ARTÍCULO

Silencing Beta2-Adrenergic Receptors Reduces Intraocular Pressure: A New Approach for Glaucoma Therapy

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Trabajo premiado con el Premio de la Real Academia Nacional de Farmacia de su Concurso Científico 2011.
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ABSTRACT

The search for new treatments for ocular hypertension and glaucoma, due to the side effects commercial products present, is inviting to look for new strategies apart from the available ones. In this sense the interference RNA technique (RNAi), also known as siRNA to selectively silence beta2 adrenergic receptors has been investigated. In this sense it has been obtained a reduction in intraocular pressure (IOP), the main factor triggering glaucoma, of 30 ± 5 %, compared to a control (scrambled siRNA). The results were in terms of IOP reduction similar to that obtained with commercial compounds but the duration of the siRNA for the beta2 adrenoceptor lasted for almost 5 days, compared to the average of 8 hours in the case of the other commercial compounds. No apparent side effects were observed in the ocular structures. In summary, the use of siRNA against the beta2 adrenergic receptors open a new perspective for the treatment of glaucoma.

Keywords: Glaucoma; Intraocular Pressure; Adrenergic Receptors; siR.

RESUMEN

Los Receptores con Silenciador Beta2-Adrenérgicos, reducen la presión intraocular: Un nuevo acercamiento a la terapia del Glaucoma

La búsqueda de nuevos tratamientos para la hipertensión ocular y el glaucoma, dado los efectos secundarios que presentan los fármacos actuales, sugieren el desarrollo de estrategias diferentes a las actualmente disponibles. En este sentido se ha puesto a punto la tecnología de los RNA de interferencia (RNAi) también llamados siRNA para silenciando selectivamente los receptores beta2 adrenérgicos tratar de obtener una reducción sustancial de la presión intraocular (PIO), principal

factor desencadenante del glaucoma. El empleo de un siRNA de origen comercial para el receptor beta2 ha producido una reducción sustancial de la PIO de 30 ± 5 %, comparado con el control, un siRNA sin sentido (scramble). Estos resultados fueron en magnitud semejantes al de los fármacos comerciales salvo que la duración del siRNA para el receptor beta2 adrenérgico duró casi 5 días en comparación de las 8 horas que suelen durar los fármacos comerciales. Los estudios en relación con los efectos secundarios no mostraron ninguna modificación en las estructuras oculares. En resumen, el empleo de los siRNA frente a los receptores beta2 adrenérgicos presenta muy buenas perspectivas como aproximación novedosa para el tratamiento del glaucoma.

Palabras clave: Glaucoma; Presión intraocular; Receptores adrenérgicos; siRNA.

1. INTRODUCTION

The aqueous humour is a transparent nutritional fluid that provides the nutrients to the inner avascular structures of the eye (1). This fluid also gives an internal pressure, termed intraocular pressure or IOP that grants the right shape of the ocular globe. The maintenance of an adequate balance between the formation and the drainage of the aqueous humour is a guarantee of ocular health (2). When changes in the aqueous humour dynamics occur concomitant alterations happen that may lead in pathological states. One problem derived from the accumulation of the aqueous humour in the eye is the degeneration of the optic nerve as a consequence of the increase in the intraocular pressure (3). This pathophysiological condition is generally termed as glaucoma and is in most of the cases due to problems in the drainage of the aqueous humour rather than a problem in its synthesis.

The control of the aqueous humour production occurs in the ciliary body and it is regulated by the sympathetic nervous system (4). Noradrenaline released from the sympathetic terminals stimulate adrenergic receptors that facilitate the production of the aqueous humour. This fact has been taken by the pharmaceutical companies to develop a series of antagonists of mainly β2-adrenoceptors although α1 agonists seem to help to reduce aqueous humour synthesis (4).

Recently, a new biochemical approach to avoid the expression of proteins has been discovered: Small interference RNA. These are double stranded RNA sequences that after being processed in the cytoplasm they destroy the mRNA to which it has been designed avoiding the synthesis of the corresponding protein (5).

In the present work, the use of siRNA, designed to silence β2-adrenoceptors has been used in order to reduce IOP and therefore consider it as a possible strategy for the treatment of ocular hypertension and glaucoma.

2. MATERIAL AND METHODS

2.1. Animals

Male New Zealand rabbits were 6-8 weeks of age at the time of the experiment. The siRNAs were topically applied in one eye, the contralateral receiving the same volume of saline. Each of the treated eyes received an amount of 250 μg of the siRNA dissolved in 40 μL of 0.9 % NaCl. The instillation was performed in a blind fashion therefore no indication of the saline. The siRNA were applied in a blind fashion Rabbit eyes were treated with 1,1 mg HPLC-purified siRNAs, applied. The siRNAs were applied in one single eye in 265 μg 0.9% NaCl drops (40 μL volume) on four consecutive days; the contralateral eye was instilled with saline solution (0.9% NaCl). Experiments were performed using a single blind design: no visible indication was given to the experimenter as to the applied solution (agent or vehicle). IOP was measured on ten days and, afterwards, animals were killed and both eyes were collected.

2.2. Intraocular pressure (IOP) measurements in rabbits

IOP was measured by means of a Tono-Pen XL contact tonometer (Mentor Massachusetts Inc., Norwell, MA). Topical anesthesia (Colircusi, Laboratorios Cusi, Spain: 0.1 mg/ml tetracaine plus 0.4 mg/ml oxybuprocaine in 0.9% saline, diluted 1:3 in 0.9% saline) was applied (10 μL) to the cornea before each measurement to avoid animal discomfort.

All the siRNA experiments were performed and measured at the same time. In each series of experiments, IOP was measured, from the first day, three times a day every four hours and, once the IOP started to change the measurement pattern moved once every two hours. The siRNA assays carried out were purchased from Santa Cruz (USA) (n=8), and siRNA scramble was also from Santa Cruz (USA)(n=4).

In the experiments performed with commercially available drugs (40 μL of commercial Xalatan, Trusopt and Timoftol), IOP levels were measured every hour for a total of 8 hours, following the same protocol as described before.

2.3. Cell treatments and tissue sections and histology

Ciliary body non-pigmented epithelial cells were used in order to see the silencing of the beta2 adrenergic receptors. These cells were kindly provided by Dr. Coca-Prados (University of Yale), and were treated with an antibody against the beta2 adrenergic receptor (Ab40834 from abcam).

After several washes in PBS and pre-incubation in PBS with 3% blocking serum for 1 h, cells were incubated with the primary goat polyclonal anti-beta2 (Ab40834 from abcam) diluted 1:100. After this incubation, cells were washed with PBS and incubated in a dark chamber with the secondary antibody donkey anti-goat IgG-FITC at 1:150 (Jackson ImmunoResearch, PA, USA) for 1 h to 37ºC. Then, the samples were observed under a confocal microscope (Axiovert 200; Carl Zeiss Meditec GmbH, Jena, Germany), equipped with a PASCAL confocal module (LSM 5; Zeiss). All images were managed with the accompanying PASCAL software.

Eyes were fixed in 4% paraformaldehyde in PBS 1X overnight at 4ºC. Eyes were washed twice in PBS 1X for 15 min, and immersed in 11% sucrose overnight at 4ºC. The following day the eyes were immersed in 33% sucrose prepared in PBS 1X. Finally, the eyes were embedded in OCT (Leica). 5-10 μm sections were made using a LEICA CM 1850 cryotome, in 3-aminopropiltrietoxisilane coated glasses.

Haematoxylin-eosin staining was performed using the following protocol: Sections were fixed and washed in water for 5 minutes. Haematoxylin (Carazzi´s Heamtoxylin, Panreac, Barcelona, Spain) was incubated for 1 minute and sections were washed in water for 10 minutes. Eosin (Eosin Bluish, Panreac) contrast was made and finally sections were washed serially with 96º and 100º ethanol. Finally, xilol treatment was performed to dehydrate the sections previous to their observation by means of the microscope.

Microscopy was performed with a confocal Zeiss Axiovert 200M microscope (Zeiss, Jena, Germany), equipped with a LSM 5 Pascal confocal module. All images were managed with the LSM 5 Pascal software.

2.4. Side effects

The assessment of the ocular tolerance of the RNAi sequences, were followed several aspects and parameters from the tear film, ocular anexa and ocular surface. The analysis were performed and graded before and after the topical application of siRNA with the Efron grading scales. A Topcon SL-8Z slit lamp and a Topcon SP-2000P specular microscope (Topcon Spain, Madrid, Spain), as well as a digital camera and a “de visu” exam, were used for every experiment. Slit-lamp images were captured under 10X augments, a diffuser filter and a medium degree of illumination. The images were recorded and analysed by IMAGEnet 2000 system software (Topcon).

2.5. Data analysis

All data are presented as the mean+s.e.m. Significant differences were determined by two-tailed Student's t-tests. The plotting and fitting of all the data was carried out with the computer programme Prism GraphPad v.4.0 (Prism, USA).

3. RESULTS

3.1. Effect of beta2 adrenergic receptor silencing

To see whether the silencing of beta2 adrenergic receptors produced changes in the IOP, the rabbits treated with the siRNA against the beta2 receptor were monitored in their IOP for 7 days. As presented in figure 1, there was a robust reduction in IOP which started to be measurable 24 hours after the instillation of the siRNA and which was statistically significant for 5 days. The maximal reduction obtained after silencing the beta2 receptor was 30 ± 5 % compared to control (n=6). This and similar values were maintained for 1-2 days, with the IOP remaining low for three more days (with a mean of 30 % of reduction) and then returning slowly towards control values (Figure 1).

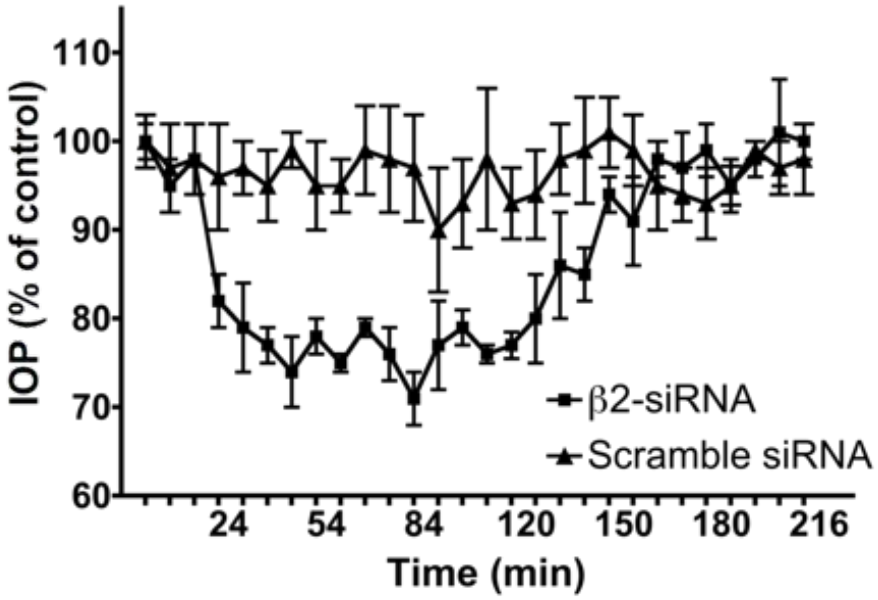

Figure 1.- Effect of a siRNA against the beta2 adrenergic receptor. Intraocular pressure was reduced 24 hours after the treatment started returning to normal values almost 5 days after the treatment started. The scrambled siRNA did not significantly modify intraocular pressure.

In order to further confirm the selectivity of the tested siRNA, a scramble siRNA was also assayed. As observed in Figure 1 after the treatment with the scramble siRNA, rabbit IOP did not significantly change during the time of the experiment.

3.2. Demonstration of the beta2 receptor silencing

When comparing the fluorescence level of non-treated cells with those treated with the siRNA designed for the beta2 adrenergic receptor, it was clear that the siRNA was able to significantly reduce the amount of receptors according to the clear fading of the fluorescence present in treated cells. As observed in Figure 2, there was a reduction in the presence of the beta2 receptors in the treated ciliary non-pigmented epithelial cells which reduction was 66.5 ± 7.2 % with respect to control (100 %; n=4).

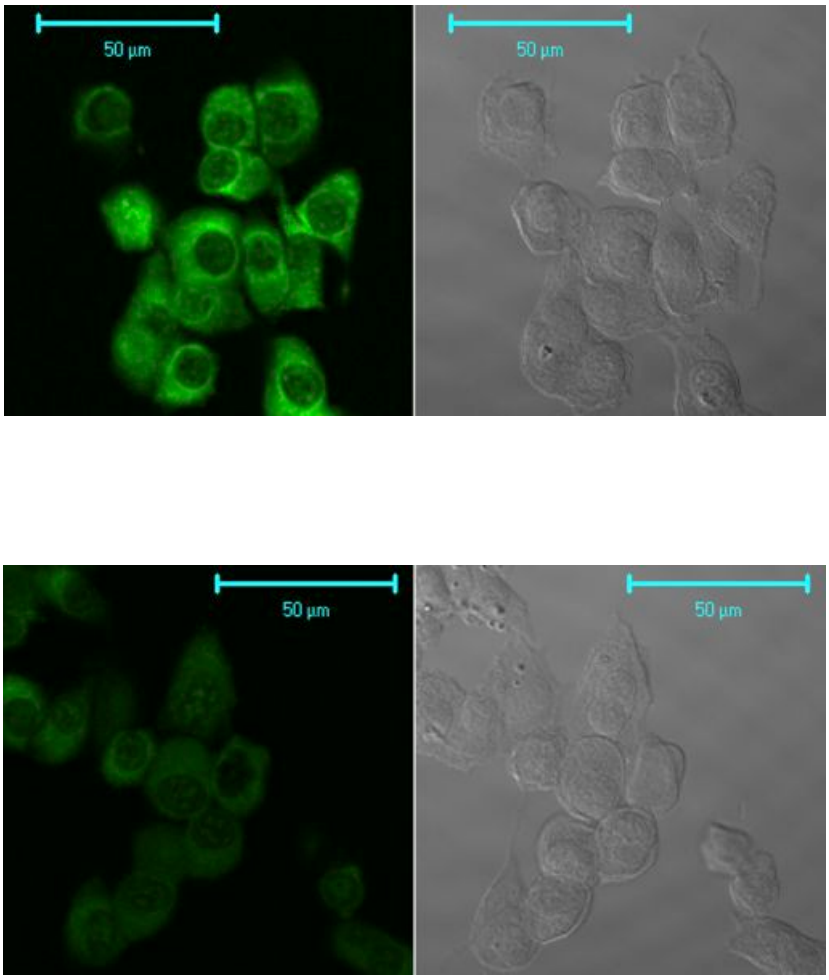

Figure 2.- Beta2 adrenergic immunostaining of ciliary non-pigmented epithelial cells before (upper panels) and after the treatment of the siRNA (lower panels). The receptor can be observed in green on the left panels while Nomarsky Interferencial Contrast can observe the same cells on the right.

3.3. Commercial drugs

The validation of our model was made using a battery of ophthalmic commercial drugs on New Zealand White rabbits. In these studies the activities on IOP of Trusopt (dorzolamide), Xalatan (latanoprost) and Timoftol (timolol) have

been assayed. A single drop (40 μL) has been instilled in rabbit eyes and IOP measures have been performed each hour for 8 hours.

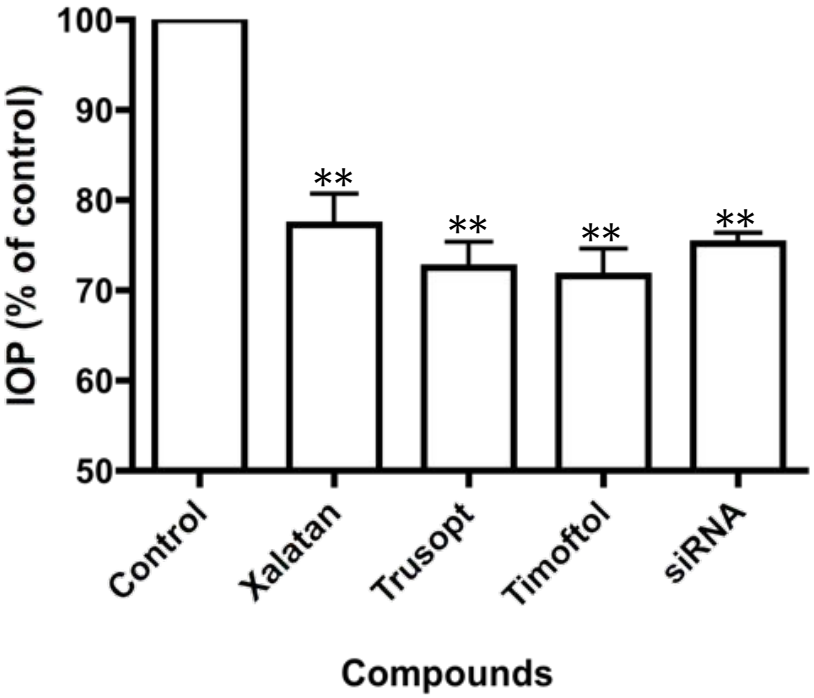

Figure 3.- Comparative effects of commercial compounds currently used for the treatment of glaucoma as well as the siRNA used along this research. (***p<0.001).

As described in Figure 3, any of those compounds produced a reduction in IOP of between 20-35% and the duration of this effect was about 6 hours. These experiments confirmed the suitability of this animal model, due to its ability and flexibility to regulate the IOP, to be used with RNAi applications.

3.4. Side effects

The integrity of the ocular structures after the siRNAs application was assessed observing the ciliary processes under microscopy by means of haematoxylin-eosin staining (Figure 4). No differences were observed between treated and non-treated animals.

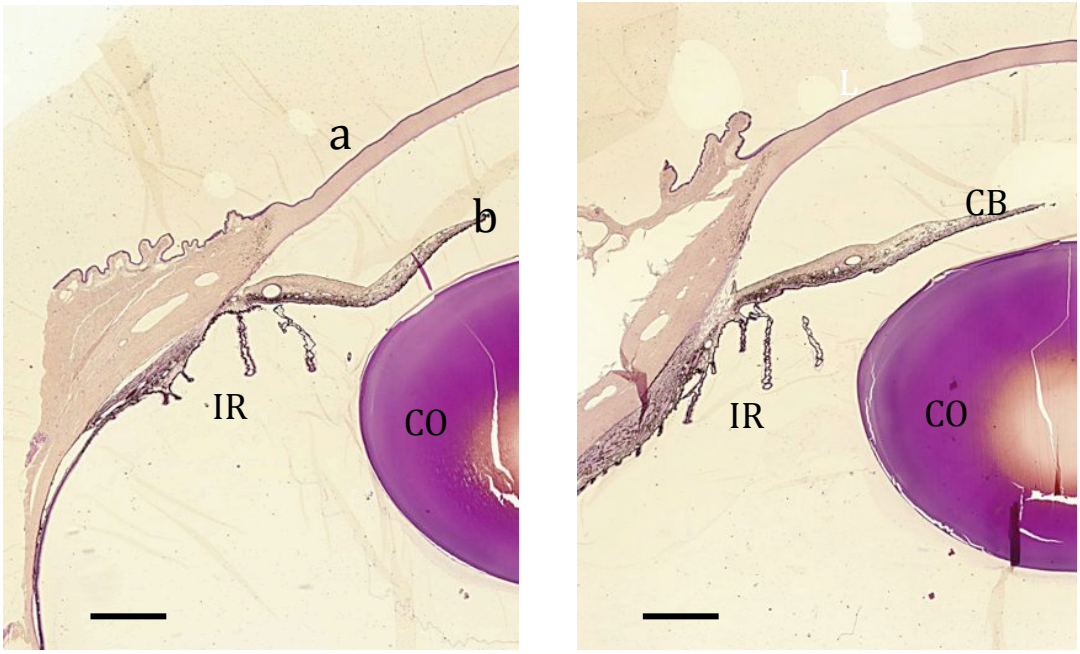

Figure 4.- Lack of changes in the ocular anatomy after the treatment with the siRNA against the beta2 adrenergic receptor. a) Control eye of an untreated animal showing the main parts of anterior and posterior chamber. b) siRNA treated eye obtained after completing the whole experimental time. CO: cornea, IR: iris, CB: ciliary body, L: lens.

4. DISCUSSION

There is an increasing interest in the use of RNAi therapeutics to treat a wide range of diseases; particularly those caused by the expression of mutant genes, including dominant-negative proteins, over-expressed genes leading to gain-of-functions effects or aberrant splicing isoforms. Gene silencing by RNA interference has been well demonstrated to efficiently knockdown targeted genes in vitro, but few reports describe similar effects in vivo (6). One of the first studies published that showed the downregulation of VEGF, a stimulatory factor in choroidal neovascularization (7), by a single intravitreal injection in the eye (8) is now being tested in patients with subfoveal choroidal neovacularization due to AMD in a Phase I Trial. siRNA (Sirna-027) injected into the vitreous cavity diffuses throughout the eye and is detectable for at least five days after administration (9).

The eye, as a relatively isolated compartment, seems as an ideal organ for RNAi treatment. RNAi mechanism of action allows targeting of specific sequences of single genes and local delivery should limit the exposure to the rest of the body, reducing the dose of siRNA to be used and minimizing side effects in other tissues. In this sense, treatment of eye diseases with this technology seems to present

some benefit with respect to other pathologies or strategies. In contrast, RNAi systemic delivery requires larger amounts of product and favours a broad delivery, sometimes affecting, non-specific tissues such as liver, lung and spleen.

In this way, one important point to be considered is to verify that the topical application of the siRNA effectively passes through the cornea to reach the targeted tissues. The eGFP mouse has been a useful model to validate the delivery of these oligonucleotides to the ciliary body. The siRNA silencing GFP are efficiently taken up by ocular tissues, reaching the ciliary body and other structures, and effectively silenced endogenous eGFP expression for at least four days, when consecutive administrations were applied. SiRNAs have been topically administered and it is presumed that it can be delivered by either transcellular transport across the corneal epithelium and stroma (9) or by the paracellular way (10). The corneal epithelium seems to be the barrier, which limits transcorneal diffusion although the existence of the paracellular way would be a mechanism that allows the transport of siRNA inside the eye. The spaces between cells, estimated to be about 60 Å, permit molecules to gain access to the anterior chamber (10). It is difficult to evaluate quantitatively how much of the applied siRNA reaches the ciliary processes, nevertheless and by means of the experiments performed with the eGFP mouse it has been possible to demonstrate that siRNA penetrates and that they can diminish the fluorescence of intracellular ocular structures. Therefore, these experiments allowed us to confirm a positive delivery in several ocular structures and to establish the proper protocol for each of them.

Once the in vivo ocular approach of RNAi technology was tried out, our main aim was to confirm this strategy as a new open angle glaucoma treatment. To this end, we focused our attention on beta2 adrenergic receptors, since their commercial inhibitors are effective in lowering IOP, the primary indication of glaucoma (11). These receptors are mainly located in the trabecular meshwork in the ciliary body.

The principal finding of this paper is the development of therapeutic siRNAs targeting the beta2 adrenergic receptors, which are involved in intraocular pressure. The use of siRNA may offer advantages and may be preferable to DNA-based strategies for in vivo applications, although the instability and the poor uptake into target tissues remain the most important handicaps. A commercial sequence was tested in vivo in rabbits with very interesting results.

According to the in vivo data obtained in the eGFP mouse model, optimal delivery time, application pattern and effective RNAi sequence dose was estimated for the normotensive New Zealand white rabbit model. For these experiments, targeting the ciliary body, 0.265 mg of beta2 adrenergic receptor siRNA were administered daily in a single dose on four consecutive days. The results confirmed the reduction of the IOP in rabbits, in a marked and sustained way.

It is inevitable to compare the results presented here with the ones obtained with commercial antiglaucomatous drugs. Commercial compounds such as Xalatan, Trusopt or Timoftol were capable of reducing IOP in our experimental models although their effects were significantly shorter in terms of time-effect than the ones obtained with the siRNA tested here. While the commercial compounds reduced IOP for a few hours, the carbonic anhydrase siRNA effect lasted for several days. This is a clear demonstration of the differences in the molecular mechanism of action of RNAi silencing sequences compared to existing drugs. When RNAi sequences are topically applied there is a delay until the effect is observed in comparison with to the commercially available drugs, but in terms of duration RNAi sequences were more effective both in time and magnitude in keeping IOP low. When dorzolamide (Trusopt) has access to the ciliary processes it can effectively inhibit carbonic anhydrases, but to keep a sustained effect it relies on the continuous application of this compound (12). On the contrary the topical application of siRNA against beta2 adrenergic receptors enabled us to obtain a significant reduction in IOP with a long-term effect. This may be one of the most interesting features of this new approach for the treatment of ocular hypertension and glaucoma. Moreover, RNAi sequence has been designed to specifically silence particular subtype of adrenergic receptor with successful results. These results confirm that the eye as an ideal target for a RNAi therapeutic approach, since they also imply that it will be possible to diminish the final RNAi sequence dose. This is a relevant point in order to minimize side-effects, both off-target effects, those non-specific RNAi-induced gene silencing ones due to cross-reactions with other targets of limited sequence similarity (13), and the activation of the interferon system (14). In this report we do not describe the analysis of either off-target effects or inflammatory response, on which we will probably provide interesting information in the near future. However, if we examine the corneal epithelium or the ocular structures, RNAi sequence treated animals have not shown any structural issue, to suggest that this technology could be an interesting new approach for the treatment of glaucoma.

In a remarkably short time since RNAi natural phenomenon was discovered in model organisms (15), this technology has emerged as a powerful tool for the study the function of the gene. However, in the last few years the therapeutic use of RNAi is taking a rising path with interesting results, the most promising being the phase I clinical trials in patients with neovascular AMD. Our results support a new generation of drugs for open angle glaucoma treatment and also provide insights into the treatment of other ocular diseases.

5. ACKNOWLEDGEMENTS

This work was supported by grants from Ministerio de Ciencia e Innovación [SAF2010-16024]; RETIC Red de Patología ocular del envejecimiento, calidad visual y calidad de vida [RD07/0062/0004]; NEUROTRANS CM [S-SAL 0253-2006]; and BSCHUCM [GR58/08].

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