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ACTIVESITE PHARMACEUTICALS

A NOVEL PLASMA KALLIKREIN INHIBITOR DECREASES RETINAL VASCULAR PERMEABILITY IN ANIMAL MODELS OF DIABETES AND HYPERTENSION.

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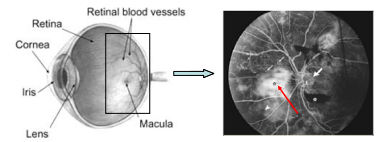
Abstract

Diabetic macular edema (DME), the swelling of the central portion of the retina, is a major vision-threatening vascular complication of both type 1 and type 2 diabetes, afflicting >600,000 people in the US alone. Underlying the development of DME is increased retinal vascular permeability (RVP), the passive efflux of plasma proteins and fluid across a compromised blood-retinal barrier. The serine protease plasma kallikrein (PK) has recently been implicated in contributing to aberrant RVP in diabetic retinopathy. PK cleaves high molecular weight kininogen to generate bradykinin, which leads to the opening of endothelial cell tight junctions and consequent plasma extravasation. Since plasma PK levels are increased in type 1 diabetes, and activation of PK in rodent vitreous leads to increased RVP, we hypothesized that one of the factors underlying the development of increased RVP in diabetes may be mediated by PK. We have developed a novel and selective small molecule inhibitor of PK, ASP-440, which was recently shown to be highly effective in an acute model of hypertension-induced elevated RVP[1]. We have since examined the effect of chronic systemic treatment with ASP-440 via a subcutaneously implanted osmotic pump on RVP in Sprague Dawley rats with 4 weeks of streptozotocin-induced diabetes. RVP was measured using Evans Blue dye permeation. RVP was increased by approximately 2.5 fold in diabetic rats compared with age-matched non-diabetic controls. ASP-440 decreased RVP in diabetic rats in a dose-dependent manner by over 80% compared with vehicle-treated DM rats ($p<0.001$) to levels not significantly different from that in non-diabetic control animals. ASP-440 did not alter blood glucose or body weight in diabetic rats compared with vehicle-treated diabetic rats. These novel results underscore the potentially key role played by PK in increasing RVP in rodent models of hypertension and diabetes, and also suggest that the pharmacological inhibition of PK by ASP-440 may be a promising new approach to the treatment of DME.

Diabetic Macular Edema

Diabetic macular edema (DME) is the principal cause of vision loss in persons with diabetes, accounting for between 12,000 - 24,000 new cases of blindness in the US every year. The underlying cause of DME is increased retinal vascular permeability (RVP). Plasma fluid, proteins and lipids leak from the microvasculature of the retina causing the retina to swell and deposition of hard exudates near the central retina, the macula[2]. Elevated blood pressure and blood glucose are independent risk factors for development of DME in diabetes, and intensive glycemic and blood pressure control can reduce the incidence of DME. However, no pharmacological treatment has been clinically approved for the treatment of DME. There is thus a great unmet need for a safe and effective therapeutic intervention in the earliest stages of DME that would robustly suppress increased RVP, and thus reduce the likelihood of development of clinically significant DME and attendant vision loss.

Increased RVP Detected as Fluorescein Leakage (Red Arrow) from Capillaries Near the Macula in a Patient with DME



Modified from Frank (2004) *NEJM*, **350**:48-58

Plasma Kallikrein: Mediator of Pathological RVP?

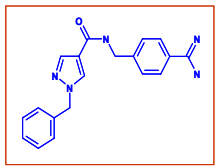
The serine protease plasma kallikrein (PK) has been recently implicated in the pathogenesis of RVP[3]. PK circulates in plasma as a zymogen, and upon activation cleaves high molecular weight kininogen to generate the vasoactive peptide bradykinin (BK). BK and its metabolites cause the opening of endothelial tight junctions lining blood vessels, resulting in increased vascular permeability. Fluid and macromolecular components of the plasma leak from the vessels into surrounding tissue potentially resulting in edema formation. Increased vascular permeability in the retina underlies the development of macular edema in diabetes.

PK levels are known to be increased in individuals with type 1 diabetes[4], and also in rodent models of type 1 diabetes[5]. In patients with type 1 diabetes, higher plasma levels correlate with increased blood pressure[4], albuminuria[4], and retinopathy[6]. Taken together, these diverse reports suggest that upregulation of PK correlates with adverse retinal and renal complications of diabetes.

Study Goals

The major goal of this study was to determine whether PK plays a role in the increased RVP underlying DME through the use of a novel small molecule inhibitor of PK, **ASP-440**. The effect of subcutaneously delivered ASP-440 on RVP is assessed in models of **hypertension** (angiotensin- II-induced) and **diabetes** (streptozotocin-induced). Elevated blood pressure and blood glucose, recapitulated in these models, are known to be independent risk factors for DME.

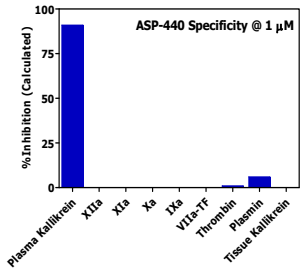
A



ASP-440

K_i = 0.1 μM

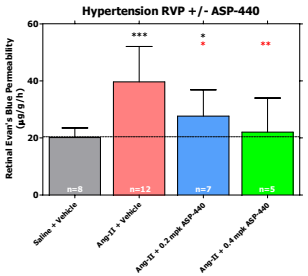
1-benzyl-1H-pyrazole-4-carboxylic acid 4-carbamimidoyl-benzylamide

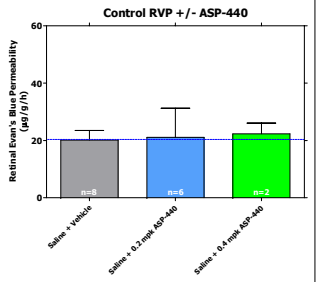


B

ASP-440 Dose-dependently Normalizes Increased Retinal Vascular Permeability (RVP) to Evans's Blue Induced by 6-day Treatment with Angiotensin-II (Ang-II), but Does not Affect Baseline RVP in Control Animals

*, p = 0.05 vs Saline + Vehicle; ***, p=0.0004 vs Saline + Vehicle;
*, p= 0.04 vs Ang-II + Vehicle; **, p = 0.017 vs Ang-II + Vehicle;

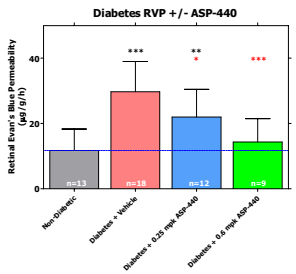


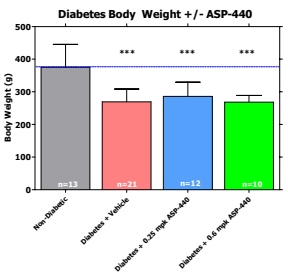


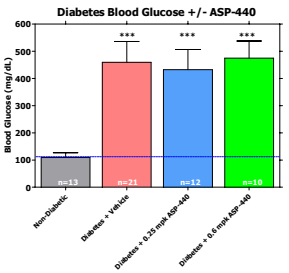
C

ASP-440 Dose-dependently Normalizes Increased Retinal Vascular Permeability (RVP) to Evans's Blue Induced by 28 Days of Diabetes, but Does Not Change Blood Glucose or Body Weight in the Diabetic Animals.

**, p = 0.003 vs Non-Diabetic; ***, p<0.0001 vs Non-Diabetic;
*, p = 0.03 vs Diabetic+Vehicle; ***, p = 0.0002 vs Diabetic+ Vehicle;







D

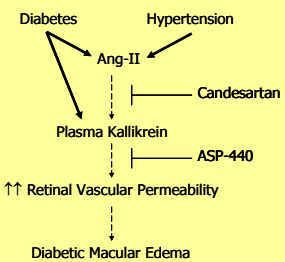
Drug	Mechanism of Action	Daily dose, Route	Animal Model	Duration of Treatment	% RVP Inhibition
Candesartan	ARB	2 mg/kg p.o.	Hypertension (Ang-II)	6 d	90%
ASP-440	PK Inhibitor	0.4 mg/kg s.c.			90%
Candesartan	ARB	4 mg/kg p.o.	Diabetes (STZ)	14 d	58%
ASP-440	PK Inhibitor	0.6 mg/kg s.c.		28 d	86%

The AT1 receptor blocker candesartan normalizes RVP in the Ang-II hypertension model, indicating PK is activated downstream of the AT1R. Candesartan is only partly effective in the diabetes model, whereas ASP-440 remains equally potent, suggesting that PK may also be activated by non-AT1R pathways in diabetes.

Summary

1. **RVP is increased in rat models of hypertension and diabetes.**
2. **A small molecule inhibitor of PK, ASP-440, dose-dependently reduces the RVP (essentially to control levels) when delivered systemically in these rodent models.**
3. **PK appears to be activated downstream of Ang-II in hypertension, and by both Ang-II-dependent and Ang-II-independent pathways in diabetes.**
4. **Inhibition of PK may be a therapeutic approach to the treatment of DME – a clinical condition whose risk is increased by both elevated blood pressure and hyperglycemia.**

Potential Molecular Pathway for DME



References

1. Phipps, J. A., Clermont, A. C. et al, (2009) " Plasma kallikrein mediates angiotensin II type 1 receptor-stimulated retinal vascular permeability" *Hypertension*, **53**, 175-81
2. Mohamed, Q., M. C. Gillies, et al. (2007) "Management of diabetic retinopathy: a systematic review." *JAMA* **298**, 902-16
3. Gao, B. B., A. Clermont, et al. (2007) "Extracellular carbonic anhydrase mediates hemorrhagic retinal and cerebral vascular permeability through prekallikrein activation." *Nat Med* **13**, 181-8
4. Jaffa, A. A., R. Durazo-Arvizu, et al. (2003) "Plasma prekallikrein: a risk marker for hypertension and nephropathy in type 1 diabetes." *Diabetes* **52**, 1215-21
5. Tschope, C., A. Reinecke, et al. (1999) "Functional, biochemical, and molecular investigations of renal kallikrein-kinin system in diabetic rats." *Am J Physiol* **277**, H2333-40
6. Kedzierska, K., K. Ciechanowski, et al. (2005) "Plasma prekallikrein as a risk factor for diabetic retinopathy." *Arch Med Res* **36**, 539-43

Methods

Ang-II Model of Hypertension:

Animals were made hypertensive by using subcutaneously implanted Alzet mini-osmotic pumps delivering Ang-II at a constant rate of 18 μg/kg/h, with control rats receiving saline. ASP-440 was also delivered via Alzet minipumps at 0.2 or 0.4 mg/kg/d, with control Ang-II animals receiving an equivalent volume of vehicle (10% PEG400, 90% PBS).

Streptozotocin Model of Diabetes:

Male Sprague-Dawley rats were made diabetic by a single intraperitoneal injection of streptozotocin (55 mg/kg). Following confirmation of hyperglycemia, ASP-440 was delivered via Alzet minipumps at a constant rate of 0.25 or 0.6 mg/kg/d for 28 days, with control diabetic animals receiving an equivalent volume of vehicle.

Measurement of RVP:

RVP was measured using Evans Blue (EB), a dye that tightly binds to albumin. Briefly, Evans blue (EB) was injected intravenously at 45 mg/kg. Following 2 hours of circulation of the dye, 0.1 ml blood is drawn from the iliac artery to obtain plasma EB concentration. Anesthetized rats were perfused for 2 min via the left ventricle at 37°C with 0.05 M, pH 3.5, citrate-buffered paraformaldehyde (1% w/v). Immediately after perfusion, both eyes were enucleated, bisected away under an operating microscope, and thoroughly dried in a Speed-Vac. EB was extracted by incubating each retina in 240 μl formamide for 18 h at 70°C and the background-subtracted absorbance of the filtered supernatant determined by measuring A_{620} (EB absorbance maximum). RVP is expressed as μg EB per gram retinal tissue per hour.