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European Journal of Pharmacology 349 (1998) 263–268

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Cardiovascular activity of WIN 65579, a novel inhibitor of cyclic GMP phosphodiesterase 5

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Received 8 January 1998; revised 5 March 1998; accepted 10 March 1998

Abstract

This study describes the phosphodiesterase inhibitory potency and cardiovascular actions of WIN 65579 (1-cyclopentyl-3-ethyl-6-(3-ethoxy-4-pyrridyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-one), a potent, new cGMP phosphodiesterase 5 inhibitor. WIN 65579 is a competitive inhibitor of phosphodiesterase 5, with IC_{50} values of 2–3 nM for phosphodiesterase 5 from human or canine vascular sources. WIN 65579 has low affinity for phosphodiesterases 1, 2 and 3 ($IC_{50} > 3–10$ μM), and is somewhat selective for phosphodiesterase 4 ($IC_{50} \sim 100$ nM). WIN 65579 is an endothelial-dependent relaxant of rat aortic smooth muscle ($EC_{50} = 60$ nM) and lowers mean arterial blood pressure in conscious spontaneous hypertensive rats following intravenous or oral dosing. WIN 65579 also increases plasma cGMP levels, and reinstates vascular responsiveness to nitroglycerin in conscious rats that are nitroglycerin-tolerant. These data show that WIN 65579 is one of the more potent phosphodiesterase 5 inhibitors, and that WIN 65579 possesses cardiovascular activities consistent with vascular phosphodiesterase 5 inhibition in vivo. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Phosphodiesterase; cGMP; Nitric oxide (NO); Nitroglycerin; Cardiovascular

1. Introduction

It is now accepted that agents which elevate intracellular cyclic GMP (cGMP) levels reduce vascular tone (Murad, 1986) and have other beneficial cardiovascular effects, including prevention of platelet aggregation and reduction in vascular smooth muscle proliferation (Radomski et al., 1990; Kariya et al., 1989). The intracellular levels of cGMP are regulated by guanylate cyclases and by cGMP phosphodiesterases. Multiple isozymes of phosphodiesterases belonging to seven major gene-related families exist, and at least 18 clones for phosphodiesterase isozymes have been identified (Beavo et al., 1994; Loughney and Ferguson, 1996). These isozymes differ with respect to tissue distribution, substrate specificity, and sensitivity to selective inhibitors.

Inhibitors of the major cGMP phosphodiesterase found in vascular smooth muscle, phosphodiesterase 5, have been shown to produce endothelial-dependent vasorelaxant activity by virtue of their ability to potentiate nitric oxide-mediated increases in intracellular levels of cGMP (for review, see Silver, 1996). Among these inhibitors are zaprinast, an early phosphodiesterase 5 inhibitor (Martin et al., 1986; Harris et al., 1989; McMahon et al., 1989), WIN 58237 (1-cyclopentyl-3-methyl-6-(4-pyridyl)pyrazolo[3,4-*d*]pyrimidin-4-(5H)-one; Silver et al., 1994), and more recent compounds such as sildenafil (Terrett et al., 1996) and E-4021 (sodium 1-[6-chloro-4-(3,4-methylenedioxybenzyl)-aminoquinazolin-2-yl]piperidine-4-carboxylate sesquihydrate; Saeki et al., 1995), which are reportedly in clinical development trials for treatment of impotence, and angina or pulmonary hypertension.

We now describe a more potent phosphodiesterase 5 inhibitor, WIN 65579 (1-cyclopentyl-3-ethyl-6-(3-ethoxy-4-pyrridyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-one) (Fig. 1). WIN 65579, which is an analog of the pyrazolo-pyrimidine

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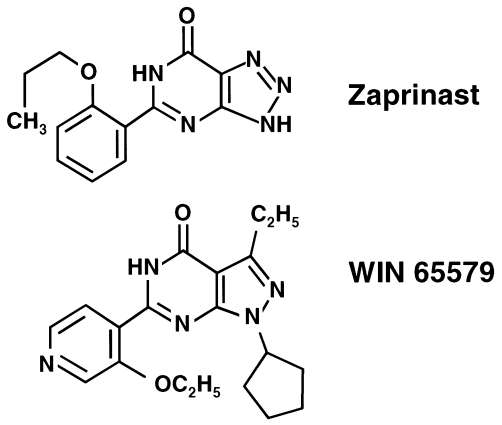

Fig. 1. Chemical structures of zaprinast and WIN 65579 (1-cyclopentyl-3-ethyl-6-(3-ethoxy-4-pyrridyl)-1H-pyrazolo[3,4-*d*] pyrimidin-4-one).

WIN 58237, is a competitive inhibitor of phosphodiesterase 5 with a K_i value of approximately 1 nM. WIN 65579 also demonstrates in vitro endothelial-dependent vasorelaxant and in vivo vasodepressor properties, and restores nitroglycerin vasoresponsiveness in tolerant animals.

2. Materials and methods

All procedures involving animals described in this manuscript were approved by the Institution Animal Care and Use Committee at Sterling Winthrop Pharmaceuticals Research Division (Collegeville, PA) and conformed with federal regulations. Sterling Winthrop Pharmaceuticals Research Division animal facilities and programs were accredited by the American Association for Accreditation of Laboratory Animal Care.

2.1. Preparation of phosphodiesterase isozymes and quantitation of phosphodiesterase inhibition

Methods for preparation of phosphodiesterase isozymes involved differential centrifugation followed by DEAE-Sephacel chromatography and subsequent affinity column chromatography as described previously (Pagani et al., 1992; Silver et al., 1994). Isozymes were prepared from cardiac and vascular tissues of dogs. Phosphodiesterase 1, phosphodiesterase 3, phosphodiesterase 4 and phosphodiesterase 5 were prepared from canine aortae (4 aortae), phosphodiesterase 2 was prepared from canine ventricles (2 ventricles), while phosphodiesterase 4 and phosphodiesterase 5 were also prepared from human saphenous veins (8 veins). Colony-bred mongrel dogs (10–15 kg, Hazelton Laboratories, Cumberland, VA) were killed by overdose with pentobarbital, and hearts and/or aortae were rapidly excised and placed on ice. Human saphenous veins were obtained from bypass procedures and likewise rapidly placed in ice for transport to the laboratory. All tissues were cleaned, minced with fine scissors and homogenized immediately in 10 volumes of extraction buffer (0–4°C) that contained 10 mM Tris acetate, pH 7.5, 2 mM $MgCl_2$, 1 mM dithiothreitol, 2000 U/l of aprotinin, 0.1 μM phenylmethylsulfonyl flouride, 0.1 μM leupeptin and 0.1 μM pepstatin. The homogenate was centrifuged at 40 000 $\times g$ for 30 min. The supernatant fraction was applied to a DEAE-Sepharose CL6B column (10 $\times$ 1 cm) that had been equilibrated with 70 mM sodium acetate and 1 mM dithiothreitol, pH = 6.5 (Buffer A). The column was washed with 4 to 5 bed volumes of the same buffer. Phosphodiesterase isozymes were eluted with a linear 70 to 1000 mM sodium acetate gradient (containing 1 mM dithiothreitol, pH 6.5). Fractions were collected and assayed for cyclic nucleotide hydrolysis with 1 μM cGMP or cAMP as substrate.

The first peak of phosphodiesterase activity (which primarily hydrolyzed cGMP) was separated further into phosphodiesterase 1 and phosphodiesterase 5 by affinity chromatography. The appropriate fractions were pooled and dialyzed vs. 40 mM Tris, 50 mM NaCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 1 mM dithiothreitol, pH 7.5, then applied to a calmodulin–Sepharose 4B column equilibrated with dialysis buffer. Phosphodiesterase 5 was eluted by washing the column with 3 bed volumes of 40 mM Tris, 200 mM NaCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 1 mM dithiothreitol, pH 7.5. A subsequent wash with 3 bed volumes of 40 mM Tris, 50 mM NaCl, 1 mM $MgCl_2$, 2 mM ethylene glycol bis(2-β-aminoethly ether)-*N*,*N*′-tetraacetic acid and 1 mM dithiothreitol, pH 7.5, eluted phosphodiesterase 1. Phosphodiesterase 5 was further purified by zaprinast-analog affinity chromatography. The zaprinast analog was attached to 6-aminohexyl-Sepharose 4B. The sample was applied to a 1-ml column in buffer containing 10 mM Tris, pH 7.5, 0.2 M sodium acetate, 2 mM $MgCl_2$, and 1 mM dithiothreitol, and the column was washed with 4 bed volumes of the same buffer. Phosphodiesterase 5 was eluted with 50 mM cGMP, which was subsequently removed by extensive dialysis vs. Buffer A.

The remaining fractions from the original DEAE-Sepharose CL6B column that hydrolyzed cAMP but not cGMP were pooled, then further purified by rolipram-analog affinity chromatography. The rolipram analog was attached to epoxy-activated Sepharose 6B. The sample was applied to a 20-ml column in buffer containing 10 mM Tris, pH 7.5, 0.5 M sodium acetate, 2 mM $MgCl_2$ and 1 mM dithiothreitol, and the column was washed with 4 bed volumes of the same buffer. The flow-through contained phosphodiesterase 3, which was used without further purification. Phosphodiesterase 4 was eluted with 50 mM cAMP, which was removed from the purified enzyme by extensive dialysis vs. Buffer A. The separation of phosphodiesterase 3 and phosphodiesterase 4 by rolipram-analog affinity chromatography was performed at room temperature, which resulted in improved recovery compared with 4°C.

After dialysis vs. buffer A, the purified phosphodiesterase isozymes were concentrated with an Amicon ultrafiltration system. The concentrated fractions were diluted with ethylene glycol to 50% and stored at −20°C. No significant changes in hydrolysis or sensitivity to inhibitors were noted for up to 6 months of storage.

At concentrations used in these studies, none of the vehicles significantly affected phosphodiesterase activity. Each assay was performed in triplicate. The concentration of compound, which produced 50% inhibition of cyclic nucleotide hydrolysis (IC_{50}) was calculated from concentration–response curves (Tallarida and Murray, 1987) by using six concentrations of inhibitor and a substrate concentration less than or equal to the K_m. The K_i value was determined by using six concentrations of the inhibitor and four concentrations of the substrate, cGMP.

2.2. In vitro studies

Male Sprague–Dawley rats (250–350 g) were anesthetized deeply with pentobarbital (50 mg/kg) and then killed by exsanguination. The thoracic and abdominal cavities were opened immediately, and the aorta was removed. In some studies, the entire length of the aorta was functionally denuded of the endothelial layer by gently scraping the luminal surface with a solid polyethylene catheter. The effectiveness of this procedure was tested in each preparation by measuring the vasorelaxant response to 1 μM carbachol at the end of the experiment. Denuded or intact rings were attached to stainless-steel ring holders immersed in 10-ml glass jacketed tissue baths containing a modified Krebs' solution (in millimolar): NaCl, 118: KCl, 4.7; $MgCl_2$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 1.6; $NaHCO_3$, 21.4; dextrose, 11.1; and EDTA, 0.026, pH 7.4 at 37°C.

Effects of WIN 65579 on vasorelaxation responses were determined as described previously (Harris et al., 1989; Silver et al., 1994). Aortic rings were stretched with a preload force of 2 g, equilibrated for 75 min, and then contracted with either 1 μM (denuded rings) or 3 μM (intact rings) phenylephrine, which produced 1.8 to 2.2 g of active force. Different amounts of phenylephrine were used to compensate for the 20% difference in active force developed between denuded and intact vascular rings. At this point, graded concentrations of WIN 65579 were added cumulatively, and the amount of relaxation was quantified after 10 min of each concentration.

2.3. Effects on blood pressure, heart rate and plasma cGMP levels in conscious, chronically instrumented rats

All procedures were as described previously (Dundore et al., 1991, 1993; Silver et al., 1994). Male spontaneously hypertensive rats (300–350 g, Taconic Farms) were anesthetized with sodium pentobarbital (50 mg/kg i.p.). By using an aseptic surgical technique, an arterial catheter fabricated from Teflon and Tygon tubing, and a venous catheter fabricated from polyethylene and Tygon tubing were inserted into the abdominal aorta and vena cava through the femoral artery and vein, respectively. The catheters were tunneled s.c., exteriorized between the scapulae, filled with a heparin solution (200 IU/ml in saline) and sealed with a stainless-steel plug. The wounds were sutured and treated with a topical antibiotic (furazolidone, Veterinary Products Laboratories, Phoenix, AR). Animals were allowed at least 2 days recovery before testing.

On the day of experiment, the rats were placed into individual Plexiglas boxes. The arterial catheter was connected to a pressure transducer (P23XL-1, Spectramed, Oxnard, CA) for the measurement of mean arterial blood pressure and heart rate.

After a 1-h adaptation period, three mean arterial pressure and heart rate measurements were taken at 5-min intervals. The mean of these three measurements represented the resting mean arterial pressure and heart rate. WIN 65579 (0.1 to 10 mg/kg) or vehicle (0.05 M NaOH, 1 ml/kg) was given as an i.v. bolus. Five min after the administration of each dose of WIN 65579 or vehicle, mean arterial pressure and heart rate was measured, and the next dose was given in a cumulative dose–response manner. In additional experiments, WIN 65579 or vehicle was administered by oral gavage. Effects on mean arterial pressure and heart rate were quantified for up to 6 h following dosing.

For determination of the cGMP concentration, a sample of arterial blood (0.5 ml) was drawn into a syringe containing 0.5 M EDTA (1% v/v) through the arterial catheter. Plasma was obtained by centrifugation ($2500 \times g$ at 4°C for 5 min) and immediately frozen at −20°C. Samples were assayed within 1 week by thawing at 4°C. After acetylation, the concentration of cGMP was determined by using a radioimmunoassay kit (New England Nuclear Research Products, Boston, MA) and expressed as fmol of

Table 1
Inhibition of phosphodiesterase isozymes by WIN 65579 and zaprinast

	Phosphodiesterase inhibition, IC_{50} (nM)				
	PDE1 (CA)	PDE2 (CV)	PDE3 (CA)	PDE4 (CA;HSV)	PDE5 (CA;HSV)
WIN 65579	> 10,000	> 10,000	3000	135; 100	2; 3
Zaprinast	8000	> 10,000	> 10,000	10,000	310; 180

Source of phosphodiesterase (PDE) isozymes.
CA, canine aorta; CV, canine ventricle; HSV, human saphenous vein.

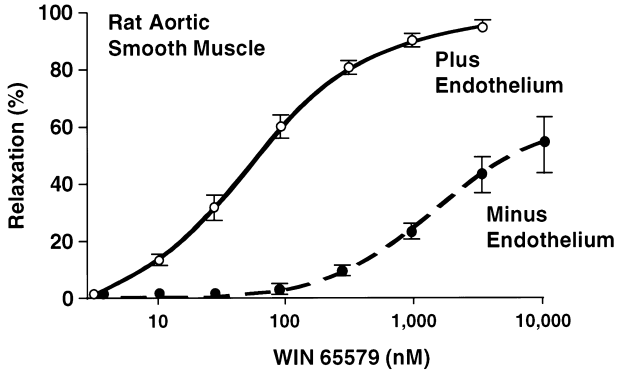

Fig. 2. Concentration-related relaxation of rat aortic smooth muscle rings by WIN 65579. Aortic rings were either intact or were denuded of endothelium as described in the text. Rings were pre-contracted with 1 μM (denuded rings) or 3 μM (intact rings) phenylephrine (which produced 1.8–2.2 g of active force) followed by graded, cumulative concentrations of WIN 65579. The amount of relaxation was determined 10 min after addition of each concentration. Values are mean $\pm$ S.E.M. for 12 rings/group. The calculated EC_{50} for WIN 65579 is 60 nM in the presence of endothelium, and 5 μM in the absence of endothelium.

cGMP per μl of plasma as described previously (Dundore et al., 1993).

Effects on nitroglycerin tolerance in vivo were assessed in spontaneously hypertensive rats as described previously (De Garavilla et al., 1993, 1996). Rats chronically instrumented with femoral arterial and venous catheters were injected with 100 mg of nitroglycerin per kg, s.c., 3 times/day for 3 days to induce hemodynamic tolerance to nitroglycerin. Decreases in mean arterial pressure in response to bolus i.v. doses of nitroglycerin (1–300 μg/kg) in the presence or absence of WIN 65579 were quantified in these tolerant rats. The dose of WIN 65579 used in this study (1 mg/kg i.v.) produced minimal decreases in mean arterial pressure after single dosing. A control, non-tolerant group was administered nitroglycerin vehicle, s.c., for 3 days and the vehicle for WIN 65579 on the day of experimentation.

2.4. Statistical analyses

Analysis of variance followed by a Dunnett's test was used to evaluate the dose-dependent reductions in mean arterial pressure due to nitroglycerin within each group. Analysis of variance followed by a Student's *t*–Newman–Keuls test was used to evaluate significant differences between groups. A Student's *t*-test was used to compare the i.v and p.o. effects of WIN 65579 vs. vehicle for mean arterial pressure and heart rate responses.

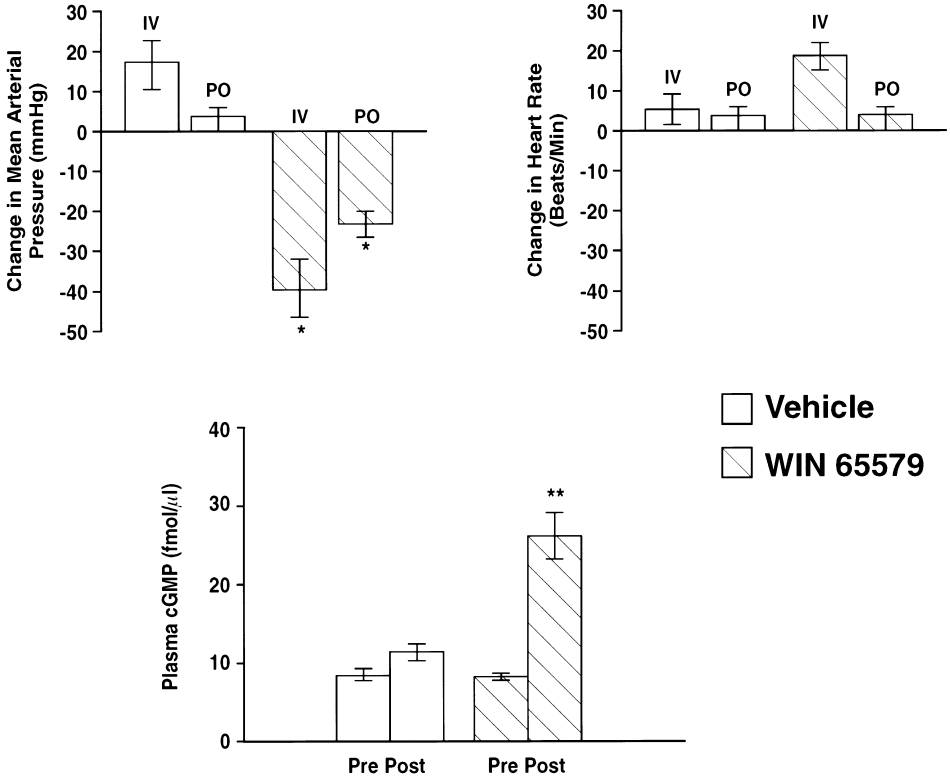

Fig. 3. Effects of WIN 65579 on mean arterial blood pressure, heart rate, and plasma cGMP levels in conscious spontaneously hypertensive rats. Top panels: WIN 65579 (10 mg/kg) or its vehicle (0.05 N NaOH, 1 ml/kg) was administered intravenously (i.v.) or by oral gavage (p.o.) to consciously instrumented rats as described in the text. Values shown represent the maximum change (mean $\pm$ S.E.M.) in mean arterial blood pressure (i.v.—up to 30 min post-dose; p.o.—up to 3 h post-dose) and corresponding change in heart rate following dosing of WIN 65579 or vehicle to 10 rats/group. $*$ indicates $P < 0.05$ relative to the vehicle group. Bottom panel: WIN 65579 (10 mg/kg) or vehicle (0.05 N NaOH) was administered intravenously to conscious rats ($n = 6$ rats/group). Cyclic GMP levels (fmol/μl) were determined by radioimmunoassay prior to (Pre) and 30 min after (Post) administration of WIN 65579 or vehicle. $**$ indicates $P < 0.05$ relative to vehicle.

3. Results

WIN 65579 is a potent inhibitor of phosphodiesterase 5 isolated from vascular tissue with an IC_{50} value of 2–3 nM (Table 1). Potency for inhibition of phosphodiesterase 5 was approximately the same whether canine aortic or human saphenous veins were used as the source of vascular tissue. Mechanistically, WIN 65579 is a competitive inhibitor, with respect to the substrate cGMP, with a calculated K_i value of approximately 1 nM (data not shown). WIN 65579 was very selective for phosphodiesterase 5 relative to phosphodiesterases 1, 2 or 3. Inhibition of cAMP phosphodiesterase 4 occurred with an IC_{50} = 100–135 nM.

WIN 65579 possesses vasorelaxant activity that is dependent on an intact endothelium (Fig. 2). With phenylephrine as a contractile agonist, the calculated EC_{50} value for WIN 65579 in the presence of an intact endothelium is approximately 60 nM, whereas the EC_{50} value in the absence of endothelium is approximately 5 μM.

WIN 65579 produces dose-related reductions in mean arterial pressure following i.v. administration. At 10 mg/kg i.v., WIN 65579 decreases mean arterial pressure by 40 ± 7 mmHg, and also slightly increases heart rate by 18 ± 3 b/min (Fig. 3). Oral administration of WIN 65579 (10 mg/kg) also decreases mean arterial pressure (by 24 ± 3 mmHg), while no significant elevation in heart rate (4 ± 3 b/min) was noted. Decreases in mean arterial pressure following p.o. administration lasted for approximately 6 h in spontaneously hypertensive rats (data not shown).

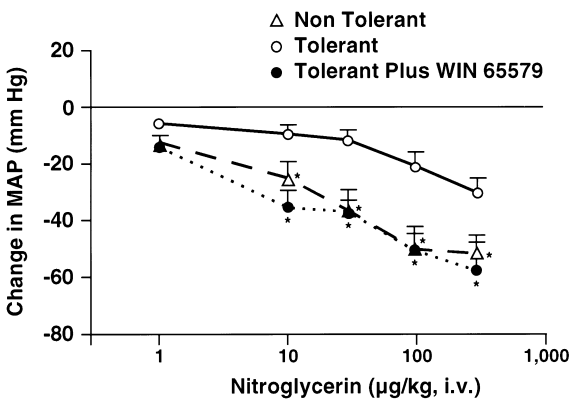

Fig. 4. Dose-related reductions in mean arterial blood pressure (mean arterial pressure) by nitroglycerin in conscious rats. Rats were injected subcutaneously for 3 days with 100 mg/kg nitroglycerin 3 times/day (tolerant group) or vehicle (0.9% saline; non-tolerant group). Animals then received cumulative i.v. injections of nitroglycerin; reductions in mean arterial pressure are the mean ± S.E.M. for 6–10 rats/group. Animals in the tolerant group either received WIN 65579 (1 mg/kg; closed circles) or vehicle for WIN 65579 (0.05 N NaOH; open circles) intravenously 10 min prior to the cumulative i.v. nitroglycerin injections. Reductions in mean arterial pressure for the non-tolerant and tolerant plus WIN 65579 groups were statistically (*; $P < 0.05$) lower than reductions in the tolerant group not receiving WIN 65579 at doses of 10, 30, 100, and 300 μg/kg nitroglycerin.

WIN 65579 (10 mg/kg i.v.) also increases plasma cGMP levels, from basal levels of 8 ± 1 to 26 ± 3 fmol/μl in rats (Fig. 3).

Tolerance to reductions in mean arterial pressure after i.v. administration of nitroglycerin was induced in rats by chronic (3 day) administration of high dosages of nitroglycerin (Fig. 4). In the tolerant group, significant reductions in mean arterial pressure were observed only at 100 and 300 μg/kg of nitroglycerin, in contrast to non-tolerant rats, which showed significant reductions in mean arterial pressure at doses of nitroglycerin of 10 μg/kg and above. Treatment of tolerant animals with WIN 65579 (1 mg/kg, i.v.) restored the hypotensive effect of nitroglycerin as evidenced by significant reductions in mean arterial pressure at doses greater than or equal to 10 μg/kg nitroglycerin.

4. Discussion

This study details the phosphodiesterase 5 inhibitory activity of a new compound, WIN 65579. Moreover, WIN 65579 also has in vitro and in vivo properties associated with phosphodiesterase 5 inhibition, including endothelial-dependent relaxation of contracted rat aortic smooth muscle, depressor effects when administered to conscious spontaneously hypertensive rats, the ability to increase plasma levels of cGMP, and the ability to effectively reinstate vascular responsiveness to nitroglycerin in animals that have been made nitroglycerin-tolerant.

WIN 65579 is among the most potent of the phosphodiesterase 5 inhibitors reported, with an IC_{50} value in the 2–3 nM range. This compares favorably with E-4021, which has a reported IC_{50} value of 4 nM for phosphodiesterase 5 (Saeki et al., 1995). WIN 65579 is also slightly more potent as an endothelial-dependent vasorelaxant, with an EC_{50} value of 60 nM, relative to the reported value of 110 nM for E-4021 (Saeki et al., 1995). WIN 65579 also has high affinity for phosphodiesterase 5 purified from a human vascular source (saphenous veins).

Win 65579 is a chemical analog of WIN 58237, which has an IC_{50} for phosphodiesterase 5 inhibition of 180 nM (Silver et al., 1994). However, WIN 58237 is only roughly 2× selective for phosphodiesterase 5 vs. phosphodiesterase 4 (IC_{50} = 300 nM), whereas WIN 65579 is approximately 50-fold more potent for phosphodiesterase 5 relative to phosphodiesterase 4 (Table 1). It is interesting that this class of pyrazolopyrimidines has affinity for inhibiting a cGMP phosphodiesterase (phosphodiesterase 5), as well as a cAMP phosphodiesterase (phosphodiesterase 4), yet is highly selective vs. the other cAMP and cGMP phosphodiesterases (Table 1).

WIN 65579 produces in vivo cardiovascular actions that are consistent with phosphodiesterase 5 inhibition. It is a depressor agent when administered either intravenously or

orally to conscious spontaneously hypertensive rats (Fig. 3). While there is a corresponding tachycardia following intravenous administration, there is none observed following oral administration. This is likely due to the smaller decrease in mean arterial pressure observed following oral administration. WIN 65579, like other phosphodiesterase 5 inhibitors (Dundore et al., 1993), increases plasma cGMP levels following intravenous administration (Fig. 3), presumably by potentiating the effect of vascular nitric oxide.

Win 65579 also reinstates vasoresponsiveness to nitroglycerin in animals made tolerant to this vasodilator by repeat exposure. Interestingly, the overall magnitude of effect of WIN 65579 in the current study was somewhat less than that previously observed with zaprinast or WIN 58237 in this same model (De Garavilla et al., 1993; Silver et al., 1994). This may be related, in part, to the differences in phosphodiesterase isozyme selectivity among the three agents. WIN 65579 is highly potent and selective for phosphodiesterase 5, while WIN 58237 has some affinity for phosphodiesterase 4, and zaprinast has some affinity for phosphodiesterase 1. It is possible that inhibition of multiple phosphodiesterases may enhance the effects of phosphodiesterase 5 inhibition in this model.

Currently, at least two selective phosphodiesterase 5 inhibitors are undergoing clinical evaluation. E-4021 is purportedly in Phase II clinical trials for the treatment of angina or pulmonary hypertension, and sildenafil is being used to treat impotence (Terrett et al., 1996), which may be linked to a deficit in nitric oxide (Rafjer et al., 1992). Other potential uses for phosphodiesterase 5 inhibitors include acute respiratory distress syndrome, as well as the aforementioned nitroglycerin tolerance (see Silver, 1996 for review). WIN 65579 represents a new, potent inhibitor of phosphodiesterase 5 with both oral and intravenous bioavailability. WIN 65579 remains to be tested in human trials to ascertain the potential clinical utility of this agent.

Acknowledgements

The authors acknowledge the fine technical assistance of Ross Bentley, Dawn Clas, Linda Hamel, Keith Jackson, Phillip Pratt, Glen Van Aller, and Marlo Volberg.

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