“Somewhere, something incredible is waiting to be known”.

Carl Edward Sagan

“I know that two and two make four and should be glad to prove it too if I could. Though I must say if by any sort of process I could convert two and two into five, it would give me much greater pleasure”.

Lord Byron

University of Alberta

NSAID Prodrugs with Improved Anti-inflammatory Activity and Low Ulcerogenicity: Wake Up Call to Pharmaceutical Companies and Health Authorities

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Pharmaceutical Sciences

Faculty of Pharmacy & Pharmaceutical Sciences

Spring 2012
Edmonton, Alberta

To my lovely mother and grandparents,

without their support this work

would not have been possible.

Abstract

The objective of this work was to synthesize and evaluate the biological properties of a new series of nitric oxide-releasing non-steroidal anti-inflammatory drugs (NO-NSAIDs) possessing a tyrosol linker between the carboxylic acid present in classical NSAIDs and a NO-releasing group (PROLI/NO) derived from the naturally occurring amino acid L-proline; however, initial screening of ester intermediates without the PROLI/NO group showed the desired efficacy/safety ratio. The NSAID prodrugs were potent selective COX-2 inhibitors and showed equipotent anti-inflammatory activity compared to the corresponding parent NSAIDs, but showed a markedly reduced gastric toxicity. Furthermore, simple NSAID ester prodrugs were able to increase the activity of phase II carcinogen-metabolizing enzymes (NQO1); however, unlike NCX-4016 (NO-aspirin), NSAID esters were not effective inhibitors of platelet aggregation. These results provide complementary evidence to assume that the use of NO-releasing groups in hybrid NSAID prodrugs *is not required to decrease the ulcerogenic profile of classical NSAIDs*.

Acknowledgements

First and foremost I would like to say a big thanks to all the persons involved in my thesis directly or indirectly and in helping me to achieve this Master's of Science degree. Secondly, I am heartily thankful to my supervisor, Dr. Carlos A. Velázquez, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. He continuously provided essential advice for all the issues that arose during the development of this work. He has made available his support in a number of ways, his immediate response to questions, his inspiration, his positive attitude and his encouragement when I was working on tough problems. All this has made the past two and a half years a very enjoyable experience. Through his continuous emphasis on quality I was motivated to strive for excellence in my work.

I am grateful to Dr. Edward E. Knaus, Dr. Ayman El-Kadi and Dr. Paul Jurasz for their guidance and support in finishing various projects related to the thesis. I would also like to thank my supervisory committee members Dr. Kamaljit Kaur and Dr. Glen Baker. I am thankful to Dr. Vishwanatha Somyaji for his support in providing the spectral data. I am indebted to my lab members and colleagues Fahad Aldawsari, Keriman Ozadali, Mohamed El Gendy and Shofiur Rahman for showing their support of me. I would also like to thank Anja Kruessel for providing helpful feedback on the thesis writing.

It is an honour for me to thank Dr. Antoine Noujaim, who endowed the Antoine Noujaim Graduate Scholarship in Pharmaceutical Sciences of which I was the recipient. I thank the University of Alberta for being a great source of motivation and encouragement. Finally, I would like to thank my mother, to whom this thesis is dedicated. I owe her and my grandparents endless thanks for their guidance in my youth and for serving as role models in my adulthood.

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List of Abbreviations

δ - In NMR context means “chemical shift values”, expressed in parts per million

MST - 3-Mercaptopyruvate sulfurtransferase

AAPCC - NPDS -American association of poison control centers, National poison data system

ARE - Antioxidant responsive element

AA - Arachidonic acid

CO - Carbon monoxide

$CDCl_3$ – Deuterated chloroform

Conc. - Concentrated

CBS - Cystathionine β-synthase

CSE - Cystathionase

COXIBS - Cyclooxygenase-2 inhibitors

COX - Cyclooxygenase

COX-1 - Cyclooxygenase-1

COX-2 - Cyclooxygenase-2

DMSO - Dimethyl sulfoxide

CH_2CL_2 - Dichloromethane

GRAS - Generally regarded as safe

GI - Gastrointestinal

GIT - Gastrointestinal tract

IFN - Interferon

IL - Interleukin

H_2S - Hydrogen sulphide

LOX - Lipoxygenase enzymes

NO - Nitric oxide

NCX-4016 - NO-Aspirin

MA - Mefenamic acid

MTT – 3-(4,5-Dimethylthiazol-2-(yl-2,5-diphenyltetrazolium bromide))

PGs - Prostaglandins

THF - Tetrahydrofuran

TEA - Triethylamine

TXs - Thromboxanes

TNF-α - Tumor necrosis factor alpha

UI - Ulcer index

1 Chapter: Introduction

Over a century has passed since the synthesis of acetylsalicylic acid (aspirin) as the first non steroidal anti-inflammatory drug (NSAID) by Felix Hoffman, working at Bayer Industries[1-2]. Since then, NSAIDs have become one of the most widely prescribed group of drugs worldwide, and drugs of first choice to treat pain, fever, and inflammation. Apart from the traditional use, recent studies have shown that NSAIDs can also be used (alone or in combination) to treat other disorders such as atherosclerosis,[3] thrombosis,[4] Alzheimer's disease,[5-8] and disorders for which chronic inflammation is an etiological factor. In this regard, there is increasing evidence suggesting potential use of NSAIDs in the prophylactic treatment of some types of cancer, for example colon cancer, breast cancer and prostate cancer[9-12].

The major pharmacological mechanism of action of NSAIDs is to inhibit the production of prostaglandins (PGs) and thromboxanes (TXs) (Figure 1). PGs have a wide variety of physiological effects. TXs are vasoconstrictors and facilitate platelet aggregation, thus, they induce a potent hypertensive effect. Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes convert arachidonic acid (AA) to produce various PGs and TXs[13].

COX-1 is generally regarded as a constitutive enzyme which is present in most tissues; it is involved in the physiological production of PGs and provides maintenance functions such as cytoprotection in the stomach. In contrast, COX-2 is regarded as an inducible enzyme (induced by cytokines, growth factor, interleukin-1-β, and carrageenan) and is expressed in inflammatory cells[14]. However, recent reports have challenged these "traditional" roles of COX-1 and COX-2 enzymes, emphasizing the importance of re-evaluating not only their roles

in the inflammatory process[15], but also their contribution to the underlying mechanisms of NSAID-induced side-effects. In this regard, epidemiological studies have shown a significant risk of gastrointestinal,[16-17] renal,[18] and hepatic[19] side-effects apparently associated with the inhibition of the COX-mediated PG synthesis[20].

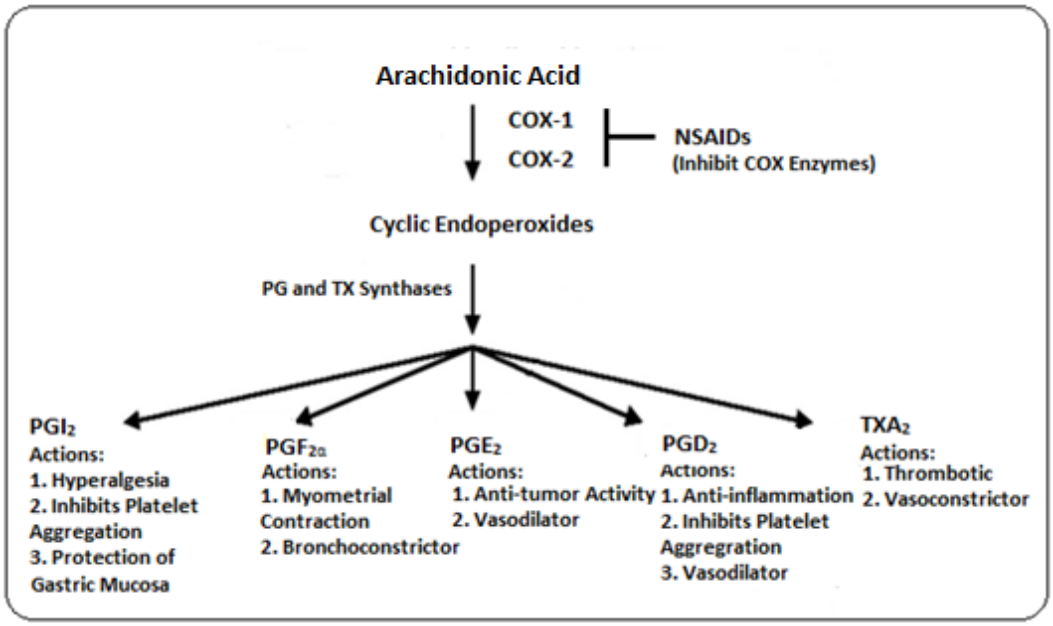


Figure 1: Cyclooxygenase Pathway

Aspirin has emerged as one of the most widely used antiplatelet agents (it inhibits COX-1–derived thromboxane A_2) in the primary and secondary prevention of cardiovascular disease, reducing the risk of myocardial infarction and stroke[21]. However, even at the low doses (10–100 mg per day) that are recommended for these purposes, aspirin can significantly increase the risk of gastrointestinal (GI) bleeding and ulceration[22]. Like aspirin, prolonged administration of other NSAIDs is also associated with a higher incidence of GI side-effects such as stomach irritation and ulceration. NSAID-related toxicity has been documented, and recognized for over 60 years[23], and has been referred to as an "epidemic"[24]. Over the years, introduction of more potent NSAIDs with an even greater propensity for toxic side-effects increased the awareness about

NSAID-induced gastro-duodenal ulceration, and provided impetus to the development of effective NSAIDs with a more favourable GI safety profile.

The development of gastrointestinal tract (GIT)-safe anti-inflammatory drugs has presented a unique challenge. Even after so many years of research, it has been difficult to separate the therapeutic advantages of NSAIDs from their GI side effects. In this regard, there have been adopted different strategies to design safer NSAIDs. To reduce their mechanism-based GI toxicity, one of the most successful alternatives developed in the early 90's were the selective COX-2 inhibitors, generically known as Coxibs. Their commercial success was based on the hypothesis that COX-1 derived eicosanoids promote gastroprotective mucosal defences; by sparing COX-1, COX-2 specific inhibitors will provide effective anti-inflammatory and analgesic activity while substantially reducing the risk of gastric and peptic ulcers and GI bleeding compared to traditional NSAIDs[25]. However, this approach is now under scrutiny due to conflicting evidence regarding the cardiovascular safety of the COX-2 inhibitors[2 26-28].

Another alternative to counteract the GI toxicity of NSAIDs was to form hybrid prodrugs with the ability to release biological mediators such as nitric oxide (NO) and hydrogen sulphide (H_2S)[29-30]. NO and H_2S are endogenously produced cellular mediators which now have been widely recognized to exert many of the same actions as PGs in the GIT. It has been proposed that co-administration of NO counteracts most of the adverse events related to NSAID-induced COX-1 inhibition, including decreased prostanoid synthesis, reduction in mucosal blood flow, and the over-expression of inflammatory mediators such as plasma tumor necrosis factor alpha (TNF-α) and leukocyte–endothelial cell adherence[31-32].

In the search of safer NSAIDs, "simple" prodrugs of NSAIDs have been studied, too. A wide variety of different prodrugs have been developed to temporarily mask the acidic group of NSAIDs. Generally, these Prodrugs have an ester or

amide group instead of the carboxylic acid group. They have become promising agents, which can be used to reduce the GI toxicity due to the local action of the carboxylic acid group. There have been reports that these ester or amide prodrugs of NSAIDs have a better therapeutic profile than their parents, examples of which are discussed later. These prodrugs still have to be further evaluated[33-34].

1.1 The Bigger Picture of the Problem

1.1.1 NSAID Gastropathy

In 2007, the US Poison Control Centers (American Association of Poison Control Centers, National Poison Data System [AAPCC NPDS]) described 307,590 cases of NSAID-related toxicity; 205,245 of these cases were correlated with single exposures to NSAIDs[35]. Financial loss due to gastric complications caused by NSAIDs is estimated to be in billions of dollars[36]. It has been reported that 15–35% of all peptic ulcer complications are caused by NSAIDs and the increase of hospitalization and deaths due to GI-related disorders parallels the increased use of NSAIDs[37-38]. It is estimated that each year more than 100,000 Americans are hospitalized and between 15,000 and 20,000 Americans die from ulcers and GI bleeding linked to NSAID use[39]. According to a study published by González *et al.,* it is difficult to estimate the actual damage exerted by NSAIDs. They suggested that the risk of upper GI bleeding/perforation varies between individual NSAIDs at the doses commonly used in the general population. They also reported that drugs with a long plasma half-life or those which are administered in sustained-release formulations are associated with a higher degree of inhibition of both COX isozymes and with a greater risk of upper GI bleeding/perforation[40]. Recent studies using capsule endoscopy and double-balloon endoscopy allowed better visualization and diagnostic evaluation of the entire small intestine. These techniques have also shown that NSAIDs can cause a wide variety of

abnormalities in the small intestine, such as ulcerations, perforation, and bleeding[41-42].

1.2 Pathogenesis of NSAID-Induced Gastropathy

1.2.1 Cyclooxygenase Enzymes

COX converts AA, which is released from the cell plasma membrane by the action of phospholipase A_2, into several types of eicosanoids. There are at least two different types of COX isoforms, namely COX-1 and COX-2; the clinical relevance of COX-3 as a drug-target is still under debate[43-44]. COX-1 is considered as a "housekeeping" enzyme due to constitutive expression in most cell types and tissues. High levels of constitutive expression of COX-1 have been detected in the stomach and platelets[45]. The inducible isoform COX-2 is expressed during inflammatory states by inflammatory mediators such as interleukin (IL)-1β, IL-2, IL-6, interferon (IFN)γ and TNF-α[46]. Most NSAIDs act by inhibiting both COX isoenzymes at different rates and extents.

The GI toxicity produced by NSAIDs is a complex process involving several etiological factors[23 47], some of which are:

a) Suppression of prostaglandin synthesis (mainly PGE_2 and PGI_2) throughout the GI tract

b) Direct irritation of the epithelial layer of gastric mucosa

c) Effects on the microcirculation by recruiting leukocytes

d) Oxidative stress and cell membrane damage

1.2.2 Inhibition of Gastric Prostaglandins

Prostaglandins play an important role in the GIT; they mediate several components of mucosal defence (blood flow, mucus and bicarbonate secretion, epithelial cell replication and mucosal immunocyte function)[48]. Thus, the inhibition of PG synthesis greatly increases gastric mucosa's susceptibility to damage induced by acid, pepsin or other luminal irritants[49].

1.2.3 Direct Irritation of Gastric Mucosa

Most NSAIDs, including aspirin, are carboxylic acid derivatives and consequently are not ionized in the acidic pH of the stomach. The non-ionized drug is readily absorbed across the gastric mucosa into the pH-neutral mucosa where it is ionized and temporarily trapped within the epithelial cells. The high intracellular concentration of the drug may induce cellular injury via mitochondrial respiration disruption, which ultimately causes cell death and produces sites of local tissue damage. These topical irritant properties were subsequently found to be predominantly associated with NSAIDs that have a carboxylic acid residue[50].

1.2.4 Effects on the Microcirculation

The ability of NSAIDs to reduce gastric mucosal blood flow has been recognized for several decades[51]. Prostaglandins of the E and I series are potent vasodilators that are continuously produced by the vascular endothelium. The inhibition of their synthesis by an NSAID leads to a reduction in vascular tone[49 51]. Several lines of evidence have suggested that damage to the local vascular endothelium is an early event following the administration of NSAIDs to experimental animals. Endothelial injury is also an early event in the pathogenesis of GI damage

associated with ischemia reperfusion, in which neutrophils have been demonstrated to play a critical role[52].

1.3 Selective COX-2 Inhibitors

The development of selective COX-2 inhibitors was based on the hypothesis that the inducible COX-2 isoform is associated with inflammatory conditions, whereas the constitutively expressed COX-1 is responsible for the cytoprotective effects of PGs; however, this assumption is now under scrutiny[15]. The prediction that only COX-2 plays a role in inflammation and hyperalgesia, whereas COX-1 is involved in gastric cytoprotection led to the idea that a selective COX-2 inhibitors would exhibit all the beneficial anti-inflammatory and analgesic properties associated with NSAIDs without causing any gastric disturbances[53-54]. The introduction of coxibs to the market in the mid-1990s came with bold promises of improved GI safety that have not been completely fulfilled[55-56].

Celecoxib, rofecoxib and valdecoxib (Figure 2) were approved by the FDA on the basis of trials that typically lasted three to six months and in which the end point was a clinical surrogate—endoscopically visualized gastric ulceration[57]. While producing less GI ulceration and bleeding than conventional NSAIDs, coxibs were still capable of triggering significant GI adverse events when given concomitantly with low-dose aspirin, in which case their GI safety over a conventional NSAID is lost[58-59]. Of course, cardiovascular toxicity issues were the main concern with these compounds and led to the withdrawal of rofecoxib (Vioxx) from the market (2004). This increased the awareness of similar cardiovascular toxicity with the entire NSAID class[28 60-61]. Thus, despite the big (but short-lived) commercial success of some selective COX-2 inhibitors, after the withdrawal of rofecoxib and other highly selective inhibitors, patients and clinicians faced the problem of having limited therapeutic options for the treatment of pain and inflammation.

This justifies the current need for safer and effective anti-inflammatory drugs with improved safety profiles over existing NSAIDs.

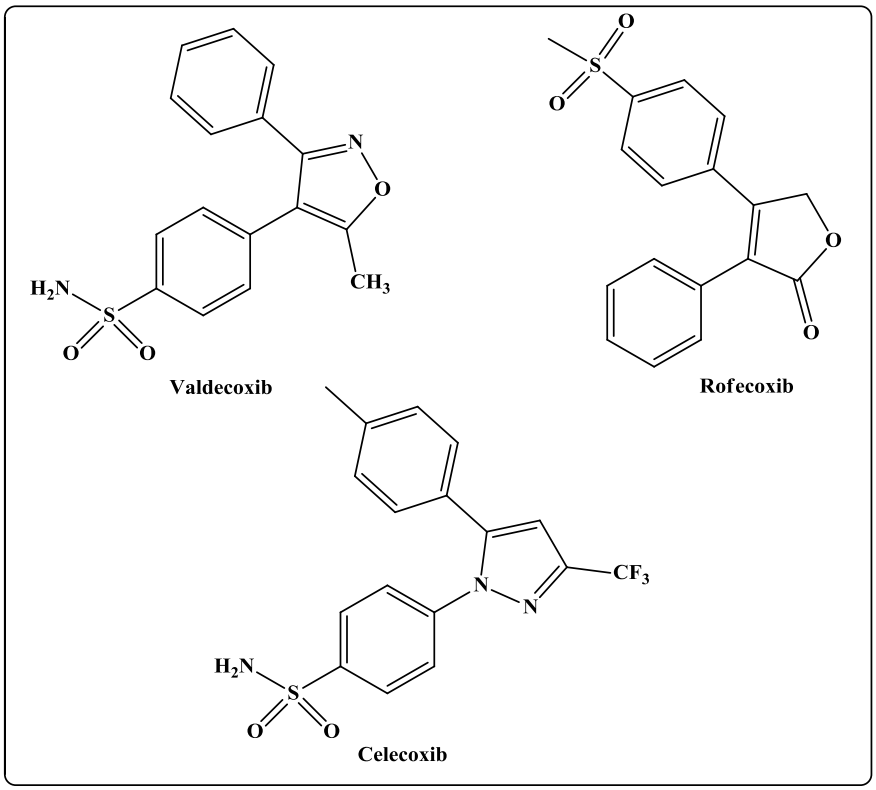


Figure 2: Molecular Structures of Valdecoxib, Rofecoxib and Celecoxib

1.4 Nitric Oxide (NO)-Releasing NSAIDs

Another widely explored and promising approach towards the development of GI-sparing NSAIDs is the formation of hybrid drugs by linking an organic nitrate NO-releasing moiety. The rationale behind the design of this class of drugs is that, small quantities of NO exert beneficial effects in the GIT by enhancing mucosal defence. In the stomach, NO modulates epithelial fluid and mucus secretion; it is an important mediator of the vascular tone of the gastric microcirculation and stimulates mucosal healing by enhancing collagen deposition through fibroblasts and by angiogenesis[62-63]. A case-control study

found that the use of medications that release NO, such as nitroglycerin and other nitro-vasodilators, is associated with a reduction in the incidence of gastric lesions in patients taking any type of NSAIDs[62 64-65].

Classical NSAIDs such as naproxen, ibuprofen, flurbiprofen, ketoprofen, and aspirin have been coupled to NO-donating moieties; the biological evaluation of NO-NSAIDs has been extensively explored in a wide variety of experimental models over the past fifteen years, demonstrating their efficacy, potency, improved safety, and broad spectrum of activities which extend beyond anti-inflammatory and analgesic applications. NO-NSAIDs such as NO-aspirin (NCX 4016), NO-diclofenac (NITROFENAC)[66], and NO-naproxen (NAPROXCINOD/HCT 3012/AZD 3582)[67-68] (Figure 3) have been shown either pre-clinically or clinically to cause minimal or insignificant gastric damage with equivalent analgesic and anti-inflammatory activity in comparison to their respective parent NSAIDs[57]. These studies provided proof of concept that is in agreement with their intended design and application (Table 1).

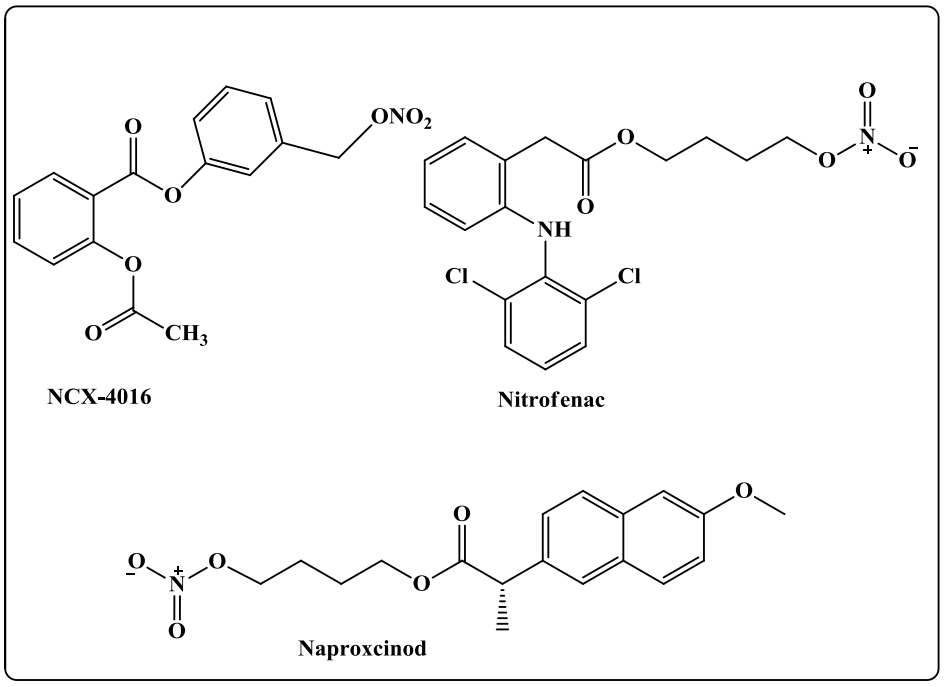
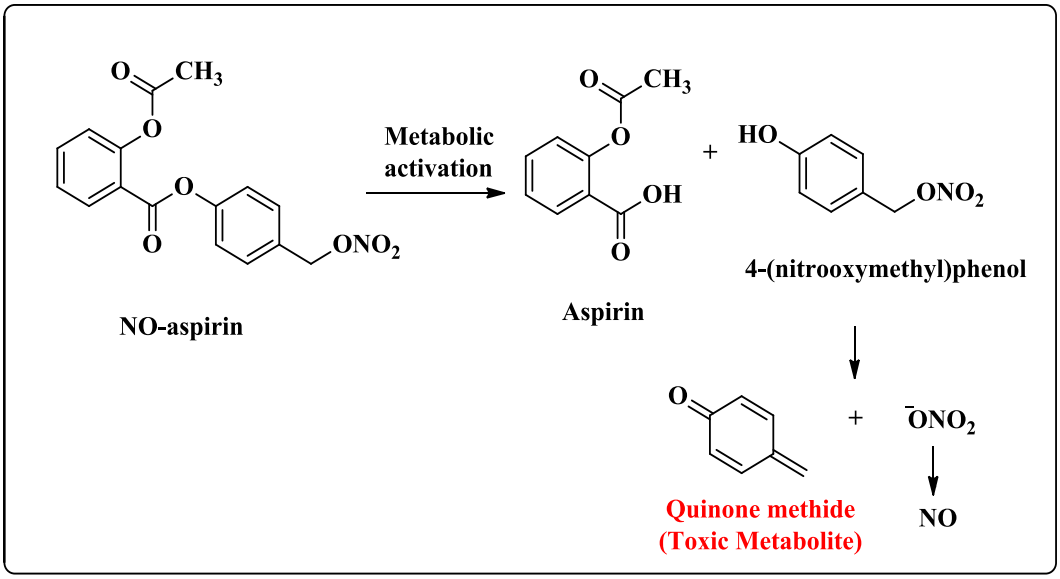


Figure 3: Molecular Structures of NCX-4016, Nitrofenac and Naproxcinod

Table 1: Current Status of Nitric Oxide (NO)-Releasing Nonsteroidal Anti-Inflammatory Drugs in Clinical Trials[69-70]

Drug	Company	Status[71]
NO-Aspirin (NO-acetylsalicylic acid)	Nicox	Program abandoned
NO-Diclofenac	Nicox	Preclinical
NO-Naproxen	AstraZeneca & Nicox	Reapplied for Regulatory Review
NCX 434	Nicox	Preclinical
NCX 1236	Nicox	Preclinical
S-NO-Diclofenac	NitroMed	Preclinical
NO-Ibuprofen	Nicox	Phase I

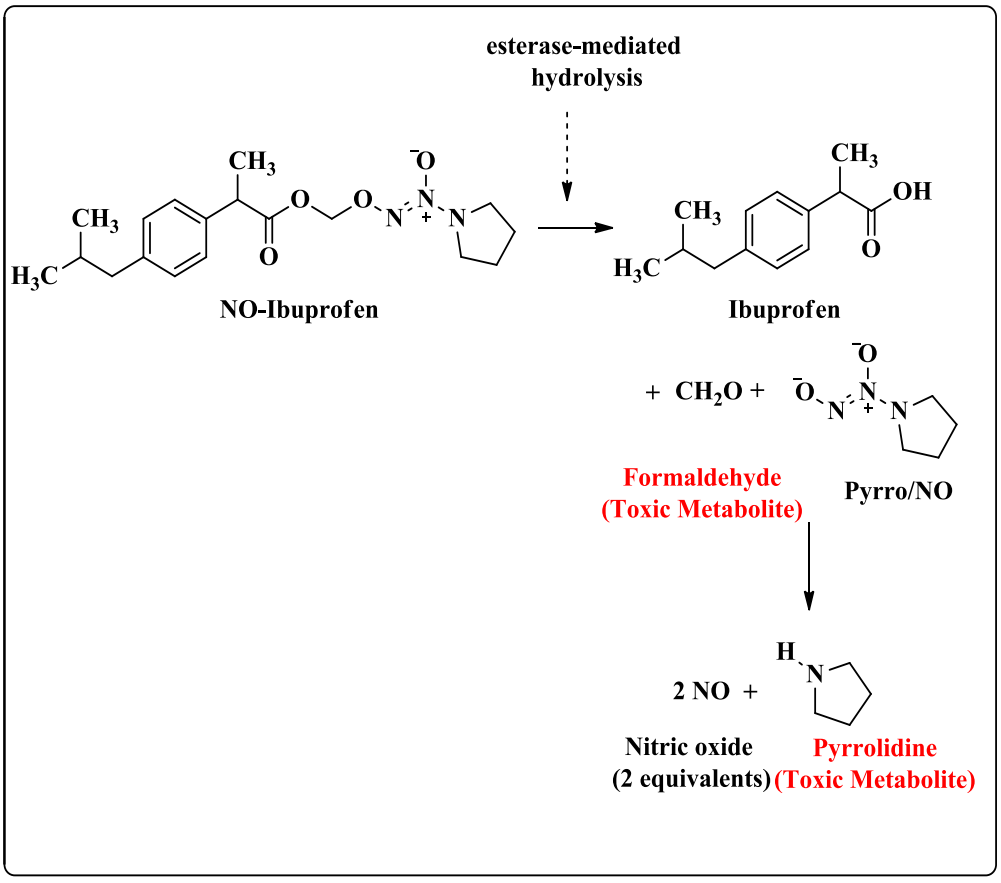
One critical issue in understanding the clinical efficacy of NO-NSAIDs is the pharmacological contribution that the NO-releasing moiety plays. There is enough evidence showing that NO-NSAIDs release NO when added to biological fluids (blood, liver homogenates, and others) via esterase hydrolysis and redox enzymes[72-74]. Recently, a study published by Kashfi *et al.* proposed that the *in vivo* metabolism of NO-aspirins (three regioisomers) involves the formation of quinone methides from its para and ortho isomers, and a carbo-cation from the meta isomer, with the NO-releasing group functioning as a leaving group. According to their study, quinone methide formation is responsible for NO-aspirin's anti-cancer activity[75] (Scheme A).



Scheme A: Mechanism of Quinone Methide Formation

Although organic nitrates were the first NO-donor groups to be reported in the literature, they are not the only ones. In 2005, Velázquez *et al.* reported the synthesis and biological evaluation of a new series of NO-NSAIDs possessing a *N*-diazen-1-ium-1,2-diolate. Because diazeniumdiolates (also called NONOates) have the ability to release twice as much NO as compared to organic nitrates, these new molecules were regarded as NONO-NSAIDs[76-78]. The first generation

of NONO-NSAIDs possessed diazeniumdiolates derived from secondary amines such as pyrrolidine and *N*,*N*-dimethylamine. Safety concerns about the release of carcinogenic nitrosamines obtained as secondary products in the metabolism of secondary amines led to the development of second-generation NONO-NSAIDs having diazeniumdiolates obtained from naturally occurring L-proline (the corresponding *N*-nitrosamine of L-proline is generally regarded as safe[79]). However, even though NONO-NSAIDs of this class were effective anti-inflammatory agents and their design addressed the safety concerns related to the generation of carcinogenic nitrosamines, they released two equivalents of formaldehyde (HCHO) per mol of drug upon metabolism, which precluded their use as potential drug candidates (Scheme B).



Scheme B: Formation of Toxic Metabolites from Second-Generation NONO-NSAIDs

Recently, a study was reported that compared NO-aspirin (NCX-4016) to NONO-aspirin (CVM-01)[80]. This study showed that both hybrid prodrugs were equipotent analgesic and anti-inflammatory agents. Importantly, this study also concluded that the gastroprotection provided by both hybrid prodrugs was not entirely dependent on the amount of NO released by them. Despite the observed higher levels of NO released from the *N*-diazeniumdiolate ion present in NONO-aspirin (confirmed by the measurement of plasma nitrate/nitrite levels), the extent of gastric protection was practically identical for both prodrugs[80], and consequently, any potential benefit related to the release of two moles of NO could not be established, and questioned if NO was really involved in the reduced ulcerogenic profile of NO-NSAIDs.

1.5 Hydrogen Sulfide-Releasing Prodrugs

The endogenous production of H_2S has recently gained the interest of the scientific community[81]. It has been considered the third gas with biomodulatory actions, along with NO and carbon monoxide (CO). Three enzymes catalyze the formation of H_2S: cystathionine β-synthase (CBS), cystathionine γ-lyase (cystathionase, CSE), and 3-mercaptopyruvate sulfurtransferase (MST). In the liver, kidney, enterocytes, vascular smooth muscle cells, and endothelial cells, H_2S is synthesized by CSE, whereas in the brain its production is attributed to CBS. MST is operative at cardiac, kidney, and brain levels. H_2S in the cardiovascular system is mainly produced by CSE[82]. The biological actions of H_2S have been uncovered in the past 10 years, which include roles in the regulation of blood pressure, inflammation, cell viability, cellular oxygen consumption, pain processing, and insulin secretion[83]. Exogenously administered H_2S, at micromolar concentrations, can induce vasorelaxation *in vitro* and *in vivo*[84]. The blood pressure lowering action is mediated by the activation of ATP-sensitive potassium channels in the vascular smooth muscle and seems to be independent

from the activation of guanylate cyclase[81]. H_2S can facilitate the vasorelaxant action of NO. All these applications of H_2S led to the investigation of various H_2S-releasing molecules[85].

The synthesis of H_2S-releasing derivatives of NSAIDs has been reported recently. As is the case of NO-NSAIDs, a H_2S-releasing derivative of diclofenac and naproxen was substantially better tolerated in terms of gastric damage than the parent drugs [85-87]. With administration of the same dose of the compound three times over 24 h, very low levels of intestinal damage were observed, at least 90% less than those observed in rats given diclofenac at an equimolar dose[85]. Moreover, there was no change in haematocrit in rats treated with the H_2S-releasing derivative, while diclofenac administration resulted in decrease in haematocrit of about 50%, consistent with the widespread bleeding that was evident in the gastrointestinal tract [85 87]. The addition of a H_2S-releasing moiety also resulted in a detectable increase in anti-inflammatory potency of the H_2S-diclofenac compound as compared to diclofenac and naproxen[85 87]. While the development of H_2S-releasing anti-inflammatory drugs is still ongoing, the pre-clinical data available thus far provide cause for optimism.

1.6 Prodrugs of NSAIDs

Considerable attention has been focused on the development of bio-reversible derivatives, such as prodrugs, to temporarily mask the acidic group of NSAIDs as a promising means of reducing or abolishing the GI toxicity due to topical irritation. Prodrugs are pharmacologically inactive derivatives of active agents, which undergo chemical and/or enzymatic biotransformation, resulting in the release of the active drug after administration. The metabolic product subsequently elicits the desired pharmacological response. Most prodrugs of NSAIDs have been prepared by derivatization of the carboxyl group. The esters

have dominated prodrug research, because they have the ideal characteristic of exhibiting reasonable *in vitro* chemical stability which allows them to be formulated with adequate shelf lives. In addition, by virtue of their ability to function as esterase substrates, esters are suitably labile *in vivo*. With this aim, different promoeities have been taken into consideration to design new efficacious NSAID prodrugs[33 88]. In the following sections, various ester, amide, and anti-oxidant derivatives of NSAIDs will be discussed.

1.6.1 Esters

Khan *et al.* have evaluated the glycolamide ester prodrugs of ibuprofen, diclofenac, naproxen, and indomethacin[89-90]. Polyoxyethylene esters of ketoprofen, naproxen, and diclofenac showed good stability in phosphate buffer (pH 7.4), and simulated gastric fluid (pH 2.0), and were readily hydrolyzed by human plasma (Figure 4). Anti-inflammatory and analgesic activities of these esters were equivalent to the parent drugs, with significantly reduced gastric irritation even at high doses[91]. Ibuprofen β-D-glucopyranoside has been reported by Khan *et al.* to possess superior anti-inflammatory and analgesic activities over the parent drug with significantly less ulcerogenicity[92]. Alkyl ester prodrugs of ibuprofen have been reported by Bansal *et al.* with significant improvement in the oral delivery of ibuprofen in terms of reduced gastro ulcerogenicity and maintenance of pharmacological activity. These esters were also evaluated for their physicochemical properties and anti-inflammatory activity in topical carrageenan induced rat paw edema. The benzyl ester prodrug showed a significantly reduced gastric ulcerogenicity at equimolar doses with retention of anti-inflammatory and analgesic activities[93-94].

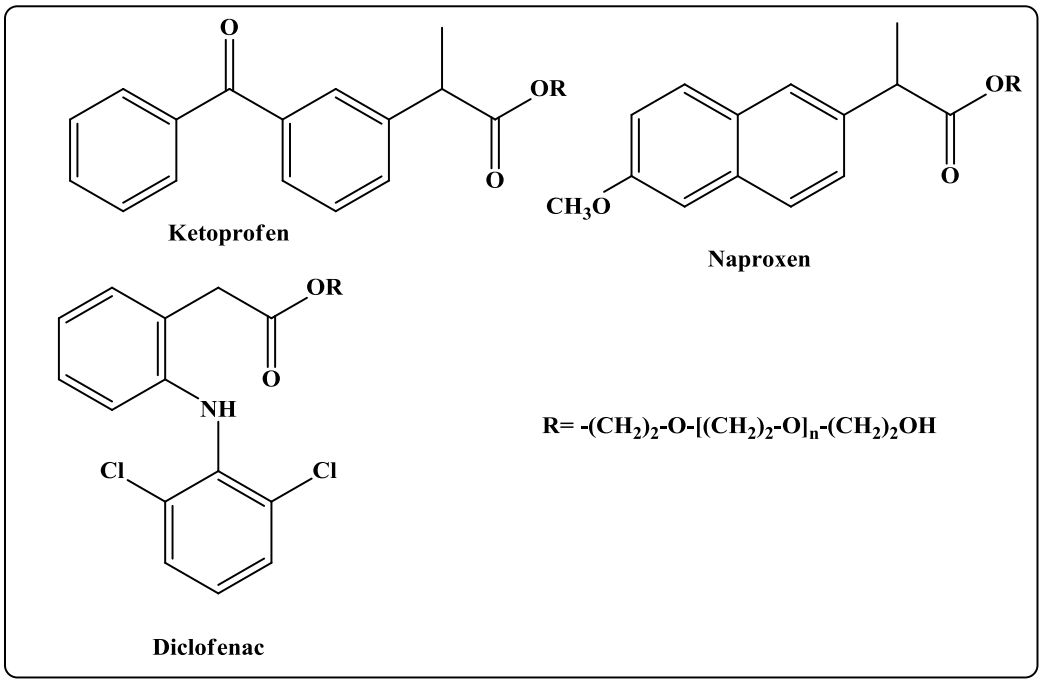


Figure 4: Polyoxyethylene Esters of Ketoprofen, Naproxen and Diclofenac

Ester prodrugs of ibuprofen which were synthesized using α-methyl, ethyl, and propyl glucopyranosides have been reported to undergo rapid cleavage inside the biological system and elicit a pharmacological profile quite similar to that of ibuprofen after oral administration, but unlike the parent drug, they display reduced gastric ulceration[95]. Ibuprofen ester prodrugs possessing a 1,2,3-trihydroxypropane-1,3-dipalmitate/stearate were also prepared and evaluated[96]. Mohan *et al.* synthesized a wide variety of alkyl ester prodrugs of flurbiprofen with significant reductions in ulcerogenicity. The reduction of the ulcer index (UI) in rats indicates that n-propyl, isopropyl, benzyl, and cyclopentyl prodrugs of flurbiprofen are significantly ($p < 0.05$) less irritating to the gastric mucosa as compared to the parent drug flurbiprofen[97]. Curcio *et al*. recently reported the anti-inflammatory activity of NSAID prodrugs formed by esterification of ketorolac with D-galactose[98]. This approach yielded ketorolac prodrugs with markedly reduced ulcerogenic side-effects compared to unprotected ketorolac. Curcio's approach involved the formation of labile molecules which upon *in vitro*

activation (esterase-mediated hydrolysis) release ketorolac and inactive D-galactose.

1.6.2 Amides

Mishra *et al.* synthesized ten prodrugs of ketorolac by amidation with ethyl esters of amino acids glycine, *L*-phenylalanine, *L*-tryptophan, *L*-valine, *L*-isoleucine, *L*-alanine, *L*-leucine, *L*-glutamic acid, *L*-aspartic acid, and β-alanine. Marked reduction in UI and comparable analgesic, anti-inflammatory activities as compared to ketorolac were obtained in all cases[99]. Galanakis *et al.* synthesized and evaluated amide derivatives of indomethacin and naproxen with an *L*-cysteine ethyl ester. The derivatives are reported to be potent anti-inflammatory, antioxidant, and hypocholesterolemic-hypolipidemic agents, with considerably reduced gastrointestinal toxicity[100] (Figure 5).

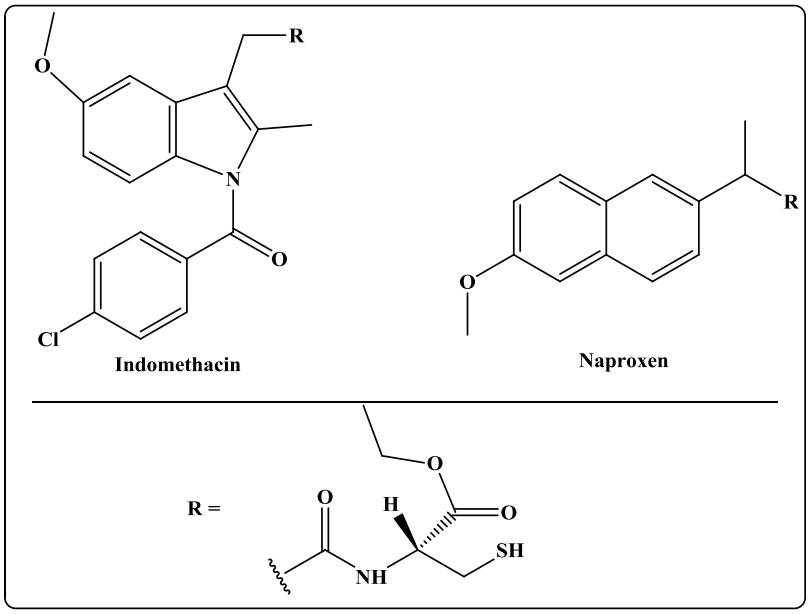


Figure 5: Amide Derivatives of Indomethacin and Naproxen with *L*-Cysteine Ethyl Ester

Kalgutkar *et al.* reported the synthesis and biological evaluation of a series of non-ulcerogenic indomethacin amide derivatives[101]. Shanbhag *et al.* reported several non-ulcerogenic amino alkyl esters of ibuprofen and naproxen[102]. Verma *et al*. synthesized acylimidazole derivatives of carboxylic acid group containing NSAIDs. All the compounds were found to retain analgesic and anti-inflammatory activities with much fewer NSAID-induced GI complications when compared to the standard[103]. Rasheed *et al.* synthesized mefenamic acid (MA) prodrugs of tyrosine and glycine. The glycine prodrug showed maximum analgesic activity of 86%, and both tyrosine and glycine prodrugs showed better anti-inflammatory activity of 74% and 81%, respectively, when compared to the 40% of MA. Further, the prodrugs showed fewer gastric ulcers compared to MA; the tyrosine and glycine prodrugs had an average UI of 9.1 and 4.5, respectively, while an average UI of 24.2 was observed with MA[104].

1.6.3 Anti-Oxidants

During inflammation, reactive oxygen species (free radicals) are produced in an uncontrolled way, causing tissue damage. Melatonin, an antioxidant, was reported to show protective effects in indomethacin induced gastric injury by virtue of its radical scavenging activity[105]. Based on this observation, Kourounakis *et al.* found it interesting to synthesize and evaluate amide derivatives of diclofenac, ibuprofen and indomethacin with a well known antioxidant, cysteamine; the derivatives exhibited good anti-inflammatory and antioxidant activities and showed a significant reduction in ulcerogenicity[106]. Doulgkeris *et al.* has designed and synthesized a series of novel molecules having a residues of classical NSAIDs (naproxen/indomethacin) and an antioxidant moiety, both attached through amide bonds to known nootropic structures like L-proline, trans-4-hydroxy-l-proline or dl-pipecolinic acid. The compounds retained anti-

inflammatory and antioxidant activities, acquired hypocholesterolemic action, and possessed greatly reduced gastrointestinal toxicity[107] (Figure 6).

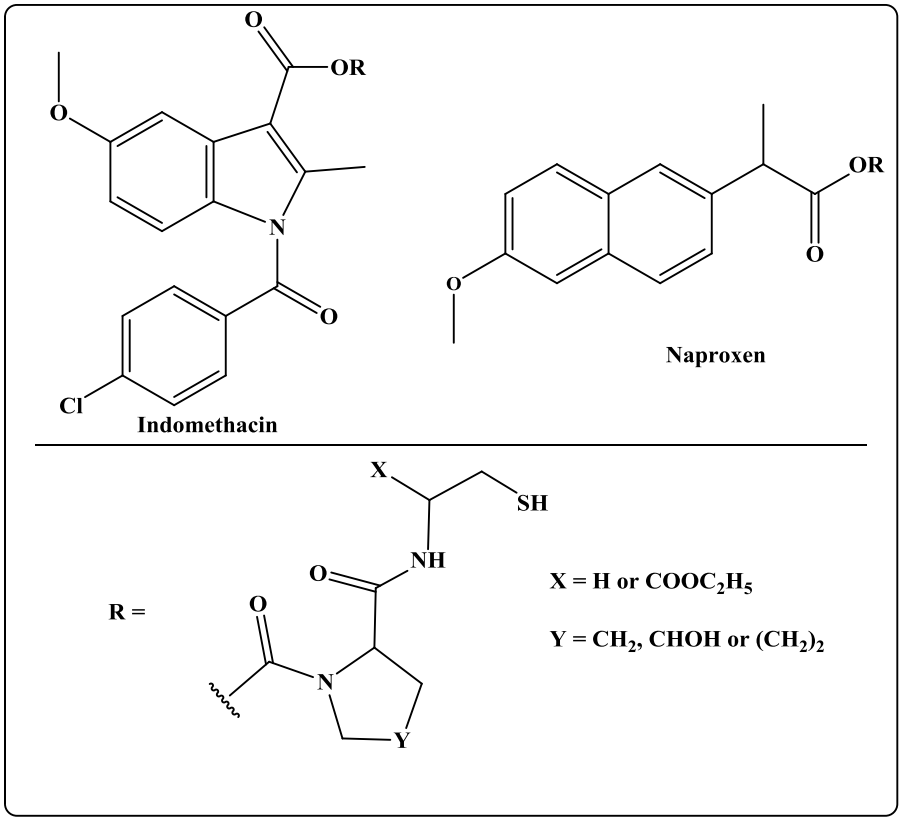


Figure 6: Molecular Structures of Naproxen & Indomethacin, Attached with an Antioxidant Moiety

In 2009 Spadaro *et al.* synthesized naproxen esters containing tocopherols. The synthesized prodrugs exhibited anti-inflammatory activity and antioxidant activity with a significant reduction in gastropathy[108]. In 2005 Zhang *et al.* reported phenolic ester prodrugs of indomethacin. These prodrugs showed significant anti-oxidant activity with remarkably low gastric toxicity[109]. Jiang *et al.* synthesized a series of novel conjugates of aspirin with natural phenolic acid antioxidants connected through a diol linker. These conjugates were found to be efficient antioxidants and many of them demonstrated much more potent anti-inflammatory activity than aspirin. They were further confirmed to significantly reduce ulcerogenic potency and toxicity in mice in comparison to aspirin.

However, it is evident that the anti-inflammatory activity *in vivo* of these dual-acting molecules was not simply consistent with their antioxidant ability *in vitro*[110].

Despite the overwhelming amount of information describing the potential benefits of hybrid prodrugs possessing NO- or H_2S-releasing moieties, the search for an ultimate NSAID with superior therapeutic advantage but devoid of complications hasn't been successful to date. In spite of extensive efforts, further research is needed to design and identify prodrugs that are appropriate for clinical use in terms of stability, metabolism, toxicology, and side effects.

1.7 Drug Design

We proposed the design and biological evaluation of new nitric oxide-releasing non-steroidal anti-inflammatory drugs (NONO-NSAIDs) possessing:

- A NO-donor (PROLI/NO) derived from a naturally occurring amino acid (L-proline)
- A naturally occurring diol as linker between the NSAID and the NO-donor

This strategy would allow us to obtain a new series of NONO-NSAIDs which upon metabolism would release the active components (NSAID and NO) as well as innocuous metabolites (L-proline, a naturally occurring alcohol, and a 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl moiety). We expected these hybrid prodrugs to be at least as effective as the parent NSAIDs, but devoid of GI side-effects (Figure 7).

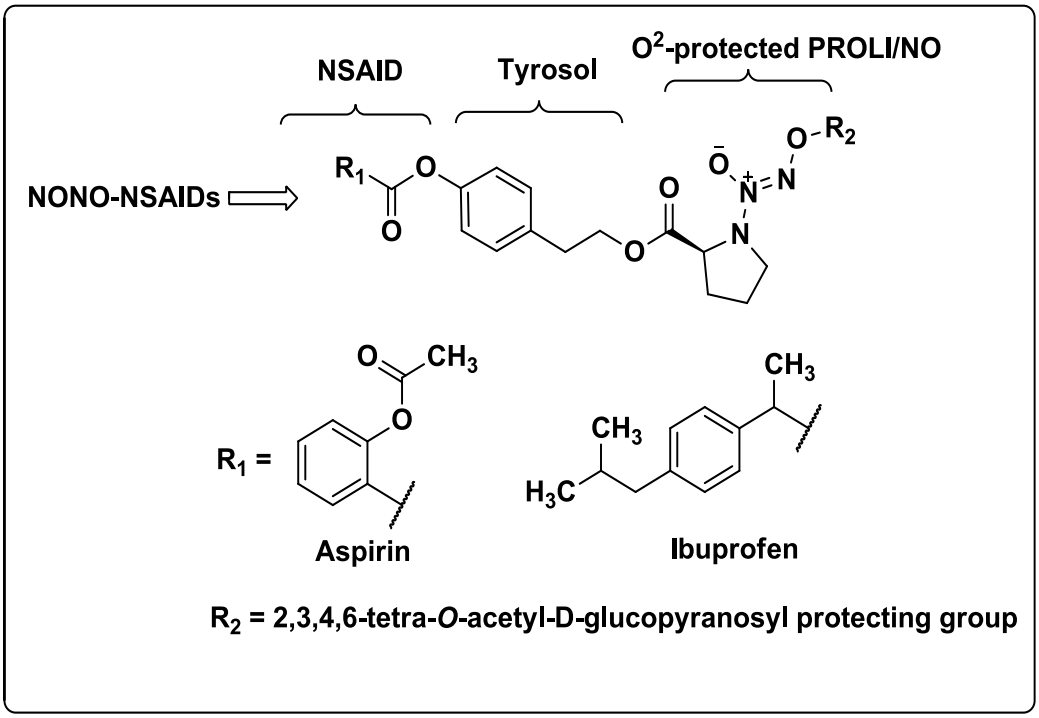
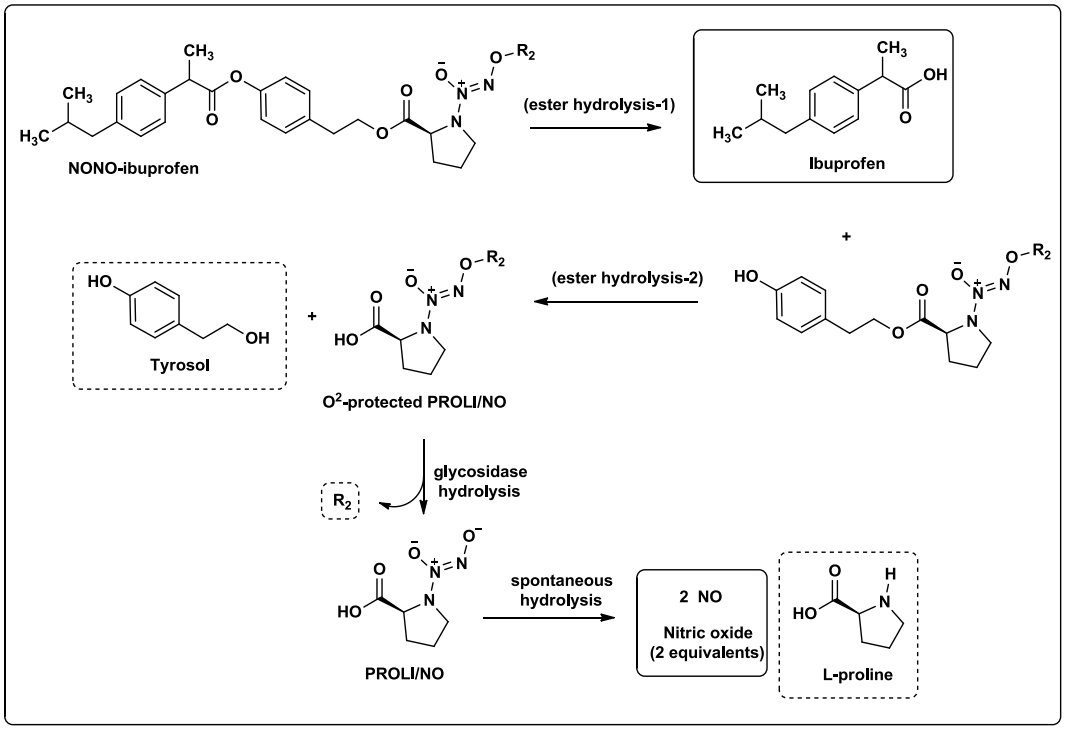


Figure 7: Design of NONO-NSAIDs Possessing N-diazeniumdiolates (PROLI/NO) and a Simple Phenol Linker (Tyrosol) Between the NSAID and the NO-Releasing Moiety**.**

The nature of the alcohol linker was the key to our design. In this regard, we considered the use of a wide variety of diols including sugars and polyphenols; however, because sugar chemistry represented a challenging approach, and the use of polyphenols would probably require a cumbersome protection-deprotection synthetic strategy, initially we decided to use simple phenols such as 4-(2-hydroxyethyl)phenol (tyrosol). Tyrosol was selected as the prototype naturally occurring diol, and two hydroxybenzyl alcohols (3-HBA, 4-HBA) as comparative additional linkers. All these alcohols will act as linkers between the carboxylic acid group of the corresponding NSAID and the carboxylic acid group present in PROLI/NO, a known NO-releasing diazeniumdiolate. Tyrosol and hydroxybenzyl alcohols (3-HBA, 4-HBA) are structurally related to hydroxytyrosol (Figure 3), a well studied natural anti-inflammatory and anti-oxidant compound.

Additionally, tyrosol and hydroxytyrosol are two common constituents found in olives and olive oil[111-112].

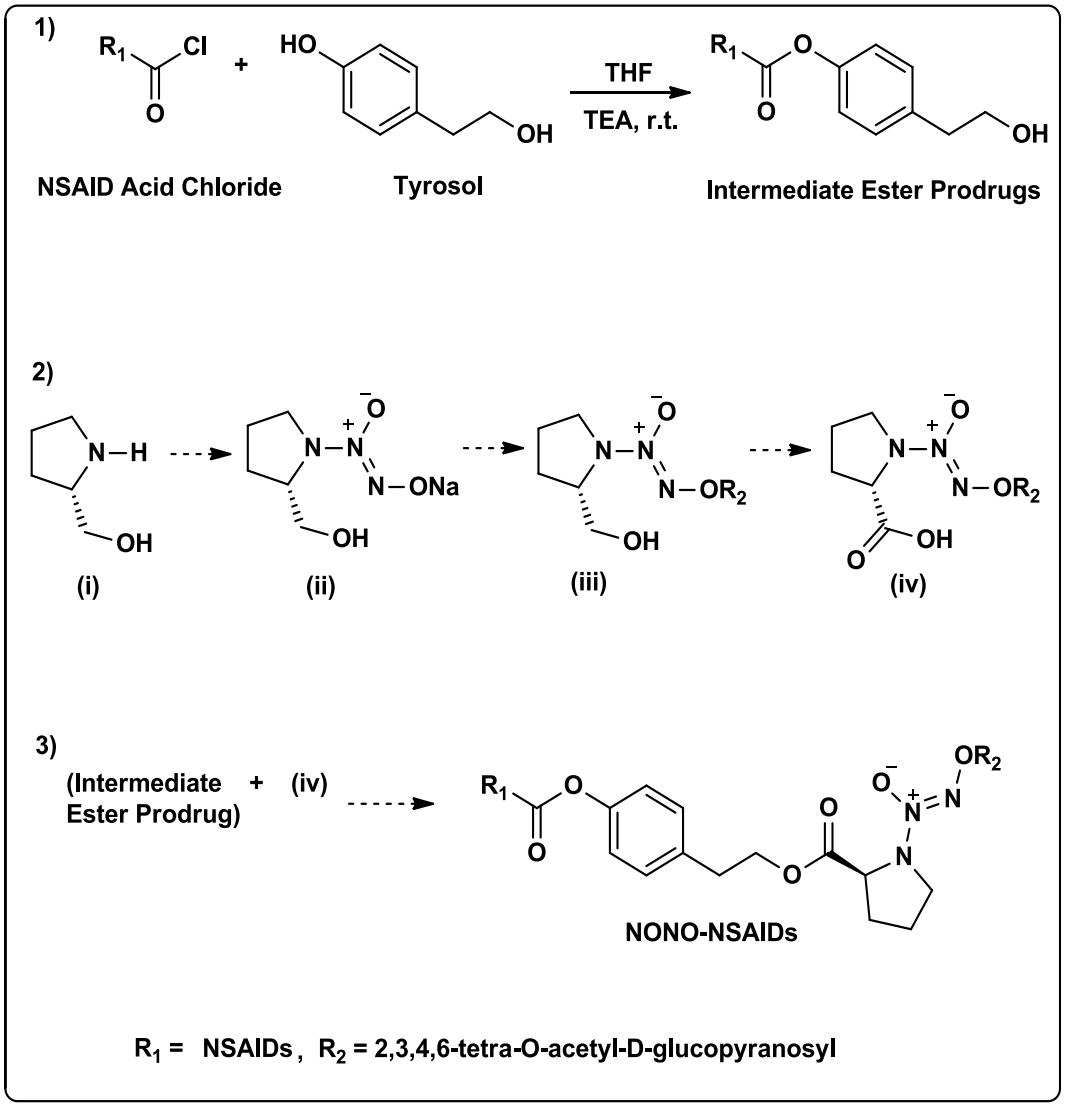
The rationale behind the design of new NONO-NSAIDs possessing a naturally occurring PROLI/NO moiety as the source of NO and a simple phenol such as tyrosol as linker, is based on the assumption that, upon metabolic activation, these ester prodrugs would release the anti-inflammatory NSAID, cytoprotective NO, and innocuous metabolites such as L-proline, tyrosol, and a 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl moiety (Scheme C). The naturally occurring simple phenols are expected to show synergistic mechanism(s) of action other than, or in addition to, COX inhibition by the NSAID. This synergistic action is supposed to improve anti-inflammatory, analgesic, anti-thrombotic, and/or chemopreventive potency, while avoiding gastrointestinal and cardiac side-effects commonly observed with classical NSAIDs.



Scheme C: Theoretical metabolic activation of NONO-ibuprofen. Compounds marked with a solid rectangle represent the two active components (ibuprofen and NO) released by metabolic activation; compounds marked with a broken-line rectangle represent innocuous metabolites produced after ester and glycosidase hydrolysis. R_2 represents a protecting 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl moiety.

The chemical synthesis of novel NONO-NSAID prodrugs possessing a tyrosol moiety involved three steps; the first one was the preparation of the corresponding ester intermediates by reacting NSAID acid chlorides with tyrosol (Scheme D1). Under the experimental conditions used for these reactions (THF/TEA, at 25° C), the phenol group reacted much faster than the aliphatic alcohol, which was in agreement with the reports describing similar esterification reactions using a structurally related 3-hydroxybenzyl alcohol[113]. The second step involved in the preparation of O^2-protected PROLI/NO derivatives is described in Scheme D2; in this regard, it has been reported in the literature that is not possible to obtain O^2-alkylation products by simple nucleophilic displacement (S_N2) using PROLI/NO (O^2-sodium salt of **iv**) and electrophiles; therefore, the

preparation of intermediate **(iv)** would require an indirect route involving the synthesis of O^2-sodium PROLINOL/NO **(ii)**, O^2-alkylation with 1-bromo-2,3,4,6-tetraacetoxyglucose to obtain **(iii)**, and subsequent oxidation to the alcohol group in L-prolinol to obtain the target carboxylic acid **(iv)**[114]. The last step involved the esterification reaction between intermediate ester prodrugs with (**iv)** to yield the corresponding NONO-NSAIDs (Scheme D3).



Scheme D: Proposed chemical synthesis of NONO-NSAIDs possessing a tyrosol linker between NSAID and the NO-releasing group PROLI/NO. 1) Preparation of intermediate ester prodrugs; 2) Synthesis of O^2-protected PROLI/NO derivatives (iv); 3) Synthesis of NONO-NSAIDs by esterification of carboxylic acids (iv) and intermediate ester prodrugs. The broken arrows represent proposed reactions which were not carried out (see discussion about *in vitro* and *in vivo* evaluation below).

2 Chapter: Synthesis and Biological Evaluation of Aspirin and Ibuprofen Ester Prodrugs

2.1 Objective

The purpose of this part of the project was to synthesize and evaluate the *in vitro* and *in vivo* anti-inflammatory profile of NONO-NSAIDs and compare it to that obtained with intermediate ester prodrugs described in Scheme D. Additionally, we assessed the ulcerogenic profile of intermediates and final products, in order to obtain essential structure-activity relationship data that would allow us to establish the minimal requirements needed to maintain potency, efficacy, and safety.

2.2 Introduction

We screened six ester intermediates of aspirin and ibuprofen ester prodrugs possessing tyrosol, 3-HBA or 4-HBA (Figure 3) as COX-1/COX-2 inhibitors *in vitro* using an enzyme immunoassay kit (Cayman, cat. 560131). The results of simple esterification of carboxyl groups in aspirin and ibuprofen produced an improved inhibitory activity compared to the parent NSAID and change in selectivity towards COX-1 or COX-2 depending on the alcohol moiety. Further, we tested these intermediate prodrugs on carrageenan-induced paw edema assay, which has long been used to assess the anti-inflammatory properties of NSAIDs. The *in vitro* results of COX inhibition were reciprocated in the *in vivo* carrageenan-induced paw edema assay. The results acquired with *in vitro* and *in vivo* anti-inflammatory assay made us very keen and we evaluated the ability of these drugs to produce gastric damage by using the acute ulcerogenesis assay.

2.3 Chemistry

Ibuprofen acid chloride **(7)** was formed by the dropwise addition of 1.2 equivalents of oxyalyl chloride to a solution of ibuprofen in tetrahydrofuran (THF) at room temperature following a reported procedure[115] (Scheme E). The corresponding ester prodrugs were synthesized by simple nucleophilic displacement reactions between tyrosol, 3-HBA, or 4-HBA and aspirin acid chloride **(1)** or ibuprofen acid chloride **(7)** dissolved in THF at room temperature in the presence of triethylamine (TEA) (Scheme F).

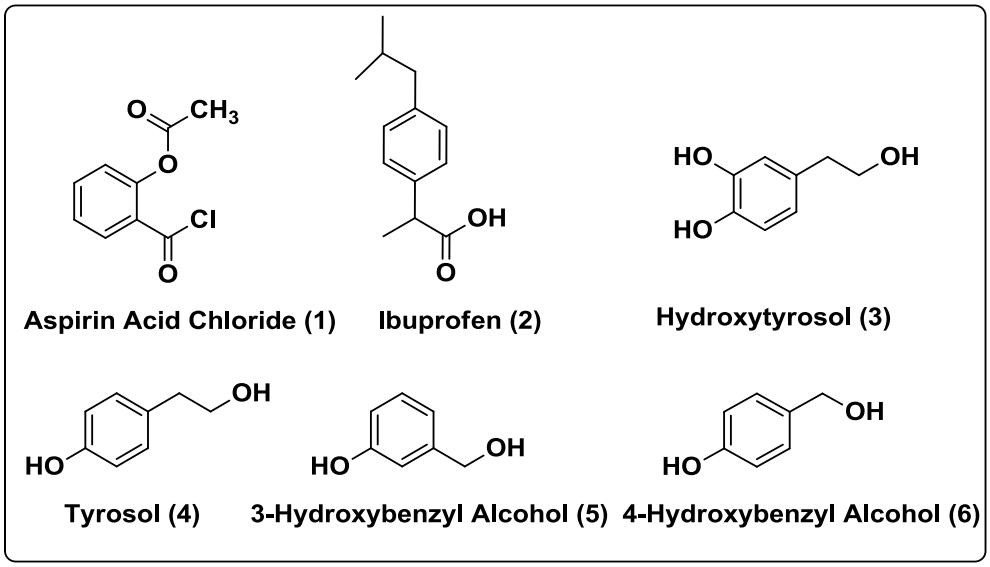
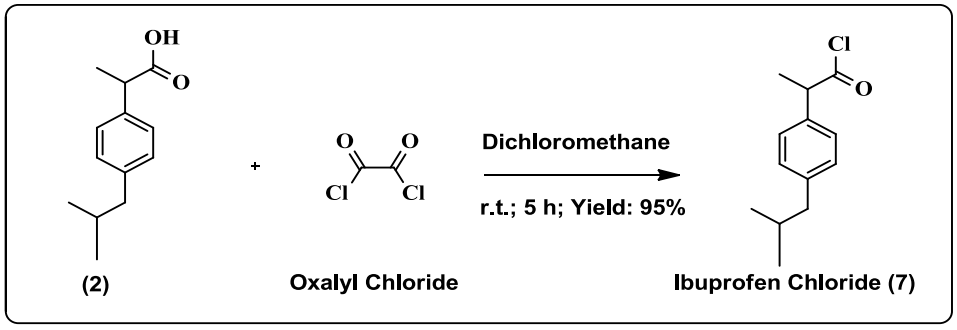
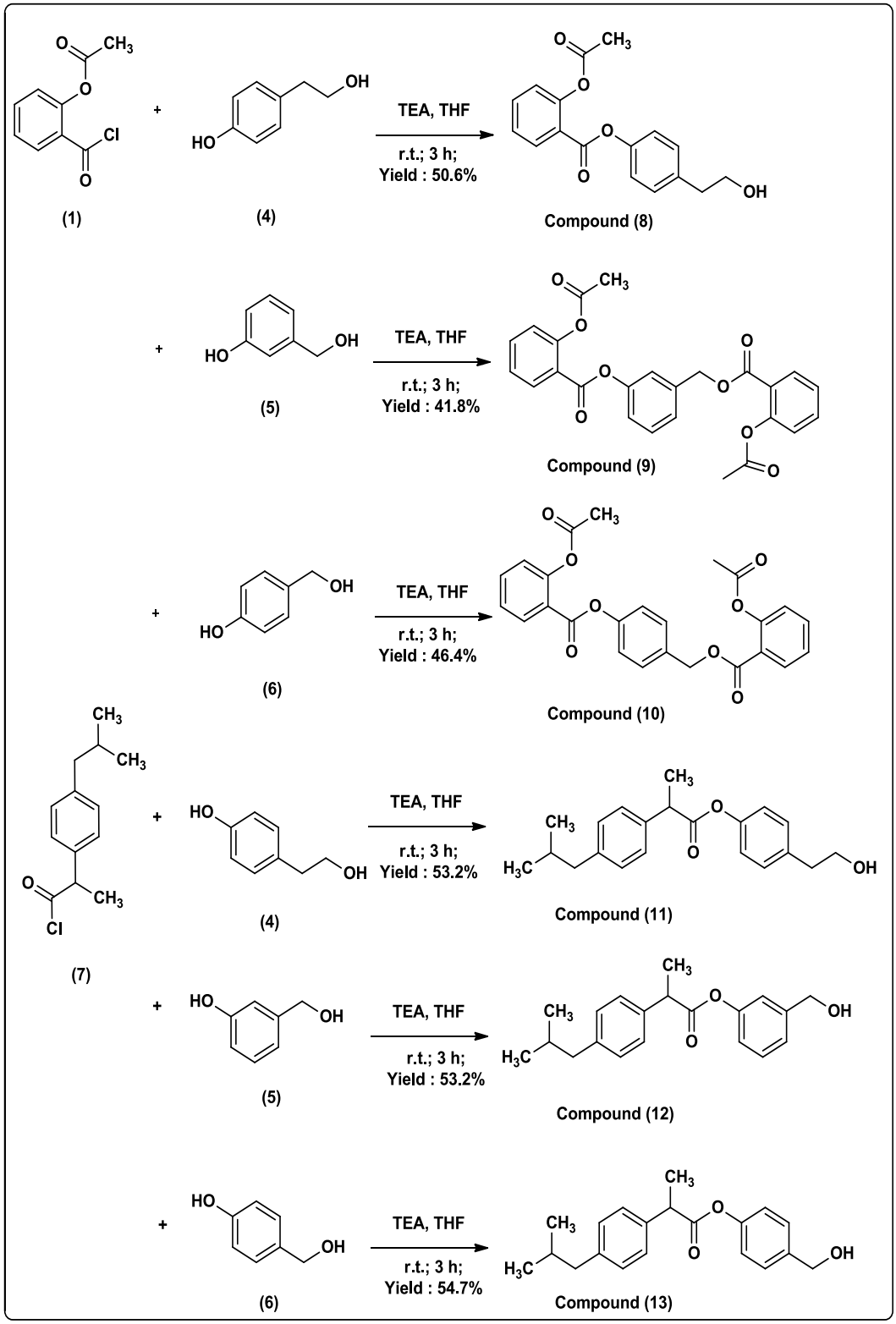


Figure 8: Molecular Structures of Aspirin Acid Chloride, Ibuprofen, and Various Phenols



Scheme E: Synthesis of Ibuprofen Acid Chloride



Scheme F: Synthesis of Prodrugs **(8-12)**

2.4 Results

2.4.1 In vitro COX-1/COX-2 Enzyme Inhibition

Esterification of carboxyl groups in aspirin and ibuprofen produced an improved inhibitory activity compared to the parent NSAID, and selectivity toward COX-1 or COX-2 depending on the alcohol moiety (Table 2). Ibuprofen prodrugs **(12)** and **(13)** were not active towards either of the enzymes at the highest test compound concentration used (100 μM). One could interpret from the data that the use of tyrosol – a known molecule with weak anti-inflammatory properties[116-117], conferred prodrugs **(8)** and **(11)** with a significantly improved COX inhibitory profile. The ibuprofen prodrug **(11)** was 5500 times more active than ibuprofen against COX-2, and about two times less potent against COX-1; this shows a selectivity index = 32200, which is remarkable for a simple ester prodrug. The complete COX inhibition assay results are shown in Table 2.

Table 2: Results of *In Vitro* COX-1/COX-2 Enzyme Inhibition for Prodrugs **(8-13)**

Compounds	COX-1 IC_{50} (µM)[a]	COX-2 IC_{50} (µM) [a]	Selectivity Index (S.I)[b]
8	0.03	0.38	0.078
9	1.49	> 100	<0.01
10	> 100	1.17	>85.47
11	6.44	0.0002	32220
12	> 100	> 100	-
13	> 100	> 100	-
Aspirin[78]	0.3	2.4	0.12
Ibuprofen[78]	2.9	1.1	2.63

[a]The test compound concentration required to produce 50% inhibition of COX-1 or COX-2 *in vitro*. The result (IC_{50}, µM) is the mean of two determinations acquired using an ovine COX-1/COX-2 assay kit (Catalogue No. 560101, Cayman Chemicals Inc., Ann Arbor, MI), and the deviation from the mean is <10% of the mean value. [b]Selectivity index (SI) = COX-1 IC_{50}/COX-2 IC_{50}.

2.4.2 Carrageenan-Induced Rat Paw Edema Assay

The test compounds were evaluated by using the *in vivo* rat carrageenan-induced foot paw edema model. The test drugs were administered at equimolar concentrations to those required for the reference compound to elicit about 50% inhibition (their corresponding ID_{50} values were published before) [78]. We noticed improvement in the anti–inflammatory activity of the test compounds **(9), (11) & (13)** compared to the parent NSAIDs (Table 3). These data correlate with the *in vitro* test results of COX inhibition.

Table 3: Results of Carrageenan-Induced Rat Paw Edema Assay for Prodrugs **(8-13)**

Compounds	Dose (µmol/kg)	% Inhibition
8	705	58.6 ± 0.1
9	705	85.0 ± 8.9
10	705	27.9 ± 9.3
11	325	63.2 ± 5.6
12	325	41.8 ± 5.6
13	325	67.1 ± 5.6
Aspirin[78]	705	50
Ibuprofen[78]	325	50

Inhibitory activity in a carrageenan-induced rat paw edema assay. The results are expressed as the ID_{50} value (mg/kg) at 3 h after oral administration of the test compound. Test drugs were administered at an equimolar dose of the parent NSAIDs required to decrease the inflammatory response by 50%. Results are expressed as ± mean SEM (n = 3).

2.4.3 Acute Ulcerogenesis Assay

One of the most common side effects correlated to NSAID therapy is gastrointestinal irritation and bleeding. It was therefore essential to evaluate prodrugs **(8) – (13)** for undesirable ulcerogenic effects, and compare them to those induced by their parent drugs. All test drugs were suspended in 1% methylcellulose solution. The severity of gastric damage was expressed as an UI. Prodrugs **(8), (9), (10), (12)** and **(13)** showed minimum ulcerogenicity in comparison to their parent drugs (Table 4). In Figure 9, the visual comparison of gastric damage caused by prodrugs and the parent NSAIDs can be observed. This observation was rather surprising and it was not in accordance with our original approach, because ester prodrugs were intermediate molecules, which were needed to synthesize the final NONO-NSAIDs by attaching the NO-releasing moiety PROLI/NO. These results suggested that the NO-releasing moiety was not essential to counteract the ulcerogenic effects of the parent NSAIDs, and dramatically changed our approach, because suddenly, it seemed that we may not need to add NO-donor groups.

Table 4: Result of Acute Ulcerogenesis Assay for prodrugs **(8-13)**

Compounds	Ulcer Index[a]
8[c]	2.6 ± 0.9
9[c]	2.6 ± 3.2
10[c]	0.3 ± 0.3
11[d]	35.6 ± 9.3
12[d]	2.3 ± 4.0
13[d]	9.6 ± 3.7
Aspirin[b]	57.4
Ibuprofen[b]	45.8

[a]The average overall length (in mm) of individual ulcers in each stomach, at 5 h after oral administration of prodrugs. [b]250 mg/kg dose. [c]Equimolar amount to 250 mg of Aspirin/kg. [d]Equimolar amount to 250 mg of 250 mg of Ibuprofen/kg. Results are expressed as ± mean SEM (n = 3).

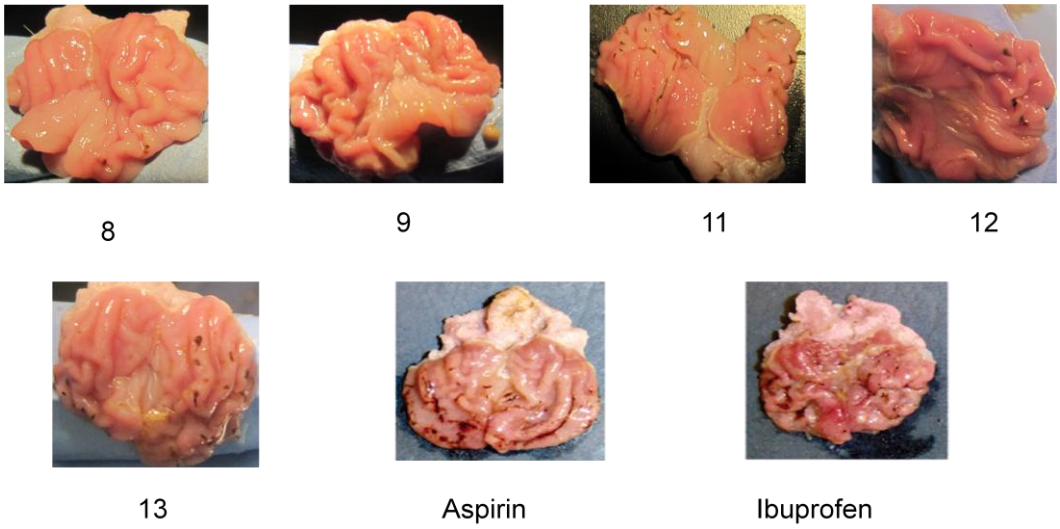


Figure 9: Acute Ulcerogenesis Assay for Prodrugs **(8**, **9**, **11**, **12** and **13)**. Pictures for Aspirin and Ibuprofen were Obtained From Archives Used in Previous Reports[78].

2.5 Discussion

It has been reported in the literature that esterification or amidation of the carboxylic acid group of NSAIDs may change the selectivity for COX inhibition [118-119] (Figure 10). Our results are in agreement with these reports. Compounds **(8), (9)** and **(11)** showed selective inhibition of COX-2 (Table 2). Compound **(11)** was the most potent (IC_{50} = 0.2 nM) and selective (SI = 32,220). Most prodrugs showed a modest improvement in their *in vivo* anti-inflammatory effect compared to their parent counterparts (except for compound **(11)** which was less potent than ibuprofen). It is important to consider that tyrosol, 4-HBA, and 3-HBA are three phenols with reported anti-oxidant and mild anti-inflammatory activity[120-121], which may explain the modest increase in anti-inflammatory activity.

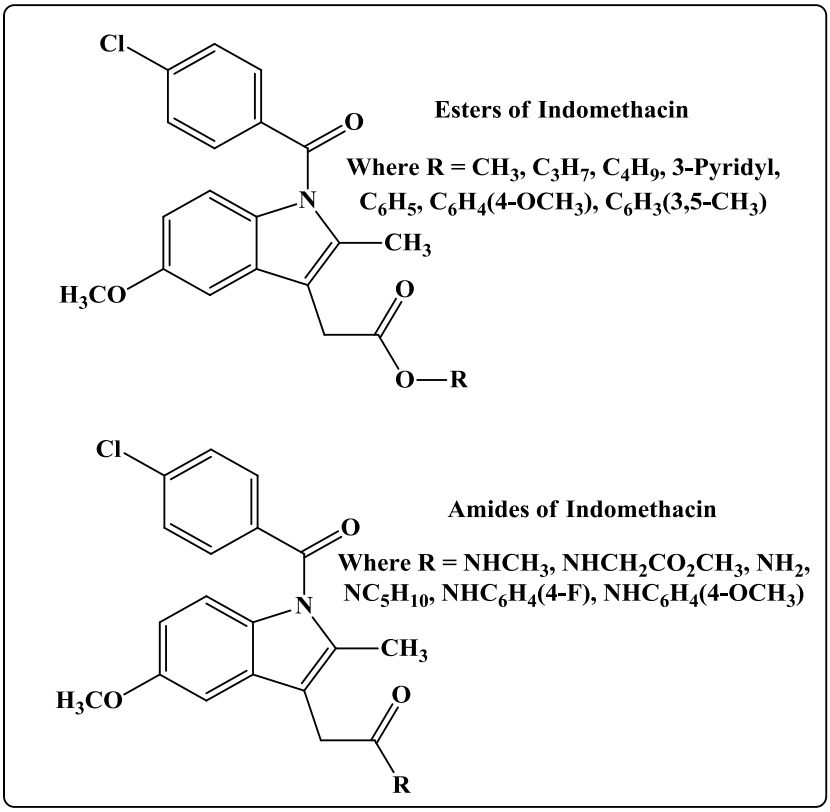


Figure 10: Molecular Structures of Various Indomethacin Ester and Amide Derivatives

In this regard, Jiang et al. recently reported the synthesis and biological evaluation of a series of novel conjugates of aspirin having one "phenolic acid antioxidant group (p-coumaric acid, ferulic acid or caffeic acid)" connected through a diol linker to the carboxylic acid group present in aspirin (Figure 11). These prodrugs showed considerable anti-inflammatory activity without significant GI side-effects[122]. One of the diol linkers used by Jiang's group was tyrosol, which is the protecting group we used in this work to form NSAID esters. Our work concludes that it is not essential to have additional phenolic acid anti-oxidants linked to tyrosol, to maintain the anti-inflammatory profile of the corresponding NSAID or to decrease its ulcerogenic effects.

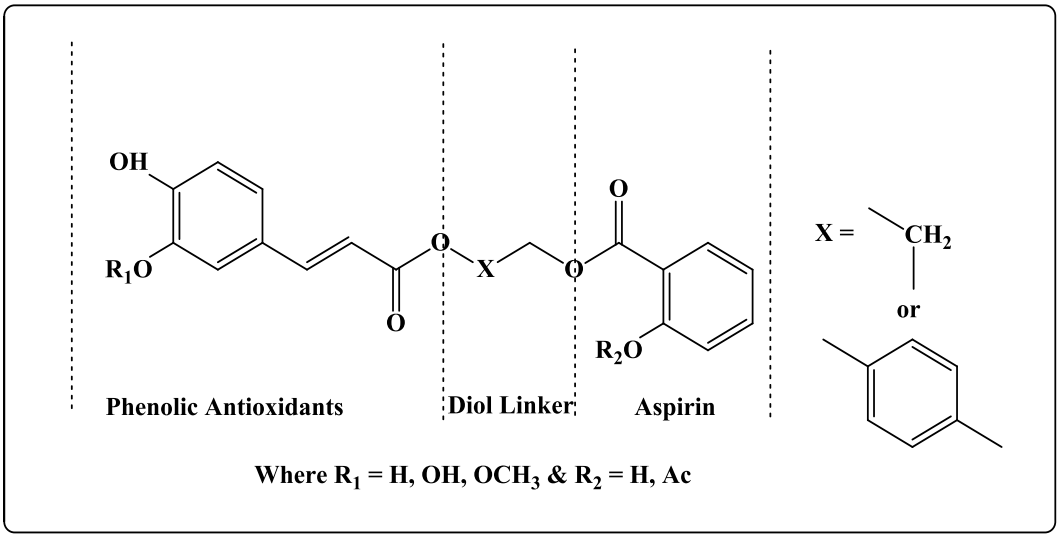


Figure 11: Molecular Structures of Conjugates of Aspirin with Phenolic Antioxidants

Results obtained in the acute ulcerogenesis assay showed a significant but unexpected reduction in ulcerogenicity by most ester prodrugs. Literature reports describe that the approach to counteract the gastroenteropathy induced by NSAIDs is by protecting NSAIDs with a biological mediator either a NO or H_2S-releasing moiety. The biological mediators were expected to counteract the ulcerogenic side-effects of the NSAID [32 57 123-124]. However, our results showed that "simple" ester prodrugs possessing a natural phenol such as tyrosol may be able to prevent the formation of ulcers. This challenged our initial approach with regard to the use of NO-releasing diazeniumdiolates (or any other NO-donor group). It has been reported in the literature that co-administration of free radical scavengers produced a decrease ulcerogenic response from NSAIDs[125-126]; therefore, it is reasonable to assume that the decreased ulcerogenic effect observed with tyrosol prodrugs may be due to the anti-oxidant properties exerted by the phenol moiety.

To obtain additional evidence in support of these observations, we decided to synthesize and evaluate tyrosol esters of indomethacin.

3 Chapter: Synthesis and Biological Evaluation of Indomethacin Ester Prodrugs

3.1 Objective

In this part of the project, our aim was to synthesise and evaluate tyrosol ester prodrugs of indomethacin and obtain additional evidence in support of the observation that ester formation is sufficient to decrease the ulcerogenic profile of indomethacin.

3.2 Introduction

To compare the potential anti-oxidant effect exerted by tyrosol in NSAID ester prodrugs, we synthesized three indomethacin esters possessing tyrosol as a reference, phenol as a representative analogue without the hydroxyethyl side chain, and ethylene glycol as a linear non-aromatic diol (Figure 12). Indomethacin was chosen based on its potent anti-inflammatory effect, and considering that it has been associated with a high incidence of gastric toxicity. Unlike aspirin or ibuprofen, indomethacin is available to patients by prescription only. Phenol and ethylene glycol are not known for their anti-oxidant activity, so their effect on causing ulcers would give us essential structure-activity relationship data needed to understand the role of ester moieties in the mechanism by which these prodrugs acquire a non-ulcerogenic profile.

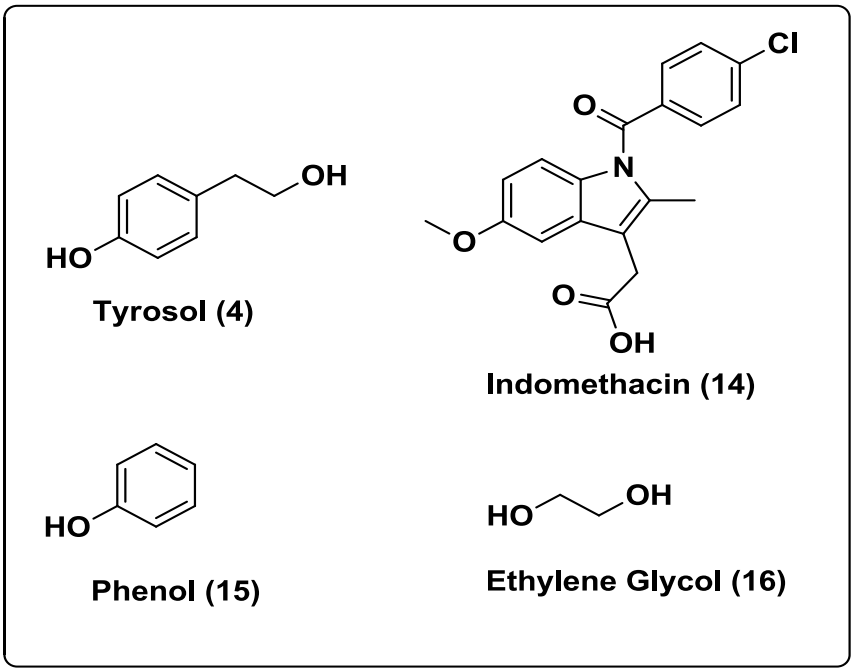
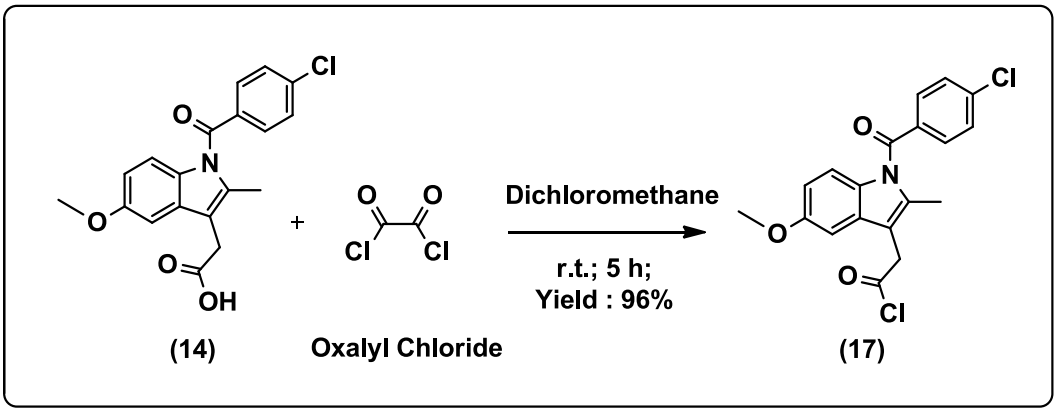


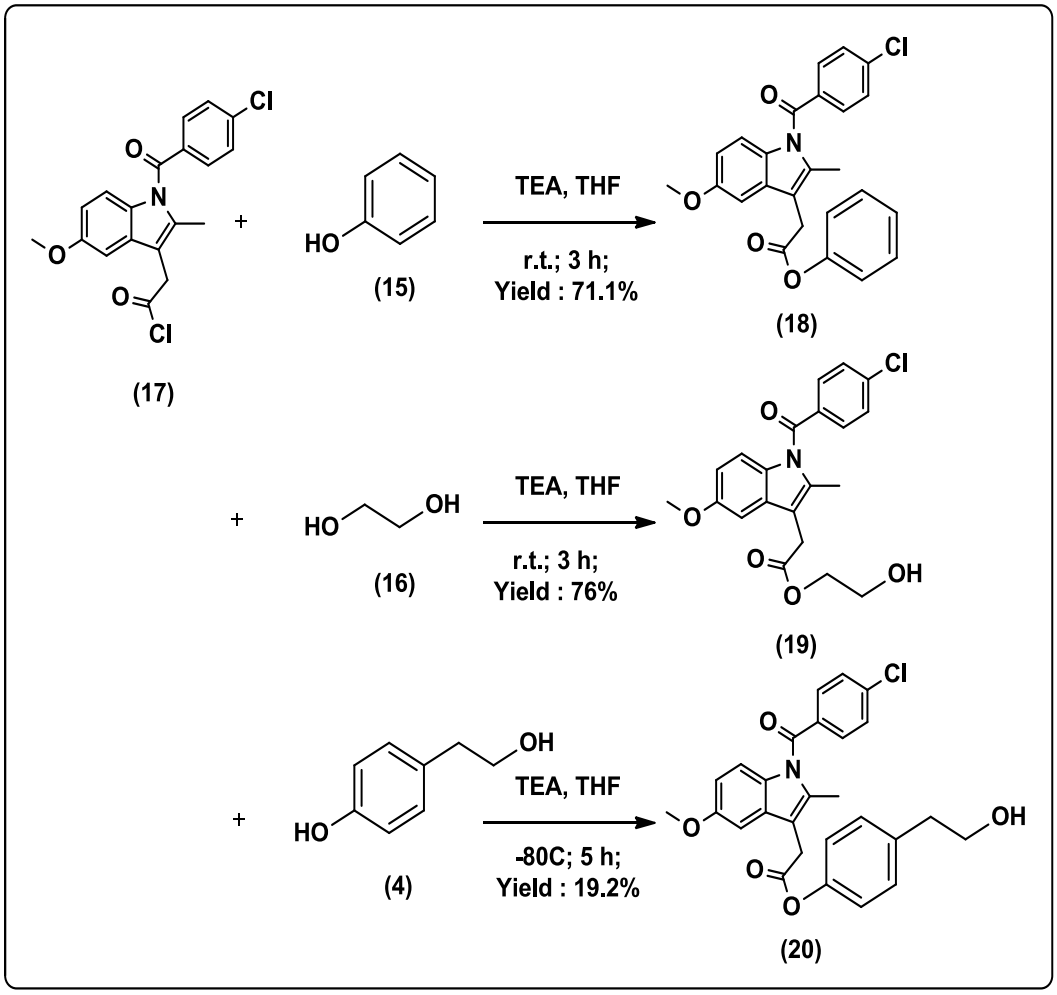
Figure 12: Molecular Structures of Various Alcohols and Indomethacin

3.3 Chemistry

Ester prodrugs were formed by the same synthetic method as described for aspirin and ibuprofen ester prodrugs. Phenols were reacted with indomethacin acid chloride to form the corresponding prodrugs. Indomethacin acid chloride **(17)** was formed by the dropwise addition of oxyalyl chloride to a solution of indomethacin in dichloromethane (CH_2Cl_2) at room temperature; Indomethacin acid chloride was used in the crude form in further reactions. The yield of this reaction was 96 % (Scheme G). The ester prodrugs were synthesized by reaction between phenol, ethylene glycol, and tyrosol with indomethacin acid chloride dissolved in CH_2Cl_2 at room temperature or at – 80 °C in the presence of TEA (Scheme H).



Scheme G: Synthesis of Indomethacin Acid Chloride.



Scheme H: Synthesis of Indomethacin Ester Prodrugs.

3.4 Results

3.4.1 In Vitro COX-1/COX-2 Enzyme Inhibition

The *in vitro* COX-1/COX-2 enzyme inhibition data obtained with indomethacin prodrugs showed a similar trend to that obtained with aspirin and ibuprofen tyrosol esters (Table 5). Prodrugs **(18)** and **(19)** showed selectivity towards COX-2 (selectivity index = 69.44 and 21.69 respectively). As described for tyrosol prodrugs of aspirin and ibuprofen, the use of this moiety conferred indomethacin-tyrosol (prodrug **20**) an improved COX inhibitory profile. Also, prodrug **(20)** was about 1239 times more selective towards COX-2 than indomethacin; however, prodrug **(20)** showed a markedly reduced inhibitory potency on COX-1 ($IC_{50} > 100$ μM) compared to indomethacin ($IC_{50} = 0.1$ μM). Interestingly, prodrug **(19)** possessing an ethylene glycol moiety was not active on either enzyme at the highest test compound concentration used (100 μM), which highlights the essential role of the aromatic group in COX inhibition. The decreased potency observed for prodrug **(19)** shows another structural feature needed to exert enzyme inhibition; Marnett's group had shown high potency and selectivity for amino ethyl derivatives of indomethacin, which suggests that amino groups may confer additional and/or stronger binding affinity to ethyl indomethacin prodrugs.

Table 5: Result for *In Vitro* COX-1/COX-2 Enzyme Inhibition for prodrugs **(18-20)**.

Compounds	COX-1 $IC_{50}(\mu M)^a$	COX-2 $IC_{50}(\mu M)^a$	Selectivity Index (S.I)[b]
18	> 100	1.44	>69.44
19	> 100	> 100	-
20	> 100	4.61	>21.69
Indomethacin[29]	0.1	5.7	0.0175

[a]The *in vitro* test compound concentration required to produce 50% inhibition of COX-1 or COX-2. The result (IC_{50}, µM) is the mean of two determinations acquired using an ovine COX-1/COX-2 assay kit (Catalogue No. 560101, Cayman Chemicals Inc., Ann Arbor, MI), and the deviation from the mean is <10% of the mean value. [b]Selectivity index (SI) = COX-1 IC_{50}/COX-2 IC_{50}.

3.4.2 Carrageenan-Induced Rat Paw Edema Assay

The indomethacin prodrugs were given in the concentration required for indomethacin to produce a 38.35 % inhibition. Remarkably, all the simple ester prodrugs showed improved anti–inflammatory activity in comparison to indomethacin (Table 6). Compounds **(18), (19)** and **(20)** showed an improved anti-inflammatory activity *in vivo* (57.35, 60.62 and 61.32% decrease in the inflammatory response respectively), which represents 1.5 to 1.6-fold increase in potency compared to indomethacin (38.35% inhibition at equimolar dose). This result correlated with the *in vitro* test for COX-1/2 enzyme inhibition. In the literature, there is sufficient evidence to support an anti-oxidant-based

moderate anti-inflammatory activity exerted by 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, and tyrosol[116-117 127]; however, since compounds **(18)** (indomethacin-phenol) and **(19)** (indomethacin-ethylene glycol) also showed improved activity *in vivo*, this mechanism is unlikely to be significant because phenol and ethylene glycol are not reported as anti-inflammatory compounds.

Table 6: Results of Carrageenan-Induced Rat Paw Edema Assay for Prodrugs **(18-20)**

Compounds	Dose (µmol/kg)	% Inhibition
18	81	57.35 ± 7.1
19	81	60.62 ± 8.3
20	81	61.32 ± 9.2
Indomethacin	81	38.35 ± 8.3

Inhibitory activity of ester prodrugs, in carrageenan-induced rat paw edema assay. The results are expressed as the ID_{50} value (mg/kg) at 3 h after oral administration of the test compound. Test drugs were administered at a dose equimolar of to the dose of indomethacin required to decrease by 50% the inflammatory response. Results are expressed as ± mean SEM (n = 3).

3.4.3 Acute Ulcerogenesis Assay

The indomethacin prodrug with tyrosol was significantly non-ulcerogenic (U.I. = 2.3 ± 2.5) in comparison to indomethacin (U.I. = 34.4 ± 4.2) or its prodrugs with phenol or ethylene glycol (U.I. = 19.0 ± 1.8and 20.3 ± 2.1 respectively). The severity of gastric damage was expressed as UI. The fact that prodrugs containing phenol and ethylene glycol (devoid of anti-oxidant activity) are considerably more ulcerogenic than indomethacin-tyrosol provides additional evidence to support our approach. Anti-oxidant ester prodrugs may play a role in preventing gastric ulceration caused by NSAIDs (Table 7).

Overall, the results obtained with indomethacin prodrugs, as well as those observed with aspirin and ibuprofen tyrosol prodrugs, strongly suggest that *NO-releasing groups are not essential to decrease the ulcerogenic profile of NSAIDs.* In this regard, it seems that simple esterification of aspirin, ibuprofen or indomethacin with an anti-oxidant phenol group is sufficient to significantly decrease the ulcerogenic response observed after an acute dose of the corresponding NSAID.

Table 7: Results of Acute Ulcerogenesis Assay for prodrugs **(18-20)**

Compounds	Ulcer Index[a]
18[c]	19.0 ± 1.8
19[c]	20.3 ± 2.1
20[c]	2.3 ± 2.5
Indomethacin[b]	34.4 ± 4.2[78]

[a]The average overall length (in mm) of individual ulcers in each stomach, at 5 h after oral administration of prodrugs. [b]29 mg/kg dose. [c]Equimolar amount to 29 mg/kg of Indomethacin. Results are expressed as ± mean SEM (n = 3).

3.5 Discussion

Our results showed that "simple" ester prodrugs possessing a natural anti-oxidant phenol such as tyrosol are significantly less ulcerogenic than unprotected NSAIDs. This statement is supported by the observation that prodrugs **(18)** (possessing phenol) and **(19)** (possessing ethylene glycol) exerted an ulcerogenic profile resembling that of indomethacin; however, prodrug **(20)** (possessing a tyrosol moiety) was significantly less toxic. There are a few possible explanations to rationalize this. One of the reasons could be related to increased lipophilicity of the esters compared to free carboxylic acids. Increased lipophilicity may be correlated to an increased rate and extent of absorption (improved bioavailability), which would decrease the time of local exposure of epithelial

cells to the NSAID. Another possible explanation could be related to the lower degree of toxicity of the ester compared to the ionizable free acid; the pH changes involved in the transport of acid drugs from the gastric lumen (highly acidic) to intracellular neutral environments creates equilibrium of ionized/non-ionized species, which has been reported to interfere with normal mitochondrial respiration. A third possible cytoprotective mechanism might involve decreased oxidative stress by the NSAID ester compared to the parent acid. In this regard, the phenols used (tyrosol, 4-HBA and 3-HBA) are reported to have mild anti-oxidant activity [120-121]. It has been reported in the literature that use of free radical scavengers with NSAIDs may decrease the ulcerogenicity of NSAIDs [125-126]. Some polyphenols create a local environment where free radical formation is inhibited. This would certainly be a contributing factor by preventing the cytotoxic effects of superoxide anion[23], which has been reported as one of the mechanisms of NSAID-induced toxicity. This part of the project proved that *we don't need NO-releasing moieties to reduce ulcerogenicity.*

Furthermore, we decided to compare NCX-4016 (**22**, a well established NO releasing aspirin) and aspirin with our prodrug (**8**). This part of the project gave us a direct insight into the differences between using the benefits of using a NO moiety or an anti-oxidant moiety.

4 Chapter: Comparison of Tyrosol Ester with NO-releasing prodrug

4.1 Objective

The objective of this part of the project was to compare tyrosol prodrug (**8**) with NCX 4016 and aspirin in different experimental set ups.

4.2 Introduction

Considering that NO-releasing groups would be non-essential to decrease the ulcerogenic profile of NSAIDs, we decided not to synthesize the original diazeniumdiolate-containing NSAIDs proposed at the beginning of our program. However, considering that NO-NSAIDs might still be significantly better than conventional NSAIDs for applications other than treatment of inflammation and pain, we decided to screen a representative NO-aspirin (NCX-4016) and compared its profile to that of aspirin and tyrosol prodrugs.

As aspirin and NO-aspirin have shown potential applications in preventing thrombosis,[128-131] we wanted to evaluate the collagen- and AA-induced platelet aggregation inhibitory profile of NSAID prodrugs **(8)** (aspirin-tyrosol) and **(21)** (aspirin-3HBA) (Figure 13). This experiment would provide essential information that would allow us to evaluate the role of anti-oxidant tyrosol moieties compared to the well established effects of NO in cardiovascular pharmacology.

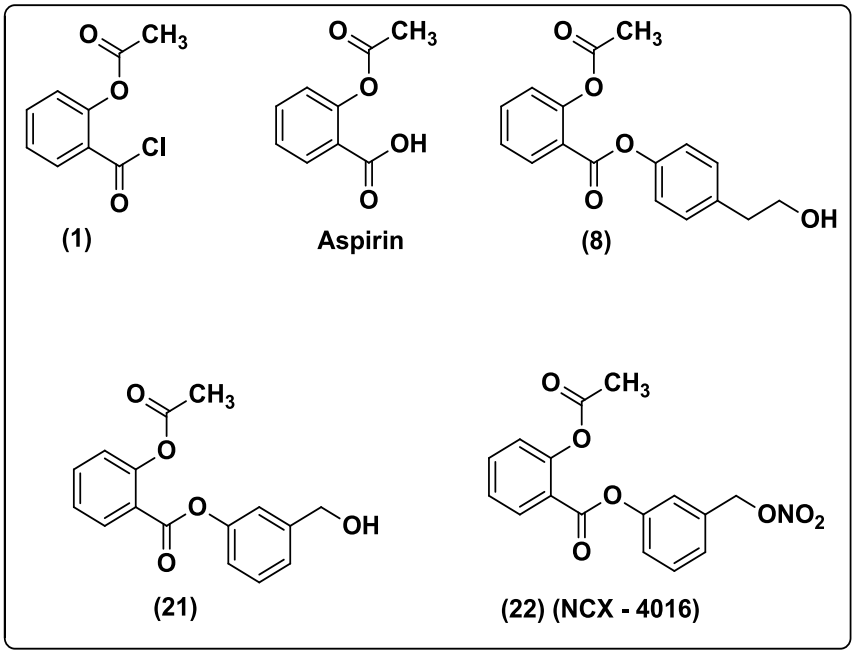
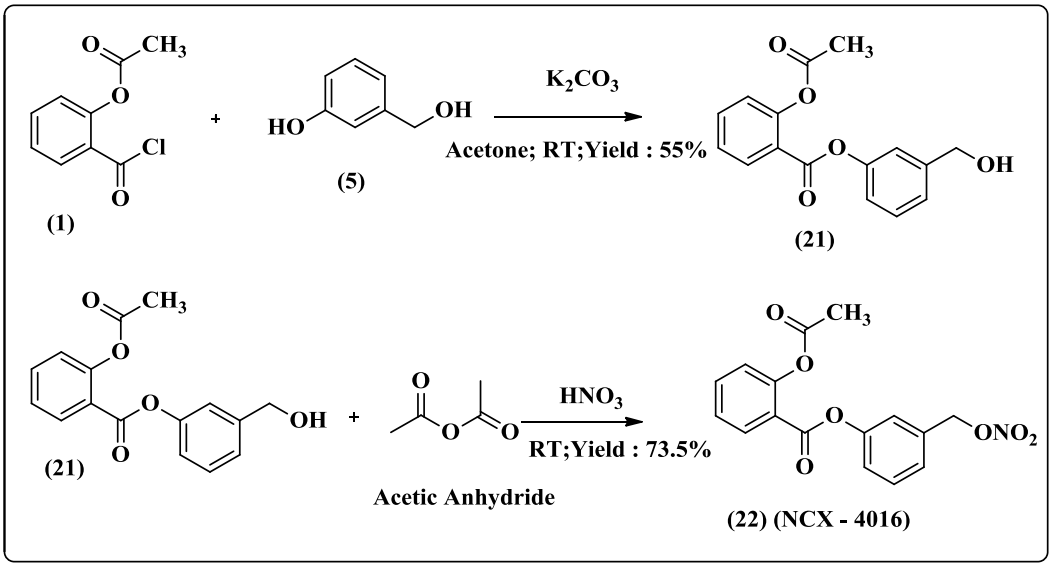


Figure 13: Molecular Structure of Aspirin and Its Various Derivatives.

4.3 Chemistry

The synthesis of prodrug (**8**) was described previously in the thesis. To synthesise NCX–4016, the intermediate ester prodrug (**21**) was formed by a simple nucleophilic displacement reaction between 3-Hydroxybenzyl alcohol (**5**) with aspirin acid chloride dissolved in acetone, using potassium carbonate as base. Compound (**21**) was nitrated by a reaction with fuming nitric acid to get NCX–4016. The yield of these reactions was 55% and 73.5% respectively (Scheme I).



Scheme I: Synthesis of Prodrug **(21)** and NCX-4016

4.4 Results

4.4.1 Carrageenan-Induced Rat Paw Edema Assay

Prodrug (**8**) and aspirin showed a concentration-dependent *in vivo* anti-inflammatory activity. These drugs exerted 58.2% and 50% inhibitory activity respectively, when administered orally at a dose of 705 µmol/Kg concentration, and 67.7% and 86.2% inhibition at 999 µmol/Kg concentration. The tyrosol prodrug showed better anti-inflammatory effect than NCX-4016; NCX-4016 decreased inflammation by 26.31%, whereas prodrug (**8**) exerted 67.7 % inhibition (Table 8).

Table 8: Results of Carrageenan-Induced Rat Paw Edema Assay for Prodrugs (**8**), (**22**) and Aspirin

Compounds	Dose (μmol/kg)	% Inhibition
8	999	67.71 ± 4.7
22	999	26.31 ± 1.68
Aspirin	999	86.23 ± 5.7

Inhibitory activity in a carrageenan-induced rat paw edema assay. The results are expressed as the ID_{50} value (mg/kg) at 3 h after oral administration of the test compound. Test drugs were administered at an equimolar dose of Aspirin. Results are expressed as ± mean SEM (n = 3).

4.4.2 Acute Ulcerogenesis Assay

The aspirin prodrug possessing tyrosol (**8**) was notably non-ulcerogenic and similar results were observed with NCX-4016; however, as expected, this was not the case for aspirin, which produced a significant ulcerogenic effect. For example, NCX-4016 showed an U.I. = 3.5 ± 1.9, prodrug (**8**) = 1.2 ± 0.9, and aspirin = 34.5 ± 6.2. These results provided complementary evidence to support the hypothesis that NO-releasing groups are not required to counteract the gastric ulcerogenic profile of NSAIDs produced by an acute dose of aspirin, ibuprofen, or indomethacin. Details of the assay are given in Table 9.

Table 9: Results of Acute Ulcerogenesis Assay for Prodrugs (**8**), (**22**) and Aspirin

Compounds	Ulcer Index[a]
8[c]	1.3 ± 0.9
22[c]	3.8 ± 1.9
Aspirin[b]	34.5 ± 6.3

[a]The average overall length (in mm) of individual ulcers in each stomach at 5 h after oral administration of prodrugs. [b]180 mg/kg dose. [c]Equimolar amount to 180 mg of Aspirin. Results are expressed as ± mean SEM (n = 3).

4.4.3 Platelet aggregation assays (in vitro)

We evaluated the collagen and AA-induced platelet aggregation inhibitory profile of NSAID prodrug **(8)** (aspirin-tyrosol) and compared it to that obtained with aspirin and NCX-4016. Platelet aggregation was initiated by collagen (1 μg /mL) or AA (0.5 mM) and monitored by AGGRO-LINK software for 6 min. Human platelets were incubated with the corresponding test compound (100 μM) at t = 0; then collagen or AA was added exactly after 2 minutes (t = 2) and the light transmittance was measured for 4 more minutes (total t = 6 minutes).

Additionally, we included another aspirin prodrug possessing a 3-hydroxybenzyl group (**21**); this prodrug is structurally related to NCX-4016 except that it does not have an organic nitrate (-ONO_2). Prodrug (**21**) would give an indication of the

anti-aggregatory properties of a non-NO-releasing aspirin which is similar to both NCX-4016 and prodrugs (**8**).

In the collagen-induced platelet aggregation experiment prodrugs (**8**) and (**21**) and aspirin exerted almost same amount of platelet aggregation (14.1 to 19.8). However, NCX-4016 showed only 4% of platelet aggregation, which is notably better than the other drugs (Table 10 and Figure 14).

The second experiment involved the use of AA (0.5 mM) as the aggregating agent. In this case we observed a similar inhibitory profile to that obtained with collagen, the NCX-4016 was the most potent platelet aggregation inhibitor (61.2% and mean aggregation 31.8), followed by prodrug (**8**) (mean aggregation 76.025), aspirin (mean aggregation 77.9); the least potent was prodrug (**21**) (mean aggregation 83.05) (Table 11 and Figure 15).

These results prove that the presence of NO-releasing groups (organic nitrates) is required to enhance the cardioprotective properties of aspirin (inhibition of agonist-induced platelet aggregation).

Table 10: Results of Collagen Aggregations

Drug	Mean aggregation	SEM	N
Control	100	0	5
8	19.8	3.5	5
21	18.9	4.3	5
22	4	1.8	5
Aspirin	14.1	3.7	5

Table 11: Result of Arachidonic Acid Aggregations

Drug	*Mean aggregation*	*SEM*	*N*
Control	100	0	4
8	76	15.3	4
21	83	4.6	4
22	31.8	14.3	4
Aspirin	78	7.4	4

Figure 14: Collagen Aggregations

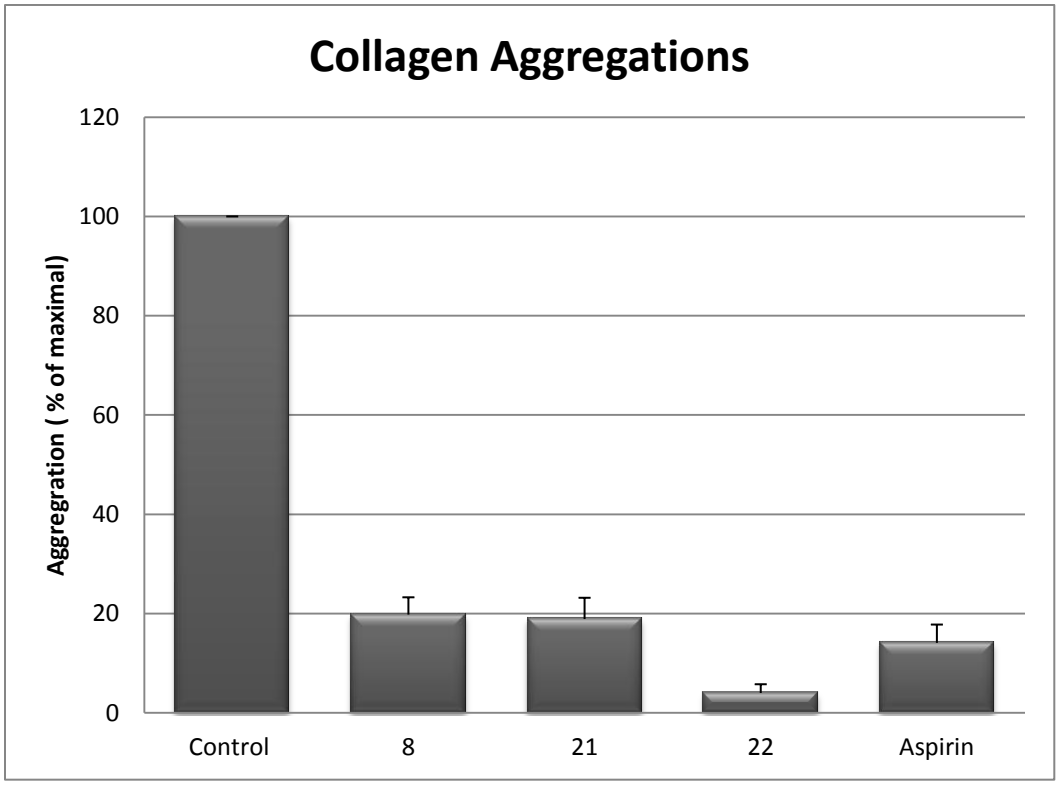
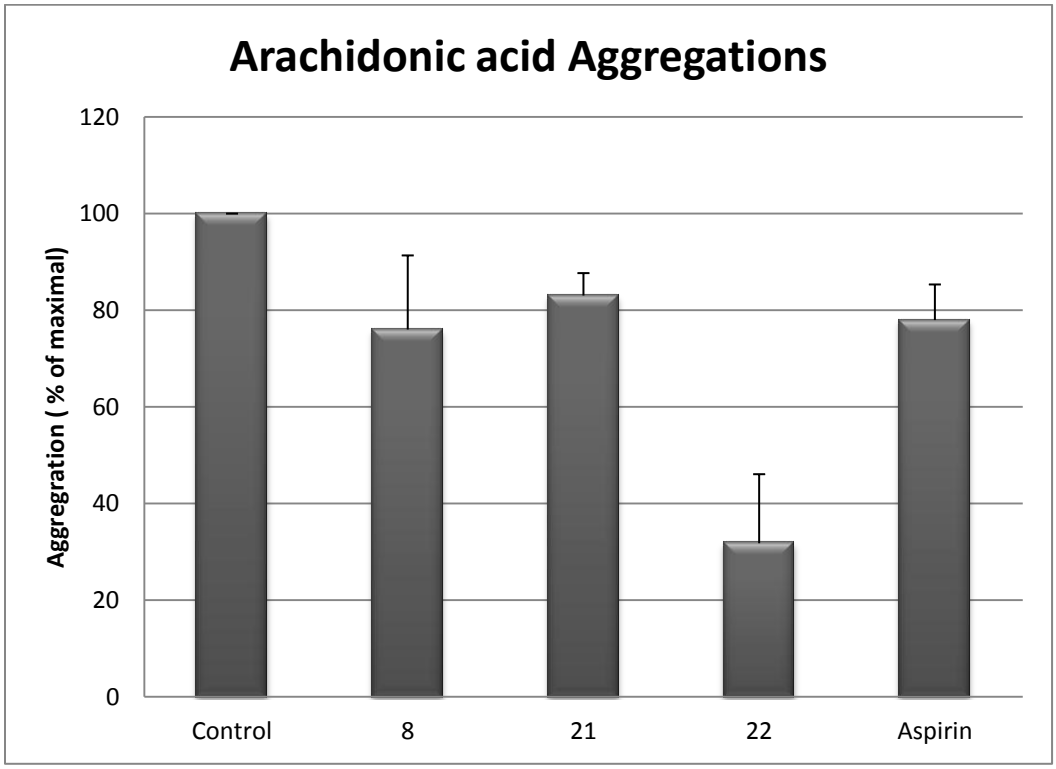


Figure 15: Arachidonic Acid Aggregations



4.5 Discussion

The comparison between the NO-releasing aspirin and a phenolic (tyrosol) prodrug of aspirin proved that we don't need a NO- releasing moiety to produce a NSAID with a better GI safety profile. A simple esterification of NSAIDs can be an alternative to deal with the relatively high incidence of several gastrointestinal toxic side-effects associated with long-term anti-inflammatory therapies. We observed that neither tyrosol nor 3-HBA had an additional effect on platelet aggregation inhibition (other than that exerted by aspirin). The increased inhibition of platelet aggregation observed with NCX-4016 was potentiated by the release of NO from the organic nitrate (-ONO_2) group, which highlights the potential applications of hybrid prodrugs in preventing stroke and other cardiovascular disease states. The use of hybrid molecules possessing NO-

releasing moieties offers an advantage compared to simple prodrugs possessing only a phenol group because bioactive NO complements the COX-1 dependent platelet aggregation inhibitory activity of aspirin[132]. So, “simple” NSAID ester prodrugs possessing phenol moieties are not a suitable replacement to NO-aspirins for the prophylactic treatment of cardiovascular events.

5 Chapter: Lipoxygenase Inhibition and Induction of NQO1 by Ester Prodrugs

5.1 Objective

The last part of the project had two objectives. The first was to evaluate the potential inhibitory profile of tyrosol ester prodrugs on 5-LOX, since LOX enzymes have also been involved in the inflammatory process in addition to the COX pathway. And the second was to determine the potential chemopreventive properties of tyrosol prodrugs via induction of phase II carcinogen-metabolizing NQO1 enzyme.

5.2 Introduction

There have been reports in literature describing that gastric ulceration can be minimized by dual COX/LOX inhibitors[134], but the mechanism by which these drugs exert a decreased gastric toxicity is not yet completely understood[133-134]. We decided to test our tyrosol ester prodrugs for potential LOX inhibition, considering that anti-oxidant groups might confer inhibitory activity by complexing with the heme moiety within the active site of the enzyme.

Additionally, NSAIDs are now considered to be one of the most promising classes of drugs with potential cancer chemopreventive properties[135-142]. For this reason, it was important for us to test tyrosol ester prodrugs. The NAD(P)H:quinone oxidoreductase (NQO1)-mediated detoxification of quinones plays a critical role in cancer prevention. Xenobiotic metabolizing enzymes are classified as phase I (oxidation, reduction) and phase II (conjugation) enzymes; the balance between

phase I (sometimes acting as carcinogen-activating enzymes) and the phase II detoxifying enzymes is important to assess an individual's risk for developing cancer[143]. In this regard, NQO1 protects cells against toxic electrophilic quinones and their metabolic precursors by reducing quinones to semiquinones; therefore, it has been suggested that modulation of drug-metabolizing enzymes could lead to facilitated elimination of endogenous and environmental carcinogens[144]. For this reason we chose to test the activity of the ester prodrugs towards NQO1 enzyme.

5.3 Results

5.3.1 LOX Inhibition Assay

The ester prodrugs did not show significant inhibition of 5-LOX (enzyme immunoassay, Cayman cat. number = 760700). Out of nine prodrugs only three were active. However, two out of these three compounds were molecules containing tyrosol i.e. prodrugs (**8**) and (**11**). Prodrug (**8**) showed an IC_{50} of 213.9 µM whereas prodrug (**11**) showed an IC_{50} of 6.1 µM. Tyrosol itself showed a remarkable inhibitory profile, with the lowest IC_{50} (1.2 µM). This shows that tyrosol is an important moiety required for the LOX inhibition, but linkage of the phenol group to a carboxylic acid decreases its inhibitory effect. The results were not conclusive enough to establish a potential role of LOX inhibition in the overall anti-inflammatory profile of tyrosol esters. The details of the LOX inhibition assay are summarized in Table 12.

Table 12: Results of LOX Inhibition Exerted by Prodrugs

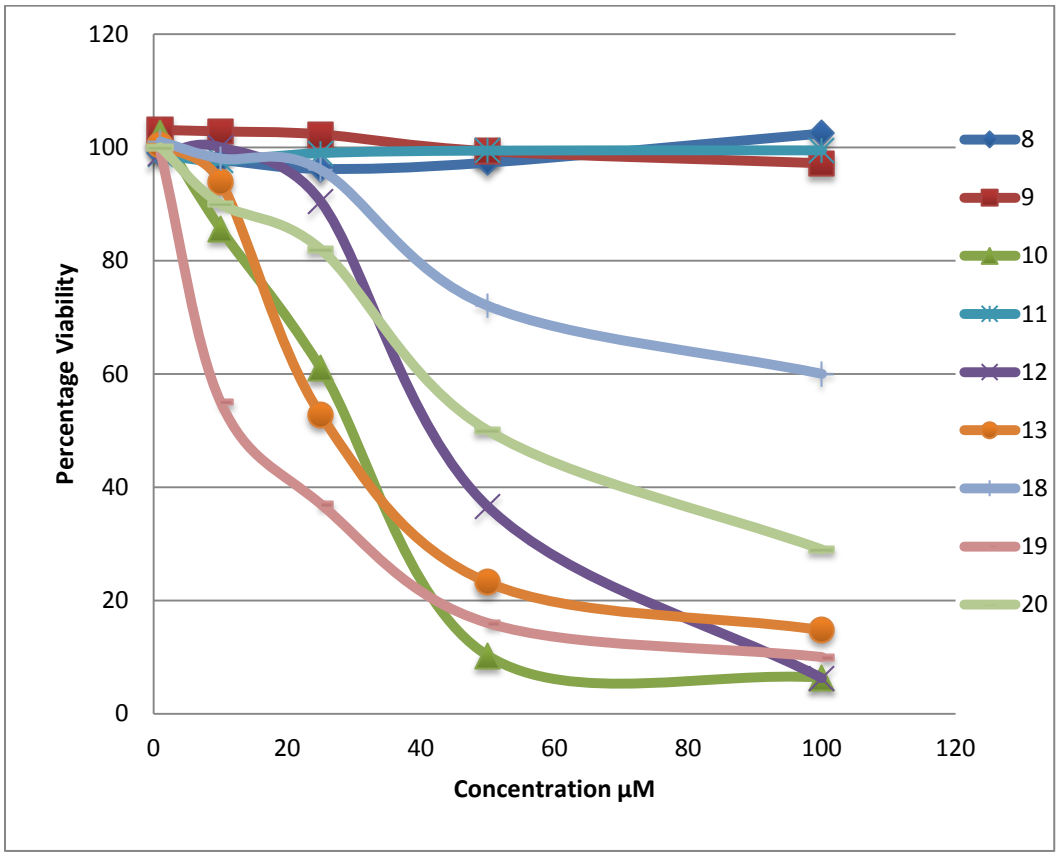
Compounds	5-LOX IC_{50}[a](μM)
8	213.9
9	N.A.
10	N.A.
11	6.1
12	N.A.
13	9.2
18	N.A.
19	N.A.
20	N.A.
21	N.A.
22	N.A.
Tyrosol	1.2
3-HBA	N.A.
4-HBA	7.71

[a]The in vitro test compound concentration required to produce 50% inhibition of potato 5-LOX (Cayman Chemicals Inc. Catalog No. 60401). The result (IC_{50}, μM) is the mean of two determinations acquired using a LOX assay kit (Catalog No. 760700, Cayman Chemicals Inc., Ann Arbor, MI, USA) and the deviation from the mean is <10% of the mean value. N.A. = Not Active, IC_{50}>500μM

5.3.2 Determination of Cell Viability (Prior to NQO1 Activity)

To determine the optimal concentrations to use in cell-based studies, prodrugs were tested for potential cytotoxicity. The cell line used was liver cancer HepG2. The effect of prodrugs on HepG2 cell viability was determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) to formazan crystals. The MTT assay showed that 10 µM was the highest test compound concentration safe enough to work with (except for compound **19**) (Figure 16).

Figure 16: Effect of Prodrugs on Cell Viability.



HepG2 cells were treated for 24 h with prodrugs (1, 10, 25, 50, and 100 µM). Cell cytotoxicity was determined using the MTT assay. Data are expressed as percentage of untreated control (which is set at 100%) ± SE (n = 8). $P < 0.05$, compared to control.

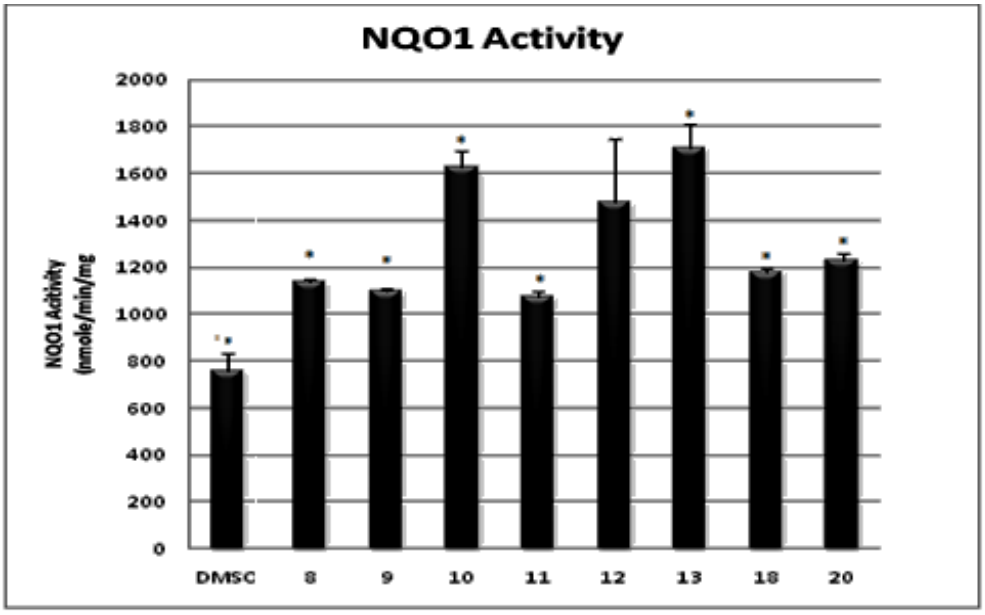
5.3.3 Determination of NQO1 Activity

Considering that classical NSAIDs do not normally increase the activity of phase II xenobiotic-metabolizing enzymes[145], and the induction of NQO1 activity was correlated to NO-release from NO-aspirins[145], the observation that simple prodrugs possessing a tyrosol moiety could induce enzymatic activity would be

relevant because it should offer new evidence suggesting that NSAID ester prodrugs could potentially induce phase II xenobiotic-metabolizing enzymes.

Previous work reported with NO-aspirins showed that NQO1 induction is dependent on the presence of good leaving groups such as nitrates ($^{-}ONO_2$) or bromides (Br^-)[146]. Considering that tyrosol prodrugs designed in our work do not possess leaving groups, we would not expect a significant induction of NQO1activity. We determined the ability of our ester prodrugs (**8**)-(**13**), (**18**) & (**20**) to increase the enzymatic activity of NQO1 in HepG2 cells. Surprisingly, all our prodrugs induced an increase in the activity of NQO1 enzyme *in vitro*, compared to control cells receiving dimethyl sulfoxide (DMSO) as vehicle (Figure 17).

Figure 17: Determination of NQO1 Activity



NQO1 activity was determined by the continuous spectrophotometric assay to quantitate the reduction of its substrate, 2,6-dichlorophenolindophenol (DCPIP). The rate of DCPIP reduction was monitored over 1.5 min at 600 nm with an extinction coefficient (ϵ) of 2.1 mM^{-1} cm^{-1}. The NQO1 activity was calculated as the decrease in absorbance per minute per milligram of total protein of the sample. The concentration used for the ester prodrugs was 10 µM. A one-way analysis of variance (ANOVA) was carried out to assess statistical significance. The differences were significant when * = $p < 0.01$ compared to DMSO; n = 3. The solvent used for all prodrugs was DMSO.

5.4 Discussion

Dunlap *et al*. reported structure-activity relationship studies for NO-aspirin possessing 2-, 3-, or 4-hydroxybenzyl alcohols, assessing the importance of the nitrate group (-ONO_2) on anti-inflammatory, antiproliferative, and chemopreventive activity. Dunlap's group concluded that "conisogenic" structures possessing good leaving groups (bromine) instead of nitrates are

equipotent agents to those possessing nitrate groups, because both types of prodrugs generate electrophilic quinone methides *in vivo*. Quinone methides rapidly react with intracellular glutathione (GSH) and thiol-rich sensor proteins to activate antioxidant responsive element (ARE) and induce NQO1 enzyme activity[146-147]. Considering that our prodrugs do not possess good leaving groups, we did not expect the formation of quinone methides, and therefore, our prodrugs would not be expected to exert the same mechanism of action as that reported for NO-aspirins or "conisogenic" compounds having good leaving groups. However, tyrosol prodrugs increased the enzymatic activity of NQO1 in HepG2 cells. These results indicate that simple NSAID ester prodrugs possessing anti-oxidant moieties such as tyrosol may be considered potential chemopreventive agents, despite the absence of good leaving groups (including organic nitrates). It is difficult to evaluate this statement without having additional data involving cell growth inhibition in specific cancer cell lines, or studies specially designed to evaluate chemoprevention; however, results obtained in this work suggest that NSAID esters should be studied along with classical NSAIDs, not only on the expression and activity of phase I and phase II metabolizing enzymes, but also in models of induced cancer (future directions).

6 Chapter: Conclusion, Limitations and Future Directions

6.1 Conclusion

The results reported in this thesis present simple ester prodrugs, which have the potential to be regarded as safe in comparison to NSAIDs in clinical use. Simple esterification of aspirin, ibuprofen, and indomethacin with phenolic antioxidant groups such as tyrosol, 3-hydroxybenzyl alcohol, and 4-hydroxybenzyl alcohol yields potent and selective COX-2 inhibitors *in vitro* with improved anti-inflammatory activity in vivo.

The work presented in this thesis offers conclusive evidence to support that NO-releasing groups are not required to decrease the ulcerogenic profile of classical NSAIDs, regardless of whether they are available over-the-counter (aspirin, ibuprofen) or by prescription only (indomethacin). Considering that H_2S-releasing NSAIDs were designed based on a similar approach as that of NO-NSAIDs, this observation might be applicable to H_2S-releasing prodrugs as well; in other words, H_2S-releasing groups may not be required to decrease gastric toxicity associated with acute doses of NSAIDs; this would have significant implications for several ongoing clinical trials involving some of these compounds.

With regards to the indomethacin prodrugs (**18**), (**19**) and (**20**) the work presented, gives support to the fact that anti-oxidant moieties play a significant role in the prevention of ulcer formation. It is evident from the results obtained

with prodrugs (**18**) and (**19**), which didn't contain an anti-oxidant moiety, that future designs should consider careful evaluation of the nature of this moiety.

Results obtained in this work, along with those reported previously by other groups, constitute a good body of evidence to propose the use of NSAID prodrugs (esters, and probably amides as well) instead of unprotected molecules. The significance of this statement is evident considering that NSAIDs are one of the most highly used group of drugs world-wide, and the relatively high incidence of gastrointestinal side-effects associated with their long-term use. In this regard, it is essential to open the debate within the scientific community to re-evaluate the potential use of new and/or existing NSAID prodrugs as a safer alternative to the use of classical NSAIDs.

This work also concludes that the advantages of using NO- and H_2S-releasing moieties in the design of hybrid NSAIDs are justified when the purpose of such prodrugs involves:

- Accelerating the healing of pre-existing ulcers.
- Disease states other than the conventional anti-inflammatory/non-ulcerogenic profile; for example, to potentiate the anti-thrombotic activity of aspirin, or the prophylactic prevention of similar cardiovascular disorders, in which the beneficial effects of NO are (until now) clearly evident.

The potential application of NSAID ester prodrugs as chemopreventive agents is still controversial. Our work showed that aspirin, ibuprofen, and indomethacin esters possessing simple phenol groups increased the activity of the carcinogen-metabolizing enzyme NQO1, which is not in agreement with previous observations correlating this effect with the extent of NO-release from NO-aspirins. This constitutes a future topic for research to determine the role of good leaving groups (including organic nitrates) in the expression and activity of

phase I and phase II metabolizing enzymes, using a more robust experimental protocol design focused exclusively on chemoprevention.

6.2 Limitations

It is worth mentioning that the anti-inflammatory profile of NSAID prodrugs discussed above constitutes only one of the biological effects attributed to these molecules, and the presence of NO-releasing moieties as well as anti-oxidant groups may have a significant effect on other potential applications, for example the use of aspirin for the prophylactic treatment of cardiovascular disorders and cancer[4]. Therefore, the scope of the current work is only limited to discussing the role of ester groups in decreasing the gastric toxicity of NSAIDs while maintaining their efficacy *in vivo*.

Moreover, this study used relatively high (acute) doses of NSAIDs administered only once, rather than the administration of lower amounts over a long period of time, which would provide data regarding the effects of NSAID ester prodrugs when administered on a long-term basis. Furthermore, another interesting setting on which simple esters could be compared to NO-NSAIDs in future experiments is on chronic animal models designed to measure the effect of drugs on healing of pre-existing ulcers[148].

6.3 Future Directions

1. Review the pharmacokinetic and pharmacodynamic profile of reported "simple" ester and amide NSAID prodrugs.
2. Conduct a structure-activity relationship study to determine the structural requirements which are essential to maintain safety and efficacy of simple prodrugs which *may substitute NSAIDs associated with a high incidence of gastrointestinal side-effects*.
3. Determine the inhibition of COX-1/COX-2 expression and activity by prodrugs using a cell-based assay.
4. Evaluate the chemopreventive properties of NSAID ester prodrugs in an *in vivo* model (Min Mice Model).
5. Evaluate the ulcerogenic properties of prodrugs in a chronic (long-term) ulcerogenic model.

7 Chapter: Experimental Procedures

7.1 Chemistry

Melting points were determined with an Electrothermal Mel-Temp® melting point apparatus (Dubuque, IA, USA) and are uncorrected. Infrared (IR) spectra were recorded as films (chloroform solutions or neat compounds) on NaCl plates using a Nicolet 550 series II Magna FTIR spectrometer. ^{1}H-NMR spectra were measured on a Bruker AM-300 spectrometer with TMS as internal standard, where coupling constants (*J*) are estimated in Hertz (Hz). Mass spectra (MS) were recorded on a Waters micromass ZQ 4000 mass spectrometer using the ESI mode. All the compounds showed a single spot on RediSep® silica gel glass plates (UV_{254}, 0.2 mm) using a high, medium, and low polarity solvent mixture and no residue remained after combustion, indicating a purity higher than 95%. Column chromatography was performed on a CombiFlash Retrieve system using RediSep Rf silica gel (40-60 μM) cartridges. Ibuprofen acid chloride (**7**, racemic) [76],Indomethacin acid chloride (**17**)[76], and NO-releasing Aspirin (NCX-4016)[149] were synthesized according to reported literature procedures. Acetyl salicyloyl chloride (**1**) was obtained from TCI America (Portland, OR); all other reagents were purchased from Aldrich chemical company (Milwaukee, WI) and were used without further purification. The *in vivo* anti-inflammatory and UI assays were carried out using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

4-(2-Hydroxyethyl)phenyl acetylsalicylate (8).

A solution of tyrosol (**4**, 1.4 g, 7.2 mmol) and TEA (0.2 g, 1.9 mmol) in dry THF (10 mL) was stirred for 10 minutes under a nitrogen atmosphere before adding (dropwise) a solution of acetyl salicyloyl chloride (**1**, 1.0 g, 7.2 mmol) previously dissolved in dry THF (5 mL). This reaction mixture was stirred 25 °C for 5 h; all solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to give **(8)** as white solid (1.1 g, 50.6 % yield); mp: 66-69°C; IR (NaCl) 3370 (OH), 2936 (C-H aromatic), 2867 (C-H aliphatic), 1740 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.51 (bs, 1H, O*H*), 2.31 (s, 3H, CH_3), 2.89 (t, *J* = 6.7 Hz, 2H, PhCH_2), 3.88 (t, *J* = 6.7 Hz, 2H, CH_2OH), 7.12 (d, *J* = 8.5 Hz, 2H, phenyl H-2, H-6), 7.17 (dd, *J* = 7.9 Hz, 1.2 Hz, salicyloate H-3), 7.28 (d, *J* = 8.5 Hz, 2H, phenyl H-3, H-5), 7.39 (td, *J* = 7.3, 1.2 Hz, salicyloate H-5), 7.64 (td, *J* = 7.3, 1.8 Hz, salicyloate H-4), 8.22 (dd, *J* = 7.3, 1.2 Hz, salicyloate H-6). ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 21.0, 38.6, 63.5, 115.4, 121.7, 122.5, 124.0, 126.1, 130.1, 132.2, 134.5, 136.4, 149.1, 151.1, 163.0; MS: 301.0$[M+1]^+$.

3-(2-Acetoxybenzoyloxy)benzyl 2-acetoxybenzoate (9).

A solution of 3-hydroxylbenzyl alcohol (**5**, 1.72g, 13.88 mmol) and TEA (1.5 g, 15.2 mmol) in dry THF (20 mL) was stirred for 10 minutes under a nitrogen atmosphere before adding (dropwise) a solution of aspirin acid chloride (**1**, 2.5 g, 13.88 mmol) in dry THF (10 mL). This reaction mixture was stirred for 6 h at 25 °C; all solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to obtain **(9)** as a white solid (2.6 g, 41.8 % yield); mp: 115-118°C;IR (NaCl) 2081 (C-H aromatic), 2851 (C-H aliphatic), 1772 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.19 (s, 3H, CH_3), 2.3 (s,

3H, CH_3), 5.3 (s, 2H, PhCH_2), 7.1 (dd, *J* = 7.9, 1.2 Hz, phenyl H′-6), 7.18 (dd, *J* = 7.9, 1.2, phenyl H′-2), 7.41(m, H=3), 7.56 (td, *J* = 7.3, 1.8 Hz, salicyloate* H-4), 7.65 (td, *J* = 7.3, 1.8 Hz, salicyloate H-4), 8.07 (dd, *J* = 7.3, 1.2 Hz, salicyloate* H-6), 8.22 (dd, *J* = 7.3, 1.2 Hz, salicyloate H-6). ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 20.8,21.0, 66.2, 121.5, 121.7, 122.4, 123.0, 123.8, 124.0, 125.88, 126.04, 126.17, 129.82, 131.92, 132.17, 134.0, 134.63, 137.45, 150.71, 151.18, 162.81, 164.19, 169.65; MS: 449$[M+1]^+$.

4-(2-acetoxybenzoyloxy)benzyl 2-acetoxybenzoate (10).

A solution of 4-hydroxylbenzyl alcohol (**6**, 1.31 g, 10.57 mmol) and TEA (1.28 g, 12.7 mmol) in dry THF (20 mL) was stirred for 10 minutes under a nitrogen atmosphere before adding (dropwise) a solution of aspirin acid chloride (**1**, 2.1 g, 10.57 mmol) in dry THF (10 mL). This reaction mixture was stirred for 6 h at 25 °C; all solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to obtain **(10)** as a white solid (2.2 g, 46.4 % yield); mp: 108-110°C;IR (NaCl) 3081 (C-H aromatic), 2890 (C-H aliphatic), 1772 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.19 (s, 3H, CH_3), 2.3 (s, 3H, CH_3), 5.3 (s, 2H, PhCH_2), 7.09 (dd, *J* = 7.9 Hz, 1.2 Hz, salicyloate* H-3), 7.12 (m, 3H), 7.33 (td, *J* = 7.3, 1.2 Hz, salicyloate* H-5), 7.39 (td, *J* = 7.3, 1.2 Hz, salicyloate H-5), 7.49 (d, *J* = 8.5 Hz, 2H, phenyl H-3, H-5), 7.56 (td, *J* = 7.3, 1.8 Hz, salicyloate* H-4), 7.65 (td, *J* = 7.3, 1.8 Hz, salicyloate H-4), 8.07 (dd, *J* = 7.9, 1.2 Hz, salicyloate* H-6), 8.23 (dd, *J* = 7.9, 1.2 Hz, salicyloate H-6). ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 21.0, 38.6, 63.5, 115.4, 121.7, 122.5, 124.0, 126.1, 130.1, 132.2, 134.5, 136.4, 149.1, 151.1, 163.0; MS: 449$[M+1]^+$.

4-(2-Hydroxyethyl)phenyl 2-(4-isobutylphenyl)propanoate (11).

A solution of tyrosol (**4**, 1.5, 11.1 mmol) and TEA (3.3 g, 13.3 mmol) in dry THF (15 mL) was stirred for 10 minutes under a nitrogen atmosphere before adding (dropwise) a solution of ibuprofen acid chloride (**7**, 2.5 g, 11.1 mmol) in dry THF (10 mL). This reaction mixture was stirred at 25 °C for 5 h; all solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to give **(11)** as white solid (1.9 g, 53.2 % yield); mp: 47-50°C; IR (NaCl) 3383 (OH), 2955 (C-H aromatic), 2867 (C-H aliphatic), 1740 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 0.91 (d, *J* = 6.6 Hz, 6H, $CH(CH_3)_2$), 1.60 (d, *J* = 7.3 Hz, 3H, $PhCHCH_3$), 1.85 (nonet, *J* = 6.7 Hz, 1H, $CH_2CH(CH_3)_2$), 2.47 (d, *J* = 7.3 Hz, 2H, $PhCH_2$), 2.83 (t, *J* = 6.7 Hz, 2H, $PhCH_2$), 3.82 (t, *J* = 6.7 Hz, 2H, CH_2OH), 3.93 (q, *J* = 7.3 Hz, 1H, PhCH), 6.93 (d, *J* = 8.5 Hz, 2H, phenyl H-2, H-6), 7.14 (d, *J* = 7.9 Hz, 2H, Tyrosol phenyl H-2, H-6), 7.18 (d, *J* = 8.5 Hz, 2H, phenyl H-3, H-5), 7.29 (d, *J* = 7.9 Hz, 2H, Tyrosol phenyl H-3, H-5); ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 18.5, 22.3, 30.1, 38.5, 45.0, 45.2, 63.5, 121.2, 121.4, 127.1, 129.2, 129.3, 129.4, 129.8, 135.9, 137.1, 140.7, 149.4, 173.3; MS 327 $[M+1]^+$.

3-(Hydroxymethyl)phenyl 2-(4-isobutylphenyl)propanoate (12).

A solution of 3-hydroxylbenzyl alcohol (**5**, 1.38 g, 11.1 mmol) and TEA (1.3 g, 13.3 mmol) in dry THF (15 mL) was stirred for 10 minutes under a nitrogen atmosphere before adding (dropwise) a solution of ibuprofen acid chloride (**7**, 2.5 g, 11.1 mmol) in dry THF (10 mL). This reaction mixture was stirred for 5 h at 25 °C; all solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to obtain **(12)** as a white

solid (1.8 g, 53.2 % yield); mp: 47-50°C; IR (NaCl) 3383 (OH), 2955 (C-H aromatic), 2860 (C-H aliphatic), 1753 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 0.91 (d, *J* = 6.6 Hz, 6H, $CH(CH_3)_2$), 1.60 (d, *J* = 6.7 Hz, 3H, CH_3), 1.85 (nonet, 1H, $CH_2CH(CH_3)_2$), 2.47 (d, *J* = 7.3 Hz, 2H, $PhCH_2$), 3.92 (q, *J* = 6.7 Hz, 1H, PhCH), 4.66 (s, 2H, CH_2OH), 6.91 (dd, *J* = 7.3, 2.4 Hz, 1H, phenyl H′-6), 7.01 (distorted t, 1H, phenyl H′-2), 7.14 (d, *J* = 7.9 Hz, 2H, phenyl H-2, H-6), 7.18 (d, *J* = 7.9 Hz, phenyl H′-4), 7.29 (d, *J* = 7.9 Hz, 2H, phenyl H-3, H-5), 7.32 (t, *J* = 7.9 Hz, 1H, phenyl H′-5); ^{13}C-NMR ($CDCl_3$) δ = 18.4, 22.3, 30.1, 45.0, 45.2, 63.6, 119.7, 120.4, 124.0, 127.1, 129.3, 129.4, 137.1, 140.7, 142.5, 150.9, 173.2; MS: 313 $[M+1]^+$.

4-Hydroxymethylphenyl 2-(4-isobutylphenyl)propanoate (13).

A solution of 4-hydroxybenzyl alcohol (**6**, 1.38 g, 11.1 mmol) and TEA (1.3 g, 13.3 mmol) in dry THF (15 mL) was stirred for 5 minutes under a nitrogen atmosphere before adding (dropwise) a solution of Ibuprofen acid chloride (**7**, 11.1 mmol) previously dissolved in dry THF (10 mL). This reaction mixture was stirred at 25 °C for 5 h; all precipitated solids (triethylammonium chloride) were filtered off and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to give **(13)** as an oil (1.9 g, 54.7% yield); IR (NaCl) 3383 (OH), 2955 (C-H aromatic), 2860 (C-H aliphatic), 1753 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 0.91 (d, *J* = 6.6 Hz, 6H, $CH(CH_3)_2$), 1.60 (d, *J* = 6.7 Hz, 3H, CH_3), 1.85 (septet, 1H, $CH(CH_3)_2$), 2.47 (d, *J* = 7.3 Hz, 2H, $PhCH_2$), 3.93 (q, *J* = 7.3 Hz, 1H, PhCH), 4.66 (s, 2H, CH_2OH), 6.98 (d, *J* = 8.5 Hz, 2H, phenyl H′-2, H′-6), 7.14 (d, *J* = 7.9 Hz, 2H, phenyl H-2, H-6), 7.21 (d, *J* = 7.9, 2H, H-3, H-5), 7.33 (d, *J* = 8.5 Hz, 2H, phenyl H′-3, H′-5); ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 18.5, 22.3, 30.1, 45.0, 45.2, 64.7, 121.4, 127.1, 127.9, 129.3, 129.4, 137.1, 138.3, 140.7, 150.2, 173.2; MS: 312 $[M+1]^+$.

Phenyl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetate (18). A solution of phenol (**15**, 0.12 g, 1.3 mmol) and TEA (0.16 g, 1.5 mmol) in dry THF (5 mL) was stirred under a nitrogen atmosphere for 10 minutes before adding (dropwise) a solution of indomethacin acid chloride (**17**, 0.50 g, 1.3 mmol) in dry THF (5 mL). This mixture was stirred at 25 °C for 3 h; all precipitated solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (3:7) as eluent to give **(18)** as a white solid (0.41 g, 71.1% yield); IR (NaCl): 2930 (C-H aromatic), 2829 (C-H aliphatic), 1753 (CO), 1683 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 2.46 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 3.91 (s, 2H,PhCH_2CO_2), 6.70 (dd, *J* = 9.1, 2.4 Hz, 1H, indolyl H-6), 6.91 (d, *J* = 9.1 Hz, 1H, indolyl H-7), 7.04-7.07 (m, 1H, phenyl H-4), 7.07 (d, *J* = 2.4 Hz, 1H, indolyl H-4), 7.26 (m, 2H, phenyl H-2, H-6), 7.36 (t, *J* = 7.9 Hz, 2H, phenyl H-3, H-5), 7.47 (d, *J* = 8.5 Hz, 2H, benzoyl H-3, H-5), 7.68 (d, *J* = 8.5 Hz, 2H, benzoyl H-2, H-6). ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 13.4, 30.6, 55.7, 101.2, 111.8, 112.0, 115.0, 121.3, 125.1, 129.4, 130.5, 130.8, 131.8, 133.8, 136.1, 139.3, 150.7, 156.1, 168.2, and 169.23; MS: 379.1 $[M+1]^+$.

2-Hydroxyethyl2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetate (19).

A solution of ethylene glycol (**16**, 0.99 g, 15.9 mmol) and TEA (0.19 g, 1.9 mmol) in dry THF (5 mL) was stirred under a nitrogen atmosphere for 10 minutes before adding (dropwise) a solution of indomethacin acid chloride (**17**, 0.60 g, 1.5 mmol) in dry THF (5 mL). This reaction was stirred at 25 °C for 3 h; all precipitated solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. To remove unreacted (excess) ethylene glycol, the residue was dissolved in EtOAc (100 mL), washed with brine (3 x 20 mL), and dried over sodium sulfate, then the solvent was evaporated under vacuum. The residue was

purified by silica gel column chromatography using EtOAc/hexane (4:6) as eluent to give **(19)** as a grey/green solid (0.49 g, 76% yield); mp: 77-79° C; IR (NaCl): 3471(OH), 2923 (C-H aromatic), 2829 (C-H aliphatic), 1734 (CO), 1690 (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 2.35 (s, 3H, C*H*$_3$), 3.68 (s, 2H, PhC*H*$_2$), 3.77 (t, *J* = 4.8 Hz, 2H, C*H*$_2$OH), 4.20 (t, *J* = 4.8 Hz, 2H, CO_2C*H*$_2$), 6.64 (dd, *J* = 9.1 Hz, 2.4 Hz, 2H, indolyl H-6), 6.84 (d, *J* = 9.1 Hz, 1H, indolyl H-7), 6.94 (d, *J* = 2.4 Hz, 1H, indolyl H-4), 7.43 (dd, *J* = 8.5 Hz, 2.4 Hz, 2H, benzoyl H-3, H-5), 7.63 (dd, *J* = 8.5 Hz, 2.4 Hz, 2H, benzoyl H-2, H-6). ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 13.3, 30.2, 55.6, 61.0, 66.5, 101.2, 111.6, 112.2, 114.9, 129.0, 130.4, 130.7, 131.1, 133.8, 135.9, 139.2, 156.0, 168.2, and 171.0; MS: 402 $[M+1]^+$.

4-(2-Hydroxyethyl)phenyl2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetate (20).

A solution of tyrosol (**4**, 0.18 g, 1.3 mmol) and TEA (0.16 g, 1.5 mmol) in dry THF (10 mL) was stirred under a nitrogen atmosphere at -80 °C for 10 minutes before adding (dropwise) a solution of indomethacin acid chloride (**17**, 0.50 g, 1.3 mmol) in THF (5 mL). This reaction mixture was stirred at -80 °C for 5 h; the precipitated salts (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (3:7) as eluent to give **(20)** as a dark green viscous liquid (0.12 g, 19.46 % yield); IR (NaCl): 3458 (OH), 2999 (C-H aromatic), 2904 (C-H aliphatic) X (CO), Y (CO) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 2.29 (s, 3H, C*H*$_3$), 2.81 (t, *J* = 6.7 Hz, 2H, PhC*H*$_2$), 3.62 (s, 2H, C*H*$_2$$CO_2$), 3.82 (s, 3H, OC*H*$_3$), 4.27 (t, *J* = 6.7 Hz, 2H, C*H*$_2$OH), 6.64 (dd, *J* = 9.1 Hz, 2.4 Hz, 2H, indolyl H-6), 6.65 (d, *J* = 8.5 Hz, 2H, phenyl H-2, H-6), 6.84 (d, *J* = 9.1 Hz, 1H, indolyl H-7), 6.91 (d, *J* = 8.5 Hz, 2H, phenyl H-3, H-5), 6.94 (d, *J* = 2.4 Hz, 1H, indolyl H-4), 7.43 (dd, *J* = 8.5 Hz, 2.4 Hz, 2H, benzoyl H-3, H-5), 7.63 (dd, *J* = 8.5 Hz, 2.4 Hz, 2H, benzoyl H-2, H-6); ^{13}C-NMR (300 MHz, $CDCl_3$) δ = 13.3, 30.4, 34.1, 55.7, 65.5, 101.4, 111.6, 112.5,

114.9, 115.3, 129.1, 129.6, 129.8, 130.6, 130.8, 131.1, 133.9, 135.7, 139.2, 154.1, 156.0, 168.3, 170.7; MS: 477 $[M+1]^+$.

7.2 Biological Evaluation

Cyclooxygenase Inhibition Assay (in vitro)

All experimental compounds and reference compounds (aspirin, ibuprofen, and indomethacin) were evaluated for their ability to inhibit human recombinant COX-2 and ovine COX-1 using a cyclooxygenase inhibitor screening assay kit (catalog number 560131, Cayman Chemical, Ann Arbor, MI, USA) following the procedure suggested by the manufacturer. Cyclooxygenase catalyzes the first step in the biosynthesis of AA to PGH_2. PGF_2R, produced from PGH_2 by reduction with stannous chloride, is measured by enzyme immunoassay (ACE competitive EIA). Stock solutions of test compounds were dissolved in a minimum volume of DMSO. Briefly, to a series of supplied reaction buffer solutions (960 µL, 0.1 M Tris-HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1or COX-2 (10 µL) enzyme in the presence of heme (10 µL) was added 10 µL of various concentrations of test drug solutions (0.001, 0.01, 0.1, 1, 10, 100, and 500 µM in a final volume of 1mL). These solution were incubated for a period of 2 min at 37 °C after which 10 µL of AA (100 µM) was added, and the COX reaction was stopped by the addition of 50 µL of 1 M HCl after 2 min. PGF_2R, produced from PGH_2 by reduction with stannous chloride, was measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells since the concentration of the PG tracer is held constant while the concentration of PGs varies. This antibody-PG complex binds to a mouse antirabbit monoclonal

antibody that had been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent, which contains the substrate to acetylcholinesterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow color that absorbs at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation: absorbance α [bound PG tracer] α 1/PGs. Percent inhibition was calculated by comparison of compound treated to various control incubations. The concentration of the test compound causing 50% inhibition (IC_{50}, μM) was calculated from the concentration-inhibition response curve (duplicate determinations).

Anti-inflammatory Assay (in vivo)

The experimental compounds (**8-13**, **18-20** and **22**) were evaluated using the carrageenan-induced rat foot paw edema model reported previously[150]. Animals were fasted and weighed 24 hours prior to the experiment. All test compounds were suspended in 1.2 mL of a 1% methylcellulose aqueous solution and administered at the following doses: Group 1) aspirin and aspirin derivatives (compounds **8, 9, 10**) = 710 μmol/kg; Group 2) ibuprofen and ibuprofen derivatives (compounds 11, 12, 13) = 325 μmol/kg; Group 3) indomethacin and indomethacin derivatives (compounds **18**, **19, 20**) = 80 μmol/kg; Group 4) Control (1% methylcellulose solution). Test compounds were administrated orally by gavage before subplantar injection of 0.1 mL of a 1% solution of carrageenan in 0.9% saline on the left hind paw. Paw volumes were measured by plethysmometer immediately before induction of oedema and after 3 hours. The increase in paw volume was calculated by subtracting the initial paw volume (basal) to the paw volume measured, and was expressed as percentage

increased in paw volume. This assay was carried out using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

Acute Ulcerogenesis Assay[151]

Ulcerogenic activity was evaluated after oral administration of Aspirin (250mg/kg), Ibuprofen (250mg/kg), Indomethacin (29mg/kg) or an equivalent amount of the corresponding test compound **(12-18)**. All drugs were suspended and administered in 1.2 mL of a 1% methylcellulose solution. Control rats received oral administration of vehicle (1.2 mL of 1.0% methylcellulose solution). Food, but not water, was removed 24 h before administration of test compounds. Six hours after oral administration of the drug, rats were euthanized in a CO_2 chamber and their stomachs were removed, cut out along the greater curvature of the stomach, gently rinsed with water, and placed on ice. The number and the length of ulcers observed in each stomach were determined using a magnifier lens. The severity of each gastric lesion was measured along its greatest length (1 mm = rating of 1, 1–2 mm = rating of 2, >2 mm = rating according to their length in mm). The UI for each test compound was calculated by adding the total length (L, in mm) of individual ulcers in each stomach, divided by the number of animals in each group (n = 3): UI = (L1 + L2 + L3)/3.

Platelet Aggregation Assays (in vitro)

Prostacyclin-washed platelet suspensions were prepared as previously described[152-154]. Briefly, upon isolation platelets were counted and resuspended (2.5×10^8/mL) in Tyrode's buffer and allowed to rest at room temperature (1 hour) for the inhibitory effects of the prostacyclin on platelets to wear off. Subsequently, platelets were pre-incubated for 2 minutes at 37 ^{o}C in a lumi-aggregometer in the presence of the corresponding prodrug, and then platelet

aggregation was induced with either collagen (10 mg/mL) or thrombin (0.3 U/mL). To give uniform platelet responses, both chosen agonist concentrations were at the top of their respective concentration-response curves. Aggregation was monitored for a further 4 minutes and the extent of aggregation was determined by Aggrolink^{TM} software.

Expression of NQO1 (in vitro)

The enzymatic activity of the xenobiotic-detoxifying enzyme NQO1 was assessed using previously reported procedures[155]. Briefly, HepG2 cells were grown for 24 h in six well plates before incubation with the test compounds for another 24 h. Thereafter, cells were washed with PBS and kept in 0.5 mL of homogenization buffer at -80 ^{o}C for at least 24 h; thawed cells were extracted and homogenized with a Kontes homogenizer and then centrifuged at 10,000×*g* for 20 min. The supernatant fractions were then removed and protein concentrations in the supernatant were determined by the method of Lowry *et al.* using bovine serum albumin as the standard[156]. The enzymatic activity of NQO1 was determined following the spectrophotometric assay described by which quantitates the reduction of 2,6-dichlorophenolindophenol (DCPIP) by 0.01 mg of cell homogenate protein in the presence of β-nicotinamide adenine dinucleotide phosphate (NADPH; 200 µM) and flavin adenine dinucleotide (FAD; 5 µM). The rate of reduction of DCPIP (40 µM) in 1 mL of Tris–HCl buffer (pH 7.8, 25 mM) containing 0.1% (v/v) Tween 20 and 0.023% bovine serum albumin was monitored for 90 seconds at 600 nm with ε = 2.1 mM^{-1} cm^{-1}, in presence and absence of 10µM dicumarol. The NQO1 activity was calculated as the decrease in absorbance per min per mg of total protein of the sample.

5-Lipoxygenase Inhibition Assay

The ability of the test compounds to inhibit potato 5-LOX (Catalog No. 60401, Cayman Chemical, Ann Arbor, MI, USA) (IC_{50} values, µM) was determined using an enzyme immuno assay (EIA) kit (Catalog No. 760700, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The Cayman Chemical lipoxygenase inhibitor screening assay detects and measures the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase. Stock solutions of test compounds were dissolved in a minimum volume of DMSO and were diluted using the supplied buffer solution (0.1 M, Tris–HCl, pH 7.4). To a 90 µl solution of 5-LOX enzyme in 0.1 M, Tris–HCl, pH 7.4 buffer, 10 µl of various concentrations of test drug solutions (0.01, 0.1, 1 and 10 µM in a final volume of 210 µl) were added and the lipoxygenase reaction was initiated by the addition of 10 µl (100 µM) of linoleic acid (LA). After maintaining the 96-well plate on a shaker for 5 min, 100 µl of chromogen was added and the plate was retained on a shaker for 5 min. The lipoxygenase activity was determined after measuring absorbance at a wavelength of 500 nm. Percent inhibition was calculated by the comparison of compound-treated to various control incubations. The concentration of the test compound causing 50% inhibition (IC_{50}, µM) was calculated from the concentration–inhibition response curve (duplicate determinations).

8 Chapter: Bibliography

1. Jack DB. One hundred years of aspirin. *The Lancet* 1997;350(9075):437-39.
2. Michael M, Lichtenstein R, Singh G. Gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs. *The New England Journal of Medicine* 1999;340(24):1888.
3. Jacob S, Laury-Kleintop L, Lanza-Jacoby S. The select cyclooxygenase-2 inhibitor celecoxib reduced the extent of atherosclerosis in apo E-/- mice. *Journal of Surgical Research* 2008;146(1):135-42.
4. Yasuda O, Takemura Y, Kawamoto H, Rakugi H. Aspirin: recent developments. *Cellular and Molecular Life Sciences* 2008;65(3):354-58.
5. ADAPT Research Group, Meinert CL, McCaffrey LD, Breitner JC. Alzheimer's Disease Anti-Inflammatory Prevention Trial: Design, methods, and baseline results. *Alzheimer's and Dementia* 2009;5(2):93-104.
6. AD2000 Collaborative Group, Bentham P, Gray R, Sellwood E, Hills R, Crome P, Raftery J. Aspirin in Alzheimer's disease (AD2000): a randomised open-label trial. *Lancet Neurology* 2008;7(1):41-49.
7. Szekely CA, Zandi PP. Non-Steroidal Anti-Inflammatory Drugs and Alzheimer's Disease: The Epidemiological Evidence. *Cns & Neurological Disorders-Drug Targets* 2010;9(2):132-39.
8. Kukar T, Golde TE. Possible mechanisms of action of NSAIDs and related compounds that modulate gamma-secretase cleavage. *Current Topic Medicinal Chemistry* 2008;8(1):47-53.
9. Spitz GA, Furtado CM, Sola-Penna M, Zancan P. Acetylsalicylic acid and salicylic acid decrease tumor cell viability and glucose metabolism modulating 6-

phosphofructo-1-kinase structure and activity. *Biochemical Pharmacology* 2009;77(1):46-53.

10. Orido T, Fujino H, Hasegawa Y, Toyomura K, Kawashima T, Murayama T. Indomethacin decreases arachidonic acid uptake in hca-7 human colon cancer cells. *Journal of Pharmacological Sciences* 2008;108(3):389-92.
11. Bakhle YS. COX-2 and cancer: a new approach to an old problem. *Br J Pharmacol* 2001;134(6):1137-50.
12. Rao CV, Reddy BS. NSAIDs and chemoprevention. *Current Cancer Drug Targets* 2004;4(1):29-42.
13. Samuelsson B, Goldyne M, Granstrom E, Hamberg M, Hammarstrom S, Malmsten C. Prostaglandins and thromboxanes. *Annual Review of Biochemistry* 1978;47:997-1029.
14. Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, Seibert K. Selective-inhibition of inducible cyclooxygenase-2 in-vivo is antiinflammatory and nonulcerogenic. *Proceedings of the National Academy of Sciences of the United States of America* 1994;91(8):3228-32.
15. Rouzer CA, Marnett LJ. Cyclooxygenases: structural and functional insights. *The Journal of Lipid Research* 2009;50:S29-S34.
16. Castellsague J, Holick CN, Hoffman CC, Gimeno V, Stang MR, Perez-Gutthann S. Risk of Upper Gastrointestinal Complications Associated with Cyclooxygenase-2 Selective and Nonselective Nonsteroidal Antiinflammatory Drugs. *Pharmacotherapy* 2009;29(12):1397-407.
17. Schaffer D, Florin T, Eagle C, Marschner I, Singh G, Grobler M, Fenn C, Schou M, Curnow KM. Risk of serious NSAID-related gastrointestinal events during long-term exposure: a systematic review. *Medical Journal of Australia* 2006;185(9):501-06.
18. Schneider V, Levesque LE, Zhang B, Hutchinson T, Brophy JM. Association of selective and conventional nonsteroidal antiinflammatory drugs with

acute renal failure: A population-based, nested case-control analysis. *American Journal of Epidemiology* 2006;164(9):881-89.

19. Mounier G, Guy C, Berthoux F, Beyens MN, Ratrema M, Ollagnier M. Severe renal adverse events with arylcarboxylic non-steroidal anti-inflammatory drugs: results of a eight-year French national survey. *Therapie* 2006;61(3):255-66.
20. Wang D, DuBois RN. Prostaglandins and cancer. *Gut* 2006;55(1):115-22.
21. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *British Medical Journal* 2002;324(7329):71-86.
22. Yeomans ND, Hawkey CJ, Brailsford W, Næsdal J. Gastroduodenal toxicity of low-dose acetylsalicylic acid: a comparison with non-steroidal anti-inflammatory drugs. *Current Medical Research and Opinion* 2009;25(11):2785-93.
23. Wallace JL. Pathogenesis of NSAID-induced gastroduodenal mucosal injury. *Best Practice & Research Clinical Gastroenterology* 2001;15(5):691-703.
24. Zeidler H. Epidemiology of NSAID-induced gastropathy. *Clinical Rheumatology* 1991;10(4):369-73.
25. Scheiman JM. Gastroduodenal Safety of Cyclooxygenase-2 Inhibitors. *Current Pharmaceutical Design* 2003;9:2197-206.
26. Huang Z, Velázquez C, Abdellatif K, Chowdhury M, Jain S, Reisz J, Dumond J, King SB, Knaus E. Acyclic triaryl olefins possessing a sulfohydroxamic acid pharmacophore: synthesis, nitric oxide/nitroxyl release, cyclooxygenase inhibition, and anti-inflammatory studies. *Organic & Biomolecular Chemistry* 2010.
27. Sun SX, Lee KY, Bertram CT, Goldstein JL. Withdrawal of COX-2 selective inhibitors rofecoxib and valdecoxib: impact on NSAID and gastroprotective drug prescribing and utilization. *Current Medical Research and Opinion* 2007;23:1859-66.

28. Melnikova I. Future of COX2 inhibitors. *Nature Reviews Drug Discovery* 2005;4(6):453-54.

29. Velázquez C, Praveen Rao PN, Knaus EE. Novel Nonsteroidal Antiinflammatory Drugs Possessing a Nitric Oxide Donor Diazen-1-ium-1,2-diolate Moiety: Design, Synthesis, Biological Evaluation, and Nitric Oxide Release Studies. *Journal of Medicinal Chemistry* 2005;48(12):4061-67.

30. Wallace JL, Reuter BK, Cirino G. Nitric oxide-releasing non-steroidal anti-inflammatory drugs: A novel approach for reducing gastrointestinal toxicity. *Journal of Gastroenterology and Hepatology* 1994;9(S1):S40-S44.

31. Calvert JW, Coetzee WA, Lefer DJ. Novel Insights Into Hydrogen Sulfide–Mediated Cytoprotection. *Antioxidants & Redox Signaling* 2010;12(10):1203-17.

32. Lanas A. Role of nitric oxide in the gastrointestinal tract. *Arthritis Research & Therapy* 2008;10(Suppl 2):S4.

33. Halen PK, Murumkar PR, Giridhar R, Yadav MR. Prodrug designing of NSAIDs. *Mini-Reviews in Medicinal Chemistry* 2009;9(1):124-39.

34. Férriz JM, Vinšová J. Prodrug design of phenolic drugs. *Current Pharmaceutical Design* 2010;16(18):2033-52.

35. Bronstein AC, Spyker DA, Cantilena LR, Green JL, Rumack BH, Heard SE. 2007 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 25th Annual Report. *Clinical Toxicology* 2008;46(10):927-1057.

36. Singh G. Recent considerations in nonsteroidal anti-inflammatory drug gastropathy. *The American Journal of Medicine* 1998;105(1, Supplement 2):31S-38S.

37. Tenenbaum J. The epidemiology of nonsteroidal anti-inflammatory drugs. *The Canadian Journal of Gastroenterology* 1999;13(2):119-22.

38. Galanakis D, Kourounakis AP, Tsiakitzis KC, Doulgkeris C, Rekka EA, Gavalas A, Kravaritou C, Charitos C, Kourounakis PN. Synthesis and pharmacological evaluation of amide conjugates of NSAIDs with L-cysteine ethyl ester, combining potent antiinflammatory and antioxidant properties with significantly reduced gastrointestinal toxicity. *Bioorganic & Medicinal Chemistry Letters* 2004;14(14):3639-43.
39. Wiley-Blackwell. Slow-release NSAIDs pose greater risk of GI bleeding, study finds. *ScienceDaily.* 2011.
40. Massó González EL, Patrignani P, Tacconelli S, Rodríguez LAG. Variability among nonsteroidal antiinflammatory drugs in risk of upper gastrointestinal bleeding. *Arthritis & Rheumatism* 2010;62(6):1592-601.
41. Fujimori S, Takahashi Y, Seo T, Gudis K, Ehara A, Kobayashi T, Mitsui K, Yonezawa M, Tanaka S, Tatsuguchi A, Sakamoto C. Prevention of Traditional NSAID-Induced Small Intestinal Injury: Recent Preliminary Studies Using Capsule Endoscopy. *Digestion* 2010;82(3):167-72.
42. Fujimori S, Seo T, Gudis K, Tanaka S, Mitsui K, Kobayashi T, Ehara A, Yonezawa M, Tatsuguchi A, Sakamoto C. Diagnosis and treatment of obscure gastrointestinal bleeding using combined capsule endoscopy and double balloon endoscopy: 1-year follow-up study. *Endoscopy* 2007;39(12):1053,58.
43. Chandrasekharan NV, Dai H, Roos KL, Evanson NK, Tomsik J, Elton TS, Simmons DL. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. *Proceedings of the National Academy of Sciences of the United States of America* 2002;99(21):13926-31.
44. Kis B, Snipes JA, Busija DW. Acetaminophen and the Cyclooxygenase-3 Puzzle: Sorting out Facts, Fictions, and Uncertainties. *Journal of Pharmacology and Experimental Therapeutics* 2005;315(1):1-7.

45. G. Perrone MS, A.; Simone, L.; Vitale, P. Selective COX-1 Inhibition: A Therapeutic Target to be Reconsidered. *Current Medicinal Chemistry* 2010;17:3769-805.
46. Wallace JL, McKnight W, Reuter BK, Vergnolle N. NSAID-induced gastric damage in rats: Requirement for inhibition of both cyclooxygenase 1 and 2. *Gastroenterol* 2000;119(3):706-14.
47. Edward J Frech MFG. Treatment and chemoprevention of NSAIDassociated gastrointestinal complications. *Journal of Therapeutics and Clinical Risk Management* 2009(5):65–73.
48. Turini ME, DuBois RN. CYCLOOXYGENASE-2: A Therapeutic Target. *Annual Review of Medicine* 2002;53(1):35-57.
49. Wallace JL, Tigley AW. new insights into prostaglandins and mucosal defence. *Alimentary Pharmacology & Therapeutics* 1995;9(3):227-35.
50. Frech EJ, Go MF. Treatment and chemoprevention of NSAID-associated gastrointestinal complications. *Therapeutics and Clinical Risk Management* 2009;5(1):65-73.
51. Ashley SW, Sonnenschein LA, Cheung LY. Focal gastric mucosal blood flow at the site of aspirin-induced ulceration. *The American Journal of Surgery* 1985;149(1):53-59.
52. Wallace JL. How do NSAIDs cause ulcer disease? *Best Practice & Research Clinical Gastroenterology* 2000;14(1):147-59.
53. Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, Seibert K. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proceedings of the National Academy of Sciences of the United States of America* 1994;91(8):3228-32.
54. Kalgutkar AS. Selective cyclooxygenase-2 inhibitors as non-ulcerogenic anti-inflammatory agents. *Expert Opinion on Therapeutic Patents* 1999;9(7):831-49.

55. Wallace JL, Ferraz JGP. New Pharmacologic Therapies in Gastrointestinal Disease. *Gastroenterology Clinics of North America* 2010;39(3):709-20.
56. Wallace JL. Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? *Physiological reviews* 2008;88(4):1547-65.
57. Fiorucci S, Santucci L, Distrutti E. NSAIDs, coxibs, CINOD and H2S-releasing NSAIDs: What lies beyond the horizon. *Digestive and Liver Disease* 2007;39(12):1043-51.
58. Fiorucci S, Santucci L, Wallace JL, Sardina M, Romano M, del Soldato P, Morelli A. Interaction of a selective cyclooxygenase-2 inhibitor with aspirin and NO-releasing aspirin in the human gastric mucosa. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100(19):10937-41.
59. Fiorucci S, de Lima OM, Jr., Mencarelli A, Palazzetti B, Distrutti E, McKnight W, Dicay M, Ma L, Romano M, Morelli A, Wallace JL. Cyclooxygenase-2-derived lipoxin A4 increases gastric resistance to aspirin-induced damage. *Gastroenterol* 2002;123(5):1598-606.
60. Sun SX, Lee KY, Bertram CT, Goldstein JL. Withdrawal of COX-2 selective inhibitors rofecoxib and valdecoxib: impact on NSAID and gastroprotective drug prescribing and utilization. *Current Medical Research and Opinion* 2007;23(8):1859-66.
61. Johnsen SP, Larsson H, Tarone RE, McLaughlin JK, Norgard B, Friis S, Henrik TS. Risk of Hospitalization for Myocardial Infarction Among Users of Rofecoxib, Celecoxib, and Other NSAIDs: A Population-Based Case-Control Study. *Archives of Internal Medicine* 2005;165(9):978-84.
62. Wallace JL, Del SP. The therapeutic potential of NO-NSAIDs. *Fundamental & Clinical Pharmacology* 2003;17(1):11-20.

63. Kitagawa H, Takeda F, Kohei H. Effect of endothelium-derived relaxing factor on the gastric lesion induced by HCl in rats. *Journal of Pharmacology and Experimental Therapeutics* 1990;253(3):1133-37.

64. Lanas A, Bajador E, Serrano P, Fuentes J, Carreño S, Guardia J, Mercedes S, Miguel M, Ricardo S. Nitrovasodilators, Low-Dose Aspirin, Other Nonsteroidal Antiinflammatory Drugs, and the Risk of Upper Gastrointestinal Bleeding. *New England Journal of Medicine* 2000;343(12):834-39.

65. Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. The chemical biology of nitric oxide: Implications in cellular signaling. *Free Radical Biology and Medicine* 2008;45(1):18-31.

66. Cuzzolin L, Conforti A, Donini M, Adami A, Del Soldato P, Benoni G. Effects on intestinal microflora, gastrointestinal tolerability and antiinflammatory efficacy of diclofenac and nitrofenac in adjuvant arthritic rats. *Pharmacological Research*;29(1):89-97.

67. Wallace JL, Reuter B, Cicala C, Mcknight W, Grisham M, Cirino G. A Diclofenac Derivative without Ulcerogenic Properties. *European Journal of Pharmacology* 1994;257(3):249-55.

68. Wallace JL, Mcknight W, Reuter B, Cicala C, Grisham M, Cirino G. Nitric-oxide releasing NSAID derivatives - antiinflammatory without gastropathy. *Gastroenterol* 1994;106(4):A208-A08.

69. Stefano F, Distrutti E. Cyclo-oxygenase (COX) inhibiting nitric oxide donating (CINODs) drugs: A review of their current status. *Current Topics in Medicinal Chemistry* 2007;7(3):277-82.

70. Fiorucci S, Antonelli E, Burgaud JL, Morelli A. Nitric oxide-releasing NSAIDs - A review of their current status. *Drug Safety* 2001;24(11):801-11.

71. http://www.nicox.com/index.asp

72. Kashfi K, Rigas B. Molecular targets of nitric oxide-donating aspirin in cancer prevention. *Prostaglandins & Other Lipid Mediators* 2006;79(1-2):160-60.

73. Muscat JE, Dyer AM, Rosenbaum RE, Rigas B. Nitric oxide-releasing medications and colorectal cancer risk: The Framingham Study. *Anticancer Res* 2005;25(6C):4471-74.

74. Rigas B, Kashfi K. Nitric-oxide-donating NSAIDs as agents for cancer prevention. *Trends in Molecular Medicine* 2004;10(7):324-30.

75. Kashfi K, Rigas B. The mechanism of action of nitric oxide-donating aspirin. *Biochemical and Biophysical Research Communications* 2007;358(4):1096-101.

76. Abdellatif KR, Chowdhury MA, Dong Y, Das D, Yu G, Velázquez CA, Suresh MR, Knaus EE. Dinitroglyceryl and diazen-1-ium-1,2-diolated nitric oxide donor ester prodrugs of aspirin, indomethacin and ibuprofen: synthesis, biological evaluation and nitric oxide release studies. *Bioorganic and Medicinal Chemistry Letters* 2009;19(11):3014-18.

77. Abdellatif KR, Chowdhury MA, Dong Y, Das D, Yu G, Velázquez C, Suresh MR, Knaus EE. Diazen-1-ium-1,2-diolated nitric oxide donor ester prodrugs of 5-(4-carboxymethylphenyl)-1-(4-methanesulfonylphenyl)-3-trifluoromethyl- 1H-pyrazole and its aminosulfonyl analog: Synthesis, biological evaluation and nitric oxide release studies. *Bioorganic and Medicinal Chemistry* 2009;17(14):5182-88.

78. Velazquez C, Rao PNP, Knaus EE. Novel nonsteroidal antiinflammatory drugs possessing a nitric oxide donor diazen-1-ium-1,2-diolate moiety: Design, synthesis, biological evaluation, and nitric oxide release studies. *Journal of Medicinal Chemistry* 2005;48(12):4061-67.

79. Hecht SS, Abbaspour A, Hoffman D. A study of tobacco carcinogenesis. Bioassay in a/J mice of some structural analogs of tobacco-specific nitrosamines. *Cancer Letters* 1988;42(1-2):141-45.

80. Chattopadhyay M, Velazquez CA, Pruski A, Nia KV, Abdellatif KR, Keefer LK, Kashfi K. Comparison between NO-ASA and NONO-ASA as safe anti-inflammatory, analgesic, antipyretic, anti-oxidant, prodrugs. *Journal of Pharmacology and Experimental Therapeutics* 2010;335(2):443-50.

81. Lefer DJ. A new gaseous signaling molecule emerges: Cardioprotective role of hydrogen sulfide. *Proceedings of the National Academy of Sciences* 2007;104(46):17907-08.

82. Perna AF, Luciano MG, Ingrosso D, Raiola I, Pulzella P, Sepe I, Lanza D, Violetti E, Capasso R, Lombardi C, De Santo NG. Hydrogen Sulfide, the Third Gaseous Signaling Molecule With Cardiovascular Properties, Is Decreased in Hemodialysis Patients. *Journal of Renal Nutrition* 2010;20(5, Supplement 1):S11-S14.

83. Ishigami M, Hiraki K, Umemura K, Ogasawara Y, Ishii K, Kimura H. A Source of Hydrogen Sulfide and a Mechanism of Its Release in the Brain. *Antioxidants & Redox Signaling* 2009;11(2):205-14.

84. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H_2S as a novel endogenous gaseous KATP channel opener. *EMBO Journal* 2001;20(21):6008-16.

85. Wallace JL, Caliendo G, Santagada V, Cirino G, Fiorucci S. Gastrointestinal Safety and Anti-Inflammatory Effects of a Hydrogen Sulfide-Releasing Diclofenac Derivative in the Rat. *Gastroenterol* 2007;132(1):261-71.

86. Rossoni G, Sparatore A, Tazzari V, Manfredi B, Soldato PD, Berti F. The hydrogen sulphide-releasing derivative of diclofenac protects against ischaemia–reperfusion injury in the isolated rabbit heart. *British Journal of Pharmacology* 2008;153(1):100-09.

87. Wallace JL, Caliendo G, Santagada V, Cirino G. Markedly reduced toxicity of a hydrogen sulphide-releasing derivative of naproxen (ATB-346). *Br J Pharmacol* 2010;159(6):1236-46.

88. Tsvetkova B, Peikov P, Tencheva J. Prodrugs and hydrolysis of esters. *Pharmacia* 2001;48(1-4):45-57.

89. Khan MSY, Khan RM. Synthesis and biological evaluation of glycolamide esters as potential prodrugs of some non-steroidal anti-inflammatory drugs. *Indian Journal of Chemistry - Section B Organic and Medicinal Chemistry* 2002;41(10):2172-75.

90. Khan MSY, Akhter M. Synthesis, biological evaluation and kinetic studies of glyceride prodrugs of diclofenac. *Indian Journal of Experimental Biology* 2004;42(11):1066-72.

91. Bonina F, Puglia C, Santagati NA, Saija A, Tomaino A, Tita B. Oligoethylene ester derivatives of ketoprofen, naproxen and diclofenac as oral prodrugs: A pharmacological evaluation. *Pharmazie* 2002;57(8):552-55.

92. Khan MSY, Khan RM. Synthesis of the prodrug ibuprofen β-D-glucopyranoside and its biological evaluation as a better moiety than the parent drug. *Indian Journal of Chemistry - Section B Organic and Medicinal Chemistry* 2002;41(5):1052-55.

93. Bansal AK, Khar RK, Dubey R, Sharma AK. Benzyl ester prodrug of ibuprofen: Pharmacological and toxicological profile. *Bollettino Chimico Farmaceutico* 2001;140(2):79-82.

94. Bansal AK, Khar RK, Dubey R, Sharma AK. Activity profile of glycolamide ester prodrugs of ibuprofen. *Drug Development and Industrial Pharmacy* 2001;27(1):63-70.

95. Zhao X, Tao X, Wei D, Song Q. Pharmacological activity and hydrolysis behavior of novel ibuprofen glucopyranoside conjugates. *European Journal of Medicinal Chemistry* 2006;41(11):1352-58.

96. Khan MSY, Akhter M. Synthesis, pharmacological activity and hydrolytic behavior of glyceride prodrugs of ibuprofen. *European Journal of Medicinal Chemistry* 2005;40(4):371-76.

97. Mohan R, Ramaa CS. Ester prodrugs of flurbiprofen: Synthesis, plasma hydrolysis and gastrointestinal toxicity. *Indian Journal of Chemistry - Section B Organic and Medicinal Chemistry* 2007;46(7):1164-68.

98. Curcio A, Sasso O, Melisi D, Nieddu M, La Rana G, Russo R, Gavini E, Boatto G, Abignente E, Calignano A, Rimoli MG. Galactosyl prodrug of ketorolac: synthesis, stability, and pharmacological and pharmacokinetic evaluations. *Journal of Medicinal Chemistry* 2009;52(12):3794-800.

99. Mishra A, Veerasamy R, Jain PK, Dixit VK, Agrawal RK. Synthesis, characterization and pharmacological evaluation of amide prodrugs of ketorolac. *European Journal of Medicinal Chemistry* 2008;43(11):2464-72.

100. Galanakis D, Kourounakis AP, Tsiakitzis KC, Doulgkeris C, Rekka EA, Gavalas A, Kravaritou C, Charitos C, Kourounakis PN. Synthesis and pharmacological evaluation of amide conjugates of NSAIDs with L-cysteine ethyl ester, combining potent antiinflammatory and antioxidant properties with significantly reduced gastrointestinal toxicity. *Bioorganic and Medicinal Chemistry Letters* 2004;14(14):3639-43.

101. Kalgutkar AS, Crews BC, Rowlinson SW, Marnett AB, Kozak KR, Remmel RP, Marnett LJ. Biochemically based design of cyclooxygenase-2 (COX-2) inhibitors: Facile conversion of nonsteroidal antiinflammatory drugs to potent and highly selective COX-2 inhibitors. *Proceedings of the National Academy of Sciences USA* 2000;97(2):925-30.

102. Shanbhag VR, Crider AM, Gokhale R, Harpalani A, Dick RM. Ester and Amide Prodrugs of Ibuprofen and Naproxen - Synthesis, Antiinflammatory Activity, and Gastrointestinal Toxicity. *Journal of Pharmaceutical Sciences* 1992;81(2):149-54.

103. Verma A, Das N, Dhanawat M, Shrivastava SK. Conjugation of some NSAIDs with 5-phenyl-2-aminothiazole for reduced ulcerogenicity. *Thai Journal of Pharmaceutical Sciences* 2010;34(2):49-57.

104. Rasheed A, Ashok Kumar CK. Tyrosine and glycine derivatives as potential prodrugs: Design, synthesis, and pharmacological evaluation of amide derivatives of mefenamic acid. *Journal of Enzyme Inhibition and Medicinal Chemistry* 2010;25(6):804-11.

105. Alarcón de la Lastra C, Motilva V, Martín MJ, Nieto A, Barranco MD, Cabeza J, Herrerías JM. Protective effect of melatonin on indomethacin-induced gastric injury in rats. *Journal of Pineal Research* 1999;26(2):101-07.

106. Kourounakis PN, Tsiakitzis K, Kourounakis AP, Galanakis D. Reduction of gastrointestinal toxicity of NSAIDs via molecular modifications leading to antioxidant anti-inflammatory drugs. *Toxicology* 2000;144(1-3):205-10.

107. Doulgkeris CM, Galanakis D, Kourounakis AP, Tsiakitzis KC, Gavalas AM, Eleftheriou PT, Victoratos P, Rekka EA, Kourounakis PN. Synthesis and pharmacochemical study of novel polyfunctional molecules combining anti-inflammatory, antioxidant, and hypocholesterolemic properties. *Bioorganic and Medicinal Chemistry Letters* 2006;16(4):825-29.

108. Spadaro A, Bucolo C, Ronsisvalle G, Pappalardo M. Design, synthesis and antiinflammatory activity of novel γ-tocopherol naproxen ester prodrug. *Journal of Pharmaceutical Sciences and Research* 2009;1(4):88-95.

109. Zhang YC, Chen PT, Guan HS, Li YX. Synthesis and pharmacological evaluation of novel conjugates of indomethacin with antioxidant activity. *Chinese Journal of Chemistry* 2005;23(11):1523-29.

110. Jiang S, Ding N, Zhang W, Zhang Y, Geng M, Li Y. Novel Conjugates of Aspirin with Phenolic Acid as Anti-inflammatory Agents Having Significantly Reduced Gastrointestinal Toxicity. *Archiv Der Pharmazie* 2010;343(4):215-21.

111. Granados-Principal S, Quiles JL, Ramirez-Tortosa CL, Sanchez-Rovira P, Ramirez-Tortosa MC. Hydroxytyrosol: from laboratory investigations to future clinical trials. *Nutrition Reviews* 2010;68(4):191-206.

112. Cornwell DG, Ma JY. Nutritional benefit of olive oil: the biological effects of hydroxytyrosol and its arylating quinone adducts. *Journal of Agricultural and Food Chemistry* 2008;56(19):8774-86.

113. Castaldi G, Oldani E, Razzetti G, Benedini F. 2001 patent WO2001004082A1.

114. Chakrapani H, Showalter BM, Kong L, Keefer LK, Saavedra JE. V-PROLI/NO, a prodrug of the nitric oxide donor PROLI/NO. *Organic Letters* 2007;9(17):3409-12.

115. Bikzhanova G, Toulokhonova I, Gately S, West R. Novel silicon-containing drugs derived from the indomethacin scaffold: Synthesis, characterization and evaluation of biological activity. *Silicon Chemistry* 2007;3(3):209-17.

116. de la Puerta R, Martinez-Dominguez E, Ruiz-Gutierrez V. Effect of minor components of virgin olive oil on topical antiinflammatory assays. *Z Naturforsch C* 2000;55(9-10):814-19.

117. Bertelli A, Migliori M, Bertelli AA, Origlia N, Filippi C, Panichi V, Falchi M, Giovannini L. Effect of some white wine phenols in preventing inflammatory cytokine release. *Drugs Under Experimental and Clinical Research* 2002;28(1):11-15.

118. Kalgutkar AS, Marnett AB, Crews BC, Remmel RP, Marnett LJ. Ester and Amide Derivatives of the Nonsteroidal Antiinflammatory Drug, Indomethacin, as Selective Cyclooxygenase-2 Inhibitors. *Journal of Medicinal Chemistry* 2000;43(15):2860-70.

119. Abdellatif KR, Chowdhury MA, Dong Y, Das D, Yu G, Velázquez CA, Suresh MR, Knaus EE. Dinitroglyceryl and diazen-1-ium-1,2-diolated nitric oxide donor ester prodrugs of aspirin, indomethacin and ibuprofen: Synthesis, biological evaluation and nitric oxide release studies. *Bioorganic and Medicinal Chemistry Letters* 2009;19(11):3014-18.

120. Dhiman SB, Kamat JP, Naik DB. Antioxidant activity and free radical scavenging reactions of hydroxybenzyl alcohols. Biochemical and pulse radiolysis studies. *Chemico-Biological Interactions* 2009;182(2-3):119-27.

121. Di Benedetto R, Varì R, Scazzocchio B, Filesi C, Santangelo C, Giovannini C, Matarrese P, D'Archivio M, Masella R. Tyrosol, the major extra virgin olive oil compound, restored intracellular antioxidant defences in spite of its weak antioxidative effectiveness. *Nutrition, Metabolism and Cardiovascular Diseases* 2007;17(7):535-45.

122. Jiang S, Ding N, Zhang W, Zhang YC, Geng MY, Li YX. Novel conjugates of aspirin with phenolic acid as anti-inflammatory agents having significantly reduced gastrointestinal toxicity. *Arch Pharm* 2010;343(4):215-21.

123. Schmassmann A, Zoidl G, Peskar BM, Waser B, Schmassmann-Suhijar D, Gebbers JO, Reubi JC. Role of the different isoforms of cyclooxygenase and nitric oxide synthase during gastric ulcer healing in cyclooxygenase-1 and -2 knockout mice. *American Journal of Physiol Gastrointest Liver Physiol* 2006;290(4):G747-56.

124. Wallace JL. Hydrogen sulfide-releasing anti-inflammatory drugs. *Trends in pharmacological sciences* 2007;28(10):501-05.

125. Del Soldato P, Foschi D, Benoni G, Scarpignato C. Oxygen free radicals interact with indomethacin to cause gastrointestinal injury. *Inflammation Research* 1986;17(5):484-88.

126. Vaananen PM, Meddings JB, Wallace JL. Role of oxygen-derived free radicals in indomethacin-induced gastric injury. *American Jouranal of Physiol Gastrointest Liver Physiol* 1991;261(3):G470-75.

127. Lim EJ, Kang HJ, Jung HJ, Park EH. Anti-angiogenic, anti-inflammatory and anti-nociceptive activity of 4-hydroxybenzyl alcohol. *Journal of Pharmacy and Pharmacology* 2007;59(9):1235-40.

128. Derhaschnig U, Schweeger-Exeli I, Marsik C, Cardona F, Minuz P, Jilma B. Effects of aspirin and NO-aspirin (NCX 4016) on platelet function and coagulation in human endotoxemia. *Platelets* 2010;21(5):320-28.

129. Fiorucci S, Santucci L, Gresele P, Faccino RM, Del Soldato P, Morelli A. Gastrointestinal safety of NO-aspirin (NCX-4016) in healthy human

volunteers: a proof of concept endoscopic study. *Gastroenterol* 2003;124(3):600-7.

130. Rossoni G, Manfredi B, Soldato PD, Berti F. NCX 4016, a nitric oxide-releasing aspirin, modulates adrenergic vasoconstriction in the perfused rat tail artery. *British Journal of Pharmacology* 2002;137(2):229-36.
131. Fiorucci S, Mencarelli A, Meneguzzi A, Lechi A, Renga B, del Soldato P, Morelli A, Minuz P. Co-Administration of Nitric Oxide-Aspirin (NCX-4016) and Aspirin Prevents Platelet and Monocyte Activation and Protects Against Gastric Damage Induced by Aspirin in Humans. *Journal of the American College of Cardiology* 2004;44(3):635-41.
132. Sogo N, Magid KS, Shaw CA, Webb DJ, Megson IL. Inhibition of human platelet aggregation by nitric oxide donor drugs: Relative contribution of cGMP-independent mechanisms. *Biochemical and Biophysical Research Communications* 2000;279(2):412-19.
133. Sud'ina GF, Pushkareva MA, Shephard P, Klein T. Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) selectivity of COX inhibitors. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 2008;78(2):99-108.
134. Celotti F, Laufer S. Anti-inflammatory drugs: new multitarget compounds to face an old problem. The dual inhibition concept. *Pharmacological Research* 2001;43(5):429-36.
135. Sarkar F. Current Trends in the Chemoprevention of Cancer. *Pharmaceutical Research* 2010 ;27(6):945-9.
136. Hail N, Jr., Cortes M, Drake EN, Spallholz JE. Cancer chemoprevention: A radical perspective. *Free Radical Biology and Medecine* 2008;0(0):0.
137. Cuzick J. Chemoprevention of breast cancer. *Breast Cancer* 2008;15(1):10-16.
138. Rao CV, Reddy BS. NSAIDs and Chemoprevention. *Current Cancer Drug Targets* 2004;4:29-42.

139. Williams JL, Borgo S, Hasan I, Castillo E, Traganos F, Rigas B. Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) alter the kinetics of human colon cancer cell lines more effectively than traditional NSAIDs: Implications for colon cancer chemoprevention. *Cancer Research* 2001;61(8):3285-89.

140. Santini DL, Rigas B, Shiff SJ. Role of diet and NSAIDs in the chemoprevention of colorectal cancer. *Biochemical Society Transactions* 1999;10(3):68-76.

141. Dannenberg AJ, Zakim D. Chemoprevention of colorectal cancer through inhibition of cyclooxygenase-2. *Seminars in Oncology* 1999;26(5):499-504.

142. Butterworth JR. Chemoprevention of colorectal cancer in inflammatory bowel disease. *Digestive and Liver Disease*;In Press, Corrected Proof.

143. Wilkinson J, Clapper ML. Detoxication enzymes and chemoprevention. *Proceedings of the Society for Experimental Biology and Medicine* 1997;216(2):192-200.

144. Kwak MK, Wakabayashi N, Kensler TW. Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* 2004;555(1-2):133-48.

145. Gao J, Kashfi K, Liu X, Rigas B. NO-donating aspirin induces phase II enzymes in vitro and in vivo. *Carcinogenesis* 2006;27(4):803-10.

146. Dunlap T, Abdul-Hay SO, Chandrasena RE, Hagos GK, Sinha V, Wang Z, Wang H, Thatcher GR. Nitrates and NO-NSAIDs in cancer chemoprevention and therapy: In vitro evidence querying the NO donor functionality. *Nitric Oxide* 2008;19:115-24.

147. Dunlap T, Chandrasena RE, Wang Z, Sinha V, Wang Z, Thatcher GR. Quinone formation as a chemoprevention strategy for hybrid drugs: balancing cytotoxicity and cytoprotection. *Chemical Research in Toxicology* 2007;20(12):1903-12.

148. Elliott SN, Mcknight W, Cirino G, Wallace JL. A Nitric Oxide-Releasing Nonsteroidal Antiinflammatory Drug Accelerates Gastric-Ulcer Healing in Rats. *Gastroenterol* 1995;109(2):524-30.

149. Li-yuan J, Li C, Yi-hua Z. Improved synthesis of 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester. *Chinese Journal of Medicinal Chemistry* 2004;14(3):178-79.

150. Winter CA, Risley EA, Nuss GW. Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine* 1962;111:544-7.

151. Cocco MT, Congiu C, Onnis V, Morelli M, Felipo V, Cauli O. Synthesis of new 2-arylamino-6-trifluoromethylpyridine-3-carboxylic acid derivatives and investigation of their analgesic activity. *Bioorganic & Medicinal Chemistry* 2004;12(15):4169-77.

152. Jurasz P, Sawicki G, Duszyk M, Sawicka J, Miranda C, Mayers I, Radomski MW. Matrix metalloproteinase 2 in tumor cell-induced platelet aggregation: Regulation by nitric oxide. *Cancer Research* 2001;61(1):376-82.

153. Jurasz P, Alonso D, Castro-Blanco S, Murad F, Radomski MW. Generation and role of angiostatin in human platelets. *Blood* 2003;102(9):3217-23.

154. Jurasz P, Santos-Martinez MJ, Radomska A, Radomskit MW. Generation of platelet angiostatin mediated by urokinase plasminogen activator: effects on angiogenesis. *Journal of Thrombosis and Haemostasis* 2006;4(5):1095-106.

155. Elbekai RH, El-Kadi AOS. Arsenite and cadmium, but not chromium, induce NAD(P)H:quinone oxidoreductase 1 through transcriptional mechanisms, in spite of post-transcriptional modifications. *Toxicology in Vitro* 2008;22(5):1184-90.

156. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 1951;193(1):265-75.

157. Ernster L. DT diaphorase. *Methods in Enzymology* 1967;10:309-17.