Elucidation of the Effects of the CYP1A2 Deficiency Polymorphism in the Metabolism of 4-Cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]pyrimidine-2-(1H)-one (YM-64227), a Phosphodiesterase Type 4 Inhibitor, and Its Metabolites in Dogs

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ABSTRACT:
The canine CYP1A2 1117 C>T single nucleotide polymorphism is responsible for a substantial portion of the interindividual variability seen in the pharmacokinetics of 4-cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]pyrimidine-2-(1H)-one (YM-64227). The purpose of this study is to investigate the contribution of CYP1A2 to the metabolism of YM-64227 and its five metabolites (MM-1 to MM-5), as well as to determine the interindividual variability between the pharmacokinetic profiles of the compounds with respect to the CYP1A2 deficiency polymorphism. α-Naphthoflavone and anti-CYP1A1/2 antibody inhibited the metabolic activities at which MM-2 and MM-3 were formed from YM-64227 in C/C- and C/T-type microsomes. In T/T type, the rate of MM-2 and MM-3 formation was lower, and α-naphthoflavone and anti-CYP1A1/2 antibody were shown to have no effect. A positive correlation between the overall metabolism of YM-64227 and phenacetin O-deethylation, a CYP1A2 activity marker, was observed in all the genotypes. The in vitro metabolic clearances in the T/T type of MM-1, MM-3, MM-4, and MM-5 were less than 50% lower than those in the C/C type. The anti-CYP1A1/2 antibody inhibited the metabolism of MM-1, MM-3, MM-4, and MM-5 in the C/C and C/T types. These results suggest that the formation of MM-2 and MM-3 from YM-64227 is catalyzed by CYP1A2, and that CYP1A2 contributes mainly to the subsequent metabolism of the primary metabolites of YM-64227, with the exception of MM-2. It is possible that the interindividual variability of YM-64227 with respect to the CYP1A2 deficiency polymorphism is caused by a decrease in the metabolic activities of both the unchanged drug and its metabolites.

4-Cyclohexyl-1-ethyl-7-methylpyrido[2,3-d]pyrimidine-2-(1H)-one (YM-64227) is a novel phosphodiesterase type 4 inhibitor. Unlike other phosphodiesterase type 4 inhibitors, YM-64227 does not induce the emetic effects that normally accompany the anti-inflammatory activity. In addition, it maintains the ability to inhibit antigen-induced airway responses. For this reason, YM-64227 promises to be a highly effective and safe drug when used as an antiasthmatic or anti-inflammatory drug (Iwata et al., 1998).

In previous studies, a novel CYP1A2 1117 C>T single nucleotide polymorphism (SNP) was found in beagle dogs, which are used extensively in pharmacology and safety assessment studies (Tennizu et al., 2004a; Mise et al., 2004a). This SNP resulted in an amino acid change from an Arg codon to a stop codon at position 373, yielding an inactive enzyme. The T-allele frequency was 0.39, which suggests that 10 to 15% of the dogs would not express the CYP1A2 protein. The anti-CYP1A1/2 antibody inhibited the metabolism of MM-1, MM-3, MM-4, and MM-5 were less than 50% lower than those in the C/C type. In the T/T type, the rate of MM-2 and MM-3 formation was lower, and α-naphthoflavone and anti-CYP1A1/2 antibody were shown to have no effect. A positive correlation between the overall metabolism of YM-64227 and phenacetin O-deethylation, a CYP1A2 activity marker, was observed in all the genotypes. The in vitro metabolic clearances in the T/T type of MM-1, MM-3, MM-4, and MM-5 were less than 50% lower than those in the C/C type. The anti-CYP1A1/2 antibody inhibited the metabolism of MM-1, MM-3, MM-4, and MM-5 in the C/C and C/T types. These results suggest that the formation of MM-2 and MM-3 from YM-64227 is catalyzed by CYP1A2, and that CYP1A2 contributes mainly to the subsequent metabolism of the primary metabolites of YM-64227, with the exception of MM-2. It is possible that the interindividual variability of YM-64227 with respect to the CYP1A2 deficiency polymorphism is caused by a decrease in the metabolic activities of both the unchanged drug and its metabolites.

The pharmacokinetics of YM-64227 after an intravenous administration were not affected by genotype. These findings suggest that the clearance of YM-64227 in dogs is hepatic blood flow-limited, and the CYP1A2 deficiency mainly affects first pass metabolism. The metabolic profiles in the T/T group were quite distinct from the others. In the C/C and C/T groups, MM-2 had the highest AUC among the metabolites, and the AUCs of MM-2, MM-3, MM-4, and MM-5 were much lower than that of MM-2. In the T/T group, the AUCs of MM-1, MM-4, and MM-5 were significantly higher than those in the C/C group.

In addition to the pharmacokinetic studies, in vitro metabolism studies using liver microsomes prepared from these dogs were performed to determine the in vitro metabolic clearance of YM-64227 (Tennizu et al., 2006). The CL<sub>int. in vitro</sub> values of MM-2 and MM-3 in the T/T type were significantly lower than those in the C/C type. No
significant differences were observed in the CL_{int \textnormal{, in vitro}} of MM-1, MM-4, or MM-5 among three groups. These in vitro studies suggest that the formation of MM-2 and MM-3 is catalyzed by CYP1A2. The finding that the CL_{int \textnormal{, in vitro}} of MM-2 was lower in the T/T type than in the C/C or C/T types agreed well with the fact that the plasma concentrations of MM-2 in the T/T group were significantly lower than those in the other groups. However, differences in vitro metabolic clearance alone could not explain the metabolic profiles of the other metabolites in the three groups. A possible explanation for the discrepancy between the in vivo and in vitro studies is that CYP1A2 plays a significant role not only in the metabolism of YM-64227, but also in the subsequent metabolism of its metabolites.

The purpose of this study is to determine the contribution of CYP1A2 to the metabolism of YM-64227 and its metabolites, as well as to elucidate the causes of interindividual variability in the pharmacokinetics of YM-64227 in dogs with respect to the CYP1A2 deficiency polymorphism.

Materials and Methods

**Chemicals.** YM-64227, its five metabolites (MM-1 to MM-5), and 4-(3-chlorophenyl)-1-ethyl-7-((1-hydroxyethyl)pyridin-2(1H)-one, the internal standard for HPLC analysis, were synthesized at the Chemistry Research Laboratories of Astellas Pharma Inc. (Ibaraki, Japan). α-Naphthoflavone and acetaminophen were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and m-acetaminophen was purchased from Aldrich Chemical (Milwaukee, WI). Phenacetin was purchased from Wako Pure Chemical (Osaka, Japan). NADPH-generating system solutions A and B were purchased from BD (San Jose, CA), and NADPH was purchased from Roche (Mannheim, Darmstadt, Germany). Human anti-CYP1A1/2 antibody was purchased from Nihon Nosan Kogyo Co. (Kanagawa, Japan). Purified water obtained from a Milli-Q system (Millipore Corp., Bedford, MA) was used throughout the study. All other chemicals used were commercially available and of the highest purity.

**Effect of α-Naphthoflavone on the Metabolism of YM-64227.** The canine liver microsomes were prepared and the CYP1A2 1117 C>T SNP genotyping method was performed as described by Tennizu et al. (2004a). YM-64227 (5 μM) was incubated in a reaction mixture (0.5 mM) consisting of 0.1 mg/ml liver microsomes, a NADPH-generating system (1.3 mM NADP, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM MgCl₂), 0.1 mM EDTA, and 100 mM Na/K-phosphate buffer (pH 7.4). Pooled liver microsomes prepared from five individuals for each genotype were used. Experiments were performed in duplicate. Enzyme reactions were initiated by adding 30 μl of the NADPH-generating system. The reaction was terminated after incubating at 37°C in a shaking water bath for 15, 30, 45, and 60 min by adding 4 ml of tert-butyl methyl ether. After stopping the metabolic reaction, the concentrations of the metabolites were quantified using HPLC with a Cosmosil-packed phenyl tert-buty methyl ether column (5 μm, 250 mm × 4.6 mm i.d., Nacalai Tesque, Inc.). Mobile phases A, consisting of 50 mM acetic acid and acetaminol (80:20), and B, consisting of 50 mM acetic acid and acetaminol (20:80), were used for gradient elution. The metabolites of YM-64227 were detected fluorimetrically at 400 nm with emission at 330 nm.

**Immunoinhibition of the Metabolism of YM-64227.** Immunoinhibition of the metabolism of YM-64227 was examined by preincubating 0.05 mg of pooled liver microsomes with various concentrations of human anti-CYP1A1/2 antibody at room temperature for 30 min. The other reaction conditions were the same as those described above (“Effect of α-Naphthoflavone on the Metabolism of YM-64227”).

**Metabolism of Phenacetin.** Phenacetin was incubated in a reaction mixture (0.5 ml) consisting of 0.1 mg/ml liver microsomes, 1 mM NADPH, 0.1 mM EDTA, and 100 mM Na/K-phosphate buffer (pH 7.4). Phenacetin concentrations of 1 to 500 μM were used to estimate the kinetic parameters. Individual canine liver microsomes (n = 5 per each group genotyped as C/C, C/T, and T/T types) were used. Experiments were performed in duplicate. Enzyme reactions were initiated by adding 50 μl of 10 mM NADPH. After incubating at 37°C in a shaking water bath for 10 min, the reaction was terminated by adding 5 ml of tert-butyl methyl ether. After stopping the metabolic reaction, the concentration of acetaminophen was quantified using an HPLC-UV column. A Chromolith SpeedROD, RP-18e (4.6 × 50 mm; Merck KGaA, Darmstadt, Germany) was used for the sample trap. Analysis was performed on a TSK gel ODS 80Ts (5 μm, 4.6 × 250 mm; Tosoh Co., Tokyo, Japan). A column switch (Campins-Falco et al., 1993; Fried and Wainer, 1997) with a minor modification was used to prevent the late-eluting substrate from interfering with the analysis. The mobile phase was a mixture of 0.5 M phosphoric acid, acetaminol, and water (10:10:80). Acetaminol and the internal standard were detected at 250 nm.

The kinetic data for phenacetin metabolism obtained with liver microsomes were fitted to eq. 1 using the SAS system version 8.2 (SAS Institute, Cary, NC) to estimate K_{m}, V_{max}, and CL_{int}. The CL_{int \textnormal{, in vitro}} values were calculated using eq. 2.

\[
V = V_{max} \cdot S/(K_m + S) + CL_{int} \cdot S
\]

\[
CL_{int \textnormal{, in vitro}} = V_{max}/K_m + CL_{int}
\]

**Metabolism of the Primary Metabolites of YM-64227.** YM-64227 primary metabolites (MM-1 to MM-5, 0.7 μM) were incubated in a reaction mixture (0.5 ml) consisting of liver microsomes (2.0 mg/ml for MM-1 and 0.5 mg/ml for the other metabolites), a NADPH-generating system, 0.1 mM EDTA, and 100 mM Na/K-phosphate buffer (pH 7.4). Pooled liver microsomes for each genotype were used. Experiments were performed in duplicate. The enzyme reactions were initiated by adding 30 μl of the NADPH-generating system. The reaction was terminated after incubating at 37°C in a shaking water bath for 15, 30, 45, and 60 min by adding 4 ml of tert-butyl methyl ether. The assay method of YM-64227 metabolites was the same as described above (“Effect of α-Naphthoflavone on the Metabolism of YM-64227”). The rate of metabolism for the primary YM-64227 metabolites using liver microsomes is described by eq. 3.

\[
X(t) = CL_{int \textnormal{, in vitro}} \cdot C(t)
\]

\[
X(t) = \text{AUC}_{0-t} \cdot \text{CL}_{int \textnormal{, in vitro}} \cdot C(t)
\]

\[
\text{AUC}_{0-t} \text{ (ng · min/ml)} \text{ represents the area under the concentration-time curve from time } 0 \text{ to } t \text{ for substrate in the mixture. The } CL_{int \textnormal{, in vitro}} \text{ value can be obtained from the slope of a plot of } X(t) \text{ versus } \text{AUC}_{0-t} \text{ (Iwatsubo et al. 1999).}
\]

Immunoinhibition of the Metabolism of the Primary Metabolites of YM-64227. Immunoinhibition of the primary metabolites of YM-64227 was examined by preincubating liver microsomes (1.0 mg for MM-1 and 0.25 mg for the other metabolites) with 50 μl of human anti-CYP1A1/2 antibody at room temperature for 30 min. The reaction conditions were the same as those described above (“Metabolism of the Primary Metabolites of YM-64227”) except that the samples were incubated for 60 min.
Results

Effect of α-Naphthoflavone on the Metabolism of YM-64227.

The formation rate for MM-1 was almost the same among the three liver microsome genotypes (Fig. 2). The addition of α-naphthoflavone increased this rate in a concentration-dependent manner in all three types of microsomes. The formation rate of MM-2 in the T/T type was 11% of that in the C/C type, which was similar to the rate in the C/T type. α-Naphthoflavone at the concentration of 1 μM decreased the rate of MM-2 formation in the C/C and C/T groups. A higher concentration (10 μM) of α-naphthoflavone was slightly less effective at inhibiting the formation of MM-2. Not only did α-naphthoflavone fail to inhibit MM-2 formation in the T/T type, it seemed to activate it. The effects of α-naphthoflavone on the metabolic formation of MM-3 were basically similar to those of MM-2. The formation rate of the T/T type was only about 23% of that of the C/C type, whereas the rate in the C/T type was comparable. In addition, α-naphthoflavone lowered the formation rate of MM-3 in both the C/C and C/T types, but not in the T/T type. The formation rates of MM-4 and MM-5 in the three types were similar, with α-naphthoflavone having only a slight effect.

Immunoinhibition of the Metabolism of YM-64227. As is shown in Fig. 3, anti-CYP1A1/2 antibody inhibited the formation of MM-2 and MM-3 in the C/C and C/T types by approximately 75%. Anti-CYP1A1/2 antibody did not exert any other inhibitory effects on the formation rates in the C/C or C/T types, nor did it have any effect on the formation rates in the T/T type.

Metabolism of Phenacetin. The Michaelis-Menten and Eadie-Hofstee plots of phenacetin O-deethylation (POD), a CYP1A2 activity marker substrate, are shown in Fig. 4. Since the Eadie-Hofstee plots suggested the contribution of multiple components, the following three models were compared: 1) one saturable component and one nonsaturable component, 2) two saturable components, and 3) two
The plasma concentrations of MM-1, MM-4, and MM-5 in than CYP1A2. However, the in vitro findings do not fully explain (MM-1, MM-4, and MM-5) is catalyzed by P450 isozymes other
activities of MM-2 and MM-3 in the presence of
immunoinhibition studies using \( \alpha \)-naphthoflavone suggest that CYP1A2 contrib-
table 1

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>C/C</th>
<th>C/T</th>
<th>T/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{max}} ) (pmol/min/mg protein)</td>
<td>886.3 ± 213.0</td>
<td>837.4 ± 180.1</td>
<td>211.0 ± 62.5**</td>
</tr>
<tr>
<td>( K_m ) (( \mu )M)</td>
<td>22.3 ± 4.4</td>
<td>22.1 ± 3.5</td>
<td>13.6 ± 5.2**</td>
</tr>
<tr>
<td>( CL_{\text{int, s}} ) (( \mu )L/min/mg protein)</td>
<td>41.5 ± 14.5</td>
<td>37.2 ± 3.7</td>
<td>16.9 ± 7.3**</td>
</tr>
<tr>
<td>( CL_{\text{int, ns}} ) (( \mu )L/min/mg protein)</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
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Statistically significant differences from the C/C type are indicated as * \( p < 0.05 \) and ** \( p < 0.01 \) using the Tukey test.

saturable components and one nonsaturable component. Evaluation of fit was carried out using the Akaike information criterion (Akaire, 1969). Model 1 showed the best fit and was therefore selected for parameter calculation (Table 1). The \( V_{\text{max}} \), \( CL_{\text{int, s}} \), and \( CL_{\text{int, ns}} \) values for the T/T type were lower than those for the C/C type by 24, 41, and 43%, respectively. The \( CL_{\text{int, ns}} \) value did not differ significantly among the three types. The correlation between YM-64227 hydroxylation and POD are shown in Table 2 and Fig. 5. The \( CL_{\text{int, in vitro}} \) value for MM-2 formation, MM-3 formation, and the overall metabolism of YM-64227 correlated significantly with that of POD (\( p < 0.01 \)).

Metabolism of the Primary Metabolites of YM-64227. The \( CL_{\text{int, in vitro}} \) values for the metabolism of MM-1, MM-2, MM-3, MM-4, and MM-5 in the T/T type were lower than that in the C/C type by 50, 73, 30, 41, and 34%, respectively (Table 3). The C/T type had \( CL_{\text{int, in vitro}} \) values similar to, to slightly higher than the C/C type for the metabolism of these metabolites.

Immunoinhibition of the Metabolism of the Primary Metabolites of YM-64227. In the C/C- and C/T-type microsomes, anti-CYP1A1/2 antibody inhibited the metabolism of MM-1, MM-3, MM-4, and MM-5 by approximately 40% of the control values (Fig. 6). The metabolic activity of MM-2 in the C/C and C/T types were not affected by anti-CYP1A1/2 antibody. In the T/T type, no inhibition of metabolism was observed at all for MM-2, MM-3, MM-4, or MM-5 in the presence of anti-CYP1A1/2 antibody. In fact, the addition of anti-CYP1A1/2 antibody increased the metabolic activity of MM-1 in the T/T group to 220% of the control.

Discussion

In vitro metabolism studies of YM-64227 performed using canine liver microsomes prepared from beagle dogs genotyped with respect to the CYP1A2 deficiency polymorphism strongly suggest that CYP1A2 contributes to the formation of MM-2 and MM-3 from YM-64227. In contrast, the formation of the other metabolites (MM-1, MM-4, and MM-5) is catalyzed by P450 isozymes other than CYP1A2. However, the in vitro findings do not fully explain the pharmacokinetic profiles of the metabolites YM-64227 in vivo. The plasma concentrations of MM-1, MM-4, and MM-5 in CYP1A2-deficient dogs were higher than those in dogs expressing active CYP1A2. In contrast to MM-1, MM-4, and MM-5, no significant differences in the plasma concentrations of MM-3 were detected between dogs lacking CYP1A2 and those expressing active CYP1A2. To elucidate the effects of the CYP1A2 deficiency polymorphism on the in vitro metabolism and in vivo pharmacokinetics of YM-64227 and its metabolites, several metabolism studies were conducted in canine liver microsomes to investigate the influence of CYP1A2 to the metabolism of YM-64227 and its metabolites.

The formation rates for MM-2 and MM-3 in the T/T type were lower than those in the C/C and C/T types, whereas the formation rates of the other metabolites (MM-1, MM-4, and MM-5) were not significantly different among the three. These findings suggested that CYP1A2 plays a significant role in the formation of MM-2 and MM-3, but not in the formation of others. \( \alpha \)-Naphthoflavone, a typical CYP1A2 inhibitor (Chang et al., 1994; Mise et al., 2004b), reduced the MM-2 and MM-3 formation rates in the C/C and C/T types, but no inhibitory effects were observed in the T/T type. Thus, the metabolic activities of MM-2 and MM-3 in the presence of \( \alpha \)-naphthoflavone were comparable in the three types. The formation rates of MM-1, MM-4, and MM-5 were not inhibited by \( \alpha \)-naphthoflavone, and activation was detectable in the formation of all MM-1 types. \( \alpha \)-Naphthoflavone is a known activator of CYP3A-mediated activities (Zhao and Ishizaki, 1997; Nakajima et al., 1999), and the activation of MM-1 formation suggests the possibility that this metabolic pathway is catalyzed by homologs of CYP3A4. Although this does not fully explain the reason why MM-1 formation is activated, previous inhibition studies using \( \alpha \)-naphthoflavone suggest that CYP1A2 contributes to the formation of MM-2 and MM-3.

Results obtained from immunoinhibition studies on the C/C and C/T types support this theory. Anti-CYP1A1/2 antibody inhibited the
inhibits POD both in canine and human liver microsomes (Nakajima et al., 1997). The CL\textsubscript{int, in vitro} values of POD significantly correlated with those of the saturable component of POD, just as it is in human CYP1A2. The active CYP1A2 protein suggests that canine CYP1A2 is involved with these activities, and it was therefore difficult to calculate CL\textsubscript{int, in vitro} accurately. Thus, pooled microsomes were used to study the metabolism of the YM-64227 metabolites. The CL\textsubscript{int, in vitro} values for the metabolism of MM-1, MM-3, MM-4, and MM-5 in the T/T type were lower than those in the C/C type by less than 50%. Meanwhile, the CL\textsubscript{int, in vitro} value for the metabolism of MM-2 in the T/T type was 73% of that in the C/C type. Anti-CYP1A1/2 antibody decreased the metabolic activities of MM-1, MM-3, MM-4, and MM-5 in the C/C and C/T types to less than 60% of the control values, but the metabolic activities of MM-2 were not affected by anti-CYP1A1/2 antibody. None of the metabolic activities of the primary metabolites of YM-64227 in the T/T type were inhibited by anti-CYP1A1/2 antibody.

Figure 7 is a schematic illustration of the involvement of CYP1A2 in the formation and elimination of each YM-64227 metabolite and the effects of CYP1A2 deficiency on the AUC of each. MM-2 is formed by CYP1A2, but CYP1A2 is not involved in its elimination; consequently, the AUC of MM-2 is less in dogs that do not express CYP1A2. The formation of MM-1, MM-4, and MM-5 is independent of CYP1A2; however, it does catalyze their metabolism. CYP1A2 deficiency decreases the rates of elimination of MM-1, MM-4, and MM-5 without affecting their formation, which, in turn, leads to an increase in AUC. Unlike other metabolites, both the formation and elimination of MM-3 is catalyzed by CYP1A2. As a result, CYP1A2-deficient polymorphism apparently does not affect the AUC of MM-3 in dogs.

The pharmacokinetic profiles of drugs are affected by several factors, such as the induction and inhibition of metabolizing enzymes caused by drugs taken concomitantly, as well as genetic polymorphism. The effects of these factors on pharmacokinetics of metabolites need to be investigated, especially if the metabolites of the drugs are pharmacologically active or potentially toxic. In such cases, evaluation of the involvement of the P450 isozyme in both the formation and elimination of the metabolites can be very informative. Poor metabolizers of CYP2C9, 2C19, and 2D6 result in higher plasma concentrations of tolbutamide (Sullivan-Klose et al., 1996), debrisoquine (Kagimoto et al., 1990), and omeprazole (Furuta et al., 1996), respectively, compared with extensive metabolizers. Concurrent with an increased amount of the unchanged form of these drugs, the concentrations of their metabolites, hydroxy tolbutamide, 4-hydroxy debrisoquine, and 5-hydroxyomeprazole, and omeprazole sulfone, were all lower in poor metabolizers. These findings may be explained by the fact that P450 isoforms contribute to the metabolism of the parent drugs and do not eliminate their primary metabolites. In contrast, the dose-corrected steady-state plasma concentrations of mesoridazine, a primary metabolite of thioridazine, was not affected by the genetic polymorphism of CYP2D6, although the formation of mesoridazine that results from the metabolism of thioridazine is catalyzed by CYP2D6 (Berecz et al., 2003; Wojcikowski et al., 2006). CYP2D6 contributes to the metabolism of mesoridazine as well (Wojcikowski et al., 2006); therefore, both the formation and elimination of mesoridazine is regulated by CYP2D6. The decrease in the formation of mesoridazine is accom-
panied by a decrease in elimination; therefore, the resulting changes in the plasma concentration level are insignificant.

The contribution of CYP1A2 to the formation and elimination of the primary metabolites of YM-64227 in canine liver microsomes was investigated. The involvement of CYP1A2 in both the formation and elimination of the metabolites of YM-64227 account for the effects of the CYP1A2 deficiency polymorphism on the plasma concentration profiles in dogs. This finding is valuable as it may explain the interindividual variability seen in the metabolism of several CYP1A2 substrates in dogs.

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References


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