Effects of the Renin Inhibitor A-64662 in Monkeys and Rats with Varying Baseline Plasma Renin Activity

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SUMMARY The efficacy of the potent, primate selective renin inhibitor A-64662 was studied in monkeys and rats with varying baseline plasma renin activity (PRA) to elucidate the relationship between PRA and the hypotensive response induced by this compound. The effect of a single bolus of vehicle or A-64662 at 0.001, 0.01, 0.1, 1.0, and 10.0 mg/kg i.v. was compared in 30 normal and 30 salt-depleted, anesthetized monkeys (n = 5/dose). Baseline mean arterial pressure (MAP) was similar among all groups, but baseline PRA was elevated in salt-depleted monkeys. A-64662 induced a comparable dose-related fall in MAP, affecting the magnitude and duration of action, accompanied by inhibition of PRA, the duration of which was dose-related in both the normal and salt-depleted groups. However, the minimum effective doses required to reduce MAP by approximately 10% were 0.01 mg/kg for the salt-depleted monkeys and 0.1 mg/kg for the normal monkeys. In a second study, three consecutive boluses of vehicle or A-64662 at 0.1, 1.0, and 10.0 mg/kg were administered to anephric monkeys, human renin–infused anephric monkeys, and normal monkeys (n = 4/group). A dose of 0.1 mg/kg was ineffective, but the 1.0 mg/kg dose lowered MAP by 11 ± 3% (mean ± SE) in the anephric monkeys. The infusion of renin into anephric monkeys restored the efficacy of A-64662 at the 0.1 and 1.0 mg/kg doses to responses comparable to those of the normal monkeys. A-64662 at 10.0 mg/kg caused a similar fall in MAP of 50 to 60% in anephric, renin-infused anephric, and normal monkeys in the absence of detectable PRA. A-64662, which is inactive against rat renin in vitro, was given as a 1.0 mg/kg i.v. bolus followed by a 0.1 mg/kg/min infusion and was without effect on MAP and PRA in sham-operated and two-kidney, one clip conscious rats (n = 6/group). We conclude that 1) PRA is involved, at least in part, in mediating the observed hypotension and 2) A-64662 may exert actions secondary to its effect on PRA. (Hypertension 11: 613-619, 1988)

KEY WORDS • renin inhibitors • hypotension • plasma renin activity • cynomolgus monkeys

RENIN is the enzyme that catalyzes the first and rate-limiting step in the formation of angiotensin II. Unlike angiotensin converting enzyme, which has multiple substrates, renin is selective for a single naturally occurring substrate, angiotensinogen.1 Although angiotensin converting enzyme inhibitors provide an effective blockade of the renin-angiotensin-aldosterone system and are successful therapeutic agents in the treatment of hypertension and congestive heart failure,2,3 renin inhibitors may afford further target specificity in treating various cardiovascular disorders. In addition, renin inhibitors will serve as precise tools to study the role of the renin-angiotensin-aldosterone system in regulating blood pressure and fluid volume under conditions characterized by suppressed, normal, and enhanced activity of this hormonal axis.

In recent years, extraordinary advances have been made in synthesizing relatively small, potent renin inhibitors that function as substrate analogues.4-7 We now report on one such renin inhibitor, A-64662, [N-(3-amino-3-methyl-1-oxobutyl)-4-methoxy-L-phenylalanyl]-N-[1S,2R,3S)-1-(cyclohexylmethyl)-2,3-dihydroxy-5-methylhexyl-L-histidinamide, which is a dipeptide with a molecular weight of 657. This com-
pound has three desirable characteristics retained in a single molecule. First, it is a selective, potent inhibitor of primate renin (IC50 = 14 nM human plasma renin, 2.3 nM cynomolgus monkey plasma renin, >10,000 nM rat plasma renin; measured at pH 7.4). Second, its only true peptide bond has been rendered relatively stable to enzymatic degradation, by incorporating an O-Me into the phenylalanine substituent. Third, it is relatively soluble (1.0 mg/ml for the base; >100.0 mg/ml for the diacetate salt).

The present studies employed A-64662 as a research tool to investigate the physiological role of plasma renin activity (PRA) in eliciting the observed hypotension induced by this compound. These studies defined the efficacy of A-64662 in monkeys and rats with varying baseline PRA; two species with vastly different sensitivities to the inhibitory action of A-64662.

Materials and Methods

Monkey Studies

Male cynomolgus (Macaca fascicularis) monkeys, weighing 3 to 6 kg, were housed under constant temperature and lighting conditions. The monkeys were obtained from Hazelton Research Products (Reston, VA, USA) and Charles River Laboratories (Wilmington, MA, USA). Each animal was employed once in this study and had not participated in other experiments for at least 4 weeks before this study.

Pretreatment

Three pretreatment regimens were used. 1) Normal monkeys were fed fresh fruit and standard, certified Purina monkey chow diet (0.325% Na = 0.138 mEq Na/g; St. Louis, MO, USA). 2) Salt-depleted monkeys were fed fresh fruit and a certified Purina monkey chow diet, from which the salt normally added during the manufacturing process had been omitted (0.054% Na = 0.0235 mEq Na/g). In addition, these monkeys were treated twice with furosemide (5.0 mg/kg p.o.) 1 week and 1 day before experimentation. 3) Anephric monkeys were prepared by undergoing unilateral nephrectomy 1 week before the experiment. Removal of the remaining kidney was performed 18 hours before study. These monkeys were maintained on the same diet fed the normal monkeys.

General Protocol

On the day of the experiment, each monkey, deprived of food overnight, was sedated with ketamine hydrochloride (Bristol Laboratories), 10.0 mg/kg i.m., and an intravenous catheter was inserted into a leg vein for the infusion of 5% dextrose in water (D5W) and anesthesia, using sodium pentobarbital (Abbott Laboratories) as a 15.0 mg/kg bolus plus a 0.1 mg/kg/min maintenance infusion. A femoral artery was catheterized with PE-90 tubing for the direct and continuous measurement of mean arterial pressure (MAP) and heart rate (HR) using a Model P23 Gould-Statham pressure transducer and a Model 7 Grass polygraph (Quincy, MA, USA). To maintain the normal body temperature of the monkey, all experiments were performed on temperature-controlled surgical tables. Blood samples were obtained for the determination of arterial PRA during control and at intervals following the administration of vehicle or A-64662, using the method described by Preibisz et al., in which PRA is determined at pH 5.7. Each animal received an i.v. bolus dose of A-64662 or vehicle at a volume of 0.1 ml/kg over 1 minute followed by a 0.5-ml flush with D5W. A-64662 was determined to be 100% pure by titration and 99.3% pure by high performance liquid chromatography analysis. A stock solution of A-64662 was prepared at 100 mg/ml in 0.3 M acetic acid and D5W. D5W was used for additional dilutions and served as the vehicle.

Single Bolus Dose-Response Study

The effects of a single i.v. bolus dose of A-64662 on MAP, HR, and PRA were studied in 60 monkeys, prepared either as normal or as salt-depleted monkeys (n = 30/prepretreatment group). After obtaining baseline measurements, each monkey received one dose of either vehicle or A-64662 and was subsequently observed for 3 hours. The doses administered were 0 (vehicle), 0.001, 0.01, 0.1, 1.0, and 10.0 mg/kg. Five normal and five salt-depleted monkeys were tested at each dose in a randomized fashion. Data are reported in Tables 1 and 2, at intervals when blood samples were collected for PRA determination.

Multiple Bolus Dose-Response Study

To minimize the number of monkeys studied in the anephric state, a multiple bolus dose-response study was designed. The effects of three consecutive i.v. bolus doses of vehicle or A-64662 at 0.1, 1.0, and 10.0 mg/kg, injected at 60-minute intervals were studied in four groups of monkeys (n = 4/group): Group 1, anephric monkeys receiving vehicle only; Group 2, anephric monkeys receiving A-64662; Group 3, anephric monkeys infused with human renin and receiving A-64662; and Group 4, normal monkeys receiving A-64662.

The renin that was infused throughout the experiment into the Group 3 monkeys was derived from trypsin-activated prorenin released by human chorionic cells in culture. To elevate PRA to the levels described herein, an average of 2.6 mGU/kg/min of human renin was infused. A steady state blood pressure response to the infused renin was achieved before the recording of control values.

Rat Study

Surgical Pretreatment

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA), weighing 150 g on average, were employed in this study. These experiments were designed to determine whether A-64662, which is relatively ineffective in inhibiting rat PRA, would lower MAP in rats with normal MAP and PRA and in rats with elevated MAP and PRA. Baseline
The rats were allowed to recuperate from the operation. A-64662 (10 mg/kg i.p., Brevital, Eli Lilly), the right renal artery was isolated and either clipped with a (inside diameter, 0.2 mm) silver band (2K1C) or left unclipped in hypertensive rats (2K1C rats). With the rats under anesthesia with methohexital sodium and catheters were placed in the carotid artery, femoral vein, and jugular vein using PE-50 tubing. The carotid artery catheter was used to record MAP and HR, as well as for the 30 min, 60 min and 120 min after clipping. Sham-operated normotensive rats served as controls and were studied between Weeks 4 and 6 after clipping. Sham-operated normotensive rats served as controls and were studied in a random order with the 2K1C rats.

**Experimental Protocol**

One day before the study, each rat was again anesthetized with methohexital sodium, 50.0 mg/kg i.p. (Brevital, Eli Lilly), the right renal artery was isolated and either clipped with a (inside diameter, 0.2 mm) silver band (2K1C) or left untouched (sham-operated controls). In either case, the left kidney was undisturbed by the surgical procedure. The rats were allowed to recuperate from the operation and were monitored weekly by tail cuff for the development of hypertension. Hypertension was achieved between Weeks 4 and 6 after clipping. Sham-operated normotensive rats served as controls and were studied in a random order with the 2K1C rats.

### Table 1. Effects of A-64662 in Normal Monkeys

<table>
<thead>
<tr>
<th>A-64662 dose (mg/kg i.v.)</th>
<th>Variable</th>
<th>Time relative to dose administration (min)</th>
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<tr>
<td>0.001</td>
<td>MAP (mm Hg)</td>
<td>82 ± 2</td>
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<td>HR (beats/min)</td>
<td>154 ± 10</td>
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<td>PRA (ng Ang I/ml/hr)</td>
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### Table 2. Effects of A-64662 in Salt-Depleted Monkeys

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Values are means ± SE.

*p < 0.05, compared with Time 0 values.
withdrawal of blood samples for PRA determinations. The jugular vein catheter was used to administer A-64662 using a Harvard infusion pump (South Na-
tick, MA, USA), and the femoral vein catheter was used for the delivery of captopril. All three catheters were tunneled under the skin and exteriorized at the back of the neck. All rats were classified as renin-
dependent or renin-independent. Renin dependency was evaluated by monitoring the MAP response to saralasin (30 μg/kg i.v.). The 2K1C rats that showed a transient hypotensive response to the saralasin bolus were initiated into the study. In all cases, the sham-
operated rats qualified as saralasin nonresponders. Each animal was allowed to recover overnight from the anesthesia.

On the day of the experiment, baseline MAP mea-
surements were recorded in the conscious sham-operated and 2K1C rats during a 30-minute vehicle infusion period. Vehicle alone was then replaced with a 1.0 mg/kg bolus of A-64662 followed by a constant 30-minute infusion of A-64662 at 0.1 mg/kg/min, in-
fused at a rate of 7.4 μl/min. Fifteen minutes into the renin inhibitor infusion, a bolus of captopril (0.1 mg/kg) was superimposed. MAP and HR were moni-
tored throughout the experiment. Blood samples were obtained during the control period, during the A-64662 infusion alone, and during the infusion of A-64662 in the presence of captopril.

Statistics
Statistical analyses of within-group changes using Time 0 as baseline control were conducted using the one-sample t test. Differences among treatment groups were determined by analysis of variance and Tukey’s multiple comparison procedure. Changes were consid-
ered significant at a p level of 0.05 or less. Values are reported as means ± SE.

Results

Monkey Studies

Single Intravenous Bolus Dose-Response Study

The MAP, HR, and PRA responses to A-64662 in the normal and salt-depleted monkeys are shown in Tables 1 and 2, respectively. Baseline MAP and HR of the normal and salt-depleted monkeys were similar, but baseline PRA values in the salt-depleted monkeys were significantly higher than those in the normal monkeys (p < 0.05). A-64662 caused a dose-related fall in MAP in both the normal and salt-depleted mon-
keys, affecting the magnitude (up to 62%) and the duration of action (maximum, >3 hours) compared with baseline, Time 0. The onset of action was consist-
tently rapid, occurring within the first 5 minutes after dosing. The statistical comparison between the normal and salt-depleted monkeys indicated that the overall MAP responses were similar with two exceptions. First, in the salt-depleted monkeys, the 0.01 mg/kg dose lowered MAP significantly below the corre-
sponding response in normal monkeys at the 5-minute time point (9 ± 2 vs 2 ± 0.7% change in MAP com-
pared with their respective baselines). This was the minimum dose in which a clear MAP response was noted in at least one pretreatment group. Second, 15 minutes after administration of 1.0 mg/kg, MAP fell to a significantly greater extent in the salt-depleted mon-
keys than in the normal monkeys (26 ± 4 vs 12 ± 4% change in MAP from their respective baselines). MAP responses to A-64662 were not significantly different from the corresponding vehicle control group values, except at the two highest doses tested. The groups receiving 1.0 mg/kg had MAP values that were signifi-
cantly lower than their respective vehicle control groups at 5, 15, 30, and 60 minutes in the salt-depleted monkeys, but only at 30 minutes in the normal
monkeys.

A short-lived suppression of PRA of less than 50% was noted in the normal monkeys and salt-depleted monkeys at the lowest dose of 0.001 mg/kg. Doses of 0.01 mg/kg and higher caused an initial 90% or greater inhibition of PRA (p < 0.05 compared with baseline and vehicle control groups). The duration of inhibition of PRA was dose-related. The recovery of MAP and PRA to pretreatment values was dose-related and not always achieved by 180 minutes. In general, A-64662 induced negligible reflex tachycardia compared with baseline or the vehicle control group. A consistent elevation of HR to 10% or more above baseline was observed in the normal and salt-depleted monkeys receiving the 10.0 mg/kg dose, although these changes were not always significant.

Multiple Intravenous Bolus Dose-Response Study

The effects of consecutive boluses of A-64662 at doses of 0.1, 1.0, and 10.0 mg/kg were studied in the absence and the presence of detectable PRA (Figure 1). Bilateral nephrectomy reduced baseline PRA val-
ues to below 1.0 ng angiotensin I (Ang I/ml/hr. Vehi-
cle alone did not significantly alter MAP, HR, or PRA in anephric monkeys (Group 1; see Figure 1). In Group 2, treatment with A-64662 in anephric monkeys ind-
ced a small, but dose-related fall in MAP of 4 ± 0.9 and 11 ± 3%, but a marked fall of 51 ± 6% below baseline in response to 0.1, 1.0, and 10.0 mg/kg doses, respectively. HR fell slightly only during the 1.0 mg/kg treatment period (<2%; p ≤ 0.05), while PRA remained unchanged. In Group 3, in which the infusion of human renin into anephric monkeys signifi-
cantly elevated baseline PRA to a mean of 80 ± 35.0 ng Ang I/ml/hr, MAP fell by 10 ± 3, 16 ± 8, and 50 ± 11% with progressive doses of A-64662. PRA was suppressed to zero at 5 minutes after the 0.1 mg/kg dose, recovering to only 10.0 ng Ang I/ml/hr after 60 minutes. The subsequent doses of 1.0 and 10.0 mg/kg suppressed and maintained PRA at zero for the duration of the experiment. HR remained unaffected. In Group 4, normal monkeys with intact kidneys, in-
creasing doses of A-64662 induced a dose-related fall in MAP of 11 ± 3, 21 ± 5, and 60 ± 11%. Baseline PRA of 10.0 ng Ang I/ml/hr was completely inhibited 5 minutes after the 0.1 mg/kg dose, but it had recover-
ed to 8.0 ng Ang I/ml/hr by 60 minutes after dosing.
FIGURE 1. Effects of A-64662 at 0.1, 1.0, and 10.0 mg/kg i.v. in the presence and absence of detectable PRA (n = 4/group). The means ± SE of the MAP (○), HR (△), and PRA (□) before and after dosing are shown for (top to bottom) Group 1 (anephric monkeys receiving vehicle only), Group 2 (anephric monkeys receiving A-64662), Group 3 (anephric monkeys receiving A-64662 during replacement of PRA by a constant infusion of human renin), and Group 4 (normal monkeys receiving A-64662). The area under the curve is shaded for MAP and PRA. Asterisk indicates significant difference (p < 0.05) compared with Time 0 values.

The subsequent doses of A-64662 completely inhibited PRA with no sign of recovery. No change in HR was observed. Multiple comparison analyses revealed that the MAP responses to 10.0 mg/kg in all three A-64662-treated groups (Groups 2–4) were similar to each other and significantly different from the vehicle-treated group (Group 1). (The magnitude of the effect of 10.0 mg/kg given as the third dose was similar to that in the single bolus protocol.) The hypotensive effect of 10.0 mg/kg was more persistent in the normal monkeys, as the MAP response at 15 minutes after this dose was greater as compared with the other three groups.

Rat Study
The effects of A-64662 in rats are delineated in Figure 2. A-64662, administered to conscious sham-operated controls and 2K1C rats, did not alter MAP or HR or PRA regardless of baseline values, at a dose that effectively lowers MAP in anesthetized and conscious (unpublished data, 1987) monkeys with intact kidneys. Superimposition of captopril induced a maximal fall in MAP below baseline of approximately 5% in the sham-operated controls and 15% in the 2K1C rats, with a slight tachycardia in the latter group. The normal reactive rise in PRA following captopril was noted in both groups, albeit to a greater extent in the 2K1C rats as compared with the sham-operated controls (309 ± 113 vs 118 ± 78% change from baseline, respectively). None of the changes, however, in any of the parameters were significantly different from baseline.

Discussion
The present studies investigated the role of the PRA in eliciting the hypotensive response to the renin inhibitor A-64662 by studying the efficacy of A-64662 in the presence of varying baseline PRA. In a major single i.v. bolus dose-response study of 60 monkeys, A-64662 caused a dose-related decrease in MAP and suppression of PRA in both normal and salt-depleted monkeys. The recovery of both MAP and PRA were dose-related but did not necessarily follow each other
temporally. This dissociation between MAP and PRA was noted earlier in dogs receiving statine-containing renin inhibitory peptide. A-64662 and other potent renin inhibitors effectively lower MAP in salt-depleted animals, as would be expected. Interestingly, in our study the overall MAP response of the normal monkeys to A-64662 was remarkably similar to that of the salt-depleted monkeys. These results may suggest that the renin-angiotensin-aldosterone system is important in regulating MAP in normal monkeys, as it is in controlling blood pressure, aldosterone secretion, and sodium balance in normal, healthy men on a normal salt diet. Another explanation could stem from the use of ketamine and sodium pentobarbital in our preparation, both of which are known to alter PRA and possibly render the normal monkeys more sensitive to the effects of A-64662.

The baseline PRA did affect, however, the minimal efficacious dose of A-64662. For example, an approximate 10% fall below baseline in MAP occurred at a dose of 0.01 mg/kg in the salt-depleted monkeys, at 0.1 mg/kg in the normal monkeys in the single bolus study, and at 1.0 mg/kg in the multiple bolus study in anephric monkeys. Others have reported that inhibiting renin by either pharmacological intervention or use of monoclonal antibodies against renin had no significant effect on blood pressure in nephrectomized animals. In our study, the efficacy of A-64662 at 0.1 and 1.0 mg/kg doses was restored in the anephric monkeys to a response that was comparable to normal monkeys by replacing the plasma renin pool through an infusion of human renin. These data suggest that inhibition of PRA is involved in the primary action of A-64662. Interestingly, the MAP response to A-64662 at 10.0 mg/kg was similar among anephric, normal, and salt-depleted monkeys. It is the latter observation, together with the finding that increasing doses of A-64662 enhanced the magnitude of the hypotensive response even though PRA was maximally suppressed, that warrants attention. These findings present several possibilities, one of which is that undetectable, residual PRA was inhibited at higher doses of A-64662. Another possible explanation is that A-64662 exerted actions that were secondary to its effects on PRA. These secondary actions may have been due to interaction with other hormonal systems or the components of the nervous system that regulate MAP. Another possibility is that low doses of A-64662, which had ready access to the plasma renin pool, inhibited this pool first, and higher doses additionally inhibited pools of tissue renin, such as vascular, heart, or brain. This possible secondary response was clearly evident at the 1.0 mg/kg dose, was exaggerated at the 10.0 mg/kg dose, and may even have been occurring at doses below 1.0 mg/kg. If this effect was nonspecific or unrelated to renin inhibition, one would expect it to be reproducible in any species. However, the actions of A-64662 did not appear to be nonspecific or unrelated to renin inhibition, since in models where PRA was not inhibited, such as the sham-operated or 2K1C rat, this compound had no effect on MAP. Further, we have found that A-64662 is a specific inhibitor of renin at a concentration of $1.0 \times 10^{-15}$ M, since it does not inhibit other aspartyl proteases such as cathepsin D, gastricsin, or pepsin, as measured by methods previously published.

Finally, similar to angiotensin converting enzyme inhibitors, renin inhibitors as a class do not induce a reflex increase in heart rate in response to their blood pressure-lowering actions. This lack of reflex tachycardia demonstrated with A-64662 may be a common property of any drug that interferes with the renin-angiotensin-aldosterone system and, as studies involving angiotensin converting enzyme inhibitors suggest, may be due to either an increase in cardiac vagal activity, perhaps modulated by prostaglandins, or an inhibition of angiotensin II presynaptic facilitation of sympathetic neurotransmission.

In conclusion, the hypotensive action of A-64662 was mediated in part by inhibiting PRA and possibly by an undefined mechanism related to renin inhibition.

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