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PROGRESS TOWARD CLINICAL APPLICATION OF THE NITRIC OXIDE–RELEASING DIAZENIUMDIOLATES[1]

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■ **Abstract** Diazeniumdiolates, compounds of structure $R^1R^2NN(O)=NOR^3$, which have also been called NONOates, have proven useful for treating an increasing diversity of medical disorders in relevant animal models. Here, I review the chemical features that make them such excellent starting points for designing materials capable of targeting reliable and controllable fluxes of bioactive NO for in vitro and in vivo applications. This is followed by a consideration of recent proof-of-concept studies that underscore what I believe to be the substantial clinical promise of such materials. Examples covered include progress toward inhibiting restenosis after angioplasty, preparing thromboresistant medical devices, reversing vasospasm, and relieving pulmonary hypertension. Together with a very recent report describing the beneficial effects of diazeniumdiolate therapy in a patient with acute respiratory distress syndrome, the results of the animal experiments support the prediction that a broad selection of problems in clinical medicine can be solved by judiciously mining the enormous variety of possible $R^1R^2NN(O)=NOR^3$ structures.

INTRODUCTION

> *. . . Our current strategy . . . is quite simple: first bring NONO-2 to the market; second, start working on the next generation of products; and, third, explore some of the other therapeutic possibilities. . . . We could become the nitric oxide powerhouse of the pharmaceutical industry!*
>
> *Musings of corporate executive Renu Krishnan in "NO" (1, p. 116)*

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It seems from the quotation above that Renu Krishnan, the entrepreneurial protagonist of pharmaceutical innovator Carl ("Father of the Pill") Djerassi's recent novel *NO*, sensed early in her career that the nitric oxide (NO)-releasing diazeniumdiolates provide an unusual opportunity for rationally designing broad-spectrum drugs and biomedical devices. In Djerassi's story line (1), Renu parlays her knowledge of diazeniumdiolate chemistry into a blockbuster drug called "NONO-2" for treating male erectile dysfunction, ultimately earning her both international acclaim and enough personal wealth to fund her own basic research aimed at conquering other challenging medical problems, including pulmonary hypertension, cancer, and infectious disease.

I think Renu is right. Here, I review the growing body of in vivo proof-of-concept reports demonstrating the diazeniumdiolates' efficacy in relevant animal models for treating a variety of clinical disorders. These reports have convinced me that within this chemistry lurks a vast opportunity for designing solutions to a host of biomedical problems.

WHAT ARE DIAZENIUMDIOLATES AND WHAT MAKES THEM SO USEFUL FOR RATIONALLY DESIGNING NEW DRUGS AND BIOMEDICAL DEVICES?

Although Renu, her company, and presumably also her ability to fund her own wide-ranging basic research program in this day and age are all fictional, the compound class at the heart of her success is not. Diazeniumdiolates (or NONOates, as she also calls them) are compounds having the generic structure shown in Figure 1. They are blessed with three attributes that make them an especially attractive starting point for designing solutions to important clinical problems, namely structural diversity, dependable rates of NO release, and a rich derivatization chemistry that facilitates targeting of NO to specific sites of need—a critical goal for therapeutic uses of a molecule with natural bioeffector roles in virtually every organ.

Regarding structural diversity, all three R groups of Figure 1 can be varied over a wide range to produce isolable materials. Salts in which R^3 is ionically bound to the diazeniumdiolate oxygen have proven especially useful for generating controlled fluxes of NO spontaneously in aqueous media. Depending on the identity of the R^1R^2N group of Figure 1, reproducible half-lives of NO generation ranging from 2 s to 20 h have been documented for the simple diazeniumdiolate salts characterized thus far. The structures of ionic diazeniumdiolates discussed in this review are shown in Figure 2 along with their half-lives at pH 7.4 and 37°C.

Importantly, these solution half-lives tend to correlate very well with their pharmacological durations of action. This suggests that they are minimally affected by metabolism and are thus fundamentally different from currently available

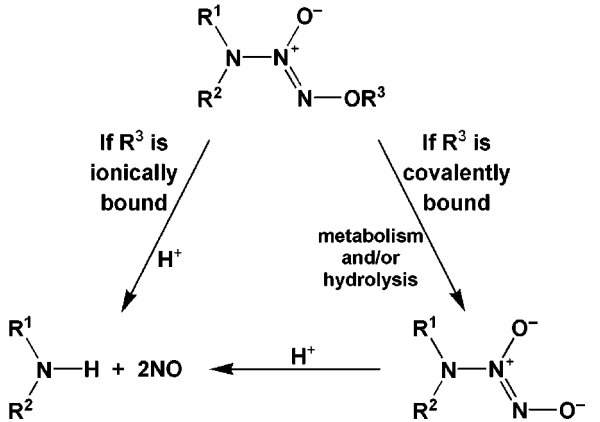

Figure 1 Mechanisms of NO release for the diazeniumdiolates discussed in this chapter. If R^3 of the generic structure shown at the top is a cation, NO is generated spontaneously on protonation of the anionic portion's R^1R^2N nitrogen (2), as shown at the left. If R^3 is covalently bound, it must be removed to free the anion (as shown on the right) before spontaneous NO generation can begin; some examples of masking the diazeniumdiolate ion in this way for pharmacological advantage are listed toward the end of the chapter, along with some specific mechanisms by which such a cleavage can be effected.

clinical nitrovasodilators that require redox activation before NO is released. Even SPER/NO, whose half-life has proven to be the most variable (2, 3) among the diazeniumdiolates investigated thus far (see Figure 2), has consistently shown pharmacological effects expected of a spontaneous NO-releaser intermediate in half-life between the rapid and slow examples of Figure 2. Of course, as ions of low molecular weight, these agents should be subject to excretion after in vivo administration, even in the absence of metabolism; thus, the 20-h in vitro half-life of DETA/NO (Figure 2) does not translate to a 20-h half-life for its in vivo biological effects, as is illustrated below.

The enormous diversity of isolable materials that can be synthesized by varying R^1 and R^2 of Figure 1 can be extended even further by covalently binding different R^3 groups to the terminal oxygen of the ionic diazeniumdiolate moiety. This can lead to a variety of well-defined outcomes. For example, when R^3 is a methoxymethyl (MOM) group and R^1R^2N is a piperazine ring, the diazeniumdiolate (MOM-PIPERAZI/NO; see Figure 3) is still a spontaneous NO releaser at physiological pH, but the rate-limiting step is hydrolytic cleavage of the MOM group; in this case, the MOM derivative's half-life for NO generation is 17 days (4). Most other diazeniumdiolates with covalently bound R^3 groups have proven effectively stable toward spontaneous hydrolysis, but by choosing R^3 groups that

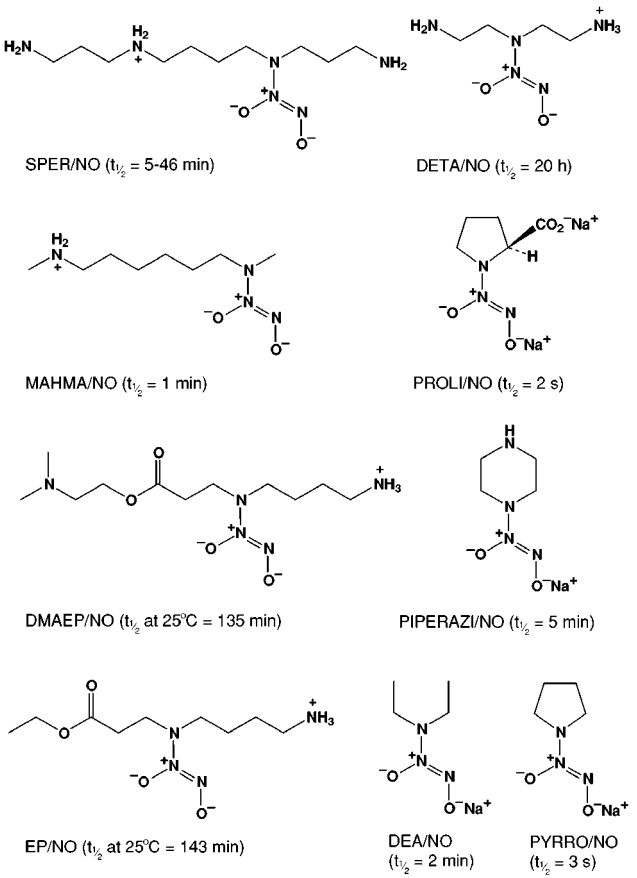

Figure 2 Structures and half-lives ($t_{1/2}$) in pH 7.4 phosphate buffer at 37°C of ionic diazeniumdiolates discussed in this chapter.

are vulnerable to cleavage by specific enzymes, agents capable of cell- or organ-selective NO generation after system-wide administration can be prepared. A case in point is AcOM-PYRRO/NO (see Figure 3), a cell-permeant neutral molecule that has proven stable in cell culture medium but undergoes hydrolysis upon catalysis by intracellular esterases. This feature was exploited in an in vitro test of NO's antileukemic activity (5) in which the ability of AcOM-PYRRO/NO to concentrate NO release inside the cell made it orders of magnitude more cytotoxic than the ion produced on esterase-induced AcOM-PYRRO/NO hydrolysis (PYRRO/NO, which generates extracellular NO homogeneously throughout the medium with a half-life of 3 s; see Figure 2). The structures of AcOM-PYRRO/NO and other neutral diazeniumdiolates bearing covalent R^3 groups that are discussed in this

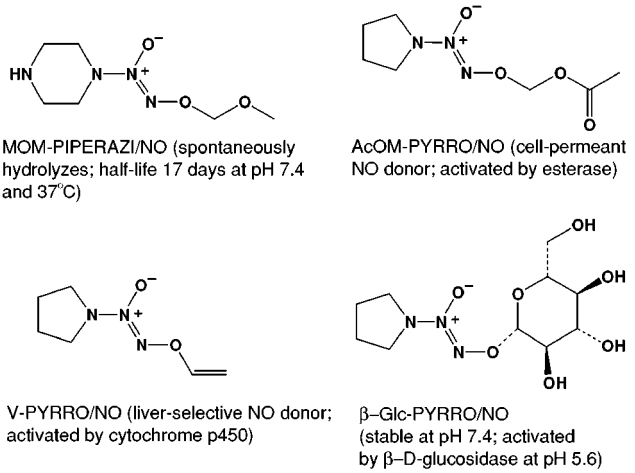

Figure 3 Structures of O^2-substituted diazeniumdiolates discussed in this chapter, along with conditions reported to convert them to NO-releasing form.

chapter are shown in Figure 3, together with the conditions under which they are known to release NO.

WHAT CLINICAL PROBLEMS HAS DIAZENIUMDIOLATE CHEMISTRY SHOWN THE POTENTIAL TO SOLVE THROUGH PROOF-OF-CONCEPT STUDIES IN RELEVANT ANIMAL MODELS?

The ease of preparing an exceptional breadth of diazeniumdiolate structures and the wide array of well-defined spontaneous and enzymatically induced NO generation patterns they provide make this class of compounds an ideal starting point for targeting NO selectively to bodily sites of need. This is a critical requisite for successful NO donor drug design, given the fact that NO affects the function of essentially every organ system we own. Several strategies for achieving such therapeutically useful targeting have been introduced, including local administration of spontaneous NO releasers having the appropriate half-life and duration of action, incorporation of the diazeniumdiolate group into insoluble polymers whose NO-derived effects are limited to the cells and physiological fluids in the immediate vicinity of the polymer surface, and design of prodrugs that are stable in the systemic circulation but activated for NO release by enzymes concentrated in the target organ or cell type. Examples of each strategy are described below.

Restenosis After Angioplasty

Blood vessel disorders are a leading cause of incapacity and death. Among the most common, and serious, of these is coronary artery disease, in which blood flow through the vessels supplying the heart muscle becomes restricted. When this happens through buildup of atheromatous plaque, compounded in some cases by acute thrombosis, a thickening of the vessel wall results; a procedure known as angioplasty has come into wide use as a means of restoring normal coronary blood flow. As illustrated in Figure 4, the procedure involves insertion of a balloon at the diseased site via a catheter. Inflating the balloon expands the artery beyond its ability to recoil, opening the vessel to its proper diameter for immediate restoration of proper perfusion.

The problem with this procedure is that the arterial overstretch that is crucial to its short-term success often gives rise to a long-term complication called restenosis. Overstretch is accompanied by extensive tearing and fracturing of the affected vessel that the body immediately seeks to heal, but in a substantial percentage of patients the healing mechanisms overreact, producing more vascular smooth muscle cells than are destroyed. This leads to further thickening of the vessel wall

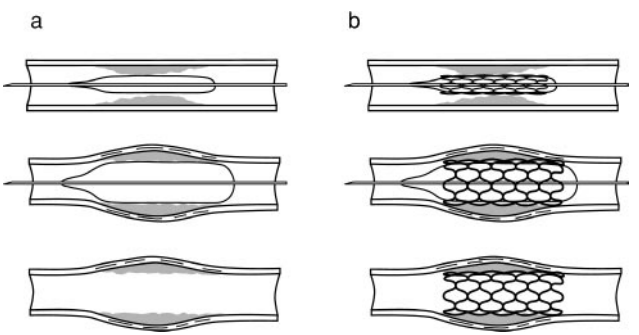

Figure 4 Balloon angioplasty with and without stent deployment. (*a*) In balloon angioplasty, a thin catheter is threaded through the circulatory system until the uninflated balloon at its tip penetrates the diseased artery at the point of blockage, as shown in the top diagram. The balloon is then inflated to expand the artery, as shown in the middle, before being deflated and withdrawn to allow blood flow to resume (*bottom panel*). (*b*) An increasingly common feature of angioplasty involves deployment of an expandable wire structure to help keep the artery from collapsing after the balloon is withdrawn. The procedure is the same as in (*a*), except that a wire stent is placed over the balloon before insertion (*top*). The stent expands when the balloon is inflated (*middle*) and retains its expanded form after the balloon and catheter are withdrawn (*bottom*), remaining in place after the procedure is complete to provide a permanent structural support for the arterial wall.

and thus renewed restriction of blood flow. It is reported that approximately 40% of the more than 1,000,000 coronary angioplasties performed each year in the United States and Europe show angiographic evidence of restenosis within six months after the procedure (6).

A possible approach to reducing the risk of restenosis following such surgical interventions has been suggested by the work of Kaul et al. (7). Recognizing that NO is an efficacious inhibitor of vascular smooth muscle cell proliferation, they hypothesized that exposing the outer (adventitial) side of the vessel to NO during and after inflation of the balloon may reduce the excessive production of neointimal cells on the inside of the vessel. To test this hypothesis, they surgically exposed a portion of a rat's iliofemoral artery and covered its adventia with a gel containing SPER/NO (an adduct of the physiological polyamine spermine with NO that regenerates NO and spermine on contact with sources of hydrogen ion; see Figure 2) before expanding the balloon and examined the vessel cross-section two weeks later. As shown in Figure 5, the SPER/NO-treated artery was similar in appearance to the normal, healthy, uninjured control, but the artery that was exposed to the carrier molecule spermine alone closely resembled the injured but untreated control.

This result clearly supported a potentially beneficial role for advential delivery of NO as an approach to promoting vascular healing after angioplasty, but it did not, at first blush, seem applicable to the problem of restenosis in the coronary arteries. If, to apply it, one would have to physically expose and directly contact a vessel on the surface of the heart, one might just as well circumvent the blockage using the time-tested approach of bypass surgery.

How, then, to localize NO delivery to the outer surface of a coronary artery? It happens that the human heart is largely enveloped by a flexible sac called the pericardium. The coronary arteries are seen as bulges on the portion of the heart within this sac and are bathed more or less continuously by the fluid it contains. With this in mind, Baek et al. (8) tested the hypothesis that injection of a long-lasting NO donor into the pericardial fluid immediately before angioplasty would minimize neointima formation after balloon overstretch. They started by considering the longest-acting spontaneous NO donor of Figure 2, DETA/NO. Unfortunately, its 20-h chemical half-life, as measured in 0.1 M phosphate buffer at 37°C, was partially canceled as an advantage by its fairly rapid clearance from the incompletely isolated pericardial compartment; experiments with a radioiodinated surrogate of this very low molecular weight (163 g/mol) agent pointed to a pericardial residence half-life of less than 5 h. Reasoning that a much larger molecule might be better retained in the pericardial space, they then considered a diazeniumdiolated bovine serum albumin derivative (D-BSA) prepared by joining 22 MOM-PIPERAZI/NO (see Figure 3) moieties to each protein molecule using standard linker chemistry. Consistent with the molecular size hypothesis, this high-mass protein derivative (74,000 g/mol) exhibited an intrapericardial residence half-life of over 22 h. Combining the chemical and biological kinetic data indicated that the initial rates (in

moles of NO per h per mole of agent dosed) of NO release from equimolar boluses of DETA/NO versus D-BSA would be almost identical, but that the flux 24 h later would be more than 30-fold greater for the diazeniumdiolated protein.

The protein derivative was thus chosen over DETA/NO for further work in which the researchers injected it into the pericardial fluid of the pig immediately before angioplasty and examined the treated vessel segment two weeks later. At a dose of 400 mg in 10 ml of buffered saline per pig, a concentration similar to that of normal albumin in serum, D-BSA reduced intimal hyperplasia by half, relative to that seen in controls receiving underivatized bovine serum albumin. Very importantly, a substantial portion of the neointimal cells produced in the healing process were seen to have concentrated themselves in the crack resulting from balloon-induced rupture of the arterial wall. This positive remodeling effect greatly enlarged the vessel diameter relative to controls treated with underivatized albumin, as illustrated in Figure 6.

These results strongly support the hypothesis that controlled dosage of NO over the outside of a coronary artery undergoing angioplasty can substantially benefit the healing process, but the procedure requires insertion of two catheters, one for delivering drug into the pericardium and a separate one for balloon deployment. To avoid this complication, one might envision dosing the NO into the inside of the artery during and for a therapeutically relevant length of time after the overstretch. One way to accomplish this in principle is to employ an NO-releasing stent. As illustrated in Figure 4, a stent is a semiflexible metallic mesh that is placed over the balloon such that inflation expands it into a rigid structure designed to oppose any tendency of the vessel to re-constrict to its pre-angioplasty diameter. Stent use has cut the restenosis rate from 40% in balloon-only patients to 20%–25% (6). Coating the stent surface with an NO-generating material could be an ideal way to reduce the restenosis risk even further by dosing the diseased portion of the artery undergoing angioplasty with NO.

Here again, the diazeniumdiolates' chemical versatility provides a convenient approach to localizing NO's effects at the site of need. Choosing selected silicon-bearing R groups from the effectively infinite variety of Figure 1 molecules, one investigation has focused on the silane-based strategy illustrated in Figure 7. In this structure, DETA/NO (the long-lived NO generator of Figure 2) molecules are covalently attached via conventional silanization reactions to the surface of the metallic stent. The spontaneous release of NO that was seen when the silanized, diazeniumdiolated metal surface was immersed in aqueous buffer (9) would be expected to occur when it is exposed to the aqueous conditions surrounding the fresh wound that is created as the balloon-driven stent struts push out sharply against the arterial wall; NO's rapid reaction with oxyhemoglobin would be expected to keep it from traveling very far downstream, and the fact that the NO carrier portion is covalently anchored to the metal surface should combine to minimize systemic effects. Diazeniumdiolated metal surfaces, such as that shown in Figure 7, have yet to be tested in a human-relevant animal model of balloon angioplasty, but further research and development may result in significant benefits with respect to postintervention healing, including the potential for reestablishment of a

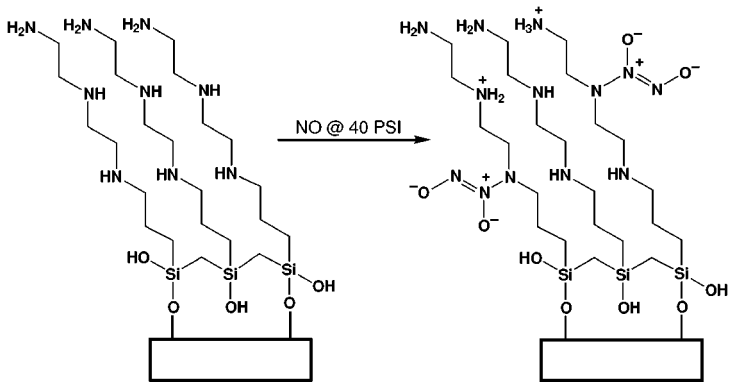

Figure 7 Preparation of NO-releasing metal surfaces (9).

functioning endothelium (the layer of cells that line the normal artery and synthesize essential vasoactive substances, such as NO, but that is often irreparably damaged in conventional vascular surgery procedures).

Thrombogenicity of Medical Devices

Foreign objects implanted in the body tend to induce a physiological response that includes adhesion of platelets to the implant surface as an unwanted, potentially life-threatening side effect (10). As one example, thrombus formation at the site of stent deployment is an occasional cause of acute angioplasty failure. Concurrent dosage with systemic anticoagulants is used to minimize this complication, but this necessarily increases the risk of uncontrolled bleeding at the point of catheter insertion or at potentially unrecognized sites in the brain or elsewhere that may be vulnerable to hemorrhage.

It happens that NO potently inhibits not only vascular smooth muscle cell proliferation, but also the adhesion, aggregation, and activation of platelets. With this in mind, it will be interesting to see whether the diazeniumdiolated metal stents derivatized as in Figure 7 will both inhibit acute thrombotic failure via NO's antiplatelet activity as well as reduce restenosis risk by way of its cytostatic effect in vascular smooth muscle.

But a stent is only one of many devices whose surfaces may be advantageously diazeniumdiolated, and covalent silanization of a metal is only one way to accomplish it. In the following paragraphs, I illustrate three different chemical approaches to diazeniumdiolating implant surfaces for three different in vivo applications.

VASCULAR GRAFTS When dealing with diseased or damaged blood vessels, surgeons often need to replace, rather than repair, the affected segment. This is usually done via transplants from elsewhere in the patient's own circulatory system, but artificial blood vessels (preferably known as synthetic vascular grafts) are often

used instead. Unfortunately, currently approved synthetic grafts are prone to clogging with thrombus, especially when their diameter is small (2–6 mm) (10).

With the goal of strictly confining the antithrombotic effect to the surface of the graft, thereby obviating the need for systemic anticoagulants, Smith et al. dipped small (4 mm)-diameter porous Teflon graft segments into a freshly prepared solution of both polyethylenimine (PEI) and a cross-linking agent such that the cross-linked PEI chains became intimately interwoven with, and thus securely fastened to, the fabric of the graft (11). Half of the segments were then exposed to NO to establish a multitude of diazeniumdiolate groups at secondary amine sites on the PEI, whereas the other half were left to serve as untreated controls. The segments were then placed into the unheparinized baboon circulatory system. The results are shown in Figure 8.

The diazeniumdiolated grafts experienced very little accumulation of thrombus during the 1-h observation period, whereas, as expected, platelet deposition steadily increased in the control grafts (11). In vitro measurement of the grafts' capacity for NO generation revealed a slowly declining rate that was observable by chemiluminescence methods for several weeks, suggesting that they might have remained thromboresistant far beyond the 1-h observation period of Figure 8. If so, and if the PEI-based coating remains biocompatible throughout the lifetime of the device, significant improvements in vascular graft technology could be forthcoming.

EXTRACORPOREAL MEMBRANE OXYGENATION (ECMO) CIRCUITS ECMO is a procedure of last resort for certain patients whose lungs do not function properly. It involves pumping their blood out of the body and through an oxygenating chamber, then back into the patient's circulatory system. A major complication of this invasive procedure is loss of platelets through adhesion to the inner surface of the tubing used to conduct the blood through its extracorporeal circuit. As with the abovementioned vascular surgery applications, systemic anticoagulant treatment with all its attendant disadvantages is a necessary part of conventional ECMO therapy (12).

M. Meyerhoff and his colleagues at the University of Michigan have exploited diazeniumdiolation chemistry by engineering significant thromboresistivity into ECMO circuitry. For one such formulation, they simply blended MAHMA/NO (structure shown in Figure 2) into poly(vinyl chloride) (PVC) to use as a coating for the internal surface of the tubing. After applying a topcoat of unblended PVC, the tubing was tested in an unheparinized rabbit model of ECMO. As shown in Figure 9, a significant reduction in platelet loss compared to heparinized, PVC-only controls was seen throughout the 4-h observation period (13).

CHEMICAL SENSORS The Meyerhoff group has demonstrated even longer periods of thromboresistivity for implantable devices that are central to their mainline research effort. As analytical chemists, they have long focused on developing electrodes that can give real-time readouts for clinically significant analytes in a patient's blood. However, platelet deposition on the electrode surface has been a major source of inaccuracy in the observed readings.

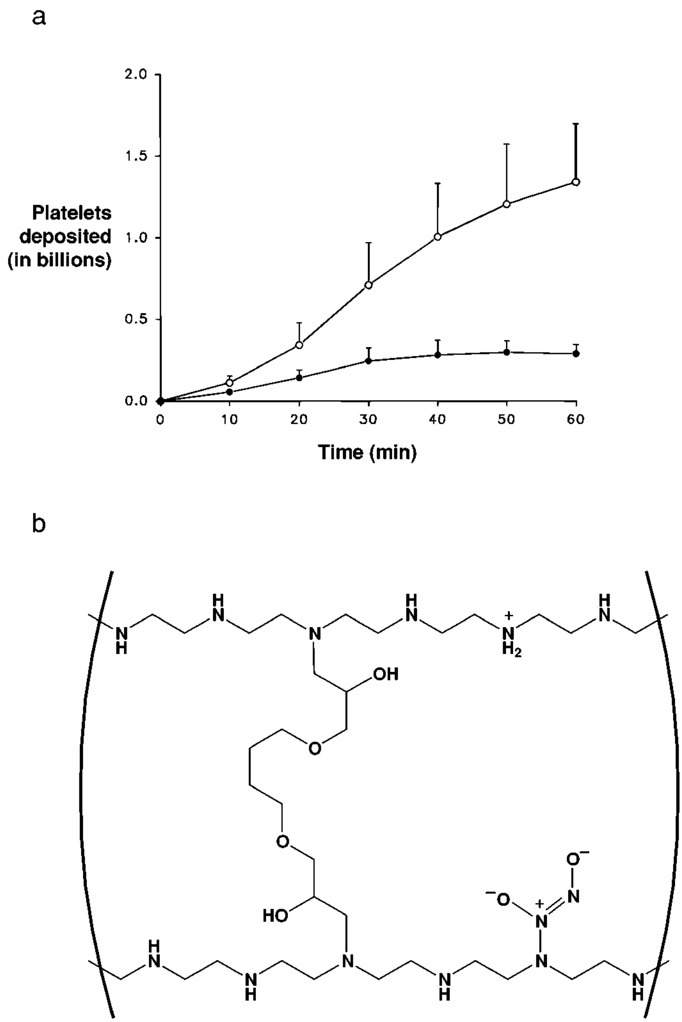

Figure 8 (*a*) Inhibition of platelet deposition in poly(tetrafluoroethylene) vascular grafts coated with cross-linked poly(ethylenimine) that was either (•) diazeniumdiolated to generate NO at a rate estimated to be 1–2 nmol/min/mg or (○) generating no NO. Data were collected by quantifying accumulation of radioactivity in grafts placed in arteriovenous shunts in baboons whose platelets had been labeled with ^{111}In but who were not treated with heparin. Data are means ± SEM (n = 5). (*b*) Representative structural unit of the crosslinked poly(ethylenimine) after incorporating it into the Teflon vascular graft and diazeniumdiolating it to produce the NO-releasing graft segments whose antiplatelet activity is summarized in (*a*). [Adapted from Reference 11 with permission.]

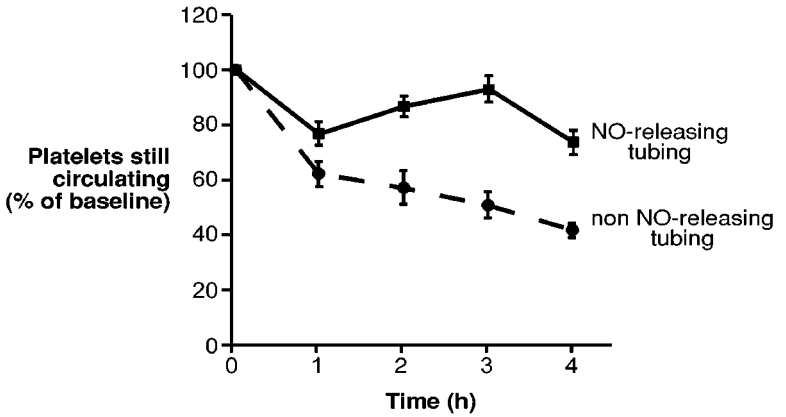

Figure 9 Degree of platelet retention in unheparinized rabbits undergoing simulated extracorporeal membrane oxygenation (ECMO) whose extracorporeal blood conduits are lined with MAHMA/NO-containing PVC to inhibit platelet adhesion (■) as compared with controls whose tubing is made of undiazeniumdiolated PVC (●). [Adapted from Reference 13 with permission.]

To address this problem, the researchers have designed a polymeric sleeve that they fit over the portion of the electrode tip that contacts blood before insertion into the blood stream. To overcome the additional problem that the MAHMA/NO in the PVC blend used for the above-mentioned ECMO study tended, as a small, hydrophilic molecule, to leach out of the polymer once deployed, they designed a sleeve made of diazeniumdiolated silicone rubber (14). As shown in Figure 10, the diazeniumdiolated sleeve not only inhibited platelet adhesion/activation and gross thrombus formation at the sensor tip, but also generated continuous readings of blood oxygen levels that are very close to those produced by standard clinical chemistry methods obtained using discrete arterial blood samples; by contrast, measurements collected with the undiazeniumdiolated control sleeve during the 16-h observation period tended to be much lower than the reference values (Figure 10).

Given the extensive variety of biomedical devices whose function may be significantly improved by fabricating them with NO-releasing surfaces, the market for insoluble polymers bearing covalently bound diazeniumdiolate groups could be very large indeed. The list of possibilities includes not only the stents, vascular grafts, ECMO circuitry, and chemical sensors mentioned above, but also dialysis shunts, penile implants, dermal patches, surgical supplies, wound dressings, and more.

Vasospasm

People reporting to hospital emergency rooms with what they describe as the worst headaches they have ever experienced often turn out to have suffered a

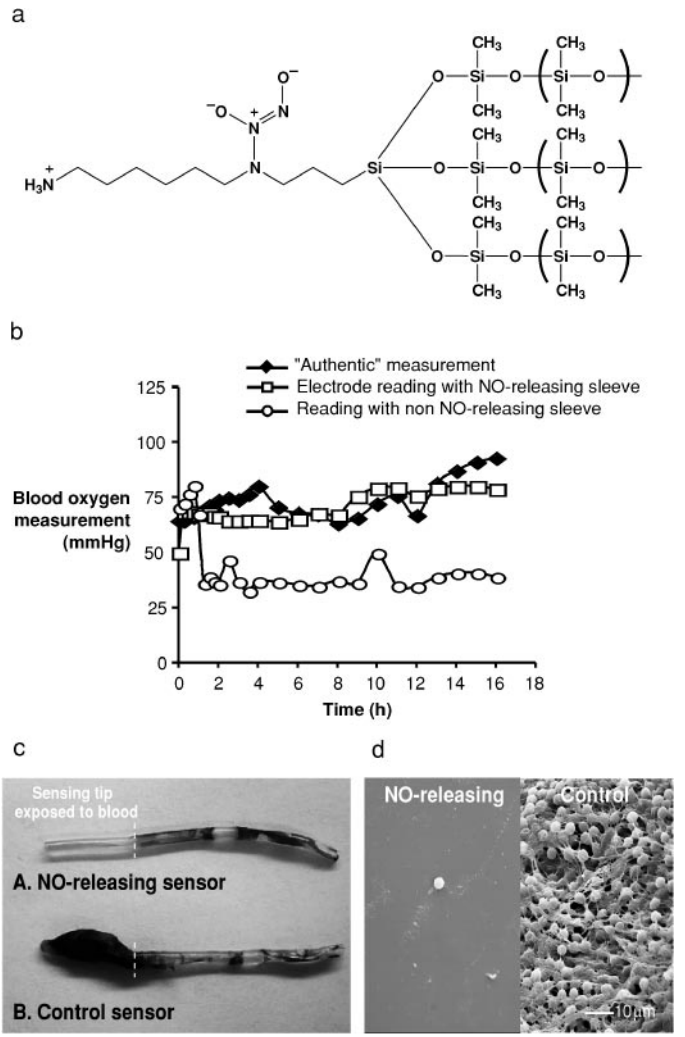

Figure 10 Improved biosensor accuracy using a diazeniumdiolated silicone rubber sleeve to cover the blood contact surface of the electrode. (*a*) Representative structural unit of the polymer. (*b*) Comparison of blood oxygen levels continuously monitored over a 16-h period with the NO-releasing intravascular electrode (□) versus both standard measurements (♦) and data collected with a control sleeve incapable of NO generation (○). (*c*) Gross view of sensor tips bearing (*top*) and devoid of (*bottom*) the NO-releasing sleeve after 16 h in the blood stream of a dog and (*d*) closeup view thereof obtained by scanning electron microscopy. $n = 1$ in all cases. (Courtesy of M. Frost & M. Meyerhoff, University of Michigan.)

subarachnoid hemorrhage, bleeding within the brain caused by rupture of a cranial artery. Neurosurgeons are able to clip off the ruptured aneurysm with remarkable proficiency, and many patients go on to full recovery and long, healthy lives thereafter.

The problem is that for a period of up to two weeks after the surgery these patients are at risk of one of the most debilitating forms of stroke, cerebral vasospasm. This is thought to occur because hemoglobin in the residual blood surrounding a cranial artery reacts with and destroys the vessel-produced NO; if this "steal" effect is sufficiently extensive, the NO that remains unreacted may be unable to oppose the vasoconstrictor factors that are normally present, allowing the artery to narrow dangerously or even collapse. This slows the flow of blood to downstream regions of the brain and can lead to serious impairment. Of the more than 28,000 Americans who suffer subarachnoid hemorrhage each year (15), 22%–40% experience the symptoms of vasospasm; of these, roughly one third are left with long-term neurological deficits and another third fail to survive the risk period (16).

Fortunately, the risk period for cerebral vasospasm lasts only a week or two after the bleeding has been stopped, ending as soon as the residual interstitial blood has been cleared by natural processes. Not so fortunately, none of the methods currently approved for treating cerebral vasospasm has proven very effective.

A very promising approach to addressing this unanswered medical need relies on the potent vasodilatory effect of NO. In a pioneering study, Pluta et al. reasoned that if the spasms are really caused by a local deficiency of NO, NO replacement therapy may be all that is needed to prevent them. To test this, they chose to infuse the ultrafast NO donor of Figure 2, PROLI/NO, immediately upstream from the affected vessel with the assumption that its 2-s half-life for NO release at the pH of blood, combined with the even shorter lifetime in the presence of red blood cells of the free NO produced via PROLI/NO dissociation, should limit the drug's vasodilatory effect to the site of the deficiency. They then prepared alkaline solutions of PROLI/NO and infused them into the carotid arteries of monkeys with induced cerebral vasospasm in such a way that the infusate was neutralized by the blood and began generating copious NO into the spasm but not much beyond that site. The degree of arterial spasm in the monkey with the least effective PROLI/NO response was less than 25% in the PROLI/NO-dosed animals, whereas all the saline-treated controls were more than 50% spastic. Very importantly, there was no observable effect on systemic blood pressure (15).

A second successful strategy employed the long-lived agent DETA/NO, whose half-life under physiological conditions is 20 h. If this material were to be infused as the PROLI/NO was, systemic effects would be unavoidable. For this reason, Wolf et al. injected the compound instead into the cerebrospinal fluid of the dog, where the blood/brain barrier kept it reasonably contained, concentrating NO release at the exteriors of the blood vessels in the brain. Normal vessels that were properly dilated were not overly dilated, but the spastic vessel was opened dramatically. Illustrating the latter result in Figure 11, part (*a*) shows the arteriogram of a normal

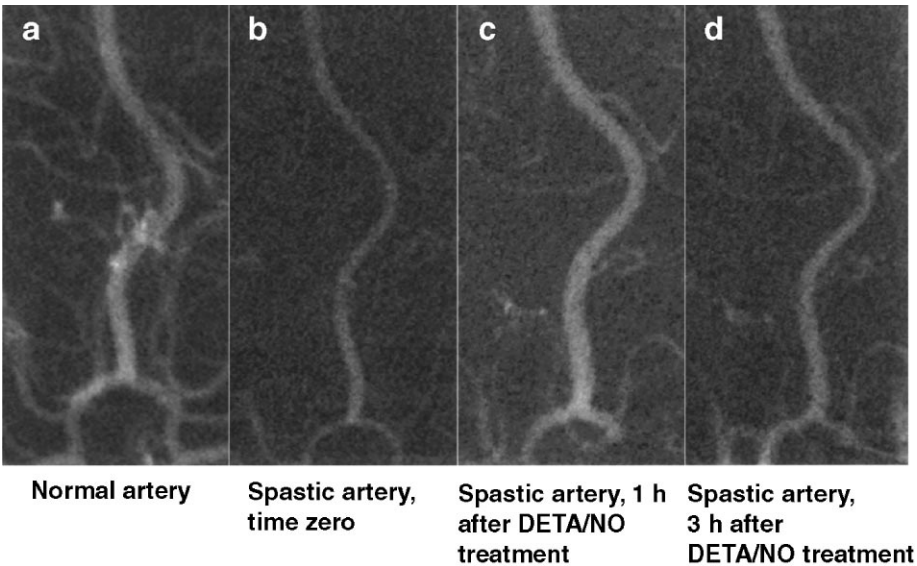

Figure 11 Reversal of cerebral vasospasm by diazeniumdiolates. Panels (*a*)–(*d*) show the results of injecting NO-releasing DETA/NO (structure in Figure 2) into the cerebrospinal fluid of a dog. Panel (*a*) shows the normal basilar artery. Lightening of the arteriogram indicative of severely restricted blood flow, the signature of a spastic vessel, is seen in (*b*). Panel (*c*) confirms that a 2-μmol dose of DETA/NO restores blood flow in the affected vessel; the photograph illustrates the dilation of the artery 1 h after DETA/NO was injected. Panel (*d*) shows the same artery 3 h after bolus intrathecal injection, demonstrating a lasting effect consistent with the compound's prolonged half-life for NO release. [Adapted from Reference 17 with permission.]

artery next to the same vessel in spasm (*b*). The next picture (*c*) shows the same vessel 1 h after administration of the DETA/NO. Finally, the same artery 3 h after injection shows that there is still a significant opening of the vessel after a single bolus administration (*d*) (17).

A third successful approach involved surgically introducing an ethylene/vinyl acetate copolymer blended with 20% DETA/NO around the artery at risk of collapse (18). In this rat femoral artery model of vasospasm, the DETA/NO-polymer blend kept the lumen of the affected vessel segment patent throughout the nine-day risk period, even when it was first implanted seven days after extra-arterial blood deposition. Although such polymeric NO sources may not be capable of therapeutically benefiting all regions of the brain at risk of spasm after subarachnoid hemorrhage, the method has the advantage of continuous, controlled NO release following a single treatment conveniently applied during the initial surgical intervention to repair the aneurysm (18).

Pulmonary Hypertension

Proper lung function depends on high-volume, low-resistance blood flow through the lung to promote adequate gas exchange. Pulmonary hypertension is a life-threatening condition in which the resistance (pulmonary vascular resistance, or PVR) and blood pressure in the lung increase to pathological levels. When this leads to sufficiently poor oxygenation, the ECMO procedure described above and other heroic interventions may be indicated.

An ingenious therapy for pulmonary hypertension involves mixing low concentrations of gaseous NO with the air these patients breathe. The concentration is kept low enough that the oxidation of NO to toxic NO_2 is negligible, and the vasodilatory effect is limited to the site of need—the pulmonary vascular bed. The procedure has been widely tested and is now approved by the FDA for treating hypoxic respiratory failure associated with persistent pulmonary hypertension of the newborn (19).

There are, however, several problems with this procedure. One is the danger of a rebound effect, in which abrupt cessation of NO inhalation leads to a rapid increase of pulmonary arterial pressure (PAP) to levels higher than those seen before treatment was begun. In addition, rigorous control of gas flows is needed to keep the NO_2 level acceptably low and the NO concentration within its narrow therapeutic range; the assortment of gas cylinders, meters, hoses, and other equipment involved in the procedure is so intricate to manage that it is not yet established in many hospitals, and in most cases demands that the patient remain on a respirator in the intensive care unit.

An important advance in NO inhalation therapy would be to develop an ambulatory treatment regime that allows the patient to leave the hospital and resume normal activities without being burdened by cumbersome gas handling equipment. With this in mind, Hampl et al. tested the ability of periodically administered DETA/NO (Figure 2) to relieve pulmonary hypertension in rats whose lungs had been injured by monocrotaline exposure (20). They found that once-a-day inhalation of aerosolized DETA/NO lowered these animals' PAP to normal levels with no observed effect on overall blood pressure (i.e., mean systemic arterial pressure, or MAP) or other signs of toxicity.

Similar results have been reported in a pig model of pulmonary hypertension with (21, 22) and without (23, 24) accompanying acute lung injury. These studies showed that intratracheally aerosolizing zwitterionic diazeniumdiolates EP/NO and DMAEP/NO (structures in Figure 2) consistently lowered PAP and PVR with little or no significant effect on MAP or SVR in animals treated intravenously with either U46619 or oleic acid. Addition of surfactant to the aqueous dosing solutions did not affect the rate of spontaneous NO generation or DMAEP/NO's in vitro vasodilatory activity, but surfactant pretreatment did improve the diazeniumdiolate's ability to lower PAP and increase oxygenation in the lung injury model (22). Other than mild systemic hypotension, no differences between diazeniumdiolate-treated animals and the saline controls were reported

in these studies (i.e., in methemoglobin levels or histological parameters). Of considerable interest from the mechanistic point of view, the researchers also reported a consistently greater degree of lung selectivity for DMAEP/NO than for EP/NO, a finding they attributed to the presence of two versus one basic amino groups in the former versus the latter as the structural feature that inhibited absorption of net cationic DMAEP/NO across the mucosal barrier into the systemic circulation (23, 24). This rationale may also explain the lung selectivity observed for cationic DETA/NO and the total lack thereof for aerosolized DEA/NO (25) (structure in Figure 2), an obligatory anion devoid of other polar functional groups.

Very recently, Lam et al. (26, 27) reported the results of administering aerosolized DETA/NO to a patient with acute respiratory distress syndrome (ARDS). As shown in Figure 12, the PVR index and the mean PAP were reduced by 8%–40% (mean 26) and 8%–29% (mean 18), respectively, as a result of nebulizing 5 ml of 0.03 M DETA/NO into the patient's airways over a 20-min period. Intrapulmonary shunting and alveolar-arterial oxygen partial pressure gradient were also temporarily reduced by DETA/NO treatment, and no significant effect on systemic hemodynamics, pulmonary artery wedge pressure, or serum methemoglobin levels was observed. The authors indicate that they are currently pursuing a Phase II clinical trial with aerosolized DETA/NO in ARDS patients.

WHERE DO WE GO FROM HERE?

Clinical studies such as the one just mentioned that was reported by Lam et al., as well as trials corresponding to protocols currently being prepared that propose to treat cerebral vasospasm using diazeniumdiolate therapy, will of course offer the most direct insights into the real clinical promise of this class of NO-releasing molecule; however, in my view, there is an equally urgent incentive to, as Renu put it in the Djerassi novel, "explore some of the other therapeutic possibilities" not mentioned above.

What of Renu's own golden focus, drugs useful for helping men achieve erections sufficiently tumescent for sexual success, for example? It happens that the ability of several different ionic diazeniumdiolates to induce erections in male cats has already been described by Champion et al. (28), and the techniques employed included transurethral administration, the same one Renu used for applying her commercially successful NONO-2. One may think that the demand for new erection-inducing drugs would have disappeared with the introduction of the oral phosphodiesterase (PDE) inhibitor sildenafil (Viagra®), which acts by slowing the enzymatic degradation of cyclic 3′,5′-guanosine monophosphate (cGMP), the agent that keeps the corpus cavernosum relaxed in a sustained erection. Because cGMP is synthesized by soluble guanylyl synthase when NO coordinates with its heme iron center, an absence of NO could result in a deficiency of cGMP, even in the presence of abundant PDE inhibitor. NO replacement therapy may, therefore,

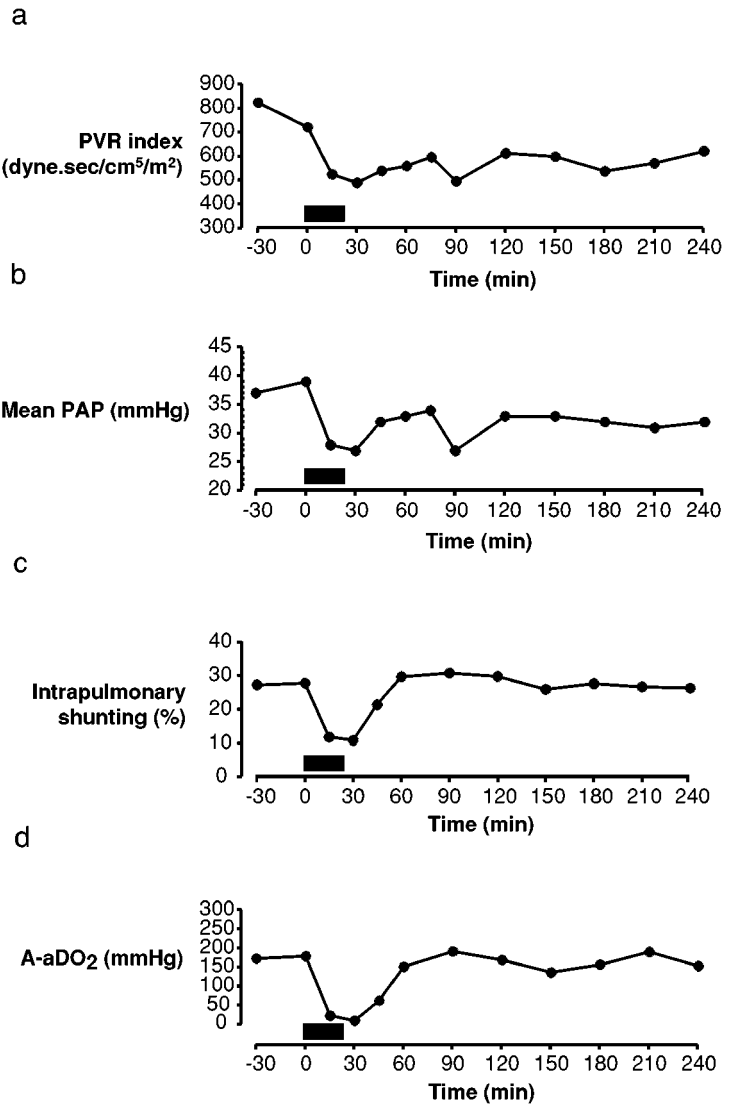

Figure 12 Changes in pulmonary hemodynamic and oxygenation parameters for an ARDS patient before and after aerosolized DETA/NO administration: (*a*) pulmonary vascular resistance (PVR) index, (*b*) mean pulmonary arterial pressure (PAP), (*c*) intrapulmonary shunting, and (*d*) alveolar-arterial oxygen partial pressure gradient (A-aDO_2). The 20-min period of DETA/NO aerosol administration is indicated by (▬). [Adapted from Reference 27 with permission.]

be an effective treatment option for the substantial proportion of impotent men who do not respond adequately to sildenafil. The experiments of Champion et al. suggest that it would not take much NO to do the trick; one of the diazeniumdiolates they tested was PROLI/NO, which generates NO with a half-life of 2 s at physiological pH, but that had a total duration of erectile response of more than 15 min per administration.

The Pro-Prodrug Approach

Although designing a penis-selective oral formulation of inherently acid-sensitive diazeniumdiolate ions poses a most difficult, perhaps insurmountable, challenge, chances of success would be greatly improved by covalently attaching a protecting group to the ion's terminal oxygen to stabilize it for delivery to that organ. Even if this "pro-prodrug" strategy does not yield an oral drug for treating erectile dysfunction, it may enable other routes of administration that are more palatable to the patient than transurethral injection. For example, it would be very interesting to see whether the cell- (and presumably tissue-) permeant pro-prodrug AcOM-PYRRO/NO (structure in Figure 3) would induce erections in animals on topical administration, presaging the possibility of a noninvasive treatment via local cream application.

Another established diazeniumdiolate pro-prodrug is V-PYRRO/NO, designed and demonstrated to be liver selective in its NO release via metabolism by enzymes concentrated in that organ. Its structure is illustrated in Figure 3. V-PYRRO/NO has been shown to be hepatoprotective in at least three settings: in rodent models of fulminant liver failure [induced on treating rats with tumor necrosis factor-α and galactosamine (29) or mice with bacterial endotoxin and galactosamine (30)]; in regional ischemia induced on preparing the livers of pigs for transplant (31); and in acetaminophen toxicity in mice (32). The drug is designed for activation as an NO donor by cytochrome P450-induced oxidative removal of the vinyl group to generate the free PYRRO/NO ion (29), whose half-life for NO release is very short (3 s; see Figure 2), a mechanism that would be consistent with similar selectivity for other organs rich in this enzyme.

Circumventing Toxicity

Just as the V-PYRRO/NO example of the previous section gave us the opportunity to illustrate the pro-prodrug concept of designing appropriately derivatized diazeniumdiolates for activation as NO donors by specific enzymes, it also serves as an excellent starting point for discussing the critical issue of toxicity. True, its hepatoprotective effects could be achieved without significant systemic hypotension, a major dose-limiting toxicity that must be avoided in any therapy in which physiological NO levels are increased. But V-PYRRO/NO also has the potential to be converted to *N*-nitrosopyrrolidine, one of the most potent experimental hepatocarcinogens known. It is not the goal of this work to cure someone's acute liver disorder one day only to increase the risk of liver cancer later.

Because conversion to N-nitroso compounds is possible for any compound having a Figure 1 structure with $R^1 \neq H \neq R^2$, it is crucial that the potential for conversion to carcinogens be explicitly addressed in any attempt to translate this work from bench to bedside. Here again, the chemical flexibility of the diazeniumdiolates provides us with a variety of strategies for doing this. One that could be applicable to PYRRO/NO derivatives, such as V-PYRRO/NO, is to use an R^1R^2N moiety whose N-nitroso derivative is not carcinogenic. In the case of V-PYRRO/NO, adding a carboxyl group to the 2-carbon of the pyrrolidine ring would convert the corresponding nitrosamine to *N*-nitrosoproline, a compound that is continuously produced in our bodies but that has been screened for carcinogenicity numerous times without once showing positive results (33). Anchoring the R^1R^2N moiety covalently to a stationary solid, as discussed in a previous section, would also make carcinogenicity of the derived N-nitroso functions a non-issue. Infusing PROLI/NO [the fast-acting, spontaneous NO releaser formed on 2-carboxylating PYRRO/NO ion that has already been shown to reverse cerebral vasospasm in monkeys (see above)] at the site of need is another approach to circumventing toxicity in general and systemic hypotension in particular. Another possibility would be to exploit carbon-bound diazeniumdiolates [$R_3C\text{-}N(O)=NOR^3$ analogues of the Figure 1 structure] that have been shown to generate NO under certain conditions, or primary amine derivatives [$R^1HN\text{-}N(O)=NOR^3$] that do not decompose to stable N-nitroso derivatives. Of course, no compound should ever be used for clinical purposes without first having been thoroughly evaluated for possible toxicity. There is no substitute for proper preclinical testing, no matter how benign the structure is predicted to be.

Chemistry is the Key

Perhaps the most general and appealing way to limit toxicity is to target NO release so efficiently to sites of therapeutic benefit that collateral effects on other tissues become insignificant. The ability to convert proteins and polysaccharides to NO-releasing form by diazeniumdiolation may offer the possibility of exploiting the homing capacity of antibodies and lectin-binding carbohydrates for this purpose. Further development of the (pro-)prodrug approach should also lead to a variety of important advances; an intriguing example recently introduced to the chemical literature involves O^2-glycosylated diazeniumdiolates such as β-Glc-PYRRO/NO (structure in Figure 3), a pro-prodrug shown to be activated for NO release by β-D-glucosidase (34). One-electron oxidation of C-bound diazeniumdiolates such as alanosine (35) and cupferron (36, 37) offers the possibility of generating NO on redox activation, and a pro-prodrug class that is activated for NO release by proteases, such as α-chymotrypsin and prostate-specific antigen, has also been characterized (38). It will be most interesting to see whether any of the strategies mentioned in this paragraph, or others yet to be described, will confirm their promise when their hypothetical activities are tested in vivo.

In closing this consideration of potential clinical applications for the diazeniumdiolates, I think it appropriate to revisit their fundamental chemical advantages

as a basis for further progress. Renu herself, when contemplating how to devote her newfound wealth to the conquest of cancer and infectious disease, started with the chemical basics. For example, if NONOates are good, would NONONOates be better? How about the hexazeniumhexolates? Although Renu did not say what specific structures these terms were meant to include, my own view is that clinical potential can best be maximized through an exhaustive understanding of fundamental diazeniumdiolate chemistry—spectral characteristics; structural considerations; acid-base behavior; coordination chemistry; analytical aspects; and reactivity studies, including mechanisms by which they dissociate to NO, their photochemistry, and their redox behavior. A growing number of laboratories are actively engaged in expanding this knowledge base by synthesizing new diazeniumdiolates and reporting on their properties. It remains only for industrious clinical innovators to convert the growing structural diversity of these compounds, their dependable rates of NO release, and their rich derivatization chemistry into means of pharmacologically targeting NO to specific sites of physiological need.

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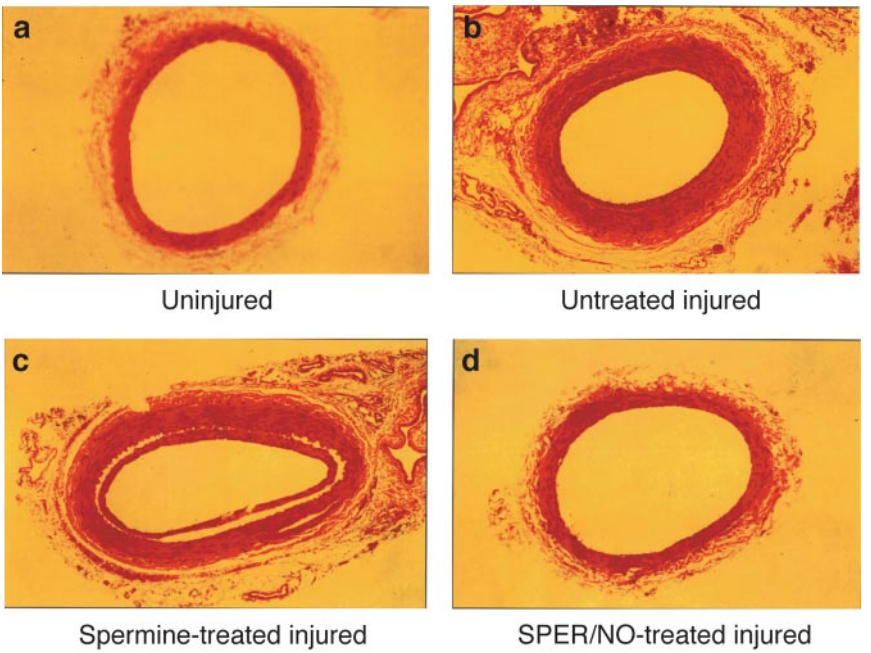

Figure 5 Beneficial effect of NO-releasing SPER/NO on healing of the rat iliofemoral artery after balloon angioplasty. Panel (*b*) shows the extensive stenosis (narrowing caused by excessive thickening of the vessel wall) of a balloon-injured artery two weeks after overinflation, relative to the normal cross-section in panel (*a*). Packing a spermine-containing gel around the outside of the vessel immediately before balloon treatment has no effect on injury-induced stenosis (*panel c*), but replacing the spermine with its diazeniumdiolate SPER/NO (structure shown in Figure 2) led to a nearly normal-looking vessel at the two-week time point (*panel d*). [Courtesy of Sanjay Kaul, Cedars Sinai Medical Center. Figure reprinted from Reference 39 with permission.]

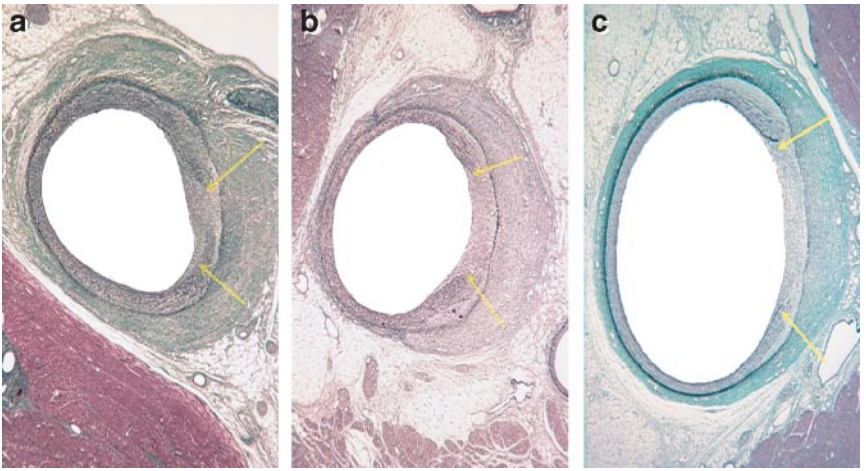

Figure 6 Effect of D-BSA, a diazeniumdiolated bovine serum albumin derivative, in promoting healing of balloon-injured pig coronary arteries. Shown are representative cross-sections of arteries taken two weeks after angioplasty from pigs dosed immediately before 30% balloon overstretch via intrapericardial instillation with 400 mg of underivatized BSA (*a*), 40 mg of D-BSA (*b*), and 400 mg of D-BSA (*c*). Arrows denote ruptured ends of the internal elastic lamina for each vessel shown. [Reprinted from Reference 8 with permission.]