

## Pharmacological Reviews: The emerging role of the innate immune response in idiosyncratic drug reactions

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## Supplemental Data

## A. Effects on Organ Weights

## 1. Rodent Studies

## a. Amodiaquine

Compared to untreated control rats, no effects on spleen, liver, or lung weights were observed when male Wistar rats were treated with amodiaquine (10 mg/kg/day, intraperitoneal (i.p.)) for up to 3 weeks (Elko and Cantrell, 1970). A second study also did not find any changes in liver weight during 7 weeks of amodiaquine (~200-300 mg/kg/day in rodent meal) treatment, although spleen weights were significantly increased from weeks 1 to 7, with peak weight at week 3 (Metushi *et al.*, 2015). In contrast, amodiaquine (10 mg/kg/day, per os (p.o.)) treatment of male Wistar rats for 4 days resulted in increased liver weights after 4 weeks compared to saline-treated rats, while heart, spleen, and kidney weights were unaffected (Ajani *et al.*, 2008).

Male Sprague Dawley rats administered amodiaquine (5, 10, or 15 mg/kg, p.o.) for 14 days, following which changes were evaluated up to 34 days (Niu *et al.*, 2016). The testis weights of amodiaquine-treated animals were found to be significantly lower than those of the control animals, although results from this study were presented as pooled groups for each drug dose and included data from 4 different endpoint times.

After 1 week of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment in male Brown Norway rats, another group noted significantly smaller livers and larger spleens, compared to control animals (Liu *et al.*, 2016).

## b. Amoxicillin

In female B6C3F1 mice dosed with 20, 100, or 500 mg/kg/day amoxicillin by oral gavage for 28 days, there were no changes in spleen weight or cellularity (Lebrec *et al.*, 1994).

## c. Nevirapine

In male rats administered 6 mg/kg nevirapine for 60 days, liver weights appeared slightly decreased compared to those of controls (Awodele *et al.*, 2015). This is unexpected as nevirapine induces liver enzymes, and an increase in liver weight is expected. In contrast, another study using male rats dosed nevirapine at 32 mg/kg for 4 weeks, an increase in liver weights was observed (Adaramoye *et al.*, 2012). The different results may be explained by the different doses used.

## d. Clozapine

To evaluate potential immunotoxicity, female BALB/c mice were treated with clozapine (1, 5, 10, or 20 mg/kg/day, i.p.) for 21 days (Abdelrahman *et al.*, 2014). Clozapine caused a dose-dependent reduction in spleen weight, reductions in both thymus and liver weight at the highest dose, and an overall decrease in body weight at 10 and 20 mg/kg, but no changes in kidney weight.

Conversely, following a week of clozapine treatment (25 mg/kg/day, p.o.) in male Sprague Dawley rats, liver weights were significantly increased compared to controls (Jia *et al.*,

2014). One study investigated the effects of clozapine (10, 15, or 25 mg/kg/day, i.p.) in male Wistar rats after 21 days of treatment (Abdel-Wahab and Metwally, 2014). At the two highest doses, clozapine caused increases in heart weight, total body weight, and overall heart to body weight ratios. Similarly, after 4 weeks of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased relative heart weight compared to control (Nikolic-Kokic *et al.*, 2018).

## B. Effects on Liver

### 1. Rodent Studies

#### a. Amodiaquine

In a study of male Wistar rats, amodiaquine was administered either orally (269  $\mu$ mol/kg [95.7 mg/kg]), intraperitoneally (269  $\mu$ mol/kg [95.7 mg/kg]), or intramuscularly (2.15, 21.5, 269, or 538  $\mu$ mol/kg [0.77, 7.7, 95.7, or 191.5 mg/kg]) for 4 days, and liver changes were evaluated from day 5 to day 18 (Clarke *et al.*, 1990). No changes were observed with doses up to 269  $\mu$ mol/kg (95.7 mg/kg) but following the final intramuscular (i.m.) injection of highest dose (538  $\mu$ mol/kg [191.5 mg/kg]), animals developed a significant increase in serum ALT levels, which remained elevated until day 17. Additionally, hepatic glutathione levels were increased following both the i.p. and i.m. doses, compared to control, but no changes in liver histology were observed.

Using a bacterial fulminant model of hepatitis in C57BL/6 mice, another group demonstrated that amodiaquine had protective effects on the liver (Yokoyama *et al.*, 2007). At 24 hours post-induction, lethality was reduced from 60% to 20% with amodiaquine co-treatment and elevated serum levels of AST and ALT were significantly attenuated at 3 and 6 hours.

In fasted BALB/c mice treated with amodiaquine (180 mg/kg, p.o.), no effects on liver function or morphology were observed, although a moderate decrease in liver GSH levels were observed from 3 to 6 hours (Shimizu *et al.*, 2009). In GSH-depleted mice (induced by L-buthionine-S,R-sulfonimine (BSO)), however, amodiaquine induced a massive increase in plasma ALT levels from 6 hours, peaking at 24 hours, which was accompanied by approximately 60% lethality by 48 hours. Liver histology revealed lipid accumulation in the centrilobular hepatocytes by 6 hours, extensive necrosis with congestion by 24 hours, and inflammatory cell infiltration by 48 hours. Covalent binding of amodiaquine to liver and plasma proteins was doubled in the BSO co-treated group compared to amodiaquine alone. Co-administration of ABT prevented the increases in ALT and lethality observed with the BSO-amodiaquine group.

One study evaluated effects of amodiaquine (6.12 mg/kg, p.o.) administered twice a day in female Wistar rats treated for 3 days (Abolaji *et al.*, 2014). On day 4, slightly elevated plasma ALT, AST, and ALP levels, in addition to increased total plasma proteins, were observed with amodiaquine compared to water-treated controls.

Following 8 days of administration of amodiaquine in male Brown Norway rats (62.5 mg/kg/day, p.o.) or mice (~300-350 mg/kg/day in rodent meal), significant hepatic covalent binding was detected (Lobach and Uetrecht, 2014b). This group also showed that treatment of female C57BL/6 mice with amodiaquine (~200-300 mg/kg/day in rodent meal) for up to 7 weeks led to natural killer cell-mediated liver injury with delayed onset, which resolved despite continued treatment (Metushi *et al.*, 2015). Specifically, serum ALT levels were increased with amodiaquine from weeks 2 to 6, peaking between weeks 3 to 4. Covalent binding of amodiaquine was greater in the centrilobular region of the liver. Covalent binding and liver injury were also observed in male mice, but to a much lower extent.

Additionally, they investigated the impact of immunizing female C57BL/6 mice with amodiaquine-modified hepatic proteins (weekly for 3 weeks, plus 2 week washout) prior to amodiaquine (~200-300 mg/kg/day in rodent meal) treatment and found that this pre-treatment was effective in preventing the ALT spike observed from weeks 3 to 4 with amodiaquine alone (Mak and Uetrecht, 2015). Interestingly, the immunized group had greater leukocyte infiltration in the liver compared to amodiaquine alone. To investigate the role of macrophages in amodiaquine-induced liver injury, we conducted another study using female C57BL/6 wildtype and CCR2<sup>-/-</sup> mice, but there was no difference in the liver injury (Mak and Uetrecht, 2019). Both genotypes were treated with amodiaquine (~200-300 mg/kg/day in rodent meal).

Moreover, several models of amodiaquine-induced liver injury were investigated using mice and rats (Liu *et al.*, 2016). Amodiaquine (~200-300 mg/kg/day in rodent meal) was fed to male C57BL/6 mice for up to 4 weeks, with or without BSO in the drinking water and a single dose of diethyl maleate to deplete GSH. After 1 week, BSO co-treated animals had significantly lower liver GSH levels compared to amodiaquine alone or control; however, BSO co-treatment paradoxically prevented the amodiaquine-induced ALT peak observed during weeks 2 to 4. Covalent binding of amodiaquine to hepatic proteins was not substantially altered with BSO co-treatment. The BSO phenomena was indicated to have been reproduced in Brown Norway and Wistar rats, although the data was not shown.

Liu *et al.* (2016) did, however, demonstrate that male Lewis, Wistar, and Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) developed elevated serum ALT levels (greatest in Wistar rats) with increased hepatic covalent binding from weeks 2 to 4. Less severe liver injury, as measured by ALT peaks, was observed with female rats. To cause immune suppression, male Brown Norway rats were co-treated with amodiaquine and cyclosporine, which effectively prevented the amodiaquine-induced ALT peak. Conversely, when rats were administered poly I:C prior to amodiaquine to stimulate immune activation, there was an earlier onset of increased ALT. Natural killer cell activation was induced in treated rats by co-administration of retinoic acid, which also exacerbated the amodiaquine-induced ALT increase.

#### b. Amoxicillin

Serum ALT was increased at day 14 in male Wistar rats administered amoxicillin at a dose of 30 mg/kg/day intraperitoneally (Oyebode *et al.*, 2019).

#### c. Nevirapine

In male rats administered 6 mg/kg nevirapine for 60 days, serum ALT was increased (Awodele *et al.*, 2015). In another study using male rats, nevirapine was dosed at 32 mg/kg for 4 weeks, an increase in ALT was observed (Adaramoye *et al.*, 2012). Other markers of liver injury were also increased (serum total bilirubin and  $\gamma$ -glutamyl transferase). Necrosis was noted in the liver histology, and liver protein was decreased.

Female Brown Norway rats administered 75 mg/kg nevirapine for 7 days did not show an increase in ALT (Brown *et al.*, 2016), nor at a dose of 200 mg/kg for up to 14 days, although there appeared to be liver injury at day 7 which had resolved by day 14 in this study (Bekker *et al.*, 2012). Both of these studies also tested the effect of co-treatment of nevirapine with a substance that can cause liver injury on its own (galactosamine and lipopolysaccharide (LPS), respectively). Nevirapine was found to attenuate the galactosamine-induced ALT increase. Co-treatment with LPS attenuated the histological findings in the liver caused by nevirapine, although the combination resulted in an increase in ALT not observed with nevirapine treatment alone.

In male C57BL/6 mice, an ALT elevation at week 3 was also observed at a dose of 950 mg/kg administered in food (Sharma *et al.*, 2012). Mild necrosis in the liver was observed by histology.

#### d. Clozapine

Using a model of liver ischemia/reperfusion (I/R) in male Wistar rats, one group evaluated the protective effects of clozapine (15 mg/kg, subcutaneous (s.c.)) administered immediately following the ischemia phase and again 6 hours into the reperfusion phase (Adachi *et al.*, 2006). Notably, clozapine attenuated I/R-induced plasma ALT and AST increases after 24 hours in comparison to control animals that underwent I/R. Another group similarly showed that clozapine (15 mg/kg, s.c.) pre-treatment at 24 hours, 12 hours and immediately prior to I/R significantly attenuated increases in plasma ALT and AST at 2 hours post-ischemia (El-Mahdy *et al.*, 2013). Moreover, clozapine pre-treatment dampened I/R-mediated vacuolar degeneration of hepatocytes and dilated central vein pathology and resulted in increased hepatic GSH levels and decreased malondialdehyde (MDA, an end-product of lipid peroxidation) levels, compared to I/R controls.

One group investigated the potential impacts of clozapine in a cecal ligation puncture model of peritoneal sepsis (Machado *et al.*, 2007). After resuscitation, rats were treated with clozapine (25 mg/kg, s.c.) twice a day for 3 days. Clozapine did not attenuate hepatic damage nor was survival significantly improved when compared to the sham-operated control group. Although serum markers of organ dysfunction, including elevated creatine kinase, creatinine, AST, and amylase appeared to be altered in the clozapine-treated group compared to saline-treated controls, statistical comparison of these groups was not included.

At the end of 6 weeks of clozapine (25 or 50 mg/kg/day, p.o. [in rodent meal]) administration in female Lewis rats, clozapine extensively modified proteins in liver subcellular fractions compared to control rats (Gardner *et al.*, 2005). To test whether selenium deficiency enhanced the immunotoxicity of clozapine, female Sprague Dawley rats were maintained on a normal or selenium-deficient diet for 54 days prior to, and during, clozapine (50 mg/kg/day, p.o. in rodent meal) administration for 62 days (Ip and Uetrecht, 2008). At the end of treatment, significant hepatic covalent binding was observed with clozapine treatment, but was not altered by selenium status.

Another group used male Wistar rats to evaluate the effects of 3 weeks of clozapine (20 mg/kg/day, i.p.) with or without social isolation for the duration of treatment (Zlatkovic *et al.*, 2014). Irrespective of isolation conditions, clozapine caused marked infiltration of leukocytes in the hepatic portal triad and increased Kupffer cells and other inflammatory cells in the sinusoids, when compared to saline treated animals. Increased serum ALT and decreased hepatic GSH were also observed in all clozapine-treated groups. Clozapine-mediated increases in hepatic MDA, protein carbonyl content and GST activity were found and were exacerbated by social isolation. In the absence of social isolation, clozapine also caused elevated hepatic levels of cytosolic nitric oxide metabolites.

Hepatotoxicity was also investigated in male Sprague Dawley rats that were given clozapine (25 mg/kg/day, p.o.) with or without pre-treatment of glycyrrhethinic acid for 1 week (Jia *et al.*, 2014). Through the inhibition of 15-hydroxyprostaglandin dehydrogenase and prostaglandin reductase 2, glycyrrhethinic acid prevents the metabolism of active of prostaglandin E2 and F2 $\alpha$ , and was proposed to have hepatoprotective activity (Ming and Yin, 2013). Clozapine caused marked increased in serum ALT and AST levels, which were enhanced by glycyrrhethinic acid co-treatment.

## 2. Clinical Studies

### a. Nevirapine

In a cross-sectional study of patients with HIV and hepatitis C virus coinfection, it was found that both NNRTIs as a class and nevirapine exposure was associated with a decreased rate of fibrosis progression, an effect that was not observed with protease inhibitor exposure (Berenguer *et al.*, 2008). This effect could be due to nevirapine exposure itself and/or due to its effects on the disease states, as HAART-naïve individuals have higher hepatic proinflammatory cytokine mRNA than do HAART-treated individuals (Sitia *et al.*, 2006).

## C. Effects on Other Organs

### 1. Rodent Studies

#### a. Amodiaquine

When amodiaquine (10 mg/kg, i.m.) was administered to fasted male Wistar rats, it caused an increase in parietal cells and a decrease in mucous cells at 50 minutes post-injection (Ajeigbe *et al.*, 2012).

Another group evaluated the effects of amodiaquine (6.12 mg/kg, p.o.) administered twice a day in female Wistar rats treated for 3 days, following which no alterations in plasma levels of electrolytes, creatinine, or urea were observed (Abolaji *et al.*, 2014).

Treatment of female C57BL/6 mice with amodiaquine (~200-300 mg/kg/day in rodent meal) for up to 6 weeks demonstrated that covalent binding of amodiaquine was detectable in the kidney, the red pulp of the spleen, and in the gut (Metushi *et al.*, 2015).

During characterization of various models of amodiaquine-induced liver injury, hypertrophic cells in lung alveolar regions of male Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) for 5 weeks, which were not observed in saline-treated controls (Liu *et al.*, 2016).

#### b. Clozapine

After cecal ligation, clozapine (25 mg/kg, s.c.) administered twice a day was ineffective in reducing renal, pancreatic, lung, or muscular damage observed in male Wistar rat, although again, statistical comparison of the non-septic clozapine group versus control was not reported (Machado *et al.*, 2007). An anti-glomerular basement membrane (GBM) antibody model of proteinuria in female Wistar rats was used to evaluate the impact of twice a day clozapine (10 mg/kg, s.c.) administration on disease progression after 6 days (Tanda *et al.*, 2007). Compared to the saline and anti-GBM antibody-treated control group, clozapine was ineffective in reducing proteinuria or the formation of crescentic glomeruli in the kidney.

In a male C57BL/6 mice model of arthritis, 2 weeks of clozapine (4 mg/kg/bid, p.o.) markedly protected mice from arthritis symptoms, including an attenuation of ankle thickness from days 2 to 10 (Nent *et al.*, 2013). After 2 weeks, no inflammation was detectable in ankle joint histology in the clozapine group, while the arthritic control group presented with strong inflammation, joint destruction, and periarticular inflammatory processes along the shaft.

Male and female Wistar rats were given clozapine (20 mg/kg/day, p.o. [administered in drinking water]) for 2 months, with or without postnatal exposure to PCP, and it was shown that male non-PCP-exposed clozapine-treated rats had increased leg fat, while female PCP-exposed clozapine-treated rats had decreased total fat content compared to their respective vehicle controls (Nikolic *et al.*, 2017).

In a prenatal methylazoxymethanol (MAM) Sprague Dawley rat model of schizophrenia, where male offspring were treated with clozapine (20 mg/kg/day, i.p.) for 8 days, clozapine ±

MAM exposure did not alter brain levels of nerve growth factor (Fiore *et al.*, 2008). Serum levels of nerve growth factor and entorhinal cortex levels of brain-derived neurotrophic factor (BDNF) were only elevated in the MAM-clozapine group, relative to both the MAM-exposed saline group as well as the non-exposed clozapine group.

Conversely, when male C57BL/6 mice that were treated with PCP, co-treatment of clozapine (6 mg/kg/day, i.p.) resulted in significantly decreased cerebral cortex BDNF levels compared to saline-treated PCP controls after 2 weeks (Barzilay *et al.*, 2011). Following 1 week of clozapine (10 mg/kg/day, p.o.) in male C57BL/6 mice that had been pre-treated with PCP for 2 weeks, clozapine significantly enhanced PCP-mediated increases in prefrontal cortex mRNA expression of GSH peroxidase, glutamate-cysteine ligase modifier subunit, and glutamate-cysteine ligase catalytic subunit mRNA expression versus PCP saline controls (Tran *et al.*, 2017). Clozapine did attenuate PCP-induced GSH decreases and oxidized GSH increases, but clozapine effects in the absence of PCP were not investigated.

In a model of neurotoxicity, where male C57BL/6J mice were administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), treatment with either clozapine (1 mg/kg/day, s.c.) or clozapine *N*-oxide (1 mg/kg/day, s.c.) for 21 days significantly attenuated MPTP-induced neuron loss and reduced microglial activation (as measured by CD11b staining), compared to MPTP controls (Jiang *et al.*, 2016).

One group characterized the impact of 20 days of clozapine (10, 30, or 60 mg/kg/day, p.o. [administered in drinking water]) in a female C57BL/6 mouse model of experimental autoimmune encephalomyelitis (EAE) (Green *et al.*, 2017). At the two highest doses, clozapine significantly attenuated EAE severity scores and at the highest dose, delayed disease onset. No effects of clozapine were observed in the absence of EAE. In a second female C57BL/6 mouse model of EAE, 20 days of clozapine (10 mg/kg/day, p.o.) initiated at disease day 15 did not affect disease progression or severity, but did slightly attenuate EAE-induced white matter loss (Chedrawe *et al.*, 2018).

In another social isolation protocol in male Wistar rats, 3 weeks of clozapine (20 mg/kg/day, i.p.) did not affect cytosolic GSH levels in the hippocampus, but did significantly reduce GSH peroxidase activity, compared to saline controls (Todorovic and Filipovic, 2017b). Both GSH peroxidase and GSH reductase activity and protein levels were reduced with socially isolated clozapine treatment. In the prefrontal cortex, no changes in GSH reductase were observed, but clozapine did reduce GSH levels and GSH peroxidase activity, irrespective of social isolation (Todorovic and Filipovic, 2017a). BDNF levels were also found to be elevated after 30 days of clozapine (10 mg/kg/day, i.p.) in the brains of male CDF-344 rats (Kim *et al.*, 2012). Moreover, numerous changes in the arachidonic acid cascade were reported in the brain, in addition to decreased body weight with this model. With clozapine, the concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and COX-1 were decreased, and thromboxane B<sub>2</sub> levels and 5- and 15-lipoxygenases (LOX) mRNA levels were increased, while leukotriene B<sub>4</sub> was unchanged. In a second study by this group, clozapine was also shown to decrease the concentration of unesterified plasma arachidonic acid, and altered the composition of a number of fatty acids in the brain (Modi *et al.*, 2013).

One study evaluated the cardiotoxicity of clozapine (5, 10, and 25 mg/kg/day, i.p.) in male BALB/c mice after up to 2 weeks of treatment (Wang *et al.*, 2008). A dose-dependent increase in myocardial histopathological scores was observed with clozapine compared to saline treated controls, where inflammatory lesions consistent with myocarditis were most severe at day

7 and had begun to resolve by 2 weeks. Co-administration of propranolol significantly dampened the severity of cardiac inflammation observed at 2 weeks.

Cardiac inflammation was also demonstrated in male Wistar rats given clozapine (10, 15, or 25 mg/kg/day, i.p.) for 3 weeks (Abdel-Wahab and Metwally, 2014; Abdel-Wahab *et al.*, 2014). Compared to the saline control group, clozapine caused distinct and dose-dependent inflammatory lesions, interstitial edema, perinuclear vacuolation, focal subendocardial fibrosis, and disorganization and degeneration of the myocardium. Clozapine also increased serum levels of creatine kinase (15 and 25 mg/kg) and LDH (all doses) and reduced cardiac GSH (25 mg/kg) and GSH peroxidase activity (15 and 25 mg/kg). All inflammatory effects were attenuated by co-administration of captopril for the duration of high-dose clozapine treatment.

Additionally, after 4 weeks of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased infiltrating leukocytes in the heart and caused an abnormal structure of cardiomyocytes that was not present in the vehicle control group (Nikolic-Kokic *et al.*, 2018).

One study found that clozapine (0.5 mg/kg/day, p.o.) administration to male Sprague Daley rats for 30 days caused noticeable aberrations in kidney, spleen, and heart morphology, and also increased expression of cardiac CYP1A2, CYP3A4, and CYP2C19 mRNA compared to saline control animals (Mohammed *et al.*, 2020). Cardiac histopathological changes induced by clozapine included vascular congestion with intermuscular edema. This was accompanied by severe histopathological lesions in the spleen, as evidenced by hemosiderosis in the red pulp and lymphoid depletion (reduction in the diameter of lymphoid follicles of the white pulp). In the kidney, clozapine-treated animals exhibited numerous hyaline casts in the lumina of the renal tubules, with marked vacuolar and hydropic degeneration in the epithelial linings, focal hemorrhage and vascular congestion. Furthermore, serum levels of cardiac troponin I, LDH, creatinine, blood urea, nitrogen, urea, and uric acid were elevated following clozapine treatment, and serum lysozyme activity levels were decreased. Most biochemical changes were attenuated when clozapine was co-administered with sulpride (another antipsychotic) for the duration of the study.

After 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats, multiple cystic atretic follicles were observed with degenerated zona pellucidum and oocyte, vacuolation of cells, and an excessive deposition of collagen fibers, concentrated around the atretic follicles that was not observed in the control group (Khalaf *et al.*, 2019).

In addition to a reduction in spleen weight at all doses of clozapine (1, 5, 10, or 20 mg/kg/day, i.p.), a marked decrease in spleen cellularity and decreased white pulp density were reported following 3 weeks of the clozapine (5, 10, or 20 mg/kg) in female BALB/c mice, compared to saline controls (Abdelrahman *et al.*, 2014). Moreover, when mice were given a single dose of sheep RBCs on after 14 days of clozapine treatment, a dose-dependent reduction in hemagglutination titres was observed at after 21 days. When mice were rechallenged with a second dose of sheep RBCs, clozapine (5, 10, or 20 mg/kg) significantly attenuated footpad swelling. This was accompanied by a decrease in inflammatory infiltrates of leukocytes in both the hypodermis and muscle layers of the foot, observed with the two highest doses of clozapine.

## 2. Clinical Studies

### a. Clozapine

To investigate cardiovascular complications, a retrospective analysis of 8,000 male and female patients who commenced clozapine therapy was conducted (Kilian *et al.*, 1999). The incidence of myocarditis or cardiomyopathy reportedly due to clozapine administration in this population was 0.23% (20 male and 3 female cases). Of the 15 myocarditis cases, the median

time to onset was 15 days of clozapine (range: 3-21 days) and 6 cases were accompanied by eosinophilia.

Among 38 cases (27 male and 11 female) of clozapine-induced myocarditis, the mean time to onset was  $18 \pm 2$  days (range: 14-33 days) and the mean dose at onset was  $232 \pm 69$  mg/day (range: 50-750 mg/day) (Ronaldson *et al.*, 2010). Eighteen patients also presented with fever up to 6 days prior to detection of elevated serum TRP levels and 20 cases developed eosinophilia in the subsequent week.

In a study of 15 clozapine-naïve schizophrenia patients (8 male and 7 female) titrated from 25 mg/day up to 100 mg/day over 4 weeks, a slight increase in serum levels of hsCRP was observed at week 3 and 3 patients developed tachycardia, although no changes in leukocytes or TRP-I were observed (Curto *et al.*, 2015).

#### D. Effects on Cell Death and Proliferation

##### 1. Rodent Studies

###### a. Amodiaquine

A dose-dependent loss of spermatogenic cells and disorganization of seminiferous tubule morphology was observed following amodiaquine (5, 10, or 15 mg/kg, p.o.) treatment in male Sprague Dawley rats for 14 days, although undifferentiated spermatogonia remained alive in the testes (Niu *et al.*, 2016). TUNEL staining suggested that these cells had undergone apoptosis, which was supported by increased protein levels of FS-7-associated surface antigen (Fas), Bcl-2-associated X protein (BAX), and caspases-3, -8, and -9 in amodiaquine-treated testes. Blood-testes-barrier damage was also reported, in addition to a significant reduction in the blood-testes-barrier-associated proteins tight junction protein-1, occludin, claudin-11, eppin, and E-cadherin. No changes in serum luteinizing hormone or testosterone were observed. Amodiaquine-induced histological changes were reversed following a 20-day recovery period.

Increased apoptosis, as measured by TUNEL staining, was noted in the livers of male Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) for 5 weeks, which was not observed in saline-treated controls (Liu *et al.*, 2016). Likewise, only in amodiaquine-treated animals was cell proliferation in the germinal center of spleen significantly increased at this time. These changes were also preceded by increases in serum osteopontin (peaking at day 12) and serum cytochrome c (peaking at week 4), detected only in the amodiaquine group.

###### b. Amoxicillin

Caspase-3 and -9 were activated in the liver of male Wistar rats treated with 30 mg/kg/day amoxicillin intraperitoneally once daily for 14 days (Oyebode *et al.*, 2019).

###### c. Nevirapine

Nevirapine was administered to Sprague-Dawley rats at a dose of 200 mg/kg once daily by oral gavage, and the effect of co-treatment with LPS was examined (Bekker *et al.*, 2012). Apoptosis was noted by histological examination at the 6- and 24-hour endpoints in the nevirapine-treated group, but the liver of the nevirapine and LPS co-treated group appeared normal. At 7 days, the histology of the nevirapine group exhibited centrilobular hepatocellular degeneration, cell swelling, and hepatocyte apoptosis which resolved by days 14 and 21; again, the liver appeared normal in the co-treated group.

In female Brown Norway rats treated with 159 mg/kg/day 12-hydroxynevirapine by oral gavage, transcripts related to apoptosis and autophagy, such as death-associated protein kinase 1 (Dapk1) and Kruppel-like factor 15 (Klf 15), or cell stress, such as lipin1 (Lpin1) and DnaJ



(Hsp40) homologue, subfamily B, member 5 (Dnajb5) were upregulated in the skin 6 hours post-dose (Zhang *et al.*, 2013).

In the liver of Brown Norway rats treated with 150 mg/kg nevirapine for 8 weeks, hepatocytes were noted to have developed necrotic cytoplasm, while some endothelial cells exhibited signs of degeneration (Sastry *et al.*, 2018).

In Wistar rats administered nevirapine for 4 weeks, the live-dead ratio of the sperm was decreased in a dose-dependent manner (Adaramoye *et al.*, 2012). This was confirmed with findings of spermatocyte necrosis observed by histology.

#### d. Clozapine

In male Sprague Dawley rats, clozapine (5 mg/kg/day, i.p.) had no effect on ketamine-induced increases in caspase-3 activity in any brain area examined (striatum, hippocampus, or prefrontal cortex) (George *et al.*, 2020). However, clozapine did attenuate the elevated Bax/Bcl-2 ratio and cytochrome c expression induced by ketamine in all brain areas, and also attenuated elevated prefrontal cortical and hippocampal MDA levels.

At 2 hours post-clozapine (10 mg/kg, i.p.) administration in male Sprague Dawley rats, elevated microtubule-associated protein 1 light chain 3-II (an autophagosome marker) staining was observed in the frontal cortex, compared to saline-treated controls (Kim *et al.*, 2018). Increased autophagosome numbers were also demonstrated via transmission electron microscopy at this time. Additionally, autophagy-related protein 5-12 conjugates were increased, further indicating that activation of autophagy had occurred.

Following 21 days of clozapine (20 mg/kg/day, i.m.) administration, Sprague Dawley rats had noticeable neutrophil toxicity, manifested by condensation and subsequent breakdown of chromatin material, although this morphology was not clearly distinguishable in the figures provided in the paper (Wasti *et al.*, 2006).

After 28 days of treatment, another group did not observe any translocation of AIF from the mitochondria to the nucleus in the striatum of male Sprague Dawley rats administered clozapine (30 mg/kg/day, i.p.), suggesting against the possibility of caspase-independent neuronal cell death with clozapine (Skoblenick *et al.*, 2006).

Furthermore, after 28 days of clozapine (10 mg/kg/day, i.p.) treatment in male Sprague Dawley rats, clozapine had no effect on apoptotic markers, including histone-associated DNA fragmentation, Bax/Bcl-2 ratio, or levels of X-linked inhibitor of apoptosis protein in the frontal cortex, compared to saline controls (Jarskog *et al.*, 2007). However, levels of cleaved-caspase 3 protein, in addition to levels of activity of caspase-3, were significantly higher with clozapine.

In contrast, no changes in striatal caspase-3 activity levels were observed in male Wistar rats treated with clozapine (10 mg/kg/day, i.p.) for 21 days (Bishnoi *et al.*, 2011).

In male Wistar rats given clozapine (10, 15, or 25 mg/kg/day, i.p.) for 3 weeks, increased cardiac caspase-3 protein levels were reported (Abdel-Wahab and Metwally, 2014). In a second study by this group, clozapine-induced increases in caspase-3 activity levels and TUNEL staining were observed and were attenuated by co-administration of captopril (Abdel-Wahab *et al.*, 2014).

Following 3 weeks of treatment in male Wistar rats, clozapine (20 mg/kg/day, i.p.) increased focal necroses and apoptotic hepatocytes in liver lobules, with or without the coaddition of social isolation (Zlatkovic *et al.*, 2014). Another study noted acidophilic degeneration (indicative of dying hepatocytes) in male Sprague Dawley rats that had been co-treated with clozapine (25 mg/kg/day, p.o.) and glycyrrhetic acid for 1 week (Jia *et al.*, 2014).

Another study noted that ovarian antral follicles were characterized by increased apoptotic cells following 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats (Khalaf *et al.*, 2019). Additionally, clozapine treatment increased ovarian p53 staining and decreased Ki67 staining in comparison to the water control group.

After 11 days of clozapine (13.5 mg/kg/day, p.o.) administration in male C57BL/6 mice, increased pancreatic apoptosis and decreased proliferation were observed, measured by TUNEL and Ki67 staining, respectively, when compared to vehicle controls (Huang *et al.*, 2012). These effects were exacerbated by clozapine administration in combination with a high fat (60% fat versus standard 12% fat) diet. Members of this group later confirmed these findings, showing that administration of clozapine (13.5 mg/kg/day, p.o.) to male C57BL/6 mice resulted in an increase in apoptosis and a decrease in proliferation in pancreatic islet cells (again, measured by TUNEL and Ki67 staining, respectively), as early as 2 weeks into treatment (Hsu and Fu, 2016).

## 2. Clinical Studies

### a. Nevirapine

To our knowledge, only one study has examined cell death using samples from patients treated with nevirapine. In peripheral blood mononuclear cells, a lesser proportion of total lymphocytes and CD4 T cells was found to be apoptotic in patients receiving nevirapine-containing HAART compared to HAART-naïve, HIV-infected individuals (Karamchand *et al.*, 2008). The proportion of apoptotic total lymphocytes was greater than the proportion of apoptotic CD4 T cells, suggesting that a different lymphocyte subset was undergoing apoptosis. Altogether, this study does not support an increase in total lymphocyte or CD4 T cell apoptosis with nevirapine treatment (in combination with stavudine and lamivudine) when compared to untreated HIV-infected individuals, although an additional comparison to healthy individuals might be informative.

### b. Clozapine

To characterize potential pathways of cell death associated with clozapine, one group examined localization of the intramitochondrial protein apoptosis-inducing factor (AIF) in striatal sections collected post-mortem from 4 clozapine-treated schizophrenic patients and 4 healthy controls (Skoblenick *et al.*, 2006). Translocation of AIF from the mitochondria to the nucleus initiates a caspase-independent cell death cascade, however, no translocation was observed in either patients or controls.

## E. Effects on Immune Cells

### 1. Rodent Studies

#### a. Amodiaquine

Compared to untreated control rats, no effects on phagocytosis activity (measured by carbon particle clearance from blood) were observed when male Wistar rats were treated with amodiaquine (10 mg/kg/day, i.p.) for up to 3 weeks (Elko and Cantrell, 1970). However, amodiaquine did shorten the duration of elevated phagocytosis activity observed in malaria-infected rats.

In the study comparing routes of administration in male Wistar rats, immediately following the final i.m. injection of amodiaquine at the highest dose (538  $\mu$ mol/kg), animals developed a significant reduction in white blood cells that lasted for 4 days, after which, counts remained higher than baseline until day 14 (Clarke *et al.*, 1990).

Male Wistar rats administered amodiaquine (10 mg/kg/day, p.o.) for 4 days developed a significant increase in fibrinogen and total leukocyte counts compared to saline-treated rats at the study endpoint of 4 weeks (Ajani *et al.*, 2008).

With amodiaquine treatment (6.12 mg/kg, p.o.) twice daily in female Wistar rats, no changes in erythrocyte MDA, GSH, or G6PD levels were reported (Abolaji *et al.*, 2014).

Another group investigated the roles of myeloperoxidase and NADPH oxidase in the covalent binding of amodiaquine to neutrophils using several models: male rats (Brown Norway) and female mice (C57BL/6J wildtype, MPO<sup>-/-</sup>, gp91 phox<sup>-/-</sup>, Rac1<sup>-/-</sup>, Rac2<sup>-/-</sup>, COX1<sup>-/-</sup>, and COX2<sup>-/-</sup>) were administered amodiaquine for up to 8 days via gavage (rats, 62.5 mg/kg/day) or mixed in rodent meal (mice, ~300-350 mg/kg/day, p.o.) (Lobach and Uetrecht, 2014b). In wildtype mice and rats, significant covalent binding was detected in neutrophils after day 1 of treatment, reaching a maximum following day 4. After day 1, significant covalent binding was also observed in the bone marrow of rats and mice; specifically, binding was observed in myeloid cells, lymphoid cells, and megakaryocytes, but not in erythroid cells. Furthermore, amodiaquine appeared to bind both intracellularly as well as to surface proteins. This covalent binding was significantly reduced in neutrophils from amodiaquine-treated MPO<sup>-/-</sup> mice, and a similar trend emerged in gp91 phox<sup>-/-</sup> neutrophils, although it did not reach significance. No difference in covalent binding was observed in neutrophils from Rac1<sup>-/-</sup>, Rac2<sup>-/-</sup>, COX1<sup>-/-</sup>, or COX2<sup>-/-</sup> mice compared to neutrophils from wildtype mice treated with amodiaquine for 8 days, indicating that the oxidative burst pathway, prostaglandin H synthase, or cyclooxygenase are not necessary for covalent binding of amodiaquine in neutrophils.

During amodiaquine (~200-300 mg/kg/day in rodent meal) treatment in female C57BL/6 mice, members of this group found that there was an elevation in lymphocytes in the cervical lymph nodes at week 2, and in the liver and spleen at week 3 of treatment (Metushi *et al.*, 2015). IHC revealed an increase in CD4<sup>+</sup>, CD8<sup>+</sup>, CD11b<sup>+</sup>, F4/80<sup>+</sup>, CD45R<sup>+</sup>, and Ki67<sup>+</sup> cells in the liver and an increase in CD11b<sup>+</sup>, Ki67<sup>+</sup>, F4/80<sup>+</sup>, and CD45R<sup>+</sup> cells in the splenic red pulp with amodiaquine, with maximal increases observed around week 3. Flow cytometry revealed an increased in NK1.1<sup>+</sup> and CD8<sup>+</sup> cells in the cervical lymph nodes at week 2 in the spleen at weeks 1 and 3, and in the liver at week 3. Amodiaquine treatment of Rag1<sup>-/-</sup> mice (B- and T-cell deficient) in this study demonstrated an even greater increase liver NK1.1<sup>+</sup> cells and ALT, and upon depletion of natural killer cells, the ALT increase was attenuated.

In the immunization protocol by this group, there was expanded white pulp in the spleens of amodiaquine hepatic protein immunized, amodiaquine-treated animals compared to the control or amodiaquine alone treated group at week 3 (Mak and Uetrecht, 2015). Additionally, immunized animals exhibited the highest number of splenic CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells.

Moreover, after week 5 of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment, this group noted that the livers of treated Wistar rats exhibited focal inflammatory infiltrates with increased ED1<sup>+</sup> cells and treated Brown Norway rats displayed hypertrophic Kupffer cells, in addition to increased ED1<sup>+</sup>, ED2<sup>+</sup>, and CD45<sup>+</sup> leukocytes (Liu *et al.*, 2016). Total liver and lymph node lymphocytes were increased in Brown Norway rats after 3 and 4 weeks of treatment, respectively, driven by increased CD4<sup>+</sup> T cells (specifically, T<sub>H</sub>17 cells in the liver). NK1.1<sup>+</sup> cells were also increased in the liver at week 3 and in the lymph nodes and peripheral blood at week 4 of treatment. Peripheral blood, spleen, and lymph node M1 monocytes/macrophages were decreased from weeks 1 to 3 but were increased in the liver at weeks 3 and 4. Activated M2 macrophages were elevated in all investigated organs (spleen, liver, lymph node, peripheral blood) around weeks 3 to 4.

In the study comparing the effects of amodiaquine treatment in female C57BL/6 wildtype and CCR2<sup>-/-</sup> mice, CD11b<sup>+</sup>/Gr-1<sup>+</sup> and NK1.1<sup>+</sup> cells were increased with amodiaquine in the livers of wildtype-treated animals, but not in CCR2<sup>-/-</sup> mice (Mak and Uetrecht, 2019).

Another group evaluated the protective effects of amodiaquine in a mouse model of intracerebral hemorrhage (ICH) induced by striatal injection of collagenase VII (Kinoshita *et al.*, 2019). At 3 days, amodiaquine significantly suppressed ICH-induced accumulation of activated microglia/macrophages (Iba1<sup>+</sup> cells) and also suppressed activation of astrocytes (GFAP<sup>+</sup> cells) in the perihematomal region, compared to saline-treated ICH controls. Conversely, amodiaquine had no effect on ICH-induced neuron loss or injury volume, although this study did not look at the effects of amodiaquine in the absence of ICH.

#### b. Amoxicillin

In female B6C3F1 mice dosed with 20, 100, or 500 mg/kg/day amoxicillin by oral gavage for 28 days, some immune changes were noted, but none appeared dose-dependent (Lebrec *et al.*, 1994). WBC counts were noted to be decreased at the highest dose, and there was no change in NK cell activity.

In male Wistar rats administered amoxicillin (30 mg/kg/day, i.p.), WBC counts were increased at day 14 (Oyebode *et al.*, 2019).

Male Sprague-Dawley rats were administered 50 mg/kg amoxicillin by oral gavage 3 hours before induction of lung injury; no change in pulmonary alveolar macrophage function was noted (Tamaoki *et al.*, 1999).

In male Sprague-Dawley rats administered an intratracheal instillation of LPS to induce mucus hypersecretion and administered 40 mg/kg amoxicillin orally for 4 days, there was no difference in leukocyte counts bronchoalveolar lavage fluid (Ou *et al.*, 2008).

#### c. Nevirapine

Hematological analysis of rats administered 6 mg/kg/day nevirapine orally revealed a decrease in white blood cell (WBC) counts, but no changes in other parameters of erythrocytes or platelets after 60 days (Awodele *et al.*, 2015). In rats administered a higher dose of 200 mg/kg nevirapine orally daily, lymphocytes and platelets were noted to be over the reference range at day 21; however, counts from control animals were not presented (Bekker *et al.*, 2012).

In male Sprague-Dawley rats administered 35–165 µg/kg nevirapine i.p. 4 hours before intravital imaging, decreased rolling velocity and increased rolling flux, adhesion, and emigration was observed (Orden *et al.*, 2014). Leukocyte emigration was confirmed by hematoxylin- and eosin-stained mesenteric tissue, in which a significantly higher number of infiltrated leukocytes, primarily PMNs, were seen.

In a study of hematological parameters of male Wistar rats, different groups were administered 0, 3, 6, 12, or 24 mg/kg drug once daily for up to 25 days; however, the drugs used were a fixed-dose combination of 150 mg lamivudine, 300 mg zidovudine, and 200 mg nevirapine and it was not stated what the dose represented (Nubila *et al.*, 2012). Some changes were found at the individual timepoints, but they did not seem to follow a dose-response pattern. In rats treated with 12 mg/kg, over time, red blood cell (RBC) counts, platelets, and lymphocytes were increased, and MCHC and neutrophils were decreased. Overall, the observed effects were quite small.

In a study using female Brown Norway rats, nevirapine was administered in food at a dose of about 150 mg/kg/day for up to 21 days (Popovic *et al.*, 2006). Ear-draining auricular lymph nodes were analyzed, which were chosen because the ears of these rats have been shown to turn red after about 7 days of nevirapine treatment, preceding the appearance of rash at 14-21

days. Total cell count CD4<sup>+</sup> T cell, CD8<sup>+</sup> T cell, B cell, and macrophage counts and percentages were elevated in these lymph nodes of treated rats at days 7, 14, and 21 by flow cytometry. Expression of ICAM-1 and MHC II was increased overall, and MHC II expression was also shown to be increased in B cells and macrophages. Immunohistochemical staining confirmed those findings for ED1 (macrophages), ICAM-1, and MHC I and II at day 7, with a greater increase at day 14. Macrophage infiltration was noted to precede T cell infiltration, which occurred at 14 days.

#### d. Clozapine

In a prenatal MAM schizophrenia model, clozapine (20 mg/kg/day, i.p.) attenuated increased peripheral blood granulocytes in the MAM-exposed group after 8 days (Fiore *et al.*, 2008). In the absence of MAM exposure, clozapine treatment did not have significant effects.

In male C57BL/6J mice treated with MPTP, co-treatment with clozapine (1 mg/kg/day, s.c.), but not clozapine *N*-oxide (1 mg/kg/day, s.c.), for 3 weeks resulted in a significant decrease in total leukocyte counts compared to the MPTP control group (Jiang *et al.*, 2016). In the absence of MPTP administration, clozapine still caused the decrease in leukocytes, compared to control animals.

In a female C57BL/6 mouse EAE model, no effect of clozapine (60 mg/kg/day, p.o. [administered in drinking water]) on immune cell populations in the spleen were observed, but clozapine attenuated EAE-induced increases in T cells and macrophages in the spinal cord by day 11 (Green *et al.*, 2017). No effects of clozapine were observed in the absence of EAE.

In a second EAE model, clozapine (60 mg/kg/day, p.o. [administered in drinking water]) significantly attenuated increases in infiltrating neutrophils and monocytes in the spinal cord, brain, blood, and spleen (Robichon *et al.*, 2020). No significant cell alterations were observed with clozapine in the absence of EAE. In this study, female C57BL/6 mice were also treated with clozapine (60 mg/kg/day, p.o. [administered in drinking water]) for 1 week, following which, animals received a single injection of CCL2 or CCL5 and immune changes in the local draining lymph nodes were evaluated 18 hours later. Compared to CCL2 treated controls, clozapine greatly attenuated increased total cell numbers, T cells, and neutrophils in draining lymph nodes. No changes were noted with CCL5 treatment.

In a K/BxN mouse arthritis model, clozapine (4 mg/kg/bid, p.o.) did not affect leukocyte counts (Nent *et al.*, 2013). Clozapine (25 mg/kg, s.c.) administered twice a day also did not modulate sepsis-mediated neutrophil infiltration in the liver, lung, or ileum of male Wistar rats, as measured by myeloperoxidase activity (Machado *et al.*, 2007). However, cardiac myeloperoxidase activity was markedly upregulated following administration of male Wistar rats with clozapine (10, 15, or 25 mg/kg/day, i.p.) for 21 days (Abdel-Wahab and Metwally, 2014).

Following 21 days of clozapine (20 mg/kg/day, i.m.) administration, Sprague Dawley rats showed a marked increase in total WBC and neutrophil counts and a decrease in lymphocyte counts compared to saline-treated controls, while no significant changes in erythrocyte morphology or haematological indices were observed (Wasti *et al.*, 2006).

Increased blood monocytes, in addition to decreased lymphocytes and total leukocytes, were reported following 3 weeks of clozapine (5, 10, or 20 mg/kg/day, i.p.) in female BALB/c mice (Abdelrahman *et al.*, 2014). At the highest dose, this was also accompanied by a significant reduction in neutrophils and erythrocytes.

Clozapine (30 mg/kg/day, i.p.) administration in female Sprague Dawley rats resulted in significantly elevated blood neutrophil and monocyte counts, beginning on days 8 and 9, respectively, while no changes in leukocytes or lymphocytes were reported (Lobach and

Utrecht, 2014a; Ng *et al.*, 2014). A transient neutrophil spike was also reported after day 1. This was accompanied by an expansion of the bone marrow myeloid, but not erythroid or lymphoid, compartment on day 10, as well as an increase in the efflux rate of less mature neutrophils from the bone marrow. Specifically, clozapine caused an increase in granulocyte production and myeloperoxidase-positive cells, concentrated in the paratrabecular zone, and an increase in megakaryocytes, in addition to more prominent sinuses. Clozapine also increased the number of mature cells in the bone marrow, as well as the number of CD18<sup>+</sup> and CD11b<sup>+</sup> cells.

Although not a rodent study, with clozapine (30 mg/kg/day, s.c.) treatment in female New Zealand White rabbits, the half-life of peripheral blood neutrophils was significantly increased after 2 days, but was significantly decreased after 10 days (Iverson *et al.*, 2010). Absolute neutrophil counts were transiently increased around day 2. Moreover, the efflux rate of neutrophils from the bone marrow was enhanced with clozapine.

In female Sprague Dawley rats treated with clozapine (30 mg/kg/day, i.p.) an increase in covalent binding to neutrophil proteins was observed, plateauing by day 4 (Lobach and Utrecht, 2014b). No binding to lymphocytes was observed. At the end of 6 weeks of clozapine (40 mg/kg/day, p.o. [in rodent meal]) treatment in female Lewis rats, substantial covalent binding to a 49 kD protein was noted in bone marrow neutrophils that was not present in rats fed normal rodent meal (Gardner *et al.*, 1998).

Following 2 months of clozapine (50 mg/kg/day, p.o. in rodent meal) treatment in female Sprague Dawley rats maintained on a selenium sufficient or deficient diet, no effect on GSH peroxidase activity in whole blood was observed, although significant covalent binding in the bone marrow of clozapine treated animals was detected, irrespective of diet (Ip and Utrecht, 2008). Weekly peripheral blood leukocyte and neutrophil counts were also unaltered.

## 2. Clinical Studies

### a. Amoxicillin

In children with presumed bacterial or mycoplasmic lower respiratory tract infections treated with 60 mg/kg/day amoxicillin for 10 days, *ex vivo* lymphocyte lytic activity did not change with amoxicillin treatment (Agostoni *et al.*, 1988).

In a phase I clinical trial, healthy male volunteers were given a single dose of 785 mg amoxicillin orally (Gomez-Lus *et al.*, 1998). The killing activity of PMNs isolated from peripheral blood were measured *ex vivo*. Amoxicillin treatment alone did not affect PMN killing, but in combination with serum, bactericidal activity was significantly enhanced, even after amoxicillin concentrations fell below inhibitory concentrations.

In a study designed to examine the effects on immune parameters in healthy individuals, adult males were administered oral amoxicillin (1 g) and clavulanate potassium (125 mg) twice daily for 5 days (Dufour *et al.*, 2005). No changes were observed in white blood cell subsets, NK cell cytokine production, or polynuclear phagocyte activity up to 61 days after starting treatment.

### b. Nevirapine

Multiple studies involving nevirapine report changes in blood cell counts, but these tend to be in combination with other drugs. Leukopenia responsive to G-CSF treatment has been described, but in these cases zidovudine was part of the treatment regimen which has been suspected of being the causative agent (Yamamoto *et al.*, 2000; Shahar *et al.*, 2004). Similarly, trials of nevirapine and zidovudine combinations for the prevention of perinatal HIV transmission have shown an increase in neutropenia with extended co-treatment of nevirapine and zidovudine (Kumwenda *et al.*, 2008), while neutropenia was attributed to zidovudine

exposure in a study in which some patients were also exposed to single dose nevirapine (Read *et al.*, 2007).

Another example of combination therapy associated with increased risk of blood cell adverse effects is nevirapine and lumefantrine. In an open-label clinical trial in Malawi, patients who were already taking antiretroviral therapy were given lumefantrine in addition to their current therapy, first at half the target dose, then increased to the full dose after 28 days (Banda *et al.*, 2018). Neutropenia was observed in all study groups, but thrombocytopenia was only observed in combination with nevirapine, leading to the recommendation not to titrate the dose of lumefantrine further.

Infants of mothers from the HIV-1 Protective Immunity and Perinatal Exposure Study trial in South Africa were studied up to 12 weeks after birth (Schramm *et al.*, 2010). Infants of HIV-positive mothers received no antiretroviral therapy, single dose nevirapine (the mother was sometimes also given single dose nevirapine at onset of labour if their HIV status was known before delivery), or triple antiretroviral therapy. At birth, nevirapine-treated infants had higher monocyte counts and percentages and basophil counts; these differences did not persist at 6 weeks. Infants whose mothers had a CD4 T cell count >500 cells/ $\mu$ L had higher WBC, monocyte percentages and monocyte and basophil counts but lower lymphocyte and eosinophil percentages if they were exposed to single dose nevirapine than those who were unexposed.

### c. Clozapine

In a retrospective analysis in the United States of 118 hospitalized schizophrenic patients treated with clozapine patients for a minimum of 3 weeks, it was found that female patients were significantly more likely to develop eosinophilia compared to male patients, which was experienced by 23% of female and only 7% of male patients (Banov *et al.*, 1993). Additionally, eosinophilia was always experienced during weeks 3 to 5 of clozapine initiation and resolved with continued treatment. A similar retrospective analysis in a United Kingdom-based population of 160 schizophrenic patients (105 male and 55 female) initiating clozapine therapy was conducted, where 13% of patients (17 male and 4 female) developed eosinophilia between weeks 2 and 4 of clozapine treatment, with peak eosinophil levels reported after 7.5 weeks (range: 3-19 weeks) (Chatterton, 1997). The mean dose at the onset and peak of eosinophilia were 188 mg/day (range: 25-350 mg/day) and 227 mg/day (range: 0-600 mg/day), respectively. All except for 1 patient had resolution of eosinophilia with continued treatment.

Following retrospective analysis of 17 cases of clozapine-induced fever, 4 cases of leukocytosis, 3 cases of bandemia, 2 cases of neutrophilia, 1 case of eosinophilia, and 1 case of monocytosis were noted during the first month of treatment (Tham and Dickson, 2002). Similarly, in a retrospective review of 31 cases (19 male and 12 female) of clozapine-induced fever, 58% of patients had an elevated leukocyte count and 10% of the patients had an elevated eosinophil count (Pui-yin Chung *et al.*, 2008).

In a 6-week study of 14 male and 13 female patients diagnosed with schizophrenia, schizophreniform disorder, or schizoaffective disorder, transient elevations in total leukocyte and granulocyte counts were observed after the second week of treatment, while monocyte and lymphocyte counts remained stable over the course of the study (Pollmacher *et al.*, 1996). Mean clozapine doses were  $162 \pm 68$ ,  $245 \pm 115$ , and  $305 \pm 160$  mg/day at weeks 1, 2, and 6 respectively. Moreover, this group noted increased granulocyte and monocyte counts in a study of 20 schizophrenic patients and these levels were most prominent 2 weeks after commencing clozapine therapy (Pollmacher *et al.*, 1997).

However, an earlier study of 10 schizophrenic patients and 8 healthy controls reported that clozapine did not significantly influence total leukocyte, granulocyte or lymphocyte counts, measured at 1, 2, and 6 weeks of treatment (Pollmacher *et al.*, 1995). Mean clozapine doses were  $188 \pm 89$ ,  $285 \pm 138$ , and  $343 \pm 169$  mg/day, respectively. Likewise, this group did not find any changes in blood cell populations in an investigation of 6 male and 6 female patients with schizophrenia who were monitored for the first 6 weeks of clozapine treatment (Hinze-Selch *et al.*, 2000). In this study, the mean clozapine dose increased from  $85 \pm 59$  mg/day at week 1 to  $231 \pm 105$  mg/day at week 6.

One study reported a negative correlation between monocyte ROS release and the dose of clozapine administered to schizophrenia patients (1 male and 7 female), occurring 3 weeks into treatment, at which time the mean dose was  $184 \pm 35$  mg/day (150–250 mg/day) (Gross *et al.*, 2003). Patients in this study were all started on clozapine at a dose of 50 mg/day, which was subsequently increased by 50 mg/day every 3 days until symptom remediation was observed.

Peaking at 2 weeks of treatment, a significant increase in circulating CD34<sup>+</sup> hematopoietic cells, neutrophils and leukocytes was observed in a study of 8 schizophrenia patients starting clozapine therapy (titrated from 50 mg/day up to doses where serum level of 350–600 ng/mL) (Loffler *et al.*, 2010).

## F. Effects on Mediators of Inflammation and Related Concepts

### 1. Rodent Studies

#### a. Amodiaquine

In the bacterial fulminant model of hepatitis, elevated serum levels of TNF- $\alpha$  were significantly dampened at 3 hours with co-administration of amodiaquine (5 mg/kg, s.c.) in C57BL/6 mice (Yokoyama *et al.*, 2007). However, this was not accompanied by any significant changes in increased mRNA levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-12, or IL-18 in the liver. In the ICH model, amodiaquine significantly suppressed ICH-induced increases in mRNA expression of IL-1 $\beta$ , CXCL2 and CCL2 at 6 hours, compared to vehicle control (Kinoshita *et al.*, 2019).

In another study with amodiaquine (~200–300 mg/kg/day in rodent meal) treatment in female C57BL/6 mice, it was found that serum cytokines IL-1 $\alpha$ , IL-12 (p40), and IL-17 were elevated at week 1 with treatment (Metushi *et al.*, 2015). Notably, IL-3, IL-5, IL-10, IL-12(p70), IL-13, IFN- $\gamma$ , TNF- $\alpha$ , and G-CSF were all significantly downregulated by week 7. Over the first 4 weeks of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment in male Brown Norway rats, this group also noted spikes in serum levels of IL-2, IL-5, IL-9, IL-12, TGF- $\beta$ 1, and CCL2, which were not observed in saline-treated controls (Liu *et al.*, 2016). After week 5, hepatic RANTES and IL-18 were significantly increased in the amodiaquine-treated group, but IL-2, IL-5, IL-6, and IL-12 levels were significantly decreased compared to control. Following amodiaquine rechallenge, additional increases in serum IL-4, IL-13, and IFN- $\gamma$  were also observed.

#### b. Amoxicillin

An acute otitis media model using male Sprague-Dawley rats found that treatment with amoxicillin, administered in drinking water to a dose of about 51 mg/kg/day for 5 days, induced changes in osteocalcin (Melhus and Ryan, 2004). Osteocalcin is produced by osteoblasts and is a marker of bone formation. The maximum level of this marker in bullar bone was decreased and delayed compared to untreated animals. No change in expression of IL-6, TNF- $\alpha$ , or IL-10 was noted.



In LPS-induced mucus hypersecretion in male Sprague-Dawley rats, 40 mg/kg amoxicillin administered orally for 4 days did not alter cytokine (IL-1 $\beta$ , IL-8, and TNF- $\alpha$ ) levels in bronchoalveolar lavage fluid (Ou *et al.*, 2008).

### c. Nevirapine

In rats administered nevirapine and/or LPS, serum IL-2, IFN- $\gamma$ , and TNF- $\alpha$  were measured (Bekker *et al.*, 2012). Nevirapine treatment did not cause a significant change in serum IL-2 levels up to 24 hours, although co-treatment with LPS attenuated the increase in IL-2 that was observed in the LPS group. No significant difference was observed in serum IFN- $\gamma$  levels up to 24 hours. Nevirapine treatment caused a significant increase in serum TNF- $\alpha$  levels at 24 hours; this was attenuated with LPS co-treatment. Interestingly, coadministration of nevirapine with LPS may have altered the pharmacokinetics of nevirapine – the 24-hour nevirapine serum level was higher than the 6-hour level with co-treatment, while the opposite was observed with nevirapine alone. With chronic treatment, nevirapine treatment caused a significant increase in serum IL-2 at day 21 compared to nevirapine and LPS, while there was a non-significant increase in serum TNF- $\alpha$  at day 7 in the nevirapine and LPS group compared to the nevirapine group. No changes were observed with IFN- $\gamma$ . Unfortunately, no results were shown for controls or LPS treatment only. Again, pharmacokinetics differed with LPS coadministration: while nevirapine serum levels were lower at weeks 2 and 3 compared to week 1 with nevirapine only, nevirapine levels were similar at weeks 1, 2, and 3 with co-treatment. The authors note that LPS has been reported to inhibit CYP450 enzymes; they also suggest that LPS has effects on the innate immune system, so the combination of these effects may explain the observations observed in the co-treated groups.

In female Brown Norway rats treated with 150 mg/kg/day nevirapine in food, serum IFN- $\gamma$  was not changed during the primary exposure up to 21 days, although it was elevated upon rechallenge (Popovic *et al.*, 2006). In a similar study, lymphocytes from auricular lymph nodes of female Brown Norway rats treated for up to 21 days showed increased production of CXCL1, CCL3, IL-10, IL-18, and CCL5 (Chen *et al.*, 2009). In the same animal model but at 6 hours following 150 mg/kg nevirapine administered by gavage, mRNA in the liver showed changes in expression of some immune-related genes, such as Zap70 (associated with control of immune tolerance), FK506 binding protein (Fkbp5 or immunophilin, involved in immunoregulation), or Irgm M; or protein folding, such as Fkbp5 and ER degradation enhancer and mannosidase alpha-like 1 (Edem 1) (Zhang *et al.*, 2013). In the skin, 12-hydroxynevirapine (159 mg/kg by gavage) treatment increased expression of over 2,000 genes after 6 hours of treatment, including TRIM63 (a ubiquitin ligase), Fkbp5, IL-22 RA2 (soluble IL-22 antagonist), and S100a7a (a DAMP).

In female mice treated with 3.3 mg/kg/day of nevirapine by oral gavage for 8 weeks, markers of neuroinflammation was characterized in the hippocampus (Zulu *et al.*, 2018). Astrogliosis was detected by increased staining for glial fibrillary acidic protein, and IL-1 $\beta$  and TNF- $\alpha$  were increased. Tenofovir was also found to have a similar effect. These results suggested that nevirapine and tenofovir could induce inflammation in the brain. Nevirapine, but not tenofovir, was also found to decrease brain-derived neurotrophic factor in the hippocampus; the authors speculate that this may be due to a difference in the ability of the two drugs to inhibit DNA polymerase, as tenofovir is a weaker inhibitor.

Cbl-b is an E3 ubiquitin ligase that is involved in regulation of activation in many leukocytes (Lutz-Nicoladoni *et al.*, 2015). In male Cbl-b<sup>-/-</sup> mice treated with nevirapine in food for 7 days at a dose approximating 950 mg/kg/day, serum IL-6 and IFN- $\gamma$  were increased with

treatment; the increase was greater if liver necrosis was noted by histology (Sharma *et al.*, 2012). These increases resolved by day 14.

#### d. Clozapine

Pre-treatment with clozapine (5 mg/kg, i.p.) completely prevented the induction of HSP70 immunoreactive cells triggered by PCP in the cingulate cortex of female Sprague Dawley rats, observed at 24 hours (Sharp *et al.*, 1994). Likewise, pre-treatment with clozapine (20 mg/kg, i.p.) significantly reduced PCP-induced increases in hsp70 mRNA levels in cortex at 3 hours (Nakahara *et al.*, 1999). In the absence of PCP treatment, clozapine also decreased hsp70 mRNA in the prefrontal cortex, striatum, and nucleus accumbens. In a similar model, the induction of both c-fos and HSP70 immunoreactive brain cells at 24 hours by dizocilpine was dose-dependently inhibited by clozapine (0.31, 0.62, 1.25 mg/kg, s.c.) pre-treatment in female BKTO mice (O'Neill *et al.*, 1998).

After 65 days of clozapine (20 mg/kg/day, p.o. [administered in drinking water]), male PCP-exposed clozapine treated rats had increased serum corticosterone concentrations and both male clozapine groups had increased serum TNF- $\alpha$  concentrations, while female PCP-exposed clozapine treated rats had decreased serum corticosterone concentrations and both female clozapine groups had increased serum IL-6 concentrations (Nikolic *et al.*, 2017).

After 90 minutes, clozapine (30 mg/kg, p.o.) significantly damped LPS- and polyI:C-induced increases in serum TNF- $\alpha$  and IL-6, but further increased IL-10 levels (Sugino *et al.*, 2009). In the absence of LPS or polyI:C, clozapine did not affect levels of proinflammatory mediators, but did increase serum levels of IL-10 compared to saline-treated controls.

In a male rat hepatic I/R model, clozapine (15 mg/kg, s.c.) pre-treatment significantly reduced hepatic IL-12 (p70) and TNF- $\alpha$  levels compared to I/R control animals at 2 hours post-ischemia (El-Mahdy *et al.*, 2013).

In a female C57BL/6 mouse model of EAE, clozapine (60 mg/kg/day, p.o. [administered in drinking water]) significantly attenuated increases in CCL2 and CCL5 protein levels in the brain, and mRNA levels in both the brain and spinal cord (Robichon *et al.*, 2020). Clozapine did not affect EAE-induced changes in CCL2 or CCL5 levels in the blood or spleen, nor did clozapine have any effect in the absence of EAE.

After 2 weeks, clozapine (5 mg/kg/day, i.p.) completely reversed social isolation-induced reductions in plasma IL-4, IL-6, INF- $\gamma$ , and kynuric acid and elevations in TNF- $\alpha$ , kynurenine, and quinolinic acid in male Sprague Dawley rats (Moller *et al.*, 2013).

No changes in IL-1 $\beta$  or COX-2 hippocampal protein levels were observed after 3 weeks of clozapine (20 mg/kg/day, i.p.) treatment in male Wistar rats, but decreased TNF- $\alpha$  levels were observed in clozapine-treated rats that were also socially isolated for the duration of the study (Todorovic and Filipovic, 2017b). In the prefrontal cortex, clozapine alone had no effect, but significantly attenuated elevated IL-1 $\beta$ , TNF- $\alpha$ , and COX-2 levels induced by social isolation (Todorovic and Filipovic, 2017a).

Clozapine (25 mg/kg, s.c.) given twice daily was not effective in reducing sepsis-induced elevations in serum IL-1 $\beta$ , TNF- $\alpha$ , or IL-10 in male Wistar rats (Machado *et al.*, 2007). It appears as though clozapine may have exacerbated the increases in TNF- $\alpha$  and IL-10, but a statistical comparison of clozapine and the appropriate control group was not provided.

In male Wistar rats, clozapine (2, 8, 32  $\mu$ mol/kg, s.c. [0.65, 2.61, 10.46 mg/kg]) paradoxically reduced core body temperature by up to 2°C (Oerther and Ahlenius, 2000).

Five hours after administration of clozapine (20 mg/kg, i.p.) to male hooded Long Evans rats, mRNA expression levels of TNF- $\alpha$  were significantly reduced in the prefrontal cortex with treatment but were not altered in other brain regions (Paterson *et al.*, 2006).

Clozapine (30 mg/kg/day, i.p.) administration in female Sprague Dawley rats resulted in transiently elevated serum G-CSF levels at 3 and 6 hours after the first dose and increased CXCL2 expression in the bone marrow on day 10 (Lobach and Uetrecht, 2014a).

No changes in striatal TNF- $\alpha$ , norepinephrine, or dopamine were detected with clozapine (5 or 10 mg/kg/day, i.p.) in male Wistar rats (Bishnoi *et al.*, 2008, 2011). Conversely, another study showed that 2 weeks of clozapine (15 mg/kg/day, i.p.) treatment in male C57BL/6 mice caused a moderate increase in cingulate cortex norepinephrine (Ookubo *et al.*, 2013).

In male BALB/c mice, significantly elevated serum levels of epinephrine and norepinephrine were observed from 1 to 2 weeks with clozapine (5, 10, and 25 mg/kg/day, i.p.) (Wang *et al.*, 2008). In the heart tissue, TNF- $\alpha$  levels were also increased with clozapine compared to saline controls but were only measured after 2 weeks of treatment and may have been higher during peak inflammatory lesion observation at day 7. Co-administration of propranolol significantly dampened all increased mediators after 2 weeks.

After 21 days, clozapine (10, 15, or 25 mg/kg/day, i.p.) elevated serum and cardiac 8-oxo-2'-deoxyguanoside levels in male Sprague Dawley rats (Abdel-Wahab and Metwally, 2014; Abdel-Wahab *et al.*, 2014). Clozapine also caused elevations in cardiac MDA, 3-nitrotyrosine, nitric oxide, nitrite, and TNF- $\alpha$ . Elevated levels of serum TNF- $\alpha$  and reduced IL-10 were also reported, and inflammatory effects were attenuated by co-administration of captopril.

After 21 days of treatment in male Wistar rats, clozapine (10 mg/kg/day, i.p.) caused a number differentially-expressed proteins in the nucleus accumbens, including increased levels of HSPA8, HSP75, and GSH synthase (Kedraka-Krok *et al.*, 2016).

Male Sprague Dawley rats treated with clozapine (20 mg/kg/day, i.p.) for 30 days were reported to have reduced cerebrospinal fluid levels of IL-8 versus saline controls, while kynurenic acid levels were unaffected (Larsson *et al.*, 2015).

Clozapine (0.5 mg/kg/day, p.o.) administration for 30 days resulted in increased expression of renal kidney injury molecule-1 and tissue inhibitor of metalloproteinase-1 mRNA (Mohammed *et al.*, 2020). Additionally, serum protein levels of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and MDA were elevated with clozapine. Except for clozapine-induced MDA increases, all parameters were attenuated by co-administered with sulpride.

## 2. Clinical Studies

### a. Amoxicillin

In healthy adult males administered oral amoxicillin (1 g) and clavulanate potassium (125 mg) twice daily for 5 days, no change in the proportion of TNF- $\alpha$ -expressing CD14<sup>+</sup> cells in the blood and no change in lactoferrin or lysozyme levels in the serum or feces was observed up to 61 days after beginning treatment (Dufour *et al.*, 2005).

### b. Nevirapine

A study examined the activation of T cells isolated from women up to 6 weeks after delivery (Shalekoff *et al.*, 2009). Women were HIV-positive and received single dose nevirapine if HIV status was known before delivery; controls were identified as HIV-positive after delivery and had therefore not received nevirapine. Nevirapine was associated with decreased plasma CCL3 and IL-8, but not IL-15 or TNF- $\alpha$ .

In a study combining patients from three clinical trials in which IL-6 levels in HIV-positive patients were studied, nevirapine exposure was correlated with lower plasma IL-6 levels

compared to efavirenz exposure (IL-6 was observed to be highest in untreated patients) (Borges *et al.*, 2015). Nevirapine treatment is associated with lower HIV RNA levels compared to other NNRTIs, which might help to explain the observed effect. Randomization to protease inhibitor- or NNRTI-based therapies was not performed in these trials.

A study examined the levels of soluble CD14 (sCD14) in plasma of HIV-positive patients in Nantes, France (Allavena *et al.*, 2013). Patients were taking triple antiretroviral therapy including either nevirapine or efavirenz. Patients on nevirapine-based therapy had a median plasma sCD14 level of 1.7 mg/mL, while those on efavirenz-based therapy had a level of 1.9 mg/mL. However, there was no control group and the study was not randomized; patients on efavirenz were significantly younger, had a shorter time on ARV than those on nevirapine. A few comparisons suggested that sCD14 levels were not related to viral load.

A study examined soluble markers of immune activation in HIV-positive mothers and infants, and examined the effect of exposure to nevirapine: some mothers had not received single-dose nevirapine before giving birth, while some had; HIV-negative mother/child pairs were also used as a control (Schramm *et al.*, 2006). Neopterin and soluble L-selectin levels in cord blood in exposed, uninfected infants were significantly higher with maternal nevirapine exposure. No differences in neopterin, soluble L-selectin, or  $\beta_2$ -microglobulin levels were observed in infants infected intrapartum (HIV-negative at birth, but HIV-positive at 6 weeks of age). Neopterin and  $\beta_2$ -microglobulin levels were increased with nevirapine exposure in infants infected *in utero* (HIV-positive at birth). The same markers were measured in maternal plasma drawn at the same time as the cord blood; the only difference was an increase in neopterin levels with nevirapine exposure in mothers whose infants were infected *in utero*. No correlations between maternal and infant levels for any of the three analytes was found, suggesting that immune activation in the infant occurs independent of the maternal environment. The authors suggest that single-dose nevirapine induces T cell anergy, which may aid in preventing HIV-1 replication. Additionally, the authors propose that nevirapine may synergize with HIV-1 to increase immune activation.

Two studies have been performed evaluating the effect of genetic variations in some immune markers. A study examining the effect of CCL3L1 (a CCR5 ligand) gene copies on HIV mother-to-newborn transmission found that maternal nevirapine was associated with decreased spontaneous and phytohemagglutinin-stimulated release of CCL3 in cord blood mononuclear cells from uninfected infants (Kuhn *et al.*, 2007). Additionally, greater infant CCL3L1 gene copies were associated with reduced HIV transmission overall, but this effect was attenuated with maternal single-dose nevirapine exposure. Another study using cohorts in Malawi, South Africa, and Uganda examined multiple chemokine receptor polymorphisms in the context of maternal HIV transmission (Singh *et al.*, 2008). Mother-to-child transmission was reduced with nevirapine treatment with CCR5-59029-G/A or CCR5-59353-T/C, in which both polymorphisms were associated with an increased risk compared to wildtype. Transmission risk was increased with nevirapine treatment for the CX3CR1-745-G/A (249-V/I) (280-T/M) polymorphism. In both cases, nevirapine appears to have an effect on the role of certain cytokines in HIV transmission.

In a study of 11 HIV-positive children initiated on triple therapy with stavudine, ritonavir, and nevirapine for up to 24 weeks, responses of blood cells to multiple stimuli were evaluated *ex vivo* (Blazevic *et al.*, 2001). At baseline, peripheral blood mononuclear cells from these children had a lower delayed-type hypersensitivity response to *Candida albicans* compared to peripheral blood mononuclear cells from healthy controls, which increased at week 24.

However, the response to tetanus toxoid was not observed in HIV-positive children at baseline (significantly lower than healthy controls) and did not change at 24 weeks. In evaluating APC function, monocytes from HIV-positive patients produced significantly less IL-12p70 in response to *Staphylococcus aureus* Cowan compared to healthy controls; at 24 weeks, IL-12p70 secretion was increased. However, these effects cannot be attributed to nevirapine therapy alone, as a combination of drugs was administered to these patients; additionally, the effect of treatment and restitution of CD4 T cells also likely plays an important role in these effects.

### c. Clozapine

In a retrospective analysis of 93 schizophrenia or schizoaffective disorder patients (65 male and 28 female) initiated on clozapine in Canada, it was noted that 20% of patients developed fever during first month treatment, lasting an average of 2 weeks (Tham and Dickson, 2002). The majority of fever onset was observed from weeks 1 to 3, although a range from 3 to 26 days was reported. Similarly, in a retrospective review of 227 patients (113 male and 94 female) commencing clozapine therapy in Hong Kong, 14% (19 male and 12 female) developed a fever in first 3 weeks of treatment that lasted around 5 days (range: 1-12 days) (Pui-yin Chung *et al.*, 2008). The mean time to fever onset was 14 days (range: 5-21 days, plus one case at day 47) and mean clozapine dosage at the onset of fever was  $166.1 \pm 73.2$  mg/day (range: 50-300 mg/day).

In a study of 10 schizophrenic patients (7 males and 3 females) and 8 healthy controls (6 males and 2 females), a marked increase in soluble IL-2R levels was observed from weeks 2-6 of treatment, when mean clozapine doses were  $285 \pm 138$  and  $343 \pm 169$  mg/day, respectively (Pollmacher *et al.*, 1995). To characterize these immune changes in more detail, a second 6-week study of 14 male and 13 female patients diagnosed with schizophrenia, schizophreniform disorder, or schizoaffective disorder was conducted (Pollmacher *et al.*, 1996). Compared to pre-clozapine baseline levels, plasma levels of TNF- $\alpha$ , soluble TNF-R p55, soluble TNF-R p75, and soluble IL-2R were elevated for the duration of the study, measured at week 1 (mean clozapine dose:  $162 \pm 68$  mg/day), week 2 (mean clozapine dose:  $245 \pm 115$  mg/day), and week 6 (mean clozapine dose:  $305 \pm 160$  mg/day) of treatment. Additionally, plasma IL-6 levels were transiently increased at week 2 of treatment and 44% of patients developed a fever that lasted up to 6 days.

This group replicated these findings in a study of 6 male and 6 female patients with schizophrenia who, again, were monitored during initiation of clozapine therapy (Hinze-Selch *et al.*, 2000). Soluble TNF-R p55 and p75 were increased with clozapine from weeks 1 to 6 and TNF- $\alpha$  and soluble IL-2R were elevated from week 2 onward. Of 20 schizophrenia patients monitored during the first 6 weeks of clozapine therapy, 55% developed a transient fever and increase in plasma G-CSF levels that was most prominent following 2 weeks of treatment (Pollmacher *et al.*, 1997). Similarly, fever was reported in 47% of schizophrenic patients monitored during the first 2 weeks of clozapine treatment (Hinze-Selch *et al.*, 1998). In this study, mean time to fever onset was  $15 \pm 5$  days (range: 2-25 days) with a mean duration of  $2.5 \pm 1.3$  days (range: 1-6 days). The average clozapine dose increased from  $178 \pm 57$  mg/day at week 1, to  $281 \pm 111$  mg/day at week 2, to  $325 \pm 152$  at week 6.

Another group conducted a study with 17 schizophrenic patients (7 male and 10 female) started on clozapine and sex- and age-matched healthy controls (Monteleone *et al.*, 1997). After 10 weeks (mean clozapine dose:  $312 \pm 120$  mg/day [range: 75-400 mg/day]), no differences in plasma IL-6 levels were observed with clozapine, but TNF- $\alpha$  levels that had been elevated in the schizophrenia group at baseline were dampened to control levels. Cytokine changes were also

evaluated in a study of 23 schizophrenic patients (13 males and 10 females) before and after starting clozapine (Maes *et al.*, 1997). Compared to healthy controls (11 males and 6 females), clozapine-naïve patients showed significantly higher plasma IL-6R and IL-1RA and lower uteroglobin levels. During the first 2 weeks of clozapine treatment, further increases in plasma IL-6 and increased uteroglobin occurred and after 5 weeks. Increased plasma levels of soluble CD8 and IL-1RA were observed.

In a study evaluating cytokine changes in 12 new clozapine users, it was demonstrated that while levels of IL-8 and IL-10 were unchanged, serum levels of soluble CD8 and leukemia inhibitory factor receptor were markedly elevated after 2 months of clozapine treatment, compared to baseline or 4-month treatment levels (Maes *et al.*, 2002). The mean clozapine doses at months 2 and 4 were  $358.3 \pm 144.3$  mg/day and  $395.0 \pm 101.2$  mg/day, respectively. A similar study compared cytokine levels of 22 new clozapine users (16 male and 6 female) to 21 patients who had been maintained on clozapine for greater than 6 months (14 male and 7 female) (Hung *et al.*, 2017). Compared to 6% of old users, 47% of new users developed fever during weeks 1 to 4, lasting an average of 2 days. Although no differences were observed in cytokine levels between new and old clozapine users, compared to new users without fever, plasma levels of TNF- $\alpha$ , INF- $\gamma$ , IL-2, and IL-6 levels were significantly different in the patients who developed clozapine-induced fever.

Another group conducted a 6-week randomized double-blinded trial to compare the effects of clozapine (mean modal dose:  $266.7 \pm 77.9$  mg/day) and olanzapine (mean modal dose:  $21.2 \pm 2.5$  mg/day) in patients with schizophrenia, schizophreniform disorder or schizoaffective disorder (Kluge *et al.*, 2009). Thirty patients were randomized to either the clozapine (7 male and 8 female) or olanzapine (5 male and 10 female) group. Five patients treated with clozapine developed fever during the first 3 weeks of treatment, which lasted up to 5 days, while no patients treated with olanzapine developed fever. Compared to baseline values, plasma TNF- $\alpha$ , soluble TNFR-2, and soluble IL-2R remained significantly elevated from week 2 to 6 with both treatments and peaked around week 3, although levels of soluble TNFR-2 were markedly higher with clozapine compared to olanzapine. Additionally, IL-6 and soluble TNFR-1 levels were only elevated with clozapine treatment during weeks 2 to 3 and weeks 2 to 6, respectively. Moreover, at week 2 of clozapine treatment, IL-6 and soluble TNFR-2 were significantly higher in patients with fever compared to those without fever. Lastly, plasma leptin levels were significantly increased in females for the entire study, while levels in males were transiently elevated during weeks 2 and 3 with clozapine.

## G. Effects on Applicable Signal Transduction Factors

### 1. Rodent Studies

#### a. Amoxicillin

In male Sprague-Dawley rats administered an intratracheal instillation of LPS to induce mucus hypersecretion and administered 40 mg/kg amoxicillin orally for 4 days, there was no difference in NF- $\kappa$ B expression in bronchial epithelium (Ou *et al.*, 2008).

#### b. Clozapine

Using the PCP pre-treatment model in male C57BL/6 mice, it was shown that 1 week of clozapine (10 mg/kg/day, p.o.) administration enhanced PCP-induced decreases in cytosolic Nrf2 and increases in nuclear Nrf2 protein levels in the prefrontal cortex (Tran *et al.*, 2017). However, after day 1, clozapine significantly attenuated PCP-induced mitochondrial translocation of phosphorylated AKT (Tran *et al.*, 2018). In the postnatal model of schizophrenia using male

C57BL/6 mice, it was found that co-administration of clozapine (6 mg/kg/day, i.p.) with PCP greatly decreased the phosphorylation ratio of AKT (also called protein kinase B) in the cerebral cortex compared to vehicle-treated PCP controls, although the phosphorylation ratio of extracellular signal-regulated kinase (ERK) was unchanged (Barzilay *et al.*, 2011).

One study evaluated the impact of 3 weeks of clozapine (1 mg/kg/day, s.c.) pre-treatment on nigral NF- $\kappa$ B levels in male Sprague Dawley rats followed by apomorphine stimulation (Saldana *et al.*, 2006). Clozapine induced a significant decrease in nigral NF- $\kappa$ B p65 and p50 protein levels compared to the apomorphine-stimulated, saline-pre-treated control group. Contrary to this, no changes in striatal NF- $\kappa$ B p65 protein levels were detected following 3 weeks of clozapine (5 or 10 mg/kg/day, i.p.) administration in male Wistar rats (Bishnoi *et al.*, 2008, 2011).

Dose-dependent elevations in cardiac NF- $\kappa$ B p65 were observed following 3 weeks of clozapine (10, 15, or 25 mg/kg/day, i.p.) treatment in male Wistar rats (Abdel-Wahab and Metwally, 2014). A similar outcome was noted in another study, where socially isolated male Wistar rats given clozapine (20 mg/kg/day, i.p.) for 3 weeks had decreased cytosolic and increased nuclear NF- $\kappa$ B p65 protein levels in the liver, versus saline controls (Zlatkovic *et al.*, 2014). This, however, was not observed in the prefrontal cortex, where clozapine significantly attenuated social isolation-induced reduced cytosolic and elevated nuclear NF- $\kappa$ B p65 protein levels (Todorovic and Filipovic, 2017a). No changes in cytosolic or nuclear NF- $\kappa$ B p65 hippocampal protein levels were observed in this model (Todorovic and Filipovic, 2017b).

One hour following clozapine (12 mg/kg) administration in female Sprague Dawley rats, hepatic eIF2 $\alpha$  phosphorylation was significantly increased, suggesting activation of the protein kinase R-like ER kinase/eukaryotic translation initiation factor 2A (PERK/eIF2 $\alpha$ ) ER stress axis (Weston-Green *et al.*, 2018). Additionally, in this model, hepatic sphingolipid homeostasis was significantly disrupted. Specifically, hepatic levels of ceramide and sphingolipids were markedly reduced with clozapine, while ceramide synthase and a fatty acid elongase enzyme were increased.

Male Sprague Dawley rats treated with a single dose of clozapine (10 mg/kg, i.p.) showed a marked increase in frontal cortex phosphorylated-Unc-51-like kinase (ULK) 1 (Ser317) and phosphorylated-Beclin1 (Ser93) was demonstrated from 2 to 4 hours and an increase in phosphorylated-AMP-activated protein kinase (AMPK)  $\alpha$  (Thr172) from 1 to 4 hours, although total levels of these proteins were unchanged (Kim *et al.*, 2018). Moreover, AMPK substrates were increased from 1 to 4 hours. Co-treatment of rats with clozapine and the AMPK inhibitor dorsomorphin resulted in attenuation of these increases at 2 hours. Together, the AMPK-ULK1-Beclin1 signaling pathway is responsible for induction of autophagy, where phosphorylation of AMPK leads to its activation and subsequent phosphorylation of ULK, together leading to the activation of Beclin1 and the activation of autophagy components. However, AMPK has been shown to play a role in a number of biochemical pathways, including mitochondrial biogenesis and lipid metabolism (Hardie *et al.*, 2016) and thus, clozapine's impact on AMPK signaling likely extends beyond autophagy.

It was demonstrated that PPAR $\alpha$  and LXR $\alpha$  (ligand-activated nuclear hormone receptors that normally control aspects of lipid homeostasis) mRNA levels were significantly decreased at 6 hours following clozapine (25 or 50 mg/kg, i.p.) administration in female Sprague Dawley rats, as compared to vehicle controls (Ferno *et al.*, 2009). Additionally, LXR $\alpha$  target gene cholesterol efflux gene ATP-binding cassette, sub-family A was significantly upregulated at this time. All changes resolved by 48 hours.

One study demonstrated that, when co-administered with ketamine in male Sprague Dawley rats, clozapine (5 mg/kg/day, i.p.) significantly attenuated decreased phosphorylated AKT and phosphorylated GSK-3 $\beta$  and attenuated increased phosphorylated  $\beta$ -catenin in several brain regions, compared to ketamine-treated controls (George *et al.*, 2020).

Thirty minutes following N-desmethylozapine (20 mg/kg, s.c.) administration in male CD1 mice, increased phosphorylated AKT and phosphorylated AKT-glycogen synthase kinase (GSK) expression levels were reported in the nucleus accumbens, compared to saline controls. This effect was significantly antagonized by pre-treatment with the  $\delta$ -opioid receptor antagonist naltrindole (Olianas *et al.*, 2011). Using C57BL/6 TLR-2<sup>-/-</sup> mice, however, it was demonstrated that the increased expression levels of phosphorylated GSK-3 $\alpha/\beta$  observed in TLR-2<sup>-/-</sup> mice were markedly reduced in several brain regions 1 hour post-clozapine (1 mg/kg, i.p.) administration (Park *et al.*, 2015). Clozapine did not alter increased levels of phosphorylated AKT.

Another study showed that 2 weeks of clozapine (15 mg/kg/day, i.p.) treatment in male C57BL/6 mice resulted in a number of brain region-specific changes in histone deacetylases (HDAC) (Ookubo *et al.*, 2013). Specifically, clozapine increased HDAC2, HDAC3, and HDAC8 in the striatum, HDAC2 and HDAC3 in the nucleus accumbens, and HDAC3 in cingulate cortex, compared to vehicle controls. Additionally, acetylated histone H3 protein expression levels were increased in several brain areas with clozapine.

Differences in histone-promoter binding in the frontal cortex were noted following administration of clozapine (10 mg/kg/day, i.p.) in male 129S6/Sv mice for 3 weeks (de la Fuente Revenga *et al.*, 2019). Clozapine induced a significant decrease of lysine-acetylated histone H3 and HDAC2 binding to the metabotropic glutamate 2 receptor, homer protein homolog 1, and glutamate receptor ionotropic, NMDA 1 gene promoter regions of wildtype, but not in clozapine-treated HDAC2<sup>-/-</sup> mice.

## H. Effects on Mitochondria and Oxidative Stress

### 1. Rodent Studies

#### a. Amoxicillin

In male Wistar rats, amoxicillin (10 or 30 mg/kg/day, i.p.) was administered once daily for 14 days (Oyebode *et al.*, 2019). The higher dose resulted in significantly increased mitochondrial permeability transition pore opening, enhanced rat liver microsome ATPase activity, cytochrome C release, and increased levels of rat liver microsome malondialdehyde generation.

#### b. Nevirapine

A study using male CF-1 mice administered 3.3 mg/kg nevirapine by oral gavage for 36 days found that mitochondrial complex IV activity was inhibited in the cerebral cortex, striatum, and hippocampus, but not striatum (Streck *et al.*, 2011).

A study using male albino rats administered 6 mg/kg nevirapine orally for 60 days found no difference in serum catalase, superoxide dismutase, or glutathione (Awodele *et al.*, 2015). Increased serum malondialdehyde was reported, which is used as a measure of lipid peroxidation. This effect was not modulated by co-treatment with antioxidants vitamin C, vitamin E, or jobelyn.

A study of multiple organs in Wistar rats treated with 18 or 36 mg/kg for 4 weeks compared to vehicle control found that nevirapine caused a dose-dependent increase in malondialdehyde in the liver, kidney, and testes, which was accompanied by a decrease in superoxide dismutase and catalase activities and glutathione (Adaramoye *et al.*, 2012).



A number of ultrastructural changes were observed in the liver of female Brown Norway rats treated with 150 mg/kg nevirapine for 8 weeks (Sastry *et al.*, 2018). Degenerate mitochondria were observed in hepatocytes of treated rats. Lipid droplets associated with smooth endoplasmic reticulum cisternae were also observed in these hepatocytes.

In female Brown Norway rats treated with 159 mg/kg/day 12-hydroxyn Nevirapine, transcripts related to mitochondria, including pyruvate dehydrogenase kinase, isozyme 4 (Pdk4), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (Hmgcs2), and uncoupling protein 3 (Ucp3), were upregulated in the skin 6 hours post-dose (Zhang *et al.*, 2013).

### c. Clozapine

One group demonstrated that coadministration of clozapine (10 mg/kg/day, i.p.) in male Sprague Dawley rats with either PCP or methamphetamine for 28 days greatly attenuated the decrease in cytochrome c oxidase activity observed in multiple brain regions observed with PCP or methamphetamine alone (Prince *et al.*, 1997b). Clozapine monotherapy also increased brain cytochrome c oxidase activity (Prince *et al.*, 1997a). Conversely, no changes in complex I activity were observed. Another group evaluated the effects of clozapine (10 mg/kg/day, p.o.) in male C57BL/6 wildtype or p47phox<sup>-/-</sup> mice that had been pre-treated with PCP (Tran *et al.*, 2018). After day 1, clozapine attenuated PCP-induced mitochondrial changes in the prefrontal cortex of wildtype mice, and attenuated elevations in ROS and protein oxidation in both genotypes. Clozapine in mice without PCP treatment did not appear to have any significant effects.

In male Sprague Dawley rats treated with ketamine, 1 week of clozapine (5 mg/kg/day, i.p.) markedly attenuated decreased hippocampal GSH levels and decreased striatal catalase activity observed in the ketamine control group (George *et al.*, 2020).

One hour following administration of clozapine (25 mg/kg, i.p.) in male Sprague Dawley rats, activating transcription factor 3 (ATF3) mRNA levels in the liver were significantly elevated compared to animals in the water control group (Lauressergues *et al.*, 2012). ATF3 is a sensor of ER stress, which can initiate UPR through several downstream pathways. By 3 hours, ATF3 levels remained elevated, and increases in 3-hydroxy-3-methylglutaryl-coA reductase and tribbles drosophila homolog 3 (a marker of the PERK pathway of ER stress) were also observed.

With social isolation, male Sprague Dawley rats were given clozapine (5 mg/kg/day, i.p.) for 14 days and it was found that clozapine completely reversed social isolation-induced reductions in frontal cortical ATP and elevations in striatal ATP (Moller *et al.*, 2013). However, in another social isolation model in male Wistar, liver cytosolic CuZn-superoxide dismutase protein levels were decreased with clozapine (20 mg/kg/day, i.p.), irrespective of housing status during the study (Zlatkovic *et al.*, 2014).

Compared to controls, male Wistar rats treated with clozapine (25 mg/kg/day, i.p.) for 28 days displayed increased AMP hydrolysis in the striatum, but not in the hippocampus, at 24 hours following the final dose (Lara *et al.*, 2001).

Male Long Evans rats treated with clozapine (10 mg/kg/day, i.p.) for 21 days had neuronal expression of nitric oxide synthase mRNA that was significantly decreased compared to control levels (Bullock *et al.*, 2008).

One group demonstrated that treatment of male Wistar rats with clozapine (25 mg/kg/day, i.p.) for 4 weeks resulted in inhibition of succinate dehydrogenase activity in striatum, but not in other areas of the brain, and cytochrome oxygenase activity was unchanged (Streck *et al.*, 2007).

After 28 days of clozapine (25 mg/kg/day, i.p.) treatment and 3 rest days, no change in mitochondrial superoxide production was observed in several brain regions of male Wistar rats when compared to saline-treated rats (Martins *et al.*, 2008). However, protein carbonyl content (a measure of oxidative damage to proteins) was found to be increased with clozapine in the hippocampus, while thiobarbituric acid reactive substances (TBARS; a measure of lipid peroxidation) were decreased in the cerebral cortex with treatment. Another group investigated similar effects following 21 days of clozapine (10 mg/kg/day, i.p.) administration in male Wistar rats and found increased levels of striatal TBARS and superoxide anion and decreased levels of non-protein thiols and total nitric oxide in comparison to vehicle control rats (Bishnoi *et al.*, 2011).

One group observed multiple stress-related metabolic pathway alterations in prefrontal cortex after 4 weeks of clozapine (21 mg/kg/day, i.p.) administration in male Sprague Dawley rats (Cai *et al.*, 2017). Specifically, clozapine increased creatine, inosine, progesterone, and phosphatidylethanolamine levels, and decreased corticosterone levels in comparison to vehicle controls.

Hippocampal protein levels of malate dehydrogenase and vacuolar ATP synthase were demonstrated to be significantly reduced after 30 days of clozapine (45 mg/kg/day, p.o.) treatment in male Sprague Dawley rats compared to water-treated control animals (La *et al.*, 2006).

After 28 days of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased the activity of cardiac SOD1, without affecting protein levels, in comparison to the vehicle control group (Nikolic-Kokic *et al.*, 2018).

After 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats, one group noted inhibition of ovarian mitochondrial complex I, but not complex III, activity, decreased SOD and GSH levels, and increased TBARS, and these changes were not observed in controls (Khalaf *et al.*, 2019).

Male Sprague Dawley rats administered clozapine (45 mg/kg/day, p.o.) for 34 days exhibited increased mRNA and protein levels of cytochrome b5 but increased mRNA and decreased protein levels of glial fibrillary acidic protein, shown in mitochondria isolated from the cerebral cortex (Ji *et al.*, 2009). Increases in gene expression of MAPK-activated protein kinase 2 and Ndufv2 (whose encoded protein is an electron transport chain member participating in oxidative phosphorylation) were not observed at the protein level.

One study noted a number of changes in mRNA expression in the brains of male C57BL/6 mice after 31 days of clozapine (10 mg/kg/day, p.o. in rodent meal pellets) versus mice on the control diet (Mehler-Wex *et al.*, 2006). Specifically, isoforms of cytochrome C oxidase, lactate dehydrogenase, ATPase, and mitochondrial uncoupling protein 1 decreased in expression, while phospholipase C increased in expression.

## 2. Clinical Studies

### a. Nevirapine

A retrospective study of HIV-infected children in Accra, Ghana found that nevirapine exposure was associated with a positive score using the Enquête Périnatale Française, a symptom-based evaluation tool (Langs-Barlow *et al.*, 2013).

A study of black South African patients infected with HIV taking a combination of stavudine, lamivudine, and either efavirenz or nevirapine for 4-24 months found that nevirapine exposure, but not efavirenz exposure, resulted in a significant time-dependent increase in mean total lymphocyte  $\Delta\psi_m^{\text{low}}$  (mitochondrial depolarization) and was also correlated with mean total

lymphocyte apoptosis (Karamchand *et al.*, 2008). However, as control patients were HAART-naïve, it is difficult to isolate the effect of nevirapine alone when it was administered as part of combination drug therapy here.

A cross-sectional study of HIV-infected adults in Tennessee compared exposure for at least 30 days to HAART with or without NNRTI to patients who had not received HAART in the last 3 months (6 months for NNRTIs) on measures of oxidative stress (Redhage *et al.*, 2009). Median plasma F<sub>2</sub>-isoprostane levels, a measure of lipid peroxidation, were found to be decreased in the NNRTI-exposed group; there was a trend toward lower levels with nevirapine exposure, but this was not statistically significant.

A prospective cohort study of HIV-uninfected and -infected pregnant women and infant pairs in Cameroon found that nevirapine treatment of HIV-exposed, uninfected infants (compared to HIV-unexposed, uninfected infants) was not associated with a lower mitochondrial to nuclear DNA ratio in dried blood spots at 6 weeks of age, although zidovudine exposure was (Jao *et al.*, 2017).

A multicenter, prospective randomized trial evaluated efficacy and safety of switching to lopinavir-ritonavir and nevirapine versus lopinavir-ritonavir and two NRTIs in HIV-infected adults who had achieved virologic suppression (Negredo *et al.*, 2009). The nevirapine-treated group experienced an increase in mitochondrial to nuclear DNA ratio in peripheral blood mononuclear cells by week 48. There was also an increase in cytochrome c oxidase IV activity at weeks 24 and 48 (presumably also in PBMCs).

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