Frontiers in Nephrology: Targeting Inflammation Using Novel Nitric Oxide Donors

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ABSTRACT
Chimeric molecules are single-chemical entities that possess at least two separate functions. In the design of new chimeric medicines, the two biologic actions are often designed to be synergistic and, thereby, complement each other in activating a specific target, such as a gene, a receptor, or an enzyme. In most chimeric molecules, one functionality is designed to provide a high affinity to a designated site, thereby permitting the targeting of the second functionality, which is usually nonspecific. This review focuses on the development of two classes of chimeric medicines, anti-inflammatory and diuretic chimeric agents, both of which incorporate a nitric oxide moiety into the parent pharmacophore.


CHIMERIC CYCLOOXYGENASE-2 INHIBITORS
Selective cyclooxygenase-2 (COX-2) inhibitors were developed because they were conceived to offer an improvement over existing nonselective nonsteroidal anti-inflammatory drugs (NSAID).9–17 The existing NSAID are widely known to cause adverse effects, such as gastrointestinal irritation and/or bleeding; in addition, their long-term use produces mild hypertension and a detriment in renal function, among other abnormalities.18–20 These unwanted actions have been shown to be a result of inhibition of COX-1, an enzyme that produces a family of prostaglandins that are considered fundamental to maintaining the health and integrity of many organs, including platelets, blood vessels, and the kidney.21–22 Inhibiting this enzyme and, thereby, eliminating prostanooids that promote normal homeostasis (beneficial prostanooids) results in a “deficient” organ whose ability to function is compromised. Reported reductions in various organ functions (e.g., platelet adhesion and aggregation, renal blood flow, endoscopic ulceration) all provide support for this mechanistic concept.23–26
The advantage of inhibiting selectively COX-2, an enzyme that is mainly induced as a result of an inflammatory stimulus, is that it will not inadvertently block the beneficial prostanooids that are produced by COX-1.10,27,28 The induced COX-2 enzyme was thought to generate excessive amounts of detrimental prostanooids at sites of injury, thereby amplifying the damage and overwhelming local host defense mechanisms. Although this simple concept is generally true, exceptions have become apparent as a result of the clinical use of selective COX-2 inhibitors.29 In particular, prostacyclin, a beneficial prostanooid that is generated by the

Published online ahead of print. Publication date available at www.jasn.org.

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endothelium and whose actions are important in maintaining vascular integrity and preventing the activation of circulating platelets and the adhesion of neutrophils, is synthesized via both COX-1 and COX-2 pathways. Moreover, the reduction of prostacyclin production in organs, such as the kidney, can lead to abnormal organ function that is indistinguishable from that caused by nonspecific NSAID.

Another enzyme that is induced along with COX-2 (and with similar kinetics) at the site of inflammation is NO synthase-2 (NOS-2). The NO that is generated by NOS-2 is part of a local host defense response and has complex biology. The freshly generated NO is capable of supplementing the local site levels of constitutive NO as the low-flux endogenous NO is rapidly oxidized and eliminated at the site of an inflammatory insult. The NO reacts with superoxide anions that are generated by COX-2 or other downstream sources that are activated by inflammatory stimuli to form an intermediate with powerful cytotoxic actions, peroxynitrite (ONOO⁻), thereby helping to eliminate the inflammatory stimulus (if a biologic agent) and creating a “noninfective” boundary around the site of injury to localize or prevent further expansion of the inflammatory event itself.

Hobbs et al. showed that certain mutations in the NOS-2 gene result in the generation of high fluxes of NO, which can have severely adverse consequences at an inflammatory site. In these cases, the levels of endogenous NO are bolstered by NOS-2, and a heightened adverse oxidant effect may be observed in the context of other inflammatory mediators. For example, if the endothelium has this mutation in its NOS-2 gene, then with inflammation and activation of COX-2 in the vessel wall, oxidative inactivation of NO would occur by virtue of its rapid reaction with superoxide and the subsequent generation of the cytotoxic peroxynitrite. In this setting, vascular relaxation would decrease and vascular leukocyte adhesion would increase. This effect would be further enhanced if a selective COX-2 inhibitor were given and local prostacyclin production also inhibited. In these cases, local homeostatic responses are virtually nonexistent. This concept is illustrated in Figure 1.

One way to combat this possible scenario and to improve the clinical usefulness of a COX-2 inhibitor is to transform it into a chimeric molecule that provides NO. The concept of linking two separate functions into such a single molecule is exemplified in Figures 2 and 3. In this example, the single-chemical entity, NMI-1093, has two biologic functions: One is the potent, selective inhibition of COX-2, and the other is the ability to generate NO rapidly (Schroeder JL, Bandarage RR, Bandarage UK, Earl R, Gzawa M, Fang X, Garvey DS, Gaston RD, Khanapure SP, Stevenson CA, and Wey SJ, NitroMed, Burlington, MA, 2001, unpublished data). In Figure 2, the des-NO analogue (NMI-1089) is also shown, to illustrate the enhanced benefits of the chimeric derivative.

NMI-1093 has a potency and selectivity for COX-2 comparable to the clinically approved drugs, such as rofecoxib. The des-NO analogue (NMI-1089) also retains excellent selectivity for COX-2 and is, thus, a good agent with which to examine the benefit of the added NO donor functionality that has been incorporated into its structure (NMI-1093). The relative potencies and selectivity of action are shown in Figure 4.

The anti-inflammatory properties that are inherent in the chimeric COX-2 inhibitor are illustrated in Figure 5. This classic animal model is often used to compare and rank the efficacies of both selective COX-2 inhibitors and nonselective NSAID. As shown, it is evident that...
NMI-1093 is a potent anti-inflammatory drug with a similar efficacy to that of rofecoxib and celecoxib. The compounds have similar duration of action and also exhibit similar potencies in a second animal model of inflammation, the rat paw edema model (data not shown; Trocha AM, Shumway M, Augustyniak M, Young D, Janero DS, NitroMed, Burlington, MA, unpublished data).

The benefit of the NO donor functionality that is inherent in NMI-1093 is illustrated in Figure 6. Figure 6A demonstrates the ability of NMI-1093 to inhibit ADP-induced platelet aggregation in vitro. The des-NO analogue and rofecoxib have no activity in this assay, whereas the widely known NO donor isosorbide dinitrate has similar activity to that of NMI-1093. Figure 6B extends this observation to an in vivo setting. In this anesthetized rat model, a short extracorporeal vascular loop is created, and a silk thread is placed in the loop for 15 min. During the time the thread is in contact with circulating blood, the foreign surface of the thread is the site of platelet deposition. This “white” thrombus on the thread can be removed and weighed, reflecting the capacity for platelet activation and adhesion. As illustrated, the chimeric molecule can prevent platelet deposition in vivo, whereas both rofecoxib and the des-NO analogue cannot.37

An important role of endothelial NO is the maintenance of vascular reactivity (Cochran E, Trocha M, Young D, Marek P, NitroMed, Burlington, MA, 2001, unpublished data).38 An adverse effect of NSAID and selective COX-2 inhibitors is a modest but significant elevation in BP.32,39 This effect can be demonstrated by measurement of the enhanced sensitivity to the vasoconstrictor angiotensin II in animals after long-term drug administration at dosages of drug that do not directly affect BP (7 d).40 As shown in Figure 7, there is an enhanced constriction response to infused angiotensin II in the presence of a selective COX-2 inhibitor that is not apparent in the presence of an equivalent dosage of a chimeric inhibitor. This lack of hypertension with chimeric NO donor drugs has also been reported with naproxcinod.41–43 Naproxcinod is a chimera of naproxen and a mononitrate donor functionality. This drug, which has recently completed phase 3 studies, is as effective an NSAID as naproxen itself; however, it

Figure 4. Inhibition of COX-1 and COX-2 thromboxane production in human whole blood by NMI-1093, NMI-1089, rofecoxib, and celecoxib. PG, prostaglandin (I2 or e2). Data are percentage of inhibition and are the mean of six experiments.

Figure 5. Effect of oral administration of equimolar doses (15 μmol/kg) of celecoxib, rofecoxib, valdecoxib, etoricoxib, NMI-1093, and NMI-1089 in the rat carrageenan air pouch model of inflammation. Results are expressed as white blood cell (WBC) content in the pouch exudates and are means ± SEM (n = 5 to 12). **P < 0.01 versus vehicle.

Figure 6. (A) Inhibition of ADP-induced aggregation of human platelet-rich plasma by isosorbide dinitrate (ISDN), NMI-1093, and NMI-1089. Data are percentage of inhibition relative to the aggregation of control platelets that were not treated with a test agent and are means ± SEM (n = 6). (B) Effect of oral administration of rofecoxib, NMI-1093, or NMI-1089 on in vivo thrombus formation in an anesthetized rat arteriovenous shunt model. Data are expressed as thrombus weight (mg) and are means ± SEM (n = 5 to 8). *P < 0.05 versus vehicle or rofecoxib.
does not cause elevations in BP and has a reduced gastric irritancy profile.\textsuperscript{13}

In summary, this new class of medicines, chimeric COX-2 inhibitors, has the potential to provide effective anti-inflammatory and analgesic activities with a better safety profile than the existing COX-2 inhibitors. The benefits of possessing bifunctional activities include minimal gastrointestinal irritation, improved vascular reactivity (antihypertensive properties), enhanced antiplatelet properties, and improved renal function, among others.

CHIMERIC DIURETICS

The second example of a chimeric pharmacologic agent is illustrated in Figure 8. In this example, NO has been linked via a nonhydrolyzable bond to the diuretic hydrochlorothiazide (HCTZ). Linking the nitrate to HCTZ targets the release of NO to the kidney.

Results and guidelines from the Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT)\textsuperscript{44} and the joint National Committee on Prevention of High Blood Pressure\textsuperscript{38} suggest that thiazide diuretics should be used as first-line therapy, either alone or in combination with other antihypertensive agents. There are more than 27 marketed HCTZ combinations, and, although their use is encouraged, these diuretics suffer from a series of adverse effects, particularly when used in higher dosages. The practice of using HCTZ combination products (e.g., Aldactazide) is intended to lower the dosage of HCTZ and to add an agent with a complementary mode of action to assist in adequate BP control, minimizing undesirable effects of either agent used alone in the usual dosages. The adverse effects of HCTZ include electrolyte imbalance, stimulation of the renin-angiotensin system, orthostatic hypertension, and renal dysfunction, as well as the onset of metabolic disturbances, such as new-onset diabetes.

The benefit of creating a chimeric HCTZ is to target NO to the kidney, where it can have a multiplicity of actions, all helping to improve the clinical profile of HCTZ itself. These actions include reduced HCTZ-induced electrolyte imbalance, enhanced systemic and renovascular homeostatic control, inhibitory actions on the release of deleterious neurohormones (renin-angiotensin-aldosterone system), reduced metabolic disturbances, and reduced diuretic tachyphylaxis.

As shown in Figure 9, the chimeric HCTZ NMI-3377 has a similar potency and duration of action as HCTZ itself. In this rat model, similar urinary volumes and time courses were measured (Kuo JT, Wong AL, Wexler RS, Zifcak BM, Ellis JL, Dube GP, Selog WM, NitroMed, Lexington, MA, 2005, unpublished data). In Figure 10, the measurement of elevated NO metabolites is plot-
As shown, increases in renal levels of the derivative metabolites of NO, nitrite, S-nitrosothiols, and NO hemes are apparent for approximately 3 h after dosing (Dhawan V, Dube GP, Ellis JL, Schwalb DS, Shumway MJ, Warren MC, Zemtseva IS, Janero DR, NitroMed, Lexington, MA, 2005, unpublished data).

The results of enhanced NO delivery to the kidney are seen in reduced circulating levels of the renin and aldosterone (Figure 11). This effect has been demonstrated in rats in which minipumps that administered equimolar doses of either NMI-3377 or HCTZ continuously for 4 wk were implanted (Melim TL, Gordon LJ, Wong HL, Wexler RS, Ellis JL, NitroMed, Lexington, MA, 2005, unpublished data). The HCTZ-induced increases in these vasoconstrictor hormones were significantly attenuated in the NMI-3377-treated animals, and these actions would be expected to translate into improved endothelial function, reduced chronic fibrotic changes in the cardiovascular system and kidney, and better BP control.

In SHR-SP-treated rats that received an NO synthesis inhibitor (to suppress endogenous NO and, thereby, better assess the benefit of the chimeric molecule), NMI-3377 produced a significant reduction in the excretion of urinary albumin when compared with HCTZ. This model involves treatment of groups of animals with vehicle or equimolar doses of HCTZ or NMI-3377. After 7 d, l-nitroarginine methylester (NOS inhibitor) was introduced, and all animals were then monitored for an additional 25 d before being killed and measurement of renal function. Figure 12 shows that the improvement in the excretion of urinary albumin in animals that were

Figure 10. Levels of nitrite (µM) (upper graph), NO-HEME (nM) (lower left graph), and S-nitrosothiols (RSNO; nM) (lower right graph) in the kidneys of rats after oral administration of equimolar doses of HCTZ and NMI-3377 at 0, 15, 30, 60, 180, and 360 min. Data are means ± SEM (n = 6 per group).

Figure 11. Plasma renin (A) and aldosterone (B) levels before (day 0) and after 29 d of oral administration of vehicle, HCTZ (10 mg/kg per d), or NMI-3377 (10 mg/kg per d HCTZ equivalent). Data are expressed as relative units (renin) or pg/ml (aldosterone) and are means ± SEM (n = 8 per group). *P < 0.05; **P < 0.001.

Figure 12. Cumulative urinary albumin in rats after oral administration of vehicle alone (black), vehicle plus l-nitroarginine methylester (L-NAME; green), HCTZ plus L-NAME (blue), NMI-3377 plus L-NAME (yellow), or valsartan plus L-NAME (red). Rats were treated for 35 d (10 mg/kg per d, HCTZ or HCTZ equivalent). Data are expressed as mg and are means ± SEM (n = 6 to 10 per group). *P < 0.05 versus L-NAME or vehicle; +P < 0.01 versus L-NAME or vehicle.
treated with the chimeric diuretic NMI-3377 is significantly better than that in animals that were treated with HCTZ and comparable to animals that were treated with valsartan.

CONCLUSIONS

This review presented the creation and benefit of two classes of chimeric pharmacologic agents. In both examples, the two functions that have been incorporated within a single molecule have resulted in the targeting of NO to a particular site of action or organ. In addition, in both examples, the clear benefit in biologic profiles and improvement in efficacies should provide significant advances in the treatment of inflammatory and cardiorenal diseases.

DISCLOSURES

G.L. is an employee of NitroMed, Inc.; and J.L. is a member of the board of directors and holds stock in NitroMed, Inc.

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