Laboratory Studies

An Orally Active Inhibitor of Renin

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SUMMARY A potent renin inhibitor, U-71038 (Boc-Pro-Phe-N-MeHis-Leu(CHOHCH₂)Val-Ile-Amp), was tested for oral effectiveness. Enzyme kinetic studies indicated that U-71038 was a competitive inhibitor of hog renin with an inhibitor constant (Kᵢ) value of 12 nM. Intravenous as well as oral administration of U-71038 to anesthetized, ganglion-blocked rats infused with hog renin elicited dose-related hypotensive responses. Intravenous administration of U-71038 to conscious, sodium-depleted monkeys caused dose-related decreases of blood pressure and plasma renin activity without affecting heart rate. Similarly, the oral administration of U-71038 at 50 mg/kg to conscious, sodium-depleted monkeys elicited a pronounced hypotension and decrease in plasma renin activity that persisted for 5 hours. The hypotensive responses elicited by intravenous and oral administration of U-71038 to hog renin-infused rats and sodium-depleted monkeys were shown to be due entirely to inhibition of the renin-angiotensin system. A comparison of the results obtained after the intravenous administration of U-71038 with the results obtained after the oral administration of U-71038 implied that at least 10% of the orally administered U-71038 must have been absorbed to cause the observed effects in hog renin-infused rats and sodium-depleted monkeys. The studies demonstrated that an inhibitor of renin with a long duration of action and with oral effectiveness is a feasible entity.

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KEY WORDS • renin inhibition • blood pressure • plasma renin activity • hog renin-infused rat • cynomolgus monkey

T HE pioneering work of Haber, Burton, and co-workers1-8 during the period from 1973 to 1980 established, with the synthesis of renin inhibitory peptide (RIP; Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys), that the inhibition of renin in vivo was feasible. Subsequently, Szelke et al.7,8 as well as Boger et al.9,10 succeeded in developing inhibitors of renin that are more potent than RIP. In spite of an extensive search, however, the goal of discovering an orally active renin inhibitor has continued to be elusive. "Potent inhibitors of renin have been developed, but none yet reported has the duration of action or oral effectiveness characteristic of medicinally useful agents."11 Typifying this dilemma is a recent report12 on CGP 29287 (Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys[Boc]-OMe), a specific and long-acting renin inhibitor in conscious, volume-depleted marmosets. The hypotension and the suppression of plasma renin activity evoked by the oral administration of 100 mg/kg of CGP 29287 was approximately equivalent to that observed after the intravenous administration of CGP 29287 at 0.1 mg/kg. One interpretation of these results is that only 0.1% of the orally administered dose of CGP 29287 had been absorbed in order to elicit the observed effects on plasma renin activity and blood pressure.

Several biochemical properties differentiated U-71038 (Boc-Pro-Phe-N-MeHis-Leu[CHOHCH₂]-Val-Ile-Amp) from other analogues evaluated for renin inhibitory activity in this laboratory. These properties and how they were evaluated are described in this article. However, the primary intent of the present study was threefold and was essentially sequential in nature. First, experiments were performed to determine an inhibitor constant (Kᵢ) value and the type of inhibition exhibited by U-71038 with hog renin. Second, based on the results of the kinetics study, experiments were performed in hog renin-infused rats to ascertain the conditions required for U-71038 to exert optimal effects through intravenous and oral administration. Finally, experiments were performed in conscious, sodium-depleted cynomolgus monkeys in an attempt to validate the observations made in rodents.

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Materials and Methods

The U-71038 was synthesized in a linear sequence: Boc-Ile was first coupled to 2-aminomethylpyridine (Amp) with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The peptide chain was then extended in a stepwise manner by removing the Boc group with trifluoroacetic acid in dichloromethane and then coupling to the next Boc amino acid with diethylphosphoryl cyanide. In this way, Boc-Leu[CHOHCHJVal(4S-tert-butyldimethylsilyloxy-5S-tert-butyloxy carboxy lamino-2S-isopropyl-7-methyl octanoic acid), Boc-N-MeHis(Ts), Boc-Phe, and then Boc-Pro were successively coupled to the peptide chain. After each coupling, the product was purified by chromatography on silica gel with ethyl acetate/hexane or methanol/ethyl acetate as eluant. The tert-butyldimethylsilyl protecting group was removed during the trifluoroacetic acid treatment before the coupling of Boc-Phe. The final step of the sequence entailed the removal of the tosyl group from the imidazoyl ring by 1-hydroxy-benzotriazole in methanol. The U-71038 was purified by chromatography on silica gel with methanol/ammonia/dichloromethane as eluant. High-performance liquid chromatography on a Brownlee RP-18 analytical column (10 µm, 25 cm × 4.6 mm inside diameter; Anspec, Ann Arbor, MI, USA) using an isocratic mixture of 90% methanol and 10% phosphate buffer (pH 3) showed a single peak. Fast atom bombardment mass spectroscopy gave [M + H]+ at m/z of 930.5789 (calculated 930.5816) for U-71038, [M + H]+ at m/z of 586 for N-MeHis-Leu[CHOHCHJVal-Ile-Amp, and [M + H]+ at m/z of 435 for Leu[CHOHCHJVal-Ile-Amp. The remnant of the N-MeHis residue appeared at 124 as the deacylated species. Thus, the fast atom bombardment mass spectrum of U-71038 indicated an N-MeHis residue at the correct position in the peptide chain.

The plasma renin inhibitory activity of U-71038 was estimated in human, cynomolgus monkey, and rat plasmas collected with 10% ethylenediaminetetraacetic acid. The angiotensin I converting enzyme inhibitory activity of U-71038 was determined with a slightly modified method of Aoyagi et al. Appropriate concentrations of U-71038 were dissolved in the buffer, and after incubation for 25 minutes at 37°C with the enzyme and substrate, perchloric acid was added, and the absorbance of the acid-soluble fractions was measured at 280 nm. The percentage of inhibition was estimated from the net absorbance of inhibited assays in relation to uninhibited control assays. A plot of percentage of inhibition versus log inhibitory concentration was constructed, with the IC50 defined as the inhibitory concentration causing 50% inhibition. The IC50 of U-71038 was determined with a slightly modified method of Aoyagi et al. Bovine cathepsin D and porcine hemoglobin were obtained from Sigma Chemical. The U-71038 was dissolved in 0.2 M acetate buffer (pH 3.2) along with the hemoglobin substrate. Incubations for 30 minutes at 37°C were terminated in ice, and 1.7 M perchloric acid was added to all of the mixtures. Extinction at 280 nm was measured, and the IC50 was determined as described for the pepsin inhibition ex-
periments. Inhibition of angiotensin I converting enzyme activity was estimated as described by Cushman and Cheung. Briefly, the enzyme was extracted from rabbit lung. The substrate was hippuryl-L-histidyl-L-leucine (Aldrich Chemical, Milwaukee, WI, USA), and the buffer was K2HPO4 (pH 8.3). The U-71038 was dissolved in 50% dimethylformamide. Incubations were for 60 minutes at 37°C and were terminated with an addition of HCl. Absorbance of the ethyl acetate–extracted hippuric acid was measured at 228 nm. The IC50 was determined as described for the pepsin inhibition experiments.

The U-71038 was assayed for renin inhibitory activity in vitro by measuring the generation of angiotensin I from hog angiotensinogen by hog renin. Hog angiotensinogen (2.6 μg of renin-releasable angiotensin I/mg) was obtained from Sigma Chemical. The hog renin (9.1 Goldblatt units/mg) was a gift from Dr. Erwin Haas and had been prepared from hog kidneys using Steps 1 through 5 of the Haas, Lamfrom, and Goldblatt procedure. Each incubation tube contained 380 μl of 0.05 M Na2HPO4, 0.10 M NaCl at pH 7.5, 10 μl of 0.3 M PMSF, and 20 μl of hog renin (0.022 GU/ml). The mixtures, with or without U-71038, were preincubated for 90 minutes at 37°C before the reactions were started with the addition of 100 μl of the appropriate dilution of angiotensinogen. Aliquots were then removed at 0, 10, 20, and 30 minutes. Quantitation of the angiotensin I generated was based on standard radioimmunoassay techniques using the Gamma Coat [125I] Plasma Renin Activity Radioimmunoassay Kit. Velocities were obtained from the slope of a plot of angiotensin I versus time for each reaction mixture. A Michaelis constant (Km) value for hog angiotensinogen and a Vmax value for the hog renin–U-71038 reaction were determined using classic methods.

Male Sprague-Dawley rats weighing approximately 200 g (Upjohn, Kalamazoo, MI, USA) were anesthetized with dial-urethane, 100 mg/kg i.p. The trachea of each animal was cannulated, bilateral vagotomy carried out, and one carotid artery cannulated for the recording of blood pressure with a Statham P23D transducer (Oxnard, CA, USA) and a Grass polygraph (Quincy, MA, USA). The jugular veins were cannulated; one cannula was used for the infusion of U-71038, and the other was connected to a Harvard infusion pump (Millis, MA, USA) to allow the infusion of hog renin. An infant feeding tube (Dovel, Cranston, RI, USA) was passed into the animals’ stomach through the mouth for oral administration of U-71038. The latter experiments were performed in animals that had fasted for 24 hours before being anesthetized. Ganglionic blockade was accomplished with mecamylamine (Merck Sharp & Dohme, West Point, PA, USA), 1.25 mg/kg i.v. Shortly thereafter, an infusion of partially purified hog renin (ICN Pharmaceuticals, Cleveland, OH, USA) at 0.15 GU/kg/min was used to maintain the blood pressure at the level present before ganglionic blockade. In some experiments, an angiotensin I (Peninsula Laboratories, Belmont, CA, USA) or an angiotensin II (CIBA-Geigy, Summit, NJ, USA) infusion at 0.30 μg/kg/min replaced the hog renin infusion. This type of preparation has been used previously by Blaine et al.21 to study the intravenous dose-response relation of statine-containing renin inhibitory peptide on blood pressure. Validation of this preparation for oral absorption studies was obtained when it was observed that the oral administration of captopril, 5 mg/kg, completely neutralized the pressor effect of the hog renin infusion within 60 minutes in each of five anesthetized, ganglion-blocked rats. Preliminary experiments had shown that U-71038 was more soluble in 0.1 M citric acid (30 mg/ml) than in any other physiologically acceptable vehicle evaluated. The U-71038 was dissolved in 0.1 M citric acid and infused intravenously at 0.05 ml/min for 10 minutes or administered into the stomach as a 5 ml/kg bolus.

Male cynomolgus monkeys (Hazleton, Alice, TX, USA) weighing 4 to 6 kg were anesthetized with ketamine, and polyvinyl catheters were implanted under sterile conditions in the abdominal aorta and the thoracic vena cava through an external iliac artery and vein, respectively. At least 1 week was allowed for recovery from the operation before initiation of an experiment. The monkeys were fed a quantity of Standard SKF Monkey Diet deleting sodium (ICN Nutritional Biochemicals, Cleveland, OH, USA) adequate to furnish potassium at 1 mEq/kg/day. Intravenously administered 0.9% saline adequate to provide sodium at 1 mEq/kg/day maintained the animals in a sodium-replete state. Sodium depletion was accomplished by omitting the daily intravenous administration of saline and substituting the intravenous administration of furosemide (1 mg/kg body weight per day) for 7 days before a study. Drinking water was allowed ad libitum. Experiments were performed while the conscious monkeys were seated in primate restraining chairs (Plas-Labs, Lansing, MI, USA). Blood pressure was recorded continuously from the arterial catheter with a Statham pressure transducer and a Grass polygraph. Heart rate was recorded continuously from a Grass tachograph triggered by the arterial pressure pulse. Blood samples (1.5 ml) were obtained before and at intervals after a 17-minute intravenous infusion of vehicle (5 ml of 0.1 M citric acid diluted with 5 ml of 5% dextrose) or of U-71038 (0.05, 0.5, or 5.0 mg/kg) in the vehicle. Similarly, blood samples were obtained before and at intervals after 0.1 M citric acid or U-71038 in 0.1 M citric acid was administered intragastrically through the nasal route with an infant feeding tube (8F, 15 in. long) as a 5 ml/kg bolus. Plasma renin activity was determined with standard radioimmunoassay techniques at pH 7.4 using the Gamma Coat [125I] Plasma Renin Activity Radioimmunoassay Kit. The magnitude of the renin-dependent blood pressure component of each animal was estimated at least 4 hours before each experiment by the blood pressure response to a bolus injection of saralasin (Peninsula Laboratories), 1 mg/kg i.v. The animals involved in this study were cared for and used in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services,
1985, NIH Publication No. 85-23) and the Animal Welfare Act and subsequent amendments.

Statistical analyses of the data were carried out using repeated-measures analysis of variance with F test, covariance analysis, or Student’s paired or unpaired t test as appropriate. Probability values less than or equal to 0.05 were considered to be significant.

Results

In vitro studies with U-71038 indicated that it exhibited an inhibitory potency (IC_{50}) against human plasma renin of 2.6 \times 10^{-10} \text{M} at pH 6 and of 3.9 \times 10^{-10} \text{M} at pH 7.4. It exhibited an inhibitory potency (IC_{50}) against rat plasma renin at pH 6 of 6.7 \times 10^{-7} \text{M}, against cynomolgus monkey plasma renin at pH 6 of 1.7 \times 10^{-9} \text{M}, and against cynomolgus monkey plasma renin at pH 7.4 of 1.6 \times 10^{-9} \text{M}.

The U-71038 was found to be completely resistant to the proteolytic actions of carboxypeptidase Y, chymotrypsin, and elastase over a 60-minute incubation period. In contrast, under identical experimental conditions, the breakdown of RIP by carboxypeptidase Y was observed to be approximately 85% at 30 minutes and 100% at 60 minutes; the breakdown of RIP by chymotrypsin was estimated to be 70% at 30 minutes and 85% at 60 minutes; and the breakdown of RIP by elastase was found to be complete (100%) by 30 minutes. Although the data are not presented in this study, U-71038 was also found to be completely resistant to breakdown by the enzymes in a rat liver homogenate.

The renin inhibitory specificity of U-71038 was demonstrated when it was shown to be much less effective as an inhibitor of pepsin (IC_{50} = 7 \times 10^{-6} \text{M}), cathepsin D (IC_{50} = 6 \times 10^{-6} \text{M}), and angiotensin I converting enzyme (IC_{50} = 1 \times 10^{-3} \text{M}).

A Lineweaver-Burk plot illustrating classic competitive inhibition of the reaction between hog renin and hog angiotensinogen by U-71038 is shown in Figure 1. The \(K_m\) value for the hydrolysis of hog angiotensinogen by hog renin was found to be 3.7 \text{ mM} at pH 7.5. This value is not appreciably different from the value of 1.65 \text{ mM} at pH 7.5 reported previously by Skeggs et al.\(^{22}\) The inhibitor constant (\(K_i\)) value was found to be 12 nM.

Figure 2 illustrates the results of experiments with U-71038 in anesthetized, ganglion-blocked, hog renin-infused rats. The "prerenin" delta blood pressure indicates that the magnitude of the hog renin-dependent blood pressure component was 61 ± 1.6 mm Hg in these animals. The 0.1 M citric acid vehicle evoked only modest effects on blood pressure when administered intravenously. The intravenous administration of U-71038 at 0.15, 0.5, 1.5, and 5.0 mg/kg in the 0.1 M citric acid vehicle elicited dose-dependent hypotensive responses. Both the magnitude of the hypotension and the duration of the response appeared to be a function of dose. It also appeared that the magnitude of the hypotension elicited by the 1.5 mg/kg dose was a maximal effect. The latter possibility could not be verified unequivocally, since the intravenous administration of U-71038 at 15 mg/kg resulted in a hypotension that was smaller in magnitude but of a greater duration than that elicited by U-71038 at 5 mg/kg. This peculiar effect appeared to be at least partially due to the physical characteristics of the intravenous solution, since the intravenous administration of U-71038 at 15 mg/kg to anesthetized, ganglion-blocked rats receiving infusions of an-
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Gliotensin I or angiotensin II caused an initial transient pressor response that, in all probability, counteracted the hypotension resulting from the inhibitory effect of U-71038 on the infused hog renin.

The oral administration of U-71038 to anesthetized, ganglion-blocked, hog renin–infused rats elicited the dose-dependent hypotensive effects illustrated in Figure 3. The magnitude of the hypotension as well as the duration of the response appeared to be a function of dose. The 150 mg/kg dose of U-71038 was adequate to transiently obliterate the renin-dependent blood pressure component in these experiments. The oral administration of U-71038 in a 0.1 M citric acid vehicle to anesthetized, ganglion-blocked rats infused with angiotensin I or angiotensin II caused consistent but apparently non-dose-related depressor responses of less than 10 mm Hg that peaked at 30 minutes and had essentially dissipated by 60 minutes. The time trend of these responses was similar to that exhibited by the 0.1 M citric acid vehicle in Figure 3 and was out of phase with the nadir of the hypotensive responses in the rats infused with hog renin.

The administration of saralasin (1 mg/kg i.v.) to conscious, sodium-replete monkeys did not significantly alter mean arterial blood pressure or heart rate. Similarly, the intravenous administration of the citric acid vehicle or of U-71038 at 5 mg/kg in the vehicle had no significant effect on blood pressure. A transient tachycardia, which occurred 10 to 15 minutes after termination of the intravenous infusion of U-71038, was paralleled by a similar transient tachycardia in the animals infused with the vehicle. Although plasma renin activity determinations were carried out on blood samples obtained from sodium-replete animals, the majority of the values were below the sensitivity of the assay when carried out at pH 7.4 and were not considered to be reliable.

The control values for blood pressure and heart rate of sodium-depleted monkeys were not significantly different from the control values for these same parameters in sodium-replete monkeys. The plasma renin activity values of the sodium-depleted monkeys were, however, approximately 20-fold greater than those of the sodium-replete monkeys. Consistent with the latter observation was the finding that the administration of saralasin (1 mg/kg i.v.) to conscious, sodium-depleted monkeys evoked a significant depressor response without a significant alteration of heart rate (Figure 4).

The magnitude of the renin-dependent blood pressure component was estimated to be at least 19 mm Hg in this series of experiments. Figure 4 illustrates the effects of the intravenous administration of vehicle and of U-71038 at 0.5 and 5.0 mg/kg in vehicle on the blood pressure, heart rate, and plasma renin activity of conscious, sodium-depleted monkeys. At 0.5 mg/kg, U-71038 elicited a pronounced but transient hypotension and reduction of plasma renin activity. In contrast, at 5 mg/kg, U-71038 caused a pronounced hypotension and a total inhibition of plasma renin activity that persisted throughout the 195-minute experimental period. For reasons of clarity, the effects of U-71038 at 0.05 mg/kg i.v. were not included. At 0.05 mg/kg i.v., U-71038 caused a small (8 mm Hg at 30 minutes), transient hypotension. Blood pressure had returned essentially to the pretreatment level at 75 minutes. Plasma renin activity was reduced by 90% at 30 minutes and by 50% at 75 minutes and had recovered to 85% of the pretreatment value at 195 minutes. The heart rate effects were similar to those shown for the vehicle and for the 0.5 and 5.0 mg/kg doses of U-71038. The effect of U-71038 was clearly dose-related on plasma renin activity but not on heart rate. Statistical analysis indi-
cated that the hypotensive effects of U-71038 in conscious, sodium-depleted monkeys was significantly dose-related.

Figure 5 illustrates that saralasin (1 mg/kg i.v.) estimated the magnitude of the renin-dependent blood pressure component to be at least 20 mm Hg in this group of conscious, sodium-depleted monkeys. Oral administration of vehicle was essentially without effect on the blood pressure, heart rate, or plasma renin activity of these animals. In contrast, oral administration of U-71038 at 50 mg/kg in vehicle elicited a significant hypotension that persisted for 5 hours. During this 5-hour period, the heart rate was not significantly different from that of the vehicle-treated animals. The hypotension was accompanied by a significant reduction in plasma renin activity that persisted for 5 hours. Oral administration of vehicle or of U-71038 at 50 mg/kg in vehicle did not significantly alter the blood pressure or heart rate of conscious, sodium-replete monkeys (Figure 6). Administration of saralasin (1 mg/kg i.v.) to this group of conscious, sodium-replete monkeys evoked an average 7 mm Hg pressor response.

Discussion

We chose U-71038 for in vivo evaluation as an inhibitor of renin for several reasons: 1) it exhibited a high in vitro inhibitory potency against human plasma renin as well as against cynomolgus monkey plasma renin; 2) it proved to be resistant to the actions of several proteolytic enzymes that were shown to have profound effects on RIP; and 3) it was shown to be more than four orders of magnitude less effective as an inhibitor of the aspartyl proteases pepsin and cathepsin D and of the metalloprotease angiotensin I to angiotensin II converting enzyme as an inhibitor of renin.

The U-71038 exhibited a reasonable affinity for hog renin in vitro and was shown to be a competitive in-
the hypotensive effects observed in sodium-depleted monkeys after intravenous or oral administration of U-71038 were due entirely to inhibition of the renin-angiotensin system.

A comparison of the hypotension elicited by the oral administration of U-71038 at 50 mg/kg and the hypotension elicited by the intravenous administration of U-71038 at 5 mg/kg to the hog renin-infused rats indicated that the responses were virtually identical. Similarly, the oral administration of U-71038 at 50 mg/kg to conscious, sodium-depleted monkeys evoked a hypotensive response that was equivalent to that caused by the intravenous administration of U-71038 at 5 mg/kg in these animals. One interpretation of these results is that at least 10% of the orally administered dose of U-71038 at 50 mg/kg had to have been absorbed in order to elicit the observed effect. Alternatively, a much larger percentage of the orally administered dose of U-71038 could have been absorbed with only a fraction of the intact drug surviving degradation as well as other removal processes and actually reaching the target enzyme, renin. Although the present investigation does not allow discrimination between these two possibilities, the latter interpretation is less attractive than the former for the simple reason that intravenous administration of U-71038 to sodium-depleted monkeys elicited hypotensive responses with long duration of action. It was previously noted that U-71038 is resistant to the proteolytic actions of carboxypeptidase Y, chymotrypsin, elastase, and the enzymes in a rat liver homogenate. Resistance to proteolytic degradation may thus contribute to the long duration of action of U-71038. Intravenous administration of the renin inhibitor Iva-Phe-Nle-Sta-Ala-Sta-OH (SR 42128) to sodium-depleted cynomolgus monkeys also elicited hypotensive responses with long duration of action. It was previously suggested that the observed anti-diuretic effect must have been due to absorption of the intact peptide, since any enzymatic cleavage of peptide bonds or the disulfide bridge in the vasopressin analogue invariably leads to biological inactivation. Exactly why U-71038 appears to be reasonably well absorbed following oral administration is not understood at the present time. In any event, the present investigation has demonstrated that an inhibitor of renin with a long duration of action and with oral effectiveness is a feasible entity.

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