Biotransformation to nitric oxide of organic nitrates in comparison to other nitrovasodilators

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Nitrovasodilators are prodrugs which, although chemically heterogeneous, exert their pharmacodynamic action via a common pathway, i.e., the release of nitric oxide (NO). The NO, which results from metabolism of nitrovasodilators in vascular and non-vascular cells, stimulates the cytosolic enzyme guanylyl cyclase leading to an increase in the concentration of intracellular cyclic guanosine monophosphate (cGMP). In general, the rate of NO generation from the individual compounds correlates well with the extent of cGMP increase and their potency to relax vascular tissue. The amounts of NO generated are sufficient to inhibit platelet aggregation and to induce disaggregation. Nitrovasodilators thus mimic the action of endothelium-derived relaxing factor (EDRF). After more than a century of empiric use, the application of nitrovasodilators today may be regarded as causal therapy, since these drugs act by substituting an endogenous factor. The production or release of which is impaired under pathophysiological circumstances associated with endothelial dysfunction. Marked differences exist between individual compound classes with regard to bioactivation mechanisms, cofactor requirements, and the extent and nature of the concomitant formation of metabolites other than NO. This review describes the discovery of the mode of action of nitrovasodilators and our current understanding of the pathways involved in their bioactivation and biodegradation with special emphasis on the enzymatic and non-enzymatic metabolism of organic nitrates. In addition, the in-vivo metabolism of NO is reviewed briefly.

Introduction

More than a century ago William Murell proposed that the sublingual application of glyceryl trinitrate (GTN) could relieve acute attacks of angina pectoris, the principal symptom of ischaemic heart disease. The assumption that GTN could be effective in angina was based on the observed similarity between the pharmacodynamic effect of this compound in healthy individuals and the vasodilator effect of inhaled nitrite described by Thomas Lader Bruntou 12 years earlier. Knowing that venoconstriction improved angina, both authors believed that the relief produced by physical and pharmacological means was due to a reduction in arterial pressure and consequently in ventricular afterload. Although this assumption was incorrect, there is no doubt today as to the efficacy of nitrovasodilators, such as organic nitrates, in the treatment of coronary heart disease. We know now that these compounds are prodrugs which, although chemically heterogeneous, exert their pharmacodynamic action via a common pathway, i.e., the release of nitric oxide (NO). The NO which results from their metabolism in vascular and non-vascular cells stimulates the cytosolic enzyme guanylyl cyclase, leading to an increase in the concentration of intracellular cGMP. This in turn affects the activity of cGMP-dependent protein kinases in a complex manner not yet fully understood, finally resulting, at least in part, in decreased intracellular free calcium concentrations. As the degree of contraction of vascular smooth muscle depends directly on the concentration of free calcium, a decrease in its concentration translates into relaxation.

In 1987 evidence was presented showing that 'endothelium-derived relaxing factor' (EDRF) is chemically and pharmacologically indistinguishable from nitric oxide. Later, it was found that NO is synthesized from the terminal guanido nitrogen of L-arginine by an enzyme called NO synthase. This novel biochemical route was termed the 'L-arginine: NO pathway' and plays a major role, not only in the control of the cardiovascular system, but also in central and peripheral neurotransmission as well as in host defence mechanisms. The increasing knowledge about the various actions of endogenously produced NO and its obvious involvement in many bioregulatory systems renewed both scientific and clinical interest in NO-donating compounds. Since from the stage of NO release onward the actions of nitrovasodilators and EDRF are identical, these drugs may be regarded as EDRF mimetics which imitate a physiological blood vessel dilatation process. An important advantage of vasodilator therapy over compounds that act by increasing the release of EDRF is that nitrovasodilators do not require an intact endothelial cell layer to become effective. They cause vasodilatation regardless of whether or not the endothelium is intact, being even more potent in its absence.

Current understanding about the mechanisms of bioactivation and biodegradation of nitrovasodilators are given below, with special emphasis on the organic
nitrates, as these compounds still represent one of the cornerstones of cardiovascular drug therapy.

**Discovery of the mode of action of nitrovasodilators**

Once the vasodilator properties of amyl nitrite and nitroglycerin had been discovered, it took more than 100 years to elucidate their mode of action at the cellular level (see Table 1). The breakdown of GTN in blood in vitro to inorganic nitrate (NO$_3^-$) was noted as early as 1889[39]. This finding was confirmed by Crandall and co-workers who reported the rapid disappearance of GTN from the circulation with the appearance of NO$_3^-$ after i.v. administration of the drug[4]. For many years, considerable effort was directed towards trying to link this cleavage to the pharmacological effects observed. The concept that the action of organic nitrates could be mediated via the release of NO$_3^-$ was contradicted by the fact that these ions displayed vasodilator effectiveness only at much higher concentrations than the parent nitrate itself. It remained unclear also why the comparably low amounts of NO$_3^-$ arising upon cleavage of an organic nitrate, should have any effect on smooth muscle tone when basal NO$_3^-$ levels within the vascular cell are assumed to be already in the μmolar range. Moreover, when GTN was subjected to alkaline hydrolysis before injection, no fall in blood pressure was observed, suggesting that the intact nitrate molecule rather than NO$_3^-$ may be essential for activity[41]. The nitrite ion remained the only known metabolite of organic nitrates until Needleman and co-workers in the mid 1960s reported that rat liver metabolized GTN to the respective glycyl nitrites.

The phenomenon of cross-tolerance between different organic nitrates without cross-tolerance to other vasodilators suggested a common site of action specific to the nitrates. Based on the knowledge that sulphhydryl groups are crucial for the relaxant response to GTN, Needleman and Johnson later proposed the interaction of organic nitrates with specific sulphhydryl groups containing 'nitrate receptors' within the smooth muscle cell as a prerequisite for their pharmacodynamic effect[49]. However, neither specific binding sites for GTN, nor antagonists to their pharmacological action have yet been identified.

In the following years it became more and more obvious that nitrates have to be metabolized in order to become effective. Having accepted this important assumption, Ignarro and co-workers developed an elegant hypothesis according to which S-nitrosothiols were regarded as the ultimate active metabolites of nitrovasodilators[14]. Although these compounds were shown to display relaxant and blood pressure lowering effects similar to those of GTN, their formation from nitrovasodilators under physiological conditions remained doubtful[11,12].

At about the same time it was shown that organic nitrates can stimulate prostacyclin release in cultured endothelial cells and in intact tissue[13,14] suggesting that the vasodilator action of nitrates might be mediated, at least in part, by stimulation of the synthesis or release of arachidonic acid metabolites. This hypothesis appeared attractive since prostacyclin is a potent vasodilator and is the major prostaglandin synthesized in the vessel wall. It was, however, challenged substantially by the lack of antagonism of the relaxant response to nitrates by either blockade of cyclooxygenase in vivo or removal of the endothelium in vascular preparations in vitro.

In spite of early suggestions from Ferid Murad's group in the late 1970s, that free NO may be the common mediator of the dilator action of organic nitrates, nitroprusside, hydroxylamine and azide[15], until the mid 1980s this concept was widely ignored by the scientific community. This may be explained by the perception at that time that NO was a toxic gas rather than a relevant biological mediator. The same group proposed the term 'nitrovasodilator' for all those compounds which elicit their dilator action via generation of NO. This expression is, however, somewhat misleading because the action of these drugs is not limited to the mere dilation of blood vessels and may thus be better referred to as 'NO donors' instead. Only a few reports appeared in the literature, in which the metabolism of nitrovasodilators to NO was measured directly. These include an EPR study in whole animals[16] and further in vitro studies on the metabolism of nitroglycerin in homogenates of coronary arterial smooth muscle cells[17] and the microsomal fraction of hepatocytes[18]. When the endogenous endothelium-derived relaxing factor (EDRF) was found to be pharmacologically indistinguishable from authentic NO, we presented evidence to suggest that the organic nitrates, sodium nitroprusside and molsidomine may act through the release of a common mediator, namely NO, and thus in a manner identical to EDRF[19]. This was later confirmed by reports from other laboratories.

**Bioactivation pathways of nitrovasodilators**

In accordance with their produg character, most nitrovasodilator compounds have to undergo metabolic activation in order to generate NO. Neither compound activates guanylyl cyclase directly. In vivo, enzymatic and non-enzymatic pathways of NO formation may be competing with each other and the significance of either
pathway will depend strongly on the prevailing enzymatic profile and the availability of necessary cofactors, such as thiols, in the particular type of vascular tissue. Nitrovasodilators can be subclassified into several groups according to their chemical structure. The individual compounds generally differ in their need for specific cofactors to release NO, the pH-dependence of decomposition, and the susceptibility to oxygen, light and temperature. Depending on the chemical nature of both nitrovasodilator and cofactor, the pathways of bioactivation are clearly different. Figure 1 outlines the different pathways of NO formation from NO donors, as determined by in vitro methods (for review see28-31).

Organic nitrates are metabolized by a combination of enzymatic and non-enzymatic processes. The activity of glutathione-S-transferase22,23) and cytochrome P450 related enzymes24-25) is thought to be involved, and both systems may compete for the nitrate as a substrate. Thiols present in the cytosol are likely to account for a major part of non-enzymatic nitrate metabolism, and in both cases an unstable thionate may be the common intermediate. For a number of reasons, nitrosothiols are now thought unlikely to be precursors of NO formation. Concomitantly produced nitrite and nitrate ions may interexchange with extracellular compartments without affecting enzyme activity. Of all thiols available within the cytosol, only cysteine appears to induce additional NO formation; hence, the decomposition to nitrite, albeit predominating, represents an inactivation pathway.

In contrast, organic nitrates, such as amyl nitrite, react with all available thiol groups to form unstable S-nitrosothiols, which rapidly decompose to NO by homolytic cleavage of their S-N bond. In vivo this reaction will almost exclusively occur with glutathione, which is the most abundant thiol compound in mammalian tissue26). S-nitrosothiols, which may also be formed from nitrates in the plasma27, decompose in the same way to NO after entering the cell. The potency of enzyme stimulation by nitrosothiols was found to be inversely correlated with the stability of the compounds. Due to rapid transnitrosation reactions between a given nitrosothiol and free SH-groups, the presence of low molecular weight thiols may accelerate the rate of NO formation from certain S-nitrosothiols by giving rise to the formation of a more unstable nitrosothiol. It should be noted that, apart from NO, thiol radicals (RS·) are formed during the decomposition of this class of compounds, which may cause irreversible protein modification.

Sodium nitroprusside spontaneously liberates NO by an as yet unknown mechanism. The relatively small amounts of NO released from this inorganic complex in vitro are not sufficient to account for its marked dilator potency, which suggests that nitroprusside may either require intracellular redox biotransformation28-29) or exert additional effects on other regulatory processes unrelated to the generation of NO.

Molsidomine does not appear to require specific cofactors to generate NO but it has first to be converted to its active metabolite SIN-1 by liver esterases. After hydrolysis to the open-ring form SIN-1A, this compound releases NO via a radical process following reaction with molecular oxygen as electron acceptor. In the course of this reaction, superoxide (O2·-) is formed, which may give rise to oxidative side reactions due to the formation of hydrogen peroxide (H2O2) and hydroxyl radicals (OH·). Neither NO formation from, nor enzyme stimulation by, syndnonimines is enhanced by thiols.
The nitric oxide generated via these pathways binds to the haem group of guanylyl cyclase, which in turn results in enzyme activation and consequent vasodilatation. As can be seen from this scheme, besides the desired NO a number of other metabolites may also arise during biotransformation of a given prodrug, and this sometimes occurs in amounts much higher than those achieved for NO. Thus, compounds with a well-known metabolism and toxicology should be the first choice when aiming for therapeutic substitution of endogenous NO deficiency. From large studies with dynamite workers in explosives plants we know that in 915,000 man-years of experience no fatalities due to long-term exposure to organic nitrates have occurred[30]. Whether or not other nitrovasodilator classes are similarly harmless is less clear at present.

Metabolism of authentic and nitrate-derived NO in vivo

What happens to NO after its formation in vivo? Taking into consideration that NO is formed in the living body on a continuous basis and that it displays a high affinity for haemoproteins, fairly high concentrations of nitrosyl-haemoglobin should be found in the blood. Because this could not be confirmed experimentally[31], NO must obviously undergo rapid metabolism. From in vivo studies on the metabolic fate of radioactive labelled[32]NO in rats, we know that NO is not kept in the respiratory tract or lung when inhaled but passes the trachea, bronchial tube and alveoli, to enter the blood stream mainly unconverted[32]. From here on the fate of inhaled, endogenously produced and nitrate-derived NO is the same. In the blood, NO reacts with oxygenated haemoglobin contained in the red cells to form methaemoglobin and NO$_3^-$ as main products. Together with a smaller amount of NO$_2^-$, which arises from the decomposition of formed nitrosylhaemoglobin, NO$_3^-$ is then transferred into the serum and mainly excreted in the urine. A small amount of NO$_2^-$ is supposed to be secreted into the saliva and via the intestinal tract into excrement and may be exhaled after bacterial reduction as nitrogen (N$_2$) or ammonia (NH$_3$). From these data it is presumed that the metabolism of NO follows basically the same route as that of inorganic NO$_2^-$ and NO$_3^-$ compounds, that are known to be present in millimolar concentrations throughout the human body. That nitrovasodilators in vivo indeed act via the release of NO is confirmed by the finding that in patients who inhaled organic nitrates or received organic nitrates slight increases in the level of methaemoglobin were accompanied by concomitant increases in the plasma level of NO$_3^-$[15,34].

Cellular and non-cellular metabolism of organic nitrates

For a long time the liver was considered to be the predominant site of metabolic breakdown of nitrates. Metabolism of nitrates was later shown also to occur in kidney, lung, intestinal mucosa, and in vascular tissue[35,36]. There appears to be considerable confusion in the literature as to which pathways are involved in the bioactivation of organic nitrates and which ones should only be regarded as inactivating ‘first pass’ metabolism. Drug metabolism per se does not necessarily lead to bioactivation, but is often associated to a major extent with biodegradation. Techniques have now become sufficiently sensitive to show that both the biotransformation of GTN to the respective nitrites and the increase in intracellular cGMP levels precedes the onset of vascular relaxation[39]. These findings are consistent with the view that metabolic activation of organic nitrates is a prerequisite for their pharmacodynamic action.

The interaction between nitrates and thiols

Sulphhydryl-containing compounds, such as cysteine, have been shown to react chemically with organic nitrates to form large amounts of inorganic nitrite ions, together with comparably small amounts of NO[40]. Further investigations on the mechanistic aspects of these reactions revealed that for a given thiol compound there is a constant ratio between the formation rates of NO and nitrite (e.g. 1:14 for cysteine), regardless of the structure of the tested organic nitrate (Fig. 2). Since the conceivable reduction of nitrite to NO was experimentally excluded, it was assumed that in the course of the reaction between thiol and nitrates a common intermediate is formed which eventually decomposes with the release of NO$_3^-$ and NO. Chemically, one of the most likely mechanisms explaining these findings is the formation of a thionitrate arising from transsulferylation between organic nitrate and thiol compound (see inset of Fig. 2). Certain structural prerequisites of the thiol are thought to account for the finding that under physiological conditions only a limited number of sulphhydryl-containing compounds react with organic nitrates to form NO, whereas virtually all thiols decompose organic nitrates to NO$_3^-$. This is in agreement with previous findings that in broken cell preparations, activation of guanylycyclase by organic nitrates specifically requires the addition of cysteine while other nitrovasodilators such as sodium nitroprusside increased enzyme activity without added thiol[17,20]. Thiols that failed to mediate enzyme activation by organic nitrates often caused considerably higher NO$_3^-$ generation than those that were effective, indicating that nitrite production is not coupled to guanyly cyclase stimulation[40,41]. However, a close correlation was always found between the rate of thiol-induced NO formation from organic nitrates and the extent of enzyme stimulation in vitro[17] (Fig. 3a)). The higher the measured rate of NO formation from the respective organic nitrate, the lower the concentration that was needed to stimulate the target enzyme half-maximal. The highest NO liberation was seen with glyceryl trinitrate, followed by the group of dinitrates and mononitrates. When the respective compounds were tested at concentrations which elicited just half-maximal enzyme stimulation, an almost identical rate of NO liberation was measured (see inset of
Fig. 3(a)). Furthermore, the addition to the incubation mixture of oxyhaemoglobin, an effective scavenger for NO, completely abolished enzyme stimulation by all nitrates. Virtually the same correlation was found between the potency of different organic nitrates to relax precontracted rat aortic rings and the formation of NO from these compounds under the same conditions (Fig. 3(b)) demonstrating that this in vitro data is indeed meaningful for the action of nitrates in intact vascular tissue. There are enough reasons to believe that the sulphhydryl-dependent pathways discussed here may reflect the sequence of events taking place at the active site of the enzyme(s) involved in organic nitrate bioactivation.

METABOLISM TO NO BY VASCULAR CELLS AND THE CONTRIBUTION OF GLUTATHIONE S-TRANSFERASE AND CYTOCHROME P450

More recently organic nitrates were demonstrated to be metabolized to NO by intact cells of the vessel wall[2]. Superfusion of porcine aortic smooth muscle cells with GTN resulted in the immediate and concentration-dependent formation of NO, indicating rapid cellular uptake and metabolism of the drug. Evidence was presented that the biotransformation of organic nitrates operates with a unique profile in the vascular cells of several animal species as well as in man[23]. The typical biphasic pattern may reflect the existence of two distinct sites of biotransformation, namely a high-affinity component of enzymatic nature which is prone to rapid desensitization, and a low-affinity component which may be represented by the non-enzymatic reaction with cysteine. These data are in agreement with the observation that the concentration-response curve for the relaxant effect of GTN is generally biphasic[43]. The contribution of either pathway to the pharmacodynamic action of organic nitrates, however, remains to be investigated.

Our laboratory is investigating cultured vascular cells which are either pretreated with enzyme inhibitors prior to challenge with organic nitrates or confused with substrate inhibitors and nitrates. In preliminary experiments, we found the amounts of NO generated upon the biotransformation of GTN in vascular smooth muscle cells is not inhibited at all by pretreatment with either the glutathione-S-transferase inhibitor sulfobromophthalene or the inhibitors of the cytochrome P-450 pathway, cimetidine and SKF-525, suggesting that none of these pathways plays a major role in nitrate bioactivation in vascular smooth muscle. One has to be extremely careful, however, in interpreting results which are solely based on the use of apparently specific enzyme inhibitors, because in our hands some of these compounds are evidently less specific than generally anticipated.

Evidence was presented that the generation of NO from organic nitrates not only results in an intracellular rise in the level of cGMP but also in the release of this nucleotide into the extracellular space[25]. In all cases,
the egress of cGMP from vascular cells closely paralleled the formation of NO. Thus, in addition to phosphodiesterase activity, egress of cGMP appears to be an alternative mechanism for the reduction of increased cyclic nucleotide levels. Whether this transport of cGMP is merely passive or controlled by an active transport system remains to be investigated.

**RELATIONSHIP BETWEEN GTN BIOTRANSFORMATION AND NITRATE TOLERANCE**

Interestingly, both NO formation and cGMP release markedly decreased upon the repeated administration of high concentrations of organic nitrates to cultured vascular cells. The typical pattern of GTN biotransformation observed in this model was paralleled by the pharmacodynamic effect of GTN in a bioassay system consisting of an isolated working rat heart preparation (Fig. 4). In this model, GTN caused a marked increase in coronary flow, indicating dilatation of coronary resistance vessels. Vasodilatation was paralleled by a respective decrease in oxygen consumption, whereas left ventricular pressure, dp/dt and heart rate remained unchanged. Increase in flow rapidly returned to baseline upon cessation of the nitrate infusion. Repeated application of GTN produced the same characteristic profile.
of pharmacodynamic action as observed for the metabolism to NO at the cultured cells. Again, the effect of GTN was biphasic with a short-lasting component that virtually disappeared after the fourth challenge (Fig. 4). The pattern of cellular NO formation in cultured vascular cells thus perfectly matched that of GTN-induced coronary vasodilation in situ, which strongly suggests that the observed decrease in the metabolism of organic nitrates to NO may be the biochemical correlate for the clinically well-known problem of ‘nitrate tolerance’. These data extend previous results which demonstrated that this particular phenomenon is mainly the result of impaired biotransformation rather than neurohumoral counterregulation or desensitization of guanylyl cyclase, as suggested earlier.\textsuperscript{44,45} The development of nitrate tolerance is likely to affect multiple sites in the nitrate biconversion cascade\textsuperscript{46} and may be related to intracellular events, such as the depletion of cysteine stores and/or the down-regulation of the enzyme system(s) that convert organic nitrates to NO.

**ENDOTHELIAL METABOLISM OF NITRATES TO NO.** IMPLICATIONS FOR THE MODULATION OF BLOOD CELL FUNCTION

Most notably, it was demonstrated that not only vascular smooth muscle cells but also endothelial cells, which have previously been regarded as an inert cell layer with respect to the metabolism of organic nitrates, actively convert organic nitrates to NO.\textsuperscript{42,47} Calculations based on protein content surprisingly revealed that endothelial cells metabolized GTN as effectively as did smooth muscle cells. The biotransformation of organic
nitrates in endothelial cells may have important implications for the modulation of blood cell function, because nitrate-derived NO is released not only toward the muscular cell layer causing vessel relaxation, but also to the luminal site leading to inhibition of adhesion and aggregation of blood cells. An important advantage of organic nitrates and other nitrovasodilators over compounds which enhance the release of EDRF is that even at the sites of endothelial injury, the NO which arises during nitrovasodilator metabolism in the smooth muscle will effectively inhibit the adhesion of leukocytes, platelets and macrophages to the vascular wall. Together with recent results suggesting that nitrates can inhibit the proliferation of smooth muscle cells, this pathway may have important implications for the prevention of atherosclerosis, the crucial pathogenic steps of which include the attachment of leukocytes to the vascular wall, their migration into the intimal layer and, as a result, an increased rate of myocyte proliferation in the subendothelial cell layer.

The implication that these pathways have for the antithrombotic potential of nitrates can be judged from results of coinoculation experiments with platelets and cultured vascular cells. The representative tracings depicted in Fig. 5 demonstrate that nitrates alone are poor inhibitors of platelet aggregation in vitro. Their anti-aggregatory effect can, however, be markedly potentiated in the presence of aortic endothelial or smooth muscle cells and can be further potentiated by addition of plasma. The effects are qualitatively similar in platelet rich plasma and washed platelets, with the only major difference being the more pronounced disaggregating effect of nitrates in the presence of plasma. The almost complete reversal of potentiation by oxyhaemoglobin confirmed that these effects were mediated by the extra-cellular release of NO. Since the concentration-response relationship for the antiplatelet effect of nitrates is shifted to the therapeutically relevant range ($10^{-7}$ M) in the presence of a small number of vascular cells, this pathway is likely to be the long-sought key to understanding why organic nitrates are effective inhibitors of platelet aggregation in vivo, whereas they generally display poor effectiveness under in vitro conditions. This is considered to be of particular importance under pathological circumstances which are known to be associated with an enhanced platelet aggregability. It is our current belief that the combination of antiplatelet activity and beneficial haemodynamic effects, both of which are brought about by NO, plays an important role in the overall anti-ischaemic effectiveness of organic nitrates.

**Summary and conclusions**

Organic nitrates and other nitrovasodilators are metabolized to NO in vascular and non-vascular cells by both enzymatic and non-enzymatic pathways. Marked differences exist between individual compounds with regard to bioactivation mechanisms, cofactor requirements, susceptibility to enzymatic breakdown, and the extent and nature of the concomitant formation of metabolites other than NO. A feature common to all nitrovasodilators is the stimulation of soluble guanylyl cyclase. The rate of NO generation from the individual compounds correlates well with the amounts of cGMP released and the potency to relax vascular tissue. NO generation from nitrovasodilators at the sites of endothelial dysfunction is likely to prevent adhesion of activated blood cells and to decrease exaggerated cell proliferation. Furthermore, the amounts of generated NO are sufficient to inhibit platelet aggregation and, in the presence of plasma constituents, also to induce disaggregation. Nitrovasodilators thus represent prodrugs of the endothelium-derived relaxing factor (EDRF). After more than 100 years these compounds have moved from empiric therapy to emerge as...
pathophysiologically oriented drugs. Their application may be regarded as causal therapy, since they act by substituting an endogenous factor, the production or release of which may be impaired under pathophysiological circumstances associated with endothelial dysfunction. A proper understanding of the structural determinants which govern pharmacokinetics, and the extent and route of biotransformation of nitrovasodilators in different vascular tissues is an important prerequisite for rational drug development. This issue surely deserves major attention and may allow the design of NO-donating compounds with tissue and organ specificities distinct from those of classical nitrovasodilators. The continuing elucidation of bioregulatory mechanisms linked to the endogenous formation of NO has thus opened up new and fascinating therapeutical avenues for NO-donating drugs in the future.

References


