Structure-based discovery of multi-target directed anti-inflammatory p-nitrophenyl hydrazones; molecular docking, drug-likeness, *in-silico* pharmacokinetics, and toxicity studies.

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ABSTRACT

Abstract We designed novel *p*-nitrophenyl hydrazones as multi-target inhibitors of COX-2, 5-LOX, and H^+/K^+ ATPase in a bid to overcome side effects associated to NSAIDs and coxibs. Specifically, compounds 1-(4-nitrophenyl)-2-[(3,4,5-trimethoxyphenyl)methylidene] hydrazine (**3**), 4-hydroxy-2-methyl-6-[(2-(4-nitrophenyl)hydraz-1-ylidene)methyl]thiochroman-1,1-dioxide (**6**), 4-methoxy-2-methyl-6-[(2-(4-nitrophenyl)hydraz-1-ylidene)methyl]thiochroman-1,1-dioxide (**8**), 2-methyl-6-[(2-(4-nitrophenyl)hydraz-1-ylidene)methyl]-4-(trifluoromethyl)thiochroman-1,1-dioxide (**11**), 4-[(2-(4-nitrophenyl)hydraz-1-ylidene)methyl]benzenesulfonamide (**13**), 4-[(2-(4-nitrophenyl)hydraz-1-ylidene)methyl]-3-(trifluoromethyl)benzenesulfonamide (**14**), 5-methyl-6-{4-[(2-(4-nitrophenyl)hydraz-1-ylidene)methyl]phenyl}-2,3,4,5-tetrahydropyridazin-3-ol (**16**), and 5-methyl-6-{4-[(2-(4-nitrophenyl)hydraz-1-ylidene)methyl]phenyl}-4,5-dihydropyridazin-3(2H)-one (**17**) indicated promise as potent multi-target inhibitors of COX-2, 5-LOX, and H^+/K^+ ATPase with potential anti-inflammatory activity devoid of adverse effects of NSAIDs. Interactions with important amino acids which are key for ant-inflammatory activity and proton pump inhibition were noticed. All the compounds are less COX-2 selective compared to celecoxib. These compounds in addition have shown druglike physicochemical properties, passed Lipinski's, Egan's, Veber's, Muegge's and Ghose's rules

for druglike small molecules and orally bioavailable drugs. The compounds also passed golden triangle's rule for potent and metabolically stable drugs. Also, these compounds passed Pfizer and GSK rules. The compounds also indicated excellent pharmacokinetic profiles complementing their potential anti-inflammatory activity with apparent safety profiles.

Keywords: *p*-nitrophenyl hydrazones, molecular docking simulation, pharmacokinetics, drug-likeness, NSAIDs gastrointestinal side effects, coxibs cardiovascular adverse effects, toxicity.

1. Introduction

Inflammation is the body's complicated biochemical response to damaging stimuli like irritants, infections, or damaged cells. It is the organism's preventive attempt to start the healing process and eliminate harmful stimuli.[1] Pathogens (bacteria, fungi, and viruses), trauma (shock or burns), toxic substances (pollutants), and immune system responses (hypersensitivity) are some of these agents.[2] Gastric toxicity is ascribed to currently marketed nonsteroidal anti-inflammatory drugs (NSAIDs). Long-term usage of these medications has been linked to GI ulcers, bleeding, and nephrotoxicity. The carboxylic acid moiety included in most NSAIDs causes local irritation, and reduces tissue prostaglandin synthesis which weakens the homeostatic role of cytoprotective prostaglandins in supporting GI health and balance.[3]

Gastric and duodenal ulcers are frequent gastrointestinal tract illnesses with significant clinical incidence rates and the potential for serious upper gastrointestinal hemorrhage. They may be caused by an imbalance between aggressive and defensive forces in the stomach and duodenum. The lowering of acid output has been shown to be an effective way of increasing ulcer healing.[4] H^+/K^+ ATPase facilitates the final stage in gastric acid production, therefore inhibiting it can result in a more significant decrease in gastric acid output.[4] As a result, the H^+/K^+ ATPase enzyme inhibition might be used to treat a variety of acid-related disorders. Proton pump inhibitors (PPIs) are the major drugs used in GI acid-related diseases, as H^+/K^+ ATPase inhibitors on the market,[4] however, their known limited efficacy, short duration of action, and relapses are the drawbacks that call for the development of new agents.

Co-administration of NSAIDs and PPI are common practices to overcome gastrointestinal events associated with NSAIDs for patients with ulcers. Although PPI recorded no significant side effects. However, drug cost and possible drug-drug interactions adverse outcomes, and ulcers recurrent are the disadvantages associated with this treatment.

COX-1 inhibition causes the majority of GI adverse effects, whereas highly selective COX-2 inhibitors generate cardiovascular side effects. LOX inhibitors, on the other hand, alleviate heart problems caused by COX-2 inhibition, and 5-LOX leukotrienes induced hypersensitivity and allergic reactions side effects of NSAIDs.[1] Therefore, the discovery of a multi-target drug that inhibits inflammatory targets COX-2 and 5-LOX, and also proton pump H^+/K^+ ATPase could solve toxicity events associated with the use of NSAIDs and coxibs. Co-inhibition of COX and 5-LOX potentially reduce side effects on the cardiovascular and gastrointestinal tract while retaining the primary activity of COX-1/2 inhibitors.[5] Studies have reported that the hydrazone moiety present in some compounds possesses a pharmacophoric character for the inhibition of COX and LOX enzymes with better safety and efficacy as compared to few available drugs on the market.[6,7] Some benzothiazole hydrazones have been reported as potent inhibitors of the H^+/K^+ ATPase enzyme in addition to COX-2 enzyme.[4] As a result, the design of multi-target inhibitors of H^+/K^+ ATPase, COX-2, and 5-LOX are being investigated in our ongoing discovery effort as a means of developing more active and cost-effective anti-inflammatory agents with less or no adverse effects.

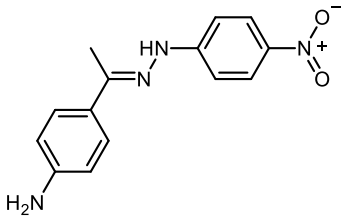
In-silico techniques are now frequently employed to investigate key characteristics that might help medicinal chemists evaluate a compound's chemical and physicochemical parameters. Because these properties have an impact on pharmacokinetic characteristics, the goal of *in-silico* investigations is to save time and money by avoiding the costs of biological tests for molecules with a high likelihood of presenting future pharmacokinetic issues.[8] The failure of drug candidates in clinical trials is mostly due to undesirable pharmacokinetic (PK) features or unacceptable toxicity. Since its inception, the idea of drug-likeness has grown in importance in the screening of molecules with optimal bioavailability throughout the early stages of drug development. As a result, identifying suitable candidates with a good balance of potency, absorption, distribution, metabolism, excretion, and toxicity (ADMET) is a critical scientific requirement.[9]

The idea of drug-likeness was introduced as a way to give relevant guidance throughout the early phases of drug development so that a chemical may enter and pass clinical trials.[10] It may be described as the total of the molecular physicochemical features that distinguish drugs from other substances. Indeed, the term "drug-likeness" is frequently used to characterize PK and safety, and it may also refer to substances that have good ADMET features.[11,12]

Importantly, *in-silico* predictions should not be used to replace or reject experimental studies; rather, they should be used in tandem. The importance of experimental *in-vitro* and *in-vivo* pharmacokinetics experiments for the assessment of a novel medicine is unquestionable. Because it is

well understood that a compound's pharmacokinetic qualities are intimately linked to its chemical structure, experimental data is saved in computer databases and a large number of experimental observations are compared to structural and physicochemical features such that computer-assisted *in-silico* screenings can make use of these properties.[8] The information included in databases, which have been increasingly shared by the pharmaceutical sector, is critical to the veracity of theoretical models. These models are also put through challenge tests to validate their confidence level.[8]

Following the promising activity of *p*-nitrophenyl hydrazones against TNF-α in a reported patent JP2012046453A which described their therapeutic effect against chronic inflammatory diseases, eighteen *p*-nitrophenyl hydrazones were designed using structure-activity-relationship (SAR) derived from the patent and other literature,[4] in addition to hybridization concept for some selected designed compounds.



Lead compound: IC_{50} = 1.2 E^{-04}

The following points were noted from the SAR study.

- The presence of two aryl groups linked together by a hydrazone moiety bridge is the primary requirement for anti-inflammatory activity.
- Structure activity relationship study showed that the presence of at least one nitro group on either hydrazine ring or aldehyde ring increases anti-inflammatory activity of hydrazones.

$$Ar_1R_1C = N\text{-}NR_2Ar_2$$

R_1= H, C_1-C_6 alkyl group. R_2= H, C_1-C_6 alkyl group.

- The presence of electron-withdrawing groups on the Ar_1 (aldehyde ring) and electron-donating group on Ar_2 (hydrazine ring) increases anti-inflammatory activity.
- The presence of an electron-donating group on the Ar_1 (aldehyde ring) and Ar_2 (hydrazine ring) increases anti-ulcer activity.
- The presence of both electron-withdrawing and electron-donating groups on Ar_1 (aldehyde ring) and electron-donating group on Ar_2 (hydrazine ring) decreases the anti-inflammatory activity.

- When the Ar_1 is benzene ring there is good anti-inflammatory activity.
- Replacement of Ar_1 with a 2-6 long aliphatic branched or straight chains decrease anti-inflammatory and anti-ulcer activities.
- Replacement of Ar_1 with heterocycles such as indole, pyridine, furan, thiophene, and pyrrole decrease anti-inflammatory and anti-ulcer activities.

In the current work, compounds **1**, **2**, **3**, **4**, **5**, **12**, **13**, **14**, and **15** were designed by substituting a nitro group on the hydrazine ring (Ar_2) at position four of the ring, while electron-withdrawing or electron-donating group(s) were substituted on the aldehyde ring (A_1) at varying positions. Compounds **6**, **7**, **8**, **9**, **10**, **11**, **16**, and **18** were designed using hybridization concept by combining active 4-nitrophenyl hydrazine with fragments of active pharmaceutical ingredients (API) or their derivatives.

2. Materials and Methods

2.1 Protein crystal structure and ligands collection

The 3D crystal structure of the proteins; rodent cyclooxygenase-2 (COX-2) co-crystalized with celecoxib, human 5-lipoxygenase (5-LOX), and human H^+/K^+ ATPase were obtained from the RCSB protein data bank with PDB ID; 3LN1, 3O8Y, and 6JXH respectively. Celecoxib, Zileuton, and Omeprazole were used as the reference drugs.

2.2 Proteins and ligands Preparation

The proteins were prepared in the UCSF chimera 1.11.2 Dock prep module wherein bond orders, formal charges, missing polar hydrogen atoms, topologies, incomplete and terminal amide groups, and missing side chains are all refined in protein structures, and water molecules were removed. The proteins were further prepared in the Autodock tool repeatedly and were converted from PDB to PDBQT file format.

The ligands were drawn using ChemAxon's Marvin sketch and were saved in their 3D format as mol. files. Ligands energy minimization was performed with entos envision and was subsequently prepared in UCSF chimera 1.11.2 wherein formal charges and polar hydrogens were added. These were repeated in Autodock tool 1.5.6 and were subsequently saved as PDBQT files.

2.3 Receptor Grid Generation

The receptor grid box module of Autodock tools 1.5.6 was used to produce the region of interaction between proteins and ligands. In terms of coordinates, the protein's binding dimensions inside which the center of a docked position is constrained as x, y, and z.

2.4 Molecular Docking Using Autodock Vina

The docking simulation was performed with Autodock vina script using bash commands in the Cygwin run time environment. The docking procedure was carried out in a flexible docking mode, which creates conformations for each input ligand automatically. The produced ligand poses were subjected to a series of hierarchical filters that assessed the ligand's interaction with the receptor. This approach penalizes steric conflicts while recognizing favorable hydrogen bonding, and hydrophobic, metal-ligation interactions. After the simulation was completed, the binding energies of the ligands were ranked using excel. Each ligand poses (conformations) was viewed in UCSF chimera 1.11.2, the most favorable complexes formed were viewed in the discovery studio wherein various interactions between the ligands and the receptors were elucidated in 2D format.

2.5 Drug-likeness, in-silico pharmacokinetics (ADME), and toxicity studies

The drug-likeness studies, *in-silico* pharmacokinetics, and toxicity studies were evaluated on ADMETlab 2.0 and Protox-II web servers. The ADMETlab 2.0 was used to evaluate detailed parameters of drug-likeness, absorption, distribution, metabolism, and excretion. The toxicity studies were conducted on Protox-II and ADMETlab 2.0 web servers. The data were analyzed statistically using a Two-way analysis of variance (ANOVA) with replication, residual error test, and Tukey’s Multiple Comparison Test.

3. Results and Discussion

3.1 Molecular docking simulation analysis

Cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), and the proton pump H^+/K^+ ATPase are clinically relevant biological targets that have been used in the development of many drugs including the coxibs (e.g celecoxib), zileuton, proton pump inhibitors (e.g omeprazole, lansoprazole, pantoprazole, and rabeprazole) respectively.

The COX-2 enzyme is a biological target used for a wide range of anti-inflammatory drugs as it is responsible for the biosynthesis of prostaglandins which are mediators of inflammation. Hence, a docking study was accomplished to explore the possible binding conformers for the newly designed

hydrazones into the COX-2 active site to predict their binding mode and explain their possible anti-inflammatory activity. It is noteworthy to know that all NSAIDs except aspirin are reversible inhibitors of COX enzymes. Aspirin covalently modifies both COX-1 and COX-2 through acetylation of Ser530 and Ser516 respectively.

5-Lipoxygenase (5-LOX) is responsible for the metabolism of arachidonic acid (AA) for the biosynthesis of leukotrienes which are potent proinflammatory mediators. Hypersensitivity and allergic reactions including asthma, airway edema, bronchospasm, etc have been associated with metabolites of the 5-LOX enzyme. These leukotrienes have also been implicated in NSAIDs induced cardiovascular and hypersensitivity side effects. Therefore, COX/5-LOX inhibitors are potential new drugs for the treatment of inflammation. Notably, zileuton is the only approved and marketed 5-LOX inhibitor. However, its associated drawbacks are low potency and poor pharmacokinetic profiles including rapid clearance and short half-life.

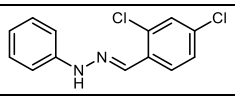
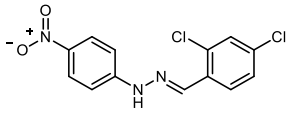
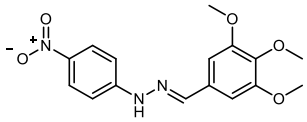
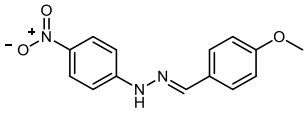
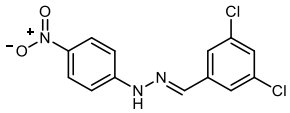
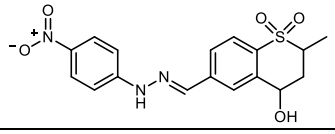
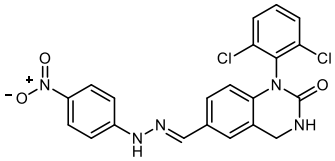
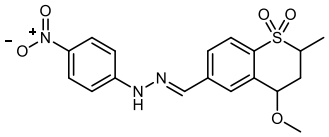
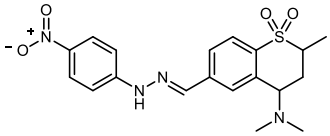
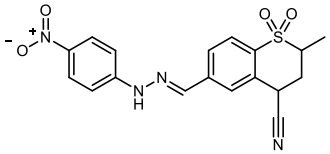
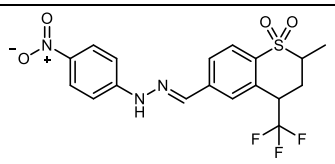
Analysis of the reversible binding conformers of the prepared compounds will give better insight into their possible anti-inflammatory activity and their ability to overcome NSAIDs-associated toxicity. H^+/K^+ ATPase catalyzes the last step of gastric acid secretion. Over secretion of this acid leads to gastric irritation and a variety of ulcers. To this note, gastrointestinal side effects of NSAIDs are associated with over secretion of gastric acid due to inhibition of biosynthesis of cytoprotective prostaglandin responsible for the production of gastrointestinal protective mucus. Proton pump inhibitors are used to manage acid-related diseases.

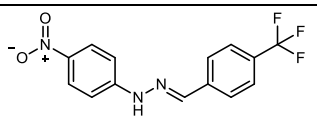
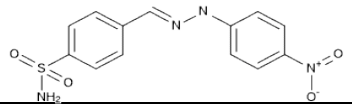
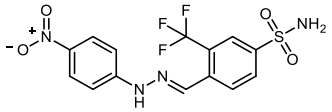
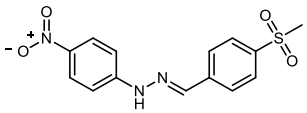
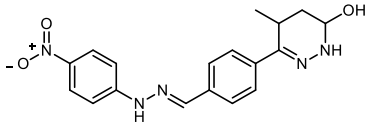
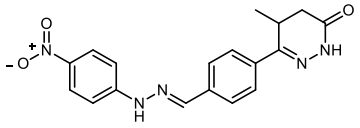
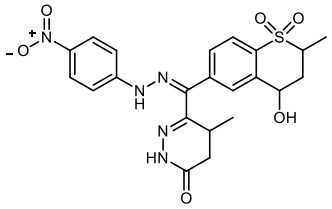
It is noteworthy to state that all PPIs are covalent inhibitors. These include omeprazole, lansoprazole, pantoprazole, rabeprazole, and tenatoprazole. Omeprazole covalently interact with Cys813 and Cys892. Lansoprazole react with Cys813 and Cys321. Also, pantoprazole and tenatoprazole covalently interact with both Cys813 and Cys822. Covalent interaction of pantoprazole and tenatoprazole with Cys822 confers a longer duration of action and irreversibility.[13] The designed hydrazones are covalent inhibitors. However, this current discussion is based on their reversible inhibition of H^+/K^+ ATPase.

All the designed hydrazones showed promise as multi-target inhibitors of COX-2, 5-LOX, and H^+/K^+ ATPase according to their binding energy in **Table 1** with less selectivity toward COX-2 compared with celecoxib. It worth mentioning that all the highly selective COX-2 inhibitors (coxibs) are associated with serious toxicity, while all excluding celecoxib have been withdrawn from the market by FDA. Celecoxib remain in the market with warnings. Except for compound 1, all the designed hydrazones bind more favorably to the 5-LOX active site than zileuton as demonstrated by

their binding energies. Also, all the designed hydrazones indicated higher affinity for the active site of H^+/K^+ ATPase in comparison to omeprazole. Exception to this observation was with compounds 1, 3, 4, and 5 with lower but comparable binding affinity within the active site of the enzyme.

Table 1 Binding Energy (Kcal/mol)

Compounds	Struture	COX-2	5-LOX	H^+/K^+ ATPase
1		-8.4	-5.3	-6.5
2		-8.9	-7.2	-7.3
3		-7.6	-6.9	-6.8
4		-8.4	-7.5	-6.7
5		-8.9	-7.7	-6.9
6		-7.8	-7.2	-8
7		-8.1	-8.1	-8.7
8		-8	-6.7	-7.8
9		-7.9	-6.8	-7.7
10		-7.9	-6.8	-8
11		-8.1	-7.6	-8.6

12		-9.5	-8.8	-8
13		-9.5	-8.1	-7.3
14		-9.6	-7.3	-7.5
15		-9	-7.9	-7.2
16		-9.7	-7.7	-7.8
17		-8.8	-7.4	-7.9
18		-8.8	-8	-8.3
Celecoxib		-12.6	----	----
Zileuton		----	-6.2	----
Omeprazole				

Further analysis indicated that compounds **1-5** and **12-15** are strong competitive inhibitors of COX-2 as celecoxib, however, with less selectivity for COX-2 compared with celecoxib, whereas compounds **6-11**,**16**, and **17** are competitive only at their most favorable binding (pose) energy. Compound **18** is a non-competitive inhibitor of COX-2. Compounds **3**, **6**, **8**, **11**, **13**, **14**, **16**, and **17** displayed plausible hydrogen bond interactions in addition to other hydrophobic interactions with the three biological targets as shown in **Table 2**. Compounds **3** and **16** had hydrogen bond interactions with Ser516 which is a NSAIDs key interaction for anti-inflammatory activity. Most of the designed hydrazones formed hydrogen bonds with His75, whereas others had hydrophobic interactions with the residue as does celecoxib. Furthermore, compounds **13**, **14**, and **16** formed hydrogen interactions with Gln178, Arg499, and Phe504 similarly as celecoxib. In addition, compounds **13** and **14** also formed hydrogen bond interaction with Ser339 while compound 16 interacted with Ser516. Compounds **2**, **3**, **5**, **12**, and **16** formed hydrogen bond interaction with Phe504. Also, compounds **8** and **17** formed

hydrogen bond interaction with Gln178 while compounds **2** and **15** formed hydrogen bond interaction with Arg499 similarly as celecoxib. 2D illustration of interactions of compound **3** with the cyclooxygenase-2 active site, 5-lipoxygenase active site and the H^+/K^+ ATPase active site can be seen below **(Fig1-3).**

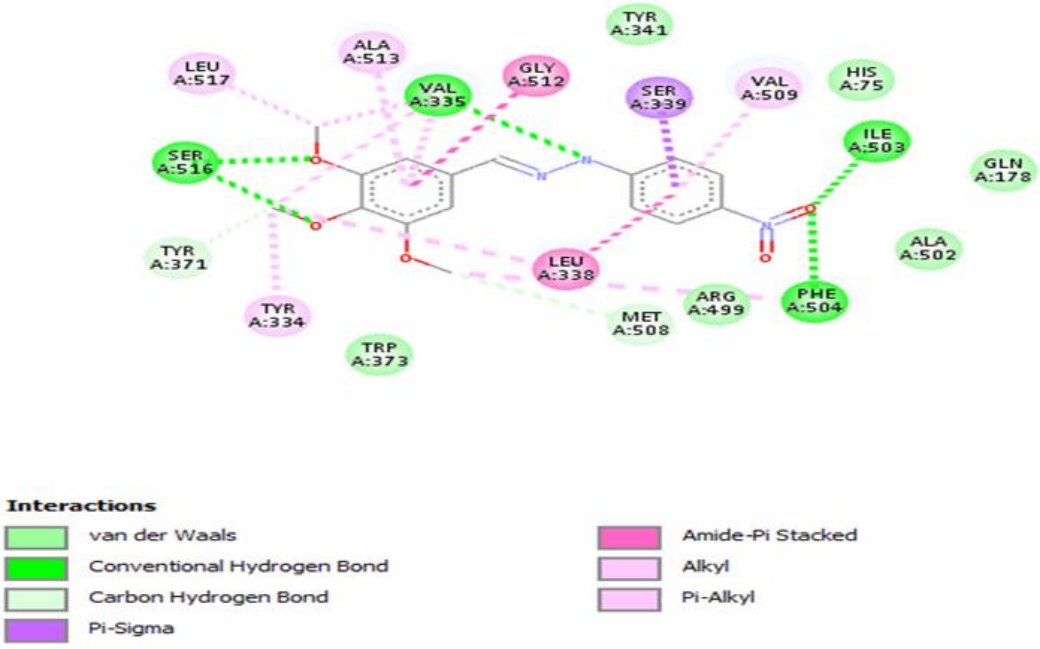


Fig 1: 2D illustration of interactions of compound 3 with the cyclooxygenase-2 active site.

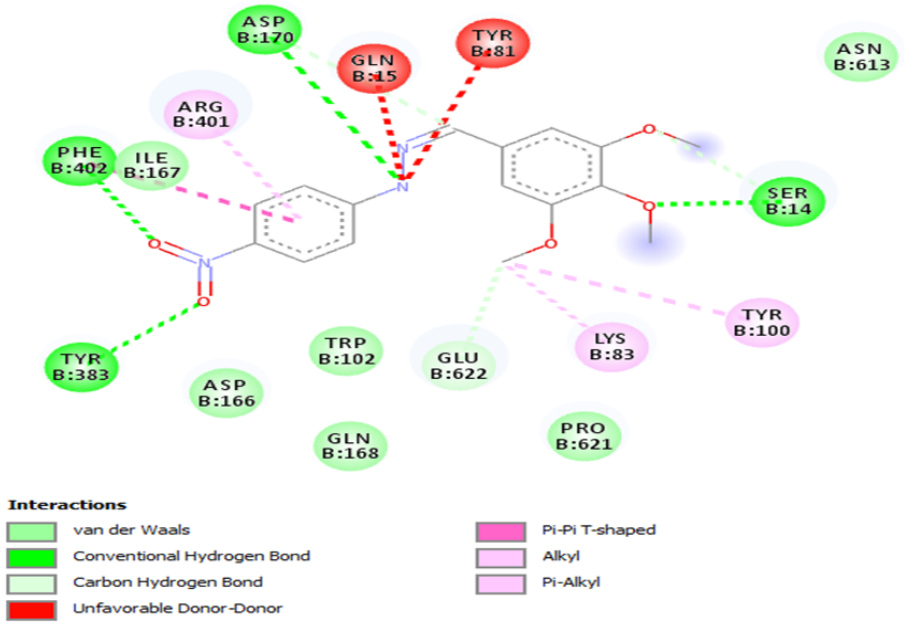


Fig 2: 2D illustration of interactions of compound 3 with the 5-lipoxygenase active site.

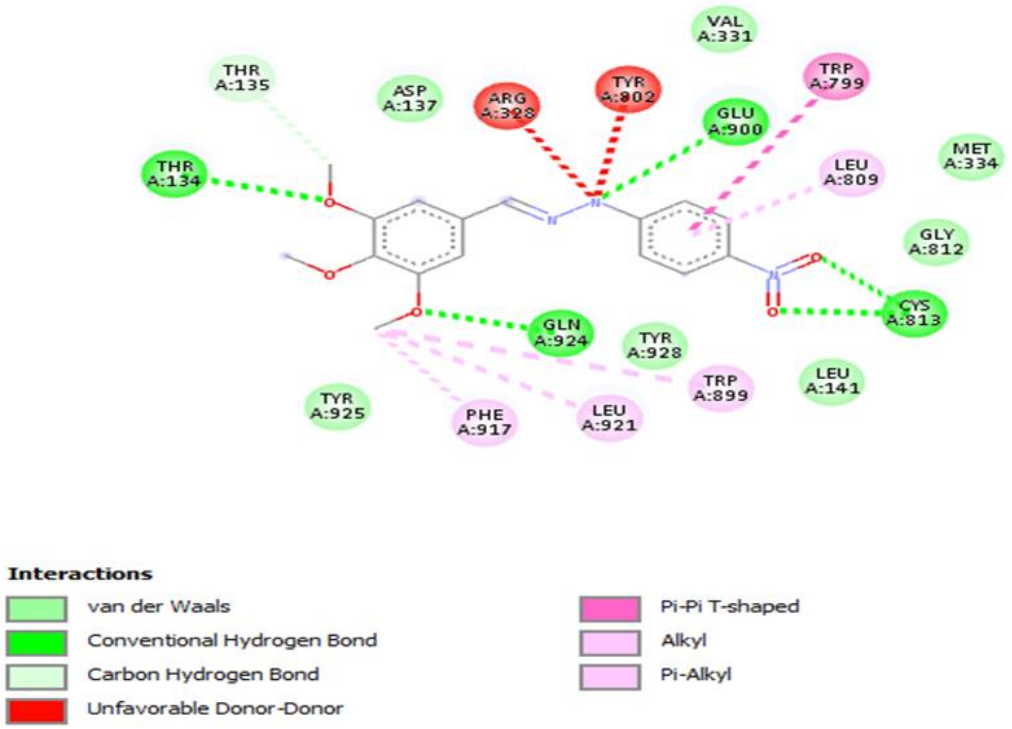


Fig 3: 2D illustration of interactions of compound 3 with the H+/K+ ATPase active site.

Table 2 Hydrogen bond interactions

Compound	**COX -2**	Residues	**5-LOX**	Residues	**H^+/K^+ ATPase**	Residues
1	**0**	-	**1**	Trp605	**0**	
2	**2**	Arg499, Phe504	**2**	Gln15, Arg401	**1**	Thr152
3	**5**	Ser516,Val335, Ile503, Phe504	**4**	Ser14, Asp170, Tyr383, Phe402	**5**	Thr134, Cys813, Glu900, Gln924
4	**0**	-	**3**	Tyr81,Tyr383, Phe402	**3**	Thr134, Cys813
5	**2**	His75, Phe504	**3**	Ser14, Arg401	**2**	Gln924, Tyr928
6	**2**	His75, Thr79	**3**	Asp170, Arg401, Gln611	**4**	Arg328, Tyr802, Cys813, Ile814
7	**1**	His75	**1**	Arg401	**3**	Ile814, Asn989
8	**2**	His75, Gln178	**3**	Gly174, Asn180, Arg401	**4**	Ile814, Gln924, Tyr928, Asn989
9	**1**	His75	**4**	Asp170, Ser171, Arg401, Asn613	**2**	Gln104, Gln159
10	**1**	Asn567	**2**	Asp422, Arg596	**1**	Ile814
11	**2**	His75, Gln178	**4**	Asp170, Ser171, Arg401, Gln611	**5**	Asn138, Arg328, Tyr802, Cys813, Ile814
12	**2**	Ile503, Phe504	**3**	His373, Ala424, Asn425	**1**	Thr152

13	**4**	Gln178, Ser339, Arg499, Phe504	**6**	Ser14, Lys83, Tyr383, Asp166, Arg401	**5**	Thr134, Asp137, Asn138, Arg328, Tyr925
14	**4**	Gln178, Ser339, Arg499, Phe504	**3**	Lys83,Ser171,Gln611	**7**	Thr134, Asp137, Arg328, Ile814, Asn989
15	**2**	His75, Arg499	**2**	Lys83, Arg401	**4**	Gln127, Asn138, Tyr925, Asn989
16	**4**	Arg106, Gln178, Phe504, Ser516	**2**	Gln15, Tyr383	**4**	Thr138, Asp137, Ile814, Asn989
17	**2**	His75, Gln178	**5**	Lys83, Asp170, Ser171, Arg401	**3**	Ile814, Tyr928, Asn989
18	**2**	Asn567, Val568	**4**	Tyr558, Asp559, Gln609	**0**	-
Celecoxib	**3**	Gln178, Arg499, Phe504	-	-	-	-
Zileuton	-	-	**2**	His195, Phe197	-	-
Omeprazole	-	-	-	-	**2**	Asp137, Ile814

On the other hand, only compound **10** is a competitive inhibitor of 5-LOX, binding at the same binding site as zileuton at its lowest binding energy. Compounds **2**, **7**, **8**, **9**, **11**, **14**, **16**, and **17** bind at three of the five binding sites of zileuton. Compounds **1**, **3**, **6**, **10**, and **18** bind at two of the binding sites of zileuton. Compounds **4**, **5**, and **13** bind at one of the five binding sites of zileuton. All these compounds also bind at 1-3 sites within the active sites where zileuton does not bind. Compound **15** does not bind at any binding sites of zileuton. All the *p*-nitrophenyl hydrazones bind at the same binding site within the active site of 5-LOX at their most favorable binding energy indicating that the para nitro group confers site directing or selectivity. Exceptions to this are compounds **12** and **17** which bind at different binding sites at their lowest binding energy. However, these two compounds bind at the nitro group directing site at their other poses where other *p*-nitrophenyl hydrazones bind.

The binding analysis revealed that all the compounds are competitive inhibitors of H^+/K^+ ATPase except for compounds **1**, **2**, **9**, **12**, and **18**. The halogens in compounds **1**, **2**, and **12** are site directing as these compounds bind at the same binding within the active site. However, this is not observed with compounds **11** and **14** which are competitive inhibitors. It can be said that compounds **11** and **14** bindings within the active site are directed by the sulphonyl (SO_2) moiety rather than the substituted halogens. Compounds **9** and **18** are also non-competitive inhibitors because they did not bind where omeprazole binds within the active site.

All the compounds except compounds **1**, **2**, and **12** interacted with Cys813 either through hydrogen bonds; compounds **11**, **6**, **4**, and **3**, or through hydrophobic interactions; all other compounds. Compounds **1**, **2**, and **12** with halogen directing moiety formed hydrophobic interactions with Cys822.

Compounds **14** and **16** formed hydrogen bond interaction with Asp137 and Ile814 similar to omeprazole in addition to hydrophilic interactions with other residues. Compound **13** formed a hydrogen bond interaction with Asp137 whereas compounds **6**, **7**, **8**, **10**, **13**, **14**, **16**, and **17** interacted with Ile814 via hydrogen bond according to results in **Table 2** complementing their binding energies and competitive binding in relation to omeprazole.

3.2 Drug-likeness and In-silico Pharmacokinetic and Toxicity (ADMET) Evaluation.

3.2.1 Physicochemical Properties

All the designed hydrazones exhibit drug-like physicochemical properties according to results in **Tables 3** and **4** having a molecular weight (MW) of less than 600 $gmol^{-}$, less than 12 hydrogen bond acceptors (nHA), and less than 7 hydrogen bond donors (nHD). The designed hydrazones comprise less than 11 rotatable bonds (nRot), less than 6 rings (nRing), less than 15 heteroatoms (nHet), less than 18 atoms within a ring, and zero charges. The compounds also exhibit good flexibility with scores ranging between 0.23-0.50 and number of rigid bonds less than 30.

Table 3 Physicochemical properties of the designed compounds

Compounds	MW	Vol.	Densi.	nHA	nHD	nRot	nRing	MaxRing	nHet	fChar
1	264	250.25	1.06	2	1	3	2	6	4	0
2	309	276.19	1.12	5	1	4	2	6	7	0
3	331	324.00	1.02	8	1	7	2	6	8	0
4	271	271.86	1.00	6	1	5	2	6	6	0
5	309	276.00	1.12	5	1	4	2	6	7	0
6	375	351.28	1.07	8	2	4	3	10	9	0
7	455	417.69	1.09	8	2	5	4	10	10	0
8	389	368.57	1.06	8	1	5	3	10	9	0
9	402	388.10	1.04	8	1	5	3	10	9	0
10	384	365.51	1.05	8	1	4	3	10	9	0
11	427	377.99	1.13	7	1	5	3	10	11	0
12	309	281.27	1.10	5	1	5	2	6	8	0
13	320	292.86	1.09	8	3	5	2	6	9	0
14	388	328.36	1.18	8	3	6	2	6	12	0
15	319	299.16	1.07	7	1	5	2	6	8	0
16	353	351.84	1.00	8	3	5	3	6	8	0
17	351	349.21	1.01	8	3	6	3	6	8	0
18	485	454.71	1.07	11	4	6	4	10	12	0
Piroxicam	316	296.90	1.07	6	1	2	3	10	7	0
Celecoxib	381	343.11	1.11	5	2	4	3	6	9	0

Table 4 Physicochemical properties of the designed compounds (cont'd)

Compounds	nRig	Flex	SC	TPSA (Å)	logS	logP	logD
1	13	0.23	0	24.39		-4.83	4.55
2	14	0.29	0	70.21		-4.85	4.24
3	14	0.50	0	97.90		-3.87	2.94
4	14	0.36	0	79.44		-3.74	3.18
5	14	0.29	0	70.21		-4.85	4.31
6	21	0.19	2	121.90		-4.67	3.25
7	26	0.19	0	99.87		-7.00	5.00
8	21	0.24	2	110.90		-5.95	3.61
9	21	0.24	2	104.91		-5.87	3.63
10	22	0.18	2	125.46		-6.55	3.65
11	21	0.24	2	101.67		-6.69	4.36
12	14	0.36	0	67.53		-6.59	4.72
13	16	0.31	0	127.69		-5.28	3.13
14	16	0.38	0	127.69		-5.80	3.79
15	16	0.31	0	101.67		-5.28	3.37
16	20	0.25	2	112.15		-4.86	3.79
17	20	0.30	0	112.95		-4.11	2.61
18	27	0.22	3	167.32		-4.15	1.84
Piroxicam	22	0.09	1	96.43		-4.67	1.30
Celecoxib	19	0.211	0	77.98		-4.87	3.47

Most of the designed hydrazones have 0 stereogenic centers (chiral centers), compounds **6**, **8**, **9**, **10**, **11**, and **16** have two stereogenic centers in their structures which are still within the threshold of ≤ 2. However, compound **18** has three stereogenic centers which is beyond the threshold. All the designed compounds have polar surface area (TPSA) less than 140 topological polar surface area (TPSA) except for compound **18** with TPSA of 167.32. All the compounds have logP less than 5 log mol/L. Other than **3** and **4**, all the compounds have poor water solubility (logS) with values ranging from -4.11 for compound 17 to -7.00 for compound **7**. Except for compound **12** with high logD equal to 4.12, all other designed compounds have logP at physiological pH 7.4 below the threshold indicating that they are soluble at the physiological pH 7.4.

3.2.2 Drug-likeness and Medicinal Chemistry Friendliness

The quantitative estimate of drug-likeness (QED) score of all the designed compounds falls between 0.64 to 0.48 which is a bit below the threshold score of 0.67 for attractive compounds according to results in **Table 5**, some of the compounds are well above 0.49 meniscus for unattractive compounds. All the compounds are within the range of easy to synthesized scores for synthetic accessibility (SA) ≤

6. The fraction of sp^3 hybridized carbon score (Fsp^3) which is the number of sp^3 carbons divided by the total number of carbon atoms in a molecule is below 0.42 suitable threshold for all the compounds. However, similar results were observed with piroxicam and celecoxib.

Table 5 Drug-likeness and Medicinal Chemistry Friendliness.

Compounds	QED	SA	Fsp3	MCE-18	Lipinski Rule	Veber Rule	Egan Rule
1	0.64	1.80	0	10.00	Pass	Pass	Pass
2	0.52	2.04	0	12.00	Pass	Pass	Pass
3	0.48	2.07	0.19	13.00	Pass	Pass	Pass
4	0.52	1.85	0.07	11.00	Pass	Pass	Pass
5	0.52	2.12	0	12.00	Pass	Pass	Pass
6	0.48	3.57	0.24	65.71	Pass	Pass	Pass
7	0.29	2.65	0.05	48.09	Pass	Pass	Pass
8	0.48	3.63	0.28	65.22	Pass	Pass	Pass
9	0.47	3.64	0.32	68.04	Pass	Pass	Pass
10	0.49	3.75	0.22	65.46	Pass	Pass	Pass
11	0.45	3.75	0.28	75.00	Pass	Pass	Pass
12	0.53	2.05	0.07	14.00	Pass	Pass	Pass
13	0.49	2.05	0.00	14.00	Pass	Pass	Pass
14	0.46	2.41	0.07	18.00	Pass	Pass	Pass
15	0.52	2.02	0.07	14.00	Pass	Pass	Pass
16	0.44	3.51	0.22	55.64	Pass	Pass	Pass
17	0.47	2.44	0.11	17.00	Pass	Pass	Pass
18	0.30	4.28	0.27	86.79	Pass	Pass	Pass
Piroxicam	0.78	3.78	0.13	58.24	Pass	Pass	Pass
Celecoxib	0.75	2.14	0.12	22.00	Pass	Pass	Pass

Compounds **7** and **16** scaffolds are ranked as trending scaffolds currently observed in medicinal chemistry by MCE-18. Compounds **6**, **8**, **9**, **10**, and **11** are ranked as compounds with high structural similarity to the compounds disclosed in patent records, whereas compound 18 with an MCE-18 score of 86.79 is considered a novel scaffold with a strong drug-like structure. All the compounds passed Lipinski's rule of five, Veber rule, and Egan rule for oral bioavailability which indicates that they are orally bioavailable. The compounds also conform with the golden triangle rule for metabolically stable, permeable, and potent drug candidates, the Ghose rule, and the Muegge rule for drug-like molecules filter. Compounds **3**, **6**, **8**, **10**, **13**, **14**, **15**, **16**, and **1**7 also passed Pfizer and GSK rules for small molecules. Exception is compound **18** which failed Muegge, Ghose, Veber, and Egan rules, it also failed the GSK rule. The reasons for failure are due to high molecular weight, high molar refractivity,

and high topological polar surface area (TPAS). However, it passed the Pfizer rule in addition to Lipinski and Golden Triangle rules.

All the designed compounds exhibited zero alerts indicating that there's no PAINS substructure incorporated in the compounds. Pan-assay interference compounds (PAINS) are chemical compounds that often give false-positive results (false hits) in high-throughput screening assays. They tend to react nonspecifically with numerous biological targets rather than specifically affecting desired targets. The PAINS compounds are undesirable hits and are often filtered off from compound libraries. The designed compounds gave zero alerts for PAINS, therefore they are more suitable for drug discovery bioassays and have drug-like potentials.

The alarm NMR rule is also used to identify potentially reactive or promiscuous compounds. Like PAINS filters, it cannot distinguish between bad or innocent suspects which include covalent inhibitors. The reactivity alerts from alarm NMR can be useful as a good indicator of possible phase I and II metabolic reactions of compounds. The hydrazone moiety comprises both nucleophilic and electrophilic centers, in addition to substitution of ring activators and deactivators on the two rings, therefore these make them reactive compounds with alarm NMR alerts. Possible reduction of nitro group was also captured by the alarm NMR filter.

All the designed compounds gave zero alerts for Bristol-Myers Squibb (BMS) rule translating that none contain undesirable reactive substructure(s). Bristol-Myers Squibb (BMS) rule is used to filter undesirable reactive compounds and reagents that could cause serious toxicities. All the compounds also gave zero alerts for the Chelator rule indicating that none of the compounds are polydentate ligands according to results in **Table 6**. Hydrazone moiety contains two nucleophilic nitrogens. Each of the nitrogens has one lone pair that can coordinate with transition metals to form a coordinate covalent bond i.e the two nitrogen can contribute two co-ordinate covalent bonds to form a complex compound. Therefore, hydrazones are bidentate (didentate) ligands. However, hydrazones do not have the ability to form crown or ball complexes that are characteristic of polydentate ligands (tridentate and above). These ligands are called chelators. Chelators such as crown-ether-like compounds are especially undesirable in drug discovery inputs and are often rejected because of their potential to act as ionophores in addition to their poor druggability.

Table 6 Drug-likeness and Medicinal Chemistry friendliness results (cont'd)

Compounds	Muegge Rule	Ghose Rule	Golden Triangle Rule	PAINS	Alarm NMR Rule	BMS Rule	Chelator Rule
1	Pass		Pass		Pass		0
2	Pass		Pass		Pass		0
3	Pass		Pass		Pass		0
4	Pass		Pass		Pass		0
5	Pass		Pass		Pass		0
6	Pass		Pass		Pass		0
7	Pass		Pass		Pass		0
8	Pass		Pass		Pass		0
9	Pass		Pass		Pass		0
10	Pass		Pass		Pass		0
11	Pass		Pass		Pass		0
12	Pass		Pass		Pass		0
13	Pass		Pass		Pass		0
14	Pass		Pass		Pass		0
15	Pass		Pass		Pass		0
16	Pass		Pass		Pass		0
17	Pass		Pass		Pass		0
18	Fail		Fail		Pass		0
Piroxicam	Pass		Pass		Pass		0
Celecoxib	Pass		Pass		Pass		0

3.2.3 In-silico Pharmacokinetic (ADME) Evaluation.

3.2.3.1 Absorption

The predictive human colon adenocarcinoma cell lines (Caco-2) *in-vivo* drug permeability values of all the compounds range between -6.00 cm/s for compound 18 and -4.33 cm/s for compound 2. Compounds 6, 9, 13, and 18 had values less than -5.15 cm/s minimum for proper Caco-2 permeability. However, these values are higher than -6.05 cm/s for piroxicam according to results in **Table 7**. The rest of the compounds are regarded as compounds with proper Caco-2 permeability with their permeability value greater than -5.15 cm/s.

Table 7 Absorption

Compounds	Caco-2	MDCK	Pgp-inhi.	Pgp-subs.	HIA	F30%	F20%	F10%
1	-4.28	12.0×10^{-6}	0.004	0.032	0.003	0.976	0.062	0.55
2	-4.33	146.0×10^{-6}	0.002	0.004	0.004	0.004	0.001	0.55

3	-4.61	55.0×10^{-6}	0.024	0.016	0.008	0.028	0.003	0.55
4	-4.41	201.0×10^{-6}	0.000	0.028	0.010	0.016	0.002	0.55
5	-4.37	139.0×10^{-6}	0.004	0.008	0.006	0.004	0.001	0.55
6	-5.51	65.0×10^{-6}	0.001	0.083	0.025	0.739	0.002	0.55
7	-4.60	125.0×10^{-6}	0.308	0.010	0.009	0.002	0.002	0.55
8	-4.86	136.0×10^{-6}	0.002	0.025	0.010	0.061	0.002	0.55
9	-5.76	73.0×10^{-6}	0.000	0.061	0.009	0.002	0.002	0.55
10	-5.08	220.0×10^{-6}	0.000	0.005	0.009	0.001	0.002	0.55
11	-4.73	143.0×10^{-6}	0.010	0.004	0.007	0.003	0.001	0.55
12	-4.42	159.0×10^{-6}	0.001	0.015	0.007	0.002	0.001	0.55
13	-5.24	201.0×10^{-6}	0.001	0.010	0.013	0.002	0.002	0.55
14	-4.83	171.0×10^{-6}	0.001	0.016	0.007	0.002	0.002	0.55
15	-5.03	116.0×10^{-6}	0.001	0.008	0.014	0.003	0.002	0.55
16	-5.12	3.0×10^{-6}	0.000	0.171	0.028	0.824	0.014	0.55
17	-5.05	45.0×10^{-6}	0.077	0.031	0.017	0.001	0.001	0.55
18	-6.00	14.0×10^{-6}	0.014	0.731	0.093	0.011	0.002	0.55
Piroxicam	-6.05	18.0×10^{-6}	0.082	0.002	0.010	0.003	0.002	0.56
Celecoxib	-4.77	23.0×10^{-6}	0.084	0.005	0.003	0.001	0.002	0.55

Empirical decision for P-gp, HIA, and F: 0-0.3: excellent; 0.3-0.7: good; 0.7-1.0: poor.

Compounds **1**, **16**, **18**, and piroxicam had medium apparent permeability with *Papp* values greater than 2 x 10^{-6} cm/s but less than 20 x 10^{-6} cm/s in Madin−Darby Canine Kidney cells (MDCK). All other compounds in the library had highly passive permeability greater than 20 x 10^{-6} cm/s.

Compounds **3**, **7**, **11**, and **17** indicated a stronger substrate affinity of P-gp than its inhibition with scores less than 0.30. This was also observed with celecoxib and piroxicam being stronger substrates

than inhibitors of the P-gp enzyme. Compound 18 is a weak or non-substrate of P-gp with a score of 0.73 according to the result in **Table 7**. All other compounds are more potent inhibitors of P-gp than their P-gp substrate tendencies. The observed disparities between Caco-2 permeability and MDCK permeability for the designed hydrazones can be explained by P-gp inhibitory and substrate affinities. Drugs that are P-gp substrates usually have disparities in their Caco-2 and MDCK permeability. Examples include Vinblastine; a P-gp substrate having low permeability in the Caco-2 model but high permeability in the MDCK model. Prazosin is another P-gp substrate that had medium permeability in the Caco-2 model but high permeability in the MDCK model. Also Quinidine, a P-gp substrate had high permeability in the Caco-2 model but medium permeability in the MDCK model. Though most of the designed hydrazones had high passive permeability in both models, however, their permeability disparities can be linked-to P-gp efflux activity. This is the case with compounds 3 and 17 with lower apparent permeability coefficient values compared to compounds 7 and 11 which are also P-gp substrates with high affinity.

The MDCK *in-vitro* permeability model is sensitive to P-gp efflux activity while Caco-2 *in-vivo* model is not. This P-gp efflux activity effect is more pronounced for compounds with high passive permeability ($>20 \times 10^{-6}$ cm/s). The effect of P-gp activity on its substrates' permeability has been reported by Xiannu *et al*.[14] in an experimental study where the permeabilities of P-gp substrates were compared using Caco-2 and MDCK models in the presence and absence of cyclosporin A; a P-gp inhibitor. The study revealed that there was a substantial increase in permeability of P-gp substrates in the MDCK model when cyclosporin A was added. However, there was no observable difference in permeability of P-gp substrates in the Caco-2 model when cyclosporin A was added. This explained why compound 17 had low Caco-2 permeability and high MDCK apparent permeability. It also explained why most of the designed hydrazones which are strong inhibitors of P-gp had high apparent permeability coefficient (*Papp*) values.

The designed compounds demonstrated plausible human intestinal absorption (HIA). All the compounds had HIA+ scores below 0.1 indicating that they are non-HIA+ i.e their human intestinal absorption far exceeds >30% absorbance. Compounds 1 had the best HIA which equal HIA of celecoxib. Compounds 3, 6, 7, 9, 10, 11, 12, and 14 had better HIA compared to piroxicam while compounds 4 and 8 had HIA equal to that of piroxicam.

The results for oral bioavailability predictive evaluation impressively indicated that all the compounds are orally bioavailable. All the compounds except compounds 1, 6, and 16 indicate excellent F30% scores which are quite below 0.1 suggesting that 30% of each of these compounds is orally

bioavailable. However, compounds 1, 6, and 16 scores are 0.976, 0.739, and 0.824 respectively which translates that 30% of each of these compounds may not be orally bioavailable. All the compounds demonstrated exceptional F20% in the predictive model with scores between 0.001 and 0.062 which suggests that 20% of each of these compounds is orally bioavailable. Also, all the compounds displayed interesting F10% scores which suggests that 10% of each of these compounds is orally bioavailable as illustrated in **Table 7** accordingly.

3.2.3.2 Distribution

The plasma protein binding (PPB) for the designed compounds was found to be between 91.28% - 100.995% which is a bit more than the 90% maximum earmarked for proper plasma protein binding (PPB) according to results 8. It was observed that compound 18 had PPB (91.28%) closest to the 90% threshold. The plasma protein binding of the compounds is notwithstanding within the admissible range as many approved drugs were found to have greater than 90% plasma protein binding. It has been noticed that many clinically successful drugs exhibit high PPB. This is indicated by celecoxib PPB (94.96%) which is greater than 90% maximum indicating that there is no fast and hard rule with PPB. Furthermore, this claim is also supported by the documented statistics of drugs approved by the United States Food and Drug Administration (FDA). Almost a third of the 260 marketed medications authorized by the US Food and Drug Administration (FDA) before 2003 have a PPB of greater than 95%. This range is usually regarded as having a high level of protein binding for drugs. Furthermore, 5% of the drugs have a PPB greater than 99 %, which is considered an extremely high PPB. PPB statistics for pharmaceuticals authorized by the US FDA from 2003 to 2013 show that 45 percent of newly approved drugs had a PPB of > 95%, while 24 percent have a PPB of > 99 percent. These findings showed that compounds with a PPB greater than 99% can still be useful medications [15].

Volume distribution of all the designed hydrazones 0.305 L/kg – 2.731 L/kg falls within the proper Vd range 0.04-20 L/kg threshold and are similar to those of reference drugs; piroxicam 0.340 L/kg and celecoxib 1.105 L/kg. Drugs may have a propensity to bind proteins throughout the body where they reach a point of equilibrium between a bound & unbound phase. Depending on the charge of a drug at physiologic pH, a drug may tend to bind macromolecules inside or outside the plasma. The volume distribution (Vd) of these compounds is therefore governed by their acid-base character and their lipophilicity. Since hydrazones are weak bases, they are to have strong interactions with negatively charged phospholipid head groups located on phospholipid membranes. The extent of this binding is also dependent on their overall lipophilicity. In general, basic molecules will leave the

systemic circulation leading to higher volume distribution (Vd) as compared to acidic molecules. Therefore, they exhibit a propensity to leave the plasma and enter the extravascular compartments of the body, meaning that a higher dose of a drug is required to achieve a given plasma concentration. (High Vd = More distribution to other tissue).

All the designed hydrazones indicated very high blood-brain barrier (BBB) permeabilities which are less than 0.1 scores according to results in **Table 8** suggesting that their permeability is in excellent category. The only odd among the designed hydrazones is compound 16 with 0.887 which suggests that the compound may not be able to pass through BBB. The blood-brain barrier (BBB) has been identified as a dynamic interface that maintains optimal conditions for neuronal and glial activity by controlling the flow of chemicals between the blood and the brain. Neurodegenerative diseases (such as Alzheimer's disease and multiple sclerosis), stroke and traumatic brain damage, infectious processes, and inflammatory pain are all thought to be linked to the BBB. As a result of BBB failure in various diseases, transport and permeability may be hindered [16].

Table 8 Distribution

Compounds	Plasma Protein Binding	Volume Distribution	BBB	Fraction Unbound
1	100.52%	2.731 L/kg	0.198	0.82%
2	100.80%	1.779 L/kg	0.107	0.61%
3	98.97%	0.714 L/kg	0.14	1.55%
4	99.25%	0.732 L/kg	0.228	0.74%
5	101.00%	2.085 L/kg	0.072	0.55%
6	98.46%	0.361 L/kg	0.025	1.23%
7	100.50%	1.198 L/kg	0.424	0.74%
8	99.30%	0.534 L/kg	0.045	0.80%
9	97.53%	1.066 L/kg	0.495	2.23%
10	98.77%	0.444 L/kg	0.012	1.14%
11	100.50%	0.942 L/kg	0.333	0.38%
12	99.99%	1.310 L/kg	0.28	0.30%
13	98.37%	0.492 L/kg	0.016	0.97%
14	99.00%	0.597 L/kg	0.181	0.54%
15	98.42%	0.305 L/kg	0.034	1.21%
16	98.30%	1.146 L/kg	0.887	0.83%
17	92.04%	1.021 L/kg	0.401	7.21%
18	91.28%	0.380 L/kg	0.028	11.05%
Piroxicam	73.60%	0.340 L/kg	0.967	27.15%
Celecoxib	94.96%	1.105 L/kg	0.586	5.01%

Empirical decision for BBB: 0-0.3: excellent; 0.3-0.7: good; 0.7-1.0: poor.

The fraction unbound or plasma free drug fraction (F_u) for most of the compounds was less than 5% minimum except for compound 18 with 11% F_u as shown in table 8. This demonstrates that most of the compounds have a low fraction unbound as a consequence of high plasma protein binding (PPB). The observed scores reflected those factors that affect PPB. F_u is determined as free drug concentration divided by the total drug concentration.

In the absence of transporters, the free drug concentration is the same on both sides of the biological membrane at a steady-state, and also the free drug or unbound concentration at the site of action is the species that exert pharmacological activity such as *in-vivo* efficacy and toxicity, according to the free drug hypothesis. This may be true for the designed hydrazones because of their high passive permeability and probable quick rate of permeation, which can accelerate the pace of attaining equilibrium across membranes. However, there are certain exceptions to the second portion of the theory that may apply to designed hydrazones, as follows;

Hydrazone moiety is a reactive center comprising both nucleophilic and electrophilic characters. This enables the moiety to interact irreversibly with the biological target forming covalent bonds with amino residues of the target in addition to reversible interactions with the therapeutic target. Therefore, the activity of hydrazones is dependent on the cumulative concentration of irreversibly bound hydrazone or possible metabolite to the target. The free drug concentration will always determine the initial binding kinetics of the hydrazone to the target or the site of action, but the subsequent time course of receptor occupancy and the pharmacodynamics events that the hydrazone triggers do not follow the time course of free drug concentration. This is because the efficacy of the hydrazone will depend on the deactivation of the biological target and the time taken for the body to resynthesize the biological target and not on the free drug concentration in plasma.

An instance is the selegiline and rasagiline inhibition of monoamine oxidase B (MAO-B) irreversibly and selectively, increasing the half-life of dopamine lowering its metabolism by MAO-B. The inactivation of MAO-B and the time it takes for the body to resynthesize MAO-B, rather than the free drug concentrations of selegiline or rasagiline in plasma, determine the inhibitors' efficacy. Omeprazole and other proton pump inhibitors (PPIs) are other examples, as they initially generate sulphenic acid under acidic circumstances before irreversibly binding with the target [17].

Most often and obviously as it appears from the results in table 4.6 that a decrease in PPB leads to an increase in F_u, however, *in-vivo* in reference to Denni *et al*., 2010 [17] the unbound concentration does

not depend on PPB after oral administration. *In-vivo,* the binding of a drug to plasma proteins does not usually change the concentration of the free drug. Therefore increasing the F_u or decreasing PPB has no effect on the free drug concentration *in-vivo* for most drugs. The exposure of the therapeutic target *in-vivo* to the concentration of the free drug, as measured by the AUC_u, which is the exposure or measurement of the quantity of unbound drug in the body, is independent of the F_u for most orally administered drugs. The total AUC (AUC_{total}) bound plus unbound) decreases as the F_u increases owing to increasing clearance.

Despite the fact that all moderately or highly lipophilic drugs have a high PPB (> 99%), several of them featured in the top 100 most prescribed drugs in 2005. This demonstrates the lack of industry consensus on PPB, with certain compounds moving on in research despite having a low F_u, while others are being eliminated. Diclofenac, ibuprofen, losartan (and its metabolite, EXP3174), naproxen, pioglitazone, rosiglitazone, and montelukast are all drugs having a high PPB. Most of these medications, such as montelukast, have therapeutic dosages in the sub-milligram level, demonstrating that F_u plays no role in their efficacy. Cardiovascular disease (losartan, warfarin, and furosemide); pain (diclofenac and naproxen); metabolic diseases (rosiglitazone, glyburide, and pioglitazone); allergy and respiratory conditions (cetirizine and montelukast); and central nervous system disorders (sertraline) are all examples of drugs with high plasma protein binding [17].

3.2.3.3 Metabolism

The cytochrome P450 (CYP) enzymes are incredibly important in terms of the number of existing drugs that they process, their substrate specificity, polymorphism, and propensity to be key determinants in drug-drug interactions (DDI) [18].

The results in **Table 9** represent the prediction of metabolism of the designed hydrazones by the CYP450 metabolizing enzymes. Among the compounds, compounds 9, 18, and piroxicam are inhibitors of CYP1A2 with scores of 0.02 and 0.01, 0.17 exhibiting potent inhibitor of the metabolizing enzyme. These compounds, however, are weak substrates of the enzyme with 0.93, 0.55, and 0.62 scores respectively. This translates that the metabolizing CYP1A2 may not metabolize compounds 9 and 18. Compounds 2, 5, 6, 10, 12, 13, 15, 16, and 17 are substrates of CYP1A2 with high affinity according to the results in table 9. All other compounds and celecoxib are weak inhibitors and substrates of the metabolizing enzyme. This means that they may not interact with the enzyme CYP1A2. Enzyme CYP1A2 generally metabolizes aromatic amines and heterocyclic compounds.

Table 9 Metabolism.

Compounds	CYP1A2		CYP2C19		CYP2C9		CYP2D6		CYP3A4	
	Inhib.	Subs.	Inhib.	Subs.	Inhib.	Subs.	Inhib.	Subs.	Inhib.	Subs.
1	0.99	0.47	0.95	0.12	0.81	0.84	0.55	0.76	0.20	0.26
2	0.95	0.21	0.91	0.09	0.67	0.92	0.51	0.87	0.27	0.30
3	0.54	0.97	0.60	0.66	0.37	0.84	0.02	0.88	0.58	0.73
4	0.87	0.68	0.57	0.22	0.43	0.94	0.24	0.90	0.61	0.51
5	0.97	0.20	0.88	0.09	0.56	0.77	0.58	0.84	0.26	0.16
6	0.34	0.11	0.38	0.41	0.39	0.95	0.10	0.56	0.21	0.42
7	0.44	0.64	0.92	0.27	0.90	0.94	0.31	0.83	0.39	0.92
8	0.54	0.55	0.48	0.67	0.48	0.92	0.20	0.67	0.33	0.58
9	0.27	0.93	0.47	0.70	0.44	0.93	0.24	0.90	0.26	0.86
10	0.44	0.13	0.38	0.07	0.53	0.96	0.17	0.22	0.43	0.50
11	0.72	0.59	0.94	0.48	0.93	0.98	0.41	0.75	0.60	0.77
12	0.91	0.18	0.81	0.08	0.64	0.91	0.28	0.85	0.44	0.22
13	0.46	0.11	0.10	0.07	0.11	0.85	0.14	0.34	0.32	0.48
14	0.82	0.59	0.28	0.13	0.49	0.88	0.21	0.47	0.21	0.25
15	0.70	0.12	0.40	0.11	0.17	0.90	0.14	0.60	0.45	0.74
16	0.76	0.12	0.34	0.08	0.44	0.87	0.03	0.78	0.41	0.31
17	0.64	0.33	0.88	0.06	0.79	0.90	0.40	0.51	0.83	0.15
18	0.01	0.55	0.23	0.28	0.58	0.98	0.03	0.16	0.72	0.84
Piroxicam	0.17	0.62	0.13	0.20	0.10	0.95	0.22	0.14	0.20	0.58
Celecoxib	0.84	0.60	0.71	0.62	0.86	0.70	0.052	0.60	0.15	0.85

Empirical decision: 0-0.3: excellent; 0.3-0.7: good; 0.7-1.0: poor.

Compounds **1**, **2**, **4**, **5**, **7**, **10**, **11**, **12**, and **15** are strong substrates of CYP2C19. On the other hand, compounds 8 and 9 are strong inhibitors of the metabolizing enzyme. Conversely, compounds 6, 10, 13, 14, 16, and piroxicam are strong inhibitors and substrates of CYP2C19. However, celecoxib and other designed p-nitrophenyl hydrazones are weak inhibitors and substrates of the metabolizing enzyme CYP2C19.

The metabolizing enzyme CYP2C19 is responsible for the inactivating metabolism of proton pump inhibitors and metabolic activation of the anticoagulant clopidogrel. It has a prominent role in antidepressant metabolism, while its endogenous substrates include progesterone and melatonin [19]. Compounds 3, 13, and 15, and piroxicam are strong inhibitors of the CYP2C9 metabolizing enzyme. Compounds 2, 4, 5, 6, 8, 9, 10, 10, 12, 14, 16, 18, and 18 are moderate to weak inhibitors of CYP2C9. Celecoxib and other compounds 17, 11, 7, and 1 are non-inhibitors of the metabolizing enzyme. Also, all the designed compounds and the reference drugs have no substrates affinities/tendencies for CYP2C9 according to results in **Table 9**.

The enzyme CYP2C9 metabolizes weakly acidic substances including anticoagulant warfarin, anticonvulsants, angiotensin receptor blockers, oral antidiabetic agents, and most nonsteroidal anti-inflammatory drugs [19]. Unlike most NSAIDs, hydrazones are weakly basic compounds, this explains why they are not metabolized by CYP2C9.

Compounds 6, 8, 14, 15, 17, and celecoxib are weak substrates of CYP2D6. 1 – 5, 7, 9, 11, 12, and 16 are non-substrate of CYP2D9. Compounds 3, 4, 7 – 18, and celecoxib are strong inhibitors of the CYP2D6 metabolizing enzyme. Compounds 2 and 5 are weak inhibitors of the metabolizing enzyme. Piroxicam is a strong inhibitor and substrate of CYP2D6.

CYP2D6 is poorly expressed in the liver, despite this fact it accounts for the metabolism of 15-25% of all drugs due to its extraordinarily broad substrate selectivity. These include antiarrhythmics, antidepressants, antipsychotics, β-blockers, opioid analgesics, and anticancer drugs. CYP2D6 shows the greatest impact of genetic polymorphism among all major xenobiotic-metabolizing CYPs. Individual polymorphs of CYP2D6 are classified as poor, intermediate, efficient, or ultrarapid metabolizers. Poor metabolizers have significantly altered the metabolism of several major drug classes. This can lead to failure of detoxification and adverse drug reactions, while extensive and ultrarapid metabolizers may exhibit poor therapeutic responsiveness to CYP2D6 substrates because of rapid clearance. As a consequence, the development of drug candidates which are metabolized by CYP2D6 is usually discontinued [19].

Compounds 3, 4, 11, 12, 15, and 16 are weak inhibitors of CYP3A4. Compounds 17 and 18 are non-inhibitors of CYP3A4. The rest of the designed compounds, piroxicam and celecoxib are strong inhibitors of the metabolizing enzyme. Likewise, compounds 4, 8, 10, 13, and piroxicam are weak substrates of CYP3A4. Compounds 3, 9, 11, 15, 18, and celecoxib are non-substrates of CYP3A4. The rest of the compounds as displayed in table 9 are strong inhibitors of the metabolizing enzyme.

CYP3A4 is the predominant CYP isoform in the human liver which can account for up to 50% of total hepatic CYP expression. CYP3A4 metabolizes drugs such as immunosuppressants, macrolide antibiotics, benzodiazepines, statins, antidepressants, opioids, and anticancer drugs. It is also involved in endogenous steroid catabolism [19].

3.2.3.4 Excretion

Drug clearance is an important pharmacokinetic parameter that defines, together with the volume of distribution, the half-life, and thus the frequency of dosing of a drug. Compounds **1**, **3**, and **4** had clearance between 5 mL/min/kg and 15 mL/min/kg which lies in the moderate clearance. Compounds **2**, 5 – 18 have clearance of less than 5 ml/min/kg by the results in **Table 10** indicating low clearance.

Compounds **3** and piroxicam have moderate half-lives which may be greater than three hours. Compound 16 half-life may be less than three hours. Celecoxib and other designed hydrazones showed very high score for half-lives therefore have half-lives greater than three hours.

Table 10 Excretion

Compounds	Clearance (mL/min/kg)	Half-life ($T_{1/2}$)
1	5.697	0.152
2	3.852	0.092
3	6.34	0.383
4	5.638	0.161
5	3.913	0.124
6	0.809	0.09
7	1.585	0.087
8	1.1	0.05
9	2.189	0.053
10	0.543	0.079
11	1.433	0.019
12	4.148	0.049
13	0.98	0.101
14	1.1	0.046
15	0.509	0.067
16	2.06	0.717
17	4.539	0.197

18	1.42	0.099
Piroxicam	1.033	0.561
Celecoxib	0.992	0.029

Empirical decision for $T_{1/2}$: 0-0.3: excellent; 0.3-0.7: good; 0.7-1.0: poor.

3.2.4 Toxicity

The results for different toxicity endpoints for the designed compounds are in tables 11 to 16. For the proper analysis of their toxicities, some approved anti-inflammatory drugs including piroxicam, celecoxib, and acetylsalicylic acid (ASA) are used as references. Also, a cardioprotective hydrazone drug levosimendan (LSD) and a simple aromatic hydrazone (SAH) were used as references for the inherent compound class toxicities. The percentage accuracy of prediction and percentage average similarity of each compound compared to the data sets of the models used are also estimated in the results in **Table 11**

Table 11 Organ toxicity continued.

Compounds	Eye corrosion	Eye irritation	Prediction-Accuracy(%)	Average Similarity(%)
1	0.61	0.99	70.97	87.77
2	0.47	0.99	69.26	70.27
3	0.22	0.82	68.07	63.74
4	0.77	0.99	69.26	70.57
5	0.78	0.99	68.07	67.42
6	0.003	0.13	54.26	46.30
7	0.003	0.01	54.26	43.93
8	0.003	0.06	54.26	45.06
9	0.003	0.02	54.26	45.53
10	0.003	0.04	54.26	48.42
11	0.003	0.01	54.26	46.53
12	0.79	0.99	68.07	61.99

13	0.003	0.92	67.38	56.53
14	0.003	0.57	54.26	48.40
15	0.004	0.32	67.38	56.53
16	0.004	0.35	67.38	52.64
17	0.003	0.03	54.26	48.99
18	0.003	0.01	23.00	36.84
Piroxicam	0.02	0.003	54.26	47.16
Celecoxib	0.003	0.08	54.26	48.08
ASA	0.01	0.99	100.00	100.00
Levosimendan	0.01	0.30	69.26	73.69
SAH	0.96	1.00	70.97	82.53

Empirical decision for Eye corrosion/irritation: 0-0.3: excellent; 0.3-0.7: good; 0.7-1.0: poor.

3.2.4.1 Organ toxicity.

The predicted lethality doses (LD_{50}) of the compounds are tabulated in **Table 12**. Compounds **12**, **13**, **14**, **15**, and aspirin (ASA) are categorized by their LD_{50} as Class III: (50 mg/kg < LD_{50} ≤ 300 mg/kg) which suggests that they are toxic if swallowed. Compounds **1**, **2**, **3**, **4**, **5**, **7**, **8**, **16**, **17**, **18**, SAH, piroxicam, celecoxib, and LSD are categorized by their predicted LD_{50} into Class IV (300mg/kg < LD_{50} ≤ 2000mg/kg). This translates that they are harmful if swallowed. Compounds 6, 10, and 11 are categorized as Class V: (2000mg/kg < LD_{50} ≤ 5000kg/kg) suggesting that they may be harmful if swallowed according to the toxic class of the globally harmonized system (GHS) of classification of labeling of chemicals.

Table 12 Organ Toxicity.

Compounds	LD50(mg/kg)	ToxicClass.	H-HT/DILI	Carcino.	Immuno.	Mutagen.	Cytotoxic.
1	1000	4	0.03	0.58(A)	0.92(I)	0.58(I)	0.81(I)

2	1000	4	0.05	0.50(A)	0.64(I)	0.57(A)	0.68(I)
3	1800	4	0.27	0.58(A)	0.96(A)	0.58(A)	0.72(I)
4	1500	4	0.13	0.67(A)	0.76(I)	0.85(A)	0.72(I)
5	800	4	0.09	0.57(A)	0.81(I)	0.73(A)	0.66(I)
6	3200	5	0.28	0.52(I)	0.55(A)	0.69(A)	0.74(I)
7	1450	4	0.21	0.56(I)	0.56(I)	0.50(I)	0.54(A)
8	1450	4	0.63	0.52(A)	0.50(I)	0.78(A)	0.54(A)
9	2000	4	0.54	0.50(I)	0.92(A)	0.62(A)	0.60(I)
10	3200	5	0.58	0.53(I)	0.81(I)	0.66(A)	0.66(I)
11	3200	5	0.96	0.54(I)	0.79(A)	0.64)(A)	0.74(I)
12	220	3	0.11	0.53(A)	0.93(I)	0.54(I)	0.61(I)
13	250	3	0.43	0.59(A)	0.99(I)	0.58(I)	0.75(I)
14	250	3	0.74	0.50(I)	0.53(A)	0.55(I)	0.82(I)
15	250	3	0.75	0.56(A)	0.88(I)	0.54(I)	0.77(I)
16	1000	4	0.03	0.68(A)	0.66(I)	0.72(A)	0.72(I)
17	1000	4	0.86	0.72(A)	0.77(I)	0.73(I)	0.68(I)
18	600	4	0.52	0.52(I)	0.74(I)	0.68(A)	0.65(I)
Piroxicam	480	4	0.25	0.71(A)	0.69(I)	0.71(I)	0.65(I)
Celecoxib	1400	4	0.64	0.56(A)	0.99(I)	0.75(I)	0.91(I)
ASA	250	3	0.26	0.80(I)	0.99(I)	0.97(I)	0.94(I)
Levosimendan	507	4	0.10	0.66(A)	0.99(I)	0.50(A)	0.78(I)
SAH	1250	4	0.02	0.73(A)	0.99(I)	0.91(A)	0.82(I)

Empirical decision for H-HT/DILI: 0-0.3: excellent; 0.3-0.7: good; 0.7-1.0: poor.

Key: A = Active, I = Inactive.

The human hepatotoxicity (H-HT) or drug-induced liver injury (DILI) prediction results are tabulated in **Table 12**. Compounds 8, 9, 10, 11,13, and celecoxib had good safety H-HT/DILI profiles compared to other designed hydrazones as indicated in table 11. Compounds **11**, **14**, **15**, and **18** may be hepatotoxic according to their predicted toxicity in **Table 12**. Hepatotoxicity is a potential complication of nearly all classes of medication. Non-steroidal anti-inflammatory drugs (NSAIDs) are also an important cause of hepatotoxicity.

Though piroxicam (0.25) and ASA (0.26) scores are within the excellent non-hepatotoxic range. However, the celecoxib score (0.64) indicated a possibly considerable hepatotoxicity risk. Studies have shown that diclofenac is glucuronylated and also subjected to cytochrome P450-mediated reactions that result in bioactive products [20]. Both its reactive metabolites and immune mechanisms mediate toxicity. The decreased prostaglandin synthesis due to cyclooxygenase (COX) inhibition may also enhance injury. Other NSAIDs including bromfenac, nimesulide, and sulindac are associated with hepatotoxicity. Nimesulide administration has been reported to elicit severe toxicity resulting in acute liver failure. Sulindac and ibuprofen are associated with cholestatic DILI that is reversible after drug withdrawal, although fatal cases have also been reported [20].

Most of the designed compounds have moderate risk of carcinogenicity, including piroxicam, celecoxib, LSD, and SAH as indicated in table 11. The carcinogenicity of the hydrazones as well as the reference drugs may be due to the presence of primary and secondary amine functional groups in their structures as the case may be. It has been reported that primary and secondary amine groups in several drugs react *in-vitro* and *in-vivo* with sodium nitrite yielding N-nitroso compounds. A typical instance is the development of hemorrhagic liver tumors in rats due to the formation of dimethylnitrosamine (DMN) from aminopyrine and nitrite in the rat stomach [21]. Piroxicam, celecoxib, and levosimendan are also predicted to be carcinogenic for the same reason. Though ASA was found to be non-carcinogenic with 0.81 probability, it has been reported that its sodium salt (sodium salicylate) is carcinogenic [22].

Compounds 3, 6, 9, 11, and 14 were predicted to be immunotoxic. The reference approved NSAIDs used were inactive with probabilities of 0.69 for piroxicam, 0.99 for celecoxib, and 0.99 for ASA. However, several experimental studies have reported the immunotoxicity of NSAIDs; Yoshioka *et al*. reported lethal immunotoxicity of co-administration of indomethacin and β-glucan in mice due to significantly elevated concentrations of Interferon (IFN)-ϒ, interleukin (IL)-6 and colony-stimulating

factor (CSF) concentrations in sera of indomethacine/L-glucan-treated mice [23]. Further studies reported similar lethality for aspirin, diclofenac, and sulindac [24].

Haematotoxicity and immunotoxicity of diclofenac and ibuprofen due to immunomodulatory effects on levels of Immunoglobulin G (IgG) and Immunoglobulin M (IgM), in addition to perturbations in immune-related organs (spleen, bone marrow, and lymph node) have been reported [25]. The drugs also induced an increase in CRP level in serum and enhanced activation of the alternative complement system that may contribute to deleterious reactions.

Most of the designed hydrazones have been predicted as mutagenic, including SAH and reference drug LSD according to the results in table 11. Though, piroxicam, celecoxib, and ASA were predicted to be non-mutagenic with probabilities of 0.71, 0.75, and 0.95 respectively. Studies have found that some NSAIDs including indomethacin, oxyphenbutazone, and methyl salicylate are mutagenic in the Ames test [23].

Nearly all the designed hydrazones are non-cytotoxic according to the results in **Table 11**. Exceptions are compounds 6 and 8 which have been predicted to be cytotoxic. Contrary to the cytotoxicity prediction of NSAIDs, experimental studies have been reported on the direct cytotoxicity (apoptosis and necrosis) of NSAIDs [26]. Furthermore, *in-vivo* analysis using both oral and intravenous administration of NSAIDs suggested that not only COX inhibition but also the COX-independent direct cytotoxic effect of NSAIDs involved in the development of gastric lesions. Reported NSAIDs with cytotoxicity include indomethacin, piroxicam, celecoxib, diclofenac, ibuprofen, etodolac, and aspirin. However, rofecoxib was found to induce neither necrosis nor apoptosis [27]. Also, naproxen was reported as non-cytotoxic in another study [28]. Also, ASA, salicylate derivatives, oxyphenbutazone, diclofenac sodium, and indomethacin were found to be carcinogenic and cytotoxic in Rec-assay [23].

All the designed hydrazones had excellent safety profile 0.03 – 0.38, and are therefore non-blocker of the human ether-a-go-go related gene as demonstrated by the results in **Table 13**. They may not cause hERG toxicities which include long QT syndrome (LQTS), arrhythmia, and Torsade de Pointes (TdP), which lead to palpitations, fainting, or even sudden death.

The predicted rat or mice oral acute toxicity (OAT) of the designed compounds in **Table 13** including SAH, and piroxicam are in the range of 0.02-0.40 which are way within excellent to good indication of safety score for low toxicity i.e >500mg/kg. However compound 17, celecoxib, LSD, and ASA with scores between 0.76 – 0.89 suggests that these compounds may exhibit oral acute toxicity in mice or rats with dose ≤ 500mg/kg.

Table 13 Organ toxicity continued.

Compounds	hERG Blocker	OAT	FDAMDD	Skin Sensit.	Respiratory Toxicity
1	0.02	0.04	0.67	0.89	0.02
2	0.14	0.03	0.74	0.92	0.81
3	0.22	0.03	0.18	0.94	0.80
4	0.20	0.02	0.64	0.95	0.86
5	0.16	0.02	0.81	0.89	0.69
6	0.04	0.05	0.94	0.25	0.03
7	0.34	0.04	0.85	0.79	0.65
8	0.19	0.03	0.93	0.29	0.03
9	0.38	0.12	0.95	0.19	0.86
10	0.01	0.22	0.98	0.42	0.96
11	0.03	0.03	0.96	0.09	0.78
12	0.07	0.02	0.57	0.36	0.93
13	0.10	0.12	0.93	0.12	0.19
14	0.03	0.08	0.95	0.08	0.94
15	0.21	0.04	0.86	0.59	0.05
16	0.11	0.28	0.85	0.53	0.96
17	0.16	0.89	0.63	0.61	0.94
18	0.04	0.25	0.94	0.13	0.14
Piroxicam	0.02	0.40	0.21	0.08	0.77
Celecoxib	0.11	0.77	0.68	0.01	0.58
ASA	0.02	0.76	0.01	0.51	0.27
Levosimendan	0.03	0.89	0.87	0.42	0.99

SAH	0.01	0.02	0.34	0.95	0.06

Empirical decision: 0-0.3: excellent; 0.3-0.7: good; 0.7-1.0: poor.

The FDA maximum recommended daily dose (FDAMDD) of compounds 1, 4, 12, 17, and celecoxib equals 0.011 mmol/kg -bw/day. The FDAMDD for compound 3, piroxicam, SAH, and ASA is greater than 0.011 mmol/kg -bw/day. However, for most of the compounds and the reference drug LSD, their FDAMDD is less than 0.011 mmol/kg -bw/day. The FDAMM provides an estimate of the toxic dose threshold of chemicals in humans.

The results in **Table 13** predicted that compounds 1 – 5, 7, and SAH are sensitive to skin and may not be formulated for topical formulations. However, compounds 10, 15, 16, 17, ASA, and LSD may be tolerant as skin topical medicine. Compounds 6, 8, 9, 11 – 14, 18, piroxicam, and celecoxib may be well tolerated by the skin.

All the reference NSAIDs had non-sensitive scores from the prediction except for ASA with a moderate skin sensitivity score of 0.51. As with hydrazones, the prediction is not tantamount to possible skin dermatological toxicity. Regardless, the dermatological toxicity of NSAIDs is well documented [29]. This involves a wide spectrum of skin disorders which include urticarial rash and other conditions as severe as toxic epidermal necrolysis and Stevens-Johnson syndrome, although the two latter skin disorders are uncommon. Pseudoporphyria, i.e. skin blistering occurring on sun-exposed areas of skin, is an interesting phenomenon that is now recognized with NSAID use.

The result of respiratory toxicity is tabulated in table 12 accordingly. Compounds 2 – 4, 9 – 12, 14, 17, piroxicam, and levosimendan (LSD) are predicted to cause respiratory toxicity. Compounds 1, 8, 13, 15, 18, aspirin (ASA), and simple aromatic hydrazone (SAH) are predicted as non-respiratory toxic compounds with excellent safety profiles. Whereas compounds 5, 6, 7, and celecoxib are predicted to be moderately safe compounds. Some reports indicated the allergic and pseudo allergic reactions of NSAIDs leading to urticaria/angioedema including anaphylactic shock and asthma [30, 31].

Although aspirin was predicted to be non-toxic with 0.27, aspirin-exacerbated respiratory disease (formerly known as aspirin-induced asthma or aspirin intolerant-asthma) has also been reported. It presents with bronchospasm which may affect the entire respiratory tract and is associated with other allergic symptoms, such as flushing, conjunctiva injection, and nasal congestion. It is also associated with an increased incidence of Reye's syndrome, an often fatal, fulminating hepatitis with cerebral edema [32]. Respiratory alkalosis and pulmonary infiltrates with eosinophilia are other documented NSAIDs reactions.

3.2.4.2 Tox21 Pathway.

3.2.4.2.1 Nuclear receptor pathway toxicity

The designed hydrazones were evaluated for their possible interactions with the nuclear receptors. It was discovered from the results in **Table 14** that the designed hydrazones may not interact with most of the nuclear receptors except for the aryl hydrocarbon receptor (NR-AhR) and estrogen receptor (NR-ER). Compounds 1 – 4, and SAH may interact with aryl hydrocarbon receptors (NR-AhR) with probabilities of 0.73, 0.52, 0.60, 0.54, and 0.66 respectively. Compounds 1, 4, and SAH may also interact with estrogen receptor with probabilities of 0.52, 0.64, and 0.67 respectively.

The aryl hydrocarbon receptor (NR-AhR) is a ligand-activated transcription factor that integrates environmental, dietary, microbial, and metabolic stimulants to control transcriptional programs in a ligand-specific, cell-type-specific, and context-specific manner [33]. Many natural compounds, synthetic drugs, and endogenous metabolites are ligands for the AhR, which have therapeutic potential for use in treating diseases through pathway "cross-talk". Interactions between AhR and NF-kB pathways in the lung strongly suggest the importance of this cross-talk in diseases such as lung carcinogenesis, inflammation of the lung; asthma [34]. As such, the interaction of the stated compounds with the NR-AhR may not lead to toxicity. In addition, there is a cross-talk between NR-AhR and NR-ER.

However, when estrogen receptors are activated by small molecules other than estrogens, the expression of the associated genes is deregulated leading to neurological, developmental, and reproductive toxicity. AhR can also increase the degradation of ERα or the androgen receptor [35].

Table 14 Nuclear receptor pathway toxicity

Compounds	NR-AR	NR-AR-LBD	NR-AhR	NR-Ar.	NR-ER	NR-ER-LBD	NR-PPAR-γ
1	0.91(I)	0.98(I)	0.73(A)	0.55(I)	0.52(A)	0.77(I)	0.96(I)
2	0.98(I)	0.89(I)	0.52(A)	0.68(I)	0.75(I)	0.73(I)	0.93(I)
3	0.85(I)	0.99(I)	0.60(A)	0.75(I)	0.55(I)	0.86(I)	0.97(I)
4	0.69(I)	0.99(I)	0.54(A)	0.61(I)	0.64(A)	0.94(I)	0.97(I)
5	0.99(I)	0.97(I)	0.59(I)	0.69(I)	0.71(I)	0.78(I)	0.93(I)
6	0.97(I)	0.97(I)	0.82(I)	0.93(I)	0.86(I)	0.89(I)	0.96(I)
7	0.93(I)	0.98(I)	0.61(I)	0.86(I)	0.89(I)	0.91(I)	0.95(I)
8	0.90(I)	0.97(I)	0.81(I)	0.86(I)	0.87(I)	0.87(I)	0.96(I)
9	0.94(I)	0.96(I)	0.85(I)	0.87(I)	0.86(I)	0.83(I)	0.96(I)
10	0.98(I)	0.98(I)	0.79(I)	0.93(I)	0.88(I)	0.76(I)	0.95(I)
11	0.97(I)	0.98(1)	0.79(I)	0.89(1)	0.88(I)	0.82(I)	0.95(I)
12	0.98(I)	0.99(I)	0.62(I)	0.79(I)	0.65(I)	0.83(I)	0.92(I)
13	0.99(I)	0.98(I)	0.97(I)	0.97(I)	0.94(I)	0.97(I)	0.98(I)

14	0.95(I)	0.97(I)	0.97(I)	0.90(I)	0.84(I)	0.95(I)	0.96(I)
15	0.97(I)	0.99(I)	0.89(I)	0.95(I)	0.90(I)	0.95(I)	0.97(I)
16	0.93(I)	0.98(I)	0.65(I)	0.90(I)	0.85(I)	0.95(I)	0.95(I)
17	0.93(I)	0.99(I)	0.64(I)	0.90(I)	0.86(I)	0.96(I)	0.96(I)
18	0.93(I)	0.98(I)	0.89(I)	0.96(I)	0.90(I)	0.95(I)	0.95(I)
Piroxicam	0.97(I)	0.99(I)	0.87(I)	0.92(I)	0.90(I)	0.97(I)	0.95(I)
Celecoxib	1.00(I)	0.99(I)	0.99(I)	0.93(I)	1.00(A)	0.98(I)	0.98(I)
ASA	0.99(I)	1.00(I)	0.99(I)	1.00(I)	0.98(I)	0.99(I)	0.99(I)
Levosimendan	0.98(I)	0.98(I)	0.66(I)	0.93(I)	0.88(I)	0.99(I)	0.98(I)
SAH	0.99(I)	1.00(I)	0.66(A)	0.84(I)	0.67(A)	0.96(I)	0.91(I)

Key: A = Active, I = Inactive.

3.2.4.2.2 Stress response pathway toxicity

Compounds 1 – 6, and 12 – 15 are predicted to interact with stress response - mitochondrial membrane potential (SR-MMP) with probabilities as indicated in **Table 15**. The role of MMP in "mito-inflammation" has been well documented [36]. Therefore, the possible interaction of the designed compounds with MMP may not lead to toxicity.

Table 15 Stress response pathways toxicity

Compound	SR-ARE	SR-ATAD5	SR-HSE	SR-MMP	SR-p53
1	0.76 (I)	0.60(I)	0.76(I)	0.69(A)	0.77(I)
2	0.82 (I)	08.2(I)	0.82(I)	0.61(A)	0.75(I)
3	0.90 (I)	0.68(I)	0.90(I)	0.64(A)	0.88(I)
4	0.94 (I)	0.56(I)	0.94(I)	0.54(A)	0.77(I)
5	0.84 (I)	0.88(I)	0.84(I)	0.73(A)	0.78(I)
6	0.94 (I)	0.85(I)	0.94(I)	0.51(A)	0.88(I)
7	0.93 (I)	0.80(I)	0.93(I)	0.60(I)	0.78(I)
8	0.94 (I)	0.85(I)	0.94(I)	0.53(I)	0.89(I)
9	0.90 (I)	0.85(I)	0.90(I)	0.56(I)	0.90(I)
10	0.91 (I)	0.80(I)	0.91(I)	0.53(I)	0.90(I)
11	0.93 (I)	0.83(I)	0.93(I)	0.50(I)	0.91(I)
12	0.87 (I)	0.90(I)	0.87(I)	0.70(A)	0.83(I)
13	0.97 (I)	0.99(I)	0.97(I)	0.63(A)	0.96(I)
14	0.96 (I)	0.97(I)	0.96(I)	0.52(A)	0.91(I)
15	0.97 (I)	0.96(I)	0.97(I)	0.59(A)	0.95(I)
16	0.89 (I)	0.88(I)	0.89(I)	0.67(I)	0.76(I)
17	0.91 (I)	0.88(I)	0.91(I)	0.61(I)	0.72(I)
18	0.91 (I)	0.92(I)	0.91(I)	0.58(I)	0.91(I)
Piroxicam	0.97 (I)	0.95(I)	0.97(I)	0.58(I)	0.87(I)
Celecoxib	0.99 (I)	0.98(I)	0.99(I)	0.87(I)	0.95(I)
ASA	0.99 (I)	0.99(I)	0.99(I)	0.97(I)	0.98(I)
Levosimendan	0.98 (I)	0.98(I)	0.98(I)	0.88(I)	1.00(A)

SAH	0.89 (I)	0.61(I)	0.89(I)	0.51(I)	0.94(I)

Key: A = Active, I = Inactive.

3.2.4.3 Environmental toxicity

The results for environmental toxicity predictions are in **Table 16**. Compounds 1, 2, 3, and 5 had bioconcentration factor (BCF) values 3.000 log10(L/kg) < BCF < 3.700 log10(L/kg) which is equivalent to 1000 L/kg < BCF < 5000 L/kg categorized as bioaccumulative by the United States Environmental Protection Agency under the Toxic Substances Control Act (TSCA). Other designed hydrazones and reference drugs had BCF < 3.000 log10(L/kg) which corresponds to BCF < 1000 L/kg categorized as non-bioaccumulative by the US EPA under the TSCA. Compounds 1, 2, and 5 with BFC values of 3.404, 3.429, and 3.345 log10(L/kg) respectively are below the 3.700 log10(L/kg) threshold by Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) threshold for very bioaccumulative chemicals. While other designed hydrazones and reference drugs are below the 3.300 log10(L/kg) REACH threshold for bioaccumulative chemicals.

Table 16 Environmental Toxicity

Compound	BCF [log10(L/kg)]	IGC_{50}	$LC_{50}FM$	$LC_{50}DM$
1	3.404	4.980	5.682	6.003
2	3.429	5.052	5.998	6.291
3	3.027	4.279	5.382	6.472
4	2.903	4.637	5.730	6.329
5	3.345	5.049	6.000	6.242
6	0.539	4.313	5.249	5.473
7	2.536	4.651	5.984	5.924
8	1.058	4.574	5.509	5.853
9	0.816	4.692	5.563	5.778
10	0.843	4.933	6.785	6.231
11	0.944	4.917	5.849	6.042
12	2.775	4.738	5.935	6.484
13	1.110	4.504	5.286	6.067
14	1.492	4.690	5.563	6.519
15	0.503	3.987	4.937	5.297
16	1.472	4.885	6.511	5.968
17	0.956	4.463	4.879	5.436
18	0.420	4.157	4.385	4.653
Piroxicam	0.359	3.991	3.521	4.382
Celecoxib	1.559	4.585	5.332	6.252
ASA	0.217	2.661	3.748	2.997

Levosimendan	0.735	4.790	5.900	5.408
SAH	2.728	4.443	5.202	5.487

Unit for IGC_{50}, $LC_{50}FM$, $LC_{50}DM$ is $-\log10[(mg/L)/(1000 \times MW)]$

The results for 48 hours of Tetrahymena pyriformis (IGC_{50}) for all the compounds are also tabulated in table 16. These results indicated the concentration of the designed hydrazones in water in mg/L that could cause 50% growth inhibition to Tetrahymena pyriformis after 48 hours. According to the results, most of the compounds are less toxic to the tetrahymena pyriformis compared to the reference drugs. Compounds 15 with an IGC_{50} value of 3.987 $-\log10[(mg/L)/(1000\times MW)]$ which is slightly lower than the IGC_{50} value of piroxicam but higher than those of celecoxib, ASA, and LSD. This suggests that compound 15 is more toxic than piroxicam but less toxic than celecoxib, ASA, and LSD.

Likewise, the 96-hour fathead minnow LC_{50} ($LC_{50}FM$) results in table 16 indicate the predicted concentration of the designed hydrazones in water in mg/L that could cause 50% of fathead minnow to die after 96 hours. Compounds 10 and 16 are the safest of all the designed compounds. Compounds 2, 5, 7, and 12 are less toxic compared to levosimendan (LSD). Compounds 11 had comparable $LC_{50}FM$ to LSD. Compounds 1, 3, 4, 8, 9, and 14 are more toxic than LSD but less toxic than celecoxib. Compounds 6 and 13 have comparable $LC_{50}FM$ to celecoxib. Compounds 15, 17, and 18 are more toxic compared to LSD and celecoxib but are safer than piroxicam and aspirin according to their $LC_{50}FM$ in table 16.

Compounds 1, 2, 3, 4, 5, 10, 12, 13, and 14 had $LC_{50}DM$ (the concentration of the designed hydrazones in water in mg/L that could cause 50% of Daphnia Magna to die after 48 hours) greater than 6.000 $-\log10[(mg/L)/(1000\times MW)]$, values comparable to celecoxib $LC_{50}DM$ of 6.252 $-\log10[(mg/L)/(1000\times MW)]$. This implies that the these compounds are as safe as celecoxib to the Daphnia Magna but nonetheless safer than piroxicam, aspirin, and LSD with $LC_{50}DM$ of 4.382 $-\log10[(mg/L)/(1000\times MW)]$, 2.997 $-\log10[(mg/L)/(1000\times MW)]$ and 5.408 $-\log10[(mg/L)/(1000\times MW)]$ respectively. Compounds 6, 7, 8, 9, 11, 15, 16, and 17 had $LC_{50}DM$ which are safer than those of piroxicam and ASA, and are safer or comparable to that of LSD $LC_{50}DM$ according to results in table 16. Compound 18 $LC_{50}DM$ is safer than that of piroxicam and ASA but less safe compared to celecoxib and LSD. Taken together, all the designed hydrazones are apparently benign to the aquatic environment. A preprint of these results has been previously reported [37].

Conclusion

This docking analysis has revealed that compounds 3, 6, 8, 11, 13, 14, 16, and 17 indicated promise as potent multi-target inhibitors of COX-2, 5-LOX, and H^+/K^+ ATPase with potential anti-inflammatory activity devoid of adverse effects of NSAIDs. These compounds demonstrate plausible pharmacokinetic profiles with apparent safety profiles.

Conflict of interest

The authors declare no conflict of interest.

Authors' declaration

The authors of this work as named in the article declare that this work was done by the authors and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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