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Towards a treatment for diabetic retinopathy: Intravitreal toxicity and preclinical safety evaluation of inducible nitric oxide synthase inhibitors

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Abstract

Purpose—To determine the maximum tolerated dose of a single intravitreal injection of aminoguanidine and 1400W, two inhibitors of inducible nitric oxide synthase, in rabbit eyes.

Methods—Groups of four New Zealand white rabbits were injected with balanced salt solution in the right eye and a single dose of either aminoguanidine (5 mg, 1 mg, 0.25 mg) or 1400W (2 mg, 0.4 mg) in the left eye. Toxicity was assessed by slit lamp and fundus examination, intraocular pressure and pachymetric measurements, and electrophysiologic and histologic analysis.

Results—Eyes injected with high doses of aminoguanidine (5 mg) or 1400W (2 mg) demonstrated severe retinal vascular attenuation and infarction. Lower doses of intravitreal aminoguanidine (1 mg) and 1400W (0.4 mg) caused no significant toxic ocular effects in rabbit eyes.

Conclusions—If the difference in vitreal volume between rabbit eyes and human eyes is taken into account, aminoguanidine (2.7 mg) and 1400W (1 mg) would be reasonable intravitreal doses to test for safety and efficacy in early clinical trials.

Translational Relevance—Inhibition of inducible nitric oxide synthase has already been shown to be beneficial in various animal models of diabetic eye disease. Dosing information is needed for preliminary testing in humans.

Keywords

inducible nitric oxide synthase; aminoguanidine; 1400W; diabetic retinopathy; intravitreal toxicity; iNOS inhibitor; nitric oxide

Introduction

Studies have shown that, besides vascular endothelial growth factor (VEGF), nitric oxide (NO) is likely to be another key signaling molecule in the pathogenesis of diabetic retinopathy. No drugs targeting this pathway are approved for human use.

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Correlative evidence that NO is involved in diabetic and other ischemic retinopathies includes:

1. Vitreous and aqueous NO levels are elevated in patients with proliferative diabetic retinopathy¹⁻⁶.
2. Inducible nitric oxide synthase (iNOS) expression is increased in models of retinal ischemia and reperfusion⁷⁻¹⁰.
3. Diabetic rats have increased iNOS levels and activity¹¹⁻¹⁵.
4. Diabetic human retinas show increased iNOS immunoreactivity^{16,17}.
5. Allelic polymorphisms of the iNOS gene alter the risk of diabetic retinopathy^{18,19}.

Studies involving pharmacologic and genetic inhibition of iNOS provide causative evidence of the involvement of NO in diabetic retinopathy and in oxygen-induced ischemic neovascularization. Pharmacologic inhibitors of iNOS include aminoguanidine and 1400W.

1. Systemic aminoguanidine prevents histological evidence of retinopathy in diabetic rats²⁰⁻²² and dogs²³.
2. Diabetic iNOS-deficient mice are protected from retinal capillary degeneration, leukostasis, and blood retinal barrier breakdown (i.e. edema)^{24,25}.
3. Systemic aminoguanidine inhibits neovascularization in rodents with oxygen-induced retinopathy (OIR)²⁶.

The mechanism by which NO is involved in diabetic retinopathy is unclear. NO regulates VEGF gene expression²⁷. Yet, VEGF is known to regulate endothelial nitric oxide synthase (eNOS) expression²⁸⁻³⁰. eNOS and neuronal nitric oxide synthase (nNOS) are constitutively expressed isoforms of NOS, unlike iNOS which is activated under hypoxic or inflammatory conditions³¹. VEGF-promoted angiogenesis depends on NO-induced endothelial cell proliferation and organization³². However, whether NO positively or negatively regulates angiogenesis depends on the specific tissue and circumstance³³. Thus, NO is involved in angiogenesis at multiple levels in a complex way.

To further complicate matters, unlike 1400W, aminoguanidine is not only an iNOS inhibitor, but also an inhibitor of advanced glycosylated endproducts (AGEs). AGEs have been implicated as an important pathologic mechanism for the harmful effects of hyperglycemia on blood vessels. Aminoguanidine binds to reactive intermediates of early glycosylated products and prevents AGE-induced protein crosslinking, a process thought to be particularly important in diabetic nephropathy.

While 1400W has not been studied in humans, oral aminoguanidine (pimagedine) has been found to be beneficial for diabetic retinopathy in a large phase 3 clinical trial looking primarily at diabetic nephropathy³⁴. Intravitreal delivery of aminoguanidine circumvents concerns about systemic side effects, as bioavailability is close to 100%, making the dose necessary for therapeutic effect miniscule compared to what is required by systemic

delivery. Ophthalmologists are already familiar with intravitreal administration of anti-VEGF agents. The purpose of this study was to characterize the ocular toxicities of two iNOS inhibitors, aminoguanidine and 1400W, and to determine the maximum non-toxic intravitreal dose of a single injection of these drugs in rabbit eyes.

Methods

All experiments in this study were conducted in accordance with the guidelines set forth by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the institutional guidelines regarding animal experimentation in ophthalmic and vision research. Groups of four New Zealand white rabbits (female, ~2 kg) were subjected to an intravitreal injection of balanced salt solution in the right eye and to different doses of either aminoguanidine hydrochloride (5 mg, 1 mg, 0.25 mg) or 1400W dihydrochloride (2 mg, 0.4 mg) in the left eye. A total of 20 rabbits were used. Drug information: aminoguanidine (alternative names: pimagedine, guanyl hydrazine; $\text{CH}_6\text{N}_4\cdot\text{HCl}$, MW 110.6), 1400W ($\text{C}_{10}\text{H}_{15}\text{N}_3\cdot 2\text{HCl}$, MW 250.2). Taking into account rabbit vitreal volume (1.5 ml), these doses equate to final concentrations of aminoguanidine (30 mM, 6 mM, 1.5 mM) and 1400W (5.3 mM, 1.1 mM). As part of the toxicological analysis, the rabbits underwent slit lamp examination and fundus photography, as well as intraocular pressure and pachymetric measurements and full-field electroretinography. The rabbits were subsequently sacrificed by intravenous pentobarbital overdose and their eyes examined histologically.

Intravitreal injections

Drugs were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile balanced salt solution, refrigerated, and used within 24 hours of preparation. Intravitreal injections were performed while the rabbits were under general anesthesia with isoflurane delivered by mask. Eyes were anesthetized with topical tetracaine then sterilized with 5% betadine. A volume of 0.05 or 0.075 cc was injected into the vitreous cavity using a 30-gauge needle attached to a tuberculin syringe approximately 2 mm from the temporal limbus. Each rabbit received only a single intravitreal injection in each eye.

Iris and Fundus Photography

Iris and fundus photographs for both eyes of each rabbit were obtained at baseline and at one and six days after injection using a handheld camera (Kowa Genesis-D). Eyes were dilated with phenylephrine 2.5%, tropicamide 1%, and atropine 1%.

Intraocular Pressure and Pachymetric Measurements

Intraocular pressure was measured using a pneumotonometer (Mentor Model 50 Classic) with the rabbits awake and topically anesthetized. Central corneal thickness was measured with an ultrasonic pachymeter (Chiron Intraoptics System CorneoGage III). The average of five measurements was used for analysis. Measurements were obtained at baseline and at one and six days after injection. Some rabbits were also measured at other time points after injection.

Electroretinography (ERG)

Full-field electroretinography was performed on isoflurane-anesthetized rabbits at baseline and at seven days after injection between the hours of 9am and noon. Eyes were dilated with phenylephrine 2.5%, tropicamide 1%, and atropine 1% prior to testing. Rabbits were then dark-adapted for one hour. Recordings performed under red light were obtained for both eyes simultaneously using a single reference electrode placed subcutaneously at the base of the dorsal neck. A subcutaneous ground electrode was placed on the tail. Contact lens jet electrodes (LKC Technologies) were placed on the eye with a hydroxypropyl methylcellulose cushion. The Espion full-field ERG system with a ColorDome (Diagnosys, Lowell, MA) was used for data acquisition. Recordings containing large spikes of heartbeat electrical interference superimposed on the waveform were discarded, and 5 to 10 responses were averaged per flash intensity. The testing procedures were performed in accordance with ISCEV standards. Dark-adapted rod (scotopic) and light adapted cone (photopic) responses were determined with 0.01 and 3.0 cd-s/m² stimuli, respectively. Cone responses were measured after 10 minutes of light adaptation. For data analysis, the average amplitude of the left eye b-waves was normalized to that of the right eye. Implicit times were also calculated from the time of the stimulus to the nadir of the a-wave or the peak of the b-wave. A nonparametric Kruskal-Wallis one-way analysis of variance with post hoc pairwise comparison was employed for statistical analysis.

Histology

On day 13 post-injection the rabbits were sacrificed with a lethal overdose of intravenous pentobarbital. The eyes were then immediately enucleated and placed in a 10% formalin solution for 7 days. They were then bisected in an anterior to posterior direction perpendicular to the medullary ray, and the lens was removed. Each calotte was dehydrated with a series of graded ethanol solutions (50%, 75%, 90%, 100%) and then cleared with xylene and embedded in paraffin wax (Leica Histoembedder Jung). A microtome (Shandon Finesse 325) was used to take 5 µm sections, which were then mounted and stained with hematoxylin and eosin. The slides were examined and images captured using a light microscope (Zeiss).

Results

Aminoguanidine

Taking into account all measures of toxicity, the maximum tolerated dose of aminoguanidine was 1 mg. With the 5 mg dose, clear toxicity was evident by funduscopy, electrophysiologic, and histologic examination. At this dose, within 24 hours after injection, three out of four eyes exhibited severe iris vascular congestion and shallowing of the anterior chamber. All four eyes demonstrated loss of pupillary reactivity and a sustained drop in intraocular pressure (Fig. 1A). Unplanned intraocular pressure measurements were obtained on days 2 and 3 to ensure that the anterior chamber shallowing did not result in painful acute angle closure glaucoma that would necessitate immediate sacrifice of the animal. This never happened although the iris vascular congestion persisted to day 6 (Fig. 1B), by which point obvious retinal vascular attenuation and retinal ischemia were noted in all eyes (Fig. 1C). In three of four eyes, this was accompanied by a mild focal peripheral vitreous reaction (Fig.

1C, last photo) that sometimes began as early as day 1. There was no observable corneal edema, anterior segment inflammation, or cataractous changes, and pachymetric measurements remained stable ($323 \pm 8 \mu\text{m}$ on day 6 vs $319 \pm 25 \mu\text{m}$ at baseline). All eyes injected with balanced salt solution were unchanged from baseline.

At the 1 mg and 0.25 mg doses of aminoguanidine, no changes in intraocular pressure or iris/retinal vascular caliber were noted. The eyes lacked inflammation and appeared completely normal funduscopically (Fig. 2A) and histologically (Fig. 2B).

Full-field ERG revealed severe reductions in the scotopic (Fig. 3A) and photopic (Fig. 3B) b-wave amplitudes for the 5 mg dose of aminoguanidine. At the two lower doses of aminoguanidine, scotopic responses were normal, and photopic responses were not significantly different from baseline. Dark-adapted combined responses (Fig. 3C) and oscillatory potentials (Fig. 3D) were obliterated for the 5 mg dose of aminoguanidine and unaffected or minimally affected for the two lower doses. Analysis of the combined response waveforms revealed statistically significant reductions in the b-wave amplitude (Fig. 3E) and increases in the a-wave implicit time (Fig. 3F) only for the highest dose of aminoguanidine.

1400W

Taking into account all measures of toxicity, the maximum tolerated dose of 1400W was 0.4 mg. With the 2 mg dose, clear toxicity was evident by funduscopy, electrophysiologic, and histologic examination. In contrast to the high dose of aminoguanidine, intraocular pressure was unchanged from baseline at one and six days after injection of 2 mg of 1400W (Fig. 4A). Despite the lack of change in intraocular pressure, within 24 hours after injection, eyes exhibited loss of pupillary reactivity (Fig. 4B) and severe retinal vascular attenuation and infarction (Fig. 4C). This was followed by re-perfusion and varying degrees of hemorrhage by day 6. Unlike with aminoguanidine, there was no appreciable iris vascular congestion or vitreous reaction. There was no observable corneal edema, anterior segment inflammation, or cataractous changes, and pachymetric measurements remained stable ($338 \pm 9 \mu\text{m}$ on day 6 vs $323 \pm 8 \mu\text{m}$ at baseline). Histologic analysis revealed loss of the inner retinal layers and disruption of the photoreceptor layer (Fig. 4D). All eyes injected with balanced salt solution were unchanged from baseline.

With the lower 0.4 mg dose of 1400W, no changes were discernible on ocular examination (Fig. 5A). Histologic analysis revealed no damage to any of the retinal or corneal layers (Fig. 5B).

Scotopic (Fig. 6A) and photopic (Fig. 6B) b-wave responses were nearly unrecordable at one week for the 2 mg dose of 1400W in striking contrast to the normal responses for the 0.4 mg dose. Dark-adapted combined responses (Fig. 6C) and oscillatory potentials (Fig. 6D) were obliterated for the 2 mg dose of 1400W and unaffected or minimally affected for the 0.4 mg dose. Analysis of the combined response waveforms revealed statistically significant reductions in both the a-wave and the b-wave amplitudes (Fig. 6E) for the high dose but not the low dose of 1400W. There was a trend towards an increase in the a-wave implicit time for the high dose of 1400W, but this did not reach statistical significance (Fig. 6F).

Discussion

At high enough concentrations, most drugs will exhibit some level of toxicity. It is important to characterize toxic effects in animal studies so that when initial human clinical trials are performed, investigators can carefully search for anticipated side effects. As expected, the highest doses of aminoguanidine and 1400W demonstrated toxic effects. Both drugs caused severe retinal vascular attenuation with associated ischemia. Reperfusion and retinal hemorrhage occurred with 1400W, but not aminoguanidine. In contrast, focal vitreal inflammation was noted only with aminoguanidine. No significant anterior segment inflammation occurred with either drug, and no toxic effects on the cornea or lens were observed.

At lower doses, a single intravitreal injection of aminoguanidine (1 mg) or 1400W (0.4 mg) caused no apparent toxicity by slit lamp and fundus examination, nor by electrophysiologic or histologic analysis. Ultrastructural examination by electron microscopy and immunohistochemical studies for markers of apoptosis and gliosis will be important future studies to confirm lack of toxicity at these doses.

That severe retinal vascular attenuation was observed with both drugs at high doses suggests that the toxic reaction is not an idiosyncratic one. Rather, it is likely due to non-specific inhibition of eNOS. Under *in vitro* conditions, aminoguanidine is 10-fold more specific against iNOS than eNOS, in contrast to 1400W which is 4000-fold more specific³⁵. eNOS is a constitutively expressed nitric oxide synthase in contrast to iNOS³¹, which is activated under inflammatory and hypoxic conditions such as occurs with diabetic retinopathy. We believe that successful treatment of human diabetic retinopathy using these compounds will only be possible if the correct dose that maximizes iNOS inhibition without compromising eNOS activity is chosen. This dose will need to be empirically determined. It is important to note that even if these compounds have the intended biological effect, any meaningful prolonged therapeutic effect would likely require repeated intravitreal injections, and that this study only evaluated toxic effects of one injection. Nonetheless, it is a starting point.

The multiple and complex pathways governing NO production at different times in the pathogenesis of diabetes are difficult to sort out and are likely the cause of seemingly conflicting reports. For example, El-Remessy et al. conclude that constitutive NOS (eNOS and nNOS) rather than iNOS is important in diabetic blood retinal barrier breakdown. They reach this conclusion because they were unable to find increased iNOS levels in their diabetic rats³⁶, results in agreement with a prior study³⁷. This is in direct contrast to the study by Du et al., who did find increased iNOS protein expression in diabetic rats¹². We propose that the difference is due to the duration of diabetes. In the first two studies, the rats had only been diabetic for two weeks by means of injection of streptozotocin. In the latter study, the rats had been diabetic for two months. Which NOS isoform is most active and what role NO plays depends on the stage of the disease.

Similarly, the finding that eNOS knockout mice develop accelerated diabetic retinopathy³⁸ might suggest that NO helps prevent retinopathy and that inhibition of NOS would be harmful. However, we believe the more correct interpretation is that eNOS null mice cannot

vasodilate in response to ischemic stress and thus suffer accelerated retinopathy due to lack of a compensatory mechanism designed to limit ischemic damage. In other words, NO coming from upregulated eNOS may compromise the blood retinal barrier initially as El-Remessy et al. suggest, but the overall vasodilatory effect is still beneficial in limiting ischemic damage, making eNOS an undesirable drug target. In contrast, inducible NO coming from inflammatory cells may be categorically harmful, making iNOS a better target.

The delicate balance of NO levels and the multiple roles NO plays in maintaining homeostasis³⁹ could make nitric oxide inhibition a tricky strategy to pursue. Geographic atrophy observed in some patients in response to repeated anti-VEGF injections⁴⁰ may be a result of suppression of NO, a known downstream effector of VEGF^{28-30,32,41}. In addition, NO is involved in keeping intraocular pressure low^{39,42}. (However, it should be noted that aminoguanidine was found to be neuroprotective in a rat model of chronic glaucoma⁴³.) At any rate, nitric oxide (over-)inhibition could potentially lead to geographic atrophy, glaucoma, and worsened ischemia, among other things. If nitric oxide inhibition is a viable strategy, the key lies in finding the correct dose.

Conclusions

This study represents the first assessment of the safety of two iNOS inhibitors delivered intravitreally. At lower doses, a single intravitreal injection of aminoguanidine (1 mg) or 1400W (0.4 mg) caused no detectable change to the structure or the function of the rabbit eye. Rabbit eyes are known to have a greater inflammatory response than human eyes⁴⁴; the lack of ocular inflammation at these doses is reassuring. It remains to be seen whether proportional human doses, aminoguanidine (2.7 mg) and 1400W (1 mg), will be safe and/or beneficial for the treatment of human diabetic retinopathy. The dosage information from this study and the pharmacokinetic information from future studies will be essential in designing a clinical trial testing just that.

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Summary Statement

Maximum tolerated intravitreal concentrations of two inducible nitric oxide synthase inhibitors, aminoguanidine and 1400W, were determined in rabbit eyes.

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Fig 1. Aminoguanidine, 5 mg.

A) Intraocular pressure at baseline and various days after injection of aminoguanidine (AG) or balanced salt solution (BSS). Error bars for the BSS line represent the standard deviation from 4 eyes. B) Iris photos 6 days after injection of either BSS or aminoguanidine. No dilating drops were given prior to these photos. The aminoguanidine-injected eye exhibits iris vascular congestion and is fixed and dilated. C) Fundus photos at baseline and 1 and 6 days after injection of aminoguanidine. Note the vascular attenuation apparent on Day 6, as well as the focal peripheral vitreous reaction (far right).

Fig 2. Aminoguanidine, 1 mg and 0.25 mg

A) Fundus photos at baseline and 1 and 6 days after injection of AG. B) Retinal (left) and corneal (right) histology at 13 days after injection of BSS or aminoguanidine (1 mg).

Fig 3. Full-field electroretinographic studies of aminoguanidine

A) Scotopic b-wave responses from the left eyes of rabbits 7 days after a single intravitreal injection of different doses of AG, expressed as a percentage of the response from the BSS-injected right eyes of the same rabbits. B) Photopic b-wave responses. C) Average waveforms of the combined (rod and cone) response as a function of time. D) Average waveforms of the oscillatory potential responses as a function of time. Low dose aminoguanidine omitted for clarity. E) Combined (rod and cone) a- and b-wave response amplitudes for different doses of AG. F) Combined a- and b-wave implicit times for different doses of AG. Error bars represent standard errors. ***p 0.005. **p 0.01.

Fig 4. 1400W, 2 mg

A) Intraocular pressure at baseline, 1 day, and 6 days after injection of balanced salt solution (BSS) or 1400W. Error bars represent the standard deviation. B) Iris photos 6 days after injection of either BSS or 1400W. No dilating drops were given prior to these photos. The 1400W-injected eye is fixed and dilated. C) Fundus photos of two eyes at baseline and 1 and 6 days after injection of 1400W. Note the vascular attenuation and retinal infarction apparent on Day 1, followed by reperfusion and either minor (top) or major (bottom) hemorrhage. The medullary ray is depicted on the far right panels. D) Retinal histology at 13 days after injection of BSS or 1400W. Note loss of the inner retinal layers and disruption of the photoreceptor layer in the 1400W-injected eye.

Fig 5. 1400W, 0.4 mg

A) Fundus photos at baseline and 1 and 6 days after injection of 1400W. B) Retinal (left) and corneal (right) histology at 13 days after injection of BSS or 1400W.

Fig 6. Full-field electroretinographic studies of 1400W

A) Scotopic responses from the left eyes of rabbits at baseline and 7 days after a single intravitreal injection of different doses of 1400W, expressed as a percentage of the response from the BSS-injected right eyes of the same rabbits. Error bars represent standard errors. B) Photopic responses. C) Average waveforms of the combined (rod and cone) response as a function of time. D) Average waveforms of the oscillatory potential responses as a function of time. E) Combined (rod and cone) a- and b-wave response amplitudes for different doses of 1400W. F) Combined a- and b-wave implicit times for different doses of 1400W. Error bars represent standard errors. **p 0.01. *p<0.05.