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**Review Article**

# PKC – a target for treating diabetic complications

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**Abstract**

Sustained hyperglycemia causes severe diabetic microvascular complications, such as retinopathy, peripheral neuropathy and nephropathy. Because of the difficulty in achieving complete euglycemia, a new approach to preventing diabetic complications is required. Protein kinase C (PKC) β, which is activated in diabetic animals and in vascular cells exposed to high glucose levels, is one of the targets for improving diabetic complications. Hyperglycemia-induced PKC activation causes vascular abnormalities such as altered hemodynamics, angiogenesis, vasoconstriction, endothelial permeability, cell growth, cytokine activation and leukocyte adhesion. In preclinical studies and clinical trials, PKCβ inhibitors were well tolerated and improved diabetic retinopathy, peripheral neuropathy and nephropathy. Selective PKCβ inhibition may therefore represent a promising approach to the treatment of diabetic complications.

## Introduction

Nearly 75% of diabetes patients have at least one of the three major diabetic microvascular complications: diabetic retinopathy, diabetic peripheral neuropathy or diabetic nephropathy. The Diabetes Control and Complications Trial (DCCT), the U.K. Prospective Diabetes Study (UKPDS) and the Kumamoto Study have established that intensive glycemic control in both type 1 and type 2 diabetes patients can delay the onset and progression of vascular complications (1-3). However, it is difficult to strictly control blood glucose levels. In the DCCT, only 44% of the patients receiving intensive therapy achieved the goal of a glycosylated hemoglobin (HbA1c) value of 6.05% or less (1). Furthermore, current therapies using blood glucose-lowering drugs have not shown sufficient efficacy to prevent the development of diabetic microvascular complications. Epalrestat (Kinedak®; Ono Pharmaceutical), an aldose reductase inhibitor, is indicated for the treatment of diabetic peripheral neuropathy in Japan. However, in the U.S., there are no medications approved to target the underlying cause of diabetic microvascular complications; therefore, a new approach to prevent these complications is urgently needed.

In the diabetic state, multiple mechanisms have been implicated in glucose-mediated vascular damage and contribute to diabetic microvascular complications: increased oxidative stress, increased polyol pathway flux, increased hexosamine pathway flux and increased advanced glycation end products (AGEs), which are produced nonenzymatically. Activation of protein kinase C (PKC) through the *de novo* synthesis of diacylglycerol (DAG) also occurs in the diabetic state (4) and is affected by some of these mechanisms. Activation of the DAG/PKC pathway in vascular tissues is associated with early abnormal functions, such as angiogenesis, vasoconstriction, endothelial permeability, cell growth, cytokine activation and leukocyte adhesion, in the diabetic state and is deeply implicated in diabetic microvascular complications. Therefore, inhibiting the DAG/PKC pathway under hyperglycemic conditions may represent a potential therapy for diabetic microvascular complications.

## PKC isoforms and structure

PKC is a family of serine/threonine kinases that consists of at least 12 isoforms. These isoforms are classified into three categories based on their structure and regulation (5, 6). Conventional PKCs (α, β1, β2 and γ) are $Ca^{2+}$-dependent and are activated by both phospholipids and DAG. Newer PKCs (δ, ε, η and θ) can be activated by phospholipids and DAG, but are independent of $Ca^{2+}$. Atypical PKCs (ι/λ and ζ) are not responsive to these activators but can be activated by insulin (7-10). PKCμ, or PKD, is a novel member of the PKC family that differs from the other isoforms in structural and enzymatic

properties, and has a substrate specificity distinct from other PKC isoforms.

PKC has an *N*-terminal regulatory region and a *C*-terminal catalytic region, comprised of conservative regions (C1-C4) and variable regions (V1-V5). In the regulatory region, the C1 site has two cysteine-rich domains that bind to DAG. The C2 site is involved in $Ca^{2+}$-dependent membrane binding. The C3 site, present at the catalytic domain, has an ATP-binding site and is the main target of some PKC inhibitors. The C4 site, also present in the catalytic domain, recognizes substrates of PKC. The catalytic domain is well preserved among PKC isoforms.

## PKC activation and diabetes

Conventional PKC isoforms are activated by DAG, which is produced by multiple pathways: hydrolysis of phosphatidylinositol 4,5-diphosphate ($PIP_2$) by phospholipase C (PLC), or hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD). Hyperglycemia-induced PKC activation plays a major role in the development of vascular dysfunction. Under hyperglycemic conditions, an excess amount of glucose flows into cells via the glucose transporter 1 (GLUT1), and *de novo* synthesis of DAG from the glycolytic intermediate dihydroxyacetone phosphate and glycerol-3-phosphate is increased. Hyperglycemia-induced DAG activates several PKC isoforms, especially PKCβ1 and β2 in retina, aorta, heart, renal glomeruli and perineural vascular tissue of sciatic nerve, all of which are strongly implicated in diabetic microvascular complications (see Figs. 1 and 2, Table I).

Modification of an upstream pathway can inhibit PKC activation. Diacylglycerol kinase (DGK) catalyzes the phosphorylation of DAG to produce phosphatidic acid and has been suggested to be a pharmacological target of D-α-tocopherol (vitamin E) for diabetic renal dysfunction (11). DGKα is translocated from the cytoplasm to the plasma membrane, with elevation of kinase activity. In addition, a peroxisome proliferator-activated receptor (PPAR) γ agonist such as troglitazone, which possesses a 'chroman ring' similarly to D-α-tocopherol, induces the subtype-specific translocation of DGKα (12, 13).

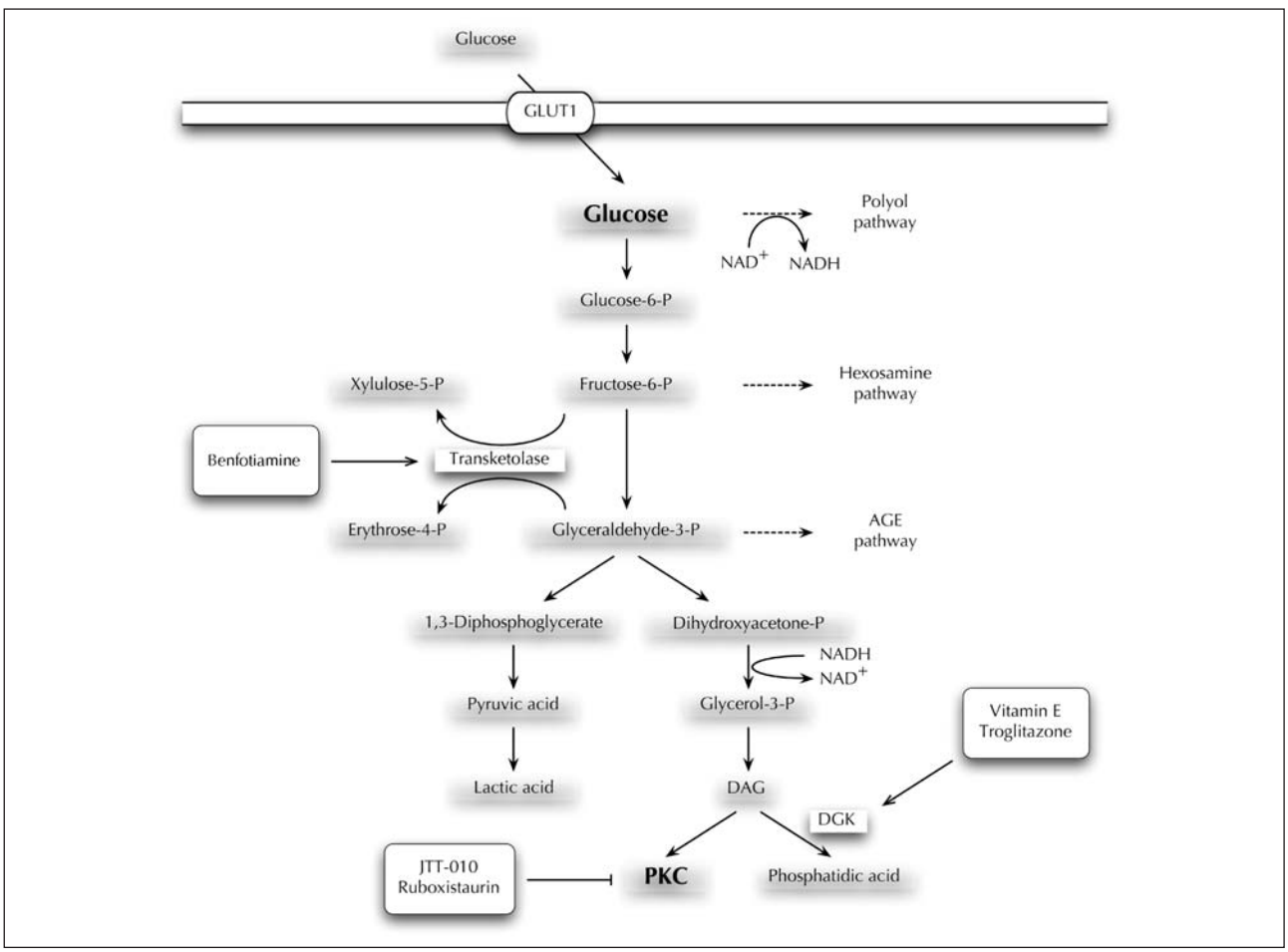


Fig. 1. Putative mechanism of hyperglycemia-induced PKC activation. Excess glucose influx via the glucose transporter 1 (GLUT1) into vascular cells leads to *de novo* synthesis of diacylglycerol (DAG) and subsequent PKC activation. Increased NADH/$NAD^+$ ratio and oxidative stress also activate PKC. Major PKC inhibitors bind to the ATP-binding site of PKC to inhibit PKC activity. Activation of transketolase and diacylglycerol kinase (DGK) overuses intermediates and reduces DAG, which results in indirect attenuation of PKC activity.

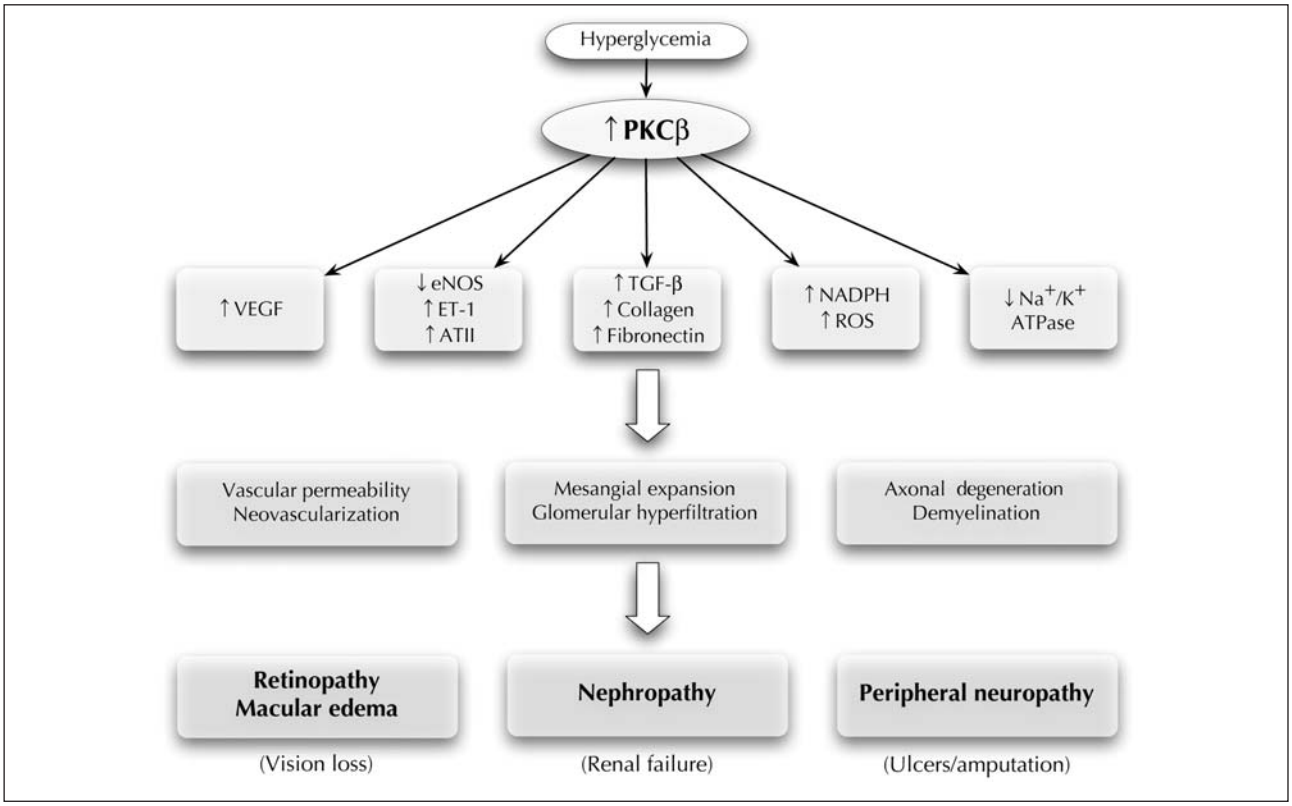


Fig. 2. Potential mechanism of diabetic microvascular complications caused by hyperglycemia-induced PKCβ activation. VEGF: vascular endothelial growth factor; eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; ATII: angiotensin II; TGF-β: transforming growth factor-β; ROS: reactive oxygen species.

*Table I: DAG levels and PKC activity in diabetes.*

| Tissue | DAG | PKC | Activated PKC isoforms |
|---|---|---|---|
| Retina | ↑ | ↑ | α, β1, β2, ε |
| Heart | ↑ | ↑ | α, β2, δ |
| Aorta | ↑ | ↑ | β2 |
| Renal glomeruli | ↑ | ↑ | α, β1, β2, δ, ε |
| Sciatic nerve | –, ↑ | ↑, ↓, – | β1, β2 (perineural vascular tissue) (PKCα decreased in endoneural tissue) |

↑: increase, ↓: decrease, –: unchanged in references cited (11, 16-31).

Activating DGK using such drugs results in attenuation of DAG levels and inhibition of PKCβ activation.

Increasing availability of the glycolytic metabolites glyceraldehyde-3-phosphate and fructose-6-phosphate activates the major biochemical pathways implicated in the pathogenesis of hyperglycemia-induced vascular damage (the hexosamine pathway, AGE formation pathway and the DAG/PKC pathway). Benfotiamine, a lipid-soluble thiamine derivative, can inhibit these pathways by activating the pentose phosphate pathway enzyme transketolase, which converts glyceraldehyde-3-phosphate and fructose-6-phosphate to xylulose-5-phosphate and erythrose-4-phosphate, respectively. In retina and renal glomeruli of diabetic animals, benfotiamine inhibits these pathways, including PKC activation and nuclear factor-κB (NF-κB) activation, by activating transketolase, and prevents experimental diabetic retinopathy and nephropathy (14, 15).

**PKC inhibitors**

Staurosporine (32) and the isoquinolinesulfonamide GF-109203X (33) are first- and second-generation PKC inhibitors, respectively (see Fig. 3, Table II). These compounds inhibit PKC isoforms nonselectively and are not specific for PKC. Because of its role in signal transduction in many tissues, it appeared likely that nonselective inhibitors of PKC isoforms would cause toxicity. Midostaurin (PKC-412, CGP-41251; Novartis; Fig. 3, Table II) is an orally available staurosporine derivative that inhibits PKC and other tyrosine kinases (34). Although midostaurin is being evaluated for use in acute myeloid leukemia and has exhibited some beneficial effects in diabetic macular edema, serious toxicity excludes its clinical application in diabetic patients (35). JTT-010 (Japan Tobacco; Fig. 3, Table II) is a new structural class of PKCβ-selective inhibitor that possesses a 3-

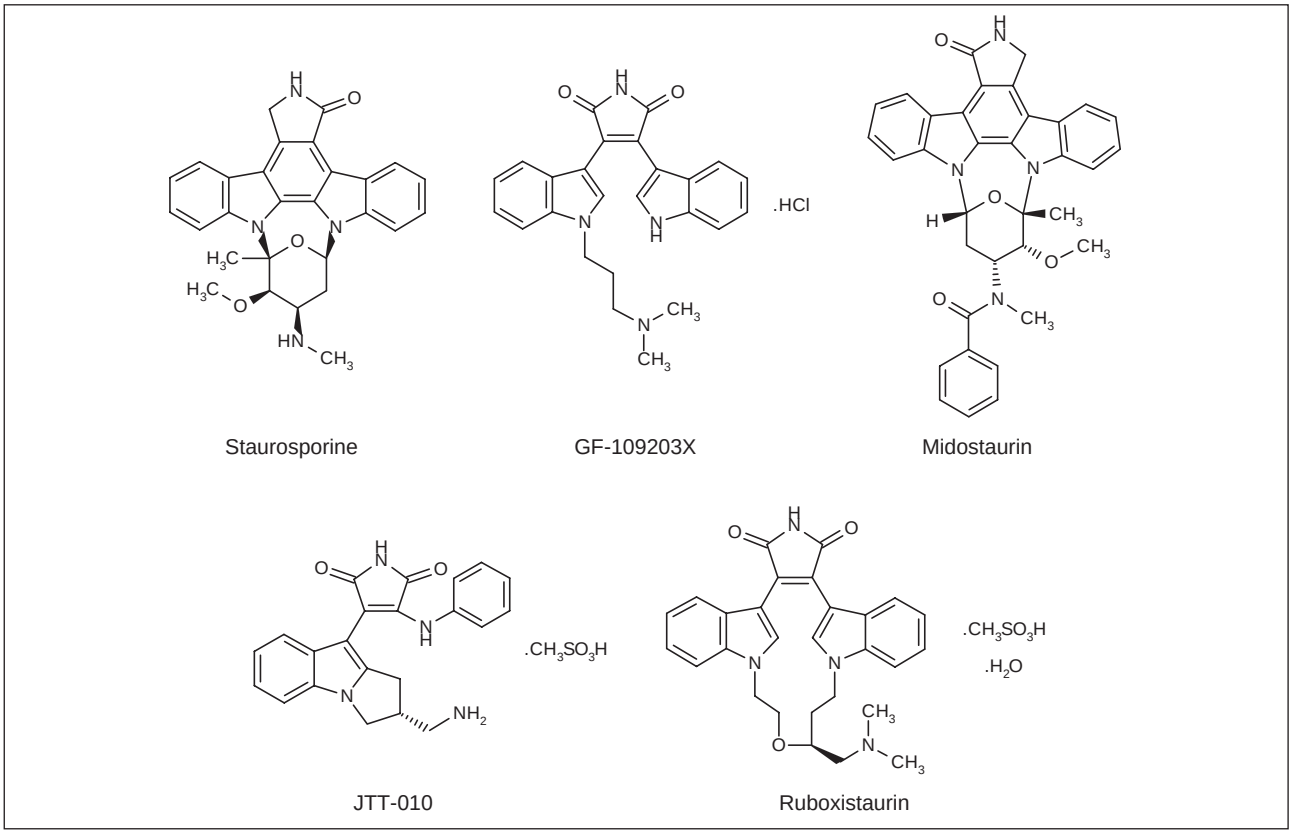


Fig. 3. Structures of PKC inhibitors.

*Table II: Isoform selectivity of PKC inhibitors.*

| Compound | PKC isoform ($IC_{50}$, nM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | α | β1 | β2 | γ | δ | ε | ζ | η | μ |
| Staurosporine | 8.7 | 11 | 4.0 | 11 | 4.3 | 7.4 | 1700 | – | 24 |
| GF-109203X | 8.4 | 18 | – | – | 210 | 132 | 5800 | – | – |
| Midostaurin | 24 | 17 | 32 | 18 | 360 | 4500 | >10,000 | 60 | – |
| JTT-010 | 86 | 4.0 | 2.3 | 110 | 54 | 490 | 1700 | – | >10,000 |
| Ruboxistaurin | 360 | 4.7 | 5.9 | 300 | 250 | 600 | >10,000 | 52 | – |

$IC_{50}$ values are cited from references 36-39.

anilino-4-(3-indolyl)maleimide pharmacophore (36). $IC_{50}$ values for JTT-010 for PKCβ1 and PKCβ2 are 4.0 and 2.3 nM, respectively, and $IC_{50}$ values for other PKC isoforms are 54 nM or greater. Ruboxistaurin mesilate (LY-333531, Arxxant™; Lilly; Fig. 3, Table II) also selectively inhibits PKCβ1 and PKCβ2 with $IC_{50}$ values of 4.7 and 5.9 nM, respectively (37). The PKCβ-inhibitory activity of ruboxistaurin has been demonstrated in various models of diabetic complications and several clinical trials are ongoing.

## PKC and diabetic retinopathy

Diabetic retinopathy is the most prevalent diabetic microvascular complication, present in approximately half of all diabetic patients (40, 41), and is a leading cause of visual loss in adults in industrialized countries (42). When abnormal new vessels proliferate on the retina in diabetic retinopathy, the stage is called proliferative diabetic retinopathy, which accounts for most of the cases of severe visual loss. In addition, retinal vessels can become permeable and cause swelling of the retina, which is called diabetic macular edema, a leading cause of moderate visual loss in diabetes (43).

Hyperglycemia-induced activation of PKCβ appears to mediate increases in retinal vascular permeability and neovascularization in animal models and causes retinal hemodynamic abnormalities in diabetic patients. Vascular endothelial growth factor (VEGF), the formation of which is stimulated by PKC, is hypothesized to play a key role in these changes in retinal vessels. Ruboxistaurin reduces VEGF-induced retinal permeability and the mitogenic response to VEGF (44). In addition, PKCβ knockout mice show a significant decrease in retinal neovascularization and PKCβ2 transgenic mice show an increased angiogenic response to retinal ischemia (45). These results indicate that PKCβ plays an important role in retinal neovascularization.

A reduction in retinal blood flow is one of the early features of diabetic retinopathy. JTT-010 inhibited retinal PKC activity and ameliorated the reduction in retinal blood flow (mean circulation time, or MCT) in streptozotocin (STZ)-induced diabetic rats (46). Ruboxistaurin also decreased retinal PKC activity and improved MCT in diabetic rats (23). The electroretinogram (ERG) is known as a sensitive indicator of early diabetic retinopathy (47). The delay in oscillatory potential in diabetic retinopathy is significantly improved by JTT-010 in Spontaneously Diabetic Torii (SDT) rats, which show severe diabetic retinopathy (48, 49, unpublished data) and by ruboxistaurin in STZ-induced diabetic rats (29).

Leukocyte adhesion is thought to be involved in the pathogenesis of diabetic retinopathy (50). PKC is highly expressed in leukocytes and platelets, and PKC activity in monocytes is correlated with plasma glucose levels (51). A PKCβ inhibitor reduced leukocyte entrapment in the retinal circulation which was increased in STZ rats (52).

The Protein Kinase C beta Inhibitor Diabetic Retinopathy Study (PKC-DRS) evaluated the safety and efficacy of ruboxistaurin (53). In this multicenter, double-masked, randomized, placebo-controlled trial, 252 patients with type 1 or type 2 diabetes and moderately severe to very severe nonproliferative diabetic retinopathy were enrolled. Subjects received doses of 8, 16 or 32 mg/day of ruboxistaurin or placebo for 36-46 months. The primary endpoint of the PKC-DRS was to prevent a minimum 3-step retinopathy progression on the Early Treatment Diabetic Retinopathy Study (ETDRS) scale or application of panretinal laser photocoagulation. There were no statistically significant differences among treatment groups in the time to progression of diabetic retinopathy or in the cumulative percentage of patients who reached this endpoint. However, the incidence of moderate visual loss and sustained moderate visual loss was lower in the group receiving 32 mg/day ruboxistaurin. The phase III retinopathy study PKC-DRS2 in diabetes patients with moderately severe to severe nonproliferative diabetic retinopathy was recently completed and preliminary results showed that 32 mg/day of ruboxistaurin produced a statistically significant reduction in the relative risk of sustained moderate visual loss (54).

The Protein Kinase C beta Inhibitor Diabetic Macular Edema Study (PKC-DMES) was designed to determine whether orally administered ruboxistaurin could delay the progression of diabetic macular edema to involve or imminently threaten the center of the macula (from > 300 μm to < 100 μm from center), or delay the application of focal laser photocoagulation for diabetic macular edema (53, 55). A total of 686 diabetes patients with mild to moderate nonproliferative diabetic retinopathy were randomly assigned to receive doses of 4, 16 or 32 mg/day ruboxistaurin or placebo for at least 30 months. At 36 months, ruboxistaurin did not show a significant effect on diabetic macular edema. However, after excluding the 18% of patients with progression to photocoagulation or patients with HbA1c > 10%, the risk was significantly reduced (23% and 31%, respectively). A phase III study evaluating the effect of ruboxistaurin on diabetic macular edema progression in patients with less severe diabetic retinopathy is ongoing and expected to be completed in 2010 (54).

**PKC and diabetic peripheral neuropathy**

Diabetic peripheral neuropathy is associated with the duration of diabetes, and overall 20-40% of diabetes patients are estimated to have peripheral neuropathy (56), which can lead to toe, foot or leg amputation. Under hyperglycemic conditions, high expression of aldose reductase induces sorbitol accumulation, reduction of $Na^+/K^+$ ATPase and decreased PKC activity in the neural tissue. Decreased PKC activity in diabetic nerves seems inconsistent with activity in other tissues; however, PKC activity of nerves differs according to the site involved. In diabetic endoneural tissue, the activity of PKCα was decreased. On the other hand, PKCβ1 and β2 activities were increased in the perineural vascular tissue (31). Thus, hyperglycemia appears to activate PKCβ in the perineural vascular tissue, and impaired microvascular function may cause nerve damage.

Motor (MNCV) and sciatic nerve conduction velocity (SNCV) defects are markers of large myelinated fiber dysfunction (57, 58). JTT-010 and ruboxistaurin ameliorated the NCV deficits in STZ rats to near normal levels (29, 36). Abnormal variations in heart rate, such as in the R-R interval, were also ameliorated by ruboxistaurin in STZ rats (29) and by JTT-010 in SDT rats (unpublished data).

There is some evidence to indicate a relationship between PKC and diabetic hyperalgesia as a small-fiber system dysfunction (59, 60). Staurosporine reduced mechanical hyperalgesia (61) and ruboxistaurin ameliorated thermal hyperalgesia in STZ rats (62). JTT-010 prevented chemical hyperalgesia caused by formalin injection in STZ rats (36). The antinociceptive effect of PKCβ inhibitors is presumably due to improvement in reduced NO-cGMP pathway activity in dorsal root ganglion (DRG) neurons (63). Although the mechanism is not clear, JTT-010 also prevented diabetic hypoalgesia induced by thermal and chemical stimuli (36).

A phase II pilot study was conducted to assess the effect of ruboxistaurin on nerve function and sensory symptoms in patients with diabetic peripheral neuropathy (64, 65). In this multinational, double-masked, randomized, placebo-controlled study, 205 diabetes patients with diabetic peripheral neuropathy were enrolled. Subjects received either 32 or 64 mg/day ruboxistaurin or placebo for 1 year. A total of 83 patients had clinically significant symptoms, such as numbness, allodynia, prickling and pain (lancinating, aching and burning), at baseline (Neuropathy Total Symptom Score-6 [NTSS-6] > 6). Abnormal measurable vibration detection threshold (VDT) assessment, neurological examination and standardized electrophysiological nerve conduction tests were conducted. The primary endpoint of the study was to evaluate the effects of ruboxistaurin on the change in

quantitative sensory testing of vibration. There were no changes in VDT upon ruboxistaurin treatment at the endpoint. However, in the patients with NTSS-6 > 6, there was a significant reduction from baseline in the NTSS-6 score with ruboxistaurin treatment. In a subgroup of patients with clinically significant symptoms and less severe diabetic peripheral neuropathy, there was a significantly greater reduction in the NTSS-6 score and improvement in VDT with ruboxistaurin. These results indicate that inhibition of PKCβ is beneficial to patients with symptomatic or less severe diabetic peripheral neuropathy by improving sensory symptoms and vibration sensation.

Two phase III clinical trials were recently completed. In these trials, statistical comparison of the changes in NTSS-6 between placebo- and ruboxistaurin-treated groups did not demonstrate significant differences. However, further results of PKCβ inhibition by ruboxistaurin on nerve dysfunction in diabetic peripheral neuropathy will be clarified in ongoing 3-year phase III neuropathy studies, which are scheduled to be completed in mid-2007 (54).

## PKC and diabetic nephropathy

Nephropathy develops in nearly 45% of diabetes patients and is the leading cause of end-stage renal disease requiring dialysis or transplantation (66). Glomerular mesangial matrix expansion and capillary basement thickening are characteristic of diabetic nephropathy. Increased type IV collagen, laminin or fibronectin is involved in diabetic mesangial expansion (67, 68). Diabetes-induced PKCβ activation contributes to accumulation of these mesangial basement components by increasing transforming growth factor-β (TGF-β) (69).

In the STZ rat, JTT-010 and ruboxistaurin normalized the abnormally elevated glomerular filtration rate (GFR) or creatinine clearance, and significantly reduced the abnormally elevated urinary albumin excretion (23, 36). Ruboxistaurin also prevented the increased mRNA expression of TGF-$\beta_1$, fibronectin and α1 (type IV) collagen in the glomeruli of STZ rats (69). In *db/db* mice, which develop diabetes and obesity, ruboxistaurin normalized elevated PKC activity in glomeruli, and ameliorated the elevation in TGF-$\beta_1$ levels and mesangial expansion (30). In the STZ-induced diabetic (mRen-2)27 rat, which is transgenic for the entire mouse renin gene and becomes hypertensive, ruboxistaurin did not affect hypertension and hyperglycemia; however, it reduced albuminuria, glomerulosclerosis and tubulointerstitial fibrosis, with a concomitant increase in TGF-β (70).

Oxidative stress is considered to play an important role in the development of diabetic nephropathy. NADPH oxidase activity, which was significantly enhanced in diabetic glomeruli and the source of reactive oxygen species (ROS) generation, was also improved by ruboxistaurin in isolated glomeruli from STZ rats (71).

A pilot study to evaluate the effect of ruboxistaurin on nephropathy in type 2 diabetes was conducted in the U.S. (72). In this multicenter, double-masked, placebo-controlled study, 123 type 2 diabetes patients with proteinuria and near-normal serum creatinine received either 32 mg/day ruboxistaurin or placebo for up to 1 year. The patients were under intensive glycemic control and blood pressure regulation with angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), or both, for at least 6 months before screening. ACE inhibitors and/or ARBs were continued throughout the study. The primary endpoint of the study was a reduction in the urinary albumin to creatinine ratio (ACR), and the estimated glomerular filtration rate (eGFR) was also evaluated as an index of renal function. After 1 year, the ruboxistaurin-treated group showed a significant reduction in ACR. This ACR-lowering effect of ruboxistaurin appeared as early as 1 month after starting treatment. The eGFR level in the placebo group showed a significant decrease over 1 year. In contrast, the reduction in eGFR in the ruboxistaurin-treated group was not significant. There were no differences in blood pressure or HbA1c between the groups. This clinical trial showed that the PKCβ inhibitor has benefits in the treatment of diabetic nephropathy, in addition to intensive glucose control and inhibition of the angiotensin system.

## Conclusions

A number of preclinical studies and clinical trials have demonstrated that hyperglycemia-induced activation of PKCβ is implicated in diabetic microvascular complications such as retinopathy, peripheral neuropathy and nephropathy. Although several molecular mechanisms of diabetic microvascular complications have been clarified, appropriate preventive therapies have not been established. Selectively inhibiting PKCβ has demonstrated the ability to prevent and/or delay the progression of diabetic microvascular complications, even in the presence of hyperglycemia, in animal models and clinical trials, with reduced toxicity. A PKCβ-selective inhibitor would therefore have great promise for the treatment of these serious diabetic microvascular complications.

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