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Mechanism of Erectogenic Effect of the Selective Phosphodiesterase Type 5 Inhibitor, DA-8159

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DA-8159, a new Phosphodiesterase (PDE) 5 inhibitor, has exhibited potent erectogenic potential in a penile erection test in rats and anesthetized dogs. In this study, we investigated the mechanism of its erectogenic activity by measuring the activity of DA-8159 against a various PDE isozymes and assessing cGMP and cAMP formation in a rabbit corpus cavernosum *in vitro*. DA-8159 inhibited the PDE 5 activity in rabbit and human platelets, which the IC_{50} was 5.84± 1.70 nM and 8.25± 2.90 nM, respectively. The IC_{50} of DA-8159 on PDE 1, PDE2, PDE 3 and PDE 6 were 870 ± 57.4 nM, 101 ± 15 μM, 52.0 ± 3.53 μM and 53.3 ± 2.47 nM, respectively. This suggests that DA-8159 is a potent, highly selective, competitive inhibitor of PDE 5-catalyzed cGMP hydrolysis. The rates of cGMP hydrolysis catalyzed by human platelets-derived PDE 5 as a function of the cGMP concentration (5~100 nM) and two-fixed DA-8159 concentration (11.3 and 18.8 nM) were investigated in order to characterize the mode of PDE 5 inhibition by DA-8159. DA-8159 increased the apparent K_m value for cGMP hydrolysis but had no effect on the apparent V_{max}, indicating a competitive mode of inhibition. DA-8159 increased the cGMP concentrations in the rabbit corpus cavernosum dose dependently. In the presence of sodium nitroprusside (SNP), DA-8159 significantly stimulated the accumulation of cGMP when compared to the control level. This indicated that the enhancement of a penile erection by DA-8159 involved the relaxation of the cavernosal smooth muscle by NO-stimulated cGMP accumulation. In conclusion, DA-8159 is a selective inhibitor of PDE 5-catalyzed cGMP hydrolysis and the enhancement of a penile erection by DA-8159 is mediated by the relaxation of the cavernosal smooth muscle by the NO-stimulated cGMP accumulation.

Key words: DA-8159, Phosphodiesterase 5 inhibitor, Rabbit corpus cavernosum, Intracavernous pressure (ICP), PDE isozymes, cGMP

INTRODUCTION

A penile erection is a hemodynamic process involving the relaxation of smooth muscles of the corpus cavernosum and its arterioles. This relaxation process results in increased blood flow into the trabecular spaces of the copora cavernosa (Lue, 1983; Andersson and Wagner, 1995) Smooth muscle relaxation is mediated by nitric oxide (NO) which during sexual stimulation, is synthesized in the nerve terminals of the parasympathetic nonadrenergic, non-cholinergic (NANC) neurons in the penis and by the endothelial cells comprising the blood vessels and the lacunar spaces of the corpora cavernosa (Burnett *et al.*, 1992; Trigo-Rocha, *et al.*, 1993; Burnett, 1995). Although the effector system has not been fully defined, NO activates the guanylate cyclase that increases the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). cGMP induces the relaxation of the smooth muscle of the corpus carvenosum and the penile arterioles (Burentt, 1995). The cGMP levels are regulated by a balance between the rate of synthesis by guanylate cyclase and the rate of hydrolytic breakdown to guanosine 5'-monophosphate (GMP) by cyclic nucleotide phosphodiesterase (PDE) isozymes (Beavo, 1995). Therefore, some agents that inhibit cGMP hydrolysis may be expected to enhance smooth muscle relaxation in the corpus cavernosum and thereby facilitate the penile erection responses.

Sildenafil was developed as a potent and competitive type 5 cGMP-specific phosphodiesterase (PDE 5) inhibitor, which is the predominant isozyme in the human corpus cavernosum, and its potential use treating male erectile dysfunction (ED) is now well-accepted since its

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first introduction in 1998. Recently, DA-8159 has been also developed as a novel selective PDE 5 inhibitor for treating ED. DA-8159 has an erectogenic potential via relaxing the smooth muscles of the rabbit corpus cavernosum *in vitro*.

In this study, the mechanism of action of DA-8159 was investigated. First, the selectivity of DA-8159 for the major PDE isozymes was examined. Second, its kinetic mode of inhibitory action was examined. Finally, its effect on the increase in the endogenous cGMP levels in the rabbit smooth muscle of the corpus cavernosum tissue in vitro was measured.

MATERIALS AND METHODS

Animals

Male New Zealand White rabbits (2.8~3.2 kg) were used in this study. All rabbits were housed in a room maintained at a constant temparature/huminity (21±2°C, 50%) on a 12/12 h light/ dark cycle with food and water available *ad libitum*.

Materials

DA-8159 and Sildenafil citrate were synthesized at Dong-A Pharmaceutical Co. (Kyunggi, Korea). The purity was above 99.8% as confirmed by HPLC and potentiometric titration in glacial acetic acid. Sodium nitroprusside (SNP), dimethylsulfoxide (DMSO), and the other reagent were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of phosphodiesterase isozymes

The PDE isozymes type 1 and 2 were isolated from bovine heart by a slight modification of the method reported by Wang, *et al.* (Wang *et al.*, 1975). Frozen bovine heart slices were thawed and homogenized in 2.5 volumes of a buffer, pH 7.5, containing 20 mM Tris and 1 mM EDTA. The homogenate was centrifuged at 1,500 rpm for 30 min. The supernatant was collected, and its pH was adjusted to 8.0. The supernatant was then brought to a 60% saturation of $(NH_4)_2SO_4$, and centrifuged at 8,500 rpm for 20 min. The pellet was suspended in an equilibration buffer (pH 7.5, 20 mM Tris, 1 mM imidazole, 10 μM $MgAc_2$, 0.05 M NaCl, 10 mM β-mercaptoethanol) and then dialyzed against the same buffer overnight. The dialyzed sample was centrifuged at 40,000 rpm for 1 h and the supernatant was placed onto a DEAE cellulose column (2 × 12 cm), which had been equilibrated with the same buffer. The column was washed with two bed volumes of the equilibration buffer and then eluted with a linear salt gradient of 0.05~0.4 M NaCl. The total volume of the gradient was 300 mL. The column fractions were tested for PDE activity using the following method. The enzyme fractions were separated into two activity peaks, pooled separately and stored in small aliquots at -20°C.

The PDE isozymes type 3 and 5 were isolated from human and rabbit platelets by the method previously described. (Hidaka and Asano, 1976). Fresh whole blood heparinated at a concentration of 25 units/mL was centrifuged at 450 g for 5 min. A careful microscopic examination showed that the platelet-rich plasma was virtually free from erythrocyte and leukocyte contamination. The platelets were isolated from the platelet-rich plasma by centrifugation at 1,200 g for 15 min. The platelet homogenates were prepared in a 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM $MgCl_2$, using a homogenization tube with a Teflon pestle. The homogenized solutions were sonicated (30 sec/mL), and a soluble PDE preparation was obtained from the sonicated homogenate by centrifugation at 105,000 g for 60 min. This supernatant was applied to a DEAE-cellulose column. DEAE-cellulose chromatography was performed as described previously on columns (2× 12 cm) with bed volumes of 35 mL. The buffer used was 50 mM Tris-acetate (pH 6.0) containing 3.75 mM 2-mercaptoethanol. The enzyme preparation was applied to the column, which was followed by elution with several column volumes of buffer. The initial wash contained no detectable phosphodiesterase activity. A linear gradient from 0~1 M sodium acetate was then applied with a flow rate of 0.5 mL/min and a total gradient volume of 300 mL. The total volume of the gradient was 300 mL. The column fractions were tested for PDE activity using the following method. The enzyme fractions were separated into two activity peaks and pooled separately and stored in small aliquots at -20°C.

The PDE isozyme type 6 was isolated from the retina of bullfrogs (*Rana catesbeiana*). The procedure used was a slightly modification of the method reported by Hurley and Stryer. (Hurley and Stryer, 1982). The retina was obtained from half of the eyeball and stored at -70°C until the PDE 6 isozyme was prepared. The retina was gently rinsed in a Ringer's solution (105 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM $NaHCO_3$, 10 mM Hepes, pH 7.5) containing 6~11% Percoll and broken up by forcing it through a 5 mL syringe. The suspension was centrifuged at 15,000 g for 15 sec to remove the remaining contamination. The supernatant was assayed for PDE 6 activity as a crude extract.

Determination of PDE activity assay

The PDE 1, 2, 3 or 5 activity was measured by the method reported by Hidaka and Shibuya (Hidaka and Shibuya, 1974). The reaction mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 400 nM cyclic [^{3}H]AMP or cyclic [^{3}H]GMP and an appropriate enzyme preparation in a total volume of 150 μL. The mixture was incubated for 40 min at 30°C, and the reaction was quenched by boiling

for 5 min. Subsequently, 0.1 units of 5'-nucleotidase was added and the mixture was incubated at 37°C for 20 min. The first product, 5'-[^{3}H]AMP or [^{3}H]GMP, formed by the PDE is converted to [^{3}H]adenosine or [^{3}H]guanosine by action of 5'-nucleotidase. 1 mL of the slurry resin (AG1-X2, 200~400 mesh, Bio-Rad) in methanol was added and incubated at 4°C for 20 min with vigorously stirring. The mixture was centrifuged at 10,000 rpm for 5 min at 4°C. The radioactivity of 500 µL of the supernatant was determined by a liquid scintillation counter.

The PDE 6 activity was measured by the method previously described (Artemyev *et al.*, 1998). Ten microliters of a 5× PDE assay buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/mL bovine serum albumin, final 1× concentrations) was mixed with 10 µL of the PDE 6-containg samples in the wells of an untreated polystyrene 96-well plate. Subsequently, 10 µL of trypsin (TPCK-treated, 20~100 µg/mL) was added at 4 for a sufficient period to an optimally activate PDE 6, and proteolysis was halted with 10 µL of a six-fold weight excess of a soybean trypsin inhibitor. Ten microliters of a 10 mM cyclic GMP stock solution and 0.1 unit of 5'-nucleotidase were added sequentially. To quantify the inorganic phosphate calorimetrically, 150 µL of a working molybdate solution (0.4 N H_2SO_4, 0.2 % ammonium molybdate, 2% sodium dodesyl sulfate, and 1.8% ascorbic acid) was added and incubated at 37 for 20 min. The absorbance was determined at a wavelength of 700 nm.

Measurement of cGMP level in the rabbit corpus cavernosum

Rabbit corpus cavernosum smooth muscle (CCSM) strips were obtained from male New Zealand White rabbits (2.8~3.2 kg). The penectomies were performed on euthanized animals. The whole penis was dissected free in Krebs-Ringer solution containing NaCl 118.3 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25.0 mM, Ca-EDTA 0.016 mM and glucose 11.1 mM. Four strips of the CCSM (about 3×3×7 mm) were obtained from each corpus cavernosum, and each strip was then mounted in an organ-bath chamber containing 10 mL of the Krebs solution. The solution was aerated with 95% O_2, and 5% CO_2, at pH 7.4 and maintained at 37°C. The bath solution was replaced every 10~15 min after being equilibrated for 30 min. The cavernous strips were then exposed to DA-8159 (10^{-5} M), sildenafil (10^{-5} M) and the nitric oxide donor, sodium nitroprusside (SNP) (10^{-5} M) for 10 min (Freelish and Noack, 1987). The tissue was rapidly frozen in liquid nitrogen in order to quench the reaction. The frozen tissue was homogenized in cold 10% trichloroacetic acid at 2~8°C. After centrifugation at 2,000 g for 10 min at 4°C, the supernatant was washed with water-saturated diethyl-ether. The aqueous extract was lyophilyzed, and the dried extract was resuspended in 50 mM sodium acetate buffer. Aliquots of the samples were assayed for their cGMP contents by radioimmunoassay (BIOTRA, Amersham Pharmacia). The Data is expressed as pmol cGMP/mg protein.

RESULTS

Effect on the activity of PDE isozymes

The cAMP or cGMP hydrolytic activities of PDE 1 to 6 were measured to determine the inhibition profiles of DA-8159 for major PDE isozymes. DA-8159 potently inhibited the PDE 5 isolated from either of the human or rabbit platelets with an IC_{50} of 8.25± 2.90 nM or 5.84± 1.70 nM, respectively (Table I, Fig. 1). The potency of DA-8159 was similar to that of sildenafil (IC_{50} = 8.50± 2.05 nM for human platelets, 6.46± 2.05 nM for rabbit platelets). However, DA-8159 had a low potency against the other PDE isozymes in the rabbit platelets, which is a similar result to that of sildenafil (Table I).

Table I. IC_{50} values for the inhibition of the major PDE isozymes by DA-8159 and Sildenafil

Compound	IC_{50}				
	PDE1 (nM)	PDE2 (µM)	PDE3 (µM)	PDE5 (nM)	PDE6 (nM)
DA-8159	870±57.4 (n = 5)	101±15.1 (n = 3)	52.0±3.53 (n = 5)	5.84±1.70 (n = 6)	53.3±2.47 (n = 8)
Sildenafil	720±66.4 (n = 5)	111±25.0 (n = 3)	30.6±1.65 (n = 5)	6.46±2.05 (n = 5)	72.4±2.94 (n = 7)

IC_{50} values were determined using 0.4 µM cGMP as substrate for PDE 1, 2, and 5 and 0.4 µM cAMP as substrate for PDE 3 and 6. Data was expressed as a mean± S.D. n = number of experiments.

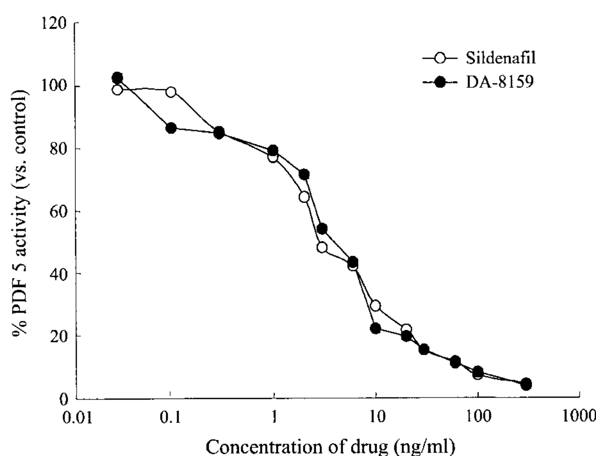

Fig. 1. The effects of DA-8159 and Sildenafil on the cGMP hydrolytic activity of PDE 5. The PDE 5 fractions from human platelets were incubated with either DA-8159 (closed circles) or Sildenafil (open circles) in the presence of 0.5 µM cGMP. Both DA-8159 and sildenafil inhibited the PDE 5 activity dose-dependently.

Mode of inhibition for PDE 5

To characterize the influence of DA-8159 on the cGMP hydrolytic activity of PDE 5, this enzyme was partially purified from human platelets using DEAE-cellulose column chromatography. Fig. 2 shows the cGMP hydrolysis profiles. Some fractions exhibiting the PDE 5 enzyme activity were pooled and used for the cGMP hydrolysis assay.

In order to characterize the mode of PDE 5 inhibition by DA-8159, the rates of cGMP hydrolysis catalyzed by the human platelets-derived PDE 5 were determined using the cGMP concentration (10~100 nM) and two-fixed DA-8159 concentration (11.3 and 18.8 nM). In the absence of DA-8159, the K_m value for PDE 5 was 7.27± 4.23 μM (n = 5). The extent of the inhibition of PDE 5 activity by DA-8159 decreased with increasing cGMP substrate concentration. DA-8159 increased the apparent K_m value for cGMP hydrolysis but had no effect on the apparent V_{max}, indicating a competitive mode of inhibition (Fig. 3). The K_i value for PDE 5 inhibition by either DA-8159 or sildenafil was a 7.28± 2.96 nM, 8.38± 3.23 nM, respectively (n = 3).

Effects on cGMP accumulation in the isolated rabbit corpus cavernosum

cGMP synthesis in the isolated corpus cavernosum of the rabbit was assess in order to further investigate mechanism of DA-8159 action. Fig. 4 showed the cGMP concentration in the rabbit corpus cavernosum tissues. In the control, without nitric oxide donor or a PDE 5 inhibitor, the cGMP concentration was 5.08 pmoles/mg protein/10 minutes. SNP (10 μM) increased the cGMP level (6.36 pmoles/mg protein/10 minutes) by 25.2% and stimulated

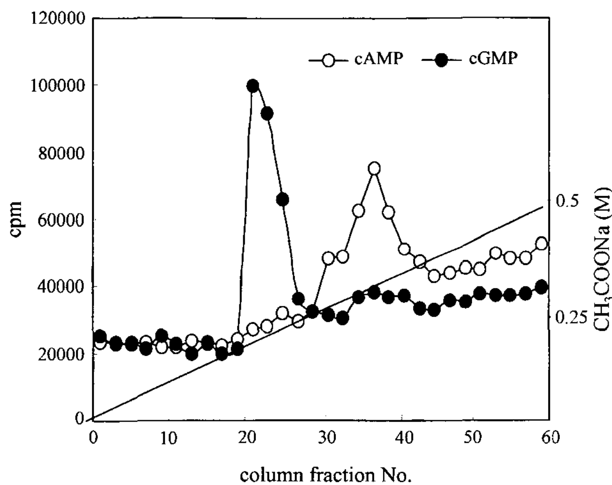

Fig. 2. DEAE-cellulose column chromatography profile for the partial purification of PDE activity from human platelets. The platelet homogenates were chromatographed on a DEAE-cellulose column, and a linear gradient from 0~1 M sodium acetate was then applied with a flow rate of 0.5 mL/min and a total gradient volume of 300 mL. The column fractions were tested for PDE activity as described in *Materials and Methods*.

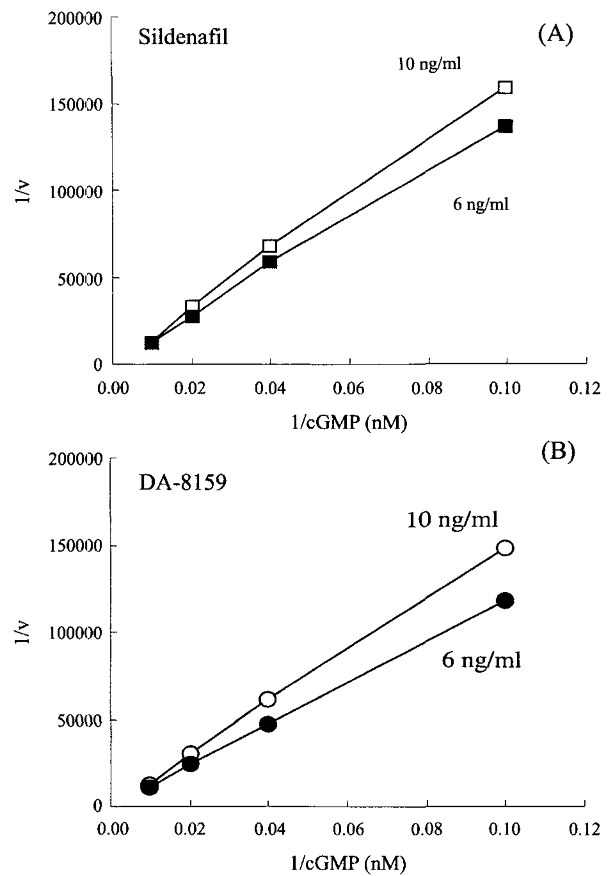

Fig. 3. Inhibition of the cGMP hydrolytic activity of PDE 5 by DA-8159 and Sildenafil. The PDE 5 fractions from human platelets were incubated with various cGMP concentrations (10~100 nM) and two fixed DA-8159 concentrations (●; 11.3 and ○; 18.8 nM, 3A) or Sildenafil (■; 12.6 and □; 21.1 nM, 3B). The extent of the inhibition of PDE 5 activity by DA-8159 or sildenafil decreased with increasing cGMP substrate concentration.

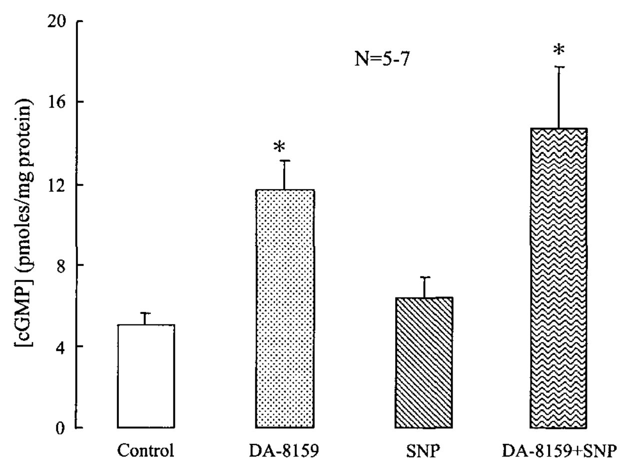

Fig. 4. Effect of DA-8159 on cGMP accumulation in the rabbit corpus cavernosum smooth muscle at 10 minutes after changing the solution for the 4 types of experimental conditions: control, DA-8159, sodium nitroprusside (SNP), and DA-8159 plus SNP. Data was expressed as mean± SD. *P < 0.05 vs. control (n = 5-7).

the accumulation of cGMP in these tissues. DA-8159 (10 μM) increased the cGMP level (11.76 pmoles/mg protein/10 minutes) by 131% when compared to the control. In the presence of SNP, DA-8159 increased the cGMP concentration by 189% when compared to the control. The cAMP concentration was unaffected by DA-8159 (data not shown).

DISCUSSION

Phosphodiesterases (PDEs) are a large group of structurally related enzymes that catalyse the hydrolysis of 3', 5'-cyclic nucleotides to their corresponding inactive nucleoside 5'-monophosphate by cleaving the phosphodiester bond between the phosphorus and oxygen atoms at the 3'-position. Eleven PDEs families have been identified with each family being the product of a separate gene. In addition, these PDEs usually have several isoforms. The PDEs differ in their primary structure, tissue distribution, affinities for the cyclic nucleotides and their sensitivity to Ca^{2+} and various inhibitors (Corbin and Francis, 1999; Dousa, 1999). PDE 4, PDE 7 and PDE 8 are highly specific for cAMP, whereas PDE 5, PDE 6 and PDE 9 are highly specific for cGMP. The other PDEs (PDE 1, PDE 2, PDE 3, PDE 10 and PDE 11) exhibit a dual specificity (Corbin and Francis, 1999; Dousa, 1999). The activity of PDEs is regulated by the substrate availability, an increase in the levels of one or both cyclic nucleotides, various intracellular signals (e.g. Ca^{2+} and calmodulin) and feedback phosphorylation via cAMP- and cGMP-dependent protein kinases (PKA and PKG respectively) (Corbin *et al.*, 2000; Lim *et al.*, 1999; Ekholm *et al.*, 1997). PDE 5 is the main cGMP-specific PDE expressed in skeletal, cardiac and smooth muscles (Corbin and Francis, 1999). PDE 5 contains two allosteric cGMP-specific binding sites in its regulatory N-terminal domain, in addition to specific cGMP-binding sites in its C-terminal domain (Fink *et al.*, 1999). cGMP binding to the allosteric sites is essential for the regulatory phosphorylation of PDE 5 by PKG or PKA (Murthy, 2001; Corbin *et al.*, 2000).

The utility of sildenafil as an efficacious, orally active agent for the treating male erectile dysfunction has created significant interest in the discovery of other phosphodiesterase type 5 (PDE 5) inhibitors. PDE 5 is the primary cGMP-hydrolyzing enzyme activity present in the corpus cavernosum, which is the smooth muscle in the penis that helps control the vascular tone (Dishy *et al.*, 2001). When a male is sexually stimulated, nitric oxide is released from the cavernosal nerve. This activates soluble guanylyl cyclase in the corpus cavernosum, causing an increase in the intracellular cGMP levels, which are normally hydrolyzed by PDE 5. Inhibiting PDE 5 elevates the levels of the cyclic nucleotide, leading to an enhanced relaxation of the smooth muscle, increased arterial inflow, venous congestion, and ultimately an erection.

DA-8159 is a synthesized inhibitor of PDE 5. In the inhibitory activity test of the PDE isozymes, from PDE 1 to PDE 6, DA-8159 competitively inhibited PDE 5 in the rabbit platelets with a K_i value of 7.28± 2.96 nM. This shows that DA-8159 may bind at the catalytic site of PDE 5 with a high affinity, preventing access to the normal substrate, cGMP. PDE 6, which is known to play a key role in light-signal photoreduction, shows high similarity to PDE 5 in terms of its substrate specificity for cGMP (Beave 1995; Gilepspie *et al*, 1989). However, PDE 6 has a less than 60% amino acid sequence homology with PDE 5 and can be distinguished from PDE 5 on the basis of its activation by trypsin and histone. PDE 6 has a significantly higher K_m for cGMP, which is on the order of 30 to 40 μM (Gilepspie *et al*, 1989; Hurwitz *et al.*, 1985). DA-8159 is almost 10-fold less potent with PDE 6 compared to PDE 5, which is similar to Sildenafil. Consequently, it is predicted that there will be little effect on the visual function as a consequence of PDE 6 inhibition at the therapeutic range of DA-8159 (Ballard *et al.*, 1998). In this study, human platelets were used as the PDE 5 source for studying the DA-8159 inhibition kinetics. The enzyme preparation had a K_m value of 7.27± 4.23 mM for cGMP, which is similar to the value reported for the PDE 5 isolated from human platelets (Ballard *et al.*, 1998).

DA-8159 increased the cGMP levels in the rabbit corpus cavernosum and in the presence of SNP, a nitric oxide donor, the cGMP levels were further increased when compared with those of DA-8159 alone. This suggests that DA-8159 enhanced cGMP accumulation in the rabbit corpus cavernosum and the cGMP levels increased up to the 3-fold higher than the basal levels at the maximum effective DA-8159 concentration. These results are closely related to the mode of DA-8159 action in the sequential erection mechanism as follows, (1) sexual arousal, (2) NO release from the NANC neurons and the endothelium in the corpus cavernosum, (3) NO-activation of guanylyl cyclase in the smooth muscle cell component of the corpus cavernosum, and (4) cGMP-mediated relaxation of the tissue (Finberg *et al*, 1993; Azadzol *et al.,* 1991; De Tajeda *et al.*, 1989; Trigo-Rocha *et al.*, 1993). On the basis of these results, DA-8159 acts principally via inhibiting PDE 5 in a similar manner to Sildenafil, and the subsequent enhancement of cGMP accumulation is linked to the NO-mediated NANC and parasympathetic drive systems (Jeremy *et al.*, 1997).

In summary, DA-8159 is a potent, highly selective and competitive inhibitor of PDE 5-catalyzed cGMP hydrolysis. Sequentially, it amplifies the NO/cGMP pathway involved in relaxing the corpus cavernosum smooth muscle. DA-

8159 may enhance a penile erection by augmenting the nitric oxide-mediated relaxation pathways *in vivo*.

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