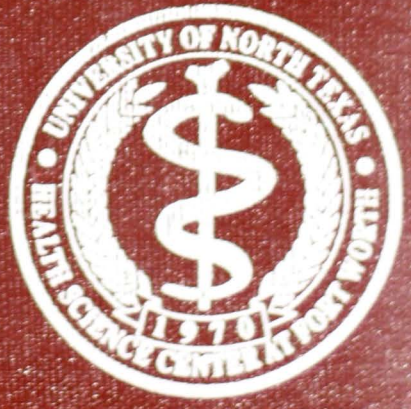
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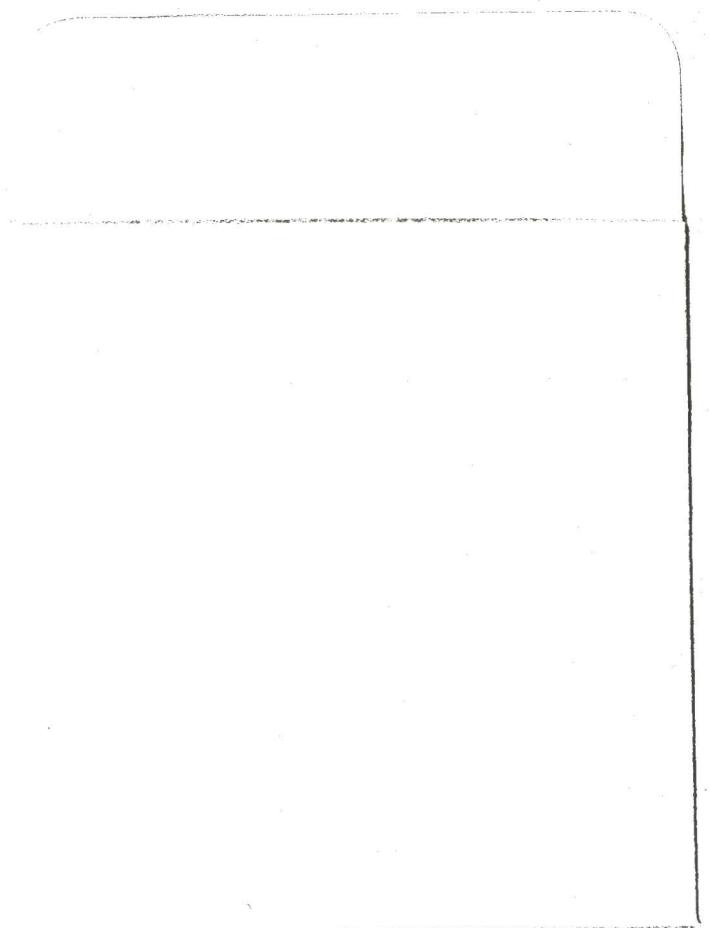

DeFaller, Joseph M., Mechanism of Action and Clinical Efficacy of AL-3789, an Angiostatic Steroid. Doctor of Philosophy (Pharmacology), December, 1996, 115 pp., 11 tables, 31 illustrations, reference list, 115 titles.

Angiogenesis, the growth of new blood vessels, is important in cancerous tumor growth, diabetes, and degenerative diseases. During angiogenesis, proliferating vascular endothelial cells from blood vessels secrete proteinases, including urokinase (uPA) and stromelysin-1 (MMP-3), which dissolve the extracellular matrix and allow them to migrate and form new blood vessels. In this investigation the angiostatic effects of AL-3789, an angiostatic steroid, were investigated in *in vitro* human cell models and human clinical trials. The angiostatic mechanism of action of this agent at 10^{-5} molar concentration was demonstrated to include: 1) inhibition of lipopolysaccharide-induced uPA and MMP-3 production by human microvascular endothelial cells (HMVEC-L), 2) dose-dependent inhibition of HMVEC-L proliferation, and 3) alteration in the expression of approximately 1% of HMVEC-L proteins visualized by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). These protein changes are similar in magnitude to those caused by glucocorticoids, which act via an intracellular/intranuclear glucocorticoid receptor. The clinical efficacy of AL-3789 in preventing re-neovascularization and recurrence of malignant pterygia following surgical excision in humans was demonstrated by topical ocular dosing of a 1.0% suspension three times daily in a double-masked, randomized, prospective, placebo-controlled trial. Pterygium is a chronic condition in which neovascularized fibrotic tissue grows over the cornea to evenutally obstruct the visual axis in some patients. Computer image analysis of serial photographs for each patient following surgery showed a re-neovascularization growth rate following pterygium excision of

1.58 mm^2/week for patients treated with placebo compared to 0.78 mm^2/week exhibited by the AL-3789 treated group ($p < 0.05$, ANOVA). Pterygia recurred in 71% of the placebo group compared to 42% of the AL-3789 treated group ($p < 0.05$, Chi-square contingency test). In conclusion, the angiostatic steroid AL-3789 inhibits neovascularization in part by decreasing vascular endothelial cell proliferation and proteinase (urokinase and stromelysin-1) secretion. AL-3789 treatment significantly inhibits re-neovascularization and recurrence rates following malignant pterygium excision in humans.

MECHANISM OF ACTION AND CLINICAL EFFICACY

OF AL-3789, AN ANGIOSTATIC STEROID

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CHAPTER I

INTRODUCTION

Statement of the Problem

Angiogenesis, the formation of new blood vessels, occurs naturally in wound healing, in endometrial hypertrophy during ovulation, and during embryogenesis. It is a biological process that is necessary for normal growth, maintenance, and overall homeostasis. The process of angiogenesis is complex and highly regulated by a diverse system of angiogenic stimulators and inhibitors. Under normal conditions these regulators maintain vascular endothelial cells in a quiescent state. Pathologic conditions such as cancerous tumor growth shift this homeostatic balance, stimulating endothelial cells to form new blood vessels. Solid tumors require new blood vessels for growth and, in many cases, metastasis. The process of new blood vessel growth has been shown to consist of several steps which include the activation, migration, and proliferation of vascular endothelial cells. The ability to control any of these essential cellular activities during angiogenesis might allow control of the process itself and therefore, control of many pathologies.

Pathologic angiogenesis can occur in all body systems and tissues. In the eye, neovascularization is a common aspect of many potentially devastating ocular diseases, including neovascularization of the retina in exudative age-related macular degeneration (ARMD) and proliferative diabetic retinopathy (PDR). The study of angiogenesis is therefore an area of intense interest in ophthalmology, as

investigators search for therapies which will retard or prevent the growth of new blood vessels that destroy visual structures or occlude the visual axis.

Agiostatic steroids are a class of compounds which have been reported to affect angiogenesis in a variety of *in vitro* and animal models of neovascularization. One of these, AL-3789 (Alcon Laboratories, Inc.), has been demonstrated to inhibit neovascularization in 1) the chick embryo chorioallantoic membrane (CAM) modelMcNatt *et al*, 1990), 2) the lipopolysaccharide (LPS)-induced rabbit model of corneal neovascularization (Griffen *et al*, 1993), 3) the kitten and rat retinopathy of prematurity (ROP) models (Penn *et al*, 1996), and 4) neovascularization associated with intraocular tumor growth (Ma *et al*, 1995).

The objective of this research was to investigate the mechanism of action of AL-3789 by systematically examining the drug's effect on some of the essential steps of angiogenesis, and to determine if the compound has utility for pharmacological treatment of neovascular ocular diseases in man. The specific aims included: 1) determination of the effect of AL-3789, or its active metabolite, AL-4940, on the regulation of enzymes and inhibitors involved in extracellular matrix turnover required during angiogenesis, i.e. expession of urokinase (uPa), plasminogen activator inhibitor (PAI-1), and stromelysin (MMP-3) in human microvascular endothelial cells (HMVEC-L), 2) determination of the effect of AL-3789 on HMVEC-L proliferation, microtubule formation, and protein expression to further elucidate its mechanism of action, and 3) conduct of human clinical trials to determine the safety and therapeutic efficacy of AL-3789 in the prevention of a pathologic neovascular process in the eye, namely, re-neovascularization following recurrent pterygium excision.

Putative Mechanisms of Angiogenesis

It has been shown that cancerous tumor growth is angiogenesis dependent (Folkman, 1990). Previously, tumor hyperemia, growth, and metastasis were thought to result from expansion of existing vessels supplying the tumor (Conan and Shelton, 1946; Day, 1964). It is now evident that tumors can only develop to a limited degree by obtaining nutrients from surrounding tissues, and that new vessel growth to the tumor is necessary once it reaches 1-4 mm in diameter (Ausprunk and Folkman, 1977; Folkman, 1990).

Tumors grown *in vitro* or in the avascular rabbit cornea do not grow and metastasize until they become vascularized. In retinoblastoma, metastastic seeds to the anterior vitreous are avascular and do not grow, but remain viable. In addition, it has been shown that breast carcinoma intratumoral endothelial cells proliferate 45 times faster than endothelial cells in the surrounding healthy stroma (Vartanian *et al*, 1994). Therefore, proliferation of vascular endothelial cells appears to be an essential feature of angiogenesis. The sequence of events involved in angiogenesis is shown in Figure 1.

It has been proposed that endothelial cells at the tips of existing venules migrate through the vessel wall into the surrounding extracellular matrix where they form new capillaries. Although the process of angiogenesis is continuous, it can be artificially divided into discreet steps that include 1) receipt of an angiogenic signal

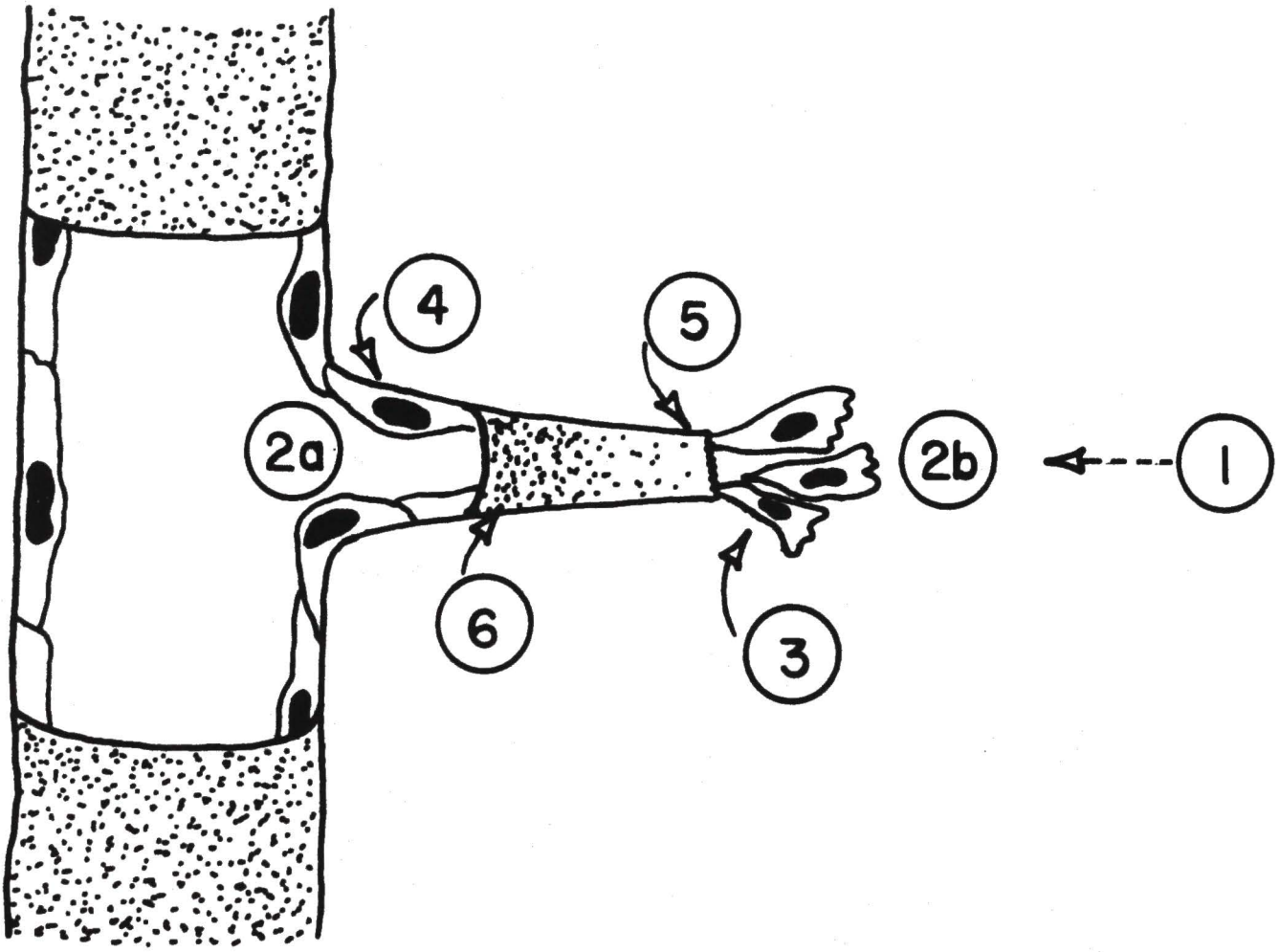


Figure 1. Sequence of events in angiogenesis: 1) receipt of an angiogenic signal (*i.e.*, VEGF, EGF, bFGF, TNF-α, etc.) that activates the cells, 2) secretion of proteases that degrade (2a) the vascular basement membrane and (2b) the interstitial matrix, 3) migration of endothelial cells into the surrounding tissue, 4) proliferation of the vascular endothelial cells, 5) formation of lumen by associated migrating cells, and 6) elaboration of a new basement membrane around the new vessel.

(*i.e.*, VEGF/VPF, EGF, bFGF, TNF-α, etc.) that activates the cells, 2) secretion of proteases that degrade (2a) the vascular basement membrane and (2b) the interstitial matrix, 3) migration of endothelial cells into the surrounding tissue, 4) proliferation of the vascular endothelial cells, 5) formation of lumen by associated migrating cells, and 6) elaboration of a new basement membrane around the new vessel. Control of neovascularization might be controlled by altering any of these essential steps of angiogensis.

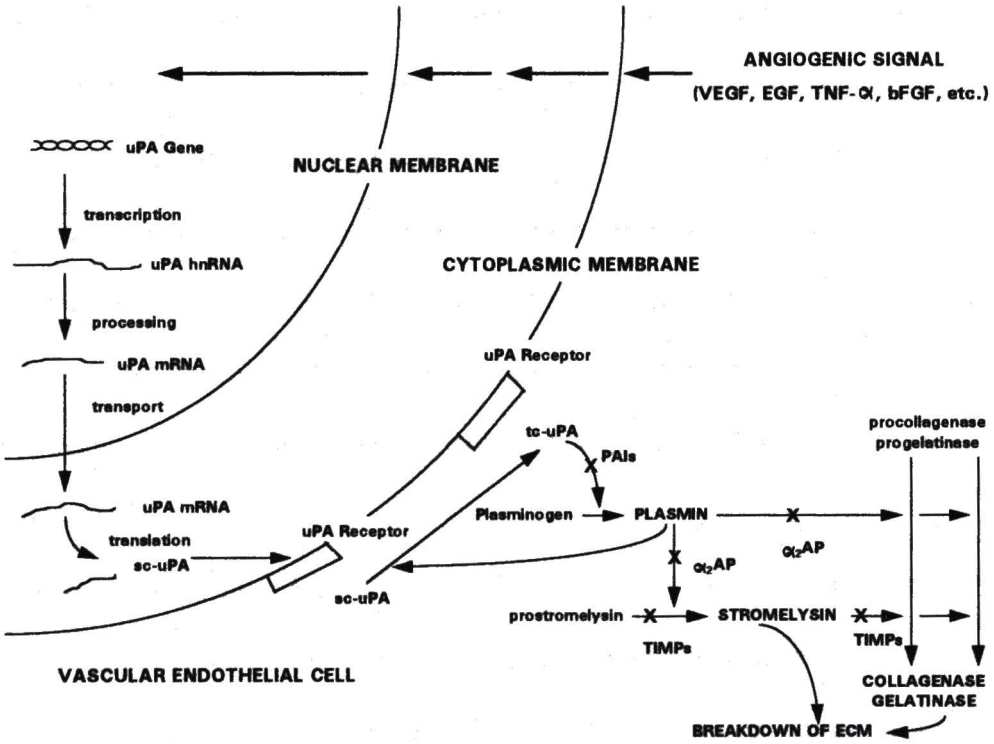


Figure 2. Hypothetical cascade of cellular and extracellular events which lead to production of proteases and degradation of the extracellular matrix. (VEGF, Vascular Endothelial Growth Factor; EGF, Epithelial Growth Factor; TNF-α, Tumor Necrotic Factor alpha; bFGF, basic Fibroblast Growth Factor; uPa, urokinase-like Plasminogen Activator; sc-uPa, single-chain uPa; tc-uPa, two-chain active uPa; PAIs, Plasminogen Activator Inhibitors; α_2AP, alpha-two antiplasmin; TIMPs, Tissue Inhibitors of Matrix Metalloproteinases; ECM, Extracellular Matrix).

The angiogenic process is initiated by angiogenic signals such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor/vascular

permeability factor (VEGF/VPF), tumor necrosis factor-alpha (TNF-α), epidermal growth factor growth factor (EGF), and others, which activate vascular endothelial cells (D'Amore and Thompson, 1987; Mauviel, 1993; Figure 2). Once activated, vascular endothelial cells elaborate proteases which allow the cells to dissolve their basement membrane. They then proliferate and migrate through the extracellular matrix, forming new capillary networks.

Two types of plasminogen activator enzymes are secreted by activated endothelial cells: urokinase (uPA), and tissue-type plasminogen activator (tPA). The fibrinolytic activity in blood, a process which is necessary to prevent fibrin deposition in blood vessels, is largely determined by the blood concentration of tPA (Wun and Cappuano, 1985). The activity of tPA is regulated primarily by PAI-1. Urokinase, expressed by vascular endothelial cells, catalyzes the conversion of plasminogen to plasmin, and plasmin formation begins the cascade of events which lead to formation of matrix metalloproteases (MMPs) which break down the extracellular matrix.

Increased tissue levels of uPA have been shown in neoplasia, metastasis, cell proliferation, invasion of tumor cells, and in angiogenesis (Dano *et al*, 1985; Saksela and Rifkin, 1988). Tissue and plasma uPA concentrations are independent prognostic indicators whose increase is associated with decreased survival times in patients with breast and gastric cancer (Janike, 1990; Duffey *et al*, 1990; Schmitt *et al*, 1991; Foekens *et al*, 1992; Graeff *et al*, 1992; Del Vecchio *et al*, 1993; Nekarda *et al*, 1994). uPA is a serine protease secreted by monocytes and activated endothelial cells as a 54 kD single-chain polypeptide (pro-uPA, scuPA).

Single-chain uPA binds to cellular surface uPA receptors which are responsible for localization of fibrinolytic activity and directional regulation of plasmin generation. The scuPA can be activated by plasmin to the active two-chain form (tcuPA) (Dano *et al*, 1985) when associated with the uPA receptor. Estreicher *et al* (1990) demonstrated that the uPA receptor polarizes protease activity to the leading edge of migrating monocytes. The location of the receptor on the cell surface therefore seems to control the cell's orientation during migration by allowing protease formation in the direction of movement towards the angiogenic signal.

The genes responsible for expression of uPA and tPA have been cloned and sequenced (Petersen *et al*, 1990; Pennica *et al*, 1983; Friezner-Degen *et al*, 1986; Riccio *et al*, 1985). The synthesized uPA polypeptide is converted by proteolytic cleavage to a two-chain active form connected by a disulfide bond; the B-chain of the molecule contains the proteolytic active site of uPA, while the A-chain determines the interaction of the enzyme with matrix proteins and the uPA receptor. Upon activation, uPA catalyzes the conversion of plasminogen to plasmin, which results in matrix degradation by plasmin itself. Plasmin also activates the matrix metalloproteinases (MMPs), which also degrade the extracellular matrix. MMPs are zinc-dependent proteases capable of degrading a wide range of extracellular matrix components.

Within the MMP family of proteases (Table 1) are collagenases and glycanases, including gelatinase, stromelysin, transin, proteoglycanase, stromelysin-2, and PUMP-1, as well as interstitial, Type I, fibroblast, PMN, and Type IV collagenases (Mauviel, 1993, van Hinsbergh and Koolwijk, 1992). Expression of

MMPs is modulated by cytokines and growth factors through the gene products of the *fos* and *jun* family of oncogenes. Following expression they are regulated by specific inhibitors known as TIMPs (tissue inhibitors of metalloproteinases).

Table 1. Matrix metalloproteases.

MMP Number	Name	MW (kD)	Substrate	Distribution
Collagenases				
1	Interstitial Collagenase	52.5	Fibrillar collagens I,II, III, V, VII, X	Connective tissue cells, monocytes, Endothelial Cells
8	Neutrophil Collagenase	75	Fibrillar collagens I,II,III Gelatin	Neutrophils (PMN)
Gelatinases				
2	Gelatinase A	72	Gelatin, Collagens IV,V,VII,X,XI, Fibronectin, Elastin	Most cell types, tumor cells
9	Gelatinase B	92	Same as Gelatin A	Connective tissue cells, monocytes, macrophages, tumor cells
Stromelysins				
3	Stromelysin-1	50/67	Proteoglycans, Fibronectin, Collagens III,IV,V,IX, Gelatins	Same as MMP-1, tumor cells
10	Stromelysin-2	53	Same as MMP-3	Macrophages, tumor cells
7	PUMP-1	28	Gelatins, Fibronectin, proteoglycans, Collagen IV, Elastin	Immature monocytes, connective tissue cells, tumor cells
Others				
	Stromelysin-3	54.6	Unknown	Stromal cells of tumors
	Metalloelastase	21	Elastin, Fibronectin	Macrophages

Together the enzymes described above dissolve and remodel the fibrous and globular proteins (collagens, as well as proteoglycans, glycoproteins, laminins, and

fibronectins) of the extracellular matrix and basal vascular membrane, allowing migration and budding of endothelial cells and subsequent angiogenesis.

Vasculature of the Eye

Neovascularization may arise from any blood vessel in the eye. Blood is supplied to the eye by the ophthalmic artery, which branches from the internal carotid artery posterior to the optic nerve head (Figure 3). The ophthalmic artery branches, in turn, into two major vessels, the central retinal artery and the long posterior ciliary artery. The central retinal artery penetrates the dura of the optic nerve posterior and infero-medially and emerges at the lamina cribrosa of the optic nervehead, where it branches into superior and inferior vessels on the nasal side of the optic disc. At this point the vessels are visible ophthalmoscopically. The superior and inferior vessels branch into nasal and temporal arterioles and then into second- and third-order arterioles to produce the typical vascular pattern of the retina.

The long and short posterior ciliary arteries supply blood to the ciliary body, the iris, and the anterior and posterior choriod. After branching from the ophthalmic artery, the short ciliary artery proceeds anteriorly along the optic nerve and penetrates the sclera, where it branches into arterioles which supply the choroid and the lamina cribrosa. The long ciliary artery traverses the chorid anteriorly where it supplies the ciliary body and iris. Neovascularization of the iris is accompanied by overgrowth of these vessels into the anterior chamber angle and trabecular meshwork to cause neovascular glaucoma.

The central retinal vein drains the retina by means of vessels which correspond roughly to the arteries and arterioles. It drains into the superior ophthalmic vein or the cavernous sinus. The choriodal veins do not follow the arterial pattern but collect into four major veins which drain into the ophthalmic veins. Retinal veins are composed only of an endothelial cell layer.

The vessel walls of retinal arterioles are composed of endothelial cells with basement membrane and a muscle coat but no internal elastic lamina. The endothelial cells form tight junctions and do not allow leakage of blood components into the retina except in disease states. This barrier is known as the "inner blood retinal barrier." The capillary retinal bed forms two layers at the level of the ganglion cells and the inner nuclear layer. The capillary bed nourishes the inner layers of the retina, while the choriocapillaris, the terminus of the short posterior ciliary arteries, supplies the photoreceptors. Neovascular vessels in exudative age-related macular degeneration (ARMD) arise from the choriocapillaris. The fovea, or macular area, is normally free of capillaries. Subfoveal neovascular membranes may arise in exudative ARMD.

Choriodal capillaries do not form tight junctions, but they are impermeable to large molecules such as proteins. The retinal pigment epithelial cells form tight junctions; this is termed the "outer blood retinal barrier." At the optic nerve head there is an additional network of capillaries called the precapillary plexus.

The circulation of the retina may be visualized by a technique known as fluorescein angiography. Generally, a 5 cc bolus of 20% sodium fluorescein is injected into the antecubital vein rapidly and serial photographs of the fluorescing

dye are taken when it reaches the retinal circulation. Abnormalities of the circulatory pattern, including constricted blood flow (ischemia), blockages (occlusions), and leakage (exudation) can be identified using this technique.

Recently, indocyanine green videoangiography has been reported to allow better visualization of retinal vessels in neovascular disease states (Guyer *et al*, 1996).

Ocular Neovascular Diseases and Current Therapies

Neovascularization accompanies many sight-threatening disease processes in the posterior segment of the eye such as diabetic retinopathy and age-related macular degeneration (Goldie, 1970; Newell, 1986; Folkman, 1987; Mulliken and Young, 1988). In the anterior segment, neovascularization is present in rubeosis iridis and following trauma, including surgical procedures such as corneal transplant and pterygium excision. Neovascularization may occur following chronic ocular inflammation and with continuous irritation in contact lens wear. Damage to the eye from neovascularization may result in visual loss following disruption of normal cellular function in the retina, or from bleeding and scar formation which may destroy vision.

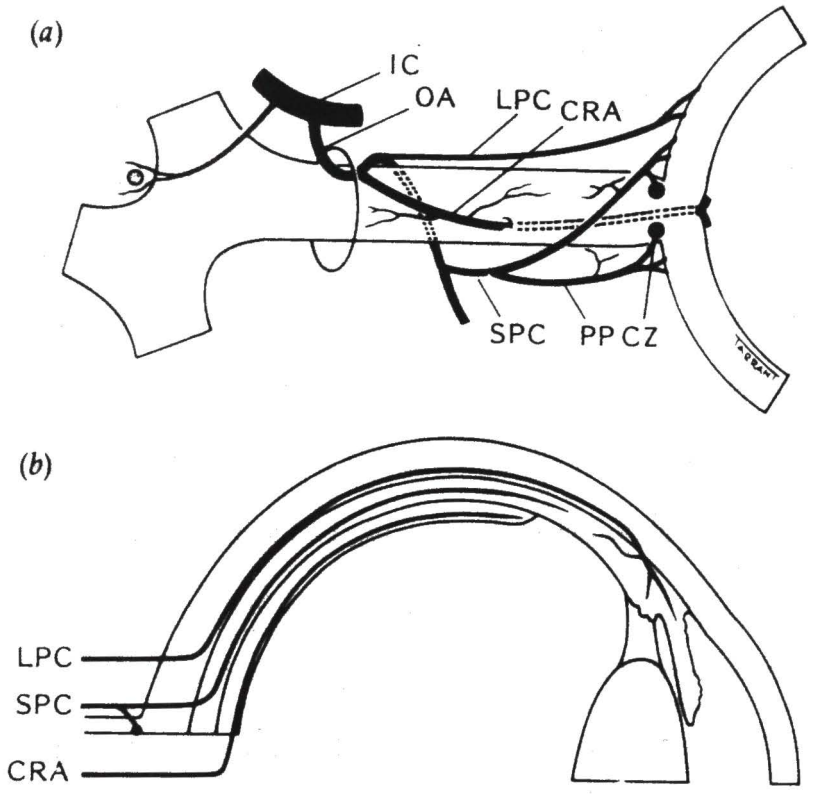


Figure 3. Arterial vasculature of the eye. (a) IC, internal carotid; OA, ophthalmic artery; SPC, short posterior ciliary artery; CZ, circle of Zinn; CRA, central retinal artery; LPC, long posterior ciliary artery; PP, pila plexus. (b) arterioles of the eye (from Grey, 1990).

Anterior Segment Neovascular Diseases

The major anterior segment diseases with a neovascular component include anterior chamber and anterior ocular surface tumors, rubeosis iridis and subsequent neovascular glaucoma, neovascularization following corneal transplant rejection, and pterygium. In addition, filtering blebs formed during glaucoma filtering surgery, or trabeculectomy, may become vascularized when they fail; it is not known, however, if neovascularization is the cause of bleb failure. For conditions which can be treated surgically, such as tumor and pterygium, current standard treatment includes excision of tissue and the use of antimetabolites such as 5-fluorouracil or mitomycin-C at the close of surgery. Beta-irradiation is also utilized, singly or in combination with antimetabolites; these treatments are given in hope of arresting proliferating cells which incite the condition. Immunomodulators such as alpha-interferon and glucocorticoids have been used without complete success to suppress the immune reaction and resultant neovascularization which results upon corneal transplant rejection.

Pterygium is a condition of unknown etiology in which fibrous vascularized tissue grows across the limbus over the cornea, eventually occluding the visual axis in some patients. Histologically, pterygium exhibits corneal subepithelial elastotic degeneration with opacification and destruction of Bowman's membrane. The body of the pterygium is composed of highly vascularized hypertrophic connective tissue, while the pterygium head is made up of atrophic conjunctival epithelium and fibroblasts (Chen *et al*, 1994). Surgical excision of pterygia is indicated if the lesion encroaches on the visual axis or if ocular irritation requires it. Pterygium may result

from irritation of the eye, either in the form of excessive ultraviolet radiation or harsh environmental conditions. The disease is more prevalent in outdoor workers and in equatorial regions. In the United States, approximately 70,000 pterygium surgeries are performed each year. Approximately 20% of primary pterygia become recurrent, or malignant, after the first excision. Hirst *et al* (1994) reported that 97% of recurrent pterygium in their patient population will recur within one year of surgical excision no matter what surgical procedure or pharmacological treatment is given; the speed of the recurrence progressively increases with each surgical excision.

The frequency of neovascular glaucoma is highest in patients with diabetic retinopathy or central retinal vein occlusion and results from overgrowth of iris vessels (rubeosis iridis) into the anterior chamber angle. Treatment includes high dose topical steroids, aqueous humor suppressants such as beta-blockers, and panretinal photocoagulation.

Posterior Segment Neovascular Diseases

By far, the most common and visually devastating ocular neovascular diseases are the posterior segment diseases. Age-related exudative macular degeneration (ARMD) and diabetic retinopathy account for a large measure of blindness in Western countries; "wet" ARMD is responsible for approximately 10% and diabetic retinopathy for 8% of registered blindness. In addition to these conditions, many vascular occlusive diseases which incite neovascularization such as central and branch retinal vein and artery occlusions contribute to many cases of lost vision (Grey, 1990).

Most people over 70 years of age show degenerative changes of the macula that are visible ophthalmoscopically consisting of "drusen", which are hyalinized lipid material and protein deposited between Bruch's membrane and the RPE. Drusen may be white and crystalline (hard) or grey and diffuse (soft). Increasing atrophy of the RPE causes secondary overlying photoreceptor loss and gradual decrease in visual acuity with age. There is no proven effective treatment available for this "dry" form of ARMD.

The pathogenesis of exudative, or "wet" age-related macular degeneration includes breakdown of Bruch's membrane and invasion of new blood vessels from the choriocapillaris between Bruch's membrane and the RPE and retinal layers. Approximately 20% of patients with ARMD develop neovascularization of the retina. The trigger causing active proliferation of blood vessels into the retina is unknown, but several hypotheses include increased material deposited in drusen, invasion of macrophages into Bruch's membrane, ischemia of the retina, altered immnological states, and accumulation of an angiogenic substance within Bruch's membrane (Grey, 1990). Subretinal and subretinal pigment epithelial fluid accumulates at the macula causing distortion and reduction of central vision; this elevation of the retina is termed serous detachment of neurosensory retina. Neovascularization develops at or close to the macula. In the case of macular neovascularization, vision disturbance is immediate and often severe. For perifoveal neovascularization, the choriodal neovascular membrane (CNM) expands to eventually affect the foveal pigment epithelium with profound central visual loss. As the neovascularization progresses, the RPE is destroyed; if untreated, the

neovascular membrane eventually becomes fibrotic and results in a disciform macular scar.

The only treatment for exudative ARMD that has been shown to be of benefit in selected cases is laser photocoagulation of the neovascular tissue to obliterate proliferating vessels and allow subretinal fluid to absorb (Macular Photocoagulation Study Group, 1991). Loss of vision in the area of photoablation, either pericentral or central, always occurs. Laser treatment must be carried out early in the disease process, especially in the case of perifoveal choroidal neovascular membranes so that the advancing membrane is stopped before central vision is affected. The aim of laser photocoagulation is to apply heavy, confluent overlapping burns over the entire neovascular lesion. If successful, the new vessels will regress within a few weeks and subretinal fluid will be absorbed, preserving remaining vision. However, recurrences of CNM are common in the treated eye, as are appearance of new membranes in fellow eyes.

Diabetic retinopathy is one of the most common causes of blindness in the United States. Risk factors for diabetes mellitus include heredity and obesity. Approximately one-third of type I diabetics exhibit retinopathy compared to 16% of type II diabetics (Grey, 1990). In patients with no retinopathy, intensive control of blood glucose concentrations close to the normal range has been shown to reduce the mean risk for development of retinopathy by 76%; in patients with mild retinopathy, intensive glucose control slowed the progression of retinopathy by 54% (Diabetes Control and Complications Trial Researach Group, 1993). Diabetic retinopathy is caused by biochemical changes in retinal capillaries that are not

completely understood. Increased aldose reductase activity and increased sorbitol levels in the retina have been suggested as a cause of reduced pericyte levels and thickening of the retinal capillary basement membrane. Retinal capillary endothelial cells proliferate, disrupting vessels and causing microaneurysms, capillary dilatation, vascular leakage, exudation, and chronic edema. Further capillary ischemia and hypoxia of the retina induces angiogenic factors such as VEGF/VPF, which cause increased endothelial cell proliferation and neovascularization with fibrosis surrounding new vessels. The fibrovascular changes are known as proliferative diabetic retinopathy (PDR), and vision is lost due to hemorrhage of new fragile vessels into the optical axis as well as growth of vessels between the vitreous and retina, resulting in retinal detachment. In the macula, changes may be exudative, edematous, or ischemic. Exudative changes consist of lipid deposition around the macula from extensive leaking of abnormal capillaries and microaneurysms. Edematous maculopathy causes reduction of vision from chronic macular edema. Ischemic maculopathy is caused by capillary closure in the macula.

Treatment of proliferative diabetic retinopathy consists of panretinal laser photocoagulation. Destruction of abnormal retinal tissue by this method is thought to decrease retinal metabolism and ischemia, resulting in decreased levels of VEGF/VPF and therefore less neovascularization. Multiple treatments are necessary in some cases, but some cases respond and become quiescent. Visual prognosis for patients that do not respond is poor.

In central retinal vein occlusion (CRVO), there in no known etiology in a large number of cases. Additionally, no physical obstruction of the vessel can be shown

by histological examination in some cases. Risk factors include systemic hypertension, glaucoma, diabetes, and hyperviscosity syndromes. The result of CRVO is ischemia of the retina, with accompanying decrease in vision which may be slight, or very profound, and may be accompanied by hemorrhage, macular edema, and neovascularization. In the perfused or nonischemic form of CRVO, blood flow continues in the majority of the retina and visual acuity loss is less severe than in the nonperfused or ischemic form. In the nonperfused form, approximately 50% of the retina exhibits lack of circulatory flow (Maragal *et al*, 1982) and severe permanent loss of vision usually results. The degree of vision loss corresponds to the size and location of the nonperfused macular area. Eventually venous circulation will be reestablished, but by 30 months post-ischemia one-third of patients will have further severe visual loss, one-third will maintain the visual acuity resulting from the ischaemic insult and one-third will have improved vision (Clarkson, 1994). In branch retinal vein occlusion (BRVO) retinal nonperfusion is generally less extensive and loss of vision less severe.

Central retinal artery occlusion (CRAO) risk factors include hypertension, diabetes, arteritis and atherosclerosis. Occlusion of the central retinal artery results in significant decrease in retinal perfusion and rapid permanent visual loss. The extensive ischemia results in neovascularization of the retina and neovascular glaucoma in about 10% of eyes if not treated with laser photocoagulation. In branch retinal artery occlusions which involve the temopral arteries, up to 80% of patients will recover much of their visual acuity with no treatment if the fovea is

spared. Again, laser photocoagulation of ischemic retina is the only treatment available for vein or artery occlusions.

A pharmacological agent capable of being administered to the eye or orbital tissues that could control or halt neovascular activity would be of great therapeutic importance in ocular posterior segment ocular neovascular diseases. Angiostatic steroids hold promise for the pharmacological treatment of neovascular processes of the eye. As noted, some forms of neovascularization in the retina are currently being treated by laser photocoagulation to inhibit new vessel growth. Laser treatment frequently arrests the neovascular process but always results in visual loss to the patient. In the case of perifoveal choroidal neovascular membranes in age-related macular degeneration, 60% of patients develop renewed neovascularization following laser treatment. Use of a drug to control the initial neovascular process or the revascularization process would be of great benefit to these patients.

New Pharmacological Treatments For Angiogenesis

As discussed above, the search for effective treatments that can control the angiogenic process is of intense interest to many researchers. Many different classes of molecules have the ability to modulate the angiogenic cascade, and several of these are active areas of investigation by individual researchers as well as the pharmaceutical industry.

There are several physiologic angiogenic inhibitors endogenous to man that act on various aspects of the neovascular process. Included in these are the interferons, heparinases, proteinase inhibitors, and molecules which inhibit endothelial cell activity. Alpha-interferon inhibits the release of angiogenic factors (*e.g.* bFGF, VEGF/VPF, PDGF), while Platelet Factor 4 (PF4) inhibits angiogenic factor binding to endothelial cells. The TIMPs, CDI (cartilage-derived inhibitor of angiogenesis), and PAIs bind with and inactivate proteinases secreted by the activated endothelial cell. Thrombospondins (TSPs) and proliferin-related protein (PRF) inhibit endothelial cell activity. None of these compounds have been approved to date for use as angiostatic agents.

Investigational physiologic and synthetic agents that inhibit the angiogenic signal prior to endothelial cell activation include α-interferon and antisense oligonucleotides to bFGF mRNA. Tecogalan, pentosan polysulfate, and suramin bind free angiogenic factors. Platelet Factor 4, oligonucleotide-based bFGF binding compounds, and heparinases block angiogenic factor binding to the endothelial cell. Several drugs inhibit endothelial cell receptor intracellular signalling; SU-101 is a PDGF receptor kinase blocker, and CAI (carboxy amido-triazole) inhibits calcium influx. Thalidomide, now in Phase II clinical trials for exudative ARMD, inhibits endothelial cell proliferation as do the fumagellin analogues. Several investigational drugs inhibit matrix metalloprotease activity, including Batimastat, TIMP-2, CDI, minocycline, and GM6001. In addition, endothelial cell integrin receptor antagonists against the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors are being investigated.

Medroxyprogesterone acetate, an angiostatic steroid, has been reported to upregulate the production of PAI-1 in cultured vascular endothelial cells, with an accompanying decrease in urokinase activity as a primary mechanism of angiostatic action.

Newer investigative strategies include the targeting of receptor tyrosine kinases and other cell surface growth factor receptors, as well as intracellular signal transduction pathways, as potential areas for pharmacological control of angiogenesis.

AL-3789, An Angiostatic Steroid

The angiogenesis inhibiting properties of a new class of steroids, in combination with the cofactor heparin, was first reported by Crum *et al* (1985), who showed that angiostatic activity of the steroids in the chicken embryo chorioallantoic membrane model was independent of glucocorticoid and mineralocorticoid activity. It has been shown that medroxyprogesterone acetate (MPA), an anti-cancer and angiostatic steroid, inhibits urokinase (uPA) activity in cultured bovine endothelial cells (Ashino-Fuse *et al*, 1989). This protease is related to metastatic capacity and plays an important role in tissue remodelling and cell migration by catalyzing the conversion of plasminogen to plasmin. In addition, it has been shown that certain angiostatic steroids inhibit the fibrinolytic response of endothelial cells to angiogenic stimulation by bFGF by increasing plasminogen activator inhibitor (PAI-1) production (Blei *et al*, 1993). The increase in PAI-1

activity was accompanied by a decrease in plasminogen activator (PA) activity; the authors suggested that the PAI-1 increase was responsible for inhibiting plasmin formation and the resultant cascade of proteolytic enzymes which break down the extracellular matrix, thereby inhibiting the angiogenic process. In this study, some steroids that upregulated PAI-1 did not possess angiostatic activity. Therefore, the degree of inhibition of PA suppression did not uniformly correlate with the efficacy of angiostatic steroids in the chick chorioallantoic membrane (CAM) model of neovascularization reported earlier (Crum *et al*, 1985);

AL-3789 is 4,9(11)-pregnadien-17α,21-diol-3,20-dione-21-acetate, and represents the lead compound of a novel class of steroids that inhibit neovascularization (Figure 4). AL-3789 has a molecular formula of $C_{23}H_{30}O_5$, a molecular weight of 386.5, and a melting point of 233-236°C. The compound is stable to heat, autoclave, and ethylene oxide sterilization, and has been formulated in a stable topical ophthalmic suspension that contains 1.0% AL-3789, mannitol, sodium chloride, Carbopol 974P, Polysorbate 80, edetate disodium, benzalkonium chloride, and water. AL-3789 has also been formulated in a 10% injectable suspension for ocular subconjunctival or intravitreal injection.

In the studies reported by Crum *et al* (1985), angiostatic steroid activity was associated with the pregnane steroid structure and additional structural components on the D ring: absence of a 17α-hydroxyl group and of carbons 20 and 21 on the D ring decreased angiostatic activity. Hydrocortisone, 17-α hydroxyprogesterone, and tetrahydrocortexolone (THS) were reported by these authors to be angiostatic in the chick embryo chorioallantoic membrane (CAM) model of angiogenesis. THS

was the most potent angiostatic molecule reported in their series of eleven steroids. It was subsequently shown that heparin is not required as a cofactor for the anti-neovascular effects of some angiostatic steroids (McNatt and Clark, unpublished observations, Blei *et al*, 1993). The mechanism of angiostatic activity of these compounds was not known at the time, but observations implicated the breakdown of the vascular endothelial cell basement membrane as a necessary first step of angiogenesis in *in vitro* models (Ingber *et al*, 1986). More recent work (Ashino-Fuse *et al*, 1989; Blei *et al*, 1993) suggests that angiostatic steroids are not involved in basement membrane breakdown.

Folkman *et al* (1990) suggested that the steroid 17-α hydroxyl group was essential for angiostatic activity, and that reduction of the A-ring by, for example, by conversion of cortisol to dihydro- and tetrahydrocortisol, enhanced angiostatic activity. McNatt *et al* (1990) confirmed that the 17-α hydroxyl group significantly enhanced angiostatic activity in their series of structural modifications of tetrahydrocortisol. There appeared to be considerably more latitude in the 17-β-modifications of angiostatic steroids that could be accomplished and still retain angiostatic activity than was postulated by Folkman's group.

McNatt *et al* found that absence of a 3-α, 11-β, or C21 hydroxyl groups had little effect on angiostatic activity. The structures for selected compounds tested in the CAM and corneal neovascularization studies by these authors, as well as the structure for AL-3789, are given in Figure 4. They also found that the A-ring oxidation state did not play a major role in angiostatic activity; the 3-keto group could be reduced to an α-OH, and the presence or absence of C1-2 and C4-5

double bonds did not affect activity. A 5-α reduction of the steroid nucleus creates a trans junction of the A- and B-rings, giving a planar structure, while a 5-β reduction of the C4-5 A-ring double bond results in a cis A-B ring junction, causing the A-ring to be perpendicular to the B ring. Angiostatic activity was found to be independent of the position of the A-ring in their study (Figure 5). Introduction of a C9-11 double bond into the C-ring appeared to enhance angiostatic activity. In addition, it was found that structural constraints on the 17-α position, such as the structure represented by 17β-methyl-5α-androstan-3α, 17α-diol-16α-acetic acid lactone, eliminated angiostatic activity. This compound has a lactone ring which spans the 17α- and 17β- positions, creating a rigid structure connecting those positions.

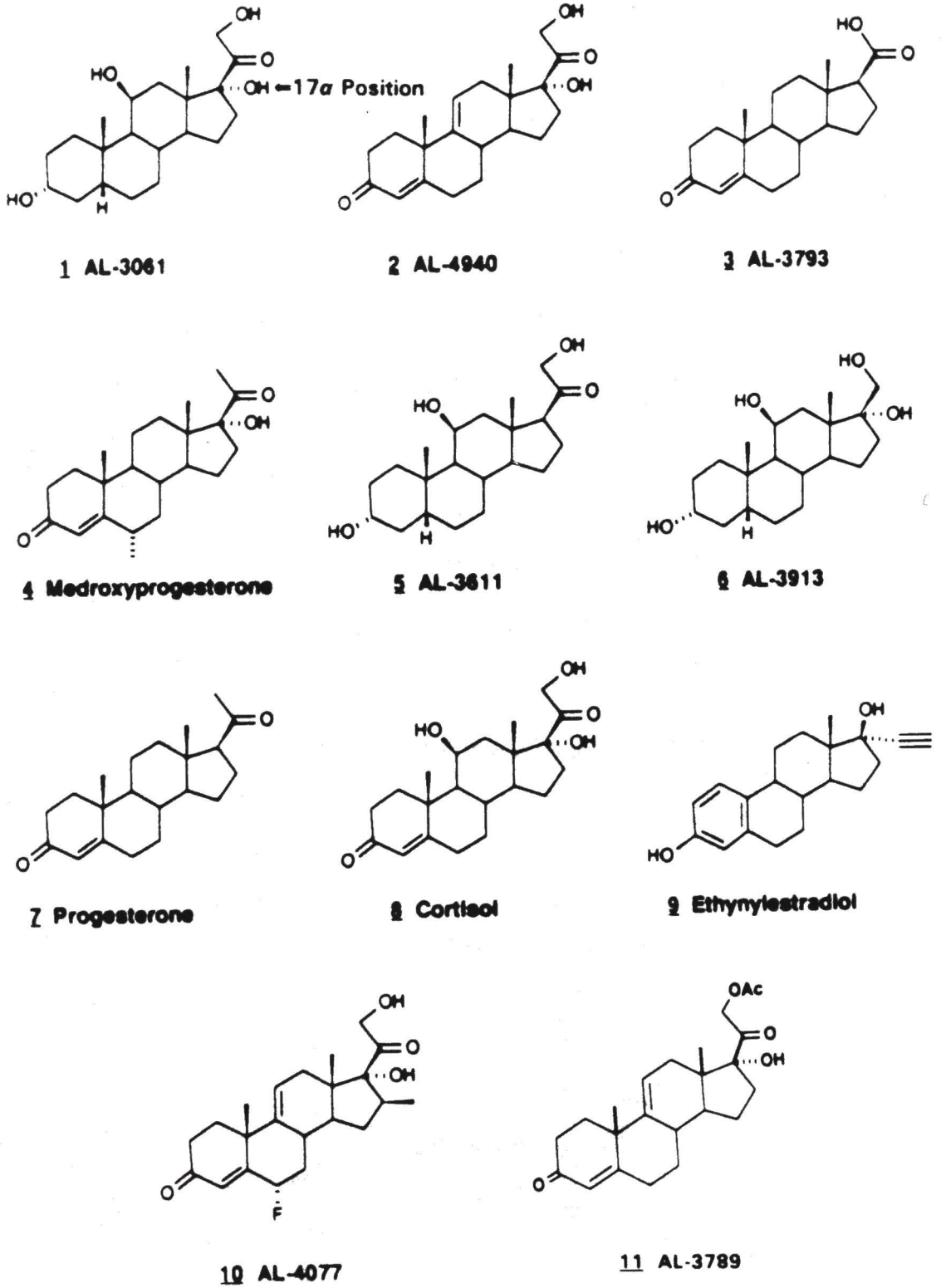


Figure 4. Structures of steroids included in the CAM study (McNatt *et al*, 1990) and the Rabbit Model of Corneal Neovascularization study (Griffin *et al*, 1993).

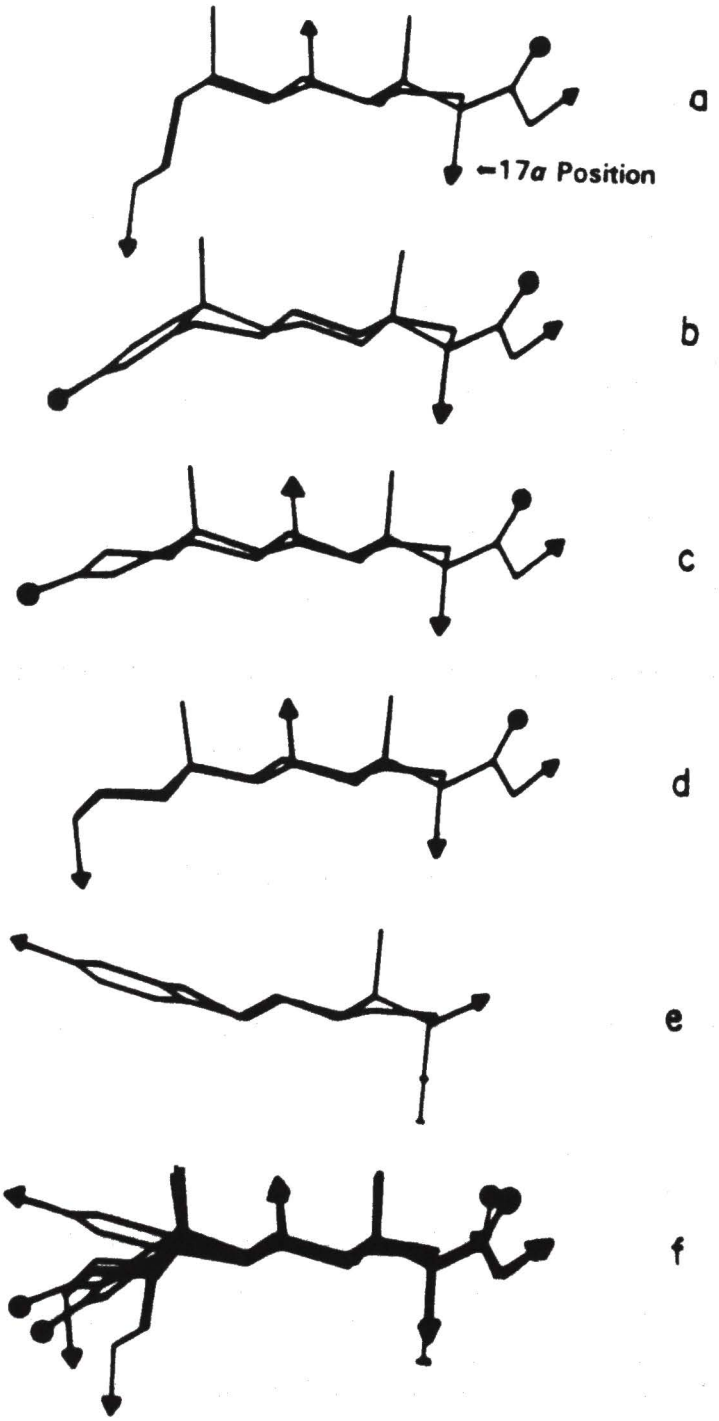


Figure 5. Variable conformation of the angiostatic steroid A-ring. Figures are planar projections of (a) tetrahydrocortisol, (b) AL-3789, (c) cortisol, (d) allo-tetrahydrocortisol, (e) ethynylestradiol, and (f) a composite of a-e (McNatt *et al*, 1990).

CHAPTER II

PRECLINICAL STUDIES OF AL-3789

Several studies have assessed the effect of angiostatic steroids on neovascularization using *in vitro* and animal models of neovascularization. These include the chick embryo chorioallantoic membrane (CAM) model, the rabbit corneal model of neovascularization, and the kitten and rat pup retinopathy of prematurity (ROP) models, and the murine intraocular tumor model. The pharmacological and pharmacokinetic profile of AL-3789 and its metabolite, AL-4940, has been studied. In addition, several toxicology studies have been accomplished on AL-3789 administered topically as well as systemically.

Chick Chorioallantoic Membrane (CAM) Model

Over 100 steroids with diverse structures (glucocorticoid, mineralocorticoid, progestin, androgen, and estrogen) have been evaluated for angiostatic activity in the chicken embryo CAM model (McNatt *et al*, 1990) discussed previously. Different concentrations of test molecules incorporated into liposomes were suspended in small agarose beads and applied to the shell-less CAM of 5-6 day old embryos. After two days, the angiostatic activity was characterized by inhibition of new vessel growth around the beads, and was quantitated by determining the percentage of responding embryos with avascular zones within a drug dose group.

The results were standardized to the activity of the angiostatic steroid tetrahydrocortisol (AL-3061) and expressed as a relative angiostatic factor (R.A.F., Table 2).

Table 2. Inhibition of Neovascularization in the CAM Model of Angiogenesis and in the Rabbit Model of Corneal Neovascularization (RCVN). AL-4940 (Compound 2) is the deacetylated active metabolite of AL-3789.

	COMPOUND	CAM R.A.F.	% INHIBITION OF RCNV
1.	THF (AL-3061)	1.00	35.0
2.	AL-4940	1.81	98.5
3.	AL-3793	1.23	27.0
4.	Medroxyprogesterone	1.12	•
5.	THB (AL-3611)	0.66	17.0
6.	AL-3913	1.42	15.0
7.	Progesterone	•	•
8.	Cortisol	•	•
9.	Ethynylestradiol	1.25	•
10.	AL-4077	1.16	88.0
11.	AL-3789	1.41	98.5

• Not determined.

Rabbit Model of Corneal Neovascularization

AL-3789 and AL-4940, as well as other angiostatic and non-angiostatic steroids were examined in a second model of neovascularization (Griffin *et al*, 1993). In the lipopolysaccharide (LPS)-induced model of corneal neovascularization, polymer (Elvax-40) implants containing 2 μg of LPS and 50 μg

Figure 6. Rabbit corneal model of neovascularization (Ben Ezra *et al*).

Figure 6a. Rabbit cornea eight days following implantation of 1 µg LPS.

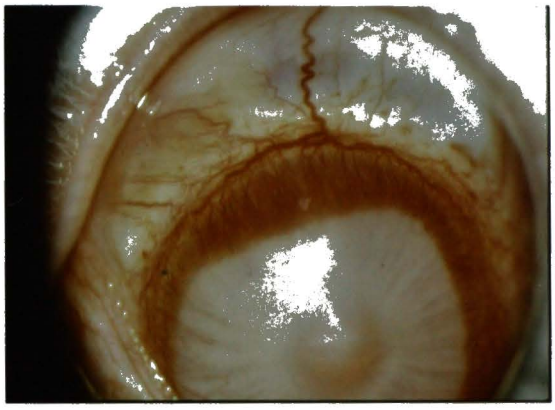


Figure 6b. Rabbit cornea eight days following implantation of LPS and treated with AL-3789 vehicle four times daily.

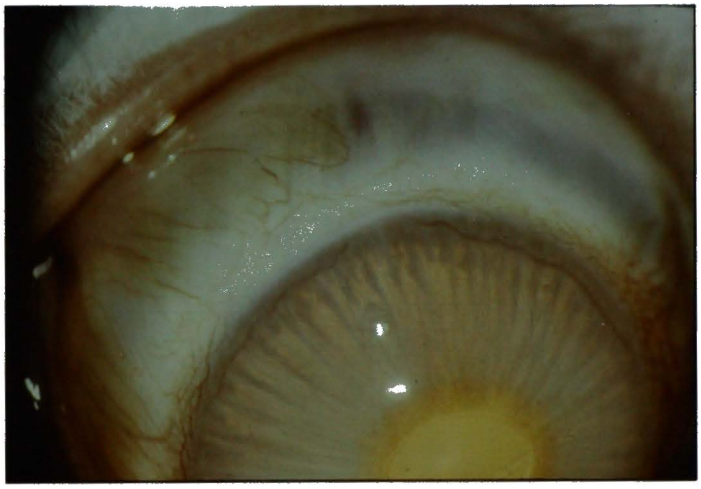


Figure 6c. Rabbit cornea eight days following implantation of LPS and treated with AL3789 1.0% four times daily.

of the angiostatic steroid being tested were surgically placed into the midstromal region of the rabbit cornea 2.5 mm from the limbus.

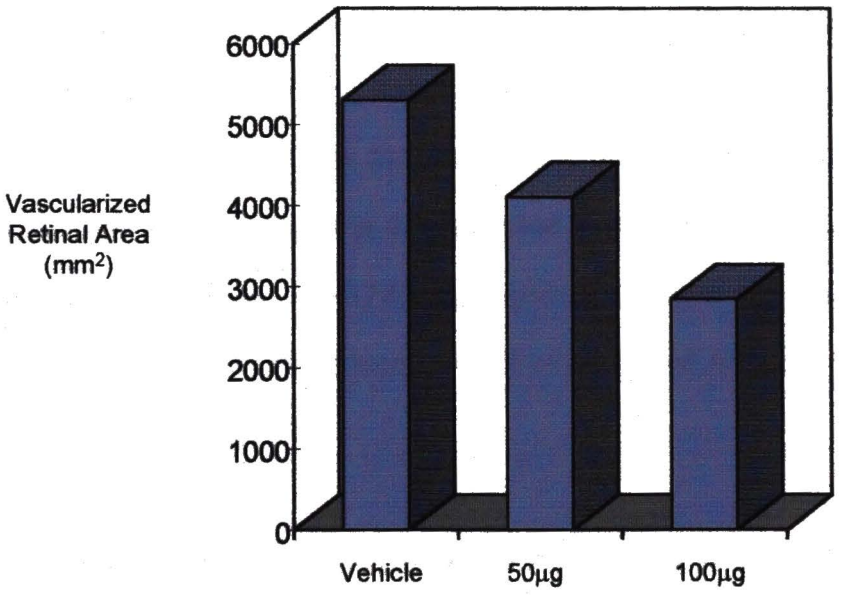


Figure 7. Effect of AL-4940 on kitten ROP retinal neovascularization.

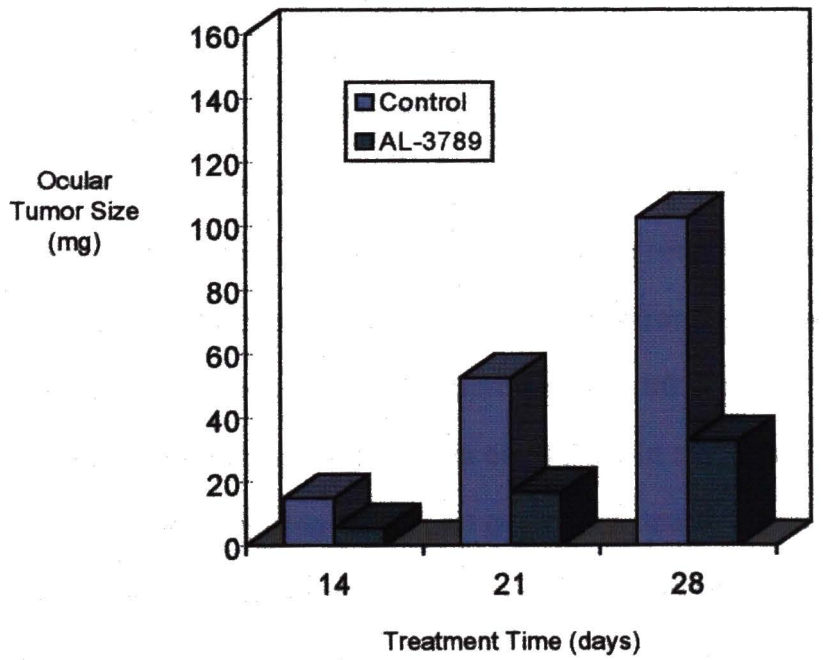


Figure 8. Effect of topical ocular AL-3789 on growth rate of mouse ocular tumor.

The neovascular growth from the limbus into the cornea was monitored over time. The ability of the test compounds to inhibit neovascular growth was determined by the reduction of new vessel growth and expressed as percent of inhibition (expressed as % RCNV, Rabbit Model of Corneal Neovascularization) of the LPS-stimulated control (Table 2). The results of this study again indicated that AL-3789, as well as AL-4940, the deacetylated metabolite of AL-3789, were potent angiostatic steroids. AL-3789 significantly inhibited corneal neovascularization in this LPS-induced model when dosed topically on the eye (BenEzra *et al*, 1994, Figure 6).

Kitten and Rat Pup Retinopathy of Prematurity (ROP) Models of Angiogenesis

AL-3789 and AL-4940 were evaluated for angiostatic activity in two models of retinal neovascularization by intravitreal administration. Retinopathy of prematurity occurs in neonates exposed to hyperoxic conditions, *i.e.*, supplemental oxygen. The developing retina fails to become vascularized due to high oxygen concentrations in ocular tissues. When the neonate is returned to normal oxygen conditions, however, the retina becomes hypoxic and retinal neovascularization occurs. AL-4940 was shown to inhibit approximately 50% of the neovascularization that occurs under these conditions in the kitten ROP model (Figure 7). In this study, neurogenesis and growth of ocular tissues were not inhibited. Additionally, intravitreal administration of AL-3789 in rat pups significantly inhibited oxygen-deprivation-induced neovascularization and vessel hemorrhage in the retina (Penn *et al*, 1996).

Murine Epithelial Carcinoma Study

Solid tumors of 1-4 mm diameter generally require their own vascular supply to continue growth and, in some cases, to metastasize. Inhibition of this neovascularization may be a viable therapeutic approach to control of solid tumor growth. In a study by Ma *et al* (1995), a murine retinal pigment epithelial carcinoma cell line (99E1) was transplanted into the anterior chamber of one eye of nude mice. The transplant generates into a rapidly growing and highly vascularized intraocular tumor. Tumor growth was assessed each week by measuring tumor mass (i.e., by subtracting the weight of the normal eye from the weight of the tumor-bearing eye). The eyes were treated topically with 1.0% AL-3789 Ophthalmic Suspension three times daily for up to 28 days. Tumor growth was inhibited by more than 50% by AL-3789 treatment (Figure 8). Neither AL-3789 nor AL-4940 inhibited the growth of this tumor cell line *in vitro*, and it is therefore possible that the inhibition was due to inhibition of neovascularization of the tumor *in vivo*.

Pharmacokinetics and Pharmacological Profile of AL-3789 and AL-4940

Aside from their angiostatic properties, AL-3789 and AL-4940 have no other known pharmacological activity. *In vitro* receptor binding studies indicate that the two compounds do not bind to a wide variety of pharmacological receptors, including the steroid family of receptors. AL-3789 and AL-4940 have moderately weak binding affinities for the human glucocorticoid receptor and exhibit marginal

carrageenan-induced footpad edema model of inflammation in rats and the endotoxin-induced uveitis inflammation model in rats, and found to be devoid of anti-inflammatory agonist or antagonist activity. AL-3789 did not affect LPS-induced TNF induction in cultured human U937 cells (Yanni *et al*, 1993).

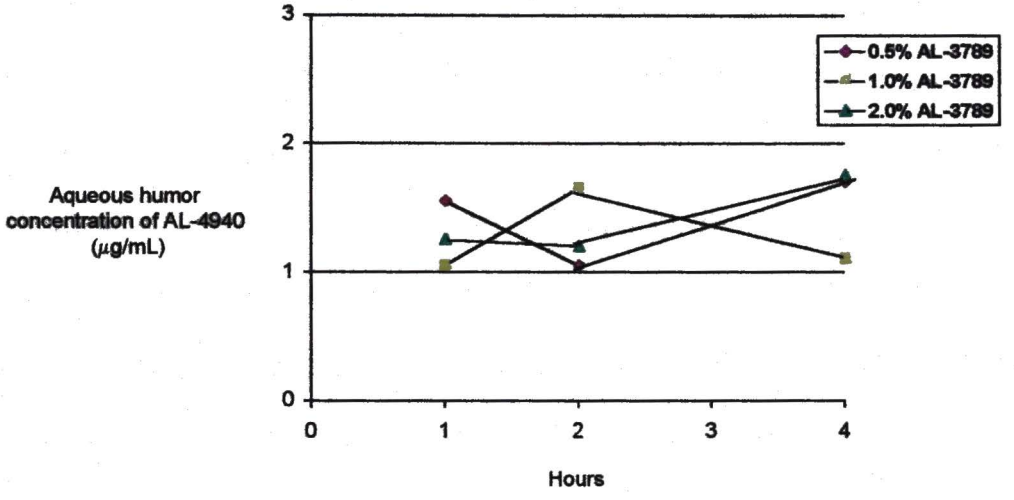


Figure 9. Topical ocular bioavailability following a single topical ocular dose of AL-3789 suspensions in rabbits.

The ocular metabolism, bioavailabillity and tissue distribution of AL-3789 and AL-4940, its deacylated metabolite, has been studied in rabbits and rats (Mattern and Sanders, 1992). After a single topical ocular dose of 1.0% AL-3789 Ophthalmic Suspension, only the metabolite, AL-4940, was detected in rabbit cornea and aqueous humor. Significant concentrations (1 μg/mL) of AL-4940 were detected in the cornea and aqueous humor at 30 minutes, 1 and 4 hours after dosing. These findings suggest that AL-3789 is rapidly metabolized to AL-4940 in the cornea. A study of topical ocular dosing of AL-4940 indicated that AL-4940 penetrates the cornea and is eliminated more rapidly than AL-3789. No significant differences in aqueous humor

penetrates the cornea and is eliminated more rapidly than AL-3789. No significant differences in aqueous humor concentrations were found following single doses of 0.5%, 1.0% and 2.0% AL-3789 Ophthalmic Suspension formulations in rabbits (Figure 9). Concentrations of the metabolite, AL-4940, did not change appreciably over the four hour sampling period in this study, suggesting relatively slow elimination of the metabolite or a corneal depot effect.

Oral dosing of AL-3789 in rats showed that it is well absorbed and rapidly metabolized (Mattern and Sanders, 1993), possibly via first pass p450-mediated oxidation in the liver to polar metabolites. One unidentified metabolite has a half-life of 40 hours. Subconjunctival injection of ^{14}C-labelled AL-3789 in rat pup eyes resulted in significant concentrations of radiolabelled compound in the choroid and retina (Mattern and Sanders, 1993).

Toxicology Studies

Topical ocular irritation and systemic toxicity evaluations of three and 12-months duration have been conducted using 0.5%, 1.0% and 2.0% AL-3789 Ophthalmic Suspension formulations in New Zealand white rabbits. Two drops of each of the AL-3789 formulations were dosed to one eye each of eight rabbits three times daily. The formulations were not irritating and no signs of phamacotoxicity were observed by biomicroscopic, indirect ophthalmoscopic, gross pathology, clinical pathology or detailed daily examinations.

An oral systemic toxicity study in rats studied AL-3789 at 25, 75 and 225 mg/kg body weight for four weeks. The detailed daily exams, gross pathology, histopathology, and clinical pathology were all unremarkable.

The intraocular toxicity of AL-3789 and AL-4940 formulated in intravitreal and subconjunctival injections has been studied in rabbits. Retinal toxicity was functionally assessed by conducting electroretinography (ERG). No toxicity was associated with intravitreal or subconjunctival injection of either compound. AL-3789 was tested for toxicity in the Pharmakon biological screening profile which includes assessment of effects on metabolic, gastrointestinal, anti-microbial, cardiovascular, immunological and pharmacological activities. AL-3789 at an oral dose of 100 mg/kg body weight did not exhibit effects on any of these systems.

Six additional safety pharmacology studies were performed including neuropharmacological profile in mice, diuretic activity in rats, gastrointestinal propulsion in mice, barbiturate sleep time potentiation in mice, neurotoxicity in mice and cardiovascular activity in dogs. AL-3789 showed no activity at oral doses of 0.1, 1.0, or 30 mg/kg body weight in the first five studies, and no cardiovascular activity in dogs at an intravenous dose of 1 mg/kg body weight (Clark, 1993). AL-3789 was not mutagenic in the modified Ames *E. coli/Salmonella* mutagenicity assay (Lawlor, 1992).

The preclinical studies above suggested that AL-3789 might have utility as an angiostatic agent, and allowed the conduct of a "proof of principle" clinical trial in man to determine its utility. Further studies were required, however, to clarify the drug's mechanism of action at the cellular level, and to determine which of the

essential steps of angiogenesis were affected by the drug. Prior studies (Ashino-Fuse *et al*, 1989; Blei *et al*, 1993; Barnathan, 1992) by other investigators suggested that angiostatic steroids such as medroxprogesterone acetate (MPA) might affect the elaboration of proteases or their inhibitors by vascular endothelial cells as part of their angiostatic mechanism of action.

The effect of AL-3789 and AL-4940 on protease expression, proliferation, microtubule formation, and overall protein expression in human microvascular endothelial cells (HMVEC-L) was therefore investigated to further elucidate its mechanism of action.

CHAPTER III

STUDIES OF THE MECHANISM OF ACTION OF AL-3789 AND AL-4940

Effect of AL-3789 and AL-4940 on uPA, PAI-1 and MMP-3 Protein Levels in Human Microvascular Endothelial Cells (HMVEC-L)

The angiostatic steroid medroxyprogesterone acetate (MPA) has been shown to inhibit the extracellular and cell-associated activity of uPA in cultured bovine vascular endothelial cells (Ashino-Fuge *et al*, 1989). uPA initiates the cascade of events resulting in proteolysis of the extracellular matrix with subsequent angiogenesis. It has also been reported that angiostatic steroids increase the production of plasminogen activator inhibitor (PAI-1) in bovine vascular endothelial cells in response to the angiogenic signal bFGF (Blei *et al,* 1993). An increase in PAI-1 level will have the effect of decreasing the level of uPA activity exhibited by these cells. In the Blei *et al* (1993) study, however, steroids which were not angiostatic also increased PAI-1 levels. The mechanism of action of angiostatic steroids remains controversial. The study reported here, therefore, examined the effect of several angiostatic steroids on the protein levels of uPA, PAI-1 and MMP-3 present in cultured human microvascular endothelial cells following LPS stimulation to determine if these compounds decrease protease levels and/or increase protease inhibitor levels.

General Methodology

All enzyme protein level studies were conducted using human lung microvascular endothelial cells (HMVEC-L) derived from a 5-year old Hispanic male and obtained from Clonetics, Inc., San Diego, CA (Figure 10). The HMVEC-L cells were grown in EGM-MV medium (Clonetics) supplemented with 20% fetal bovine serum (Hyclone), epidermal growth factor (EGF), 0.01 μg/mL, hydrocortisone, 1 μg/mL, gentamycin, 0.05 mg/mL, amphotericin-B, 0.05 μg/mL, and 4 μL/mL bovine brain extract with 10 μg/mL heparin. Cells were grown at 37°C in a humidified incubator equilibrated with 5% CO_2/95% air, and were subcultured weekly when 90-100% confluent. Either cryopreserved or proliferating cells from Clonetics were used as stock cultures. Each lot obtained was characterized by Clonetics, Inc., to be positive for indicators characteristic of endothelial cells (acetylated LDL uptake, PECAM staining, and expression of von Willebrand's (Factor VIII) antigen). In addition, Factor VIII staining was accomplished in our laboratory for positive identification of these cells.

Experimental Design

Cells of passage 3-8 were used for all experiments. To examine the effect of test compound treatment on protein levels of proteolytic enzymes expressed, approximately 1 X 10^4 cells were seeded into 6-well culture plates in the presence of 2 mL medium per well and allowed to attach overnight. The cells were washed with serum free medium (EGM without serum and hydrocortisone) and the medium replaced with serum free medium containing the test compounds. Culture was continued for 48 hours.

Figure 10. HUVEC and HMVEC-L cells.

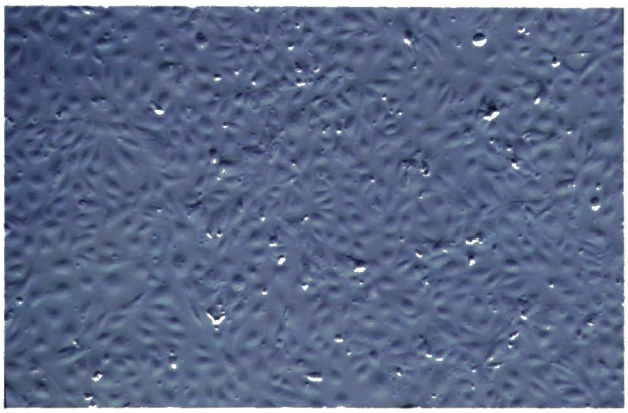


Figure 10a. Photomicrograph of human vascular endothelial cells (HUVEC) derived from umbilical cord at confluency. Magnification 65X.

Figure 10b. Photomicrograph of human microvascular endothelial cells (HMVEC-L) derived from lung. Magnification 165X.

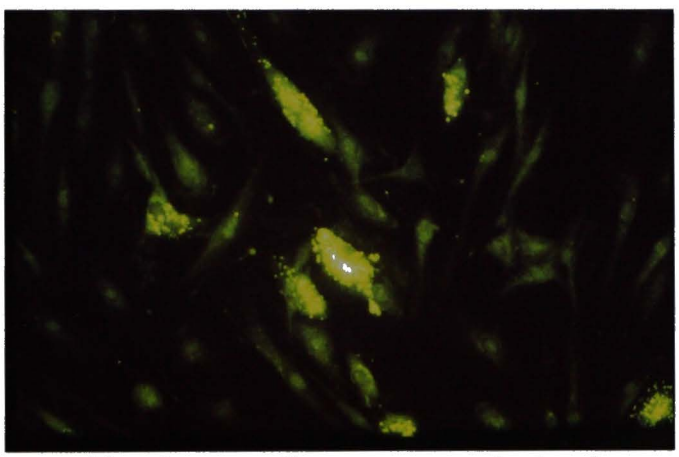


Figure 10c. Immunofluorescence photomicrograph of HMVEC-L cells stained for human Factor VIII (von Willebrand's) antigen. Magnification 340X.

Three separate experiments were conducted utilizing seven treatments to HMVEC-L cells cultured in 6-well plates: 1) control (treated with ethanol, final concentration 0.1%), 2) LPS 30-60 μg/mL (depending on the experiment), 3) 10^{-5}M AL-3789 plus LPS, 4) 10^{-5}M AL-4940 plus LPS, 5) 10^{-5}M dexamethasone (DEX) plus LPS, 6) 10^{-5}M medroxyprogesterone acetate (MPA) plus LPS, and 7) 10^{-5}M tetrahydrocortisol (THF) plus LPS. The first two experiments were conducted using 60 μg/mL LPS in all test wells except the controls; the third experiment utilized 30 μg/mL LPS. A stock solution of each steroid was prepared in absolute ethanol and appropriately diluted in media to attain the final test concentration in each well. The order of treatments to wells was randomized. Three wells in each experiment were treated with the seven test articles above, and cells and/or media from each well was analyzed in duplicate, or, in some cases, triplicate.

After 48 hours of incubation, the medium was removed and centrifuged to remove any cellular debris. TWEEN-80 was added to the media to make a final concentration of 0.01%. The remaining cells were washed with 0.5 mL of Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS), treated with 0.5%Triton X-100 in PBS, and allowed to stand for 30 minutes before removing by scraping. Media and cell lysates were stored at -70°C until assayed. Protein levels of uPA, PAI-1 and MMP-3 were accomplished on both media and cellular samples using enzyme-linked immunoassay (ELISA) with either kits obtained from American Diagnostica, Inc. (uPA and PAI-1) or plates constructed in our laboratory for stromelysin-1 (MMP-3) with purified antibodies from Oncogene Research Products. The lower detection limits for the uPA and PAI-1 ELISA assays were 10 pg uPA/mL and 50 pg PAI-1/mL;

upper limits were linear to 10 ng uPA/mL and 1.0 ng PAI-1/mL. The limits for the MMP-3 assay were 0.25 to 10 ng MMP-3/mL.

uPA and PAI-1 ELISA Analysis

These assays employed a murine monoclonal antibody against human uPA or PAI-1 as the capture antibody bound to the ELISA plate. Samples of the media or cell extract were added to the plate, and a second antibody conjugated with horseradish peroxidase (HRP) bound to the captured uPA molecules in the micro-test wells. This complex formed the antibody-enzyme detection complex (Figure 11). HRP converted the perborate/3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate to a blue colored solution, which was transformed to yellow by addition of sulfuric acid stop solution. uPA or PAI-1 protein levels were quantitated by measuring solution absorbances at 450 nm using the BIO TEK EL340 Microplate Reader and compared to the values obtained in a standard curve. The uPA assay recognizes single-chain uPA, two-chain uPA, receptor-bound uPA and uPAc omplexed with PAI-1 and PAI-2. The PAI-1 assay detects inactive and active PAI-1 and PAI-1 complexes.

MMP-3 ELISA Analysis

This analysis was again based on an ELISA "sandwich" format (Figure 11), as were the uPA and PAI-1 ELISA analysis. Anti-MMP-3 murine monoclonal antibody was precoated to the wells of the micro-well test plate and MMP-3 from the sample was bound to the antibody. A second antibody (polyclonal rabbit anti-

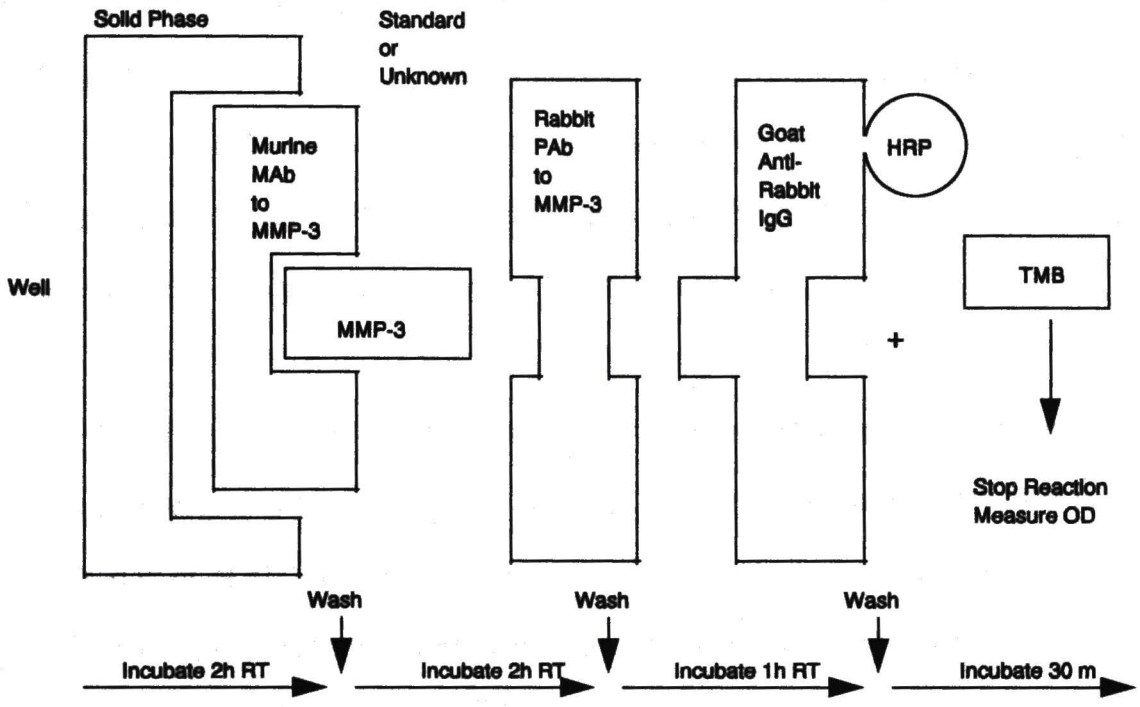


Figure 11. MMP-3 enzyme-linked immunoassay (ELISA) design.

MMP-3) was complexed with the bound MMP-3. A third (detection) antibody, goat anti-rabbit IgG complexed to HRP was bound to the antibody-antigen complex, and TMB substrate was used to produce a blue solution which was changed to yellow by sulfuric acid stop solution. MMP-3 levels were quantitated by measuring sample absorbance at 450 nm and compared to a standard curve of pro-MMP-3. The assay recognized total human MMP-3, in pro- and activated forms.

Results

Results from three separate experiments were combined and analyzed by Two-way Analysis of Variance using PROC MIXED, a standard SAS statistical analysis (Littell *et al*, 1989). The results are given in Table 3. Values represent the total concentration present in cells plus media samples for each enzyme tested. Figures 12-14 present the results of each treatment compared to the untreated control values for uPA (Figure 12), PAI-1 (Figure 13), and MMP-3 (Figure 14).

LPS has been reported to stimulate uPA production in a variety of cell types, including HUVEC and HSVEC (Amman *et al*, 1995), peripheral blood monocytes (Schwartz and Bradshaw, 1992), and macrophages (Page, 1991). Conversely, LPS has been reported to decrease uPA mRNA levels in murine proximal and distal tubule tissue sections (Moll *et al*, 1994). In our study using HMVEC-L, LPS induced a 42% average increase in cell-associated and extracellular uPA protein levels. LPS did not stimulate production of MMP-3 in these cells.

Treatment with AL-4940 at 10^{-5} molar concentration significantly inhibited expression of MMP-3 and uPA, decreasing the protein level of these enzymes compared to the LPS-treated controls by 33% and 15%, respectively. These

results were statistically significant ($p = 0.0001$ and $p = 0.0005$, two-way ANOVA, Table 3). HMVEC-L cells treated with AL-3789 at the same concentration exhibited a 19% decrease in MMP-3 protein level from control, also a statistically significant decrease ($p = 0.003$, Table 3), but did not significantly decrease uPA levels. MPA decreased MMP-3 and uPA protein levels approximately the same magnitude as the decrease seen with AL-4940 treatment (21% for MMP-3 and 32% for uPA). THF decreased protein levels of MMP-3 by 17%, a statistically significant decrease, but did not decrease uPA protein levels. Dexamethasone significantly decreased protein levels of uPA (32%) but did not decrease MMP-3 levels (Table 3).

LPS is known to increase PAI-1 and PAI-2 protein levels in several cell types, including HUVEC and HSVEC (Amman *et al*, 1991), peripheral blood monocytes (Eggsboro *et al*, 1995), and mesothelioma cells (Shetty *et al*, 1995). In our study, LPS stimulated production of PAI-1 in HMVEC-l to 239% of control level. However, none of the angiostatic compounds tested significantly affected PAI-1 protein levels compared to the LPS-stimulated control. All treatments significantly increased PAI-1 levels above the untreated control level by 194% to 239% (Figure 13).

Figure 12.

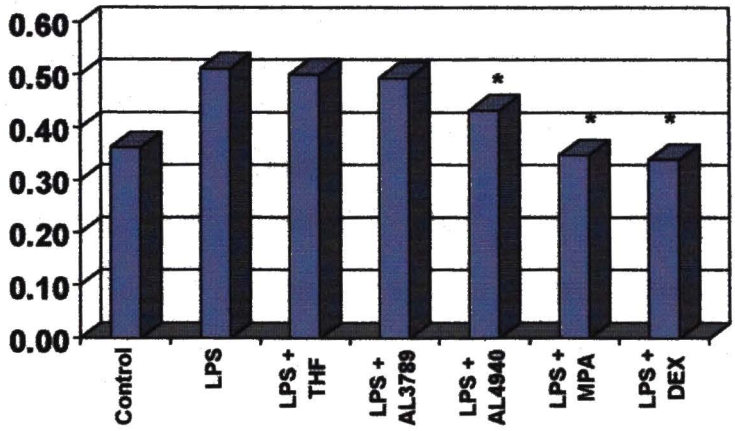


Figure 13.

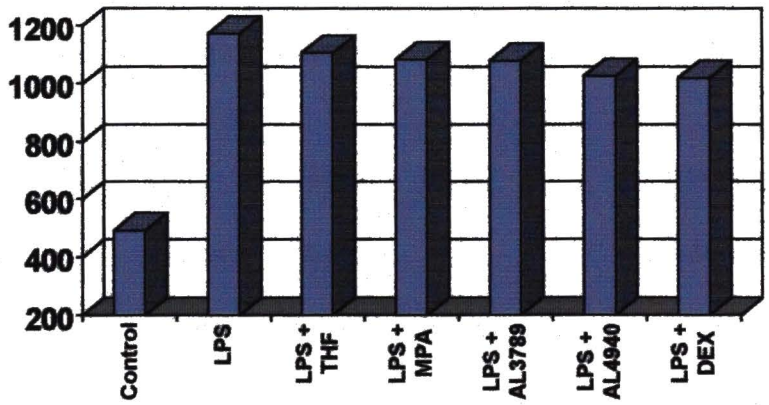
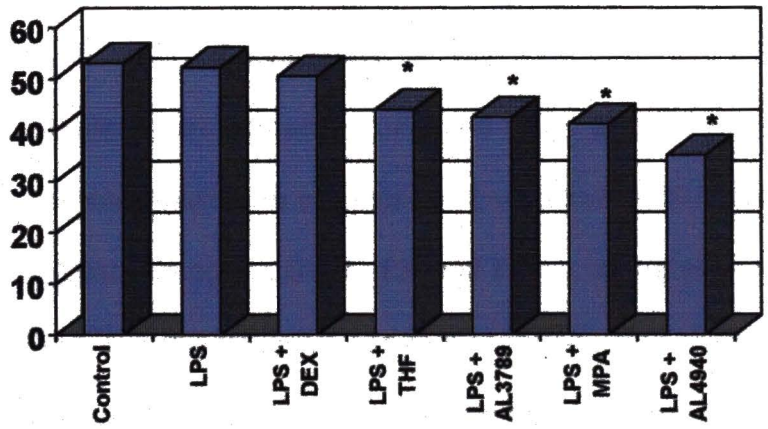


Figure 14.



Figures 12 - 14. Effect of 10^{-6}M AL-3789, AL-4940, MPA, DEX and THF on uPA, PAI-1 and MMP-3 expression in HMVEC-L stimulated with LPS (* statistically different from LPS Control, $p < 0.01$).

Table 3. Effect of 10^{-5} M AL-3789, AL-4940, DEX, MPA and THF on LPS-Stimulated Protease Expression in HMVEC-L (ELISA Assay)

	MMP-3			PAI-1			uPA		
Treatment	**Average ng/well**	**SE[2]**	**p[3]**	**Average ng/well**	**SE**	**p[3]**	**Average ng/well**	**SE**	**p[3]**
Control	53.14	2.18	-	491.5	93.2	0.0001	0.3622	0.014	0.0001
LPS	52.23	2.18	-	1172.6	93.2	-	0.5122	0.014	-
AL-3789 + LPS	42.42	2.18	0.0028	1079.9	93.2	-	0.4950	0.014	-
AL-4940 + LPS	35.02	2.18	0.0001	1027.0	93.2	-	0.4337	0.014	0.0005
DEX + LPS	48.90	2.35	-	953.1	100.6	-	0.3401	0.015	0.0001
MPA + LPS	41.18	2.18	0.0009	1084.7	93.2	-	0.3479	0.014	0.0001
THF + LPS	43.44	2.35	0.0091	1118.90	100.6		0.5048	0.015	

[1] Average of 3 separate experiments
[2] From analysis of Varience (SAS, PROC MIXED)
[3] Signigicant differences from LPS treatment

Conclusions

LPS treatment stimulated an increase in uPA and PAI-1 protein levels in HMVEC-L in our study. These results are in agreement with many investigations in several cell types that indicate upregulation of uPA and PAI-1, along with their mRNAs, by LPS. AL-4940, the deacetylated active metabolite of AL-3789, and MPA both significantly inhibited the quantity of uPA and MMP-3 present in HMVEC-L after stimulation with LPS. These results also agree with those of Ashino-Fuse *et*

al, 1989, who reported that MPA inhibited extracellular and cell-associated uPA For activity following treatment of three types of bovine endothelial cells with bFGF and PMA. In our study, MPA at 10^{-5} molar concentration significantly inhibited LPS-stimulated uPA protein levels. Other authors have reported that MPA upregulates PAI-1 levels following bovine endothelial cell stimulation with bFGF (Blei *et al*, 1993) as the primary mechanism of angiostatic steroids to decrease uPA activity. Our study in human cells did not confirm these results, but indicated that neither AL-4940 nor MPA increased PAI-1 levels above those attained by LPS stimulation. both of these angiostatic steroids, as well as AL-3789 and THF at the same concentration, PAI-1 levels in HMVEC-L remained very close to those of the LPS-stimulated control. Dexamethasone has been reported to decrease uPA activity and mRNA levels in a variety of cell types (Yamamoto *et al*, 1994, Hackett and Campochiaro, 1992), including bovine pulmonary artery cells, retinal pigment epithelial cells, and HUVEC. This was also seen in our study, with dexamethasone lowering uPA protein levels approximately 32% from the LPS-stimulated level.

Effect of AL-4940 on HMVEC-L Proliferation

Vascular endothelial cell proliferation is essential for the formation of new blood vessels in both benign and pathological states. Vascular endothelial cells remain quiescent during normal physiological states, and, upon activation, migrate out of existing blood vessels after basement membrane dissolution and proliferate to form new vessels. Cellular proliferation is mediated by several angiogenic

factors, including growth factors and cytokines. These factors, along with extracellular matrix components, appear to determine alternate phenotypes of endothelial cells: one phenotype favors protease secretion, cellular migration, and proliferation, while another favors capillary differentiation (Hackett and Campochiaro, 1993). During the pre-angiogenic phase of tumor development, tumor growth is limited and there is no activation of endothelial cells. Once tumors have reached 1-4 mm in diameter, angiogenic signals released from the tumor itself or from host macrophages and mast cells stimulate proliferation of vascular endothelial cells and initiate the angiogenic process.

Experiments were therefore conducted to determine if AL-4940 affects the growth rate of HMVEC-L and HUVEC *in vitro* and exhibits an antiproliferative effect as part of its mechanism of action. The cells used in the experiments reported here were proliferating HUVEC or HMVEC-L of passage 3-8 from cryopreservation recovery. Cells were cultured in 25 cm^2 flasks and maintained every 2-3 days with 5-6 mL of EGM-MV and recultured upon reaching 75-90% confluence.

Experimental Method

The media from proliferating cells in 25 cm^2 flasks was aspirated and the remaining cell layer washed with 2 mL of Ca^{++}- and Mg^{++}-free PBS. Attachments of the cells to the flask were loosened by treating with 5 mL of 0.25 mg/mL trypsin in PBS, and the cells observed under the microscope until approximately 90% were detached from the flask. The trypsin was neutralized by the addition of 5 mL trypsin neutralizing solution (TNS) or EGM-MV culture media containing serum. The

solution containing the cells was then centrifuged at 200 x *g* for 5 minutes and the supernatant aspirated and discarded. The cells were resuspended in 3-5 mL of EGM-MV; a 20 μL aliquot of suspended cells was taken, diluted 2X in 0.04% trypan blue solution, and counted using the hemocytometer to determine the appropriate dilution which would yield 10^4 cells in 1-2 mL. Each well of 6-well plates was seeded with 1-2 mL containing 10^4 cells and an additional 3 - 4 mL of EGM-MV media added to each well. The plates were incubated at 37°C in 5% CO_2/95% air for 72 hours in the presence of the test compounds or the appropriate volume of absolute ethanol as control (final concentration 0.1%). Each concentration of test compound was tested in triplicate on a single 6-well plate, with three wells treated and three wells as control. To harvest the cells, the media was aspirated and discarded and 1 mL of 0.25 mg/mL trypsin in PBS added to each well. The cells were observed under the light microscope until they were detached. One mL of TNS or EGM-MV was then added to neutralize the trypsin, and the solution containing the cells aspirated into a 15 mL centrifuge tube. The solution was centrifuged at 200 x *g* for 5 minutes and the supernatant aspirated and discarded.

The remaining pellet of cells was resuspended in 0.5 mL of PBS. A 0.4 mL sample of this solution diluted in 20 mL of Isoton was analyzed for number of cells using the Coulter Counter Model ZM (Coulter Electronics, Inc.).

Four separate experiments were conducted to study the effect of AL-4940 on HMVEC-L proliferation. The first two experiments were direct comparisons of cells treated with 10^{-5} M AL-4940 to vehicle-only control. In the first experiment, two plates had 6 wells treated with AL-4940 and 6 served as control wells. In the

second experiment, three plates had 9 wells treated with AL-4940 and 9 wells served as controls. The third experiment was a dose-response study of three concentrations of AL-4940, MPA and dexamethasone (10^{-5}, 10^{-7}, and 10^{-9} M for each compound), compared to an ethanol-only treated control. This experiment utilized a single 6-well plate for each concentration of the tested compounds, with three treated wells and three control wells per plate. The final experiment was a repeat of the third experiment with AL-4940 and MPA, both at a molar concentrations of 10^{-5}, 10^{-7}, and 10^{-9} M.

Results

AL-4940 at a concentration of 10^{-5} M statistically significantly decreased HMVEC-L proliferation rates during the 72 hour incubation time compared to the control cell rates in all four experiments (Table 4, Figure 15).

Table 4. Effect of 10^{-5} M AL-4940 on HMVEC-L Proliferation.

Treatment	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	No. Cells x 10^4	% Control	No. Cells x 10^4	% Control	No. Cells x 10^4	% Control	No. Cells x 10^4	% Control
Control	2.33	100	4.66	100	3.23	100	4.31	100
AL-4940 10^{-5} M	1.77	76.0	3.03	65.1	1.87	57.9	2.75	63.8

The average difference in number of cells across the four experiments was 34.3% lower with AL-4940 treatment compared to ethanol-only treatment. The

raw data, prior to transforming to total cells/well, were analyzed using analysis of variance (SAS, PROC MIXED). The least squares mean for the control group was 321.05 cells (s.e. = 17.445) and for the treated group was 207.27 cells (s.e. = 18.49). The standard deviation estimate from this analysis was 59.83 cells and the difference between treatments statistically significant at $p = 0.0001$.

Table 5. Effect of 10^{-5}, 10^{-7}, and 10^{-9} M AL-4940, MPA, and DEX on HMVEC-L Proliferation

Treatment	Concentration 10^{-5} M		10^{-7} M		10^{-9} M	
	Cells/well ± SD (x 10^4)	% Control	Cells/well ± SD (x 10^4)	% Control	Cells/well ± SD (x 10^4)	% Control
Control	3.27[1]	100.0	3.46 ± 0.55	100.0	3.92 ± 0.77	100.0
AL-4940	2.11[1]	65.7 [1,3]	3.33 ± 0.42	96.4[2]	3.80 ± 0.66	96.8[2]
Control	4.42 ± 1.09	100.0	3.11 ± 0.16	100.0	3.37 ± 0.52	100.0
MPA	3.55 ± 0.93	80.4[2,4]	3.55 ± 0.54	114.3[2,4]	3.13 ± 0.59	93.0[2]
Control	3.51 ± 0.66	100.0	2.97 ± 0.34	100.0	3.48 ± 0.29	100.0
DEX	3.02 ± 0.72	86.1	2.86 ± 0.49	96.4	3.45 ± 0.28	99.4

[1] Least Squares Mean from ANOVA of 4 experiments (SAS, PROC MIXED); reported numbers are a linear transformation from raw data (Coulter Counter counts) to total cells/well
[2] Average of 2 experiments
[3] $p = 0.0001$, ANOVA
[4] $p < 0.05$, Two-sample t-Test

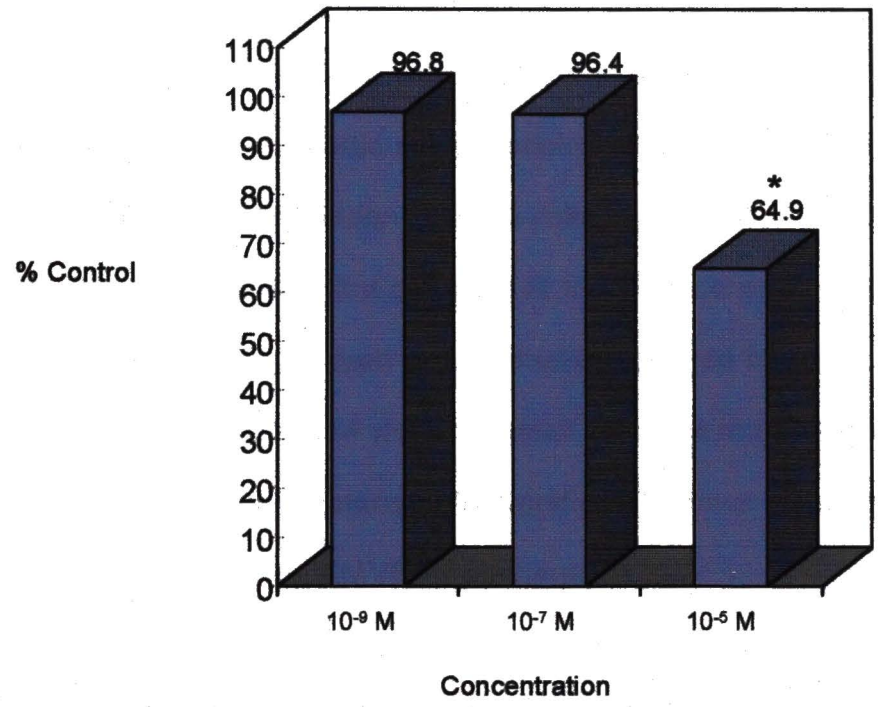


Figure 15. Effect of AL-4940 on HMVEC-L proliferation expressed as percent of the vehicle-only treated control. (*The inhibition at 10^{-5} M is statistically significant at p = 0.0001 by ANOVA).

The average treatment differences for experiments 3 and 4 are presented in Table 5 for all concentrations of MPA tested and AL-4940 at 10^{-7} and 10^{-9} M. No difference was found between treatments and controls at the lower concentrations of 10^{-7} and 10^{-9} M for AL-4940. MPA likewise produced a modest decrease in the proliferation rate of HMVEC-L at a concentration of 10^{-5} M; this effect was statistically significant at $p < 0.05$ (Table 5). At 10^{-7} M, MPA produced an apparent increase in number of cells compared to control which was also statistically significant, and no effect was seen at the lowest concentration of 10^{-9} M.

statistically significant, and no effect was seen at the lowest concentration of 10^{-9} M.

Ashino-Fuse *et al* (1989) examined the effect of MPA on proliferation of bovine adrenal cortical capillary, aortic and pulmonary artery endothelial cells and found no effect of MPA on proliferation of these cells at concentrations up to 10^{-5} molar. Our results indicate a different response at this high concentration, perhaps due to the difference in cell types used in their study compared to ours. Dexamethasone treatment at 10^{-5} M yielded a small decrease in number of cells compared to control that was not statistically significant. Lower concentrations of dexamethasone had no effect on HMVEC-L proliferation.

Table 6. Effect of 10^{-5} M AL-4940 on HUVEC Proliferation

Treatment	Experiment 1[1]		Experiment 2[2]	
	Cells/well ± SD (x 10^4)	% Control	Cells/well ± SD (x 10^4)	% Control
Control	5.36 ± 0.60	100.0	5.19 ± 0.93	100.0
AL-4940	3.34 ± 0.81	62.3[3]	3.48 ± 0.71	67.0[3]

[1] Average of two repetitions
[2] Average of three repetitions
[3] $p < 0.0001$, Two-sample t-Test

Two additional experiments tested the effect of 10^{-5} M AL-4940 on proliferation of cultured HUVEC (human vascular endothelial cells drived from umbilical cord). These experiments were conducted in the same manner and with the same protocol

as with the HMVEC-L. AL-4940 (10^{-5} M) again significantly decreased the number of cells after 72 hours of incubation by 62.3% and 67.0% compared to control values. These results were statistically significant ($p < 0.0001$, two-sample t-Test, Table 6).

CONCULSIONS

AL-4940 significantly inhibited HMVEC-L proliferation at a high concentration (10^{-5} M) in our study. While this is a high concentration in terms of most systemic drug therapy, topical ocular dosing produces high concentrations of drug at the application site in comparison to overall systemic concentrations achieved with oral, intravenous, subcutaneous or intramuscular administration of most drugs. The bioavailability study described in Chapter II indicates that AL-4940 aqueous humor concentrations after a single topical ocular administration of AL-3789 1.0% Ophthalmic Suspension remained stable at 1 μg/mL for at least four hours. This represents a molar concentration of 0.3×10^{-5}, indicating that the concentration of AL-4940 used in the proliferation studies was reasonable. It is conceivable that corneal tissue levels with topical ocular dosing of AL-4940 might be higher than 10^{-5} M with topical ocular dosing of AL-3789 1.0%.

Several angiostatic compounds are known to inhibit endothelial cell or tumor cell proliferation. Among these are suramin analogues (Firsching *et al*, 1995), thrombospondin-1 (Weinstat-Saslow *et al*, 1994), 2-methoxyestradiol (Fotsis *et al*, 1994), IP-10 (Luster *et al*, 1995), TAN1120 (Nozaki *et al*, 1993), and fumagillin (LeQuerreac *et al*, 1993). The HMVEC-L and HUVEC proliferation studies presented

here indicate that AL-4940 exerts at least part of its angiostatic influence by inhibiting vascular endothelial cell proliferation.

Effect of AL-4940 on HMVEC-L Microtubule Formation on Matrigel

Matrigel is a solubilized tissue basement membrane preparation available from Becton Dickinson that contains laminin, collagen type IV, heparan sulfate proteoglycan and entactin. Many different types of cells grown on Matrigel assume morphological structures similar to their *in vivo* forms. Acinar cells from the pancreas, for example, and mammary cells form duct-like structures with a lumen (Bendayan *et al*, 1986, Li *et al*, 1987) when grown on Matrigel. Both HUVEC and HMVEC-L cells form capillary tube structures with a lumen when cultured on Matrigel; this activity appears to mimic the capillary formation seen *in vivo* by these cells during angiogenic processes.

The formation of tubules *in vitro* on Matrigel by HUVEC and HMVEC-L has been thoroughly investigated (Grant *et al*, 1989). Endothelial cells attach to several extracellular matrix molecules, including laminin and collagen IV via specific active sites on the ECM components that bind endothelial cell surface receptors, provoking many different cellular responses. A cysteine-rich domain in the B1 chain of laminin consisting of a five-amino acid sequence (Tyr-Ile-Gly-Ser-Arg or YIGSR) has been shown to mediate differentiation of endothelial cells into tubule formation while another site named PA 21 on the A chain containing the sequence Arg-Gly-Asp (RGD) mediates cellular attachment to basement membrane. These capillary-like

tube formations occur spontaneously for endothelial cells cultured on Matrigel. Cells attach rapidly, cease DNA synthesis, align, and form tubules within 12-18 hours. In this experiment we investigated the effect of AL-4940 treatment (10^{-5} M) on tubule formation by HMVEC-L cells cultured on Matrigel-coated plates.

Experimental Methods

Six-well thin layer Matrigel plates (Becton Dickinson) were rehydrated prior to seeding by incubating each well with 1 mL of EGM-MV media at 37°C for 30 minutes. Prior to seeding, the EGM-MV was carefully aspirated from each well. Confluent HMVEC-L cells from one 75 cm^2 flask were used to seed the plates. The media was aspirated and discarded, and the cells washed with 5 mL of Ca^{++}- and Mg^{++}-free PBS. Six mL of a 0.25 mg/mL trypsin in PBS solution was introduced into the flask and the cells observed under the light microscope until approximately 90% had detached. Six mL of TNS was introduced into the flask and the solution containing the cells centrifuged in a 15 mL centrifuge tube for 5 minutes at 200 x *g*. The supernatant was discarded and the cells resuspended in 2 mL of EGM-MV media. A 20 μL aliquot of suspended cells was diluted 2X in 0.04% tryphan blue and counted using the hemocytometer. The cellular density was 4.58×10^5 cells/mL; 9 mL of media was added to the cell solution and one-half of the cell solution was treated with 10^{-5} M AL-4940 while the other half were controls receiving ethanol alone at a final concentration of 0.1%. Each well of a 6-well Matrigel-coated plate was seeded with 1.5 mL of cell solution, with wells 1-3 receiving the control cell solution and wells 4-6 receiving the AL-4940-treated cell

solution. An additional 0.5 mL of EGM-MV media was added to each well for a final cell density of approximately 1.5 x 10^5 cells/well. The Matrigel plate was incubated at 37°C in 5% CO_2/95% air for 24 hours and each well photographed at the12:00 position, the center of the well and at the 6:00 position, using a Nikon Diaphot-DPM microscope with Hoffman Modulation optics. Photographs from each control well position were compared to the corresponding treated well position to determine differences in amount of tubule formation by the control and treated cells.

Results

Each well of AL-4940-treated cells was photographed at three locations and compared to the corresponding locations on the control cell wells, as well as all other locations on the control plates, to determine if a difference in amount of tubule formation existed between the two groups. No difference in the degree of tubule formation between treated and control wells could be observed. Representative photographs are presented in Figure 16.

Conclusions

Treatment of HMVEC-L cells with 10^{-5} M AL-4940 or 0.1% ethanol (control) did not result in observable differences in tubule formation between the two treatments after 24 hours of incubation on Matrigel, a synthetic basement membrane. The cells in treated or control wells all produced significant numbers of tubules with well-defined cell clusters between short tube-like structures.

Figure 16. HMVEC-L tubule formation on Matrigel synthetic basement membrane.

Figure 16a. Photomicrograph of HMVEC-L Control treated with ethanol only. Magnification 80X.

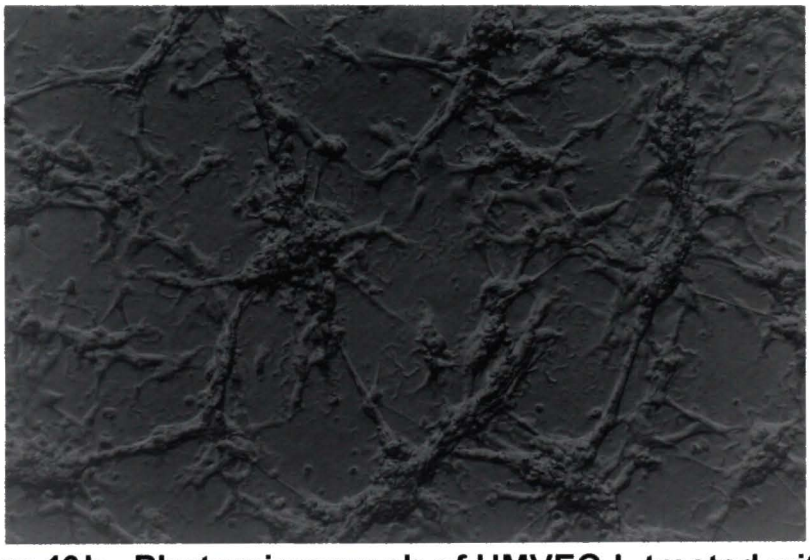


Figure 16b. Photomicrograph of HMVEC-L treated with 10^{-5}M AL-4940. Magnification 80X.

Effect of AL-4940 on HMVEC-L Protein Expression

Treatment with glucocorticoids has been shown to alter protein expression in a variety of cultured cell types (Vanderekhove *et al*, 1990; Datiles *et al*, 1992). This experiment was conducted to determine if AL-4940, the active deacetylated metabolite of AL-3789, alters or modulates protein expression in HMVEC-L or HUVEC cells. Evidence of alteration of protein expression might give insight into the possible mechanism by which angiostatic steroids inhibit neovascular processes.

Experimental Methods

HUVEC or HMVEC-L cells obtained from Clonetics were incubated in EGM-MV containing 20% fetal bovine serum (FBS, Hy Clone, #11152169), epidermal growth factor (EGF), 0.01 μg/mL, hydrocortisone, 1 μg/mL, gentamycin, 0.05 mg/mL, amphotericin-B, 0.05 μg/mL, and 4 μL/mL bovine brain extract with 10 μg/mL heparin under a humidified atmosphere of 5% CO_2 and 95% air. At confluency, test cells were treated with 10^{-5} M AL-4940 while control cells were treated with an equivalent volume of ethanol (0.1%). After 24 hours, each well was washed with phosphate-buffered saline (PBS) at 0° C and the cells pelleted at 650 x *g*. Each cell pellet was then extracted with 150 μL of 9 M urea/4% Nonidet NP-40 and assayed for ^{35}S radioactivity by liquid scintillation counting. Urea-and-detergent soluble proteins from each of the six samples were analyzed by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) which involved separating proteins in the first dimension by isoelectric focusing and in the second

dimension by 10% SDS PAGE as described by Hochstrasser *et al* (1988a). The gels were fixed in 40% methanol/10% acetic acid for 16 hours, washed briefly in H_2O and cross-linked in 10% glutaraldehyde for 30 minutes. After further washing in H_2O, the gels were soaked in 1 M sodium salicylate for 30 minutes, dried and exposed to x-ray film (Kodak Biomax MR, Sigma, St. Louis, MO). After varying periods of exposure time, the gel images on the resulting 2D autofluorograms were Fourier-filtered, quantitated, calibrated, and compared using an interpretive densitometer (CSPI, Scanalytics, Billerica, MA) to create databases using two-dimensional Gaussian spot modelling. The experimental details are described below. The overall protocol scheme was as follows:

Grow confluent HUVEC and HMVEC-L cells in MEM media with 20% FBS and antibiotics

↓

Treat cells with 10^{-5} M AL-4940 or 0.1% EtOH)

↓

Radiolabel cells for 24 h with ^{3}H-leucine
(HUVEC) or ^{35}S-methioninine (HMVEC) in amino acid-deficient MEM media

↓

Harvest cells and solubilize in 9M urea/4% NP40;
assay urea-soluble supernatants for total radioactivity

↓

Perform high-resolution isoelectric focusing
on normalized samples in polyacrylamide tube gels

↓

Apply focused gels to 10% SDS polyacrylamide slabs
and electrophorese

↓

Fix and cross-link proteins in acrylamide matrix and soak in 1M salicylate

↓

Dry gels and expose to x-ray film

↓

Image and quantify resulting protein spots for each
autofluorogram to create six separate gel files

↓

Overlay gel files for congruent spot comparison to create databases for comparison of
control and steroid-treated cells

Radiolabelling and Extraction of Cells and Tissue for Urea/Detergent-Soluble Protein

A six-well plate of confluent HUVEC or HMVEC-L cells was dosed for 24 hours as follows: 0.1% ethanol (control, wells 1-3), or 10^{-5} M AL-4940 (treated, wells 4-6). After 24 hours, the media was switched from EGM-MV to MEM (Selectamine, Gibco) containing no methionine but supplemented with 2% FBS, antibiotics and ^{35}S-Methionine (100 μCi/mL, Amersham) for HMVEC-L or MEM (Selectamine, Gibco) containing no leucine but supplemented with 2% FBS, antibiotics and ^{3}H-leucine(15 μCi/mL, Amersham) for HUVEC.

To prepare proteins for analysis in polyacrylamide gels, cultured cells were washed three times with 1 mL of Dulbecco's phosphate-buffered saline (PBS, Gibco) and harvested at 4 °C by scrapping in a total volume of 1.5 mL of PBS (3 x 0.5 mL) containing 100 μM phenylmethylsulfonyl fluoride (PMSF, Sigma). Individual wells were then examined by light microscopy at 65X to insure that all cells and associated extracellular material were removed prior to centrifugation. The cells were pelleted at 650 x *g*for 10 min. Each pellet was extracted, with occasional gentle vortexing, for 1 h with 150 μL of 9M urea containing 4% Nonidet NP-40 (Sigma). Following centrifugation at 12,000 x *g*, the radioactivity of the protein extract was determined using the LKB 1219 scintillation counter. Remaining extract was stored at -80°C. The radioactivitiy for control and treated MVEC extracts are given in Table 7. Average counts per minute (cpm) per μL ranged from 1.78-2.26 x 10^5 for controls and 3.40-3.83 x 10^5 for treated cells.

Isoelectric Focusing (IEF)

Ten grams of ultrapure urea was dissolved in 7 mL of 18 megaohm ddH_2O and incubated at 30°C until dissolved. Incubation was continued as 2.5 mL of acrylamide:PDA (30:0.8 g per 100 mL) was added. PDA (piperazine diacrylamide, Sigma) was used as the cross-linker to strengthen the IEF gels (Hochstrasser *et al*, 1988b). The solution was degased without precipitating the urea and 1.0 mL of a solution containing 0.3 g CHAPS and 0.1 mL Nonidet NP-40 in ddH_2O was added along with 1.0 mL of ampholines (Pharmacia, 80% pH range 3.5-10 and 20% pH range 5-7). Polymerization was initiated by adding 20 μL of TEMED (N, N, N', N' - tetramethylethylenediamine) and 40 μL of 10% ammonium persulfate. To the inside surface of 12 x 75 mm test tubes was added 0.75 mL of the mixture, which was then drawn up into 18 cm IEF tubes (1.5 mm inside diameter, Biorad). The IEF gels were allowed to polymerize for 2 hours at RT. Dithiothreitol (DTT, 20 μg) was added to 20 μL of ddH_2O in a 0.5 mL Eppendorf tube, and 2 μL of this solution and 2 μL of ampholines (above) were added to each 45 μL of sample and incubation continued for 2 hours. The anolyte was prepared by dissolving 0.7 mL of 85% phosphoric acid in 1.0 L of ddH_2O. The catholyte was prepared by dissolving 1.6 g of NaOH in 2.0 L of ddH_2O. Both solutions were degased until dissolved air was removed. The IEF tubes were inserted into the IEF apparatus (Biorad Model 155 Gel Electrophoresis Cell) and anolyte added to the lower reservoir. The reduced samples were added to the top of the polymerized gel with a Hamilton syringe and topped off by overlaying with catholyte. The gels were focused at 400 V for 2

hours, 800 V for 5 hours, 1200 V for up to 16 hours, and 2900 V for 1 hour. The IEF gels typically were electrophoresed for a total of 17,000 volt-hours.

2D High-resolution, Two-dimensional Polyacrylamide Gel Electrophoresis

2D High-resolution, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) of radiolabelled, VEC cell proteins was accomplished as follows: the focused IEF gels were briefly exposed to 0.5 M Tris buffer (pH 6.8) and applied along with ^{14}C-radiolabelled molecular mass standards in agarose (Amersham) to the second dimension which consisted of polyacrylamide slabs (10% T, 5% C, pH 8.8, Laemmli, 1970), using PDA as the cross-linker. The slab gels were electrophoresed at 4°C in matched sets of six gels at 50 mA per gel. Subsequent to electrophoresis in the second dimension, the gels were fixed with 40% methanol/10% acetic acid for 16 hours, cross-linked in 10% gluteraldehyde for 30 minutes, and soaked in 1 M sodium salicylate (Chamberlain, 1979) for 30 minutes. The gels were then dried for 2.5 hours at 60°C, and exposed to x-ray film at -80°C.

Autofluorographic protein profiles of the HUVEC/HMVEC-L treated and control groups were generated on X-ray film using multiple exposure times in order to create a set of 2D profiles balanced for overall exposure. Each autofluorographic image contained approximately 950 discrete spots (HMVEC-L) or 300 spots (HUVEC). Individual spots on each image were localized and marked. The profiles were then overlayed on an X-ray lightbox to determine both spots congruent with the controls and spots unique to the treated groups.

Separate gel images were first Fourier-filtered using SCANALYTICS software to remove irrelevent background noise. An example (HMVEC-L control gel 2) is shown in Figure 17. Individual spots on the autofluorographic images were identified and then calibrated for both mass and isoelectric point using 2D protein standards (Biorad). The calibrated images were used to generate congruent two-dimensional Gaussian spot model images which could be overlayed electronically to ceate two master databases (Tyson and Haralick, 1986) for HMVEC-L and HUVEC cell types. Figure 18 shows an example of a 2D Gaussian spot model generated from the corresponding section of a control gel film image. The treated and control images were analyzed for each protein spot that appeared significantly different between the two groups. Integrated optical densities for each protein spot of interest were tabulated and the ratio of integrated optical density, which is a quantitative estimate of the synthetically active protein present, calculated. The protein present in the three treated samples was calculated as percent of the analogous control sample values.

Results

Optical densities of proteins from the individual gel spot models were analyzed to determine differences between protein expression in controls and AL-4940-treated samples. The results are given in Table 8. It was found that only ten of approximately 950 total visualized proteins showed significant changes between treated and control gels. This result is in agreement with reports that steroids, both glucocorticoids and steroid hormones, alter protein expression in many cell types

(Sivo *et al*, 1996, Hogan *et al*, 1996, Wong *et al*, 1995, Lim *et al*, 1996, Kunz *et al*, 1996, Paspaliaris *et al*, 1995, Guller *et al*, 1995, Sapi *et al*, 1995, Ren *et al*, 1994, Blais *et al*, 1994, Guller *et al*, 1994, Deterding *et al*, 1994, Lamas *et al*, 1993, Takimoto *et al*, 1993, Baumann *et al*, 1991).

One 41 kD protein, spot 117 (Figure 19) appeared in HMVEC-L controls as 0.081% of total cellular protein with an isoelectric point (pI) of 4.77. This spot disappeared in HMVEC-L treated with 10^{-5} M AL-4940, being replaced with a protein of same molecular mass (spot 898, Figure 20) but with a different pI (4.84). A close-up of the area in control and AL-4940-treated gels visualizing these two proteins is shown in Figure 21. Spot 117 appears to shift position to spot 898 in the treated gels. The shift of spot 117 to spot 898 may represent charge modification of the protein or an isoform shift. Most of the proteins noted in Table 8 were downregulated in the treated gels compared to the control gels. Spots 385 and 898 represent upregulation from control values, while spots 18, 85, 64, 161, 98, 179, and 181 are present at only 6% to 75% of their control values. The downregulation of spot 161 may also be noted in Figure 21 in the control versus treated gel comparisons.

The ten proteins in the HMVEC-L gels that showed changes with AL-4940 treatment were examined in the HUVEC control and treated gels. Figures 22 (HMVEC-L) and 23 (HUVEC) show an example of one of two proteins (spot 18) that exhibited apparent downregulation with AL-4940 treatment in both cell types. The common changes in the two vascular endothelial cell types induced by AL-4940 suggests that they may be affected by a similar mechanism(s).

Table 7: Urea-soluble extract of HMVEC-L cell proteins for 2D-PAGE.

Sample #	Condition	cpm per 5μL	cpm per μL	average cpm per μL	μL for 8.012482 E+06 cpm	μL of 9M Urea for 45 Total	IEF Tube #
1	Con	841,185	168,237				
2	Con	939,365	187,873	178,055	45.00	0.00	1
3	Con	1,083,273	216,654				
4	Con	1,134,719	226,943	221,799	36.12	8.88	2
5	Con	1,163,040	232,608				
6	Con	1,106,376	221,275	226,941	35.31	9.69	3
7	AL4940	1,694,178	338,835				
8	AL4940	1,713,228	342,645	340,740	23.51	21.49	4
9	AL4940	1,535,804	307,160				
10	AL4940	1,826,383	365,276	336,218	23.83	21.17	5
11	AL4940	2,009,723	401,944				
12	AL4940	1,828,861	365,772	383,858	20.87	24.13	6

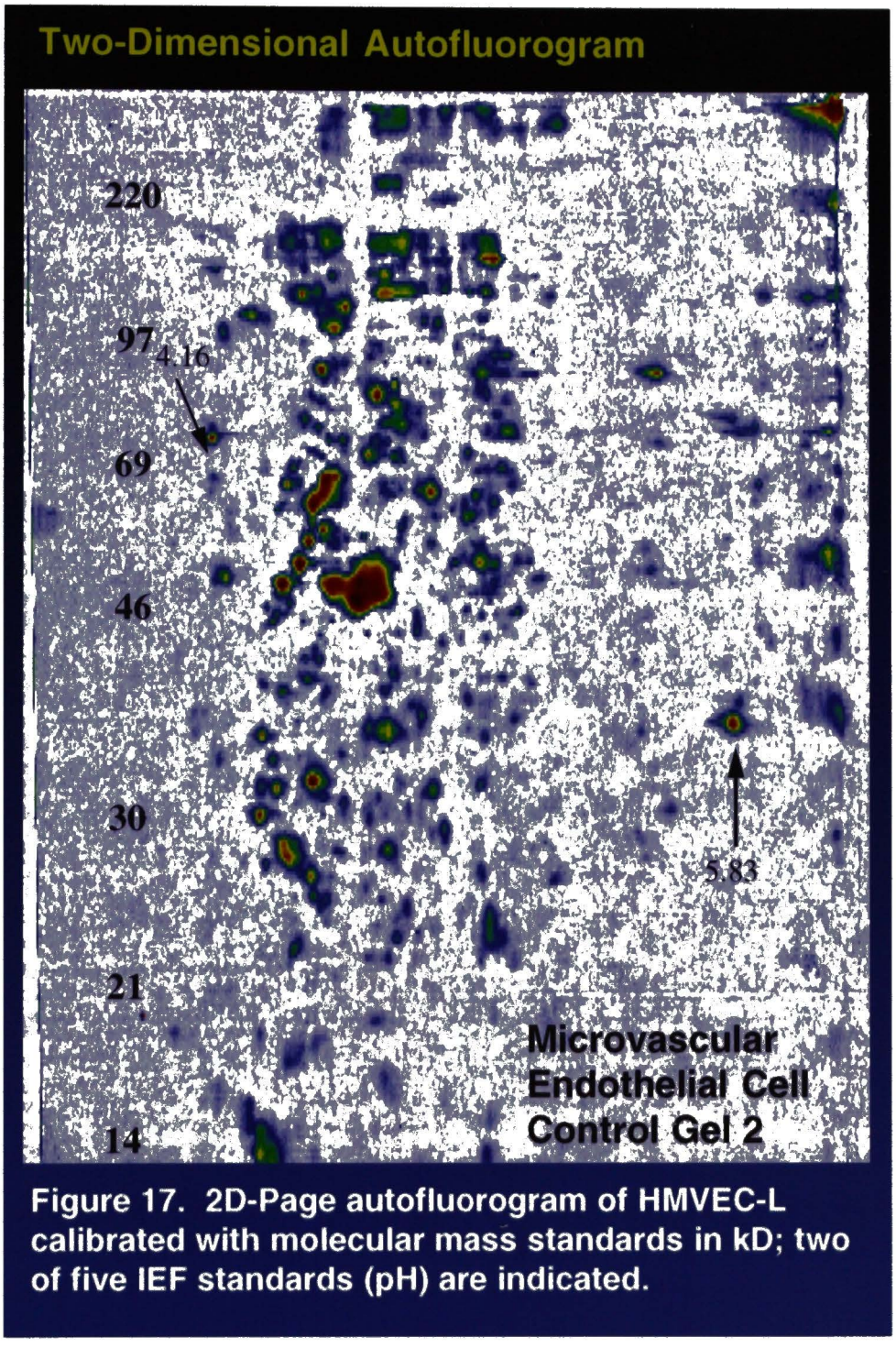


Figure 17. 2D-Page autofluorogram of HMVEC-L calibrated with molecular mass standards in kD; two of five IEF standards (pH) are indicated.

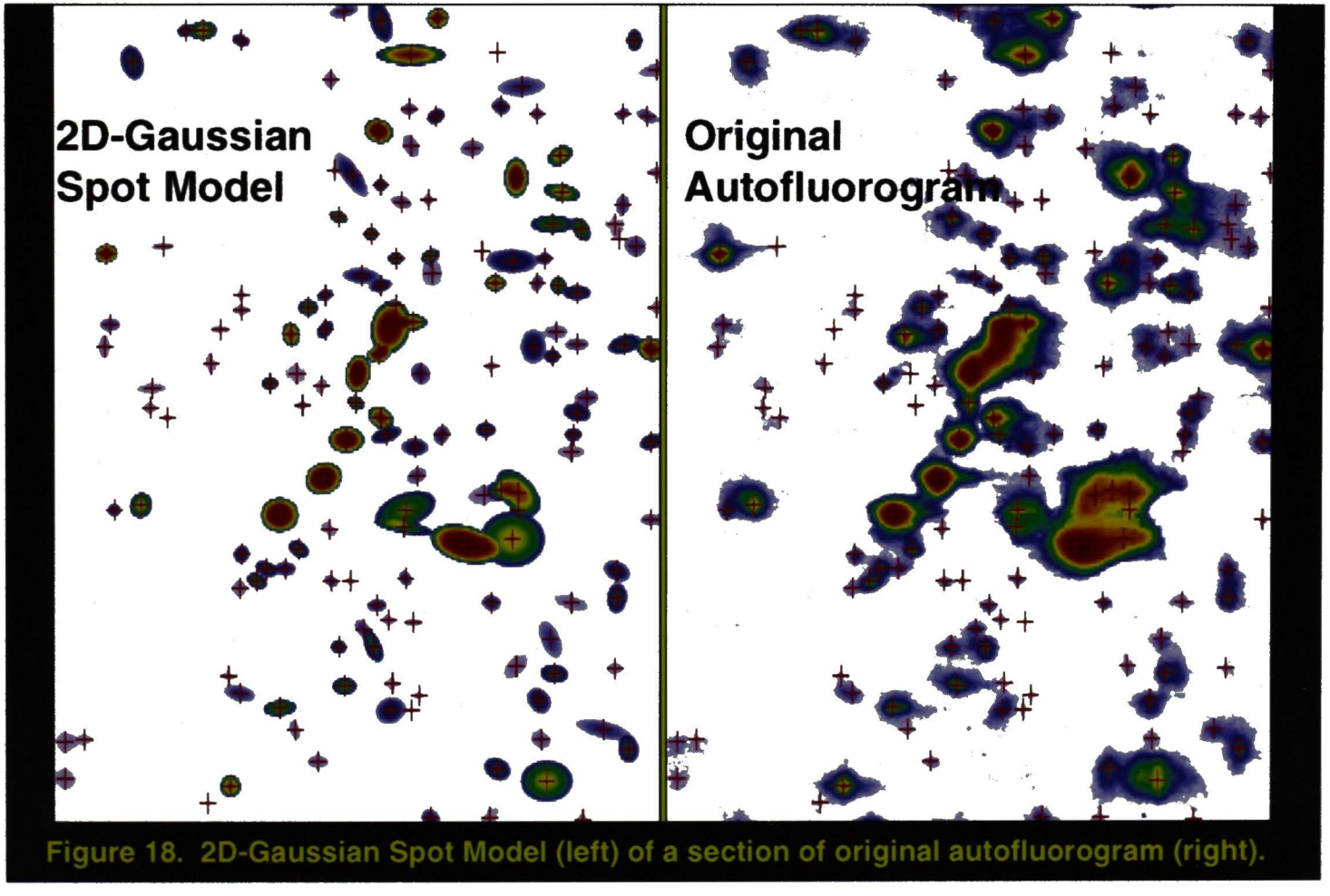


Figure 18. 2D-Gaussian Spot Model (left) of a section of original autofluorogram (right).

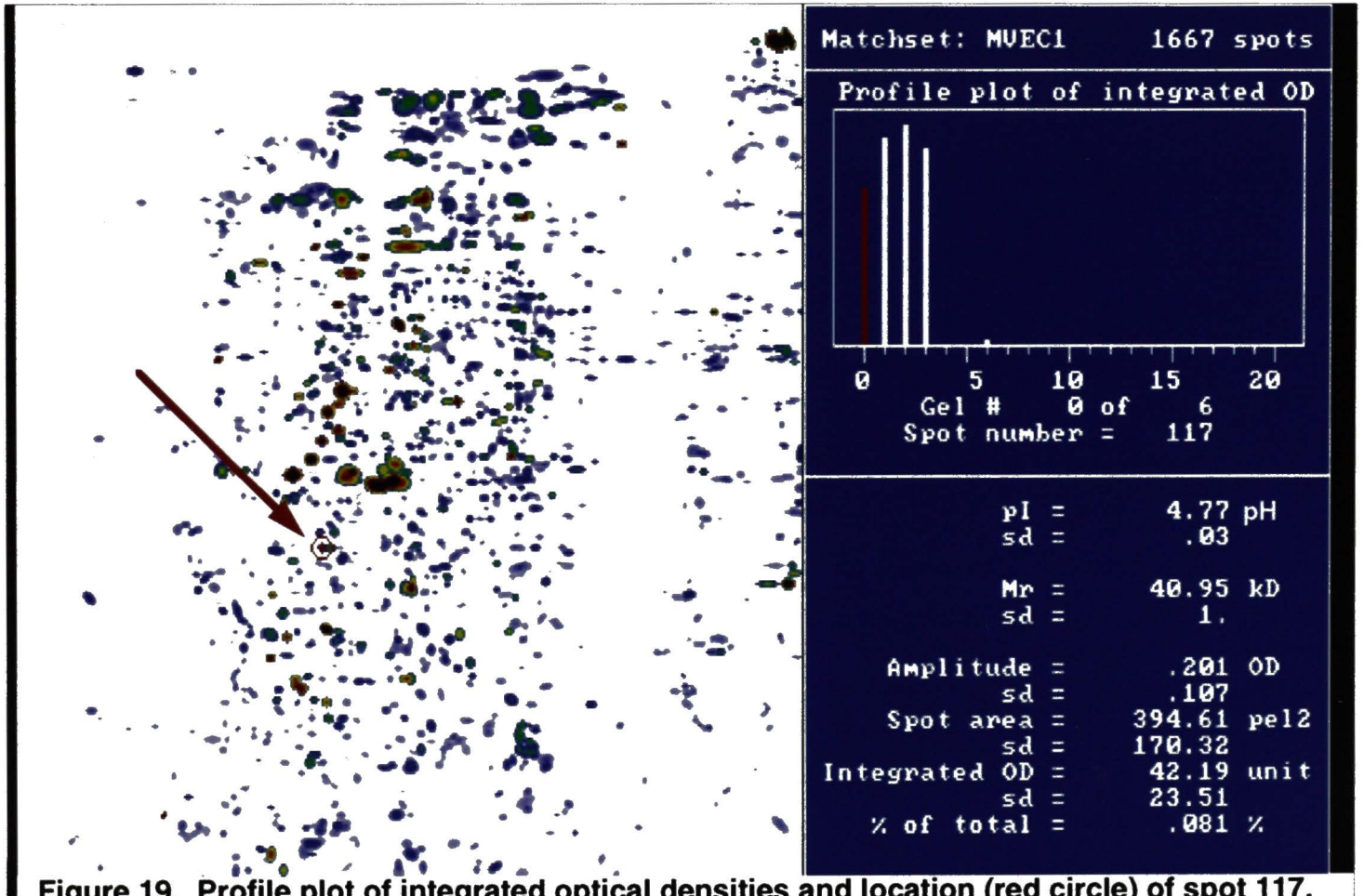


Figure 19. Profile plot of integrated optical densities and location (red circle) of spot 117.

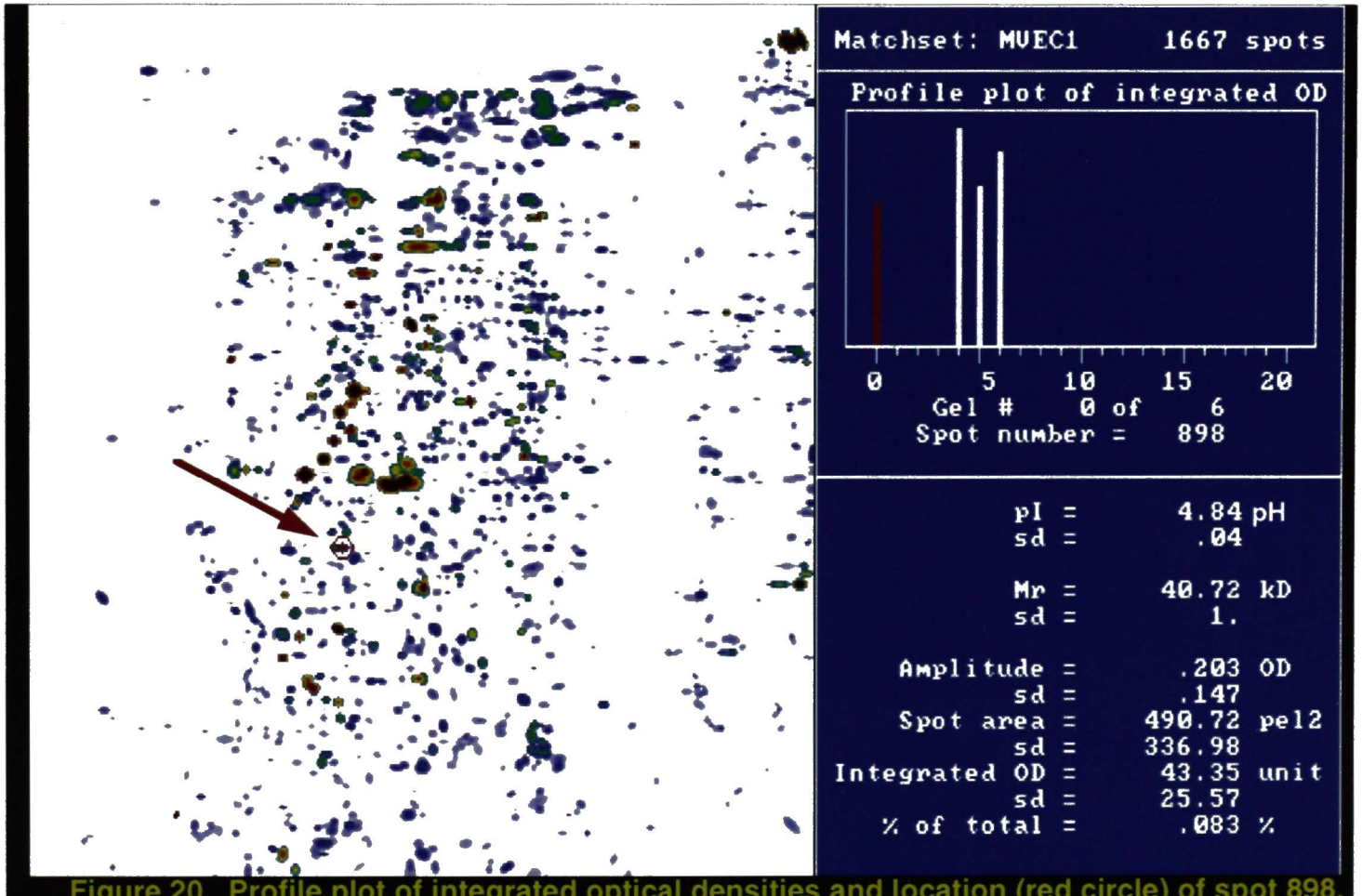


Figure 20. Profile plot of integrated optical densities and location (red circle) of spot 898.

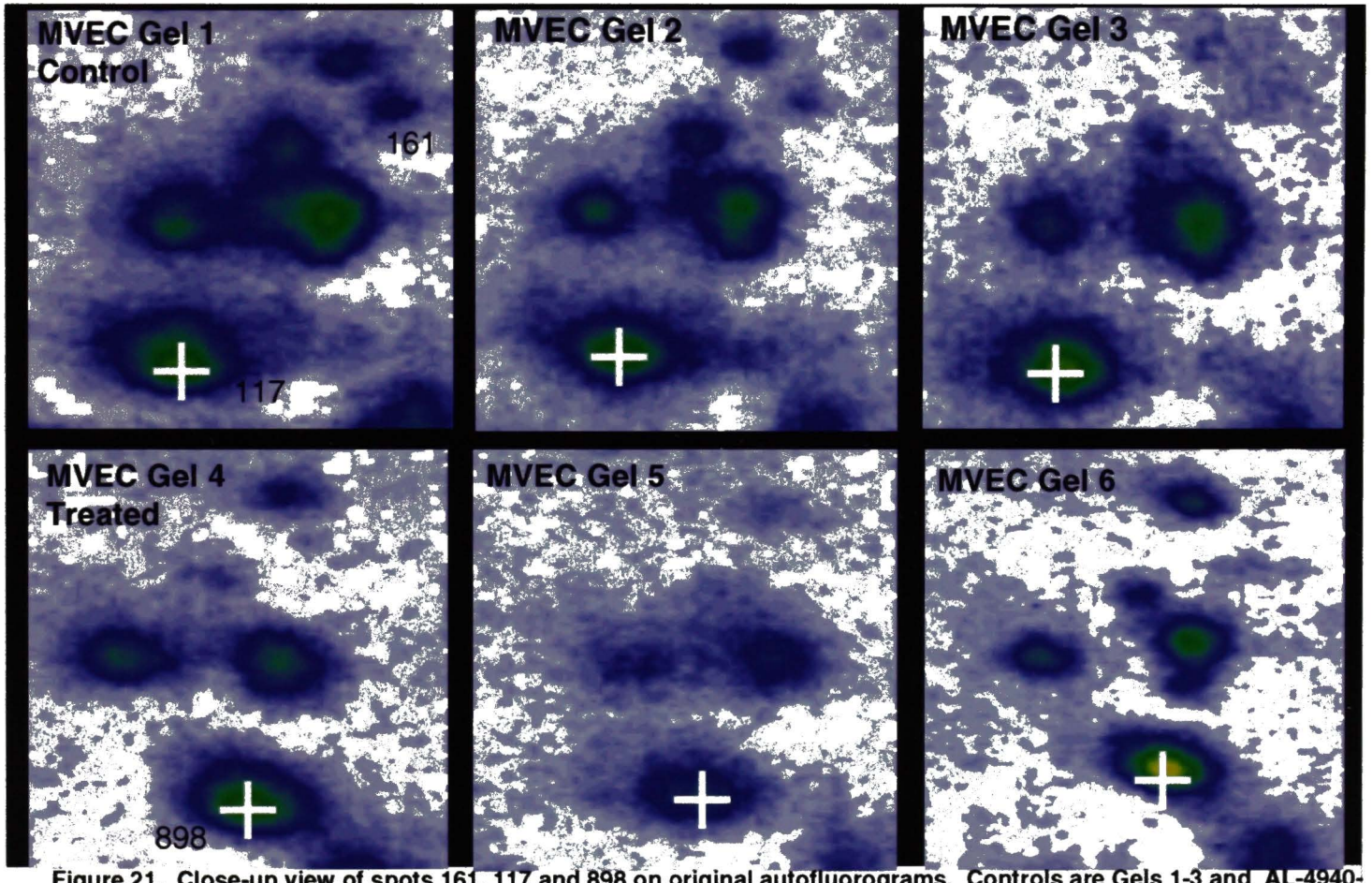


Figure 21. Close-up view of spots 161, 117 and 898 on original autofluorograms. Controls are Gels 1-3 and AL-4940-treated are Gels 4-6.

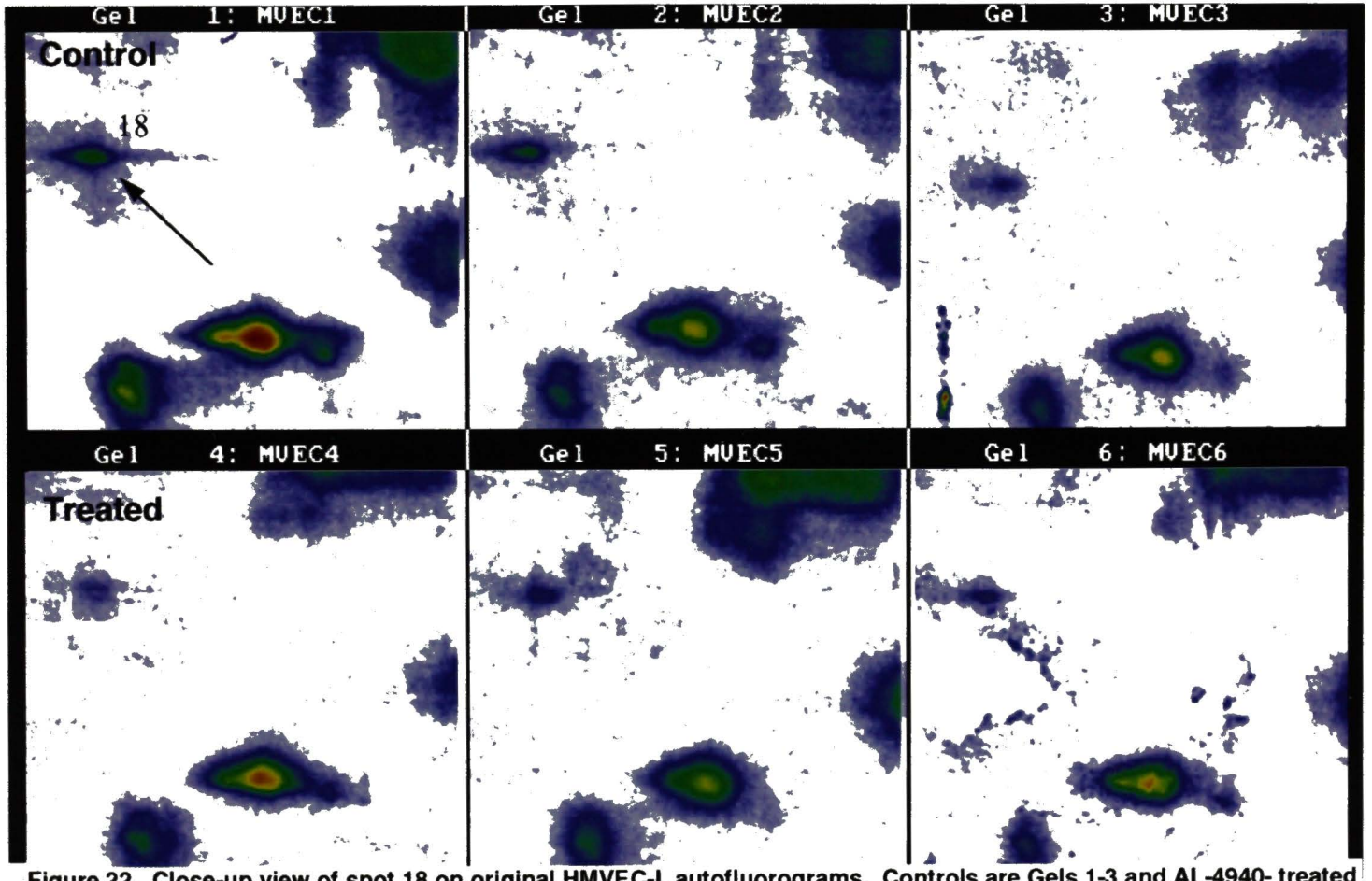


Figure 22. Close-up view of spot 18 on original HMVEC-L autofluorograms. Controls are Gels 1-3 and AL-4940- treated are Gels 4-6.

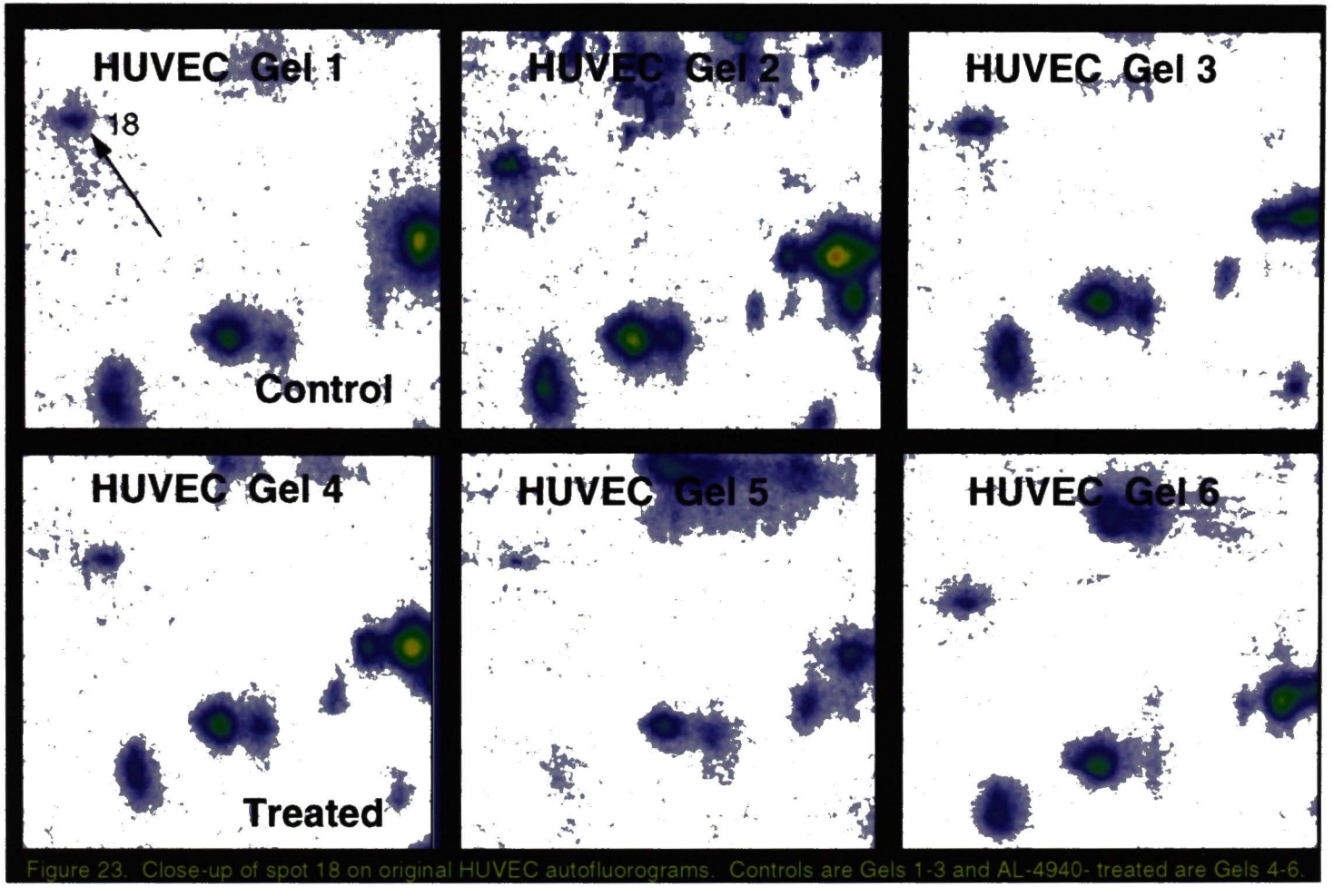


Figure 23. Close-up of spot 18 on original HUVEC autofluorograms. Controls are Gels 1-3 and AL-4940- treated are Gels 4-6.

Table 8. Changes in Protein Expression in HMVEC-L with AL4940

Spot	Database Spot Number	IEP (pH)	Mass (kD)	Control Int. O.D.	AL4940 Int. O.D.	Ratio Con/4940	% of Control
1	18	4.16 ± 0.00	127 ± 3	32.53 ± 11.23	15.28 ± 2.71	2.12	46.97
2	85	4.65 ± 0.03	63 ± 3	130.29 ± 69.0	89.04 ± 61.7	1.463	68.33
3	64	4.51 ± 0.04	46 ± 1	20.03 ± 15.10	2.88 ± 4.93	6.95	14.38
4	161	4.88 ± 0.03	43 ± 0	6.20 ± 4.45	0.86 ± 1.20	7.21	13.87
5a	117	4.77 ± 0.03	41 ± 1	55.72 ± 2.84	0.54 ± 0.93	104.17	0.96
5b	898	4.84 ± 0.04	41 ± 1	0.16 ± 0.28	57.64 ± 9.05	0.0028	36025
6	98	4.72 ± 0.04	36 ± 2	5.00 ± 1.84	2.87 ± 0.94	1.74	57.4
7	179	4.90 ± 0.03	29 ± 1	10.33 ± 1.67	0.63 ± 0.70	16.39	6.1
8	181	4.92 ± 0.03	76 ± 2	9.38 ± 9.96	7.08 ± 5.44	1.32	75.48
9	385	5.43 ± 0.01	86 ± 1	18.84 ± 9.33	25.04 ± 22.01	0.75	132.91

Conclusions

Using high-resolution, two-dimensional polyacrylamide gel electrophoresis, the expression of approximately 700 - 1000 urea-soluble proteins can be evaluated simultaneously. Here, we examined about 900 of those proteins. AL-4940 (10^{-5} M) treatment of cultured HMVEC-L and HUVEC cells significantly altered the expression of approximately 1.0% (9 of 954) of protein expression in the examined databases. The expression of most proteins did not change with drug treatment. The results of these studies indicate that AL-3789 and its active metabolite, AL-4940 might affect neovascular processes by alteration of genetic expression of proteins in vascular endothelial cells. The identity of these proteins remains to be determined.

CHAPTER IV

HUMAN STUDIES OF AL-3789

There is currently no pharmacological treatment to prevent neovascular processes in ocular diseases. Neovascularization of the retina is treated by laser ablation of new vessels, resulting in visual loss to the patient; other treatments such as surgical excision in malignant pterygium and laser ablation of subretinal neovascular membranes only slow the progression of ocular neovascularization and do not prevent re-neovascularization. There is therefore a need for a new, pharmacological approach to the safe and effective treatment of ocular neovascularization.

Studies of the potential efficacy of angiostatic steroids on retinal neovascular processes will require large numbers of patients studied for many years, since the neovascular process in many of these patients is slow. Efficacy studies of neovascular disease in the anterior segment such as pterygium recurrence, however, could be accomplished quickly since the angiogenic process proceeds relatively rapidly in these patients. The clinical study reported here constituted a "proof-of-principle" trial to determine if the angiostatic steroid AL-3789 could control human neovascular processes in the anterior segment prior to conducting retinal studies.

The study of AL-3789 in humans first required a safety study in normal human volunteers to verify the preclinical toxicology studies and ensure that the

compound was safe for dosing in humans. In addition, a comfort study was accomplished to characterize the ophthalmic dosing formulation in comparison to commonly used ophthalmic preparations.

Comfort and Safety Studies of AL-3789 in Humans

AL-3789 Ophthalmic Suspension was evaluated in two clinical studies to assess 1) the ocular comfort of 1.0% and 2.0% formulations relative to vehicle, and 2) the effect of topical chronic ocular dosing of the 1.0% and 2.0% formulations of AL-3789 for two weeks in normal humans to assess the safety and steady-state plasma concentrations of the deacetylated metabolite. The comfort study was a two week, single-masked study which employed a three-period crossover design. Subjects were assigned to a treatment order according to a randomization schedule and received one drop of 1.0% AL-3789, 2.0% AL-3789, and vehicle on a random basis. The subjects graded comfort of the topical ocular formulations according to psychometric scales, and the results were compared to previously conducted studies of ocular formulations. The safety study evaluated the safety of 1.0% and 2.0% AL-3789 when dosed three times daily (TID) in normal humans for two weeks. For both studies, volunteers were of any race or sex, ages 18-65, asymptomatic and free of any concomitant ocular or systemic disease.

Experimental Design

Comfort Study: Thirty subjects were enrolled into the study and signed an informed consent to particpate. A comprehensive ophthalmic examination was conducted for baseline examination, and three examinations were conducted during test article treatment. In addition, a post-treatment ophthalmic examination was performed. Subjects received one drop of the test solutions (AL-3789 Ophthalmic Suspension 1.0%, 2.0%, or vehicle) in their nondominant eye and graded the comfort of the drop using psychometric scales.

Safety Sudy: Twenty-one (21) subjects were enrolled into the study and signed an informed consent to participate. Volunteers received ophthalmic and systemic examinations on Days 0 and 15 of the study. An abbreviated ophthalmic examination was performed on Day 8. Ten patients dosed two drops of 1.0% AL-3789 and ten patients dosed two drops of 2.0% AL-3789 in a double-masked fashion TID in both eyes (OU) for 15 days. The ophthalmic examination included visual acuity (best corrected Snellen), slit-lamp examination of the eyelids and conjunctiva, cornea, iris, anterior chamber, pupillary response, and fluorescein staining of the cornea. Applanation tonometry was performed in addition to color vision testing with standard pseudoisochromatic plates. A dilated fundus examination was performed on Days 0 and 15 including examination of the lens, vitreous, retina, macula, choroid, and optic nerve. Blood samples were drawn on Days 0, 1, 8, and 15 for pharmacokinetic analysis of AL-4940 levels by HPLC, as well as for SMA-24, CBC, and hemotological and differential analysis. Urinalysis was performed on Days 0 and 15.

Results

Comfort Study: Analysis of the burning profile exhibited by patients who participated in the comfort study revealed that ocular and membrane discomfort changes were minimal with both 1% and 2% AL-3789 Ophthalmic Suspensions. No seroius related or unrelated safety events to the two AL-3789 formulations or their vehicle alone were reported, and no clinically significant decrease in visual acuity, worsening of ocular signs, or increase in intraocular pressure was observed in any volunteer. Both formulations were safe and well tolerated by all subjects participating in the study.

Safety Study: Twenty-one subjects were enrolled into the study. There were 8 males and 13 females, between the ages of 19 and 59 (mean 36.5 years). Twelve were Caucasian and 9 were Black; 8 subjects had blue, 11 had brown and 2 had hazel irides. Ten subjects were assigned to treatment with AL-3789 1.0% Ophthalmic Suspension, and 11 to the 2.0% formulation. One of the 10 (10%) subjects receiving the 1.0% formulation and 4 of the 11 (36.4%) receiving the 2.0% formulation experienced adverse events (Table 9). These consisted of one incident of increased intraocular pressure, 2 incidents of hypotension, and 2 of tachycardia. These events were mild, nonserious, resolved without treatment and did not preclude continuance in the study. There were no seroius events reported. No clinically significant decrease in visual acuity, color vision, worsening of ocular signs, fluorescein staining of the cornea, pupillary response, or dialated fundus

examination was observed during the study. Likewise, there were no clinically significant changes in blood chemistry values (SMA-24), hematological parameters or urinalysis values in either treatment group. Plasma samples from subjects were analyzed for concentrations of AL-4940, the active metabolite of AL-3789, by high performance liquid chromatography with tandem mass spectrometry detection (HPLC/MS/MS). All samples measured below the limit of quantitation (0.5 ng/mL), but in seven subjects at least one plasma sample showed detectable but not quantifiable AL-4940 levels. The estimated limit of detection for the assay was 0.2 ng/mL; the response in these samples was clearly distinguishable from control samples. These results indicate systemic exposure to AL-4940 at very low concentrations following topical ocular dosing with AL-3789.

Conclusions

Two ophthalmic formulations of AL-3789, 1.0% and 2.0%, were found to be comfortable for topical ocular dosing. Both formulations were evaluated for ocular and systemic safety in normal, healthy volunteers and found to be safe, well tolerated and recommended for therapeutic evaluation.

Table 9. Adverse Events by Subject (Comfort and Safety Studies).

Inv. No.	Subj. No.	Age	Sex	Treatment	Coded Adverse Event	Study Day	Intensity	Duration of Event	Outcome of Event	Serious	Causality Assessment	Subj. D/C Due to AE
648	116	33	F	AL-3789 1.0%	Intraocular Pressure Increased	8*	Mild	1 Hour	Resolved	No	Related	No
648	114	44	F	AL-3789 2.0%	Tachycardia	8*	Mild	6 Hours	Resolved	No	Related	No
648	112	35	F	AL-3789 2.0%	Hypotension	8*	Mild	4 Hours	Resolved	No	Not Related	No
648	121	19	M	AL-3789 2.0%	Hypotension	8*	Mild	N/A	Resolved	No	Not Related	No
648	120	26	F	AL-3789 2.0%	Tachycardia	8	Mild	8 Hours	Resolved	No	Not Related	No

Related = Possibly, Probably or Definitely Related
Not Related = Unlikely or Definitely Unrelated
N/A = Not Available
* Event occurred intermittently

Efficacy of AL-3789 in Preventing Re-neovascularization in Humans Following Pterygium Excision

Experimental Design

This clinical "proof-of-principle" trial determined the angiostatic efficacy of 1.0% AL-3789 Ophthalmic Suspension when dosed chronically in patients who underwent recurrent pterygium excision. The study design was double-masked, randomized, and placebo-controlled. High rates of pterygium recurrence are experienced by patients who have had a second surgical exicision procedure, with most experiencing a third recurrence within one year (Hirst *et al*, 1993). Patients were enrolled into this trial if they had undergone at least one prior surgical excision of recurrent pterygium and required a further excision. Patients were followed for a period of one year and were examined monthly. The area of surgical excision was photographed for computer image analysis of re-neovascularization during the treatment period.

Experimental Method

The double-masked, randomized, prospective clinical trial (Protocol C-94-02) was conducted by three investigators: Carmen Santos, M.D. (Puerto Rico), John Zeiter, M.D. (California), and Mark Speaker, M.D. (New York). Protocol C-94-02 allowed these investigators to enroll patients who had experienced at least one previous pterygium excision into the study. Following bare scleral surgical excision

of the pterygium, without receiving β-irradiation, mitomycin-C, 5-fluorouracil or a conjunctival flap procedure, patients were assigned treatment with either AL-3789 Ophthalmic Suspension 1% or Vehicle (Placebo), 1-2 drops TID in the affected eye for up to one year. Patients were terminated from the study and classified as "treatment failure" if the investigator determined that a pterygium had recurred and surgical excision was indicated. Follow-up examinations included best corrected Snellen visual acuity, slit-lamp examination of the anterior segment and anterior vitreous, slit-lamp photography of the area of surgical excision, applanation tonometry, and dilated fundus examination upon entering and exiting the study. Follow-up examinations were given on postoperative Days 1 and 2, Weeks 1-4 and 6, and Months 3-12. Slit-lamp photography was accomplished by a protocol which standardized photographs for all patients with regard to magnification, ocular aspect, and distance. The serial photographs for each patient were subsequently analyzed using computer image analysis by Dr. H.F. Edelhauser at Emory University (Figure 24). These photographs allowed quantification of the growth of new blood vessels into the area of surgical excision and documentation of pterygium recurrence. Briefly, serial photographs were traced on an acetate sheet beginning with tracing of the limbus in each sequential photograph.

Limbal alignment and reference blood vessels in each photograph were used for proper alignment of subsequent tracings. A unique calibration box was traced for each patient series of photographs so that neovascular areas within each patient series could be compared with time. Photographs were imaged using a Macintosh

Figure 24. Serial photographs of Patient 213 in Protocol C-94-02 (Figures 24a-e) and composite computerized image of advancing neovascular area (Figure 20f).

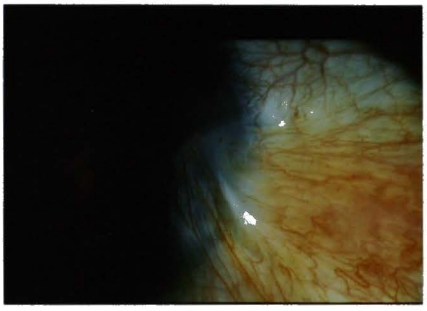


Figure 24a. Preoperative

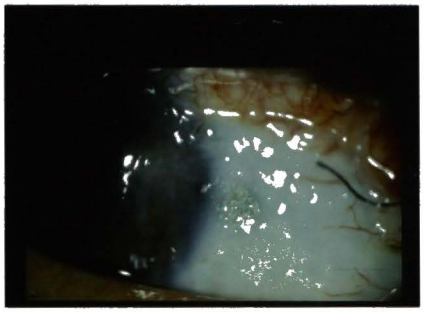


Figure 24b. Week 1 Postoperative

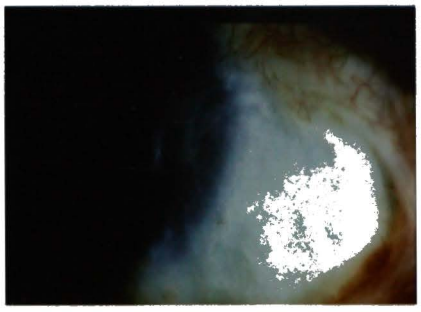


Figure 24c. Week 4 Postoperative

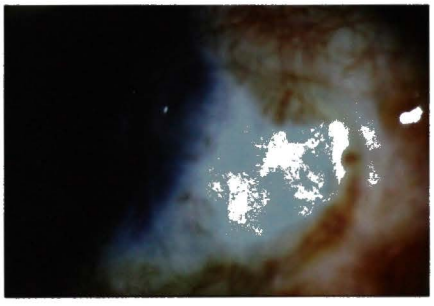


Figure 24d. Week 6 Postoperative

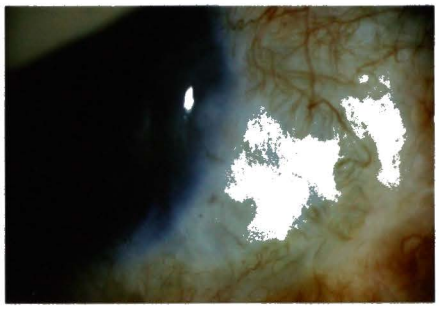


Figure 24e. Week 8 Postoperative

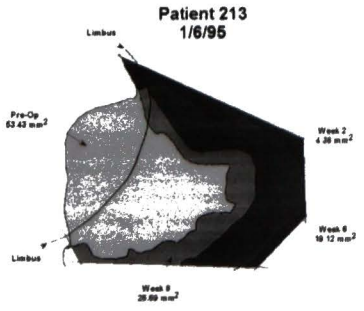


Figure 24f. Computer Image generated from Figures 20a-e.

Quadra 840AV using Ofoto® scanning software, and IMAGE 1.54 (National Institutes of Health) was used to analyze serial images.

Protocol C-94-03 was a compassionate, open-label protocol which allowed patients who experienced pterygium recurrence and were treated with placebo in the masked study to be treated with AL-3789 Ophthalmic Suspension 1% TID for up to one year. The conduct of this protocol was identical to that of C-94-02 with regard to clinical examinations and computer image analysis of serial photographs. The patients were examined monthly.

Results

Seventy-nine (79) patients were enrolled into Protocol C-94-02, of whom 12 did not receive surgery. Of the remaining 67 patients, 33 were randomized to the AL-3789 treatment group and 34 received placebo. Demographically similar patient populations were enrolled into the two treatment groups (Table 10), with the exception that a greater percentage of older patients were enrolled into the AL-3789 treatment group, however, this difference was not statistically significant ($p = 0.0933$).

Computer image analysis of serial photographs for each patient indicates that patients who were treated with placebo in the double-masked trial experienced an average neovascular growth rate of 1.52 mm^2/week, compared to 0.78 mm^2/week exhibited by the patients treated with AL-3789 Ophthalmic Suspension 1% (Littell *et al*, 1989, Intent-to-treat analysis using ANOVA results from a random coefficients model, SAS, $p = 0.0160$, Figure 25).

Table 10. Demographic comparisons of treatment groups, Protocol C-94-02

	AL-3789	Placebo
No. Patients Enrolled	39	40
No. Patients Evaluative	33	34
Completed Study	13	6
Did Not Have Surgery	6	6
Patients Discontinued		
Adverse Event	1	1
Lost to Follow-up	3	3
Patient Decision to D/C	1	1
Treatment Failure	13	23
Non-Compliance	1	0
Medical (Accident)	1	0
Age		
< 65 years	26	32
> 65 years	7	2
Sex		
Male	20	25
Female	13	9
Race		
Caucasian	7	10
Black	0	4
Hispanic	25	19
Asian	0	1
Eye Color		
Brown	30	31
Hazel	1	1
Green	1	0
Blue	1	

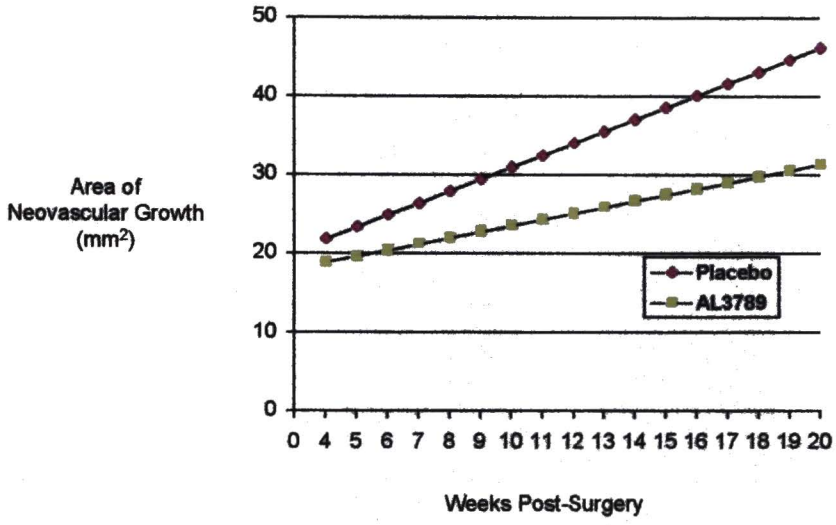


Figure 25. Longitudinal analysis of serial photographs for all patients; (Efficacy Image Analysis ,Protocol C-94-02). The overall average rate for placebo treatment was 1.52 mm^2 / week; for AL-3789 treatment, 0.78 mm^2 / week.

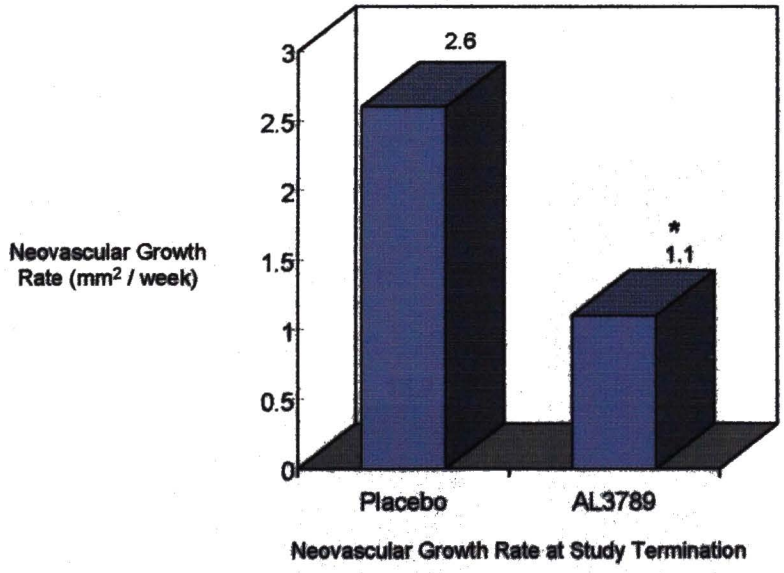


Figure 26. Effect of AL-3789 on pterygium growth rate; End Point Analysis (Protocol C-94-02; * statistically different from placebo $p<0.05$).

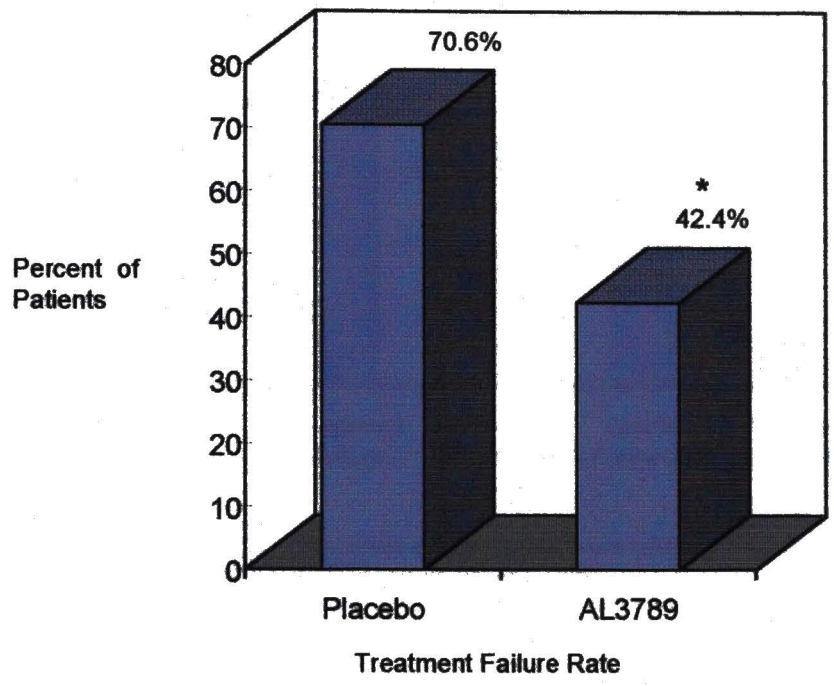


Figure 27. Percentage of patients experiencing pterygium recurrence (Protocol C-94-02; * statistically different from placebo $p<0.05$).

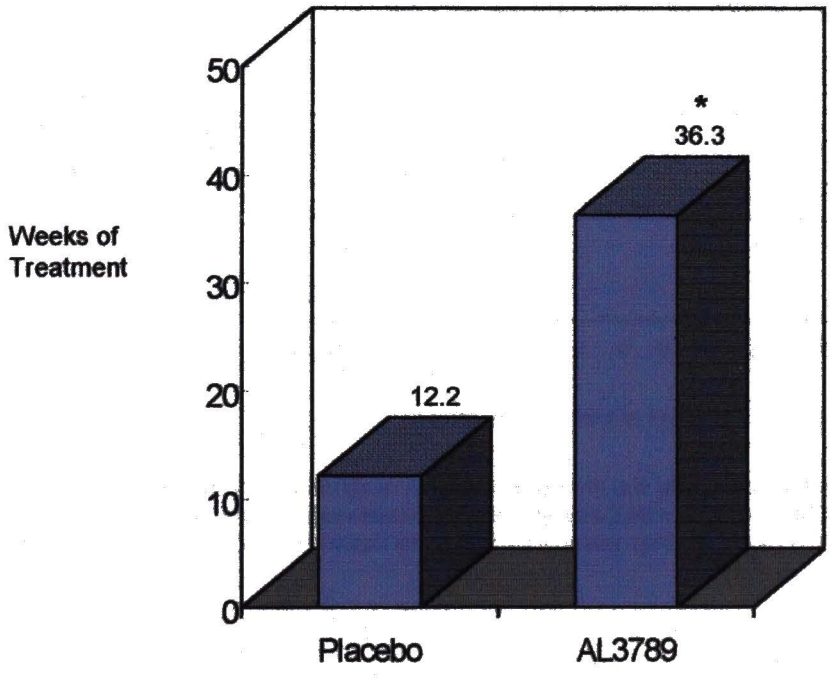


Figure 28. Mean survival time to pterygium recurrence for patients treated with placebo or AL-3789 (Protocol C-94-02;* statistically different from placebo, $p<0.05$).

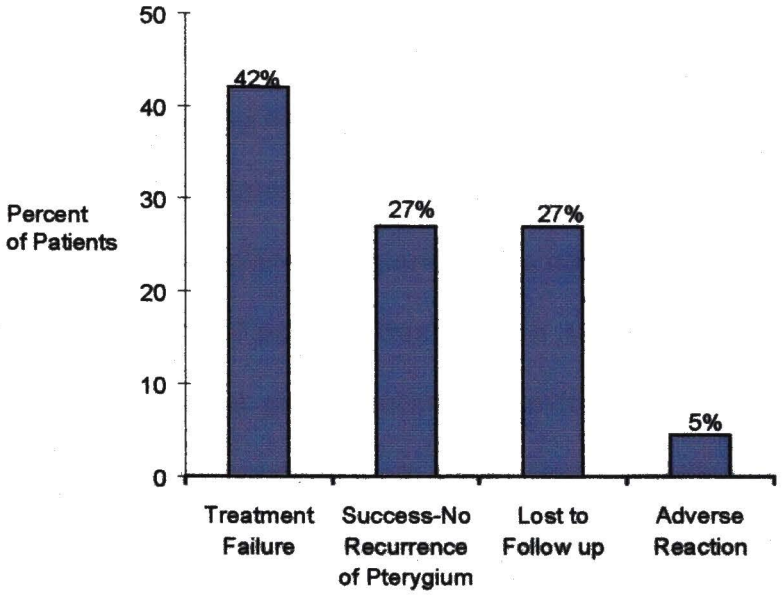


Figure 29. Disposition of placebo treatment failures from Protocol C-94-02 treated with AL-3789 under Protocol C-94-03.

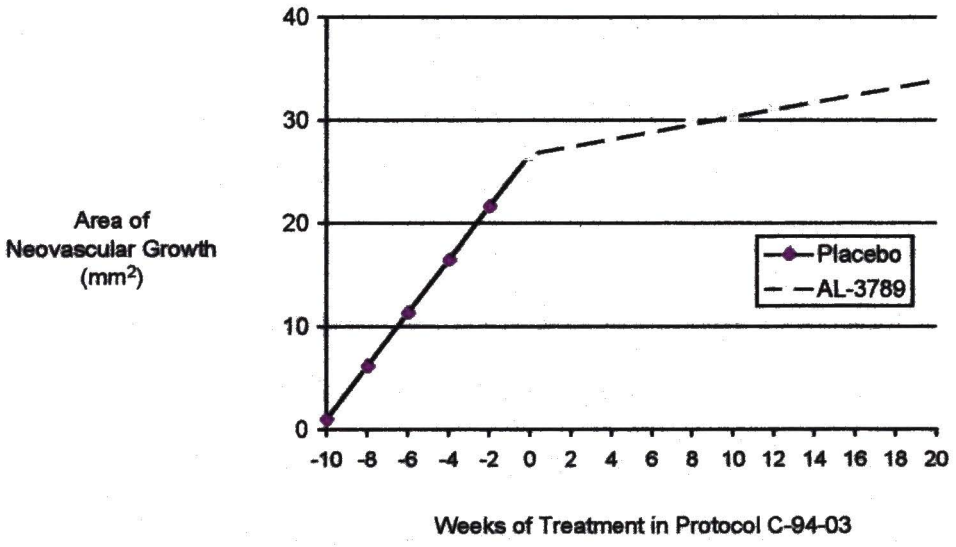


Figure 30. Change in neovascular growth rate after initiating treatment with AL-3789. The average neovascular growth rate was 2.58 mm^2 / week for placebo treatment and 0.36 mm^2 / week for AL-3789 treatment ($p<0.05$; Protocols C-94-02 and C-94-03).

An efficacy analysis (excluding patients who were non-compliant, lost to follow-up, etc.) revealed regrowth rates of 1.89 mm^2/week and 1.09 mm^2/week for placebo and AL-3789 treatment, respectively ($p = 0.0648$). These results indicate that patients treated with placebo experienced a neovascular growth rate approximately twice that of patients treated with AL-3789 Ophthalmic Suspension 1%. The mean neovascular growth rates upon exiting the study for any reason (treatment failure, study completion, etc.) statistically significantly favored treatment with 1.0% AL-3789 Ophthalmic Suspension compared to placebo (2.56 mm^2/week vs 1.12 mm^2/week, Intent-to-treat analysis, $p = 0.0212$, Figure 26).

In the double-masked study, 70.6% of patients treated with placebo experienced recurrence of the pterygium compared to 42.4% of patients treated with AL-3789 (Intent-to-treat analysis, $p = 0.027$, Figure 27). When only evaluative patients are analyzed, the treatment failure rate was 76.7% for the placebo group and 40.0% for AL-3789 group (Efficacy analysis, $p = 0.0082$). Patients were considered to be treatment failures if the pterygium recurred to the extent that the investigator felt surgical removal was indicated.

LIFETEST (SAS) survival analysis (Littell *et al*, 1989) showed that the mean survival time for patients treated with AL-3789 Ophthalmic Suspension 1% was 36.29 weeks compared to 12.22 weeks for placebo patients (Intent-to-treat analysis, $p = 0.0008$, Figure 28). The mean time to treatment failure defined as pyerygium recurrence and need for surgery significantly favored treatment with AL-3789 compared to placebo.

Twenty-two (22) of the 34 placebo patients evaluated in the double-masked study (Protocol C-94-02) experienced treatment failure, i.e. pterygium recurrence, and were entered into the open-label compassionate trial under Protocol C-94-03. These patients received treatment with AL-3789 Ophthalmic Suspension 1% TID for up to one year. Forty-two (42) percent of these patients went on to complete recurrence of the malignant pterygium, however, 27% experienced arrest of the neovascular process and did not develop recurrence (Figure 29).

The longitudinal neovascular growth rate analysis of these patients indicates that discontinuation of placebo treatment and initiation of treatment with AL-3789 significantly altered their neovascular growth rates ($p = 0.0001$, Figure 30). Patients treated with placebo experienced a mean growth rate of 2.58 mm^2/week until the time of treatment failure and rollover to AL-3789 treatment. The mean growth rate following treatment with AL-3789 was 0.36 mm^2/week.

Ocular safety events related to treatment with AL-3789 1% Ophthalmic Suspension in both studies were mild, infrequent, nonserious, resolved without treatment and did not interrupt continuation in the study (Table 11). Reported ocular safety events possibly related to treatment with AL-3789 included discomfort, pain, cataract formation and blurred vision upon instillation. None of the reported events were judged to be probably or definitely related to treatment with AL-3789.

Table 11. Incidence of adverse events in the double-masked pterygium trial (Protocol C-94-02) and the compassionate trial (Protocol C-94-03).

Incidence of Adverse Events Protocol C-94-02 and C-94-03				
	AL-3789 N=55		Placebo N=34	
	N	%	N	%
Related				
Ocular				
Discomfort Eye	3	5.5	3	8.8
Pain Eye	2	3.6	1	2.9
Cataract	1	1.8	0	
Vision Blurred	2	3.6	0	
Diplopia	1	1.8	0	
Hyperemia Eye	1	1.8	0	
IOP Increase	1	1.8	2	5.9
Edema Eye	0		2	5.9
Photophobia	0		1	2.9
Not Related				
Ocular				
Cicatrix Corneal	18	32.7	11	32.4
Discomfort Eye	7	12.7	3	8.8
Hyperemia Eye	6	10.9	1	2.9
Dellen	5	9.1	2	5.9
Pain Eye	5	9.1	6	17.6
Cyst Conjunctival	4	7.3	2	5.9
Eye Disorder	4	7.3	0	
Foreign Body Sensation	4	7.3	1	2.9
Photophobia	4	7.3	3	8.8
Pruritus Eye	4	7.3	2	5.9
Visual Acuity Decrease	4	7.3	1	2.9

Conclusions

Topical ocular treatment with AL-3789 Ophthalmic Suspension 1% significantly inhibited recurrence of malignant pterygium in patients enrolled into the double-masked trial. Many of these patients had experienced multiple surgeries with application of β-irradiation, mitomycin-C, or 5-fluorouracil prior to entry into this study. AL-3789 affected the outcome of surgery in these patients by 1) reducing the rate of re-neovascularization following the surgery and 2) reducing the rate of pterygium recurrence as judged by the necessity for additional surgery. When patients treated with placebo in the double-masked trial were treated with AL-3789 in the open-label compassionate study, 27% experienced arrest of the pterygium recurrence.

These results signify that treatment with AL-3789, while not completely effective in preventing pterygium recurrence, has a beneficial effect on this difficult disease which may be useful when applied in conjunction with accepted treatment modalities (i.e., β-irradiation, mitomycin-C, and 5-fluorouracil) which are not completely effective.

CHAPTER V

DISCUSSION

Mechanism of Action of AL-3789

AL-3789 or its metabolite AL-4940 was seen to affect vascular endothelial cell function in three different ways in the experiments described above. The compounds were found to 1) significantly decrease the quantity of two proteolytic enzymes expressed by these cells and thought to be essential for angiogenesis to occur, namely, urokinase and stromelysin, 2) significantly affect the ability of two types of vascular endothelial cells, HMVEC-L and HUVEC, to proliferate *in vitro*, and 3) significantly alter the expression of specific proteins in HMVEC-L cells. However, AL-4940 did not appear to alter Matrigel-induced "*in vitro* capillary" formation. Thus, AL-3789 and AL-4940 have been shown to affect two of the steps essential for angiogenesis to occur as outlined in Chapter I (Figure 31).

These actions may be explained by several known pharmacological mechanisms. The most straightforward explanation for the action of AL-3789 and AL-4940 is that they behave in a mechanistic manner analogous to glucocorticoids, acting through an as yet undiscovered intracellular and intranuclear receptor to affect genetic expression of protein at the level of messenger RNA synthesis. These compounds, associated with a putative angiostatic steroid receptor, might associate with steroid response elements or promoter regions of genes that produce RNA messages for proteolytic enzymes, thereby affecting the cell's ability

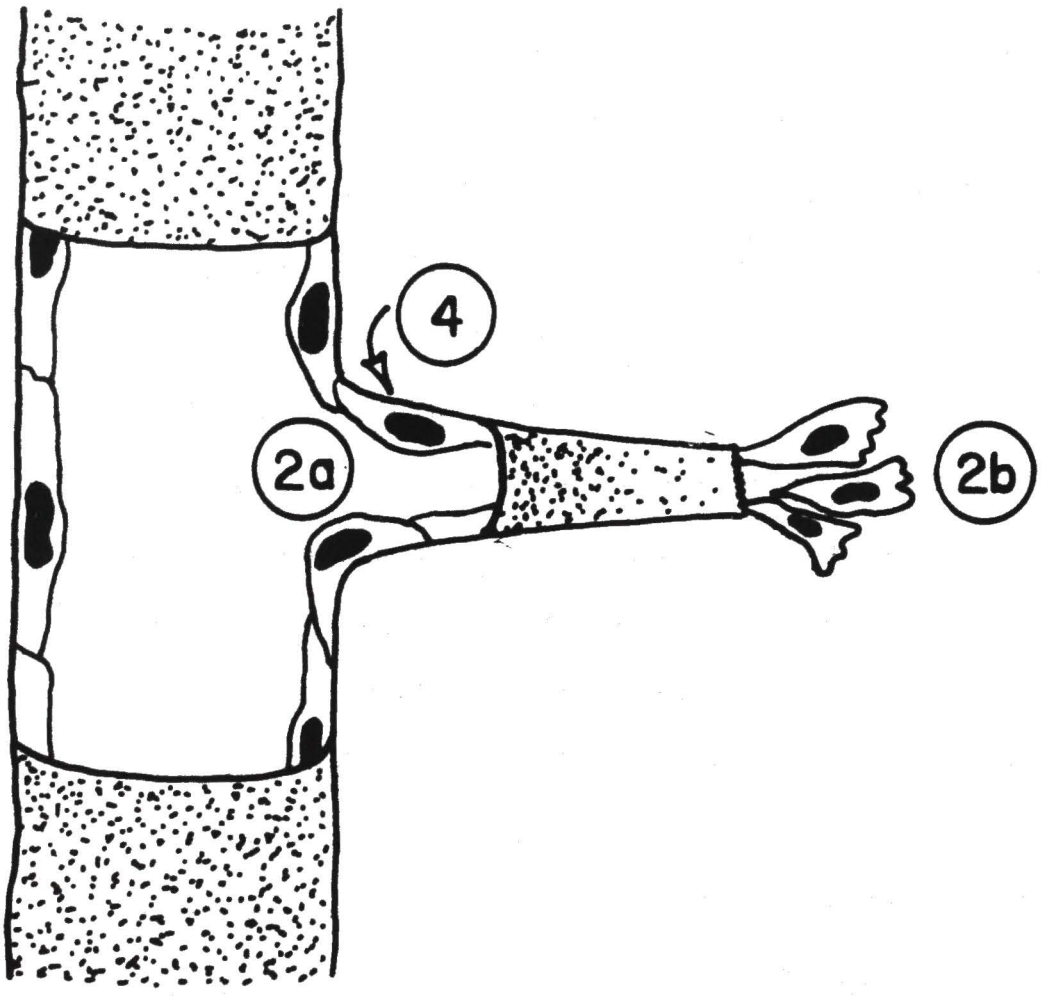


Figure 31. Points in the sequence of events in angiogenesis inhibited by AL-3789: 2a) and 2b) secretion of uPA and MMP-3 that degrades the vascular basement membrane and extracellular matrix, and 4) proliferation of vascular endothelial cells (see Figure 1).

to secrete proteases necessary for dissolution of the vascular basement membrane and movement through the extracellular matrix to produce new blood vessels. Similarly, the expression of cell cycle proteins necessary for the control of cellular proliferation to form new blood vessels might be affected by AL-3789 and AL-4940 in such a manner. Glucocorticoids generally alter the expression of 1-5% of expressed proteins in a tissue and cell-specific manner by means of receptor-mediated alterations in gene expression (Yang-Yen *et al*, 1990). This class of steroids ameliorates inflammatory processes by producing small alterations in protein expression across a number of different protein classes, resulting in many small changes which produce an overall major effect. AL-4940 was seen to alter the expression of approximately 1% of visualized proteins in HMVEC-L by 2D-PAGE analysis. This magnitude of proteins is on the order of that known to be affected by glucocorticoids, underscoring the possibility that AL-3789 and AL-4940 might act via glucocorticoid receptor-like mediated gene induction or inhibition. It is also possible that these compounds affect, either directlly or indirectly, the stablity of the protease messenger RNA to decrease urokinase and stromelysin levels. They might also affect the messages encoding growth factor and cytokine receptors of endothelial cells that control expression of the proteins studied.

The unidentified proteins which were affected by AL-4940 treatment might alter endothelial cell gene expression in a variety of ways. Glucocorticoids are known to affect the expression of cellular proteins for many cell types. In particular, the activated glucocorticoid receptor has been shown to directly repress AP-1, the transcription factor which induces expression of the matrix

metalloproteases, through protein-protein interaction (Jonat *et al*, 1990; Yang-Yen *et al*, 1990; Schule *et al*, 1990). It is possible that AL-4940 affects protein expression in a similar manner by binding to a putative receptor similar in structure to the glucocorticoid receptor. AL-4940 might also inhibit directly the *c-fos* and *c-jun* oncogenes which encode AP-1 to prevent matrix metalloprotease expression. Alternatively, AL-4940 might cause upregluation of regulatory inhibitor proteins which in turn down-modulate AP-1 function or *c-fos* and *c-jun* expression.

Nuclear factor κB is an important ubiquitous transcription factor which regulates many important cellular functions, including vascular endothelial cell adhesion molecule (CAM) expression important in cell migration and cell-cell alignment (Baeuerle and Henkel, 1994; Marui *et al*, 1993). The proteins altered by AL-4940 treatment might well be proteins which affect NFκB function, or that of its inhibitor, IκB.

The results presented here indicate that AL-3789, medroxyprogesterone acetate (MPA), and dexamethasone (DEX) inhibit expression of urokinase in human microvascular endothelial cells. These results are in agreement with those of Ashino-Fuse *et al* (1989), who reported that MPA, an angiostatic steroid, inhibited total extracellular and cell-associated urokinase activity in bovine adrenal cortical capillary, aortic, and pulmonary artery endothelial cells following stimulation with bFGF and 4β-phorbol-12-myristate-13-acetate (PMA). These authors measured plasminogen-dependent proteolytic activity expressed in relation to a standard urokinase curve, and not urokinase protein levels. They also reported that MPA had no effect on bovine endothelial cellular proliferation even at a high (10^{-5} M)

concentration. MPA is a progestin used orally and parenterally as an antineoplastic agent to treat breast carcinoma and endometrial hyperplasia, and parenterally to treat metatastic renal or endometrial carcinoma and endometriosis. Its mechanism of action has not been fully ellucidated, but it is known to inhibit tumor growth (*i.e.* cellular proliferation), neovascularization and collagenolysis (Gross *et al*, 1981). AL-4940, however, and MPA were both seen to affect human microvascular endothelial cell proliferation at 10^{-5} M in the study reported here. While these results conflict with those of Ashino-Fuse *et al*, different cell types (bovine *vs* human) were used for the two experiments.

Blei *et al* (1993) reported that MPA caused a decrease in total (media plus cell extract) urokinase activity of 83% at 10^{-6} M in their experiments using bovine aortic endothelial cells following stimulation with bFGF. They simultaneously measured a significant increase in plasminogen activator inhibitor type-1 (PAI-1) production as determined by reverse fibrin zymography and immunoprecipitation. In the study reported here, however, none of the angiostatic steroids tested (AL-3789, AL-4940, MPA, dexamethasone, or tetrahydrocortisol (THF) decreased protein levels of PAI-1 below those stimulated by lipopolysaccharide (LPS). Again, different cell types (human *vs* bovine) and different angiogenesis stimulators (LPS *vs* bFGF) were used in the two studies.

AL-4940 did not affect matrigel-induced vascular endothelial cell tubule formation for many possible reasons. Each well in the experiment was seeded with approximately 7.5×10^5 cells, which may be more cells than the number required in this model to visualize small treatment differences. In addition, the experimental

system used was very simple, with only treatments, vascular endothelial cells, and matrigel extracellular matrix molecules as components. Angiogenesis occurring *in vivo* includes many more factors which, in conjunction with AL-4940, might affect endothelial cell differentiation. These include additional extracellular matrix components not present in the matrigel preparation, as well as blood components, pericytes, monocytes and macrophages which are present in the extravascular environment. Finally, AL-4940 might not completely suppress the level of proteinases required for successful capillary tubule formation in this model, but might rely on another mechanism of action for its primary angiostatic effect.

Clinical Safety and Efficacy of AL-3789

The clinical trials reported here demonstrate that topical dosing with AL-3789 will inhibit pathological neovascularization of the ocular surface in humans. To my knowledge, this is the first report of significant effect on malignant pterygium by an angiostatic steroid. The efficacy of AL-3789 in this neovascular disease was clearly demonstrated in the "proof-of-principle" trial.

The three clinical trials involving over one hundred patients with up to one year of topical ocular dosing illustrated the ocular comfort and safety of AL-3789 1.0% Ophthalmic Suspension. Pharmacokinetic studies showed systemic exposure to the drug to be limited and no drug-related systemic effects were seen with chronic dosing. In the therapeutic clinical trial, AL-3789 was shown to decrease

the rate of re-neovasculrization, as well as overall pterygium recurrence rate, after malignant pterygium excision.

Current treatment of pterygium, both recurrent and primary, consists of surgical excision with concomitant application of β-irradiation or mitomycin-C. Both concomitant treatments are toxic to cells and their use is intended to produce an antiproliferative and antimigratory effect on fibroblasts in the area of excision. Fibroblast strains isolated from autologous and heterologous pterygial and normal conjunctival specimens have been studied to determine their growth characteristics, and it was found that pterygial fibroblast strains exhibited reduced serum and exogenous growth factor requirements for growth compared to normal conjunctival fibroblasts (Chen *et al*, 1994). All pterygial fibroblast strains tested in this study were able to form colonies in soft agar in 5% fetal bovine serum at 6.0 to 7.5% efficiency, which correlates to cell transformation and tumorigenicity. None of the conjunctival fibroblast strains were able to grow under the same experimental conditions. These results imply that pterygial fibroblasts are preneoplastic and exhibit transformed phenotype. One characteristic of transformed phenotype is increased proliferation; AL-3789 might have inhibited the proliferation rate of both activated vascular endothelial cells as well as pterygial fibroblast cells to reduce the recurrence rate in the clinical study reported here.

The reported recurrence rates following pterygium surgery vary widely and no surgical procedure or concomitant therapy produces completely satisfactory results (Hirst *et al*, 1994). Surgical procedures used to remove pterygia include simple excision leaving bare sclera, or varoius procedures which cover the excision

area with conjunctiva. These include free or swinging conjunctival flaps and conjunctival autografting. Postoperatively, patients have been treated with a variety of pharmacological agents, including topical corticosteroids, antibiotics, artificial tears, and vasoconstrictors, as well as antineoplastic agents in an attempt to arrest proliferating pterygial fibroblasts and prevent recurrence. The antiproliferative agents currently being used are mitomycin-C and β-irradiation; thiotepa and 5-fluorouracil have been used in the past. However, no single combination of a single surgical procedure with an adjunctive peri- or post-operative therapy has been proven clearly superior to any other. Recurrence rates after simple excision alone of primary pterygium range from 5% to 83% (MacKenzie *et al*, 1991; Frucht-Pery and Ilsar, 1994; Anduze and Merritt, 1985; Zauberman, 1976). Hirst *et al* (1994) reported that the chance of recurrence following primary or recurrent pterygium excision was 97% within 12 months of the removal in their experience, and that there was a trend for recurrences to happen more quickly with each subsequent removal regardless of the type of surgery used. After conjunctival autografting recurrences of 5% to 21% have been reported (Kenyon *et al*, 1985; Lewallen, 1989), while use of β-irradiation has produced recurrence rates of 0.5% to 33% after primary pterygium excision (Haik *et al*, 1962; MacKenzie *et al*, 1991). Postoperative use of mitomycin-C with varying regimens and concentrations ranging form 0.02% to 1.0% has reportedly reduced the rate of recurrence in primary pterygium to 2% to 16% (Singh *et al*, 1988; Singh *et al*, 1989; Hayasaka *et al*, 1988; Hayasaka *et al*, 1989). Both mitomycin-C and β-irradiation, however, have been reported to produce severe postoperative complications even at low doses.

Reported complications following β-irradiation include scleral necrosis and thinning, ulceration, and endophthalmitis (Cameron, 1972; MacKenzie *et al*, 1991, Farrell and Smith, 1989; Tarr and Constable, 1980). Rubenfeld *et al* (1992) have reported scleral thinning, corneal edema, secondary glaucoma, corneal perforation, iritis, and cataract formation following treatment with 0.4% mitomycin-C for several weeks following pterygium removal. The current adjunctive treatments (i.e., mitomycin-C and β-irradiation) to prevent pterygium recurrence, therefore, are effective, but not completely so, and have the potential to produce serious postoperative complications. In comparison, topical treatment with AL-3789 produced significant reduction in the recurrence rate following malignant pterygium excision without producing significant side effects attributable to drug treatment.

Treatment with AL-3789 was not completely effective in preventing pterygium recurrence in this study. However, published results of recurrence rates as high as 97% for malignant pterygium (Hirst *et al*, 1994) indicate that AL-3789 has potential as a concomitant pharmacological treatment in this difficult disease, and should be investigated in other ocular neovascular diseases as well.

Future Studies

The mechanism of action of AL-3789 and AL-4940 should be thoroughly investigated based on the positive results of the "proof-of-principle" clinical trial in pterygium. The proteins identified by 2D PAGE to be changed in these studies by

AL-4940 treatment require identification. This may be accomplished by sequencing of tryptic or CNBr (cyanogen bromide) digests of peptides of interest isolated from the polyacrylamide gels (Vandekerhove *et al*, 1990). This method, in combination with protein databank searching, would suggest the identity of exact or homologous proteins. The identity could then be verified by antibody-mediated biochemical methods such as immunoprecipitation or immunoblotting (Western Blotting). Alternatively, vascular endothelial cell gene expression altered by AL-3789 could be identified using techniques such as differential display reverse-transcriptase PCR (Liang and Pardee, 1992). As stated previously, the affected proteins/genes might be transcription factors, differentially expressed receptors, modified receptors, and so on. This information would be of great use in determining the primary mechanism of action of AL-3789.

In addition, a clinical trial to determine the effect of AL-3789 on neovascular processes in retinal diseases such as exudative age-related macular degeneration or proliferative diabetic retinopathy is mandated. There are no pharmacological agents presently available to abolish or slow these visually devastating neovascular diseases of the retina. A potential study worthy of consideration is administration of AL-3789 by 10% suspension subconjunctival or sub-Tenon's injection to patients who develop subfoveal choroidal neovascular membranes and refuse laser surgery. Progress of neovascularization in these patients can be visualized by fluorescein angiography or indocyanine green videoangiography (Guyer *et al*, 1996), and the

advancing membrane area quantitated after treatment by computer image techniques such as the one described in Chapter IV.

Summary

Topical ocular treatment with AL-3789 is comfortable, safe and effective in decreasing the neovascular response after malignant pterygium excision. The mechanism of action of this angiostatic steroid includes inhibition of proteolytic enzyme production by human microvascular endothelial cells, specifically, urokinase and stromelysin-1, as well as inhibition of proliferation of these cells. AL-3789 significantly affects protein expression in human microvascular and umbilical cord-derived vascular endothelial cells.

AL-3789 is a pharmacological agent capable of ameliorating pathological neovascular processes in human disease and deserves further investigation to determine its full therapeutic utility.

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