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Therapeutic targets of ataciguat

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Abstract

The endothelium regulates vascular and cardiac function by the release of endothelium-derived vasoactive compounds, the most important of which is nitric oxide (NO), which activates soluble guanylyl cyclase (sGC), inducing the formation of the second messenger cyclic guanosine monophosphate (cGMP). cGMP induces vasorelaxation, inhibits smooth muscle cell migration and proliferation, and tonically inhibits platelet activation, thereby preventing excessive aggregate formation and thrombosis. Furthermore, cGMP modulates myocardial oxygen metabolism, contractile function, ventricular hypertrophy and apoptosis, as well as fibrosis in the heart. Several cardiovascular diseases, such as congestive heart failure (CHF), coronary artery disease (CAD), hypertension, hypercholesterolemia, diabetes and early stages of atherosclerosis, are associated with a phenomenon commonly referred to as endothelial dysfunction. This is characterized by a significant reduction in NO bioavailability due to decreased formation or enhanced degradation. The common result is attenuated stimulation of sGC. In addition to impaired NO bioavailability, sGC itself can become dysfunctional, *e.g.*, under conditions of increased oxidative stress. Ataciguat, formerly referred to as HMR-1766, a novel anthranilic acid derivative, belongs to a new structural class of sGC activators capable of activating the oxidized form of sGC. Ataciguat has been shown to improve endothelial function and to reduce platelet activation in experimental diabetes. Chronic regular treatment with sGC activators such as ataciguat could therefore eventually constitute an effective therapy targeting deficient NO/cGMP signaling in hypertension, peripheral and coronary artery disease, heart failure, thrombosis and erectile dysfunction.

Endothelial function and nitric oxide-mediated guanylyl cyclase activation

The endothelium plays a crucial role in the control of vascular tone by releasing endothelium-derived autocoids, the most important of which is nitric oxide (NO) (1). The necessity of intact endothelium as the source of the "endothelium-derived relaxing factor" (EDRF) was discovered in 1980 by Furchgott and Zawadzki (2). Seven years later, Moncada and Palmer demonstrated that EDRF is NO generated by an endothelial enzyme, endothelial NO synthase (eNOS), which converts the amino acid arginine to citrulline either under resting conditions or after stimulation (3).

NO is a ubiquitous, cell-permeable intercellular messenger which diffuses through the cell membrane to the underlying smooth muscle cells (SMCs). There it interacts with its specific molecular target, soluble guanylyl cyclase (sGC), which partially associates with the plasma membrane in a state of enhanced NO sensitivity (see Fig. 1) (4).

The mammalian NO-sensitive sGC is a heterodimeric heme protein existing in two isoforms with similar enzymatic properties (5). Binding of NO to the ferrous heme of sGC rapidly catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). sGC heme iron has to be in the ferrous (Fe^{2+}) state for activation by NO. Upon NO binding, the iron is slightly moved out of the porphyrin plane, which is considered to trigger subsequent intramolecular rearrangements influencing the catalytic center (6).

The NO-induced cGMP signal exerts its effects through several intracellular mechanisms: cGMP-dependent protein kinases (cGKs), cGMP-regulated phosphodiesterases (PDEs) and cGMP-gated ion channels (5). cGK activation is the most prominent effector of NO in the cardiovascular system, controlling smooth muscle relaxation (7-9) and inhibition of platelet activation (10-16).

A common cGK substrate in SMCs and platelets is the vasodilator-stimulated phosphoprotein (VASP) (17-20). NO/cGMP-dependent phosphorylation of VASP plays a pivotal inhibitory role in the regulation of platelet activation (21). Phosphorylation of VASP correlates closely with inhibition of fibrinogen binding to platelet glycoprotein IIb/IIIa (gpIIb/IIIa) (11, 22). Increased cGMP induces phosphorylation of VASP preferentially at its serine residues 239 (Ser239) (21) and 157 (Ser157) (23), and

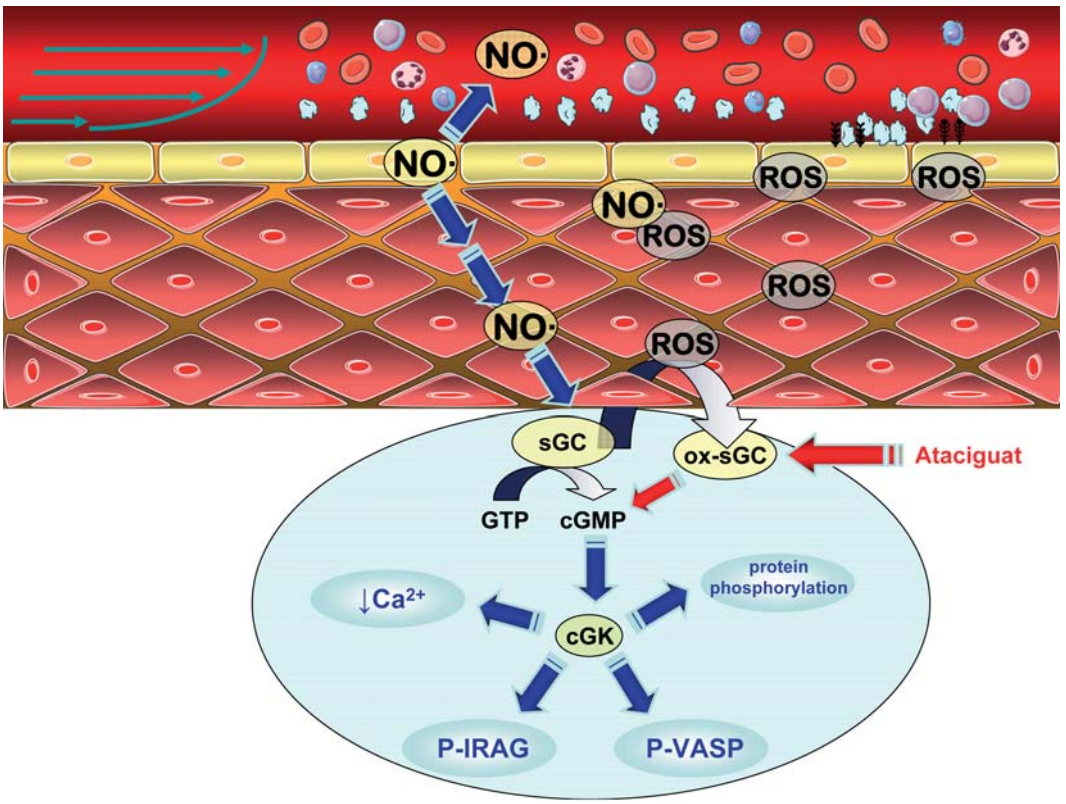


Fig. 1. Blood flow (green arrows) induces formation of nitric oxide (NO) in functional endothelial cells. Endothelium-derived NO exerts its effects within the vascular wall, targeting smooth muscle cells (SMCs) and on the luminal side interacts with blood cells, *e.g.,* it inhibits platelets and prevents their adhesion on the endothelium. In the target cell, NO activates NO-sensitive soluble guanylyl cyclase (sGC). Functional sGC catalyzes the reaction from guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which in turn activates the cGMP-dependent kinase (cGK). cGK ultimately reduces calcium influx (Ca^{2+}) and phosphorylates several substrate proteins*, e.g.,* the vasodilator-stimulated phosphoprotein (VASP) and the inositol-1,4,5-trisphosphate receptor I-associated protein (IRAG). Several cardiovascular diseases are associated with an increase in the generation of vascular reactive oxygen species (ROS) throughout the vessel wall. These scavenge NO and oxidize sGC (ox-sGC), such that the impaired remaining NO signal cannot activate sGC because ox-sGC is NO-insensitive. The novel direct sGC activator ataciguat is able to stimulate ox-sGC and therefore can improve cGMP-mediated activation of cGK in cardiovascular disease states with increased formation of ROS. See main text for details.

modulates platelet actin filament interactions (24). VASP phosphorylation affects initial sequences in platelet adhesion and activation (12, 25, 26). Using specific antibodies, VASP phosphorylation provides a sensitive monitor of defective NO/cGMP signaling, and reduced NO bioavailability in several pathophysiological states correlates with reduced VASP phosphorylation (27).

Endothelial dysfunction

Endothelial dysfunction was initially described as impaired vasodilatation in response to agonists such as acetylcholine and bradykinin, *e.g.*, in the forearm vasculature of patients with hypertension (28). Whereas the infusion of an NOS inhibitor blunts the vasodilator response to acetylcholine in control subjects, the arginine analogue does not significantly alter the response to acetylcholine in hypertensive patients. This indicates that hypertensive patients have a defect in the endothelium-derived NO system that accounts for the impaired response to endothelium-dependent vasodilators (29). Compelling evidence suggests that endothelial dysfunction results from increased vascular production of superoxide anions in several pathophysiological states such as hypertension, heart failure and diabetes (30-33).

The renin-angiotensin system (RAS) is a central component of the physiological and pathological responses of the cardiovascular system. Its primary effector, angiotensin II, is implicated in inflammation, endothelial dysfunction, atherosclerosis, hypertension and congestive heart failure (CHF), in addition to its physiological vasocontractile and blood pressure-regulating effects (34). Inhibition of angiotensin-mediated signaling in patients with atherosclerosis reverses endothelial dys-

function by improving NO availability (35). Angiotensin II causes endothelial dysfunction and reduces vascular NO bioavailability by increasing vascular superoxide anion formation, enhanced vascular protein kinase C (PKC) activity and expression of the NADPH oxidase subunits nox1, $gp91^{phox}$ and $p22^{phox}$ (36). Because superoxide rapidly scavenges NO within the vascular wall, a reduction of bioactive NO might occur despite a compensatory increased NO generation.

Normal endothelial function plays a pivotal role in the suppression of SMC proliferation. NO and prostacyclin induce the formation of cGMP and cAMP, respectively. Both cyclic nucleotides regulate cell cycle molecules in human SMCs and suppress mitogenesis, proliferation and migration (37, 38). Chronic inhibition of NOS in rats changes SMC gene expression in favor of a shift towards cell proliferation (39). NO has antiproliferative properties and inhibits the angiotensin II-induced migration of SMCs (40). Gene transfer of cGK sensitizes cultured SMCs to the antiproliferative effects of NO/cGMP (41). Organic nitrates, which liberate NO, suppress proliferation and mitogenesis, an effect enhanced by PDE5 inhibition (42, 43). YC-1, an NO-independent activator of sGC (44), exerts vascular protection through inhibition of SMC proliferation (45).

In addition to these intramural effects of deficient NO signaling, impaired luminal NO bioavailability precipitates arterial thrombosis in animal models and in individuals with endothelial dysfunction (46). Chronic inhibition of NO formation in animal models is associated with impaired fibrinolysis, enhanced thrombin and tissue factor generation (47). Platelet activation is increased in disease states with impaired NO bioavailability, such as acute coronary syndromes (48), heart failure (49, 50), diabetes (51-53) and hypercholesterolemia (54). In patients with advanced atherosclerosis, an impaired endothelium-dependent release of NO leads to reduced platelet cGMP formation (55).

We previously demonstrated that acute reduction of NO bioavailability *in vivo* rapidly increases platelet activation in humans (16) and mice (52), which is immediately reversed by exogenous NO. This direct relationship between NO bioavailability and human platelet function *in vivo* suggests that platelet activation in healthy individuals is suppressed by tonic NO production, resulting in immediate platelet activation when NO production is inhibited (16).

The relevance of the NO/cGMP signaling pathway for platelet inhibition was demonstrated by several *in vitro* and *in vivo* studies: genetic deletion of the sGC β1 subunit completely prevented NO-mediated inhibition of platelet aggregation (56), and platelet cGK proved essential to prevent platelet-endothelium adhesion and platelet-platelet aggregation after ischemia (25). Similarly, deletion of VASP increased platelet adhesion to the endothelium of $ApoE^{-/-}$ mice and to the subendothelial matrix following endothelial denudation, which could not be rescued by exogenous NO (26). These results strengthen the central functional role for NO/cGMP/cGK/VASP signaling in tonic platelet inhibition. The significance of disruption of this pathway for enhanced platelet activation in endothelial dysfunction is underlined by several studies, which demonstrate reduced platelet activation following positive pharmacological modulation of endothelial function: angiotensin or aldosterone blockade (49, 57), HMG-CoA reductase inhibition (50, 58) or direct stimulation of sGC with ataciguat (HMR-1766; sanofi-aventis) (53) reduced platelet activation and enhanced platelet VASP phosphorylation parallel to improved endothelial function in experimental CHF and diabetes.

Nitrate tolerance and eNOS uncoupling

The logical method of treating reduced NO bioavailability would be the supplementation of exogenous NO using NO donors such as organic nitrates. While this is a useful short-term approach during acute states of deficiency, prolonged nitrate treatment exerts several unfavorable effects, *e.g.,* it induces tolerance to nitrates and cross-tolerance to nitrovasodilators and endothelium-derived NO (59). Long-term treatment leads to enhanced superoxide anion production, at least partially facilitated by dysfunctional eNOS itself contributing to the so-called eNOS uncoupling (60), a phenomenon observed in several cardiovascular disease states, where eNOS generates superoxide instead of NO and enhanced eNOS activity might be deleterious (33, 61-63). In addition to scavenging of NO by superoxide, the reaction product peroxynitrite can oxidize the eNOS cofactor tetrahydrobiopterin, leading to uncoupling of eNOS. Restoration of endothelial tetrahydrobiopterin levels by transgenic approaches in genetic models of atherosclerosis reduces superoxide generation, normalizes endothelial function and attenuates disease progression (62). Similarly, normalization of tetrahydrobiopterin is generally able to restore eNOS-mediated NO formation and endothelial function in hypertension, hypercholesterolemia and diabetes (64-66). eNOS uncoupling and endothelial dysfunction are apparent in experimental diabetes and in diabetic patients (33, 67) despite the fact that eNOS expression is actually increased. Therefore, approaches merely aimed at enhanced eNOS activity would not improve NO bioavailability, but would aggravate endothelial dysfunction.

sGC dysfunction

In principal, two therapeutic approaches remain to enhance defective cGMP signaling: 1) preventing the degradation of the remaining cGMP by PDE inhibitors; or 2) increased stimulation of sGC by direct activators of the enzyme.

sGC heme iron has to be in the ferrous (Fe^{2+}) state for activation by NO. While NO increases sGC activity, reactive oxygen species (ROS) exert opposite effects on the enzyme and functionally counteract NO (68). Activation by NO is lost but basal activity is preserved if the heme

iron is oxidized to the ferric (Fe^{3+}) state (69). Increased oxidative stress decreases the expression and impairs NO-induced activation of heme-containing sGC, making vasodilator therapy with NO donors less effective. Under conditions of enhanced oxidative stress *in vivo* in several disease states, including diabetes, sGC is indistinguishable from the *in vitro* oxidized/heme-free enzyme (69). The oxidized/heme-free sGC variant is unresponsive to NO and prone to degradation.

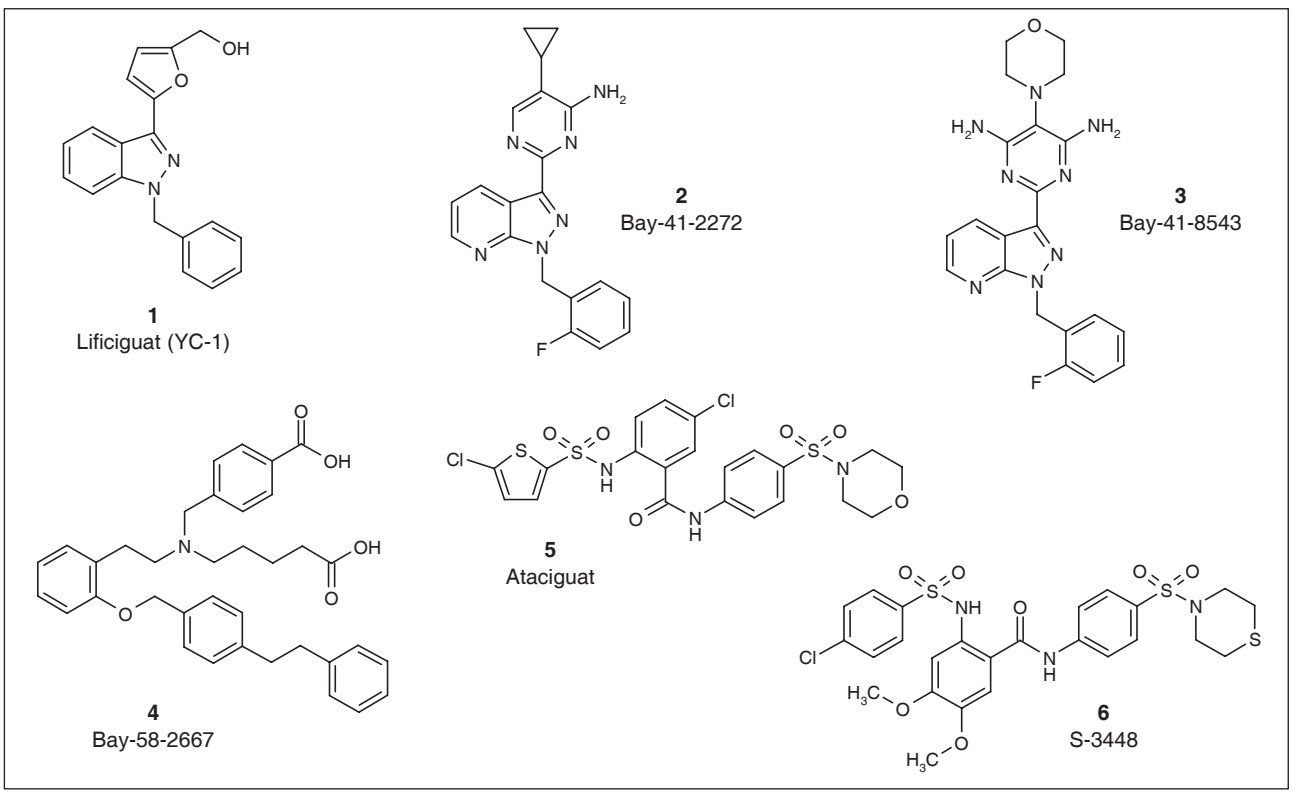
Thus, trying to enhance cGMP signaling by PDE inhibition is hampered by the dependence on a sufficiently high remaining cGMP signal. As described above, this signal is strongly affected by reduced formation of NO, uncoupling of eNOS, enhanced scavenging of NO by ROS and reduced sensitivity of sGC for NO. Therefore, heme-dependent and -independent activators of sGC have recently emerged as potential modulators of defective NO/cGMP-mediated signaling (70). Novel NO-independent sGC activators such as Bay-58-2667 or ataciguat stabilize and/or activate the NO-insensitive sGC variant (69, 71). These drugs may represent a useful therapeutic approach to preferentially dilate diseased blood vessels.

sGC activators

The first substance characterized as an NO-independent, heme-dependent stimulator of sGC was YC-1 (lificiguat, **1**) (72). YC-1 potentiates the efficacy of nitrovasodilators (44) and causes persistent elevation of intravascular cGMP levels *in vivo* by activating sGC and inhibiting cGMP breakdown (73). Other heme-dependent sGC stimulators were synthesized based on YC-1, including Bay-41-2272 (**2**) and Bay-41-8543 (**3**), which are both approximately two orders of magnitude more potent than YC-1 (70).

Activation of sGC with Bay-41-2272 reverses hemodynamic and structural changes associated with monocrotaline- and chronic hypoxia-induced experimental pulmonary hypertension. This effect is partially dependent on endogenous NO generated by eNOS (74). Chronic sGC activation in mice inhibited endothelial P-selectin expression and leukocyte recruitment in mesenteric postcapillary venules following an inflammatory response elicited by IL-1β (75). NO and prostacyclin exhibit synergistic activity with Bay-41-2272 to attenuate platelet aggregation and significantly reduce blood pressure (76). Chronic sGC activation avoided the hypertension induced by systemic NOS inhibition in rats and prevented cardiac hypertrophy and fibrosis (77).

Two sGC activators, Bay-58-2667 (**4**) and Bay-41-2272, have been investigated in pacing-induced heart failure. Acute intravenous sGC activation in dogs potently unloaded the heart, increased cardiac output and renal blood flow, and preserved renal function without further neurohumoral activation (78, 79). Because the oxidized/heme-free sGC variant is unresponsive to NO and prone to degradation, novel NO-independent sGC activators such as Bay-58-2667, which stabilize and activate the NO-insensitive sGC variant, might represent a useful therapeutic approach, preferentially dilating diseased blood vessels (69).



Ataciguat

Ataciguat (**5**) is a novel anthranilic acid derivative activating heme-oxidized sGC (71). Ataciguat and S-3448 (**6**) comprise a new structural class of sGC activators capable of activating the oxidized and/or heme-free forms of sGC (71). The potential interesting application of this class of substances is indicated by the efficacy in initial pharmacological studies: both compounds increased cGMP levels in cultured rat aortic SMCs and relaxed isolated endothelium-denuded rat aorta (71). Similarly, chronic treatment with ataciguat improved endothelial function and reversed the change to a noncontractile phenotype in aortae from diabetic rats (80). In $ApoE^{-/-}$ mice, ataciguat improved endothelium-dependent vasorelaxation and reduced atherosclerosis (81).

As observed with other sGC activators, ataciguat attenuated the development of pulmonary hypertension and right heart hypertrophy, preserved systemic arterial blood pressure, improved gas exchange and cardiac output in monocrotaline-induced pulmonary hypertension (82). In a model of transforming growth factor-β (TGF-β)-induced cardiac fibroblast extracellular matrix synthesis, ataciguat inhibited fibronectin protein synthesis in a concentration-dependent manner and prevented the differentiation of fibroblasts into myofibroblasts (83).

Pharmacological sGC activation exerts antiplatelet properties *in vitro* by increasing platelet cGMP, the main mediator of NO effects in platelets (13, 76, 84). Ataciguat elicited phosphorylation of the cGK substrate VASP at Ser239 and reduced platelet reactivity (53). The results from *in vitro* and acute *in vivo* experiments in diabetic rats suggest that the stimulation of platelet sGC by ataciguat was independent of sGC dysfunction (53). Chronic sGC stimulation *in vivo* attenuated the activation of gpIIb/IIIa (determined by platelet fibrinogen binding), as well as platelet degranulation (determined by P-selectin surface expression). P-selectin can participate in platelet adhesion to the endothelium and is responsible for platelet-leukocyte adhesion (85, 86). P-selectin-expressing platelets play a pivotal role in the interaction of activated platelets with leukocytes and the exacerbation of atherosclerosis (87). Earlier studies reported inhibition of P-selectin and CD40 ligand surface expression by cAMP/cGMP-dependent protein kinases (88), the activation of which can be monitored by VASP phosphorylation (23).

Patients with diabetes have an increased risk of thrombosis and accelerated atherogenesis. Platelet degranulation in patients with diabetes is associated with progression of proatherosclerotic vessel wall modification (89). Ataciguat induces vasorelaxation of porcine coronary arteries (71) and might therefore –together with inhibition of platelet activation— exert potential beneficial effects in stable and unstable CAD. Activation of oxidized sGC by ataciguat might be useful to overcome impaired platelet NO responsiveness, which in addition to decreased endothelial formation of NO is a predictor of increased mortality and cardiovascular morbidity in patients with acute coronary syndromes (90).

Deficient endothelium-dependent and -independent vasodilatation occurs often before the development of other overt functional or structural systemic vascular disease in patients with erectile dysfunction (91). Erectile dysfunction in diabetic men correlates with endothelial dysfunction, and reduced NO bioavailability/activity might provide a unifying explanation (92). Similar to PDE5 inhibitors, which prevent the degradation of cGMP, ataciguat sufficiently relaxes human corpus cavernosum (71).

Currently, the ACCELA study (ClinicalTrials.gov Identifier: NCT00443287) is the first clinical trial assessing the efficacy and safety of ataciguat in patients with peripheral arterial occlusive disease (PAOD). The primary objective is to investigate whether a 26-week treatment with ataciguat (three different doses) in addition to clopidogrel results in an improvement in walking capacity in patients suffering from intermittent claudication due to Fontaine stage II peripheral arterial disease, compared to placebo. The effects will be compared with cilostazol, which reversibly inhibits platelets via selective antagonism of PDE3.

Conclusions

Chronic regular treatment with sGC activators such as ataciguat may constitute an effective therapy targeting hypertension, peripheral and coronary artery disease, as well as improving cardiac function, inhibiting platelet activation and thrombosis, and preventing erectile dysfunction. sGC activation with ataciguat combines enhancement of cGMP signaling independent of NO bioavailability. Given its ability to activate the heme-oxidized NO-insensitive form of sGC, ataciguat might still be active under certain conditions of oxidative stress, when other sGC activators or NO itself are ineffective because of sGC dysfunction. Ataciguat might therefore represent a useful treatment approach in atherosclerotic cardiovascular disease.

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