ANNEXURE I Ethical Committee Certificate

J.S.S. College of Pharmacy, Ootacamund, Tamil Nadu, India.
Committee for the Purpose of control and Supervision of Experiments on
Animals (CPCSEA)
Institutional Animal Ethics committee (IAEC)

CERTIFICATE

Title of the Project: Novel Drug Targeting Approach for Diabetic Retinopathy
Proposal Number: JSSCP/IAEC/PH-D/PH:CENTRES/01/2012-13

Date received after modification (if any) : 
Date received after second modification : 10.05.2012
Approval date : 04.06.2012
Animals : New Zealand White Rabbits
Male/Female
No. of animals sanctioned : 54 RABBITS

Expiry date (Termination of the Project) : 2 MONTHS
Name of IAEC/CPCSEA chairperson : Dr. K. Elango

Dr. K. Elango

Name/Signature of Chairperson
Chairperson
Institutional Animal Ethics Committee
JSS College of Pharmacy
Rocklands, Ooty-643 001

Date : 04.06.2012
Place : OOTY,
ANNEXURE II. Awards/Research Papers Published, Communicated/Patents and Presented

During the tenure of this thesis (2009-2012), the following awards and grants received to the Deepa Pathak.

AWARDS RECEIVED

Award of the Women Scientist-A to the Deepa Pathak of amount 21.36 lakhs by Department of Science and Technology (DST), New Delhi, India.

Travel grant from Indian Council of Medical Research (ICMR), New Delhi, India to attend the 4th International Conference on Drug Discovery and Therapy, held at Dubai, 12th -15th February 2012.

Best Poster award with a prize money of 1000/- US$ in 4th International conference on Drug Discovery & Therapy, 12th Feb.-15th Feb., 2012 held at Dubai in Poster session entitled “Nanogel: A Promising tool for diabetic retinopathy.

Best Poster award in International conference on Recent Advances in Pharmaceutical Sciences, 21st March-23rd March, 2012 held at Rayat Institute of Pharmacy, Punjab in Poster session entitled “Cost effective herbal based PKC-βII receptor targeting nanogel for management of Diabetic Retinopathy”.

LIST OF PUBLICATION


LIST OF PATENTS

1. Naturally derived polysaccharides of fenugreek seed and its uses thereof in the preparation of nanoparticulated system (Filed).

2. Naturally derived polysaccharides of isphagula seed and its uses thereof in the preparation of nanoparticulated system (Filed).

3. Naturally derived polysaccharides of mango gum and its uses thereof in the preparation of nanoparticulated system (Filed).
LIST OF PRESENTATIONS


Oral Targeting of Protein Kinase C Receptor: Promising Route for Diabetic Retinopathy?

Deepa Pathak1,*, Ankur Gupta2, Bhagyashree Kamble3, Gowthamarajan Kuppusamy1, Bhojraj Suresh1,2,3

1Department of Pharmaceutics, J.S.S. College of Pharmacy, Ootacamund, T.N.-643001, India; 2Department of Pharmaceutical Chemistry, J.S.S. College of Pharmacy, Ootacamund, T.N.-643001, India; 3Department of Pharmacognosy, J.S.S. College of Pharmacy, Ootacamund, T.N.-643001, India

Abstract: In patients with diabetes, hyperglycemia is known to promote high levels of diacylglycerol which activates protein kinase C (PKC) in the vascular tissues and leads to the production of vascular endothelial growth factor (VEGF) in the retina. PKC activation and increased concentration of VEGF are likely to play a key role in diabetic microvascular complications, particularly change in vascular permeability, inflammation, fluid leakage and ischemia in the retina. PKC comprises a super-family of isoenzymes that is activated in response to various stimuli. The PKC family consists of 12 isomers that possess distinct differences in structure, substrate requirement, expression and localization. PKC isomer selective inhibitors and VEGF trap are likely to be new therapeutics, which can delay the onset or stop the progression of diabetic vascular disease. A new promising therapy for diabetic retinopathy is undergoing Phase III trials, in which they proposed to target PKC βII isomer using Ruboxistaurin by oral administration. Besides retina, PKC βII isomer is found in higher concentration in brain, spleen, etc. So, oral targeting may be a questionable approach since generalized inhibitors may prove toxic in the treatment of diabetic retinopathy and ocular delivery may be a better alternative approach.

Keywords: Diabetic Retinopathy, PKC, VEGF, inhibitors, novel drug delivery approach.

INTRODUCTION

Diabetic retinopathy is a complication of diabetes and a major cause of unavoidable blindness in both the developing and the developed countries. Diabetic patients with retinopathy are expected to become blind 25 times more than nondiabetics [1, 2]. Diabetic retinopathy is characterized by the formation of primitive, leaky and disorganized vascular networks, which grow into the vitreous and reflect the unique aspects of vascular endothelial growth factor (VEGF) function which in turn is activated by the active protein kinase C (PKC) receptor. In hyperglycemic patients there is an increase in diacylglycerol generation (DAG), advance glycosylation end product (AGE) and free radical generation which activate PKC receptor by DAG-PKC pathway. The expression level of VEGF gets influenced by binding with hypoxia inducible factor (HIF-1α). In diabetic retinopathy, supply of oxygen to the part of retina is decreased which blocks the blood vessels causing hypoxic and ischemic condition. To compensate this condition, there is a formation of new blood vessels in the presence of VEGF and HIF-1α known as neovascularization. These newly formed blood vessels are leaky, fragile and have thin wall which leak the blood on the surface of eye and blindness takes place [3-6]. Diabetic retinopathy progresses through various stages, the two main stages of visual loss/impairment in patients with diabetic retinopathy are: proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME). Retinal neovascularization, a hallmark of proliferative diabetic retinopathy (PDR), is a major risk factor for severe vision loss in patients. Depending on the degree and severity of newly formed vessels, presence of vitreous or pre-retinal hemorrhage and retinal detachment, PDR can be categorized as nonproliferative diabetic retinopathy (Pericyte loss, basement membrane thickening, vascular leakage, alteration in blood flow, tissue hypoxia), preproliferative diabetic retinopathy (Hypoxia, oedema, microaneurysms, soft exudates, venous beading) and proliferative diabetic retinopathy (Angiogenesis, fibrovascular ridge, breakdown of inner blood-retinal barrier, retinal detachment, blindness). Diabetic macular edema (DME) is the most common cause of moderate visual loss which may be associated with any of the stages of retinopathy [7]. DME is defined as retinal thickening or presence of hard exudates within one disc diameter of the center of the macula shown in Fig. (1).

PATHOPHYSIOLOGY AND BIOCHEMICAL CHANGES

The DAG-PKC pathway contributes to vascular function in many ways, such as regulation of endothelial permeability, vasoconstriction, extracellular matrix synthesis/turndover, cell growth, angiogenesis, cytokine activation and leucocyte adhesion [8,9] as shown in Fig. (2).

RECEPTORS AND ACTIVITY

Mainly three proteins PKC, VEGF and HIF-1α are responsible for retinopathy and these proteins are interrelated
with each other [10]. 12 isomeric forms of PKC receptor are available throughout the body wherein PKC beta II gets activated due to high glucose level in various animal tissues namely brain, aorta, kidney, retina and heart as shown in Table 1 [11, 12]. PKC delta is present in brain, heart, spleen, lung, liver, ovary, pancreas, and adrenal tissues. PKC epsilon is present in brain, kidney, and pancreas but predominantly present in brain. PKC zeta is present in most tissues, particularly lungs, brain, and liver. Both PKC delta and PKC zeta showed some heterogeneity of size among the different tissues. PKC alpha is present in all the organs and tissues examined but predominantly in brain and spleen. Further, PKC beta I and beta II are also present in great amount in the brain and spleen. In case of diabetic retinopathy, PKC beta II isoform is more active than other isoforms and PKC beta II isoform is present in high percentage in the retina [13].

VEGF are important signaling proteins involved in vasculogenesis and angiogenesis. Currently, the VEGF family consists of seven members, VEGF A, VEGF B, VEGF C, VEGF D, VEGF E, VEGF F, and P1GF with distinct individual monomeric forms consisting of 121, 145, 165, 183, 189, and 206 amino acids respectively. These VEGF isomers act via three specific tyrosin kinase receptors- VEGFR1/Fit-1,
VEGFR2/Flk-1, and VEGFR3/Flt-4 [14]. The detailed function of each receptor has not been completely determined however these VEGFRs have been targeted due to their role in angiogenesis. Increased concentration of VEGF in various organs causes serious pathological conditions like cerebral venous infarcts and vasogenic edema in brain, retinal /choroidal neovascularization and macular edema in eyes, and increased glomerular permeability in kidney. Where as decrease in VEGF level also causes various pathological conditions like stroke and impaired reparative neurogenesis in brain, impaired development of collateral vessels in heart, vascular hypertension, collapse of capillary loops and impaired podocyte function in kidney, neuropathy and impairment of wound healing [15]. Table 2 describes the biochemical changes due to impaired activity of various factors and drugs for the treatment of hyperglycemic stage.

**CURRENT MARKETED DRUGS FOR DIABETIC RETINOPATHY**

**Bevacizumab**

Bevacizumab (trade name Avastin®) is a recombinant humanized monoclonal IgG1 antibody that binds to and inhibits the biologic activity of human vascular endothelial growth factor (VEGF). Bevacizumab contains human framework regions and the complementarily-determining regions of a murine antibody that binds to VEGF (Fig. (3)). Bevacizumab is produced in a Chinese Hamster Ovary mammalian cell expression system and has a molecular weight of approximately 149 kilodaltons [31]. 2.5 mg is recommended to be administered by intravitreal injection every two weeks.

**Ranibizumab**

Ranibizumab (trade name Lucentis®) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (Fig. (4)). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an E. coli expression system. Ranibizumab is derived from the same parent murine antibody as bevacizumab. It is much smaller than the bevacizumab and has been affinity matured to provide stronger binding to VEGF-A. It is an anti-angiogenic that has been approved to treat the "wet" type of age-related macular degeneration (ARMD), a common form of age-related vision loss. 0.5 mg (0.05 mL) is recommended to be administered by intravitreal injection once a month (approximately 28 days). If monthly injections are not feasible, the regimen may be reduced to 1 injection every 3 months after the first 4 months [32].

**Limitations of Ranibizumab/Bevacizumab and their Intravitreal Formulation**

Since these drugs have high molecular weight and proteinous in nature, there are various issues related to their stability and permeability through ocular barriers like tear
Table 2. Biochemical changes due to impaired activity of various factors and drugs used in hyperglycemic stage

<table>
<thead>
<tr>
<th>Targets</th>
<th>Mechanism</th>
<th>Effective Drugs</th>
<th>Disease Stage</th>
<th>Dosages Form</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>VEGF increase permeability and simulates vasoproliferation. PKC is a signal transducer switch to activate or inhibit the activity of VEGF and VEGF in turn activate PKC and cause fluid leakage, cell proliferation and inflammation.</td>
<td>Bevacizumab (Avastin®, Genentech Inc.), Ranibizumab (Lucentis®, Genentech, Inc.), Pegaptanib (Macugen®, Eyetech), VEGF Trap-eye (Regeneron Pharmaceuticals Inc.)</td>
<td>DME and PDR</td>
<td>Intravitreal injection</td>
<td>Bevacizumab (1.25 mg to 2.5 mg) [16-18], Ranibizumab (0.5 mg) [19], Pegaptanib (0.3mg) VEGF trap (0.5 mg)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Diabetes leads to chronic inflammatory response in retinal endothelial and neural cells, resulting in VEGF production and recruitment of inflammatory mediators causing increased vascular permeability, capillary non-perfusion, neurodegeneration, and neovascularization.</td>
<td>Steroids reduce inflammation, fluid leakage and close the tight junctions between endothelial cells. e.g. Triamcinolone acetonide, Triamcinolone acetonide implant (l-vation®), Flucinolone acetonide implant (Retisert®), Dexamethasone implant (Posidurex®)</td>
<td>In Short term benefited but in the long term, needs repeated application of intravitreal injection due to recurrence of DME.</td>
<td>Intravitreal injection</td>
<td>Triamcinolone acetonide (1-8 mg, common dose 4 mg) [20].</td>
</tr>
<tr>
<td>Renin-angiotensin-aldosterone system (RAAS)</td>
<td>In diabetes intraocular RAAS system gets up-regulated and stimulates VEGF expression in retinal vascular endothelial cells.</td>
<td>RAAS inhibitors reduce blood pressure, ameliorating the hydrostatic process that exacerbates fluid leakage. E.g. ACE inhibitors: lisinopril enalapril, ramipril, candesartan, losartan and spironolactone.</td>
<td>PDR</td>
<td>Intravitreal injection</td>
<td>Enalapril (10 mg/day) [21].</td>
</tr>
<tr>
<td>Enzymes</td>
<td>The vitreous in diabetic patients undergoes structural changes leading to collagen cross-linking and vitreomacular traction worsening the DME. Newly formed vessels use the posterior hyaloid face as a scaffold to grow. The retracting vitreous pulls on these vessels and is responsible for both vitreous hemorrhage and retinal detachment in PDR.</td>
<td>Hyaluronidase, Plasmin, Microplasmin</td>
<td>PDR and DME</td>
<td>Intravitreal injection, (Under Phase III trial)</td>
<td>Hyaluronidase (5 U) alone is ineffective, whereas plasmin (0.25 U) alone induces partial posterior vitreous detachment (PVD), a very dangerous state for the diabetic eye. Combination of both can induce complete PVD in 12-week old diabetic rats [22].</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>Extracellular carbonic anhydrase increases retinal vascular permeability by increasing pH, leading to kallikrein-mediated proteolytic activation of kinin.</td>
<td>Acetazolamide</td>
<td>DME and PDR</td>
<td>Oral</td>
<td>Acetazolamide [23].</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>Hyperglycaemia increases production of reactive-oxygen species (free radicals), leading to activation of protein kinase C, formation of advanced glycosylation end-products (AGE), activation of the polyol pathway and VEGF production.</td>
<td>The antioxidants suppress production of the growth factor VEGF, which promotes abnormal blood vessels in the retina eg. Vitamin C, Vitamin E, Benfotiamine.</td>
<td>DME and PDR</td>
<td>Intravitreal injection</td>
<td>Benfotiamine has been used for the past 12 years in Europe for the treatment of neuropathy, retinopathy as well as heart and circulatory conditions and has shown no adverse effects [24].</td>
</tr>
</tbody>
</table>
fluid-eye barrier, cornea, conjunctiva and blood ocular barriers. Moreover, they are available in the form of intravitreal injection (IVT) which itself has various usage limitations like elevation in intraocular pressure from baseline up to 25 mm Hg which leads to iridocyclitis, endophthalmitis and ischemic central retinal vein occlusion. Other ocular adverse events occurring in > 10% of ranibizumab treated patients were conjunctival hemorrhage, iridocyclitis, iritis, retinal hemorrhage, retinal detachment, traumatic cataract and reduction in visual acuity. Ranibizumab has course duration of 24 months, this suggests that the cost of treatment may exceed $58,488.00 and the delivery of IVT should be carried out by a licensed and qualified physician under controlled aseptic technique through a 5-micron 19-gauge filter attached to a 1-cc tuberculin syringe. The filter needle is replaced with a sterile 30-gauge x 1/2-inch needle for the intravitreal injection. 0.05 ml is injected into the eye under aseptic conditions using sterile gloves, a sterile drape and a sterile eyelid speculum. The eye is ordinarily prepared with Betadine and adequate anesthesia. Each vial is used for only one eye. All these procedures lead to patient non-compliance [33]. There are various approaches to overcome these limitations by: 1.) increasing the absorption through the application of physical methods (iontophoresis/phonophoresis), co-administration with permeation enhancers (BL-9, Brij 78, Brij 99, Fusidic acid, Saponin), incorporation into liposomes, niosomes, or other carriers. 2.) minimizing metabolism through covalent attachment to a polymer, chemical modification of primary structure, targeting to specific tissues and co-administration with enzyme inhibitor. 3.) prolonging blood levels through use of bioadhesives, liposomes, niosomes, proniosomes, polymeric nanoparticulate systems, solid-lipid nanoparticulated system or other carrier systems [34].
Fig. (3). Pymol view (PDB id.1BJ1) of the interaction of Bevacizumab (Chain H, J, K and L) with VEGF-A (Chain W,V) receptor (Solid Mesh form and Ribbon form).

Fig. (4). Pymol view (PDB id 1CZ8) of the interaction of Ranibizumab (Chain H, X, Y and L) with VEGF-A (Chain W,V) receptor (Solid Mesh form and Ribbon form).
Ruboxistaurin (LY333531)

LY333531 (32mg OD for 1 month, under phase III trial) is a highly selective potent reversible PKC β inhibitor which shows hydrophilic interactions with VAL 423, GLU421, THR404 in the active site of PKC βII (Fig. (5)). LY333531 is present in the form of seven salts namely hydrochloride, sulphate, mesylate, succinate, tartrate, acetate and phosphate. 43% of FDA approved marketed salts are hydrochloride and only 2% have been marketed as mesylate salts. Apart from mesylate salt, rest of the salts were eliminated either due to poor crystallinity, low solubility and impurity issues. The AUC 3.89 and Cmax 896±243ng/ml of mesylate salt are higher than its hydrochloride salt i.e 1.62 and 400±142ng/ml. The metabolite of LY333531 is N-desmethyl ruboxistaurin (LY338522) was found to be equally active with Cmax value of 2455±930ng/ml [35, 36]. LY333531 is highly selective inhibitor with an IC50 value 5.9nm and 4.7nm for PKC βII and β respectively but PKC isoforms namely α, γ, δ, ε and η get inhibited by an IC50 values 360nm, 300nm, 250nm, 600nm and 52nm respectively [37, 38].

EXPECTED PROBLEMS ASSOCIATED WITH ORAL TARGETING

PKC is a signal transducer which activates or inhibits VEGF and VEGF in turn activates or inhibit PKC. Therefore, PKC and VEGF form a vicious cycle in an intracellular process of biochemical pathophysiology of neovascularization (Fig. (6)). Since PKC family is widely distributed throughout the body and the inhibition of PKC isoforms would also suppress VEGF therefore, oral dosage form of Ruboxistaurin is likely to have serious, perhaps fatal and systemic consequences [39] like increased risk of stroke and impaired reparative neurogenesis in brain, impairment of collateral vessels development in heart, vasculature hypertension, collapse of capillary loops, impaired podocyte function and protein urea in kidney, neuropathy and impaired motor sensation in peripheral nervous system and impairment of wound healing. Comparatively, since PKC β is present at high level in retina, ocular targeting of Ruboxistaurin using novel formulation approaches will be more beneficial in the treatment of diabetic retinopathy without any systemic and peripheral side effects.

FUTURE PERSPECTIVES

Since conventional drug delivery systems (Oral or IVT) have many limitations in diabetic retinopathy, novel formulation approaches like nanoparticulate system, liposomes, niosomes, ocular inserts, in-situ gelling system (Fig. (7)) etc may be a better alternative for the treatment of diabetic retinopathy. By using these techniques we can produce cost effective patient friendly formulations and also eliminate the complications associated with oral delivery and intravitreal injection. The advantages of these approaches may extend to long term contact along with targeted drug delivery and reduced dosing frequency, easy passage across blood retinal barrier due to nano size, direct targeting of drug to PKC/VEGF receptors, easy administration and patient compliance with low cost treatment. Therefore, novel non-invasive drug delivery may be a better approach for the treatment of diabetic retinopathy. We are presently engaged in the development of herbal nano formulations, using green pharmacy approach (Preparation of formulation using herbal drugs and biopolymers extracted from plant source without organic solvents) to target PKC/VEGF receptors for the treatment of diabetic retinopathy.
CONFLICT OF INTEREST

The authors do not have any conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank Department of Science and Technology, New Delhi, India for providing financial assistance for carrying out this project. We also thank J.S.S. College of Pharmacy, Ooty, India for providing facilities to carry out research in the field of diabetic retinopathy.

ABBREVIATIONS

AGE = Advanced glycosylation end-products
VEGF = Vascular endothelial growth factor
GH-IGF = Growth factor–insulin growth factor
PDR = Proliferative diabetic retinopathy
VH = Vitreous hemorrhage
RD = Retinal detachment
PDGF = Platelet derived growth factor
PKC = Protein kinase C
RAS = Renin-angiotensin system
CA = Carbonic anhydrase
IVT = Intravitreal injection
PVD = Posterior vitreous detachment

REFERENCES


[19] Diabetic Retinopathy Clinical Research Network. A randomized trial comparing intravitreal triamcinolone acetonide and focal/grid...


[27] Raskin, P.; Catran, D.; Williams, M. Pimagedine Reduces Progression of Retinopathy and Lowers Lipid Levels in Patients with Type 1 Diabetes Mellitus was presented as a poster session on Sunday; November 7, 1999.