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European Journal of Pharmacology 325 (1997) 69–80

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Pharmacology of ABT-491, a highly potent platelet-activating factor receptor antagonist

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Received 24 October 1996; revised 31 January 1997; accepted 7 February 1997

Abstract

ABT-491 (4-ethynyl-*N*,*N*-dimethyl-3-[3-fluoro-4-[(2-methyl-1*H*-imidazo-[4,5-*c*]pyridin-1-yl)methyl]benzoyl]-1*H*-indole-1-carboxamide hydrochloride) is a novel PAF (platelet-activating factor) receptor antagonist with a K_i for inhibiting PAF binding to human platelets of 0.6 nM. Binding kinetics of ABT-491 to the PAF receptor is consistent with a relatively slow off-rate of the antagonist when compared to PAF. Inhibition of PAF binding is selective and is correlated with functional antagonism of PAF-mediated cellular responses (Ca^{2+} mobilization, priming, and degranulation). Administration of ABT-491 in vivo leads to potent inhibition of PAF-induced inflammatory responses (increased vascular permeability, hypotension, and edema) and PAF-induced lethality. Oral potency (ED_{50}) was between 0.03 and 0.4 mg/kg in rat, mouse, and guinea-pig. When administered intravenously in these species, ABT-491 exhibited ED_{50} values between 0.005 and 0.016 mg/kg. An oral dose of 0.5 mg/kg in rat provided > 50% protection for 8 h against cutaneous PAF challenge. ABT-491 administered orally was also effective in inhibiting lipopolysaccharide-induced hypotension (ED_{50} = 0.04 mg/kg), gastrointestinal damage (0.05 mg/kg, 79% inhibition), and lethality (1 mg/kg, 85% vs. 57% survival). The potency of this novel antagonist suggests that ABT-491 will be useful in the treatment of PAF-mediated diseases. © 1997 Elsevier Science B.V.

Keywords: ABT-491; PAF (platelet-activating factor); PAF receptor antagonist; Ca^{2+} flux; Superoxide anion; Vascular permeability; Degranulation; Endotoxemia

1. Introduction

PAF (platelet-activating factor) is a potent mediator that may play an important role in inflammatory diseases. PAF exerts its pro-inflammatory activities by acting on specific cell-surface receptors found on platelets, neutrophils, eosinophils, macrophages and other inflammatory cells. PAF interaction with its receptor leads to a multitude of biological responses that include cell activation, increased vascular permeability, hypotension, ulcerogenesis, bronchoconstriction, and the triggering of airway hyper-responsiveness (for reviews, see Prescott et al., 1990; Snyder, 1987). These pathophysiological effects of PAF, coupled with the observation that PAF levels increase in many disease states, implicate PAF as an important mediator in disease conditions having inflammatory components such as asthma, allergic rhinitis, sepsis and related disorders, pancreatitis, inflammatory bowel disease, and ischemia/reperfusion injury (reviewed by Braquet et al., 1987b).

The potential clinical utility of PAF receptor antagonists has been well recognized, which has led to the discovery and development of numerous PAF receptor antagonists over the past 15 years (for reviews see Koltai et al., 1991; Summers and Albert, 1995). Nevertheless, the therapeutic utility of PAF receptor antagonists in human disease remains largely unproved. In asthma trials, for example, clinical studies with several early receptor antagonists failed to demonstrate beneficial effects on lung function (Bel et al., 1991; Dermarkarian et al., 1991; Freitag et al., 1993; Kuitert et al., 1993; Wilkens et al., 1990). Two recent preliminary reports indicate that WEB-2086 (4-(2-chlorophenyl)-9-methyl-2[3(4-morpholinyl)-3-propanon-1-yl[6*H*-thienol[3.2*f*[[1.2.4]triazolo]4,3-1]]1.4]diazepine), given at higher doses than studied previously, and SR-

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PII S0014-2999(97)00109-X

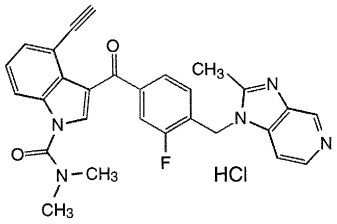

Fig. 1. Structure of ABT-491.

27,417 (*N*-(2-dimethylamino ethyl)-*N*-(3-pyridinyl methyl)[4-(2,4,6-triisopropylphenyl) thiazol-2-yl]amine), a more potent antagonist, are capable of providing improvement in lung function (Tamura et al., 1994). These later results raise the possibility that more potent antagonists may exhibit greater clinical efficacy.

In our search for a more potent PAF receptor antagonist suitable for human studies, we have synthesized and characterized ABT-491 (Fig. 1). The molecule was selected from a series of imidazopyridine-indole containing receptor antagonists that were synthesized based on the concept of merging the lipophilic indole portion of Abbott's prodrug ABT-299 with the phenyl spacer and imidazapyridine heterocycle of British Biotechnology's PAF receptor antagonist BB-882 (*N*-methyl-*N*-4-(1*H*-2-methylimidazo[4,5-*c*]pyridylmethyl) phenylsulphonyl-L-leucine ethyl ester) (Curtin et al., 1996). Optimization of the resulting series led to the discovery of ABT-491, an aqueous soluble receptor antagonist that possesses improved pharmacological properties compared to its predecessors. Based upon its favorable preclinical profile, ABT-491 has been selected for human studies. The present communication describes the pharmacological profile of this molecule: (1) characterization of in vitro activity, (2) in vivo potency in PAF challenge models, and (3) efficacy in experimental acute endotoxemia.

2. Materials and methods

2.1. Chemicals

[^{3}H]C_{18}-PAF (120–180 Ci/mmol) and [^{14}C]serotonin (55 mCi/mmol) were purchased from Amersham (Arlington Heights, IL, USA). ABT-491 (4-ethynyl-*N*,*N*-dimethyl-3-[3-fluoro-4-[(2-methyl-1*H*-imidazo-[4,5-*c*]pyridin-1-yl)methyl]benzoyl]-1*H*-indole-1-carboxamide hydrochloride) was synthesized at Abbott Laboratories. Mono-Poly Ficoll-Hypaque medium was obtained from Flow Laboratories (McLean, VA, USA). *N*-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-amino-4-methylcoumarin was purchased from Peninsula Laboratories (Belmont, CA, USA). Other reagents were purchased from Sigma (St. Louis, MO, USA). Two forms of ABT-491, the free base and hydrochloride salt, have been used to generate the pharmacology profile presented in this report. Comparative studies indicated that in vitro and in vivo potency and duration of action of the two forms are indistinguishable under the conditions of the assays.

2.2. Laboratory animals

Male, CD1/ICR albino mice (25–30 g) and Hartley guinea pigs (200–300 g) were purchased from Charles River Labs (Wilmington, DE, USA). Sprague-Dawley rats (190–220 g) were purchased from SASCO (Oregon, WI, USA), and New Zealand white rabbits from Hazelton Research Products (Hazelton, WI, USA). Animals were maintained in light/dark cycle for at least one week after purchase. Animals were given continuous access to food and water with the exception of animals for oral studies, which were fasted overnight prior to use. All studies were performed in accordance with protocols approved by the Abbott Laboratories Animal Use and Care Committee and met guidelines approved by the American Veterinary Medical Association.

2.3. Statistical methods

Drug effect was expressed as percent inhibition of the response for each treatment group based on responses of vehicle-dosed control groups. Each set of dose–response data was fitted with a straight line using the logarithm of the dose as the *x*-value. No transformation was applied to the *y*-value, and data were excluded at the extreme responses (0–15% and 90–100%) so that there was no significant deviation from a straight line. In all sets the slope term was strongly significant in the analysis of variance. The dose where the regression line crossed the 50% response line (ED_{50}) was calculated and Fieller's theorem was used to compute a 95% confidence interval for this point. Potency for antagonizing cellular responses to PAF is expressed as an A_2 value determined by Schild plots using least squares analysis to achieve the best linear fit of the data (Schild, 1947). The Student's *t*-test was used for statistical comparisons. The χ^2-test was used for analysis of results from lethality studies.

2.4. Binding assays

The PAF receptor binding assays with rabbit platelet membranes and washed human platelets were based upon the procedures described by Hwang et al. (1983, 1985a) and Hwang and Lam (1986), and detailed elsewhere (Albert et al., 1996a). In brief, the standard membrane binding assay, conducted in Millititre-GV microtiter filter plates (Millipore, Milford, MA, USA), contained 10 μg platelet membrane protein, 0.6 nM of [^{3}H]C_{18}-PAF and test compound in buffer containing 0.25% bovine serum albumin in a final volume of 100 μl. The human platelet assay, conducted in 96-well glass fiber filtration plates (Multiscreen-FC, type C, Millipore, Bedford, MA, USA), con-

tained 30×10^6 washed platelets, 0.6 nM [^{3}H]C_{18}-PAF and test compound in a total volume 250 μl assay buffer. Test compounds were dissolved in dimethyl sulfoxide and diluted into buffer vehicle (final concentration $< 0.1\%$). Both assays were conducted at ambient temperature for 60 min. After the incubation period, the filter plates were subjected to vacuum filtration, washed with 1 ml of ice-cold assay buffer, and assayed for radioactivity with a microtiter scintillation counter (Top Count Packard Instrument, Meriden, CT, USA). Specific binding was defined as the difference between total binding of 0.6 nM [^{3}H]C_{18}-PAF (^{3}H radioactivity in the absence of added PAF) and nonspecific binding (^{3}H radioactivity in the presence of 1 μM PAF). Compound binding is ^{3}H radioactivity in the presence of the test compound. Percent inhibition is calculated as [(Total binding − Compound binding)/Specific binding] × 100%.

2.5. PAF-induced platelet responses

For the rabbit platelet release assay, washed rabbit platelets labeled with [^{14}C]serotonin were prepared as described previously (Ostermann et al., 1983; Albert et al., 1996a). Aliquots of the platelet suspension were incubated at 37°C with various concentrations of test compounds or vehicle (Tyrode buffer with 1.3 mM $CaCl_2$) for 5 min. Various concentrations of PAF or vehicle buffer were then added and the reaction mixture was incubated for an additional 6 min. To assess specificity of inhibition, thrombin (0.05–0.2 U/ml) or Ca^{2+} ionophore A23187 (0.3 μM) was substituted for PAF. The release reaction was terminated by cooling on ice and by the addition of 3 mM EDTA (final concentration) in saline. The platelet suspension was then centrifuged ($1500 \times g$, 15 min, 4°C) and the supernatant was collected for measurement of released ^{14}C radioactivity by liquid scintillation spectrometry.

To assay β-thromboglobulin release in blood peripheral human blood was obtained from healthy human volunteers and treated with heparin (20 U/ml) and incubated with 0.2% bovine serum albumin in normal saline (vehicle) or ABT-491 at the indicated concentration for 5 min at room temperature. Aliquots of the blood were then incubated with PAF or vehicle for an additional 5 min at 37°C and placed in ice-water. The blood samples were centrifuged ($1000 \times g$ for 15 min) and the plasma was removed and stored frozen until analyzed. Plasma levels of β-thromboglobulin were measured using commercially available immunoassay kits following the manufacturer's recommendations (Diagnostica Stago, American Bioproducts, Parsipanny, NJ, USA).

2.6. PAF-induced neutrophil responses

The assays for PAF-induced elastase release, reactive oxygen species production and intracellular Ca^{2+} were described in detail elsewhere (Albert et al., 1996a). In brief, for the elastase release assay, neutrophils (1 million/ml in Tyrode buffer, pH 7.4) were pretreated with drug for 6–8 min at room temperature and then exposed to cytochalasin B (1 μg/ml) for 15 min at 37°C (Dewald and Baggiolini, 1987). Various concentrations of PAF in Tyrode buffer containing 0.2% bovine serum albumin were then added and the incubation was continued for an additional 10 min until terminated by rapid cooling. The cell suspensions were then centrifuged at 5°C ($700 \times g$ for 15 min). Elastase activity in the supernatant was then measured with a fluorescence plate reader (Fluoroskan II, Titertek, ICN Flow, Costa Mesa, CA, USA) using the synthetic substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-amino-4-methylcoumarin (0.25 mM) at excitation and emission wavelengths of 370 and 460 nm, respectively. Relative activity was computed as the ratio of the rate of fluorescence increase obtained with stimulated and non-stimulated cells.

For the reactive oxygen species assay, cells were resuspended (5×10^6 cells/ml) in Dulbecco's phosphate-buffered saline containing 0.25% bovine serum albumin and 20 μM luminol (Allen, 1986). The cells (70 μl) were preincubated in microtiter trays at room temperature with 10 μl drug or vehicle (1% dimethyl sulfoxide in Hanks' balanced salt solution). After 5 min, 10 μl PAF or vehicle was added and the preincubation continued for an additional 2 min at room temperature. At the end of the preincubation, 10 μl formyl-methionyl-leucinyl-phenylalanine (fMLP) or vehicle was added. The resulting light output was recorded in a microtiter scintillation counter (Top Count Packard Instrument) at 20-s intervals for 5 min. Response to PAF and fMLP challenge was measured by calculating the area under the curve (using the trapezoidal rule) for the response to the challenge over the 5-min period.

To measure intracellular Ca^{2+}, neutrophils suspended in Hanks' balanced salt solution containing 0.035% $NaHCO_3$, pH 7.0, were incubated with 5 μM indo-1 AM for 30 min at room temperature, washed, and resuspended at a concentration of $(1–2) \times 10^6$ cells/ml. The cells were pretreated with receptor antagonist for 60 s followed by the addition of agonist at the indicated concentrations. Fluorescence was measured at an excitation wavelength of 350 nm and emission wavelengths of 485 nm (low Ca^{2+}) and 410 nm (high Ca^{2+}) using an SLM 8000 spectrofluorometer (SLM-Aminco Instruments, Urbana, IL, USA). Intracellular Ca^{2+} concentration was calculated from the ratio of fluorescence at the two emission wavelengths (Grynkiewicz et al., 1985). Values are expressed as percent inhibition relative to control response to PAF in the absence of antagonist.

2.7. PAF-induced vascular permeability and paw edema

The dorsal region of rats was shaved for preparation of intradermal injections of PAF essentially as previously

described (Hwang et al., 1985b). Ten minutes prior to PAF challenge, the animals were injected with Evans blue dye (1% in normal saline, 2 ml/kg) via a tail vein (5–6 animals per group). 100 μl of 0.5 μg/ml PAF in PBS (phosphate-buffered saline) containing 0.25% bovine serum albumin, pH 7.3, was injected intradermally into the dorsal skin at four sites evenly spaced on each side of the midline (bovine serum albumin alone was injected and found to have no significant effect). Test compounds were administered intravenously or orally 1 h prior to PAF challenge unless stated otherwise. 60 min after the PAF challenge the animals were killed and the dorsal skin was reflected back to reveal the extravasation areas. The diameter of each spot was measured and used to calculate area of extravasation. For studies with guinea-pigs, dosing and analysis procedures were as described above with the exception that the Evans blue dye solution was injected via a dorsal digital vein of either a thoracic or pelvic limb of animals ($n = 6$).

PAF-induced edema was measured using a mercury plethysmograph (Model 7150, Ugo Basile, Varese, Italy). After determining the pre-challenge volume of the right hind paw of mice ($n = 6$), PAF (50 μl of 1 μg/ml PAF in PBS buffer containing 0.25% bovine serum albumin, pH 7.3) was injected subcutaneously into the paw (Qu et al., 1990). Fifteen minutes after the PAF injection, the right hind paw volume was again measured. Paw edema was calculated for each mouse, in each treatment group, by subtracting paw volume at the pre-challenge time from paw volume at 15 min. Test compounds were administered intravenously or orally 30 min prior to PAF challenge.

2.8. PAF and endotoxin-induced shock

To measure PAF or endotoxin-induced hypotension, the carotid artery of guinea pigs and rats maintained under light to moderate anesthesia by inhalation of Penthrane (Abbott Laboratories, North Chicago, IL, USA) was cannulated with PE-50 tubing. The tubing was tunneled subcutaneously to the posterior neck, exteriorized and connected to a pressure transducer and polygraph (Model MI^2, Modular Instruments, Southeastern, PA, USA) for monitoring arterial pressure. After cannulation, the animals were allowed to recover for at least 1 h. After the recovery period a baseline pressure for each animal was established by monitoring arterial pressure over a 15 min period. There was no significant difference between baseline values among control and experimental groups.

For pre-treatment studies antagonist or vehicle (0.9% saline) was given 15 min (intraarterial) or 1 h (p.o.) prior to agonist challenge. Following pretreatment, guinea pigs and rats were challenged with PAF (0.6 μg/kg) or endotoxin (LPS, 25 mg/kg in PBS, pH 7.4), administered as an intraarterial bolus. For studies of the ability of ABT-491 to reverse ongoing hypotension, drug was administered 60 min following LPS challenge. Measurements of arterial pressure were taken at 1 min intervals until the conclusion of the experiment. To correct for the small degree of animal to animal variation ($< 10\%$), pressure values were expressed as percent initial baseline. Response to PAF or LPS challenge and drug was determined by calculating the area under the curve of percent baseline vs. time (using the trapezoidal rule) for the response to the challenge over the experimental period. From these areas, percent inhibition of the agonist-induced response was calculated as (drug − agonist)/(vehicle − agonist).

Intestinal bleeding induced by LPS treatment was determined by measuring hemoglobin which leaked into the gastrointestinal lumen. ABT-491 was given p.o. to conscious rats (6–7 per group) followed 60 min later by LPS (25 mg/kg). Rats were killed 30 min after LPS challenge and a 15 cm segment of intestine, starting from the duodenum, was collected from each rat. Luminal content was collected by rinsing the intestine segment. Hemoglobin concentration in the rinse was measured with a hemoglobinometer (Coulter Electronic, Hialeah, FL, USA) by comparison to a calibration curve obtained with standard hemoglobin solutions.

Lethality studies were conducted with PAF (3–1000 μg/kg) and LPS (8.5 mg/kg) in mice and rats, respectively. In both cases ABT-491 was administered orally 30 min prior to PAF or LPS challenge. The lethal effects of PAF, which generally occurred within 30 min following PAF challenge, were monitored over an 8 h period. LPS-induced mortality generally occurred 6–10 h following LPS challenge. Rats were closely monitored over a period of 2 weeks following LPS to determine the total number of deaths.

3. Results

3.1. Inhibition of PAF binding

The intrinsic PAF binding activity of ABT-491 has been evaluated by measuring the inhibition of [^{3}H]C$_{18}$-PAF binding to the PAF receptor of isolated rabbit platelet membranes and intact human platelets. Results, shown in Fig. 2A, illustrate the potency of ABT-491 in these assays. The inhibitory binding constants (K_i) computed from these results for ABT-491 are 1.8 and 0.57 nM, respectively and are within 3-fold the potency of PAF itself (K_d, 0.6 nM). The Hill coefficients obtained from the binding results (1.1 and 0.8) approach unity, indicating, as has previously been shown for PAF (Hwang and Lam, 1986), that ABT-491 interacts with a single class of binding sites.

The nature of inhibition of PAF binding exhibited by ABT-491 was examined using rabbit platelet membranes. In a competition binding experiment, membrane preparations were incubated (1 h) with increasing concentrations of [^{3}H]PAF in the absence and presence of ABT-491 (0.5–1.9 nM). Scatchard analysis of these results revealed that the maximal number of receptor sites (B_{max}, x-inter-

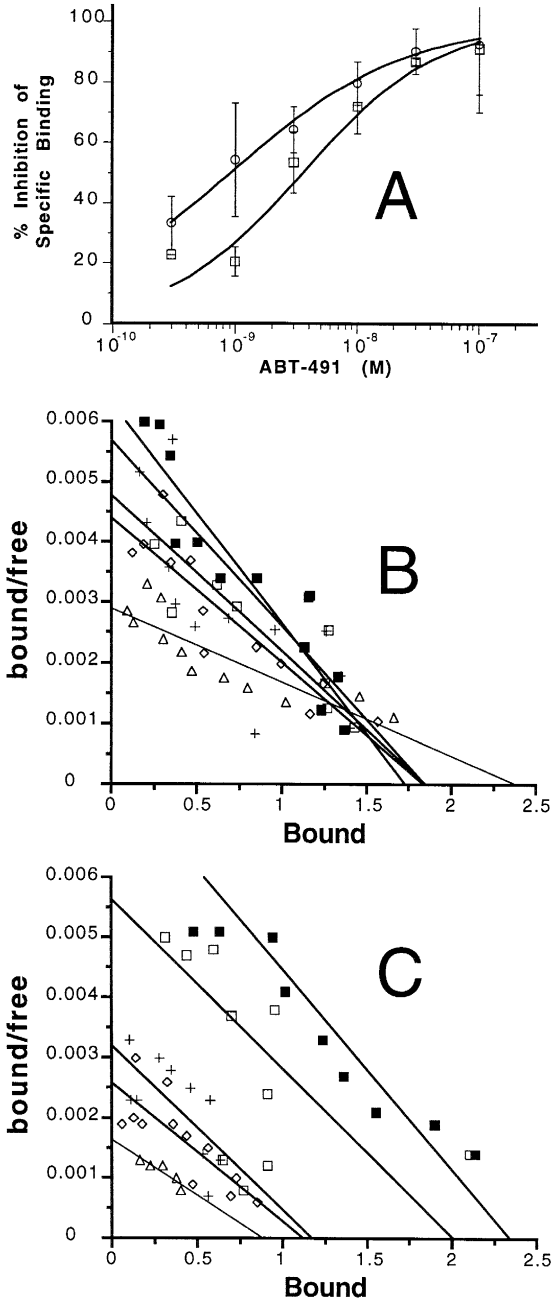

Fig. 2. Effect of ABT-491 on PAF binding. (A) Inhibition of specific binding of [^{3}H]PAF to rabbit platelet membranes and washed human platelets. Rabbit platelet membranes (square) or washed human platelets (circle) were incubated with 0.3–100 nM ABT-491 and 0.6 nM [^{3}H]PAF for 1 h at 25°C. Specific binding was determined from the difference between [^{3}H]PAF binding in the absence and presence of 1 μM PAF. Each point represents the mean ± standard deviation from two experiments performed in duplicate. (B and C) Effect of preincubation with ABT-491 on Scatchard plots of [^{3}H]PAF binding. Rabbit platelet membranes were incubated with increasing concentrations of [^{3}H]PAF (0.15–4.8 nM) for 30 min at 25°C in the presence of vehicle (closed square) or 0.5 nM (open square), 1 nM (+), 2 nM (diamond) and 4 nM (triangle) ABT-491. Drug was added to the membrane preparation either simultaneously with [^{3}H]PAF (B) or was added 60 min prior to [^{3}H]PAF (C). Specific binding of [^{3}H]PAF calculated from the saturation isotherms were subjected to Scatchard analysis. Each point represents the mean of duplicate determinations. Variation of the duplicates was within 10% of the mean.

cept) was not affected whereas the apparent dissociation constant (K_d, $-1/\text{slope}$) increased with respect to increasing concentrations of ABT-491 (Fig. 2B). These results strongly indicate that ABT-491 is a competitive inhibitor of PAF binding to the high-affinity receptor. However, although ABT-491 acts as a competitive receptor antagonist under equilibrium conditions, there is a time-dependent component to its inhibitory action. This is apparent from the Scatchard analysis of results from a binding assay in which membranes were preincubated for 1 h with ABT-491 prior to a 30 min incubation with [^{3}H]PAF. Under these experimental conditions, the B_{max} was altered with little effect on the apparent K_d, indicative of non-competitive inhibition (Fig. 2C). These results suggest that ABT-491, in comparison to PAF, may have a relatively slow off-rate on the PAF receptor.

To assess the specificity of inhibition for PAF binding, ABT-491 was evaluated in a wide variety of receptor, ion channel, and membrane binding assays. ABT-491 did not exhibit significant interaction at test concentrations up to 10 μM in any of these 45 different binding assays (data not shown).

3.2. Antagonism of PAF-induced platelet responses

Platelet degranulation and release of granular constituents is a consequence of PAF-mediated platelet activation (McManus et al., 1981). The ability of ABT-491 to inhibit platelet degranulation was demonstrated using PAF-induced [^{14}C]serotonin release from rabbit platelets. In the presence of increasing concentrations of ABT-491 (1–3 nM 5 min prior to PAF), a rightward and parallel shift in the PAF dose–response curves for [^{14}C]serotonin release was observed (Fig. 3A). From Schild analysis of the rightward shift, the A_2 value for ABT-491 for functional inhibition of PAF binding was calculated as 0.9 nM. Similar results ($A_2 = 1.3$ nM) were obtained in an additional experiment, yielding a mean A_2 value of 1.1 nM (not shown). At concentrations of ABT-491 greater than 3 nM the maximal response to PAF was not restored with agonist concentrations as high as 10 μM. These results, suggestive of non-competitive antagonism, are consistent with the kinetics observed in the binding studies with platelet membranes preincubated with ABT-491. In other experiments, ABT-491 (10 μM) did not cause release of [^{14}C]serotonin and did not inhibit release in response to thrombin (0.2 U/ml) or Ca^{2+} ionophore (0.3 μM). Taken together these results indicate that ABT-491 selectively and potently inhibits functional activity coupled to the platelet PAF receptor.

The appearance in plasma of platelet-specific granular constituents can serve as an index of activation and degranulation of platelets in blood. When incubated with human peripheral blood, PAF causes an increase in the plasma concentration of the platelet constituent β-thromboglobulin (Fig. 3B). Pretreatment of the blood with ABT-

491 inhibited the release of β-thromboglobulin in a concentration-dependent fashion. The rightward shift in the potency of PAF in the presence of increasing concentrations of the antagonist was similar to that observed with washed rabbit platelets. Schild analysis of these data and data from an additional experiment not shown yielded a mean A_2 value of 5.2 nM (Fig. 3B, insert). This value is consistent with the potency of ABT-491 for inhibiting PAF binding to platelet PAF receptors ($K_i = 0.6–2$ nM). These results demonstrate the effectiveness of ABT-491 against a PAF-induced platelet response in a complex biological milieu.

3.3. Antagonism of PAF-induced neutrophil responses

Three PAF-mediated neutrophil responses were examined in this study: intracellular Ca^{2+} mobilization, degranulation, and priming (Korchak et al., 1988; Dewald and

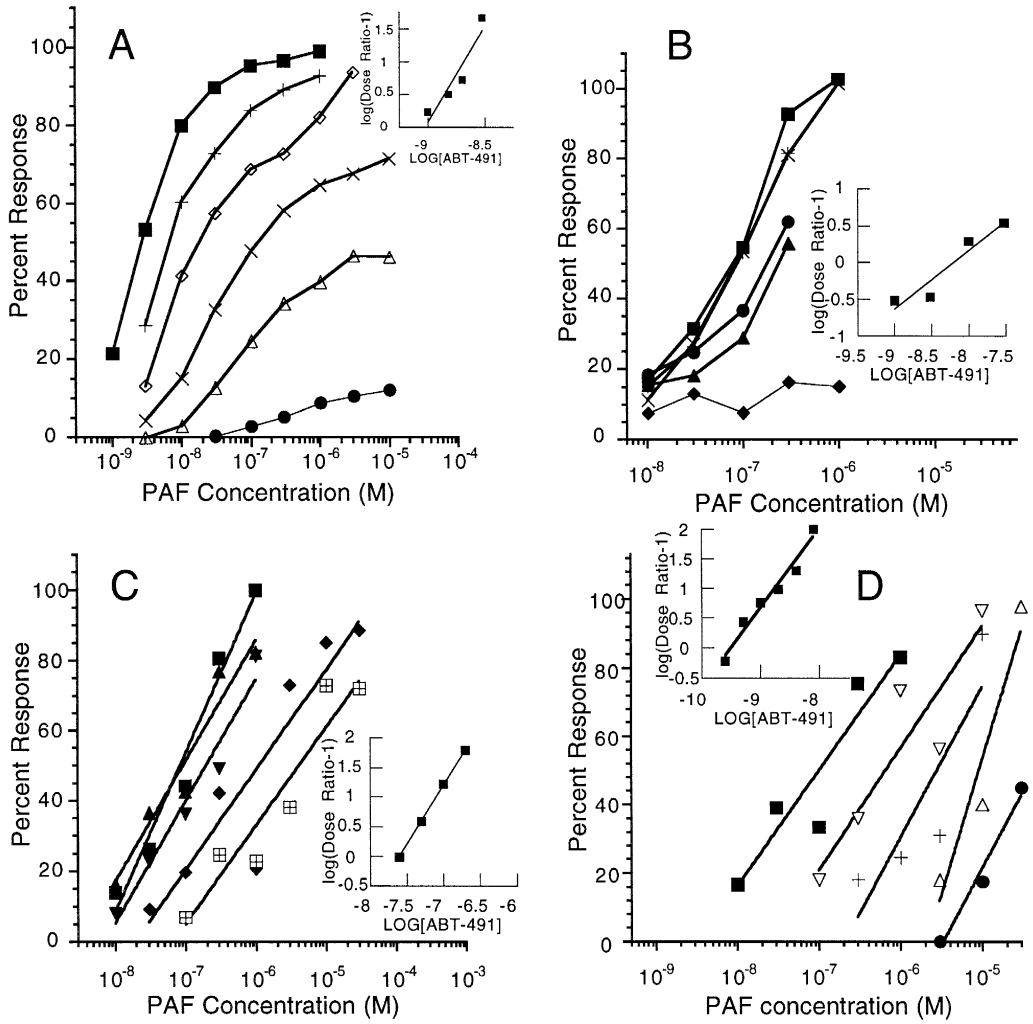

Fig. 3. Effect of ABT-491 on PAF-induced cellular responses. (A) Rabbit platelet degranulation. Rabbit platelets labeled with [^{14}C]serotonin were pre-incubated in the absence (closed square) or presence of 1 nM (+), 2 nM (diamond), 3 nM (×), 4 nM (triangle) or 6 nM (solid circle) ABT-491 and then incubated with PAF. The release of [^{14}C]serotonin was monitored and expressed as percent of maximal response to PAF in the absence of drug. Values are the mean of duplicate determinations of an experiment representative of two experiments. Schild analysis (inset) yielded an A_2 value of 0.9 nM (slope = 2.9). (B) β-Thromboglobulin release in human blood. Blood was preincubated for 5 min in the absence (closed square) or presence of 1 nM (+), 3 nM (×), 10 nM (solid circle), 30 nM (solid triangle) or 100 nM (closed diamond) ABT-491 and then exposed to PAF at the indicated concentration for an additional 5 min. β-Thromboglobulin released was measured and expressed as percent of maximal response to PAF in the absence of drug. Values are the mean of duplicate determinations of an experiment representative of two experiments. Schild analysis (inset) yielded an A_2 value of 7.3 nM (slope = 1.0). (C) Ca^{2+} mobilization in human neutrophils. Intracellular Ca^{2+} was monitored in the absence (closed square) or presence of 25 nM (closed triangle), 50 nM (inverted closed triangle), 100 nM (closed diamond) or 200 nM (hatched square) ABT-491 and expressed as percent of maximal response to PAF in the absence of drug. Values are the mean of duplicate determinations. Schild analysis (inset) yielded an A_2 value of 25 nM (slope = 2.0). (D) Degranulation of human neutrophils. Neutrophils preincubated in the absence (closed square) or presence of 0.25 nM (inverted triangle), 1 nM (+), 4 nM (triangle) or 8 nM (closed circle) ABT-491 were exposed to PAF at the indicated concentration. Elastase released into the media was measured and expressed as percent of maximal response to PAF in the absence of drug. Schild analysis (inset) yielded an A_2 value of 0.29 nM (slope = 1.2). In each panel variation of the duplicates was within 10% of the mean.

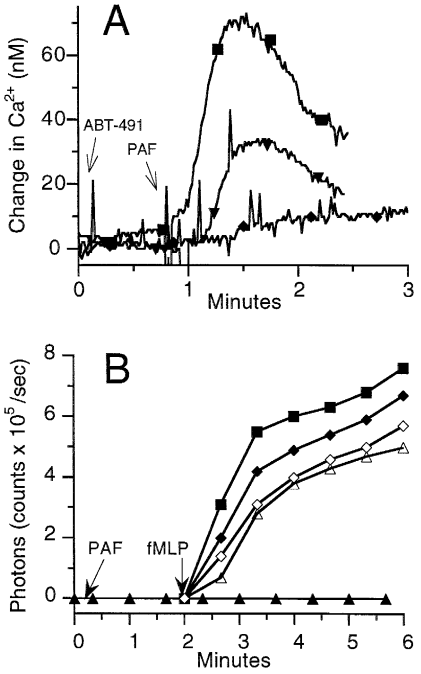

Fig. 4. Time-course of PAF-induced neutrophil responses and inhibition with ABT-491. (A) Ca^{2+} mobilization. Human neutrophils prelabeled with indo-1 were incubated 60 s (37°C) with buffer (closed square) or with 50 nM (inverted closed triangle) or 100 nM (closed diamond) ABT-491. The cells were then exposed to PAF (30 nM) and fluorescence (excitation 350 nm) was monitored. Change in intracellular Ca^{2+} levels was derived from changes in the ratio of fluorescence at 410 nm and 485 nm. (B) PAF priming of fMLP-induced release of superoxide. ABT-491 was preincubated with human neutrophils for 60 s (37°C) prior to addition of PAF (300 nM). fMLP (100 nM) was added 2 min after PAF. Reactive oxygen species formation was measured by chemiluminescence. Values are mean of duplicates. Ranges were within 10% of the mean. Symbols: closed triangle, PAF; open triangle, fMLP; closed square, PAF + fMLP; closed diamond, 125 nM ABT-491; diamond, 500 nM ABT-491.

Baggiolini, 1987; Baggiolini and Dewald, 1986; Gay, 1993). The Ca^{2+} response induced by PAF is rapid and transient, reaching a maximum in 30–45 s then returning to near-basal levels within 120 s (Fig. 4A). The response was inhibited in a concentration-dependent manner by preincubation with ABT-491 that resulted in a rightward shift in the PAF dose–response curve, suggestive of competitive antagonism (Fig. 3C). Schild analysis of the data yielded an A_2 value of 25 nM. The antagonist appears to be selective since ABT-491, at a concentration (1 μM) that completely inhibited the PAF-induced response, did not inhibit the Ca^{2+} response to leukotriene B_4 and C5a (not shown).

Neutrophil degranulation induced with PAF can be assessed by measuring elastase, an azurophil granule constituent released into the media in the presence of cytochalasin B. The release is dependent upon the PAF concentration (EC_{50} approximately 100 nM) and is complete within 5 min after exposure to the agonist. As was observed with the Ca^{2+} response, preincubation with increasing concentrations of ABT-491 resulted in a rightward shift in the dose–response relationship to PAF (Fig. 3D). Schild analysis yielded an A_2 value of 0.30 nM. Similar results were obtained in two additional experiments, yielding a mean A_2 value of 0.29 ± 0.02 nM (not shown).

In addition to its direct effects, PAF is capable of priming cells for enhanced responses to other agonists. As shown in Fig. 4B, prior exposure of human neutrophils to PAF resulted in enhanced reactive oxygen species production induced by subsequent exposure to the chemotactic peptide fMLP. PAF alone (300 nM) had virtually no effect ($< 0.1\%$ of maximal). ABT-491 (125 and 500 nM) inhibited the priming effect of PAF in a dose-related manner. Schild analysis of the data obtained from these and other experiments with additional drug concentrations yielded an A_2 value of 83 nM (slope = 1.3, not shown). These results provide further evidence of the ability of ABT-491 to functionally antagonize PAF-mediated responses in highly relevant cell populations.

3.4. In vivo models of PAF-induced inflammation

PAF induces localized cutaneous vascular permeability and edema characteristic of acute inflammation (Hwang et al., 1985b; Qu et al., 1990). ABT-491 possessed potent activity as an antagonist of these responses. In rat, an orally administrated dose of 1 mg/kg of ABT-491 resulted in 90% inhibition of the PAF-induced permeability response (Fig. 5A). The inhibition was dose related and regression analysis yielded an ED_{50} value of 0.094 mg/kg. In addition, this compound exhibited potent activity when administered intravenously ($ED_{50} = 0.003$ mg/kg). In guinea-pig, ABT-491 was several fold less potent. Nevertheless, when administered intravenously, a dose of 50 μg/kg produced near-maximal antagonism (75%) of the permeability response (Fig. 5B). As in the rat, the inhibition in guinea-pig was dose related for both intravenous and oral administration ($ED_{50} = 0.016$ and 0.29 mg/kg, respectively). Administration of ABT-491 to rats at a dose (1 mg/kg i.v.) sufficient to provide maximal (87%) inhibition of the PAF-induced response had no significant effect ($< 5\%$) on the permeability responses induced by serotonin and histamine (not shown).

The effect of ABT-491 on PAF-induced edema was assessed in mice. Pre-administration of ABT-491 to mice subjected to subcutaneous injections of PAF in the paw inhibited the resulting edema formation in a dose-related manner (Fig. 5C). The potency (ED_{50}) of ABT-491 for blocking edema was 0.069 mg/kg when administered intravenously and 0.38 mg/kg when administered orally. These values are comparable to those for inhibiting PAF-induced responses in the guinea pig and rat, and further illustrate the effectiveness of ABT-491 in blocking PAF-mediated inflammatory responses.

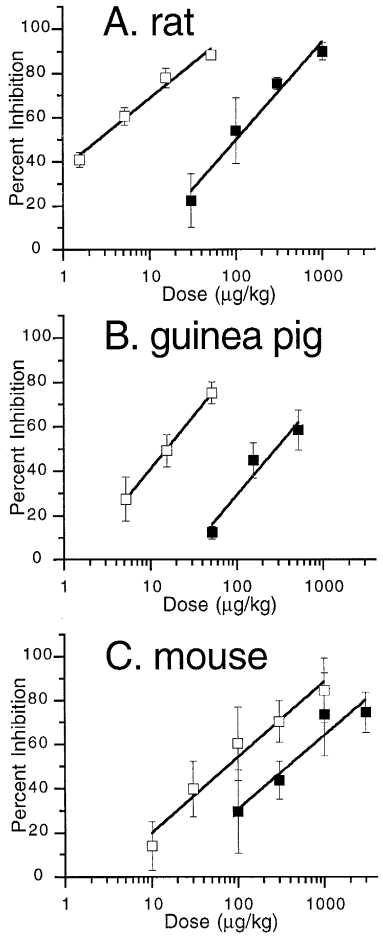

Fig. 5. Inhibition of PAF-induced cutaneous vascular permeability and edema by ABT-491. ABT-491 was administered intravenously (open square) or orally (solid square) to rats (A), guinea pigs (B), and mice (C) prior to PAF (0.05 μg) challenge. Changes in cutaneous vascular permeability (A and B) or edema (C) were determined and are expressed as mean percent inhibition of the control ± standard deviation (5–6 animals per group). Vehicle control response to subcutaneous PAF resulted in skin lesions 14.2 ± 0.5 mm (rat) and 15.1 ± 0.3 mm (guinea pig) in diameter. The vehicle control response to PAF in the paw resulted in a 93 ± 9 μl increase in volume.

3.5. Duration of action

The model for PAF-induced cutaneous permeability was used to assess the duration of activity of ABT-491. The compound was administered orally at a dose expected to produce 70–80% inhibition 1 h after administration. The level of inhibition of PAF-induced permeability was then assessed at various times after compound dosing. As shown in Fig. 6, ABT-491 (0.5 mg/kg p.o.) provided protection ($> 50\%$) against cutaneous PAF challenge in the rat for greater than 8 h.

3.6. PAF- and LPS-induced shock

Following systemic administration, PAF causes an acute drop in systemic arterial pressure (Blank et al., 1979). At higher doses of PAF, death results from acute cardiopulmonary failure and complement activation (Carlson et al., 1987; Sun and Hsueh, 1991). The hypotensive response induced in guinea pig by intraarterial administration of PAF was antagonized in a dose-dependent fashion by pretreatment with ABT-491 (Fig. 7A). The ED_{50} values derived from regression analysis for intraarterial and oral administration of ABT-491 were 0.005 and 0.026 mg/kg (Fig. 7A, insert).

LPS given intraarterially to rats also results in hypotension. The initial response is transient but is followed by a sustained decrease in systemic blood pressure (Fig. 7B). ABT-491, when administered intraarterially (0.1 mg/kg) or orally (1 mg/kg) 1 h prior to LPS, blocked the second-phase response. Inhibition was dose-dependent and analysis of the dose–response relationship (Fig. 7B, insert) yielded ED_{50} values for intraarterial and oral administration of 0.004 and 0.036 mg/kg respectively. In addition to being effective when given prior to LPS, ABT-491 was also capable of reversing hypotension when administered post LPS. As shown in Fig. 7B, administration of ABT-491 restored blood pressure as late as 1 h after LPS challenge.

Other hallmarks of septic shock, including gastrointestinal injury and lethality, are evident following the administration of LPS. Marked hyperemia and overt luminal bleeding are prominent in the small bowel of the rat within 30 min post LPS. Microscopically, congestion of mucosal microvessels, single cell necrosis and isolated loss of villi are evident (Wallace et al., 1987). In the current study the extent of damage was followed by scoring the gross appearance of the small intestine and by measuring intraluminal hemoglobin. ABT-491 (0.050 mg/kg), given orally 60 min prior to LPS challenge, inhibited intestinal bleeding by 79% ($P < 0.05$). A 10-fold higher dose resulted in complete protection and a normal appearing intestine.

Studies on the lethal effect of PAF were conducted with mice. Intravenous administration of PAF (3–1000 μg/kg) resulted in an increase in mortality that was dose-depen-

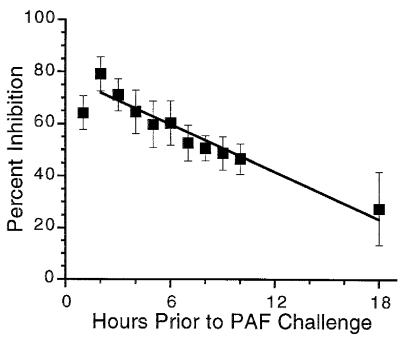

Fig. 6. Duration of inhibition by ABT-491 after oral dosing. ABT-491 (0.5 mg/kg, solid square) was administered orally at the indicated interval prior to PAF (0.05 μg) challenge. PAF-induced changes in vascular permeability were measured and expressed as mean percent inhibition of control response ± standard deviation (four animals per group).

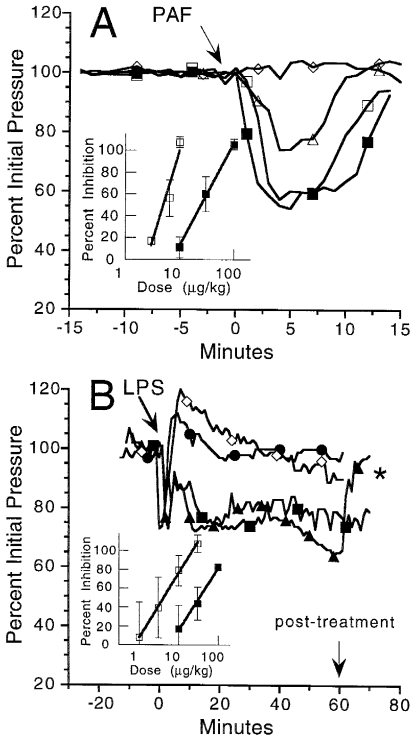

Fig. 7. Inhibition of PAF and LPS-induced systemic hypotension with ABT-491. (A) PAF. Vehicle (closed square) or 10 μg/kg (square), 30 μg/kg (triangle) or 100 μg/kg (diamond) ABT-491 was administered orally to conscious guinea pigs 1 h prior to PAF (0.6 μg/kg) challenge (time = 0). (B) LPS. Vehicle (closed square) or ABT-491 was administered intraarterially (0.1 mg/kg, diamond) or orally (1 mg/kg, closed circle) to conscious rats 15 min and 1 h prior to LPS challenge or ABT-491 (0.1 mg/kg) was administered intraarterially 60 min after receiving LPS challenge (solid triangle). Mean arterial pressure was monitored at 1 min intervals throughout both experiments. Data are expressed as mean percent initial pressure (4–7 animals per group). Standard deviations, omitted for clarity, were within 10% of the mean. The dose–response relationships obtained from addition experiments with ABT-491 administered intraarterially (open square) and orally (closed square) are illustrated in the inserts. $^{*}P < 0.05$ vs. LPS (64–70 min).

dent (LD_{100} approximately 30 μg/kg, Fig. 8). Pre-treatment with ABT-491 (1, 10 and 100 μg/kg, p.o.) resulted in a rightward shift in the dose–response curve, indicative

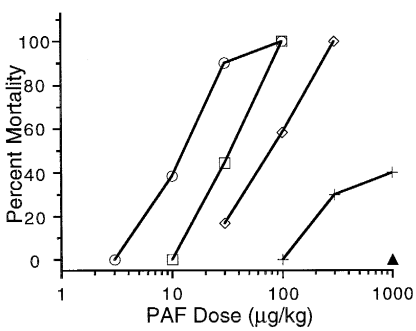

Fig. 8. Competitive antagonism of PAF-induced lethality by ABT-491 in mouse. Vehicle (circle) or 1 μg/kg (square), 10 μg/kg (diamond), 100 μg/kg (+) or 1000 μg/kg (triangle) ABT-491 was administered orally to mice (10 animals per group) 30 min prior to PAF challenge. Survival was monitored for 18–24 h.

of competitive antagonism. Administration of 1 mg/kg ABT-491 provided total protection against lethality induced by a PAF challenge as high as 1 mg/kg, greater than 30-fold the PAF LD_{100}. These observations are consistent with potent antagonism of the cardio-pulmonary responses to PAF and provide additional evidence that in vivo administration of ABT-491 results in antagonism of systemic PAF responses.

The effect of ABT-491 on long-term survival was also evaluated in the rat endotoxemia model. LPS (8.5 mg/kg, i.v.) resulted in 43% mortality within 24 h. Pretreatment with ABT-491 (1 mg/kg, p.o.) produced a marked reduction in mortality (15% vs. 43%, $P < 0.05$).

4. Discussion

The intrinsic potency of ABT-491 is in the nanomolar and sub-nanomolar range (K_i of 3.8 and 0.3 nM respectively) for rabbit and human platelets. Thus the potency of ABT-491 is comparable or superior to previously described PAF receptor antagonists and rivals that of PAF (Summers and Albert, 1995). The potent inhibition of PAF binding exhibited by ABT-491 is selective and appears to be competitive when the antagonist is co-incubated with PAF. However, longer exposure of the platelet membranes with ABT-491 in the absence of PAF results in non-competitive inhibition kinetics. This apparent lack of reversibility of inhibition under these conditions suggests that ABT-491 has a slow disassociation rate from the receptor in comparison to PAF. It is interesting to note that SR-27417, another potent PAF receptor antagonist, also has an irreversible aspect to its binding kinetics (Herbert et al., 1993b).

At the cellular level, ABT-491 is a potent antagonist of responses linked to the PAF receptor in platelets and neutrophils, two cell populations that are highly relevant to PAF pathophysiology. Platelet activation may be a trigger of coagulation activation and thrombi formation associated with disseminated intravascular coagulation, a clinical syndrome linked to septic shock (Rackow and Astiz, 1993). PAF-mediated activation and degranulation of neutrophils may play a central role in tissue damage associated with sepsis, pancreatitis, ischemia/reperfusion injury and other diseases with inflammatory components (Braquet et al., 1987b, 1989).

The platelet response assayed in our studies, degranulation, was inhibited by ABT-491 in a selective manner. With isolated platelets, the close correlation between potency for functional antagonism ($A_2 = 1.1$ nM) and binding potency ($K_i = 1.8$ nM) is consistent with interaction of ABT-491 with the PAF receptor being a mechanism of action of the drug. ABT-491 was also effective in blocking platelet activation in blood ($A_2 = 5.2$ nM), indicating that the presence of high concentrations of protein and other serum factors only slightly (5-fold) alters the ability of ABT-491 to interact with the PAF receptor.

The three neutrophil responses (degranulation, Ca^{2+} mobilization and priming) examined in this study were inhibited by ABT-491 ($A_2 = 0.3$, 25 and 83 nM, respectively), which serves to illustrate the effectiveness of the antagonist with PAF-mediated responses in pro-inflammatory cells. Interestingly, the antagonist was somewhat less potent at blocking the priming effect than the direct effects of PAF. A similar shift in potency for blocking the priming and direct effects of PAF in neutrophils has been reported previously with a structurally related receptor antagonist, ABT-299 (Albert et al., 1996a). The relatively wide range of potency values for inhibiting responses within a single cell population raises the possibility of receptor subtypes for PAF. However, only highly homologous members of a single class of PAF receptors have been cloned (Nakamura et al., 1991; Honda et al., 1991). Thus to date there is little evidence to support the existence of multiple genes expressing receptor subtypes, although differences in the 5′ untranslated region of heart and leukocyte cDNA have been reported (Mutoh et al., 1993; Shimizu et al., 1992). Another possibility to account for the range in potencies may be the existence of a distinct signal transduction pathway for the priming effect of PAF. Such a pathway has been previously suggested based upon the observation that, unlike the direct effects of PAF, primed stimulation was not desensitized by sequential exposure of neutrophils to PAF (Gay, 1993). The relative importance to PAF pathophysiology of the priming versus direct effects remains unclear since no receptor antagonist reported to date exhibits selectivity for the priming response.

The in vivo efficacy of ABT-491 was evaluated in PAF-challenge models of localized inflammatory responses (cutaneous vascular permeability and edema), a systemic response (hypotension) and a generalized response (acute lethality) across three species. When administered orally, ABT-491 was most potent for inhibiting PAF-induced permeability and hypotension in the rat and guinea-pig (ED_{50} values of 0.094 and 0.026 mg/kg, respectively). The antagonist was 4- to 10-fold less potent, although still very effective, in inhibiting PAF-induced permeability in the guinea-pig and edema in the mouse (ED_{50} values of 0.29 and 0.38 mg/kg, respectively). Approximately the same rank order in potency (ED_{50} values of 0.003–0.069 mg/kg) was observed when the antagonist was administered parenterally. Thus when given in sub-mg/kg doses either orally or parenterally, ABT-491 serves as a potent receptor antagonist of localized and systemic PAF responses.

The potent in vivo activity of ABT-491 is accompanied by long duration of action. With the PAF cutaneous vascular permeability model in rat, an oral dose of 0.5 mg/kg provided $>50\%$ protection against PAF challenge for >8 h. This duration of activity compares favorably to other PAF receptor antagonists administered at equal or higher doses, with the exceptions of SR-27,417 and ABT-299, which exhibit duration comparable to ABT-491 in this model (Albert et al., 1996a). The duration of bioactivity exhibited by ABT-491 is somewhat longer than might be expected based on the relatively rapid rate of elimination from circulation of ABT-491 obtained from pharmacokinetic studies with ABT-491 in the rat ($t_{1/2} = 0.8$ h, unpublished observation). Other PAF receptor antagonists have also been reported to exhibit surprisingly long duration of action in vivo. For example administration of SR-27417 (1 mg/kg) produces significant inhibition of PAF-induced hypotension for at least 48 h (Bernat et al., 1992). Irreversible inhibition of PAF binding to its receptor would lead to extended in vivo bioactivity. Indeed, time-dependent and non-competitive inhibition has been reported for SR-27417 and has been offered as an explanation of the antagonist's long duration of activity (Herbert et al., 1993a). A similar situation may exist for ABT-491, which exhibits competitive antagonism at the receptor and cellular level but an apparent slow off rate from the receptor.

ABT-491 was also effective in blocking the acute, shock-like responses induced by LPS in rat that are thought to be, at least in part, PAF-mediated (Sanchez Crespo and Fernandez-Gallardo, 1991; Braquet et al., 1987a; Summers and Albert, 1995). The potency of ABT-491 for inhibiting LPS-induced hypotension (i.a. and p.o. ED_{50} values of 0.004 and 0.036 mg/kg respectively) correspond with the potency of the antagonist for blocking PAF-induced hypotension (0.005 and 0.026 mg/kg) in the same species. The effectiveness in blocking the LPS hypotension is in agreement with the activity of a wide range of structurally diverse PAF receptor antagonists having varying degrees of efficacy for blocking hypotension (Doebber et al., 1985; Chang et al., 1987; Qi and Jones, 1990; Torley et al., 1992; Albert et al., 1996b). ABT-491 was also capable of reversing established LPS-induced hypotension. Administration of a single bolus of PAF, which is known to have a short half-life in vivo (Blank et al., 1981), did not result in a prolonged hypotensive response. Thus the effectiveness of the PAF receptor antagonist on reversing the LPS-induced response suggests an ongoing synthesis of PAF for at least 1 h following exposure to LPS. These results, along with those previously reported for other antagonists (Torley et al., 1992; Terashita et al., 1992; Albert et al., 1996b), help to confirm the role of PAF in the hypotension associated with endotoxic shock in the rat.

PAF has also been previously implicated as a mediator of endotoxin-induced gastrointestinal damage and lethality in the rat (Albert et al., 1996b; Torley et al., 1992; Wallace et al., 1987; Terashita et al., 1985). In the present study 0.050 mg/kg ABT-491 given orally 60 min prior to LPS challenge was effective in blocking by 79% LPS-induced damage. Thus the oral potency for blocking gastrointestinal damage correlates well with activity for the hypotensive response and places ABT-491 as one of the most potent PAF receptor antagonists evaluated in this model. ABT-491 also was effective in increasing the long-term survival rate of rats administered LPS; however, the oral

dose (1 mg/kg) necessary to achieve maximum effect was higher than that required for protection against gastrointestinal damage. Furthermore, the effect was somewhat less than that achieved in the PAF-induced lethality model (85% vs. 100% survival). The higher dose and less efficacy in the LPS lethality model may be a reflection of the longer coverage time required for protecting against LPS-induced mortality (most deaths occur 6–8 h post LPS) compared to the acute hypotensive and organ injury responses (30–60 min). They may also reflect the role of other mediators in the response to LPS.

In conclusion ABT-491 is a novel antagonist that is active at nanomolar and sub-nanomolar concentrations in selectively inhibiting PAF binding and PAF-mediated cellular responses. The receptor antagonist is orally active at sub-mg/kg doses and exhibits a bioduration of >8 h. Its oral activity coupled with its aqueous solubility provide a variety of dosing options and make ABT-491 an attractive candidate for clinical studies. Thus ABT-491, along with several other structurally distinct antagonists (e.g., BBT-882 and SR-27,417), may prove useful in defining the role of PAF in human diseases such as asthma, allergic rhinitis and sepsis.

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