Toxicity Screening of Novel Myeloperoxidase Inhibitors Using Zebrafish Model

by

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Abstract

Myeloperoxidase (MPO) is a heme-containing enzyme, which is stored and released by neutrophils and monocytes. MPO catalyzes the production of hypochlorous acid from hydrogen peroxide and chloride ion. Hypochlorous acid (HOCl) is a potent oxidizer and microbicidal chemical. In previous studies, we found that compounds that contain hydrazine moieties were effective inhibitors of MPO and, thereby, had the ability to decrease the production of HOCL. In our study, we tested hydrazine derivatives on zebrafish embryos to observe the toxicity effect in the circulatory system of the embryos including heart rate changes and cardiac edema. Furthermore, in situ hybridizations of liver cells using liver probe was applying to determine the safety of these compounds on the liver of the embryos. We found that isoniazid, 2-aminobenzoic acid hydrazide (2-ABAH), 4-aminobenzoic acid hydrazide (4-ABAH), 3-(dimethylamino) benzoic acid hydrazide (3-DMABAH), and 4-flurobenzoic acid hydrazide (4-FBAH) have little or no effect on heart rate. While sodium azide (NaN_3), 4-nitrobenzoic acid hydrazide (4-NBAH), and 4-(triflurομethyl) benzoic acid hydrazide (4-TFMBAH) caused severe edema and lowered heart rates as well as the delay of the liver development. The toxic effect of benzoic acid hydrazide (BAH) appeared only in a very high concentration. However, more experiments on mammalian model organisms will be needed to support the toxicity results.

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List of Abbreviations

2-ABAH	2-aminobenzoic acid hydrazide
4-ABAH	4-aminobenzoic acid hydrazide
BAH	benzoic acid hydrazide
4-FBAH	4-fluorobenzoic acid hydrazide
4-NBAH	4-nitrobenzoic acid hydrazide
4-TFMBAH	4-(trifluoromethyl) benzoic acid hydrazide
3-DMABAH	3-(dimethylamino) benzoic acid hydrazide
CAD	coronary artery disease
Cl^-	chloride ions
dpf	days post-fertilization
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
hpf	hours post-fertilization
IL	Interleukin
INH	Isoniazid
MPO	myeloperoxidase
MS	multiple sclerosis
NO	nitric oxide
NaN_3	sodium azide
PMNs	polymorphonuclear leukocytes
TNF	tumor necrosis factor

1. Literature review

1.1. Introduction

Human polymorphonuclear leukocytes (PMNs) are the first type of innate immune system cells that are arrived when the host body is targeted by an infectious organism (Oh et al. 2008). Neutrophils migrate to inflammatory loci via a process called chemotaxis using amoeboid movement (Allen & L. Stevens 1992; Edwards 1994). The receptors on the surface of neutrophils allow them to identify chemical gradients of molecules, such as interleukin-8 (IL-8), interferon gamma (IFN-γ), C3a, C5a, and Leukotriene B4, which point the neutrophil to the right direction of migration. PMNs have four different types of granules, each with several enzymes and proteins that have a specific antimicrobial effect (Borregaard et al. 1992; Sengeløv et al. 1993). Upon cell activation, the granule content is secreted into the extracellular space or into the formed phagosomes. Degranulation and release of the content of secretory vesicles and tertiary granules can be activated by a slight increase in intracellular calcium to 0.25 μM. Secretory vesicles and tertiary granules mainly contain albumin, collagenase, cathepsin, gelatinase, and other proteins. These proteins and enzymes play a vital and valuable role in facilitating the transportation of PMNs across the dense tissue to the inflammatory loci. Alternatively, Degranulation and release of the content from the primary (azurophilic) and secondary granules require a higher increase in intracellular calcium (0.7 μM). Azurophilic granules contain a unique heme-containing enzyme called myeloperoxidase (MPO) in large amount. MPO is involved in lipid peroxidation and released in periods of proinflammatory oxidative stress by polymorphonuclear neutrophils (Ky et

al. 2014; Meuwese et al. 2007). While secondary granules contain another enzyme called nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Both NADPH oxidase and MPO are peroxidase enzymes that have the ability of oxidize hydrogen donors especially hydrogen peroxide (Nicolas et al. 2003). MPO and NADPH oxidase both contribute to the formation of reactive oxygen species and oxidation of biological molecules resulting in their involvement in several pathologies. NADPH oxidase is a membrane-bound flavoprotein enzyme that is responsible for $O_2^{\bullet-}$ generation by using NADPH. In the vacuolar lumen of phagocytes, NADPH oxidase generates $O_2^{\bullet-}$ and H_2O_2. Although $O_2^{\bullet-}$ has antimicrobial effect, iron molecule in MPO enzyme increase the toxicity of H_2O_2 by its interaction with it resulting in the formation of hydroxyl radical which is more potent than $O_2^{\bullet-}$ (Kalyanaraman et al. 2012).

Myeloperoxidase (MPO) has an important role in the inflammation process. It is released during degranulation of neutrophils and monocytes so it is used as a biomarker of neutrophil presence at the inflammatory site.

Myeloperoxidase is a heme enzyme that is capable of catalyzing the formation of hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) and a chloride ion (Cl^-). This product has a greater bactericidal effect than hydrogen peroxide but also can be harmful to the host if the free radicals overwhelm the defense mechanism of the host. Myeloperoxidase can also form a hydroxyl radical (•OH) and oxidize a number of substrates leading to the progression of chronic inflammatory diseases. MPO may catalyze oxidation of tyrosine and nitrate to form a tyrosine radical and nitrogen dioxide. This initiates lipid/lipoprotein oxidation at the site which may stimulate the inflammatory vascular disease, atherosclerosis. When released outside the cell, MPO can damage extracellular targets, such as DNA, which may lead to tumorigenesis. Furthermore, MPO is released from neutrophils in lung tissue in response to pulmonary insult such as damage

from tobacco smoke exposure. Here MPO converts the metabolites of benzopyrene from tobacco smoke into a highly reactive carcinogen.

A previous study (Huang et al. 2014), compared the potency of MPO inhibitors focused on systemic approach to examine these inhibitors. They work on hydrazine compounds began with classic MPO inhibitors such as NaN3 and 4-ABAH, determine their Ki values and compared them to the original phenotypic IC50 values. 2-ABAH and 4-ABAH demonstrated the most effective MPO inhibition, acting through a two-step mechanism which is different than other BAH derivatives and NaN3. Furthermore, 2-ABAH and 4-ABAH produced their action by a slow-tight binding mechanism with a Ki 0.16 and 0.23 μM respectively, in accordance with their IC50 values of 0.71 and 0.50 μM respectively.

In this study, we hypothesize that MPO inhibition as a treatment for chronic inflammation will prevent unnecessary tissue damage. The experiments outlined here aim to further analyze the compounds for toxicity using the zebrafish model system. We did the experiment on nine hydrazine derivatives and used heart rate, cardiac edema, and survival rate as indicators of toxicity of these compounds.

1.2. Inflammation

Inflammation is the body's immunovascular protective response to irritation, infection, injury, or any other harmful stimuli (V. Stankov 2012). It can be stimulating by inappropriate immunological responses to foreign bodies (Subramanian et al. 2003; Auquit-Auckbur et al. 2011), chemical or physical agents such as blunt trauma (Cohen et al. 2009; Ramlackhansingh et al. 2011), chronic pressure of low intensity (Davis & Handy 1996), and vibrations (Abi-Hachem et al. 2010; Dina et al. 2010), or by cell death. There are two types of inflammation: acute

inflammation, the initial response to the stimuli, and chronic inflammation, the prolonged response. In both cases, there is a trigger. In the presence of harmful stimuli such as chemical or biological toxins, the innate immunity will rapidly begin within minutes, and inflammation will occur. Immune cells (neutrophils, monocytes, mast cells dendritic cells, and lymphocytes) (Akira et al. 2006), as well as non-immune cells (fibroblasts, endothelial cells, and epithelial cells), participate in the inflammatory process to target the phagocyte and destroy the pathogen. This destruction is mediated by a number of chemical mediators such as vasoactive amines (serotonin and histamine), peptides(bradykinin), and eicosanoids (prostaglandins, leukotrienes, and thromboxanes) (Abdulkhaleq et al. 2018). Inflammatory mediators (inflammatory cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) and chemokines) may also contribute in the inflammatory process by regulate the immunity response and induce acute-phase response (Guo et al. 2013) depending on activation of pathogen-specific receptors to modify the vascular permeability and recruitment of neutrophils and excess plasma into the infected site carrying antibodies and complement factors (Ahmed 2011).

1.2.A. Acute Inflammation

Acute inflammation is a short-term response; it begins within minutes or hours and terminates when the cause or stimuli is eradicated to rapid degradation of the inflammatory mediator. It is a part of the innate immunity of the host.

The primary mediators involved in acute inflammation are vasoactive amines and eicosanoids that increase vascular permeability leading to increase the inflammatory mediators' entry causing interstitial edema and increase prostaglandins synthesis causing local pain (Larsen & Henson 1983; Abdulkhaleq et al. 2018; Medzhitov 2008). In addition, neutrophils, basophils,

eosinophils, and mononuclear cells (monocytes, macrophages) are involved in stimulation of inflammatory process and removing foreign bodies by interact with immunological and non-immunological cells (Abdulkhaleq et al. 2018; Abbas et al. 2014). Furthermore, various cellular biochemical cascade systems function as initiators and promoter of the inflammatory response. These systems involve the fibrinolysis and coagulation systems triggered by necrosis, such as burn or trauma in addition to the complement system triggered by bacteria. Resolution, abscess formation, or chronic inflammation may result from acute inflammation.

The first response to the stimuli is vasoconstriction of the small blood vessels, which followed by vasodilatation to increase the blood flow to the infected site. This vasodilatation may be remaining for 15 minutes to hours. Thus, the permeability of the blood vessels walls increases allowing exudate to release into the injured tissue carrying clotting factors. White blood cells then start to adhere to the wall of the blood vessels to migrate and accumulate into extravascular space of the tissue. Neutrophils are the main leukocyte that involved in the acute inflammatory process. Mostly, after 24 hours monocytes start to flow to the injured site. The number of mature monocytes (macrophages) elevated after days or weeks which make them a marker for chronic inflammation.

1.2.B. Chronic Inflammation

Persistent acute inflammation due to non-degradable pathogens, viral infection, persistent foreign bodies, or autoimmune reactions may lead to chronic inflammation. Normally, the homeostatic state follows the inflammatory response rapidly by a process called resolution of inflammation. Anti-inflammatory mediators eicosanoids such as lipoxins, and some endogenous mediators such as resolvins and protectins (Serhan et al. 2008) are controlling this process. Also, monocytes are involved in cleaning cell debris (Serhan & Savill 2005). Chronic inflammation

occurs when the resolution of inflammation fails to happen, for example, in chronic cellular injury (Majno & Joris 2004; Kumar et al. 2003).

Chronic inflammation is characterized by consecutive destruction and reconstruction of the tissue from the inflammatory process. Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells) and fibroblasts, in addition to the mediators (Interferon gamma (IFN-γ) and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes), are involved in chronic inflammation. Chronic inflammation is strongly connected to the pathogenesis of inflammatory diseases. Major damage from inflammation affects the host because of the inflammatory response, not the pathogens; and it may cause necrosis, fibrosis, or tissue destruction. Understanding chronic inflammation is important because of its involvement in critical diseases, such as type 2 diabetes, rheumatoid arthritis, atherosclerosis, asthma, inflammatory bowel diseases (Garrett et al. 2010), cancer, neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and multiple sclerosis (Glass et al. 2010), and glomerulonephritis (Fakhouri et al. 2010).

1.3. Role of Myeloperoxidase in Inflammation

Host response to any injury is characterized by migration and accumulation of PMNs in the injured tissue (Menezes et al. 2008; Muller 2002) to release antimicrobial and inflammatory mediators (Genco & Slots 1984; Van Dyke et al. 1993). MPO is one of the active elements released by azurophilic granules of PMNs, and it is used as a marker of the presence of neutrophils in the inflamed tissue. MPO is a heme enzyme that catalyzes the oxidation of chloride ions (Cl^-) in the presence of hydrogen peroxide (H_2O_2) to form hypochlorous acid (HOCl), which is more potent than hydrogen peroxide in killing the bacteria (Hampton et al. 1998). Myeloperoxidase has been commonly used as an indicator of acute and chronic inflammation (Faith et al. 2008), and it has

been shown that changes in its levels are related to the severity of diseases (Hampton et al. 1998; Smith et al. 1986; Schierwagen et al. 1990).

At the beginning of the inflammatory process, after stimulation, neutrophils are the first immune cell that arrives at the inflamed site (Savill et al. 1989). Through phagocytosis, neutrophils release MPO into the microenvironment during its degranulation, followed by inactivation of MPO (iMPO) (Bradley et al. 1982). As a result, respiratory burst will induce and stimulate the secretion of TNF-α and cause tissue damage (Clark et al. 1987; Lefkowitz et al. 1993; Lefkowitz et al. 1989). TNF-α with MPO will initiate the cytokine cascade. Interleukin-8 attracts more neutrophils, and Granulocyte-macrophage colony-stimulating factor (GM-CSF), along with IL-8, causes degranulation of neutrophils. This leads to the release of MPO and starts the cycle again (Lefkowitz & Lefkowitz 2001). Myeloperoxidase can form tyrosine radicals, reactive oxygen species (ROS), or reactive nitrogen species (RNS), which can augment different diseases, such as atherosclerosis, and cancer.

1.4. Myeloperoxidase Biosynthesis

As a member of the peroxidase-cyclooxygenase family, myeloperoxidase is encoded by a gene of 14kb in size and made up of 12 exons and 11 introns positioned on the long arm of chromosome 17 in segment q12–24. Through co-translational N-glycosylation, which is followed by restricted deglucosylation (Nauseef 1987), the primary 80kDa translation product, preproMPO, is converted into a 90kDa apoproMPO. In the endoplasmic reticulum, apoproMPO transiently interacts with molecular chaperones and combines with a heme to produce enzymatically active proMPO, which is then released into the Golgi compartment (Hansson et al. 2006). ProMPO consists of a 116-amino acid N-terminal pro-region, which is required for the stability of proMPO, while its loss leads to retention of proMPO in the endoplasmic reticulum and failure of the

proteolytic process in producing mature MPO (Andersson et al. 1998). In azurophil granules, the propeptide is eliminated from the proMPO before the last proteolytic processing. In the post-endoplasmic reticulum section, a proconvertase removes the pro-region making a 74-kDa intermediate species. This is later processed by cysteine proteases to the L-chain and H-chain (McCormick et al. 2012) and dimerized afterward. The processed MPO protein is a glycosylated cationic 146kDa dimer which is mainly α-helical containing single disulfide bridge connecting the symmetry-related halves (73kDa). Each half has two polypeptides of 108 (14.5kDa, light chain) and 466 amino acids (58.5kDa, heavy chain). The two heavy chains are connected covalently by a single Cys-319– Cys-319 bridge. Both heavy and light chains have the same specific peroxidase activity as the holoenzyme (Andrews et al. 1984), indicating that dimerization has a slight impact on the whole enzymatic activity of mature MPO which is located in neutrophils and monocytes. Mostly, structural studies on MPO inhibitors used mature dimeric MPO (Zeng & Fenna 1992; Fiedler et al. 2000; Blair-Johnson et al. 2001).

1.5. Myeloperoxidase Special Effects

Bactericidal effect:

MPO has a critical role in killing bacteria by being excreted into phagocytic vacuoles where the destruction of microorganisms occurs (Klebanoff 2005). hypochlorous acid/hypochlorite ($HOCl/OCl^-$), the main product of the MPO– H_2O_2–Cl system has a potent antimicrobial effect. In pathological situations, activated phagocytes stimulate the MPO-H_2O_2 system resulting in the production of HOCl. HOCl is capable of targeting DNA, lipids, and lipoproteins through halogenation, nitration, and oxidative crosslinking (Malle et al. 2007).

The reaction of hypochlorous acid:

The oxidative products created by MPO have an important role in many processes involved in tissue damage. HOCl oxidizes sulfhydryl and thioether groups of proteins at a substantial rate (Winterbourn et al. 1985; Arnhold et al. 1993). It also chlorinates amino groups to chloramines (Test et al. 1982; Thomas et al. 1983). Since MPO has a strong cationic property, it can easily be attached to negatively charged membranes (Johansson et al. 1997). The hypochlorous acid and MPO-H_2O_2-Cl system also produces chlorohydrins and lysophospholipids in unsaturated phosphatidylcholines (Arnhold et al. 2001; Panasenko et al. 2003). Hypochlorous acid initiates lipid peroxidation via hydroperoxides that are stored in lipids (Panasenko & Arnhold 1999; Arnhold et al. 2001). Some radical products that initiate lipid peroxidation, such as tyrosyl radicals, undergo single-electron oxidation by complex I of MPO (Heinecke et al. 1993; Savenkova et al. 1994).

Attachment of myeloperoxidase to membranes:

As a result of MPO strong cationic charge, it binds to the negatively charged endothelial plasma membrane. After binding, myeloperoxidase localized at the basolateral site of the endothelium closed to interstitial matrix proteins transcytose the intact endothelium and locate along with fibronectin in the albumin (Baldus et al. 2001).

Protein nitration by myeloperoxidase**:**

In inflammatory conditions, compound I of MPO oxidizes nitrite to the nitrating species, nitrogen dioxide (NO_2) (Burner et al. 2000). During inflammation, the formation of nitrotyrosine in subendothelial and epithelial tissue regions is correlated to immunoreactivity of MPO (Baldus et al. 2002). MPO act as a scavenger of nitrotyrosine to prevent its damaging effects (Ichimori et al. 2003).

Modulation of the vessel tonus:

The endothelial secretion of MPO and the high rate of nitric oxide (NO) oxidation performed by radical products of MPO catalysis regulate the nitric oxide availability in inflammatory sites by weakening NO-dependent blood vessel relaxation and guanylate cyclase activation.(Baldus et al. 2002; Abu-Soud & Hazen 2000; Podrez et al. 1999; Eiserich et al. 2002).

Cessation of PMN responses:

MPO participates in the cessation of the influx of PMNs into the inflammation site. The inactivation of certain secreted granule contents by the MPO-H2O2-halide system reduce the attachment of the formylated peptide to chemotactic receptors and affects several functions in activated PMNs (Nauseef 1988).

1.6. Role of Myeloperoxidase in Different Pathologies

MPO products are not restricted to a specific pathogen. They are involved in the pathology of several diseases such as multiple sclerosis, Alzheimer's disease, atherosclerosis, and cancer (Podrez et al. 1999; Jolivalt et al. 1996). MPO is also found in arteriosclerotic plaques (Daugherty et al. 1994; Malle et al. 2000).

1.6.A. Cardiovascular Diseases

Multiple studies have indicated that increased levels of MPO could be a predictor of cardiotoxicity (Ky et al. 2014; Meuwese et al. 2007). Furthermore, a prospective study has linked the increased MPO levels with the risk of coronary artery disease (CAD) development in initially healthy populations (Meuwese et al. 2007). Likewise, various cross-sectional and prospective studies have established the role of MPO as significant circulating inflammatory indicator in heart failure (HF) (Tang et al. 2007), acute coronary syndrome (ACS) (Sawicki et al. 2011), and CAD

(Cavusoglu et al. 2007). The overexpression of MPO in atherosclerosis was displayed by (Daugherty et al. 1994). Additionally, the products of MPO, 3-chlorotyrosine, and some specific epitopes were found in the lesions of atherosclerosis (Hazen & Heinecke 1997; Malle et al. 2000). MPO plays a role in atherosclerosis through a variety of different mechanisms. For instance, it has a vital function in the oxidation of lipoproteins where LDL oxidation stimulates the accumulation of foam cells in artery walls causing the generation of the necrotic center of atherosclerosis. Then, oxidized HDL results in the incapability to remove excess cholesterol in atherosclerotic lesions (Heinecke 1998). The other mechanism of MPO is the dysfunction of endothelial cells as a result of reduced bioavailability of NO. MPO has high affinity toward NO, which is the endogenous substrate of MPO, leading to its uptake and resulting in a remarkable reduction of NO bioavailability (Huang et al. 2013). MPO also prevents the production of NO by halogenating arginine, which is a substrate for NO synthase (NOS). Location of NOS is changed by oxidized HDL on the plasma membrane, leading to inhibition of NOS. Oxidized LDL activates endothelial cells and accentuates their ability to release interleukine-8, which activates monocytes. Additionally, MPO participates in the development of susceptible plaque by HOCl, which causes the apoptosis of endothelial cells resulting in ischemia (Hazen 2004). Furthermore, MPO is also involved in atherosclerotic plaque instability by the activation of protease cascade, pro-apoptotic, and pro-thrombotic phenomena. Sugiyama et al. demonstrated that MPO may escalate the risk of thrombus formation because of its role in causing plaque erosion (Hazen 2004; Fu et al. 2001).

1.6.B. Central Nervous System Diseases

MPO participates in nervous system diseases caused by neuronal damages like multiple sclerosis (MS), Alzheimer's, and Parkinson's disease (Gellhaar et al. 2017; Gray et al. 2008). In autoimmune diseases, such as multiple sclerosis, neutrophils and macrophages are found in brain

tissue; thus, a significant amount of MPO will be detected in multiple sclerosis lesion (Pulli et al. 2015; Gray et al. 2008; Forghani et al. 2012; Compston & Coles 2002; Nagra et al. 1997). Furthermore, MPO seems to play a considerable role in Alzheimer's disease due to its presence at significant amounts in brain tissues of patients suffering from Alzheimer's. The presence of 3-chlorotyrosine and dityrosine in brain tissues suggests that MPO also accelerates the symptoms of the disease (Green et al. 2004; Tzikas et al. 2014; Schreitmüller et al. 2013). Moreover, MPO levels has been found to be increased in the brain of Parkinson's disease patients (Gellhaar et al. 2017; Hirsch & Hunot 2009; Teismann 2013). Additionally, different studies showed that MPO also contributes in neurodegeneration by oxidizing serotonin, a physiological substrate of MPO, into two compounds: serotonin dimer and tryptamine-4,5-dione (Gellhaar et al. 2017; Ximenes et al. 2009).

1.6.C. Renal Diseases

As a consequence of the adherent properties of monocytes and neutrophils, they bind to glomerular basement membranes, leading to oxidative damages in the attachment sites. The damaged membrane, as well as the high level of MPO in the inflamed kidney tissues, confirmed that MPO participates in glomerular diseases. In patients with glomerulonephritis, increased levels of MPO specific anti-neutrophil cytoplasmic antibody (MPOANCA) verify the direct relation of MPO and renal injury (D'Agati 2002; Hoshino et al. 2008; Anders et al. 1999; Ohtani et al. 2007).

1.6.D. Tumor Progression

MPO is one of the endogenous enzymes that produce oxidative damages to DNA/RNA by direct oxidation. Oxidative damage to DNA/RNA causes mutations that lead to the development of tumors. Through halogenation of uracil nucleoside or deamination of halogenated cytosine

nucleosides, MPO can produce 5-chlorouracil and 5-bromouracil, which are high mutagenic lesions. Additionally, by halogenating the free nucleobases, MPO produces deoxynucleosides that interfere with DNA replication. Furthermore, MPO also can metabolite xenobiotics and their carcinogenic products. For example, MPO can oxidize benzene and pro-carcinogens benzopyrene into carcinogens. Generally, many studies have confirmed that MPO can contribute to numerous types of malignancies, such as bladder cancer (Hung et al. 2004), epithelial ovarian cancer (Castillo-Tong et al. 2014; Nagra et al. 1997; Saed et al. 2010), hepatoblastoma tumor (Pakakasama et al. 2003), and lung cancer by converting benzopyrene metabolites to highly reactive carcinogens (Taioli et al. 2007; Le Marchand et al. 2000; Kantarci et al. 2002; Schabath et al. 2002; Feyler et al. 2002), larynx cancer (Cascorbi et al. 2000), breast Cancer (Qin et al. 2013; Lin et al. 2005), colorectal cancer (Al-Salihi et al. 2015; Droeser et al. 2013) and esophageal carcinomas (Lee et al. 2001). MPO can be released outside the cell, risking damage to extracellular targets, such as DNA, which may lead to tumorigenesis. If the level of free radicals surpasses the antioxidant defense mechanism, reactive oxygen species such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals can cause oxidative damage to biomolecules, potentially leading to formations of xenobiotic carcinogen and activation of procarcinogen to genotoxic intermediate

Pathology location	Role of MPO	Refrences
Cardiovascular System	Predictor of Cardiotoxicity	(Ky et al. 2014; Meuwese et al. 2007)
	Coronary Artery Disease (Cad)	(Meuwese et al. 2007)
	Heart Failure (HF)	(Tang et al. 2007)
	Acute Coronary Syndrome (ACS)	(Sawicki et al. 2011)
	Atherosclerosis	(Daugherty et al. 1994)
Central Nervous System	Multiple Sclerosis (MS)	(Pulli et al. 2015; Forghani et al. 2012)
	Alzheimer's Disease	(Green et al. 2004; Tzikas et al. 2014)
	Parkinson's Disease	(Gellhaar et al. 2017; Teismann 2013)
Renal System	Glomerulonephritis	(D'Agati 2002; Hoshino et al. 2008; Ohtani et al. 2007)
Tumor	Bladder Cancer	(Hung et al. 2004)
	Epithelial Ovarian Cancer	(Nagra et al. 1997; Castillo-Tong et al. 2014)
	Hepatoblastoma Tumor	(Pakakasama et al. 2003)
	Lung Cancer	(Le Marchand et al. 2000; Taioli et al. 2007)
	Larynx Cancer	(Cascorbi et al. 2000)
	Breast Cancer	(Qin et al. 2013; Lin et al. 2005)
	Colorectal Cancer	(Droeser et al. 2013; Al-Salihi et al. 2015)
	Esophageal Carcinomas	(Lee et al. 2001)

Table 1. Role of Myeloperoxidase in Different Pathologies.

1.7. Myeloperoxidase Deficiency

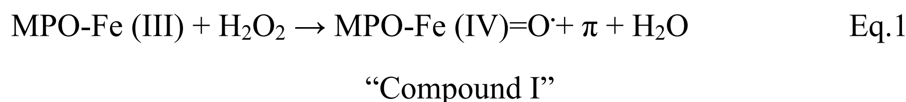
Usually, myeloperoxidase deficiency is inherited, but in some cases, it is caused by medical conditions, such as diabetes mellitus, lead poisoning, thrombotic diseases, acute or chronic myeloid leukemia, or anemia; and it resolves once the condition is treated (Dinauer & Newburger 2009; Lanza 1998; Arumugam & Rajagopal 2004). In general, because of the ability of MPO-deficient neutrophils to perform phagocytosis on bacteria, patients are asymptomatic and do not

have higher incidences of infection. In normal individuals, activated neutrophils undergo a process called respiratory burst. In this process, hydrogen peroxide, superoxide, and other reactive oxygen derivatives are produced by activated NADPH oxidase to kill the microorganisms. Myeloperoxidase has a key role in respiratory burst cessation by converting hydrogen peroxide to hypochlorous acid, a more effective compound in the microbial killing but more harmful to host cells. However, patients with MPO-deficient neutrophils have longer respiratory bursts with less hydrogen peroxide toxicity (Dinauer & Newburger 2009; Lanza 1998). Myeloperoxidase plays a role in adaptive immune response suppression, as confirmed by two models of enhanced T cell-mediated skin delayed-type hypersensitivity and antigen-induced arthritis in MPO−/− mice. Mechanistically, MPO secreted from neutrophils inhibit LPS-induced dendritic cell (DC) stimulation, as identified by decreased interlukin-12 (IL-12) formation and CD86 expression, subsequently limiting proinflammatory cytokine formation and T cell proliferation. In contrast, using MPO-deficient mice in the K/BxN arthritis and collagen-induced arthritis (CIA) models showed a reduction in the severity of the disease (Pahwa et al. 2017). In response to pulmonary insult like in tobacco smoke exposure, MPO is released from neutrophils into lung tissue in order to catalyze the formation of free radicals (Kiyohara et al. 2005) and activate highly reactive carcinogenic benzopyrene metabolites (4-aminobiphenyl and the arylamines), heterocyclic amines (Williams et al. 1998), aromatic amines (Culp et al. 1997; Kadlubar et al. 1992), and polycyclic aromatic hydrocarbons (PAHs) (Mallet et al. 1991; Trush et al. 1985). Hence, MPO-deficiency may decrease the risk of lung cancer (Rymaszewski et al. 2014).

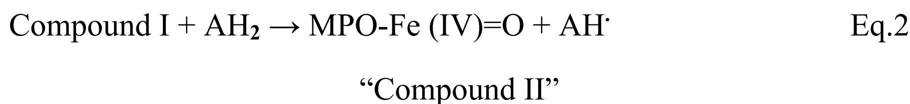
1.8. Myeloperoxidase Mechanism

A sulfonium linkage in MPO allows production of HOCl by twisting the heme from its planar state. Moreover, a ferric heme of MPO [MPO-Fe(lll)] reacts with a 100–10,000-fold lower

relative concentration of H_2O_2 and oxidizes to Compound I, a transitory intermediate (half-life~100 ms (Harrison et al. 1980), leading to the production of a ferryl porphyrin π cation radical MPO-Fe(IV) = $O^{\bullet+\pi}$ (Equation 1) (Kettle et al. 1997; Everse 1998). By two single-electron steps, MPO can produce its ferric state and a classic peroxide electron donor (AH_2) in a typical peroxidase catalytic cycle (Equations 2–3). In the first step, Compound II will be formed by the reduction of the porphyrin radical to a ferryl heme (Equation 2). Then, by AH_2, Compound II is reduced back to ferric enzyme A in the next step (Equation 3), together with the oxidation of AH2 to the free radical ($AH^{\bullet}$) (Andrews & Krinsky 1982a; Andrews & Krinsky 1982b).

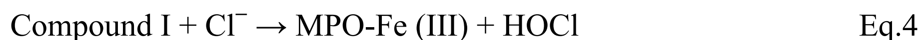


$$\text{MPO-Fe (III)} + H_2O_2 \rightarrow \underset{\text{“Compound I”}}{\text{MPO-Fe (IV)=}O^{\cdot} + \pi} + H_2O \qquad \text{Eq.1}$$

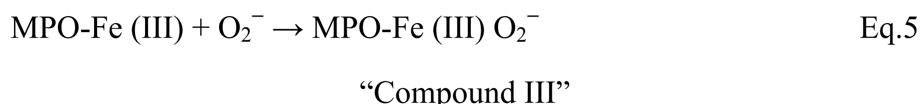


$$\text{Compound I} + AH_2 \rightarrow \underset{\text{“Compound II”}}{\text{MPO-Fe (IV)=O}} + AH^{\cdot} \qquad \text{Eq.2}$$

$$\text{Compound II} + AH_2 \rightarrow \text{MPO-Fe (III)} + AH^{\cdot} + H_2O \qquad \text{Eq.3}$$



$$\text{Compound I} + Cl^{-} \rightarrow \text{MPO-Fe (III)} + HOCl \qquad \text{Eq.4}$$



$$\text{MPO-Fe (III)} + O_2^{-} \rightarrow \underset{\text{“Compound III”}}{\text{MPO-Fe (III) }O_2^{-}} \qquad \text{Eq.5}$$

Compound II (Equation 2) and Compound III (Equation 5) do not play a role in Cl^- oxidation. In fact, Cl^- ions are oxidized to HOCl by Compound I in the chlorination cycle of MPO, and in the process, the MPO ferric state is regenerated (Equation 4) (Huang et al. 2016). MPO importance has been considered in different studies because of the vital role it has in the inflammatory responses as it oxidize DNA and protein. In addition, MPO exist at prominent level at inflammation sites making it as an important biomarker for inflammation (Klebanoff 2005).

1.9. Inhibition of Myeloperoxidase

Because of the role of MPO in the development of several chronic diseases, its inhibition may be helpful in reducing such pathologies. Different studies have shown that lipid peroxidation of LDL is at the same level in monocytes of MPO-deficient patients as well as that induced by normal cells (Hiramatsu et al. 1987; Brennan et al. 2001). MPO is a new target of much anti-inflammatory research because of its harmful role in many inflammatory diseases. A major adverse effect of inhibition of MPO is the impairment of the immune system to several pathogens (Hazen 2004). To avoid this side effect, MPO inhibitors should target the extracellular MPO, which harm the host cell. The polarity of the inhibitor determines its ability to penetrate the neutrophil membrane. Compounds with high polarity were not allowed to penetrate the cell membrane leading to inhibit extracellular MPO only. Unfortunately, most of potent MPO inhibitors have low polarity due to their high chance to penetrate the neutrophil cell membrane (Soubhye et al. 2013).

1.10. Myeloperoxidase Inhibitors

1.10.A. Compounds that support compound II formation

Multiple studies have shown that hydroxamic acid, indole, and tryptamine derivatives can stimulate the production of compound II and redirect MPO from the chlorination cycle. Many of these drugs are already used in clinical applications such as diclofenac (Zuurbier et al. 1990), salicylate, phenylbutazone, olsalazine, piroxicam, sulfasalazine, benzocaine (Kettle & Winterbourn 1991), chlorpromazine (van Zyl et al. 1990), deferoxamine (Klebanoff & Waltersdorph 1988), and acetaminophen (van Zyl, Basson & van der Walt 1989). However, this inhibitory effect was reduced in the presence of electron donor systems such as superoxide anion (Kettle et al. 1993). Dapsone and indomethacin are competitive inhibitors that act by inhibiting

HOCl production. Accordingly, MPO will remain as inactive compound II (Stendahl et al. 1978; Shacter et al. 1991). Hydroxamic acid, indole and tryptamine derivatives do not show significant ability in reducing compound II. At the same time, they are susceptible to oxidation by compound I. Hence, indicating that they are incompetent to contest with high-affinity peroxidase substrates.

1.10.B. Compounds that Support Compound III Formation

Since compound III is placed outside the peroxidation and chlorination cycle, drugs that act by stimulating compound III synthesis may prevent HOCl formation by MPO. Example of such drugs are hydroquinones, which are a good substrate for both MPO compounds I and compounds II (Kettle & Winterbourn 1992; Burner et al. 2000). Semiquinone radicals are formed in high concentration leading to the reduction of ferric MPO to ferrous MPO, which promptly form compound III by binding to the oxygen molecule consequently inhibiting the production of HOCl.

1.10.C. Benzoic Acid Hydrazides

Generally, benzoic acid hydrazides inhibit the peroxidation and chlorination activities of myeloperoxidase (Kettle et al. 1995). Derivatives of benzoic acid hydrazide with nitrogen or oxygen substituents are better inhibitors than unsubstituted benzoic acid hydrazide or its 4-chloroderivatives. Benzoic acid hydrazides are oxidized by compound I with relatively no effect of the substituents on the aromatic ring to the rate constant (Burner et al. 1999). On the other hand, there is a strong correlation between the substituents and the Hammett rule to the reduction of compound II. There is also an important correlation with Brown–Okamoto substituent constants (Hansch & Leo 1979; Burner et al. 1999) . Isoniazid (INH), which is an anti-tuberculous agent has an irreversible inhibitory effect on both peroxidation and chlorination action of MPO. The absorbance of heme related to compound III formation will be diminished by oxidation of isoniazid

or hydrazine sulfate between pH values 6.5 and 7.8 by the MPO–H_2O_2 system (van Zyl, Basson, Uebel, et al. 1989). 4-Aminobenzoic acid hydrazide (4-ABAH) has a potent inhibitory effect on peroxidation, and it is a good electron donor to compound II. In the peroxidase cycle, ABAH is oxidized by compound I and compound II. This reaction leads to the production of radicals, which transform the enzyme into compound III and ferrous MPO. Because compound III does not have any role in chlorination or peroxidation cycle, the oxidation of reducing substrates will be delayed reversibly. ABAH or ABAH radicals reduce compound III in a similar way of reduction of oxyhemoglobin by phenylhydrazine (Misra & Fridovich 1976). Irreversible inactivation will be aggravated by the disintegration of the heme group causing ferrous MPO to accumulate (Kettle et al. 1997).

1.10.D. Aromatic Hydroxamic Acids

The two major compounds in this group are salicylhydroxamic and benzohydroxamic acids. The mechanism of action of hydroxamic acids is the reduction of compound I and compound II and substitution of H_2O_2 in the formation of compound I (Schonbaum & Lo 1972; Schonbaum 1973). Additionally, in low concentrations of H_2O_2, hydroxamic acids attach to the same binding site of H_2O_2 on the enzyme and disallow the formation of compound I and II (Davies & Edwards 1989). Specifically, salicylhydroxamic acid has two inhibitory actions in peroxidase catalysis: prevention of hydroperoxide attachment to iron and contention with other donors in reactions with greater oxidation states. Still, this is considered to be a reversible inhibition that depends on contending electron donors and H_2O_2 concentration as well (Malle et al. 2007).

1.10.E. Tryptamines and Indoles

Indole derivatives are reversible inhibitors of MPO (Jantschko et al. 2005). These molecules' mechanism of action is the donation of one electron to compound I and II of heme peroxidases (Jantschko et al. 2002). However, tryptamines have a stronger inhibitory effect on chlorination than indoles, and this controls the ratios of rates of compound I to compound II reduction. The maximum inhibition of chlorination is achieved by 5-Fluorotryptamine and 5-chlorotryptamine. Indole rings may bind to the pyrrole ring D and 5-OH group, as in serotonin, or 5-OCH3, as in melatonin, targeting the heme center. Even though the indole ring appears in the same place in both cases, the orientation of the side chain is the main difference between the two molecules. In serotonin, a strong substrate, it goes to the access channel. While In melatonin, a weak substrate, it goes to a pocket alongside the periphery of the distal cavity (Hallingbäck et al. 2006). The rate of the reduction of compound I and compound II with electron-donating groups is determined by the ring substituent through increased reactivity of the aromatic ring (Jantschko et al. 2005). On the other hand, the electron-withdrawing substituents, such as Cl or F, inhibit indole derivatives from acting as a substrate for peroxidase. Due to highly positive reduction potentials of the redox couple compound I/compound II (Arnhold et al. 2006), these substituents can be an electron donor for compound I but not for compound II (Jantschko et al. 2005), leading to accumulation of compound II and deterring of MPO from its chlorination cycle.

1.11. Zebrafish

1.11.A. Uniqueness of Zebrafish

Zebrafish have been widely accepted as a model in toxic research. Use of zebrafish as an experimental model includes both adult and embryonic models. This wide acceptance is due to the many unique characteristics of zebrafish such as small size, high productiveness, and transparency of the embryos and larvae that make the experiments easy to perform (Strähle & Grabher 2010; Chakraborty & Agoramoorthy 2010). Furthermore, only small amounts of the compound studied are needed in the surrounding medium, which is absorbed by their skin or through their gastrointestinal tract, allowing for rapid estimation of pharmacological activity amongst vertebrate animal models (Fleming et al. 2005; Crawford et al. 2008). The zebrafish is small in size among vertebrate. The mature zebrafish length is about 3 cm, while embryo and larvae length range between 2–3 mm. Zebrafish are simple and non-costly, and larvae are able to survive for days in separate wells of standard 96-well or 384-well plates using essential nutrients kept in their yolk sacs. Thus, even a small laboratory can generate many embryos (Anderson et al. 2003; MacRae & Fishman 2002). However, a major challenge is in collecting sufficient tissue to apply traditional assays in histology or ex vivo cell biology, but this can be solved by combining large numbers of zebrafish. Oviparous species like zebrafish have the ability to achieve eggs fertilization outside the female, which eases both examination and analysis of its progression (Corley-Smith et al. 1999; Poleo et al. 2001). In addition, the zebrafish is known to lay large numbers of eggs. Females spawn around 300 eggs per week under ultimate conditions. Additionally, it can spawn in laboratory aquariums by adding flora and gravel to the tank (Hsu et al. 2007; Spence et al. 2007). It has also been observed that the eggs hatch rapidly, and organogenesis occurs quickly in about 48 hours post-fertilization (hpf). As a result, the major organs are developed within five to six days

post-fertilization (dpf). Another advantage is the transparency of the embryo that allows the researchers to integrative the biology of multiple organ systems at high-resolution crossways and detects any changes early (Berry et al. 2007; Hill et al. 2005; Hsu et al. 2007; Parichy et al. 2009). Interestingly, there is strong evidence of the similarity of zebrafish genetically, physiologically, and pharmacologically to mammals, specifically to humans. Their cardiovascular, nervous, and digestive systems are basically comparable to mammals (Hsu et al. 2007), and their genes are more than seventy-five percent similar (Chakraborty et al. 2009).

1.11.B. Characteristics of Zebrafish Heart

Anatomically, the human heart consists of four compartments divided by a septum and valves. On the other hand, the heart of zebrafish is composed of one atrium and one ventricle divided by an atrioventricular valve. Regardless of this difference, the zebrafish is considered as beneficial for cardiovascular studies as a useful vertebrate model system (Staudt & Stainier 2012). Early cardiac morphological events between zebrafish and humans, such as the creation of cardiac progenitor, myocardial plate compilation, development of heart tube, cardiac looping, and valve development, are vastly alike, with the advantage of the shorter time needed by zebrafish to complete these processes. Zebrafish and humans have resemblances in several important genes and regulatory networks for cardiogenesis. Because zebrafish embryos can last their first seven days without an efficiently working cardiovascular system by receiving oxygen through passive diffusion through the skin, roles of many unidentified genes in vertebrate cardiac development and function have been identified by studying zebrafish (Stainier 2001). Moreover, several electrical aspects of zebrafish and human hearts are alike (Staudt & Stainier 2012). The zebrafish embryonic heart rate range between 140–180 beats per minute (bpm) which is considered to be comparable to the normal human fetal heart rate 130–170 bpm (De Luca et al. 2015). Lately, zebrafish have

also grown in their acceptance for cardiovascular research because of their abilities to regenerate the cardiovascular tissue (Foglia & Poss 2016), which offers promising potentials for development of new therapies for cardiac damage (Sarmah & Marrs 2016).

1.11.C. Organ-Specific Toxicology in Zebrafish

The most common cause of failure in drug development or in drug withdrawals from the markets is organ-specific toxicities. In the past few years, zebrafish was frequently utilized as a model in drug development processes to improve the identification of toxicity at an early stage.

The organogenesis in larval zebrafish is completed within 48 hpf. Then, when humoral and neural communications are established in the next few days, different cell types migrate into every organ. For example, peripheral innervation by the somatic and autonomic nervous systems is incomplete for a few weeks after fertilization (Olsson et al. 2008). As in other vertebrates, during development, there is significant functional plasticity that can be detected in every organ system. The accessibility of these features is a unique characteristic of the zebrafish model. However, special care is necessary during assay development (Macrae 2010).

1.11.D. Zebrafish and Cardiotoxicity

Multiple studies have shown that cardiac defects in zebrafish are parallel to those seen in adult human patients caused by prenatal exposure to many teratogens (Loucks & Ahlgren 2012; Keshari et al. 2016). Simple heart-rate estimation of zebrafish can give excellent data associated with known adult human repolarization cardiac toxicity and drug-drug interactions (Milan et al. 2003). After 48 hours post fertilization, zebrafish will have a beating heart with a complex repertoire of ion channels and active metabolism (Milan et al. 2003; Warren & Fishman 1998). Action potential prolongation, novel genes lately linked to cardiac repolarization, and known ion

channel mutations all can be investigated using the zebrafish model. Scientists have demonstrated that medications that cause repolarization abnormalities in humans reliably re-created bradycardia in zebrafish, highlighting the resemblance of both systems and the great utility of zebrafish in anticipating chemicals harmful to cardiovascular systems. These data suggest significant similarities between zebrafish and humans at multiple levels, which can establish the basis of cardiotoxic drugs assays by using screening technologies in zebrafish (Ky et al. 2014; Meuwese et al. 2007).

1.11.E. Zebrafish and Hepatotoxicity

Many hepatotoxicants that harm mammals cause a similar effect in zebrafish. For example, exposure to mercury triggers the hepatic transcriptional response in zebrafish which is close to Hep2 cells in human (Ung et al. 2010). Although zebrafish may have an advantage over other recent models, more research is required to recognize the pathophysiological parallels.

1.11.F. Zebrafish and Nephrotoxicity

Currently, acute nephrotoxicity has been discovered in zebrafish kidney such as tubular toxicity of gentamicin. Its effects have been confirmed histologically and functionally on extracellular volume homeostasis that parallel aminoglycoside toxicity in more complex organisms (Hentschel et al. 2005). Nonetheless, more research is crucial in comprehending the renal function in the larval stages that will help validate the association between human and zebrafish kidney diseases.

1.11.G. Zebrafish and Neurotoxicity

Behavioral changes in seizures, feeding, flight responses, involuntary movement, addiction, and sleeping can be identified and monitored in zebrafish (Best et al. 2008; Rihel et al.

2010). In addition, both genetic and chemical effects of these behaviors in humans can be studied in zebrafish. Furthermore, focusing on the screen of truly incorporated behaviors will shed additional light on the efficacy of the zebrafish for the analysis of neurotoxicity.

1.11.H. Zebrafish and Muscle Toxicity

Many drugs such as the statins have myotoxicity as a side effect. Statin-related myopathy has been studied in zebrafish. In patients who used a statin, atrogin-1, which is a skeletal muscle atrophic signaling molecule, is upregulated in their muscles. Similarly, the atrogin ortholog is also upregulated in zebrafish skeletal muscle when it is exposed to statin and knockdown of atrogin-1 obviously reduces the associated myopathy (Hanai et al. 2007). However, ongoing work on exploring the usefulness of zebrafish in the interaction between the diverse toxicity pathway and the role of other genes will be needed.

1.11.I. Zebrafish and Gastrointestinal Toxicity

In zebrafish, gut motility emerges around day 4-5 post-fertilization. Yet, they have not been used for gastrointestinal toxicity screening, but some other higher-throughput assays have been developed. Assays of endocrine function, secretory function, absorption, and other gut functions can be imagined. Advanced assay design is important for these physiological endpoints, and the advantages of chemical and genetic screening will be valuable (Wallace et al. 2005; Olsson et al. 2008).

2. **Materials and Methods**

2.1. Zebrafish

Zebrafish were raised in Auburn University College of Veterinary Medicine. Casper zebrafish were used in this experiment.

2.2. Embryo collection

Embryos were collected after I hour of mating to ensure proper development uniformity. Embryos were then sorted for fertilization, pooled, and then divided into petri dishes at ~50 embryos per dish. Subsequently, the embryos were grown in the incubator at 28.5^{o} C. Between 50% epiboly stage (5 hpf) and shield stage (6 hpf), embryos were placed in 6-well plates at ~20 embryos in each plate and divided into control and treatment groups. All embryos were then incubated at 28.5^{o} C overnight.

2.3. Embryo Treatment

Reagent: 2-aminobenzoic acid hydrazide (2-ABAH), 4-(trifluoromethyl) benzoic acid hydrazide (4-TFMBAH), 3-(dimethylamino) benzoic acid hydrazide (3-DMABAH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 4-aminobenzoic acid hydrazide (4-ABAH), benzoic acid hydrazide (BAH), 4-fluorobenzoic acid hydrazide (4-FBAH), 4-nitrobenzoic acid hydrazide (4-NBAH), sodium azide (NaN_3), Dimethyl sulfoxide (DMSO) were obtained from Alfa Aesar (Ward Hill, MA). The stocks of these compounds were 20mM in 5% DMSO, so the compounds further diluted to prepare 10 ml of each compound in 0.4% DMSO. The buffer was 0.5x E2 embryo

growth medium (7.5mM NaCl, 0.25mM KCl, 0.5mM $MgSO_4$, 75µM KH_2PO_4, 25µM Na_2HPO_4, 0.5mM $CaCl_2$, 0.35mM $NaHCO_3$, pH 7.2-7.6) plus 10mM HEPES and 0.4% DMSO.

The control group was treated by the buffer while treatment groups were treated by adding 5 ml from each concentration of the nine compounds (NaN_3, Isoniazid, BAH, 2-ABAH, 4-ABAH, 4-FBAH, 4-NBAH, 4-TMABAH, 3-DMABAH) in each well and incubated at 28.5° C overnight. Six concentrations from each compound were used (20 µM, 40 µM, 80 µM, 120 µM, 200 µM, and 300µM). embryos were monitored daily and all dead embryos were removed and documented to eliminate potential damage to developing embryos. At 4 days post fertilization, all deformed, dead, and cardiac edemic embryos were observed and videos were taken for heart rate analysis.

2.4.Measurement of heart rates

Embryos were raised at 28.5° C incubation but heart rates calculated at room temperature. Before measurements, embryos were removed from the incubator to acclimate heart rate to temperature variation. Heart rates were calculated by recording short videos (30fps as vsi files around 20 seconds) followed by counting the heart beats in ten seconds by slowing the speed of the videos to half of their original speed. Then, multiplying that number by twelve to get the heart rate as beat per minute (bpm). Heart rates of all groups were measured by MVX10 research Macro Zoom Microscope. Heart rates of control groups were measured at the beginning and the end of the experiment to make sure that, the time spent calculating other groups did not affect the heart rate as it may be increased naturally during developmental stages. The experiment was repeated four times. Due to the variability in the heart rate of some of matching concentrations between experiments, we compared the heart rates of all groups to the control (normalized).

2.5.Florescence reaction for measuring MPO activity

To determine the stability of the MPO-inhibitors throughout the four days of experimentation, the inhibitory compounds were analyzed at a 120µM concentration in egg water at days zero and four. This analysis was performed three times for each compound. For this experiment black 96-well plates were used to measure the fluorescence at 650nM wavelength. In day 0 of the experiment 10µl of diluted MPO of human neutrophil, diluted by sodium acetate buffer (1:200µl), added to 80 µl of every inhibitor followed by 10 minutes waiting. After 10 minutes, 150 µl of water was added by a multichannel pipette to every well. Immediately before putting the plate in a SpectraMax plate reader, 60µl of 3,3',5,5'-Tetramethylbenzidine (TMB) reagent was added to each well.

2.6.Statistical analysis

Every experiment repeated at least three times. Each experiment included comparing groups (control vs. treatment) and counting at least five embryos per group. Unpaired t test was used to compare the mean of every concentration of each compound to the mean of the control group. Significance level is 0.05. All the analysis and graphs were produced using Graphpad software (version seven).

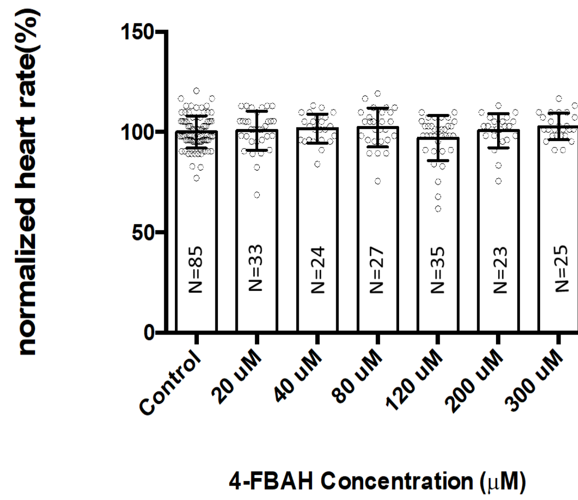
3. Results

3.1. Compounds that produced no significant decrease in heart rate in zebrafish embryos

The heartbeats were counted for every embryo at day four post fertilization individually then we calculate the mean of the heart rate for every group and normalized them to the control. As shown in figure (1), there is no significant decrease in group treated with 4-FBAH, 4-ABAH, isoniazid, 2-ABAH, and 3-DMABAH, at any concentration. The p value of these comounds was $0.0001<p<0.001$. But, 4-FBAH, had high IC50 and Ki values at 22.45 and 0.83 μM respectively (Jiansheng Huang, Forrest Smith, and Peter Panizzi,2014), which diminishes its safety and limits its use in further experimentation. 4-ABAH had an IC50 of 0.71 and a Ki is 0.23 μM. According to this data, 4-ABAH is considered a safe MPO inhibitor. 4-ABAH is already used in many lab experiments by different researchers. Isoniazid, which is used as a treatment for tuberculosis, has a good inhibitory effect on MPO enzymatic activity with IC_{50} and K_i 277.10 and 10 respectively. So, it should be noted that even though isoniazid is already used in tuberculosis, its utility as an MPO inhibitor is diminished due to its high IC_{50} value. The IC_{50} and K_i for 2-ABAH and 3-DMABAH are 0.5 and 0.16 μM for 2-ABAH, and 1.27 and 0.56 μM for 3-DMABAH, respectively which make them considered as safe compounds at the reasonable dose range.

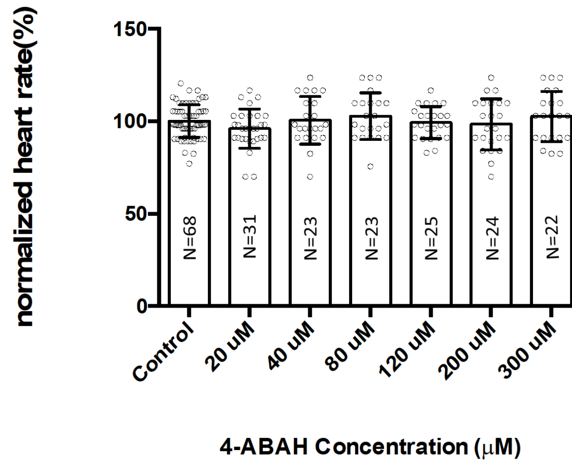
A

Heart Rate at 4 dpf after Exposure to 4-FBAH



B

Heart Rate at 4 dpf after Exposure to 4-ABAH



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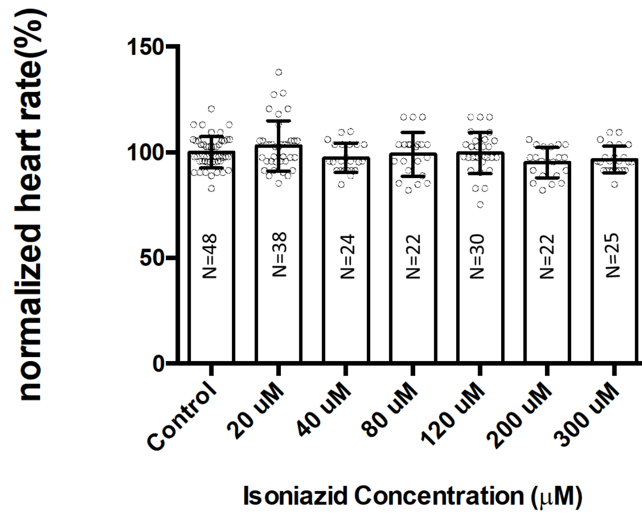

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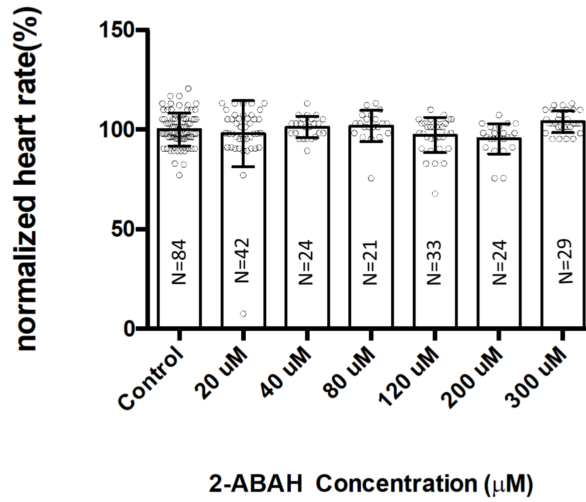

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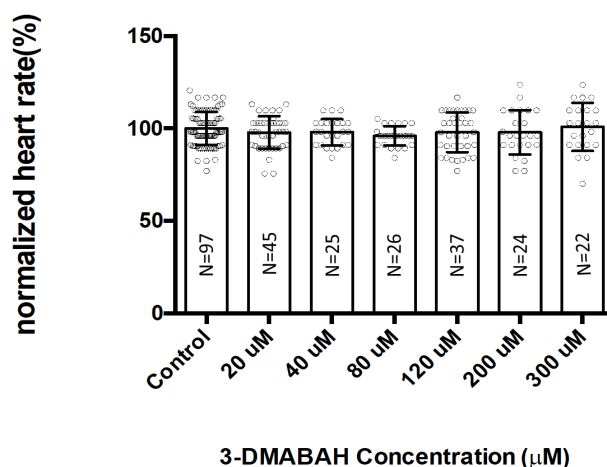


Figure 1. Effect of hydrazine containing compounds on the developing zebrafish heart. Shown are normalized heart rates of zebrafish embryos after exposure to 20 μM, 40 μM, 80 μM, 120 μM, 200 μM, and 300μM) plus the control. **(A)** The compound is 4-FBAH. By using unpaired t test p values>0.001 which means there is no significantly different in heart rates (0.6889, 0.3343, 0.2265, 0.1020, 0.7399, and 0.1254 respectively). **(B)** is 4-ABAH. P values >0.05 (0.0515, 0.8566, 0.2794, 0.7213, 0.4942, and 0.3238 respectively). (C) is isoniazid which has p values (0.7475, 0.0439, 0.4362, 0.8758, 0. 0.4417, and 0.4417 respectively). (D) is 2-ABAH group. The p values of the different concentrations are (0.3448, 0.5090, 0.3678, 0.1095, 0.0146, and 0.0185 respectively). (E) Is 3-DMABAH group which has p values (0.1727, 0.2725, 0.0298, 0.2569, 0.3422 and 0.7198 respectively).

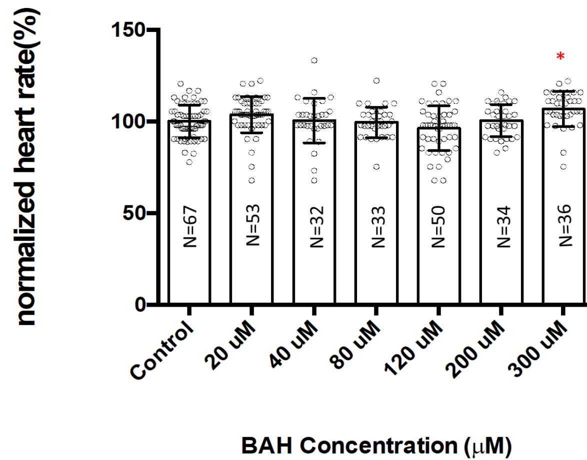
3.2. The highly toxic compounds

As shown in figure (2), BAH, NaN_3, 4-TFMBAH, and 4-NBAH have the highest toxicity among these tested hydrazine compounds. Embryos treated with BAH, demonstrated a significant change in heart rate at 300μM concentration. The IC_{50} and K_i of BAH are40.83 and 0.69 μM.

It is known that NaN_3 is a neurotoxic compound and our result reinforced its toxic pathology in developing embryos. It causes a severe decrease in the heart rate in treated embryos at a 200 μM

and 300μM concentrations. The IC50 of NaN3 is 6.57 μM and its Ki is 0.84 μM. 4-TFMBAH and 4-NBAH also significantly decreased heart rate in concentrations 120 μM, 200 μM, and 300μM. In 300 μM of 4-NBAH nearly all embryos were dead at 4dpf and the remainder had developmental abnormalities and low heart rates that prevented measurement. IC50 and Ki of 4-TFMBAH and 4-NBAH are (142.70 μM, 3.08 μM) and (54.62 μM, 3.7 μM) respectively. These compounds severely decreased embryonic heart rate, and increased the number of defected and deformed embryos.

A



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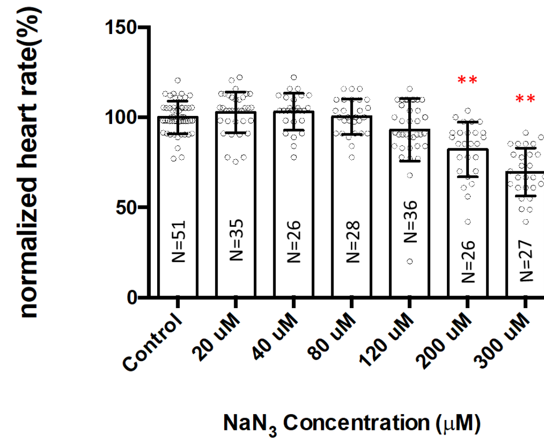

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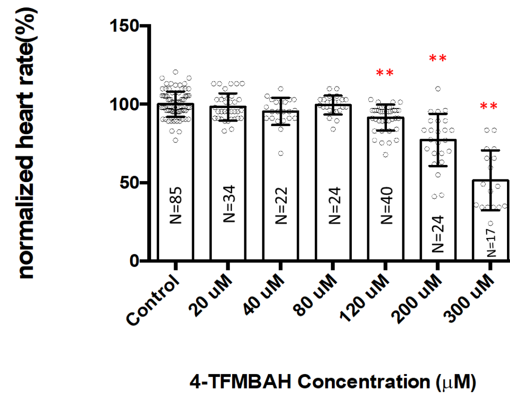


D

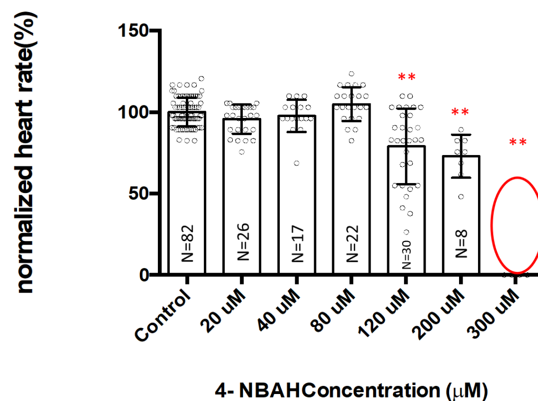


Figure 2. Effect of hydrazine containing compounds on the developing zebrafish heart. Shown are normalized heart rates of zebrafish embryos after exposure to 20 μM, 40 μM, 80 μM, 120 μM, 200 μM, and 300μM) plus the control. (A) In BAH group p values are (0.0350, 0.8453, 0.7947, 0.0640, 0.8098 and 0.0006 respectively). The result of 300μM is significantly deferent with p value ($0.0001<p<0.001$). (B) The compound is NaN_3 and by using unpaired t test p values are (0.2126, 0.1856, 0.8604, 0.0181, < 0.0001, and < 0.0001 respectively) (C) In 4-TFMBAH group p values are (0.3039, 0.0221, 0.7542, < 0.0001, < 0.0001, and < 0.0001 respectively). The result of 300μM 300μM is significantly deferent with p value ($p<0.0001$). (D) is 4-NBAH group. The p values of the different concentrations are (0.0310, 0.3453, 0.0300, < 0.0001, < 0.0001, and < 0.0001 respectively). P value is severely decreased in the highest concentrations 120 μM, 200 μM, and 300μM ($p<0.0001$).

3.3. Survival rate of the embryos

The survival rate percentage in Table (2) shows that, in the most highly toxic compounds, a large majority of embryos did not survive until the end of the experiment due to either severe cardiac edema or other developmental dysfunction.

Drug/concentration	20 µM	40 µM	80 µM	120 µM	200 µM	300 µM
Isoniazid	100 %	100 %	100 %	100 %	98.31 %	100 %
BAH	100 %	100 %	100 %	100 %	100 %	100 %
2-ABAH	100 %	100 %	100 %	100 %	100 %	100 %
4-ABAH	98.91 %	100 %	98.92 %	98.91 %	96.70 %	98.90 %
4-FBAH	100 %	100 %	100 %	100 %	100 %	100 %
3-DMABAH	100 %	100 %	100 %	100 %	100 %	98.88 %
NaN_3	100 %	100 %	98.41 %	100 %	98.41 %	95.24 %
4-TFMBAH	100 %	98.88 %	100 %	95.59 %	98.82 %	79.55 %
4-NBAH	100 %	100 %	100 %	96.67 %	77.11 %	7.69 %

Table 2. Survival rate of embryos at 4 dpf of hydrazine compounds

3.4.Florescence reaction for measuring of MPO activity

Comparing the efficacy of the inhibitors between day 0 and day 4 showed the same result in each compound. 4-ABAH, 2-ABAH, NaN3, isoniazid, 4-NBAH, 4-TFMBAH, BAH, 4-FBAH, and 3-DMABAH showed a stable efficacy by inhibiting MPO with almost same concentration That means all of these compounds are stable during these four days and have the same inhibitory effect on MPO in day 4 as well as in day 0.

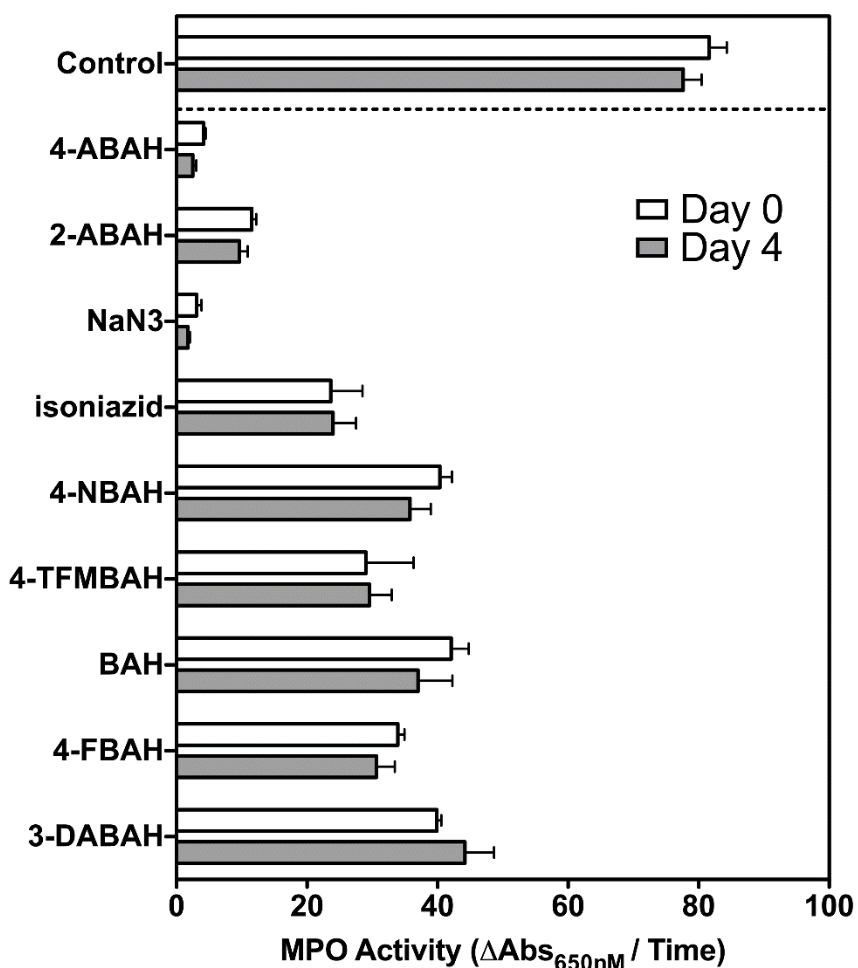


Figure 3. The result of MPO activity essay on the nine inhibitors in day 0 and day 4 at 650nM. Comparing to the control 4-ABAH, 2-ABAH, NaN3, isoniazid, 4-NBAH, 4-TFMBAH, BAH, 4-FBAH, and 3-DMABAH showed a stable efficacy on inhibition of MPO.

3.5. In Situ Hybridization of Liver Cells using Liver Probe (fatty acid binding protein 10a, fabp10a)

By using thisse and a thisse in situ hybridization full-length antisense probe for liver fabp10a, the following results were found: there is no effect on liver size in low concentration (20µM) treatment groups across the majority of compounds excluding NaN_3, 4-TFMBAH, and 4-NBAH which resulted in a slight reduction in liver size. At higher concentrations (120µM) these three compounds produce significant reduction in liver size indicating that these compounds disrupt liver organogenesis when using in high concentration. Otherwise, no significant changes occur in the remaining compounds as shown in figure (6).

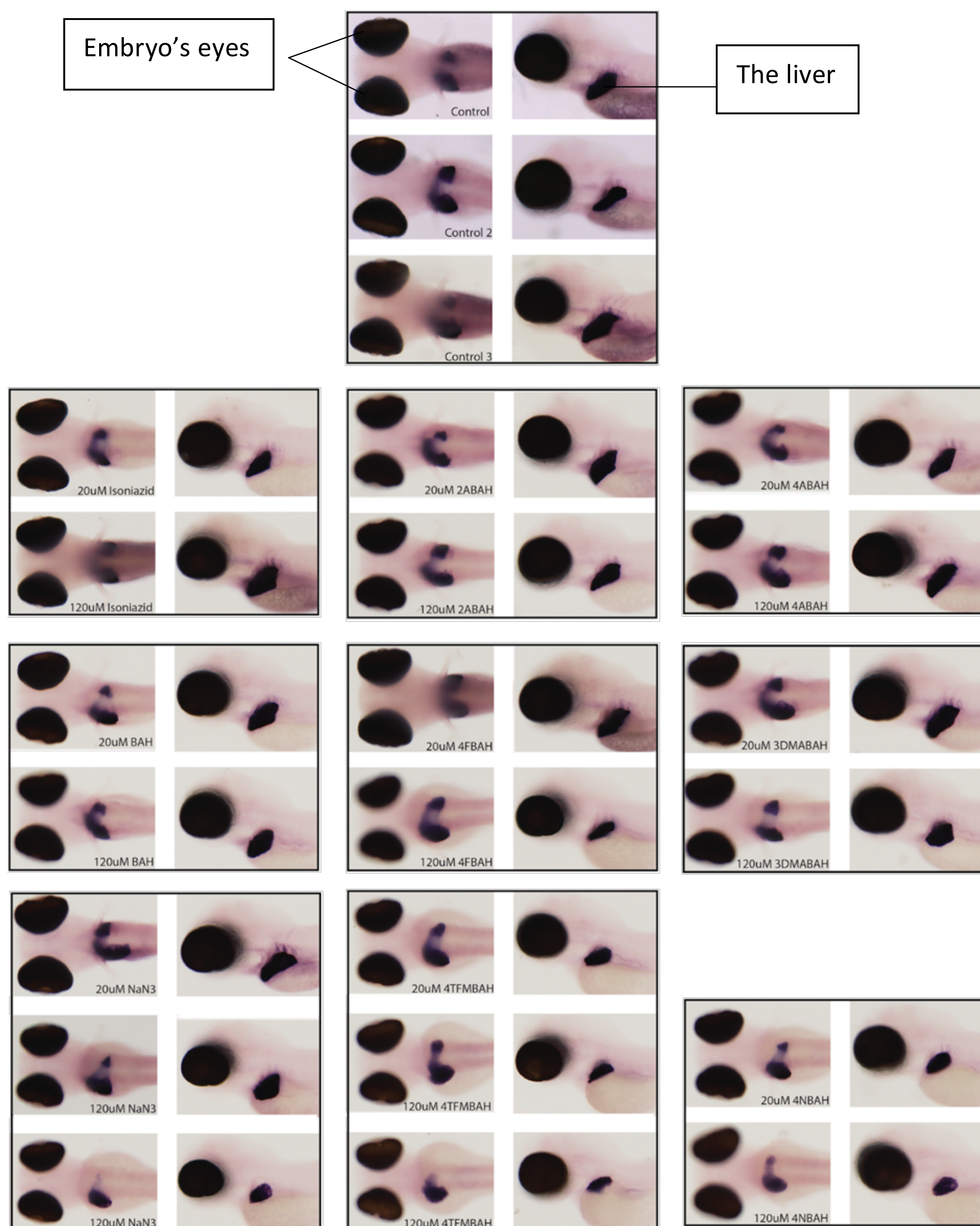


Figure 4. A dorsolateral image of in Situ Hybridization of Liver Cells using Liver Probe (fatty acid binding protein 10a, fabp10a). Isoniazid, BAH, 2-ABAH, 4-ABAH, 4-FBAH, AND 3-DMABAH showed no significant changes in liver size with both concentrations (20µM and 120µM). NaN_3, 4-TFMBAH, and 4-NBAH showed shrink in liver size appearing mostly in (120µM).

4. Discussion

4.1. Effect of different Hydrazine Compounds on Zebrafish Embryos

At 5-6 hpf, healthy and fertilized embryos were treated with the addition of different hydrazine compounds. The embryos were placed in six-well plates (20 embryos in each well). Each plate containing a different compound with each well containing a different concentration. After 24 h of treatment, embryos were evaluated and dead/defective embryos were removed. This was repeated in 24h increments between 0-4 dpf. At 4 dpf, a short video (~20 seconds, 30fps as vsi files) was recorded for every treatment group. The heart beats were counted for 10 seconds by reducing the video speed by a factor of 2 then multiplying by twelve to derive a heart rate in beats/second. Isoniazid, 2-ABAH, 4-ABAH, 3-DMABAH, and 4-FBAH did not show any significant effect on heart rate at any concentration demonstrating a good safety profile. Yet, the high IC_{50} and K_i of 4-FBAH and isoniazid limit their utility as an MPO inhibitors in further studies even though isoniazid is already used as a tuberculosis drug. In contrast, the low IC_{50} and K_i values of 2-ABAH and 4-ABAH make them good MPO inhibitors and attractive targets for further experimentation. BAH showed a toxicity effect only with the highest concentration (300 μM) which is not usually used. NaN_3, 4-TFMBAH, and 4-NBAH showed the highest toxicity effect in the embryos with high IC_{50} and K_i values. In the 300 μM treatment groups of these compounds some of embryos died due to sever cardiac edema and poor circulation. Remaining embryos had very low heart rates and a high rate of deformation. In addition to alterations in embryonic heart rates, other developmental abnormalities were detected. For example, in groups treated with BAH

many embryos developed a dorsalized tail. Result vary between slightly dorsalized in the 80 μM group to severe dorsalized tail in the 300 μM group figure (5).

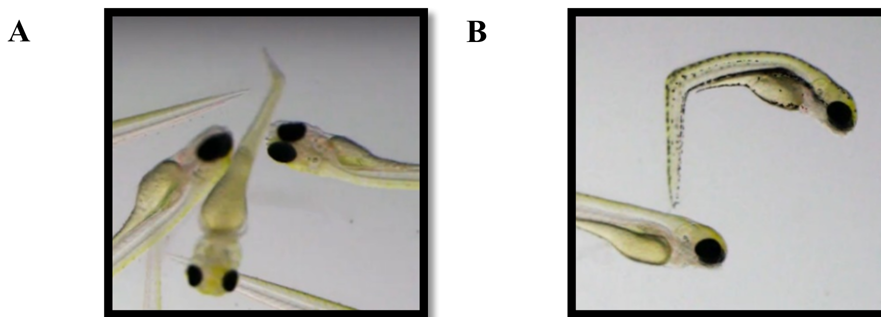
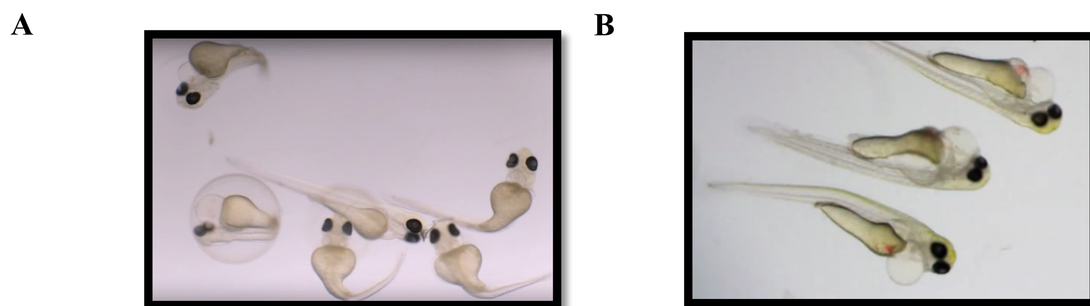


Figure 5. Dorsalized tail of embryos treated by BAH (A) Day 4 after fertilization in group of 80 μM BAH (B) Day 4 after fertilization in group of 300 μM BAH.

In the highly toxic compounds embryos could not exit chorions as development progress in the highest concentration due to the weakness of the embryos and the severity of the cardia edema that affected them. Figure (6) showed embryos treated with 300 μM NaN_3 at day 4 post fertilization.



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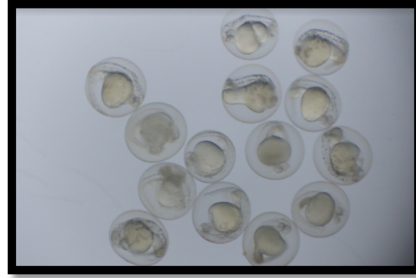
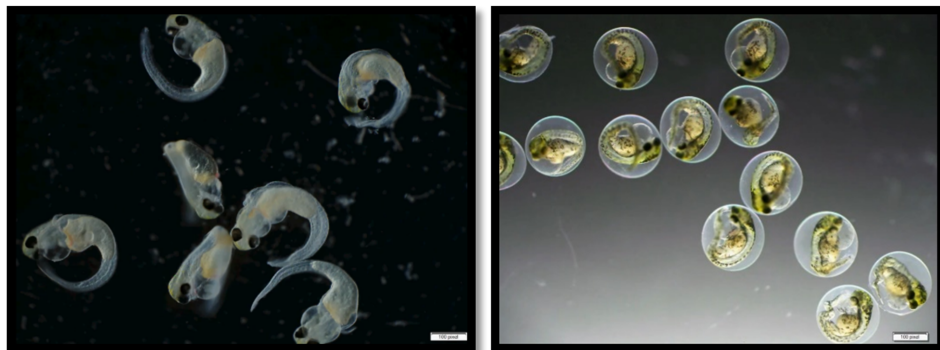


Figure 6. Group of embryos treated by 300 µM NaN_3 at 4dpf (A) unhatched embryo due to its weakness and deformities (B) embryos with sever cardiac edema (C) dead embryos

Furthermore, 4-TFMBAH and 4-NBAH at the 300 µM concentration, produced embryos displaying severe cardiac edema which negatively affected embryonic heart rate and circulation. Accordingly, decreased heart rates and poor circulation were detected as shown in figure (7).

A



B

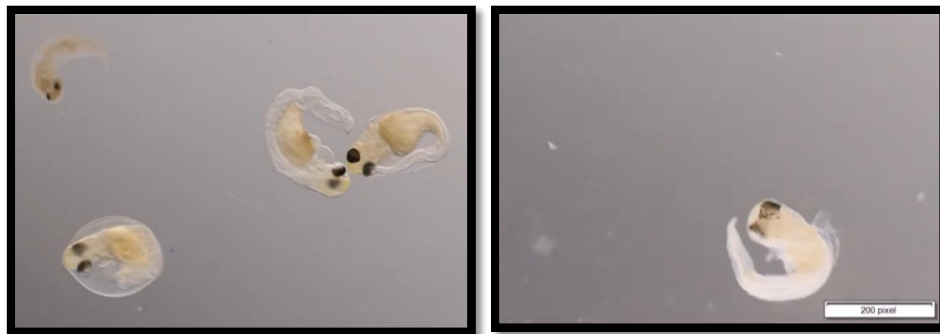


Figure 7. (A) Group of embryos treated by 300 µM 4-TFMBAH at 4dpf showing sever cardiac edema in both hatched and unhatched embryos. **(B)** Group of embryos treated by 300 µM 4-NBAH at 4dpf showing sever cardiac edema in both hatched and unhatched embryos leading to their death.

Furthermore, Fluorescence reaction for measuring MPO activity showed a stable inhibition of MPO by some hydrazine compounds (isoniazid, NaN3, 2-ABAH, 4-ABAH, and 4-NBH). That means these compounds have the same inhibitory effect on MPO through the four days and there are no physical or chemical changes affect their inhibitory effect. In contrast, there is a reduction in BAH, 4-FBAH, and 4-TFMBAH inhibitory effect on day 4. The reaction of 3-DMABAH showed an unstable decrease in its inhibitory effect with an obvious increase in MPO. There are some explanations of this reduction including the degradation of these compounds or decrease their solubility and forming participation. Yet, the exact reason is not known.

4.2. In Situ Hybridization of Liver Cells using Liver Probe (fatty acid binding protein 10a, fabp10a)

After fixing the treated embryos, 20 and 120µM from each compound, fabp 10a used to detect the toxic effect of hydrazine compounds on the liver. Fabp 10a is a liver basic protein, so the developmental delay of the embryos' livers can be detected easily. In groups that exposed to NaN3, 4-TFMBAH, and 4-NBAH these delays were dose-dependent as shown in shrinking the liver size with 120µM more than 20µM. The other compounds showed a little or no deferent in the liver size comparing to the control group.

5. Conclusion

Myeloperoxidase, a heme enzyme, can catalyze hypochlorous acid (HOCl) formation in a presence of hydrogen peroxide (H_2O_2) and chloride ion (Cl^-). The bactericidal effect of hypochlorous acid is greater than that of hydrogen peroxide. Unfortunately, its toxic effect can reach the host cell if the free radicals overcome the host defense mechanism. In addition, MPO plays a role in chronic inflammatory diseases by catalyzing the oxidation of a number of substrates such as tyrosine and nitrate leading to lipid/lipoprotein oxidation and stimulate some inflammatory vascular diseases. Furthermore, MPO can harm some extracellular targets (protein/lipid) if released outside the cell which explains its carcinogenic effect.

In our study, we hypothesize that inhibition of MPO may lead to avoiding its damaging effect on the host tissues when using MPO inhibitors as a treatment of chronic inflammation diseases. Depending on our previous study, we tested the toxicity of nine MPO inhibitors from hydrazine derivatives using the zebrafish model system. Heart rate, cardiac edema, survival rate, and in situ hybridization of liver cells were the indicators of the toxicity of these compounds in this experiment.

In conclusion, Isoniazid, BAH, 2-ABAH, 4-ABAH, 3-DMABAH, and 4-FBAH showed little or no effect on heart rate heart rate or heart morphology, suggesting they may be safe to use as MPO-inhibitors. While NaN_3, 4-NBAH, and 4-TFMBAH were the most toxic compounds tested, causing edema and lowered heart rates. The toxicity of these compounds was demonstrated

by the low survival rate of embryos within those treatment groups. 4-NBAH treatment yielded the lowest survival rate, and 4-TFMBAH also caused a notable decrease in survival rate. The in situ for the liver indicates that NaN_3 , 4-NBAH, and 4-TFMBAH treatment resulted in smaller regions of staining, possibly due to delayed development and disruption of organogenesis of the liver. As 2-ABAH has the lowest Ki (160nM) and a low toxicity, 2-ABAH may the best candidate for future in vivo studies.

More quantitative analysis of liver size should be done to better understand the reason for delayed development. Drug competence over the four days without refreshing the buffers may help to determine the effect of the buffer on the activity of these inhibitors. Furthermore, other MPO inhibitory drug screening is needed with subsequent testing in a mammalian model system.

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