD11b/CD18) and effectively immobilize the cells. Neutrophils cross the vessel wall by passing through the endothelial cell junctions, and it is speculated that they penetrate the basement membrane with the aid of digestive enzymes. Finally, the cells are released into the interstitial fluid where they migrate along chemotactic gradients towards the site of infection, mediated by a combination of host (i.e. IL-8) and pathogen-associated (i.e. fMLP) inflammatory stimulants.

In the process of tissue recruitment, neutrophils encounter a number of activating factors that prime them for prompt destruction of pathogens upon arrival at their destination. Neutrophils are initially transformed into their activated state upon the interaction of PSGL-1 and L-selection with the endothelial selectins. Next, engagement of the β2 integrins, combined with continuing stimulation from inflammatory cytokines and chemoattractants, primes the neutrophil by initiating the neutrophil oxidative burst. Once in the tissues, as the neutrophil follows the chemotactic gradient, the chemoattractants in turn bind to receptors on the cell surface, prompting the assembly of the components of the oxidative burst pathway. In addition, recognition of various pathogen-associated molecular patterns (i.e. lipopolysaccharide, flagellin) through neutrophil Toll-like receptors further induces the oxidative burst. Upon arrival at its end target, the neutrophil is in its highest activated state, primed to eliminate pathogens through phagocytosis, degranulation, and the release of extracellular traps (Figure 1-5).
1.3.3.2.1 The oxidative burst

The neutrophil oxidative burst is a pathway in which molecular oxygen is converted into a variety of reactive oxygen species, through the activation of NADPH oxidase and the peroxidase activity of myeloperoxidase. The first step in this pathway involves the assembly of NADPH oxidase into its active form. Extensive research has demonstrated that the NADPH oxidase is comprised of two oxidase-specific cytosolic proteins (p47phox and p67phox), a low molecular weight GTPase (Rac1/2), as well as a membrane bound flavocytochrome b (a heterodimer of p22phox and gp91phox). Two other proteins, the cytosolic p40phox and GTPase Rap1A, also contribute to the regulation of NADPH oxidase activity, but their definitive roles have not yet been identified. Upon activation of the neutrophil, phosphorylation of the p47phox subunit in the cytosol leads to migration of the entire cytosolic complex to the cell membrane, where it associates with flavocytochrome b, forming the active oxidase. The distinct compartmentalization of the enzymatic components ensures that in the resting cell the oxidase remains inactive, resulting in the necessary tight regulation of oxidant production. Active NADPH oxidase catalyzes the oxidation of NADPH and univalent reduction of molecular oxygen, leading to the production of superoxide.
Equation 1. \[ \text{NADPH} + 2 \text{O}_2 \rightarrow \text{NADP}^+ + 2 \text{O}_2^- + \text{H}^+ \]

The oxidative burst generates significant amounts of superoxide, which is an important precursor to the formation of a variety of potent reactive oxygen species, as well as reactive nitrogen species.\textsuperscript{62} The next step in the neutrophil oxidative burst pathway involves conversion of superoxide to hydrogen peroxide, either catalyzed by superoxide dismutase or through spontaneous dismutation.

Equation 2. \[ 2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

Hydrogen peroxide is limited in its ability to destroy pathogens and has shown to be bactericidal only at high concentrations.\textsuperscript{64} As such, the neutrophil primary granules contain high concentrations of myeloperoxidase, which efficiently convert hydrogen peroxide into the strong oxidant hypochlorus acid.

Equation 3. \[ \text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \]

Hypochlorus acid is the most potent oxidant produced by neutrophils, cytotoxic to a vast range of pathogens, and in fact, the bulk of hydrogen peroxide produced in neutrophils is consumed by myeloperoxidase to generate this molecule.\textsuperscript{63, 64} Hypochlorus acid is membrane permeable, and therefore can enter microorganisms, where it generates a number of toxic compounds including chloramines, reactive aldehydes, and tyrosyl radicals. In addition, it can oxidize iron centers, react with pyridine nucleotides, and crosslink proteins, just to name a few of its activities.\textsuperscript{63, 64} Despite the efficiency of this strong oxidant, patients with myeloperoxidase deficiencies who cannot produce hypochlorus acid do not seem to have markedly increased susceptibility to infections. In contrast, patients with chronic granulomatous disease who lack functional NADPH oxidase have an increased incidence of infection, indicating that products upstream of myeloperoxidase are able to compensate in the absence of hypochlorus acid.\textsuperscript{60} For example, hydroxyl radicals can be generated by the superoxide-driven Fenton reaction between hydrogen peroxide and a redox-active transition metal, such as iron, but the relevance of this reaction in vivo is controversial. Likely of more importance is the generation of peroxynitrite, through reaction of superoxide with nitric oxide synthase-generated nitric oxide; peroxynitrite is a potent oxidant that is accepted to play an important role in host defense.\textsuperscript{62}
1.3.3.3 Drug metabolism by neutrophils

Although drug metabolism by neutrophils is unlikely to contribute significantly to the overall clearance of drugs, the ability of these cells to generate metabolites of xenobiotics, particularly those that are reactive, is important in the context of toxicology and IDRs. The cytochrome P450 enzymes are the most abundant and most common enzymes associated with drug oxidation, although they are mainly concentrated in the liver. A number of cytochrome P450 isoforms have been detected in leukocytes, but in general the expression levels are between 100 – 10 000-fold lower than that measured in the liver. Specifically, the CYP 3A5 isoform has been identified in human neutrophils, as well as CYP 4F3. On the other hand, peroxidases are more commonly found in neutrophils, and also possess some drug metabolizing capacity, particularly the ability to oxidize electron-rich molecules. The catalytic cycle of peroxidases first involves the oxidation of the enzyme by a hydroperoxide to a ferryl iron-oxo species that contains a radical cation on the porphyrin ring, referred to as Compound I. Compound I can
oxidize substrates in two ways: a) a direct two-electron oxidation to regenerate the native enzyme; or b) a two step process involving single electron oxidations, where the porphyrin radical cation is first reduced (Compound II), followed by regeneration of the native peroxidase (Figure 1-7). Prostaglandin H synthase is one peroxidase present in neutrophils with metabolic capabilities. The enzyme first oxidizes arachidonic acid to prostaglandin G, which is a hydroperoxide. Secondly, it converts prostaglandin G to prostaglandin H, and in the process of hydroperoxide reduction, drugs can be oxidized. Myeloperoxidase is the most abundant peroxidase in the neutrophil, and in the case of this enzyme, the major substrate is chloride ion, which undergoes the direct two-electron oxidation and utilizes only Compound I. This process generates the very strong oxidant hypochlorus acid, that has been shown to metabolize numerous drugs to reactive species, such as clozapine, amodiaquine, aminopyrine, and vesnarinone.

Figure 1-7. The catalytic cycle of peroxidases (adapted from Uetrecht and Trager)

1.4 Topics in immunology

The basic tasks of the immune system, to recognize, contain, and eliminate infection, although simple in their description, involve a highly orchestrated and complex network of cells, vessels, and organs. It is suspected that drug-induced agranulocytosis and IDR, in general, involve an immune component, and therefore only a few relevant topics in immunology will be reviewed below.

1.4.1 Innate immunity

The primary response to infection involves the rapid action of a variety of innate immune cells to contain and eliminate invading pathogens. Macrophages, present in almost all tissue types, are
considered the first line of defense of the innate immune system, as they are often the first cell type to encounter pathogens in the periphery. Upon detecting the presence of an invading organism, macrophages engulf and destroy the culprit. This is followed by the secretion of an array of signaling proteins (i.e. cytokines and chemokines) to recruit neutrophils and monocytes to the site of infection to aid in pathogen destruction.\(^\text{51}\) Dendritic cells are another type of innate phagocyte; however, they are primarily involved in antigen presentation to T cells, providing a key link between the innate and adaptive immune responses. These cells are constantly surveying their surroundings for the presence of pathogens by macropinocytosis, or directly engulf pathogens by receptor-mediated phagocytosis. Once activated, dendritic cells migrate to the peripheral lymphoid organs, where they encounter and activate lymphocytes via presentation of the pathogen antigen on its cell surface (this topic will be expanded on below).\(^\text{51}\) Natural killer T cells (large granular lymphocytes) are the only lymphoid cells of the innate immune response and are responsible for nonspecific killing of abnormal cells, such as tumour cells, or cells infected by viruses. These cells do not require activation and carry out their function by releasing granzymes that induce apoptosis.\(^\text{51}\) The last cell type of the innate immune system is the mast cell, which are primarily located at the boundaries between the body and the external environment, for example, the skin. In general, mast cells are involved in allergic responses, wound healing, and pathogen defense.\(^\text{51}\)

1.4.1.1 The NLRP3 inflammasome

Cells of the innate immune system utilize an array of inherent pattern-recognition receptors to detect the presence of pathogen-associated molecular patterns associated with nonself materials. One specific type of pattern-recognition receptor is the NOD-like receptor (NLR), which in addition to recognizing pathogen-associate patterns, can also respond to host-derived danger signals, or danger-associated molecular patterns.\(^\text{69}\) These receptors have recently been shown to assemble into highly complex molecular entities called inflammasomes, which are implicated in generating caspase-1-mediated inflammatory responses. To date, a number of different types of inflammasomes have been described, each displaying a distinct profile of pathogens and molecules that activate them. The NLRP3 inflammasomes are activated by the widest array of stimulants, including whole pathogens, peptide aggregates, bacterial toxins, and environmental stimulants, and therefore appear to be most relevant to the study of IDRs.\(^\text{69, 70}\) In the hematopoietic system, the NLRP3 inflammasome is expressed most extensively in dendritic cells
and monocytes, is highly inducible in macrophages, and is present at lower levels in neutrophils.\textsuperscript{71}

When the NLRP3 comes into contact with either a pathogen or damage-associated molecular pattern, this triggers a complex activation cascade leading to the assembly of a functional inflammasome. The first step involves oligomerization of the NLRP3 through interaction of its NACHT domains. This exposes the pyrin domain (PYD) on NLRP3, allowing it to associate with the PYD of the apoptosis-associated specklike protein (ASC). ASC also contains a caspase-associated recruitment domain (CARD), which is responsible for engaging the CARD domains of two pro-caspase 1 monomers. By bringing the pro-caspase 1 monomers into close proximity, this leads to self-cleavage and the generation of active caspase 1. Finally, this enzyme converts pro-IL-1\(\beta\) or pro-IL-18 into IL-1\(\beta\) and IL-18, and promotes their release from the cell (Figure 1-8). Cytokines of the IL-1 family are highly proinflammatory, and serve to recruit and activate other immune cells, such as neutrophils.\textsuperscript{69, 70}

\textbf{Figure 1-8. NLRP3 inflammasome activation}\textsuperscript{72}

(Permission has been granted by the Nature Publishing Group to include this image in this thesis.)
1.4.2 Adaptive immunity

Adaptive immunity is characterized by antigen-specific immune responses, classified as either cell-mediated or humoral, and involves the coordinated actions of a number of different lymphocytes. In the absence of infection, the majority of lymphocytes circulate through the blood and lymphatic system as small featureless cells with no specific function. However, in the presence of infection, both T and B lymphocytes are signaled to transform into a variety of effector cells that are directly responsible for host defense.51

Cell-mediated immune responses involve the action of a number of different T lymphocytes, which mainly detect the presence of pathogens inside host cells. The cytotoxic T cells are specific to virally infected cells and can recognize an invasion due to viral antigens presented on the host cell surface in the context of MHC I. Cytotoxic T cells directly destroy the infected cells by activating caspases that cleave both host and viral DNA. These cells can be identified from other types of T cells due to the expression of the cell surface protein CD8.51 The second major class of T lymphocytes is the T helper cells, which for the purposes of this review can be broadly divided into the subtypes T\textsubscript{h}1, T\textsubscript{h}2, and T\textsubscript{h}17. Naïve CD4\textsuperscript{+} T cells that encounter antigens in the context of MHC II on antigen presenting cells (dendritic cells or macrophages) expand and differentiate into the required T helper subtype. T\textsubscript{h}1 cells are the primary subtype involved in cell-mediated immune responses, and upon activation, they generally release cytokines to aid the inflammatory response and stimulate macrophages to increase their destructive efficiency.51 On the other hand, T\textsubscript{h}2 cells are entirely dedicated to activating B cells, which will be expanded on below. T\textsubscript{h}17 cells were more recently identified, and appear to play an important role in host defense against extracellular pathogens that are not effectively cleared by T\textsubscript{h}1/T\textsubscript{h}2 responses. These cells are highly proinflammatory and release cytokines (mainly IL-17) that recruit neutrophils, and also act on a broad range of other cell types to induce the expression of a variety of proinflammatory mediators.73 The last T cell type associated with cell-mediated responses is the T regulatory cell. These cells suppress the activity of other lymphocytes and thereby help moderate immune responses. They are important in maintaining homeostasis and tolerance to self-antigens, and in a very simplified description, exist as both CD4\textsuperscript{+} and CD8\textsuperscript{+} populations.74

Humoral, or antibody-mediated immunity, is dependent on the ability of B lymphocytes to recognize soluble antigens or extracellular antigens present on the surface of pathogens. This
leads to receptor-mediated endocytosis and presentation of the antigen on the surface of the B cell in the context of MHC II. Interaction of an effector T\(_h2\) cell through its T cell receptor with the antigen on the B cell leads to proliferation and differentiation of the B cells into plasma cells. Plasma cells synthesize and secrete antibodies with identical antigen specificity as the B cells, and these antibodies aid in the removal of pathogens through neutralization, opsonization, and complement activation.\(^{51}\) Humoral immune responses are most often associated with immune memory because B cells often differentiate into memory cells that will readily respond upon a second exposure to the specific antigen. Similarly, some T cells are also able to differentiate into memory cells; however, there is evidence of some adaptive immune responses do not have any immune memory, particularly cases involving idiosyncratic adverse drug reactions.\(^{13}\)

### 1.4.3 Peripheral immune tolerance

The ability of the immune system to regulate and resolve immune responses is important in minimizing damage to the host and is governed by a fine balance between proinflammatory and tolerizing actions. T regulatory cells are the classic immune cell responsible for suppressing excessive immune responses and mediating immune tolerance.\(^{74}\) Dendritic cells also contribute to immune tolerance; particularly through their role as antigen presenting cells and their ability interact with T cells. Dendritic cells exist in an immature state in the absence of inflammation, yet they are constantly surveying their environment for the presence of pathogens and encounter numerous nonpathogenic antigens. In the process, these immature cells become loaded with antigen and are able to silence responsive T cells, or alternatively induce regulatory T cells to suppress the immune response.\(^{75}\) On the other hand, natural killer T cells have been implicated in tolerizing certain myeloid antigen presenting cells, restricting their abilities to induce immune responses.\(^{76}\) Recently, a population of macrophages was identified, the alternatively activated macrophages, that appear to focus primarily on contributing to wound healing and resolving inflammation. These macrophages arise from activation by IL-4 and IL-13, cytokines that arise from T\(_h2\)-type responses to parasitic and extracellular pathogens. These unique cells are believed to play a role in immune tolerance.\(^{77}\)
1.5 Specific drugs that cause agranulocytosis

1.5.1 Clozapine

Clozapine (Clozaril), a dibenzodiazepine derivative, is an antipsychotic agent used to manage the symptoms of treatment-resistant schizophrenia. It was one of the first effective antipsychotic agents devoid of extrapyramidal side effects (i.e. acute dystonia, Parkinsonian syndrome), and demonstrates great efficacy in patients that develop resistance to other neuroleptic agents.\(^7\) Clozapine is classified as an atypical antipsychotic because it exhibits a different neurotransmitter receptor binding profile than the conventional or typical antipsychotics. Specifically, clozapine has a high affinity for the D\(_1\) and D\(_4\) dopamine receptors, but a low affinity for D\(_2\), in contrast to typical agents that target D\(_2\).\(^7\) Furthermore, clozapine has a high affinity for a broad range of serotonin (5-HT) receptors, most importantly, strong antagonism of the 5-HT\(_2\) receptor.\(^7\) These factors combined are believed to be responsible for the decrease in extrapyramidal side effects seen with clozapine compared to typical agents. However, the major drawback with clozapine is that it causes potentially life-threatening agranulocytosis in approximately 0.5 – 1.0 % of patients during the first 3 – 6 months of treatment.\(^8\) Clozapine was first introduced to the clinic in the early 1970s, but a series of deaths in Europe, later attributed to agranulocytosis, led to its prompt withdrawal from the U.S. market in 1974. Due to the unique ability of clozapine to effectively treat patients who had developed resistance to conventional antipsychotics, it was in fact reapproved in 1990 by the FDA.\(^7\) However, this approval came with strict prescribing guidelines, and to date, a patient must have failed on two prior antipsychotics before being considered for clozapine therapy. Furthermore, neutrophil counts must be monitored weekly in order to minimize the risk of agranulocytosis. Clozapine remains an important and effective treatment option for patients suffering from schizophrenia, and recent studies have indicated that it reduces the risk of suicide in this highly susceptible patient population.\(^8\)

1.5.1.1 Drug metabolism and pharmacokinetics

In order to minimize the side effects associated with commencing clozapine therapy, it is initially administered at very low doses (25 – 50 mg/day) and titrated up slowly to daily doses of 300 – 600 mg.\(^7\) Clozapine plasma concentrations range from 100 – 1000 ng/mL, and a concentration of 350 ng/mL is suspected to be the threshold level for efficacy.\(^7,7\) Clozapine is well absorbed
(90 – 95%), but it is subject to a high first-pass metabolism, resulting in an absolute bioavailability of about 50 – 60%. Protein binding is high (95%), and peak blood concentrations occur quickly from 30 min to 4 h. Clozapine is extensively metabolized in the liver prior to excretion, primarily by CYP 1A2, as well as CYP 3A4. The des-methyl metabolite is the only active metabolite of clozapine; however, its activity is much weaker and the duration of action is much shorter.

1.5.2 Amodiaquine

The 4-aminoquinoline antimalarial agent, amodiaquine, is currently a central component of artemisinin combination therapy, which is presently one of the most globally employed antimalarial therapies. Amodiaquine has been particularly useful in treating patients with low level chloroquine-resistant \textit{P.falciparum} infections. However, in the mid 1980s a number of serious adverse drug reactions, including agranulocytosis and hepatotoxicity, were reported in travelers that were utilizing amodiaquine prophylactically long term. In response to these events, the manufacturer removed prophylaxis as an indication on the labeling, and the World Health Organization withdrew the drug from its malarial control programs. The development of parasite resistance to the antimalarial chloroquine brought about the emergence of artemisinin combination therapies. Artemisinin and its derivatives have potent antiparasite properties, but due to their extremely short half lives, are more effective when paired with an antimalarial that has slower elimination properties. As a result, amodiaquine has seen a revival in its use, administered at very low doses (10 mg/kg) in combination with artesunate, a derivative of artemisinin. The risk of developing an IDR to amodiaquine at these doses is quite low, and even when considering the data from its previous prophylactic use, the risk of agranulocytosis was 1:2000 and 1:30 000 for hepatotoxicity. The mechanism of action is still not well understood, although it is thought to inhibit heme polymerase activity in the parasite, leading to the accumulation of free heme, which is toxic to the parasite.

1.5.2.1 Drug metabolism and pharmacokinetics

Upon oral administration, amodiaquine is rapidly absorbed, reaching peak concentrations about 30 min following the dose. Amodiaquine is subject to a high first pass metabolism in the liver, and is extensively oxidized by CYP 2C8 to a pharmacologically active metabolite desethylamodiaquine. Amodiaquine has a short terminal half-life of about 5 h, and after
administering doses of 10 mg/kg, plasma concentrations of the drug in humans range between 300 – 680 ng/mL.\(^\text{84}\) Although amodiaquine is three times more potent than its desethyl metabolite, the plasma concentrations of the parent drug are low.\(^\text{82}\) On the other hand, the elimination half-life of desethylamodiaquine ranges from 3 – 12 days, so although it may be less potent, the long therapeutic effect of this metabolite is, in fact, responsible for most of the antimalarial activity.\(^\text{82}\) It is suspected that the long half-life of desethylamodiaquine is likely associated with adverse reactions that develop during prolonged usage of amodiaquine.

1.6 Research focus and rationale

As described in detail in the Introduction, the mechanisms of IDR s are not well understood, particularly those that affect the hematopoietic system. It is suspected that drug-induced agranulocytosis is induced by the combination of reactive metabolites and immune activation; therefore, it was the purpose of this body of work to: a) characterize the involvement of the NADPH oxidase/myeloperoxidase system in the covalent binding of drugs that cause agranulocytosis to neutrophils, and; b) study the response of the neutrophils and the overall immune system to clozapine at the start of treatment.

It was previously hypothesized that myeloperoxidase-generated hypochlorus acid was responsible for the covalent binding of drugs that cause agranulocytosis to neutrophils, due to the strong oxidative nature of the compound and presence within the cells that are the end target of the reaction. This proposal was supported by extensive in vitro evidence, as well as a few reports of drugs binding to neutrophils in patients.\(^\text{38, 41}\) However, the mechanism of this binding was not demonstrated in vivo, and the fact that patients with myeloperoxidase deficiency are not protected from developing clozapine-induced agranulocytosis challenges this hypothesis. With the availability of relevant mouse knock out strains, we set out investigate this hypothesis using clozapine and amodiaquine as test compounds (Chapter 2).

Due to the fact that clozapine is so effective at treating cases of treatment-resistant schizophrenia, its prescribing rate is often higher than other drugs that cause agranulocytosis. Furthermore, the requirement for patients to have weekly blood counts makes it easier on both the patient and investigator to collect study samples. Our initial plan was to further characterize
the immune response to clozapine at the start of treatment, as it has been demonstrated that clozapine induces a proinflammatory state in the majority of patients at the start of treatment.\textsuperscript{85} Unfortunately, due to a number of factors, we were not able to recruit any patients to complete this work. Instead, we set forth to expand on the studies of Iverson et al. that investigated the effect of clozapine on neutrophil kinetics in rabbits,\textsuperscript{86} by designing a rat model of early clozapine treatment and characterizing the response of the neutrophils (Chapter 3). Findings in this model led us to investigate the ability of clozapine to induce a proinflammatory response at the start of treatment, specifically the ability of clozapine to activate inflammasomes (Chapter 4). These studies combined have led us to propose a novel hypothesis, stating that the proinflammatory response to clozapine at the start of treatment is mediated by inflammasome-generated IL-1β.
CHAPTER 2

Involvement of myeloperoxidase and NADPH oxidase in the covalent binding of amodiaquine and clozapine to neutrophils: Implications for drug-induced agranulocytosis


All work was completed by Alexandra R. Lobach in this chapter.
2.1 Abstract

Amodiaquine (AQ) and clozapine (CLZ) are associated with a relatively high incidence of idiosyncratic agranulocytosis, a reaction that is suspected to involve covalent binding of reactive metabolites to neutrophils. Previous studies have shown that both AQ and CLZ are oxidized to reactive intermediates in vitro by activated neutrophils or by the combination of hydrogen peroxide and myeloperoxidase (MPO). Neutrophil activation leads to an oxidative burst with activation of NADPH oxidase and the production of hydrogen peroxide. However, the importance of this pathway in covalent binding in vivo has not been examined. In this study, we found that the binding of both AQ and CLZ to neutrophils from MPO knockout mice ex vivo decreased approximately 2-fold compared to neutrophils from wild-type mice, whereas binding to activated neutrophils from gp91 knockout (NADPH oxidase null) mice decreased 6 – 7-fold. When the AQ studies were performed in vivo, again the binding was decreased in MPO knockout mice to about 50% of the binding in wild-type mice; however, covalent binding was significant in the absence of MPO. Surprisingly, there was no significant decrease in covalent binding of AQ to neutrophils in vivo in gp91 knockout mice. In addition, there was extensive binding of AQ to many types of bone marrow cells and to peripheral lymphocytes. These results indicate that MPO is not the only neutrophil enzyme involved in the oxidation of AQ and that NADPH oxidase is not the major source of peroxide. There was also no decrease in AQ binding to neutrophils in COX-1 or COX-2 knockout mice. We were not able to readily reproduce the AQ in vivo studies with CLZ because of its acute toxicity in mice. These are the first studies to examine the enzymes involved in the bioactivation of AQ by neutrophils in vivo.

2.2 Introduction

The agranulocytosis associated with the use of clozapine (CLZ) and amodiaquine (AQ) is classified as an idiosyncratic adverse drug reaction. Although drug-specific incidence rates of idiosyncratic drug-induced agranulocytosis are generally less than 1%, the life-threatening infections that follow the drop in neutrophil counts put patients at great risk. The mechanism of drug-induced agranulocytosis is not well understood, but like many idiosyncratic adverse drug reactions, it is suspected to involve an immune mechanism. Bioactivation of these drugs to
reactive intermediates is likely key to the reaction, and according to the hapten hypothesis, the covalent binding of these metabolites to endogenous proteins could stimulate an immune response that may ultimately be responsible for causing the adverse reaction. The metabolism of numerous drugs associated with relatively high incidences of agranulocytosis has been studied extensively in vitro, and it was previously determined that these drugs are commonly metabolized by components of the neutrophil oxidative burst pathway and myeloperoxidase (MPO) to reactive species. In particular, MPO can produce hypochlorous acid, which is a very strong oxidant that is capable of oxidizing these drugs to reactive intermediates. Further studies have shown that activated neutrophils can metabolize drugs such as CLZ and AQ to reactive intermediates in vitro, which in turn covalently bind to neutrophil proteins. Moreover, binding of CLZ to the neutrophils of patients taking the drug was detected in vivo. The ability of neutrophils to generate reactive metabolites via the NADPH/MPO pathway could explain why they are the target of this adverse reaction. However, if neutrophil activation leading to an oxidative burst is required for covalent binding in vitro, then it is unclear if the degree of neutrophil activation in vivo would be sufficient to explain the extent of CLZ covalent binding that was observed to neutrophils from patients taking the drug. In addition, neither decreased MPO nor decreased NADPH oxidase activity in humans is associated with a decreased risk of CLZ-induced agranulocytosis. With the availability of knockout animals, we set out to definitively test the roles of MPO and NADPH oxidase in the bioactivation of AQ and CLZ.

2.3 Materials and Methods

2.3.1 Chemical materials

AQ-hydrochloride was purchased from Ipca Laboratories Ltd. (Mumbai, India), and CLZ was generously provided by Novartis Pharmaceuticals Inc. (Dorval, QC). Ammonium chloride, anhydrous sodium sulfate, bovine serum albumin (BSA), Dextran-500, EDTA, horseradish peroxidase (HRP), manganese dioxide, sodium dodecyl sulfate (SDS), Trizma base, and 2-iminothiolane (2-IMT) were purchased from Sigma-Aldrich (St. Louis, MO). Potassium bicarbonate, sodium chloride, Tween-20, and 3,3',5,5'-tetramethylbenzidine (TMB) were bought from BioShop Canada Inc. (Burlington, ON), and blue carrier protein (BCP), the lane marker reducing sample buffer, and SuperSignal West pico chemiluminescent substrate were purchased
from Pierce Biotechnology (Rockford, IL). Acrylamide/bis solution (30%), nitrocellulose membrane (0.2 μm), and nonfat blotting-grade milk powder were obtained from BioRad (Mississauga, ON). Hanks’ balanced salt solution (HBSS), PBS (pH 7.4) and α-modified Eagle’s medium (α-MEM) were purchased from Gibco (Life Technologies Inc., Burlington, ON). Hydrogen peroxide was obtained from EMD Chemical Inc. (Gibbstown, NJ). Antibodies were obtained from the following companies: HRP-conjugated goat-anti-rabbit IgG (H+L chains) was from Cedarlane (Burlington, ON), HRP-conjugated goat-anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA), and monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Sigma.

2.3.2 Preparation of the AQ-BSA protein conjugate

The synthesis of the AQ-immunogen is described in Scheme 1. The first step was to convert AQ to its reactive quinone imine (AQQI). AQ (0.4 g) was dissolved in 90 mL of chloroform over anhydrous sodium sulfate (0.1 g) and oxidized by manganese dioxide (1 g) for 30 min at room temperature. The production of AQQI was confirmed by LC/MS analysis using a PE Sciex API 3000 quadrupole system with an electrospray ionization source (Sciex, Concord, ON) interfaced with a HPLC system (Shimadzu, Columbia, MD) and a Zorbax 300SB-C18 5 μm 4.6 × 150 mm column (Agilent, Mississauga, ON). The second step was to add terminal sulfhydryl groups to BSA. The thiolating agent, 2-IMT, was dissolved in distilled water and reacted with BSA in 50 mM sodium phosphate buffer, pH 8.0, for 1 h at room temperature. The optimal ratio for the overall reaction was 3:1, 2-IMT to the number of lysine groups available for conjugation on BSA (30 – 35 groups), which yielded the maximal addition of 19 2-IMT groups to BSA. The number of sulfhydryl groups added to BSA was quantified using Ellman’s reagent (Pierce) and confirmed by mass spectrometry (10 – 19 of the 2-IMT groups added, depending on the reaction). Mass spectrometry was carried out by the Advanced Instrumentation for Molecular Structure Laboratory at the University of Toronto using an AB/Sciex QStar mass spectrometer with an ESI source and an Agilent 1100 capillary LC system with a desalting column. The thiolated protein was immediately reacted with AQQI to form the AQ-BSA conjugate. AQQI reconstituted in dimethylformamide was added to the 2-IMT-BSA solution (2:1, AQQI/sulfhydryl groups) and mixed vigorously for 1 h at room temperature followed by overnight dialysis against distilled water at 4 °C. Products from the thiolation and conjugation reactions were analyzed by SDS-PAGE to confirm the increase in protein mass. The theoretical
mass ranges of thiolated BSA and AQ-BSA were calculated on the basis of the number of free thiols measured with Ellman’s reagent. The measured mass ranges from the gels for both proteins were found to correspond with the theoretical masses, confirming the reaction of AQQI with the sulphydryl groups on BSA.

Scheme 1. Chemical synthesis of the AQ-BSA conjugate

2.3.3 Production of the anti-AQ antiserum

Polyclonal antibodies against AQ were raised in a female New Zealand white rabbit (Charles River, St. Constant, QC) by the Division of Comparative Medicine at the University of Toronto according to the experimental protocols approved by the University of Toronto Animal Care Committee. Briefly, the initial immunization contained 400 µg of protein conjugate mixed with Freund’s complete adjuvant. The first and second boosts were administered at 3 and 6 weeks and contained 250 µg of protein conjugate mixed with Freund’s incomplete adjuvant. Blood was collected 2 weeks after each boost (titers 1 and 2, respectively), and the terminal titer was collected 3 weeks after the final boost.
2.3.4 Anti-AQ antibody ELISA

Nunc MaxiSorp ELISA plates (Bethyl Laboratories, Montgomery, TX) were coated overnight with antigen, AQ-BCP, at 4 °C. AQ-BCP was produced using a similar reaction scheme as described for AQ-BSA except that the 2-IMT ratio was increased to 34:1. All of the following steps were carried out at room temperature. Plates were washed three times with wash buffer (50 mM Tris-HCl, 0.05% Tween-20, pH 7.5) and blocked for 1 h with 0.1% casein (Pierce). Following three washes, anti-AQ antiserum samples were serially diluted 2-fold in wash buffer with 0.1% casein and incubated against the antigen for 1 h. Plates were washed four times and incubated for 1 h with goat-anti-rabbit IgG HRP (diluted 1:20 000 with wash buffer, 0.1% casein). Following five washes, TMB was added to the plates and incubated for 5 – 10 min, after which the reaction was stopped with 1 N HCl. The absorbance was read at 450 nm using a SpectraMax Plus 384 microplate reader with SoftMax Pro 5 software (Molecular Devices, Sunnyvale, CA). Furthermore, the specificity of the antibodies for AQ in the terminal titers was evaluated by adding a preincubation step of the AQ antiserum with antigen before loading it on the plate. This preincubation was carried out for 30 min using AQ, AQ-BCP, and BCP as antigens to block the binding of the AQ antibodies to the AQ-modified proteins. Antigen stocks were prepared at 1 mg/mL in distilled water and serially diluted 10-fold with antiserum (diluted 1:20 000 with wash buffer, 0.1% casein). All samples were analyzed in triplicate.

2.3.5 Neutrophil and lymphocyte isolation from humans

Venous blood was collected from healthy male and female volunteers into heparin-coated vials, as approved by the University of Toronto Research Ethics Board. Neutrophils were isolated as previously described\textsuperscript{87} with the following modifications: leukocyte separation using 3% Dextran-500 was carried out for 15 min, after which the supernatant was carefully layered on top of cold Ficoll-Paque (ρ =1.077 g/mL, GE Healthcare, Waukesha, WI) and centrifuged at 610g for 20 min at 15 °C. Contaminating erythrocytes were removed by adding red cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 10 min. Isotonicity was restored with excess PBS, and the cell suspension was centrifuged at 500g for 5 min at 4 °C. The neutrophils were washed and resuspended in HBSS. Lymphocytes were isolated from blood using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer’s instructions.
2.3.6 Neutrophil and lymphocyte isolation from rodents

Neutrophils were isolated from the blood of rats and the bone marrow of both rats and mice following CO₂ euthanasia. Heparin anticoagulated venous blood from rats was mixed with an equal volume of 3% Dextran-500, and the erythrocytes were allowed to settle for 20 min. The supernatant was centrifuged at 610g for 10 min at 4 °C and resuspended in α-MEM. Femurs and tibias were obtained from rats and mice, and the bone marrow was flushed out with cold α-MEM and centrifuged at 750g for 5 min at 4 °C. Both blood and bone marrow α-MEM suspensions were layered onto discontinuous Percoll (GE Healthcare) gradients of 80/65/55%. Samples were centrifuged at 750g for 30 min at 4 °C, and the neutrophils were collected at the 80/65% interface. Contaminating erythrocytes were removed with red cell lysis buffer, and the neutrophils were washed and resuspended in HBSS. Lymphocytes were isolated from heparin anticoagulated rat blood using Lymphoprep according to the manufacturer’s instructions. Trypan blue exclusion showed the viability to be > 75% for both rodent and human cells used in the ex vivo incubations. For some preparations, a slide of cells was prepared and stained with Giemsa-Wright-like stain to confirm that > 85% of cells had characteristic neutrophil or lymphocyte morphology.

2.3.7 Covalent binding of AQ to leukocytes and erythrocytes ex vivo

Intact neutrophils and lymphocytes (4.5 × 10⁶ cells in HBSS) were incubated ex vivo with 10 µM AQ. Neutrophils were activated by adding 40 ng of phorbol 12-myristate-13-acetate (PMA, Sigma). Human and rodent heparin anticoagulated blood samples (3 mL) were also incubated ex vivo with 10 µM AQ. Following the incubation, blood was centrifuged at 500g for 10 min to separate the erythrocytes from the leukocytes in the buffy coat. All incubations were for 45 min at 37 °C. All cell types were washed with PBS or HBSS to remove unbound AQ and then lysed with 1× cell lysis buffer (Cell Signaling Technologies, Pickering, ON) containing 1× Halt protease inhibitor cocktail (Pierce).

2.3.8 Treatment of animals with AQ and CLZ

All rodents were acclimatized to a 12/12 h light/dark cycle at 22 °C for a minimum of 1 week prior to starting AQ treatment and were given regular access to rodent meal (Harlen Teklad, Madison, WI) and tap water ad libitum. The experimental protocols were approved by the University of Toronto Animal Care Committee. (A) Female Sprague-Dawley rats (200 – 250 g,
Charles River) housed in pairs were administered CLZ in saline (30 mg/kg/day) by ip injection for up to 4 days. (B) Male brown Norway rats (200 – 250 g, Charles River) housed in pairs were administered AQ in saline (62.5 mg/kg/day) by gavage for up to 8 days. (C) Female C57BL/6J mice (15 – 20 g, 6 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME) and housed four to a cage. AQ was administered in the food for up to 8 days. (D) Female MPO knockout, female gp91 phox knockout, and corresponding wild-type mice were obtained from Jackson Laboratories (15 – 20 g, 7 weeks) and treated with AQ for 8 days. (E) Female cyclooxygenase (COX)-1 knockout, female COX-2 knockout, and corresponding wild-type mice were obtained from Taconic (Germantown, NY) (12 – 15 g, 6 weeks) and treated with AQ for 8 days. All mouse strains were treated with 300 – 350 mg/kg/day of AQ.

2.3.9 SDS-PAGE and immunoblotting
Protein samples (20 – 60 μg) were combined with DTT reducing sample buffer and boiled for 5 min. Proteins were separated at 30 – 40 mA on 8% polyacrylamide gels using the Bio-Rad PROTEAN II mini-gel system followed by transfer to nitrocellulose at 250 mA in a mini Trans-Blot transfer cell. CLZ blots were incubated in 3% hydrogen peroxide for 15 min. All membranes were blocked with 5% skim milk in wash buffer (50 mM Tris-HCl, 0.9% NaCl, 0.1% Tween-20, pH 7.5) for 1 h. After washing three times, membranes were incubated overnight at 4 °C with primary antibody (rabbit-anti-AQ IgG or rabbit-anti-CLZ IgG) diluted in wash buffer, 2% milk. Membranes were washed five times and incubated for 1.5 h at room temperature with goat-anti-rabbit-IgG HRP diluted 1:20 000 in wash buffer, 2% milk. Following three washes, membranes were incubated with SuperSignal West pico chemiluminescent substrate and imaged using a FluoroChem Alpha Innotech imager with AlphaEase FC software, version 6.0.0 (ProteinSimple, Santa Clara, CA). Each immunoblot was repeated at least twice, and GAPDH was used as the loading control. In addition, the specificity of the AQ antibodies in the terminal titers was evaluated by adding a preincubation step to the immunoblot described; before addition to the blot, the primary antibody (rabbit anti-AQ IgG) was preincubated with AQ (10 μg/mL) for 30 min.

2.3.10 Histopathology and immunohistochemistry
Formalin-fixed, paraffin-embedded femur longitudinal sections were stained with hematoxylin and eosin by the pathology department at the Centre for Modeling Human Disease at The
Toronto Centre for Phenogenomics. For immunohistochemical analysis, the primary antibody was the rabbit-anti-AQ antibody, and the secondary antibody was goat-anti-rabbit IgG-HRP from Sigma. Signal was developed using NovaRed (Vector, Burlington, ON), with Mayers hematoxylin (Sigma) as the counter stain. Each experiment was repeated at least twice.

2.3.11 Statistical analysis

GraphPad Prism (GraphPad, San Diego, CA) was employed for all statistical analyses by the paired Student’s $t$ test or one-way ANOVA. Data sets were expressed as the mean ± SEM, and results were considered statistically significant if $p < 0.05$. 
2.4 Results

2.4.1 Characterization of the anti-AQ antiserum

An increasing amount of AQ antibodies was detected by ELISA post immunization (Figure 2-1A). The antibodies from the final bleed were confirmed to be specific for AQ because this signal could be blocked by preincubation with either AQ or the AQ-BCP conjugate but not BSA, BCP, or 2-IMT alone (Figure 2-1B). The covalent binding of AQ to liver protein was previously demonstrated in rats treated with AQ for 4 days; therefore, AQ-treated liver (8 days) was employed as a positive control to test the ability of the AQ antibody to detect endogenous AQ-protein conjugates. Binding to a wide array of rat liver proteins was detected by immunoblots utilizing the anti-AQ antiserum as primary antibody (Figure 2-2A). No binding was detected in the control livers or when the preimmunization rabbit antiserum was used as the primary antibody (Figure 2-2C). Furthermore, the binding could be blocked by preincubating the liver blot with AQ or AQ-BCP but not with BCP alone. Mice treated for 8 days with AQ were likewise found to have extensive binding of AQ to their liver proteins, and similar to the rat, this binding could be blocked by AQ or AQ-BCP primary antibody preincubation (Figure 2-2B).

Figure 2-1. ELISA analysis of the anti-AQ antiserum

ELISA analysis of the anti-AQ antiserum to (A) detect the presence of AQ antibodies after the first immunization (titer 1) and two subsequent boosts (titers 2 and 3) and (B) test the specificity of the antiserum for AQ by preincubating the antiserum with increasing concentrations of AQ, AQ-BCP conjugate, or BCP before loading on the plate. Wells were coated with the AQ-BCP conjugate. Data represent the mean ± SEM from three analyses.
2.4.2 Detection of covalent binding of AQ and CLZ to rodent neutrophils in vivo

Covalent binding of CLZ to neutrophil proteins with molecular masses between 40 – 50 kDa was detected in neutrophils isolated from rats treated with CLZ; however, there were also artifact bands in lanes with protein from untreated controls (Figure 2-3A). After 3 days of treatment, the amount of binding increased slightly but did not appear to increase further after that time. In
contrast, there were no significant artifact bands in the blots from AQ-treated animals, and neutrophil proteins over a range from 20 to 100 kDa were modified in both rats and mice (Figure 2-3B,C). Binding increased after 2 days of AQ treatment and appeared to be maximal by 3 – 4 days. We were not able to detect covalent binding of CLZ to neutrophils in mice in vivo because mice are more sensitive to the acute toxicity (extreme sedation) of CLZ than rats; therefore, we were unable to achieve blood concentrations comparable to therapeutic concentrations in humans, and, in addition, the anti-CLZ antibody was not as sensitive as the anti-AQ antibody.

Figure 2-3. Immunoblot analysis to detect binding of AQ or CLZ to neutrophil proteins

Immunoblot analysis of (A) rat neutrophil protein over 4 days of treatment with CLZ (30 mg/kg/day, ip), (B) rat neutrophil protein over 8 days of treatment with AQ (62.5 mg/kg/day, gavage), and (C) mouse neutrophil protein over 4 days of treatment with AQ (350 mg/kg/day, in food). Protein loading was 30 μg per lane for all blots; the primary anti-CLZ antiserum was diluted 1:1000, and the primary anti-AQ antiserum was diluted 1:2000.
2.4.3 Lack of MPO and functional NADPH oxidase decreased covalent binding of AQ and CLZ to neutrophils ex vivo

Binding of AQ was detected in neutrophils from MPO knockout mice ex vivo; however, it was slightly less (1.8-fold) than that to neutrophils from wild-type mice. When PMA was added to stimulate the oxidative burst, binding increased in both neutrophils from MPO knockout and wild-type animals, but again, it was to a lesser extent in the MPO knockouts (2.5-fold less; Figure 2-4A). These ex vivo results suggest that PMA activation promotes MPO-dependent AQ oxidation, but there is likely another source of oxidant in the neutrophil. To study the pattern of binding in the complete absence of the neutrophil oxidative burst pathway, gp91 phox knockout mouse neutrophils were incubated ex vivo in a similar manner as the MPO knockout neutrophils. Lack of functional NADPH oxidase led to significantly less binding of AQ to gp91 knockout neutrophils than wild-type (5.8-fold less; Figure 2-4B). As expected, PMA activation did not change the extent of covalent binding to the gp91 knockout neutrophils; yet, significant covalent binding still did occur.

The binding of CLZ to neutrophils ex vivo was investigated in a comparable manner, and a similar pattern of binding was observed as with AQ; however, binding of CLZ to wild-type neutrophils was only detectable when the cells were activated with PMA. When MPO knockout neutrophils were activated with PMA, CLZ was found to bind 1.6-fold less compared to neutrophils from wild-type animals, but it was still detectable (Figure 2-4C). Covalent binding of CLZ in neutrophils from gp91 knockout mice was not significant compared to control (Figure 2-4D).

Focusing on binding observed to wild-type neutrophils, a notable difference was observed between the covalent binding patterns of the two drugs with respect to the necessity for PMA activation. AQ was found to bind significantly to wild-type neutrophils ex vivo both in the absence and presence of PMA, whereas CLZ required PMA activation in order for binding to be detected (Figure 2-4E). It is important to note though that the AQ antibody is more sensitive than the CLZ antibody, and there are also artifact bands in the blots using the CLZ antibody, which makes it a less sensitive tool, and this may be largely responsible for the apparent difference.
Figure 2-4. Covalent binding of AQ or CLZ to neutrophils ex vivo from MPO or NADPH oxidase knockout mice

Neutrophils (4.5 × 10^6 cells) isolated from mouse bone marrow were incubated with AQ (10 μM), CLZ (10 μM), or drug + PMA (40 ng) at 37 °C for 45 min. AQ and CLZ immunoblots were carried out with 5 and 15 μg per well of protein, respectively, and the AQ and CLZ antisera as primary antibodies at dilutions of 1:2000 or 1:1000, respectively. Neutrophils were obtained from MPO knock out mice (A, C) and gp91 phox knockout mice (B, D). (E) Integrated density values were calculated from at least three separate immunoblots and presented graphically to highlight changes in neutrophil covalent binding of AQ and CLZ. Statistical significance was determined using the paired Student’s t test and one-way ANOVA, where *, p > 0.05; **, p > 0.01; and ***, p > 0.001. KO, knockout.
2.4.4 MPO is responsible for ~50% of the covalent binding of AQ to neutrophils in vivo

With the availability of MPO and gp91 phox (NADPH oxidase null) knockout mice, we were able to study the enzyme contributions to AQ covalent binding in vivo. MPO knockout mice treated with AQ for 8 days (300 mg/kg/day) had a 2.0-fold decrease in the amount of covalent binding to their neutrophils compared to wild-type mice treated with AQ. This decrease was seen consistently to the proteins in the 20 – 60 kDa range over three separate immunoblots (Figure 2-5A). However, despite the 50% decrease in binding, there was still a considerable amount of binding observed in the knockouts. Unfortunately, mice are extremely sensitive to the sedative effects of CLZ, limiting our ability to give a high enough dose to produce CLZ blood levels in the human therapeutic range. In addition, the anti-CLZ antibody is not as sensitive and specific as the anti-AQ antibody. As a result, we were unable to detect CLZ covalent binding in vivo in mice for comparison to AQ.

Surprisingly, gp91 knockout mice treated with AQ for 8 days (350 mg/kg/day) showed no significant decrease in neutrophil binding compared to AQ-treated wild-type mice (Figure 2-5B). To investigate the possibility that compensation by other NADPH oxidase isoforms in the gp91 knockouts may have led to a false negative result, the same experiment was carried out in Rac1 and Rac2 knockout mice. Lack of the gp91 phox protein only renders the NOX2 isoform null, whereas NOX1, NOX2, and possibly NOX3 are all dependent on Rac. Despite the inactivation of three NOX isoforms in vivo, still no change in binding was observed (data not shown). This lack of dependence on the neutrophil oxidative burst for AQ to covalently bind in vivo indicates that an oxidative burst is not required for covalent binding of AQ in vivo. An alternative peroxidase present in the neutrophil that we considered as a possible source of AQQI production was prostaglandin H synthase, specifically COX-1 and COX-2. Xenobiotics can be oxidized directly by the COX enzyme during the reduction of prostaglandin G2 to prostaglandin H2. Furthermore, we hypothesized that in neutrophils the prostaglandin G2 hydroperoxide could also oxidize MPO to compound I, which would allow MPO itself to directly oxidize AQ to AQQI, independent of hydrogen peroxide. Nevertheless, as seen in Figure 2-5 D,E, both COX-1 and COX-2 knockout mice treated with AQ for 8 days (350 mg/kg/day) showed no decrease in the extent of covalent binding compared to wild-type neutrophils, eliminating prostaglandin H synthase as an alternative source of AQQI production.
Figure 2-5. In vivo covalent binding of AQ to mouse neutrophils

Mice were treated for 8 days with AQ (300 – 350 mg/kg/day), and covalent binding of AQ to neutrophils isolated from the bone marrow was detected by immunoblot. Protein loading was 30 μg per lane, and the anti-AQ antiserum was used as primary antibody at a dilution of 1:2000. (A) MPO knockout compared to wild-type mice. (B) Gp91 phox knockout compared to wild-type mice. (D) COX-1 knockout compared to wild-type mice. (E) COX-2 knockout compared to wild-type mice. Negative (Neg) and positive (Pos) control samples on COX-1 and COX-2 immunoblots were neutrophils obtained from C57BL/6J mice, untreated or treated with AQ, respectively (as seen in Figure 3C). (C) Integrated density values were calculated from three separate immunoblots and presented graphically to highlight changes in AQ covalent binding between wild-type and knockout neutrophils. Statistical significance was determined using the paired Student’s t test, where *, p > 0.05. KO, knockout.
2.4.5 AQ covalently binds extensively to bone marrow cells and lymphocytes

A significant amount of MPO-independent AQ binding was found to be present in the MPO knockout mice in vivo; therefore, we tested the ability of AQ to bind to other cell types in the blood and bone marrow. The binding of the reactive AQQI to different types of blood cells was previously evaluated, and it was reported that the greatest binding was to neutrophils, followed closely by lymphocytes, with no binding observed to erythrocytes. In this study, after only one dose of AQ, significant levels of AQ binding to a variety of bone marrow cells was observed (Figure 2-6). Binding was observed to myeloid and lymphoid cells as well as megakaryocytes; however, no binding was observed within the erythroid colonies. Furthermore, AQ appeared to bind both intracellularly as well as to surface proteins.

Given the widespread ability of AQ to bind bone marrow proteins, next we evaluated the circulating blood cells. Due to the larger blood volume of rats compared to mice these studies were carried out in rats. The first experiment examined the ability of AQ to bind ex vivo to a mixture of leukocytes collected from the buffy coat of heparin anti-coagulated blood. Binding to a large range of proteins was observed (20 – 150 kDa) independent of the need for cellular activation (Figure 2-7A). Lymphocytes isolated from whole blood were found to bind AQ extensively ex vivo (Figure 2-7B), whereas no binding to circulating erythrocytes was detected (Figure 2-7C). This binding preference was confirmed in vivo, where after 4 days of AQ treatment (62.5 mg/kg/day) strong binding to lymphocytes was observed, but not to the erythrocytes (Figure 2-7D). A similar study was carried out with CLZ in vivo in which rats were treated for 4 days (30 mg/kg/day) and covalent binding was assessed to the lymphocytes to determine if CLZ was able to bind to cell types other than neutrophils. In contrast to AQ, no binding of CLZ to the lymphocytes was detected (Figure 2-7E).
Figure 2-6. Immunohistochemistry staining of rodent bone marrow for AQ

Immunohistochemistry of bone marrow longitudinal sections from rats (62.5 mg/kg) and mice (350 mg/kg) treated with AQ for 1 day. Slides were incubated with the anti-AQ antiserum as the primary antibody (1:10 000) and counterstained with Mayer's hematoxylin. AQ-modified proteins stain brown.
Figure 2-7. Covalent binding of AQ or CLZ to rat blood cells

Cell types were isolated from whole blood (heparin anticoagulated) and incubated ex vivo with AQ (10 μM) at 37 °C for 45 min. Covalent binding was assessed by using the anti-AQ antiserum as primary antibody at 1:2000. (A) Leukocytes were isolated from the buffy coat of whole blood following the incubation step (30 μg/well). (B) Lymphocytes (10 μg/well). (C) Erythrocytes (50 μg/well). (D) Covalent binding of AQ to lymphocytes (10 μg/well) and erythrocytes (50 μg/well) was evaluated in vivo following 4 days of AQ treatment (62.5 mg/kg/day). (E) Covalent binding of CLZ to lymphocytes (30 μg/well) was evaluated for comparison in vivo following 4 days of CLZ treatment (30 mg/kg/day). The anti-CLZ antiserum was used at a dilution of 1:1000 as the primary antibody for immunoblot analysis.
2.4.6 Evaluation of the binding of AQ to human blood cells ex vivo

To determine whether the covalent binding of AQ to blood cells from rats and humans was similar, human venous blood was tested. First, heparin anticoagulated blood was incubated with AQ (10 μM) followed by centrifugation to isolate the leukocytes in the buffy coat. Extensive binding of AQ to human leukocytes was observed over the same wide range of proteins similar to that observed with rat blood (Figure 2-8A). Neutrophils were isolated from the blood prior to the ex vivo incubations, and, as with the rodent neutrophils, the cells were incubated with AQ alone as well as AQ combined with PMA to activate the oxidative burst pathway. PMA activation was found to increase the amount of AQ bound to neutrophils significantly when compared to AQ alone; yet, as was observed with the rodent neutrophils ex vivo, significant covalent binding of AQ was observed in the absence of activation (Figure 2-8B). There was also significant covalent binding to human lymphocytes (Figure 2-8C) but not to erythrocytes (Figure 2-8D). Overall, the covalent binding of AQ to human and rodent blood cells ex vivo was found to be comparable.
Figure 2-8. Covalent binding of AQ to human blood cells ex vivo

Whole blood (3 mL) was incubated with AQ (10 μM) at 37 °C for 45 min. The leukocytes in the buffy coat (A) and the erythrocytes (D) were separated following the incubation for independent immunoblot analysis. (B) Neutrophils (4.5 × 10^6 cells) isolated from the peripheral blood were incubated with AQ (10 μM) or AQ + PMA (40 ng) at 37 °C for 45 min. (B) Lymphocytes (4.5 × 10^6 cells) isolated from the peripheral blood were incubated with AQ (10 μM) at 37 °C for 45 min. Covalent binding was assessed by immunoblot (30 μg protein per well for blood leukocytes, 50 μg for erythrocytes, and 10 μg for lymphocytes and neutrophils) employing the anti-AQ antiserum as primary antibody at a dilution of 1:2000.
2.5 Discussion

The bioactivation of AQ and CLZ by neutrophils was studied extensively in vitro. This provided a simple hypothesis for the initial step in the mechanism of AQ- and CLZ-induced agranulocytosis. Specifically, the combination of NADPH oxidase and MPO oxidized these and several other drugs that cause agranulocytosis to reactive metabolites that covalently bind to neutrophils. These reactive metabolites could be formed either directly from compound 1 of MPO or by hypochlorous acid that is generated by MPO. The covalent binding of these reactive metabolites could, in turn, lead to agranulocytosis. However, the observation that patients with decreased MPO activity can develop CLZ-induced agranulocytosis raised doubts about this simple hypothesis. If this observation is correct, then either the mechanism of agranulocytosis does not require covalent binding of the drug or some other enzyme is responsible for bioactivation of the drugs. In addition, if neutrophil activation were required to produce an oxidative burst and NADPH oxidase activation, then it seemed unlikely that many neutrophils would be activated in the circulation of normal individuals. In the absence of activation, what would account for the observed in vivo covalent binding of CLZ to neutrophils in patients taking the drug? In the present work, we used MPO and gp91 phox (NADPH oxidase null) knockout mice to determine the contribution of these enzymes to the in vivo covalent binding of AQ and CLZ to neutrophils.

First, we compared the covalent binding of AQ and CLZ to wild-type and MPO knockout neutrophils ex vivo. In both cases, there was a decrease in covalent binding; however, significant binding was still observed in the neutrophils from the MPO knockout animals (Figure 2-4). NADPH oxidase, upstream of MPO in the neutrophil oxidative burst pathway, contains heme, which itself can act as a peroxidase. We found that the lack of functional NADPH oxidase led to an even more pronounced decrease in binding for both AQ and CLZ in ex vivo experiments (Figure 2-4), supporting the involvement of both MPO and NADPH oxidase in the mechanism of binding. In vivo, there was also a decrease in AQ covalent binding in MPO knockout mice; yet, significant covalent binding remained. Surprisingly, there was no significant decrease in the in vivo covalent binding of AQ in NADPH knockout mice. This indicates that it is not the major source of peroxide for the activation of MPO under normal circumstances. Even during an infection, most of the activated neutrophils would be at the site of the infection rather than in the
circulation. Unfortunately, we were not able to investigate CLZ binding in mice in vivo because of their extreme sensitivity to the sedative effects of CLZ and the lower sensitivity of the anti-CLZ antibody. The contrast between the ex vivo and in vivo results with the NADPH oxidase knockout neutrophils highlight the complexities of in vivo systems and demonstrate the importance of confirming ex vivo findings in relevant animal models.

This lack of dependence of AQ covalent binding on NADPH oxidase in vivo indicates that activation of the cells is not essential for binding to occur. This conflicts with previous reports that found that activation of human neutrophils was required for covalent binding of AQ or CLZ ex vivo. This may simply be a matter of the sensitivity of the assay, although we were previously able to detect covalent binding of CLZ in vivo in patients (the antibody is the same one used in these previous experiments, but that was more than a decade ago and the antibody appears to have degraded to some degree). Our ex vivo studies with wild-type neutrophils revealed a difference between AQ and CLZ; CLZ binding was only detectable following PMA activation, whereas AQ displayed significant binding in the absence of PMA (Figure 2-4). However, the fact that the anti-AQ antiserum is more specific and sensitive than the anti-CLZ antiserum may be one reason for this difference, and we cannot be certain that the oxidation of CLZ is qualitatively different from that of AQ. Furthermore, in the paper that reported that activation of the neutrophils was required for detection of AQ binding, the antibody employed for detection of the AQ-protein conjugates was obtained by treating rats with AQ, which directly induces the production of a low titer of AQ antibodies. This is in contrast to our high-titer AQ antibodies that were generated via immunization of rabbits with a highly conjugated AQ-BSA immunogen. It is also possible that CLZ requires a stronger oxidant for reactive metabolite generation and therefore MPO may play a larger role in the oxidation of CLZ than in the oxidation of AQ. However, covalent binding of CLZ to neutrophils from MPO knockout mice was observed, and the clinical data suggests that CLZ can cause agranulocytosis in MPO-deficient patients. This implies that other enzymes are also involved in the bioactivation of CLZ. It was interesting that both in vivo and ex vivo AQ bound extensively to a variety of bone marrow cell types, including myeloid and lymphoid, but not erythroid cells (Figure 2-6). In the circulating blood of rats, lymphocytes bound AQ extensively both ex vivo and in vivo, but in contrast, no binding of CLZ to lymphocytes was detected (Figure 2-7). The observation that AQ did not bind to red cells or their precursors is consistent with the observation that even when the
reactive metabolite, AQQI, was incubated with various cell types covalent binding was observed to neutrophils and lymphocytes but not to erythrocytes. This appears to be due to the lysosomotropic nature of the drug and higher levels of glutathione and other antioxidants in red cells could also contribute to this difference. Ultimately, we are not certain what enzymes and oxidants are responsible for the covalent binding of AQ and CLZ in vivo. MPO appears to play some role, but other enzymes also catalyze these oxidations. As for the actual oxidant, if MPO plays any role in the oxidation, then there must be a source of peroxide, and other than cyclooxygenase, we are not familiar with a significant cellular source of peroxide. There may be sufficient levels of ambient hydrogen peroxide to provide the oxidant, but, of course, that is speculation.

The findings of this study further complicate the picture of how drugs that cause agranulocytosis can be activated to reactive metabolites by neutrophils and other leukocytes. Predicting the susceptibility of a drug candidate to cause agranulocytosis before it reaches the market remains a challenge; however, it is probably still true that if a drug candidate is readily oxidized to a reactive metabolite by relatively weak oxidants such as peroxidases it will be more likely to cause agranulocytosis. The formation of covalent adducts with neutrophils in vitro has been demonstrated by many drugs with a high incidence of agranulocytosis and is considered a flag for potential to cause the reaction, but the enzymes and/or oxidants responsible for bioactivation and covalent binding in vivo are not clear.

2.6 Acknowledgements

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Note: Integrated density values for select immunoblots from this chapter, as well as the immunoblots for the Rac1 and Rac2 knockout mice experiments are reported in Appendix I, section A.3.6.
Clozapine promotes the proliferation of granulocyte progenitors in the bone marrow leading to increased granulopoiesis and neutrophilia in rats.


All work was completed by Alexandra R. Lobach in this chapter.
3.1 Abstract

Clozapine is an atypical antipsychotic that is limited in its use due to the risk of idiosyncratic agranulocytosis. The bone marrow is suspected to be the site of the reaction, and indirect measurements in patients suggest that neutrophil production and maturation are altered in the marrow by clozapine. Specifically, the majority of patients present with elevated neutrophil counts at the start of treatment, often paired with increased serum granulocyte-colony stimulating factor (G-CSF). Employing a rat model of clozapine treatment, we set out to determine if the neutrophilia observed at the start of treatment is characteristic of G-CSF-associated bone marrow stimulation. Female Sprague-Dawley rats were treated with 30 mg/kg/day of clozapine for 10 days, and sustained neutrophilia was evident after 1 week of treatment paired with spikes in G-CSF. Within the bone marrow, clozapine was found to induce proliferation of the granulocyte progenitor colonies as measured by a methylcellulose assay. This led to elevated granulopoiesis observed by H&E and myeloperoxidase staining of bone marrow slices. Increased release of neutrophils from the marrow to the circulation was measured through 5-bromo-2′-deoxyuridine labeling in vivo, and these neutrophils appeared to be less mature based on: a) a decrease in the nuclear lobe count and; b) increased expression of surface CD62L. Furthermore, faster transit of the neutrophils through the marrow was suggested by a shift towards elevated numbers of neutrophils in the bone marrow maturation pool, and increased CD11b and CD18 staining on the less mature neutrophils residing in the marrow. Taken together, these data indicate that clozapine stimulates the bone marrow to produce more neutrophils in a manner that is characteristic of endogenous G-CSF stimulation, and it is consistent with the inflammatory response observed in patients treated with clozapine.

3.2 Introduction

Clozapine is an effective atypical antipsychotic agent, which is widely employed in cases of treatment-resistant schizophrenia, but its use is restricted due to the risk of agranulocytosis in 0.5 – 1.0% of patients. The mechanism of this reaction has been difficult to study, in part, due to the lack of a relevant animal model of drug-induced agranulocytosis, and it remains difficult to predict which patients will react adversely to clozapine. Examination of the bone marrow from
patients with clozapine-induced agranulocytosis usually shows a complete absence of myeloid
precursors, indicating that precursor cells are likely the end target of the reaction rather than
peripheral blood neutrophils.\textsuperscript{101} There is evidence suggesting altered bone marrow function in
the peripheral blood of patients, including elevated numbers of CD34\textsuperscript{+} hematopoietic stem and
progenitor cells, and decreased neutrophil nuclear lobe count.\textsuperscript{102,103} Furthermore, the majority of
patients experience elevated neutrophil counts in the first weeks of treatment, paired with
increases in serum granulocyte-colony stimulating factor (G-CSF).\textsuperscript{104} Given the known functions
of G-CSF to regulate the proliferation, differentiation, and activation of granulocytes, it has been
suggested that G-CSF is causally linked to the neutrophilia observed at the start of clozapine
treatment.\textsuperscript{105} However, given the invasive nature of obtaining such samples from patients, this
has not been confirmed by examination of bone marrow in clinical studies. G-CSF elevates the
number of neutrophils in the circulation by first stimulating the proliferation of neutrophil
precursors in the bone marrow. Secondly, it characteristically decreases the transit time of
neutrophils through the post-mitotic pool of the marrow, leading to the release of less mature
neutrophils into the circulation.\textsuperscript{106} Furthermore, G-CSF has also been shown to activate
neutrophils as determined by a number of markers, particularly increased expression of the
surface adhesion protein CD11b.\textsuperscript{107} Given the characteristic manner in which G-CSF leads to
neutrophilia, and prior studies demonstrating that clozapine alters neutrophil kinetics in rabbits,\textsuperscript{86}
we set out to determine if rats treated with clozapine develop neutrophilia mediated by G-CSF
stimulation, similar to that observed in patients.

3.3 Materials and Methods

3.3.1 Chemical materials

Clozapine was provided by Novartis Pharmaceuticals Inc. (Dorval, QC) and DMP406, a
clozapine analogue, was obtained from DuPont-Pharma (Wilmington, DW). Ammonium acetate,
ammonium chloride, 5-bromo-2’-deoxyuridine (BrdU), calcium chloride, Dextran-500, EDTA,
magnesium chloride, and Trizma base were bought from Sigma-Aldrich (St. Louis, MO).
Potassium bicarbonate, sodium chloride, and Tween-20 were purchased from BioShop Canada
Inc. (Burlington, ON). Iscove’s modified Dulbecco’s medium (IMDM), phosphate buffered
saline (PBS, pH 7.4), and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco
and DAPI from Invitrogen (Life Technologies Inc., Burlington, ON). Hydrochloric acid (12 N) and HPLC-grade methanol were obtained from Caledon Laboratories Inc. (Georgetown, ON), sodium hydroxide (10 N) was from VWR International (Mississauga, ON), and acetic acid was from EMD Chemical Inc. (Gibbstown, NJ). The following antibodies were purchased from BD Biosciences (Mississauga, ON): mouse-anti-rat CD32 (D34-485); APC-anti-CD11b (WT.5); PE-anti-rat granulocytes (RP1); and nucleic acid stain 7-AAD. FITC-anti-CD62L (CLO85F) and FITC-anti-CD71 (CLO71F) were bought from Cedarlane (Burlington, ON). PE-anti-CD45 (OX-1) was purchased from BioLegend (San Diego, CA), RPE-anti-CD18 was from AbD Serotec (Raleigh, NC), rabbit-anti-myeloperoxidase was from Abcam Inc. (Cambridge, MA), and rabbit-anti-rat macrophage inflammatory protein-2 (MIP-2) was from Bioss, Inc. (Woburn, MA).

### 3.3.2 Animal treatment

Female Sprague-Dawley rats (225-250 g) were purchased from Charles River (St. Constant, QC) and housed in pairs or triplets with a 12/12 h light/dark cycle at 22 °C. All animals were acclimatized for a minimum of 1 week prior to commencing any experimental procedures, and were given regular access to rodent chow (Harlen Teklad, Madison, WI) and tap water. All experimental protocols were approved by the University of Toronto Animal Care Committee. Clozapine was dissolved in a small amount of 1 N HCl, followed by dilution in saline, and the pH was adjusted to approximately 5 using 1 N NaOH. Clozapine was administered by ip injection at 30 mg/kg/day for up to 10 days.

### 3.3.3 Blood and bone marrow collection

Blood samples were collected from the tail vein at specified time points to obtain serum, measure leukocyte counts in whole blood, and analyze leukocytes by flow cytometry. Total leukocyte counts were obtained manually from whole blood samples mixed with Turk’s blood diluting fluid (Ricca Chemical Company, Arlington, TX). Leukocyte differentials were calculated from blood smears stained with Giemsa-Wright-like stain (CAMCO Stain Pak, Cambridge Diagnostic Products, Inc., Fort Lauderdale, FL) by manually classifying a minimum of 100 leukocytes per slide. Furthermore, the number of nuclear lobes in the neutrophils were quantified manually following the method described by Delieu et al. Briefly, a minimum of 50 neutrophils were classified per slide as having one to six nuclear lobes. Some serum samples were analyzed for G-CSF by ELISA according to the kit protocol (R&D Systems Inc., Minneapolis, MN).
The bone marrow was flushed from the femurs and tibias using cold IMDM/2% FBS. Cells were carefully resuspended before filtering through a 70 μm cell strainer and centrifuging at 750g for 5 min at 4 °C. The cells were resuspended in IMDM/2% FBS and the number of nucleated cells were counted manually with Turk’s solution. Trypan blue (0.4%, Gibco) exclusion showed the viability to be > 90% for all bone marrow preparations.

3.3.4 Measurement of clozapine serum concentrations

Clozapine standards were prepared in methanol (0.01 – 1.5 μg/mL), diluted two-fold with blank rat serum, and then combined with internal standard DMP406 (0.1 μg/mL final concentration). Clozapine serum samples were collected from the rats at 0.5, 1, 3, 5, and 24 h post-clozapine injection. As was done for the standards, clozapine serum samples were diluted two-fold with blank rat serum, followed by addition of DMP406 (0.1 μg/mL). Protein was allowed to precipitate for 30 min at -20 ºC. The supernatant was isolated by centrifugation at 16 000g, diluted two-fold with water, and analyzed using a PE Sciex API 3000 quadrupole mass spectrometer with an electrospray ionization source (Sciex, Concord, ON) interfaced with a HPLC system (Shimadzu, Columbia, MD). The mobile phase consisted of 50% methanol, 1% acetic acid, and 2 mM CH₃CO₂NH₄ and a Kinetex 2.6μ C18 100A 50 x 3.0 mm column (Phenomenex, Torrance, CA) was employed.

3.3.5 Assessment of the bone marrow progenitor cells

Rat MethoCult medium (Stem Cell Technologies, Vancouver, BC) for the optimal growth of colony forming unit (CFU)-Granulocyte (G), CFU-Macrophage (M), and CFU-GM colonies, was combined with nucleated cells from the bone marrow at a 10:1 v/v ratio. Specifically, 1.5 x 10⁴ cells in 1.1 mL of medium were added to 35 x 10 mm suspension plates in duplicate, and incubated for 10 days at 37 °C, 5% CO₂, > 95% humidity. Total colonies were counted on each plate and classified manually according to characteristic CFU-G, CFU-M, and CFU-GM morphology.
3.3.6 Identification of the myeloid, lymphoid, and erythroid populations in the bone marrow

The myeloid, lymphoid, and erythroid populations in the bone marrow were identified by modifications to the method of Saad et al.\textsuperscript{108} Nucleated cells from the bone marrow (1 x 10^6 cells/sample) were resuspended in PBS/5% FBS and blocked with CD32 antibody for 15 min. All incubations were carried out at 4 °C. Cells were washed with PBS/5% FBS and stained with FITC-anti-CD71 and PE-anti-CD45 for 30 min. Following three washes, cells were incubated for 25 min with 1× fixation/permeabilization buffer (eBioscience), washed again, and then resuspended in staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl_2, 0.5 mM MgCl_2, 0.1% Tween-20). A final concentration of 2.5 μM DAPI was added to all samples to identify the nucleated cells. Samples were analyzed by flow cytometry using a BD FACSCanto II configured with FACSDiva software. Compensation was carried out before sample analysis using beads from BD Biosciences.

3.3.7 Bone marrow histopathology

Femurs were fixed in 10% formalin, and the paraffin sections, hematoxylin/eosin (H&E), unstained slides, and MIP-2 stained slides were prepared by the pathology lab at the Centre for Modeling Human Disease at The Toronto Centre for Phenogenomics. Immunohistochemistry was carried out on unstained slides to detect myeloperoxidase in the bone marrow according to the protocol provided with the rabbit-anti-myeloperoxidase antibody from Abcam. Binding was visualized using NovaRed (Vector, Burlington, ON) with Mayers hematoxylin (Sigma) as the counter stain.

3.3.8 Leukocyte and neutrophil isolations

Leukocytes in whole blood were separated from the erythrocytes by Dextran-500 (3%) sedimentation for 20 min at room temperature. All subsequent steps were carried out at 4 °C, unless stated otherwise. The supernatant was centrifuged at 610g for 10 min, and contaminating erythrocytes were removed with red cell lysis buffer (155 mM NH_4Cl, 10 mM KHCO_3, 0.1 mM EDTA) for 10 min at room temperature. Isotonicity was restored with excess PBS, and the cell suspension was centrifuged at 500g for 5 min. Neutrophils were isolated from the bone marrow by density centrifugation through a Percoll (GE Healthcare) gradient of 80/65/55% prepared in PBS. Samples were centrifuged at 750g for 30 min, and the neutrophils were collected at the
80/65% interface. Contaminating erythrocytes were removed as described above for the leukocytes, followed by one wash with PBS/5% FBS before analysis. For some preparations, a slide of cells was prepared and stained with Giemsa-Wright-like stain to confirm that > 85% of cells had characteristic neutrophil morphology.

### 3.3.9 Measurement of neutrophil release from the bone marrow

Clozapine was dosed daily, and 1 h following the 4th dose, BrdU dissolved in saline was administered in a single ip injection to all rats (100 mg/kg). Blood was collected at 24, 48, and 72 h post BrdU injection in the first study; 96, 120, and 144 h in the second. Leukocytes were isolated from whole blood as described previously, fixed and permeabilized using the BD Pharmingen FITC BrdU flow kit (BD Biosciences, San Jose, CA), and frozen at -80 °C. All samples were prepared for flow cytometry analysis according to the kit protocol and stained with PE-anti-rat RP1 and FITC-anti-BrdU antibodies. Flow cytometry analysis was carried out using a BD FACSCalibur configured with FlowJo software (Tree Star Inc., Ashland, OR), and compensation was carried out manually using fixed cells before sample analysis.

### 3.3.10 Assessment of neutrophil surface markers of activation and maturation

Leukocytes isolated from the blood and neutrophils isolated from the bone marrow (1 x 10^6 cells/sample) were resuspended in PBS/5% FBS and blocked with CD32 antibody for 15 min. All incubations were carried out at 4 °C. Cells were washed with PBS/5% FBS and stained with FITC-anti-CD62L, RPE-anti-CD18, APC-anti-CD11b, and 7-AAD for 30 min. Following two more washes, cells were incubated for 15 min in intracellular fixation buffer (eBioscience, San Diego CA), washed again, and then resuspended in staining buffer (eBioscience). Samples were analyzed by flow cytometry using the BD FACSCanto II. Signals in fluorescence minus one control samples were utilized to determine proper gating positions to distinguish between positively and negatively stained populations.

### 3.3.11 Statistical analysis

Prism 5 (GraphPad, San Diego, CA) was employed for all statistical analyses by two-way ANOVA, one-way ANOVA, or the unpaired Student’s t test. Data sets were expressed as the mean ± SEM, and results were considered statistically significant if p < 0.05.
3.4 Results

3.4.1 Serum concentrations of clozapine

Following a 30 mg/kg ip injection of clozapine, serum levels of clozapine were found to peak at 1.25 μg/mL 30 min post-injection, and fall to 0.41 μg/mL after 5 h (Figure 3-1). Clozapine was no longer detectable in the serum after 24 h. The blood levels in the rats were comparable to those seen in patients treated chronically with clozapine (0.35 – 1.30 μg/mL).109

3.4.2 Changes in leukocyte counts and G-CSF concentrations with clozapine treatment

Total leukocyte counts in the peripheral blood were found to remain stable over 10 days of daily clozapine treatment, as did the number of lymphocytes. Neutrophil counts spiked transiently after one dose of clozapine, followed by a steady increase until the 10th day of treatment. A sharp increase in monocytes was also noted during the last two days of therapy (Figure 3-2). Serum G-CSF was analyzed over 8 days of clozapine treatment, and large spikes in G-CSF were observed in different animals at different time points, particularly those animals that exhibited the highest neutrophil counts. Because the G-CSF was released in spikes, there was no statistically significant difference between the control and treated groups (Figure 3-3).

Figure 3-1. Measurement of clozapine blood levels in rats by LC-MS/MS

Serum was collected from rats after a single ip injection of clozapine, 30 mg/kg. Protein was removed by methanol precipitation, and the supernatant was analyzed by LC-MS/MS using a mobile phase of 50% methanol, 1% acetic acid, and 2 mM CH₃CO₂NH₄ and a Kinetex 2.6μ C18 100A 50 x 3.0 mm column. Results are shown from individual animals, and expressed as the mean ± SEM. The grey shaded area represents the average therapeutic range reported in patients.
Figure 3-2. Leukocyte counts over 10 days of clozapine treatment

Rats were treated with clozapine for 10 days by ip injection (30 mg/kg/day). Total leukocyte counts were determined manually and differential counts were obtained from freshly prepared blood smears. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Figure 3-3. Serum concentrations of G-CSF in response to clozapine

Serum concentrations of G-CSF were measured by ELISA over 8 days of clozapine treatment (30 mg/kg/day). Results are expressed as the mean ± SEM.
3.4.3 Clozapine promotes granulocyte production in the bone marrow

The effect of clozapine on the growth of the granulocyte and macrophage colony forming units (CFU-GM, CFU-G, and CFU-M) was assessed by the methylcellulose assay. Bone marrow samples obtained from clozapine-treated and control animals were cultured ex vivo and colony growth was evaluated. Overall, a considerable increase in the total number of colonies was present on the clozapine-treated plates compared to control (Figure 3-4), of which a significant portion could be attributed to the CFU-G colony type. The percent of CFU-G colonies on the clozapine-treated plates was significantly elevated, whereas the percent of CFU-GM colonies decreased. The growth of the CFU-M colonies appeared unchanged by clozapine.

Figure 3-4. Growth of progenitor cells from clozapine-treated bone marrow

Granulocyte/macrophage progenitor cell growth was assessed by the methylcellulose assay. Bone marrow was obtained from rats treated with clozapine (30 mg/kg/day) for 10 days. Nucleated bone marrow cells (1.5 \times 10^4) were incubated ex vivo in MethoCult medium (Stem Cell Technologies) for 10 days. Colonies were classified and quantified manually on each plate. (A) Number of colonies. (B) Percent of total colonies. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by one-way ANOVA, where *, \( p < 0.05 \); and ***, \( p < 0.001 \). CFU, colony forming unit; G, granulocyte; M, macrophage.
To determine if the increase in granulocyte progenitors led to changes in the number of cells residing in the erythroid, lymphoid, and myeloid compartments of the bone marrow, rats were treated with clozapine for either 5 or 10 days, at which point the bone marrow was collected for flow cytometry analysis. The nucleated cells were identified by DAPI staining, and the surface markers CD45 and CD71 were employed to distinguish between the erythroid and leukocyte lineages. The leukocytes were further separated into the lymphoid and myeloid compartments based on characteristic differences in side scatter (Figure 3-5A). After 5 days of clozapine treatment, a shift towards increased myeloid cell production was observed, and following 10 days, this difference was found to be statistically significant. The lymphoid and erythroid compartments were unaffected by clozapine (Figure 3-5B).

Figure 3-5. Measuring the myeloid, lymphoid, and erythroid compartments of the bone marrow

Following 10 days of clozapine treatment (30 mg/kg/day) in rats, bone marrow was isolated from the femurs. Cells were fixed, followed by DAPI staining to identify nucleated cells. A minimum of 40,000 cells were collected in the DAPI positive gate. (A) Representative plots of DAPI positive cells. Cells from the erythroid lineage (R4) were separated from the myeloid and lymphoid cells (R3) based on CD71 and CD45 staining of nucleated cells. The myeloid and lymphoid cells in R3 were separated based on characteristic differences in side scatter. (B) The number of cells in the erythroid, lymphoid, and myeloid compartments was quantified based on this gating strategy. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by one-way ANOVA, where **, p < 0.01.
H&E staining of the bone marrow revealed similar findings in the myeloid compartment, more specifically, there was an increase in granulocyte production with clozapine treatment (Figure 3-6A). Elevated numbers of granulocytes were observed throughout the marrow, but most notably in the paratrabecular region, leading to confinement of the erythroid cells to the central area of the marrow. Furthermore, the bone marrow sinuses were more prominent in the treated animals, and a slight increase in the number of megakayocytes was noted. Elevated granulocyte production was confirmed by immunohistochemical labeling of myeloperoxidase; increased staining was observed in the bone marrow of clozapine-treated animals, most notably in the paratrabecular region (Figure 3-6B).

Figure 3-6. Histology of the bone marrow following clozapine treatment

Rats were treated with clozapine for 10 days (30 mg/kg/day). Femurs were fixed in formalin and paraffin embedded prior to staining with (A) H&E and (B) myeloperoxidase. Myeloperoxidase protein stains dark red/brown.
3.4.4 Clozapine promotes the release of neutrophils from the bone marrow to the circulation

Neutrophil release from the bone marrow was assessed by labeling dividing cells in the bone marrow with BrdU, and observing their appearance in the peripheral circulation by flow cytometry. On the 4th day of clozapine treatment a single BrdU injection was administered. Due to blood volume restrictions in the rat, two studies were carried out to allow for daily blood collection up to 144 h following the BrdU injection, and the data were combined after analysis. The group of control rats was found to exhibit normal neutrophil kinetics. The percentage of BrdU-labeled neutrophils present in the blood of the clozapine-treated group significantly increased 72 h following the BrdU injection, compared to control (Figure 3-7A). Given the increase in the neutrophil count observed with clozapine treatment, it was not surprising to find that the total number of BrdU-labeled neutrophils also increased in the periphery (Figure 3-7B). From this data it was not possible to determine if the neutrophils were exiting the bone marrow faster due to clozapine; therefore, the number of nuclear lobes (a measure of neutrophil maturity) in the circulating neutrophils was quantified manually on blood smears. Following 10 days of treatment, a left shift in the number of nuclear lobes was observed, i.e. significantly more neutrophils were present with three lobes and fewer with four. Overall, the average lobe count was decreased indicating that the neutrophils were less mature (Figure 3-8).

The expression levels of neutrophil surface markers of activation and maturation (CD62L, CD11b, and CD18) were assessed on peripheral blood neutrophils by flow cytometry. CD11b and CD18 were unchanged by clozapine treatment, suggesting that the neutrophils were in an unactivated state. On the other hand, CD62L expression was found to increase significantly on day 10 (Figure 3-9). Although this is not reflective of the characteristic changes reported with G-CSF stimulation related to activation, the elevation in CD62L is in fact suggestive of faster release of neutrophils from the bone marrow. Studies by Lund-Johansen et al. report that neutrophils most recently released from the bone marrow express higher levels of CD62L, which in the case of clozapine suggests that a higher portion of neutrophils present in the circulation have recently exited the marrow.
Figure 3-7. The effect of clozapine on neutrophil release from the bone marrow

Dividing neutrophils in the marrow were labeled with BrdU and their appearance in the peripheral circulation was detected by flow cytometry. On the 4th day of clozapine treatment a single BrdU injection was administered and blood was collected from the tail vein every 24 h, up to 144 h post BrdU injection. (A) The percentage of BrdU-labeled neutrophils was measured by flow cytometry using a FITC-anti-BrdU antibody combined with a PE-anti-granulocytes antibody to gate the neutrophils. (B) The total number of BrdU-labeled neutrophils was obtained by multiplying the percentage of BrdU-labeled cells by the total neutrophil count (Figure 3-2). Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where *, p < 0.05; and ***, p < 0.001.

Figure 3-8. Neutrophil nuclear lobe counts following clozapine treatment

Rats were treated for 10 days with clozapine (30 mg/kg/day). (A) The percentage of neutrophils with 1-6 lobes. (B) The average neutrophil nuclear lobe count. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where *, p < 0.05; and ***, p < 0.001; or the unpaired Student’s t test, where Φ, p < 0.05.
Figure 3-9. Measuring neutrophil surface markers of activation and maturation

Rats were treated with clozapine for 10 days (30 mg/kg/day) and leukocytes were isolated from the peripheral blood via Dextran-500 sedimentation and stained with CD62L, CD11b, and CD18. Live cells were gated based on 7-AAD staining, and the neutrophil population was identified based upon its characteristic forward and side scatter in the peripheral blood. A minimum of 20,000 events were collected in the live cell gate. (A) Representative plots of CD62L staining in control and clozapine-treated neutrophils. (B) The percentage of neutrophils stained with CD62L, CD11b, or CD18. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where *, p < 0.05.

3.4.5 Clozapine promotes differentiation and maturation of neutrophils in the bone marrow

Neutrophils residing in the bone marrow obtained from femurs of clozapine-treated and control rats were analyzed by flow cytometry for markers of maturation and mobilization. Due to the lack of commercial antibodies available to specifically identify rat neutrophils, we instead isolated the neutrophils from the bone marrow by density centrifugation prior to flow cytometry analysis. Numerous studies have characterized the maturation of granulocytes in the bone marrow by flow cytometry based on cellular granularity (side scatter) and the expression of various cell adhesion molecules. Focusing first on the morphological traits of the cells, Lund-Johansen and Terstappen determined that the myelocytes, metamyelocytes, band cells, and
segmented neutrophils can be separated from the less mature granulocytes based on their high side scatter. In this study, two populations of neutrophils in the bone marrow were identified in a similar manner; one with higher granularity or side scatter (SSC^high) representing the more mature cells, and the other with lower side scatter (SSC^low) corresponding to the less mature cells (Figure 3-11A). Clozapine treatment significantly increased the percentage of SSC^high neutrophils but had no effect on the percentage of SSC^low cells. This suggests that although clozapine promoted neutrophil maturation, the bone marrow was able to keep up with the increased demand for less mature cells.

Changes in the expression of surface adhesion proteins as granulocytes differentiate and mature in the bone marrow has also been well characterized by flow cytometry, using the markers CD18, CD11b, and CD62L. CD18 is down-modulated during early granulocyte maturation, followed by a steady increase until it reaches its maximum expression level at the end of granulocyte proliferation. CD11b is first detected at the early myelocyte stage and increases rapidly with cellular maturation. Progenitor cells highly express CD62L, which decreases as cells commit to the granulocyte lineage, followed by a steady increase as these cells continue to mature. Focusing first on comparing the control groups between the SSC^low and SSC^high populations, the percentage of cells staining positive for CD18 was moderately higher in the SSC^high population (46% versus 59%, \( p < 0.001 \), one-way ANOVA) whereas CD11b staining was significantly higher (18% versus 92%, \( p < 0.001 \)) (Figure 3-11B,C). This characteristic shift in CD18 and CD11b expression confirms that the SSC^high population contained more mature granulocytes. On the other hand, CD62L expression was moderately lower in the SSC^high cells (8% versus 1%, \( p < 0.05 \)). Although the SSC^high cells were expected to express higher levels of CD62L than the SSC^low cells, it is possible that the granulocyte progenitor cells were included in the lower granularity gate, which would upwardly skew the amount of CD62L staining.

Clozapine was found to alter the expression of these surface markers in both populations of granulocytes. With the less mature cells (SSC^low), CD11b and CD18 staining increased significantly with clozapine treatment (Figure 3-11B), whereas CD62L expression was unchanged. The distinct elevations of CD11b and CD18 indicate that clozapine promoted neutrophil differentiation and maturation of the less mature cells within the bone marrow. As the neutrophils transitioned to the SSC^high population, a modest increase in CD62L was apparent, but CD11b and CD18 were unchanged (Figure 3-11C). Previous studies have shown that CD62L
increases as neutrophils transit through the maturation pool. Based on the staining pattern of these three markers, it appears that clozapine caused the less mature neutrophils in the bone marrow to differentiate and mature, which resulted in an increased number of cells in the maturation pool.

Further analysis of these markers, as seen in Figure 3-11D, revealed that the number of cells staining double positive for CD62L and CD11b increased significantly in both the SSC\textsuperscript{high} and SSC\textsuperscript{low} populations. This is an interesting observation in the context of neutrophil mobilization because CD62L\textsuperscript{high}/CD11b\textsuperscript{+} granulocytes have been identified as the sole cellular source of constitutive MIP-2 in murine bone marrow. MIP-2 is a neutrophil chemoattractant, which is induced at early stages of inflammation, and is thought to be involved in neutrophil mobilization from the bone marrow. In fact, immunohistochemistry staining for MIP-2 in the bone marrow increased in all treated animals compared to control (Figure 3-10), indicating that clozapine stimulated the production of this chemoattractant. On the contrary, MIP-2 was not detectable by ELISA in the blood. This confirms that the increased numbers of the CD62L\textsuperscript{+/CD11b\textsuperscript{+}} granulocytes seen in the bone marrow of clozapine-treated rats is likely a true observation.

![Figure 3-10. Immunohistochemistry staining of MIP-2 in the bone marrow](image)

Rats were treated with saline (control) or clozapine for 10 days (30 mg/kg/day). Femurs were fixed in formalin and paraffin embedded prior to staining with an anti-rat MIP-2 antibody. MIP-2 proteins stain dark red/brown.
Figure 3-11. Characterizing the effects of clozapine on bone marrow neutrophils

(A) Neutrophils were separated into two distinct populations based on granularity/side scatter (SSC\textsuperscript{high} and SSC\textsuperscript{low}), an indication of maturity. (B) Expression of CD62L, CD11b, and CD18 in the SSC\textsuperscript{low} population was calculated as a percentage of the total number of cells within the SSC\textsuperscript{low} gate. (C) Expression of CD62L, CD11b, and CD18 in the SSC\textsuperscript{high} population was calculated as a percentage of the cells within the SSC\textsuperscript{high} gate. (D) Percentage of neutrophils within the SSC\textsuperscript{high} and SSC\textsuperscript{low} populations staining double positive for CD62L and CD11b. All gates were established based on staining in fluorescence minus one control samples. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by one-way ANOVA, where **, p < 0.01; and ***, p < 0.001; or the unpaired Student’s t test, where Φ, p < 0.001. SSC, side scatter.

3.5 Discussion

Clozapine remains an important therapy option to treat schizophrenia, particularly for patients who develop resistance to first line therapeutics; however, the propensity to cause agranulocytosis severely limits its use. To work towards understanding the mechanism of this reaction, clozapine has been widely studied in the clinic, in animal models, and also in vitro.\textsuperscript{19, 101} Even though clozapine treatment does not lead to agranulocytosis in most patients, it does have significant effects on neutrophils.\textsuperscript{4-7} The availability of an animal model would greatly contribute to unraveling the mechanistic unknowns, yet despite extensive efforts, development of such a tool has been unsuccessful.\textsuperscript{43, 116} In this study, we found that female Sprague-Dawley rats treated with 30 mg/kg/day of clozapine, although they do not develop agranulocytosis, appear to respond to clozapine in a similar manner as patients at the start of treatment. Neutrophil counts were significantly increased after 8 days and remained elevated until the end of the treatment period (10 days). The transient peak in the neutrophil count 24 h following the first dose of clozapine was likely due to a rapid efflux of mature cells from the bone marrow or possibly demargination. This spike returned quickly to baseline, followed by a slow and steady increase in neutrophil count during the first week of treatment. The fact that it took 8 days for the neutrophil
count to rise significantly suggests that the production changes in the bone marrow commence at the level of the granulocyte progenitor cells; it takes approximately 5 – 6 for a granulocyte progenitor to transit through the mitotic and post-mitotic pools of the bone marrow.\textsuperscript{117} As the neutrophil counts were rising over 8 days, spikes in serum G-CSF were observed in the rats; however, a persistent elevation as seen in patients was not apparent. This is likely due to the short half-life of G-CSF in the serum,\textsuperscript{118} and measurements taken more acutely following a single dose of clozapine in this rat model showed a significant increase at 3 and 6 h, but not at 24 h (see Chapter 4).

Changes in the rat bone marrow following clozapine treatment were found to be reflective of stimulation by endogenous G-CSF. First of all, increased proliferation of the progenitor cells was observed in the bone marrow obtained from clozapine-treated animals; specifically, elevated production of CFU-G colonies (Figure 3-4). This, in turn, led to elevated numbers of cells in the myeloid compartment of the marrow (Figure 3-5) and increased granulopoiesis observed throughout the marrow on H&E-stained bone marrow slides, most notably in the paratrabecular region, which constitutes the granulocytic generation zone.\textsuperscript{119} Myeloperoxidase immunohistochemical staining was found to increase in parallel, supporting the notion of increased granulocyte production (Figure 3-6). Overall, these direct bone marrow measurements indicate that clozapine elevates the production of granulocytes, commencing at the level of the progenitor cells.

The fact that clozapine changes neutrophil kinetics in the bone marrow has been demonstrated previously in rabbits by Iverson et al., and we were able to show a similar effect in female Sprague-Dawley rats treated with 30 mg/kg/day of clozapine.\textsuperscript{86} A distinct increase in the percent of BrdU-labeled neutrophils compared to control at 72 h post BrdU injection was observed, with elevated total cell numbers sustained between 72 – 120 h (Figure 3-7). From this data we were unable to determine if the peak in BrdU-labeled cells occurred earlier than the control group. Therefore, to investigate if clozapine decreased the transit time of neutrophils through the post-mitotic pool, the number of nuclear lobes in the circulating neutrophils was examined, and clozapine treatment was, in fact, found to decrease the lobe count (Figure 3-8). Furthermore, the expression of CD62L increased on the surface of the peripheral blood neutrophils, which is an indicator of early release from the bone marrow; active bone marrow release leads to elevated levels of CD62L on circulating neutrophils, likely due to the premature release of cells from the
maturation pool.\textsuperscript{113} CD11b and CD18 expression did not change with clozapine treatment, suggesting that neutrophils in the blood were circulating in an unactivated state (Figure 3-9). Although the neutrophils did not appear to be in an activated state, the clozapine-induced release of less mature neutrophils into the circulation, suggesting decreased transit time through the post-mitotic pool, is reflective of bone marrow stimulation by G-CSF.

Lastly, we focused on the neutrophils residing in the bone marrow to determine if there were changes evident that corresponded to the changes in circulating population with respect to maturation. Two populations of neutrophils were identified in the marrow based on differences in cellular granularity (SSC); SSC\textsuperscript{high} representing the more mature cells and SSC\textsuperscript{low} corresponding to the less mature cells. The gating strategy was confirmed by the characteristic staining of CD18 and CD11b in less mature versus more mature neutrophils in the marrow.\textsuperscript{111, 112} The fact that clozapine significantly increased the percentage of SSC\textsuperscript{high} neutrophils, but had no effect on the SSC\textsuperscript{low} cells, indicates that more cells resided in the maturation pool, and yet the marrow was able to keep up with the production of the less mature cells. Specifically, within the SSC\textsuperscript{low} population, distinct elevations of CD11b and CD18 staining were found, indicating that the less mature neutrophils in the marrow were influenced by clozapine to differentiate and mature. This aligns with the shift towards elevated cell numbers in the maturation pool. As the neutrophils transitioned to the SSC\textsuperscript{high} population, a modest increase in CD62L was apparent; a marker that has been shown to increase as neutrophils transit through the maturation pool.\textsuperscript{113}

These studies provide direct evidence in the bone marrow that clozapine stimulates neutrophil production, likely mediated by G-CSF; however, the reason why clozapine specifically targets this population of cells and the events leading to this bone marrow stimulation remain unknown. Previous studies suggest that clozapine induces apoptosis in peripheral blood neutrophils, and as a result this loss of cells would signal the bone marrow to ramp up production to maintain homeostasis.\textsuperscript{120} Yet despite numerous attempts, evidence of clozapine-induced neutrophil apoptosis in vivo has proven difficult to demonstrate.\textsuperscript{86, 116} Alternatively, increased neutrophil production may be due to indirect effects such as inflammatory stimulation, which has been well documented at the start of clozapine treatment.\textsuperscript{85} Cytokines induced by inflammation, such as IL-6, TNF-\textgreek{a}, and IL-1\textgreek{b}, have been shown to induce the bone marrow to cycle, leading to increased granulocyte production.\textsuperscript{121, 122} Observation of a unique population of neutrophils in the bone marrow: those staining double positive for CD62L and CD11b, was an interesting finding
particularly in the context of inflammation and neutrophil mobilization. Studies by Sigrid et al. identified CD62L+/CD11b+ granulocytes as the sole cellular source of constitutive MIP-2 in murine bone marrow.\cite{114} MIP-2 is a chemoattractant that is implicated as a modulator of neutrophil mobilization from the bone marrow,\cite{115} and we were able to detect its increased production in clozapine-treated rat bone marrow by immunohistochemistry (Figure 3-10). The fact that MIP-2 is induced at early states of inflammation\cite{114} supports the hypothesis that clozapine-induced granulopoiesis is the result of immune stimulation.

Overall, the results from this study demonstrate that clozapine stimulates the bone marrow in a manner that is characteristic of G-CSF, specifically through the increased proliferation of granulocyte progenitor cells. This leads to an increase in the neutrophil maturation pool in the bone marrow, a faster transit of neutrophils through the post-mitotic pool, and increased release of cells into the peripheral blood. These changes in neutrophil kinetics are ultimately observed in the patient as clozapine-induced neutrophilia. Although it remains unknown how these changes in the bone marrow might be related to agranulocytosis, these observations indicate that clozapine significantly modulates neutrophil production, and they are analogous to changes observed in patients treated with clozapine. The observation that clozapine treatment leads to an immune response with an increase in inflammatory cytokines such as IL-6, could provide a link between the observed neutrophil kinetic changes and agranulocytosis. Specifically, this immune response could lead to an increase in G-CSF, which mediates the change in neutrophil kinetics, and in some patients, the immune response could cause the destruction of mature neutrophils and/or neutrophil precursors. Understanding how the immune system plays a role in clozapine-induced G-CSF production leading to neutrophilia may provide clues to help unravel this mechanism.

### 3.6 Acknowledgements

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CHAPTER 4

Clozapine induces a proinflammatory response in rats that is mediated by inflammasome activation and IL-1β

All work was completed by Alexandra R. Lobach in this chapter; however Kyle J. Weston was responsible for the maintenance of the THP-1 cells, and therefore contributed to the generation of Figure 4-3, Figure 4-4 and Figure 4-5. Furthermore, IL-1β ELISAs were carried out by both parties to generate Figure 4-3B.
4.1 Abstract

The atypical antipsychotic agent clozapine is associated with idiosyncratic agranulocytosis, severely limiting its use in patients. Both toxic and immune components are suspected to be key factors in the mechanism of this reaction. Previous studies have demonstrated that the majority of patients starting clozapine therapy display evidence of immune activation during the first weeks of therapy; however, it remains unclear how clozapine is inducing these immune changes. Employing a rat model of early clozapine treatment, clozapine was found to stimulate the release of proinflammatory markers in the blood, represented by significant elevations in neutrophil count, α-1-acid glycoprotein (α-1-AGP), granulocyte-colony stimulating factor (G-CSF), and chemokine ligand 1 (CXCL1). These findings led us to look for evidence of inflammasome activation. Although interleukin (IL)-1β was not detected in vivo, clozapine was found to stimulate IL-1β production in human THP-1 monocytes and macrophages in vitro that could be specifically blocked by the caspase inhibitor Z-VAD-fmk. Interestingly, in rats, coadministration of the IL-1 receptor antagonist anakinra was found to significantly suppress the clozapine-induced increases in neutrophil count, α-1-AGP, G-CSF, and CXCL1. These results indicate for the first time that inflammasome activation may be the route by which clozapine stimulates a proinflammatory response in vivo, and that IL-1β is likely a key inflammatory mediator in this process.

4.2 Introduction

The atypical antipsychotic agent clozapine is associated with a 0.8% risk of agranulocytosis, and despite its unique efficacy, the risk of this adverse reaction severely limits its use. If there were a better understanding of the mechanism of this reaction, it might make it possible to predict who is at greatest risk and could significantly improve clozapine therapy. Both toxic and immune components are suspected to be key factors in the mechanism of clozapine-induced agranulocytosis. It is likely that this reaction is caused by the reactive nitrenium ion of clozapine, which can be formed by myeloperoxidase-generated oxidants in neutrophils, and furthermore, has been shown to form covalent adducts with neutrophil proteins. Clozapine was shown to irreversibly bind to neutrophils in patients; patients that had been treated with clozapine...
for an extended time period without developing agranulocytosis.\textsuperscript{41} Therefore, it is likely that the binding of the reactive metabolite is necessary, but not sufficient, to cause agranulocytosis. The idiosyncratic nature and delay in onset of the reaction are most easily explained by an immune mechanism, and are typical of other idiosyncratic drug reactions that are clearly immune-mediated.\textsuperscript{123} Studies by Pollmächer et al. found that approximately 50\% of patients treated with clozapine developed a mild fever and neutrophilia. Most also had elevated levels of serum interleukin (IL)-6, tumor necrosis factor (TNF)-α, granulocyte-colony stimulating factor (G-CSF), and soluble IL-2 receptor at the start of treatment; all strong indicators of immune activation.\textsuperscript{104, 105} Significant elevations in C-reactive protein, as well as soluble CD8, and IL-8 have also been reported.\textsuperscript{124,125} IL-6, TNF-α, and IL-8 are mainly produced by activated macrophages/antigen presenting cells, which are required for the induction of an immune response. On the other hand, T-cell activation is implied by the increases in soluble IL-2 receptor and CD8. Yet, despite continued clozapine treatment all of these inflammatory markers were reported to return to baseline levels, which suggest the involvement of some form of immune tolerance. It remains unclear how clozapine induces immune changes in patients; therefore, we set out to investigate the onset of the proinflammatory state using our rat model of early clozapine treatment. Findings in this model led us to investigate the ability of clozapine to activate inflammasomes in human THP-1 cells in vitro, and the involvement of IL-1 in the proinflammatory response in vivo. Results from this study suggest for the first time that clozapine-induced immune activation is mediated by inflammasome activation and IL-1β.

4.3 Materials and Methods

4.3.1 Chemical materials

Clozapine was kindly provided by Novartis Pharmaceuticals Inc. (Dorval, QC) and olanzapine was purchased from Toronto Research Chemicals Inc. (Toronto, ON). Sodium hydroxide (10 N) was bought from VWR International (Mississauga, ON) and lipopolysaccharides (LPS) and phorbol 13-myristate 12-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was bought from BioShop Canada Inc. (Burlington, ON) and 2-mercaptoethanol, phosphate buffered saline (PBS, pH 7.4), and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco (Life Technologies Inc., Burlington, ON).
4.3.2 Animals and blood collection

Female Sprague-Dawley rats (225 – 250 g) were purchased from Charles River (St. Constant, QC) and housed in pairs or triplets with a 12/12 h light/dark cycle at 22 °C. All animals were acclimatized for a minimum of 1 week prior to starting any experimental procedures, and all protocols were approved by the University of Toronto Animal Care Committee. Rats were given regular access to rodent chow (Harlen Teklad, Madison, WI) and tap water. Clozapine was dissolved in a small amount of 1 N HCl, diluted in saline, and pH adjusted to approximately 5 with 1 N NaOH. Clozapine was administered by ip injection at 30 mg/kg/day for up to 10 days. Blood samples were collected from the tail vein at specified time points to obtain whole blood and serum. Rats were also cotreated with clozapine and the IL-1 receptor antagonist anakinra (Amgen, Thousand Oaks, CA). Stock anakinra was diluted in PBS and administered sc (50 mg/kg), first as a predose 24 h before the start of the study, and again 1 h before a single clozapine injection (30 mg/kg, ip). Serum concentrations of clozapine were measured by LC-MS/MS (method described in Chapter 3).

4.3.3 Leukocyte counts

Total leukocyte counts were determined manually from whole blood mixed with Turk’s blood diluting fluid (Ricca Chemical Company, Arlington, TX). Leukocyte differentials were calculated manually by classifying at least 100 leukocytes on blood smears stained with Giemsa-Wright-like stain (CAMCO Stain Pak, Cambridge Diagnostic Products, Inc., Fort Lauderdale, FL).

4.3.4 Inflammatory marker profiling in the serum

Serum samples were obtained from acute 24 h studies and a chronic 10 day study. ELISAs were carried out to test for rat G-CSF, IL-1β, IL-6, TNF-α, and chemokine ligand 1 (CXCL1) according to the kit protocols from R&D Systems Inc. (Minneapolis, MN). Serum concentrations of rat α-1-acid-glycoprotein (α-1-AGP) were measured using an ELISA kit from Life Diagnostics Inc. (West Chester, PA). Concentrations of human IL-1 receptor antagonist were measured by ELISA in the samples from the anakinra cotreatment study using a kit from R&D Systems Inc.
4.3.5 Measurement of cytokine production and clozapine binding in THP-1 cells

THP-1 monocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in ATCC high glucose RPMI-1640 media, supplemented with 10% FBS and 2-mercaptoethanol. Monocytes were stimulated with 1 μg/mL of LPS for 3 h, washed with PBS, and resuspended in media without 2-mercaptoethanol. Clozapine and olanzapine were dissolved in DMSO (0.25%) and added to the cells over a concentration range of 1 – 25 μg/mL. To inhibit IL-1β production, the caspase inhibitor Z-VAD-fmk (InvivoGen, San Diego, CA; 10 μg/mL) was coincubated with both drugs. Cells were incubated for 24 h at 37 °C, after which they were spun down, and the cell culture media was then tested for IL-1β by ELISA (Life Technologies Inc., Burlington, ON). In some cases, to detect covalent binding of clozapine to the cells, protein was extracted for western blot analysis. Cells were lysed with 1× cell lysis buffer (Cell Signaling Technologies, Pickering, ON) containing 1× Halt protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL). Western blots were carried out using an anti-clozapine antibody, as described in Chapter 2.

Alternatively, monocytes were differentiated to macrophages with PMA (25 ng/mL) over 3 days at 37 °C. Cells were washed once and resuspended in fresh media without 2-mercaptoethanol. Clozapine and olanzapine were dissolved in DMSO (0.25%) and added to the cells over a concentration range of 1 – 10 μg/mL. Both drugs were coincubated with Z-VAD-fmk (10 μg/mL) to block caspase activation. Cells were incubated at 37 °C for 6 – 24 h, at which point media was collected for IL-1β analysis, as well as measurement of TNF-α, CXCL1, G-CSF, and IL-6 using ELISA kits from R&D. As was done for the monocytes, in some cases protein was extracted from the cells for western blot analysis to detect covalent binding to clozapine.

4.3.6 Statistical analysis

Prism 5 (GraphPad, San Diego, CA) was employed for all statistical analyses by two-way or one-way ANOVA, or the Student’s t test. Data sets were expressed as the mean ± SEM and results were considered statistically significant if p < 0.05.
4.4 Results

4.4.1 Clozapine increases neutrophil counts after acute and chronic treatment

Total leukocyte counts were found to remain stable for 24 h after a single dose of clozapine (acute study, Figure 4-1A) as well over 10 days of daily clozapine treatment (chronic study, Figure 4-1B). In the acute study, a significant spike in neutrophils was observed 3 h after the dose, which remained elevated over the remainder of the 24 h period. This was paired with a significant decrease in lymphocytes at 3 and 6 h, which returned to control levels 24 h post-dose. Chronic treatment with clozapine led to a continuing increase in neutrophil counts, whereas no change in the lymphocyte counts was noted over this same time period. Overall, an increasing state of neutrophilia was observed in the rats over 10 days of treatment, which is common in approximately 50% of patients at the start of treatment.\textsuperscript{104}
Figure 4-1. Leukocyte counts in rats treated acutely and chronically with clozapine

Leukocyte counts (A) over a 24 h period following a single dose of 30 mg/kg clozapine, or (B) chronically over 10 days of clozapine treatment (30 mg/kg/day). Whole blood was collected from the tail vein, and total leukocyte and differential counts were determined manually. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
4.4.2 Proinflammatory markers are elevated after acute and chronic clozapine treatment

Increases in numerous proinflammatory markers, such as C-reactive protein, IL-6, and TNF-α have been reported at the start of treatment in clozapine patients.\(^{125,126}\) Of particular interest, G-CSF has also been shown to increase in the serum and is likely related to the transient neutrophilia observed in patients.\(^{104}\) Therefore, we set out to find evidence of inflammation in our rat model, based upon the observations reported in patients. C-reactive protein was unchanged in the clozapine-treated rats over 10 days of treatment, likely because this acute phase marker is not highly expressed in rats after an inflammatory stimulus (Life Diagnostics Inc.). Alternatively, the acute phase protein α-1-AGP, a much better marker of inflammation in rats, was significantly elevated 24 h post-clozapine dose (Figure 4-2A). Over long-term treatment, an increasing trend in α-1-AGP was observed during the first week that peaked significantly at 8 days, and then began to drop off 2 days later (Figure 4-2B). The presence of IL-6 was detectable only in serum from the acute study, but no difference between the control and treated groups was found. TNF-α and IL-1β were not detectable in the serum from either study, and similarly IL-6 was undetectable in the chronic serum samples. G-CSF was significantly elevated at 3 and 6 h post-clozapine dose, which dropped to baseline levels by 24 h. This short half-life of G-CSF is likely why chronic treatment led to no change in this marker; serum was collected immediately before the next daily dose, which corresponds to the acute 24 h time point. Due to the significant changes observed in the neutrophil counts with clozapine treatment, the neutrophil chemoattractant CXCL1 was also tested for in the same serum samples. This chemokine was strongly upregulated 3 h post-dose, but sharply disappeared from the blood by 6 h. Over 10 days of treatment CXCL1 levels were unchanged, which was not surprising given the short half-life of the protein observed in the acute study.
Figure 4-2. Serum concentrations of proinflammatory markers following acute and chronic clozapine treatment

Serum concentrations of proinflammatory markers were measured by ELISA (A) over a 24 h period following a single dose of clozapine (30 mg/kg), or (B) chronically over 10 days of clozapine treatment (30 mg/kg/day). Blood was collected from the tail vein and serum was collected by centrifugation at 10 000 g for 5 min. All protein concentrations were measured using commercial ELISA kits. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by two-way ANOVA, where **, p < 0.01; and ***, p < 0.001.
4.4.3 Clozapine stimulates IL-1β production in THP-1 monocytes and macrophages

Human THP-1 cells were employed to assess the ability of clozapine to activate inflammasomes in monocytes and macrophages. Clozapine was found to induce IL-1β production and release in LPS-stimulated monocytes that were incubated with 25 μg/mL of the drug for 24 h. Production of IL-1β was completely blocked by the caspase inhibitor Z-VAD-fmk, confirming that the cytokine was generated by caspase-dependent inflammasome activation (Figure 4-3A). In the absence of LPS stimulation IL-1β was undetectable. PMA-differentiated macrophages were found to be more sensitive to clozapine, and IL-1β levels were significantly elevated at much lower clozapine concentrations (5 and 10 μg/mL). Likewise, addition of Z-VAD-fmk blocked production of IL-1β in the macrophages. Furthermore, in a time course analysis, IL-1β was detected as early as 3 h and remained elevated until 24 h (Figure 4-3B). Olanzapine, which is structurally similar to clozapine, was used as a negative control in all experiments, and did not induce IL-1β production in either cell type.

The media from the macrophage incubations was also analyzed for the production of other proinflammatory cytokines downstream of IL-1β, particularly those found to be associated with clozapine treatment in patients and rats (Figure 4-4). Following a 24 h incubation with clozapine, CXCL1 was detected in response to 2 – 10 μg/mL of the drug, and likewise, in a time course analysis, CXCL1 was detected at 18 h and 24 h (10 μg/mL clozapine). TNF-α appeared to be slightly elevated after 24 h over the same concentration range (2 – 10 μg/mL clozapine); however, no significant difference compared to DMSO was found. Furthermore, no changes were seen with TNF-α or IL-6 in the time course analysis, and G-CSF was undetectable.

Lastly, the covalent binding of clozapine to the THP-1 macrophages and monocytes was assessed by western blotting. Although some background binding was observed in the DMSO-treated macrophages, increased binding was evident as the clozapine concentration increased from 2 to 10 μg/mL (Figure 4-5). In contrast, very little binding to monocytes was observed when the cells were incubated with 20 μg/mL of clozapine, and in fact, appeared to be no stronger than the background DMSO level to the macrophages.
Figure 4-3. Clozapine-induced production of IL-1β in THP-1 monocytes and macrophages

(A) THP-1 monocytes were stimulated for 3 h with LPS (1 μg/mL) followed by incubation with 1 – 25 μg/mL of clozapine for 24 h. (B) Left; macrophages were differentiated from monocytes over 3 days using 25 ng/mL of PMA, and then incubated with 1 – 10 μg/mL of clozapine or olanzapine for 18 – 24 h. Right; macrophages were incubated with 10 μg/mL of clozapine for 3, 6, 18, and 24 h. In both cell types, the caspase inhibitor Z-VAD-fmk (10 μg/mL) was employed to block inflammasome activation against 10 μg/mL of clozapine or olanzapine. IL-1β concentrations were measured in the media by ELISA. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by one-way ANOVA, where ψ, p < 0.05; †, p < 0.01; and ‡, p < 0.001.
Figure 4-4. Production of proinflammatory cytokines by clozapine-treated THP-1 macrophages

Macrophages were differentiated from monocytes over 3 days using 25 ng/mL of PMA, and (A) incubated with 1 – 10 μg/mL of clozapine for 24 h. The caspase inhibitor Z-VAD-fmk (10 μg/mL) was employed to block inflammasome activation against 10 μg/mL of clozapine. Olanzapine (10 μg/mL) was used as a negative control. (B) Macrophages were incubated with 10 μg/mL of clozapine for 3, 6, 18, or 24 h. All cytokine concentrations were measured in the cell culture media by ELISA using commercial kits. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by one-way ANOVA, where ψ, p < 0.05; and †, p < 0.01.
4.4.4 Coadministration of anakinra blocks the clozapine-induced inflammatory response in vivo

Rats were coadministered clozapine and the IL-1 receptor antagonist anakinra to assess the involvement of IL-1 in the proinflammatory response observed at the start of clozapine treatment. The serum concentrations of clozapine 3 h post-injection were measured in all rats treated with clozapine, and in fact, two rats out of five cotreated with clozapine and anakinra were found to have significantly reduced clozapine concentrations. During clinical trials no interactions between a variety of other drugs and anakinra were found, and more specifically, anakinra was not reported to be an inducer of CYP 1A2, the primary pathway of clozapine elimination. However, IL-1 itself has been shown to significantly depress cytochrome P450 levels and activities in mice, specifically within 24 h of a single injection of recombinant IL-1. In theory, blocking the IL-1 receptor could suppress this inhibitory effect on the P540 enzymes, leading to increased P450 metabolism of clozapine in the anakinra cotreated animals. Nonetheless, not all cotreated animals had significantly depressed clozapine blood levels. A plot of neutrophil count versus clozapine blood concentration at 3 h indicated that neutrophil counts...
were in fact suppressed by anakinra, particularly in those animals with the highest clozapine blood levels (Figure 4-6A). However, to be certain that the two animals with the low blood levels did not falsely skew the data in favor of proinflammatory suppression; they were removed from the analysis. This led to clozapine blood levels between the clozapine alone (n=3) and anakinra cotreated (n=3) groups that were not statistically different (Figure 4-6A).

Serum concentrations of human IL-1 receptor antagonist were measured in all animals treated with anakinra, and the cotreated group was found to have slightly higher concentrations of anakinra, specifically at 3 h (Figure 4-6B). Anakinra is not a substrate of P450 and is primarily eliminated by the kidneys.129 Although it is just speculation, cotreatment with clozapine could lead to competitive inhibition of anakinra clearance in the kidney, leading to increased anakinra concentrations. Despite this difference, anakinra alone was found to have no effect on any of the parameters measured, so it is unlikely that the slightly higher anakinra concentrations in the cotreated group impacted the results.

Cotreatment of anakinra with clozapine appeared to decrease the total leukocyte and lymphocyte counts at 3 and 6 h; however, these changes were not statistically different from the anakinra alone or clozapine alone groups (Figure 4-7). As observed in the previous acute study, the neutrophil count was elevated at 3 and 6 h in the clozapine-treated group, which was suppressed significantly at both time points with coadministration of anakinra. Similarly, anakinra was found to block the clozapine-induced increase in CXCL1 at 3 h, and α-1-AGP at 24 h (Figure 4-8). G-CSF was significantly elevated in the clozapine group at 3 and 6 h, and although anakinra cotreatment did not significantly block this increase, G-CSF concentrations in the cotreated group were in fact found to be no different from anakinra alone. Overall, the concentrations of G-CSF in all samples were noted to be lower than those measured in the previous study, which may have led to a reduction in the ability to detect subtle changes in this marker.
Figure 4-6. Serum concentrations of clozapine and anakinra

(A) Serum concentrations of clozapine were measured by mass spectrometry 3 h following a 30 mg/kg ip injection. Some animals were cotreated with 50 mg/kg of anakinra, sc. (B) Serum concentrations of human IL-1 receptor antagonist (anakinra) were measured by ELISA over a 24 h period following injection of anakinra alone (50 mg/kg, sc) or cotreatment with clozapine (30 mg/kg, ip). Blood was collected from the tail vein and serum was collected by centrifugation at 10 000g for 5 min. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by the Student’s unpaired t test, where *, p < 0.05. CLZ, clozapine; AK, anakinra.
Figure 4-7. Leukocyte counts in rats cotreated with clozapine and anakinra

Leukocyte counts over a 24 h period following cotreatment of clozapine (30 mg/kg, ip) and anakinra (50 mg/kg, sc). A predose of anakinra was administered the day before clozapine treatment, as well as 1 h before clozapine administration. Whole blood was collected from the tail vein and total leukocyte and differential counts were determined manually. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by two-way ANOVA, where *, $p < 0.05$; and ***, $p < 0.001$. 
Figure 4-8. Serum concentrations of proinflammatory markers following cotreatment of clozapine with anakinra

Serum concentrations of biomarkers of inflammation were measured by ELISA over a 24 h period following cotreatment of clozapine (30 mg/kg, ip) with anakinra (50 mg/kg, sc). A predose of anakinra was administered the day before clozapine treatment, as well as 1 h before clozapine administration. Blood was collected from the tail vein and serum was collected by centrifugation at 10 000g for 5 min. All protein concentrations were measured using commercial ELISA kits. Results are expressed as the mean ± SEM and statistical difference compared to control was determined by two-way ANOVA, where *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
4.5 Discussion

The ability of clozapine to stimulate a proinflammatory response in patients at the start of therapy has been well established over the last decade, yet, the mechanism behind this response is still not well understood.\textsuperscript{85} The elevation of a wide array of proinflammatory markers, neutrophil counts, as well as body temperature, strongly indicate that a state of inflammation is present following clozapine administration.\textsuperscript{85} Recently, we established a model of early clozapine treatment in female Sprague-Dawley rats, and found that neutrophil counts were elevated due to stimulation of the bone marrow to produce more granulocytes (Chapter 3). We suspect that the response in the bone marrow may be the result of inflammation provoked by the drug. Therefore, we set out to determine if a proinflammatory response to clozapine could be detected in our rat model, in both acute and chronic scenarios. We found that in addition to the significant elevation of neutrophil counts after 10 days of clozapine treatment, they also increased acutely at 3, 6, and 24 h post-injection (Figure 4-1). A sensitive marker of inflammation in rats, the acute phase protein $\alpha$-1-AGP, was strongly elevated both acutely and chronically. This change is likely analogous to the elevated levels of C-reactive protein present in patients during the first 8 weeks of treatment.\textsuperscript{125} Patients also experience increased levels of G-CSF, most prominently during the second week of treatment.\textsuperscript{104} Although some animals displayed spikes in this marker over 10 days of treatment (see Chapter 3 for 8 day study), overall no significant increase was observed. On the other hand, when we tested for G-CSF acutely, we found that it was strongly elevated 3 – 6 h following clozapine injection, which returned to baseline levels by 24 h (Figure 4-2); this short half-life of G-CSF provides an explanation for why it was not detectable chronically. Although changes in other proinflammatory markers seen in patients, such as TNF-$\alpha$ and IL-6 were not present in the rats, we did find a significant spike in CXCL1 3 h post-dose. CXCL1 is a neutrophil chemoattractant present in the blood, and is thought to play a role in recruiting neutrophils from the bone marrow to the circulation.\textsuperscript{114}

The three markers that we found significantly elevated following a single dose of clozapine, namely, $\alpha$-1-AGP, G-CSF, and CXCL1, all share a common induction pathway, specifically stimulation by IL-1$\beta$. This potent immune stimulator is generated by the NLRP3 inflammasome, which plays an important role in innate immune responses.\textsuperscript{70} Briefly, upon activation by pathogen associated or endogenous danger signals, the inflammasome associates into its active
form, which ultimately allows for active caspase-1 to convert pro-IL-1β into its active form.⁷² Therefore, we set out to determine if clozapine could activate inflammasomes in THP-1 cells; a human monocytic cell line commonly used to study inflammasome activation.¹³⁰ In fact, we found that both THP-1 monocytes and macrophages produced significant concentrations of IL-1β when incubated with clozapine for 18 – 24 h (Figure 4-3). These responses were shown to be inflammasome-dependent, as they could be blocked by coincubation with the caspase inhibitor Z-VAD-fmk. Moreover, the negative control olanzapine, which is structurally similar to clozapine, was not able to induce a positive response. Further testing of the macrophages revealed that clozapine induced significant production of CXCL1, and possible elevations of TNF-α and IL-6 after 18 – 24 h (Figure 4-4). A time course analysis revealed that IL-1β was significantly elevated after 3 h, whereas it took at least 18 h for CXCL1 to increase, suggesting that clozapine-induced CXCL1 production in THP-1 macrophages was downstream of IL-1β.

Given the evidence that clozapine activated inflammasomes in THP-1 cells, we set out to demonstrate the relevance of these findings in vivo. We attempted measuring IL-1β by ELISA, but found that it was undetectable in the serum from both the acute and chronic clozapine rat studies. This was not surprising, as it is documented that very low levels of IL-1β in vivo are sufficient to induce inflammation.¹³¹ A cotreatment study in vivo with Z-VAD-fmk and clozapine was attempted, but in this case we found that overall inhibition of caspase led to elevated neutrophil counts in the Z-VAD-fmk treated group, presumably by inhibiting the apoptosis of neutrophils. This masked the effects of clozapine itself in the cotreated group. Our next approach was to inhibit IL-1 signaling by administering the IL-1 receptor antagonist anakinra, and interestingly, anakinra cotreatment was found to block the increased neutrophil counts observed at 3, 6, and 24 h with clozapine alone. Similarly, the elevations in CXCL1 at 3 h and α-1-AGP at 24 h were negated by anakinra; however, the changes in G-CSF at 3 – 6 h were not significantly different from either the clozapine alone nor anakinra alone groups. The concentrations of G-CSF measured in this study were significantly lower than those observed previously, and although clozapine treatment alone did lead to a significant increase at 3 – 6 h, the ability to detect subtle changes in the cotreated group may have been lost due to this decrease in the concentration range. Most importantly, the increase in G-CSF observed in the cotreated group was not statistically significant compared to anakinra alone, indicating that anakinra did appear to have a moderate repressive effect on the production of this factor. These results taken
together indicate that the proinflammatory response induced acutely by clozapine in rats is mediated by IL-1, as cotreatment with the IL-1 receptor antagonist was able to significantly prevent the increases in neutrophil count, α-1-AGP, and CXCL1.

The ability of clozapine to activate inflammasomes and induce IL-1β production provides further evidence that this drug is able to activate the immune system; this is an important finding in the context of clozapine-induced agranulocytosis. It is well established that clozapine is oxidized to a reactive nitrenium ion that covalently binds to neutrophils, and that such metabolism and binding are common amongst many drugs that cause agranulocytosis. However, patients that never developed agranulocytosis were shown to have clozapine covalently bound to their neutrophils, indicating that the reaction required more than just binding to be induced. Endogenous proteins that become modified with exogenous molecules have the potential to induce immune responses, and it is suspected that clozapine-bound neutrophil proteins could act in this way. Individual differences in immune responses could explain why covalent binding was present in patients that did not develop agranulocytosis. To date, however, very little evidence has been reported to support the involvement of the immune system in clozapine-induced agranulocytosis. The delay in onset of the reaction is inherently suggestive of an immune mechanism, yet, some cases of drug rechallenge following agranulocytosis have led to a faster onset of a second reaction, whereas others have not. Tests for drug-specific or anti-neutrophil antibodies in patients with clozapine-induced agranulocytosis were negative, suggesting that the immune component of this reaction is unlikely to be antibody-mediated. On the other hand, some hypotheses have been put forward suggesting that clozapine-induced agranulocytosis may involve an autoimmune component. Chronic elevations of proinflammatory cytokines, such as those seen at the start of clozapine treatment, are often indicative of autoimmune type reactions. Of particular relevance, the IL-1 family of ligands and receptors has been shown to be involved in a variety of inflammatory conditions, and more specifically IL-1β has been associated with numerous autoinflammatory disorders. Interestingly, Maes et al. reported increased levels of IL-1 receptor antagonist in clozapine patients after at least 6 weeks of treatment. Our findings that clozapine induced IL-1β production in THP-1 cells, and that coadministration of an IL-1 receptor antagonist in rats with clozapine blocked cytokine elevation, strongly suggest that clozapine activates inflammasomes in vivo. It could be hypothesized that inflammasome activation in clozapine patients leads to a proinflammatory response, which in the majority of patients
resolves with a form of cellular tolerance. Whereas, in less than 1% of patients, the combination of inflammasome activation and covalent binding of clozapine to neutrophil proteins could lead to induction of an autoinflammatory state, which ultimately could be responsible for the destruction of myeloid precursors in the bone marrow and agranulocytosis in the circulation. Although this is just speculation, interestingly, over a decade ago Guest et al. proposed that clozapine-induced agranulocytosis may be the result of an autoimmune T cell reaction against bone marrow granulocytes; such a mechanism would be consistent with the delayed response that is observed upon reexposure to clozapine.$^{134}$

Overall, we were able to demonstrate for the first time that clozapine activates inflammasomes, and that IL-1β is likely an important mediator of clozapine-induced proinflammatory responses present at the start of therapy. To fully understand the implications of these findings, inflammasome activation needs to be investigated in clozapine-treated patients as well as with other drugs that cause agranulocytosis.

### 4.6 Acknowledgements

We thank Novartis Pharmaceuticals Inc. for supplying clozapine. Portions of this work were presented by A. R. Lobach at the Society of Toxicology Annual Meeting in San Antonio, TX, in 2013.
CHAPTER 5

Summary and conclusions
5.1 Summary of findings and discussion

Drug-induced agranulocytosis has been a challenging IDR to study, particularly with the lack of a relevant animal model of the reaction. As such, investigating the mechanism of the reaction in vivo in the absence of an actual IDR was best studied by focusing independently on the two suspected contributing components, reactive metabolite formation and immune activation. Despite the lack of an animal model or access to appropriate patient populations, significant insight into the mechanism of drug-induced agranulocytosis was in fact provided by the studies reported in this thesis.

Investigating the involvement of the NADPH oxidase/myeloperoxidase pathway in the covalent binding of amodiaquine and clozapine to neutrophils led to a number of interesting findings, and has presented an opportunity for future studies on this subject. The compelling in vitro evidence implicating myeloperoxidase as the primary source of oxidant for drug bioactivation in neutrophils led to widespread acceptance of this hypothesis in vivo. Our desire to confirm the involvement of myeloperoxidase in the covalent binding of amodiaquine to neutrophils in vivo highlights the importance of studies that bridge in vitro and in vivo observations. The results from our in vivo investigations did not simply support in vitro findings, but rather elevated the complexity of the metabolic scenario. The observation that covalent binding to neutrophils decreased 2-fold in amodiaquine-treated myeloperoxidase knockout mice supports the hypothesis that this enzyme is involved in generating the amodiaquine quinone imine metabolite. However, the involvement of other pathways or oxidants was apparent due to the fact that a) binding to neutrophils was not completely abolished in the absence of myeloperoxidase, and; b) extensive binding to circulating lymphocytes was detected both in vivo and in vitro. Despite extensive efforts, we were unable to identify an alternative source of oxidant, but effectively eliminated NADPH oxidase (NOX1/2) and the peroxidase prostaglandin H synthase (COX-1/2) as contributors. Similar studies were carried out with ex vivo incubations of knockout neutrophils with clozapine, and covalent binding was also found to decrease in the absence of myeloperoxidase, but again was not completely abolished. On the other hand, in vitro lymphocyte incubations with clozapine did not lead to any covalent binding. This highlights the largest limitation encountered with these studies; it was not possible to effectively compare the differences in binding between amodiaquine and clozapine in vivo due to the sensitivity of mice
to the extreme sedative effects of clozapine. This, combined with an anti-clozapine antibody that lacked specificity and sensitivity, made it impossible to detect covalent binding in vivo. Overall, these studies did provide conclusive evidence that myeloperoxidase is responsible for a portion of the covalent binding of amodiaquine to neutrophils in vivo, yet complicated the picture by suggesting that other pathways/oxidants can also metabolize the drug, and NADPH oxidase is not the source of peroxide required for myeloperoxidase-mediated oxidation.

Establishing a rat model of early responses to clozapine proved to be a useful tool in the absence of an animal model of clozapine-induced agranulocytosis. When employing animal models of human conditions, it is of utmost importance that the model be reflective of the true scenario in patients. Therefore, we exerted a considerable effort to confirm that the rats were responding to clozapine at the start of treatment in a similar manner as patients, which is summarized in Table 5-1. First off, the dose was optimized to generate clozapine blood levels in the rats that were within the therapeutic range. Administering a daily dose of 30 mg/kg by ip injection led to neutrophilia in the circulation, and these neutrophils were found to have a decreased nuclear lobe count, which have both been reported in patient studies. Elevated levels of G-CSF were detected acutely in the rats 3 – 6 h following a single dose of clozapine, as well as spikes during the first week of treatment, which in patients occurs later in the course of treatment at 6 weeks. The covalent binding of clozapine to neutrophils was also assessed in the rats, and binding was detected to a similar range of proteins as previously reported in patients.

Table 5-1. A rat model of early changes induced by clozapine

<table>
<thead>
<tr>
<th>Effect on circulating neutrophils</th>
<th>Humans</th>
<th>Sprague-Dawley Rats (female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clozapine dose</td>
<td>300-600 mg/day</td>
<td>10 mg/day</td>
</tr>
<tr>
<td>Blood levels</td>
<td>0.35-1.3 ug/mL</td>
<td>0.4-1.3 ug/mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect on the bone marrow (evidence in the serum)</th>
<th>Humans</th>
<th>Sprague-Dawley Rats (female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ neutrophil lobe count (blood)</td>
<td>↑ in CD34+ cells (blood)</td>
<td>↓ neutrophil lobe count (blood) spikes in serum G-CSF @ 3-6 h and during week 1</td>
</tr>
<tr>
<td>↑ in serum G-CSF @ week 6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Covalent binding to neutrophils</th>
<th>Humans</th>
<th>Sprague-Dawley Rats (female)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bands detected between 58-106 kDa</td>
<td>bands detected between 40-80 kDa</td>
</tr>
</tbody>
</table>
Extensive investigation with this model, which initially focused on the effects of clozapine on neutrophils and bone marrow, led us to a novel finding regarding the ability of clozapine to activate the immune system. To begin with, clozapine was found to stimulate the bone marrow to produce more neutrophils, starting at the level of the progenitor cells and influencing them to generate more granulocyte colonies. This led to increased numbers of myeloid cells in the bone marrow, and more specifically, an increase in granulopoiesis. By labeling the dividing neutrophils in the bone marrow with BrdU, increased release of the neutrophils from the bone marrow was confirmed in vivo, leading to significantly elevated neutrophil counts after 8 days of clozapine treatment. Overall, this alteration of neutrophil production and release was found to be typical of G-CSF-induced bone marrow stimulation. This appears to reflect the situation in patients because elevated levels of G-CSF and neutrophilia are common during the first weeks of clozapine therapy. In addition, other effects such as an increase in CD34+ cells and decreased neutrophil nuclear lobe count are evidence of bone marrow stimulation. However, the extent to which these findings in rats can be extrapolated to patients is limited, and the difficulty of directly studying the bone marrow in patients further restricts the ability to confirm these findings.

Since increased G-CSF production is often accompanied by elevated levels of proinflammatory cytokines, and this has in fact been demonstrated in clozapine patients, the next set of studies focused on investigating how clozapine induces a proinflammatory state at the start of treatment. Although most of the classic inflammatory markers found in clozapine patients, such as IL-6, TNF-α, and C-reactive protein, were not detected in the rats, significantly increased concentrations of the acute phase protein α-1-AGP (a robust inflammatory marker in rats) was indeed indicative of inflammation. Furthermore, the neutrophil chemoattractant, CXCL1, was also strongly upregulated. Upstream analysis of G-CSF, α-1-AGP, and CXCL1 production suggested a common induction pathway by IL-1. This led to a combination of in vitro and in vivo studies to determine if clozapine could activate inflammasomes to produce IL-1β, and if the proinflammatory response observed in rats was in fact induced by IL-1. Investigations with THP-1 macrophages revealed that clozapine is a potent activator of inflammasomes, leading to both IL-1β and CXCL1 production by these cells in vitro. Coadministration of clozapine and the IL-1 receptor antagonist anakinra in vivo appeared to block the strong upregulation of α-1-AGP and CXCL1 by clozapine, in addition to suppressing the increase in neutrophil counts. These studies
combined suggest that the proinflammatory response observed at the start of clozapine treatment is mediated by inflammasome-generated IL-1β.

The evidence that clozapine activates inflammasomes in vitro is a novel finding that in the context of IDRs proposes a unique pathway by which drugs, or their reactive metabolites, may activate the immune system. A variety of danger signals have been shown to activate the NLRP3 inflammasome, of particular relevance, damage-associated molecular patterns generated endogenously that are indicative of cellular damage. Production of such danger signals could be the result of covalent binding of a drug reactive metabolite, such as the clozapine reactive nitrenium ion, to cellular proteins. Interestingly, in addition to its ability to activate inflammasomes in the THP-1 macrophages, clozapine also covalently bound to the cells; whether or not the two findings are related remains to be elucidated. In the context of the danger hypothesis, in order to stimulate an immune response both signal 1, represented by the presentation of drug-modified peptides to T cells, and signal 2, a danger signal, are required. Inflammasome activation may represent the source of signal 2. Although drugs that cause agranulocytosis were the focus of these studies, many of the characteristics of other types of IDRs are similar. Therefore, inflammasome activation by drugs may represent a general mechanism by which many drugs cause IDRs.

Using clozapine as a model compound to summarize the overall results from the studies described in this thesis (Figure 5-1), we hypothesize that myeloperoxidase and probably other unknown oxidants mediate the generation of a clozapine nitrenium ion in neutrophils, which in turn, covalently binds to neutrophil proteins. These clozapine-modified proteins, or possibly the parent compound itself, activates inflammasomes in macrophages, for instance, leading to the production and release of active IL-1β from these cells. IL-1β induces a proinflammatory state through interaction with the IL-1 receptor, leading to increased release of acute phase proteins (i.e. α-1-AGP, C-reactive protein) and proinflammatory cytokines (i.e. IL-6, TNF-α) at the start of treatment. The production of the neutrophil mediator G-CSF is also induced by IL-1β, resulting in increased production of CFU-G colonies in the bone marrow and elevated granulopoiesis. Mobilization of neutrophils from the bone marrow is mediated by the chemokines CXCL1 (circulation) and MIP-2/CXCL2 (bone marrow), leading to neutrophilia. However, as observed in patient studies, the circulating inflammatory markers and neutrophil counts return to baseline levels with continued treatment, suggesting the involvement of some
form of immune tolerance. Since we suspect that the inflammatory cascade is initiated by IL-1β, this tolerance may involve suppression of IL-1β signaling through the IL-1 receptor, or simply increased production of the IL-1 receptor antagonist. It is possible that in the small percentage of patients that develop agranulocytosis, this tolerance does not occur, thereby allowing IL-1β to continue its proinflammatory insult. This sustained innate immune response could stimulate an adaptive immune response that targets neutrophil precursors in the bone marrow, leading to agranulocytosis in the circulation.

Figure 5-1. Insights into the mechanism of clozapine-induced agranulocytosis
5.2 Implications and future directions

Elucidating the other pathways/oxidants capable of metabolizing amodiaquine to the quinone imine in neutrophils and lymphocytes is an important area to investigate in future studies. The fact that amodiaquine binds to neutrophils and lymphocytes, but not erythrocytes, may provide a starting point to identify other oxidants to investigate. Although cytochrome P450s are not expressed at very high levels in leukocytes, the ability of these enzymes to oxidize amodiaquine has been demonstrated in vitro, and it may not be necessary for large quantities of enzyme to be present to lead to detectable covalent binding. Determining how these drug-modified proteins stimulate an immune response and making a link between their generation and agranulocytosis is also an area where much research remains to be done. More importantly, a new anti-clozapine antibody needs to be generated so that the binding of clozapine can be investigated in vivo and compared to amodiaquine. The approach employed to generate the amodiaquine immunogen, specifically, the thiolation of a carrier protein that was then reacted with the amodiaquine reactive quinone imine, could theoretically be employed to synthesize a similar clozapine immunogen. The clozapine nitrenium ion also reacts with thiol groups, which may lead to an immunogen with a higher hapten density than obtained previously and therefore a stronger antibody titer. With a high titer and specific clozapine antiserum it may be possible to detect covalent binding in mice at the lower dose required for murine studies.

Designing studies to determine how clozapine activates inflammasomes, and whether or not this is dependent on covalent binding, is a new and important area to investigate. Is the activation due to direct interaction of the drug with the cell, or the downstream generation of damage-associated molecular patterns? Understanding what cell types can be activated, and where these cells are located will likely provide valuable mechanistic insights. Furthermore, the ability of other drugs that cause agranulocytosis to activate inflammasomes, as well as those that cause other types of IDRs, may prove to be an important predictor of IDR propensity. Interestingly, preliminary studies with amodiaquine have already demonstrated that this drug strongly activates inflammasomes in THP-1 cells (K. Weston, unpublished data). Most drugs that cause IDRs form reactive metabolites, but the opposite is not always true; not all drugs that form reactive metabolites are associated with a significant incidence of IDRs. The ability of a drug, or more likely a reactive metabolite of a drug, to activate inflammasomes may be a biomarker to predict IDR risk.
In order to understand the differences between the small percentage of patients who develop agranulocytosis versus the majority of patients who we speculate, develop immune tolerance, requires an animal model of the reaction, or access to an extensive number of patients. Unfortunately, neither is a viable option at the moment, so research into developing an animal-model of drug-induced agranulocytosis, although it has been met with great challenge in the past, remains a relevant task. Possibly the recent successes with blocking immune tolerance to generate a mouse model of idiosyncratic liver injury may be applied to drugs that cause agranulocytosis, because we speculate that previous failed attempts at developing an animal model of drug-induced agranulocytosis were due to tolerance.\textsuperscript{116}

In conclusion, the ability of clozapine to activate inflammasomes is a novel finding that we suspect is related to its propensity to cause agranulocytosis. This is supported by the fact that the structural analogue, olanzapine, which does not cause agranulocytosis did not activate inflammasomes in THP-1 cells. However, to confirm this speculation, it will be necessary to link inflammasome activation to the induction of agranulocytosis; a task that will prove difficult in the absence of an animal model of the reaction. Yet, if this connection can be confirmed, the ability of drug candidates to activate inflammasomes to generate IL-1\(\beta\) could serve as a predictive tool to identify drugs that may potentially cause agranulocytosis, and possibly other IDR\textsubscript{s}. 
Appendix I

The synthesis of amodiaquine-protein conjugates to generate anti-amodiaquine antibodies
A.1 Background

The covalent binding of amodiaquine (AQ) to endogenous proteins was studied using immunochemical methods as reported in Chapter 2. To carry out these binding studies AQ-specific antibodies were required. This Appendix describes in detail the successful synthesis of AQ-protein conjugates that were employed as immunogens in rabbits to generate the AQ-specific antibodies utilized in Chapter 2.

A.2 Materials and methods

A.2.1 Chemical materials

Amodiaquine-hydrochloride (AQ) was purchased from Ipca Laboratories Ltd (Mumbai, India). Bovine serum albumin (BSA), 2-iminothiolane (2-IMT), N-acetylcysteine (NAC), N-α-acetyl-L-lysine (NAL), SDS, and Trizma base were purchased from Sigma-Aldrich (St. Louis, MO). Blue Carrier Protein (BCP), GelCode Blue, tris(2-carboxyethyl)phosphine (TCEP), and dithiolthreitol (DTT) reducing buffers were purchased from Pierce Biotechnology (Rockford, IL) and acrylamide/bis solution (30%) was obtained from BioRad (Mississauga, ON).

A.2.2 Trapping the AQ quinone imine (AQIQI) with NAC

The synthesis of AQIQI was described in Chapter 2. AQIQI was reconstituted in methanol and analyzed by mass spectrometry. To confirm the reactivity of AQIQI with thiol groups, an aqueous solution of NAC was layered on top of the original chloroform solution containing AQIQI, at an approximate ratio of 2:1, NAC:AQIQI. After 45 min the water phase containing the AQ-NAC conjugate was collected. LC-MS analysis was carried out using a PE Sciex API 3000 quadrupole mass spectrometer with an electrospray ionization source (Sciex, Concord, ON) interfaced with a HPLC system (Shimadzu, Columbia, MD) and a Zorbax 300SB-C18 5 μm 4.6 x 150 mm column (Agilent, Mississauga, ON). The mobile phase was 60% water containing 0.1% acetic acid/40% methanol.
A.2.3 Testing the reaction of AQSI with 2-IMT-modified NAL

2-IMT dissolved in distilled water was added to a phosphate buffered NAL solution (pH 8.0) at a 2:1 ratio of 2-IMT to NAL. After reacting for 1 hour at room temperature, the NAL-2-IMT mixture was combined with AQSI in 30% methanol (2:1 approximately). The reaction mixture was tested for presence of the NAL-2-IMT-AQ conjugate by LC-MS.

A.2.4 Confirming the synthesis of the AQ-BSA conjugate

Thiol groups were added to BSA by reacting with 2-IMT and quantified using the method of Ellman, as described in Chapter 2. To confirm the successful addition of multiple 2-IMT molecules to BSA, thiolated BSA was purified by centrifugal filtration (30 kDa molecular weight cutoff) and submitted for mass spectrometry analysis by the University of Toronto Advanced Instrumentation for Molecular Structure (AIMS) Laboratory. This was carried out with an AB/Sciex QStar mass spectrometer with an ESI source and an Agilent 1100 capillary LC system with a desalting column. The AQ-BSA conjugate (synthesis described in Chapter 2) was also submitted for mass spectrometry analysis. Thiolated BSA and AQ-BSA were further characterized by SDS-PAGE. Proteins (20 - 30 μg) were combined with either DTT (20 mM) or TCEP (25 mM) reducing buffer and incubated for 5 min at 95 °C. Samples were loaded onto 8% polyacrylamide gels (5% stacking), stacked for 10 min at 20 mA and separated for approximately 45 min at 30 mA using the BioRad Power Pac HC and mini-gel system. The gel was washed 3 x 10 min with distilled water and proteins were stained with GelCode Blue.

A.2.5 Preparation of the AQ-BCP conjugate

BCP was reacted with 2-IMT in 50 mM sodium phosphate buffer (pH 8.0), at a 34:1 ratio of 2-IMT to the number of lysine groups available for conjugation on BCP, for 1 hour at room temperature. The number of free thiol groups was measured immediately using the method of Ellman (approximately 1700 per protein). AQSI reconstituted in dimethylformamide was reacted with thiolated BCP at a 2:1 ratio of AQSI to free thiol on BCP. The reaction was mixed vigorously for 1 hour at room temperature, and dialyzed overnight against water at 4°C.
A.2.6   ELISA and immunoblot testing of anti-AQ antibodies generated by AQ-BCP immunization

AQ-BCP was utilized as an immunogen in one rabbit, whereas the second rabbit was immunized with AQ-BSA, as described in Chapter 2. Detecting the presence of AQ-specific antibodies in the serum of the AQ-BCP and AQ-BSA immunized rabbits was carried out by ELISA and immunoblot. Details describing these methods are outlined in Chapter 2.

A.3   Results

A.3.1   AQ was oxidized to AQQI and trapped with NAC

Both AQ and AQQI were analyzed by LC-MS to confirm the success of the oxidation reaction. At a retention time of 8.8 min, peaks were detected at m/z of 356 and 358, corresponding to AQ with the isotopes Cl^{35} and Cl^{37}, respectively (Figure A-1A). An additional peak was found at m/z of 178.5, which most likely represents Cl^{35}-AQ with a second charge (z = 2). The retention time for AQQI was found to be much longer (20.5 min) than AQ itself, and two peaks for the Cl^{35} and Cl^{37} AQQI isotopes were detected at m/z of 354.2 and 356.1, respectively, indicating a loss of two hydrogen atoms per molecule as expected (Figure A-1B). The presence of AQQI was confirmed by trapping the reactive species with NAC in a 2-phase reaction, and the aqueous phase was tested for the AQ-NAC conjugate. At the retention time of 11.5 min, peaks corresponding the Cl^{35} and Cl^{37} isotopes of AQ-NAC were detected at m/z of 517.3 and 519.3, respectively (Figure A-1C). Similar to AQ, an additional peak was noted at m/z of 259.0, which likely represents Cl^{35}-AQ-NAC with a second charge (z = 2).
Figure A-1. LC-MS analysis of AQ, AQQI, and the AQ-NAC conjugate

A) AQ was dissolved in methanol. B) AQ was oxidized by manganese (IV) oxide to AQQI in chloroform. The solvent was evaporated and AQQI was reconstituted in methanol. C) AQQI was trapped with NAC in a 2-phase reaction of water containing NAC over chloroform containing AQQI. The mobile phase was 60% methanol with 0.1% acetic acid/40% methanol and the column was a Zorbax 300SB-C18 5 μm 4.6 x 150 mm column from Agilent. Background was subtracted based on the green highlighted area.

A.3.2 NAL was reacted with 2-IMT and successfully conjugated to AQQI

To provide proof-of-concept for the thiolation of BSA with 2-IMT and subsequent conjugation with AQQI, NAL was first reacted with 2-IMT and analyzed by LC-MS. At a retention time of approximately 8 min, a mixture of 2-IMT, NAL, and the NAL-2-IMT conjugate were detected. Specifically, the highest m/z detected was 289.8, which corresponds to the NAL-2-IMT conjugate minus the chloride ion from 2-IMT. The reactants alone, NAL as well as 2-IMT (minus the chloride ion), were also found at m/z of 189.0 and 102.3, respectively (Figure A-2A). Given the successful thiolation of NAL, the NAL-2-IMT conjugate was reacted with AQQI and the reaction mixture was analyzed by LC-MS. At a retention time of about 7.8 min, evidence of a
peak at m/z of 643.2 was found, which corresponds to the molecular weight expected for NAL-2-IMT-AQ with the loss of the 2-IMT chloride ion (Figure A-2B). These results indicate that AQQI successfully reacted with the free thiol group present on the NAL-2-IMT conjugate. Therefore adding extra thiol groups to proteins appears to be a feasible approach to conjugate multiple AQ moieties to a single protein molecule.
Figure A-2. LC-MS analysis of NAL-2-IMT and the NAL-2-IMT-AQ conjugate

A) NAL was reacted with 2-IMT in phosphate buffer (pH 8.0). B) NAL-2-IMT was combined with AQQi in 30% methanol. The mobile phase was 60% methanol containing 0.1% acetic acid/40% methanol and the column was a Zorbax 300SB-C18 5 μm 4.6 x 150 mm column from Agilent. Background was subtracted based on the green highlighted area.
A.3.3 Conjugation of AQ to BSA was confirmed by mass spectrometry and SDS-PAGE

As described in Chapter 2, BSA was thiolated by reacting it with 2-IMT. In order to confirm the addition of multiple 2-IMT groups to a single BSA molecule, the 2-IMT-BSA conjugate was analyzed by mass spectrometry. The conjugate submitted to the AIMS laboratory for analysis had a maximum of 20 2-IMT units added according to the Ellman assay, corresponding to an expected molecular weight range of 66,567 – 69,170 Da. Three peaks were observed by mass spectrometry for the 2-IMT-BSA conjugate: peak 1 at 66,373 Da, for the parent BSA; peak 2 at 67,804 Da, for 10 units of 2-IMT added and; peak 3 at 69,245 Da, for 20 units of 2-IMT added to BSA (Figure A-3B). In a separate reaction, thiolated BSA was reacted with AQQI and this conjugate was also submitted to the AIMS laboratory for analysis. Despite many attempts to optimize the analysis, no signal could be obtained in the mass spectrometer, suggesting that the compound never reached the detector. Due to solubility issues experienced during the reaction of AQQI with the protein, it is highly likely that the conjugate precipitated in the column due to incompatibility with the mobile phase. Because of this, SDS-PAGE was employed as an alternative method to confirm the conjugation of AQQI to BSA (Figure A-4A). Qualitatively, the protein conjugates increased in molecular weight as expected, with the AQ-BSA conjugate displaying a much larger weight than both BSA and 2-IMT-BSA. The calculated molecular weight ranges for the two conjugates corresponded roughly with the theoretical values. To see if the higher molecular weight bands (> 130 kDa) could be removed with a stronger reducing agent, TCEP was employed in the reducing buffer instead of DTT (Figure A-4B). TCEP successfully reduced the high molecular weight bands in BSA, which represent globulins; however, the same was not observed with the AQ-BSA conjugate, even when a 30 minute TCEP pre-incubation step was added. Some of the high molecular weight bands in this sample may represent dimers or polymers of AQ-BSA, and may be another reason why the mass spectrometry analysis was not successful.
Figure A-3. Mass spectrometry analysis of BSA and the 2-IMT-BSA conjugate

Mass spectrometry was carried with an AB/Sciex QStar mass spectrometer with an ESI source and an Agilent 1100 capillary LC system with a desalting column. A) BSA. B) 2-IMT-BSA (maximum of 20 2-IMT units added per molecule of protein).
A) BSA, 2-IMT-BSA, and the AQ-BSA conjugate were reduced with 25 mM DTT and loaded onto an 8% polyacrylamide gel (30 μg protein per well). B) BSA and the AQ-BSA conjugate were reduced with 25 mM TCEP and loaded onto an 8% polyacrylamide gel (20 μg protein per well).

**Figure A-4. SDS-PAGE analysis of the AQ-BSA conjugate**

<table>
<thead>
<tr>
<th></th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
</tr>
<tr>
<td>BSA</td>
<td>66.4</td>
</tr>
<tr>
<td>BSA-SH</td>
<td>66.6 – 68.2</td>
</tr>
<tr>
<td>BSA-AQ</td>
<td>66.9 – 72.8</td>
</tr>
</tbody>
</table>

**A.3.4 Conjugation of AQ to BCP**

BCP was reacted with 2-IMT at various ratios of excess 2-IMT to the number of lysine groups available for conjugation on BCP (300-600). It was found that a 34:1 ratio of 2-IMT to lysine groups led to the optimal thiolation of BCP for subsequent reaction with AQQI. The number of thiol groups added to BCP was quantified using Ellman’s reagent by normalizing against unmodified BSA. The number of thiols added to BCP was about 1700, which suggests that more than 600 lysine groups were available for reaction with 2-IMT; however, further amino acid composition data was not available from Thermo Scientific to confirm this speculation. The maximum amount of AQQI that could be reacted with thiolated BCP, without precipitate formation was a 2-fold excess of AQQI over thiol groups. The AQ-BCP conjugate employed as the immunogen was modified with 1,700 extra thiol groups, and therefore a maximum of 1700
units of AQ (Table A-1). The coupling ratio, expressed as the number of hapten molecules per amino acids in one molecule of protein, could not be calculated based upon the limited data available for BCP, but the ratio can be estimated based upon the results for AQ-BSA. Since the molecular weight of BCP is 100-fold greater than BSA, and 100-fold more units of AQ were theoretically added to BCP, it appears that the coupling ratio between the two conjugates is likely quite similar (1:31). The optimal coupling ratio reported in the literature is 1:50.137

A.3.5 ELISA and immunoblot testing of anti-AQ antibodies generated by AQ-BCP immunization

The presence of antibodies recognizing AQ in the serum of the rabbits immunized with AQ-BSA and AQ-BCP were tested for by ELISA. The results for the AQ-BSA immunization were reported in Chapter 2 (rabbit 1). To detect antibodies in the serum from the rabbit immunized with the AQ-BCP conjugate, a capture ELISA was carried out where plates were coated with the AQ-BSA conjugate. AQ antibodies were detected in the antiserum at the first and second titer checks, as well as the terminal titer (Figure A-5A), and the antibody response appeared to be maximal by the second titer check. The specificity of the antibodies for AQ in the terminal titer was evaluated by adding a preincubation step to the capture ELISA, where the antiserum was incubated with either AQ alone, AQ-BSA, or BSA before adding it to the plate. Antiserum derived from AQ-BCP immunization showed no interaction with BSA, but was inhibited with increasing concentrations of AQ-BSA or AQ alone. The inhibition observed with these two molecules was equivalent, indicating a specific interaction of the antibody with AQ (Figure A-5B).

Covalent binding of AQ to rat liver proteins in vivo has been previously reported using serum obtained from rabbits treated with AQ as primary antibody on an immunoblot.95 Therefore, AQ-treated (62.5 mg/kg/day, 8 days) rat liver protein was used as a positive control to evaluate the ability of the synthesized AQ antibodies to detect AQ bound to protein. Mouse liver protein was also analyzed (300 mg/kg/day, in the food, 8 days). When the AQ-BCP-immunized antiserum (terminal) was utilized as the primary antibody, covalent binding to both rat and mouse liver protein was also detected (Figure A-6). However, weak bands were detected in the control samples (both species), indicating that this antiserum was binding non-specifically to some of the
liver proteins. Furthermore, the antiserum from the AQ-BCP immunization did not display as strong a binding signal when compared to the intensity observed with the AQ-BSA derived antiserum (blots were carried out side-by-side under identical conditions). Specificity of the AQ-BCP antiserum was further tested by employing the baseline serum (rabbit 2) as the primary antibody, and interestingly, a significant amount of non-specific binding was detected towards both rat and mouse liver protein. In contrast, the baseline serum from rabbit 1 showed no binding when it was employed as the primary antibody (Chapter 2). Therefore, it appears that the anti-AQ antibodies generated in rabbit 1 following AQ-BSA immunization were more sensitive and specific for detecting AQ bound to endogenous protein, due to superior background of the baseline serum. As a result, the anti-AQ antibodies from the AQ-BSA immunization were employed for all future binding studies reported in Chapter 2.

Table A-1. Properties of the AQ-BSA and AQ-BCP conjugates

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Structure</th>
<th>Max AQ units conjugated</th>
<th>Max molecular weight</th>
<th>Coupling ratio (AQ:amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1</td>
<td>AQ-BSA conjugate</td>
<td>20</td>
<td>76 K</td>
<td>1:31</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>AQ-BCP conjugate</td>
<td>1700</td>
<td>6008 K</td>
<td>1:31*</td>
</tr>
</tbody>
</table>

* Estimated from BSA-AQ
Figure A-5. ELISA analysis of the anti-AQ antiserum (AQ-BCP immunization, rabbit 2)

(A) Anti-AQ antibodies were measured after the first immunization (titer 1) and two subsequent boosts (titers 2 and 3). (B) The specificity of the antiserum for AQ was evaluated by preincubating the antiserum with increasing concentrations of AQ, AQ-BSA conjugate, or BSA before loading on the plate. Wells were coated with the AQ-BSA conjugate. Data represent the mean ± SEM from 3 analyses.

Figure A-6. Immunoblot analysis of liver proteins obtained from rodents treated with AQ

Rats were treated with AQ for 8 days, 62.5 mg/kg/day by gavage. Mice were treated with AQ for 8 days, 300 mg/kg/day, mixed in the food. Controls were treated with saline or received food with no drug. Antiserum used as the primary antibody came from the AQ-BCP immunized rabbit, either the terminal titer or the baseline serum. Protein loading was 30 μg per lane for all samples and primary antibodies were diluted 1:10000.
A.3.6 Density calculations for blots reported in Chapter 2 and AQ binding in Rac1 and Rac2 knockout mice

Density calculations are reported for blots from AQ and CLZ binding experiments in rats and mice, reported previously in Figure 2-3 and Figure 2-5. See Chapter 2 for a full description of the experimental conditions. Furthermore, as mentioned in Section 2.4.4, Rac1 and Rac2 knockout mice were treated with AQ to investigate the possibility that compensation by other NADPH oxidase isoforms in the absence of gp91 phox (NOX2) may have led to a false negative result with respect to AQ covalent binding. Rac1 and Rac2 knockout mice were obtained from the laboratory of Dr. Michael Glogauer. Rac1 conditional knockout mice lack Rac1 only in the cells of the granulocyte/monocyte lineage, however these cells are still capable of an oxidative burst and therefore have active NADPH oxidase. Conversely, Rac2 protein is required for activation of neutrophil NADPH oxidase, and therefore the neutrophils in Rac2 knockout mice are not capable of an oxidative burst. Rac1 and Rac2 knockout mice were treated with AQ for 8 days (350 mg/kg/day) as previously described for the mouse studies in Chapter 2.
Figure A-7. Integrated density values for immunoblots previously reported in Chapter 2

A – C: See Figure 2-3 for a full description of the experimental conditions. D: See Figure 2-5 for a full description of the experimental conditions. Integrated density values were calculated and reported as a percentage of the lane with the highest density value (100%).
Figure A-8. In vivo covalent binding of AQ to Rac1 and Rac2 knockout mice

Mice were treated for 8 days with AQ (350 mg/kg/day), and covalent binding of AQ to neutrophils isolated from the bone marrow was detected by immunoblot. Protein loading was 30 μg per lane, and the anti-AQ antiserum was used as the primary antibody at a dilution of 1:2000. A) Rac1 knockout mice. B) Rac2 knockout mice. Integrated density values were calculated and reported as a percentage of the wildtype lane treated with AQ that had the highest binding (100%). KO, knockout.

A.4 Summary and conclusions

Drug-specific antibodies are a useful tool to study the interaction of drugs with proteins; however, synthesis of these antibodies is not a trivial process. The first and most important step is the creation of drug-protein conjugates with a high drug to protein ratio or sufficient hapten density. Proteins are not inherently reactive with small molecules, and vice versa, small molecules are generally not designed to be reactive in their parent form. Therefore, the successful generation of drug-protein conjugates with high hapten densities requires that both reactants be converted into reactive species. Given that AQ has been shown to be easily oxidized to the AQQI\textsuperscript{98} and that this metabolite is highly reactive with free thiol groups, we decided to add thiol groups to proteins using the reagent 2-IMT. We found that reaction of AQQI with 2-IMT thiolated proteins generated AQ-protein conjugates with sufficient hapten densities to illicit immune responses in rabbits towards AQ itself. This led the successful generation of anti-AQ antibodies that were demonstrated to be highly sensitive and specific for the detection of AQ covalently bound to protein. This is the first time that this approach has been used to generate amodiaquine-protein immunogens, and we believe that this may be a viable approach for the synthesis of other drug-specific antibodies.
References


