Pharmacokinetics and Biologic Activity of the Novel Mast Cell Inhibitor, 4-(3′-Hydroxyphenyl)-amino-6,7-dimethoxyquinazoline in Mice

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Purpose. The purpose of the present study was to examine the pharmacodynamic and pharmacokinetic features of the novel mast cell inhibitor 4-(3′-Hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P180) in mice.

Methods. A high performance liquid chromatography (HPLC)-based quantitative detection method was used to measure plasma WHI-P180 levels in mice. The plasma concentration-time data was fit to a single compartment pharmacokinetic model by using the WinNonlin program to calculate the pharmacokinetic parameters. A cutaneous anaphylaxis model was used to examine the pharmacodynamic effects of WHI-P180 on anaphylaxis-associated vascular hyperpermeability.

Results. The elimination half-life of WHI-P180 in CD-1 mice (BALB/c mice) following i.v., i.p., or p.o. administration was less than 10 min. Systemic clearance of WHI-P180 was 6742 mL/h/kg in CD-1 mice and 8188 mL/h/kg in BALB/c mice. Notably, WHI-P180, when administered in two consecutive nontoxic i.p. bolus doses of 25 mg/kg, inhibited IgE/antigen-induced vascular hyperpermeability in a well-characterized murine model of passive cutaneous anaphylaxis.

Conclusions. WHI-P180 is an active inhibitor of IgE-mediated mast cell responses in vitro and in vivo. Further preclinical characterization of WHI-P180 may improve the efficacy of WHI-P180 *in vivo* and provide the basis for design of effective treatment and prevention programs for mast cell mediated allergic reactions.

KEY WORDS: WHI-P180; pharmacokinetics; quinazolines; mast cell inhibitor.

INTRODUCTION

Mast cells have been recognized as the major effector cell of the allergic or type I hypersensitivity reactions by virtue of their high affinity receptors for IgE. These allergic reactions are mediated by the activation of mast cells through the cross-linking of high affinity IgE (FcεRI) receptors by antigen on their cell surface (1,2). This IgE receptor crosslinking initiates the release of preformed granule-associated proinflammatory mediators (e.g., histamine, serotonin, proteases), newly synthesized arachidonic acid metabolites [e.g., leukotriene (LT) C_4, prostaglandin D_2 and platelet activating factor], and a number of proinflammatory cytokines [e.g., Tumor necrosis factor (TNF)α, Interleukin (IL)−1, −3, −4, −5, −6, −8, −9, −13] (3). The released mediators alone or in combination are responsible for the symptoms of allergic or asthmatic responses (4).

In a systematic search for effective mast cell inhibitors, we discovered that the novel quinazoline derivative 4-(3′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P180) is a potent inhibitor of mast cell functions. A quantitative high performance liquid chromatography (HPLC)-based quantitative detection method was developed, which allows the measurement of WHI-P180 levels in plasma. Here, we first report the biologic activity profile of WHI-P180 as a potent inhibitor of IgE-mediated mast cell responses in vitro as well as in vivo and describe its pharmacokinetics in mice.

MATERIALS AND METHODS

Synthesis and Characterization of Quinazoline Derivatives

WHI-P180 [*4-(3′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline*], WHI-P258 [*4-(phenyl)-amino-6,7-dimethoxyquinazoline*] and WHI-P154 [*4-(3′-bromo-4′-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline*] were synthesized as previously described (5, 6) and their structures (Fig. 1A) and physical properties were previously reported.

In Vitro Mast Cell Biology Experiments

RBL-2H3 cells (a rat origin mucosal mast cell line kindly provided by Dr. R.P. Siraganian, NIH, Bethesda, MD) in 48-well tissue culture plates were sensitized with a monoclonal anti-dinitrophenyl (DNP)IgE antibody (0.24 mg/ml) by overnight incubation at 37°C in a humidified 5% CO_2 atmosphere. Unbound IgE was removed by washing cells with PIPES buffered saline containing 1 mM calcium chloride.

RBL-2H3 cells were treated for 1 h at 37°C with the test or control compounds and then challenged with 20 ng/ml DNP-BSA for 30 min at 37°C. The plates were centrifuged at 200 × g for 10 min at 4°C, supernatants were removed and saved. The pellets were washed once with PIPES buffered saline and solubilized in PIPES buffered saline containing 0.1% Triton-X-100. Degranulation of RBL-2H3 cells was monitored by measuring β-hexosaminidase activity in cell free supernatants and Triton X-100 solubilized pellets by the colorimetric assay using *p*-nitrophenyl-2-acetamide-deoxy-β-glucopyranoside as previously described (7). LTC_4 was estimated in cell free supernatants by ELISA as previously described (8).

Pharmacokinetics in Mice

Female CD-1 mice and BALB/c mice from Charles River Laboratories (Wilmington, MA) were housed in a controlled environment (12-h light/12-h dark photoperiod, 22 ± 1°C, 60 ± 10% relative humidity), which is fully accredited by the USDA (United States Department of Agriculture). All mice

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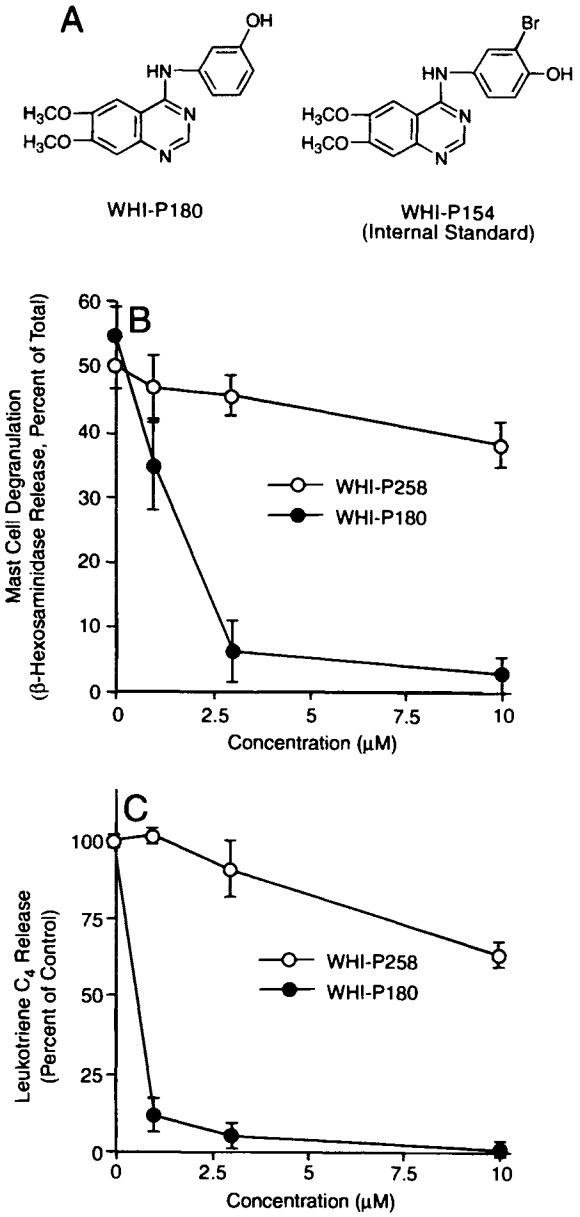

Fig. 1. (A) Chemical structures of WHI-P180 and WHI-P154 (=internal standard). (B) Effect of quinozoline derivatives on β-hexosaminidase release. β-hexosaminidase release data are presented after subtraction of spontaneous release (between 5 to 7.5%). (C) Effect of quinozoline derivatives on leukotriene C_4 release. The results of leukotriene C_4 release are expressed as percent of control. Control cells released 2.7 to 9.02 ng leukotriene $C_4/10^6$ cells in 6 different experiments. Data represent the mean ± SEM value from 3–6 independent experiments.

were housed in microisolator cages (Lab Products, Inc., New Jersey) containing autoclaved bedding. The mice were allowed free access to autoclaved pellet food and tap water throughout the experiments. Animal studies were approved by Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the Principles of Laboratory Animal Care (NIH Publication #85-23, revised 1985). In pharmacokinetic studies, mice (Study #1:CD-1 mice; Study #2: BALB/c mice) were injected either intravenously (i.v.) via the tail vein or intraperitoneally (i.p.) with a bolus dose of 20 mg/kg of WHI-P180. Blood samples (200 μL) were obtained from the ocular venous plexus by retro-orbital venipuncture prior to and at 3 min (in Study #2 only), 5 min, 10 min, 15 min, 30 min, 45 min and 1 h, 2 h, 4 h and 8 h (in Study #2 only) after administration of WHI-P180.

In order to determine the pharmacokinetics of WHI-P180 after oral administration, 12 hr fasted CD-1 mice were given a bolus dose of 200 mg/kg of WHI-P180 via gavage by using a No. 21 stainless-steel ball-tipped feeding needle. Blood sampling time points were at 3, 5, 10, 15, 30, 45 min and 1, 2, 4 h following oral administration. All collected blood samples were heparinized and centrifuged at 7,000 ×g for 10 min in a microcentrifuge to obtain plasma. The plasma samples were stored at −20°C until analysis. Aliquots of plasma were used for extraction and HPLC analysis as described below.

HPLC Determination of WHI-P180 Plasma Levels

Acetonitrile/water containing 0.1% of trifluoroacetic acid (TFA) and 0.1% triethylamine (TEA) (28:72, v/v) was used as the mobile phase. The mobile phase was degassed automatically by the electronic degasser system. The analytical column was equilibrated and eluted under isocratic conditions utilizing a flow rate of 1.0 ml/min at ambient temperature. The wavelength of detection was set at 335 nm. Peak width, response time and slit were set at >0.03 min, 0.5 s and 8 nm, respectively.

For determination of WHI-P180 levels, 10 μL of internal standard WHI-P154 (50 μM) was added to a 100 μL plasma sample. For extraction, 7 ml chloroform was then added to the plasma sample, and the mixture was vortexed thoroughly for 3 min. Following centrifugation (300 × g, 5 min), the aqueous layer was frozen using acetone/dry ice and the organic phase was transferred into a clean test tube. The chloroform extracts were dried under a slow steady stream of nitrogen. The residue was reconstituted in 100 μL of methanol: water (9:1) and 50 μL aliquot of of this solution was used for HPLC analysis using a recently reported HPLC system (9). Replicate (N = 5) plasma samples (100 μL/sample) were spiked with known amounts of WHI-P180 to yield a final concentration of 1 μM and 10 μM of WHI-P180. The samples were extracted following the above extraction procedures. The extraction recovery (ER) was calculated using formula: %ER = {Peak Area [WHI-P180]$_{extracted}$/Peak Area [WHI-P180]$_{unextracted}$} × 100.

In order to generate a standard curve, WHI-P180 was added to plasma to yield final concentrations of 0.25, 0.5, 1, 5, 10, 20, 50 and 100 μM. Subsequently, 10 μL of the internal standard (WHI-P154, 50 μM) (=0.5 nmol) was added to each sample. The plasma samples with known amounts of WHI-P180 and its internal standard WHI-P154 were extracted as previously described, and the standard curves were generated by plotting the peak area ratios (WHI-P180/WHI-P154) against the drug concentrations tested. Linear regression analysis of the standard curves was performed by using the CA-Cricket Graph III computer program, Version 1.1 (Computer Association, Inc., Islandia, NY). The standard samples of WHI-P180 (0.75 μM and 7.5 μM in plasma) were used as calibrators.

To evaluate the intra-assay accuracy and precision, WHI-P180 was added to drug-free plasma yielding final concentrations of 0.75 μM and 7.5 μM. These standard samples were prepared and analyzed within a single day following addition

of the internal standards. The concentrations were calculated using a standard curve. The ratio of the calculated concentration over the known concentration of WHI-P180 was used as the accuracy of the analytical method, and the coefficient of variance was used as an index of precision. The inter-assay accuracy and precision were determined in 5 independent experiments.

Pharmacokinetic and Statistic Analysis

Pharmacokinetic modeling and pharmacokinetic parameter calculations were carried out using the pharmacokinetics software, WinNonlin Program, Version 1.1. (Scientific Consulting Inc., Cary, NC) (10). Concentration data were weighted by 1/concentration. An appropriate pharmacokinetic model was chosen on the basis of lowest weighted squared residuals, lowest Schwartz Criterion (SC), lowest Akaike's Information Criterion (AIC) value, lowest standard errors of the fitted parameters, and dispersion of the residuals. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration profile. The area under the concentration time curve (AUC) was calculated by the trapezoidal rule between first (0 h) and last sampling time plus C/k, where C is the concentration of last sampling and k is the elimination rate constant. Systemic clearance (CL) was determined by dividing the dose by the AUC. Bioavailability (F) was estimated using the equation $F(\%) = AUC_{ip/po} \cdot Dose_{iv}/AUC_{iv} \cdot Dose_{ip/po}$. Statistic analysis was performed using the Instat program V2.03 (GraphPad Software, San Diego, CA). The significance of differences between pharmacokinetic parameters was analyzed using a two tailed t test and P values < 0.05 were considered significant.

Cutaneous Anaphylaxis Model

In order to examine the effect of WHI-P180 on passive cutaneous anaphylaxis in mice, dorsal sides of the ears of BALB/c mice were injected intradermally with 20 ng of DNP-IgE (left ears) or PBS (right ears) in 20 μL volume using a 30-gauge needle, as previously described (11). After 20 hours, mice were treated with WHI-P180 (25 mg/kg i.p.) twice at 1 hour and 30 min prior to the antigen challenge. Control mice were treated with an equal volume of vehicle. Thirty minutes after the last dose of WHI-P180 or vehicle, mice were challenged with 100 μg antigen (DNP-BSA) in 200 μl 2% Evans blue dye intravenously. Mice were sacrificed by cervical dislocation 30 minutes after the antigen challenge. For quantitation of Evans blue dye extravasation as a measure of anaphylaxis-associated vascular hyperpermeability, 8 mm skin specimens were removed from the ears of mice, minced in 2 ml formamide and incubated at 80°C for 2 hours in water bath to extract the dye. The absorbance was read at 590 nm.

RESULTS AND DISCUSSION

Mast Cell Inhibitory Effects of WHI-P180

When IgE sensitized RBL-2H3 mast cells were preincubated with increasing concentrations of the test compounds or vehicle for 1 h before challenge with antigen, we found that the novel dimethoxyquinazoline derivative WHI-P180 (Fig. 1A) effectively inhibits IgE/antigen-induced mast cell degranulation (as measured by β-hexosaminidase release)(Fig. 1B) as well as secretion of newly synthesized mediators (as measured by LTC_4 release)(Fig. 1C) in a dose-dependent fashion. WHI-P180 was much more potent than the unsubstituted dimethoxyquinazoline control compound WHI-P258 in inhibiting mast cell degranulation or leukotriene C_4 release (Fig. 1B & 1C).

Sensitivity and Accuracy of HPLC-Based Detection Method

Under the chromatographic separation conditions described in the Materials and Methods section, the retention times for WHI-P180 and for WHI-P154 (a structurally similar dimethoxyquinazoline derivative which was used as an internal standard) were 5.4 minutes and 9.5 minutes, respectively. At the retention time, the WHI-P180 and WHI-P154 were eluted without any interference peaks from the blank plasma (Fig. 2). With the described extraction conditions, the extraction recovery of WHI-P180 from plasma was 90.6%. The calibration curve was linear over the concentration-dose ranges tested and could be described by the regression equations: $Y = 4.0582 * X - 0.0953$ ($r > 0.999$), in which Y is the agent recovered in μM in plasma, and X is the peak area ratio (WHI-P180/WHI-P154). The lowest limit of detection of WHI-P180 in 100 μl

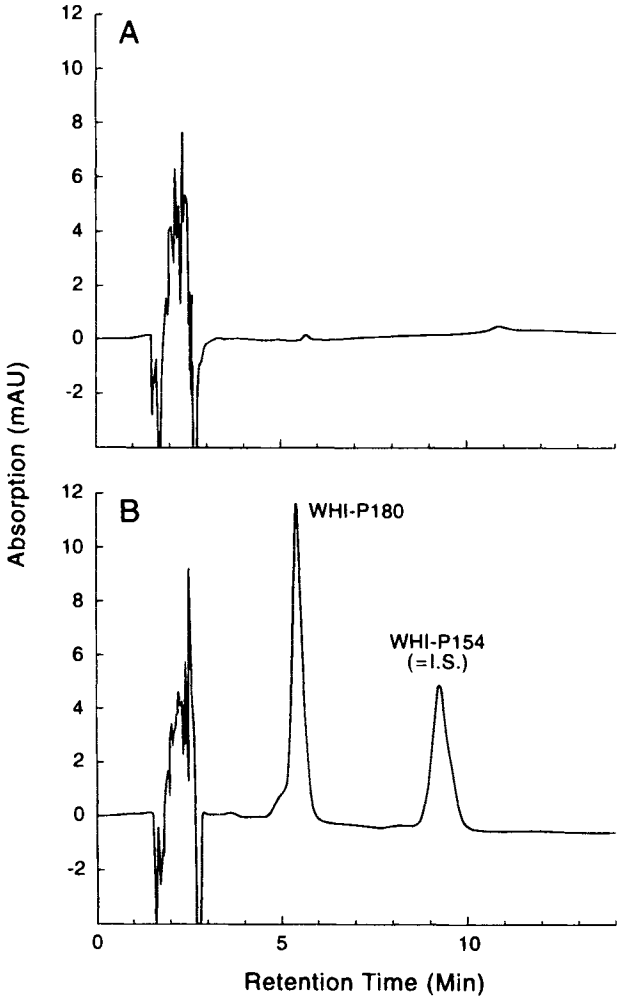

Fig. 2. Representative chromatograms from (A) blank plasma, (B) plasma sample 10 min after i.p. injection of 20 mg/kg WHI-P180. I.S. = internal standard.

plasma was 0.1 μM. The obtained results indicate that the intra-assay and inter-assay coefficient of variance (C.V.) in plasma was less than 6%. The accuracy of this HPLC detection method was 98.5% in plasma.

Pharmacokinetic Features of WHI-P180 in Mice

First, we obtained whole blood from CD-1 mice (N = 3) 30 min after i.v. administration of 40 mg/kg bolus dose of WHI-P180. Plasma was separated from the cell fraction by centrifugation in a microcentrifuge for 10 min at 7000 × g. These fractions were individually extracted and analyzed for their WHI-P180 content by quantitative HPLC. The measured WHI-P180 concentrations were 7.4 ± 1.3 μM in the plasma fraction and 2.8 ± 0.8 μM in the cell fraction with an estimated plasma/whole blood ratio of 0.73:1. The mean plasma protein binding of WHI-P180 was 81.7% and its *in vitro* binding to 5% albumin was 81.2% (data not shown) (12,13). The plasma concentration-time curves of WHI-P180 in CD-1 mice following i.v. injection of a non-toxic 20 mg/kg bolus dose are depicted in Fig. 3A. The plasma WHI-P180 concentrations were below the detection limit 45 min after the i.v. administration. Similar results were obtained in BALB/c mice. A single compartment pharmacokinetic model was used to analyze the plasma concentration time curves obtained in both mouse strains. The pharmacokinetic parameters are shown in Table 1. The central volume of distribution (Vc) was 634 mL/kg in CD-1 mice and 620 mL/kg in BALB/c mice. WHI-P180 also showed rapid elimination ($t_{1/2}$ = 3.9 min in CD-1 mice and 3.2 min in BALB/c mice) and a rapid systemic clearance (CL: 6742 mL/h/kg in CD-1 mice and 8188 mL/h/kg in BALB/c mice). The values for AUC and C_{max} were 10.4 μM·h and 110.4 μM in CD-1 mice versus 8.5 μM·h and 112.8 μM in BALB/c mice. The large volume of distribution and fast clearance suggest that WHI-P180 is rapidly distributed into extravascular compartments after i.v. administration.

In CD-1 as well as BALB/c mice, a one compartment pharmacokinetic model was fit to the pharmacokinetics data obtained following the intraperitoneal administration (i.p.) of a single 20 mg/kg bolus dose of WHI-P180 (Fig. 3B). The computer-fitted pharmacokinetic parameters are shown in Table 1. In CD-1 mice, the estimated maximum plasma concentration (C_{max}) of WHI-P180 after i.p. administration was 25.7 μM, and its bioavailability was estimated at 26.7%. WHI-P180 demonstrated rapid absorption after i.p. administration with an absorption half-life of 0.1 min, and the estimated time to reach maximum plasma WHI-P180 concentration (t_{max}) was only 0.7 min. WHI-P180 also had a rapid elimination rate with half-life of 4.0 min. In BALB/c mice, the predicted C_{max} was 7.3 μM and the bioavailability was estimated to be 19.2%. Similar to the results in CD-1 mice, WHI-P180 demonstrated rapid absorption in BALB/c mice with an absorption half-life of 1.6 min, and the estimated t_{max} was 4.0 min. The elimination half-life of WHI-P180 following i.p. injection in BALB/c mice was 5.8 min. The values for AUC were 2.8 μM·h in CD-1 mice versus 1.6 μM·h in BALB/c mice. The central volume of distribution of WHI-P180 was 648 mL/kg in CD-1 mice and 1130 mL/kg in BALB/c mice, which is almost equal to whole body water volume (14), indicating that this agent has extensive extravascular distribution. Wide distribution of WHI-P180 in mice may be partially attributed to its moderate plasma protein binding capacity, which may facilitate the escape of most of the drug from vascular compartments to other extravascular tissues and relatively rapid elimination from the animal body may result in lower concentration of WHI-P180 in plasma.

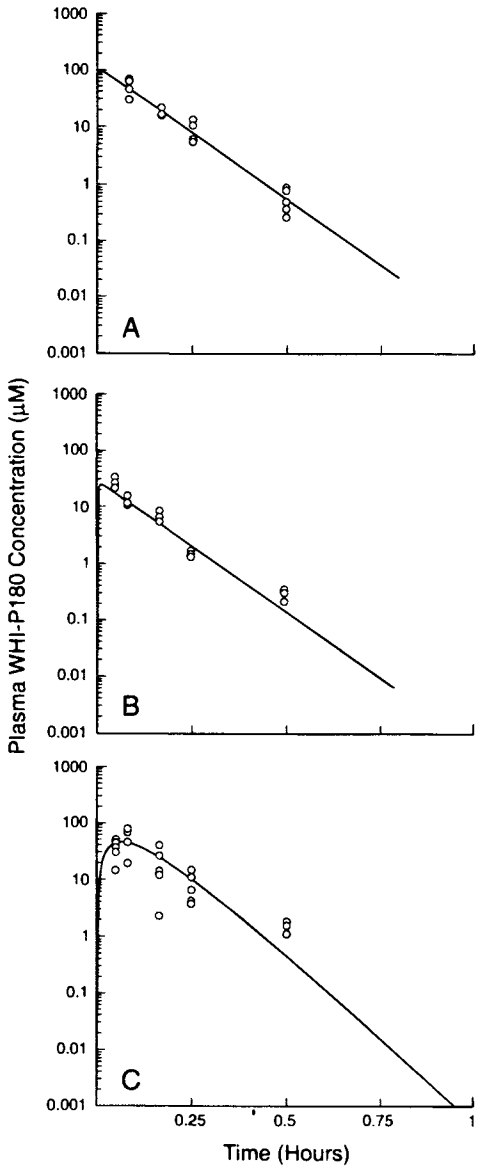

Fig. 3. Plasma concentration-time profiles of WHI-P180 in CD-1 mice following i.v. bolus injection (20 mg/kg; 5 mice per group) (A), following i.p. administration (20 mg/kg; 4 mice per group) (B), and following p.o.administration (200 mg/kg; 5 mice per group) (C).

The observed plasma concentrations following oral administration of 200 mg/kg of WHI-P180 can be best described by a one compartment model (Fig. 3C). No WHI-P180 was detected after 45 min following oral administration. The oral pharmacokinetic parameters were presented in Table 1. The predicted C_{max} of WHI-P180 after oral administration of a 200 mg/kg bolus dose was 48.0 μM and its bioavailability was estimated to be 8.3%. WHI-P180 showed a rapid absorption profile with an estimated absorption half-life of 2.7 min and a t_{max} of only 4.0 min. The estimated values for volume of distribution and elimination half-life were 450 mL/kg and 2.8 min, respectively.

Table 1. Pharmacokinetic Parameters of WHI-P180 in Mice

	Dose (mg/kg)	Vc[a] (mL/kg)	CL_s (mL/h/kg)	AUC (μM·h)	F (%)	C_{max} (μM)	$t_{1/2}$ (min)	$t_{1/2k\alpha}$ (min)	t_{max} (min)
CD-1 mice (n = 5)	i.v., 20	634 ± 149	6742 ± 665	10.4 ± 1.3	ND	110.4 ± 16.8	3.9 ± 0.5	ND	ND
CD-1 mice (n = 4)	i.p., 20	648 ± 59	25214 ± 1536[b]	2.8 ± 0.2	26.7%	25.7 ± 2.6	4.0 ± 0.2	0.1 ± 0.1	0.7 ± 0.1
CD-1 mice (n = 5)	p.o., 200	450 ± 63	81591 ± 17033[b]	8.6 ± 1.4	8.3%	48.0 ± 6.9	2.8 ± 0.7	2.7 ± 0.2	4.0 ± 0.5
BALB/c mice	i.v., 20	620	8188	8.5	ND	112.8	3.2	ND	ND
(n = 2)		(541, 713)	(7891, 8363)	(8.4, 8.8)		(98.1, 129.4)	(2.8, 3.5)		
BALB/c mice	i.p., 20	1130	42719[b]	1.6	19.2%	7.3	5.8	1.6	4.0
(n = 2)		(997, 1293)	(28455, 53435)	(1.3, 2.4)		(6.9, 7.3)	(4.6, 8.3)	(0.4, 4.6)	(1.7, 7.0)

Note: Pharmacokinetic parameters in CD-1 mice are present as estimates from pooled data (±SEM). In BALB/c mice, pharmacokinetic parameters are present as estimates from pooled data as well as the individual values.

[a] Vc has been corrected for bioavailability.

[b] Apparent systemic clearance not corrected by bioavailability (F). *Abbreviations*: $t_{1/2}$ is terminal elimination half-life; $t_{1/2k\alpha}$ is absorption half-life; t_{max} is the time required to reach the maximum plasma drug concentration following i.p. and p.o. administration. ND: no determination.

Pharmacodynamic Features of WHI-P180 in Mice

We next examined if effective mast cell inhibitory concentrations of WHI-P180 can be achieved in vivo after intraperitoneal administration of WHI-P180 at a nontoxic 25 mg/kg dose level, which was predicted to yield a C_{max} of 9 μM in a simulation model based on the pharmacokinetics data on WHI-P180 in BALB/c mice. The experimentally derived C_{max} value was 13.5 μM after intraperitoneal administration of 25 mg/kg WHI-P180 (Fig. 4), which is higher than the target concentration of 10 μM, at which WHI-P180 abrogates mast cell responses *in vitro*. This finding prompted the hypothesis that effective mast cell inhibitory plasma concentrations of WHI-P180 can be achieved *in vivo* at non-toxic dose levels. To test this hypothesis, we next studied the effects of WHI-P180 on vascular permeability in a well-characterized murine model of passive cutaneous anaphylaxis. Increased vascular permeability induced by mast cell mediators such as histamine and leukotrienes is a hallmark of anaphylaxis (15). This condition is developed by the local injection of antigen specific IgE into the mice ear and subsequent systemic challenge with antigen with Evan's blue dye. The injection of dye with antigen helps to visualize and quantitate the vascular permeability changes. Pretreatment of mice with WHI-P180 resulted in 50 percent reduction of vascular permeability changes. WHI-P180 inhibited the IgE/antigen induced plasma exudation, as measured by extravasation of systemically administered Evan's blue dye, in mice (N = 5) that had been presensitized with antigen specific IgE (Fig. 5). Thus, WHI-P180 is a potent inhibitor of IgE-mediated mast cell responses *in vivo*.

Histamine released from degranulating mast cells is known to induce the anaphylaxis-associated vascular permeability changes by causing vasodilation and partial dissociation of the endothelium (16). A number of studies have further demonstrated that arachidonic acid metabolites such as leukotrienes secreted by mast cells also contribute to enhanced vascular permeability in anaphylaxis (17,18). In a systematic search for a potent mast cell inhibitor, we identified the novel quinazoline derivative WHI-P180 (=4-(3′-Hydroxyphenyl)-amino-6,7-dimethoxyquinazoline) as an effective inhibitor of mast cell degranulation and leukotriene release. Our findings presented herein indicate that WHI-P180 is quickly absorbed following either intraperitoneal or oral administration. The time to reach the maximum plasma concentration was less than 5 min in mice following intraperitoneal or oral administration. However, the bioavailability of WHI-P180 following intraperitoneal

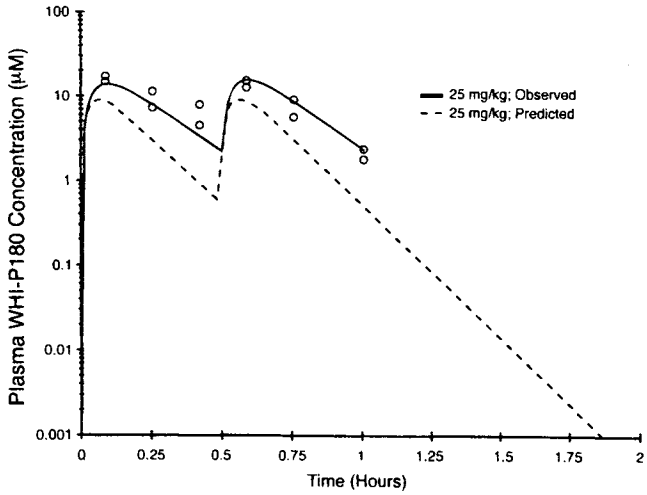

Fig. 4. Predicted and observed pharmacokinetic features of WHI-P180 in BALB/c mice following i.p. administration of two consecutive 25 mg/kg at 30 min interval.

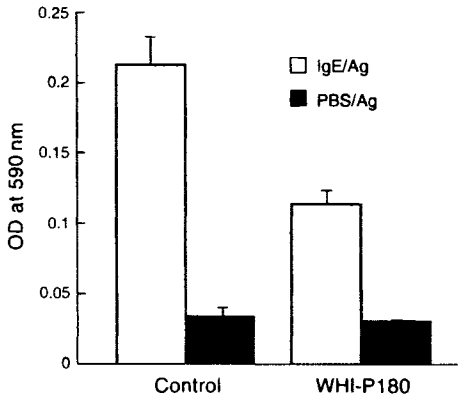

Fig. 5. Effect of P180 on passive cutaneous anaphylaxis. Cutaneous anaphylaxis was measured as Evan's blue dye extravasation into the ear skin of the mice. The data are expressed as mean ± SEM (N = 5 mice).

administration was less 30%, which is much lower than that of the quinazoline derivatives, ICI D1694 (19) which showed full bioavailability following intraperitoneal administration. The oral bioavailability of WHI-P180 was similar to that of ICI D1694. Our studies also demonstrated that WHI-P180 is very rapidly eliminated from circulation. The elimination half-life of WHI-P180 in CD-1 mice and BALB/c was less than 10 min. The short elimination half-life of WHI-P180 may be caused by its high clearance rates (~150 mL/h) which were ~1.5-times faster than the blood flow in liver (108 mL/h) (14) or ~2-times faster than blood flow in kidney (78 mL/h) (14), suggesting WHI-P180 is eliminated via both liver and kidney. Alternatively, this drug may be rapidly metabolized in vivo, which will be the subject of future studies. In summary, WHI-P180 has been demonstrated to be a potent inhibitor of IgE-mediated mast cell degranulation and mediator release *in vitro* and *in vivo*. Further preclinical characterization of WHI-P180 may improve the efficacy of WHI-P180 *in vivo* and provide the basis for design of effective treatment and prevention programs for mast cell mediated allergic reactions.

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