Inhibition of PKC β by Oral Administration of Ruboxistaurin Is Well Tolerated and Ameliorates Diabetes-Induced Retinal Hemodynamic Abnormalities in Patients

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PURPOSE. To assess ocular and systemic safety and pharmaco-dynamic effects of the oral PKC β selective inhibitor ruboxistaurin (RBX; LY333531) mesylate in patients with diabetes.

METHODS. This was a double-masked, placebo-controlled, parallel, randomized, single-center clinical study evaluating the effect of oral administration of RBX (8 mg twice a day, 16 mg per day, or 16 mg twice a day) or placebo for 28 days in patients with no or very mild diabetic retinopathy on mean retinal circulation time (RCT), retinal blood flow (RBF), treatment-emergent adverse events, and other safety parameters.

RESULTS. Twenty-nine persons aged 18 to 65 years with type 1 or 2 diabetes were evaluated. The only treatment-emergent adverse event with a statistically significant difference among treatment groups was abdominal pain, which was more common in placebo-treated subjects (P = 0.049). Statistically significant effects of RBX were observed on several hematologic and laboratory parameters, but values were within the normal reference range and none of the changes was deemed clinically meaningful. In patients receiving 16 mg RBX twice daily, the diabetes-induced increase in RCT was ameliorated, with a baseline-to-endpoint difference of −0.84 seconds (P = 0.046) relative to placebo. Increasing RBX dose was linearly associated with greater effect on RCT (P = 0.05). Similar results were observed with RBF.

CONCLUSIONS. RBX was well tolerated at doses up to 16 mg twice daily for 28 days in patients with diabetes. It ameliorated diabetes-induced RCT abnormalities. No serious safety problems were identified in this patient population. Compared with prior published data, these findings represent the first direct human evidence of both bioavailability of RBX to retinal vessels and amelioration of diabetes-induced retinal hemodynamic abnormalities by an oral PKC β inhibitor. (Invest Ophthalmol Vis Sci. 2006;47:86–92) DOI:10.1167/iovs.05-0757

The link between metabolic alterations in diabetes mellitus and the incidence of diabetic microvascular complications, such as neuropathy, retinopathy, and nephropathy, has long been suspected.1–3 However, the molecular mechanisms underlying these pathologic processes are incompletely understood. Substantial data suggest that diabetes-induced activation of protein kinase C (PKC) is an important event in the genesis of these microvascular abnormalities.4–9

Hyperglycemia increases de novo synthesis of diacylglycerol (DAG), a physiological activator of PKC. Diabetes activates multiple isoforms of PKC in animals. However, the PKC-β2 isoform is preferentially activated in tissues that usually demonstrate diabetes-induced damage such as retina, nerve, kidney, aorta, and heart.4,6,10

Activation of PKC-β is associated with basement membrane thickening, leukocyte vascular adhesion, vascular permeability, and prolonged retinal circulation time.6,9–11–15 All classic pathologic changes observed in diabetes. In addition, PKC-β activity is critical for vascular endothelial growth factor (VEGF) signaling14 and VEGF is believed to be the primary mediator of retinal vascular permeability in diabetic macular edema and intraocular neovascularization in proliferative diabetic retinopathy.15,16 As well as being involved in a variety of other ischemic retinal disorders.17,18 The PKC-β inhibitor ruboxistaurin (RBX; LY333531) mesylate and its single equipotent metabolite N-desmethyl-RBX19–21 have been shown to ameliorate diabetes-induced abnormalities in retinal circulation in the rat.21 In other animal models, RBX reduced intraocular neovascularization caused by retinal ischemia22 and suppressed VEGF-induced retinal vascular permeability.18 It also decreased leukocyte adhesion in the retinal microcirculation of diabetic rats.23

In a recently published randomized, placebo-controlled, multicenter clinical trial evaluating the progression of diabetic retinopathy as the primary endpoint, treatment with RBX for 36 to 46 months reduced the relative risk of moderate visual loss by 63% (P = 0.012) even accounting for potential confounding variables and despite having no effect on progression of diabetic retinopathy.24 This effect was primarily evident in eyes with definite diabetic macular edema at baseline, reducing the risk of moderate visual loss sustained over 6 months from 25% in placebo to 10% in the 32 mg/d RBX-treated group (P = 0.017).

Such findings suggest that an oral PKC-β inhibitor may be an effective novel therapeutic agent for concurrently suppressing diabetic microvascular complications in a variety of body systems, despite suboptimal glycemic control. The goal of the present study was to provide the initial evaluation of RBX safety in patients with diabetes during repeated administration and to provide evidence that oral administration of this compound could reach the retinal microvasculature and demonstrate a positive pharmacodynamic effect. Indeed, these data are the first to provide human evidence of amelioration of diabetes-induced retinal hemodynamic abnormalities by an oral PKC-β inhibitor.
METHODS

Subjects

Individuals were eligible for this study if they had type 1 or 2 diabetes mellitus, were 18 to 65 years of age, and had an Early Treatment Diabetic Retinopathy Study (ETDRS) retinopathy severity grade of $\leq 20$ in at least one eye (none to very mild nonproliferative diabetic retinopathy), as determined by grading of seven ETDRS standard field stereoscopic color fundus photographs at the University of Wisconsin Fundus Photograph Reading Center. Subjects had to be free of severe or chronically disabling conditions other than diabetes and have an HbA1c $\leq 11.0$% at baseline. Subjects were excluded if, in their otherwise eligible eye, they had clinically significant macular edema, a history of intraocular surgery, laser photocoagulation, or a vitreous hemorrhage. In addition, patients could not have an allergy to fluorescein dye, uncontrolled diabetes mellitus, impaired renal function (creatinine $>2.0$ mg/dL), anemia (hematocrit $<35\%$ or Hb $<12$ g/dL), impaired hepatic function (more than twice the upper normal limit for aspartate transaminase, alkaline phosphatase, lactate dehydrogenase, alkaline phosphatase, and/or total bilirubin), or any other clinically significant abnormal laboratory value. Pregnant or lactating women or those of childbearing potential who were not practicing birth control were excluded. Subjects could not have participated in any other investigational trial within the 12 weeks before their baseline visit.

Study Design

In this double-masked, placebo-controlled, parallel, randomized, single-center study, the subjects were assigned to one of four groups after a 2- to 5-week screening and entry phase. The treatment groups were (1) 8 mg RBX twice daily ($n = 7$); (2) 16 mg RBX per day ($n = 7$); (3) 16 mg RBX twice daily ($n = 8$); or (4) placebo ($n = 7$). RBX-placebo was provided so that all subjects took two identical-appearing capsules twice daily. RBX was taken orally with a meal to maximize absorption. The dose regimen lasted 28 days for all treatment groups.

During the screening and entry period of the study, patients were evaluated using the tests listed below to determine eligibility. On the day of treatment group assignment (randomization visit), subjects received the first dose of study medication and stayed at the clinic for the remainder of the day for safety assessments. Subjects returned weekly for further safety and pharmacodynamic assessments until the 28-day follow-up visit was completed.

The following systemic safety assessments were performed before randomization and at each subsequent visit: collection of adverse events, pulse, respiration, systolic blood pressure, diastolic blood pressure, temperature, urinalysis, serum creatinine, blood urea nitrogen, hematocrit, hemoglobin, mean cell volume, mean cell hemoglobin, platelet count, electrolytes, serum albumin, total protein, calcium, phosphorous, electrocardiogram, creatinine phosphokinase, lactate dehydrogenase, alanine aminotransaminase, aspartate aminotransaminase, alkaline phosphatase, and/or total bilirubin, or any other clinically significant abnormal laboratory value. Pregnant or lactating women or those of childbearing potential who were not practicing birth control were excluded. Subjects could not have participated in any other investigational trial within the 12 weeks before their baseline visit.

The following ocular safety assessments were performed before randomization and at each subsequent visit: visual acuity (by ETDRS protocol), D15 color testing, visual field 30-2 testing ( Humphrey; Carl Zeiss Meditec, Inc., Dublin, CA), clinical cataract assessment, ETDRS retinopathy level (assessed by stereoscopic fundus photography), fluorescein angiography, comprehensive eye examination, intraocular pressure, and contrast sensitivity (Vistech, Dayton, OH). An immunology panel (see the Methodology section) was measured before randomization and at the final visit. Tetanus toxoid was administered 2 weeks after randomization, and the tetanus titer response was mea-
ures at the last visit. Video fluorescein angiographic assessment of mean retinal circulation time and retinal blood flow was performed before randomization and at the final visit.

The trial was conducted in accordance with the Declaration of Helsinki and guidelines for Good Clinical Practice. The protocol was reviewed and approved by the Joslin Diabetes Center institutional review board, and all patients signed a written informed consent form.

Methodology

The University of Wisconsin Fundus Photograph Reading Center confirmed eligible retinopathy during the screening and entry phase. Clinical laboratory measurements were performed at a central laboratory, and immunity testing was performed at Boston Children's Hospital. The immunology panel included counts of total, T, and B lymphocytes; the CD4, CD5, and CD8 lymphocyte subclasses; IgG, IgG2, IgG3, IgM, IgA, IGE, and IGD levels; hepatitis A titers; and Neisseria meningococcus titers.

All patients ingested their initial RBX dose along with a standardized approved dietary allowance (ADA) 1800 calorie per day diet breakfast consisting of 360 calories, 57 g carbohydrate, 12 g protein, and 8 g fat. ECG testing was performed before the initial dose as well as 4, 9, and 24 hours after the dose(s).

Retinal hemodynamic parameters were measured at baseline and study conclusion by dye dilution technique, as previously described26 with a scanning laser ophthalmoscope (model 101; Rodenstock, Munich, Germany) with a 40° field. Video output was digitized in real time at 30 frames per second and stored with a data storage system (TRAPIX Plus/DataStore; Recognition Concepts, Carson City, NV). The analog video signal was simultaneously recorded on a videotape recorder (U-Matic; Sony, Park Ridge, NJ) for archival storage. Digitized images were analyzed on a frame-by-frame basis on a computer (VAX 4000/300; Digital Equipment Corp., Maynard, MA), to determine retinal circulatory parameters, and the resultant data were analyzed as previously described.26

Statistics

Statistical analyses were performed using a two-sided test with a nominal significance level of 0.05 unless otherwise noted. Least-square means were used for all comparisons.

The group comparisons for retinal hemodynamic parameters were performed on computer (Sigmastat; SPS, Chicago, IL). One-way repeated-measures analysis of variance (ANOVA) was used to compare the same patients at different blood glucose levels. Group comparisons were performed using one-way ANOVA. Populations were tested for normality using the Kolmogorov-Smirnov test for normality and the Levene median test for equality of variance. If the distributions failed the normality or equal variance test, then the Kruskal-Wallis ANOVA on ranks was performed. All pair-wise multiple comparisons were performed with the Student-Newman-Keuls test. P < 0.05 was considered statistically significant.

For pharmacodynamic analyses, a random-effects ANOVA model was used to characterize the dose–response relationship for the response variables (PROC MIXED in SAS, 1989; SAS Institute Inc., Cary, NC). Because it is likely that measurements on the left and right eye of the same person will correlate, a random effects model is appropriate to account for this correlation. The ANOVA model fit to the data is given as: Yijk = μ + Di + eijk or eijk where: μ is the overall mean, Di is the treatment effect for dose group i, i = 1, 2, 3 (note that the three dose groups were defined as dose = 0 for placebo, dose = 16 for RBX administered as 8 mg twice daily or 16 mg per day, or dose = 32 for RBX per day administered as 16 mg twice daily). eijk is the random subject effect for patient j in group 1 (assume eijk is distributed independently as a normal random variable with mean 0 and variance σπ2), and Yijk is the random error for patient j in group i in the left or right eye k (assume eijk is distributed independently as a normal random variable with mean 0 and variance σπ2), and Yijk is the change from baseline response for patient j in group i in eye k.

In addition, a random-effects analysis of covariance (ANCOVA) model was used to assess the impact of baseline values on the dose-

Table 3. Ocular Safety Assessment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo n = 7</th>
<th></th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Better</td>
<td>No Change</td>
<td>Worse</td>
<td></td>
</tr>
<tr>
<td>Diabetic retinopathy</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
<td>5 (71.4)</td>
<td></td>
</tr>
<tr>
<td>Clinical lens grading</td>
<td>0</td>
<td>7 (100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D15 color</td>
<td>0</td>
<td>6 (85.7)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Ophthalmic examination</td>
<td>1 (14.3)</td>
<td>5 (71.4)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Visual fields</td>
<td>1 (14.3)</td>
<td>5 (71.4)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fluorescein angiography</td>
<td>0</td>
<td>7 (100)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

A patient was classified as better at endpoint if one or both eyes improved and no eye worsened; worse at endpoint if one or both eyes worsened, even if one eye improved; or no change at endpoint if all eyes measured did not change. Probabilities were calculated with the two-tailed Fisher exact test. Data are expressed as the number of subjects (percentage of total group).
was reported as an adverse event in six patients receiving RBX.

TABLE 5. Visual Acuity and Intraocular Pressure Response

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group (n)</th>
<th>Baseline Mean (SD)</th>
<th>Endpoint Mean (SD)</th>
<th>Change Mean (SD)</th>
<th>P* (from baseline)</th>
<th>P† (vs. placebo)</th>
<th>P‡ (linear trend)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual acuity (letters correct)</td>
<td>Placebo (14)</td>
<td>59.9 (3.21)</td>
<td>62.5 (4.52)</td>
<td>2.6 (3.39)</td>
<td>0.269</td>
<td>0.843</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 mg RBX/day (28)</td>
<td>60.0 (2.40)</td>
<td>60.1 (10.9)</td>
<td>0.1 (11.6)</td>
<td>0.949</td>
<td>0.571</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32 mg RBX/day (16)</td>
<td>60.0 (7.87)</td>
<td>62.0 (7.06)</td>
<td>2.0 (2.19)</td>
<td>0.369</td>
<td>0.969</td>
<td></td>
</tr>
<tr>
<td>Intraocular pressure (mm Hg)</td>
<td>Placebo (12)</td>
<td>13.7 (1.56)</td>
<td>13.1 (3.53)</td>
<td>-0.6 (2.78)</td>
<td>0.480</td>
<td>0.469</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 mg RBX/day (28)</td>
<td>13.9 (2.32)</td>
<td>13.3 (2.45)</td>
<td>-0.6 (1.71)</td>
<td>0.265</td>
<td>&gt;0.999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32 mg RBX/day (16)</td>
<td>14.1 (2.43)</td>
<td>12.7 (2.39)</td>
<td>-1.4 (1.71)</td>
<td>0.062</td>
<td>0.666</td>
<td></td>
</tr>
</tbody>
</table>

The 8-mg RBX twice daily and 16-mg RBX daily groups were pooled for the trend tests. n, number of eyes assessed. IOP measurements from both eyes of one patient were missing.

* Within-group test of a mean difference from zero for change from baseline.
† Between-group comparisons to placebo with the Dunnett-Hsu procedure used to adjust for multiple comparisons.
‡ Tests for linear trend for change from baseline.
The baseline-to-endpoint changes in serum uric acid concentrations were significantly higher in the 16-mg RBX daily and 16-mg RBX twice daily groups (20.4 ± 24.5 and 29.0 ± 23.5 μM, respectively; mean ± SD) than was observed in the 8-mg RBX twice daily and placebo groups (−32.3 ± 31.4 and −22.1 ± 35.0 μM, respectively; mean ± SD). However, no abnormally high serum uric acid concentrations were detected in any subject and all mean endpoint serum uric acid concentrations were within the normal reference range (167–547 μM). Creatine phosphokinase levels were elevated in four subjects on one occasion each. None of these subjects reported chest pain or muscle pain at the visits when the enzyme levels were elevated and the elevations were not associated with any ECG abnormalities.

Ocular safety assessments are summarized in Table 3. No statistically significant differences were noted between placebo and RBX for any of these parameters, including AREDS clinical lens grading, D15 color testing, visual field 30-2 testing (Humphrey; Carl Zeiss Meditec), and fluorescein angiography. There was an overall trend toward an increase in fluorescein leakage from baseline to endpoint with increasing RBX dose, as determined by vitreous fluorophotometry ($P = 0.042$). However, there were no statistically significant differences compared with placebo in the change from baseline for any of the RBX doses individually (baseline-to-endpoint results for each dose level were: placebo: 0.78 ± 0.53 arbitrary units (AU) to 0.63 ± 0.93 AU; 16 mg/d: 0.43 ± 0.37 to 0.77 ± 0.60 AU ($P = 0.110$ vs. placebo); 32 mg/d: 0.42 ± 0.56 to 0.39 ± 0.35 AU ($P = 0.856$ vs. placebo). Contrast-sensitivity results (Vistech) were not significantly different between placebo and RBX treatment, except at 3 cyc/deg (Table 4) where the difference was largely driven by improvement in test results in all placebo-treated patients. No changes from baseline were observed in visual acuity or intraocular pressure in patients receiving RBX compared with placebo (Table 5).

There were no statistically significant differences between placebo and RBX treatment in any of the immunology panel parameters or in the titer response to tetanus toxoid. There were no clinically relevant differences among treatment groups in any electrocardiogram parameters, as evaluated by an expert masked grader. There were no statistically significant differences among treatment groups in any of the other safety assessments listed previously.

Three patients discontinued participation in the study because of adverse events. A subject in the 8-mg RBX twice daily group discontinued after the appearance of neutropenia. A subject in the 16-mg RBX daily group discontinued because of an episode of hypoglycemia. A subject in the placebo group discontinued because of hallucinations.

### Pharmacodynamic Effects on Retinal Hemodynamics

In this patient population with no to very mild diabetic retinopathy, retinal mean circulation time (RCT) is prolonged approximately 1 second compared with nondiabetic patients. As shown in Figure 1 and Table 6, RBX-treated patients (16 mg twice daily) experienced a RCT baseline-to-endpoint change of −0.68 ± 0.73 seconds compared with a +0.16 ± 0.80-second change in patients treated with placebo ($P = 0.046$ RBX versus placebo; $P = 0.004$ RBX versus baseline). Baseline-to-endpoint RCT change in the 16 mg/d group was intermediate at −0.15 ± 0.82 seconds ($P = 0.532$ RBX versus placebo). Overall, there appeared to be an RBX dose-dependent effect on RCT ($P = 0.027$ for linear trend). Baseline RCT correlated with baseline-to-endpoint change in RCT ($r = −0.278$, $P = 0.035$). Similar although not statistically significant effects on RBF were observed (Fig. 1B and Table 6) with an increase of 4.14 ± 5.25 AU in the 32-mg RBX per day group compared with a −1.05 ± 5.96-AU change in patients receiving placebo ($P = 0.211$ RBX versus placebo, $P = 0.043$ RBX versus baseline, $P = 0.133$ for linear trend). Baseline RBF did not correlate with a baseline-to-endpoint change in RBF. Only in patients receiving 16 mg RBX twice daily was arterial diam-
TABLE 6. Retinal Hemodynamic Parameters

<table>
<thead>
<tr>
<th>Parameter Group</th>
<th>n</th>
<th>Mean (SD) Baseline</th>
<th>Mean (SD) Endpoint</th>
<th>Change (from baseline)</th>
<th>P (linear trend)</th>
<th>P (vs. placebo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinal blood flow (AU)</td>
<td>9</td>
<td>35.48 (8.59)</td>
<td>34.43 (8.94)</td>
<td>1.05 (5.96)</td>
<td>0.762</td>
<td>0.133</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>31.33 (6.48)</td>
<td>34.01 (9.88)</td>
<td>2.68 (7.65)</td>
<td>0.093</td>
<td>0.395</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>34.81 (9.83)</td>
<td>38.94 (10.27)</td>
<td>4.14 (5.25)</td>
<td>0.043</td>
<td>0.211</td>
</tr>
<tr>
<td>Arterial diameter (pixels)</td>
<td>11</td>
<td>7.52 (0.50)</td>
<td>7.17 (0.60)</td>
<td>0.35 (0.33)</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>7.51 (0.59)</td>
<td>7.17 (0.77)</td>
<td>0.34 (0.36)</td>
<td>0.003</td>
<td>0.003</td>
</tr>
</tbody>
</table>

The 8-mg RBX twice daily and 16-mg RBX daily groups were pooled for the trend tests. n the number of eyes assessed. Measurements from seven eyes of five patients could not be calculated due to poor image quality.

* Within-group test of a mean difference from zero for change from baseline. † Between-group comparisons to placebo with the Dunnett-Hsu procedure used to adjust for multiple comparisons. ‡ Tests for linear trend for change from baseline.

The safety profile of a systemically administered compound is of considerable importance, especially when it inhibits a key signaling enzyme such as PKC where substantial toxicity might arise. Indeed, treatment of diabetic patients for 3 months with a multitargeted kinase inhibitor, which also acts as a nonspecific PKC inhibitor, resulted in significant gastrointestinal side effects (nausea, vomiting, and diarrhea) and dose-related problems with glycemic control and liver toxicity. Of interest, the compound also led to reductions in some measures of retinal thickening, as evaluated by optical coherence tomography. In contrast, RBX is selective for the β isoform of PKC and highly selective for PKC compared with other kinases. This selectivity for a single PKC isoform that is hypothesized to be involved in mediating diabetes-induced ocular complications may account for the lack of side effects attributable to RBX in this study and in an additional 937 patients treated with placebo or one of three doses of RBX for 30 to 50 months.

The observed effect of RBX on retinal hemodynamics in the present study is consistent with the hypothesis that hyperglycemia-induced activation of PKC-β may mediate diabetes-induced retinal vascular dysfunction and these are the first human data demonstrating an effect of PKC-β inhibition on retinal vascular function. There are considerable data in animal models that support the hypothesis that major diabetic microvascular complications may be mediated through overactivation of PKC-β.

Recent clinical trial data support this possibility as well, particularly with regard to prevention of visual loss and beneficial effects on macular edema. Although RBX had no effect on the primary endpoint of retinopathy progression in a randomized, placebo-controlled, multicenter clinical trial, RBX treatment for 36 to 46 months reduced the relative risk of moderate visual loss by 63% (P = 0.012), with adjustment for potential confounding factors, and reduced the risk of moderate visual loss sustained over 6 months in eyes with definite diabetic
macular edema at baseline from 25% in placebo to 10% in the 32-mg/d RBX-treated group (P = 0.017).24 Indeed, a recently completed 3-year, phase-3 randomized, placebo-controlled, multicenter clinical trial evaluating the effect of RBX on preventing sustained moderate visual loss has been initially reported to have met this primary endpoint.31 A phase-3 study evaluating the effect of RBX on the progression of diabetic macular edema is ongoing.

Thus, results of these multiple studies suggest that the oral administration of RBX in patients may have positive effects on the nonocular as well as other ocular diabetic microvascular complications, such as macular edema and nephropathy, previously shown to be mediated by PKC-β in animals.10,21-28,30 If such activity is substantiated in clinical trials primarily assessing these endpoints, selective inhibition of PKC isozymes may offer a unique therapeutic approach to retinopathy and other diabetes-related microvascular disorders.

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References