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PHOSPHO-NAPROXEN: A NOVEL AGENT FOR THE CONTROL OF ARTHRITIS AND COLON CANCER

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Abstract of the Dissertation

Phospho-naproxen: A novel agent for the control of arthritis and colon cancer

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Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used to treat various inflammatory diseases. In particular, the NSAID naproxen is a major first-line treatment for rheumatoid arthritis, an inflammatory disease affecting 1% of the population worldwide.

The potential use of NSAIDs also spans to other debilitating and chronic diseases, including cancer. Epidemiological studies have shown that the use of naproxen significantly correlates with a lower risk of colon cancer, the third most common type of cancer in the United States.

Although conventional naproxen can subdue arthritic inflammation and reduce the risk of developing colon cancer, its long-term use is associated with significant side effects, including gastrointestinal toxicity, which limit its potential to effectively control these

chronic diseases. To overcome this limitation and potentially increase its efficacy, we have developed phospho-naproxen (P-N), a novel naproxen derivative.

The objective of this work was to determine the efficacy, safety, and potential mechanism of action of P-N in two clinically relevant models, rheumatoid arthritis and colon cancer.

In an adjuvant rat model of rheumatoid arthritis, P-N proved to be more efficacious than its parent compound. P-N exhibited a 60% ($p<0.001$) enhanced safety over naproxen. In addition, P-N also significantly reduced rheumatoid arthritis inflammation, inflammatory cell infiltration, bone resorption, cartilage damage, pro-inflammatory cytokine (IL-1β and IL-6) levels, cycloxygenase-2 expression, and NF-κB levels.

P-N was also effective in preventing colon cancer in $APC^{Min/+}$ mice. P-N prevented the development of intestinal tumors up to 99% ($p<0.001$) in a dose-dependent manner compared to vehicle-treated control. Furthermore, P-N exhibited significantly enhanced potency over naproxen in reducing cell growth of various colon cancer cell lines in vitro by inhibiting cellular proliferation, blocking the cell cycle at G_1/S, and inducing apoptosis. Mechanistically, P-N inhibits NF-κB activity in colon cancer cells in a concentration-dependent manner.

In conclusion, the novel drug, P-N, is a promising candidate agent for rheumatoid arthritis therapy and colon cancer chemoprevention, satisfying two essential parameters for a drug given chronically: efficacy and safety.

This dissertation is dedicated to my parents.
Thank you for your unconditional love and unwavering support.

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List of Abbreviations

A	Annexin V
ACF	Aberrant Crypt Foci
ADHP	10-acetyl-3, 7-dihydroxyphenoxazine
APC	Adenomatous Polyposis Coli
Aq	Aqueous
ASK1	Apoptosis-Signal Regulating-Kinase 1
AUC	Area Under the Curve
BAX	Bcl-2-associated X protein
BNP	bis-*p*-nitrophenyl phosphate
CFA	Complete Freud's Adjuvant
C_{max}	maximal plasma concentration
COX	Cyclooxygenase
DCM	Dichloromethane
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
FAP	Familial Adenomatous Polyposis
GAP	GTPase activating protein
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factors
GI	Gastrointestinal

GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine Triphosphate
HNPCC	Hereditary Non-Polyposis Colon Cancer
HPLC	High pressure Liquid chromatography
HRP	Horseradish Peroxidase
Hx-EA	Hexane-Ethyl acetate
IC_{50}	Half maximal inhibitory concentration
IV	Intravenous
K-ras	Kirsten rat sarcoma
LC	Liquid Chromotography
MAP3K	Mitogen-Activated Protein 3 Kinase
MCR	Mutation Cluster Region
MMR	Mis-Match Repair
MS	Mass Spectrocopy
MSI	Microsatellite instability
Nap	Naproxen
NF-kB	Nuclear Factor Kappa B
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
P	Propidium iodide
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
P-N	Phospho-Naproxen

sPGE	Stable PGE_2 derivative
$t_{1/2}$	Elimination half-life
THF	Tetrahydrofuran
T_{max}	Time to reach maximal concentration
TMB	3, 3′, 5, 5′-tetramethylbenzidine
TP53	Tumor protein p53
Trx-1	Thioredoxin-1

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INTRODUCTION

Inflammation plays a central role in several seemingly disparate diseases. Rheumatoid arthritis and cancer are two prominent examples of the fundamental pathogenic role of inflammation. Viewed in the context of our conventional understanding of inflammation this statement may seem paradoxical. However, the current understanding of inflammation and of the complex signaling pathways that lead to its clinical manifestations are providing the essential elements of a deeper appreciation of inflammation as the common thread of diseases such as cancer, atherosclerosis, neurodegenerative, and rheumatologic diseases. In addition, to providing an insight into the pathogenesis of these clinical entities, the importance of inflammation has prompted new therapeutic strategies based on agents with strong anti-inflammatory properties. These considerations have provided the overall rationale for the work presented here.

Here, I describe a novel anti-inflammatory agent, phospho-naproxen (P-N) and examine its pharmacological properties in the control of two clinical entities that exemplify aspects of inflammation: rheumatoid arthritis and colon cancer. Below, I summarize the key features of inflammation; review the salient points of rheumatoid arthritis and colon cancer; and provide an overview of anti-inflammatory agents with emphasis on non-steroidal anti-inflammatory compounds (NSAIDs).

1.1 Inflammation

i. Definition of inflammation: Ancient and modern

The body's response to harmful stimuli is an adaptive response manifesting as inflammation. Inflammation is fundamentally protective as it serves to destroy, dilute, or entrap the harmful stimulus in order to initiate repair and regeneration. However, unregulated inflammation as seen in allergic reactions, rheumatoid arthritis, and potentially even cancer, can be life threatening. Classically, inflammation was characterized during 1st century AD by the Roman encyclopaedist Aulus Cornelius Celcus. In his description, the four cardinal signs of inflammation were: *calor* (heat), *dolor* (pain), *rubor* (redness), and *tumor* (swelling). Since that time the model of inflammation has evolved significantly to account for two major types of inflammation, acute inflammation and chronic inflammation.

Acute inflammation is well characterized as a short-term host response (1-3 days) to infection or tissue injury to serve the physiological purpose of defense and tissue repair. In general, this acute response is associated with infiltration of leukocytes and plasma proteins to the affected region, removal of the noxious stimulus, and repair of damage. This is sometimes characterized by exudation of fluid and edema.

Chronic inflammation is a long-term host response (months to years) that is characterized by the promotion of inflammation and tissue damage, and by failed attempts at tissue repair which is associated with a dysregulated inflammatory system. This chronic inflammation is

characterized by the presence of lymphocytes and macrophages, fibrosis, angiogenesis, and necrosis. Chronic inflammation is largely responsible for various debilitating conditions including arthritis and likely cancer.

ii. Inflammation and Cancer: An evolving concept

Inflammation and cancer are significantly linked. Inflammation supports a microenvironment that promotes the initiation of new tumors and/or the transition of pre-malignant cells into mature malignancies. Extrinsic inflammation can initiate the development of precancerous cells. Mature malignancies promote an intrinsic inflammatory microenvironment, which helps the maintenance and progression of established cancer cells.

Extrinsic factors such as constant chronic inflammation caused by disease or infection are significantly linked to tumorigenesis at specific anatomical sites. Chronic inflammation is responsible for 25% of cancer-related deaths in the world [3-7]. For example, infection with *Helicobacter pylori* results in constant chronic inflammation in the stomach thereby significantly increasing the risk of gastric cancer by promoting the formation of precancerous cells [8]. In fact, the strongest known risk factor for gastric cancer is infection with *Helicobacter pylori* [9].

Intrinsic factors of inflammation are usually present in premalignant cells and in established malignancies where genetic alterations activate oncogenes or inactivate tumor suppressor genes to support survival, growth, proliferation, angiogenesis, metastasis and the production of

chemokines. Often, genetic mutations can activate important pathways such as NF-κB, which can promote cell proliferation and survival as well as the expression of inflammatory cytokines. For example, aberrant activation of the Ras-Raf signaling pathway, an intrinsic insult, alone can only promote tumor inflammation to a certain extent by promoting the expression of tumor promoting cytokines and chemokines. NF-κB activation has been associated with the initiation and progression of tumor development. Overexpression of NF-κB can result in the expression of inflammatory cytokines, adhesion molecules, COX-2, iNOS (angiogenic factor), and Bcl-2 (anti-apoptotic gene). NF-κB is thus a major player associated with innate immunity, inflammation, and carcinogenesis [10].

Myc activation has been associated with the release of pro-inflammatory cytokines such as IL-1β. Release of IL-1β from B-cells (also known as B lymphocytes) is a major factor in the development of a tumor microenvironment that promotes tumor progression and metastasis through the induction of angiogenesis [11]. Tumor progression has also been associated with the induction of inflammatory mediators by the inactivation of tumor suppressor genes PTEN, p16, p53 [12, 13] and VHL [14].

In other instances such as pancreatitis, an extrinsic factor can be combined with an intrinsic factor such as mutated K-ras to drive the induction of intra-epithelial neoplasia and invasive ductal carcinoma [15].

Thus it is clear from the foregoing that the processes of inflammation and tumorigenesis can work together to form a circular feed-forward loop that supports carcinogenesis.

iii. Non-steroidal anti-inflammatory drugs (NSAIDs): Efficacy and limitations

NSAIDs best recognized pharmacological effect is to inhibit the activity of cyclooxygenase (COX) thus blocking the formation of prostaglandins, some of which are strongly pro-inflammatory. Prostaglandins (PGs) are synthesized from arachidonic acid by the bi-functional COX enzyme. The initial step of PG production starts with arachidonic acid (the precursor of prostaglandin) which is cleaved from the phospholipid cell membrane by the enzyme phospholipase A_2 [16]. Initially, arachidonic acid is converted to hydroperoxy endoperoxide prostaglandin G_2 (PGG_2) by the addition of two oxygen molecules via the cyclooxygenase activity inherent in one the active sites of COX. This is followed by the reduction of PGG_2 to PGH_2 by peroxidase activity inherent at another active site within COX [16]. The unstable intermediate, PGH_2, now serves as a precursor for thromboxane, prostacyclin, and various prostaglandins. The fate of the precursor, PGH_2, is dependent on tissue-specific synthases such as thromboxane synthase, prostaglandin E synthase, prostaglandin D synthase, and prostaglandin I synthase [17].

There are two major isoforms of COX: COX-1 and COX-2. COX-3 has been reported; however, this variant of COX-1 is considered to be non-functional in humans and rodents due to intron retention which results in a frame-shift [18-20]. Each COX isoform (COX-1, COX-2) varies depending mostly on the tissue distribution and expression. COX-1 is constitutively active and largely distributed within the gastric mucosa, kidneys, and platelets to generate prostaglandins used to control basic homeostatic functions such as mucosal integrity, renal water excretion, and platelet formation. In contrast, COX-2 is widely distributed and is inducible by inflammatory

stimuli, which generate prostaglandins that modulate platelet aggregation, vasodilation, pain, and inflammation.

There are many chemical classes of NSAIDs that variably inhibit each of the major COX isoforms. These major chemical classes include salicylates, propionic acid derivatives, acetic acid derivatives, oxicam derivatives, fenamate derivatives, ketones, and sulfonic acid derivatives.

A common *salicylate NSAID derivative* includes aspirin, which acts irreversibly on COX-1 and COX-2 by acetylating serine found in the active site of each cyclooxygenase. The acetylation feature of COX-1 within platelets, which lack nuclei, by aspirin results in the complete inhibition of thromboxane (TxA_2) synthesis. This inhibition of thromboxane synthesis leads to a loss in platelet aggregation. Aspirin also affects endothelial cells; however, only temporarily due to their ability to resynthesize cyclooxygenases. Thus endothelial cells can continue to produce prostacyclin's (PGI_2), via COX-2, resulting in vasodilation and inhibition of platelet aggregation. Together, aspirin promotes a PGI_2-rich environment thereby inducing vasodilation and anti-thrombogenesis thereby making it useful in preventing and managing myocardial infarction and stroke. Unfortunately, long-term aspirin treatment is associated with gastrointestinal (GI) ulceration, bleeding (hemorrhage), nephrotoxicity, and hepatic injury. Use aspirin of has been associated with nephropathy leading to chronic renal failure. The risk of chronic renal failure is 2.5-fold higher in regular users of aspirin than non-NSAID users [21]. Aspirin is also dangerous to patients with asthma, inducing aspirin-induced airway hyper-reactivity. Young patients who take aspirin are also susceptible to Reye's syndrome which results in hepatic encephalopathy and liver stenosis. Another salicylate derivative includes diflunisal which is a reversible inhibitor of COX-1

and COX-2. It is more potent than aspirin due to increased half-life thus making it a more effective anti-inflammatory agent, although without the positive anti-thrombotic effects. Unlike aspirin, diflunisal is not metabolized into salicylate. Therefore, it does not cause salicylate intoxication, although patients are still susceptible to GI toxicity and children are still susceptible to Reye's syndrome.

Propionic acid NSAID derivatives include fenoprofen, flurbiprofen ibuprofen, ketoprofen, oxaprozin, and naproxen. This class of NSAIDs is composed of reversible inhibitors of COX-1 and COX-2. They are effective anti-inflammatory agents which are relatively safe compared to other classes of NSAIDs, such as aspirin, therefore making them prime candidates for long-term treatment of chronic conditions such as rheumatoid arthritis and osteoarthritis. The most common adverse effects from propionic acid NSAID derivatives range from GI dyspepsia to bleeding.

Acetic acid NSAID derivatives include diclofenac, etodolac, indomethacin, ketorolac, and sulindac. These acetic acid derivatives reversibly inhibit COX-1 and COX-2 and are used to treat various diseases such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and other musculoskeletal disorders. Although effective, acetic acid NSAID derivatives are associated with a wide variety of adverse effects. Indomethacin, for example, is more potent than aspirin and is one of the most effective NSAIDs at reducing inflammation; however, its toxicity limits its use for the treatment of inflammatory diseases such as rheumatoid arthritis. Adverse effects of indomethacin include various GI complications ranging from ulceration and perforation, to diarrhea and vomiting. Indomethacin usage is also associated with pancreatitis, neutropenia, and

thrombocytopenia. Sulindac, another acetic acid derivative, is less potent than indomethacin and has less adverse effects. Sulindac is a prodrug which is metabolized by liver microsomal enzymes to its active form, sulindac sulfide. Its long duration of action is useful in the treatment of various inflammatory diseases such as acute gout, ankylosing spondylitis, osteoarthritis, and rheumatoid arthritis; however, it is still associated with several adverse effects which are common to most NSAIDs. Etodolac, a preferential COX-2 inhibitor, is also an effective acetic acid NSAID derivative; however, its adverse effects include abnormal kidney and liver function. Moreover, there is an increased risk of adverse events when used with other drugs such as methotrexate, digoxin, and lithium due their reduced metabolism and subsequent increase in serum levels. Overall acetic acid NSAID derivatives are mostly used to treat rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and other musculoskeletal disorders; however, they commonly suffer GI toxicity and can also induce hepatotoxity, nephrotoxicity, renal toxicity when taken with certain drugs.

Oxicam NSAID derivatives include the drug piroxicam, a non-selective reversible COX inhibitor. Among the inhibition of prostaglandin and thromboxane A_2 synthesis, piroxicam also has alternate mechanisms of action such as modulation of neutrophils by inhibiting collagenase, proteoglycanase, and oxidative burst. Piroxicam has a long half-life of 50 hours which permits once daily dosing for the treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. As seen with many other NSAID classes, piroxicam also suffers from adverse GI effects and prolonged bleeding due to its anti-thrombotic effect.

Fenamate NSAID derivatives include mefenamate and meclofenamate. These agents are competitive inhibitors of COX-1 and COX-2. They also act as antagonists at prostanoid receptors.

Compared to other NSAIDs, fenamates do not have any significant advantage due to their low anti-inflammatory effect and toxicity resulting in severe diarrhea and bowel inflammation.

Ketone NSAID derivatives include nabumetone which is a prodrug that is metabolized in the liver to its active metabolite, 6-methoxy-2-naphthyl acetic acid. The active metabolite preferentially inhibits COX-2. Although nabumetone exhibits lowered GI adverse effects, headaches and dizziness are common.

Sulfonic acid derivatives such as celecoxib, rofecoxib, valdecoxib, and meloxicam are selective COX-2 inhibitors. Similar to non-selective NSAIDs, these COX-2 specific inhibitors are anti-inflammatory and are generally effective for treatment of rheumatoid arthritis and osteoarthritis. The use of COX-2 selective inhibitors, however, is under scrutiny due to safety concerns. Although these drugs possess improved gastrointestinal safety they suffer from negative cardiovascular events. This cardiovascular toxicity has resulted in the withdrawal of rofecoxib and valdecoxib from the market [22].

It is evident that the inhibition of COX isoforms can considerably affect both the safety and the efficacy of NSAIDs. The major side effects of NSAIDs include toxicity to the kidney [23-26], liver [27], heart [28-30], and especially to the gastro-intestinal (GI) tract [31, 32]. Of these side effects, GI, cardiac, and renal toxicities are clinically the most important [33, 34].

NSAID-related GI complications are responsible for 100,000 hospitalizations and 16,500 deaths per year [35]. Inhibition of COX-1 can result in gastrointestinal ulcers or perforation with consequential bleeding. In fact, NSAID use increases the risk of gastric ulceration by 3 to 5 fold

[35]. This increased risk of GI ulceration and perforation is primarily due to inhibition of prostaglandin (PGE_2) and prostacyclin (PGI_2) synthesis which results in increased gastric acid ion secretion (due to PGI_2 inhibition), diminished bicarbonate secretion (due to PGE_2 inhibition), diminished mucus secretion (due to PGE_2 inhibition), reduced mucosal blood flow (due to PGE_2 and PGI_2 inhibition) [35]. The end result of this array of changes is that the GI lining becomes highly susceptible to the acidic environment of the gut.

NSAID users are at 35% increased risk of adverse cardiovascular events, such as myocardial infarction, compared to non-NSAID users [36, 37]. Specific inhibition of COX-2 can lead to adverse cardiovascular events due to unopposed thromboxane activity [36]. Specifically, increased thrombogenicity by selective COX-2 inhibitors is two-fold: 1) the prolonged inhibition of COX-2 in endothelial cells results in reduced levels of PGI_2, a vasodilator and inhibitor of platelet aggregation, and 2) COX-1 is not affected thus allowing for thromboxane A_2, a vasoconstrictor and platelet aggregator, production. Together, this promotes a shift to a prothrombotic state increasing the likelihood of thrombogenicity and adverse cardiovascular events.

Inhibition of COX-2 can also induce renal dysfunction which can lead to renal failure and subsequently increase the risk of death [38]. In the United States, 2.5 million people per year suffer from NSAID related renal events [39]. The inhibition of COX-2 by NSAIDs can result in increased sodium retention and hyperkalemia due to the inhibition of PGE_2 and PGI_2 whose normal function is to oppose vasoconstriction by angiotensin [38, 40].

Given the clinical usefulness of NSAIDs and the limiting nature of their side effects, there has been a great effort, spanning decades, to mitigate their toxicity [41-47]. Unfortunately, these efforts have their own limitations such as low efficacy and adverse drug interactions. For example, H_2 (histamine) receptor antagonists, used to block histamine from binding to H_2 receptors on parietal cells thereby inhibiting gastric acid secretion, have been used in conjunction with NSAIDs to prevent their GI toxicity. Although in some studies H_2 receptor antagonists were effective in treating the symptoms of GI toxicity, they failed to address the root cause: GI ulcerations [41-43]. Moreover, patients with diseases, such as rheumatoid arthritis, taking H_2 receptor antagonists as a preventive measure are at significantly higher risk of GI complications than patients that do not [44].

An alternative approach includes co-therapy with proton pump inhibitors which inhibit H^+/K^+ ATPase on gastric parietal cells thereby preventing H^+ secretion into the gastric lumen. Unfortunately, this therapy also comes with significant disadvantages. Proton pump inhibitors have been associated with dependency, in the sense that their discontinuation leads to significant acid secretion and dyspeptic symptoms [45]. Furthermore, drug combinations with proton pump inhibitors can lead to significant adverse events. The usage of cardiac medications in conjunction with NSAIDs is common in elderly patients. Unfortunately, proton pump inhibitors have been shown to interfere with cardiac medications used to inhibit platelet aggregation, thus making patients more susceptible to cardiac infarction [46]. More recent studies also highlight the inefficiency of proton pump inhibitors in the distal small intestine and may even exacerbate NSAID-induced intestinal damage [47].

Thus the "*safe NSAID*" which is effective remains as elusive as ever. It is because of the great need for such a compound that has prompted us to pursue the present work.

1.2 Rheumatoid arthritis: The epitome of an inflammatory disease

i. Overview

Inflammatory arthritis manifests itself in many clinical forms including: reactive arthritis and septic arthritis, both due to infectious processes; psoriatic arthritis, likely having a genetic basis; and rheumatoid arthritis (RA), caused by a chronic immune disorder [48-51]. Of these diverse forms of arthritis, RA is the most common inflammatory arthritis observed by physicians, affecting about one percent of the adult population worldwide [51]. In the United States, RA affects 1 out of 28 females and 1 out of 59 males [52] and is responsible for an estimated 250,000 hospitalizations per year [53].

RA is primarily characterized by significant inflammation and irreversible erosion of the synovial joint tissue, bone, and cartilage [51]. These changes can cause severe pain and limit mobility diminishing, as a consequence, the patient's quality of life. Left untreated, 20-30% of patients with RA become permanently work-disabled within the first 2-3 years of its onset [54].

RA primarily affects the small joints of the hands and feet, where the normally smooth synovium becomes thickened and hyperplastic, known as pannus. This inflamed synovial tissue is infiltrated by T cells, plasma cells, and macrophages from the local vascular system and the synovial fluid can be infiltrated by neutrophils. The bone is subsequently subject to perturbations such as juxta-articular erosion, subchondral cysts, and osteoporosis from osteoclastic activity. As a result of pannus formation, cartilage degradation is also associated with RA. Once cartilage has been destroyed, the pannus has access to bones which can initially cause fibrous ankylosis and progress to bone fusion (bone ankylosis). RA is also characterized by increased vascularity due to angiogenesis and vasodilation. Roughly 25% of patients with RA may also suffer from rheumatoid nodules which can develop under the skin, in the lung, pericardium, myocardium, cardiac valves, aorta and spleen negatively affecting organ function and inducing nerve pressure. Overall, these perturbations associated with RA can result in significant pain, swelling, and deformities resulting loss of function.

ii. The pathogenesis of RA

Although the cause of RA is not yet fully understood, many pieces of evidence can help explain its pathogenesis. RA may be initiated by a combination of genetic and/or environmental factors that leads to the failure of immune tolerance and significant synovial inflammation [55].

Genetic factors can play a significant role in an individual's disposition of developing RA affecting about 50% of RA cases. Genes such as human leukocyte antigen (HLA) genes, which

encode major histocompatibility complex (MHC), and PTPN22, which encodes protein tyrosine phosphatase non-receptor type 22, play a major role in RA-associated autoimmunity [56, 57]. Other genes such as cytokine promoters and T cell signaling genes can determine the severity of RA [58, 59]. Various environmental factors also play a significant role in RA. For example, smoking, one of the best-defined environmental stimuli, contributes to the development of RA, especially in predisposed individuals by promoting citrullination of vimentin [60, 61]. It is evident that RA is a multifactorial disease. Ultimately, these varied factors culminate to its cardinal clinical manifestations: symmetric inflammation and erosion of the synovial tissue, bone, and cartilage.

Various parts of the immune system play a central role in RA (Figure 1-1). Often there is an appreciable lag between the gradual increase in the amount of autoantibodies and cytokines and the clinical manifestations of RA. Following the complete activation of the autoimmune system, the synovial tissue becomes invasive and can promote cartilage and bone degradation. In RA, synovial cells have been associated with the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) which can promote cytokine production [62, 63]. Also, the expression of metalloproteinases (MMPs), which are responsible for the break down bone, cartilage, and tendons, are activated by the induction of Fos and Jun via NF-κB [64] and JNK via MAPK [65].

Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) play a significant role in RA [66]. IL-6, particularly, appears to have a prominent role in RA. Deficiency in TNF-α or IL-1 only slows the onset of RA, whereas IL-6 deficiency completely inhibits the development of RA in mice [67]. IL-6 functions in immune

response regulation, haematopoiesis, acute phase response, and bone metabolism [68]. The pleiotropic functions of IL-6 on the maturation and activation of B and T cells, macrophages, osteoclasts, and chondrocytes can affect the acute phase response in RA [69]. Patients with RA commonly have increased IL-6 production, which can result in systemic inflammation and many of the symptoms of RA [70-73]. This is also evident in rodent models, where point mutations in mice that increase IL-6 transcription induce RA-like autoimmune arthritis [74]. The dysregulation of IL-6 can activate autoreactive T cells and B cells and induce adhesion molecules on immunocompetent cells. In RA, IL-6 can induce angiogenesis via activation of vascular endothelial growth factor (VEGF) [75], and also promote bone degradation via activation of osteoclasts [76]. In fact, in humans with RA, promoter polymorphism of IL-6 strongly correlates with disease onset, activity, and therapeutic response [77-79]. Notably, IL-6 -/- mice are resistant to the development of RA [80, 81].

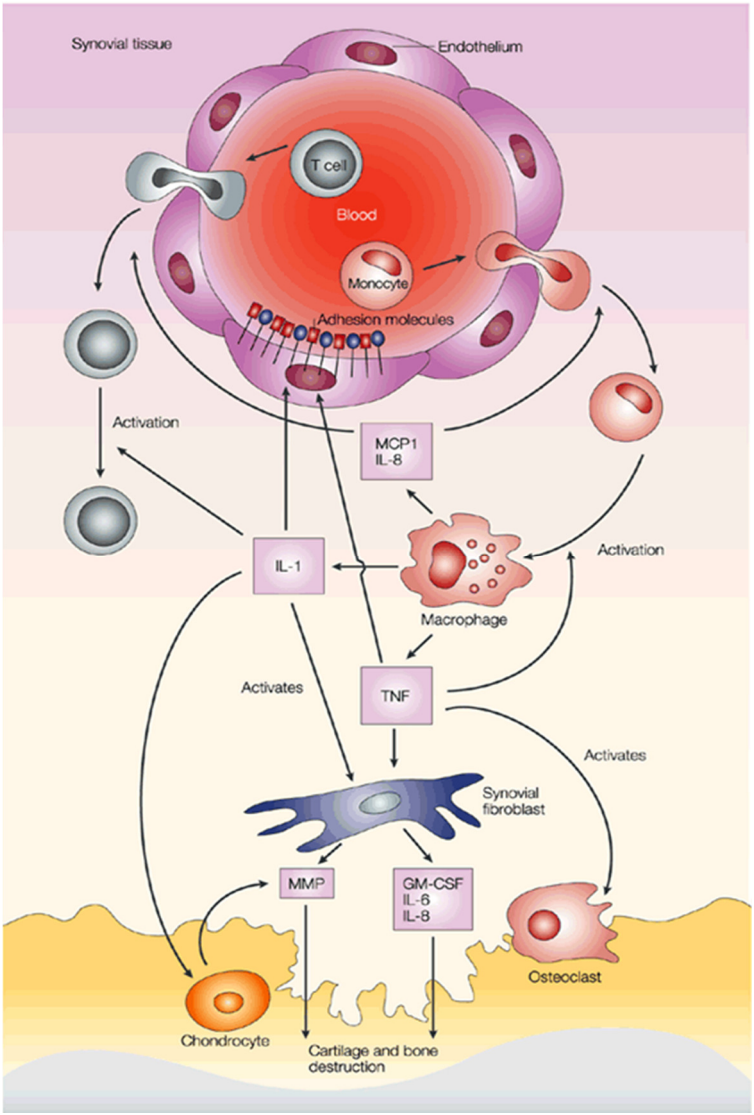

Figure 1-1: Inflammatory pathways in RA. Monocytes are attracted to the rheumatoid arthritis (RA) joint, where they differentiate into macrophages and become activated. They secrete tumor-necrosis factor (TNF) and interleukin-1 (IL-1). TNF increases the expression of adhesion molecules on endothelial cells, which recruit more cells to the joint. Chemokines, such as monocyte chemotactic protein 1 (MCP1) and IL-8, are also secreted by macrophages and attract more cells into the joint. IL-1 and TNF induce synovial fibroblasts to express cytokines (such as IL-6), chemokines (such as IL-8), growth factors (such as granulocyte–macrophage colony-stimulating factor; GM-CSF) and matrix metalloproteinases (MMPs), which contribute to cartilage and bone destruction. TNF contributes to osteoclast activation and differentiation. In addition, IL-1 mediates cartilage degradation directly by inducing the expression of MMPs by chondrocytes. Adapted from Pope et al. [2].

iii. NSAIDs as therapeutic agents for RA

Many treatments for RA exist. These treatments include: non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying anti-rheumatic drugs, and biologics. Of these therapies, NSAIDs remain the mainstay of treatment due to their rapid onset of action and low cost. Although NSAIDs are effective in reducing arthritic inflammation, they come with significant drawbacks in that their side effects can significantly compromise the health of the patient.

1.3 Colon cancer: An inflammatory disease?

Colon cancer is the third most common type of cancer diagnosed in the United States and the third most common cause of cancer-related death [82]. The cost of care for colon cancer in the US is $4.5 to $9.6 billion per year [83]. The majority of cases are sporadic, with hereditary colon cancer accounting for up to 15% of the total number of colon cancer diagnoses [84]. In 2010 in the United States, an estimated 102,900 new cases of colon cancer were diagnosed (49,470 male, 53,430 female) and 51,370 patients (26,580 male, 24,790 female) died from colon cancer [85]. Outside the United States, the incidence of colon cancer in developing countries continues to rise, potentially due to increased exposure to risk factors [86]. Although there are many screening methods available for the detection of colon cancer, compliance remains a major

hurdle. Post- diagnosis, surgical intervention is required and is associated with several drawbacks. Currently, there are no approved agents for the prevention of colon cancer.

i. Colon cancer: Clinical manifestations and risk factors

Colon cancer can remain asymptomatic for years. More often than not, the early clinical manifestations of colon cancer may be overlooked due to their mundane features that can be attributed to other causes. The location of colon cancer within the colon determines to some extent its clinical presentation. Right colonic cancer, which includes the cecum, ascending colon, hepatic flexure, and transverse colon, can often present itself as fatigue, weakness, and iron deficiency anemia. Left colonic cancer, which includes the splenic flexure, descending colon, sigmoid colon, and rectum, can present itself as irregular bowel movements or cramps. Given that these symptoms are non-specific, colon cancer usually progresses significantly by the time of detection. Colon cancers metastasize to local lymph nodes, liver, lungs, and bone while later they can infiltrate the peritoneal cavity and brain.

Genetic predisposition plays a significant role in colon cancer, affecting its prevalence and mortality [87]. Hereditary forms of colon cancer include Lynch Syndrome (or Hereditary Non-Polyposis Colon Cancer; HNPCC) and Familial Adenomatous Polyposis (FAP).

Lynch syndrome (HNPCC) is characterized by microsatellites, short repeats of DNA due to a mutation in mismatch repair genes (hMSH2, hMLH1, hPMS1, hPMS2, hMSH3, and hMSH6), which

results in their inactivation [84]. Mutations of the mismatch repair pathway result in an increase of polymerase generated replication errors. Thus the DNA replication is negatively affected at microsatellite repeat sequences resulting in microsatellite instability (MSI). Patients with Lynch syndrome have an 80% lifetime risk of developing colon cancer [88].

Familial Adenomatous Polyposis is the result of a mutation in the tumor suppressor gene, adenomatous polyposis coli (APC), which is part of the WNT signaling pathway. This mutation can be a result of a germline mutation in APC, located on the long arm of chromosome 5q21, as well as of somatic mutations of the APC gene at the mutation cluster region (MCR) between codons 1280 and 1500. Left untreated, patients with FAP are at 95% risk of developing colon cancer by age 50 [84].

Race and gender are also important contributors to colon cancer. Compared to whites, African-American women with large colonic polyps are at 62% risk of having the polyps become cancerous. Moreover, mortality among African-Americans with colon cancer is about 40% higher than that of whites [89]. Also, patients with inflammatory bowel diseases (ulcerative colitis and Crohn's disease) are 18% more likely to develop colorectal cancer by age 30 [90, 91].

ii. ***The adenoma-carcinoma sequence and its molecular correlates***

The development of colon cancer can be a lengthy process; its evolution is well mapped both in terms of phenotype and the genetic changes that drive the adenoma-carcinoma sequence (Figure 1-2). An important early advance in our understanding of colon carcinogenesis, the adenoma-carcinoma sequence denotes the phenotypic progression from normal colon

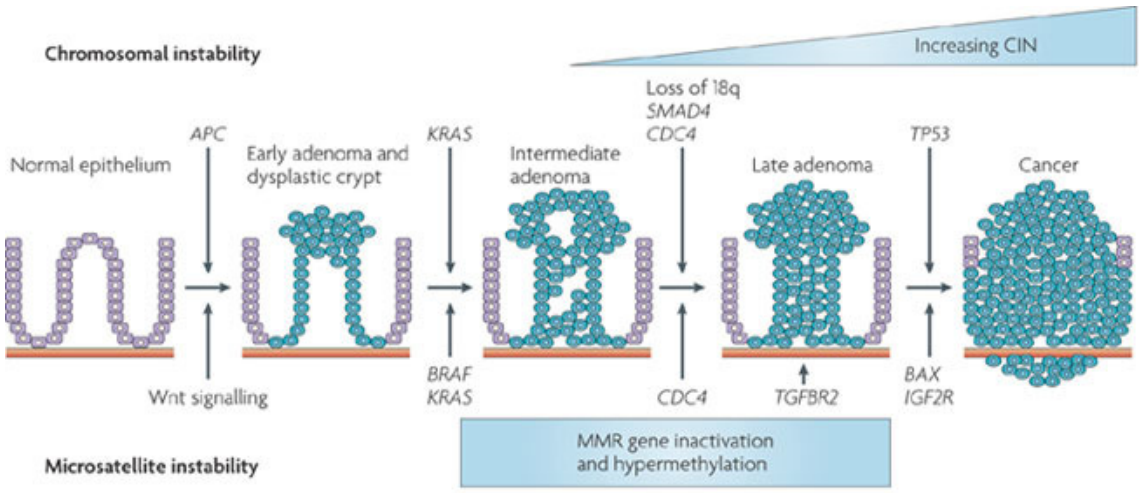

Figure 1-2: Adenoma-to-carcinoma sequence and the associated molecular alterations of colon cancer. The initial step, adenoma formation, is associated with loss of APC. Larger adenomas and early carcinomas acquire mutations in the small GTPase K-ras, followed by loss of chromosome 18q with SMAD4, and mutations in TP53 in frank carcinoma. Microsatellite instability (MSI) positive colon cancers (CCs) only carry the above changes infrequently. MSI is uncommon in adenomata and the initial step likely involves alteration in Wnt signaling. Mutations in BRAF, common in MSI CC, are likely to occur in the place of KRAS mutations. Mismatch repair (MMR) deficiency in sporadic CC occurs predominantly by downregulation of MLH1 through promoter methylation, and MSI status is increased by positive selection of cells with mutated microsatellites in MSH3 and MSH6. Further positive selection occurs for mutations affecting microsatellites in TGFBR2, iGF2R and BAX, which in turn provides a TP53-independent mechanism of progression to carcinoma. Adapted from Walther et al. [1].

epithelium to dysplastic adenoma (dysplastic aberrant crypt foci; ACF), to early adenoma, to late adenoma, and finally to carcinoma. This sequence of events reflects the combined effects of the mutational activation of oncogenes and the inactivation of tumor suppressor genes, due to environmental insults and/or genomic instability. The various gene alterations are promoted by microsatellite instability (MSI) and aberrant signaling by chromosome instability (CIN).

The primary step is adenoma formation. Dysplastic adenoma formation or dysplastic ACF has been correlated to environmental factors and mutations, both germline and somatic. Environmental factors include exposure to mutagens, reactive oxygen species (ROS), polyamines, and bacterial/viral invasion. Germline factors include those observed in hereditary diseases such as FAP and HNPCC. Briefly, the germline mutations of APC affect the APC protein, which is involved in the regulation of the Wnt signaling pathway, where it controls the proto-oncogene β-catenin [92, 93]. More specifically, APC functions as a scaffold protein that helps build a complex with β-catenin and other proteins (axin, casein kinase 1, and glycogen synthase kinase 3; GSK3) to assist in the phosphorylation and subsequent ubiquitination of β-catenin for its proteasomal degradation [93]. Mutational loss of APC function; however, increases the activation of β-catenin and its target genes, such as c-Myc [94] and cyclinD1 [95], which are involved in cellular proliferation and eventually colon carcinogenesis.

The primary step in adenoma formation has also been associated point mutations (missense mutations) or deletions of β-catenin. Furthermore, mutations in the MMR system, as seen in Lynch syndrome (HNPCC), inactivate mismatch repair genes (hMSH2, hMLH1, hPMS1, hPMS2,

hMSH3, and hMSH6), increasing polymerase generated replication errors particularly at microsatellite repeat sequences due to slippage at repetitive sequences [84].

The transition from dysplastic adenoma to intermediate adenoma is largely characterized by mutation in K-ras (Kirsten rat sarcoma). K-ras is part of the epidermal growth factor receptor (EGFR) signaling pathway involved in many pro-oncogenic pathways such as cell growth, proliferation, invasion, and metastasis. In its inactive state, K-ras is bound to guanosine diphosphate (GDP). In its active state, K-ras is bound to guanosine triphosphate (GTP). The formation of the active GTP-bound form of RAS is promoted by guanine nucleotide exchange factors (GEFs). The intrinsic enzymatic activity of K-ras allows it to inactivate by converting GTP to GDP via the removal of the terminal phosphate from GTP. This rate inactivation from GTP to GDP can be increased significantly by GAP (GTPase activating protein). Mutations of K-ras on codons 12, 13, and 61 adversely affect the ability of GAP to inhibit the inactivating hydrolysis of GTP to GDP, thereby promoting aberrant activation of the EGFR pathway [96]. K-ras mutations affect multiple cellular pathways that control cellular growth, differentiation, survival, apoptosis, cytoskeleton organization, cell motility, proliferation, and inflammation [97]. Another proto-oncogene involved in the EFGR pathway at the same intermediate adenoma progression point is BRAF (v-Raf murine sarcoma viral oncogene homolog B1), which acts downstream of K-ras [98, 99]. Mutations in BRAF are commonly found in MSI positive colon cancers [98].

Transition into late adenoma from intermediate adenoma has been associated with aberrant, hMLH1 (substitution, deletion, and hypermethylation of CpG sites), hMSH3 (deletion), hMSH6 (deletion and frameshift mutation) and MutS Homolog-2 (hMSH2; deletion) mutations.

The late adenoma is also characterized by aneuploidy, polyploidy due to chromosomal instability, LOH (loss of heterozygosity) and alterations in DNA methylation (hypermethylation). The loss of chromosome 18q is also associated with late adenoma formation in colon cancer. One major gene in 18q that is affected includes SMAD4. SMAD4 is a tumor suppressor gene which is part of the TGF-β signaling pathway. SMAD4 is subject to point mutations and deletions resulting in the activation of pro-tumorigenic functions such as proangiogenesis by increasing levels of VEGF [100].

The late adenoma to carcinoma progression is often characterized by alterations of the tumor suppressor gene, p53. These modifications are due to LOH at chromosome 17p or point mutations [101-103]. The tumor suppressive role of the p53 nuclear transcription factor is due to its ability to activate target genes such as those involved in apoptosis and cell cycle arrest. Moreover, p53 non-nuclear function in the cytoplasm involves maintaining genomic stability by centrosome duplication and alternative apoptotic pathways at the mitochondrial outer membrane [104].

The late adenoma to carcinoma transition is also characterized by frameshift mutations in Bax by MSI [105]. Bax, a pro-apoptotic protein in the Bcl-2 family, is involved with p53 induced apoptosis at the mitochondria. Upon translocation of Bax into the mitochondrial membrane, Bax undergoes conformational activation which results in the release of pro-apoptotic factors such as cytochrome c and Smac/Diablo to promote caspase activation and subsequent cell death by nuclear fragmentation [106].

iii. Colon cancer screening and treatment limitations: The argument for prevention

There are several diagnostic tests for the detection and/or prevention of colon cancer. Each of these screening procedures is associated with its own set of significant drawbacks which are limited due to sensitivity, specificity, and invasiveness. *Fecal occult blood tests* are used to detect blood loss from the gastrointestinal tract. A positive result of blood loss however does not indicate colon cancer only, as blood loss may also be caused by other GI disorders [107]. *Sigmoidoscopy* is limited by the assessment of lesion in the distal colon. *Colonoscopy*, can access the entire length of the colon, however the instrument can perforate the wall of the colon or the rectum which can lead to infection and require surgery. *CT colonography*, an imaging method with good sensitivity and specificity, is limited because it cannot remove polyps and requires a radiologist to interpret the results. *Stool DNA testing* suffers from low sensitivity for early lesion detection [108].

The current treatment options for colon cancer are dependent on the stage of the cancer. These stages range from 0 to IV, with 0 denoting cancer that has not grown past the basement membrane of the colon and IV indicating cancer that has metastasized to the other organs. The current treatment options for colon cancer at various stages are associated with significant drawbacks. For all stages of colon cancer, the first line of treatment is removal of the tumor, and potentially surrounding tissue or organs depending on invasiveness. Depending on the stage of the cancer, adjuvant therapy, chemotherapy and/or radiation therapy may be required. Surgery,

chemotherapy, and radiation therapy all can be accompanied by significant side effects and life altering limitations such as colostomy.

Given the less-than-optimal status of colon cancer treatment, it is evident that colon cancer prevention may be the best method for controlling this disease.

iv. NSAIDs and the prevention of colon cancer

The past several years have witnessed increasing emphasis on the pharmacological prevention of colon cancer (chemoprevention). Chemoprevention aims to decrease morbidity and mortality from colon cancer by lowering the risk of developing invasive or clinically significant disease by administering pharmacological or natural agents to subjects at risk.

Candidate chemopreventive agents must be both effective and safe. Agent safety becomes paramount if we consider that such an agent will be given for decades to an otherwise healthy person at risk for colon cancer. The relatively recent failure of COX-2 inhibitors is a stark reminder of the importance of agent safety [109, 110].

Epidemiological studies and interventional trials have established non-steroidal anti-inflammatory drugs (NSAIDs) as colon cancer chemopreventive agents [111-113]. The chemopreventive effect of NSAIDs has been demonstrated in several clinical studies [114-121] and their mechanistic attributes have been explored extensively both in vitro and in vivo [122].

Conventional NSAIDs are well known to inhibit the inflammatory pathway, as their name attests. A ready explanation for their antineoplastic effect has been the ability of NSAIDs to suppress prostaglandin synthesis by inhibiting the activity of COX, a crucial enzyme in the eicosanoid cascade [123, 124]. It has long been debated whether the inhibition of COX by NSAIDs is responsible for the apoptotic cell death of colon cancer [125]. Strong evidence suggests that prostaglandin inhibition by NSAIDs is not required for colon cancer cell death in human colon cancer cells and in mouse models of colon cancer [126]. Indeed, there is general consensus that NSAIDs exert their antineoplastic effect through several cyclooxygenase-independent pathways [127-130]. Several pathways mediating the antineoplastic action of NSAIDs are highlighted below. Although a complete mechanism of action is still not defined, considerable progress has been made.

Mechanistic work has centered on few of the nearly 50 conventional NSAIDs, such as aspirin, sulindac, indomethacin, and ibuprophen to elucidate their antineoplastic and pro-apoptotic effect. Aspirin was one of the first preliminary agents shown to have chemopreventive potential. Aspirin a) induced caspase-dependent phosphotidylserine externalization in HT-29 human colon adenocarcinoma cells [131]; b) induced activation, not inhibition, of NF-κB in HCT-116 and HT-29 colon cancer cells independent of COX-2 expression, mutation status of adenomatous polyposis coli, β-catenin, p53, or DNA mismatch repair (MMR) genes [132-134]); c) inhibited β-catenin transcription and downregulated the target gene, cyclin D1 in colon cancer cell lines; d) stabilized phosphorylated β-catenin, thereby increasing its ubiquitin-dependent degradation in SW948 and SW480 [135]; e) through its active metabolite it induced ROS production followed

by dissipated mitochondrial membrane potential resulting in cytochrome c release and activation of caspase-9 and caspase-3 in HT-29 and HCT-116 colon cancer cells [136].

Studies with indomethacin showed that the inhibition of prostaglandin synthesis was not dependent on its ability to promote apoptosis in HCT-116 and SW-480 colon cancer cell lines [137]. Rather, the pro-apoptotic effect of indomethacin was suggested to be from the induction of sphingomyelinase by arachidonic acid, leading to increased levels of ceramide, a known mediator of apoptosis[137]. Furthermore, the induction of growth inhibition of Caco-2 and HCT-116 human colon cancer cells by indomethacin is associated with the downregulation of ornithine decarboxylase activity and upregulation of spermidine/spermine-acetyltrasferase activity, thereby reducing intracellular pools of polyamines and promoting degradation by acetylation, respectively [138]. The overall reduction of polyamines, essential for neoplastic colon cell growth, is thereby associated with significant growth inhibition [138]. Similar to aspirin, indomethacin inhibits β-catenin transcription, downregulates the target gene, cyclin D1, in colon cancer cell lines, and stabilizes phosphorylated β-catenin in SW948 and SW480 colon cancer cells [135].

Sulindac and its active metabolite, sulindac-sulfide, were shown to induce apoptosis and cell cycle inhibition in the HT-29 colon cancer cell line [127]. Studies with sulindac revealed that its ability to promote apoptosis in colon cancer cell lines, was not dependent on the inhibition of prostaglandin synthesis [137]. Part of the pro-apoptotic effect of sulindac was likely due to the subsequent synthesis of ceramide by upregulating sphingomyelinase [137]. Additional mechanisms of the antiproliferative and pro-apoptotic effects of sulindac and its metabolite,

sulindac sulfide, on colon cancer cells include the upregulation of the membrane death receptor 5 and activation of caspase 8 [139]. Another metabolite of sulindac, sulindac sulfone, promotes peroxisome proliferator-activated receptor (PPAR) dependent transcriptional activation of spermidine/spermine-acetyltransferase (SSAT) which lowers intracellular polyamine levels in Caco-2 colon cancer cells [140]. In other cell lines such as SW-480 colon cancer cells, sulindac sulfone has been shown to downregulate Wnt/ β-catenin signaling by reducing β-catenin protein levels and subsequent transcription of cyclin D1 [141].

Ibuprofen, another conventional NSAID, also inhibits colon cancer growth. Ibuprofen inhibits the nuclear localization of β-catenin, an effect that correlated with the suppression of cyclin D1 expression in human colon adenomas [142]. Furthermore, in SW-480 cells ibuprofen decreased the levels of β-catenin and increased its phosphorylation [142]. Ibuprofen also promoted the nuclear localization of NF- κB by inducing the degradation of IκBα [142]. Surprisingly, in this case, the nuclear translocation of NF-κB did not result in the transcriptional activation of its target genes Bcl-2, survivin, and cyclin D1 [142].

The performance of conventional NSAIDs, however, is suboptimal due to reduced potency and safety. This motivated us to pursue an approach that through their chemical modification, renders them more efficacious and/or safer. This approach is discussed in detail in a later section. Phospho-naproxen (P-N) has been synthesized following this approach.

1.4 Modified NSAIDs: Towards improved safety and efficacy

i. ***Overview***

NSAID-related GI toxicity spurred the development of selective inhibitors of COX-2, since GI toxicity is associated with COX-1 inhibition [143, 144]. However, selective COX-2 inhibitors increased, among others, the incidence of cardiovascular events [145-147], necessitating the withdrawal of some of them from the market [148, 149].

Modified NSAIDs such as nitrous oxide-donating NSAIDs (NO-NSAIDs), H_2S-donating NSAIDs (HS-NSAIDs), phosphatidylcholine-NSAIDs (PC-NSAIDs) and phospho-NSAIDs have been developed in an effort to overcome the limitations of conventional NSAIDs. The rationale for each varies. For example, NO-NSAIDs were developed in the hopes of protecting the gastric mucosa from the ravages of the NSAIDs by locally releasing the gastroprotective NO. Although this assumption is in serious doubt [150], these compounds were shown to spare the gastric mucosa at least to some extent [151].

The development of phospho-NSAIDs was driven by two objectives: a) to chemically modify the carboxylic moiety, the carboxylic group is considered responsible for much of the GI toxicity of conventional NSAIDs (nearly all of the available NSAIDs are carboxylic acids); and b) to provide a chemical group that facilitates their uptake by the cell, such as the diethylphosphate moiety. The inhibition of COX-1 and COX-2 has been implicated in the induction of various adverse events. Therefore, it is plausible that the prevention of this interaction would reduce the side effects

associated with COX inhibition [152, 153]. More specifically, the carboxylic moiety interacts with arginine 120 of the COX-1 and 2 enzymes [154]. It is possible that the addition of the bulky phospho-group to the carboxylic end of an NSAID may inhibit its interaction with the smaller binding pocket of COX-1, thereby attenuating its effect on mucosal integrity, thus rendering the phospho-NSAID safer [155-157].

Previously, our lab successfully synthesized phospho-NSAIDs to potentiate and/or retain their anti-inflammatory and chemopreventive properties while significantly reducing their toxicity. Furthermore, these modified NSAIDs have also been effective in models of breast, lung, liver, pancreatic, and colon cancer [153, 158-161].

ii. Modified NSAIDs

Summarized below is the main published work on the use of modified NSAIDs for the treatment of arthritis and for colon cancer prevention.

Modified NSAIDs for Arthritis: Of the NO-NSAIDs **,** NO-naproxen was shown in an adjuvant arthritis model to reduce arthritis similar to naproxen [162]. NO-naproxen was equipotent to naproxen in reducing inflammatory edema [163] and inflammation of the paw [164]. NO-naproxen had greater gastric tolerability than naproxen [162]. Phosphatidylcholine-naproxen had greater gastric safety in arthritic rodent models than naproxen, but was less effective in reducing arthritis-related joint edema [165]. Hydrogen sulphide-releasing naproxen was as

effective as naproxen in reducing Freud's adjuvant-associated paw edema and had greater gastric safety than naproxen as well as promoted gastric ulcer healing [166]. Phospho-aspirin, phospho-ibuprofen and phospho-sulindac, synthesized by our lab, showed reduced gastrointestinal toxicity over their parental counterparts and strong anti-inflammatory effects which reduced joint related edema [152].

Modified NSAIDs for colon cancer chemoprevention: Several NO-NSAIDs such as NO-aspirin, NO-sulindac, and NO-ibuprofen have been shown to induce apoptosis and inhibit cellular proliferation in colon cancer more effectively than their parental counterparts [167]. The antineoplastic effect of NO-NSAIDs is mediated mainly through COX-independent pathways. These COX-independent pathways vary between compounds; in general, however, they are associated with the inhibition NF-κB and β-catenin target genes such as cyclin D1 [168-171]. Furthermore, NO released from these compounds has been associated with the inhibition of NF-κB binding [168], oxidative stress [172, 173], and the activation apoptosis-signal regulating-kinase (ASK1) [173].

NOSH-NSAIDs, which release nitric oxide and hydrogen sulfide, induce apoptosis and inhibit cellular proliferation. NOSH-aspirin and NOSH-naproxen have been shown to effectively inhibit colon cancer cell growth by inducing apoptosis and inhibiting the cell cycle at the G_0/G_1 phase [174-176]. NOSH-sulindac was also shown to induce apoptosis and inhibit cellular proliferation G_2/M, respectively, in colon cancer cells [176].

Another class of modified NSAIDs is hydrogen sulfide (HS)-releasing NSAIDs (HS-NSAIDs). This group of NSAIDs consists of HS-aspirin, HS-sulindac, HS-naproxen and HS-ibuprofen [177]. They were shown to inhibit colon cancer cell growth more significantly than their parental counterparts by inhibiting cellular proliferation and inducing apoptosis [177]. Their mechanism of action in colon cancer cells remains to be determined, but in breast cancer cells HS-aspirin increased the levels of reactive oxygen species and attenuated NF-κB signaling [178].

The effectiveness of phospho-NSAIDs, such as phospho-sulindac and phospho-aspirin is COX-independent. Phospho-sulindac and phospho-aspirin were observed to inhibit cellular proliferation in colon cancer cell lines at G_1/S and at G_2/M. [161, 179]. Phospho-sulindac and phospho-aspirin induce apoptosis by increasing mitochondrial superoxide anion (0_2^-) and reducing mitochondrial membrane potential which triggers apoptosis [161, 179]. Both phospho-sulindac and phospho-aspirin reduced binding of NF-κB to DNA [161, 179]. Moreover, phospho-sulindac directly downregulated the expression of Trx-1 (inhibitor of ASK1). Phospho-sulindac was also able to enhance polyamine catabolism, a promoter of carcinogenesis, by increasing SSAT (spermidine/spermine N (1)-acetyltransferase) enzymatic activity [179]. The antineoplastic effect of phospho-sulindac was also evident in animal models of colon cancer where the growth of HT-29 xenografts and the formation of intestinal tumors of Apc/Min mice were significantly inhibited [153, 180].

Phospho-aspirin increased reactive oxygen species in colon cancer cells and was accompanied by a depletion of glutathione levels [161]; glutathione scavenges reactive oxygen species by donating electrons from the thiol of cysteine to reactive oxygen species. The reduced antioxidant

ability by phospho-aspirin treatment made colon cancer cells more susceptible to reactive oxygen species. Phospho-aspirin was also effective in increasing F_2-isoprostane levels, a marker of oxidative stress, in colon cancer animal models [180]. Phospho-aspirin treatment was also marked by an increase in the MAPk family involved in apoptosis; the activation of p-38 and JNK [161].

iii. Why phospho-naproxen?

Naproxen and its side effects

Naproxen is a commonly used NSAID, primarily because of its efficacy and safety profile. Naproxen has been used effectively as a primary treatment and co-treatment of several forms of arthritis such as RA, reactive arthritis, osteoarthritis, juvenile arthritis, and septic arthritis. Compared to other NSAIDs, naproxen has been associated with a *lowered risk of myocardial infarction* [145, 181, 182] with some studies suggesting that it lowers the risk of adverse cardiovascular events by inhibiting platelet aggregation [183, 184]. Naproxen is also associated with the *lowest risk of NSAID-related hepatoxicity* [185]. Up until 2005, naproxen was associated with one liver-related hospitalization (out of 37,671 patients) and 1 liver-related death (out of 51,942 patients) [186].

Naproxen, however, is not completely free of side effects. Although renal-related adverse events are low [187], a study by Whelton *et al.* (2000) observed detrimental changes in the

kidney's glomerular filtration rate with naproxen use in healthy elderly patients [25]. Furthermore, the most common and major side effect of naproxen is gastric toxicity leading to significant gastric bleeding and ulceration. At the recommended dose for rheumatoid arthritis, naproxen has been associated with a significantly high rate of gastric and duodenal ulcers [43, 188, 189]; the cumulative incidence of gastric and duodenal ulcers for naproxen is 41% [188, 189].

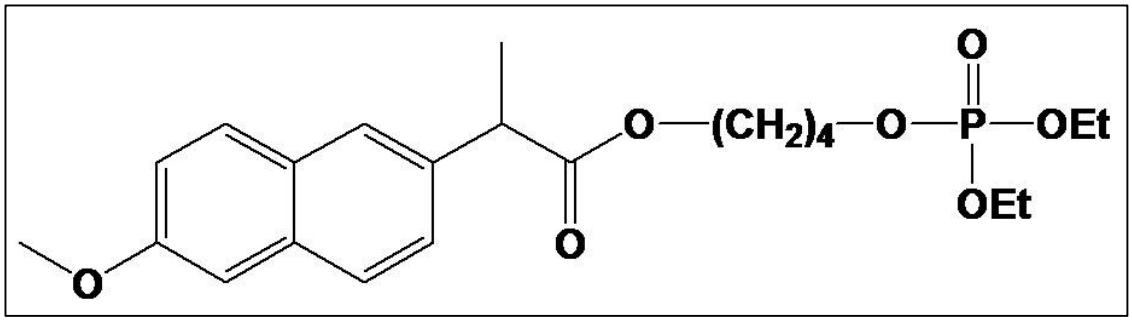

Figure 1-3: The structure of phospho-naproxen. Phospho-naproxen consists of naproxen conjugated to a butane-diethylphosphate moiety via a carboxylic ester bond

Phospho-naproxen

The synthesis and development of P-N was based on a series of important considerations. First, out of all NSAIDs, naproxen has the most favorable safety profile, especially with regards to the feared cardiac and renal toxicities. Second, it has been clear that chemically modifying the carboxylic group of conventional NSAIDs abrogates much of their GI toxicity. Third, the phospho modification of NSAIDs appears to be the most successful to date. This modification consistently shows superb safety over their parent compounds and strong efficacy in preclinical models. As

shown in Figure 1-3, P-N consists of a naproxen residue and a butane-diethylphosphate moiety bound through a carboxylic ester bond.

Both arthritis and colon cancer chemoprevention are in substantial need of safe and efficacious agents, a consideration made even more relevant by the fact that for both indications pharmacological agents will be on a long-term basis. Following the synthesis and essential pharmacology of P-N, we assessed its efficacy and safety in preclinical models of arthritis and colon cancer.

Chapter 2: Synthesis and Pharmacology of P-N

This section presents, in addition to the synthesis of P-N, its basic pharmacological properties as well as aspects of its efficacy in the treatment of rheumatoid arthritis and in the prevention of colon cancer.

2.1 Synthesis of Phospho-naproxen

The synthesis of P-N, described below, is relatively straightforward and lends itself to scale-up for the case when larger quantities of this compound will be needed for its preclinical evaluation in anticipation of regulatory submissions. The work on the synthesis of P-N was led by Dr. K. Vrankova and mass spectrometry was conducted at our core facility by R. Rieger and T. Koller.

Briefly, the starting material (compound **1**) was chlorinated in the presence of $SOCl_2$ to give compound **4**. Compound **4** was then reacted in the presence of excess 1, 4-butandiol to obtain

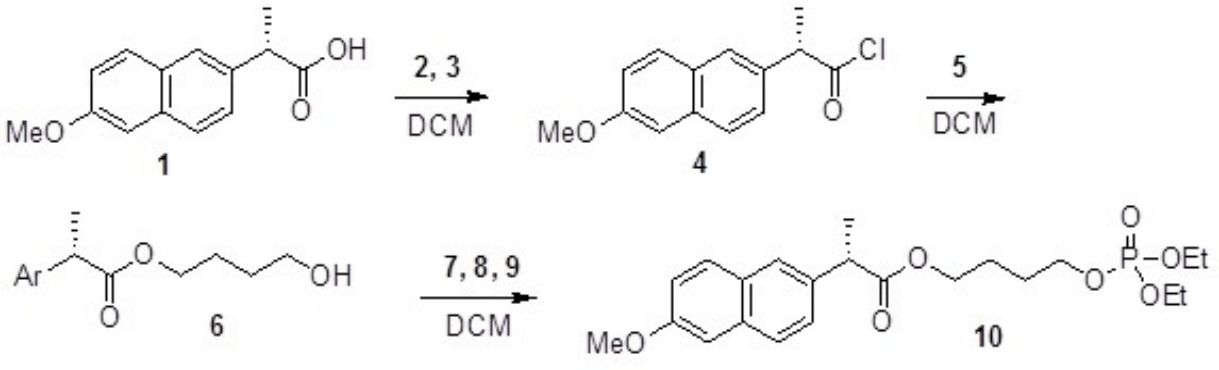

Figure 2-1 Scheme of the synthesis of phospho-naproxen

compound **6**. Compound **6** was then reacted with diethyl chlorophosphate in anhydrous conditions to obtain the final compound **10** (P-N) (Figure 2-1).

Synthesis of 4-Hydroxybutyl (+)-(*S*)-2-(6-methoxynaphthalen-2-yl) propanoate or 4-Hydroxybutyl "naproxenate" (6): FW ~ 302.40, Batch no.1: Neat thionyl chloride (**2**: 1.02 mL, 1.67 g, 14.0 mmol, 1.4 eq) was added drop-wise to a solution of **1** (2.30 g, 10.0 mmol, 1.0 eq) and Dimethylformamide (DMF) (**3**: 3 drops) in anhydrous Dichloromethane (DCM; 10 mL) at 0 ºC. The mixture was allowed to warm up to room temperature and let to stir at this temperature for 16 h after which the solvent was evaporated and the residue was co-evaporated with toluene (2 × 5 mL) and hexane (2 × 5 mL). The crude yellow solid **4** (2.50 g) was used without further purification or the solution was used directly.

4: **^{1}H NMR** ($CDCl_3$, 300 mHz) δ 1.87 (d, *J* = 7.2 Hz, 3H), 3.93 (s, 3H), 4.25 (q, *J* = 7.2 Hz, 1H), 7.14 (s, 1H), 7.17 (ddd, *J* = 8.9, 8.5, 2.5 Hz, 1H), 7.35 (dd, *J* = 8.7, 1.8 Hz, 1H), 7.69 (d, *J* = 1.2 Hz, 1H), 7.74 (d, *J* = 8.4 Hz, 1H), 7.76 (d, *J* = 7.5 Hz, 1H).

A solution of chloride **4** (2.49 g, 10.0 mmol, 1.0 eq) in anhydrous DCM (20 mL) was added drop-wise (during 2 h period, from a pressure-equalizing addition funnel) to a solution of 1, 4-butandiol (**5**: 4.43 mL, 4.51 g, 50.0 mmol, 5 eq) in anhydrous DCM (80 mL) at room temperature and the mixture was stirred at room temperature for 2 h after the addition was finished. Then the reaction was quenched with K_2CO_3 (5 % aq, 30 mL) and extracted with DCM (2 × 20 mL). The combined organic layers were washed with water (30 mL), brine (30 mL), dried over Na_2SO_4, and evaporated. The crude material was purified on a silica gel column (200 mL) with a gradient of

Hexane-Ethyl acetate (Hx-EA; 80:20 → 60:40 → 50:50) to afford colorless thick oil **6** (2.38 g, 7.88 mmol, 79 %) which solidified upon standing.

6: **^{1}H NMR** ($CDCl_3$, 300 MHz) δ 1.47-1.56 (m, 2H), 1.57 (d, *J* = 7.2 Hz, 3H), 1.60-1.68 (m, 2H), 3.54-3.57 (m, 2H), 3.84 (q, *J* = 7.2 Hz, 1H), 3.91 (s, 3H), 4.11 (t, *J* = 6.5 Hz, 2H), 7.10-7.16 (m, 2H), 7.40 (dd, *J* = 8.4, 1.5 Hz, 1H), 7.66-7.72 (m, 3H); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 18.4 (CH_3), 25.0 (CH_2), 29.0 (CH_2), 45.5 (CH), 55.3 (CH_3), 62.2 (CH_2), 64.5 (CH_2), 105.6 (CH), 119.0 (CH), 125.9 (CH), 126.2 (CH), 127.1 (CH), 128.9 (C), 129.2 (CH), 133.6 (C), 135.7 (C), 157.6 (C), 174.7 (C); **MS (GC-EI)** *m/z* (%) 302 ($M^{\bullet+}$, 20), 185 (100).

Synthesis of 4-(Diethoxyphosphoryloxy) butyl "naproxenate" (10): FW ~ 438.50, Batch no.1: Neat diethyl chlorophosphate (**7**: 1.90 mL, 2.28 g, 13.2 mmol, 2.0 eq) was added drop-wise to a solution of **6** (2.00 g, 6.61 mmol, 1.0 eq), di-(*iso*-propyl)ethylamine (**8**: 2.54 mL, 1.88 g, 14.6 mmol, 2.2 eq) and 4-Dimethylaminopyridine (DMAP; **9**: 5 pellets, catalytic amount, approx. 5 mol %) in anhydrous DCM (20 mL) at room temperature in a two neck flask equipped with a condenser under an inert atmosphere. The mixture was refluxed for 2 h, stirred at room temperature for 15 h and refluxed for additional 3 h. The reaction was then quenched with water (100 mL), acidified to pH ~ 6 with HCl (5 % aq) and extracted with DCM (3 × 30 mL). The combined organic layers were washed with sat. $NaHCO_3$ (50 mL), dried over Na_2SO_4 and evaporated. The crude material was purified on a silica gel column (150 mL) with a gradient of Hx-EA (50:50 → 20:80) to afford colorless oil **10** (2.28 g, 5.20 mmol, 79 %). Starting material **6** was also recovered (140 mg, 0.463 mmol, 7 %).

10: **$[\alpha]_D^{21}$** (*c* = 1, $CHCl_3$) = 21.6; **^{1}H NMR** ($CDCl_3$, 300 MHz) δ 1.30 (tt, *J* = 7.2, 0.9 Hz, 6H), 1.56 (d, *J* = 7.2 Hz, 1H), 1.57-1.71 (m, 4H), 3.84 (q, *J* = 7.2 Hz, 1H), 3.90 (s, 3H), 3.96 (q, *J* = 6.4 Hz, 2H), 4.01-4.11 (m, 6H), 7.10-7.15 (m, 2H), 7.38 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.65-7.71 (m, 3H); **^{13}C NMR** ($CDCl_3$, 125.7 MHz) δ 16.1 (d, *J* = 25.0 Hz, 2×CH_3), 18.4 (CH_3), 24.8 (CH_2), 26.7 (d, *J* = 26.5 Hz, CH_2), 45.4 (CH), 55.3 (CH_3), 63.7 (d, *J* = 23.0 Hz, 2×CH_2), 64.0 (CH_2), 66.8 (d, *J* = 23.0 Hz, CH_2), 105.6 (CH), 118.9 (CH), 125.9 (CH), 126.1 (CH), 127.1 (CH), 128.8 (C), 129.2 (CH), 133.6 (C), 135.7 (C), 157.6 (C), 174.6 (C); **^{31}P NMR** ($CDCl_3$, 121.5 MHz) δ 0.26; **IR** ν 1032, 1218, 1267, 1392, 1456, 1607, 1633, 1731, 2907, 2980; **MS (ESI+,** MeCN/H_2O**)** *m/z* (%) 899 [$(2M+H^+)^+$, 40], 540 [$(M+102)^+$, 100]; **MS (ESI+,** MeCN/H_2O**)** *m/z* (%) 899 [$(2M+H^+)^+$, 70], 894 [$(2M+NH_4^+)^+$, 60], 540 [$(M+102)^+$, 100], 456 [$(M+NH_4^+)^+$, 25], 439 [$(M+H^+)^+$, 20]; **MS (ESI–,** MeCN/H_2O**)** *m/z* (%) 453 [$(M+15)^-$, 100], 437 [$(M-H^+)^-$, 80].

2.2 Pharmacology of phospho-naproxen

The metabolism, essential pharmacokinetics and in vivo safety of P-N were assessed as well as its ability to inhibit the enzymatic activity of COX-1 and COX-2, the best-understood molecular targets of all conventional NSAIDs, including naproxen [190]. Following a description of the methods that were employed for this segment of the work, specific results are presented.

MATERIALS AND METHODS

Pharmacokinetic studies in mice: P-N was administered as a single treatment (60 mg/kg) by intravenous (iv) tail vein injection to 6-week-old female CD-1 mice (n=16) weighing approximately 24 g (Charles River Laboratories, Wilmington, MA, USA). P-N was prepared with the poloxamer, Pluronic F127 (Sigma-Aldrich, St. Loius, MO), to increase the aqueous solubility making it suitable for iv administration. Poloxamers (one of them is Pluronic F127) are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene flanked by two hydrophilic chains of polyoxyethylene. Because of their amphiphilic structure, these polymers have surfactant properties that make them useful in pharmacological applications. Among others, they can be used to increase the water solubility of hydrophobic, oily substances or otherwise increase the miscibility of two substances with different hydrophobicities [191].

Briefly, Pluronic F127 and P-N at a weight ratio of 4:1 were dissolved in tetrahydrofuran (THF). The solution was added drop-wise in excess amount of water during sonication to form micelles. The organic solvent was evaporated under reduced pressure and the resulting Pluronic solution

was analyzed by HPLC to determine the final concentration of P-N. After iv administration, mice were euthanized at the 5 min, 15 min, 30 min, 1h, 2h, 4h, 6h, and 24h time points. Blood was collected and immediately deproteinized by adding a two-fold volume of acetonitrile (CH_3CN). After centrifugation for 10 min at 12000g, supernatants were analyzed by HPLC.

Pharmacokinetic parameters were determined by non-compartmental pharmacokinetic data analysis using Pharmacokinetics Solutions 2.0 software (Summit Research Services, Montrose, CO, USA). The pharmacokinetic parameters determined were maximal plasma concentration (C_{max}), time to reach maximal plasma concentration (T_{max}), elimination half-life ($t_{1/2}$) and the area under the concentration–time curve (AUC).

Tissue distribution study: The heart, lung, liver, kidney, spleen, and colon of mice (n=16) given a single iv dose of P-N (60 mg/kg) were collected at 5 min, 15 min, 30 min, 1h, 2h, 4h, 6h, and 24h. To prevent hydrolysis during tissue preparation, tissues were homogenized and sonicated in an aqueous solution of bis-*p*-nitrophenyl phosphate (BNP; a carboxylesterase inhibitor). P-N was extracted by adding a two-fold volume of CH_3CN to the tissue homogenate. After centrifugation for 10 min at 12,000 g, the supernatants were analyzed by HPLC.

HPLC analysis: The HPLC system consisted of a Waters Alliance 2695 Separations Module (Milford, MA, USA) equipped with a Waters 2998 photodiode array detector (230 nm) and a Thermo Hypersil BDS C18 column (150 × 4.6 mm, particle size 3 µm). The mobile phase consisted of a gradient between solvent A [formic acid, CH_3CN, H_2O (0.1:4.9:95 v/v/v)] and solvent B (CH_3CN) at a flow rate of 1 $mL \cdot min^{-1}$ at 30°C. A gradient elution from 30 to 100% solvent B was applied from 0–6 min, and maintained at 100% solvent B until 8 min. Authentic compounds,

naproxen and naproxen glucuronide (Toronto Research Chemicals, North York, ON), were used to confirm and quantitate peaks from analyte.

P-N metabolism by rat liver microsomes: P-N (200 μM) was preincubated at 37°C for 5 min with a NADPH-regenerating solution (1.3 mM $NADP^+$, 3.3 mM d-glucose 6-phosphate, 3.3 mM $MgCl_2$ and 0.4 $U \cdot mL^{-1}$ glucose-6-phosphate dehydrogenase) in 0.1 M potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of rat liver microsomes (protein concentration 0.5 $mg \cdot mL^{-1}$; Sigma Aldrich, St. Louis, MO, USA). Samples were maintained at 37°C for 20 min. At the end of each of the incubations, 0.3 mL aliquots were mixed with 0.6 mL of CH_3CN, vortexed and then centrifuged for 10 min at 12,000 *g*. The supernatants were analyzed by LC-MS/MS.

LC-MS/MS analysis: The LC-MS/MS system consisted of Thermo TSQ Quantum Access (Thermo-Fisher) triple quadrupole mass spectrometer interfaced by an electrospray ionization probe with an Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA, USA). Chromatographic separations were achieved on a Luna C_{18} column (150 × 2 mm), and the mobile phase consisted of a gradient from 10 to 95% CH_3CN.

Lipophilicity of P-N and metabolites: The lipophilicity of P-N and its respective metabolites was determined by OSIRIS Property Explorer and expressed as logP.

Enzymatic activity assay: The COX fluorescent inhibitor screening assay (Cayman Chemicals, Ann Arbor, MI) based on the peroxidase component of COXs was used for this study. In this assay, the reaction between PGG_2 and ADHP (10-acetyl-3, 7-dihydroxyphenoxazine) by the peroxidase component in each COX isoform produces the highly fluorescent compound resorufin. Briefly, 75

µl/well buffer, 5 µl/well heme, 5 µl/well drug (P-N or naproxen), 5 µl/well of ovine COX-1 or human recombinant COX-2 (5 µl Enzyme + 45 µl Buffer) were added to each well in this order and incubated for 10 minutes at room temperature (RT). After incubation, 5 µl/well flourometric substrate (5 µl + 45 µl Buffer) and 5 µl/well arachidonic acid (5 µl AA + 5ul KOH + 45 µl H_20) were added in that order and incubated for 2 min at room temperature. Blank consisted of 80 µl/well Buffer + 5ul heme + 5ul dimethylsulfoxide. Flourometric analysis of resorufin was completed at the excitation wavelength (530-540nm) and emission wavelength (585-595nm). Samples were analyzed using GraphPad Prism Software.

Drug toxicity based on body weight changes: Drug toxicity was defined as body weight reduction $\geq$ 10% of baseline. Three groups of female LEW/CrlBR Lewis rats (140-190 g, n= 9/group) were treated by oral gavage once daily for 3 consecutive days as follows: 1) Vehicle: corn oil 1ml; 2) naproxen 50 mg/kg; and 3) P-N 95mg/kg (equimolar to naproxen). Animals were weighed on days 1, 3, and 5.

GI toxicity in rats: Following a standardized protocol [192], four groups of female Sprague Dawley rats (body weight = 151-182 g) were treated for 4 days by oral gavage as follows: 1) Vehicle: corn oil 1 ml (n=2); 2) Indomethacin 4.75 mg/kg/d (n=3); 3) naproxen 50 mg/kg/d (n=5); and 4) P-N 95 mg/kg/d (n=5) (equimolar to naproxen). Indomethacin was used as a reference compound (positive control) for GI toxicity [192]. On day 5, 30 min before sacrifice, animals were injected with 1% Evans blue solution in the tail vein and the number and size of small intestinal ulcerations were counted by an investigator blinded to the treatment protocol. GI toxicity was rated based on the number and size of ulcerations: small= < 1 mm diameter; medium= 1-4 mm diameter; and

large= >4 mm diameter. Scoring guidelines are as follows: 0= no ulcerations or mucosal damage; 1= 1-15 small mucosal ulcerations, 2= small mucosal ulcerations with ≤10 medium ulcerations and no large ulcerations; 3= small and medium ulcerations with ≤5 large ulcerations and no intestinal adhesions; 4= predominantly medium and large ulcerations (>5 total); large ulcerations exhibit signs of perforations and adhesions, which make it difficult to remove the intestine intact; 5= on necropsy, massive peritonitis resulting from intestinal perforation(s) [192].

Statistical analyses: Data, obtained from at least three independent experiments, were expressed as means ± SEM. Statistical comparisons, for these and subsequent studies, were performed with Student's t-Test or one-factor analysis of variance followed by Tukey test for multiple comparisons. Values of $p<0.05$ were considered statistically significant. Power analysis was conducted using Statistical Solutions software, to confirm appropriate sample size.

Animal studies: All animal care and experimental procedures, for these and subsequent studies, were approved by the Institutional Animal Care and Use Committee at Stony Brook University.

RESULTS

Pharmacokinetics and biodistribution of P-N in mice

To determine the pharmacokinetic properties and biodistribution of P-N in mice and to study its in vivo metabolism, mice were administered a single dose of P-N (60 mg/kg) by intravenous tail vein injection. Intravenous injection was utilized to simulate conditions where 100% of P-N is bioavailable. Blood samples obtained at various time points were analyzed by HPLC to determine relevant pharmacokinetic parameters (Table 2-1, Figure 2-2).

Table 2-1: The pharmacokinetic parameters of P-N and its metabolites in the blood. P-N (60 mg/kg) was administered to mice as a single iv dose.

Compound	AUC_{0-24} (μM*h)	T_{max} (min)	C_{max} (μM)	$t_{1/2}$ (min)
P-N	16	5	89	9
Naproxen	630	15	153	146
Naproxen glucuronide	38	15	30	58

In these experiments we detected by HPLC in blood P-N and two major metabolites, naproxen and naproxen glucuronide; their respective elution times were 6.3 min, 4.7 min, and 3.5 min. Naproxen and naproxen glucuronide were identified by using their corresponding authentic compound. Many drugs with a bulky acyl moiety are initially metabolized in the liver by carboxylesterases in the liver which cleave drugs at their carboxylic ester in preparation for the addition of more polar groups [193]. Furthermore, the process of glucuronidation of drugs is another common metabolic pathway supported by the liver [194]. In the liver, a drug is processed

by glucuronosyltransferase, which conjugates glucuronic acid to the drug. These drug conjugates are referred to as glucuronides, which are more hydrophilic, i.e. they can be excreted more easily through aqueous media such as blood and urine [194].

Several different pharmacokinetic parameters were determined for P-N and its metabolites,

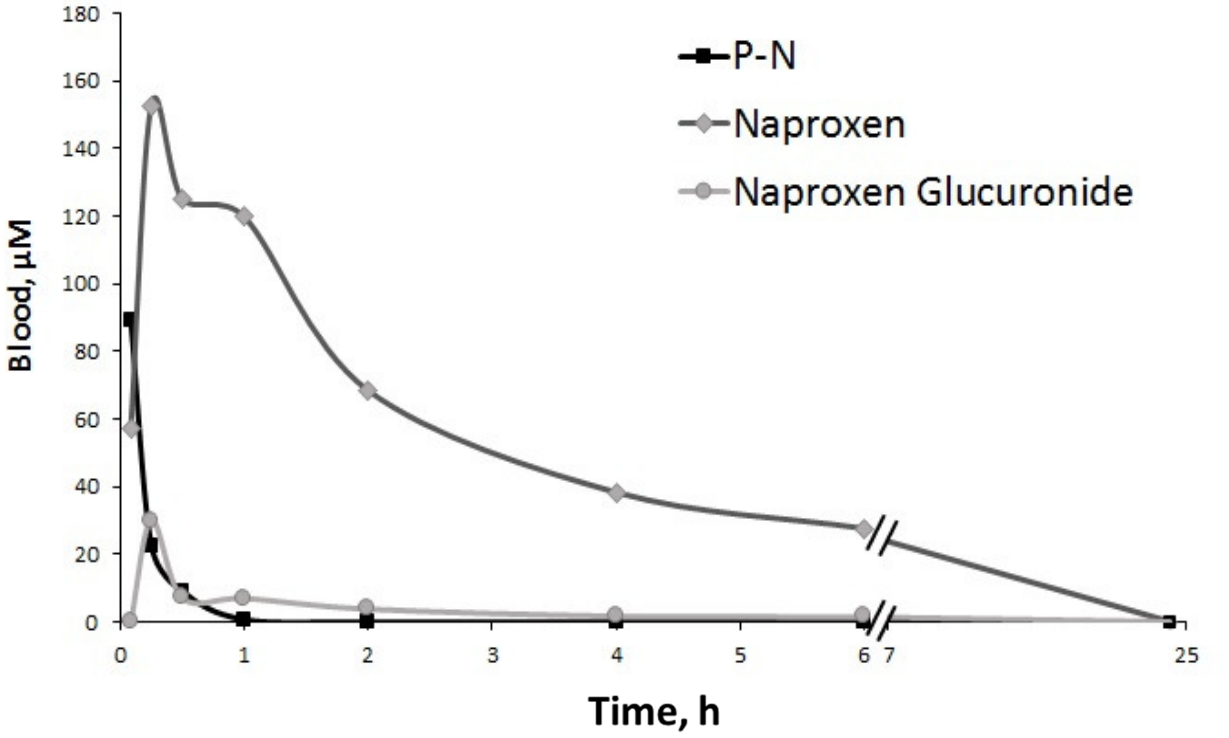

Figure 2-2: Blood levels of P-N and metabolites. Levels of P-N and the metabolites, naproxen and naproxen-glucuronide, were determined in mice treated with P-N (60mg/kg iv).

naproxen and naproxen glucuronide. The total drug exposure of P-N, determined by the area under the curve (AUC) was 16 µM*h. The peak concentration of P-N, the C_{max}, was 89 µM. This concentration peaked within 5 min (T_{max}) of administration. The terminal half-life ($t_{1/2}$) of P-N in

the blood was 9 min. Naproxen was observed to have: an AUC of 630 µM*h, T_{max} of 15 min, C_{max} of 153 µM, and $t_{1/2}$ of 146 min. Naproxen glucuronide was observed to have: an AUC of 38 µM*h, T_{max} of 15 min, C_{max} of 30 µM, and $t_{1/2}$ of 58 min (Figure 2-2 and Table 2-1). Overall, the AUC, T_{max}, C_{max}, and $t_{1/2}$ of P-N were markedly less than those of its metabolites, naproxen and naproxen glucuronide, indicating massive and rapid metabolic transformations of P-N.

To evaluate the biodistribution of P-N in mice, the drug levels in heart, lung, liver, kidney, spleen, and colon were determined by HPLC (Table 2-2, Figures 2-3, 2-4). As seen previously in the blood, P-N is rapidly hydrolyzed by esterases in the body. Therefore, during tissue preparation it was highly likely that active esterases would start to hydrolyze P-N, which would present lower than actual levels of P-N in organs. To this end, to a carboxylesterase inhibitor (BNP) was added during tissue homogenization to prevent the hydrolysis of P-N during tissue preparation. BNP is a known inhibitor of carboxylesterase, the enzyme that has been previously confirmed to be largely responsible for the hydrolysis of P-N [193].

In these biodistribution experiments we observed two major peaks by HPLC at the 6.3 min and 4.7 min elution time points. These peaks were determined to be P-N and naproxen, respectively, which were identified by using their corresponding authentic compound. In this study, naproxen glucuronide was not quantifiable due to the simultaneous elution of the BNP peak around elution time point of naproxen glucuronide at 3.5 min.

In these experiments the biodistribution of P-N was observed to greatest in the heart, which was observed as significantly high AUC of 44 µM*h. The rapid absorption of P-N is suggested by its C_{max} of 382 µM and T_{max} 5 min. These values were the most significant compared to the other

Table 2-2: Biodistribution and pharmacokinetic parameters of P-N and its metabolite, naproxen, in major organs. P-N (60 mg/kg) was administered to mice as a single dose iv.

Organ	Compound	AUC_{0-24} (µM*h)	T_{max} (min)	C_{max} (µM)	$t_{1/2}$ (min)
Heart					
	P-N	44	5	382	4
	Naproxen	168	5	82	61
Lung					
	P-N	24	5	151	9
	Naproxen	497	30	145	107
Liver					
	P-N	4	5	26	4
	Naproxen	300	15	128	65
Kidney					
	P-N	16	5	70	7
	Naproxen	176	15	117	58
Spleen					
	P-N	5	15	13	6
	Naproxen	41	15	38	41
Colon					
	P-N	4	5	15	7
	Naproxen	53	15	34	190

organs (Table 2-2). Although, these values are significantly greater than those observed for other organs it is important to note that the rate of clearance is one of the fastest, compared to other organs (Figure 2-3 and 2-4). These kinetic parameters for the heart may vary considerably to

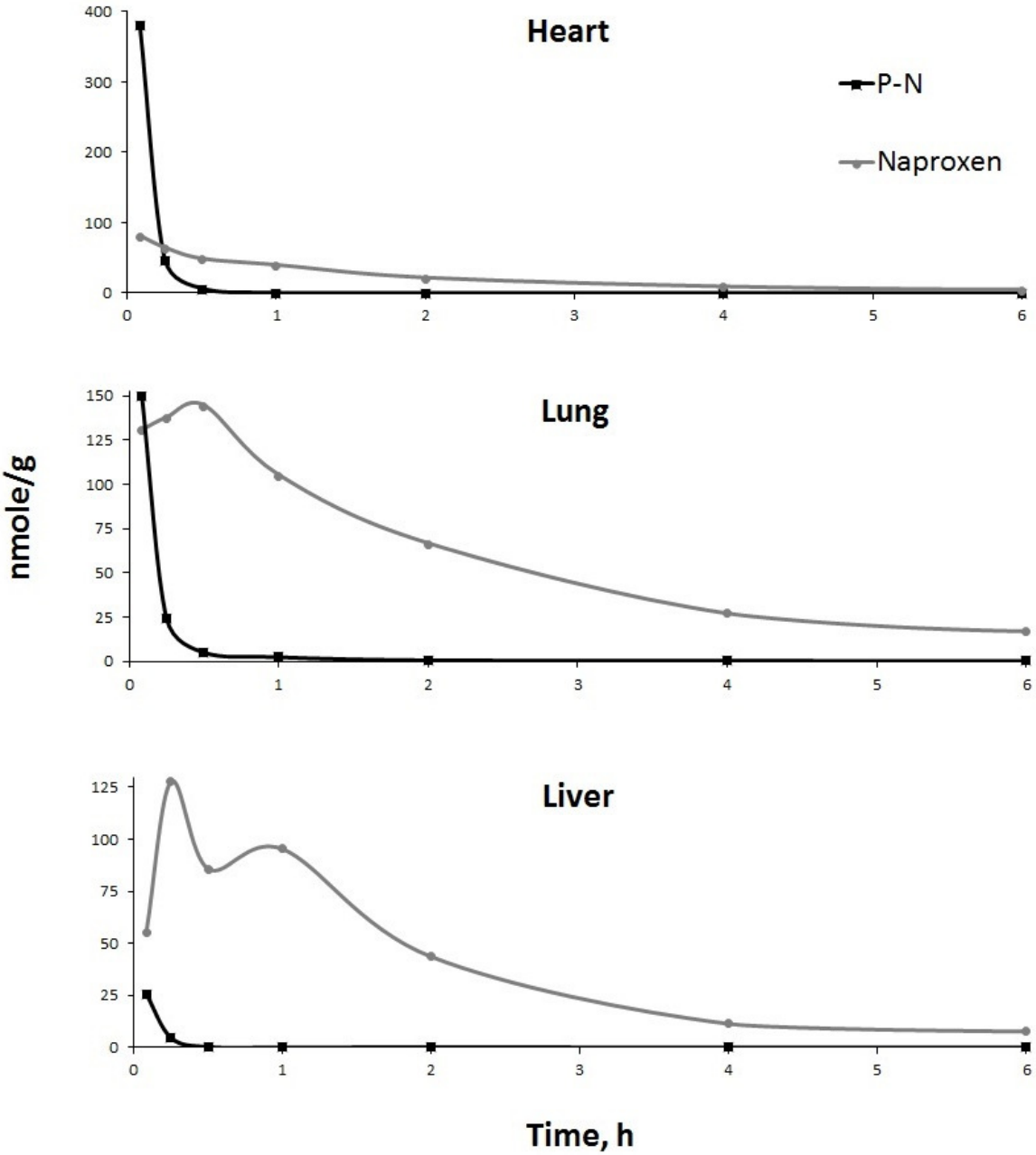

Figure 2-3: Levels of P-N and naproxen in heart, lung, and liver. Levels of P-N and the metabolite, naproxen, were determined in mice treated with P-N (60 mg/kg iv).

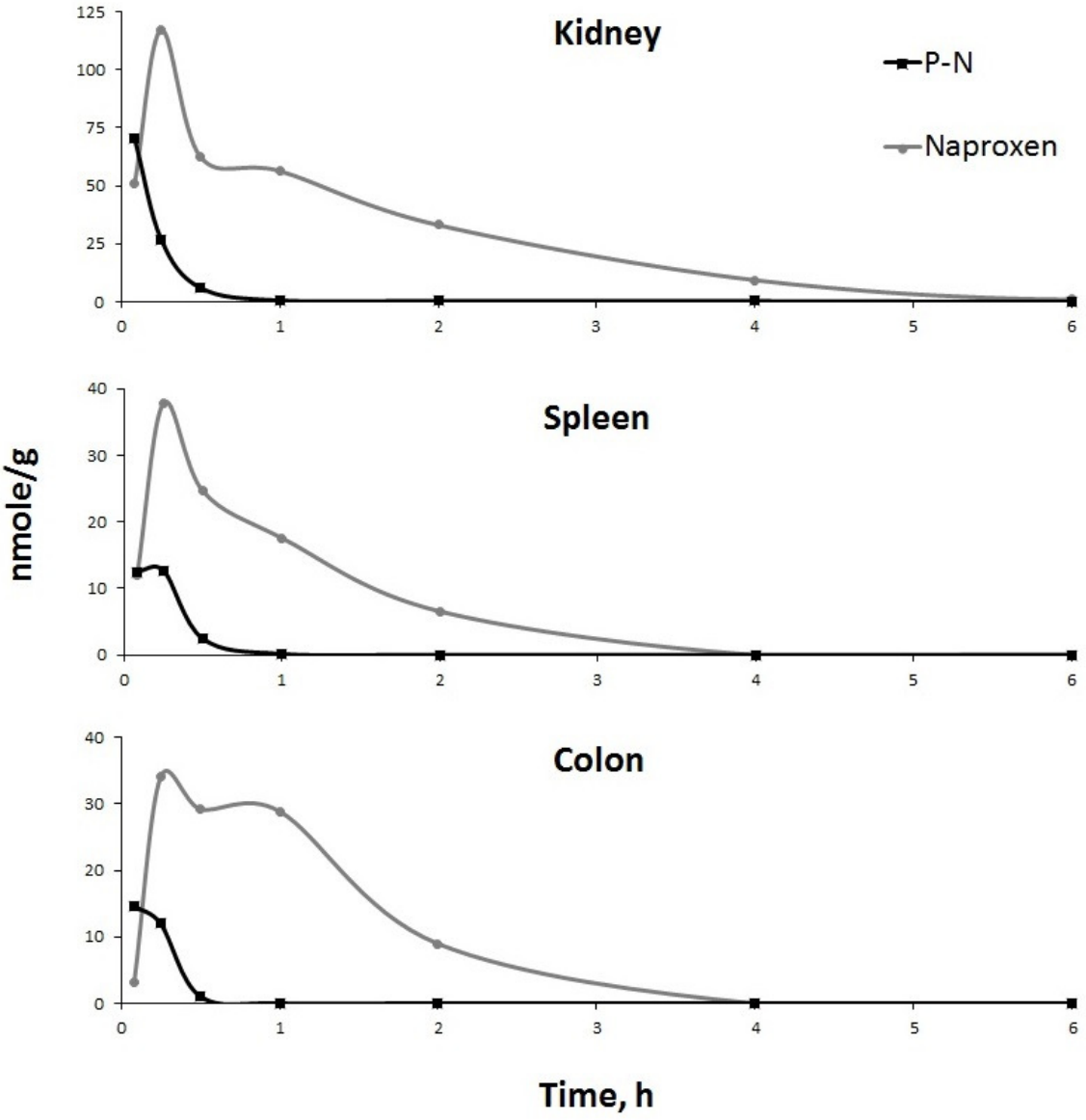

Figure 2-4: Levels of P-N and naproxen in kidney, spleen, and colon. Levels of P-N and the metabolite, naproxen, were determined in mice treated with P-N (60 mg/kg iv).

other organs due to the direct intravenous administration of P-N. Furthermore, as seen in the blood (Figure 2-2), other organs also show rapid conversion of P-N to naproxen (Figures 2-3 and 2-4).

Metabolism of P-N by rat liver microsomes

Since glucuronidation occurs mainly in the liver, especially when a is drug administered orally [195], we examined the metabolism of P-N in liver microsomes, which represent an enriched source of the major metabolic enzymes that render drugs more polar and, therefore, easier to eliminate from the body [194]. These potential metabolites were investigated by LC-MS/MS

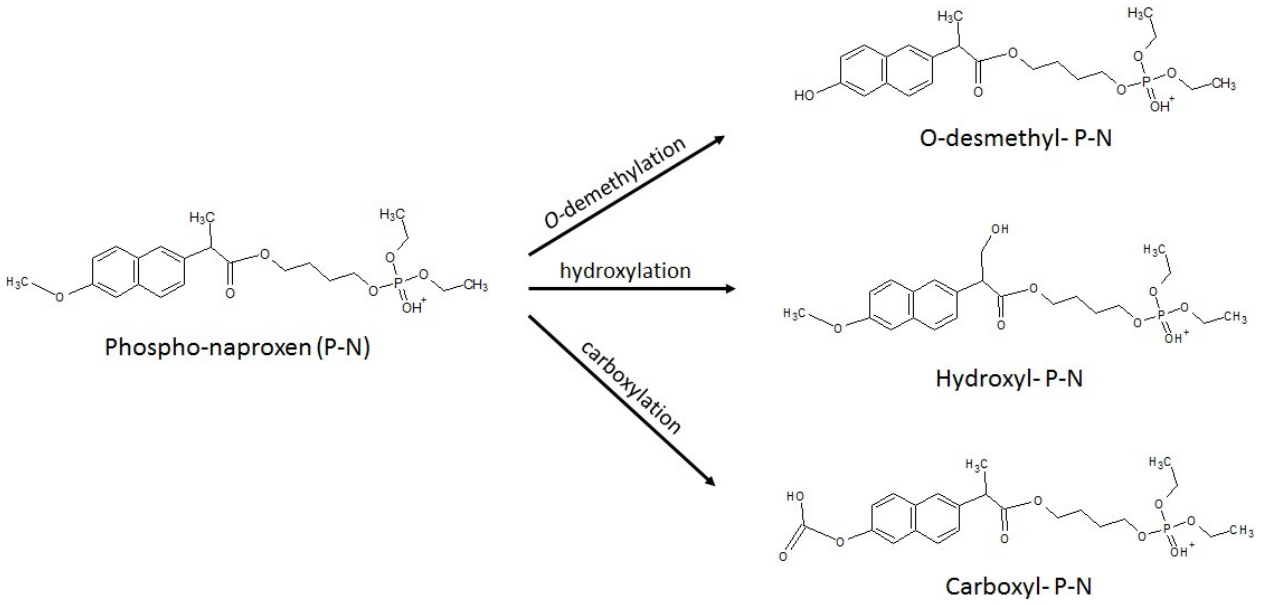

Figure 2-5: Major metabolic transformations of P-N in liver microsomes.

rather than HPLC, which is limited by the availability of synthetic standards and also by its inability to detect non-UV absorbing metabolites. In other words, LC-MS/MS provided the ability to determine potentially active metabolic products of P-N, some of which could be undetectable by HPLC.

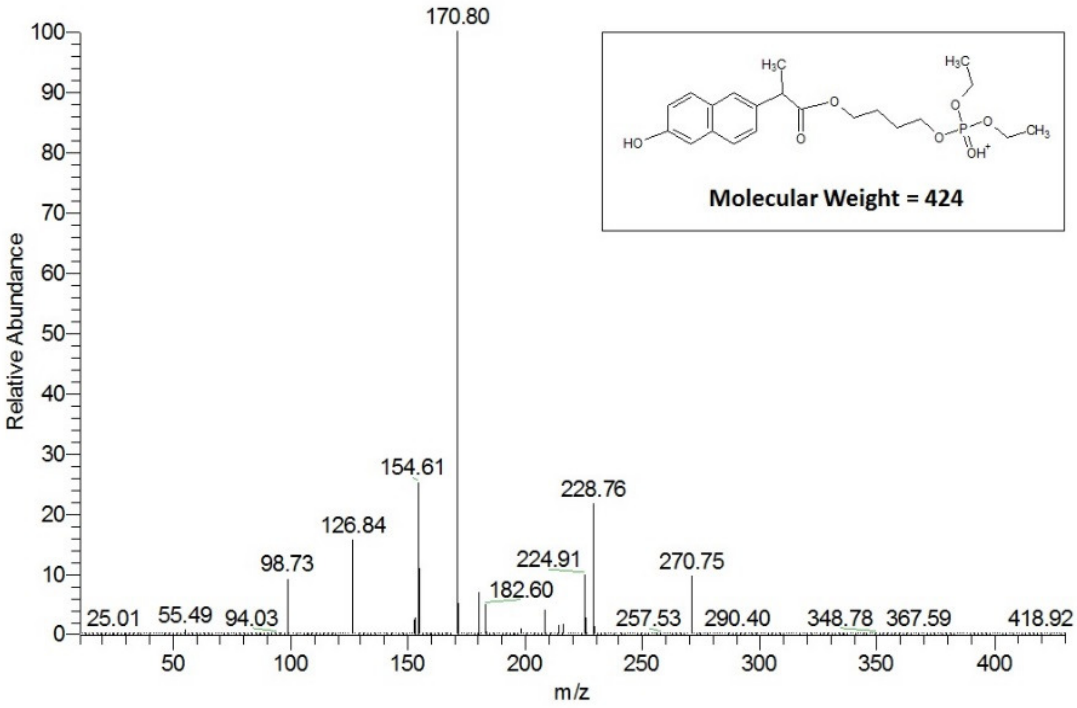

Figure 2-6: Identification of P-N metabolite, *o*-desmethyl- P-N, by liver microsomes.

As shown in Figure 2-5, the metabolism of P-N by rat liver microsomes generated three major metabolites by its demethylation, hydroxylation, and carboxylation, which generated *o*-desmethyl- P-N, hydroxyl- P-N, and carboxyl-P-N, respectively. Each of these three metabolites of P-N was identified by LC-MS/MS analysis. As shown in Figures 2-6, 2-7, and 2-8, LC-MS/MS analysis revealed the following: the protonated *o*-desmethyl-P-N was observed at *m/z* 425.3, which was fragmented to generate ions at *m/z* 170.8 and 270.75; the protonated hydroxyl-P-N was observed at *m/z* 455, which was fragmented to generate ions at *m/z* 200.79, 228.64, and 154.43; and the protonated carboxyl-P-N was observed at *m/z* 469, which was fragmented to generate ions at *m/z* 261.17, 315.18, and 155.08.

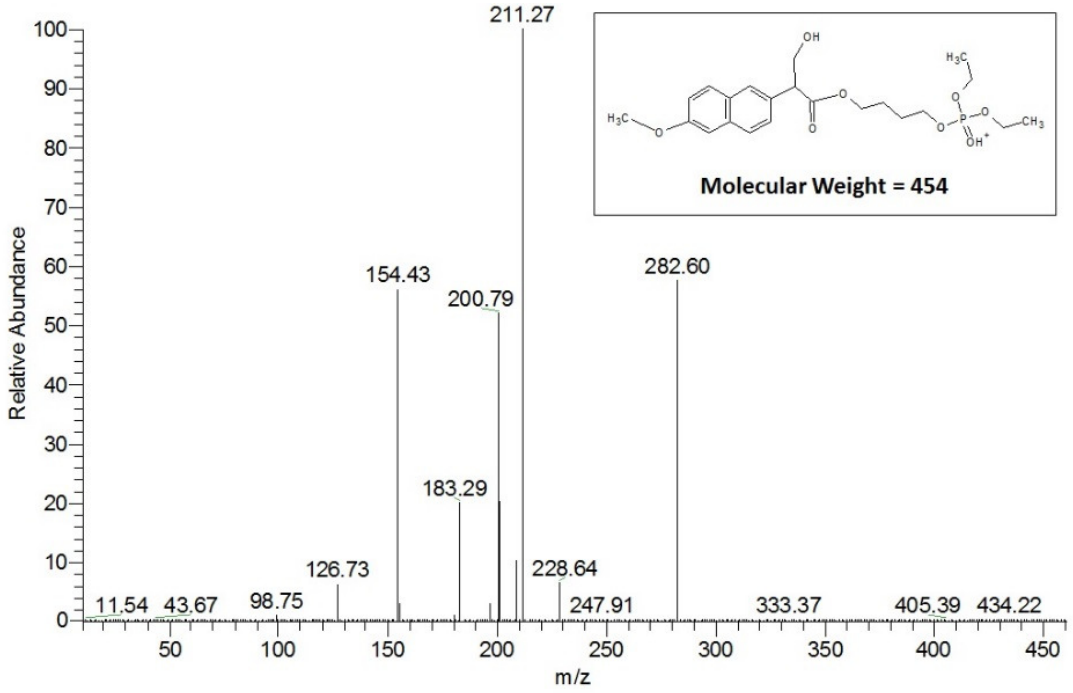

Figure 2-7: Identification of P-N metabolite, hydroxyl- P-N, by liver microsomes.

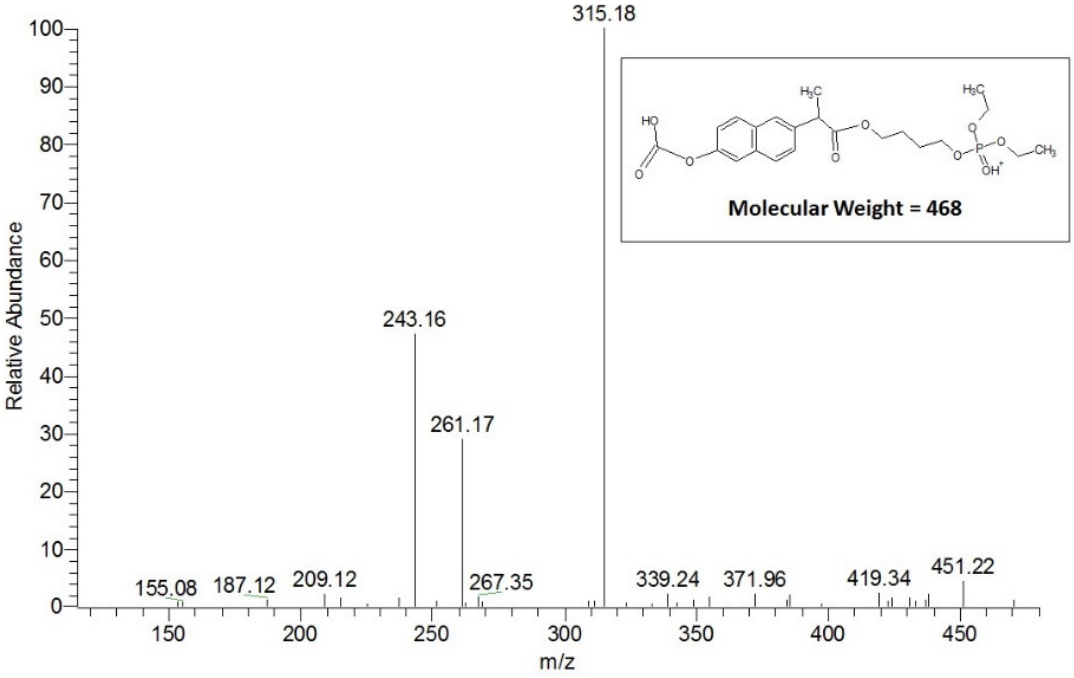

Figure 2-8: Identification of P-N metabolite, carboxyl- P-N, by liver microsomes.

Calculated Lipophilicity (LogP)

The LogP value of a compound, which is the logarithm of its partition coefficient between n-octanol and water log ($c_{octanol}/c_{water}$), is a well-established measure of the compound's lipophilicity. This study examines the calculated LogP, of each metabolite of P-N based on a validated computer algorithm which distinguishes 368 atom types which are composed of various properties of the atom itself and its direct neighbors.

Table 2-3: Calculated lipophilicity (LogP) of P-N and its respective metabolites

Compound	LogP
Phospho-naproxen	3.96
Carboxyl-phospho-naproxen	3.79
O-desmethyl-phospho-naproxen	3.68
Hydroxyl-phospho-naproxen	3.03
Naproxen	2.53
Naproxen-glucuronide	0.34

As shown in Table 2-3, P-N and three of its direct metabolites are highly lipophilic (Figure 2-5), a property attributed to their non-polar structural components. Naproxen is much less lipophilic than P-N whereas naproxen glucuronide is hydrophilic, a property that accounts for its role in the elimination of P-N from the body.

Effect of P-N on the enzymatic activity of COX-1 and COX-2

The inhibition of COX-1 and COX-2 by NSAIDs accounts for a significant portion of their pharmacological efficacy but also for their adverse events on the major organs of the body. One of the most common adverse effects of NSAIDs associated with the inhibition of COX-1 in the gut, is the formation of ulcers, which can lead to hemorrhage and even intestinal perforation that can lead to death [31, 32, 35]. On the other hand, the inhibition of COX-2 by NSAIDs plays a significant role in the development of thrombogenic events, which can lead to myocardial infarction, stroke, and death [28-30, 36]. P-N is a derivative of an NSAID. It was therefore prudent to investigate its ability to inhibit COX-1 and COX-2, which would give insight into its safety and mechanism of action.

Table 2-4: Half maximal inhibitory concentration (IC_{50}) of P-N and naproxen on COX-1 and COX-2 enzymatic activity

Cyclooxygenase Enzyme	Naproxen (IC-50, µM)	Phospho-naproxen (IC-50, µM)	Potency Reduction of P-N
COX-1	56	2922	52x
COX-2	10	2394	230x

To this end, the effect of P-N on the enzymatic activity of purified COX-1 and COX-2 was identified. The outcome was *the half maximal inhibitory concentration* of P-N, which was dependent on the functional ability of COX-1 and COX-2 to convert the substrate, arachidonic

acid, to prostaglandin G_2 (PGG_2, the intermediate precursor to prostaglandins, thromboxane, and prostacyclins). In this study, the half maximal inhibitory concentration of P-N was compared to conventional naproxen to determine potential differences in potency.

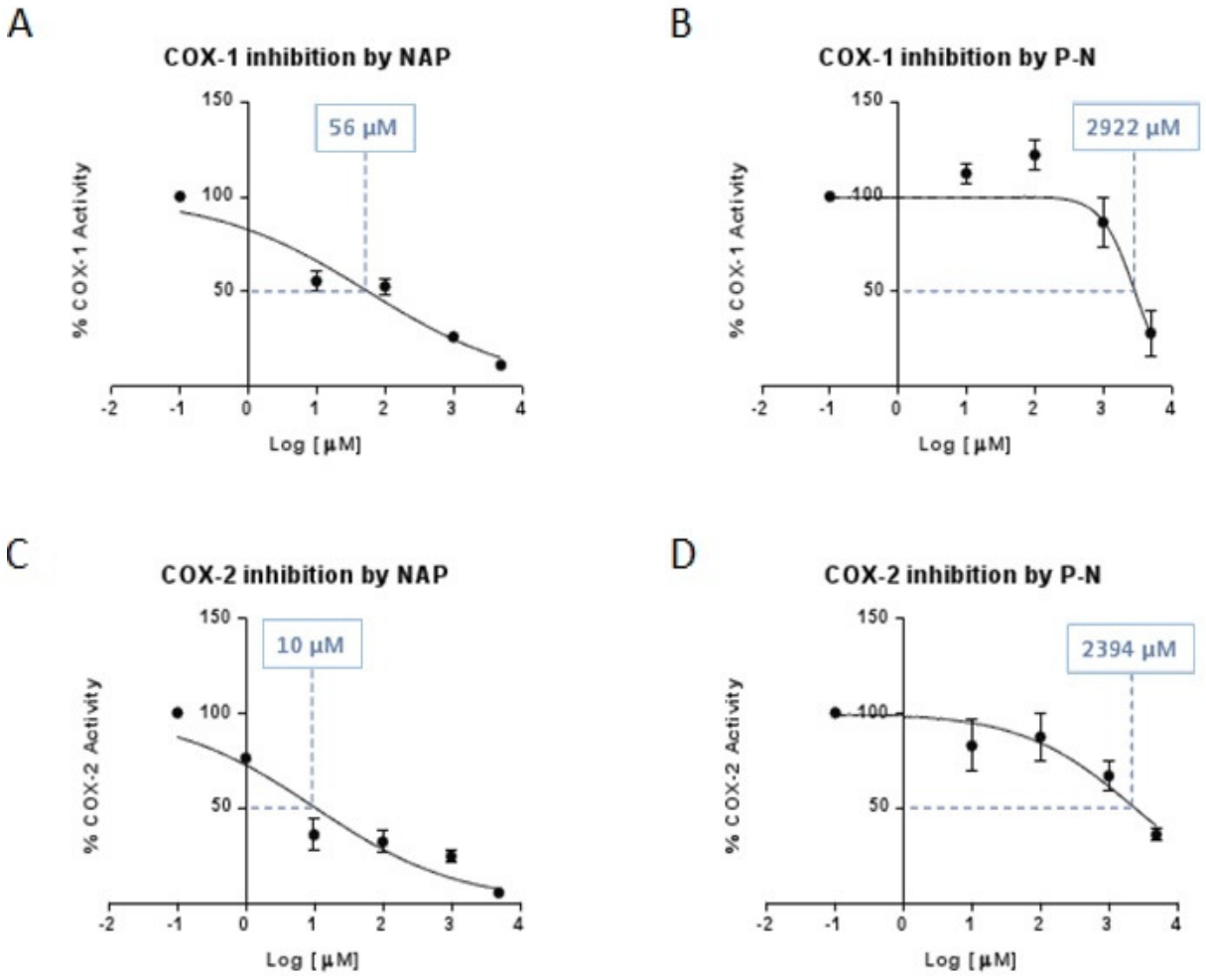

Figure 2-9: Enzymatic activity of COX-1 and COX-2 with P-N and Naproxen. **(A)** COX-1 treated with naproxen (NAP) **(B)** COX-1 treated with P-N **(C)** COX-2 treated with naproxen (NAP) **(D)** COX-1 treated with P-N. Values are mean + SEM. Boxes denote half-maximal inhibitory concentration.

As shown in Figures 2-9A and 2-9B, the half maximal inhibitory concentration of P-N (2,922 μM) was 52 times less than that of naproxen (56 μM) for COX-1. The half maximal inhibitory concentration of P-N and naproxen for COX-2 was 2,394 μM and 10 μM, respectively (Figure 2-9C, 2-9D). Thus, the potency of P-N for the inhibition of COX-2 was 230 times less than that of naproxen. Data are also summarized in Table 2-4.

Body weight toxicity of P-N

As noted previously, P-N has a significantly reduced potential to inhibit COX-1 and COX-2 compared to naproxen. COX inhibition is associated with significant adverse events therefore the inability of P-N to inhibit COX effectively may afford it increased safety over naproxen.

To identify potential differences in toxicity between P-N and naproxen, a safety study based on body weight was conducted. The universally accepted measure of toxicity is signified by ≥10% loss in body weight [196]. This study also assisted in the determination of a safe dosing paradigm of P-N and naproxen for use in subsequent animal studies, which utilized animal models of rheumatoid arthritis and colon cancer.

In an acute 5-day toxicity study, P-N and naproxen were administered to rats at equimolar doses by oral gavage, a mode of administration used in subsequent studies (Figure2-10). P-N

(95 mg/kg) caused no significant reduction in body weight over time. In fact, P-N treated animals

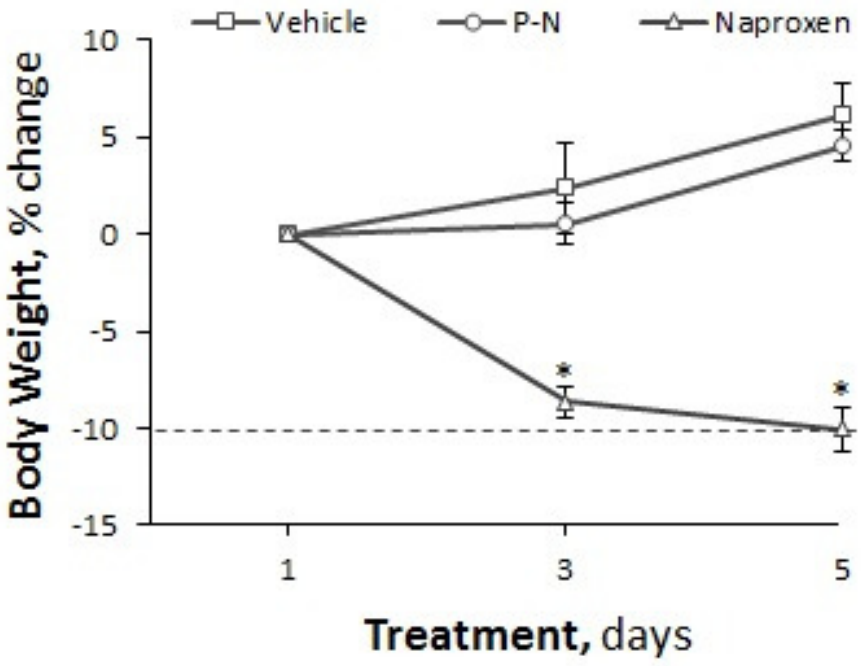

Figure 2-10: Percent change in body weight in study groups. Vehicle (n=9), Naproxen 50mg/kg (n=9), or P-N (Phospho-naproxen 95 mg/kg; n=9). Values are mean + SEM. (*=p<0.001, compared to original body weight)

increased their body weight over time, a trend which followed closely that of animals treated with vehicle alone. In contrast, rats treated with an equimolar dose of naproxen (50 mg/kg) experienced a significant loss in body weight by day 3 compared to day 1, a weight loss of 8.6% ($p<0.001$). Treatment for 5 days induced toxicity by this criterion (10.1% weight loss; $p<0.001$).

GI toxicity in rats

The cumulative incidence of gastric and duodenal ulcers among NSAID users is 41% [43, 188, 189]. To determine the potential GI toxicity of P-N, a specialized GI toxicity study was conducted; this study included a naproxen group. As presented in Methods, this study uses a positive inducer of GI toxicity, indomethacin, and utilizes a well-established clinically relevant scoring system to quantify the size and number of ulcers and intestinal perforations [192].

In this study, gross examination of the GI tract of the indomethacin (positive control) treated group showed significant medium and large ulcerations, perforations, and adhesions. The P-N treated group showed no significant signs of ulcerations or mucosal damage, being similar to the vehicle treated group. In contrast, the naproxen treated group exhibited mucosal ulcerations up to 1 mm in size (Figure 2-11B). The average clinical score of the vehicle (corn oil) group was 0 (no GI toxicity), and of the positive control (indomethacin) was, as expected, 4 (Figure 2-11A). Treatment with P-N 95 mg/kg led to a score of 0.4±0.24, which was 90% lower than that of indomethacin ($p<0.001$) and not significantly different than vehicle ($p>0.1$) . In contrast, the equimolar dose of naproxen produced substantial mucosal damage (score: 1.4±0.24), compared to vehicle ($p<0.01$). In fact, naproxen was associated with a 3.5-fold increase in toxicity, compared P-N ($p<0.05$) (Figure 2-11A, 2-11B). Overall, this study established the superior GI safety of P-N.

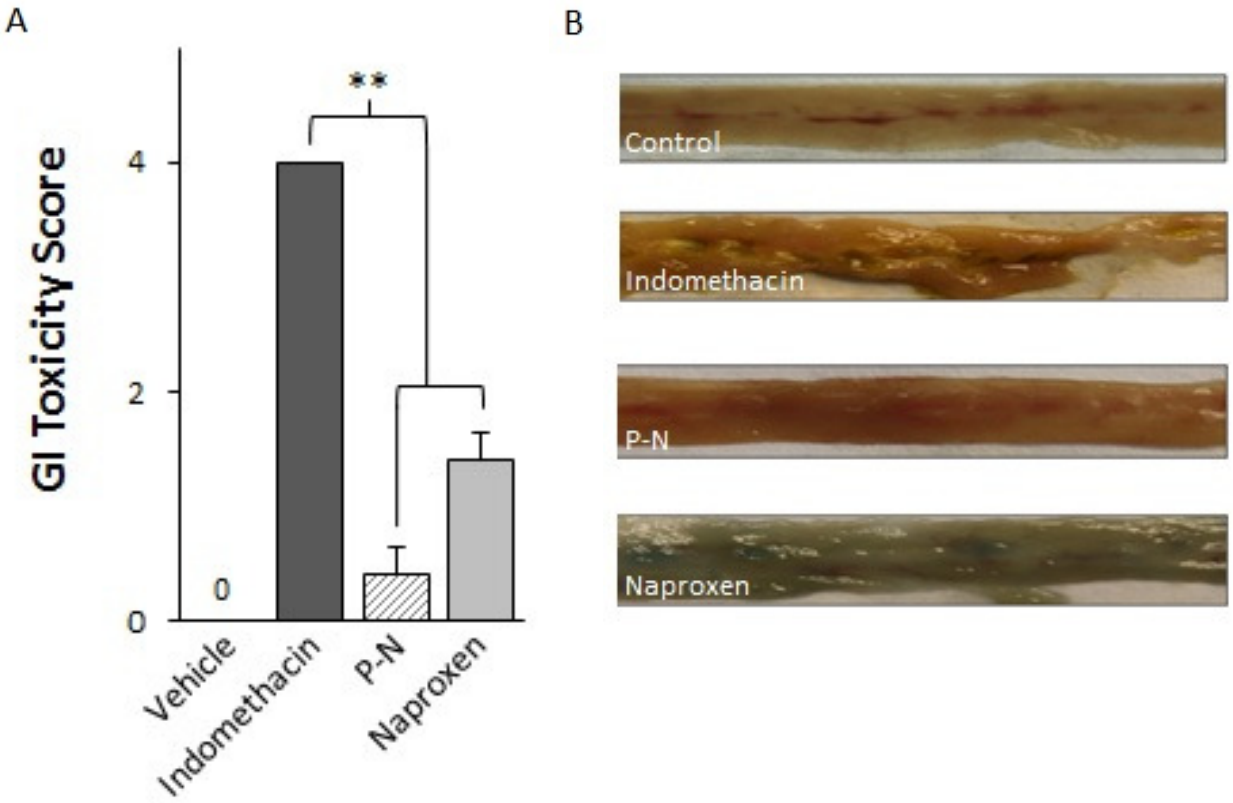

Figure 2-11: GI toxicity evaluation in normal rats: Control (n=2), Indo (Indomethacin 4.75mg/kg/d n=3), Naproxen 50mg/kg/d (n=5), or P-N (Phospho-naproxen 95 mg/kg/d; n=5). **(A)** Total mean gastrointestinal toxicity score. Values are mean + SEM on day 5 after 4 days of treatment. (*= $p<0.001$) **(B)** Images of the small bowel (mucosal surface) representing each study group.

DISCUSSION

Pharmacokinetics- Biodistribution- Metabolism

The pharmacokinetic analysis of P-N in mice indicates rapid ester hydrolysis and significant glucuronidation of naproxen, one of the hydrolysis products. Enzymatic ester hydrolysis of P-N by esterases has been confirmed in vitro and in cultured cells [193]. Such hydrolysis in vivo is entirely plausible in this case, a conclusion strengthened by observations of the metabolite products and by reports on other members of this class of compounds [193].

The pharmacokinetic analysis makes it clear that P-N is rapidly metabolized to naproxen; their respective T_{max} values only differ by 10 min. It is also clear from the nearly 40-fold higher values of the $AUC_{0\text{-}24h}$ of naproxen compared to P-N and the far lower $AUC_{0\text{-}24h}$ value of the glucuronide that there are additional P-N metabolites that have not been detected. This is consistent with the metabolic profile of P-N generated by the liver. The lack of appropriate authentic compounds has precluded direct assessment of the presence of such metabolites in the circulation; their apparent lipophilicity would suggest that they may be bound to proteins, even though their carboxylic ester bond makes them susceptible to enzymatic hydrolysis.

Glucuronidation is also a rapid process, evidenced by its T_{max} of 15 min. It is likely that P-N is first converted to naproxen, which then undergoes glucuronidation. As already mentioned, the liver is known to be the primary site for the glucuronidation of naproxen. The two methyl groups of the naproxen part of the P-N molecule are metabolized and the target of three reactions that we identified, demethylation, hydroxylation, and carboxylation.

These findings are supported by biodistribution data, which show significant abundance of naproxen over P-N in the liver, a great source of carboxylesterase 1 [197].

Thus it appears that phase I metabolism of P-N is followed by phase II metabolic changes that include naproxen. The major phase II metabolic pathway of NSAIDs in the human liver occurs by glucuronidation [198]. Glucuronosyltransferases, of the liver, conjugate acyl glucuronides to COOH groups to increase water solubility in preparation for their elimination [194]. Previous data demonstrated that naproxen, the hydrolyzed product of P-N, is glucuronidated by UDP-glucuronosyltransferases in the liver [195]. Therefore, it is likely that a major part of the hydrolysis of P-N to naproxen and the subsequent glucuronidation of naproxen occur in the liver [193, 195, 197]. In the case of P-N, naproxen undergoes demethylation at the same methyl group as P-N [199]. The detection of *o*-desmethyl-P-N, carboxyl-P-N, and hydroxyl-P-N confirms that various other dominant metabolites of P-N exist.

Of interest, as P-N is metabolized the lipophilicity of its metabolites is progressively reduced, with naproxen glucuronide having the lowest lipophilicity value, compared to the other metabolites. This is, in fact, expected, as the conjugation of glucuronide is a final step in the elimination of P-N through altered polarity [194, 195]. Interestingly the significant lipophilicity of P-N is maintained in its direct metabolites, *o*-desmethyl-P-N, carboxyl-P-N, and hydroxyl-P-N. This high level of lipophilicity perhaps facilitates their binding to hydrophobic sites on proteins and membranes [200].

Although, direct metabolites of P-N such as *o*-desmethyl-P-N, carboxyl-P-N, and hydroxyl-P-N were detected, it is evident that hydrolysis of P-N still occurs in abundance in rodent models. It is, however, important to keep in mind that in a clinical setting P-N given orally would have a higher propensity to remain intact in the GI tract [201] and the blood [202], where the activity of carboxylesterase is significantly lower than in rodents. Such reduction in P-N hydrolysis in humans would increase the bioavailability and biodistribution of P-N, likely increasing its efficacy.

It is clear from the totality of our data that P-N undergoes transformations aiming to both inactivate and eliminate it from the body. The corollary to these findings is that if a large fraction of the compound is needed to be delivered intact to a target site, then it ought to be protected from the action of carboxylesterases, a task that will be easier in humans with their low carboxylesterase activity. Methods to achieve this include, for example, its incorporation into liposomes or, when practically feasible, the co-administration of carboxylesterase inhibitors. Both approaches have given promising results with structurally similar members of this class of compounds [193, 203].

COX Enzymatic Activity

The inhibition of COX-1 and COX-2 plays a significant role in producing the adverse events associated with NSAID usage, of which GI, heart, and renal toxicities are the most prominent. [23-26, 28-32]. Gastric and duodenal ulcers occur at a very high rate; the cumulative incidence of gastric and duodenal ulcers for naproxen is 41% [43, 188, 189].

As already mentioned, the specific inhibition of COX-1 in the gut by NSAIDs plays a critical role in GI safety which can result in formation of ulcers or perforation of the GI, which can lead to bleeding and even death [35]. This effect is primarily due to inhibition of prostaglandin and prostacyclin synthesis by NSAIDs, which results in increased gastric acid secretion, diminished bicarbonate secretion, diminished mucus secretion, and reduced mucosal blood flow thereby making the GI lining highly susceptible to the acidic environment of the gut [35].

The heart is also susceptible to the adverse conditions imposed by NSAIDs, increasing the risk of adverse cardiovascular events by 35% versus non-NSAID users [36, 37]. Inhibition of COX-2 plays a significant role in the development of cardiac toxicity leading to an unrestricted shift in thromboxane activity. As noted previously, an increase in thromboxane results in increased vasoconstriction and platelet aggregation thereby increasing the probability of an adverse cardiovascular event [36]. This side effect of cardiovascular toxicity has resulted in the withdrawal of previously approved COX-2 specific inhibitors from the market [22].

Furthermore, renal dysfunction and renal failure are also associated with NSAID inhibition of COX-2 leading to increased sodium retention and hyperkalemia due to the inhibition of PGE_2 and PGI_2 whose normal function is to oppose vasoconstriction by angiotensin [38, 40]. Inhibition of COX-2 can also induce renal dysfunction which can lead to renal failure and subsequently increase the risk of death [38]. In the United States, 2.5 million people per year suffer from NSAID related renal events [39]. The inhibition of COX-2 by NSAIDs can result in increased sodium retention and hyperkalemia due to the inhibition of PGE_2 and PGI_2 whose normal function is to oppose vasoconstriction by angiotensin [38, 40].

Due to the significant side effects of NSAIDs by the inhibition of COX-1 and COX-2, it is plausible that the attenuation of this interaction may in fact be beneficial in increasing their potential safety while promoting their efficacy via COX-independent effects. The interaction of NSAIDs with COX-1 and COX-2 is largely dependent on their carboxyl group, which forms a salt bridge with the positively charged guanidinium moiety of arginine 120 of the COX-1 and COX-2 enzymes [154]. This interaction positively effects the binding affinity of NSAIDs to COX-1 and COX-2. The addition of the bulky phospho-group to the carboxylic end of NSAIDs may inhibit its interaction with COX-1 and COX-2 thereby increasing its safety.

In this regard, it was important to identify potential differences in the enzymatic inhibition of COX by P-N, which has the addition butane-diethylphosphate moiety to carboxyl group of naproxen. The half maximal inhibitory concentration of P-N for COX-1 and COX-2 was determined by an enzyme activity assay, which utilizes the inherent peroxidase activity of COX (responsible for the reduction of PGG_2 to PGH_2 in vivo) [16]. The enzymatic assay demonstrated that the addition of a butane-diethylphosphate moiety of P-N negatively affects its potential to inhibit COX-1 and COX-2. Specifically, the potency of P-N to inhibit COX-1 was 52 times less than that of naproxen. Furthermore, the potency of P-N to inhibit COX-2 was 230 times less than naproxen.

The inherent attenuation of COX-1 and COX-2 inhibition conferred by the butane-diethylphosphate moiety of P-N may have a potential positive effect on its safety profile compared to naproxen.

Safety

As noted previously, toxicity of NSAIDs play a significant factor in their ability to work effectively. To determine the broad safety of the compound, P-N, versus the parent form, naproxen, a study of dosage safety was conducted. This study also afforded the data required to determine a safe dosage paradigm for P-N and naproxen in the subsequent studies. The global benchmark for the determination of tolerant dose in the investigation of new compounds is represented by a 10% loss in body weight [196]. P-N was as safe as the vehicle, corn oil. In contrast, naproxen (the parent compound; given at equimolar doses to P-N), exhibited a rapid and significant loss in body weight by day 3, which reached the 10% toxicity threshold by day 5. These results demonstrate that P-N is significantly safer than the parent compound, naproxen. This stark difference in safety suggests P-N is significantly more tolerable than naproxen, thereby allowing for a significantly greater treatment dose of P-N than naproxen.

To determine if this significant decrease in body weight toxicity by naproxen was due to GI side effects, a specialized toxicity study to determine the GI health of investigational new compounds was conducted. This study included the use of a positive inducer of GI toxicity (indomethacin) and a well-established scoring system of the gastric system, which is clinically relevant [192].

As noted previously, NSAIDs are associated with several side effects; those afflicting the GI tract being the most common. NSAIDs have been associated with a 3 to 5 fold increase in risk of gastric ulceration, which can develop into perforation, excessive bleeding, and even death [35]. In fact, the most common and major side effect of naproxen is gastric toxicity leading to

significant gastric bleeding and ulceration. Gastric and duodenal ulcers occur at a very high rate; the cumulative incidence of gastric and duodenal ulcers for naproxen is 41% [43, 188, 189].

In this study, treatment with naproxen was associated with a significant 3.5-fold increase ($p < 0.05$) in GI toxicity compared to P-N. P-N was not only significantly safer that naproxen, but it also exhibited no significant signs of toxicity, compared to vehicle. These data confirm that a major target organ for toxicity by naproxen is the GI tract. Due to this inherent increase in safety by P-N, high dosage concentration and long-term treatment or prevention are feasible, which is necessitated by any chronic disease.

The resulting increase in safety can be explained in part by the alteration of the carboxylic region of P-N by the conjugation of a butane-diethylphosphate moiety. As seen previously, this moiety significantly reduces the ability of P-N to inhibit COX-1 and COX-2. The prostaglandins produced by COX play a pivotal role in regulating mucus secretion, bicarbonate secretion, mucosal blood flow, and epithelial cell proliferation and repair [35, 204]. P-N's inability to effectively inhibit COX may limit its ability to prevent prostaglandin synthesis in the GI, thereby significantly reducing its potential to induce GI toxicity. Overall, the reduced inhibition of COX-1/2 by P-N can permit maintenance of homeostatic production of prostaglandins in the GI system.

Furthermore, the reduction in GI toxicity by P-N compared to naproxen may also be related to epithelial absorption and the release of these compounds in gastric cells. Conventional NSAIDs with the carboxylic residue, such as naproxen, have been associated with the direct damage of

the epithelial cells of the GI tract [205]. This damage occurs when these conventional NSAIDs, with an exposed carboxylic end, enter the gastric epithelial cell and become ionized in the neutral intracellular environment [205]. Once ionized, their diffusion out of the cell is prevented, which results in significant accumulation [205]. This accumulation of ionized conventional NSAIDs in gastric epithelial cells promotes osmotic movement of water into the cell thus promoting cellular swelling and consequently lysis of gastric cells [205, 206].

Together, these data demonstrate that the conjugation of a butane-diethylphosphate moiety to carboxyl group of naproxen, to form P-N, promotes significant gastric safety over its parent compound, which can to a large extent be explained by two cardinal effects: a) its significantly reduced ability to inhibit COX; and b) the covalent modification of the carboxyl group of P-N [152, 153].

Chapter 3: P-N in Arthritis

To investigate the efficacy, safety, and mechanism of action of P-N in arthritis, animal models of RA were employed. Following a description of the methods used in this segment of the work, specific results are presented.

MATERIALS AND METHODS

Animal model of arthritis: Arthritis was induced using complete Freud's adjuvant (CFA) as described by Whiteley et al [207]. Female LEW/CrlBR Lewis rats 140-190g (Charles River Laboratories, Wilmington, MA) were housed with free access to food and water under a 12hr dark/ light cycle (lights on at 0700h) in a temperature (23±2°C) and humidity controlled room (55±15%). Rats were given a single 100 µl subcutaneous tail injection of *Mycobacterium butyricum* (DIFCO Laboratories; Detroit, MI) 10 mg/ml, suspended in incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). Animals were divided into the following groups (6-9 animals/group), and treated daily by oral gavage: 1) No adjuvant: corn oil 1ml/animals; 2) Vehicle + CFA: corn oil 1ml/animals; 3) Naproxen 35 mg/kg/d + CFA; 4) P-N (equimolar to naproxen) 66.5 mg/kg/d + CFA. Treatment was initiated on the day of CFA administration and continued for 18 days. Arthritis was evaluated every 3 days by an investigator blinded to the treatment protocol using a scoring system based on the intensity of the edema in the front and hind paws and tail [208]. Briefly, each paw was assigned a score of 0 to 4: 0 = no swelling or redness in any joint; 1-

3 = intermediate scores; 4 = very severe swelling and redness in large and small joints. In addition, the tail of each animal was assigned a score of 0 to 3: 0 = no signs of inflammation, even at the site of injection; 1-2 = intermediate scores; 3 = > 25% of the tail exhibiting edema and necrosis. The sum of the paw and tail scores of each animal represented the *total clinical arthritis score* (range 0-11) and represented as mean ± SEM for each treatment group. At the completion of the study, animals were euthanized, with CO_2, 1 h after the final dose of the test agent. The heart, liver, kidney, spleen and stomach were preserved in formalin for further analyses.

Paw weight: Hind paws were transected using a guillotine above the talocrural joint. Paws were photographed and weighed. Weights were represented as mean ± SEM for each treatment group.

Histological examination: Following 48 h in formalin at 10°C, the right hind paw from each arthritis study group was placed in decalcifier (Surgipath, Grayslake, IL) for 7 days. Once the decalcification was complete, the talocrural joint was transected along its longitudinal plane to give approximately equal halves and paraffin embedded. 4 µM-thick joint sections were stained with H&E. Sections were examined under an Olympus BX41 microscope (20×; Olympus, Center Valley, PA, USA) and photographed with an Olympus DP25 digital camera. A pathologist blinded to sample identity evaluated and scored sections for inflammation, bone resorption, and cartilage damage as previously described [209]. Briefly, inflammation was scored using a scale from 0-3: 0= normal, 1= mild infiltration of inflammatory cells in periarticular tissue, 2= moderate inflammation with moderate edema, 3= severe inflammation with severe edema. Bone resorption was scored using a scale from 0-5: 0 = normal; 1 = minimal (small areas of resorption

in distal tibial trabecular or cortical bone, not readily apparent on low magnification, rare osteoclasts); 2 = mild (numerous areas of resorption in distal tibial trabecular or cortical bone, not readily apparent on low magnification, numerous osteoclasts); 3 = moderate (obvious resorption of medullary trabecular and cortical bone without full thickness defects in cortex, loss of some medullary trabeculae, lesion apparent on low magnification, numerous osteoclasts); 4 = marked (full thickness defects in cortical bone, often with distortion of profile of remaining cortical surface, marked loss of medullary bone of distal tibia, numerous osteoclasts, no resorption in smaller tarsal bones); 5 = severe (full thickness defects in cortical bone, often with distortion of profile of remaining cortical surface, marked loss of medullary bone of distal tibia, numerous osteoclasts, resorption also present in smaller tarsal bones). Cartilage damage was scored from 0 to 5 according to the following criteria: 0 = normal; 1 = minimal (no obvious chondrocyte loss or collagen disruption); 2 = mild (focal mild chondrocyte loss and/or collagen disruption); 3 = moderate (multifocal moderate chondrocyte loss and/or collagen disruption); 4 = marked (multifocal marked chondrocyte loss and/or collagen disruption); 5 = severe (multifocal severe chondrocyte loss and/or collagen disruption).

GI toxicity in rats with arthritis: The same subgroups of animals were used from the arthritis efficacy study (see Methods- Arthritis Model). Briefly, these groups included: 1) No adjuvant: corn oil 1ml/animals; 2) Vehicle + CFA: corn oil 1ml/animals; 3) Naproxen 35 mg/kg/d + CFA; 4) P-N (equimolar to naproxen) 66.5 mg/kg/d + CFA. At the end of the 18 day treatment, animals were sacrificed and evaluated for GI toxicity. The scoring protocol used in the section of the study is detailed in the previous section.

Major organ toxicity*:* The heart, liver, kidney, stomach, and spleen were obtained from rats of the arthritis efficacy study. Organs were cut into 3 sections, equal in length, separating the anterior and posterior ends. Each section was paraffin embedded, 4 μm thick sections were stained with H&E, and examined for necrosis and degeneration of cells by a pathologist blinded to sample identity. Sections were examined under an Olympus BX41 microscope (20×; Olympus, Center Valley, PA, USA) and photographed with an Olympus DP25 digital camera.

Cytokine blood levels: Blood from animals of the arthritis study was collected in serum separating tubes (BD; Franklin Lakes, NJ) and allowed to clot. Samples were centrifuged at 1,200 x g for 10 minutes at 4C. The serum levels of IL-1β and IL-6 were assayed by enzyme-linked immunosorbent assay (Invitrogen; Carlsbad, CA), following the manufacturer's protocols. Briefly, a biotinylated antibody was utilized as a detection antibody. Detection antibody was then labeled with horseradish peroxidase (HRP) tagged with streptavidin. Colorimetric measurement of biotin bound streptavidin-HRP was determined by TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate. Chromogenic change was stopped at 30 minutes with sulfuric acid and measured at 450nm.

PGE_2 activity in blood plasma: Blood from animals of the arthritis study was collected in EDTA-treated vacutainers supplemented with 10 μM indomethacin, to prevent ex vivo formation of prostaglandins. Samples were centrifuged at 1,500 x g for 10 minutes at 4C. The very short half-life of PGE_2 in plasma required the measure of PGE_2 metabolites (13, 14-dihydro-15-keto PGE_2 and 13, 14-dihydro-15-keto PGA_2), which were converted into a stable PGE_2 derivative (sPGE), following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). The measure

of sPGE was determined by competitive enzyme-linked immunosorbent assay. Briefly, sPGE (extracted from plasma samples) are added with PGE_2 conjugated to acetylcholinesterase (PGE-Ach) to each well, both of which compete for a limited amount of pre-plated PGE_2 metabolite-specific rabbit anti-serum. In turn, increasing levels of sPGE results in less PGE-Ach binding, and vice-versa. PGE-Ach concentrations, inversely proportional to sPGE, are determined by addition of Ellman's reagent, which is converted to thiocholine by the acetylcholinesterase. The final product of the reaction results in 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm.

PGE_2 activity in talocrural joint tissue: Talocrural joint tissue from arthritis study was pulverized under cryogenic conditions maintained with liquid nitrogen. Tissue was homogenized with modified homogenization buffer (10mM Tris-HCl pH 7.5, 10mM EDTA, 0.5M NaCl, 0.1 Phosphate buffer pH 7.4), 10uM Indomethacin, and inhibitors PMSF, Leupeptin, Aprotinin, Pepstatin) on ice using 2mm Zirconia beads and Beadbeater (Bio Spec Products, Bartlesville, OK). Samples were frozen at -80°C overnight until further analysis. Samples were thawed at 4°C and spun for 10 min at 4°C. PGE_2 concentration of supernatant was determined by competitive enzyme-linked immunosorbent assay, according to manufacturer directions (Cayman Chemical, Ann Arbor, MI, USA), and normalized to protein concentration.

COX-1/2 expression: Immunohistochemical staining for cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) was performed on joint tissue samples. Briefly, paraffin-embedded sections (4 µm thick) were deparaffinized, rehydrated, and microwave-heated for 15 min in 0.01

mol/L citric buffer (pH 6.0) for antigen retrieval. Then, 3% hydrogen peroxide was applied to block endogenous peroxidase activity. After 30 min of blocking with normal serum (Invitrogen, Carlsbad, CA), rabbit anti-COX-1 antibody (Cayman Chemical, Ann Arbor, MI) or rabbit anti-COX-2 polyclonal antibody (Cayman Chemical, Ann Arbor, MI) and the corresponding control isotype IgG were applied and incubated overnight at 4°C. Slides were washed thrice with PBS, each for 5 min. The biotinylated secondary antibody and the streptavidin-biotin complex were applied, each for a 60 min incubation at room temperature. After rinsing with PBS, the slides were immersed for 10 min in 3,3'-diaminobenzidine (Sigma, St. Louis, MO) solution (0.4 mg/mL, with 0.003% hydrogen peroxide), and monitored under the microscope. The reaction was terminated with distilled water. Slides were then counterstained with hematoxylin, dehydrated, and cover-slipped. Five fields per section were photographed and the percentage of positive cells in was determined. The intensity of cytoplasmic staining of COX-1/2 was assessed semi-quantitatively as previously described [210]. *Scoring system:* negative: no staining or <10% positive cells; 1+: weak staining or 10–25% positive cells; 2+: moderate staining or 25–50% positive cells; 3+: strong staining or >50% positive cells.

Synovial NF-κB activation: NF-κB activation was evaluated in the synovium of the talocrural joint animals using an antibody to the phospho-p65 (Ser276) chain of activated NF-κB. Paraffin-embedded joints were deparaffinized, rehydrated, and microwave-heated for 15 min in 0.01 mol/L citrate buffer (pH 6.0) for antigen retrieval. H_2O_2 3% was applied to block endogenous peroxidase activity. After 15 min of blocking with normal serum (Invitrogen, Carlsbad, CA), the primary rabbit anti-phospho-NF-κB p65 ser276 antibody (Cell Signaling Technology, Beverly, MA)

or control isotype IgG (dilution 1:50) was applied and incubated overnight at 4°C. Slides were washed three times with PBS, each for 5 min. The biotinylated secondary antibody and the streptavidin-biotin complex (Vector Laboratories, Burlingame, CA) were applied, each for 30 min at room temperature with interval washing. After rinsing with PBS, the slides were immersed for 5 min in the coloring substrate 3, 3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) solution (0.4 mg/mL, with 0.003% hydrogen peroxide), and monitored under the microscope. The reaction was terminated with distilled water. Slides were then counterstained with hematoxylin. Tissue sections were examined using an Olympus BX41 microscope (40×) and photographed with an Olympus DP25 digital camera. Five different fields per slide from synovium were counted and scored; positive staining appears brown. Results were scored from 0 to 4 based on the percentage of positive cells among total synovial cells. *Scoring system:* negative: no staining or <10% positive cells; 1+: weak staining or 10–25% positive cells; 2+: moderate staining or 25–40% positive cells; 3+: moderate to strong or 40-60% positive cells; 4+: strong staining or >60% positive cells

RESULTS

Efficacy of P-N in rats with experimental arthritis

To evaluate the anti-inflammatory effect of P-N, the adjuvant-induced model of RA was utilized, which closely mimics the pathology of human RA [209]. Animals with arthritis were treated daily for 18 days with vehicle, naproxen or P-N, the last two at equimolar doses. The progression of arthritis was closely monitored every 3 days using a validated scoring system which incorporates the macroscopic features of arthritis (redness, swelling, edema, and necrosis at the joints and tail) [207].

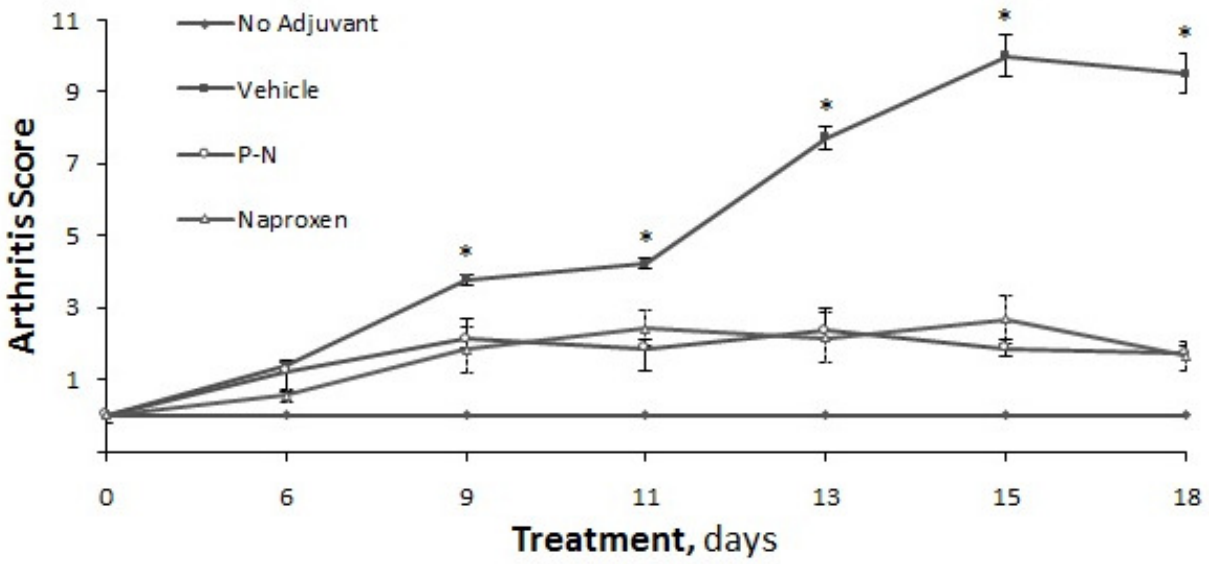

Figure 3-1: The total mean arthritic score for each treatment group over time. No Adjuvant; (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7) or Naproxen (35mg/kg/d; n=6).Values are mean + SEM over time (Day 0 to Day 18). (*p<0.01)

Compared to the vehicle-treated control group, P-N significantly reduced the arthritis score by day 9 of treatment ($p<0.01$; Figure 3-1). This significant reduction in arthritis score was maintained to day 18, which marked the conclusion of the live animal study. More specifically, at the end of the study period, on day 18, arthritis score was 9.5±0.55 (mean ± SEM for this and all subsequent values) for the vehicle-treated control group and 1.71±0.2 for the P-N group, represented by an 82% reduction ($p<0.001$). Naproxen generated essentially identical results (Figure 3-2).

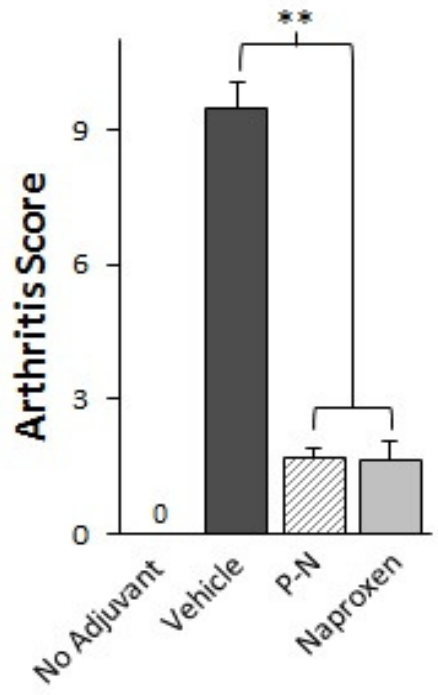

Figure 3-2: The total mean arthritic score for each treatment group at end of study. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7) or Naproxen (35mg/kg/d; n=6).Values are mean + SEM on day 18. (**p<0.001)

Paw weight in arthritis model

The paws of each animals group were transected at the end of the study period and weighed as a relative measure of swelling and edema. In addition to the significant reduction in the arthritis score, P-N reduced the hind paw weight of the rats by 30% (score: 1.09±0.01; $p<0.001$) compared to the vehicle control (Figure 3-3). Of note, the hind paw weights of the P-N treated group were virtually identical to those of the negative (no adjuvant) control group (score: 1.11±0.02; $p>0.05$), indicating normalization of the affected joints in this regard. Naproxen generated similar results (score: 1.14±0.02; $p>0.05$), although slightly higher.

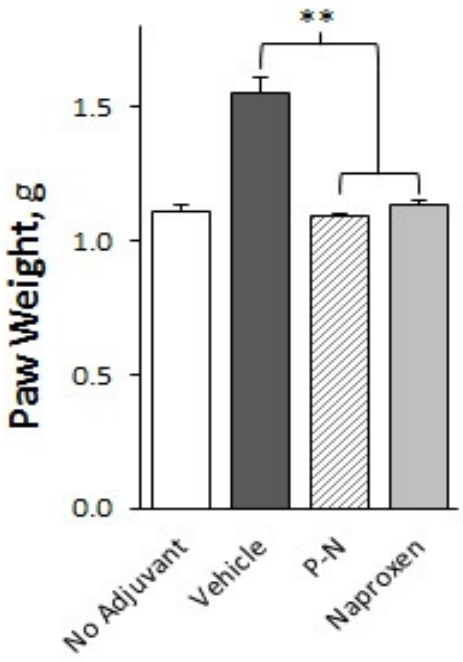

Figure 3-3: Hind paw weight from animals representing each treatment group. No Adjuvant; (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7) or Naproxen (35mg/kg/d; n=6). Values are mean + SEM on day 18. (**p<0.001)

Visual inspection of paw and joint

As noted previously, patients with RA suffer from significant swelling, pain, edema, and stiffness at the joints. Untreated and treated animals at the end of the 18 day study were grossly examined at the paws and joint, after segmental resection. As expected in the untreated arthiritic animals (Veh), displayed significant joint inflammation and edema at the talocrural joint including the surrounding area of the paw pad and digits, Figure 3-4.

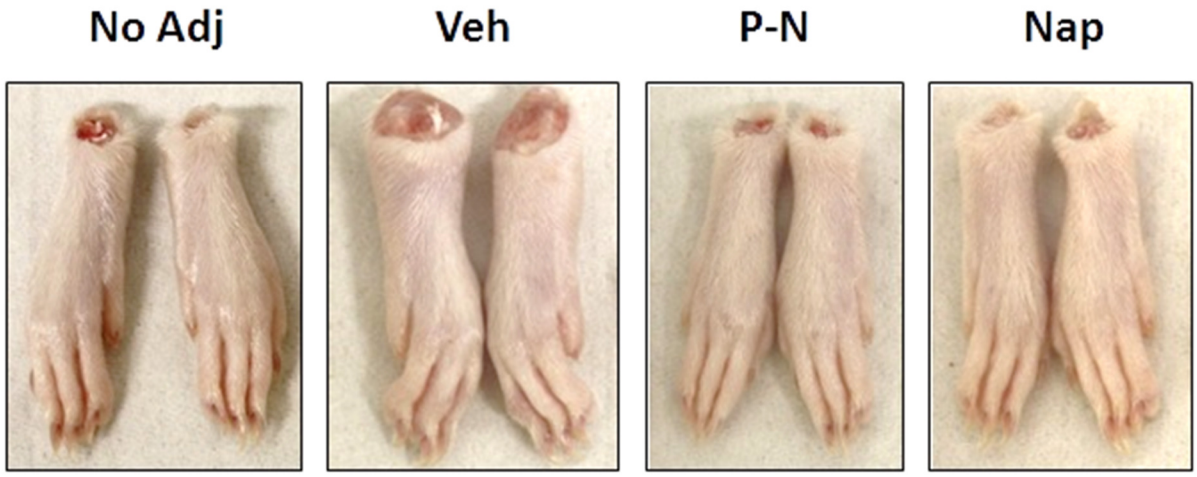

Figure 3-4: Photograph representation of the talocrural joint in hind paws of one animal representative of each treatment group on day 18. No Adj (No Adjuvant; n=6), Veh (Vehicle + Adjuvant; n=9), P-N (phospho-Naproxen 66.5 mg/kg/d; n=7) or Nap (naproxen 35mg/kg/d; n=6).

Arthritic animals treated with P-N, showed significant reductions in joint inflammation and edema, compared to untreated arthritic animals. In fact, paws of animals treated with P-N were indistinguishable from animals without arthritis. Some naproxen treated arthritic animals showed some slightly more swelling than P-N treated animals.

Histology of the talocrural joint

Closer study of the talocrural joint was completed by microscopic examination of joint sections stained with hematoxylin and eosin.

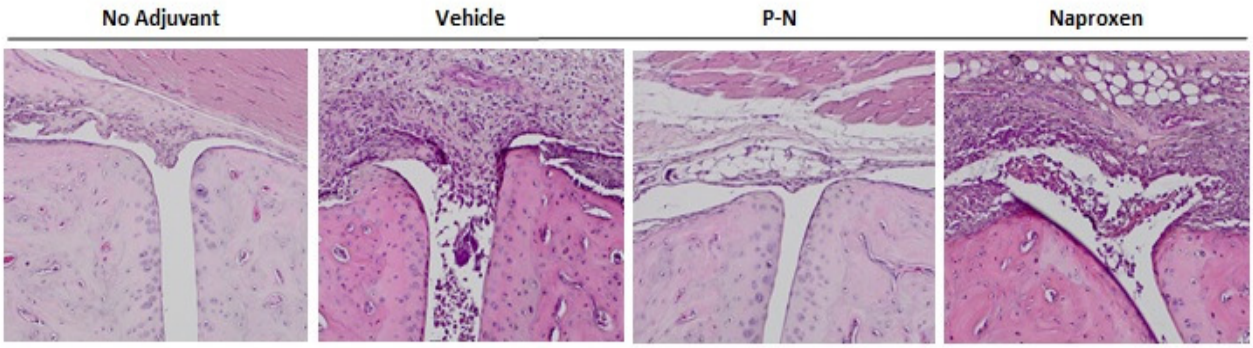

Figure 3-5: Histological representation of the talocrural joint on day 18. No Adjuvant (n=6), Vehicle (n=9), P-N (Phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6).

As expected, histological examination of the talocrural joint of arthritic animals revealed significant inflammatory cell infiltration accompanied by synovitis, pannus formation, and edema (Figure 3-5). These symptoms were normalized in animals treated with P-N. Although naproxen treated animals showed similar reductions, a moderate increase in inflammatory cell infiltration into the joint space was observed in some animals.

Inflammation of the talocrural joint

In order to evaluate all the animals in each group, a robust and validated scoring system was used to account for the infiltration of inflammatory cells in periarticular tissue as well as edema of the joint.

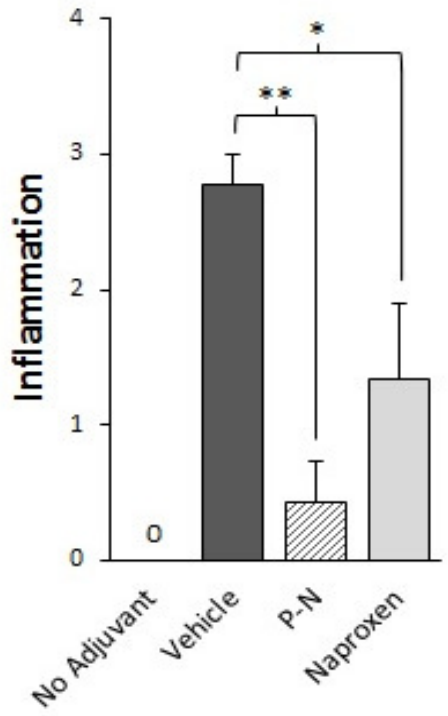

Figure 3-6: Inflammation score in the talocrural joint. No Adjuvant (n=6), Vehicle (n=9), P-N (Phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean ± SEM on day 18. (*p<0.05; **p<0.0001)

P-N significantly reduced inflammation in the talocrural joint by 85% (score: 0.43±0.30), compared to the vehicle control (score: 2.77±0.22; p<0.0001), Figure 3-6. This reduction was also evident in naproxen treated animals; however, these animals exhibited only a 52% (1.33±0.56) reduction in inflammation compared to the vehicle control (p<0.05).

Bone resorption of the talocrural joint

Bone loss is common in patients with RA. Bone resorption in RA is mainly attributable to the abnormal activation of osteoclasts. In this study, bone resorption was scored based on a validated scoring system, which accounts for the amount to bone resorption and the number of osteoclasts.

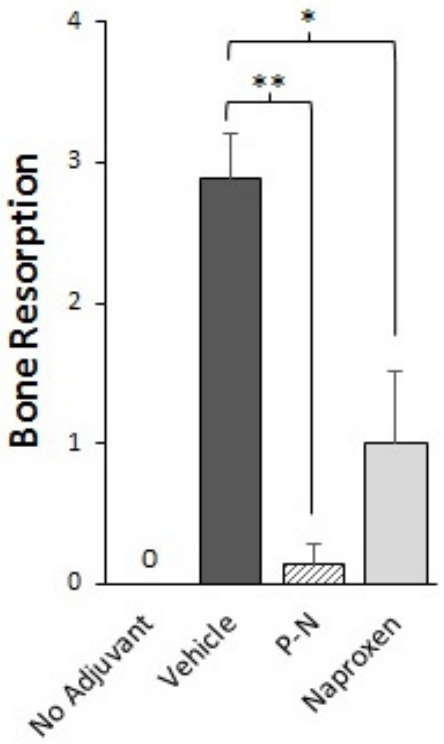

Figure 3-7: Bone resorption score in the talocrural joint. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean ± SEM on day 18. (*p<0.05; **p<0.0001)

As expected, untreated arthritic animals (Veh) exhibited moderate bone resorption (score: 2.89± 0.31), whereas healthy non-arthritic animals exhibited normal bone structure (score: 0), Figure 3-7. P-N treated animals was significantly reduced by 95% (score: 0.14±0.14; p<0.0001),

compared to the vehicle control (score: 2.89± 0.31), essentially normalizing bone resorption to normal levels. In contrast, naproxen treated animals exhibited only a 65% (score: 1.00±0.52; $p<0.05$) reduction, compared to the vehicle control (score: 2.89± 0.31).

Cartilage damage of the talocrural joint

Another feature of RA is cartilage damage, which occurs during the later stages of RA. The loss of chondrocytes in RA is associated with the narrowing of the joint space, which was not observed by histological examination.

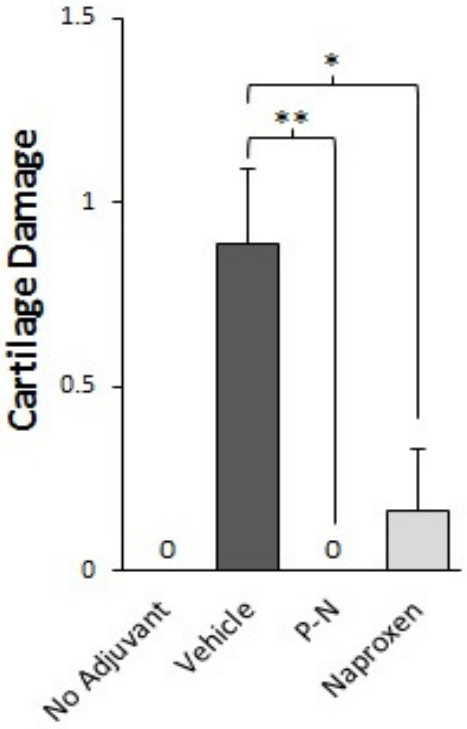

Figure 3-8: Cartilage damage score in the talocrural joint. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean ± SEM on day 18. (*p<0.05; **p<0.01)

Overall, cartilage damage score for P-N treated animals resulted in a 100% reduction (score: 0; $p<0.01$), compared to the vehicle control (score: 0.89±0.2) (Figure 3-8). Naproxen demonstrated an 81% (score: 0.17±0.17; $p<0.05$) reduction, compared to the vehicle control (score: 0.89±0.2).

Although these differences were significant compared to untreated arthritic animals, it is important to note that the overall score of cartilage damage for all groups was less than 1, which is represented by normal to minimal cartilage damage. Given the 18 day study period, it is likely that cartilage damage during this stage of RA had not significantly progressed.

GI toxicity in arthritic rats

As noted previously, at the recommended dose for rheumatoid arthritis, naproxen has been associated with a significantly high rate of gastric and duodenal ulcers [43, 188, 189]. The cumulative incidence of gastric and duodenal ulcers for naproxen is 41% [188, 189].

In this study, the average clinical score of GI toxicity was determined by a validated scoring system which accounts for the number of ulcerations, size of ulcerations, and overall integrity of the intestinal tract.

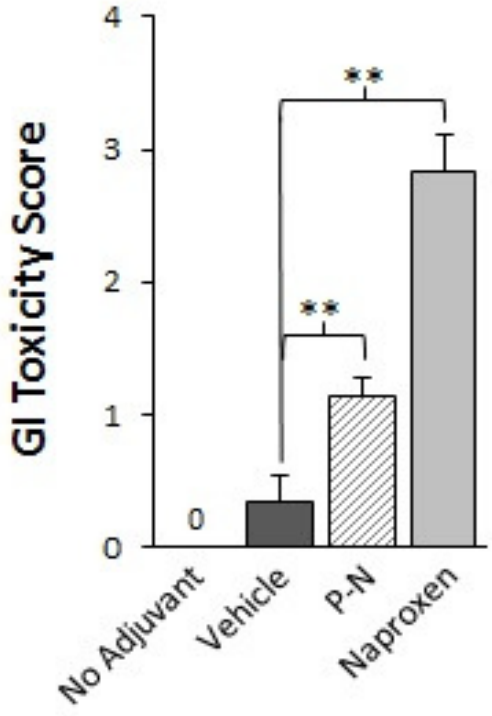

Figure 3-9: Gastrointestinal toxicity scores for each treatment group. No adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or naproxen (35mg/kg/d; n=6). Values are mean ± SEM on day 18. (**p<0.001)

The GI toxicity score of the vehicle-treated arthritis group exhibited essentially no mucosal damage (score: 0.33±0.2), with their score being slightly higher than the healthy, no adjuvant, group (score: 0; p>0.05) (Figures 3-9). GI mucosal damage in naproxen treated animals (score: 2.83±0.28) was significantly greater, associated with a 2.5-fold increase, than P-N treated animals (score: 1.14±0.14; p<0.001).

Histological analysis of major organs

As noted previously, naproxen can induce significant GI toxicity. To further investigate the potential GI safety of P-N over the conventional compound naproxen, during the treatment of RA, resected stomachs from each treatment group were sectioned and examined microscopically for signs of drug induced damage.

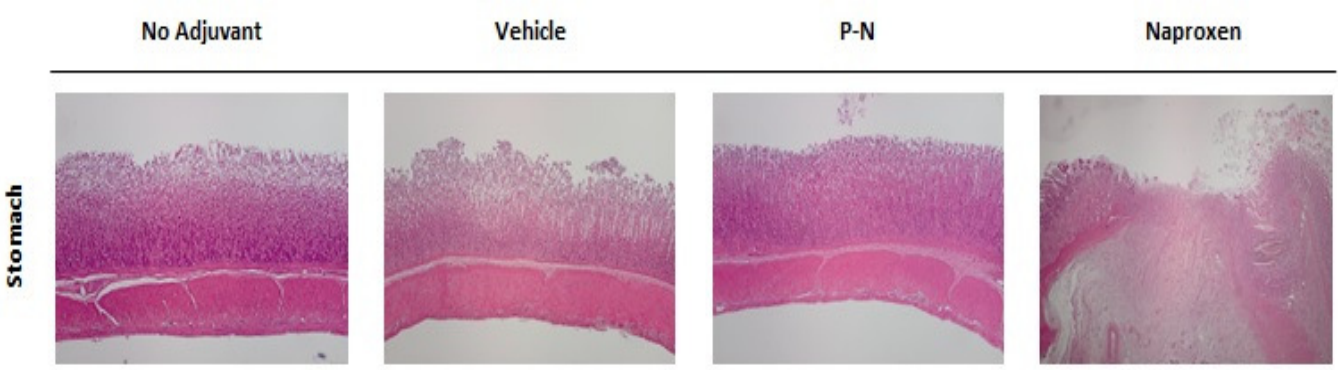

Figure 3-10: Representative sections of the stomach gastric mucosa of each treatment group after 18 days of treatment. One animal from each treatment group. No adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or naproxen (35mg/kg/d; n=6).

The gastric mucosa of the stomach in the vehicle-treated arthritis group and in the healthy, no adjuvant group was normal. Normal mucosa was also present in arthritic animals treated with P-N. In stark contrast, arthritic animals treated with naproxen showed mucosal ulcerations, some of them penetrating (Figure 3-10).

As for any new drug the heart, kidney, liver, and spleen which are all susceptible to NSAID toxicity were also examined microscopically.

These data showed no significant degeneration of cardiomyocytes, renal tubulin epithelial cells, hepatocytes, and splenocytes (Figure 3-11).

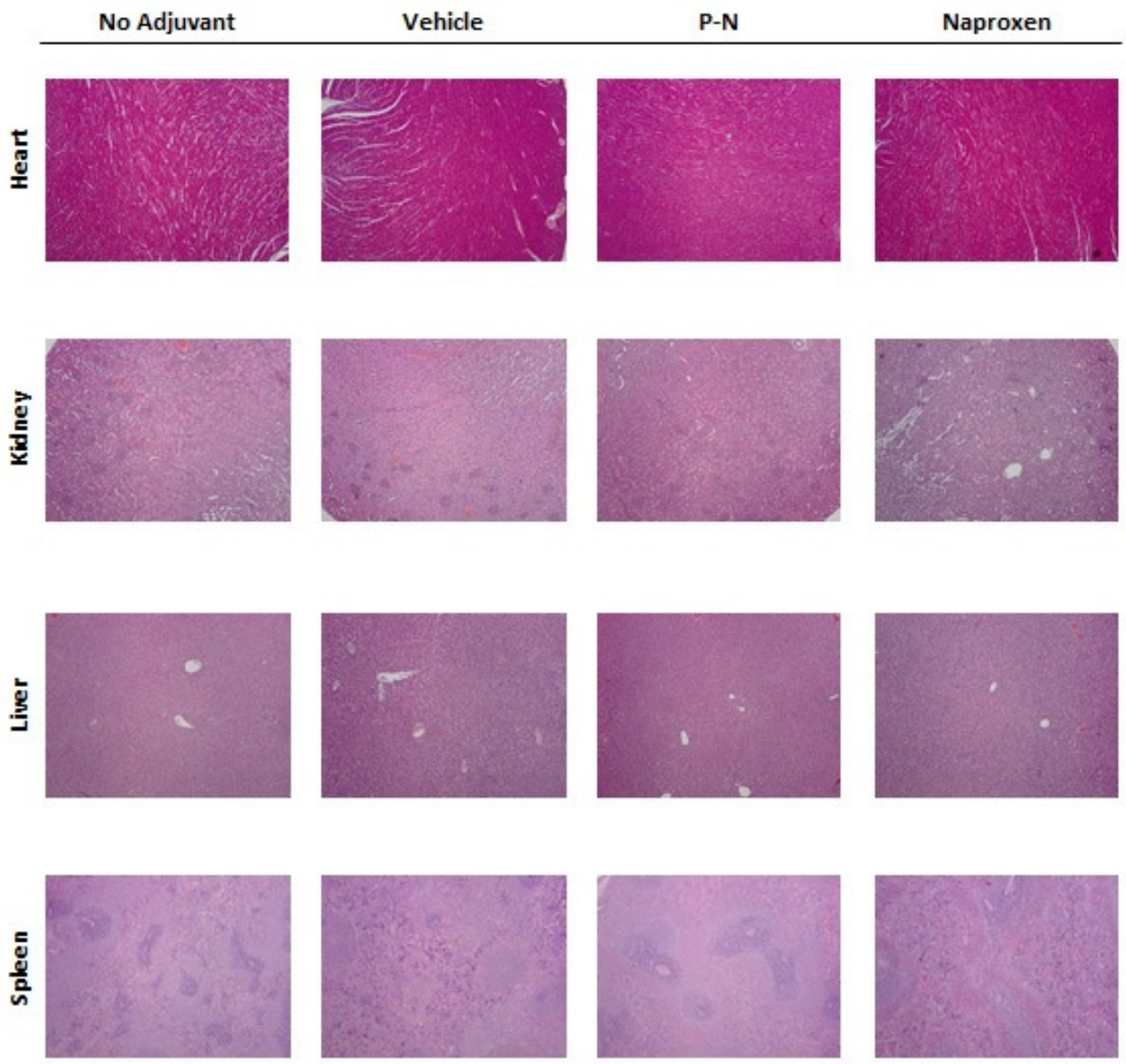

Figure 3-11: Sections of the heart, kidney, liver, spleen after 18 days of treatment. One animal from each treatment group. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or naproxen (35mg/kg/d; n=6).

Inflammatory cytokines

As previously noted, pro-inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-1 Beta (IL-1β), play a significant role in the onset and progression of RA. Levels of each of these pro-inflammatory cytokines were determined in each treatment group.

Interleukin-6

Pro-inflammatory IL-6 levels were determined in treatment groups by enzyme-linked immunosorbent assay. CFA treatment alone (Vehicle) significantly increased IL-6 serum levels from 83.56±2.28 pg/ml in animals without arthritis (No Adjuvant) to 106.40±6.73 pg/ml, which was representative of 27% ($p<0.01$) increase (Figure 3-12A). Treatment with P-N significantly reduced IL-6 levels to 84.16±1.62 pg/ml, which was represented by a 21% ($p<0.01$) reduction in IL-6 levels, compared to the vehicle treated arthritis group. Naproxen-treated animals also showed similar results, reducing IL-6 to 78.13±2.06 pg/ml, which was represented by a 26% ($p<0.01$) reduction in IL-6 levels, compared to the vehicle treated arthritis group.

Together, P-N and naproxen restored IL-6 production levels to normal levels seen in healthy non-adjuvant animals.

Interleukin-1β

Pro-inflammatory IL-1β levels were determined in treatment groups by enzyme-linked immunosorbent assay. The levels of the pro-inflammatory cytokine IL-1β significantly increased from 43.44±1.10 pg/ml in the no adjuvant animals to 51.75±3.08 pg/ml in vehicle treated arthritic

animals, which was representative of a 19% ($p<0.05$) increase (Figure 3-12B). Treatment with P-N significantly reduced IL-1β levels to 37.46±1.03 pg/ml, which was represented by 28% ($p<0.01$) reduction in IL-1β levels, compared to the vehicle treated arthritis group. Naproxen treated animals also showed similar results, reducing IL-1β to 39.62±1.03 pg/ml, which was represented by a 23% ($p<0.01$) reduction in IL-1β levels, compared to the vehicle treated arthritis group.

Together, P-N and naproxen restored IL-1β production levels to normal levels seen in healthy non-adjuvant animals.

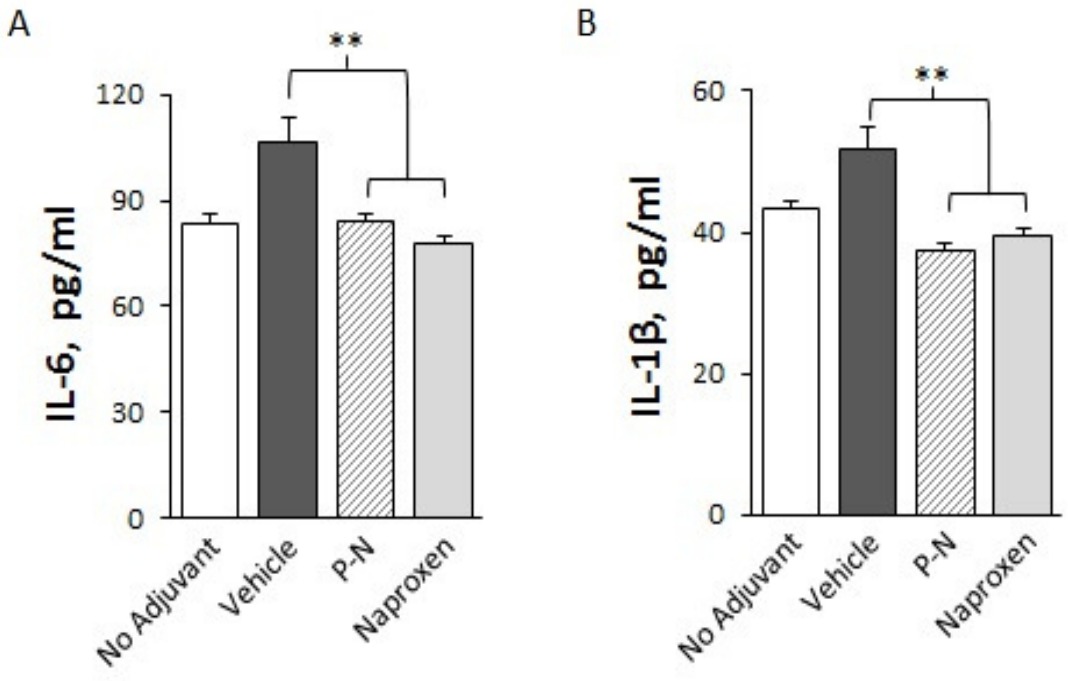

Figure 3-12: Blood serum cytokine expression in each treatment group. (A) Interleukin-6, **(B)** Interleukin-1 Beta. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean ± SEM on day 18. (** $p<0.01$)

PGE_2 metabolite in blood plasma

PGE_2 is a major pro-inflammatory eicosanoid in RA, which exhibits a host of pleiotropic pro-inflammatory actions.

Plasma levels of the most prominent metabolite of PGE_2, which reflect PGE_2 production in an animal over a significant period of time (e.g., hours), was determined. PGE_2 metabolite plasma levels increased from 87.18±9.61 pg/ml in healthy non-adjuvant treated animals to 172.09±10.97

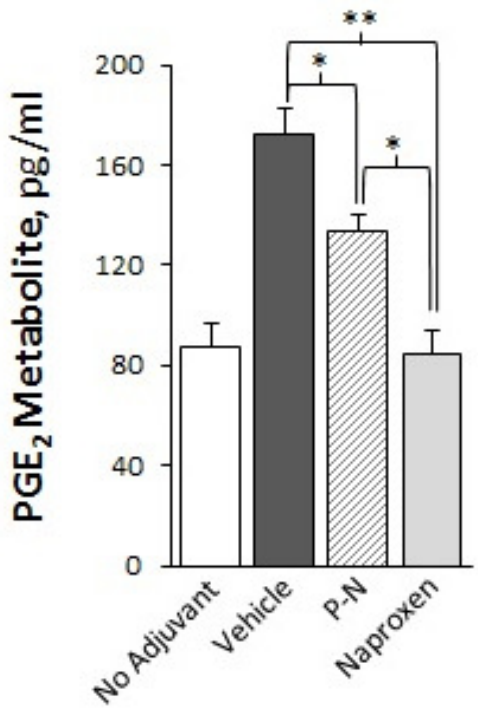

Figure 3-13: PGE_2 levels in rat plasma for each treatment group. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean ± SEM on day 18. (*p<0.01, **p<0.001)

pg/ml in vehicle treated arthritic animals, which was representative of a 97% (p<0.001) increase (Figure 3-13). Treatment with P-N significantly reduced PGE_2 blood plasma metabolites levels to

133.34±6.52 pg/ml, which was represented by 23% ($p<0.01$) reduction in PGE_2 metabolite levels, compared to vehicle treated arthritic animals. This reduction in PGE_2 metabolite levels was significantly greater in naproxen treated animals (84.5±9.33 pg/ml), which was represented by a 51% ($p<0.001$) reduction, compared to vehicle treated arthritic animals. Naproxen essentially reduced blood plasma levels to basal levels seen in healthy non-adjuvant animals. In comparison to P-N, levels of PGE_2 metabolite in naproxen treated arthritic animals was 37% ($p<0.01$) less than that of P-N treated arthritic animals.

Together, naproxen was more effective in reducing PGE_2 metabolite levels in the blood than P-N.

PGE_2 in talocrural joint tissue

PGE_2 levels in the affected joint tissue was also determined. As expected, PGE_2 levels in joint tissue increased from 11.17±1.74 pg/ml in healthy non-adjuvant animals to 42.70±8.40 pg/ml vehicle treated arthritic animals, which was representative of a 282% ($p<0.05$) increase (Figure 3-14).

Interestingly, treatment with P-N significantly reduced PGE_2 levels in joint tissue to 16.55±1.8 pg/ml, which was representative of a 61% ($p<0.05$) reduction in PGE_2 levels, compared to vehicle-treated arthritic animals (an effect than was not observed in the blood). Moreover, naproxen also

reduced PGE_2 levels in joint tissue to 17.25±3.73 pg/ml, which was represented by 60% ($p<0.05$) reduction in PGE_2 levels, compared to vehicle treated arthritic animals.

Together, P-N and naproxen reduced PGE_2 levels in joint tissue, close to those observed in healthy non-adjuvant animals.

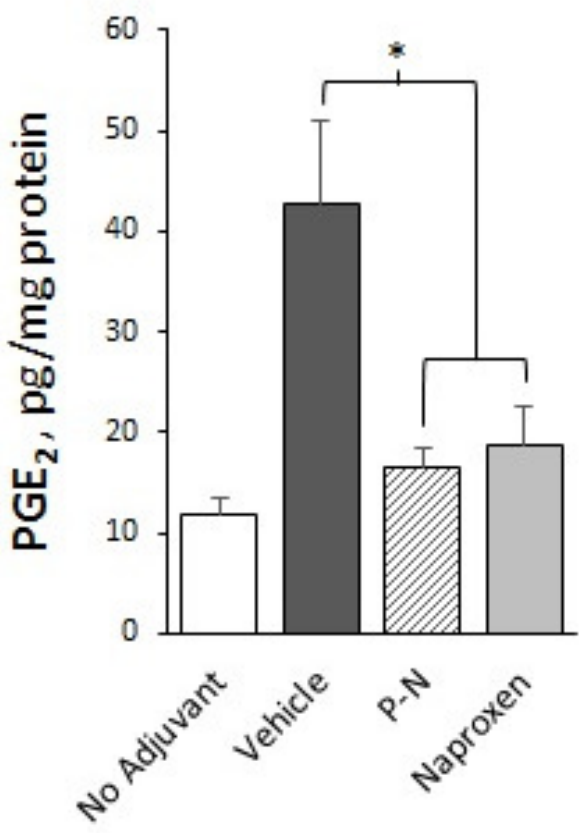

Figure 3-14: PGE_2 levels in affected joint tissue for each treatment group. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean + SEM on day 18. (*$p<0.05$)

Expression of COX-1 and COX-2

High levels of COX, the enzyme which catalyzes the formation of PGE_2, is commonly observed in high levels in patients with RA.

COX-1 expression in the synovial endothelial tissue of all groups, did not change significantly compared to healthy non-arthritic animals (score: range from 2.7 to 3.0; $p>0.05$) (Figure 3-15A). This finding is in keeping with the role of COX-1 constitutive expression, not affected by inflammation.

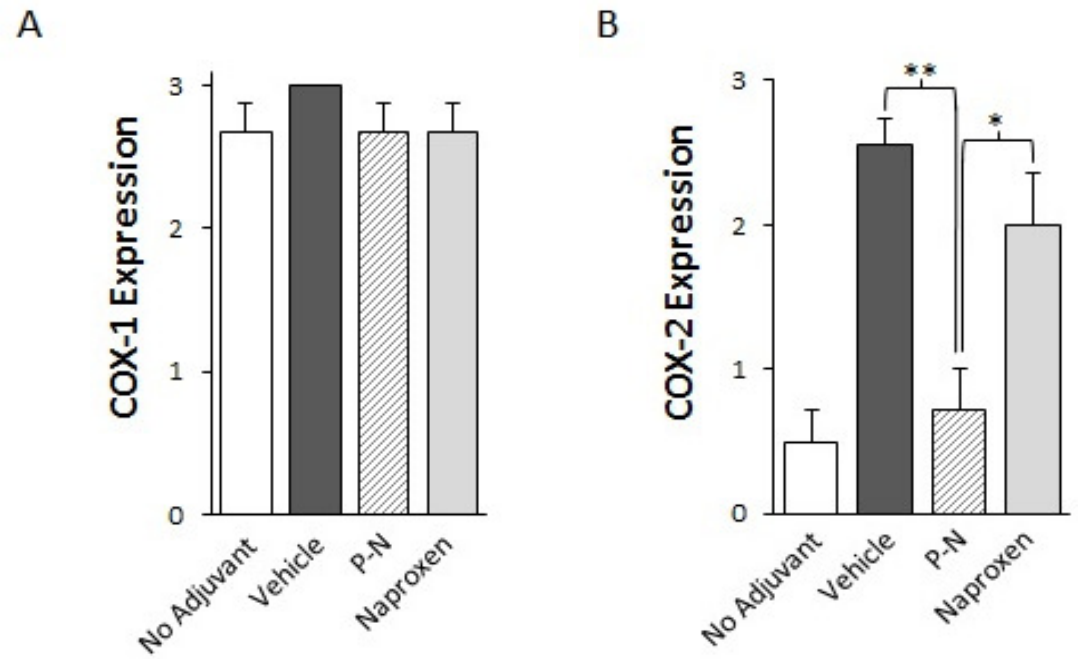

Figure 3-15: Synovial endothelium COX expression in each treatment group. (A) COX-1, **(B)** COX-2. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean ± SEM on day 18. (**p<0.001) (*p<0.01)

As shown in Figures 3-15B and 3-16, the induction of arthritis in vehicle treated animals (score: 2.6±0.18) was accompanied by marked (5.2-fold) overexpression of COX-2 versus the healthy non-adjuvant group (score: 0.5±0.22) in the synovial endothelial tissue of the talocrural joint. P-N significantly reduced the overexpression of COX-2 in vehicle treated animals by 72% (score: 0.7±0.29; $p<0.001$). In contrast, naproxen reduced COX-2 expression by only 22% (score: 2.0±0.37; $p>0.10$), an effect that was not statistically significant. Overall, P-N was significantly effective in reducing COX-2 expression in arthritic animals, bringing COX-2 expression levels close to those observed in healthy non arthritic animals.

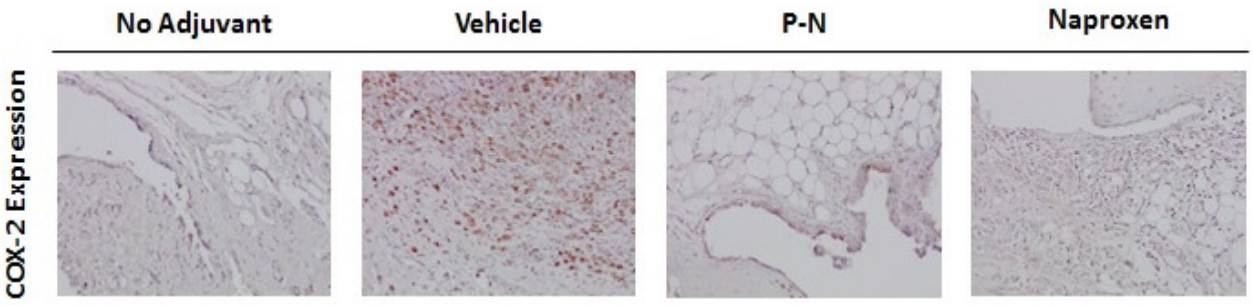

Figure 3-16: Representative immunohistochemistry of COX-2 expression in the synovial endothelium of the talocrural joint on day 18: No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6).

Overall, P-N was significantly effective in reducing COX-2 expression in arthritic animals, bringing COX-2 expression levels close to those observed in healthy non arthritic animals.

NF-κB activation in synovial joint tissue

NF-κB activation in joint tissue of vehicle-treated arthritic animals was increased by 94% ($p<0.0001$, score: 2.8±0.22) compared to healthy non-adjuvant animals (score: 0.17±0.07; Figures 3-17 and 3-18). P-N (score: 0.38±0.17) suppressed NF-κB activation by 87% versus the vehicle-treated arthritic group ($p<0.001$). This was in contrast to the rather limited effect of naproxen (score: 1.20±0.42) which was associated with a 58% inhibition in NF-κB activation versus the vehicle-treated arthritis group ($p<0.01$). P-N was associated with 69% ($p<0.05$) greater inhibition of NF-κB in activation in joint tissue than naproxen.

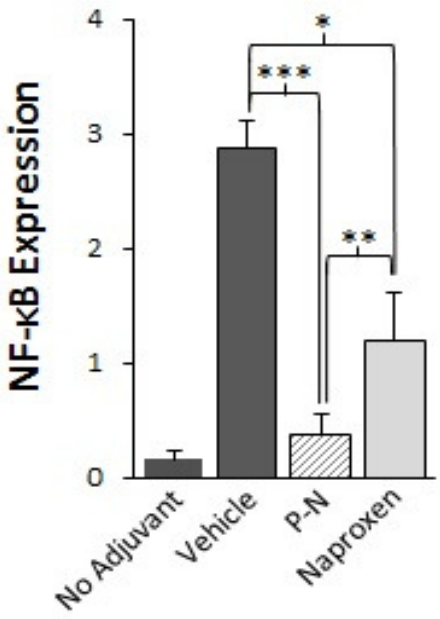

Figure 3-17: Synovial NF-κB activation in each treatment group. No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6). Values are mean + SEM on day 18. (*$p<0.01$) (**$p<0.05$) (***$p<0.001$)

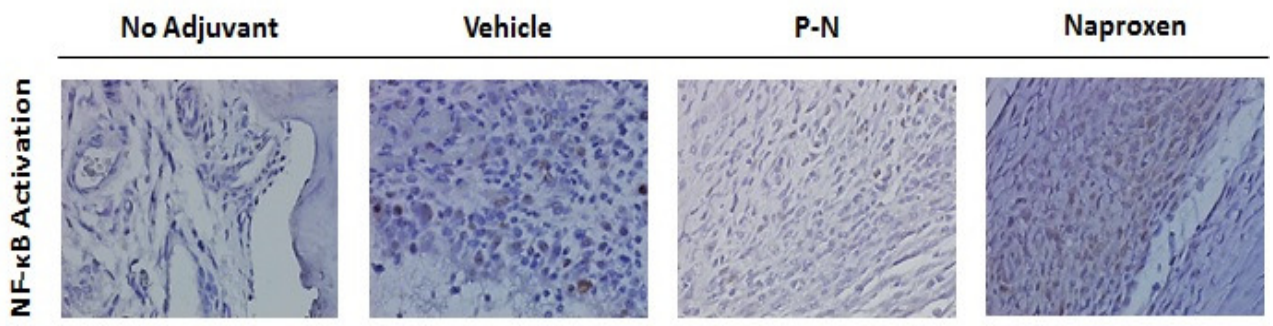

Figure 3-18: Representative immunohistochemistry of NF-κB activation in the synovial endothelium of the talocrural joint on day 18: No Adjuvant (n=6), Vehicle (n=9), P-N (phospho-naproxen 66.5 mg/kg/d; n=7), or Naproxen (35mg/kg/d; n=6).

Overall, P-N suppressed the relative activation of NF-κB induced by adjuvant in vehicle treated arthritic animals by 92% ($p<0.05$), whereas naproxen reduced the relative activation by only 62% ($p<0.05$).

DISCUSSION

Efficacy in Arthritis

To evaluate the efficacy of P-N, animal's representative of RA in a clinical setting was required. In vivo arthritis models have been previously developed using the immunopotentiator- complete Freund's adjuvant (CFA; emulsified solution of *Mycobacterium butyricum*), which closely represents RA in humans [211]. This model provides reliable onset and progression of robust articular inflammation, which is measurable in a live rodent, permitting data collection during disease progression [212]. In this model of RA, treatment with P-N was observed to be significantly effective in reducing the development of arthritic symptoms such as redness, swelling, edema, and necrosis at the joints and tail. Treatment with P-N significantly reduced arthritis scoring by 82%, compared to untreated arthritis animals.

As noted previously, arthritis inflammation is also accompanied by significant swelling of the extremities, which is represented by an increase in paw weight [209]. Treatment with P-N significantly reduced hind paw weight by 30%, compared to untreated arthritis animals. In fact, P-N essentially normalized swelling, showing no significant difference from healthy animals without arthritis. This reduction of swelling was also confirmed by visual inspection of the talocrural joint, post resection. The edema of the talocrural joint in arthritic animals was prevented with P-N treatment, showing no significant difference compared to healthy animals. Interestingly, the observed visual swelling of the joint was marginally more in animals treated

with naproxen than P-N, although this effect was not significant. To effectively determine, potential internal effect of P-N treatment, further examination was required.

Swelling of the joint in RA is associated with excessive fluid retention and edema due to synovial inflammation, known as synovitis [207]. Furthermore, during the progression of RA within the joint, various features manifest such as: infiltration of inflammatory cells, pannus formation, bone resorption, and cartilage degradation, all hallmarks of RA [212-214].

As expected, in-depth histological analysis demonstrated that animal models of RA, were presented with significant infiltration of inflammatory cells and synovial thickening, which was accompanied by a significant reduction of the free joint space. This increase was also confirmed by 85% reduction of the inflammation score, which showed significant infiltration of inflammatory cells, edema and pannus formation.

Osteoclast differentiation and survival is a significant factor associated with RA. In RA, altered rates of osteoclast precursor differentiation and apoptosis are responsible for bone destruction induced by terminally differentiated cells [215]. As expected, arthritic animals, in this study, presented resorption of medullary trabecular and cortical bone. This induction of bone matrix resorption has been previously demonstrated in the same animal model of RA [209, 212]. Interestingly, arthritic animals treated with P-N demonstrated significantly reduced bone resorption by 95%, compared to untreated arthritic animals. Treatment with naproxen; however, reduced bone resorption by only 65%, compared to untreated arthritic animals. In addition, the induction of cartilage damage is observed in the later stages of RA. Significant reductions in cartilage damage were observed in P-N and naproxen treated animals; however, it is important

to note that the overall observed cartilage damage induced by RA in the adjuvant model was minor, as seen previously [209].

One significant transcription factor involved in osteoclast survival and differentiation is NF-κB [216]. More specifically, the activation of the p65 NF-κB subunit has been previously demonstrated to play a significant role in arthritis-associated osteoclastogenesis and survival [217]. Therefore, it is feasible that the significant inhibition of bone resorption induced by P-N may in part be due to an inhibitory effect on NF-κB activation. A potential mechanism of action, which was explored later in this study.

Overall, P-N treatment induced significant reductions in inflammation, bone resorption, and cartilage damage. Naproxen-treated groups showed similar results but a less pronounced effect associated with mild infiltration of inflammatory cells in periarticular tissue, edema, and greater bone resorption. Together, these data show that P-N is efficacious in ameliorating the hallmarks of clinical RA.

Safety

Although P-N was effective in reducing RA-associated features (greater than naproxen in some parameters), it is important to consider its safety. As noted previously, the clinical usefulness of a drug also depends upon its safety. This is especially important when a drug is administered over a long period of time that, as in case of RA can span from years to decades.

Therefore, an in-depth assessment of the clinical potential of P-N in RA requires the examination of its safety profile.

As noted previously, NSAID use is associated with toxicity of several organs, most notably GI toxicity, which compromises their utility [31, 32]. Clinical endoscopy studies of naproxen demonstrated significant increases in GI toxicity at recommended treatment doses [43, 188, 189]. In fact, patients using naproxen for arthritis treatment have a 41% increase in the cumulative incidence of GI ulcers [188]. This increased risk of GI ulceration is principally due to COX inhibition, which results in the inhibition of prostaglandin and prostacyclin synthesis in the GI.

This study demonstrates that P-N is significantly safer than naproxen in the rat adjuvant model of arthritis. This increased safety was manifest by a 60% reduction in GI toxicity compared to naproxen, which was confirmed by histological examination of the gastric mucosa. The P-N-treated arthritis group was healthy, whereas the naproxen treated group presented with perforating ulcers; left untreated, these ulcers lead to death. As noted earlier, the enhanced GI safety of P-N can likely be accounted for by the attenuation of COX-1 and COX-2 inhibition by P-N, compared to naproxen. In addition, to increased safety of the GI tract, P-N did not show signs of toxicity in other major organs including the heart, liver, kidney, stomach, and spleen. Together these data show that P-N is much safer than naproxen when used in the treatment of RA. Thus, safety seems to be one of the cardinal differentiating features between these two compounds in the treatment of RA.

Mechanism of action

To determine the mechanism of action of P-N, various parameters that control or contribute to the inflammatory response in RA were investigated. In the model of RA used in these studies, the inflammatory reaction induced by immune system comprises infiltration with mononuclear cells and production of pro-inflammatory chemokines, the latter playing a significant role in the induction of histopathological changes and edema of the joints.

Two pro-inflammatory cytokines, IL-1β and IL-6, play a central role in the pathophysiology of RA [66, 218, 219]. IL-6, in particular, appears to have a prominent role in RA. Deficiency in IL-1 slows the onset of RA, whereas IL-6 deficiency completely inhibits the development of RA [67]. In fact, in humans with RA, IL-6 promoter polymorphism strongly correlates with disease onset, activity, and therapeutic response [77-79]. Notably, IL-6 -/- mice are resistant to the development of RA [80, 81]. The prominent role of cytokines also play a central role in the RA animal model utilized in this study [212, 213].

This study demonstrated P-N to be significantly effective in reducing pro-inflammatory cytokine levels in RA [68-73]. Specifically, P-N effectively returned IL-6 to the levels of healthy non arthritic animals; these levels were reduced by 21% compared to untreated arthritic animals. The effect of naproxen was similar to that of P-N. In addition, P-N significantly reduced IL-1β levels by 28%, compared to untreated arthritic animals. This effect which was also associated with a restoration of IL-β levels to basal levels observed in healthy non-arthritic animals. Naproxen also demonstrated a similar effect.

PGE_2 is a major pro-inflammatory eicosanoid, the role of which in inflammatory arthritis is well-established; COX catalyzes a key step in its formation [220]. PGE_2 synthesized by COX plays a significant role in RA [221]. Conventional NSAIDs, such as naproxen, used in the treatment of RA inhibit COX enzymatic activity thereby reducing PGE_2 [222]. Interestingly, P-N does not reduce blood PGE_2 levels to the levels of healthy non arthritic animals. In sharp contrast and as expected, naproxen restored PGE_2 blood levels to those of healthy non arthritic animals. The reduced ability of P-N to normalize PGE_2 blood levels, can be explained in part by its lack of effect on COX enzyme activity. Nevertheless, P-N was highly effective in treating RA in this animal model, as efficaciously, if not marginally better, as naproxen.

The pleiotropic pro-inflammatory actions of PGE_2 is especially evident in the synovial fluid of patients with RA [223]. In fact, PGE_2 stimulates the production of pro-inflammatory cytokines in RA [224]. Study of PGE_2 directly in the affected joints showed that, unlike in the blood, P-N significantly reduced PGE_2 levels in the affected joint tissue returning them to those of non-arthritic animals. Since P-N does not inhibit COX enzymatic activity, the explanation of this finding lies in the reduced expression of COX expression in the synovium. Untreated arthritic animals expressed high levels of COX-2 (5.2 fold increase compared to healthy non arthritic animals), as expected [225, 226]. Animals treated with naproxen maintained their robust COX-2 expression, but those treated with P-N had markedly reduced levels of COX-2 expression, compared to untreated arthritic animals. As expected, expression levels of COX-1, which is constitutively expressed in tissue was not significantly affected in all groups,

These findings provide a mechanistic distinction between P-N and naproxen in their anti-inflammatory action: naproxen inhibits the activity of COX in the joint, whereas P-N inhibits its expression.

One major regulatory transcription factor involved in the expression COX and pro-inflammatory cytokines, including osteoclastogenesis is NF-κB. Previously, studies have demonstrated that NF-κB plays a role in the expression of COX-2 in RA [227]. More specifically, phospho-p65 NF-κB subunit binds to the NF-κB consensus sequence of the COX-2 promoter [228]. In addition, the activation of p65 NF-κB subunit also mediates the synthesis of pro-inflammatory cytokines, such as IL-1β and Il-6, in RA [227, 229, 230]. Furthermore, the activation of p65 NF-κB in arthritis is associated osteoclastogenesis and survival [217, 231].

In this study, untreated arthritic animals exhibited significant activation of p65 NF-κB, compared to normal healthy controls. This increase of NF-κB activation in untreated arthritic animals was significantly suppressed by 87% in arthritic animals treated with P-N. This reduction in NF-κB activation was similar to that of normal healthy animals, a difference which was not statistically different. Moreover, P-N was significantly more effective than naproxen in controlling NF-κB activation, another critical distinction between these two molecules.

Overall, these studies suggest that P-N's mechanism of action is partially distinct than that of naproxen. An effect, which was suggested by differences observed in bone resorption, PGE_2 levels, and COX expression of the affected joint in arthritic animals. It is feasible that P-N's distinct inhibitory effect on NF-κB may in part play role in its positive effect in RA. As noted previously,

NF-κB activation plays a significant role in the arthritis, which has resulted in the investigation of NF-κB inhibitors for the treatment of RA [232]. It is conceivable that a complex regulatory circuitry including NF-κB, COX, and IL's is at play here and P-N interacts with it in a way that leads to reduced inflammation.

Chapter 4: P-N in Colon Cancer

To investigate the potential efficacy, safety, and mechanism of action of P-N in cancer an animal model of colon cancer was employed as well as various human colon cancer cell lines.

The animal model utilized in this study, $APC^{Min/+}$, closely resembles the development of intestinal adenomas in humans. As noted previously, mutation of the APC gene is a critical driving factor in colon cancer development, as seen in patients with FAP. Left untreated, patients with FAP are at 95% risk of developing colon cancer by age 50 [84]. NSAIDs have been demonstrated to be partially effective in the chemoprevention of colon cancer. Initial studies with the NSAID, aspirin, has demonstrated significant efficacy in the prevention of intestinal adenomas in $APC^{Min/+}$ mice [233].

To further investigate the potential efficacy and mechanism of action of P-N, in vitro studies were performed with human derived colon cancer cell lines, HT-29, SW-480, and LoVo. Similar to the $APC^{Min/+}$ mouse model, these human derived colon cancer cell lines possess an APC gene mutation. Following a description of the methods that were employed for this segment of the work, specific results are presented. Results are summarized below.

MATERIALS AND METHODS

Efficacy in Animal Models of Colon Cancer: $APC^{Min/+}$, 47 days old, were divided into the following groups: 1) Vehicle (Corn oil, n=15); 2) Naproxen 11mg/kg (n=8); 3) Naproxen 35mg/kg (n=17); 4) P-N 20.9 mg/kg (n=8); 5) P-N 66.5 mg/kg (n=11); 6)P-N 1600 mg/kg (n=11). Min mice have a germ-line nonsense mutation at codon 850 of the APC gene, a thymine to adenosine transversion at nucleotide 2549, resulting in a conversion of leucine to a stop codon. $APC^{Min/+}$ were treated by gavage daily 75 days. On day 75 of treatment, all animals were euthanized; intestines were subsequently removed and opened longitudinally. Tumors were counted under a magnifying lens.

Histological examination: Intestinal tracts of vehicle and P-N 1600 mg/kg mice were resected at the medial (jejunum) and distal (ileum) small intestine and the colon. Intestinal sections were then paraffin embedded, cut into 4 µM-thick sections, and stained with H&E. Sections were examined under an Olympus BX41 microscope (Olympus, Center Valley, PA, USA) and photographed with an Olympus DP25 digital camera.

Safety of P-N at high doses: $APC^{Min/+}$ mice treated with vehicle or P-N 1600 mg/kg were observed for potential GI toxicity, as highlighted previously. In addition, the stomach was resected and preserved in 10% formalin. For analysis, the stomachs were dissected longitudinally and the contents of the stomach were washed away with PBS. The stomach mucosal lining was examined for ulcers and perforations under a dissection microscope.

Cell Culture: Human colon cancer (HT-29, SW-480, and LoVo) cell lines were grown as monolayers in the specific medium suggested by the American Type Culture Collection (Manassas, VA) and supplemented with 10% fetal calf serum (Mediatech, Herndon, VA), penicillin (50 U/ml), and streptomycin (50 µg/ml; Life Technologies, Grand Island, NY). Cells were incubated at 37°C in 5% CO_2.

Cell viability: Cell viability of HT-29, SW-480, and LoVo colon cancer cell lines was determined using the MTT assay (Roche Diagnostics, Indianapolis, IN) following the manufacturer's instructions. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, cells were seeded into 96 well plates at a concentration of 10,000 cells/100 µL per well. The following day, cells were treated with P-N or naproxen for 24 h. Following drug treatment, cells were then incubated with MTT for 4 h. MTT is reduced to formazan by viable cells. Therefore, the amount of formazan dye formed directly correlates with the number of metabolically active cells in culture. Formazan formed by viable cells were then dissolved with 10% SDS in 0.01 M HCl for 8 h. After solubilization, levels of formazan dye was measured using colorimetric analysis at the absorbance wavelength of 570 nm and reference wavelength of 690nm.

Cell Proliferation: Cell proliferation was evaluated through the incorporation of the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU). Briefly, HT-29 cells were seeded in 60 mm well plates at a concentration of 1,000,000 cells/4 ml per plate. Cells were allowed to adhere overnight and then treated with P-N for 24 h. After treatment, cells were incubated with 10 µM BrdU for 30 min to allow for incorporation into newly synthesized DNA. Cells were harvested by trypsinization, centrifuged at 500 g for 5 min, and washed twice with 1.0% bovine serum albumin. Cells were

fixed with 70% ethanol. Fixed cells were added drop wise, under vortex, to 2N HCL with 0.5% Triton X-100 in order to denature DNA to single strands thereby providing an antibody to BrdU (Anti-BrdU FITC) access to incorporated BrdU in DNA. Cell were then centrifuged (500 g for 5 min). The cell pellet was resuspend in 0.5% Tween 20/ 1%BSA solution with Anti-BrdU FITC and incubated for 30 min. Unbound Anti-BrdU FITC was removed by centrifugation (500 g for 5 min) and the pellet was resuspend in 5µg/ml of propidium iodide (PI) to stain total DNA. Analysis was completed by flow cytometry (FACScaliber, BD Bioscience).

Cell cycle analysis: For cell cycle analysis, cells were stained with PI, a fluorescent staining dye that intercalates with DNA, to determine the proportion of cells in each stage of the cell cycle. Briefly, HT-29 cells were seeded in 60 mm well plates at a concentration of 1,000,000 cells/4 ml per plate. Cells were allowed to adhere overnight and then treated with P-N for 24h. Cells were harvested by trypsinization, centrifuged at 500 g for 5 min, and washed twice with PBS. Cells were fixed by adding a 70% ethanol solution drop-wise under agitation and incubated for 30 min at 4°C. Cells were centrifuged for 5 min at 850 g and then washed twice with PBS. Ribonuclease A was added to degrade RNA to prevent interference with PI-DNA binding. DNA was stained by adding 400 µl of PI and analyzed by flow cytometry (FACScaliber, BD Bioscience).

Apoptosis assay: Cellular apoptosis was determined by Annexin V-fluorescein isothiocyanate-conjugated (FITC) and PI staining. HT-29 cells were seeded in 6 well plates at a concentration of 300,000 cells/2 ml per well. Cells were allowed to adhere overnight and then treated with P-N for 24 h. After treatment, cells were harvested by trypsinization and stained with a buffered solution of Annexin V-FITC (100X dilution; Invitrogen, Carlsbad, CA) and PI (0.5 µg/ml; Sigma, St

Louis, MO). Staining by Annexin V-FITC on cell surfaces expressing phosphatidylserine (a marker of early apoptosis), PI bound to DNA of permeabilized cells (indicative of necrosis), or dual staining of Annexin V-FITC and PI (indicative of later stage apoptosis) were quantified by flow cytometry analysis (FACScaliber, BD Bioscience).

NF-κB – DNA binding assay: HT-29 cells were treated with P-N or naproxen at increasing doses for 4h. Treatment with P-S at 100 µM for 4 h served as positive indicator of nuclear NF-κB DNA binding inhibition, as demonstrated previously [153]. Nuclear fractions were isolated from 3×10^6 cells following standard protocol. Cells were collected by centrifugation at 800 g for 10 min, the pellet was resuspended in 200 µl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% Igepal), incubated for 10 min at 4°C, and centrifuged for 1 min at 12,000 g. The supernatant fraction was discarded, and the nuclear pellets were resuspended in 60 µl of buffer B (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF). Samples were incubated for 20 min at 4°C and centrifuged at 10,000g for 15 min at 4°C. The protein concentration of the supernatant was determined by the method of Bradford assay. The NF-κB activity was determined using the LightShift chemiluminescent EMSA (Thermo Fisher Scientific, Rockford, IL). Briefly, the nuclear extracts were incubated with biotin-labeled NF-κB consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') at 37°C for 20 min, then loaded onto the polyacrylamide gel and transferred to a nylon membrane. The membrane was exposed to UV-light for 10 min for cross-linking of the transferred DNA, incubated with stabilized streptavidin-horseradish peroxide conjugate in blocking buffer for 15 min and covered with substrate working solution, followed by exposure to X-ray film.

RESULTS

Effect of P-N in prevention of tumorigenesis in $APC^{min/+}$ mice

The potential anti-cancer effect of P-N and naproxen was evaluated in the $APC^{min/+}$ mouse model of colon cancer. At the two lowest doses used, P-N and naproxen were given at equimolar doses. At the highest dose of P-N (1600 mg/kg), naproxen could not be administered at an equimolar concentration due to significant toxicity.

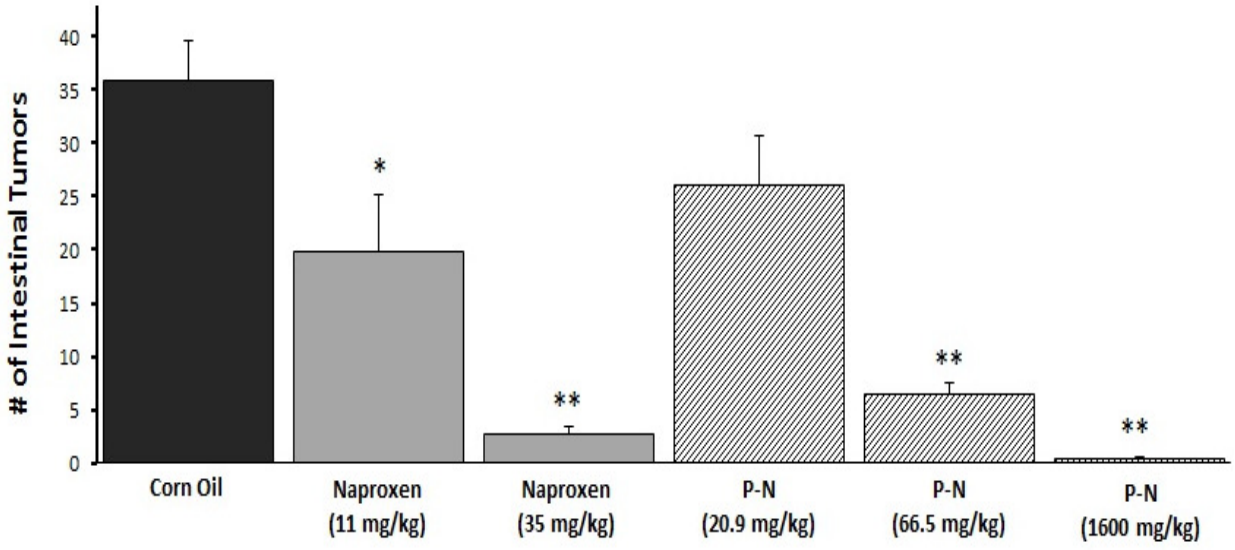

Figure 4-1: Tumors in the intestinal tract of $APC^{min/+}$ mice. Treated for 75 days with Corn oil, naproxen (11, 35 mg/kg), or P-N (20.9, 66.5, or 1600 mg/kg). Values are mean $\pm$ SEM. (*$p<0.05$) (**$p<0.001$)

P-N at 66.5 mg/kg and 1600 mg/kg significantly inhibited the number of intestinal adenomas by 82% ($p<0.001$) and 99% ($p<0.001$), respectively, in a dose-dependent manner compared to corn oil control (Figure 4-1).

Conventional naproxen at 11 mg/kg and 35 mg/kg resulted in 45% ($p<0.05$) and 92% ($p<0.001$) inhibition, respectively, compared to corn oil control. P-N administered at the lowest dose of 20.9 mg/kg inhibited tumor development by 27% ($p>0.05$) compared to control. However, it is clear that the greater safety of P-N compared to naproxen allows for high dosing over long periods of time, which practically eliminates tumor development in these mice.

Histological examination of the intestinal tract of $APC^{min/+}$ mice

To determine morphological characteristics and differences between P-N treated and untreated animals, the jejunum, ileum, and colon was examined by microscopy. Histological examination of the intestines of $APC^{min/+}$ mice treated with P-N 1600 mg/kg demonstrated normal intestinal tissue devoid of neoplastic features (Figure 4-2).

In contrast, untreated $APC^{min/+}$ mice showed high grades of dysplasia in the medial intestine (jejunum). Untreated $APC^{min/+}$ mice showed broad-based sessile polypoid adenomas in the distal

intestine (ileum), with medium-grade dysplasia. Furthermore, the colon of untreated $APC^{min/+}$ mice showed pedunculated adenomas with a high-grade dysplasia.

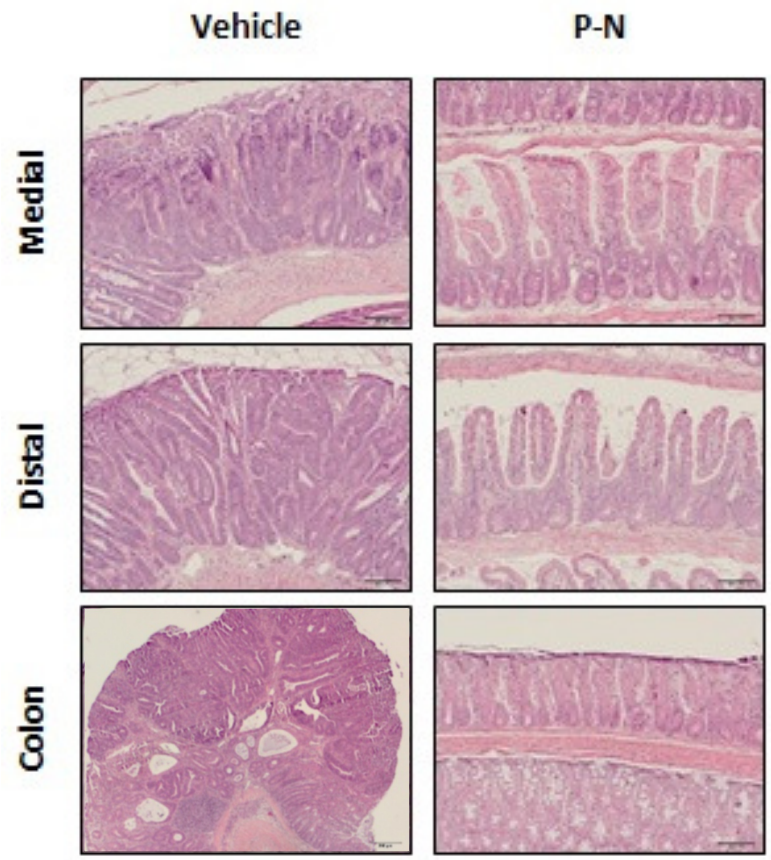

Figure 4-2: Histological examination of intestinal and colon sections of $APC^{min/+}$ mice. Representative images are presented from medial, distal small intestine and colon for vehicle control and P-N (1600 mg/kg) treated $APC^{min/+}$ mice.

Effect of P-N on the cell viability of human colon cancer cells

The remarkable effect of P-N in the prevention of tumorigenesis in $APC^{min/+}$ mice prompted studies in human derived colon cancer cell lines (HT-29, SW-480, LoVo) to determine its efficacy in vitro. A notable feature of each of the human derived colon cancer cell lines is that they have

APC mutations, similar to that of the $APC^{min/+}$ mouse model. The half maximal inhibitory concentration value for cell viability (IC_{50}) of these various human colon cancer cell lines treated with varying concentrations of P-N or naproxen was determined by MTT assay, which measures the production of formazan (reduced MTT) in live cells.

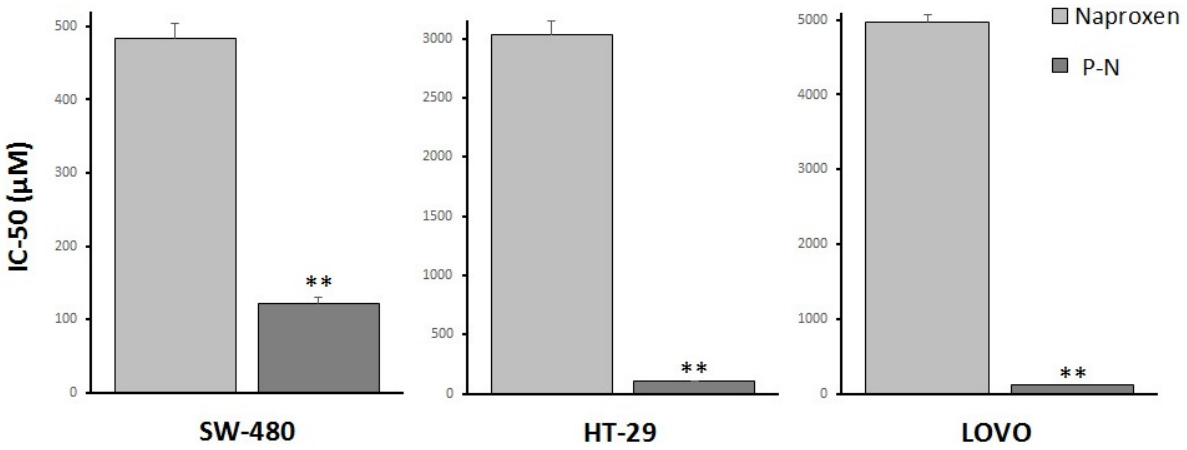

Figure 4-3: IC_{50} values for colon cancer cells. Treated with P-N or naproxen for 24 h. Values are mean ± SEM

The IC_{50} values of P-N for cell viability of these cell lines, after 24 h of treatment, were significantly ($p<0.001$) lower than those of naproxen. The modification of naproxen to generate P-N reduced the IC_{50} of conventional naproxen by 96%, 75%, and 97% ($p<0.001$) in HT-29, SW-480, and LoVo human colon cancer cells, respectively (Figure 4-3). Thus the potency enhancement of P-N over naproxen is as high as 39-fold (Table 3-1).

Table 4-1: Potency enhancement and IC_{50} values for colon cancer cells. Treated with P-N or naproxen for 24 h. Values are mean ± SEM

Colon Cancer Cell Line	P-N (IC-50, µM; 24h)	Naproxen (IC-50, µM; 24h)	Potency Enhancement of P-N
HT-29	109 ± 1	3029 ± 115	28x
SW-480	122 ± 8	484 ± 21	4x
LOVO	126 ± 5	4971 ± 104	39x

Body weight change of $APC^{min/+}$ mice

As noted previously, the treatment with NSAIDs at high doses over a long period of time can result in significant toxicity. To investigate potential induction of general toxicity over time, $APC^{min/+}$ mice were weighed during the 75 day treatment period.

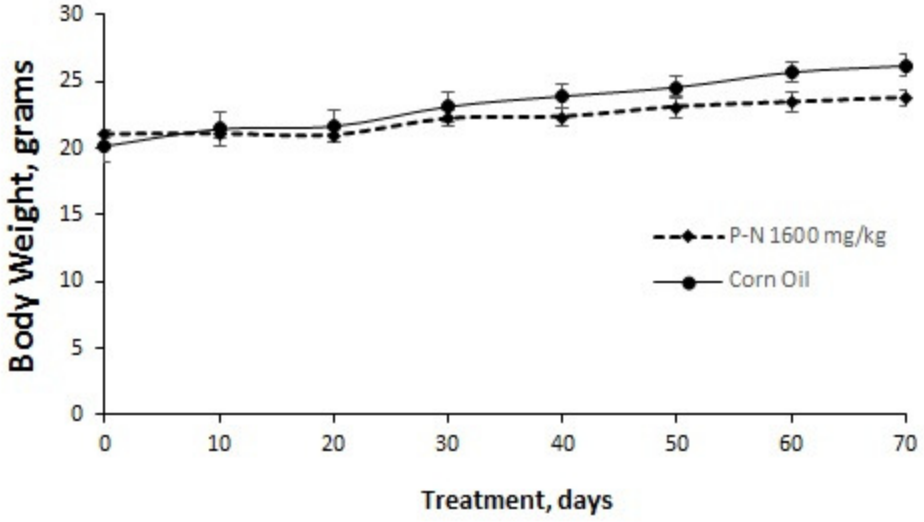

Figure 4-4: Body weight over time in $APC^{min/+}$ mice. Treated with corn oil or P-N 1600 mg/kg

Animals treated with high doses of P-N (1600mg/kg) did not show any signs of toxicity, which would normally be represented by a 10% loss in body weight (Figure 4-4). High dose treatment with naproxen (842 mg/kg), equimolar to the highest dose of P-N (1600 mg/kg), induced significant toxicity directly after treatment thereby precluding the use of this dose of naproxen during the 75 day study period. The significant safety of P-N at high doses during the course of the treatment period is demonstrated by the steady increase in weight of P-N treated animals, which followed a similar trend as animals treated with vehicle (corn oil) (Figure 4-4). Thus, no overall toxicity was observed by treatment of $APC^{min/+}$ mice with P-N even at high doses.

Gastric safety of P-N in $APC^{min/+}$ mice

The safety of P-N, a critical consideration for its clinical application, was assessed in $APC^{min/+}$ mice treated with the highest effective dose of P-N. As demonstrated previously with the parent

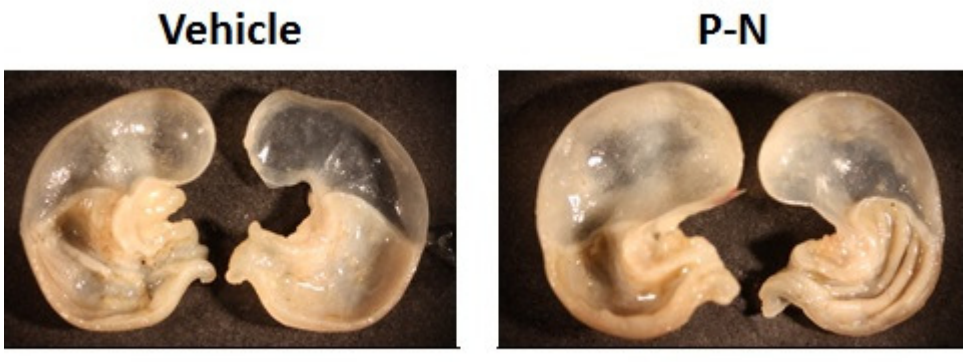

Figure 4-5: Stomach lining of $APC^{min/+}$ mice treated with vehicle or P-N (1600 mg/kg).

compound (naproxen), the organ with major toxicity is the GI, which was associated with significant ulceration of the stomach lining and GI tract.

To investigate the potential of GI toxicity by P-N, the stomach and the intestinal tract of mice treated with the highest dose of P-N (1600 mg/kg) was examined. There was no ulceration or perforation of the stomach, which was as healthy as the stomach of animals treated with vehicle (Figure 4-5). Naproxen treatment, as seen previously, at significantly lower doses showed significant ulcerations and even perforations of the stomach (Figure 3-9).

The health of the colon was also examined. Histological examination of colon, and the small intestine demonstrate that P-N at high doses (1600mg/kg) had no apparent GI toxicity (Figure 4-6).

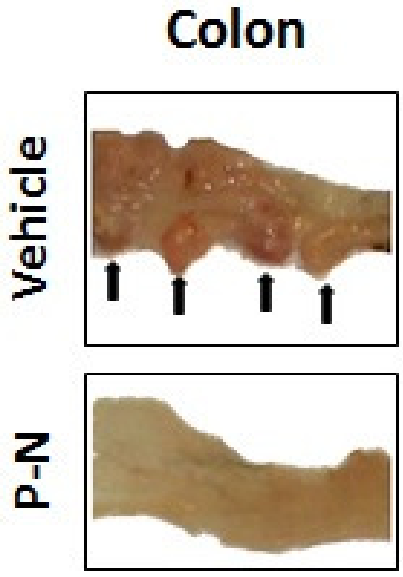

Figure 4-6: Colon section of in APC$^{min/+}$ mice. Treated with vehicle or P-N (1600 mg/kg). Arrows denote adenomas.

Untreated APC$^{min/+}$ had a significant number of intestinal tumors (Figures 4-1, 4-2). Indeed, towards the end of the study period, untreated APC$^{min/+}$ mice experienced rectal prolapse and rectal bleeding due to large adenomas (Figures 4-2, 4-6). APC$^{min/+}$ mice treated with P-N 1600 mg/kg were healthy throughout the study period and had no rectal prolapse. Importantly, APC$^{min/+}$ mice treated with naproxen (842mg/kg) at equimolar doses had significant drug toxicity (data not shown). In contrast, APC$^{min/+}$ mice treated with P-N (1600 mg/kg), a dose which was 145 times greater than the lowest dose of naproxen (11 mg/kg), had no apparent side effects.

Effect of P-N on colon cancer cell proliferation in S-phase

To investigate the potential anti-proliferative effect of P-N on human colon cancer cells, HT-29 cells were treated with P-N for 24h, as indicated. Newly synthesized DNA of replicating

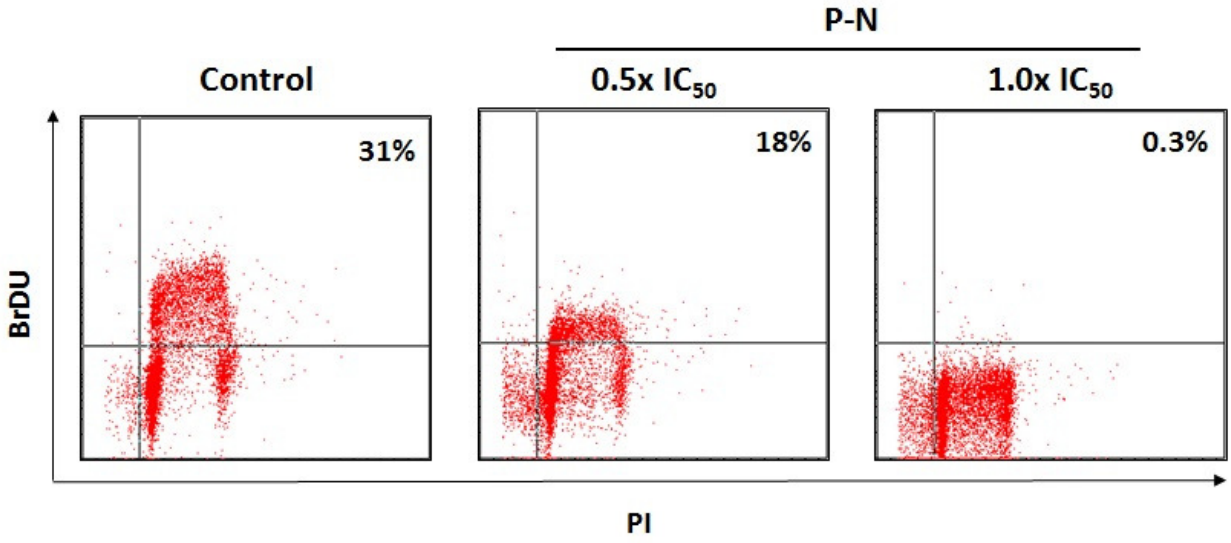

Figure 4-7: Histograms of BrdU incorporation in HT-29 cells. Cells were treated for 24 h without (control) or with 0.5×, 1× IC_{50} P-N. The percentage of bromodeoxyuridine positive cells is shown in the right upper corner of each panel.

HT-29 cells during the S-phase of the cell cycle was determined by BrdU incorporation during DNA replication. P-N markedly reduced the percentage of proliferating colon cancer cells in S-phase from 31% in controls to 18% and 0.3%, at 0.5x IC_{50} and 1x IC_{50} of P-N (Figure 4-7). Compared to untreated controls, this accounted for 42% and 99% reduction of cells in the S-phase at 0.5x IC_{50} and 1x IC_{50} of P-N, respectively (Figure 4-8).

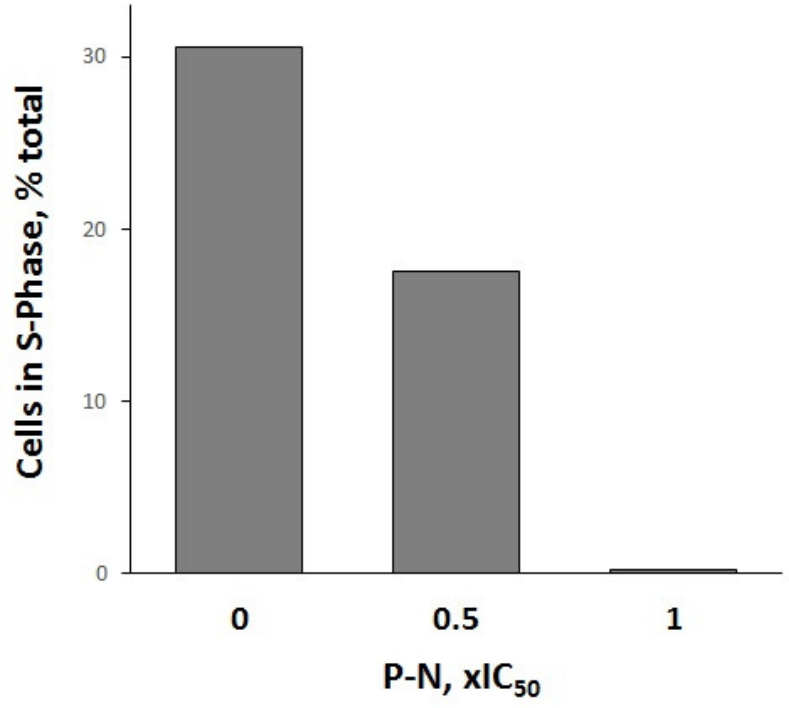

Figure 4-8: Percentage of proliferating HT-29 cells in S-phase. HT-29 cells treated for 24 h with 0, 0.5, 1 xIC_{50} of P-N. Representative percentage of proliferating cells in S-phase are shown, derived from flow cytometry histograms represented in Figure 4-7.

Effect of P-N on cell cycle phase transitions

To investigate the potential effect of P-N on cell cycle kinetics, HT-29 colon cells were treated with P-N for 24 h, as indicated. P-N blocked the G_1 to S transition after 24 h of incubation, which was represented by an increase in the percentage of cells in the G_1-phase (Figure 4-9). Untreated control cells had 66% of cells in G_1-phase. Of cells treated with P-N 0.5 x$IC_{50,}$ 75% were in G_1-phase (Figure 4-10).

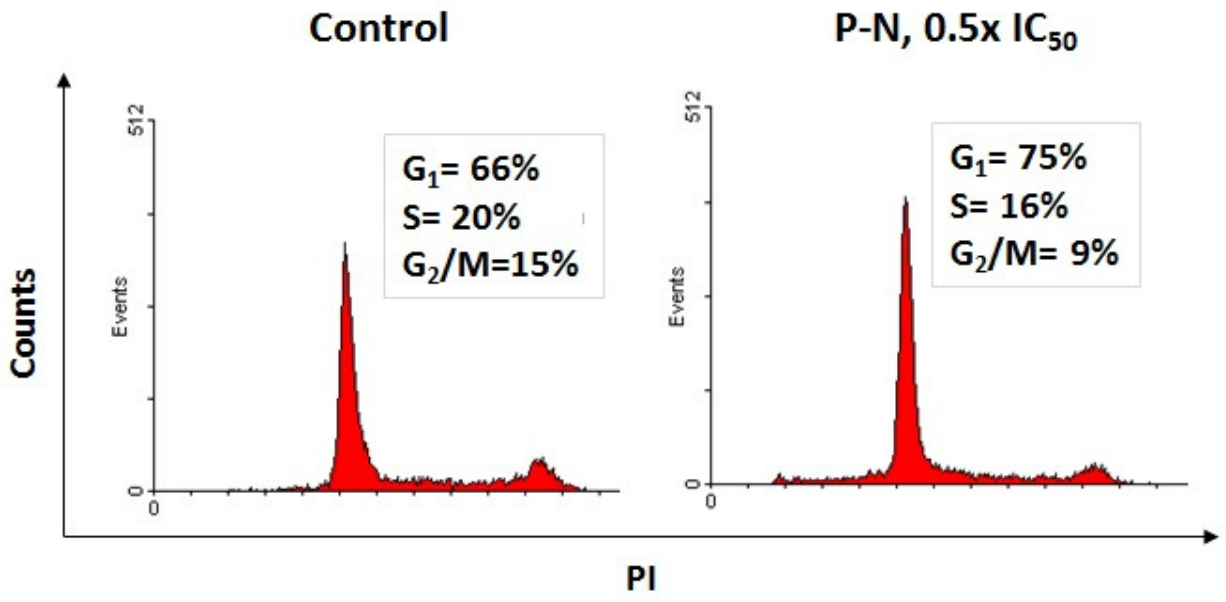

Figure 4-9: Histograms of DNA content in HT-29 cells treated for 24 h without (control) or with 0.5× IC_{50} P-N. The percentage of cells in each phase of the cell cycle is shown in the right upper corner of each panel.

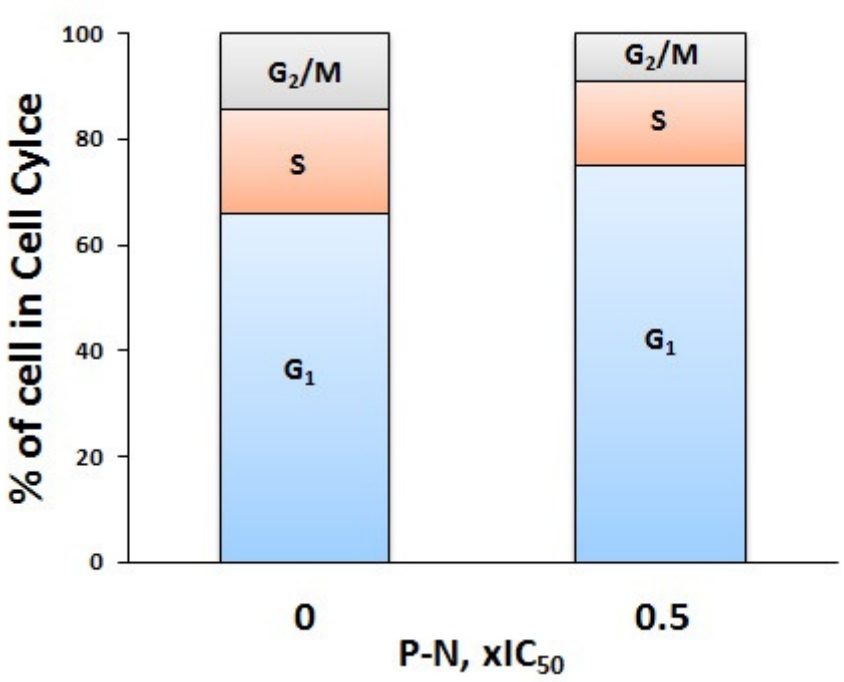

Figure 4-10: Cell cycle progression in HT-29 cells. HT-29 cells treated for 24 h with 0, 0.5 xIC_{50} of P-N. Representative profiles of the distribution of cells in G_1, G_2/M and S phases are shown, derived from flow cytometry histograms represented in Figure 4-9.

Effect of P-N on apoptosis in HT-29 cells

To investigate the potential apoptotic effect of P-N on colon cancer cells, HT-29 cells were treated with P-N for 24h. Annexin V protein demonstrates high affinity, specificity, and sensitivity for phosphatidylserine which is translocated to the cell surface of an intact plasma membrane indicating the initiation of apoptosis. PI staining, a dye that intercalates into DNA, indicates a loss of membrane integrity, which is present in necrotic cells. Thus, Annexin V^-/PI^- cells are viable

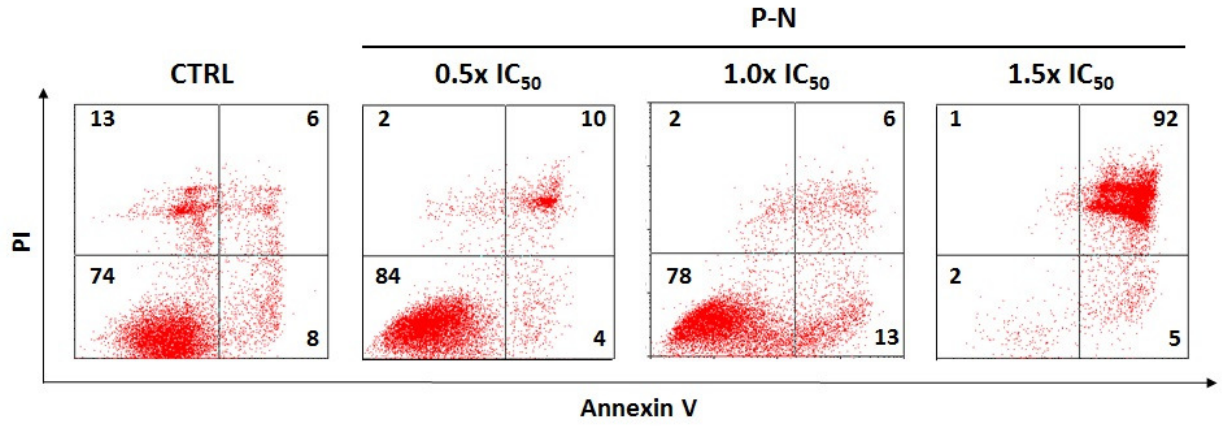

Figure 4-11: Histograms of HT-29 cells stained with PI and Annexin V. The percentage of cells are indicated in each quadrant for cells treated without (control) or with 0.5, 1.0, 1.5 xIC_{50} of P-N.

cells; Annexin V^+/PI^- are early apoptotic; Annexin V^+/PI^+ are late apoptotic, and Annexin V^-/PI^+ are necrotic (Figure 4-11).

Total percentage of Annexin V staining for controls was 13%. The percent of Annexin V positive cells in HT-29 cell treated 0.5, 1.0, or 1.5× IC_{50} was 14%, 20%, and 97%, respectively. In other words, treatment with 0.5, 1.0, 1.5 xIC_{50} of P-N for 24 h, respectively, increased the proportion of Annexin V^+ cells in a dose-dependent fashion up to 7.3-fold, compared to controls (Figures 4-12).

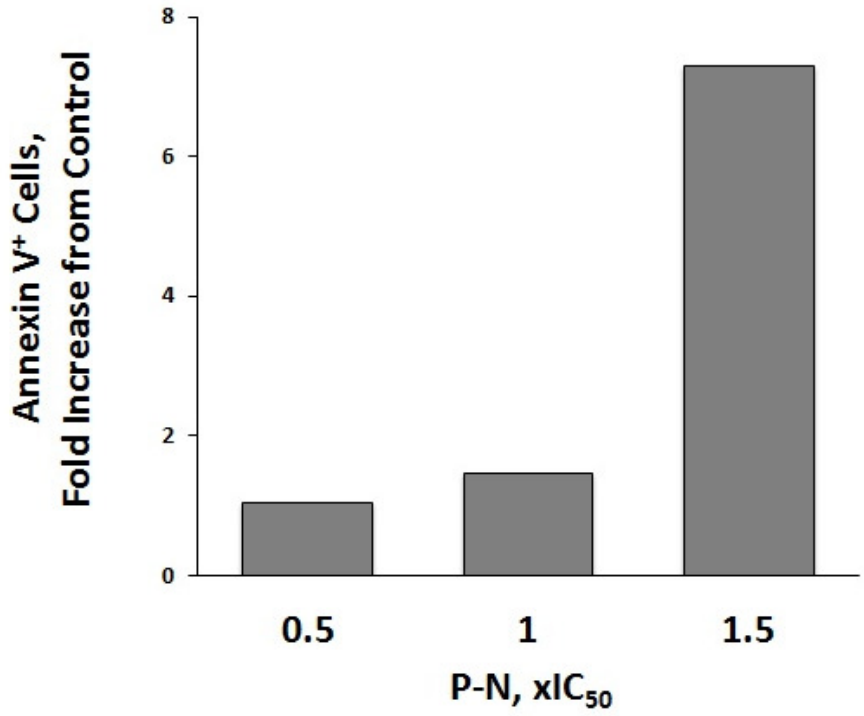

Figure 4-12: Fold increase of Annexin V⁺ staining from control in HT-29. Annexin V⁺ staining (apoptosis) of HT-29 cells treated 0.5, 1.0, 1.5 xIC_{50} of P-N for 24h. Values are the Annexin V⁺ cells from P-N treated cells divided by Annexin V⁺ cells from control. These values are derived from flow cytometry histograms, represented in Figure 4-11.

Nuclear NF-κB binding in HT-29 treated with P-N

The activation of the transcription factor NF-κB involves its translocation into the nucleus, where it binds to DNA to initiate transcription of target genes. Given the dominant role of NF-κB in colon carcinogenesis, the potential inhibitory effect of P-N on nuclear NF-κB binding was evaluated in a human derived colon cancer cell line.

HT-29 colon cancer cells were treated with P-N or naproxen for 4 h. To determine whether P-N affects the NF-κB–DNA interaction, electrophoretic mobility shift assays EMSA on nuclear extracts from HT-29 colon cancer cells.

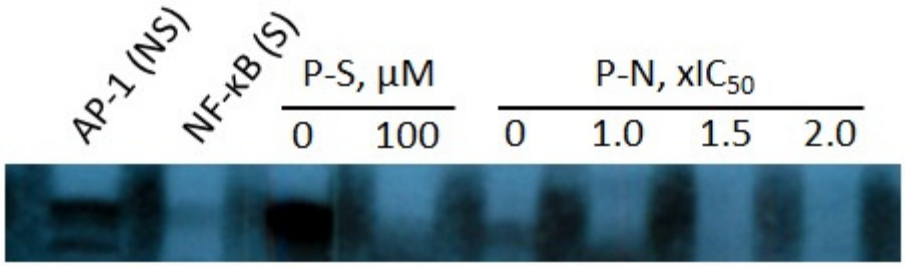

Figure 4-13: EMSA for NF-κB nuclear fraction of P-N treated HT-29 cells. Cells were treated with 4 h with P-N at 0, 1.0, 1.5, 2.0 xIC_{50}. Treatment with P-S served as a positive indicator of NF-κB inhibition. AP-1, served as unlabeled non-specific (NS) competitive oligonucleotide. NF-κB, served as unlabeled specific (S) competitive oligonucleotide.

Specificity of the nuclear NF-κB probe was confirmed EMSA by incubating nuclear extracts with a non-specific oligonucleotide sequence (AP-1 NS) that does not bind biotin-labeled NF-κB consensus sequence resulting in normal band intensity (Figure 4-13). In addition, an unlabeled NF-κB specific competitive oligonucleotide was added in excess of labeled biotin-labeled NF-κB consensus sequence resulting in significantly reduced band intensity, thereby confirming specificity of the labeled biotin-labeled NF-κB. Furthermore, the functionality of EMSA was also confirmed in cells treated with P-S, as previously demonstrated 100 μM P-S effectively reduces nuclear NF-κB binding in HT-29 cells after 4 h [153].

EMSA demonstrated that P-N inhibited nuclear NF-κB binding. This inhibition was marked by a significant reduction in NF-κB DNA binding in HT-29 cells treated with P-N for 4 h at increasing concentrations (Figure 4-13). Colon cancer cells treated with P-N at 1.0x IC_{50} showed reduced levels of nuclear NF-κB binding, compared to untreated control. This effect was increased at P-N 1.5x and 2.0x IC_{50}, which showed the greatest inhibition.

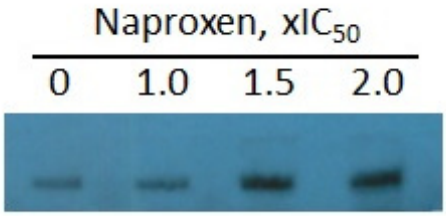

Figure 4-14: EMSA for NF-κB nuclear fraction of naproxen treated HT-29 cells.
Cells were treated with 4 h with naproxen at 0, 1.0, 1.5, 2.0 xIC_{50}.

In contrast to P-N, treatment of colon cancer cells with naproxen at 1.0x IC_{50} showed no significant change in nuclear NF-κB binding, compared to untreated control. This difference between P-N and naproxen was also evident at higher concentrations. In sharp contrast to P-N, naproxen treated colon cancer cells at 1.5x and 2.0x IC_{50} showed an increase in nuclear NF-kB binding at naproxen (Figure 4-14).

Overall, treatment with P-N significantly inhibited nuclear NF-κB binding in a concentration-dependent fashion, whereas treatment with naproxen increased nuclear NF-κB binding.

DISCUSSION

The chemoprevention of colon cancer is clinically significant due to the significant burden associated with colon cancer screening and patient compliance. The prevention of colon cancer by pharmacological intervention, especially in high risk individuals, has significant potential to decrease morbidity and mortality rates. The novel chemopreventive agent, P-N, is explored in this study.

The development of a chemopreventive agent differs significantly than that of agents for the treatment of established cancer. Often the utility of agents in the treatment of cancer outweighs their significant side effects, which can lead to significant illness. These treatment agents often mitigate cancer burden or extend lifespan in the range of months. The development of a chemopreventive agent, however, which is intended for chronic usage in individuals without cancer requires both significant efficacy and safety.

Conventional NSAIDs have been previously reported to be effective in mitigating colon tumor incidence [111-113]; however, many of these conventional NSAIDs induce significant adverse events when used chronically as a chemopreventive [109, 110]. This study explores the utility and potential mechanisms of action the novel agent, P-N, for the chemoprevention of colon cancer. As noted previously, the structure of P-N is based on the NSAID, naproxen.

The efficacy and safety of novel agent for the treatment and/or prevention of colon cancer is commonly established in the $APC^{min/+}$ colon cancer mouse model. This $APC^{min/+}$ mouse model is utilized due to the close resemblance to the human gene mutation of APC, which results in the

development of intestinal adenomas in both species. Here the utility and safety of P-N was initially observed in this well-established mouse model of colon cancer ($APC^{min/+}$). In addition, the efficacy and mechanism of action of P-N was also explored in established cancer cell lines derived from human patients with colon cancer.

$APC^{Min/+}$ mice possess a germ-line nonsense mutation at codon 850 of the APC gene, a thymine to adenosine transversion of nucleotide 2549, resulting in the conversion of leucine to a stop codon. This mutant APC protein greatly contributes to the development of gastrointestinal tumors due to loss of control over the proto-oncogene, β-catenin [234]. The APC protein normally functions as a scaffolding protein, which assists in the degradation of β-catenin in the absence of Wnt signaling [92, 93]. Mutation in APC leads to its loss of function, which increases the activation of β-catenin and its target genes, such as c-Myc [94] and cyclin D1 [95], which are involved in cellular proliferation and eventually colon carcinogenesis. Patients with this APC mutation, as seen in FAP, have a 95% risk of developing colon cancer [84].

In this study, the potential efficacy of P-N in the prevention of colon cancer in this $APC^{min/+}$ model was investigated. Untreated animals were affected by significant tumors in the intestine, including the formation of broad sessile polypoid adenomas and pedunculated adenomas, which exhibited moderate to high grades of dysplasia. Treatment of these $APC^{min/+}$ mice with P-N led to a marked dose-dependent prevention of tumor progression. Treatment with P-N 20.9 mg/kg, 66.5 mg/kg, or 1600 mg/kg, respectively, reduced intestinal tumor burden by 27%, 82%, or 99%, compared to untreated control.

The animal model utilized in this study, $APC^{Min/+}$, closely resembles the development of intestinal adenomas in humans. As noted previously, mutation of the APC gene is a critical driving factor in colon cancer development, as seen in patients with FAP. Left untreated, patients with FAP are at 95% risk of developing colon cancer by age 50 [84]. NSAIDs have been demonstrated to be partially effective in the chemoprevention of colon cancer. Initial studies with the NSAID, aspirin, has demonstrated significant efficacy in the prevention of intestinal adenomas in $APC^{Min/+}$ mice [233].

It is important to note that even at the highest dose of P-N, daily treatment did not induce any signs of toxicity. It has been previously demonstrated that the major target organ responsible for toxicity in NSAIDs is the GI tract. Thorough examination of the intestine and stomach demonstrated that daily treatment with P-N 1600 mg/kg did not induce GI toxicity, whereas an equimolar dose of naproxen induced significant drug toxicity. Together, these data confirmed that P-N is much safer than naproxen. Previously, it has been demonstrated that P-N has a significantly attenuated ability to inhibit COX enzymatic activity, therefore it is plausible that this marked improvement in safety is due to its reduced ability to inhibit COX.

To investigate the potential mechanism of action of P-N, in vitro studies were performed in human colon cancer lines with APC mutations. This APC mutation is responsible for the induction of colon cancer in the mouse model, $APC^{min/+}$. In vitro studies in three cell lines demonstrated an important cytokinetic effect of P-N, likely accounting for its chemopreventive efficacy. These studies also showed a remarkable, nearly 40 fold, potency enhancement of P-N compared to its parent compound. The reason these differences are not maintained in vivo is likely attributed to

the extensive hydrolysis of P-N by esterases, ubiquitous in rodents (and much less so in humans). As already alluded to earlier, such potency can be maintained in vivo by circumventing the inactivating action of carboxylesterases by using specific inhibitors.

NSAIDs have long been considered to be effective in preventing cancer via their enzymatic inhibitory effect on COX-2. In fact, 80% of colon cancer tissue overexpresses COX-2, therefore making COX-2 a feasible target [235]. Previous studies have demonstrated that COX-2 plays a significant role in tumor progression. $APC^{min/+}$ mice with COX-2 knockouts results in reduced intestinal tumorigenesis, similar to that observed with treatment by a COX-2 specific inhibitor [236]. However, it is important to note that the knockout of COX-2 or treatment with a COX-2 specific inhibitor did not completely inhibit tumorigenesis, leaving behind a significant number of adenomas in the intestine. It is clear, that there are other significant driving factors, which are involved in the development of colon cancer that are COX-independent. In this study, the potential of P-N to act independently of COX was observed in the human derived colon cancer cell line, SW-480, which does not express COX-2. In this cell line, P-N demonstrated 4-times greater potency in reducing cell viability at a concentration which was 75% lower than naproxen. It is feasible that P-N's significantly greater efficacy over naproxen in this COX-2 negative colon cancer cell line is due to COX-independent effects. As observed previously, several modified NSAIDs modulate their chemopreventive efficacy via COX-independent mechanisms of action lending further support to the concept of COX-independent cancer prevention by P-N.

The in vitro studies defined to a first approximation the mechanism of action of P-N in colon cancer. The cytokinetic effect by P-N was extensive and strong. It encompassed cell proliferation,

cell cycle and apoptosis. Notable was the inhibition of the G_1 to S phase transition consistent with P-N's antiproliferative effect. The induction of apoptosis was robust and concentration-dependent and likely represents the dominant kinetic effect of this compound that is highly relevant to its chemopreventive properties.

Finally, of significance is the inhibitory effect of P-N on NF-κB binding. P-N demonstrated significant inhibition of NF-κB binding to DNA in a concentration-dependent manner. In stark contrast, naproxen induced NF-κB binding to DNA. This mechanistic difference may in part explain P-N's significant cytokinetic effect. NF-κB is a major transcription factor involved in the development and progression of cancer where it modulates cellular proliferation and cell survival [237]. NF-κB and the signaling pathways that are involved in its activation are important for tumor development. The constitutive activation of NF-κB in cancer from genetic mutation and deletions can result in the overexpression of NF-κB or functional loss of its regulators such as IκB. The well-known hallmarks of tumorigenesis include: self-sustaining proliferative signaling; insensitivity to growth suppressors; tissue invasion and metastasis; replicative immortality; angiogenesis; and resistance to apoptosis [238]. Notably, NF-κB can induce many of these cellular modifications in various cancers which is associated with promoting cancer-cell proliferation, increasing angiogenesis and metastasis, and inhibiting apoptosis [239].

More specifically, in oncogenesis, NF-κB modulates cellular proliferation by regulating cyclin D1, which is involved in the G_1 to S transition [240]. Moreover, cyclin D1 is overexpressed in patients with adenomatous polyps, primary colorectal adenocarcinoma, and familial adenomatous polyposis [241]. Recent studies demonstrated that inhibition of NF-κB reduces p65

expression and enhances apoptotic cell death in HT-29 colon cancer cells [242]. Furthermore, recent work by Williams et al., demonstrated that modified NSAIDs induced inhibition in NF-κB binding to DNA [170]. This inhibitory effect on NF-κB binding to DNA was associated with significant inhibition effect on cell growth, indicating that the growth inhibitory effect may be mediated by the effect on NF-κB. It is, therefore, conceivable that NF-κB is a major molecular target of P-N. Indeed, clinical studies have demonstrated significant expression of p65 NF-κB in colon cancer tissue compared to normal tissue [243].

Taken together these data indicate that P-N is efficacious in the prevention of colon cancer and that the high doses of this compound that can be employed due to its superior safety, offering a real clinical advantage. It should be noted that although these conclusions are derived from preclinical data from an animal model of colon cancer and human derived colon cancer cell lines, their clinical validation is required. Overall, the chemopreventive effect of P-N is due to a combined effect on inhibition of cellular proliferation, blockade of cell cycle at the G_1 to S phase transition, and the promotion of apoptosis. These features of P-N are likely COX independent, while the inhibitory effect of P-N on NF-κB may be a genuine molecular target of major mechanistic importance.

Conclusion and Future Directions

These studies confirm that P-N is highly effective in the treatment of RA and in the prevention of colon cancer.

In the treatment of RA, P-N was effective in reducing pro-inflammatory cytokines, PGE_2 synthesis, COX expression, and NF-κB activation. In colon cancer, P-N was highly effective in the prevention of colon cancer, which was associated with its ability to inhibit of cell growth and proliferation, induce cell cycle blockade and apoptosis, as well as inhibit nuclear NF-κB binding. Through these various COX-independent mechanisms of action, it is evident that P-N has significant potential to function as a treatment for RA and as a chemopreventive agent for colon cancer. One common modulator of these various COX-independent effects induced by P-N is NF-κB. NF-κB has been shown to control the expression of cytokines and COX, as well as regulate cell cycle kinetics and apoptosis. It is therefore likely that the modulation of NF-κB by P-N plays a significant role in its efficacy in the treatment and the prevention of these two diseases. Studies have demonstrated, previously, that NF-κB is a major factor in the development of colon cancer and the pathogenesis of RA. In fact, the therapeutic potential of NF-κB inhibition in the treatment of RA and cancer has been highlighted previously [232, 240].

It is clear that although P-N is structurally based on conventional naproxen, it is a new molecular entity with a distinct mechanism of action for the treatment of RA and prevention of colon cancer. P-N's pharmacological potential is significantly related to its considerable safety

over naproxen. P-N's significant safety far exceeds that of naproxen and which is present even at high drug doses makes it a strong candidate for clinical application. Notably, P-N's safety and efficacy provide an advantage in long-term clinical applications such as the treatment of RA and the prevention of colon cancer.

Interestingly, the strong inflammatory component in the pathogenesis of RA and colon cancer suggests that P-N may be effective in other inflammatory diseases such as various arthritides and various types of cancer. P-N's therapeutic potential for the treatment of cancer is a distinct possibility, as demonstrated by its efficacy in pre-established human cancer cells.

Given the susceptibility of P-N to ester hydrolysis by carboxylesterases, several options could be pursued to overcome this limitation. They include encapsulation in liposomes [203] or the use of carboxylesterase inhibitors [193]. Topical application of P-N is another avenue to be explored, especially in view of the successful topical use of phospho-NSAIDs formulated in a hydrogel and used to treat skin cancer [244].

Ultimately, the place of P-N in clinical pharmacology will be decided based on human studies. The present work provides a strong rationale and the background information required to pursue its clinical development.

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