

STATE-OF-THE-ART PAPER

Cyclic Guanosine Monophosphate Signaling and Phosphodiesterase-5 Inhibitors in Cardioprotection

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Cyclic guanosine monophosphate (cGMP) is an important intracellular second messenger that mediates multiple tissue and cellular responses. The cGMP pathway is a key element in the pathophysiology of the heart and its modulation by drugs such as phosphodiesterase (PDE)-5 inhibitors and guanylate cyclase activators may represent a promising therapeutic approach for acute myocardial infarction, cardiac hypertrophy, heart failure, and doxorubicin cardiotoxicity in patients. In addition, PDE-5 inhibitors may prove to be innovative therapeutic agents for enhancing the chemosensitivity of doxorubicin while providing concurrent cardiac benefit. (J Am Coll Cardiol 2012;59:1921–7) © 2012 by the American College of Cardiology Foundation

Cyclic guanosine monophosphate (cGMP) is a critical intracellular second messenger regulating fundamental physiological processes in the myocardium, from acute contraction/relaxation to chronic gene expression, cell growth, and apoptosis. Several studies have shown that cGMP inhibits hypertrophy, reduces ischemia-reperfusion (I/R) injury, and regulates contractile function and cardiac remodeling (1–3). cGMP is generated from the cytosolic purine nucleotide guanosine triphosphate by guanylyl cyclases (GCs) using Mg^{2+} or Mn^{2+} as cofactors. Two isoforms of GCs exist in vertebrate cells and tissues: a nitric oxide (NO)-sensitive cytosolic or soluble guanylyl cyclase (sGC) and natriuretic peptide (NP)-activated plasma membrane bound, particulate guanylyl cyclase (pGC). Once produced, the effects of cGMP occur through 3 main groups of cellular target molecules: cyclic guanosine monophosphate-dependent protein kinases (PKGs), cGMP-gated cation channels, and phosphodiesterases (PDEs). cGMP positively regulates PKG but inhibits/activates PDEs, which are predominant in the cardiovascular system (4,5). This paper reviews many of the latest findings on cGMP related to the cardioprotection.

Regulation of cGMP by PDEs

The cGMP pool in the cell is tightly controlled by PDEs, which specifically cleave the 3',5'-cyclic phosphate moiety

of cyclic adenosine monophosphate (cAMP) and/or cGMP to produce the corresponding 5' nucleotide. Currently, 21 PDE genes have been cloned and are classified into 11 families according to their sequence of homology, biochemical, and pharmacological properties (6). The PDEs vary in their substrate specificity for cAMP and cGMP: PDE-5, PDE-6, and PDE-9 are specific for cGMP; PDE-4, PDE-7, and PDE-8 are specific for cAMP; and PDE-1, PDE-2, PDE-3, PDE-10, and PDE-11 have mixed specificity for cAMP/cGMP (7). PDE-5 selectively hydrolyzes cGMP, and its inhibition increases cGMP bioavailability. The abundance of PDE-5 in smooth muscles and its role in regulating their contractile tone has made PDE-5 an important drug target for the treatment of erectile dysfunction (6), leading to the development of potent PDE-5 inhibitors, such as sildenafil (Viagra and Revatio, Pfizer, New York, New York), vardenafil (Levitra, Bayer Schering Pharma AG, Leverkusen, Germany), and tadalafil (Cialis and Adcirca, Eli Lilly Canada Inc., Toronto, Ontario, Canada). Revatio and Adcirca have also been approved for the treatment of pulmonary hypertension. Earlier studies found that PDE-5 is not present in normal cardiomyocytes (8,9), although later investigations revealed its expression in canine (10), mouse cardiomyocytes (1,11,12), and human heart (13,14). PDE-5 expression is increased in hypertrophic human right ventricle, as well as failing left ventricular tissue (13–15). A gene silencing model also confirmed PDE-5 protein expression (16), whereas a recent report still questioned its presence in adult mouse cardiac myocytes (17). Because cGMP-hydrolytic activity is also attributable to PDE-1 and PDE-3, Vandeput et al. (14) suggested that the effects of sildenafil on cGMP hydrolysis were due to inhibition of both PDE-5 and PDE-1 in the left ventricles of normal and failing mouse hearts.

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Abbreviations and Acronyms

ANP	= atrial natriuretic peptide
BNP	= B-type natriuretic peptide
cAMP	= cyclic adenosine monophosphate
cGMP	= cyclic guanosine monophosphate
DOX	= doxorubicin
DMD	= Duchenne muscular dystrophy
GC	= guanylyl cyclase
I/R	= ischemia-reperfusion
mdx	= dystrophin-deficient
MI	= myocardial infarction
mitoK_{ATP}	= mitochondrial K _{ATP}
NO	= nitric oxide
NOS	= nitric oxide synthase
NP	= natriuretic peptide
PDE	= phosphodiesterase
pGC	= particulate guanylyl cyclase
PKG	= cyclic guanosine monophosphate-dependent protein kinases
sGC	= soluble guanylyl cyclase

cGMP in Pre- and Post-Conditioning

NO triggers various physiological responses by binding and activating sGC to produce cGMP from guanosine triphosphate (18). NO-cGMP-PKG signaling pathway is involved in the cardioprotective action in I/R injury as a survival signal (19,20). In cardiomyocytes, cGMP reduced the effects of myocyte stunning after simulated I/R (21). The NO donor S-nitroso-N-acetyl-L,L-penicillamine mimicked the pre-conditioning-like effect by inducing cGMP (22). Moreover, the activation of the NO/cGMP/PKG pathway inhibited the elevation of intracellular Ca²⁺ concentrations by phosphorylating target proteins responsible for intracellular Ca²⁺ homeostasis during I/R injury in Chinese hamster ovary cells (23).

Ischemic pre-conditioning rapidly increased cGMP levels via sGC during ischemia, leading to delayed protective effect (24 h later) against myocardial stunning and infarction in conscious rabbits (24). Bradykinin, one of

the triggers of pre-conditioning, caused receptor-mediated production of NO resulting in cGMP production, activation of PKG, and opening of mitochondrial K_{ATP} (mitoK_{ATP}) channel in rabbit heart and cardiomyocytes (25). Opening of mitoK_{ATP} channels causes partial compensation of the membrane potential, which enables additional protons to be pumped out to form a H⁺ electrochemical gradient for both adenosine triphosphate synthesis and Ca²⁺ transport (26). The cGMP/PKG pathway also confers ischemic post-conditioning protection in part by delaying normalization of pH during reperfusion, probably via PKG-dependent inhibition of Na⁺/H⁺-exchanger in rat heart (27).

cGMP Modulatory Drugs for Cardioprotection

cGMP modulatory drugs induce cardioprotective effect through PKG as outlined in Figure 1. NPs exert biological effects by binding to membrane-associated pGC. Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type NP are 3 structurally related, but genetically distinct, signaling molecules that regulate the cardiovascular, skeletal, nervous, reproductive, and other systems by activating pGC and elevating intracellular cGMP

concentrations. ANP is primarily stored in atrial granules and secreted in response to atrial stretch. BNP is also in atrial granules but is found in the highest level in ventricles of stressed hearts (28). C-type NP is found at lower concentration in vascular endothelium and is present in higher concentration in chondrocytes where it stimulates long bone growth (29). In the cardiovascular system, NPs exhibit growth suppressive, antiproliferative, and antihypertrophic actions on vascular smooth muscle cells, cardiomyocytes, and fibroblasts (30). ANP/BNP exert myocardial protective effects against I/R injury through a cGMP-PKG-dependent modulation of mitoK_{ATP} channels (31). ANP also protects against reoxygenation-induced hypercontracture in cardiomyocytes by stimulating cGMP synthesis (32). Administration of ANP at reperfusion protected against I/R injury (33,34) and exerted antiapoptotic effects in rat cardiomyocytes through cGMP-PKG and by inducing phosphatidylinositol 3-kinase-protein kinase B (PI3K/AKT) signaling (35). cGMP analogue, 8-Br-cGMP, or elevation of intracellular cGMP concentration via the sGC activator NO or BNP exerted cardioprotective effects through PKG activation (36).

Cinaciguat (BAY 58-2667) activates sGC independent of NO (37). This drug preferentially activates sGC when the

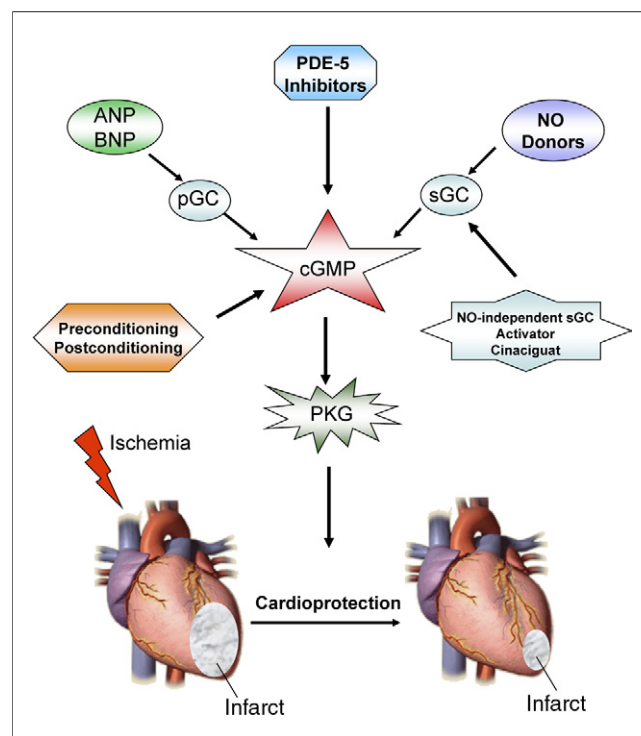


Figure 1 Myocardial Protection by Up-Regulation of cGMP

Cardioprotective modalities including pre-conditioning, post-conditioning, atrial natriuretic peptide (ANP)/B-type natriuretic peptide (BNP), nitric oxide (NO) donors, and Cinaciguat generate cyclic guanosine monophosphate (cGMP) through activation of soluble guanylyl cyclase (sGC)/particulate guanylyl cyclase (pGC). cGMP exerts cardioprotective effects against ischemia/reperfusion injury through activation of cyclic guanosine monophosphate-dependent protein kinase (PKG).

heme iron is oxidized (Fe^{3+}) or the heme moiety is missing, which makes this enzyme insensitive to both its endogenous ligand NO and exogenous nitrovasodilators. These unique attributes make Cinaciguat an attractive molecule for protection against reperfusion injury. Indeed, Cinaciguat induces an effect similar to pre-conditioning or post-conditioning against I/R in rabbit, rat (38), mouse (39), and dog (40) heart by activating PKG and opening of the mitoK_{ATP} channels (39). Intriguingly, this drug caused PKG-dependent generation of hydrogen sulfide in mouse cardiomyocytes and protected against simulated ischemia/reoxygenation injury, similar to tadalafil in the heart (3). Hydrogen sulfide causes cardioprotection through opening of sarcolemmal K_{ATP} channels in rat heart and cardiomyocytes (41).

PDE-5 inhibitors are promising drugs for cardiovascular protection. Our laboratory first demonstrated the cardioprotective effect of sildenafil against I/R injury (42). Rabbits treated with sildenafil before ischemia showed significant reduction in infarct size, which was mediated by opening of mitoK_{ATP} channels. Sildenafil also reduced cell death due to necrosis and apoptosis in isolated cardiomyocytes, suggesting that the cytoprotective effect of this drug was independent of its vascular/hypotensive effect (11). Vardenafil is 20-fold more potent than sildenafil for inhibiting purified PDE-5 (43), and it also displayed a similar protective effect against I/R injury in rabbits (44). Moreover, both drugs reduced infarct size when infused at reperfusion through opening of mitoK_{ATP} channels (45). Tadalafil is a long-acting PDE-5A inhibitor that has a half-life of 17.5 h (46) and is effective for up to 36 h for improving erectile function. Tadalafil also reduced infarct size and improved cardiac function after I/R in mice (3).

Mechanistically, the cardioprotective effect of sildenafil was dependent on enhanced NO generation through endothelial nitric oxide synthase/inducible nitric oxide synthase (47), activation of protein kinase C (48), and opening of mitoK_{ATP} channels (42). Sildenafil also increased cGMP accumulation and PKG activation in mouse cardiomyocytes and heart (11,47). The PKG-dependent cytoprotective mechanism of sildenafil involves phosphorylation of ERK and GSK3 β , induction of Bcl-2, and opening of mitoK_{ATP} channels (49) (Fig. 2). Interestingly, sildenafil increased Bcl-2 expression, which was absent in inducible NOS-deficient cardiomyocytes, thereby suggesting a link of NO signaling with the expression of antiapoptotic protein (49). Overexpression of PKG1 α was also reduced in adult rat cardiomyocyte injury after ischemia, which involved inhibition of active caspase-3, phosphorylation of Akt, ERK, and JNK, and increased expression of NOS and Bcl-2 as well as decreased Bax expression (50).

cGMP Signaling in Hypertrophy and Heart Failure

Sildenafil exerts antihypertrophic effects in mice with pressure overload in the absence of vascular unloading (1). The

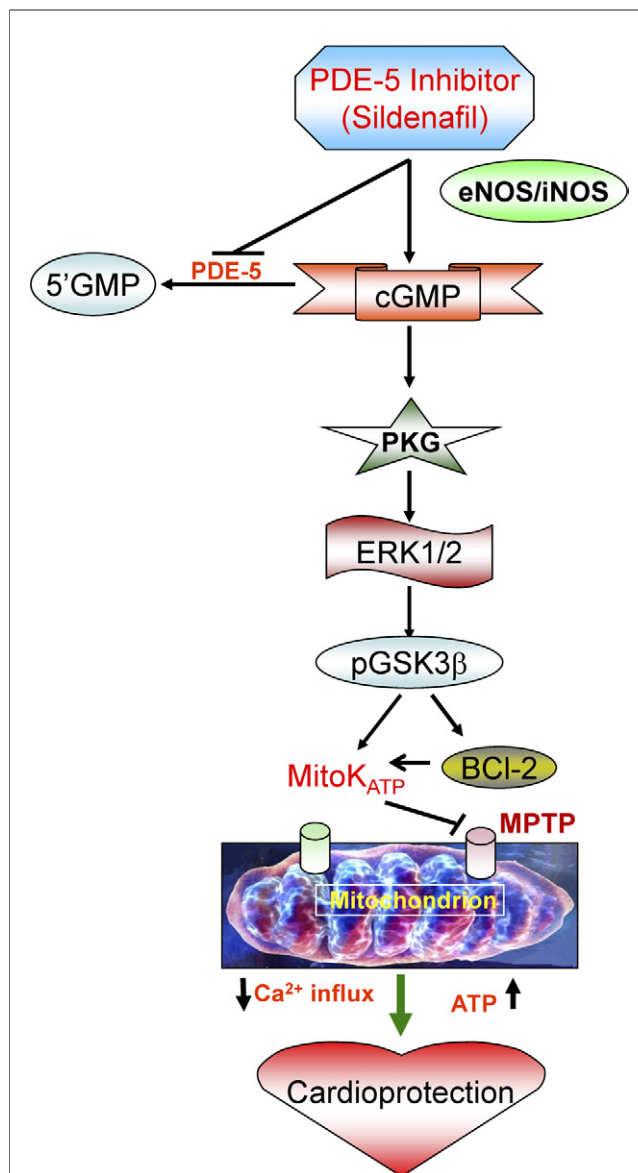


Figure 2 Mechanism of Cardioprotection by the PDE-5 Inhibitor Sildenafil

Sildenafil treatment triggers signaling cascade resulting in increased expression of endothelial nitric oxide synthase/inducible nitric oxide synthase (eNOS/iNOS) and activation of cGMP-dependent PKG. PKG subsequently causes phosphorylation of ERK1/2 and pGSK3 β in conjunction with an increase in the Bcl-2, which inhibits apoptosis through attenuation in cytochrome C release and inhibition of the mitochondrial permeability transition pore (MPTP). PKG also opens mitochondrial K_{ATP} (mitoK_{ATP}) channels, which limits against ischemia/reperfusion injury through preservation of adenosine triphosphate (ATP) and decrease in Ca²⁺ influx in the mitochondria.

antihypertrophic effects coexisted with PKG activation, and its targets included regulator of G protein-coupled signaling-2 (51), as well as calcineurin-NFAT-TRPC6 (52). In the hypertrophied right ventricular myocardium, PDE-5 is up-regulated, PKG activity is inhibited, and cGMP is preferentially shifted to inhibition of PDE-3 (15). This leads to an increase in cAMP, protein kinase A

activation, increased intracellular calcium, and increased contractility. The increased PDE-5 expression predisposed mice to adverse left ventricular remodeling after myocardial infarction (MI). Left ventricular systolic and diastolic dysfunction were more marked in PDE-5-TG (transgenic mice with cardiomyocyte-specific overexpression of PDE-5) than in wild-type mice, associated with enhanced hypertrophy and reduced contractile function in isolated cardiomyocytes from remote myocardium (13). Chronic treatment with sildenafil immediately after MI or beginning 3 days post-MI attenuated ischemic cardiomyopathy (53), suggesting that PDE-5 inhibition may be a promising therapeutic tool for patients with advanced heart failure. Interestingly, PKG activation with sildenafil was associated with the inhibition of Rho kinase (54), which is known to suppress left ventricular remodeling post-MI in mice (55).

Cardiac Dysfunction in Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a progressive and fatal genetic disorder of muscle degeneration. Patients with DMD lack dystrophin as a result of mutations in the

X-linked dystrophin gene. The loss of dystrophin leads to severe skeletal muscle pathologies and cardiomyopathy, a delayed symptom of the disease that usually develops by the second decade of life, with >90% of patients presenting clinical symptoms by 18 years of age (56). Reduced NO-cGMP signaling is a key contributor to DMD cardiac pathogenesis. Dystrophin-deficient (mdx) mice exhibit cardiac dysfunction with a decrease in diastolic function followed by systolic dysfunction later in life. Loss of dystrophin prevents normal neuronal nitric oxide synthase (nNOS) expression and/or signaling in all (skeletal, smooth, and cardiac) muscle systems (57). The stimulation of cGMP synthesis by overexpression of cardiac-specific nNOS reduced impulse-conduction defects in mdx mice (58,59). Similarly, increased pGC activity in young mdx mice decreased susceptibility to cardiac damage during sympathetic stress (60). Chronic treatment with sildenafil reduced functional deficits in cardiac performance of aged mdx mice without any effect on normal cardiac function in wild-type controls (57). When sildenafil treatment was started after cardiomyopathy had developed, the established symptoms were rapidly reversed within a few days. These results suggest

Table 1 Pharmacological Agents That Target the cGMP Signaling Pathways

Drug/Agent	Target	Model/Species	Results	Ref. #
Atrial natriuretic peptide	pGC	Rabbit and rat heart, adult rat ventricular myocytes	Protects against I/R injury and RO-induced hypercontracture by cGMP-dependent nuclear accumulation of zyxin and Akt	32-35
Bradykinin	B2 receptor	Rabbit heart and cardiomyocytes	Mimics ischemic preconditioning by NO-cGMP-PKG dependent opening of mitoK _{ATP} channels	25
B-type natriuretic peptide	pGC	Rat heart	Protects against I/R injury through cGMP-PKG dependent modulation of mitoK _{ATP} channels	31
8-Br-cGMP	cGMP analogue	Neonatal rat ventricular myocytes	Protects against SI-RO via PKG	36
Cinaciguat (BAY 58-2667)	sGC	Rabbit, rat, mouse, and dog heart	Induces pre- or post-conditioning-like effect against I/R by PKG mediated opening of mitoK _{ATP} channels	38-40
Sildenafil	PDE-5	Rabbit and mice heart, and adult mouse cardiomyocytes	Protects against I/R and SI-RO injury through eNOS/iNOS, activation of PKC, increased accumulation of cGMP, activation of PKG, phosphorylation of ERK, and opening of mitoK _{ATP} channels	2,11,42,47-49,53
		Mice, adult mouse cardiomyocytes	Inhibits DOX-induced cardiomyocytes apoptosis, preserved mitochondrial membrane potential ($\Delta\Psi_m$), and myofibrillar integrity and prevents DOX-induced left ventricular dysfunction	57,63
		mdx mice	Reduced functional deficits in the cardiac performance of aged mdx mice	57
		Mice prostate cancer xenograft	Sensitizes DOX-induced tumor reduction and provides concurrent cardioprotective benefits.	67
S-nitroso-N-acetyl-L, L-penicillamine	NO donor	Neonatal rat ventricular myocytes	Mimics preconditioning effect against SI-RO, which is cGMP dependent but independent of PKC or mitoK _{ATP} channels	22
Vardenafil	PDE-5	Rabbit heart	Protects against I/R injury via opening of mitoK _{ATP} channels	3,45
		Brain tumor-bearing rat	Improved survival and reduced DOX-induced tumor size	66
Tadalafil	PDE-5	Mice heart	Limits I/R injury by hydrogen sulfide signaling in a PKG-dependent fashion	3
			Prevents DOX-induced cardiomyopathy and improved left ventricular function through up-regulation of cGMP, PKG activity, and MnSOD level without interfering with chemotherapeutic benefits of DOX	57,64

cGMP = cyclic guanosine monophosphate; DOX = doxorubicin; eNOS/iNOS = endothelial nitric oxide synthase/inducible nitric oxide synthase; I/R = ischemia/reperfusion; mdx = dystrophin-deficient; mitoK_{ATP} = mitochondrial K_{ATP} channel; MnSOD = manganese superoxide dismutase; NO = nitric oxide; NOS = nitric oxide synthase; PDE = phosphodiesterase; pGC = particulate guanylyl cyclase; PKC = protein kinase C; PKG = cyclic guanosine monophosphate-dependent protein kinases; sGC = soluble guanylyl cyclase; SI-RO = simulated ischemia-reoxygenation.

that PDE-5 inhibitors may be useful in the treatment of cardiomyopathy in patients with DMD.

cGMP and Doxorubicin-Induced Cardiotoxicity

Doxorubicin (DOX) is one of the most powerful and widely used anticancer drugs in clinics. Specifically, the cumulative doses >550 mg/m² increase the risk of developing cardiac adverse effects, including congestive heart failure and dilated cardiomyopathy (61). DOX cardiotoxicity involves increased oxidative stress, inhibition of nucleic acid and protein synthesis, release of vasoactive amines, alteration of mitochondrial energetics, and altered adrenergic function. Reduction in fractional shortening and abnormalities in the nonspecific T-wave and ST-segment of electrocardiography are typically observed in DOX-induced ventricular dysfunction (62). Treatment with sildenafil before DOX administration inhibited cardiomyocyte apoptosis, preserved mitochondrial membrane potential ($\Delta\Psi_m$), and myofibrillar integrity and prevented left ventricular dysfunction as well as ST segment prolongation (63). Similarly, tadalafil also improved left ventricular function and prevented cardiomyocyte apoptosis in DOX-induced cardiomyopathy through mechanisms involving up-regulation of cGMP, PKG activity, and manganese superoxide dismutase levels without interfering with the chemotherapeutic benefits of DOX (64). Thus, prophylactic treatment with PDE-5 inhibitors might become a promising therapeutic intervention for managing the clinical concern of DOX-induced cardiotoxicity in patients.

cGMP in Cancer Chemotherapy

Sildenafil and vardenafil induce caspase-dependent apoptosis and antiproliferation effects in B-cell chronic lymphatic leukemia (65). The combination of vardenafil and DOX significantly improved survival and reduced the tumor size in brain tumor-bearing rats (66). Oral administration of vardenafil and sildenafil increased the rate of transport of compounds across the blood-tumor-brain and improved the efficacy of DOX in treatment of brain tumors. We recently reported that sildenafil is both a powerful sensitizer of DOX-induced killing of prostate cancer and a provider of concurrent cardioprotective benefit (67). Co-treatment with sildenafil and DOX enhanced apoptosis in PC-3 and DU145 prostate cancer cells through enhancing reactive oxygen species generation compared with normal prostate cells in which such combination attenuated DOX-induced reactive oxygen species generation. The basic difference in mitochondrial respiration between normal and cancer cells (which produce large amount of lactate regardless of the availability of oxygen) seems to make cancer cells more sensitive to oxidative stress (68). DOX-induced apoptosis is mainly initiated by oxidative DNA damage, although this apoptosis may involve topoisomerase II inhibition as well. The increased apoptosis by sildenafil and DOX was associated with enhanced expression of proapoptotic proteins Bad

and Bax and suppression of antiapoptotic protein, Bcl-2, and Bcl-xL. Moreover, treatment with sildenafil and DOX in mice bearing prostate tumor xenografts resulted in significant inhibition of tumor growth.

Conclusions

It is clear from the studies summarized here that the NO-cGMP-PKG pathway plays a key role in protection against MI, pre- and post-conditioning, hypertrophy, heart failure, and DOX-induced cardiotoxicity (summarized in Table 1). A number of clinically relevant therapeutic modalities including GC activators and PDE-5 inhibitors are promising agents in modulating the cGMP pathway in these disease states. Research over the past 9 years on the cardiac uses of PDE-5 inhibitors have helped initiate human trials, including the National Institutes Health multicenter trial (RELAX: Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure [NCT00763867]) in patients with heart failure and a clinical trial of sildenafil (Revatio) (REVERSE-DMD [NCT01168908]) to treat DMD and Becker muscular dystrophy patients with cardiac disease, which is currently recruiting patients at the Johns Hopkins medical institutions (Baltimore, Maryland). The role of sildenafil in enhancing the chemotherapeutic efficacy of DOX in prostate and other cancer cell lines, while alleviating the cardiotoxic effects of DOX, suggests that a new paradigm may be emerging for a safer use of this agent in the treatment of various types of cancer.

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