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Guanylate Cyclase Activators, Cell Volume Changes and IOP Reduction

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Key Words

Nitric oxide • Soluble guanylate cyclase • Protein kinase G • Cell volume • Trabecular meshwork • Schlemm's canal • Glaucoma • Aqueous humor outflow

Abstract

Glaucoma afflicts millions of people worldwide and is a major cause of blindness. The risk to develop glaucoma is enhanced by increases in IOP, which result from deranged flow of aqueous humor. Aqueous humor is a fluid located in the front of the eye that gives the eye its buoyancy and supplies nutrients to other eye tissues. Aqueous humor is secreted by a tissue called ciliary processes and exits the eye via two tissues; the trabecular meshwork (TM) and Schlemm's canal. Because the spaces through which the fluid flows get smaller as the TM joins the area of the Schlemm's canal, there is resistance to aqueous humor outflow and this resistance creates IOP. There is a correlation between changes in TM and Schlemm's canal cell volume and rates of aqueous humor outflow; agents that decrease TM and Schlemm's canal cell volume, increase the rate of aqueous humor outflow, thus decreasing IOP. IOP is regulated by guanylate cyclase activators as shown in humans, rabbits and monkeys. There are two distinct groups of guanylate cyclases, membrane guanylate cyclase and soluble guanylate cyclase (sGC); activation of both have been shown to decrease IOP. Members of the membrane guanylate cyclase family of receptors bind to peptide ligands, while the sGC responds to gases (such as NO and CO_2) and compounds (such as YC1, [3-(5'-hydroxymethyl-2'furyl)-1-benzyl indazole), a benzyl indazole derivative, and BAY-58-2667); activation of either results in formation of cyclic GMP (cGMP) and activation of protein kinase G (PKG) and subsequent phosphorylation of target proteins, including the high conductance calcium activated potassium channel (BKca channel). While activators of both membrane guanylate cyclase and sGC have the ability to lower IOP, the IOP lowering effects of sGC are noteworthy because sGC activators can be topically applied to the eye to achieve an effect. We have demonstrated that activators of sGC increase the rate at which aqueous humor exits the eye in a time course that correlates with the time course for sGC-induced decreases in TM and Schlemm's canal cell volume. Additionally, sGC-induced decrease in cell volume is accompanied by both K^+ and Cl^- efflux induced by

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activation of K^+ and Cl^- channels, including the BKca channel and/or K^+/Cl^- symport. This suggests that parallel K^+/Cl^- efflux, and resultant H_2O efflux result in decreases in cell volume. These observations suggest a functional role for sGC activators, and suggest that the sGC/cGMP/PKG systems are potential therapeutic targets in the treatment of glaucoma.

Introduction

Elevated intraocular pressure (IOP) puts a patient at an increased risk for developing visual field loss, in the progressive blinding disease, glaucoma. IOP results from the balance between aqueous humor secretion by the ciliary processes and outflow through the TM and Schlemm's canal (the conventional outflow pathway). The major route for the outflow of aqueous humor is the TM [1, 2] comprising of uveal and corneoscleral TM [1] and the juxtacanalicular cells (JCT-TM) [3], in conjunction with the Schlemm's canal. An improper balance of aqueous humor secretion and outflow, in particular increased resistance to the outflow of aqueous humor through the conventional outflow pathway, will yield high IOP. Through an unknown mechanism, this elevated IOP increases the likelihood of retinal ganglion cell death. As retinal ganglion cells are lost, the patients vision is progressively lost as well, both of which are irreversible. Since we are currently unable to repair the damage from the loss of the retinal ganglion cells, the only viable therapy for patients with ocular hypertension is management of IOP; because it has been demonstrated that prolonged and substantial lowering of IOP in those with glaucoma (whether accompanied by elevated IOP or not) slows or prevents vision loss [4-6].

Currently IOP can be managed by two major methods; ocular hypotensive drugs or surgery. In general, both protocols are intended to increase the drainage of aqueous humor from the eye. Current therapies for lowering IOP include inhibiting the secretion of aqueous humor or enhancing the drainage of aqueous humor from the eye. There are two drainage areas in the eye; the uveoscleral and the conventional outflow pathway. While medical therapeutics available for daily use effectively reduce secretion, only one of the two outflow pathways is currently targeted to improve drainage from the eye; the uveoscleral pathway. Unfortunately, there are no effective daily treatments that act primarily on the conventional pathway, that accounts for the majority (up to 90%) of total aqueous humor drainage in humans. Therefore understanding the cellular and molecular mechanisms that regulate the conventional outflow pathway is of importance to the development of novel therapeutic strategies for the treatment of glaucoma. This review will discuss the role of guanylate cyclase activation in the regulation of aqueous humor outflow in the conventional outflow pathway. Specifically, we will discuss the role of activation of sGC by nitric oxide (NO)-dependent and independent mechanisms in regulating cell volume in the conventional outflow pathway (Fig. 1). Additionally, we will discuss the signaling mechanism(s) and the ion channel(s) involved.

Changes in Cell Volume Regulate Aqueous Humor Outflow and Subsequently IOP

Once thought to be a passive process, the outflow of aqueous humor is now known to be actively controlled by the cells in the aqueous humor outflow pathway. Several mechanisms have been characterized by which changes in aqueous humor outflow can occur. These include TM and Schlemm's canal cell volume changes [7-15], ciliary muscle contractility [16-19], alterations of the extracellular matrix in the TM and pore formation and vacuoles in the inner wall of the Schlemm's canal [20-22]. This discussion focuses on TM and Schlemm's canal cell volume and the role the changes in cell volume play in regulating outflow facility.

Cell volume changes in the aqueous humor outflow pathway are of interest to study as they have been correlated with changes in outflow facility. Previous studies demonstrated that hypotonic or hypertonic challenge to perfused eye anterior segments will decrease or increase outflow facility respectively [7]. To hypothesize a mechanism by which changes in cell volume in the outflow pathway would affect aqueous humor drainage we must understand the architecture of the aqueous humor outflow pathway. The TM is comprised of three anatomical regions; the uveal, corneoscleral and the juxtacanalicular regions [3, 23]; with two distinct cell populations [15, 24]. The uveal and corneoscleral TM are composed of interwoven collagen covered elastin "beams" surrounded by spaces through which fluid flows. As aqueous humor flows deeper into the juxtacanalicular TM, towards the Schlemm's canal, the open spaces

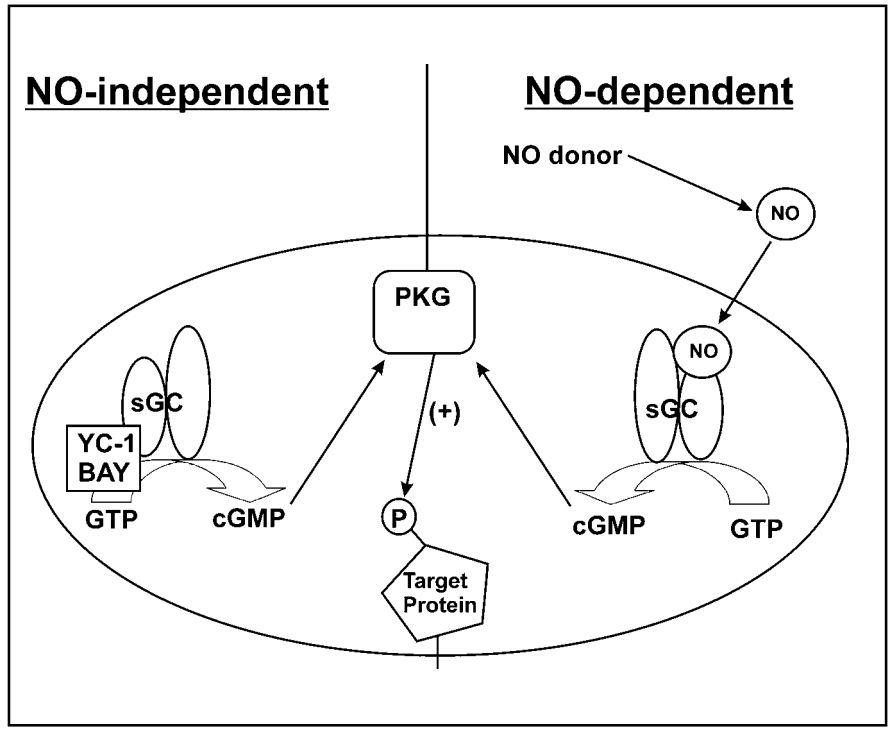

Fig. 1. Summary diagram of the pathway involving nitric oxide (NO) -dependent and -independent regulation of trabecular meshwork and Schlemm's canal endothelial cell volume. NO donors cause the release or the formation of NO, which then binds to and activates soluble guanylate cyclase (sGC). NO- independent activation of sGC is achieved using YC-1 and BAY-58-2667 (BAY). Activation of sGC results in the synthesis of cGMP from GTP. cGMP activates protein kinase G (PKG) which phosphorylates (P) target proteins including the BKca channel, with subsequent K^+ efflux.

available for fluid to flow through decrease. As the space sizes for fluid flow decreases, the volume and/or contractility of the cells lining these openings would begin to exert more influence over the extracellular matrix associated with these cells and subsequently influence outflow facility. Therefore, increasing or decreasing the volume of the cells in the juxtacanalicular TM, and the endothelial cells lining the wall of the Schlemm's canal would therefore decrease or increase the rate by which fluid flows exits the eye, respectively. To understand the components involved in maintaining and/or returning to resting cell volume and basal outflow facility, investigators have utilized perfused eye anterior segments.

Using this technique, several ion channels have been implicated in mediating the recovery to baseline outflow facility following a hypotonic or hypertonic challenge [12]. Recovery from a hypotonic challenge was slowed and the magnitude of the decrease in outflow facility was greater in the presence of the BKca channel inhibitor iberiotoxin. The opposite effect was seen when the BKca channel activator NS1619 was included with the hypotonic challenge. Recovery to baseline outflow quickened and the magnitude of the outflow facility reduction was decreased. This study also implicated the chloride swell channel in mediating a recovery for hypotonic challenge as the channel blocker tamoxifen produced effects similar to iberiotoxin [12]. Additionally, the Na, K, 2Cl co-transporter blocker bumetanide, which has been shown to decrease TM cells volume [10], increased outflow facility in an eye anterior segment perfused with isotonic medium [7]. While these mechanisms have been experimentally shown to contribute to the regulation of aqueous humor outflow, the possibility exist that other unknown mechanisms also contribute.

Nitric Oxide Synthase in the Aqueous Humor Outflow Pathway

Nitric oxide (NO), a diatomic gas with a very short half-life of just a few seconds [25], is produced by a family of enzymes, the nitric oxide synthases (NOS), through the conversion of L-Arginine to NO and L-citrulline. The active enzyme is composed of a heme-bound homodimer assembled from heme-deficient monomers. There are 3 major types of NOS in mammalian tissue; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are generally expressed constitutively, and are capable of producing low levels of NO, <1uM [26]. The production of NO is highly regulated in the body, as NOS form complexes with associated proteins that may be inhibitory or stimulatory [27]. NO production is thought to have two distinct roles in the body;

production of an autocrine/paracrine signaling molecule and production of a toxic free-radical gas. With all three isoforms producing the same molecule, the pathophysiological implications of any inappropriate NO production must be kept under consideration.

Postmortem human tissue aqueous humor outflow pathway has been shown to express eNOS [28], however in cultured human TM cells, we observed a ~160kDa band with a polyclonal nNOS antibody, indicating nNOS expression [29]. These observations need further confirmation. Nitrinergic nerve terminals have also been localized in the TM [30]. Other studies have determined that activation of NOS in response to increased perfusion pressure[31, 32] or in response to kappa opioid receptor activation [33] resulted in increased NO production in the outflow pathway.

NOS expression in the TM suggests that endogenous NO production would influence aqueous humor outflow. In fact, studies demonstrate that the NOS inhibitor L-NAME, dose-dependently reduced outflow facility in perfused human eye anterior segments [34] further suggesting a functional role for NOS activity in outflow facility. Similarly, we have shown a functional effect of NOS activity on TM and Schlemm's canal cell volume in culture as L-NAME dose-dependently increased cell volume [15].

Nitric Oxide Regulation of IOP

Numerous studies have demonstrated the effects of NO on IOP and aqueous humor outflow facility. Topical application of NO donors to rabbit eyes [35] reduced IOP by increasing the rate of aqueous humor outflow through the conventional outflow pathway [36]. Studies have also shown that intravitreal and intracameral injections of NO donors in rabbits caused a drastic decrease in IOP, which was correlated with nitrite production indicating that NO was released [37]. Additionally it has been shown that NO donors reduce IOP in monkeys through an action on outflow resistance [38]. Most recent studies have demonstrated decreased IOP and increased aqueous humor outflow in mice over expressing eNOS when compared to wild type animals [39].

NO generated by nNOS and eNOS has been shown to activate cyclic-nucleotide-gated channels, protein kinases, and phosphodiesterases [40]. These physiological processes may be considered 'low concentration' effects, requiring less than 1 μM of NO [26]. High concentrations of NO produced by iNOS can result in auto-oxidation and the production of dinitrogen trioxide [41], which is the primary mechanism of nitrosylation in which nitrosothiols of cysteines are formed. In addition, strong oxidants such as superoxide (a reactive oxygen species) can act with NO to form peroxynitrite [42] which reacts with the phenol moiety of tyrosine resulting in nitration of tyrosine residues in proteins. Both nitrosylation and nitration of tyrosine residues have been shown to affect protein function. The implications of these findings on the effect of high concentrations of NO to increase aqueous humor outflow and NOs effect on retinal ganglion cells are unknown. This is outside the scope of our discussion, but must always remain under consideration. Here we will focus on the effects of low, physiologically relevant concentrations of NO such as produced by nNOS or eNOS.

NO mediates its physiological effects by binding to its receptor, sGC. Once bound to sGC, it causes the conversion of guanosine 5'-triphosphate (GTP) to 3'-5'-cyclic guanosine monophosphate (cGMP). cGMP will then go on to interact with various cyclic-nucleotide gated channels, protein kinases and protein phosphodiesterases to produce physiological effects. Because the binding of NO to sGC results in decreased IOP, the study of the activation sGC in the tissues of the aqueous humor outflow pathway, becomes critically important since it may provide new targets for the development of ocular hypotensive drugs. In fact, inhibition of sGC abolished the NO-induced increases in aqueous humor outflow [43], suggesting the involvement of sGC in mediating the NO-induced decreases in IOP.

Guanylate Cyclase and IOP

The role of guanylate cyclase and cGMP in regulating IOP is well documented. There are two distinct groups of guanylate cyclases, membrane guanylate cyclase (mGC) [44-48] and sGC [40, 49-54]. The members of the mGC family of receptors bind peptide ligands; atrial natriuretic peptide, brain natriuretic peptide and c-type natriuretic peptide. sGC has binding sites for gases such as NO and carbon dioxide and other ligands that are NO-independent, such as YC1. Activators of both mGC and sGC have been shown to decrease IOP in humans, rabbit and monkey [35-38, 55-63] thus providing a physiological role for the guanylate cyclases in IOP regulation. Because the natriuretic peptides cannot be absorbed in the eye, our study focuses on activators of sGC.

sGC comprises an α subunit and a smaller heme-containing β subunit, both of which constitute the active enzyme. The heterodimers are activated by NO binding to the heme moiety [64-66], whereas homodimers exhibit little or no synthetic activity, even in the presence of NO. Binding of NO to ferrous heme activates sGC whereas oxidation of heme to ferric heme results in complete attenuation or inhibition of the sGC response. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) is a specific inhibitor of sGC [67-69] and oxidizes sGC in a highly selective manner, therefore ODQ is used to inhibit the NO-dependent response.

We have pharmacologically characterized sGC in bovine TM and biochemically characterized sGC in primary human TM cells [43]. The α and β sGC subunits are expressed in an equivalent ratio and addition of the NO donor, DETA-NO, increased cGMP levels in low passage human and porcine TM cells. Taken together these data would suggest a functional sGC heterodimer in regulating aqueous humor outflow and IOP. We further characterized sGC in transformed human TM cells, however, the addition of DETA-NO did not result in increases in cGMP levels. Additionally, Western blots of fractionated TM cell lysates would suggest that while the sGC heterodimer is necessary for sGC activity, it is not sufficient and may require other factors that are not present in transformed cells [43].

Activation of sGC is involved in the NO-induced increases in outflow facility, as addition of ODQ to the perfusate abolished the NO-induced increases in aqueous humor outflow facility [43]. Furthermore, inhibition of sGC in TM and Schlemm's canal cells, abolished the NO-induced decreases in TM and Schlemm's canal cell volume suggesting a functional role for sGC in regulating conventional aqueous humor outflow and IOP.

Protein Kinase G (PKG)

Increased cGMP activates PKG, which phosphorylates target proteins. NO/PKG phosphorylates specific proteins at either serine or threonine residues changing their conformation and thus altering their function. There are two PKG isoforms, PKGI and PKGII, that are tissue specific. PKGI is expressed in cerebellum, dorsal root ganglia, lung, smooth muscle, platelets, hippocampus and olfactory bulb. PKGII is expressed in the intestinal mucosa, kidney, chondrocytes and several brain nuclei (see review [70]). Pharmacological evidence suggests that PKG mediates NO function in TM [14] and Schlemm's canal [15], suggesting a functional role for PKG in both TM and SC. Specifically, inhibition of PKG, by the specific inhibitor, Rp-8-Br-PET-cGMPS, abolished the NO- induced decreases in TM and Schlemm's canal cell volume [14, 15].

Other Activators of Soluble Guanylate Cyclase

In addition to the endogenous sGC activator NO, compounds such as YC-1 have been shown to activate sGC independent of NO [71] and decrease IOP [72]. Though the mechanism by which YC-1 activates sGC is not fully understood, it is thought to affect the positioning of the heme in the enzyme [73] or enhance the activation of sGC by NO [74] in a synergistic manner. With evidence of a decreased ability to produce NO in the TM of glaucomatous eyes [75], it is also of interest to note the reported synergistic effect of YC-1 on the NO-induced stimulation of sGC [76]. This suggests the possibility that drugs like YC-1 may be used, not only to activate sGC, but also to amplify the diminished, endogenous NO production capacity suggested by the above mentioned study. In addition to YC-1-like, heme-dependent sGC activators, several heme-independent sGC activators have been characterized. These compounds, like BAY-58-2667, are reported to activate sGC even though the heme may be absent or oxidized [77], a state in which NO cannot activate sGC. With the complexities of NO's participation in the redox state of a cell, as well as the ability of YC-1 to act synergistically with NO and BAY-58-2667 to activate sGC independent of heme, it is of significant clinical interest to investigate the ability of NO-independent sGC activators to affect outflow facility and volume changes in TM cells.

Our laboratory provided evidence that YC-1 and BAY-58-2667 decrease human TM cell volume through the involvement of the BK_{Ca} channel. Specifically, YC-1 and BAY-58-2667 decreased TM cell volume; these decreases are mediated by the sGC/cGMP/PKG pathway in a manner dependent on the BK_{Ca} channel [78]. The actions of YC-1, however, are biphasic, with lower concentrations causing increases in TM cell volume, while higher concentrations elicit a cell volume reduction. The biphasic effects of YC-1 and BAY-58-2667 could be explained by the possible existence of two binding sites for these compounds on sGC that may

involve heme-dependent and independent moieties of the sGC [79].

In our hands, the specific inhibitor of sGC, ODQ attenuated the YC-1-induced decreases in TM cell volume, however, ODQ potentiated the BAY-58-2667 effects. While the precise mechanisms are unclear, experimental evidence suggests that removal of the heme prosthetic group or oxidation to its ferric form by ODQ causes conformational changes in sGC such that it no longer responds to NO or YC-1 but does respond to BAY-58-2667 [80]. While we were not able to demonstrate sGC involvement in the BAY-58-2667-induced decreases in TM cell volume, other studies have demonstrated that BAY-58-2667 binds to and activates sGC with subsequent increases in cGMP levels [81]. Similarly, we demonstrated that higher concentrations of BAY-58-2667 caused increases in cGMP levels that correlated with decreases in TM cell volume, suggesting sGC involvement in BAY-58-2667-induced decreases in TM cell volume.

BK_{Ca} Channel Modulation of TM and Schlemm's Canal Cell Volume

The BK_{Ca} channels are activated by increased calcium [82, 83], changes in cell membrane voltage [84] or neurotransmitters including NO [85, 86]. In cerebral artery smooth muscle cells the phosphorylation of the α subunit of the BK_{Ca} channel by PKG has been shown to mediate the NO-induced activation the BK_{Ca} channel [85].

Studies demonstrating the cGMP-induced activation of the BK_{Ca} channel in bovine TM cells [87] and the channel's involvement in cGMP-induced relaxation of isolated bovine TM strips [88] provide evidence demonstrating the influence of the BK_{Ca} channel in regulating aqueous humor outflow. Therefore, we investigated the role BKca channel activation plays in regulating TM cell volume and outflow facility. To do this we utilized a well characterized, BK_{Ca} channel blocker, iberiotoxin [89]. In addition to the endogenous BK_{Ca} activity modulators mentioned above, several compounds have been synthesized that are reported to directly activate the channel, we utilized the benzimidazolone derivative, NS1619 as our BK_{Ca} channel activator [90, 91].

We demonstrated, for the first time the ability of NS1619 to increase outflow facility in a time course that correlates with the NS1619-induced decreases in TM cell volume. Interestingly, the decreases in TM cell volume in response to NS1619 were equivalent to the decreases in cell volume observed in response to the NO donor, DETA-NO only, but in combination, NS1619 and DETA-NO produced no additive cell volume decrease. Released NO from DETA-NO is thought to activate the BK_{Ca} channel via protein phosphorylation through protein kinase G [85, 92]. This PKG-dependent phosphorylation of the BKca channel increased the outward currents in rabbit arterial smooth muscle cells by increasing the voltage-dependent open probability of the channel [85]. A similar mechanism is suggested to underlie the actions of NS1619 on BK_{Ca} channel activity [90, 93].

These reductions in TM cell volume with NS1619 in our experiments may be due to potassium efflux, as studies in the TM have demonstrated that inhibition of the BK_{Ca} channel with iberiotoxin is associated with inhibition of potassium conductance [94]. Potassium efflux through the BK_{Ca} channel with possible parallel chloride efflux would tend to drive water out of the cytosol thus decreasing cell volume [95]. Taken together, these observations suggest that activating the BKca channel with either DETA-NO or NS1619, presumably by altering the open probability of the channel, is sufficient to elicit a reduction in cell volume.

To further demonstrate the ability of the BKca channel to affect changes in TM cell volume, we included NS1619 in a hypotonic challenge and examined the drugs effect on regulatory volume decrease. NS1619 reduced the hypotonic-induced increase in TM cell volume. Previously, we demonstrated that inhibition of the BKca channel with iberiotoxin potentiated the hypotonic-induced increases in cell volume and delayed the recovery to resting cell volume [14]. Similar results were demonstrated in another study; NS1619 when included in a hypotonic challenge to a perfused bovine eye reduced the decrease in outflow facility and quickened recovery to baseline. Conversely, iberiotoxin potentiated the hypotonic-induced decreases in outflow facility and delayed recovery to baseline [12]. Taken together, these data provide further evidence that BKca channel activity modulates TM cell volume and this modulation of TM cell volume may affect outflow facility.

The TM cell volume is also influenced by the activities of other transporters; the Na-K-2Cl exchanger [7, 10, 11] the Na^+/H^+ transporter [96], and the K^+ and Cl- channels [12, 96]. Further, it is possible that both the cellular contractile mechanisms and the cell volume regulatory mechanisms are functionally linked [97-99] as the BK_{Ca} channel, have been shown to regulate TM cell volume and contractility [12, 18] and

outflow facility [12]. Thus, decreasing volume and/or regulating the contractility of the TM and Schlemm's canal endothelial cells would influence the extracellular matrix associated with these cells and subsequently influence the resistance to aqueous humor outflow and subsequently IOP.

Abbreviations

IOP (Intraocular pressure); TM (trabecular meshwork); sGC (soluble guanylate cyclase); YC1 (3-(5'-hydroxymethyl-2'furyl)-1-benzyl indazole); cyclic GMP or cGMP (3'-5'-cyclic guanosine monophosphate); PKG (protein kinase G); BKca channel (high conductance calcium activated potassium channel); NO (nitric oxide); NOS (nitric oxide synthase); ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one); DETA-NO (diethylenetriamine); GTP (guanosine 5'-triphosphate); Rp-8-Br-PET-cGMPS (Rp- isomer 8- Bromo- β- phenyl-1, N^2- ethenoguanosine- 3', 5'- cyclic monophosphorothioate); P (phosphate); Bay (BAY-58-2667).

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