LEADING ARTICLE

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Nitric Oxide–Releasing NSAIDs

A Review of Their Current Status

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drugs worldwide owing to their anti-inflammatory, antipyretic and analgesic properties. However, their use is hampered by gastrointestinal (GI) toxicity, the most common drug-related serious adverse event in industrialised nations.

Nitric oxide (NO)–releasing NSAIDs, a recently described class of drugs, are generated by adding a nitroxybutyl or a nitrosothiol moiety to the parent NSAID via a short-chain ester linkage. While efficacy of nitrosothiol-NO-NSAIDs still awaits investigation, nitroxybutyl-NO-NSAIDs have been extensively studied in animals, thus the abbreviation NO-NSAIDs used here refers to the latter group of NSAID derivatives.

NO-NSAIDs retain the anti-inflammatory and antipyretic activity of original NSAIDs, although they exhibit markedly reduced gastrointestinal toxicity. NO-NSAIDs are nonselective cyclo-oxygenase (COX) inhibitors, and they also exert COX-independent activities that are NO-dependent. Indeed, NO-NSAIDs suppress production of the cytokines interleukin (IL)-1β, IL-18 and interferon-γ by causing the S-nitrosilation/inhibition of caspase-1. In acute and chronic animal models of inflammation, it has been demonstrated that NO-NSAIDs abrogated prostaglandin E_2 as well as thromboxane B_2 generation. In a murine model, NO-naproxen was approximately 10-fold more potent than naproxen in reducing animal writhing after intraperitoneal injection of acetic acid. Similar data have been obtained in chronic models of pain such as rat adjuvant arthritis. *In vivo* and *in vitro* studies suggest that NO-aspirin (acetylsalicylic acid) exerts more potent antithrombotic action than aspirin, probably by coupling the ability to inhibit COX-1 with the anti-adhesive effect of NO. Moreover, in a model of renal injury NO-flurbiprofen not only has been demonstrated to be devoid of nephrotoxicity but also to ameliorate renal function. Finally, in an animal model of chronic neurodegenerative disease, NO-flurbiprofen and NO-aspirin attenuated the brain inflammatory response. The GI toxicity of NO-flurbiprofen and NO-naproxen is currently being investigated in healthy individuals.

1. Nitric Oxide (NO): An Ubiquitous Mediator

Nitric Oxide (NO) is a highly diffusible and lipid-soluble free radical which reacts with other free radicals, oxygen, and transition metals such as iron. Binding of NO with the iron group of haemoglobin facilitates the rapid degradation of NO. The interaction between NO and oxygen results in NO_2 production which rapidly consumes more NO to form N_2O_3, an excellent nitrosating agent of thiol-containing proteins. Under normal conditions, the enzymatic sources of NO are limited to what are called the constitutive, calcium-dependent forms of NO synthase (NOS): endothelial NOS (eNOS-, type III) and neuronal NOS (nNOS, type I). The other NOS isoform is referred to as inducible NOS (iNOS, type II), which is calcium/calmodulin independent and usually occurs in states of inflammation and immune activation. iNOS is generally expressed in macrophages and neutrophils, but it can also be detected in epithelial cells.[1,2]

NO is now recognised as an important modulator of an enormous number of physiological functions. Synthesis of NO in the endothelial cells which line the inner walls of blood vessels in response to physical and chemical stimuli, has been found to play a crucial role in maintaining vasodilation and is essential for the regulation of blood pressure.[3,4] Moreover, NO inhibits aggregation and adhesion of platelets to the inner walls of blood vessels and significantly reduces the formation of blood clots. Taken together, these effects account for the major role NO plays in protecting against stroke.

In the central nervous system, NO is a neurotransmitter that underpins several functions, including the formation of memory. In the periphery, there is a widespread network of nerves, previously recognised as nonadrenergic and noncholinergic, that operate through a NO-dependent mechanism to mediate some forms of neurogenic vasodilatation and regulate various gastrointestinal (GI), respiratory and genitourinary functions. Part of these actions are mediated by the activation of soluble guanylate cyclase and the subsequent increase in the concentration of cyclic guanosine monophosphate (cGMP) in target cells.[5]

Emerging evidence suggests that some diseases are related to defects in the generation or action of NO. A polymorphism of the eNOS gene has been described recently in patients with hypertension. In addition, NO is produced in large quantities during host defence and immunological reactions.

Because it exerts cytotoxic properties and is generated by activated macrophages, NO seems to play a role in nonspecific immunity. NO also modulates the activity of bone cells (osteoblasts and osteoclasts), immune cells, endothelial cells and stromal cells. Furthermore, NO is involved in the pathogenesis of conditions such as septic shock and the hyperdynamic state of cirrhosis and in inflammation.

In the GI tract, small quantities of NO exert beneficial effects by enhancing mucosal defence, whereas high levels of NO can be detrimental. In the stomach, NO modulates epithelial fluid and mucus secretion; it is an important mediator of vascular tone of the gastric microcirculation and stimulates mucosal healing by enhancing collagen deposition by fibroblasts and by angiogenesis.[6] Moreover, NO plays a major role in gastric mucosal defense, perhaps via the inhibition of leucocyte adherence to the vascular endothelium, and protects against mucosal injury by nonsteroidal anti-inflammatory drugs (NSAIDs) and by ischaemia-reperfusion.[7] A recently published case-control study found that the use of medications that release NO, such as nitroglycerin and other nitrovasodilators, was associated with a reduction in the incidence of gastric lesions in patients taking any type of NSAID.[8]

2. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) and Cyclo-Oxygenase (COX) Inhibition

NSAIDs are among the most widely prescribed drugs worldwide as the drugs of first choice in the treatment of rheumatic disorders and other degenerative inflammatory joint diseases. However, their use is hampered by the fact that NSAID-induced

GI toxicity is among the most common drug-related serious adverse events in industrialised nations.[7] As shown by endoscopic examinations, the prevalence of ulcers in the stomach or duodenum of patients who take NSAIDs regularly approaches 20%, and the annual incidence of clinically important lesions (i.e. GI bleeding and perforation) approaches 2%.[7,9]

NSAIDS inhibit cyclo-oxygenase (COX) and, subsequently, prostaglandin (PG) synthesis.[10] COX converts arachidonate freed from the plasma membrane by the action of phospholipase A_2 into several types of eicosanoids. It is now clear that at least 2 distinct COX isoforms exist. The constitutive isoform is termed COX-1 and is expressed in the stomach and platelets, and in fetal hearts, kidneys, lungs and brains, as well as in the decidual lining of the uterus. The inducible isoform, termed COX-2, appears during inflammation and is stimulated by potent inflammatory mediators such as the cytokines interleukin (IL)-1β, IL-2, IL-6, interferon (IFN)γ and tumour necrosis factor (TNF)α. However, the dichotomy between COX-1, acting predominantly as a constitutive enzyme, and COX-2, acting predominantly as proinflammatory molecule, is less clear cut than what was hypothesised a few years ago. COX-1 plays a role in inflammation, and COX-2 exerts a protective role in the stomach, intestine and kidney.[11,12] In any case, with few or no exceptions, all classical NSAIDs are able to suppress both COX-1 and COX-2.

While the therapeutic anti-inflammatory effects of these agents are attributable to their ability to inhibit COX-2, their tolerability profiles correlate with inhibition of COX-1 and decreased synthesis of gastric mucosal PGs. For this reason, COX-2-selective drugs, which are associated with significantly fewer clinically important upper GI adverse events, have recently been developed.[13] Several clinical studies have shown that COX-2-selective NSAIDs cause less GI toxicity and effectively suppress inflammation.[13]

3. NO-NSAIDS

The NO-NSAIDs are a recently described class of NSAID derivatives generated by chemically coupling a NO-releasing moiety to the parent NSAID via a short-chain ester linkage (fig. 1 and table 1).[14-17] The rationale behind this coupling is that NO and nitrogen oxide compounds released from these derivatives would enhance GI mucosal defenses and prevent the pathogenic events that occur with suppression of PG synthesis, such as reduced gastric mucosal blood flow, increased TNFα plasma levels and leucocyte-endothelial cell adherence.[14-17] Thus, NO released by these compounds may counteract the detrimental effect of NSAIDs on COX inhibition.

3.1 Pharmacokinetic Properties

NO-NSAIDs exhibit markedly reduced GI toxicity, while retaining the anti-inflammatory and antipyretic activity of the parent NSAID. Animal data

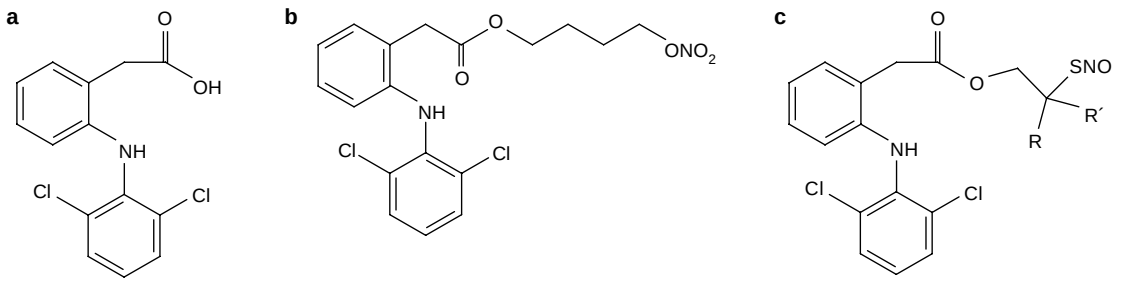

Fig. 1. Structure of diclofenac and its nitric oxide (NO)-releasing derivatives: (**a**) diclofenac, the parent compound; (**b**) nitroxybutyl diclofenac; and (**c**) nitrosothiol diclofenac.

Table I. Current status of nitric oxide (NO)-releasing nonsteroidal anti-inflammatory drugs in development

Drug	Company	Status
NO-aspirin (acetylsalicylic acid)[18]	Nicox	Phase I
NO-diclofenac[18]	Nicox	Preclinical
NO-naproxen[18]	AstraZeneca	Phase II
NO-ketoprofen[18]	Nicox	Preclinical
NO-flurbiprofen[18]	Nicox	Phase I
S-NO-diclofenac[19]	Nitromed	Preclinical

S-NO = nitrosothiol-nitric oxide-releasing.

demonstrate that NO-NSAIDs are metabolised differently than their parent NSAID. For example, nitrofenac, an NO-derivative of diclofenac, produces only 23% diclofenac and other metabolites measured as nitrite and nitrate.

Moreover, peak plasma concentrations of NO-NSAIDs are usually delayed compared with the parent NSAID. Nitrofenac peaks 7 hours after drug administration, while diclofenac has 3 peaks at 2, 5 and 10 hours.[15] Preliminary data indicate that after their absorption, NO-NSAIDs such as nitrofenac, NO-naproxen and NO-aspirin (acetylsalicylic acid) dissociate into 2 components, the parent NSAID and the NO-releasing derivative. Indeed, NO-aspirin administration results in a time-related increase in plasma nitrate/nitrite and salicylate concentrations. It has been demonstrated in humans and animals that hydrolysis of NO-aspirin might occur in the GI wall and fluids, and during the first pass of NO-aspirin through the liver.[14,16] Because the initial cleavage of NO-aspirin is relatively slow, the time required to obtain the salicylate peak plasma concentration after NO-aspirin administration is doubled compared with aspirin (6 and 3 hours, respectively), but the elimination rate of salicylic acid is similar for both compounds.[14,16] However, it is unlikely that this difference in NO-NSAIDs pharmacokinetics explains the reduced toxicity of these drugs, although it may account for the lack of hypotension seen with these compounds compared with equimolar doses of a standard NO donor.[16]

A potential limitation of NO-NSAIDs arises from their intrinsic nature as indirect sources of NO, suggesting that repeated administration of NO-NSAIDs may cause tolerance, a phenomenon frequently observed in nitrate-taking NSAIDs. This issue is particularly relevant for nitrosothiol-NO-NSAIDs. Indeed, this class of NO-NSAIDs is generated by adding a nitrosothiol (S-NO) moiety to conventional NSAIDs (such as diclofenac) through an ester linkage. These S-NO-NSAIDs, similarly to endogenous S-nitrosothiol compounds, act as NO donors without the need for metabolic transformation.[17,19,20] It is known that S-nitrosothiols can directly modulate cell physiology through S-transnitrosation reactions, by which the NO group is effectively transferred from the S-nitrosothiol to the thiol of a target biomolecule in exchange for a hydrogen.[21] Although nitrosothiol catabolism is incompletely understood, transition metal-dependent redox processes and/or enzyme-catalysed decompositions likely predominate biological pathways for NO release from nitrosothiols *in vivo*.

Oral administration of all forms of S-NO-diclofenac esters deliver diclofenac to the plasma to varying degrees, likely depending upon some salient structural features, particularly the chain length of the alkyl spacer and the substituent at the tertiary amine group. In all cases, however, plasma diclofenac levels peak within 30 minutes following oral administration, as would be expected if the S-NO-diclofenac esters were NSAID prodrugs *in vivo*. Comparable with the other NO-NSAIDs, these compounds exert anti-inflammatory activity similar to the parent NSAID, but are devoid of apparent GI toxicity.[21]

3.2 Mechanism of Action

NO-releasing NSAIDs are nonselective COX inhibitors. Their efficacy in reducing pain and inflam-

mation is largely attributable to suppression of PG synthesis. However, an increasing body of evidence indicate that NO-coupled NSAIDs exert COX-independent activities.[22-25] Indeed, we have recently demonstrated that NCX-4016, a NO-aspirin derivative, inhibits caspase activity and exerts anti-apoptotic and anti-inflammatory effects by reducing proinflammatory cytokine generation (fig. 2).[24]

Caspases are a family of intracellular cysteine proteases that share sequence homology with *Ced-3*, a nematode gene involved in the execution phase of apoptosis.[25] The mammalian counterparts of the *Ced-3* gene products include at least 14 different endoprotease that have been renamed caspases to denote cysteine proteases acting after an aspartic acid residue. Caspase-1 denotes the original IL-1 converting enzyme (ICE) that cuts the IL-1β and IL-18 (or IFNγ inducing-factor) precursor into an active mature form and has the greatest specificity for cleaving pro-IL-1β and pro-IL-18. The comparison of molecular structures suggests that the caspase family falls into 3 major groups. These include caspases that function primarily in cytokine maturation (e.g. caspase-1, -4 and -5); initiator caspases, involved in signalling early steps of extracellular regulated apoptosis (e.g. caspase-8, -9 and -10); and effector proteases involved in the execution phase of apoptosis (e.g. caspase-3, -6 and -7).

In support of this caspase functional specialisation, specific ICE inhibitors administered to mice exert poor anti-apoptotic effects, although they reduce inflammation as effectively as does blocking IL-1β activity with specific antagonists. In the past few years we have provided evidence that NCX-4016 inhibits the release of the proinflammatory cytokines (IL-1β, IL-18 and IFNγ) *in vitro* and *in vivo* through a mechanism that involves the S-nitrosylation/inhibition of proteases required for cellular processing/maturation of IL-1β and IL-18.[23] This effect is COX-independent since it cannot be reproduced by selective and nonselective COX-1 and/or COX-2 inhibitor. The mechanism through which NCX-4016 inhibits cytokine generation is largely dependent on the ability of this compound to inhibit ICE-like cysteine proteases involved in cutting pro-IL-1β and pro-IL-18.[21-25]

Likewise, endogenous NO derived from eNOS and/or iNOS inhibits T helper cell 1-type cytokine

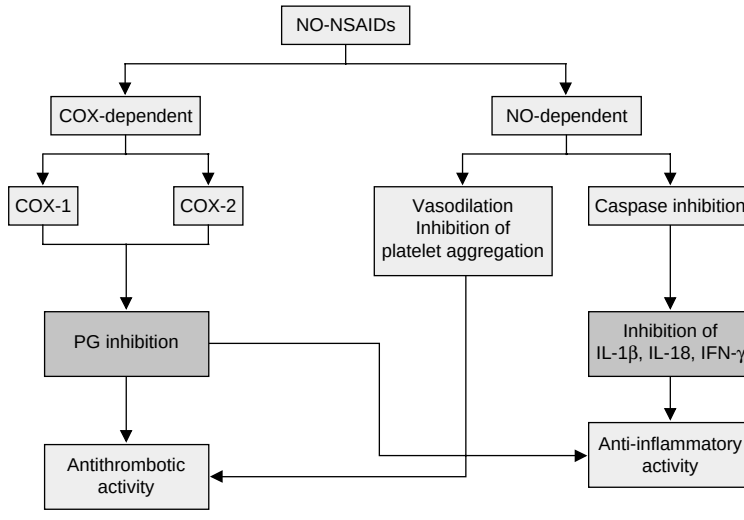

Fig. 2. Mechanism of action of nitric oxide (NO)-releasing nonsteroidal anti-inflammatory drugs (NSAIDs). NO-NSAIDs act via cyclo-oxygenase (COX)-dependent and COX-independent, NO-dependent mechanisms. Inhibition of cytokine generation may also contribute to their anti-inflammatory activity. **IL** = interleukin; **IFN**γ = interferon gamma; **PG** = prostaglandin.

production *in vitro* and *in vivo*. Selective ICE inhibitors have recently been demonstrated to be effective in reducing inflammation in patients with rheumatoid arthritis,[26] and caspase inhibitors are being developed by various pharmaceutical companies as anti-inflammatory and anti-apoptotic drugs. However, these anti-apoptotic compounds have consistent limitations due their intrinsic toxicity.[26] The fact that NO-NSAIDs inhibit caspase activity raises the possibility that they might have, in contrast to conventional NSAIDs, disease-modifying properties. The ability to inhibit proinflammatory and pro-apoptotic caspases is likely responsible for the extended range of activities of NO-NSAIDs in comparison with parent compounds.

4. Effects on the Gastrointestinal Tract

Our knowledge about the GI safety of NO-NSAIDs is mainly derived from animal studies. Available data show that NO-releasing NSAIDs spare the GI mucosa and that, at least in rats, any dosage of nitrofenac, nitrosodiclofenac, NO-naproxen, NO-flurbiprofen, NO-ketoprofen or NO-aspirin, causes significantly less gastric mucosal injury than standard NSAIDs.[14-17,19-25] A similar reduction of GI toxicity is observed if the compounds are given parenterally rather than orally, suggesting that the reduced injury is not simply attributable to reduced topical irritant properties.

Animal studies have demonstrated that NO-NSAIDs spare the stomach yet also inhibit gastric mucosal COX activity.[23,27-30] These results suggest that mechanisms other than PG inhibition are responsible for GI protection. Indeed, it has been demonstrated that topical application of NO-aspirin to the stomach markedly increased rat gastric mucosal blood flow, which remained significantly elevated for 30 minutes after removal of the NO-compound and did not exert any effect on potential difference and pH.[21,26-31] The administration of other NO-NSAIDs (NO-diclofenac and NO-flurbiprofen) induces the same effect on mucosal blood flow, suggesting that NO-NSAIDs are capable of protecting the GI mucosa from injury, possibly through preservation of mucosal blood flow.

Despite the effect of NO-NSAIDs on gastric microcirculation, it is noteworthy that, in experimental animals, they do not alter systemic arterial blood pressure, even when administered intravenously, in large doses or when administered during an experimental model of endotoxic shock.[14,30] In contrast, an equimolar dose of a conventional NO donor causes a profound hypotension. Although after NO-NSAIDs administration there is a significant increase in nitrate/nitrite plasma levels consistent with the release of NO in the systemic circulation, the absence of a hypotensive effect can be explained by the fact that the kinetics of hydrolysis of NO-compounds is very slow.

Not only are NSAIDs by themselves ulcerogenic in the stomach, but they also potentiate the ulcerogenic response to various stimuli, including stress. It has been demonstrated that, unlike aspirin, NO-aspirin does not potentiate the gastric ulcerogenic response to stress and confers a dose-dependent protection against hydrochloric acid- and ethanol-induced gastric damage.[28] Taken together, these data suggest that NO-NSAIDs are devoid of topical irritant action, are not ulcerogenic and do not potentiate the gastric ulcerogenic response to stress.[26] The absence of toxic GI effects is also confirmed in either normal or diabetic rat stomachs, which are known to be more vulnerable to NSAID-induced damage.[27]

Concerning the mechanism responsible for the lower rates of GI toxicity with NO-NSAIDs, we have recently demonstrated, *in vivo* and *in vitro*, that NO-aspirin protects the stomach by inhibiting gastric mucosal cell apoptosis and caspase activity (see section 3.2)[22]

Currently, 2 NO-NSAIDs, NO-flurbiprofen and NO-naproxen are being evaluated for GI toxicity in healthy volunteers. Thus far, the former compound was found to be significantly less injurious than flurbiprofen in healthy volunteers.[32] Data on NO-naproxen have not yet been published.

5. Effects on Renal Function

NSAIDs can severely depress glomerular filtration rate in patients with chronic renal failure by

inhibiting the synthesis of vasodilator PGs. Chronic NO inhibition leads to progressive arterial hypertension, glomerular ischaemic injury, glomerulosclerosis and interstitial inflammation, suggesting that the haemodynamic and cellular effects of NO are essential to maintain renal integrity and circulatory homeostasis.

NO-NSAIDs have reduced renal toxicity compared with parent NSAIDs.[33] Indeed, it has been demonstrated that NO-flurbiprofen is devoid of nephrotoxicity and ameliorates structural injury in remnant kidney of rats after surgical reduction of renal mass.[33] However, the mechanism underlying these protective effects are still unclear. It can be speculated that NO-flurbiprofen acts as a kidney NO donor, as indicated by the increase of the urinary excretion of nitrite/nitrate.[33,34]

6. Effects on Platelet Aggregation

Aspirin is a widely prescribed agent with antithrombotic effects at lower dosages (75 to 320 mg/day) than that necessary for anti-inflammatory activity. The antithrombotic effect of aspirin is attributable to its ability to irreversibly block thromboxane A_2 (TxA_2) in platelets, thus preventing platelet aggregation and vasoconstriction. However, even at antithrombotic doses, aspirin is associated with increased risk of GI damage.[35] Thus, it is clear that despite the effectiveness of prophylactic aspirin treatment in reducing major cardiovascular events, alternative antithrombotic drugs with lower risk of GI adverse effects are needed.

NO-aspirin, specifically NCX-4016, which spares the GI tract, is approximately 7 times more potent than aspirin as an inhibitor of thrombin-induced human platelet aggregation *in vitro*.[17] In *ex vivo* studies of ADP-, collagen- or thrombin-induced rat platelet aggregation, aspirin and NO-aspirin have been found to have comparable inhibitory effects.[17] Despite the fact that NO-aspirin inhibits platelet TxA_2 synthesis similarly to aspirin, this NO-NSAID has enhanced antithrombotic activity, suggesting that NO-aspirin inhibits platelet aggregation either by COX-1 inhibition or NO release.[17,36] In fact, haemoglobin, an NO-chelating agent, partially reverses the effect of NO-aspirin on platelet aggregation.

It is known that NO, by activating soluble guanylyl cyclase, acts primarily on the early phase of platelet activation,[17,36] thus modulating the expression of adhesion molecules. NO-aspirin, but not aspirin, at concentrations ranging from 2.5 to 500 μmol/L inhibits platelet adhesion by modulating the expression of these adhesion receptors. Moreover, in a manner similar to nitroprusside, NO-aspirin increases platelet cGMP levels and reduces the cellular calcium increase induced by thrombin. These data suggest that NO-aspirin has an anti-adhesive effect linked to the NO release.

7. Effects on Pain

The major indication for prescribing an NSAID is the treatment of pain. NO-NSAIDs, particularly NO-naproxen and NO-flurbiprofen, inhibit COX-1 and COX-2 and, therefore, their analgesic effect is largely dependent on the suppression of these COX activities. However, in several animal models NO-NSAIDs have demonstrated an increased analgesic activity in comparison with conventional NSAIDs, raising the question of whether additional mechanisms are involved in this effect. In a model of acute pain induced by intraperitoneal injection of acetic acid in mice, NO-naproxen was found approximately 10-fold more potent than naproxen in reducing animal writhing.[28] Similar data have been obtained in more chronic models of pain such as adjuvant arthritis in rats.[37,38]

8. Effects on Inflammation

The biological activity of NO-flurbiprofen and NO-naproxen has been evaluated in different experimental models of acute inflammation. In the zymosan-induced peritonitis model, polymorphonuclear leucocyte accumulation in the peritoneum was unaffected by flurbiprofen or NO-flurbiprofen (HCT-1026).[39] However, monocyte recruitment was selectively affected by NO-naproxen (49.6% inhibition), but not by flurbiprofen. Flurbiprofen and NO-flurbiprofen suppressed PGE_2 production in 4-hour inflammatory exudates as well as TxB_2 gen-

eration by activated platelets.[29,36,37] Similar results have been obtained with NO-naproxen in acute and chronic models of inflammation.[37] It is likely that the ability to suppress cytokine production is the underlying mechanism responsible for the increased anti-inflammatory potency of NO-aspirin in comparison with aspirin (see section 3.2).[23,37,38]

9. Effects in Neurodegenerative Disorders

Alzheimer's disease is associated with a distinct pattern of neuropathological changes and a dense distribution of highly activated astrocytes and microglia. Another pathological hallmark of Alzheimer's disease is increased levels of the pro-inflammatory cytokines, IL-1β, IL-6 and TNFα. Thus, it is not surprising that conventional NSAIDs have been shown to slow the progress, or delay the onset of Alzheimer's disease.[40] In addition, recent evidence suggested that neuronal COX-2 is elevated in the brains of patients with Alzheimer's disease, which suggests that long term inhibition of this enzyme might underlie the beneficial effects of NSAIDs in these patients.

In an animal model of brain inflammation, NO-flurbiprofen and NO-aspirin administered peripherally attenuated the extensive brain inflammation induced by continuous infusion of lipopolysaccharide (LPS) into the fourth ventricular space of the rat brain for 30 days.[41-43] Daily administration of NO-flurbiprofen (1, 5 and 15 mg/kg) significantly, and in a dose-dependent manner, attenuated brain inflammation as indicated by decreased density and reactivity of microglial cells. Daily administration of NO-aspirin (100 mg/kg) also attenuated the brain inflammation, but to a much lesser degree than NO-flurbiprofen.[41,42] These results suggest that NO-NSAIDs could reduce brain inflammation in rat model of brain inflammation.

In other experiments, NO-NSAIDs seem to protect from memory lost in Alzheimer's disease model.[41-43] Chronic LPS infusions impaired performance of young rats but not adult or old rats. Daily treatment with NO-flurbiprofen (15 mg/kg subcutaneously) improved the performance of LPS-infused young rats, but not LPS-infused adult or old rats. LPS infusions increased the number of activated microglia in young and adult rats but not old rats. NO-flurbiprofen treatment attenuated these changes. These results suggest that NO-NSAID therapies designed to influence the onset of Alzheimer's disease should be initiated in adults before age-associated inflammatory processes develop within the brain.

10. Effects on Bone

Bone remodelling depends on the coupled activity of osteoclasts, which are responsible for bone resorption and osteoblasts, which are associated with the bone formation. It has been demonstrated that eNOS is expressed in both osteoblasts and osteoclasts.[44] Since osteoblasts are significantly more abundant in bone and apparently express higher eNOS levels, their NO production is quantitatively more prevalent. As in other tissues, eNOS seems to be the main isoform expressed in bones in the basal state, while iNOS is expressed in osteoblasts during inflammation and is likely to be the main source of NO.

It has been shown that NO modulates the activity of both osteoblasts and osteoclasts *in vitro*. Moreover, a number of studies suggests that NO may have an anabolic effect on bone tissue.[44] Thus, NO donors increase osteocalcin synthesis and the formation of a mineralised matrix by osteoblasts *in vitro*, while NOS inhibitors have an antiproliferative effect on osteoblastic cells *in vitro*. On the other hand, the release of large amounts of NO by cytokine-stimulated cells may have an antiproliferative effect on osteoblasts. Therefore, NO appears to have a biphasic effect on bone forming cells: at low concentrations it promotes bone formation, whereas at high concentrations NO has an inhibitory effect on osteoblasts. Similarly, both stimulatory and inhibitory effects have also been reported in osteoclasts.

Based on this evidence, NO-NSAIDs have been tested *in vivo* to investigate their potential to treat or prevent bone loss. These preclinical studies have demonstrated that NO-flurbiprofen is significantly

more potent than flurbiprofen in inhibiting IL-1β release from murine osteoblast/bone marrow co-culture assay.[45,46] Since osteoblast-derived IL-1β is essential for osteoclast activation, it might be speculated that NO-flurbiprofen inhibits osteoclast formation by acting on bone-remodelling cytokines. Confirming this view, the NO-flurbiprofen effectively protected against bone mass loss induced by ovariectomy in mice.[45,46] Taken together, these data suggest that NO-flurbiprofen could be of clinical value in the treatment of bone diseases.

11. Effects on Neoplastic Cells

Numerous reports from human epidemiological studies, animal models, and *in vitro* cell culture experiments have suggested that NSAIDs may potentially be chemopreventive agents.[1] The only common denominator for the broad spectrum of nonspecific and COX-2-specific effects of the NSAIDs with chemopreventive potential is the ability to inhibit COX-2.[47] However, it is possible that non-COX actions could also be important targets of the antineoplastic effect of these drugs. Thus, the identification of the non-COX 2 pathways may reveal promising new targets for the design of antineoplastic agents. Hanif et al.,[48] observed that extremely high concentrations of sulindac sulfide and piroxicam decreased proliferation and increased apoptosis in both HCT-15 and HT29 cell lines. Supporting this view, it has been demonstrated that the NO-aspirin derivative NCX-4016, without suppressing systemic COX-1 and COX-2 activity, reduced the number of aberrant crypt foci in an animal model of colon cancer and exhibited chemopreventive effects superior to aspirin.[49] More recently the effect of NO-aspirin, NO-sulindac, and NO-ibuprofen has been tested on cultured HT-29 colon adenocarcinoma cells, and the authors have reported that NO-NSAIDs, by inducing apoptosis, reduce cell growth much more effectively than the corresponding NSAIDs.[50] Their superior effectiveness compared with traditional NSAIDs, makes NO-NSAIDs promising candidates for chemopreventive agents against colon cancer.

12. NO-Paracetamol (Acetaminophen)

Currently, paracetamol (acetaminophen) is a first-line agent for pain management and antipyresis in a variety of patients, including children, pregnant women and those with osteoarthritis and noninflammatory musculoskeletal conditions. Although paracetamol is not a true NSAID, it possesses part of the complement of NSAID therapeutic actions (analgesia and antipyresis), but it is devoid of anti-inflammatory and antithrombotic activity. When administered to humans, it reduces levels of PG metabolites in urine but does not reduce synthesis of PGs by blood platelets or by the stomach mucosa.[51]

Paracetamol is a weak inhibitor of COX-1 and COX-2 and displays some selectivity toward COX enzymes from different organs. Thus, it is a more potent inhibitor of COX from dog and rabbit brain than that those from dog spleen. The *in vitro* activity of paracetamol also depends on the addition of co-factors (e.g. in the presence of glutathione and hydroquinone, paracetamol inhibited COX activity). Recently, a variant of COX-2, known as COX-3, that is induced by high concentrations of the NSAID diclofenac and is more susceptible to inhibition by paracetamol than either COX-1 or COX-2, has been identified.[49] Inhibition of NSAID-induced COX activity by paracetamol but not aspirin, coupled with reduced sensitivity to competitively acting NSAIDs, suggests that large changes in the COX-2 active site have occurred because of chronic NSAID treatment.[51] Therefore, COX-3 may be a product of the same gene that encodes COX-2, but has specific and distinct enzymatic activities.

Owing to its relatively high safety profile and a low incidence of adverse effects, paracetamol is one of the most widely used analgesics, both in adults and children. However, paracetamol has been associated with hepatotoxicity, usually as a result of deliberate self-poisoning or accidental overdose. Nitroparacetamol (NCX-701), a newly synthesised NO-paracetamol, exhibits augmented antinociceptive activity in both rats and mice and exerts anti-inflammatory effects over the same dose range.[32] Moreover, high dosages of NO-paracetamol did not cause liver damage in mice.[52] These results in-

dicate that NO released from NO-paracetamol might exert hepatoprotective effects, suggesting that the drug may be considered a safer alternative to paracetamol in specific clinical conditions.

13. Conclusions

NO-NSAIDs are a new class of anti-inflammatory drugs. Although they were originally designed to spare the GI tract, the NO moiety appears to confer a broad range of activities to these compounds. NO-NSAIDs appear to exert their effect either through COX-dependent and COX-independent, NO-mediated pathways. The ability to inhibit ICE-regulated cytokines might explain the increased potency of these compounds in reducing inflammation. Together with the lack of hepato- and nephrotoxicity in animals, the lower incidence of GI toxicity with NO-NSAIDs suggests that clinical use of these drugs could contribute to reducing the burden of adverse events associated with selective and nonselective COX inhibitors. Clinical trials are indicated before the definitive role of NO-NSAIDs can be determined.[53]

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