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# EVALUATION OF ACUTE HEPATIC TOXICITY AND INFLAMMATION INDUCED BY NITROSAMINES *IN Mus musculus* AND *Danio rerio*

**ISABELLY ANNUNCIATO**

**SÃO VICENTE - SP**
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# Evaluation of acute hepatic toxicity and inflammation induced by nitrosamines in *Mus musculus* and *Danio rerio*


## Abstract

Nitrosamines are emerging contaminants known for their carcinogenic and oxidative properties. This study investigates the hepatotoxic and acute inflammatory effects of two nitrosamines, N-Nitrosodiethylamine (NDEA) and N-Nitrosodimethylamine (NDMA), on *Mus musculus* (mice) and *Danio rerio* (fish). Mice were exposed to 0,10 mg/kg and 0,20 mg/kg of NDEA and NDMA. Subsequent biochemical, histological, and hematological analyses were conducted to detect potential liver damage induced by these contaminants. Blood samples were collected from exposed and non-exposed groups, and liver tissues were evaluated for histological evaluation. Blood aliquots were used for hematological analysis, employing smear techniques and cell counting in a Neubauer chamber. Biochemical monitoring of liver function markers such as lactate dehydrogenase (LDH), oxalacetic transaminase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and C-reactive protein (CRP) was performed. Additionally, levels of interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), malondialdehyde (MDA), and lipoprotein-associated phospholipase A2 (Lp-PLA2), also known as platelet-activating factor acetylhydrolase (PAF-AH), were evaluated. PAF-AH is crucial in controlling inflammation, as it regulates plasma levels of PAF, a key lipid factor in the progression and development of inflammation. Our project aimed to demonstrate that nitrosamines can induce hepatocyte dysfunction, affect energy metabolism, and highlight the role of PAF in causing hepatotoxicity. In addition to *in vivo* and biochemical investigations, molecular docking, and molecular dynamics simulations were performed at BioSIM (University of Porto) to explore the potential of nitrosamines as non-selective inhibitors of Land's Cycle important enzymes (lysophosphatidylcholine acyltransferase 3 (LPCAT3), Cytosolic Phospholipase A2 (cPLA2) and PAF-AH). Considering nitrosamines are commonly found in water environments, we also monitored hematological and hepatic histological modifications induced by these compounds in *Danio rerio* (zebrafish), the hematological analysis was related to nuclear abnormalities in erythrocytes. This comprehensive study integrates biochemical, histological, and computational approaches to elucidate the hepatotoxic effects of nitrosamines and underscores the significance of PAF in mediating these effects. Through these investigations, we aim to provide deeper insights into nitrosamine-induced liver damage and inflammation mechanisms. Our results demonstrated that the nitrosamines evaluated caused increased liver damage in mice during a 24-hour exposure. Biochemical analysis of liver function markers, lipid peroxidation, and cytokines indicated elevated levels related to compound metabolism during both 4-hour and 24-hour exposures. In fish, damage was observed in liver tissue, gills, and nuclear abnormalities in erythrocytes; *In vitro* studies on PAF-AH inhibition by nitrosamines confirmed the results obtained from molecular simulation assays, demonstrating that the compounds have low affinity for the enzyme. The Molecular Dynamics results showed affinity between NDMA and cPLA2 and NDEA and LPCAT3.



**Keywords**: Nitrosamines, acute inflammation, liver function, *Mus musculus*, *Danio rerio*.

**Table of Contents** (TOC)

# 1. Introduction

## 1.1. N-nitrosamines, oxidative stress and mechanisms of inflammation

Nitrosamines became the focus of toxicological studies in 1954, when Magee and Barnes linked these compounds to liver damage in rats exposed to N-nitrosodimethylamine (NDMA). Subsequently, in 1956, the same researchers observed that rats fed NDMA developed liver tumors. The hepatotoxicity of nitrosamines was further confirmed in 1978, when Cassens et al. demonstrated liver damage in animals treated with nitrite. Nitrosamines, characterized by a nitroso group attached to an amine, encompass at least 300 documented types of hydrophilic molecules (Gushgari and Halden, 2018).

Classified as probably carcinogenic (group 2A) by the International Agency for Research on Cancer (IARC) and the United States Environmental Protection Agency (US EPA), N-nitrosamines are formed through various mechanisms involving nitrogen oxide and secondary amines in chemical and fermentative processes (IARC 1978; Park et al. 2015). These compounds can be synthesized endogenously and are found in processed foods, cigarette smoke, and as metabolites of certain drugs. Drinking water treatment is a significant source of nitrosamine production, particularly during the chloramination process, where chlorine and ammonia react to form chloramine, subsequently leading to the formation of NDMA and NDEA (Mitch et al. 2003). According to the US EPA and the Integrated Risk Information System (IRIS), concentrations of 0.7 ng/L of NDMA or 0.2 ng/L of NDEA in drinking water are associated with a lifetime cancer risk of $10^{-6}$ (US EPA 1987a, b). This highlights the significant health risks posed by these contaminants even at very low concentrations, underscoring the importance of monitoring and regulating their presence in drinking water.

**Table 1: source of nitrosamines formation**

| Amine | Nitrosating source | Contaminant |
|---|---|---|
| Triethylamine | Sodium nitrite in acid medium (dealkylation) | diethylnitrosamine - NDEA |
| Amida | Nitrosating source | Contaminant |
| Dimethylformamide (DMF) undergoes hydrolysis in ($H^+$ or $OH^-$) generating DMA | Sodium nitrite (NaNO2) | dimethylnitrosamine - NDMA |

**Source**: adapted from Paiva and Paiva, 2022

Nitrosamines became the focus of toxicological studies in 1954 when Magee and Barnes linked these compounds to liver damage in rats exposed to N-nitrosodimethylamine (NDMA). By 1956, they further discovered that rats fed NDMA developed liver tumors. This hepatotoxicity was corroborated in 1978 by Cassens et al., who observed similar liver damage

in animals treated with nitrite. Characterized by a nitroso group attached to an amine, nitrosamines are a family of hydrophilic compounds consisting of at least 300 different molecules (Gushgari and Halden, 2018).

The International Agency for Research on Cancer (IARC) and the United States Environmental Protection Agency (US EPA) classify N-nitrosamines as probably carcinogenic (group 2A) (IARC 1978). These compounds can form via mechanisms involving nitrogen oxide and secondary amines in chemical and fermentative processes (Park et al. 2015). Nitrosamines can be synthesized endogenously and are found in processed foods, cigarette smoke, and as metabolites of certain drugs. Drinking water treatment is a significant source of nitrosamine production, particularly during chloramination, where chlorine and ammonia react to form chloramine, leading to the formation of NDMA and NDEA (Mitch et al. 2003). According to the US EPA and the Integrated Risk Information System (IRIS), concentrations of 0.7 ng/L of NDMA or 0.2 ng/L of NDEA in drinking water are associated with a lifetime cancer risk of $10^{-6}$ (US EPA 1987a, b). This underscores the significant health risks posed by these contaminants even at very low concentrations, emphasizing the need for strict monitoring and regulation.

Economic development has exacerbated the contamination of water sources with emerging contaminants, both chemical and biological (Hespanhol, 2015). Studies have shown that the presence of NDMA and NDEA in water is often correlated with high levels of organic matter, typically in areas affected by sewage, nitrogen-based fertilizers, and industrial waste (Krasner et al., 2012). A recent study by the State University of Campinas analyzed eighteen drinking water samples from thirteen cities in the Campinas metropolitan region, São Paulo, Brazil. Seven types of nitrosamines were quantified, with NDMA detected in 89% of the samples and at the highest concentration of 67 ng/L (Vizioli, Hantao & Montagner, 2021). The Brazilian Ministry of Health stipulates a maximum allowed NDMA concentration in water of 0.0001 mg/L (Ordinance GM/MS No. 888, May 4, 2021).

NDMA and NDEA, being polar compounds, do not bioaccumulate but are resistant to biodegradation (Wilczak et al. 2003; Charrois et al. 2007). These compounds have garnered increasing attention due to their significant potential for inducing tumor development in humans (Gushgari and Halden, 2018). Studies have demonstrated that nitrosamines can cause various biochemical and genotoxic changes, including the production of reactive oxygen species (ROS), lipid peroxidation, increased nitric oxide release, decreased total antioxidant capacity, oxidative DNA damage, and inhibition of antioxidant enzymes such as catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase. They also exhibit pronounced

pro-inflammatory activity (Waly et al., 2018). Nitrosamines induce oxidative stress in a dose-dependent manner, affecting membrane integrity and antioxidant enzyme activity, leading to increased lipid peroxidation (Ahotupa et al., 1987; Bilal et al., 2017; Sheweita et al., 2017). Lipid peroxidation occurs when membrane phospholipids encounter ROS, resulting in hydroperoxidized lipids and alkyl radicals. This process alters membrane fluidity and integrity (Juan et al., 2021).

Polyunsaturated fatty acids (PUFAs) in phospholipids undergo metabolic recycling via the "deacylation-reacylation cycle" or "Lands Cycle," involving phospholipases A2 (PLA2s) and lysophospholipid acyltransferases (LPATs). Changes in membrane phospholipids activate this cycle (Juan et al., 2021). Cytosolic phospholipase A2 (cPLA2) releases PUFAs, forming platelet-activating factor (PAF) and arachidonic acid (AA). This cycle can be intensified by ROS, increasing the production of PAF, AA, eicosanoids, and pro-inflammatory mediators (Wu et al., 2016; Adebiyi et al., 2019). PAF, a lipid mediator, contributes to inflammatory and immune responses and is regulated by the enzyme PAF-AH (Ulambayar et al., 2019).

PAF is involved in various physiological processes, but excessive amounts can cause inflammation and oxidative stress (Papakonstantinou et al., 2017; Travers et al., 2021). PAF biosynthesis occurs via remodeling, initiated by cPLA2 acting on phosphatidylcholine, producing arachidonic acid, eicosanoids, and lysophosphatidylcholine (LPC), which is then converted to PAF by an acetyltransferase (Lys-PAFAT) (Wykle et al., 1980). Under normal conditions, PAF synthesis is minimal but increases during inflammation via the remodeling pathway. Various agents can stimulate PAF synthesis, including antigen-antibody interactions and inflammatory mediators (Owen et al., 2007; Kono et al., 2019; Ashraf et al., 2022). This project aims to investigate nitrosamines' potential to increase lipid peroxidation and ROS, thereby enhancing cPLA2 and LPCAT activity, leading to elevated arachidonic acid production and oxidative stress (Gawel et al., 2004; Su et al., 2019; Ferrara et al., 2021). Although less studied, PAF is a potent pro-inflammatory agent generated during cPLA2 action by the remodeling pathway (Linkous and Yazloviskaya, 2010; Stanca et al., 2013; Oehler et al., 2021). Monitoring PAF concentration is crucial for controlling inflammation progression. Inhibition of extracellular PAF-AH is a key point in the inflammatory process (Toyama et al., 2022). Therefore, this project will evaluate nitrosamines' ability to interact with extracellular PAF-AH.

### 1.2. Liver system in Swiss mice

Mice were introduced as animal models in laboratories in the 19th century and have since become a classical choice for in vivo testing. Swiss mice (Mus musculus) are particularly favored due to their ease of care, minimal space requirements, and short life cycles. Additionally, as mammals, mice share significant physiological and genetic similarities with humans (Harkness and Wagner, 1993). The liver in mice performs critical metabolic functions, absorbing, transforming, and storing both nutrients and toxic substances, which are later released into the bloodstream. This makes the liver essential for biotransformation processes. Moreover, it is the organ responsible for bile production and storage (Guillouzo, 1998).

Nitrites used in food preservation can transform into nitrosamines under the influence of heat and gastric acid. Once absorbed in the intestine, nitrosamines enter hepatocytes via the portal venous system, disrupting the liver's detoxification processes by interfering with cytochrome P450 enzymes. This interference can lead to cirrhosis and liver cancer (Magee and Barnes, 1956). The biotransformation of nitrosamines occurs within the microsomal system of liver P450. Initially, nitrosamines undergo hydroxylation of the alpha carbon of the alkyl group, catalyzed by P450, resulting in the formation of an aldehyde or ketone and an unstable primary N-nitrosamine. In the subsequent stage, unstable carbocations are produced that can bind to or modify genetic material (Paiva and Paiva, 2022). In this project, mice will be utilized for in vivo testing to ensure a comprehensive assessment of plasma liver markers following exposure to nitrosamines. This approach is designed to better understand the hepatotoxic effects and the biotransformation mechanisms involved.

### 1.3. Liver system in teleost fish

The literature describes the liver as a multifunctional organ, capable of acting in digestion, storage, detoxification, serum protein synthesis and vitellogenin production. In addition, the liver is fundamental for performing the deposition and metabolization of fats and carbohydrates and is also responsible for maintaining the metabolic balance of the body (Sales, C.F. et al., 2017). Most teleost fish have a liver that is reddish-brown in color and divided into lobules, located cranially and ventrally. In the digestion process, liver cells are responsible for secreting bile into the duodenum (Shtewi and Abusre, 2000, Sales, C.F. et al., 2017).

In many teleosts the liver has the so-called melanomacrophage centers (MMC), formed by aggregates of macrophages. Melanomacrophage centers can be formed in the kidneys, liver and spleen of fish when they are exposed within the aquatic ecosystem to various chemical agents,

such as contaminants, pollutants and biological agents. MMCs contain lipogenic pigment, lipofucsin, melanin, and hemosiderin, which may assist in the defense mechanism of teleosts (Roberts, 2012). In fish, after filtration of blood by the gills, the liver is responsible for performing the elimination and detoxification of various substances that have been absorbed by the body, which is extremely important for the maintenance of life of these animals (Girón-Pérez et al., 2007 and Covantes-Rosales et al., 2019).

The slower blood flow in fish relative to cardiac output causes the bile flow to circulate fifty times slower, which slows the clearance of toxic products, resulting in a longer time of action of chemicals in the body (Goessling and Sadler, 2015). Studies have suggested the hypothesis that toxic substances at lower concentrations can cause severe liver disorders when compared to mammals (Campos et al., 2008 and Goessling and Sadler, 2015). Exposure of liver tissue to chemical agents constitutes an important health risk, causing injury by the production of free radicals that initiate the process of lipoperoxidation (Faheem and Lone, 2017 and Xu et al., 2021). As in mammals, the fish liver is capable of producing humoral compounds, such as acute phase proteins (APPs) of the inflammatory response, among them inflammatory mediators and c-reactive protein (CRP). APPs are synthesized by various cells, among them hepatocytes. It is considered that these proteins have a universal character in animals, because their structure and acute phase response are very similar in all animals (Hoole et al., 2003, Roy et al., 2017 e Charlie-Silva et al., 2019).

### 1.4. The zebra fish as water polution bioindicator of toxicity

As a freshwater fish that can grow to around five centimeters in size as an adult, zebrafish (*Danio rerio*) are more cost-effective than rodents commonly used in research. Additionally, zebrafish require less space compared to rodents (Gheno, 2015). The zebrafish genome shares a 70-80% homology with the human genome, which is a significant similarity to mammals, making them valuable for various research applications, including behavioral, toxicological, and genetic studies (Lieschke & Currie, 2007). The use of *Danio rerio* in this project is further justified by the National Council for the Control of Animal Experimentation (CONCEA), which, through Normative Resolution No. 34, considers this species suitable for scientific studies. Numerous clinical and toxicological studies in the literature have successfully employed this teleost fish as an animal model, highlighting its efficacy and relevance in research. This approach ensures that the zebrafish can serve as an effective model for studying

the effects of nitrosamines, given their genetic similarities to humans and practical advantages in laboratory settings.

## 1.5. Molecular Anchoring and Molecular Dynamics of Nitrosamines with Extracellular PAF-AH (Lipoprotein-associated PLA2)

### 1.5.1. Molecular docking

Molecular docking, also known as anchoring, is a well-established in silico technique primarily aimed at drug discovery and understanding the interaction between drugs and macromolecules. This technique is instrumental in identifying new therapeutic compounds, predicting target-ligand interactions at the molecular level, and delineating the relationship between chemical structures (Pinzi & Rastelli, 2019). Additionally, docking can predict the effects of emerging contaminants in aquatic environments by considering the homology between human enzymes and those of aquatic animals. In studies involving various aquatic vertebrates, researchers have demonstrated interactions between anti-inflammatory drugs and their respective enzymes in these animals (Walker & McEldowney, 2013; Kumar et al., 2019).

The docking process involves two fundamental steps: predicting the conformation of the ligand, including its position and orientation within the molecular structure, and assessing the binding affinity. These steps are closely related to the data acquisition methods and their scoring schemes. Understanding the location of catalytic sites significantly enhances the efficiency of docking. Often, the catalytic site is known beforehand, allowing researchers to obtain information by comparing the target protein with a protein family or established protein structures (Meng et al., 2011). This prior knowledge helps in accurately predicting the interactions and potential impacts of the ligands. By leveraging molecular docking techniques, researchers can effectively explore the biochemical interactions of new drugs and contaminants, thereby advancing both pharmaceutical development and environmental toxicology studies.

### 1.5.2. *Molecular Dynamics Simulations*

Molecular dynamics (MD) is a technique used to provide detailed data on the movement of individual particles over time, describing their interactions empirically through a potential energy function (Karplus & McCammon, 2002). Several programs are widely used for MD simulations, including AMBER (Assisted Model Building with Energy Refinement) (Case et al., 2005), CHARMM (Chemistry at Harvard Macromolecular Mechanics) (Brooks et al., 2009), GROMOS (GROningen Molecular Simulation) and GROMACS (GROningen Machine for Chemical Simulation) (Van Der Spoel et al., 2005). In this project, we hypothesize that the

studied nitrosamines can induce acute liver inflammation and toxicity in Swiss mice. To monitor potential liver changes, we will conduct biochemical and histological investigations of the mice following exposure to NDMA and NDEA. Additionally, molecular dynamics studies were employed to evaluate the interaction between nitrosamines and extracellular PAF-AH, aiming to uncover potential mechanisms of enzyme inhibition. These MD simulations were carried out in collaboration with Prof. Dr. Sergio Filipe Sousa's laboratory, BioSIM, at the University of Porto in Portugal. By integrating these advanced techniques, we aim to gain a comprehensive understanding of the biochemical and molecular impacts of nitrosamines, providing insights into their hepatotoxic effects and interactions with key enzymes.

### 1.5.3. *In silico* analysis contextualization

The initial aim of this research project was to analyze the enzyme PAF-AH and the compounds NDEA and NDMA to assess the impact of these nitrosamines on the regulation of PAF levels. However, the initial results did not show a significant interaction, suggesting a possible absence or weak interaction between the nitrosamines and PAF-AH. This finding necessitated an expansion of the analyses after the initial phase of molecular dynamics, which included both PAF-AH and the main compounds, NDMA and NDEA. Advancing the investigation, the research entered a more detailed phase, incorporating docking studies and molecular dynamics for the metabolites of these nitrosamines. The goal was to investigate the previously observed lack of a direct relationship between nitrosamines and PAF-AH. The new findings raised questions about the possible interaction of nitrosamines and their metabolites with other enzymes linked to PAF, as well as the potential of these compounds to influence membrane remodeling under oxidative stress. Given the ability of nitrosamines, such as NDEA and NDMA, to act as pro-oxidants, increasing the production of reactive oxygen species (ROS) or reducing the cell's antioxidant capacity, a new dimension of analysis emerged. This aspect is particularly relevant because the metabolism of these substances in the liver, through cytochrome P450 enzymes, results in the formation of free radicals and other reactive species capable of damaging essential cellular components. This process may contribute to both carcinogenesis and other pathologies associated with oxidative stress. It raises the hypothesis that, in addition to possible direct interaction with PAF-AH or other enzymes related to PAF, nitrosamines may exert significant effects through the induction of oxidative stress. Therefore, the research expanded to include molecular dynamics assays involving key enzymes in the control and generation of PAF, as well as in membrane remodeling, considering the potential

pro-oxidant role of nitrosamines. This holistic and adaptive approach, guided by the acquisition of results and the evolution of research hypotheses, is essential for a deep and comprehensive understanding of the mechanisms involved. It also explores all possible implications of these interactions in the fields of biochemistry and pharmacology.

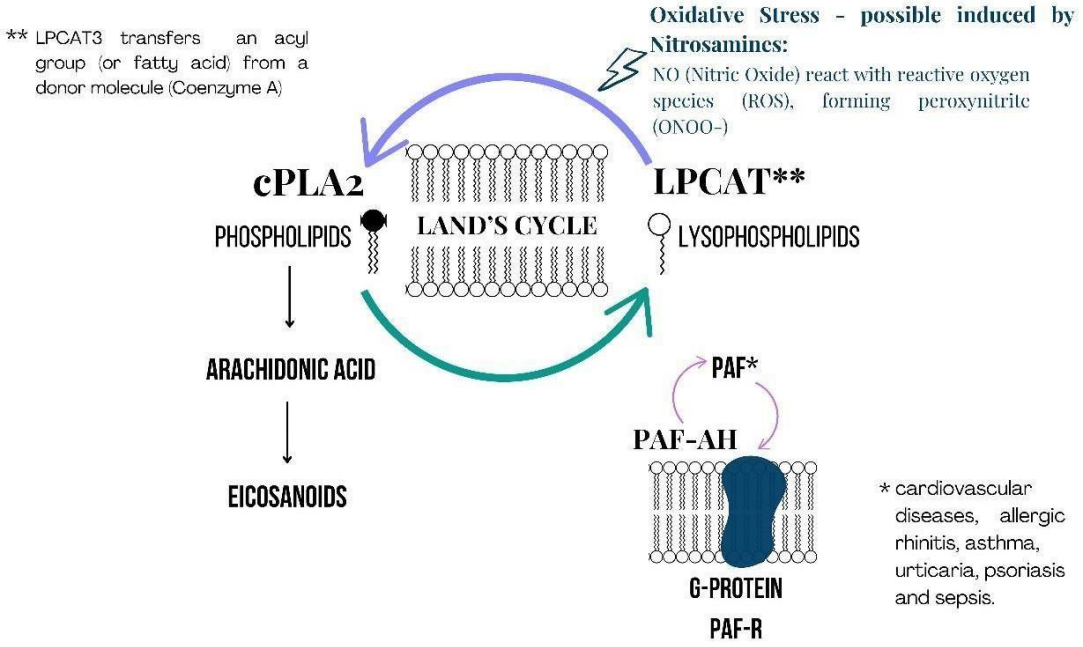


**Figure1:** The Land's cycle is a process that responds to plasma membrane damage by promoting membrane remodeling. Cytosolic phospholipase A2 (cPLA2) cleaves membrane phospholipids at the sn2 position, releasing arachidonic acid (a precursor to inflammatory processes) and lysophospholipids. Lysophospholipids lead to the production of platelet-activating factor (PAF), which is associated with cardiovascular diseases and allergic reactions. The enzyme platelet-activating factor acetylhydrolase regulates PAF levels. Membrane remodeling is facilitated by lysophosphatidylcholine acyltransferase 3, which reintroduces a fatty acid into the sn2 position of the membrane phospholipid. Source: own authorship.

## 1.6. Presentation of the Ligand and Target Molecule Files.

### 1.6.1. PAF-AH.

PAF-AH (lipoprotein-associated phospholipase A2) is a blood plasma enzyme that degrades oxidized and modified phospholipids, including Platelet Activating Factor (PAF), a mediator involved in inflammation from tissue injury, infection, and ischemia (Travers et al., 2021). PAF-AH binds to circulating lipoproteins like LDL and HDL. It consists of two subunits, with the alpha subunit containing the catalytic site (Samanta & Bahnson, 2008). The enzyme features an active site microenvironment that interacts with various inflammatory molecules, such as C-reactive protein and protein S, underscoring its biological significance (Travers et al., 2021; Kono & Arai, 2019). The histidine residue in the active site is crucial for its function and is highly conserved (Kono & Arai, 2019). PAF-AH, a 44 kDa protein, includes a catalytic triad

(serine, aspartic acid, and histidine) typical of αβ-hydrolases and a GXSXG motif characteristic of neutral lipases (Samanta & Bahnson, 2008). The enzyme's structure features an alpha/beta-hydrolase lipase fold with the catalytic triad in a hydrophobic pocket. The residual catalytic site comprises amino acids Phe110, Leu153, Phe274, Trp298, His272, Tyr160, Gln352, and Phe322, with Phe274 and Leu153 stabilizing the tetrahedral intermediate's negative charge, acting as the oxyanion hole (Samanta & Bahnson, 2008). This site stabilizes the catalytic triad. Their crystallography study (PDB:3D5E) showed a covalent interaction between PAF-AH and the ligand paraoxon (DEP), used as a reference to determine the catalytic site's coordinates and compare results with other ligands.

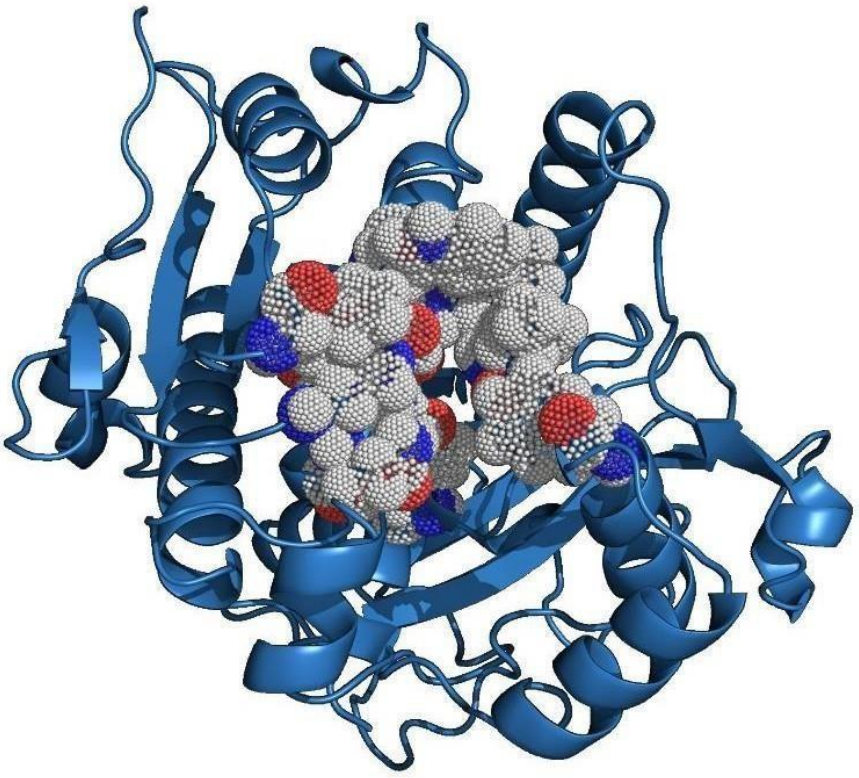

**Figure 2:**PAF-AH enzyme (chain A). The active site cavity is visualized using PyMOL software, with dots representing the cavity. Different colors indicate various elements: white for carbon, red for oxygen, and blue for nitrogen (This color coding is only applied to the dots, not to the cartoon representation of the rest of the enzyme structure). The enzyme is displayed in cartoon representation using PyMOL. The structure of PAF-AH (PDB ID: 3D5E) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org).

### 1.6.2. cPLA2

Cytosolic phospholipase A2 (cPLA2) is crucial for regulating inflammatory responses by releasing arachidonic acid from membrane phospholipids. Arachidonic acid is a precursor to bioactive lipid mediators like prostaglandins and leukotrienes, which are essential for inflammation and immune responses (Nojima, 1991). cPLA2 removes polyunsaturated fatty

acids from the sn-2 position of phospholipids, forming platelet-activating factor (PAF) and arachidonic acid (AA). Reactive oxygen species and oxidative stress can intensify cPLA2 activity, increasing PAF, arachidonic acid, eicosanoids, and pro-inflammatory mediators (Adebiyi et al., 2019). The enzyme's catalytic activity is centered in the α/β hydrolase domain, specifically involving Ser228 and Asp549. cPLA2 activity is also regulated by calcium ions, which bind to the enzyme, causing a conformational change that enhances its affinity for phospholipids (Dessen et al., 1999). Understanding cPLA2's molecular mechanisms and regulation is key for developing targeted therapies to modulate inflammation. Giripladib, a selective cPLA2α inhibitor, has been used as a reference ligand in studies (Duvernay, 2015). This knowledge supports designing therapies to precisely control inflammation and associated diseases.

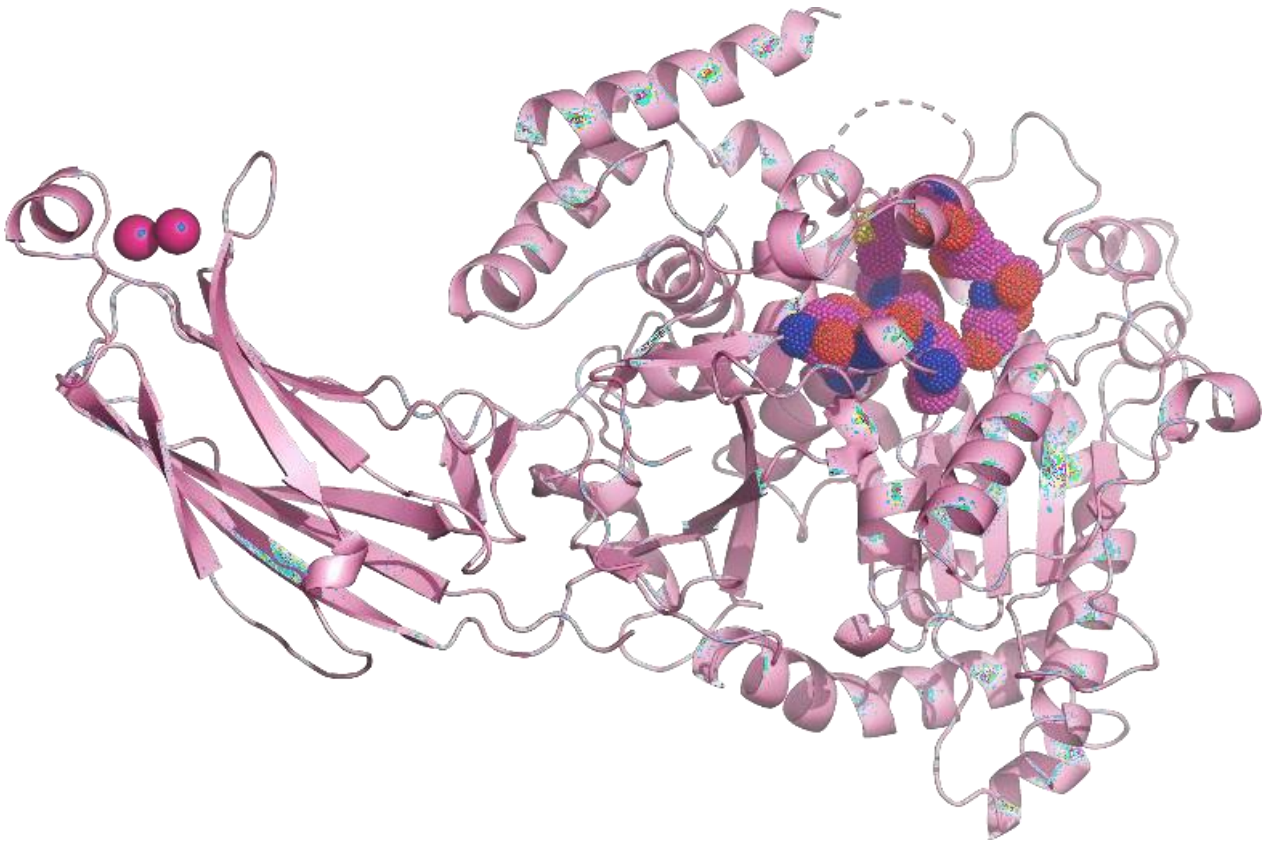

**Figure 3:** cPLA2 enzyme. The dots represent the active site cavity, and the two purple spheres depict calcium ions. The active site cavity is visualized using PyMOL software, with dots representing the cavity. Different colors indicate various elements: pink for carbon, red for oxygen, and blue for nitrogen (This color coding is only applied to the dots, not to the cartoon representation of the rest of the enzyme structure). The enzyme is displayed in cartoon representation using PyMOL. The structure of cPLA2 (PDB ID: 1CJY) was obtained from the RCSB Protein Data Bank.

### 1.6.3. LPCAT3.

Phosphatidylcholine (PC) is the primary constituent of cell membranes, synthesized de novo through the Kennedy pathway and extensively remodeled via the Land's cycle, which involves significant deacylation-reacylation. The enzyme lysophosphatidylcholine acyltransferase (LPCAT) catalyzes the re-acylation step. Among the four LPCAT members in humans, LPCAT3 specifically incorporates polyunsaturated acyl groups onto the sn-2 position of lysophosphatidylcholine. This activity modulates membrane fluidity and influences membrane protein functions (Zhang et al., 2021). LPCAT3 is crucial in lipid metabolism, particularly in the biosynthesis of phosphatidylcholine, a major phospholipid in cell membranes. Belonging to the lysophospholipid acyltransferase (LPLAT) family, LPCAT3 catalyzes the acylation of lysophospholipids to produce phospholipids. The enzyme's catalytic site consists of His388 and Asn352, with a tunnel formed by tyrosines (Tyr394, Tyr298, Tyr143, and Tyr151), all of which play important roles in the catalytic process (Zhang et al., 2021). By facilitating the incorporation of polyunsaturated fatty acids, LPCAT3 helps maintain the proper function and flexibility of cellular membranes, highlighting its essential role in cellular physiology and lipid metabolism. Lysophosphatidylcholine (LPC), a substrate of LPCAT3, was crystallized in complex with this enzyme (Zhang, et. al. 2021). Therefore, LPC was employed as the reference ligand for LPCAT3.

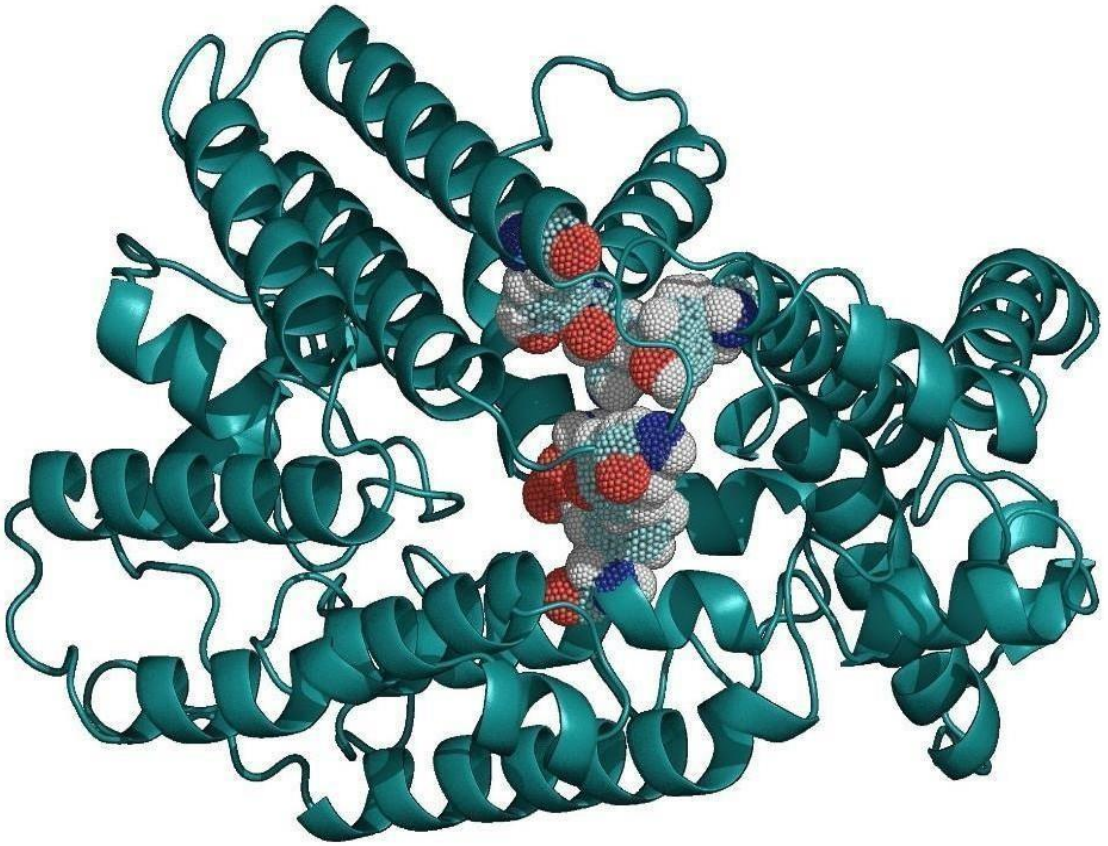

**Figure 4:** LPCAT3 enzyme. The circles show the active site cavity and the important tyrosines. The active site cavity is visualized using PyMOL software, with dots representing the cavity. Different colors indicate various elements: white for carbon, red for oxygen, and blue for nitrogen (This color coding is only applied to the dots, not to the cartoon representation of the rest of the enzyme structure). The enzyme is displayed in cartoon representation using PyMOL. The structure of LPCAT3 (PDB ID: 7F3X) was obtained from the RCSB Protein Data Bank.

### 1.7. Ligands (NDEA, NDMA and Metabolites).

N-nitrosamines, identified by the International Agency for Research on Cancer (IARC) in 1978 as probable carcinogens (Group 2A), are produced through various mechanisms involving the interaction of nitrogen oxides with secondary amines in industrial chemical processes and natural fermentations (Park et al., 2015). These compounds can form endogenously in the body, be present in processed foods such as meats and cheeses, be emitted by cigarette smoke, and result from the metabolism of certain drugs. They are also frequently detected in drinking water (Mitch et al., 2003). The persistence of N-nitrosamines in aquatic environments is particularly concerning. Unlike their rapid degradation in outdoor air through solar photolysis and other atmospheric reactions, nitrosamines such as NDMA and NDEA are more resistant in water. Photolysis can degrade these substances in the surface layers of bodies of water exposed to

sunlight, but this process is often limited by the absorption of light by dissolved substances (Papakonstantinou et al., 2017; Park et al., 2015). The primary catalyst for the bioactivation of NDMA to reactive intermediates in human liver microsomes is P450 2E1. The oxidation of the methyl group, known as α-methyl hydroxylation, results in the formation of α-hydroxy-NDMA, an unstable and mutagenic intermediate. This intermediate decomposes spontaneously, producing formaldehyde and methyl diazohydroxide. Formaldehyde can undergo further oxidation to formic acid and CO2, while methyl diazohydroxide forms the highly electrophilic methyldiazonium ion, which can alkylate DNA or undergo solvolysis to produce methanol (Li and Stephen, 2022).

The mutagenicity and genotoxicity of NDMA are well-established. NDMA induces alkylation of DNA and protein through two reactive intermediates: methyldiazonium ion and formaldehyde. Methyl DNA adducts formed by the methyldiazonium ion play a significant role in NDMA-induced carcinogenesis. Additionally, formaldehyde can generate DNA adducts, including cross-links or hydroxymethylene adducts (Li and Stephen, 2022). This comprehensive understanding of NDMA's pathways and impacts underscores the importance of monitoring and mitigating nitrosamine contamination in various environments to protect public health (Figure 5). The metabolic activation of N-nitrosodiethylamine (NDEA) for carcinogenicity is primarily facilitated by cytochrome P450 enzymes, specifically P450 2E1 and P450 2A6. During the P450-catalyzed hydroxylation at the α-carbon of the ethyl group in NDEA, an unstable intermediate, ethyl diazohydroxide, decomposes to generate the electrophilic ethyldiazonium ion (Erkekoglu and Baydar, 2010; Li and Stephen, 2022). This reaction also produces acetaldehyde as a byproduct through α-hydroxylation of the ethyl group. Moreover, when the β-carbon of NDEA undergoes hydroxylation, the reactive intermediate 2-hydroxyethyldiazonium ion can be formed through secondary α-hydroxylation on the other ethyl group of NDEA. This process adds to the complexity of NDEA metabolism, where denitrosation reactions can compete with bioactivation, indicating a dual role played by P450 enzymes in the biotransformation of NDEA (Li and Stephen, 2022). Understanding these metabolic pathways is crucial for developing strategies to mitigate the carcinogenic risks associated with NDEA exposure, highlighting the significant role of P450 enzymes in both activating and detoxifying this compound. The figure illustrates the mechanisms of biotransformation of N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) mediated by cytochrome P450 enzymes (P450s), resulting in the formation of different metabolites and final products.

For NDMA, the process begins with oxidation by P450s, forming the hydroxy-nitrosamine intermediate. This intermediate can decompose to form formaldehyde (HCHO), which is further oxidized to formic acid (HCOOH) and finally to carbon dioxide (CO2). Alternatively, the intermediate can decompose to form the methyldiazonium ion (CH3N2+), which decomposes into methanol (MeOH). The methyldiazonium ion can react with DNA, forming methyl DNA adducts, which are genotoxic lesions capable of causing mutations and potentially contributing to cancer development (Hemminki, 1993; Shuker & Margison, 1997). For NDEA, the biotransformation process also involves oxidation by P450s, which can occur at either the alpha or beta position. Alpha-hydroxylation results in the formation of a hydroxy-nitrosamine intermediate, which can decompose into aldehyde or ethyldiazonium ion. The latter can convert into ethyl-carbocation, a highly reactive species that can interact with DNA, proteins, and other biological macromolecules. Beta-hydroxylation follows a similar path, resulting in the formation of an intermediate that can decompose into 2-hydroxyethyldiazonium ion (Verna et al., 1996) Figure 5.

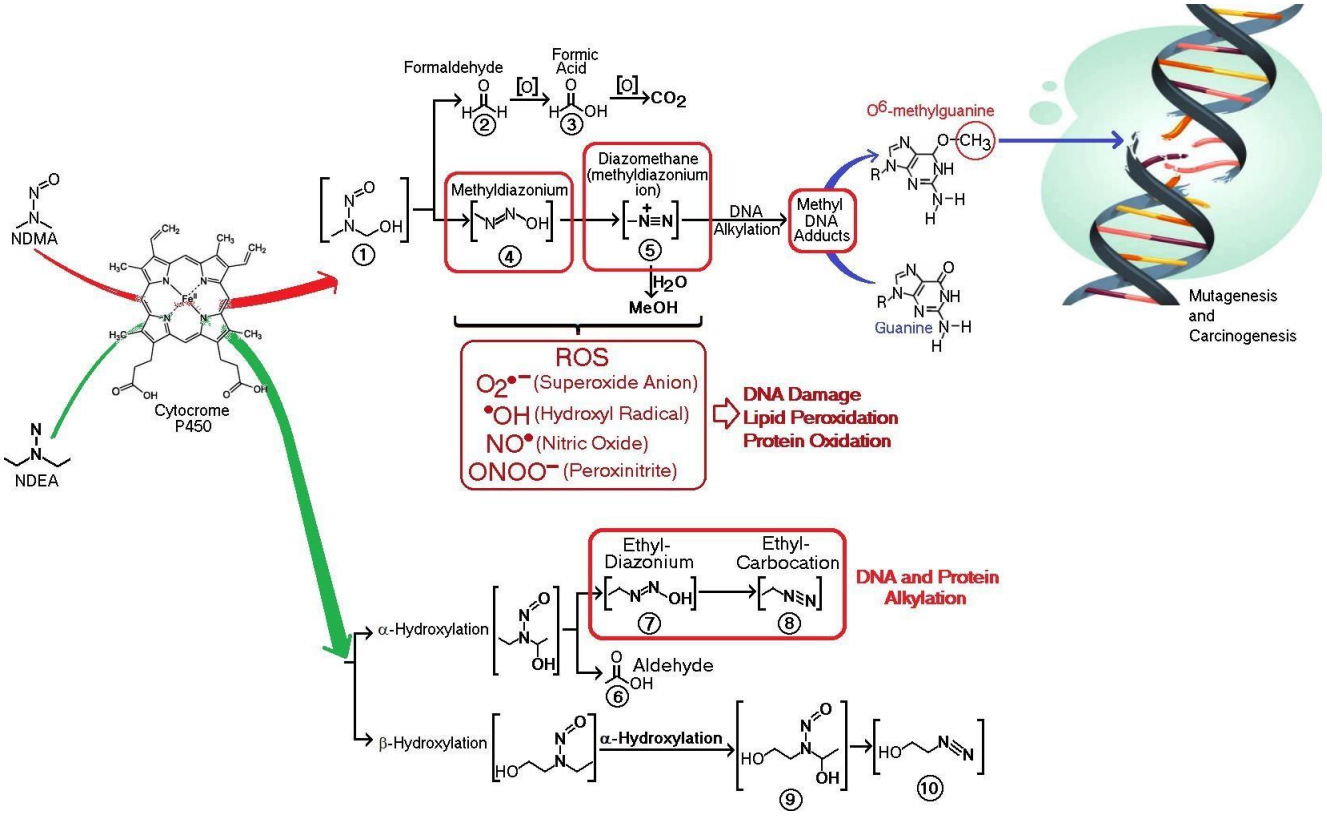


**Figura 5**. The figure illustrates the biotransformation mechanisms of two nitrosamine compounds, N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), mediated by cytochrome P450 enzymes (P450s), resulting in the formation of different metabolites and end products. Numbers not indicated in the figure: 1- α-hydroxyNDMA; 9: α- hydroxylation on the other ethyl group of NDEA; 10: 2-hydroxyethyldiazonium ion. **Source:** Own autorship

These reactive products, especially diazonium ions, can interact with DNA, forming adducts that can cause massive DNA damage. Additionally, these compounds can increase cellular oxidative stress, promoting the production of reactive oxygen species (ROS) and potentially inducing the Fenton reaction. This process generates highly reactive hydroxyl radicals that can cause severe damage to DNA, proteins, and cell membranes (Klaunig & Kamendulis, 2004; Valko et al., 2006; Marnett, 2000; Halliwell & Gutteridge, 2015). The presence of these free radicals can lead to genetic mutations and, eventually, the development of chronic diseases, including cancer. In summary, the figure highlights the complexity of the biotransformation of nitrosamines such as NDMA and NDEA, elucidating their metabolic pathways and the potential toxic and carcinogenic effects associated with their reactive metabolites. This understanding is crucial for developing effective strategies to monitor and mitigate nitrosamine contamination, thereby protecting public health.

This work analyzed the interactions between NDMA and NDEA and some of the most known and stable metabolites with the enzymes cPLA2, PAF-AH and LPCAT3. These metabolites structures were obtained from the PubChem platform (Table 2 and Figure 6).

**Table 2:** Ligands used in molecular Docking and MD

| Compound Name | Chemical Formula | Molecular weight | PubChem ID |
|---|---|---|---|
| NDMA | $C_2H_6N_2O$ | 74.08 g/mol | 6124 |
| NDEA | $C_4H_{10}N2O$ | 102.14g/mol | 5921 |
| 2-Hydroxyethanediazonium | $C_2H_5N2O^+$ | 73.07g/mol | 91426341 |
| Methanol | $CH_4O$ | 32.042g/mol | 887 |
| Formaldehyde | $CH_2O$ | 30.026g/mol | 712 |
| Methyldiazonium Ion | $^{3}\ \ ^{2}+$ | 43.048g/mol | 115287 |
| Ethanediazonium | $^{2}\ \ ^{5}\ \ ^{2}+$ | 57.07g/mol | 158606 |
| Acethaldehyde. | $C_2H_4O$ | 44.05g/mol | 177 |
| Giripladib* | $C_{14}H_{36}ClF_3N_2O_4S$ | 745.2g/mol | 11721842 |
| Lysophosphatidylcholine* | $C_{10}H_{22}NO_7P$ | 299.26g/mol | 5311264 |
| DEP (paraoxon)* | $C_4H_{10}O_3P^+$ | 137.09g/mol | 6327654 |

***Reference Ligands**

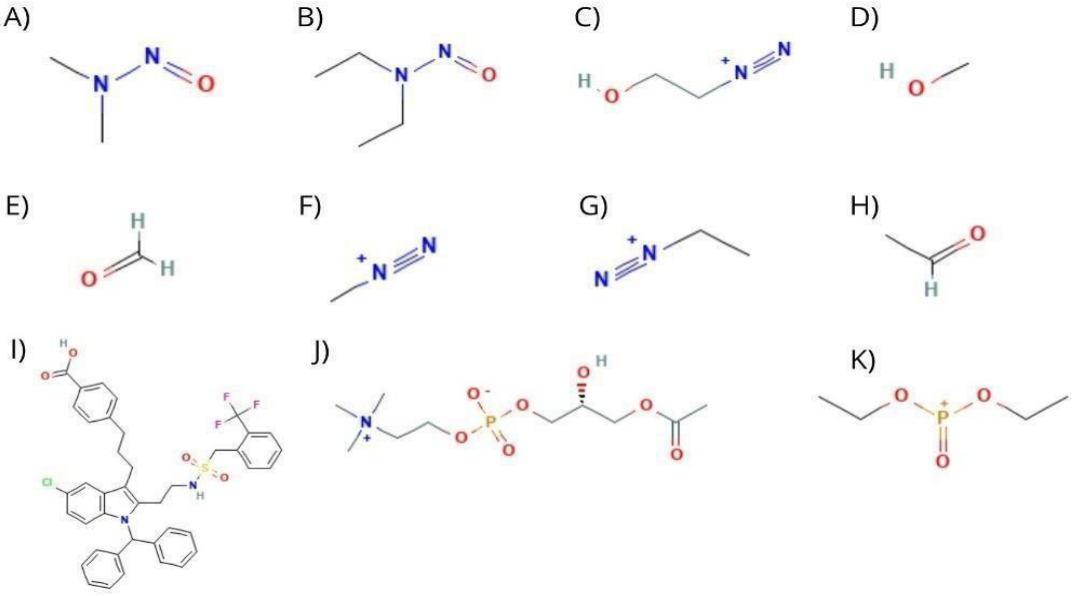


**Figure 6.** 2D structure available on the PubChem platform of the ligands.: A) NDMA; B) NDEA; C) 2-Hydroxyethanediazonium; D) Methanol; E) Formaldehyde; F) Methyldiazonium Ion; G) Ethanediazonium; H) Acethaldehyde; I) Giripladib; J) Lysophosphatidylcholine; K) DEP.

# 4. Conclusions

*4.1. Behavioral assays*

The behavioral assays with zebrafish did not show any discernible behavioral changes, according to the criteria employed, when compared to the control group (which was not exposed to the drugs). When comparing this result with the other results obtained, such as the micronucleus test and histopathological analyses, the evidence collected during the research suggests that the studied compound does not have effects related to the nervous system and the equilibrium of fish at the studied concentrations. Thus, the observed damages were systematically related to hepatic damage and nuclear abnormalities in erythrocytes.

*4.2. Micronucleo and Histological analysis in Danio rerio*

The micronucleus and histopathological analyses suggest a strong relationship between exposure to NDEA, the analyzed concentrations, and the development of pathologies and liver lesions. The visualization of nuclear abnormalities in erythrocytes suggests the interaction of the compound or its metabolites with genetic material. In the histopathological analysis shows, mainly, the hepatocyte degerneration (necrosis and liver vacuolization).

*4.3. In vivo – Mus musculus*

The results show that exposure to nitrosamines caused an increase in white blood cells (leukocytes), especially neutrophils, possibly indicating a significant inflammatory response. The increase in atypical lymphocytes suggests that the nitrosamines affected the animals' immune function.

Our biochemical results demonstrated that mice exposed to nitrosamines during 4 and 24 hours showed significantly higher levels of AST, ALT, PCR, LDH, and GGT in almost all groups when compared to the control group (saline). the specificity of NDMA action on the liver is greater than NDEA at its highest studied concentration. In a comparison relating the observed levels of biochemical markers analyzed in mice exposed to NDEA for 4 and 24 hours, it is possible to observe their progression and increase.

However, experiments with the NDMA compound at the same concentration of (0.10 mg/kg and 0.20 mg/kg) demonstred to be so toxic that it resulted in the death of all exposed animals in

less than 24 hours. This event prevented the collection of blood. But the liver was collected and it was necrosed with totally different colour than the livers collected from control group and NDEA exposed groups. Histopatological analysis suggests a several hepatic vacuolization, vascular obstruction, signs of inflammation and hemosiderin also were observed. Increases in MDA levels during NDMA exposures, show lipid peroxidation and can be related to tissue damage, resulting in necrosis. An analysis of the results according to the literature suggests that the systemic increase in TNF-α (tumor necrosis factor-alpha) can significantly impact anaphylaxis and vascular congestion, often leading to severe outcomes, including death. In severe cases, the overwhelming inflammatory response and vascular leakage can lead to disseminated vascular congestion, where multiple organs become engorged with blood. This can cause multi-organ failure, contributing to the lethality of anaphylactic reactions. These findings may possibly justify the vascular congestion observed during histological analysis and even elucidate a process that possibly contributed to the lethality found in animals exposed to NDMA.

### *4.4. In vitro*

Our in vitro test for analyzing the inhibition of PAF-AH enzyme activity confirms the results obtained from the in silico molecular dynamics tests, as both suggest the absence of interaction between nitrosamines and the enzyme's active site. The lack of this interaction is an interesting result because it speaks to the influence of nitrosamines, which are contaminants present in our daily lives, on the regulation of PAF. This work suggests that the damage caused by nitrosamines in the body is linked to their metabolism, which occurs in the liver, and to the hepatic damage that would be caused and subsequently lead to possible damage in other areas of the body, as suggested by various works in the scientific literature.

### *4.5. In silico*

This work emphasizes the selectivity of interactions between compounds and enzymes, suggesting distinct patterns of molecular recognition. Through an integrated approach of Docking, Molecular Dynamics, and MM/GBSA analyses, it contributes to a deeper understanding of ligand-protein interactions and emphasizes the relevance of optimal distance between ligands and catalytic residues, varying according to the specific enzymatic system.

In summary, the data obtained after the *in silico* analyses carried out so far suggestthat the

NDEA and NDMA compounds did not interact in a relevant way with the majority of the enzymes in focus. It is, also, evident from the results that the studied metabolites do not exert any influence on any of the enzymes.

This analysis considers PLP Normal Scores and MM/GBSA analyses. Otherwise, the compound NDMA possibly interact with the enzyme LPCAT3, while MD simulations suggest that NDEA interact with the enzyme cPLA2.

The results suggest that the nitrosamines studied do not have a significant impacton the regulation of PAF. Therefore, further studies need to be conducted to understand the strength of the interaction between NDEA and cPLA2, as well as LPCAT3 and NDMA. In both cases, these interactions can potentially alter the inflammation levels in the organism and the cell membrane remodeling.

These results not only underscore the importance of a more in-depth analysis of the complex effects of nitrosamines and related metabolites on human health but alsoemphasize the need for relevant mitigation strategies and regulations. Prolonged exposure to these compounds requires ongoing attention and risk assessment.

Our study offers valuable insights into the molecular interactions between nitrosamines, some of their metabolites, and key enzymes associated with the inflammatory process and membrane remodeling. The results underscore that nitrosamines possess a distinct toxic profile, broadening our comprehension of the array of biological responses elicited by these compounds.

### *4.6. General Conclusions*

Based on the results presented, it is concluded that exposure to nitrosamines, even at low concentrations and for relatively short periods, can cause significant damage to both *Danio rerio* fish and *Mus musculus* mice. The observed alterations in erythrocytes and liver damage highlight the toxicity of this contaminant, which is present in significant quantities in water and food, posing a risk not only to humans but also to aquatic ecosystems. The metabolism of these compounds is identified as the main cause of damage, including hepatotoxicity, oxidative stress, and interactions with genetic material, emphasizing the need for monitoring and control of these compounds in the environment.

The study showed that there are likely no direct relationships between nitrosamines and PAF control, as both in vitro and in silico assays highlighted the lack of interaction of these compounds with the PAF-AH enzyme, responsible for the degradation and regulation of PAF.

However, other relevant findings were reported, such as the influence of these compounds on hematological and genetic alterations, and especially hepatotoxic effects. Our study suggests that the biochemical and tissue changes caused are associated with the metabolization of the compounds. We suggest nitrosamines act as potential metabolic disruptors. During their metabolism by cytochrome P450, these compounds appear to increase oxidative stress by interfering with mitochondrial function. This interference possibly can lead to the generation of reactive oxygen species (ROS) and initiate the Fenton reaction.

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