Dosing-Time-Dependent Effect of Temocapril on the Mortality of Stroke-Prone Spontaneously Hypertensive Rats

Masahiko Nozawa, Koh-ichi Sugimoto, Masami Ohmori, Hitoshi Ando, and Akio Fujimura

Department of Clinical Pharmacology, Jichi Medical School, Tochigi, Japan.
Running title: Chronotherapy of Temocapril

Correspondence to Akio Fujimura, MD.
Department of Clinical Pharmacology,
Jichi Medical School, Tochigi 329-0498, Japan.
Telephone number: +81 285 58 7387
Fax number: +81 285 44 7562
E-mail address: akiofuji@jichi.ac.jp

The number of text pages is 27.
The number of tables is 2.
The number of figures is 7.
The number of references is 40.
The number of words in the Abstract is 155.
The number of words in the Introduction is 363.
The number of words in the Discussion is 1054.

Abbreviations: ACE, angiotensin-converting-enzyme; HALO, hours after lights on; HPLC, high-performance liquid chromatography; SHRSP, stroke-prone Spontaneously Hypertensive Rat.
ABSTRACT

This study was undertaken to evaluate a dosing-time-dependent effect of temocapril, an angiotensin-converting enzyme (ACE) inhibitor, on the mortality of stroke-prone spontaneously hypertensive rats (SHRSP). Temocapril (1 mg/kg/day) prolonged the survival rate of these animals, with a maximum effect after dosing at the early resting period and a minimum effect after dosing at the early active period. The pharmacokinetics of temocaprilat, an active metabolite of temocapril, did not differ significantly between the two dosing times. However, the inhibition of ACE activity in serum and organs (brain and aorta) and the reduction of blood pressure were significantly greater after dosing at the early resting period than at the early active period. These data suggest that the effect of temocapril on the mortality of SHRSP depends on the time of dosing, with a maximum effect seen after dosing at the early resting period. Dosing-time-dependent differences in the pharmacodynamics of temocapril might be involved in explaining this phenomenon.
Introduction

Angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of hypertension and related diseases. These agents have been shown to improve the morbidity and mortality of patients with hypertension (Kostis, 1995; Hansson et al., 1999) and are recommended as a first-line drug for the treatment of hypertension (Guidelines Committee, 2003).

There is increasing evidence that the effectiveness and/or toxicity of many agents are affected by their time of dosing (Cui et al., 2004; Filipski et al., 2004; Matsunaga et al., 2004; Tsuruoka et al., 2004). A chronopharmacological approach seems to be desirable for developing a more effective and safe dosage regimen. There are precedents for this approach in clinical practice (Tsuruoka et al., 2003; Debon et al., 2004; Shimonov et al., 2005).

Previous animal (Oosting et al., 1999) and human (Palatini et al., 1992; Witte et al., 1993; Sunaga et al., 1995) studies have shown dosing-time-dependent differences in the blood pressure-lowering effects of ACE inhibitors. In addition, the preventive effect of an ACE inhibitor for cardiac hypertrophy in hypertensive animals was reported to depend on its dosing time (Sugimoto et al., 2001). However, it remained to be determined whether the morbidity and mortality of patients with hypertension are affected by the dosing time of ACE inhibitors. The present study aimed to address this issue using stroke-prone spontaneously hypertensive rats (SHRSP), an animal model of human stroke (Okamoto et al., 1974; Yamori et al., 1976). Temocapril is a non-sulphhydryl ACE inhibitor, and its active metabolite (temocaprilat) is eliminated by the renal and biliary routes.
Renal function decreases with age; however, our recent observation that the plasma accumulation of temocaprilat is low during repeated treatments in the elderly indicates that temocapril is a safe antihypertensive drug for these patients (Arakawa et al., 2005). Temocapril was chosen for this study because it is expected that it will be widely used in the future in elderly patients and in patients with renal damage. To examine the dosing-time-dependency of the effects of temocapril, the drug was given to SHRSP once daily at one of four different time points for 200 days, and the survival of these animals was compared among the four trials.
Materials and Methods

Animals. Male SHRSP/Izm were obtained from Japan SLC Co. (Sizuoka, Japan). The animals were maintained for more than 2 weeks in two rooms under a specific-pathogen-free environment and controlled light-dark cycle (light:dark, 12:12 h). The lights were switched on and off at 07.00 and 19.00 h, respectively, in room 1 and at 19.00 and 07.00 h, respectively, in room 2. The rats had free access to standard rat chow and water during the acclimatization period. The experiments were performed in accordance with the Use and Care of Experimental Animals Committee of Jichi Medical School, Japan.

Drugs and Dosing. After the acclimatization period, drinking water was replaced with a 1% NaCl solution. Temocapril hydrochloride or vehicle (1% tragacanth gum solution) was given to salt-loaded SHRSP once daily by gastric gavage. The rats were randomly assigned to one of four groups, each with a different time of dosing [2, 8, 14, or 20 h after lights on (HALO); Fig. 1]. Each group was further divided into three subgroups according to the dose administered: (1) 1 mg/kg/day temocapril, (2) 10 mg/kg/day temocapril, and (3) vehicle. Temocapril was suspended in the vehicle, and the dose of temocapril was adjusted to body weight measurements twice a week.

Experiment 1: Effect of Dosing Time on the Survival Period. Following the acclimatization period, SHRSP at 8 weeks of age were divided into 12 (4 x 3) groups (n = 10 in each group) according to the above protocol. Salt-loading and dosing with temocapril (1 or 10 mg/kg) or vehicle were initiated simultaneously at each of the four different times. After treatment, survival was checked daily for 200
days. The survival period of animals that lived for more than 200 days was considered to be 200 days.

**Experiment 2: Effect of Dosing Time on the Pharmacokinetics of Temocaprilat.** Experiment 1 showed that temocapril at 1 mg/kg/day had a dosing-time-dependent effect on the survival of salt-loaded SHRSP; therefore, the chronopharmacokinetic profile of temocapril (1 mg/kg/day) was determined after oral dosing in salt-loaded SHRSP. Following the acclimatization period, SHRSP at 8 weeks of age were divided into two groups (n = 7 in each group). SHRSP were salt-loaded and temocapril (1 mg/kg) was given at 2 or 14 HALO once daily by gastric gavage for 4 weeks. For the measurement of the plasma concentration of temocaprilat, tail-vein blood was collected into heparinized tubes just before and at 1, 2, 4, 8, 12, and 24 h after the final dosing. After centrifugation (3,500 rpm, 10 min, 4˚C), the plasma samples were stored at -80˚C until assayed.

**Experiment 3: Effect of Dosing Time on Serum ACE Activity.** Following the acclimatization period, SHRSP at 8 weeks of age were divided into four groups. Salt-loading was initiated, and 1 mg/kg temocapril (n = 7 in each group) or vehicle (n = 6 in each group) was given at 2 or 14 HALO once daily by gastric gavage for 4 weeks. For the measurement of the serum ACE activity, blood was collected from the tail vein at 1, 4, 8, and 24 h after the final dosing. After centrifugation (3,500 rpm, 10 min, 4˚C), the serum samples were stored at -80˚C until assayed.

**Experiment 4: Effect of Dosing Time on Tissue ACE Activities and Heart Weight.** Following the acclimatization period, SHRSP at 8 weeks of age were divided into four groups (n = 7 in each group). Salt-loading was initiated, and
temocapril (1 mg/kg) or vehicle was given at 2 or 14 HALO once daily by gastric
gavage for > 4 weeks. For the measurement of the tissue ACE activity, the rats
were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and subjected to
whole body perfusion with cold saline through a polyethylene catheter inserted into
the abdominal aorta at around 24 (22–26) h after the final dosing. After the blood
was replaced by saline, the brain, heart and thoracic aorta were excised. The heart
was blotted dry, and the weight of the whole heart, including the atria, was
measured. The heart weight was normalized to the body weight.

**Experiment 5: Effect of Dosing Time on Blood Pressure.** Following the
acclimatization period, SHRSP at 8 weeks of age were divided into four groups
\( n = 6 \) in each group). Salt-loading was initiated, and temocapril (1 mg/kg) or
vehicle was given at 2 or 14 HALO once daily by gastric gavage for > 2 weeks. For
blood pressure measurements, the rats were anesthetized with pentobarbital sodium
(50 mg/kg, i.p.), and a polyethylene catheter filled with heparinized saline
(10 U/ml) was inserted into the left common carotid artery and connected to a
pressure transducer. The mean blood pressure was continuously recorded under
conditions of alertness, unrestraint, and free access to food and water. Blood
pressure recording was initiated at > 12 h after surgery and was performed for 24 h.

**Plasma Temocaprilat Measurement.** The plasma temocaprilat
concentration was measured by high-performance liquid chromatography (HPLC),
as previously described (Shioya et al., 1991). The assay is based on the principle
that the amount of inhibitor (temocaprilat) bound to ACE is inversely related to the
amount of hippuric acid liberated by the hydrolysis of the synthetic substrate
hippuryl-L-histidyl-L-leucine. The plasma samples were diluted 20 times with phosphate buffer (1/15 M, pH 7.4). The diluted plasma sample (100 µl) was heated at 60°C for 15 min to inactivate endogenous ACE and was pre-incubated with 100 µl of pooled rat plasma containing ACE at 37°C for 3 min. The hippuryl-L-histidyl-L-leucine (5.75 mg/ml in phosphate buffer, pH 8.3, containing 0.5 M NaCl; Sigma, St. Louis, MO, USA) solution (100 µl) was added, and the mixture was incubated at 37°C for 3 min. The reaction was terminated by the addition of 100 µl of 2 N HCl. Methylparaben (10 mg/ml in methanol; Wako, Osaka, Japan) was diluted in ethyl acetate, and 2 µg was added to the mixture as an internal standard. Ethyl acetate (1.5 ml) was added to the plasma mixture containing the internal standard. The organic solvent/plasma mixture was vortexed for 5 min and centrifuged at 5,000 x g for 5 min. The upper organic layer was collected and dried under a stream of NO₂ gas. The dried residue was dissolved in 200 µl of the mobile phase as described below, and 10 µl of the solution was injected into the HPLC system.

The HPLC system consisted of a chromatography pump (PU-880; Jasco, Tokyo, Japan) fitted with a Rheodyne manual sample injector (Model 7125, Rheodyne, Rohnert Park, CA, USA) equipped with a 10-µl sample loop, an ultraviolet detector (Uvidec 875, Jasco), and a column (TSK gel ODS-80TM 150 mm × 4.6 mm I.D., 5-µm particle size; Tosoh, Tokyo, Japan). The column was set at room temperature. The mobile phase consisted of acetonitrile/H₂O/2 N HCl (30:70:1, v/v/v) and was pumped at a flow rate of 1 ml/min. The absorbance of the effluent was monitored at 228 nm.
Plasma calibration curves were prepared with temocaprilat (Sankyo, Tokyo, Japan) diluted with rat pooled plasma to concentrations of 0–200 ng/ml.

The recoveries of hippuric acid and methylparaben were 80.8 and 81.5%, respectively. Intra-assay and inter-assay variations for 100 ng/ml hippuric acid were 5.6 (n = 4) and 11.7% (n = 4), respectively.

**Tissue Extracts for the Measurement of ACE Activity.** Tissue extracts were prepared according to the method of Takai et al. (2001). The tissue was minced and homogenized in ten volumes (w/v) of 20 mM Tris-HCl buffer, pH 8.3. The homogenate was centrifuged at 20,000 x g for 30 min at 4°C. The supernatant was discarded, and the pellet was homogenized in five volumes (w/v) of 20 mM Tris-HCl buffer containing 5 mM Mg(CH3COO)2, 30 mM KCl, 250 mM sucrose, and 0.5% NP-40. The homogenate was centrifuged (10,000 rpm, 30 min, 4°C), and the supernatant was used for the measurement of ACE activity and protein concentration. Tissue ACE activity was determined by colorimetry (Kasahara and Ashihara, 1981) and was normalized to protein concentration, which was measured using the Advanced protein assay reagent (Cytoskeleton, Denver, CO, USA).

**Pharmacokinetic Calculations.** The pharmacokinetics were characterized by the maximum concentration in the plasma (Cmax), the time to maximum plasma concentration, the elimination half-life (t1/2), and the area under the plasma concentration-time curve between zero and 24 h after dosing (AUC0-24). The elimination rate constant (Ke) was determined by a linear regression analysis of the log-linear phase of the plasma drug concentration-time curve. The elimination t1/2 was calculated as follows:
Elimination $t_{1/2} = \ln 2/K_e$.

The AUC$_{0-24}$ was calculated by the trapezoidal rule.

**Statistical Analysis.** Data are shown as the mean ± S.E. The comparison of survival periods was performed by Kaplan-Meier analysis, and other comparisons between groups were done by *post hoc* analysis following analysis of variance, using StatView (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered to indicate significance.
Results

Survival did not differ significantly among the vehicle-treated subgroups of the four dosing-time groups of SHRSP (Table 1). Temocapril prolonged survival in the 8, 14, and 20 HALO trials in a dose-dependent manner, but the survival effect in the 2 HALO trial was not dose-dependent. The survival rate with temocapril at 1 mg/kg/day was significantly greater in the 2 HALO trial than in the other trials, but a dosing-time-dependent effect was not seen with temocapril at 10 mg/kg/day (Fig. 2).

The pharmacokinetic profile of temocaprilat did not differ significantly between the 2 and 14 HALO trials after oral dosing with temocapril (1 mg/kg/day) (Fig. 3 and Table 2). The serum ACE activity was significantly inhibited at 1 h after dosing with temocapril (1 mg/kg/day) at 2 HALO and returned to the control level at 4 h (Fig. 4A). On the other hand, the inhibitory effect was not detected after dosing with temocapril (1 mg/kg/day) at 14 HALO (Fig. 4B). There was no significant difference in the tissue ACE activity (cerebrum and thoracic aorta) between the 2 and 14 HALO trials in the vehicle-treated rats (Fig. 5). Repeated treatment with temocapril (1 mg/kg/day) significantly inhibited ACE activity in the 2 HALO but not in the 14 HALO trials.

Heart weight in SHRSP significantly decreased during repeated treatment with temocapril (1 mg/kg/day) in the 2 and 14 HALO trials (Fig. 6). The variation in heart weight was significantly lower for rats treated with temocapril at 2 HALO than at 14 HALO. Blood pressure was significantly decreased after dosing with temocapril (1 mg/kg/day) in the 2 and 14 HALO trials (Fig. 7). The blood
pressure-lowering effect of temocapril persisted for up to 10 and 4.5 h after oral
dosing in the 2 HALO and 14 HALO trials, respectively.
Discussion

SHRSP is an animal model of severe hypertension with subsequent development of stroke (Okamoto et al., 1974; Yamori et al., 1976). By replacing drinking water with a 1% NaCl solution, organ damage such as stroke, cerebral edema, renal dysfunction, and cardiac hypertrophy are accelerated (Inada et al., 1995; Blezer et al., 1998). Sodium-loading enhances the activity of ACE in the brain and aorta in these animals (Mizuno et al., 1981; Fernandes-Alfonso et al., 1994) which, in turn, can exaggerate organ damage (Bader, 2002; Hanes et al., 2004).

Although many chronopharmacological profiles have been reported for antihypertensive agents, information concerning the dosing-time-dependent prevention of organ damage is limited, and no information is available concerning the dosing-time-dependent effect on survival rate. For example, the preventive effect of the ACE inhibitor trandolapril against cardiac hypertrophy in hypertensive rats with aortic banding is greater after dosing at the resting period than at the active period (Sugimoto et al., 2001). On the other hand, the beneficial effect of the calcium channel blocker nitrendipine on cardiac hypertrophy is reported to be greater after dosing at the active period in SHR (Shiga et al., 1999). The present study shows that the survival rate of sodium-loaded SHRSP depended on the dosing-time of temocapril, with a peak at 2 HALO (early resting period) and a trough at 14 HALO (early active period). Thus, these data lead us to speculate that the mortality of hypertensive animals depends on the dosing time of antihypertensive agents.
During the past two decades, numerous studies have shown that the pharmacokinetic properties and therapeutic effects of many drugs vary with dosing time in both animals and humans (Belanger et al., 1999). To evaluate the dosing-time-dependency of temocapril effects, a pharmacokinetic study was performed in SHRSP during repeated treatment for > 4 weeks. Although dosing-time-dependent differences in the pharmacokinetics of the ACE inhibitor enalaprilat were reported in patients with hypertension (Witte et al., 1993; Sunaga et al., 1995), such a chronological difference in the pharmacokinetics of temocaprilat was not detected in our animal study. These data suggest that a chronopharmacokinetic mechanism might not be involved in this event.

Elevated activity of the tissue renin-angiotensin system enhances organ damage (Bader, 2002; Hanes et al., 2004). Sodium loading stimulates tissue ACE activity in SHRSP (Mizuno et al., 1981; Fernandes-Alfonso et al., 1994); therefore, it may be that the tissue renin-angiotensin system is activated in these animals, leading to exaggerated organ damage. Takai et al. (2001) report that, although blood pressure is not lowered, the ACE inhibitor trandolapril reduces tissue ACE activity and prolongs survival in SHRSP with salt loading, which is compatible with the above hypothesis. In our study, ACE activity in the brain and aorta was significantly inhibited in the 2 HALO trial and was slightly decreased in the 14 HALO trial during repeated treatment with temocapril at 1 mg/kg/day. These data indicate that the suppression of the tissue angiotensin II concentration was greater in the 2 HALO than in the 14 HALO trial, and this may explain the dosing-time-dependent difference in the survival rate of SHRSP. ACE inhibitors
cause not only a reduction in angiotensin II but also an elevation in bradykinin, which may be another important mediator of their effects (Campbell et al., 1994). Therefore, it remains to be shown whether the effect of temocapril on bradykinin concentration depends on the time of dosing.

Previous clinical studies have shown that the blood pressure-lowering effect of ACE inhibitors (quinapril, enalapril) is more sustained after treatment at night than in the morning in patients with hypertension (Palatini et al., 1992; Sunaga et al., 1995). Animal studies have also show that the hypotensive effect of enalapril is observed after dosing at the beginning of the resting period, while blood pressure is unchanged after dosing at the beginning of the active period (Lemmer et al., 1994). These observations suggest that the hypotensive effect of ACE inhibitors depends on dosing time, with greater effects after dosing at the resting period, a conclusion that is confirmed in our study.

The regression of cardiac hypertrophy, which is partly caused by a reduction in blood pressure (Shimamura et al., 2002), was significantly greater in the 2 HALO trial in our study. This finding supports the hypothesis that there is a dosing-time-dependent difference in the effect of temocapril. The chronopharmacological profile of temocapril may contribute to the dosing-time-dependent difference in the survival rate of SHRSP. Several mechanisms are proposed for the drug-induced prolongation of life span in SHRSP: (1) reduction of blood pressure (Shimokawa et al., 1988; Inada et al., 1995; Ju et al., 2003), (2) modulation of inflammation (Ju et al., 2003; Kawashima et al., 2003), (3) prevention of extracellular matrix accumulation (Liebetrau et al., 2005),
(4) inhibition of superoxide production (Kawashima et al., 2003), and (5) reduction of platelet aggregation (Shimokawa et al., 1998). In this study, we determined the dosing-time-dependent effect of temocapril on blood pressure in SHRSP. Further studies are needed to evaluate the mechanism in more detail.

Although the plasma temocaprilat concentration did not differ significantly between the 2 and 14 HALO trials, the inhibition of serum and tissue ACE activities in the 2 HALO trial was significantly greater than that in the 14 HALO trial. Thus, it is speculated that there is a diurnal rhythm in the effect of temocaprilat on ACE activity. A diurnal rhythm in drug responsiveness has also been reported for the pituitary response to corticotrophin-releasing factor and arginine vasopressin (Graf et al., 1988). Because the flow of blood to various organs and the transport of drugs within organs have diurnal rhythms (Labrecque and Belanger, 1991), it remains to be shown whether the organ accumulation of temocaprilat was greater in the 2 HALO than in the 14 HALO trial.

Dry cough is an unexpected and troublesome side effect of ACE inhibitors. We previously demonstrated that ACE inhibitor treatment at night reduces cough compared with treatment in the morning (Ohmori and Fujimura, 2005). In the present study, the mortality of SHRSP was improved more after dosing with an ACE inhibitor at the resting period, which leads us to speculate that mortality in patients with hypertension would be greater after treatment with ACE inhibitors at night. Thus, ACE inhibitor treatment at night may be a safer and more effective dosage regimen and may provide an excellent outcome in patients with hypertension.
References


hypertension. *Hypertension* **24:**280-286.


Figure legends

Fig. 1. Schematic representation of two reversed lighting regimens to provide four different dosing-times at two points. By reversing the lighting conditions in two different rooms, dosing at two points (9:00 and 15:00 h) approximates the treatment at four circadian stages: 2, 8, 14, and 20 h after lights on (HALO).

Fig. 2. Effects of dosing time of temocapril at 1 mg/kg/day (A) and 10 mg/kg/day (B) on the survival of salt-loaded SHRSP. In each group, n = 10. *P < 0.05 vs. 2 HALO group.

Fig. 3. Plasma temocaprilat concentration in salt-loaded SHRSP during repeated dosing with temocapril (1 mg/kg/day) at 2 (○) or 14 HALO (●). Mean ± S.E.; n = 7 in each group.

Fig. 4. Changes in serum ACE activity in salt-loaded SHRSP during repeated dosing with vehicle (○) or temocapril (1 mg/kg/day, ●) at 2 (A) or 14 HALO (B). Mean ± S.E.; n = 6 or 7 in each group. ***P < 0.0001 vs. vehicle.

Fig. 5. Tissue ACE activity in the cerebrum (A) and thoracic aorta (B) of salt-loaded SHRSP after repeated dosing with temocapril (1 mg/kg/day) at 2 or 14 HALO. Mean ± S.E.; n = 7 in each group.

Fig. 6. Heart weight of salt-loaded SHRSP after repeated dosing with temocapril
(1 mg/kg/day) at 2 or 14 HALO. Mean ± S.E.; n = 7 in each group.

Fig. 7. Changes in mean arterial pressure in salt-loaded SHRSP during repeated dosing with vehicle (○) or temocapril (1 mg/kg/day, ●) at 2 (A) or 14 HALO (B). Mean ± S.E.; n = 6 in each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.
TABLE 1
Survived days of salt-loaded SHRSP after the repeated dosing of temocapril (1 or 10 mg/kg/day) at 2, 8, 14 or 20 HALO

<table>
<thead>
<tr>
<th>Dosing-time</th>
<th>2 HALO</th>
<th>8 HALO</th>
<th>14 HALO</th>
<th>20 HALO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>71 ± 7</td>
<td>59 ± 4</td>
<td>54 ± 3</td>
<td>51 ± 8</td>
</tr>
<tr>
<td>Temocapril 1 mg/kg/day</td>
<td>174 ± 17 **</td>
<td>125 ± 8 **</td>
<td>75 ± 4 **</td>
<td>80 ± 15 *</td>
</tr>
<tr>
<td>Temocapril 10 mg/kg/day</td>
<td>176 ± 16 **</td>
<td>200 ± 0 ***</td>
<td>151 ± 18 ***</td>
<td>170 ± 20 ***</td>
</tr>
</tbody>
</table>

Mean ± S.E., n=10 in each group. * P < 0.05, ** P < 0.001, *** P < 0.0001 vs. each vehicle.
**TABLE 2**
Pharmacokinetic parameters of plasma temocaprilat after the repeated dosing of temocapril (1 mg/kg/day) in salt-loaded SHRSP at 2 or 14 HALO

<table>
<thead>
<tr>
<th>Dosing time</th>
<th>2 HALO</th>
<th>14 HALO</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}} ) (ng/ml)</td>
<td>292.1 ± 31.7</td>
<td>312.6 ± 27.8</td>
<td>0.6535</td>
</tr>
<tr>
<td>( t_{\text{max}} ) (h)</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0</td>
<td>0.1473</td>
</tr>
<tr>
<td>( \text{AUC}_{0-24} ) (ng·h/ml)</td>
<td>525.9 ± 29.1</td>
<td>522.5 ± 53.4</td>
<td>0.9549</td>
</tr>
<tr>
<td>( t_{1/2} ) (h)</td>
<td>5.5 ± 1.1</td>
<td>5.8 ± 1.0</td>
<td>0.8490</td>
</tr>
</tbody>
</table>

\( C_{\text{max}} \), maximum concentration of plasma temocaprilat; \( t_{\text{max}} \), time to maximum concentration; \( t_{1/2} \), elimination half-life; \( \text{AUC}_{0-24} \), area under the plasma temocaprilat concentration-time curve from 0 to 24 h. Mean ± S.E., n=7 in each group.
**Figure 1**

Room 1
(standard lighting)

Drug dosing

Room 2
(reversed lighting)

<table>
<thead>
<tr>
<th>Time</th>
<th>Room 1</th>
<th>Room 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>07:00</td>
<td>off</td>
<td>on</td>
</tr>
<tr>
<td>09:00</td>
<td>on</td>
<td>off</td>
</tr>
<tr>
<td>15:00</td>
<td>off</td>
<td>on</td>
</tr>
<tr>
<td>19:00</td>
<td>on</td>
<td>off</td>
</tr>
</tbody>
</table>

Local hour

07:00 19:00

Lights on
(resting period)

Lights off
(active period)

2HALO (09:00)  
14HALO (15:00)

8HALO (15:00)  
20HALO (19:00)

off on

on off
Figure 2A

A

Temocapril 1 mg/kg/day

Survival rate

0 25 50 75 100 125 150 175 200

Time after dosing (day)

1.0

0.8

0.6

0.4

0.2

0

2 HALO

8 HALO *

14 HALO *

20 HALO *

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 2B

Temocapril 10 mg/kg/day

Survival rate vs. Time after dosing (day)

- 0 HALO
- 2 HALO
- 8 HALO
- 14 HALO
- 20 HALO

Time after dosing (day)

0 25 50 75 100 125 150 175 200

Survival rate

0 0.2 0.4 0.6 0.8 1.0
Figure 3

![Graph showing plasma temocaprilat concentration over time after dosing.](image-url)

- X-axis: Time after dosing (h)
- Y-axis: Plasma temocaprilat concentration (ng/ml)

Legend or key elements specifying data points or lines.
Figure 4A

A

2 HALO

Serum ACE activity (mIU/ml)

Time after dosing (h)

***
Figure 4B

B

14 HALO

Serum ACE activity (mIU/ml)

Time after dosing (h)
Figure 5A

Tissue ACE activity in cerebrum (mU/mg protein)

Vehicle  Temocapril  Vehicle  Temocapril

2 HALO  14 HALO

P < 0.01
Figure 5B

Tissue ACE activity in thoracic aorta (mU/mg protein)

Vehicle  | Temocapril  | Vehicle  | Temocapril
2 HALO    |            | 14 HALO  |

$P < 0.01$  | $P < 0.05$  |
Figure 6

The graph illustrates the heart weight (mg/g BW) for different treatments at two different time points: 2 HALO and 14 HALO. The treatments compared are Vehicle and Temocapril. The statistical significance is indicated for each comparison with:

- $P < 0.01$ between Vehicle and Temocapril at 2 HALO
- $P < 0.05$ between Vehicle and Temocapril at 14 HALO
- $P < 0.05$ between Vehicle at 2 HALO and 14 HALO
Figure 7B

14 HALO

Mean arterial pressure (mmHg)

Time after dosing (h)

0 3 6 9 12 15 18 21 24