In Vitro and In Vivo Inhibition of the 2 Active Sites of ACE by Omapatrilat, a Vasopeptidase Inhibitor

Michel Azizi, Christine Massien, Annie Michaud, Pierre Corvol

Abstract—The vasopeptidase inhibitor omapatrilat inhibits both neutral endopeptidase and angiotensin-converting enzyme (ACE). The in vitro and in vivo inhibitory potency of omapatrilat and the specific ACE inhibitor fosinopril toward the 2 active sites of ACE (called N- and C-domains) was investigated with the use of 3 substrates: angiotensin I, which is equally cleaved by the 2 ACE domains; hippuryl-histidyl-leucine, specific synthetic substrate of the C-domain in high-salt conditions; and a newly synthesized specific substrate of the N-domain designed by acetylyating the lysine residue of AcSDKP. In vitro, omapatrilat was 5 times more potent than fosinopril in inhibiting angiotensin I hydrolysis. Omapatrilat inhibited similarly both N- and C-domain hydrolysis, whereas fosinopril was slightly more specific for the N-domain. The in vivo selective inhibitory potency of single oral doses of 10 mg omapatrilat and 20 mg fosinopril were investigated in a double-blind, placebo-controlled, cross-over study in 9 mildly sodium-depleted normotensive subjects. In accordance with the in vitro results, fosinopril appeared to be more specific for the N-domain than the C-domain in vivo, since plasma and urine AcSDKP concentrations were significantly higher than those observed with omapatrilat. This study shows that it is possible to assess separately in vitro and in vivo the selectivity of ACE or ACE/neutral endopeptidase inhibitors. A differential selectivity may explain some peculiar properties observed with some ACE inhibitors. (Hypertension. 2000;35:1226-1231.)

Key Words: angiotensin-converting enzyme, angiotensin-converting enzyme inhibitors, AcSDKP, human

Angiotensin-converting enzyme (ACE) has 2 distinct active catalytic sites, hereafter called the N- and C-domains.1 The 2 domains of ACE have both common and different characteristics. Both sites hydrolyze angiotensin (Ang) I and bradykinin with almost the same catalytic efficiency.2 The N-domain cleavages specifically 2 physiological substrates, Ang-(1-7)3 and N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), a hemoregulatory peptide.4 There is no known specific physiological substrates of the C-domain, but the C-domain activity can be assessed specifically in vitro by the use of the synthetic tripeptide hippuryl-histidyl-leucine (HHL), because its hydrolysis in high chloride concentration depends mostly on this catalytic site.5 Finally, some ACE inhibitors display different inhibitory potency toward the 2 active sites.6 For example, captopril appears to be 16 times more efficient for blocking the N-domain than the C-domain, whereas lisinopril is equally potent toward the 2 active sites.6 The inhibitory potency of ACE inhibitors toward ACE can be studied with the use of synthetic7 and physiological substrates8 or with the use of a competitive assay against radiolabeled ACE inhibitors.9 In addition, it is possible to explore the characteristics of the inhibitors toward the 2 ACE domains separately by selecting appropriate substrates and markers. Such studies will allow us to specifically relate their in vitro/in vivo inhibitory potency to their in vivo pharmacological actions.

Combined neutral endopeptidase (NEP) and ACE inhibition has been proposed as a new therapeutic strategy in hypertension and congestive heart failure. Because ACE and NEP share some common catalytic mechanisms, it has been possible to design dual NEP/ACE inhibitors, also called vasopeptidase inhibitors, with K, in the nanomolar range for both enzymes.10 Omapatrilat (4S-[4α(R*)]-octahydro-4-[(2-mercapto-1-oxo-3-phenylpropyl) amino]-5-oxo-7H-pyrido[1,3-b][1,3]-thiazepine-7-carboxylic acid) is a potent, conformationally constrained peptidomimetic vasopeptidase inhibitor with a similar nanomolar inhibitory constant for both NEP and ACE.11 In vivo, omapatrilat behaves as an ACE inhibitor, as shown by the antagonism of the blood pressure response to Ang I in rats, and as an NEP inhibitor by enhancing the natriuretic effects of exogenous atrial natriuretic peptide in rats.12 It has also been shown to be effective in lowering blood pressure in different models of experimental hypertension with high or low renin levels.12 The inhibitory activity of omapatrilat against NEP and ACE has been recently shown in normal subjects.13 The drug is currently evaluated in phase III studies in hypertensive patients.14 The aims of this study were (1) to study in vitro the ACE-inhibitory constants of omapatrilat toward the N- and C- domains of ACE with the use of site-specific substrates,
(2) to compare these in vitro results with the acute blockade of the 2 domains of ACE in vivo by omapatrilat in mildly sodium-depleted healthy subjects, and (3) to compare the in vitro and in vivo inhibition of ACE by omapatrilat with that of a single oral dose of a pure ACE inhibitor, fosinopril. Fosinopril was chosen as the reference compound because it has some N-domain ACE selectivity, being 6 times more N-selective than C-selective.6

Methods

In Vitro Studies

Materials

Enzymes
Wild-type human somatic ACE and 2 ACE mutants containing either an N- or a C- active site were used as previously described6; each full-length mutant contains a single intact site; the other site was inactivated by mutation of the 2 histidine zinc ligands into lysine residues. Mutants were designed as N-domain and C-domain ACE, according to the inactivation of the other domain. Mutants were stably expressed in Chinese hamster ovary cell lines.

Peptides
HHL and Ang I were purchased from Bachem. Ang I and hippuric acid were purchased from Sigma. Acetyl-seryl-aspartyl-(N-acetyl)-lysyl-proline (AcSDAcKP) and Acetyl-lysyl-proline (AcKP) were synthesized by NeoSystem. The purity of these last peptides was >90%.

In Vivo Evaluation

Subjects
Part of the in vivo data were published previously13 and will be summarized here. In brief, 9 healthy normotensive male volunteers 18 to 35 years of age completed the study after they gave written informed consent to participate to the protocol. The protocol was approved by the “Comité Consultatif de Protection des Personnes se prétendant à des Recherches Biomédicales” (Paris-Cochin, France). The procedures followed were in accordance with the institutional guidelines.

Study Design
A single-dose, double-blind, randomized, 3-way cross-over study design was used. Each period was separated from the previous one by a 2-week washout interval. Treatments were assigned according to a Latin square design. Each subject received 10 mg omapatrilat, 20 mg fosinopril, and matched placebo on 3 separate occasions. Subjects were instructed to arrive for each phase at the Broussais Clinical Investigation Center at 7PM on the prestudy evening (D0). To induce mild sodium depletion, subjects were given 40 mg furosemide at 9PM on D0 and received a sodium-restricted diet (30 mmol/d) over the 36 hours of each phase. On the study day (D1), volunteers were given a single oral dose of the assigned treatment at 9AM and remained in a semirecumbent position for 6 hours. Fluid intake throughout each study day was unrestricted. Blood was sampled before and at 2, 4, 6, 12, and 24 hours after the dose for plasma Ang I, Ang II, ex vivo ACE activity, and AcSDKP determinations. Before oral dosing, subjects voided their bladder to complete the minimal volume of urine collected after drug intake (from 9 AM to 9 PM on D1 and from 9 PM on D1 to 9 AM on D2). AcSDKP was determined in each urine sample.

Laboratory Methods

In Vitro Kinetic and Inhibition Studies of Recombinant ACE
Kinetic and inhibition studies of recombinant ACE and ACE mutants were performed with the use of Ang I, HHL, and the new synthetic peptide AcSDAcKP as substrates. Ang I is used as a substrate equally cleaved by the N- and C-domains, whereas HHL and AcSDAcKP are specific substrates for the C- and N-domains, respectively. The rate of hydrolysis of all the substrates used was quantified by high-performance liquid chromatography (HPLC) on a Waters apparatus directed by a millennium chromatography manager. Kinetic parameters were calculated from Michaelis-Menten plots, and inhibitor potency was determined by calculation of apparent Ki values with the use of ENZFITTER software. Plot of apparent Ki versus [S] (substrate concentration) gives the Ki.

Kinetic Studies
The method for measuring the hydrolysis of Ang I and HHL by ACE has been previously described.6 For measuring N-domain activity, AcSDAcKP, a new peptide, was designed and synthesized. The activity of the N-domain has been previously studied by using its natural substrate, AcSDKP. However, the product of the reaction, KP, is difficult to resolve from the injection peak by HPLC because of its high polarity. Therefore, a more hydrophobic peptide AcSDAcKP was designed by acetylation of the lysine residue. The hydrolysis of AcSDAcKP by the wild-type ACE and the N- and C-domains was calculated from the production of AcKP. The hydrolysis was performed with the use of 0.5 · 10−9 mol/L to 10 · 10−9 mol/L enzyme in 50 mmol/L HEPES, pH 7.0, 50 mmol/L NaCl, 1 mg/mL BSA, and 10 μmol/L ZnSO4. The reaction was initiated by the addition of AcSDAcKP in a total volume of 250 μL, and the mixture was incubated at 37°C to produce 5% to 10% substrate hydrolysis. The reaction was stopped by the addition of 0.1% trifluoroacetic acid (final concentration). AcKP and AcSDAcKP were resolved and quantified by reverse-phase HPLC on a 5-μm Puresil C18 column (Waters) with a gradient of increasing concentrations of CH3CN in H2O/0.1% trifluoroacetic acid from 1% to 25% in 10 minutes and to 50% in 5 minutes, at a flow rate of 1 mL/min. Retention time was 13 and 15.6 minutes for AcKP and AcSDAcKP, respectively, with a detection at 200 nm. Initial velocities were measured over a substrate concentration range of 10 to 2000 μmol/L.

Inhibition Studies
The inhibitory potency of omapatrilat and fosinopril toward recombinant wild-type ACE was determined by establishing dose-dependent inhibition curves at equilibrium, as previously described.6 Inhibition of Ang I, HHL, and AcSDAcKP hydrolysis was determined with the use of 0.1 nmol/L, 0.1 nmol/L, and 0.4 nmol/L of wild-type ACE respectively. After preincubation with 0.05 to 2.5 nmol/L of inhibitor at 37°C for 1 hour, reactions were initiated by substrate addition at 2 different concentrations (0.5×Km and 3×Km) and performed for 5, 10, 30, and 60 minutes.

Ex Vivo Plasma ACE Activity
To determine ex vivo the separate activity of the C- and N-domains, HHL and AcSDAcKP were used as C- and N-domain substrates, respectively. Reactions were performed at 37°C with 10 or 20 μL of plasma during 60 and 120 minutes, respectively. Substrate concentration was 2.5×Km.

Plasma Ang II, Ang I, and AcSDKP Determinations
The methods used for blood samplings and for angiotensins and AcSDKP determinations were as previously described.14 The time course evolution of plasma and urinary AcSDKP concentrations were used as very sensitive markers of in vivo inhibition of the N-domain activity.16

Statistical Methods
The area under the curve (AUC) versus time was calculated according to the trapezoidal rule and integrated from 0 to 24 hours. Data were analyzed by ANOVA: The crossed factor was the subject and the within factors were treatment and period. Because the order of the treatments was randomized for each subject and a 2-week
was to measure the velocity of hydrolysis at optimal pH over 0 to 1000 mmol/L NaCl concentration and optimal velocity (v_{opt}) calculated for each point. Percentage of v_{o}/v_{opt} ratio was plotted against NaCl concentration.

**Figure 2.** Chloride dependence of AcSDAcpK hydrolysis by recombinant ACE: Wild-type ACE (x), C-domain (○), and N-domain (●). Velocity (v_{o}) was measured at optimal pH over 0 to 800 mmol/L NaCl concentration and optimal velocity (v_{opt}) calculated for each point. Percentage of v_{o}/v_{opt} ratio was plotted against NaCl concentration.

**Results**

**Kinetic Characteristics of the Hydrolysis of AcSDAcpK, a New Specific Substrate of the N-Domain of ACE**

The wild-type ACE and the N- and C-domains displayed an optimal cleavage of AcSDAcpK at pH 6.0 to 7.0 (Figure 1). The optimum chloride concentration for the hydrolysis of this substrate by the N-domain was 50 mmol/L. (Figure 2) and was selected for the determination of kinetic parameters of all ACE molecules to ensure maximum selectivity for the N-domain versus the C-domain.

The K_{m} values, calculated from Michaelis-Menten plots of AcSDAcpK hydrolysis by wild-type ACE and the N- and the C-domains, and the k_{cat} values are given in Table 1. Wild-type ACE and the N-domain exhibit a similar k_{cat}/K_{m} value, which is 16-fold higher than that of the C-domain. The relative efficacy of the hydrolysis of this substrate by the N-domain versus the C-domain (N-domain efficacy/C-domain efficacy) is 16 and compares well with that of 50 found for the natural peptide AcSDKP.

**In Vitro Inhibition by Omapatrilat and Fosinoprilat of the Hydrolysis of 3 Selective ACE Substrates**

The inhibitory constants of omapatrilat and fosinoprilat toward 3 selective ACE substrates were compared under the same conditions for each substrate (ACE concentration, pH, and NaCl concentration). Omapatrilat was 5 times more potent (K_{i}=0.06±0.05 nmol/L) than fosinoprilat (0.37±0.24 nmol/L, data from Michaud et al) in inhibiting Ang I hydrolysis. The K_{i} of omapatrilat for inhibition of HHL hydrolysis (0.45±0.28 nmol/L) was close to that of fosinoprilat for this substrate (0.29±0.11 nmol/L, data from Michaud et al). AcSDAcpK hydrolysis was mildly better inhibited by fosinoprilat (K_{i}=0.13) than by omapatrilat (K_{i}=0.31).

**In Vivo Studies**

No period effect was detected for any of the results, therefore only treatment effects are reported in the text and tables.

**Ex Vivo ACE Activity**

Ex vivo plasma ACE activity of the N- and the C-domains decreased rapidly after drug intake (Table 2). At peak, omapatrilat and fosinopril induced 90±4% and 88±13% inhibition of ex vivo ACE activity of the C-domain (HHL hydrolysis) and 88±8% and 97±3% inhibition of ex vivo ACE activity of the N-domain (AcSDAcpK hydrolysis, NS), respectively. Twenty-four hours after the dose was given, ex vivo ACE activity of the N- and the C-domains differed significantly between the 2 active drugs: For the C-domain, ACE activity was significantly lower after omapatrilat than after fosinopril intake (0.67±0.19 versus 1.14±0.3 nmol·mL^{-1}·min^{-1}, respectively, P<0.05), whereas the reverse was observed for the N-domain (10.4±6.3 versus 0.5±0.5 pmol·mL^{-1}·min^{-1}, respectively, P<0.05).

**TABLE 1. Kinetic Parameters of AcSDAcpK Hydrolysis by 3 Recombinant Forms of ACE at pH 7.0 and NaCl 50 mmol/L**

<table>
<thead>
<tr>
<th>ACE</th>
<th>K_{m}, μmol/L</th>
<th>k_{cat}, s^{-1}</th>
<th>k_{cat}/K_{m}, μmol·L^{-1}·s^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>480.9±23.1</td>
<td>7.54±0.92</td>
<td>0.016±0.002</td>
</tr>
<tr>
<td>N domain</td>
<td>347.4±26.5</td>
<td>7.75±1.59</td>
<td>0.021±0.003</td>
</tr>
<tr>
<td>C domain</td>
<td>1682.7±542.9</td>
<td>1.75±0.44</td>
<td>0.0013±0.0002</td>
</tr>
</tbody>
</table>

Values are mean±SD from 3 or 4 experiments.
**Table 2.** Effects of Omapatrilat, Fosinopril, and Placebo on Ex Vivo Plasma ACE Activity and Plasma and Urine AcSDKP in 9 Mildly Sodium-Depleted Normotensive Subjects

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Baseline</th>
<th>Value at Peak</th>
<th>Dose</th>
<th>AUC&lt;sub&gt;0-24&lt;/sub&gt; h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ex vivo ACE activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HHL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omapatrilat</td>
<td>2.34±0.54</td>
<td>0.24±0.08*</td>
<td>0.67±0.19†</td>
<td>14.10±3.70*</td>
</tr>
<tr>
<td>Fosinopril</td>
<td>2.24±0.40</td>
<td>0.25±0.26*</td>
<td>1.14±0.30*</td>
<td>16.40±7.00*</td>
</tr>
<tr>
<td>Placebo</td>
<td>1.94±0.27</td>
<td>1.93±0.37</td>
<td>2.03±0.27</td>
<td>47.10±7.30</td>
</tr>
<tr>
<td><strong>F test</strong></td>
<td>227</td>
<td>87[/]</td>
<td>149[/]</td>
<td></td>
</tr>
<tr>
<td>AcSDKP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omapatrilat</td>
<td>39.1±16.5</td>
<td>4.3±3.4*</td>
<td>10.4±6.3†</td>
<td>21.6±11.9†</td>
</tr>
<tr>
<td>Fosinopril</td>
<td>44.0±19.2</td>
<td>0.9±0.9*</td>
<td>0.5±0.5*</td>
<td>6.3±1.9*</td>
</tr>
<tr>
<td>Placebo</td>
<td>37.0±14.9</td>
<td>36.1±15.6</td>
<td>39.1±17.4</td>
<td>94.3±36.9</td>
</tr>
<tr>
<td><strong>F test</strong></td>
<td>34[/]</td>
<td>22[/]</td>
<td>38[/]</td>
<td></td>
</tr>
<tr>
<td>Plasma AcSDKP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omapatrilat</td>
<td>0.51±0.27</td>
<td>3.2±1.3*</td>
<td>2.14±1.02*</td>
<td>57±21*</td>
</tr>
<tr>
<td>Fosinopril</td>
<td>0.74±0.46</td>
<td>4.2±1.1*</td>
<td>1.96±1.10*</td>
<td>73±18*</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.46±0.27</td>
<td>1.1±0.6</td>
<td>0.73±0.34</td>
<td>18±10</td>
</tr>
<tr>
<td><strong>F test</strong></td>
<td>28[/]</td>
<td>8§</td>
<td>30[/]</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean±1 SD.

*P<0.05 vs placebo, †P<0.05 vs fosinopril after Bonferroni correction. ANOVA F(2,14), †P<0.05, §P<0.01, ||P<0.001.

**Plasma and Urine AcSDKP**

Plasma AcSDKP levels increased significantly after the intake of the active treatments, whereas they remained low and stable after placebo intake (Table 2 and Figure 3). Peak plasma AcSDKP levels and the AUC<sub>0-24</sub> of plasma AcSDKP versus time were higher after fosinopril than after omapatrilat intake, but the difference between the active drugs was not statistically significant when the Bonferroni correction was used. Twenty-four hours after the dose was given, plasma AcSDKP levels were significantly higher than placebo for both active drugs, and there was no difference between the 2 active drugs. Both active treatments also induced a much higher excretion of AcSDKP in urine during the 2 collection periods after drug intake than that observed after placebo intake (F<sub>11,24</sub>=61, P<0.001, Figure 3). The 24-hour cumulative urinary AcSDKP excretion was significantly higher after fosinopril than after omapatrilat intake (268±60 versus 165±66 pmol/24 h, P<0.05 respectively).

**Plasma Angiotensins**

Plasma angiotensin results have been previously reported and will be summarized. Omapatrilat and fosinopril induced a similar inhibition of ACE in vivo as assessed by the changes in plasma Ang II/Ang I ratio achieving similar plasma Ang II concentrations over a period of 24 hours.

**Discussion**

Vasopeptidase inhibitors are a new class of antihypertensive agents characterized by their ability to block both ACE and NEP. Their inhibitory effect on each enzyme can be evaluated in vitro by the use of specific substrates. The discovery of the presence of 2 active catalytic sites in ACE adds another degree of complexity in their mechanism of action, as ACE inhibitors may preferentially act on either ACE domain. The N-domain of ACE cleaves preferentially to the C-domain substrates such as AcSDKP, Ang-(1-7), and the N-terminal tripeptide LHRH. To a lesser extent, the C-domain hydrolyzes faster Leu<sup>5</sup>- and Met<sup>5</sup>-enkephalins as well as substance P. Whether these in vitro kinetic differences may account for some of the differences observed between different ACE inhibitors is not known. Indeed, some ACE inhibitors exhibit a preference for either domain, such as captopril or ketro-ACE. It has even been possible to design a specific ACE inhibitor that blocks the N-domain 1000 times more effi-

**Figure 3.** Left, Changes in plasma AcSDKP concentration with time after intake of 10 mg omapatrilat (●), 20 mg fosinopril (◆), and placebo (○) in 9 mildly sodium-depleted healthy subjects. Right, 12-hour urine excretion of AcSDKP after intake of 10 mg omapatrilat (open bar), 20 mg fosinopril (closed bar), and placebo (hatched bar) in 9 mildly sodium-depleted healthy subjects (***P<0.001; †P<0.05).
ciently than the C-domain activity. Therefore, it is important to evaluate separately the inhibitory activity of any new ACE or ACE/NEP inhibitor on the 2 ACE domains.

In this study, we report for the first time an in vitro and in vivo selective assessment of a dual NEP/ACE inhibitor, omapatrilat, and of a pure ACE inhibitor, fosinopril, in humans. Selective substrates were used in vitro and ex vivo to measure ACE inhibition on recombinant and plasma ACE, respectively. A new substrate for N-domain was designed to monitor easily the N-domain activity. The results obtained were compared with plasma and urinary AcSDKP levels, a reflection of in vivo N-domain inhibition, and with the Ang II/Ang I ratio, an indicator of both N-domain and C-domain activity.

Omapatrilat was found to be 5 times more efficient than fosinopril in inhibiting in vitro Ang I hydrolysis. It inhibited equally well in vitro the N- and C-domain activities, whereas fosinopril was twice more specific on the N-domain than on the C-domain of ACE.

The potency of omapatrilat and fosinopril to inhibit ACE was studied in vivo in healthy subjects. N- and C-domain ACE substrates were used to evaluate separately the ex vivo inhibitory potency of these 2 inhibitors toward the 2 ACE active sites. We used a state of mild sodium depletion in healthy subjects, which provides an experimental condition in which a 2- to 3-fold increase in plasma active renin, Ang I, and Ang II concentrations is reproducibly obtained. This approach has been used previously to investigate the additive effects of captopril and losartan because it gives optimal experimental conditions for quantifying in vivo ACE inhibition.

In this model, we have previously shown that a single oral dose of the vasopeptidase inhibitor omapatrilat (10 mg) and of the specific ACE inhibitor fosinopril (20 mg) had a similar potency to inhibit ACE in vivo over a period of 24 hours, as assessed by changes in plasma Ang II/Ang I ratio, giving similar plasma Ang II levels over 24 hours. There are, however, subtle differences between the 2 drugs. The initial inhibitory effect of omapatrilat and fosinopril for the N- and C-terminal active sites of ACE were similar, as shown by the initial changes in plasma AcSDKP and the ex vivo plasma ACE activity on the hydrolysis of HHL. In contrast, apparent dissociation from the C-domain appeared to occur significantly earlier for fosinopril than for omapatrilat: 24 hours after drug intake, the residual ex vivo ACE inhibition, assessed by HHL hydrolysis, was only 52 ± 12% for fosinopril compared with 78 ± 6% for omapatrilat. In addition, fosinopril appears to have higher affinity than omapatrilat for the N-domain of ACE because urinary AcSDKP concentrations were significantly higher than those observed after omapatrilat intake. These results compare well with those of Michaud et al., in which fosinopril was 6 times more efficient for inhibiting in vitro AcSDKP hydrolysis than Ang I hydrolysis. Interestingly, these differences between the enzyme-inhibiting properties of omapatrilat and fosinopril did not affect the changes in plasma Ang II levels over time because Ang I is a natural substrate equally cleaved by both domains. Altogether, these results show that it is possible to assess in vitro and in vivo the selectivity of an ACE inhibitor toward the 2 domains of ACE. A similar strategy has been recently used for showing in vivo the relative selectivity of captopril for the N-domain in rats. The possibility of assessing in vivo the 2 ACE domains in humans may help to elucidate some of the properties of ancient or new ACE inhibitors.

Acknowledgments

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References


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