Potential Inhibitory Effect of LASSBio-596, a New Thalidomide Hybrid, on Inflammatory Corneal Angiogenesis in Rabbits

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Key Words

Thalidomide hybrid • Inflammatory corneal angiogenesis • Rabbits

Abstract

Aims: Evaluate the effect of LASSBio-596, structurally designed as a new hybrid of thalidomide, on inflammatory corneal angiogenesis. **Methods:** Eighteen rabbits were submitted to an alkaline cauterization in the right cornea. The animals were randomly allocated to three groups: vehicle, dexamethasone and LASSBio-596. Drugs were administered by eyedrops 3 times a day for 21 days. Evaluations were performed on days 3, 6, 9, 12, 15, 18 and 21 after cauterization. At these time points, digital images of the cornea were captured in a standard fashion. The angiogenic response was measured using software that was developed specifically for this purpose. It calculated the following parameters: neovascularization area (NA), total vascular length (TVL) and blood vessel number (BVN). **Results:** It was observed that dexamethasone significantly decreased NA, TVL and BVN during all assessments. From the NA the angiogenesis rate (AR) was calculated in each group. Therefore, dexamethasone completely inhibited the inflamma-

tory corneal angiogenesis with an AR of -0.001 ± 0.006 mm²/day, which was significantly lower ($p < 0.001$) than that observed after treatment with vehicle (0.078 ± 0.024 mm²/day) and LASSBio-596 (0.054 ± 0.012 mm²/day). Although LASSBio-596 reduced angiogenesis in relation to vehicle, according to NA, TVL and BVN values, this difference was not statistically significant. However, it was found that the AR as measured in the LASSBio-596 group was significantly lower ($p < 0.05$) than that seen in control animals, indicating a potential antiangiogenic effect. **Conclusion:** We conclude that topical application of LASSBio-596 at 1.0% has a potential inhibitory effect on inflammatory corneal angiogenesis in rabbits.

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Introduction

Angiogenesis or neovascularization is the development of new capillaries from preexisting blood vessels [1, 2]. Several regulatory mechanisms of angiogenesis control both physiological processes and the spread of diseases [3]. Abnormal angiogenesis contributes to the pathogenesis of angiogenesis-dependent diseases such

as inflammatory disorders [4]. During the inflammatory process, the release of chemokines recruits leukocytes that produce angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and tumor necrosis factor α (TNF- α), among others. These growth factors attract endothelial cells, smooth muscle cells, fibroblasts, leukocytes and platelets, triggering the process of neovascularization [5].

Corneal neovascularization from the limbic vascular plexus is a common feature of several diseases of the cornea, either of infectious, traumatic, degenerative or inflammatory origin [6–8]. The main stimuli for neovascularization are inflammation, hypoxia and limbic stem cell destruction [9]. There is exacerbation of the angiogenic process in various other diseases, namely diabetic retinopathy, macular degeneration and solid tumors [4–9]. For the treatment of these diseases, several drugs with antiangiogenic properties were identified, and their mechanisms of action were studied. Thalidomide is a potent inhibitor of angiogenesis caused by TNF- α and FGF-2. However, due to its teratogenic effect in humans, its use is restricted in medical practice, engendering interest in the discovery of similar potent antiangiogenic analogs without this adverse effect [4].

LASSBio-596, structurally designed as a new hybrid of thalidomide and arylsulfonamide derivatives, is an agent that displays important anti-inflammatory and immunomodulatory properties [10–12]. The compound is obtained through the hydrolysis of the phthalimide ring, which is present in the structure of prototype LASSBio-468. But LASSBio-596 is considered safer than thalidomide because of the absence of phthalimide and glutarimide subunits, which are present in thalidomide and possibly responsible for its teratogenic profile (fig. 1). Modulation of the inflammatory process, as previously described, inhibits the recruitment of leukocytes induced by lipopolysaccharide, an effect that is accompanied by reduced levels of TNF- α [11]. The aim of this work is to evaluate the effect of the drug LASSBio-596 in a model of inflammatory corneal angiogenesis.

Materials and Methods

Animals

The study was conducted in accordance with federal and institutional guidelines approved by the Ethical Committee in Animal Research at the Federal University of Ceara (UFC) and with adherence to the tenets of the NIH Statement for the Use of Animals in Research. Male, white New Zealand rabbits aged between

Fig. 1. Chemical structure of LASSBio-596.

90 and 120 days and weighing from 1,800 to 2,700 g were used. Rabbits were from the Department of Animal Science of the UFC and had previously been examined to exclude the possibility of external eye diseases. Rabbits were housed in cages designed specifically for their species. They remained in an air-conditioned environment, with appropriate conditions of temperature, humidity and lighting, with cycles of light and dark, alternating every 12 h. Water and food were offered ad libitum. The latter consisted of a balanced diet for rabbits (Fri-Rabbit®). The animals were subjected to an adjustment period of at least 7 days prior to the procedures.

Experimental Eyedrops

LASSBio-596 was synthesized by the Laboratory of Evaluation and Synthesis of Bioactive Substances (LASSBio®) at the Federal University of Rio de Janeiro. In this study, LASSBio-596 had to be administered in an aqueous solution for efficacy as an eyedrop. The drug solubilization process was performed in the Department of Chemistry at the UFC. At first, a 1.0 wt% (w/v) solution of LASSBio-596, made with benzalkonium chloride (0.1% w/v), was subjected to slow magnetic stirring for 5 min. To this solution was added the block copolymer of ethylene oxide and ϵ -caprolactone, E114CL20 (where E represents an oxyethylene unit and CL ϵ -caprolactone; 1.0% w/v) and Tween 80 (0.02% w/v). The homogenization was conducted on an Ultrasonic-Homogenizer Sonifier II W-450 (70 W, 0 cycle, Ms73 tip, 60 s) with the sample cooled in an ice bath. The sample was finally filtered through a 0.45- μ m membrane filter (Millex-HA Filter Units, Millipore™). To control for any confounding effect of the LASSBio-596 vehicle on ocular shedding, a vehicle eyedrop (a solution of benzalkonium chloride 0.1% added to the block copolymer E114CL20 1.0% and Tween 80 0.02%) served as control. Dexamethasone eyedrop 40 mg/ml (4.0%) was prepared in distilled water and added to the solution of benzalkonium chloride 0.1%.

Inflammatory Corneal Angiogenesis Assay

We used the model of inflammatory corneal angiogenesis induced by punctual alkaline cauterization, by which neovascularization is induced by an inflammatory response, i.e. a neovascular nonspecific stimulus [13]. Prior to cauterization and all subsequent assessments, the animals were anesthetized with a combination of 12 mg/kg ketamine hydrochloride and 4 mg/kg xylazine hydrochloride i.m., complemented by 2 drops of 0.5% proxymetacaine hydrochloride applied topically to the conjunctival sac. The anesthetized animal was placed in a Styrofoam tray in left lateral

decubitus with the dorsum toward the examiner. Subsequently the eyelids were held apart with a blepharostat, exposing almost the entire corneal surface and the upper limbal area. The rabbits were submitted to a punctual cauterization on the top periphery of the right cornea with the aid of a microscope, using a magnification of $\times 16$. For this, we used a circular piece of filter paper (3 mm diameter), previously soaked in a solution of sodium hydroxide (NaOH) 1 M for 30 s (excess solution was blotted away with gauze). The filter paper was left for 2 min at approximately 1 mm from the conjunctival limbal tissue in the 12-o'clock position near the insertion of the upper rectus muscle. The eye was subsequently rinsed with 5 ml sodium chloride at 0.9% solution to remove excess NaOH. The technique produced a 3.5-mm-wide, homogeneous and circular cauterization site with well-defined borders. The animals were returned to their cages only after complete emergence from anesthesia.

Experimental Design

The experiments were performed at the Laboratory of Experimental Surgery at the UFC. The rabbits were randomly allocated to three treatment groups: a vehicle group, consisting of 6 rabbits that were treated with 1 drop (40 μ l) of the vehicle eyedrop, instilled in the conjunctival sac 3 times per day; a dexamethasone group, consisting of 6 rabbits that were treated with topical instillation of 1 drop (40 μ l) of dexamethasone 4.0% eyedrop 3 times per day; a LASSBio-596 group, composed of 6 rabbits that were treated with 1 drop (40 μ l) of the drug LASSBio-596 (diluted in solution at a concentration of 1.0%) in the conjunctival sac 3 times per day. Treatments were initiated on the day of cauterization (day zero) and continued for 21 days.

Image Acquisition and Processing

The animals were evaluated on days 3, 6, 9, 12, 15, 18 and 21 following cauterization. During exposure the cornea was kept hydrated with frequent instillations of liquid ocular gel based on polyacrylic acid, a lacrimal fluid substitute. Images of the cornea including the area of angiogenesis were then acquired in standard fashion with a video camera coupled to a surgical microscope that transferred data to a microcomputer. The microscope axis was positioned perpendicularly to the tangent of the corneal periphery to minimize spatial distortion. In addition, a green filter was used to increase the contrast of the blood vessels. Images were acquired at $\times 25$ magnification and saved as Windows® bitmaps measuring 320 $\times$ 240 pixels.

The angiogenesis response was measured by the Angiogenesis Quantifier System, a software developed specifically for this purpose [13]. The system processed the digital images of the cornea containing the area of angiogenesis and determined the following parameters: neovascularization area (NA), total vessel length (TVL) and blood vessel number (BVN) (fig. 2). From the NA, it was possible to calculate the angiogenesis rate (AR) and the inhibitory effect (IE) of each treatment in relation to vehicle at day 21 using the following equations:

$$AR = \frac{NA(day21) - NA(day3)}{18}$$

$$IE(drug) = \frac{NA(vehicle) - NA(drug)}{NA(vehicle)} \times 100$$

Evaluation of these parameters provided spatial and temporal tracking of the angiogenic response *in vivo*. Then a quantitative analysis of the inhibitory effect of each substance was performed.

Statistical Analysis

Quantitative variables were initially examined by the Kolmogorov-Smirnov test to verify normality of distribution. Mean and standard deviation were then calculated as descriptive statistics. Parametric tests were used for data analysis. To compare the three treatment groups, we used analysis of variance associated with Tukey's multiple comparison test. The unpaired t test was used to compare two variables that were not matched. For matched variables, comparisons between paired variables were performed using the t test. In all cases, the level of statistical significance was set at <0.05 . For data analysis, as well as for preparation of graphics, we used the statistical software Graph Pad Prism® version 5.00 for Windows® (Graph Pad Software, San Diego, Calif., USA, 2007).

Results

The evolution of the angiogenesis parameters (NA, TVL and BVN) in the vehicle group reveals a biphasic pattern characterized by a period of intense vascular growth up to day 12, followed by a period of stability up to day 21 (fig. 3, 4). The maximum NA, TVL and BVN values recorded were 1.998 ± 0.622 mm² (day 21), 31.956 ± 10.535 mm (day 21) and 205.333 ± 54.503 vessels (day 18).

In the dexamethasone group, there was a significant reduction ($p < 0.05$) compared with vehicle on day 3 in NA, TVL and BVN. During all subsequent assessments, the parameters for dexamethasone decreased further ($p < 0.001$) in comparison with the vehicle group (fig. 3, 4). The maximum NA, TVL and BVN values were 0.229 ± 0.089 mm² (day 18), 3.618 ± 1.367 mm (day 18) and 26.000 ± 20.396 vessels (day 9).

With regard to the antiangiogenic effect of the drug in this study, LASSBio-596 elicited the same trend as vehicle during the first 6 days, which was true for all the parameters evaluated. From the sixth day, LASSBio-596 reduced angiogenesis progression as compared with vehicle. This trend was maintained until the end of the experiment. However, the observed reduction was not statistically significant. All parameters remained constant in the LASSBio-596 group from the sixth day onward (fig. 3, 4). The maximum NA, TVL and BVN values recorded were 1.680 ± 0.322 mm² (day 18), 27.202 ± 6.216 mm (day 15) and 178.667 ± 44.889 vessels (day 15), respectively.

The vascular growth rate was measured throughout the study as AR (fig. 5). In the dexamethasone group, we

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Fig. 3. Angiogenic response observed in experimental groups: vehicle, dexamethasone and LASSBio-596. Images acquired from days 3, 9, 15 and 21 after cauterization. Magnification $\times 25$.

observed a complete inhibition of the inflammatory corneal angiogenesis with a negative AR ($-0.001 \pm 0.006 \text{ mm}^2/\text{day}$), which was significantly lower ($p < 0.001$) than vehicle ($0.078 \pm 0.024 \text{ mm}^2/\text{day}$) and LASSBio-596 ($0.054 \pm 0.012 \text{ mm}^2/\text{day}$).

Moreover, the AR measured in the LASSBio-596 group ($0.054 \pm 0.012 \text{ mm}^2/\text{day}$) was significantly lower ($p < 0.05$) than that seen in the vehicle group ($0.078 \pm 0.024 \text{ mm}^2/\text{day}$), suggesting a potential antiangiogenic effect, mainly during the second half of the angiogenic process (fig. 5).

It is noteworthy that no adverse effects were observed in the animal groups evaluated.

Fig. 2. Measurement of the angiogenesis response by the Angiogenesis Quantifier System (SQAN). First of all, it determined the region of interest, then it calculated the parameters: neovascularization area (NA), total vessel length (TVL) and blood vessel number (BVN).

Discussion

Our main purpose was not to investigate the cellular and molecular events involved in corneal neovascularization but to study the kinetics of the angiogenic process. Thus, according to the angiogenesis measured, we can infer that the temporal progression of the angiogenesis response followed a biphasic pattern, correlating with the phases of proliferation and maturation observed in a model of inflammatory neovascularization in the murine cornea [14]. The first phase is characterized by intense vascular proliferation. Vascular density is high, there are numerous vascular buds, and the rate of angiogenesis reaches maximum values. The second phase is characterized by vascular maturation. The new vessels lengthen with reduced branching and the rate of angiogenesis is stabilized. Therefore, this is a better representation of the phenomenon under study.

Several studies have demonstrated the antiangiogenic activity of glucocorticoids in models of corneal angiogen-

Fig. 4. Quantification of angiogenic response in the vehicle, dexamethasone and LASSBio-596 groups, based on NA, BVN and TVL parameters. The data, expressed in millimeters, correspond to the mean and standard deviation of measurements made on 6 animals in each group on days 3, 6, 9, 12, 15, 18 and 21 after cauterization. To compare the three treatment groups on the same day, we used the analysis of variance associated with Tukey's multiple comparison test, which showed differences between paired groups. * $p < 0.05$, ** $p < 0.01$.

esis induced by both inflammation and by specific factors. In fact, glucocorticoids and nonsteroidal anti-inflammatory drugs constitute the main pharmacological approaches to the treatment of corneal neovascularization [15]. Dexamethasone is widely used in the treatment of corneal inflammation. Several authors had previously showed the antiangiogenic effect of dexamethasone, including on cauterization-induced corneal neovascularization [16–19]. In 1987, Folkman and Ingber [16] report-

Fig. 5. AR in square millimeters per day, for the period between days 3 and 21, calculated for the vehicle, dexamethasone and LASSBio-596 groups. The data represent the mean and standard deviation of measurements made on 6 animals in each group. The analysis of variance was used to compare the three treatment groups, supplemented by Tukey's multiple comparison test, which showed differences between paired groups. * $p < 0.05$, ** $p < 0.01$.

ed the antiangiogenic function of a class of steroids, including dexamethasone, which they named the angio-static steroids. However, because of its side effects, including cataract and intraocular hypertension, steroid therapy in the management of some corneal diseases remains controversial. Dexamethasone blocks the transcription of inflammatory proteins by prohibiting the activity of the nuclear factor κ B (NF- κ B) [18]. NF- κ B plays an important role in interleukin (IL)-1 β -related inflammatory diseases, including various corneal diseases. Nakao et al. [19] showed that IL-1 β -induced NF- κ B activation is inhibited by dexamethasone and that a selective NF- κ B inhibitor diminished inflammatory corneal angiogenesis. In our study, dexamethasone eyedrops (4.0%) were administered topically 3 times a day and fully inhibited the angiogenic response throughout the experiment. In the dexamethasone group, NA, TVL and BVN were significantly ($p < 0.05$) reduced compared with vehicle on day 3. In all subsequent evaluations, there was a further decrease ($p < 0.001$) compared with vehicle (fig. 3, 4). Thus, dexamethasone completely inhibited the inflammatory corneal angiogenesis, yielding a negative AR (-0.001 ± 0.006 mm²/day) that was significantly lower ($p < 0.001$) than that observed with vehicle (0.078 ± 0.024 mm²/day) and LASSBio-596 (0.054 ± 0.012 mm²/day; fig. 5). It should be emphasized that, although the inflammatory response has not been assessed from the histological

point of view, visual inspection of animals treated with dexamethasone revealed that signs of inflammation were much less intense in treated animals compared with those that received vehicle.

Thalidomide, a synthetic derivative of glutamic acid, is a drug with immunomodulatory, anti-inflammatory, antiangiogenic and antitumoral properties [20–24]. In addition to its inhibitory effect on corneal neovascularization induced by VEGF and FGF-2, the bioactivity of thalidomide is related to its ability to inhibit the production of TNF- α , a proangiogenesis and proinflammatory cytokine produced by monocytes/macrophages [18–20]. It is likely that the activity of thalidomide is in part controlled by NF- κ B. Notably, TNF- α is one of the inducers of NF- κ B activation. NF- κ B regulates the expression of several genes involved in cell proliferation, inflammation, angiogenesis and the inhibition of apoptosis. Two of the genes regulated by activation of NF- κ B induced by TNF- α express the proangiogenic factors VEGF and IL-8. Thus, it was postulated that thalidomide would block the activation of NF- κ B induced by TNF- α . Its antiangiogenic effect would result from inhibition of VEGF, IL-8 and other angiogenic factors regulated by NF- κ B. Other proangiogenic factors are also inhibited by thalidomide – for example IL-6, a potent growth factor for malignant cells [20].

Initially introduced as a sedative, thalidomide was subsequently withdrawn from the market due to its teratogenicity, causing malformation of limbs in newborns, a condition denoted as phocomelia. Years later, D'Amato et al. [24] postulated that this malformation resulted from the inhibition of vascular growth in the developing buds of the members, during the embryonic period. With the discovery of its potent anti-inflammatory and immunomodulatory effects, thalidomide is currently used in certain pathologies with rigorous restrictions and control. So the search for new thalidomide analogs that combine high-power antiangiogenic and anti-inflammatory effects without severer adverse effects is an important goal.

Recently, LASSBio-596 was reported as a new anti-inflammatory and antifibrotic drug candidate, originally designed to mimic thalidomide and arylsulfonamide hybrids. The absence in the structure of LASSBio-596 of the two subunits that compose the thalidomide structure (i.e. phthalimide and glutarimide rings) yields a drug that is free of teratogenic effects [10].

Previously, in a model of pulmonary inflammation, LASSBio-596 demonstrated potent inhibitory activity in the recruitment of leukocytes induced by lipopolysaccharide, correlating with its effect in attenuating the levels of

TNF- α [11]. Demonstrating potent inhibitory activity in the recruitment of leukocytes induced by lipopolysaccharide, LASSBio-596 also attenuated the levels of TNF- α during pulmonary inflammation, which aroused our interest in studying its role in inflammatory angiogenesis. We hypothesized that the drug exerted an antiangiogenic effect. Moreover, LASSBio-596 was reported to effectively prevent mechanical and morphometric changes in the lung, and it was observed to block fibroproliferation in a BALB/c mouse model of asthma [12]. It was also reported that LASSBio-596 prevented lung and hepatic inflammation and completely blocked pulmonary functional and morphological changes induced by microcystins [25, 26]. Because of this important anti-inflammatory action, we begin the study of LASSBio-596 in inflammatory angiogenesis, believing in its potential antiangiogenic effect.

In this study, the values of NA, TVL and BVN quantifiers in the LASSBio-596 group were similar to those in the vehicle group during the first 6 days of the experiment. From the sixth day, LASSBio-596 reduced proliferation of new vessels compared with vehicle; this effect was maintained until the end of the experiment, although the difference was not significant. It was also observed that all parameters remained constant in the LASSBio-596 group from the sixth day (fig. 3, 4). By visual inspection, the decrease on vascular thickness was noteworthy in the LASSBio-596 group compared with the vehicle group (fig. 3). However, analyzing the AR in the LASSBio-596 group (0.054 ± 0.012 mm²/day), we observed a significant reduction ($p < 0.05$) compared with the vehicle group (0.078 ± 0.024 mm²/day). These findings demonstrate the drug's potential inhibitory effect on angiogenesis, mainly during the second half of the angiogenic process (fig. 5).

In evaluating the effectiveness of the final treatments, it was observed that dexamethasone had an inhibitory effect of 90.81%, calculated on the basis of data from the vehicle group on day 21. These findings confirmed the drug's potent antiangiogenic effect. Regarding LASSBio-596, there was an inhibitory effect of 20.74% compared with the vehicle group. Despite the lower inhibitory effect of LASSBio-596 compared with dexamethasone, we can expect an inhibitory effect because it is a thalidomide hybrid, which probably exhibits analog properties. D'Amato et al. [24] investigated the inhibitory effect of thalidomide and its analogs in rabbits using a model based on angiogenesis induced by pellets containing FGF-2. Their results showed a significant inhibitory effect of thalidomide, despite the fact that the nonterato-

genic analog suplidimide has not presented any antiangiogenic activity. Furthermore, Joussen et al. [27] correlated the antiangiogenic effect of thalidomide and its derivatives suplidimide and EM12 in a model of corneal neovascularization induced by VEGF and basic FGF with its potent teratogenic effects. They concluded by stating that further emphasis should be given to the investigation of thalidomide analogs that topically have the potential antiangiogenic effect. According to our results, we observed the potential inhibitor effect of LASSBio-596 on neovascularization, verifying that its AR was significantly lower than that of the vehicle group ($p < 0.05$).

Looking retrospectively into the possible limitations of the study, we report the difficulty during the initial dilution of the drug under study, LASSBio-596. It was difficult to obtain a collyrium, necessary to maximize the drug's effect by topical application in the cornea. Furthermore, we did not investigate treatment with other concentrations to establish dose-response curves. We believe that the next studies might reveal promising results, including unmasking LASSBio-596's molecular mechanism of action in angiogenesis.

According to the presented data, we concluded that LASSBio-596, a new hybrid of thalidomide, had a potential inhibitory effect, as determined by topical application at 1.0%, on inflammatory corneal angiogenesis in rabbits. At this time, we present initial results demonstrating the potential inhibitory effect of LASSBio-596 on inflammatory corneal angiogenesis. We would like to express our interest in continuing to study the effect of this new thalidomide hybrid in other experimental models involving neovascularization, allowing a greater understanding of its effect on the process of angiogenesis. Additionally, we would like to contribute to the science related to the discovery of new drugs that are capable of assisting in the fight against angiogenesis-dependent diseases.

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