Inhibition of Mammalian Mitochondrial Protein Synthesis by Oxazolidinones

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The effects of a variety of oxazolidinones, with different antibacterial potencies, including linezolid, on mitochondrial protein synthesis were determined in intact mitochondria isolated from rat heart and liver and rabbit heart and bone marrow. The results demonstrate that a general feature of the oxazolidinone class of antibiotics is the inhibition of mammalian mitochondrial protein synthesis. Inhibition was similar in mitochondria from all tissues studied. Further, oxazolidinones that were very potent as antibiotics were uniformly potent in inhibiting mitochondrial protein synthesis. These results were compared to the inhibitory profiles of other antibiotics that function by inhibiting bacterial protein synthesis. Of these, chloramphenicol and tetracycline were significant inhibitors of mammalian mitochondrial protein synthesis while the macrolides, lincosamides, and aminoglycosides were not. Development of future antibiotics from the oxazolidinone class will have to evaluate potential mitochondrial toxicity.

Several classes of antibiotics function by binding to the bacterial ribosome and inhibiting bacterial protein synthesis. These include aminoglycosides, macrolides, tetracyclines, lincosamides, and chloramphenicol. Linezolid (Zyvox), an oxazolidinone recently approved for clinical use, represents an important new class of antibiotic that has been very effective in treating multidrug-resistant gram-positive pathogens (for reviews see references 5 and 20).

The mitochondrial protein synthesis machinery is in many ways similar to the prokaryotic machinery and as a result may be a target for antibiotics that function by binding to the bacterial ribosome (8). Significant evidence has shown that bone marrow suppression, often reported as a dose-dependent and reversible toxic side effect of chloramphenicol therapy in humans, is caused by inhibition of mitochondrial protein synthesis (for reviews, see references 33 and 39). The oxazolidinones have been shown to bind to the large bacterial ribosomal subunit at a site that overlaps the chloramphenicol binding site and to inhibit bacterial protein synthesis (12, 24). Thus, oxazolidinones have the potential to bind to mitochondrial ribosomes and to inhibit mitochondrial protein synthesis. Dose-dependent and reversible bone marrow suppression has been noted as a side effect of treatment with linezolid (17, 22), consistent with inhibition of mitochondrial protein synthesis, as has been noted for chloramphenicol (15, 39). Pharmacia (now Pfizer) has synthesized newer oxazolidinones with increased antibiotic potency, in particular ones that would be effective against gram-negative bacteria (6, 16). While linezolid was essentially nontoxic in a rat toxicity assay (100 mg/kg of body weight, twice daily for 30 days) (10), as noted herein, some of the newer compounds were significantly more toxic, leading to rat deaths within the 30-day assay period. We hypothesized that the animal toxicity exhibited by some of the more potent oxazolidinone antibiotics, as well as the mild side effects of linezolid, was caused by inhibiting mammalian mitochondrial protein synthesis. To test this hypothesis, a variety of oxazolidinones with widely varying degrees of antibiotic potency, including linezolid and eperezolid, were evaluated for their abilities to inhibit mitochondrial protein synthesis. These results were compared to those of other clinically approved antibiotics that function by inhibiting bacterial protein synthesis.

The mitochondrial ribosome is identical in all tissues, which suggests that antibiotics would inhibit synthesis more or less equally in all cells and could cause pathology in many tissues. However, the side effects noted for chloramphenicol and linezolid appear to preferentially target the bone marrow compartment. To address issues of tissue specificity, these compounds were tested in mitochondria isolated from a variety of tissues, including rat heart and liver and rabbit heart and bone marrow.

MATERIALS AND METHODS

Isolation and incubation of mitochondria from rat and rabbit heart and rat liver. Heart mitochondria were isolated with a Polytron-type homogenizer exactly as described previously (26). Liver mitochondria were isolated identically to those from heart except that the liver was perfused briefly in situ with cold isolation buffer to remove blood and was not perfused with Nagarse (subtilisin). All other steps in the liver mitochondrial preparation were identical to those for the heart. The intactness of each preparation was demonstrated by measuring the respiratory control ratio as previously described (26). Preparations with values of <5 (liver) or <6 (heart) were discarded.

Preparation of rabbit bone marrow mitochondria. Mitochondria were isolated from rabbit bone marrow according to the method of Abou-Khalil et al. (1). Briefly, the rabbit was euthanized with an overdose of pentobarbital (intravenously) and the long bones of all four legs were removed, cleaned of tissue, and
Mitochondrial protein synthesis assay. Mitochondria, regardless of origin, were incubated at 4 mg protein/ml in 75 ml of a previously characterized protein synthesizing medium (26). The incorporation of [35S]methionine into mitochondrial protein was determined by a filter paper disk assay as described previously (26). Each oxazolidinone and classical antibiotic was tested at widely varying concentrations. Since the oxazolidinones and most of the classical antibiotics were not very soluble in water, they were dissolved at high concentrations in dimethyl sulfoxide (DMSO) and added in a 1-μl volume (1.33%). This level was chosen from a dose-response study with DMSO that indicated that levels up to 1.5% had no effect on heart mitochondrial translation. Levels of DMSO above 1.5% became increasingly inhibitory to translation (data not shown). A rate of mitochondrial protein synthesis was calculated for each incubation by following the time course of [35S]methionine incorporation at 15, 30, 45, 60, 90, and 120 min. The rate of incorporation was typically linear through 60 min of incubation, with the exception of bone marrow mitochondria, in which the rate of incorporation was linear for 30 to 45 min. To plot the dose response for each compound, a best-fit slope through the linear portion of each time course was used to calculate a per-hour rate. The controls and vehicle controls for each mitochondrial protein was determined by a filter paper disk assay as described previously (26) using glutamate and malate as substrates.

Materials. All oxazolidinones and other antibiotics used in this study were provided by Pharmacia and Upjohn, Kalamazoo, MI.

RESULTS

Rat toxicity of oxazolidinones. Linezolid, eperezolid, and PNU-100480 were shown to be essentially nontoxic in earlier rat studies (100 mg/kg twice daily, 30 days) (10, 14). However, some of the newer oxazolidinones with considerably enhanced antibacterial potency were observed to have significantly increased toxicity in rats. For example, the rat study with PNU-140693 (2) was terminated at day 10 because of rat deaths. Four early deaths were also noted for PNU-141059. While cause of death was unknown, the animals displayed bone marrow atrophy, lymphoid atrophy, leukopenia, and decreased organ weights. An attempt to understand this increased toxicity in the rat led to the work presented below.

Time course of incorporation and dose-response curves. Typical results are illustrated in Fig. 1 for rat heart mitochondria incubated with the antibiotic chloramphenicol or with the oxazolidinone eperezolid (PNU-100592). As shown, control incorporation in rat heart mitochondria was linear for at least 60 min. The vehicle (1.33% DMSO) did not have a significant influence on mitochondrial protein synthesis. Results were similar for rabbit heart and rat liver mitochondria, while rates of incorporation in rabbit bone marrow mitochondria were...
linear for 30 to 45 min (data not shown). While a time course was followed for every compound to detect compounds that might have a delayed onset or might become inactive during incubation, these events were not detected for any of the compounds used. A best-fit slope through the linear portion of the time course was obtained for each sample as a per-hour rate, expressed as a percentage of the vehicle control. Using the data from Fig. 1, a dose response for chloramphenicol is shown as a panel of Fig. 2 and data for eperezolid are shown as a panel of Fig. 3. The dose-response curves obtained were remarkably consistent, even though the absolute rate of incorporation in the controls and vehicle controls varied significantly from preparation to preparation.

Effect of clinically approved antibiotics on mitochondrial protein synthesis. The effects of eight well-characterized antibiotics with well-described toxicities were tested in the mitochondrial assay as described above. The dose-response curves and the calculated IC₅₀ₐₕs are shown in Fig. 2, with each symbol representing a separate experiment for heart (closed symbols) and liver (open symbols) mitochondria. Of these eight, kasugamycin, lincomycin, clindamycin, streptomycin, azithromycin, and erythromycin had little or no effect on mitochondrial protein synthesis from either tissue, with IC₅₀ₐₕs of >400 µM. Tetracycline was most inhibitory with IC₅₀ₐₕ of 2.1 µM in both heart and liver, while the IC₅₀ₐₕ of chloramphenicol were 9.8 and 11.8 µM in heart and liver, respectively. None of the antibiotics tested had any effect on mitochondrial respiration or coupling (data not shown).

Effect of oxazolidinones on mitochondrial protein synthesis. The time courses for incorporation of [³⁵S]methionine into mitochondrial protein were determined, and dose-response curves for eight different oxazolidinones of various antibiotic potencies were constructed (Fig. 3). Structures and MICs of selected oxazolidinones used in Fig. 3 are shown in Table 1. The symbols in each dose-response curve in Fig. 3 represent a separate experiment, closed for heart and open for liver, demonstrating that results were quite reproducible between mitochondrial preparations and that there was no significant difference between heart and liver mitochondrial data. The results also show that the curve-fitting procedure described in Materials and Methods fitted the data well. IC₅₀ values for the representative set of oxazolidinones shown in Fig. 3 range from 0.60 to 17.9 µM. PNU-140693 and PNU-141059 are shown here to be potent inhibitors of mitochondrial protein synthesis with an IC₅₀ less than 1/10 of that of linezolid. As noted above, both PNU-140693 and PNU-141059 were also considerably more toxic in rats than linezolid. Compared to linezolid, the antibacterial activity of PNU-140693 was four times more potent against gram-positive strains (Table 1) and four times more potent against Escherichia coli, in vitro studies (2), and PNU-140693 had a threefold-lower IC₅₀ for inhibiting bacterial cell-free translation (2).
The effect of oxazolidinones on mitochondrial protein synthesis is stereospecific. Published reports have established that the $5S$ configuration (noted by the arrow for eperezolid in Table 1) is necessary for the antibacterial activity of oxazolidinones (5) and for binding to the bacterial ribosome (40). If the compounds bind to the mitochondrial ribosome in the same way, then the inhibition of mitochondrial protein synthesis should have the same $5S$ configuration requirement. Four pairs of oxazolidinones were tested in which each pair differed only in the $S$ or $R$ configuration of carbon 5 (selected structures shown in Table 1). The data in Table 2 clearly demonstrate that only the $S$ configuration is active in inhibiting mitochondrial protein synthesis. These results correlate well with results using H nuclear magnetic resonance to test the binding of eperezolid ($5S$)/PNU-107112 ($5R$) and PNU-177553 ($5S$)/PNU-184414 ($5R$) to the bacterial ribosome (40).

Correlation of oxazolidinone inhibition of mitochondrial translation with antibiotic potency. If oxazolidinone toxicity is related to a ribosomal binding site that is highly similar in both bacteria and mitochondria, then one would predict that antibiotic potency and mitochondrial inhibition of protein synthesis should correlate. Antibiotic potency was measured by the MIC against three standard strains of bacteria as described in Materials and Methods. Consistent with the hypothesis, we noted that all of the oxazolidinones that were poor mitochondrial protein synthesis inhibitors ($IC_{50}$ values of $>30 \mu M$, $n = 6$) were also not effective as antibiotics at the highest doses tested and accurate MICs were not determined. To further correlate antibiotic activity with mitochondrial protein synthesis inhibition, we selected 36 different oxazolidinones of various abilities to inhibit mitochondrial protein synthesis with $IC_{50}$s between 0.22 and 25.7 $\mu M$ (including the eight used in Fig. 3) and compared their MICs to the mitochondrial protein synthesis inhibition $IC_{50}$ values for each bacterial strain (Fig. 4). While there is considerable scatter in the data, there was a weak correlation between the two measurements. However, the main reason for this correlation is the fact that, as the antibiotic potency of oxazolidinones increased (moving toward the left side of each panel), the spread in the observed mitochondrial protein synthesis $IC_{50}$s decreased dramatically. Oxazolidinones with the highest antibacterial potency (lowest MICs) were uniformly potent in inhibiting mitochondrial protein synthesis (lowest $IC_{50}$s). Conversely, as you move to the right in each panel the spread in the data increased dramatically. This is not a surprising finding, since oxazolidinones that are strong inhibitors of mitochondrial protein synthesis could be weak antibiotics because they lack other important requirements that prevent them from reaching the bacterial ribosome, including absorption, distribution, metabolism, and avoidance of efflux pumps. Four specific oxazolidinones are identified in Fig. 4: 1 is PNU-97456 (10), 2 is linezolid (10), 3 is PNU-100480 (7, 14), and 4 is PNU-140693 (2). The first three were found in this
investigation to be modest inhibitors of mitochondrial protein synthesis with IC₅₀ values of >10 μM and were reported to be essentially nontoxic in 30-day rat studies (10, 14). Of these three, linezolid is in present use and PNU-100480 (7) has been proposed as a possible treatment for *Mycobacterium tuberculosis* infection (14). The fourth, PNU-140693, is a member of the group with significantly increased antibacterial activity and a potent inhibitor of mitochondrial protein synthesis (IC₅₀, 0.95 μM). As noted earlier, this compound was also quite toxic in the 30-day rat study.

**Effects of oxazolidinones on mitochondrial protein synthesis in mitochondria from rabbit bone marrow.** The side effects noted for chloramphenicol, and toxicities noted for members of the oxazolidinone family, tended to be observed first as suppression within the bone marrow compartment. The goal here was to determine if this initial toxicity was related to

<table>
<thead>
<tr>
<th>Oxazolidinone (reference[s])</th>
<th>MIC* (μg/ml)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. pneumoniae</em></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>Eperezolid 5S (10, 40)</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Linezolid 5S (10)</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>PNU-97456 5S (10)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>PNU-140693 5S (2)</td>
<td>0.125</td>
<td>1</td>
</tr>
<tr>
<td>PNU-108812 5S (16)</td>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>PNU-177553 5S (40)</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>PNU-184414 5R (40)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PNU-107112 5R (40)</td>
<td>&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>PNU-100480 5S (7)</td>
<td>0.5</td>
<td>2</td>
</tr>
</tbody>
</table>

*IC₅₀ values were determined as described for Fig. 3. Structures of eperezolid, PNU-1107112, PNU-177553, and PNU-184414 and MICs are shown in Table 1.

**TABLE 2. Oxazolidinone inhibition of rat heart mitochondrial protein synthesis is stereospecific**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5S compounds</strong></td>
<td></td>
</tr>
<tr>
<td>PNU-177553</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Eperezolid</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>PNU-141659</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>PNU-179759</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td><strong>5R compounds</strong></td>
<td></td>
</tr>
<tr>
<td>PNU-184414</td>
<td>161 ± 46</td>
</tr>
<tr>
<td>PNU-107112</td>
<td>181 ± 48</td>
</tr>
<tr>
<td>PNU-184415</td>
<td>Not detected</td>
</tr>
<tr>
<td>PNU-244967</td>
<td>65 ± 8</td>
</tr>
</tbody>
</table>

*IC₅₀ were determined as described for Fig. 3. Structures of eperezolid, PNU-1107112, PNU-177553, and PNU-184414 and MICs are shown in Table 1.
marrow-specific differences in the oxazolidinone inhibition of mitochondrial protein synthesis or, alternatively, whether oxazolidinone inhibition was the same across mitochondria from all tissues but the pathological outcome was realized first within the rapidly dividing cells of the bone marrow. Since mitochondria could not be prepared from rat bone marrow, rabbit bone marrow was used and compared to rabbit heart mitochondria to control for the species difference. Isolation of mitochondria from bone marrow was difficult, and the respiratory control ratios varied from 2.7 to 3.7, versus 9.7 ± 1.1 for rabbit heart. Protein synthesis in isolated rabbit heart mitochondria was similar to that in rat heart mitochondria, while synthesis in isolated bone marrow mitochondria was less robust, being linear for only 30 to 45 min and reaching levels that were 45% lower (P < 0.005) than that of rabbit heart mitochondria. A group of representative oxazolidinones was tested on bone marrow mitochondria and the results compared to mitochondria isolated at the same time from rabbit heart (Table 3). With the exception of compound PNU-143702, which was not very active, there was no significant difference in the IC50 values between rabbit and rat heart mitochondria. However, the IC50 values obtained from bone marrow mitochondria were all somewhat higher than those from rabbit heart mitochondria, three compounds reaching significance (P < 0.05). Although these higher values may be related to the decreased quality of the bone marrow preparation associated with a decreased control rate of protein synthesis, oxazolidinones may have a lesser effect on bone marrow mitochondria rather than preferential toxicity. Thus, the reduction in mitochondrial synthetic function, as a result of exposure to oxazolidinones, may be similar in all tissues but may compromise the function of the rapidly growing bone marrow compartment first.

**DISCUSSION**

The results presented here demonstrate that a general feature of oxazolidinones with antibacterial properties is the ability to inhibit mitochondrial protein synthesis. The data further indicate that oxazolidinones with the strongest antibacterial properties (lowest MICs) are also the most potent inhibitors of mitochondrial protein synthesis (lowest IC50s) (Fig. 4). Thus, the site on the bacterial ribosome that binds an oxazolidinone appears to be highly conserved on mitochondrial ribosomes. However, some oxazolidinones that were potent inhibitors of mitochondrial protein synthesis were not especially good as antibacterial agents. Since oxazolidinones readily enter isolated mitochondria (E. McKee and M. Ferguson, unpublished data), the degree of inhibition is likely to be determined by their respective abilities to bind and inhibit the mitochondrial ribosome. However, many of these oxazolidinones may not readily access the bacterial ribosome. A clear example is shown by the fact that linezolid is ineffective as an antibiotic against *E. coli*. Yet the mechanism of action of linezolid was demonstrated by studying the binding of this antibiotic to *E. coli* ribosomes (2, 5). Since linezolid sensitivity could be conferred on an *E. coli* strain that contained an inactive AcrAB transmembrane pump via site-directed mutagenesis (5), an insufficient intracellular concentration is likely responsible for the lack of efficacy against *E. coli*. Thus, some oxazolidinones may contain structures that prevent uptake, stimulate active removal, or result in inactivation by bacterial enzymes.

Of the eight clinically approved antibiotics studied, only chloramphenicol and tetracycline inhibited protein synthesis in

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**TABLE 3. Effect of oxazolidinones on protein synthesis in mitochondria from rabbit heart and bone marrow**

<table>
<thead>
<tr>
<th>Oxazolidinonea</th>
<th>IC50 (µM) for tissue:</th>
<th>Rat heart</th>
<th>Rabbit heart</th>
<th>Rabbit bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNU 97786</td>
<td>1.1 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>2.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>PNU-93936A</td>
<td>2.1 ± 0.4</td>
<td>2.9 ± 0.1</td>
<td>6.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Eperezolid</td>
<td>4.2 ± 0.4</td>
<td>5.7 ± 1.4</td>
<td>14.9 ± 1.96</td>
<td></td>
</tr>
<tr>
<td>PNU-100480</td>
<td>11.8 ± 2.6</td>
<td>8.3 ± 1.0</td>
<td>18.6 ± 2.96</td>
<td></td>
</tr>
<tr>
<td>PNU-143702</td>
<td>184 ± 24</td>
<td>77 ± 10</td>
<td>192 ± 36</td>
<td></td>
</tr>
</tbody>
</table>

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*a* The structures and MICs of eperezolid and PNU-100480 are shown in Table 1.

*b* P < 0.05 compared to the IC50 of rabbit heart mitochondria.

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**FIG. 4.** Correlation of inhibition of mitochondrial protein synthesis with antibiotic potency. The IC50 for mitochondrial protein synthesis of each oxazolidinone in a varied group of 36 oxazolidinones was compared to its MIC (obtained from Pharmacia and Upjohn) against three different strains of bacteria as described in Materials and Methods. (Left panel) Penicillin-susceptible *S. pneumoniae* 9912; (middle panel) *S. aureus* 9213; (right panel) ampicillin-resistant *H. influenzae* 30063. The line in each plot represents a linear regression analysis of the relationship of the two measurements. Values for r2 equal 0.568 (left panel), 0.470 (middle panel), and 0.469 (right panel). 1, PNU-97456 (10); 2, linezolid (5, 10); 3, PNU-100480 (7, 14); 4, PNU-140693 (2).
intact mitochondria. Chloramphenicol has been associated with three well-established toxicities: (i) a dose-dependent and reversible bone marrow suppression, (ii) so-called gray baby syndrome observed in infants given high doses of chloramphenicol (100 mg/kg/day), and (iii) fatal aplastic anemia in certain genetically sensitive individuals (1 in 25,000 to 40,000) (15, 39). The effect of chloramphenicol on mitochondrial protein synthesis has been well documented, and both the bone marrow suppression and gray baby syndrome have generally been accepted to be caused by inhibition of mitochondrial protein synthesis (39). Tetracycline was previously shown to inhibit mitochondrial protein synthesis in a variety of systems (21, 30, 34, 35, 36). The tetracyclines have been associated with a host of toxicities (32), but the degree to which these toxicities are the result of inhibition of mitochondrial protein synthesis is unknown. Bacteria that are susceptible to the tetracyclines typically concentrate the antibiotic (11), which does not occur in mammalian cells (34). This difference may provide a margin of safety for human cells. The most common side effects of linezolid are nausea, diarrhea, and headache (20). Less common, but more serious, are several toxicities that are quite likely to be of mitochondrial origin. These include dose-dependent and reversible bone marrow suppression, most often leading to anemia and thrombocytopenia, analogous to that observed with chloramphenicol (17, 20, 22). Linezolid has also been associated with lactic acidosis (3) and peripheral and optic neuropathy (9, 23).

Chloramphenicol and linezolid are most often associated with bone marrow suppression, yet, as demonstrated in this study, these drugs inhibit protein synthesis more or less equally in all tissues tested. One potential explanation for this observation is that tissues like the bone marrow may concentrate these drugs within the tissue. However, both chloramphenicol and linezolid are lipophilic small molecules that are reported to have high volumes of distribution and excellent tissue penetration (4, 25). While levels of linezolid in bone marrow have not been reported, levels of chloramphenicol tend to be lower in bone marrow than in other tissues (4). Therefore, it seems more likely that the relationship between inhibition of mitochondrial protein synthesis and specific tissue toxicity is related to the overall rate of mitochondrial biogenesis specific for each tissue and each tissue’s energy demands. Typical therapeutic doses of chloramphenicol, tetracycline, and linezolid yield blood and tissue levels (4, 25, 34) of antibiotic that are at, or in some cases above, the IC_{50} values for inhibiting mitochondrial protein synthesis observed in this study. Thus, there is significant potential for toxic problems related to mitochondrial biogenesis. The extent to which tissues are able to compensate for this inhibition is unknown.

The development of new oxazolidinone compounds as antibacterials (6, 16, 18, 19, 29, 31, 37, 38) and as monooxygenase A inhibitors (13, 27) remains an active area of research involving several companies. However, since the results of the study reported here indicate that oxazolidinones that are highly potent as antibacterials are likely to be potent inhibitors of mitochondrial protein synthesis and may display increased toxicity in animals, a strong case can be made for evaluating the effects on mitochondria during the development of drugs of the oxazolidinone class.

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REFERENCES


