EFFECTS OF ROKITAMYCIN ON YOUNG RATS
WITH HYPERBILIRUBINEMIA
— DETERMINATION OF UNBOUND AND BRAIN BILIRUBIN
LEVELS AND EXAMINATION FOR LOCALIZED
YELLOW DISCOLORATION OF BRAIN TISSUE —

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Abstract——The effects of rokitamycin (RKM), a macrolide antibiotic,
on young rats with hyperbilirubinemia were investigated. RKM at a dose of
1,000 mg/kg was orally administered to 14-day-old rats with hereditary,
non-hemolytic hyperbilirubinemia (homozygous Gunn rats, total plasma
bilirubin concentration : about 7 mg/dl). Animals given 10 ml/kg of 0.5%
carboxymethyl cellulose (CMC) were used as control.

Plasma total bilirubin concentration, plasma unbound bilirubin concen-
tration and cerebellar bilirubin level did not significantly change during 1, 3,
6 and 24 hours after administration of RKM or CMC. There was no
significant difference in plasma total bilirubin concentration, plasma un-
bound bilirubin concentration and cerebellar bilirubin level between RKM-
treated and control animals at 1, 3, 6 and 24 hours after the administration.
No localized yellow discoloration of the brain tissue (non-cerebellar parts)
was noted at 1, 3, 6 and 24 hours after administration of either RKM or
CMC.

Key words : Rokitamycin, Gunn rat, hyperbilirubinemia, unbound bili-
rubin, cerebellar bilirubin level, kernicterus.

INTRODUCTION

Some drugs contribute to the promotion of bilirubin transfer from the blood to
the brain tissue by competing with bilirubin for binding to albumin (Brodersen,

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Sulfisoxazole administered to human premature infants (Silverman et al., 1956) and sulfisoxazole, sulfadimethoxine (Johnson et al., 1961; Schutta and Johnson, 1969), sulfadiazine (Johnson et al., 1961), salicylate (Johnson et al., 1961), and bucolome (Semba et al., 1978) administered to rats with hyperbilirubinemia (homozygous Gunn rats) have been reported to induce kernicterus (bilirubin encephalopathy). Therefore, for drugs that may be administered to neonates, mothers during lactation, pregnant women, or women immediately before delivery, animal experiments should confirm that they do not increase the blood unbound bilirubin and brain bilirubin concentrations or induce kernicterus.

In this study, we evaluated the effects of rokitamycin (RKM), a macrolide antibiotic, on these bilirubin levels and brain tissue.

**MATERIALS AND METHODS**

1. **Test drug.**

RKM is 3”-propionyl-leucomycin A5 (code number: TMS-19-Q) produced by propionylation of the 3” position of leucomycin A5 that is a composition of kitasamycin, a macrolide antibiotic. The chemical structure is shown in Fig. 1.

This agent is a white to light yellow powder and nearly insoluble in water. For experiment, RKM (Lot: TMS-25) suspended in 0.5% CMC solution was used.

2. **Animals and maintenance conditions.**

Homozygous Gunn rats with lifetime hyperbilirubinemia occurring several hours after birth and heterozygous Gunn rats with normal blood bilirubin concentrations, the lineage of which has been maintained in the Second Department of Anatomy, Mie University School of Medicine, were used. The homozygous rats were produced by non-sib mating between a male homozygote and a female heterozygote in the 8–9th generation produced by sib mating. The day of birth was designated as day 0 after birth. The infants were nursed by their mothers. However, when the number of infants was more than 8, heterozygotes were removed to adjust the number to 8.

The animals were maintained in an animal room at a temperature of 22 ± 1°C.
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with a humidity of 55 ± 15% and a light/dark cycle of 14/10 hours (light period: 8 a.m. -10 p.m.). Diets of pellets for mice and rats (CMF, Oriental Yeast Mfg. Ltd.) and tap water purified using a water-purifying device were given ad libitum.

3. Experimental methods.

According to the schedule shown in Fig. 2, RKM or CMC was administered, and blood was collected immediately before and at various times after the administration. Immediately after the second blood collection, animals were perfused, and the brain was removed. The homozygous rats were divided at 5 p.m. 13 days after birth into 5 groups: Group I, untreated rats; II, rats undergoing blood collection 1 hour after the dosing; III, rats undergoing blood collection after 3 hours; IV, rats undergoing blood collection after 6 hours; and V, rats undergoing blood collection after 24 hours. Two rats of the same litter with similar weights were allocated to the same group as a pair, and one was treated with RKM and the other with CMC. Lean infants (body weight: under 20 g) with poor general condition were excluded.

1) Time, dose, and route of administration.

At 9 a.m. 14 days after birth, RKM (1,000 mg/kg) or 0.5% CMC solution as a control (10 ml/kg) was orally administered. After the administration, infants were returned to their mothers. The infants were nursed and thus not in the fasting state before as well as after the dosing. The timing of administration was determined by the following reasons. The human neonatal period is considered to correspond to the period between 7-14 days after birth in rats (PMA Guidelines, 1981). In

![Fig. 2 Schedule of experiment]
addition, in this study, cerebellar bilirubin concentrations were measured, and kernicterus in non-cerebellar regions of the brain was examined. In experiments of induction of kernicterus in Wistar rats, localized yellow discoloration caused by bilirubin has been reported to occur in the cerebellum and a limited non-cerebellar part of the brain following treatment 13–15 days or 20–22 days after birth (Nakatsu et al., 1976; Yamamura and Torii, 1981). In terms of the administration dose of RKM, the LD₅₀ following oral RKM administration to Wistar rats 10 days and 5 weeks after birth were 10,000 mg/kg or more (unpublished data in Research Laboratories, Toyo Jozo Co., Ltd.) and 4,000 mg/kg or more (Matsumoto et al., 1984), respectively. Based on these findings and for easy administration of this agent suspended in 0.5% CMC solution in a volume of 10 ml/kg to rats weighing 25 g using a thin esophageal tube, the dose was determined to be 1,000 mg/kg.

2) Blood collection.

Blood (120 μl) was collected from each animal via the caudal blood vessel into a heparinized capillary glass tube immediately before as well as 1 (Group II), 3 (Group III), 6 (Group IV) or 24 hours (Group V) after the dosing. In the untreated group (Group I), blood was collected only at 9 a.m. 14 days after birth. The blood in the capillary glass tube was centrifuged by a centrifuge for hematocrit at 12,000 rpm for 5 minutes to obtain plasma. For removal of turbidity of the plasma, the glass tube was cut, and the portion containing the plasma was centrifuged again using the same centrifuge at 12,000 rpm for 10 minutes.

3) Determination of plasma total and unbound bilirubin concentrations.

The plasma total bilirubin was determined by the diazo method described by Malloy and Evelyn (1937). The unbound bilirubin was measured by the method described by Jacobsen and Wennberg (1974), but bovine albumin was used instead of human serum albumin (fraction V). The peroxidase rate constant (Kp) and oxidation rate of albumin-bound bilirubin were 4.1 ΔA/min/μM of bilirubin and 0.0011 ΔA/min/μM of bound bilirubin, respectively.

4) Quantification of cerebellar bilirubin.

Immediately after the second blood collection (blood collection at 9 a.m. 14 days after birth in Group I), infants were perfused with physiological saline via the heart for 15 minutes under ether anesthesia. After cerebral blood was removed, the brain was excised. The cerebellum was isolated, and the wet weight was measured. Extraction and quantification of cerebellar bilirubin was done by the method described by Katoh et al (1975). The recovery rate of bilirubin from the cerebellum was 90–95%. The non-cerebellar portion of the brain was immersed in 10% formalin and stored in a refrigerator for morphological evaluation.

5) Morphological evaluation of the brain.

Frontal sections at 0.5–0.8 mm of the non-cerebellar portion that was immersed in 10% formalin immediately after the removal and stored in a refrigerator for 3–5 days were prepared and observed by a dissecting binocular microscope.

4. Statistical analysis.
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Mean values were compared by Student's t-test.

RESULTS

1. Body weight.

The mean body weights immediately before and 1, 3, 6 or 24 hours after the dosing (Groups II–V), and the mean difference between these values are shown in Table 1. In any group, no significant change in the mean weight was observed after the dosing in the treated as well as control animals, and no significant difference was observed in the weight immediately before the dosing, that after the dosing, or their difference between the treated and control animals.

Table 1 Change in body weight of homozygous Gunn rats after oral administration of 1,000 mg/kg of RKM or 10 ml/kg of CMC at 9 a.m. on day 14.

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immed. before adm. (A)</td>
<td>1 h. after adm. (B)</td>
<td>Difference (B – A)</td>
</tr>
<tr>
<td>RKM</td>
<td>24.75±2.45</td>
<td>24.64±2.25</td>
<td>-0.11±0.33</td>
</tr>
<tr>
<td>1,000 mg/kg</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>Control</td>
<td>24.77±2.50</td>
<td>24.77±2.23</td>
<td>0.00±0.41</td>
</tr>
<tr>
<td>(0.5% CMC)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

Immed. before adm. : Immediately before administration. h. : hour(s). Values are mean (g) ± S.D. Number of animals examined is given in parentheses.

Table 1 (continued)

<table>
<thead>
<tr>
<th>Group</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immed. before adm. (A)</td>
<td>6 h. after adm. (B)</td>
</tr>
<tr>
<td>RKM</td>
<td>25.22±2.34</td>
<td>25.52±2.44</td>
</tr>
<tr>
<td>1,000 mg/kg</td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>Control</td>
<td>25.74±1.76</td>
<td>26.18±2.03</td>
</tr>
<tr>
<td>(0.5% CMC)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

2. Plasma total bilirubin concentration.

Table 2 shows the mean plasma total bilirubin levels immediately before and 1, 3, 6 or 24 hours after the dosing (Groups II–V), and the mean difference between these values. No significant difference was observed in the mean level before as well as after the dosing between the RKM-treated animals and the control animals in any group. In addition, no significant change was observed in the plasma total bilirubin level after the dosing in any rat in any group. However, the mean difference...
between the pre- and post-administration levels in the RKM-treated animals that underwent blood collection 1 and 24 hours after the dosing (Groups II and V) significantly differed from that in the control animals (p<0.05).

3. Plasma unbound bilirubin concentration.

The mean plasma unbound bilirubin levels immediately before and 1, 3, 6 or 24 hours after the dosing (Groups II–V), and the mean difference between these values in the same rat are shown in Table 3. No significant difference was found in the pre- and post-administration level as well as their difference between the RKM-treated animals and the control animals in any group. In addition, the plasma unbound bilirubin level immediately before the dosing did not significantly differ from that after the dosing in any rat in any group.

4. cerebellar bilirubin level.

Table 4 shows the mean cerebellar bilirubin levels in the untreated animals at 9 a.m. 14 days after birth (Group I) and in the treated animals 1, 3, 6 or 24 hours after the dosing (Groups II–V). No significant difference was observed in the mean level between the RKM–treated animals and the control animals in any group. In addition, no significant difference was seen between the RKM–treated rats killed at each time after the dosing (Groups II–V) and the controls killed at 9 a.m. 14 days after birth.
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**Table 3** Change in plasma unbound bilirubin concentration of homozygous Gunn rats after oral administration of 1.000mg/kg of RKM or 10ml/kg of CMC at 9 a.m. on day 14.

<table>
<thead>
<tr>
<th>Group</th>
<th>Immed. before adm. (A)</th>
<th>1 h. after adm. (B)</th>
<th>Difference (B - A)</th>
<th>3 h. after adm. (B)</th>
<th>Difference (B - A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKM 1,000mg/kg</td>
<td>32.15±8.49</td>
<td>29.42±6.19</td>
<td>-2.74±9.72</td>
<td>31.16±17.02</td>
<td>-1.17±10.98</td>
</tr>
<tr>
<td>Control (0.5% CMC)</td>
<td>33.75±12.58</td>
<td>25.45±5.99</td>
<td>-8.30±10.13</td>
<td>29.75±19.52</td>
<td>-2.70±14.05</td>
</tr>
</tbody>
</table>

Values are mean (µg/dℓ) ± S.D. Number of animals examined is given in parentheses.

**Table 3 (continued)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immed. before adm. (A)</th>
<th>6 h. after adm. (B)</th>
<th>Difference (B - A)</th>
<th>24 h. after adm. (B)</th>
<th>Difference (B - A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKM 1,000mg/kg</td>
<td>32.15±8.49</td>
<td>29.42±6.19</td>
<td>-2.74±9.72</td>
<td>31.16±17.02</td>
<td>-1.17±10.98</td>
</tr>
<tr>
<td>Control (0.5% CMC)</td>
<td>33.75±12.58</td>
<td>25.45±5.99</td>
<td>-8.30±10.13</td>
<td>29.75±19.52</td>
<td>-2.70±14.05</td>
</tr>
</tbody>
</table>

**Table 4** Change in cerebellar bilirubin level of homozygous Gunn rats after oral administration of 1,000mg/kg of RKM or 10ml/kg of CMC at 9 a.m. on day 14.

<table>
<thead>
<tr>
<th>Group</th>
<th>Untreated (9 a.m.)</th>
<th>1 h. after adm. (µmol/g)</th>
<th>3 h. after adm. (µmol/g)</th>
<th>6 h. after adm. (µmol/g)</th>
<th>24 h. after adm. (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKM 1,000mg/kg</td>
<td>2.63±0.45</td>
<td>2.92±0.84</td>
<td>2.65±0.40</td>
<td>2.63±0.73</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean (µmol/g) ± S.D. Number of animals examined is given in parentheses.

5. **Localized yellow discoloration.**

Localized yellow discoloration was not observed in any of the serial sections (0.5–0.8mm) of the non-cerebellar portion of the brain from any RKM-treated as well as control animal in any group.
DISCUSSION

Some antibiotics (including those that are not presently used) such as cephalothin (Malaka-Zafiri and Strates, 1969), novobiocin (Malaka-Zafiri and Strates, 1969; Bratlid, 1972a; Windorfer and Karitzky, 1975), rifamycin (Malaka-Zafiri and Strates, 1969), gentamicin (Stern, 1972), oxacillin (Malaka-Zafiri and Strates, 1969; Stern, 1972; Windorfer and Mihailova, 1973; Windorfer and Karitzky, 1975), cloxacillin (Bratlid, 1972a), and carbenicillin (Bratlid, 1972a) have been demonstrated in vivo or in vitro to compete with bilirubin for binding to albumin. In this study, RKM, a macrolide antibiotic, was orally administered to homozygous Gunn rats with hyperbilirubinemia 14 days after birth, but the unbound bilirubin concentration was not significantly increased, suggesting that competition of RKM with bilirubin is extremely weak or negligible. This finding is compatible with the results of in vitro experiments on RKM effects on the bilirubin-albumin binding that were performed in parallel with this test; when the plasma from hyperbilirubinemic homozygous Gunn rats, the serum from a newborn infant with icterus gravis neonatorum and the bilirubin-added sera separated from the human cord blood were incubated with RKM at the concentration from 10 to 500 µg/ml, the unbound bilirubin concentration was not increased in any incubation mixture (Yamamura et al., in preparation). To our knowledge, macrolide antibiotics with effects on the bilirubin-albumin binding have not been reported, yet there is no evidence that the microlide antibiotics do not affect this binding.

Oral administration of RKM did not significantly increase the cerebellar bilirubin concentration. In addition, localized yellow discoloration was not observed in the section of non-cerebellar parts of the brain in any of the examined rats. Therefore, oral RKM administration may not promote penetration of bilirubin into the brain at least in homozygous Gunn rats 14 days after birth. Kernicterus can be experimentally induced in normal Wistar rats 0 or 20 days after birth by induction of hyperproduction of bilirubin by hemolysis, inhibition of glucuronidation of unconjugated bilirubin, and inhibition of bilirubin-albumin binding (Yamamura et al., 1974; Yamamura and Torii, 1981). When these treatments are given to rats 13–15 days after birth, yellow discoloration due to bilirubin occurs in the parafascicular nucleus, subthalamic nucleus, inferior olivary nucleus, nuclei of the oculomotor nerve, nucleus of the trochlear nerve, vestibular nuclear complex, intracerebellar nuclei, and cerebellar cortex (internal granular layer) (Nakatsu et al., 1976; Yamamura and Torii, 1981). Subcutaneous administration of bucolome to 15-day-old homozygous Gunn rats also induces yellow discoloration in the subthalamic nucleus, some thalamic nuclei, the floor of the fourth ventricle, and nucleus colliculi inferioris (Semba et al., 1978). Therefore, administration of the test drug to rats with hyperbilirubinemia 14 days after birth followed by determination of cerebellar bilirubin concentrations and examination for localized yellow discoloration in non-cerebellar parts seems to be appropriate to evaluate kernicterus induction by the
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drug.

Kernicterus has been considered to be caused by transfer of blood unbound bilirubin to the brain (Diamond and Schmid, 1966; Odell, 1970; Zamet and Chunga, 1971; Maisels, 1972; Bratlid, 1972b; Yamamura and Torii, 1981). On the basis of this theory, no significant increase in cerebellar bilirubin concentrations and absence of localized yellow discoloration following oral RKM administration in this study are consistent with unchanged unbound bilirubin levels. However, recently, children with low blood unbound bilirubin levels who showed kernicterus were reported (Ritter et al., 1982). These children were premature infants with a birth weight of 1,500 g or less and developed acidosis, hypoxemia, or hypothermia before the blood unbound bilirubin concentration reached the maximum. Experimentally, blood bilirubin and albumin or albumin-bound bilirubin has been shown to be transported to the brain by increasing blood osmolality (Levine et al., 1982; Bratlid et al., 1983) or inducing respiratory acidosis by allowing inhalation of excessive CO₂ (Bratlid et al., 1984). This is considered to result from opening of the blood-brain barrier for albumin-bound bilirubin. On the other hand, since uptake of 2-deoxyglucose into isolated brain capillaries was inhibited by bilirubin in a noncompetitive manner (Katoh-Semba and Kashiwamata, 1980), functional impairment of the brain capillaries by bilirubin was suggested to contribute to the occurrence of kernicterus (Kashiwamata et al., 1984). Another study suggested an association between augmentation in blood flow in the subcortical regions of the brain and deposition of unbound bilirubin in these regions (Burgess et al., 1985). Thus, there are various factors associated with the development of kernicterus. Therefore, to evaluate the safety of a drug that might contribute to the occurrence of this disease, in addition to examination of the competition of the drug with bilirubin for binding to albumin, promotion of the penetration of bilirubin into the brain and occurrence of localized yellow discoloration should be evaluated following administration of the drug to animals with hyperbilirubinemia.

Our results show that RKM is not likely to induce kernicterus in homozygous Gunn rats with hyperbilirubinemia.

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