Pharmacokinetics and Safety of an Anti-Vascular Endothelial Growth Factor Aptamer (NX1838) Following Injection into the Vitreous Humor of Rhesus Monkeys

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Received July 7, 2000; accepted September 9, 2000

Purpose. The objective of this study was to determine the pharmacokinetics and safety for NX1838 following injection into the vitreous humor of rhesus monkeys.

Methods. Plasma and vitreous humor pharmacokinetics were determined following a single bilateral 0.25, 0.50, 1.0, 1.5, or 2.0 mg/eye dose. In addition, the pharmacokinetics and toxicological properties of NX1838 were determined following six biweekly bilateral injections of 0.25 or 0.50 mg/eye or following four biweekly bilateral injections of 0.10 mg per eye followed by two biweekly bilateral injections of 1.0 mg per eye.

Results. Plasma and vitreous humor NX1838 concentrations were linearly related to the dose administered. NX1838 was cleared intact from the vitreous humor into the plasma with a half-life of approximately 94 h, which was in agreement with the plasma terminal half-life. Vascular endothelial growth factor (VEGF)-binding assays demonstrated that the NX1838 remaining in the vitreous humor after 28 days was fully active. No toxicological effects or antibody responses were evident.

Conclusions. The no observable effect level was greater than six biweekly bilateral 0.50 mg/eye doses or two biweekly bilateral 1.0 mg/eye doses. These pharmacokinetic and safety data support monthly 1 or 2 mg/eye dose regimens in human clinical trials.

KEY WORDS: VEGF; VPF; SELEX.

INTRODUCTION

NX1838 is an oligonucleotide-aptamer inhibitor of vascular endothelial growth factor (VEGF)/vascular permeability factor (1) identified by the systematic evolution of ligands by exponential enrichment (SELEX™) process (for review, see references 2 and 3). Following SELEX and post SELEX-process modifications (1,4), the 2′-position of all pyrimidines consists of a fluorine moiety and the same position of all but two purines consists of a 2′-O-methyl group. The remaining two purines are unmodified ribose adenosines. NX1838 also contains a 40 kDa polyethylene glycol (PEG) moiety conjugated to the 5′-terminus of the aptamer and a deoxythymidine linked to the 3′-terminus via a 3′-3′ linkage (1). The modified 2'-position of the sugar ring along with the inverted 3′-3′ cap dramatically reduces the nuclease susceptibility of oligonucleotides (5–7) and addition of a large molecular weight conjugate has been shown to reduce plasma clearance in rats (8,9). NX1838 specifically recognizes $VEGF_{165}$, the major soluble isoform of VEGF, and can block $VEGF_{165}$ binding to both the fms-like tyrosine kinase (FLT-1) and kinase insert domain-containing region (KDR) VEGF-receptors and inhibit $VEGF_{165}$-mediated cellular responses *in vitro* (1,10). In animal models, NX1838 can inhibit both vascular permeability and growth of solid tumors (1,11).

NX1838 is currently in clinical trials for the treatment of age-related macular degeneration (ARMD). ARMD is a progressive bilateral degeneration of the macula and is the leading cause of loss of vision in the elderly (12–14). Choroidal neovascularization is a severe complication of ARMD. Blood vessels grow from the choriocapillaris through Bruchs membrane and ultimately, into the subretinal space (14,15). These new blood vessels can leak and hemorrhage, leading to scars or to a rapid loss of vision. Patients with subretinal neovascularization, including those with ARMD, show increased expression of VEGF and other angiogenic growth factors in the vitreous, subretinal pigmented epithelial space, and the outer layer of the macula (16–18). Thus, it is believed that antiangiogenic and/or antivascular permeability factors could delay or reverse the pathogenesis of ARMD (14,19).

To support the clinical development of NX1838, the pharmacokinetic parameters and toxicological potential of NX1838 were determined following administration by injection into the vitreous humor of primates.

MATERIALS AND METHODS

General Animal Protocols

Rhesus monkeys were obtained and cared for in accordance with all applicable state and federal guidelines and we adhered to the *Principles of Laboratory Animal Care* (NIH publication no. 85-23, revised 1985). Body weights were measured prior to dosing. For dose administration, animals were sedated with a combination of intravenous ketamine and diazepam and a mydriatic was instilled in each eye. Injections (~66 μL) were performed with a 29-gauge needle passed through the sclera and pars plana approximately 4 mm posterior to the limbus and directed posterior to the lens into the mid-vitreous. Venous blood samples were collected while the animals were restrained by a squeeze-back cage mechanism. Following euthanasia, vitreous humor was collected separately from each eye and placed on ice. Ethylenediaminetetraacetic acid (EDTA)-plasma, serum, and vitreous humor samples were frozen (–70°C) within 30 min of collection.

Toxicological Studies

A 3-month, multiple-dose pharmacokinetic and toxicological study was performed when NX1838 was administered as six semimonthly bilateral injections into the vitreous humor. Groups 3 and 4 received 0.25 mg/eye and 0.50 mg/eye, respectively, while Group 2 received four doses of 0.10 mg/eye followed by two doses of 1.0 mg/eye. A control group (Group 1) received vehicle alone (phosphate-buffered saline [PBS, pH 6.4]).

Animals were assigned to groups by a stratified random-

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ization scheme designed to achieve similar group mean body weights, and the groups were randomly assigned to treatment. All animals underwent a health screen prior to assignment to the study. The four treatment groups each consisted of three males and three females except for Group 4 (0.50 mg/eye), which consisted of four males and two females.

Animals were evaluated for changes in clinical signs, appetite, and condition of the eyes. Electrocardiograms, and blood pressure/heart rate measurements were obtained prior to the first dose and prior to necropsy.

Prior to first dose, at week 8 and week 13, blood samples were collected for evaluation of standard hematology (red and white blood cell counts, hemoglobin, hematocrit, mean corpuscular volume, mean cell hemoglobin, mean corpuscular hemoglobin concentration, platelet count, polysegmented neutrophil count, lymphocyte count, monocyte count, eosinophil count, basophil count, reticulocyte count), serum chemistry (chloride, bicarbonate, sodium, potassium, calcium, phosphorus, carbon dioxide, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, blood urea nitrogen, creatinine, uric acid, total bilirubin, direct bilirubin, indirect bilirubin, total protein, globulin, albumin, albumin/globulin ratio, glucose, cholesterol, and triglycerides) urinalysis (color, character, glucose, bilirubin, ketones, specific gravity, occult blood, pH, protein, urobilinogen, and nitrate) and coagulation parameters (prothrombin time and activated partial thromboplastin time).

Animals underwent funduscopic and biomicroscopic examinations prior to the first dose, on Day 2, and prior to doses 2, 3, 4, 5, 6, and on week 13. Indirect ophthalmoscopy and biomicroscopic exams were conducted 1 day after doses 3 and 4 as well as after doses 2, 5, and 6 for some animals. Two weeks following the final dose, a necropsy was conducted and organ weights were obtained.

Electroretinograms (ERGs) were obtained prior to doses 1, 4, and 6, and prior to necropsy. ERGs were recorded using an electroretinograph (Epic 2000) with a ganzfeld apparatus. Approximately 30 min prior to recording, each animal was dark-adapted and sedated with intramuscular ketamine. A mydriatic was instilled in each eye approximately 10–15 min prior to the ERG procedure and the animal was placed in a prone position. An intravenous combination of 10 mg/kg ketamine and 0.5 mg/kg diazepam was given to maintain sedation. ERG probes were placed subcutaneously beneath each eye and another on top of the head posterior to the brow. Lubricant (carboxymethylcellulose) was applied to each lens and contact lenses were placed on each eye. ERG tests consisted of a series of four light sequences (blue, red, white scotopic, and white flicker) which were each repeated at least five times. After completion of the test, an antibiotic ointment (Trobrex or equivalent) was placed in each eye. Instrument-averaged amplitude and latency of the b-wave was tabulated.

Intraocular pressure measurements were made with an applanation tonometer following mild sedation with intramuscular ketamine and application a topical anesthetic to the eye.

Pharmacokinetic Analyses

Pharmacokinetic parameters were determined using noncompartmental analysis (WinNonLin, version 1.5) using the linear/log trapezoidal method.

Assays

Concentrations of NX1838 are given as aptamer (oligonucleotide) weight only (8824 g/mol) and are based on an approximate extinction coefficient for the aptamer of 37 μg/ml/A_{260} unit.

NX1838 concentrations above 130 ng/mL were determined by an anion exchange HPLC method (20). Vitreous humor samples were diluted at least 5-fold in human EDTA-plasma and processed exactly as plasma samples. Concentrations below 130 ng/mL were determined by a dual hybridization assay. Briefly, NX1838 was allowed to hybridize with two DNA-oligonucleotide probes, a capture probe [5′-TTCA-CTGATTCCGTTTTTTTTLL-3′ (L = amino linker)] and detect probe [5′-BBTTTTTTTTCGGATGTATAAGCA-3′ (B = biotin)]. The capture probe was attached via the amino-linker to the wells of 96-well plates (DNA-Bind opaque white, Costar, Cambridge, MA) by adding 100 μl/well linking solution (1 M Hepes [pH 7.5], 1 mM EDTA, 100 pmol/ml capture probe) and incubating overnight at 4°C. Plates were blocked with 100 mM Hepes (pH 7.5), 0.1 mM EDTA, and 1% w/v bovine serum albumin. Duplicate samples were diluted 10-fold with 4 × SSC, containing 0.5% sarcosyl and 40 pmol/ml detect probe and heated for 10 min at 95°C. One hundred microliters was transferred to capture plates and incubated for 2 h at 45°C. Plates were washed three times (350 μl) with NTT buffer (10 mM Tris [pH 7.5], 150 mM NaCl 0.1% v/v Tween 20). Alkaline phosphatase conjugated streptavidin was added and incubated at room temperature for 30 min. Wells were washed (five times) as before, followed by the addition of a 100 μl alkaline phosphatase substrate (0.1 M diethanolamine [pH 10], 10% v/v Sapphire solution [Tropix Inc., Bedford, MA], 17 μl/mL CSPD [Tropix, Inc.], 1 mM $MgCl_2$, and 0.02% NaN_3). After 20 min, chemiluminescence was measured with a Berthold (Nashua, NH) LB 96P luminometer. Luminescence was integrated over 1 s and standards were fit to a four-parameter logistic equation.

Competition-binding assays were performed as previously described (21) with the following modifications. Plates (Microfluor "W" U bottom, Dynatech Technologies, Inc., Chantilly, VA) were coated for 2 h at room temperature with 100 μl/well 2×10^{-8} M human $VEGF_{165}$. Blocking was performed with 200 μl Superblock in Tris buffered saline (TBS; Pierce Chemical Co., Rockford, IL) and bovine serum albumin (0.1%) was used in place of fish skin gelatin. Tracer was prepared by mixing alkaline phosphatase conjugated neutravadin (Pierce Chemical Co.) and biotinylated anti-VEGF DNA-aptamer, 5′-BBCCGTCTTCCAGACAAGAGTGC-AGGG-3′ (B = biotin), at final concentrations of 1.8 μg/ml and 0.4 μg/ml, respectively. Vitreous humor samples were strained through cheesecloth and quality control samples (QCs) were prepared by diluting NX1838 standards into strained blank vitreous humor.

Serum samples taken before the first dose and 1 and 2.5 months post first dose were screened for antibodies directed against NX1838 with an enzyme-linked immunoassay (ELISA). Immulon 4 plates were coated overnight (4°C) with 100 μl of 2.5 μg/ml NX1838 in Dulbecco's PBS (DPBS). Wells were blocked with 0.1% porcine gelatin in DPBS and plates washed three times with DPBS containing 0.02% Tween 20 and 0.01% sodium azide (wash buffer). All subsequent incubations were performed at room temperature and plates were

washed three times with wash buffer between incubations. Samples, diluted 1:100 in wash buffer, were added to duplicate wells (100 μl/well) and allowed to incubate for 1 h. Alkaline phosphatase conjugated goat anti-rhesus monkey IgG-Fc antibody or goat anti-rabbit IgG-Fc antibody (Sigma Chemical Co., St. Louis, MO), diluted 10,000-fold in wash buffer, was added to each well (100 μl/well) and allowed to incubate for 30 min. Alkaline phosphatase was quantified by addition of para-Nitrophenyl phosphate (pNPP) (see manufacturer's directions, Sigma Chemical Co.). After 60 min, optical densities at 405 nm (630 nm reference wavelength) were determined with a Bio-Tek Instruments 312e microplate reader and the 1 and 2.5 month to prebleed ratio was determined.

Statistical Analyses

For tonometry and ERG, repeated measures analysis of variance (rmANOVA) with Dunnett comparisons was performed to compare each group to its own baseline and, where applicable, to control. Parameters for which the data deviated from either normality or homogeneity of variance ($p > 0.05$ for either the Levene or the Shapiro-Wilks test, $p < 0.05$) were analyzed using the ranks of the data rather than the values themselves. Comparisons were considered to be statistically significant if $p < 0.05$. For plasma concentrations and clearance, groups were compared with a one-way ANOVA.

RESULTS

Single Dose Vitreous Humor and Plasma Pharmacokinetics

Plasma concentrations versus time profiles were obtained for six female monkeys that received a single bilateral 0.50 mg/eye dose (1 mg/animal). For analyses of vitreous humor, three animals were euthanized after 7 days and three were euthanized after 28 days (see below). In addition, plasma concentration versus time profiles were obtained for two female monkeys that received a single bilateral 1.5 mg/eye dose and for two male animals that received a single bilateral 2.0 mg/eye dose. Average monkey plasma concentrations are shown in Table 1 and the plasma concentrations versus time profiles are shown in Fig. 1. All dose groups exhibited time to apparent peak plasma concentration of between 8 and 24 h with an apparent maximum achieved concentration (±SD) of 0.42 ± 0.09, 1.05 ± 0.33, and 1.99 ± 0.51 μg/mL for the 0.50, 1.5, and 2.0 mg/eye dose groups, respectively. These data indicated a linear relationship between apparent plasma C_{max} and dose.

Table 1. Mean Plasma NX1838 Concentrations in Rhesus Monkeys Following a Single Bilateral 0.5, 1.5, or 2.0 mg/eye Dose

Dose	0.5 mg/eye			1.5 mg/eye			2.0 mg/eye		
time (h)	Mean ng/ml	CV %	*n*	Mean ng/ml	CV %	*n*	Mean ng/ml	CV %	*n*
2	31	74	6	93.5	101	2	193	108	2
4	154	54	6	340	112	2	765	75	2
8	357	34	6	650	83	2	1505	32	2
12	382	25	6	905	59	2	1915	32	2
24	373	14	6	1015	29	2	1955	24	2
32	328	15	6	NS	–	–	NS	–	–
48	240	17	6	775	16	2	1285	15	2
72	183	10	6	570	20	2	800	0	2
168	94	35	6	270	31	2	455	1.6	2
336	32	13	3	71.5	29	2	160	8.8	2
504	14	21	3	20.5	38	2	30.5	39	2
672	3.3	34	3	5.75	31	2	10	42	2

NS: No sample; CV: coefficient of variation.

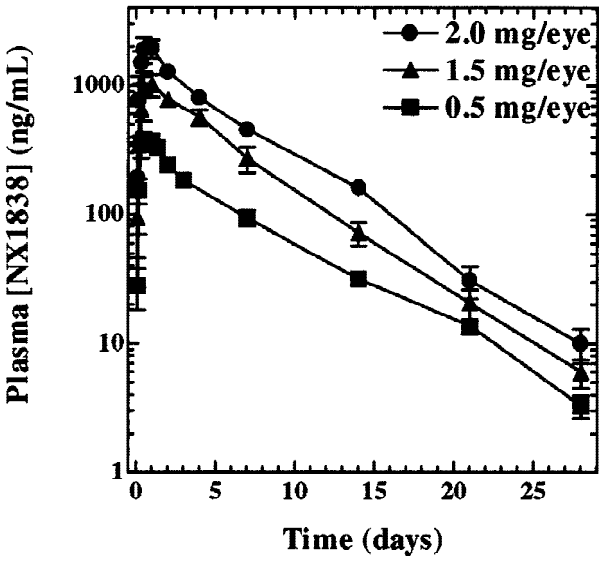

Fig. 1. NX1838 plasma concentration versus time curves following a single bilateral administration of 0.50 ($n = 6$), 1.5 ($n = 2$), or 2.0 mg/eye ($n = 2$) of NX1838 into the vitreous humor. Data are shown as the mean ± the standard error of the mean.

On the basis of a comparison of the dose-adjusted area under the plasma concentrations versus time curve ($AUC_{0\rightarrow\infty}$) obtained following intravenous administration (20) to the dose-adjusted AUCs obtained following administration into the vitreous humor, all of the NX1838 delivered to the eye entered the plasma compartment ($F = 1.2$). With $F = 1$, the calculated plasma clearance values obtained for the three dose groups (range 4.7–6.4 ml/[h/kg]) were not significantly different from one another ($p = 0.2848$).

The estimated plasma terminal half-lives following injection into the vitreous humor were 102.2 ± 21.5, 87.4 ± 0.31, and 88.9 ± 10.5 h for the 0.5, 1.5, and 2.0 mg/eye groups, respectively. Comparison to the terminal half-life of 9.3 h obtained by intravenous administration (20) suggests that the rate-determining step for the plasma terminal half-life following administration into the vitreous humor was absorption from the eye into the plasma.

Vitreous humor concentrations were determined 7 and 28 days following a single 0.50 mg/eye dose and 2 and 31 days following a single 1.0 or 2.0 mg/eye dose. The mean (±SD) NX1838 concentration 7 and 28 days post 0.50 mg/eye dose was 49.2 ± 12.7 μg/ml ($n = 6$) and 1.50 ± 0.64 μg/ml ($n = 6$), respectively (Table 2). Average concentrations 2 days post 1 and 2 mg/eye doses were 268 ± 86.8 μg/ml ($n = 2$) and 499 ± 119.0 μg/ml ($n = 8$), respectively. At 31 days post dose, NX1838 concentrations were 1.56 ± 0.21 μg/ml ($n = 4$) and 2.86 ± 1.90 μg/ml ($n = 8$) for the 1.0 and 2.0 mg/eye groups, respectively. These data were indicative of a linear relationship between dose and vitreous concentration (Fig. 2).

Half-lives of NX1838 in the vitreous humor were estimated to be 98.7, 94.1, and 89.9 h for the 0.5, 1.0, and 2.0

Table 2. Vitreous Humor NX1838 Concentrations Determined by HPLC and VEGF-Binding Competition Assays Following a Single Bilateral 0.5 mg/eye Dose in Female Rhesus Monkeys

Animal number or QC	Days post dose	Eye	HPLC assay (μg/ml)	Comp. assay (μg/ml)	Percent activity
6788F	7	OS	57.17	50.60	88.5
6788F	7	OD	33.71	31.09	92.2
5896F	7	OS	62.80	53.09	84.5
5896F	7	OD	57.73	51.69	89.5
5319F	7	OS	33.77	29.59	87.6
5319F	7	OD	50.09	47.86	95.6
QC50	–	–	62.01	46.49	75.0
5746F	28	OS	1.15	1.54	134.0
5746F	28	OD	1.24	1.39	111.7
5785F	28	OS	2.17	2.32	106.9
5785F	28	OD	2.39	2.77	115.9
6251F	28	OS	0.76	0.82	107.9
6251F	28	OD	1.26	1.29	102.0
QC1	–	–	0.91	0.92	101.1

QC50 and QC1 are 50 μg/ml and 1 μg/ml quality control solutions, respectively. OD: Oculus dexter (right eye); OS: Oculus sinister (left eye).

mg/eye dose groups, respectively (Fig. 2). Estimates were based on the assumption of a first-order elimination from the eye. First-order elimination was observed for the clearance of NX1838 from the vitreous humor of New Zealand White (NZW) rabbits (S.C.G., unpublished results). In addition, the estimated half-lives in vitreous humor were in agreement with the estimated terminal half-lives in plasma, supporting the argument for first-order elimination from the eye.

NX1838 concentrations in the vitreous humor 1 month post dose showed some eye-to-eye variability. A concentration range of 0.89–5.24 μg/ml was observed 31 days post a single 2.0 mg/eye dose ($n = 8$), values that differed by 5.9-fold. Although differences were not observed (1.3-fold) for the vitreous concentrations obtained 31 days following a single 1.0 mg/eye dose ($n = 4$), the range obtained 28 days post a single 0.50 mg/eye dose ($n = 6$) was 3.1-fold (Table 2). The reason for such differences is not clear, although it may be related to animal-to-animal variation in clearance. This explanation is supported by the fact that at 1 month post dose, differences between left and right eyes were less than those between animals (Table 2 and data not shown).

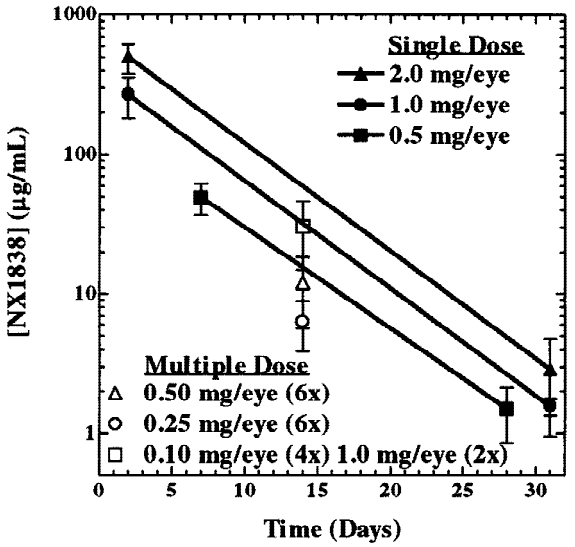

Fig. 2. Vitreous humor NX1838 concentrations following a single intravitreous injection of 0.5, 1.0, or 2.0 mg (closed symbols); or 14 days following 6 biweekly injections (6×) of 0.25 or 0.50 mg; or after 4 biweekly injections (4×) of 0.10 mg followed by two (2×) biweekly injections of 1.0 mg (open symbols). Assuming a first-order rate of elimination, lines that best fit the data were used to estimate the half-life of NX1838 in the vitreous humor.

$VEGF_{165}$ Binding Assay

Vitreous humor NX1838 concentrations following a single 0.50 mg/eye dose were also determined by a VEGF-binding competition assay that measures the ability of NX1838 to bind to human VEGF protein. By comparing the concentrations obtained by this assay to those obtained by the HPLC assay, the percent active fraction of NX1838 could be estimated. Average percent active fraction was 85% at 7 days post dose and 112% at 28 days post dose, indicating that the NX1838 remaining in the eye at these time points was fully active (Table 2). Furthermore, peak identity experiments (20) demonstrated that the measured NX1838 HPLC peak from the 7-day post dose vitreous humor samples was intact NX1838 (data not shown).

A Multiple-Dose Pharmacokinetic and Toxicological Study

Vitreous humor samples were collected 14 days following the sixth biweekly bilateral dose and assayed for NX1838

Table 3. NX1838 Vitreous Humor Concentrations (μg/ml) 2 Weeks After Receiving Six Biweekly Bilateral Injections into the Vitreous Humor

Group	Animal	Sex	Dose 6 + 2 weeks OS	Dose 6 + 2 weeks OD
Group 1, Control	R7912F	F	BLOQ	BLOQ
	R7913F	F	BLOQ	BLOQ
	R7923F	F	BLOQ	BLOQ
	R7934F	F	BLOQ	BLOQ
	R7953M	M	BLOQ	BLOQ
	R7967M	M	BLOQ	BLOQ
Group 2, 0.1 mg/eye doses 1–4, 1.0 mg/eye doses 5–6, NX1838	R7903F	F	18.39	13
	R7915F	F	30.05	35.12
	R7919F	F	25.34	7.4
	R7952M	M	18.94	21.29
	R7956M	M	42.63	47.46
	R7962M	M	55.34	50.6
Group 3, 0.25 mg/eye, NX1838	R7904F	F	7.02	8.93
	R7916F	F	10.44	7.51
	R7920F	F	1.98	5.54
	R7929M	M	8.35	7.97
	R7946M	M	5.06	5.41
	R7949M	M	3.99	3.85
Group 4, 0.5 mg/eye, NX1838	R7906F	F	18.87	20.42
	R7909F	F	14.45	7.78
	R7925M	M	9.58	12.44
	R7926M	M	3.97	4.44
	R7927M	M	6.86	6.23
	R7928M	M	20.86	17.7

BLOQ: Below limit of quantitation (0.65 μg/ml).
OD: Oculus dexter (right eye).
OS: Oculus sinister (left eye).

Table 4. NX1838 Repeated Dosing Intravitreal Toxicity Study in Primates; Tonometry Analysis Summary Table

Group		Prestudy	Week 1	Week 3	Week 3 + 24 h	Week 5	Week 5 + 24 h	Week 7	Week 7 + 24 h	Week 9	Week 9 + 24 h	Week 11	Week 11 +24 h	Week 13
Group 1,	Median	14.0	12.0	16.5	17.0	15.5	14.0	15.0	15.0	14.0	15.5	12.5	13.0	16.0
Control	Min, max	11.0, 19.0	8.0, 19.0	11.0, 19.0	7.0, 19.0	13.0, 20.0	9.0, 19.0	11.0, 17.0	10.0, 18.0	12.0, 19.0	14.0, 17.0	11.0, 17.0	11.0, 14.0	8.0, 19.0
	N	12	12	12	6	12	12	12	12	6	6	6	6	12
	Significant													
Group 2,	Median	14.0	12.5	16.5	13.0	14.0	16.0	16.0	15.5			14.0	13.0	15.0
0.1 mg/eye doses 1–4,	Min, max	11.0, 18.0	9.0, 15.0	13.0, 21.0	9.0, 17.0	8.0, 18.0	12.0, 19.0	13.0, 23.0	13.0, 19.0			9.0, 19.0	8.0, 18.0	13.0, 20.0
1.0 mg/eye doses 5–6,	*N*	12	12	12	12	12	12	12	12	0	0	12	12	12
NX1838	Significant													
Group 3,	Median	14.0	13.0	16.0	12.0	12.5	15.0	14.0	15.0			13.5	12.0	16.0
0.25 mg/eye,	Min, max	10.0, 17.0	10.0, 16.0	12.0, 22.0	8.0, 18.0	8.0, 21.0	8.0, 17.0	11.0, 20.0	13.0, 17.0			11.0, 16.0	5.0, 13.0	15.0, 20.0
NX1838	*N*	12	12	12	12	12	12	12	12	0	0	12	12	12
	Significant					A							A*T	T
Group 4,	Median	12.5	13.0	12.0	15.5	13.0	11.0	14.5	16.5	14.0	15.0	14.0	11.5	14.0
0.5 mg/eye,	Min, max	8.0, 22.0	9.0, 25.0	8.0, 21.0	15.0, 16.0	10.0, 16.0	8.0, 24.0	8.0, 23.0	10.0, 23.0	10.0, 20.0	8.0, 20.0	14.0, 14.0	11.0, 12.0	6.0, 19.0
NX1838	*N*	12	11	12	2	12	12	12	12	10	10	2	2	12
	Significant			A										A

Comparisons declared significant when $p \leq 0.05$.

A: Significantly different from control, parametric test.

A*: Significantly different from control, nonparametric test.

T: Significantly different from prestudy baseline, parametric test.

T*: Significantly different from prestudy baseline, nonparametric test.

(Table 3). The average concentration (±SD) for Groups 2 (4 × 0.10 mg/eye followed by 2 × 1.0 mg/eye), 3 (6 × 0.25 mg/eye), and 4 (6 × 0.50 mg/eye) was 30.5 ± 15.7, 6.33 ± 2.44, and 12.0 ± 6.33 μg/ml, respectively. These values were consistent with values expected following a single dose and suggested that no accumulation occurred in the vitreous humor (Fig. 2). Likewise, no plasma accumulation was observed. For Group 4 (0.50 mg/eye), animal weight-adjusted plasma concentrations were 1.73 ± 0.32 and 1.68 ± 0.40 μg·kg/ml for the 24 h time point following the first and sixth dose, respectively ($p = 0.7385$). Similarly, for Group 3 (0.25 mg/eye), the animal weight-adjusted average (±SD) plasma NX1838 concentration 24 h post initial dose (0.81 ± 0.14 μg·kg/ml) and 24 h post sixth dose (0.77 ± 0.17 μg·kg/ml) was not significantly different ($p = 0.5357$).

No animals died during the course of the study, and no treatment-related clinical signs or changes in body weight or food consumption were observed that would indicate systemic effects due to NX1838. No NX1838-related abnormalities in intraocular pressure were detected by tonometry measurements (Table 4). The pressures for all groups were within the expected range of normal for eyes that have been subjected to an injection into the vitreous humor. No compound-related effects on ERGs in weeks 6, 10, and 12 were observed (Table 5 and data not shown).

No effects on blood pressure and heart rate were present at week 12 when compared to those measurements taken prestudy, and there were no alterations in serum chemistry, hematology, coagulation, or urinalysis parameters (data not shown).

No evidence for the development of anti-NX1838 antibodies was observed (IgG) with the NX1838 antiserum ELISA. Prestudy to 1-month and prestudy to 2.5-month absorbance ratios ranged from 0.72 to 1.16. In similar experiments, this assay was able to detect rabbit NX1838 antiserum (ratio = 5.7) that was raised against NX1838 conjugated to Keyhole Limpet hemocyanin (data not shown).

There were no NX1838-related gross necropsy findings or changes in organ weights. Lesions were observed in the eyes of 4 of 6 animals in the 0.50 mg/eye group and consisted of minimal extravasation of erythrocytes in the iridal stroma and minimal mixed cell infiltrates in the iris. There were no evident alterations in blood vessels within the iris. Two of the four males in the 0.50 mg/eye group had minimal mixed mononuclear cell infiltrates consisting of lymphocytes, plasma cells, and occasional macrophages in some sections of the left iris. Infiltrates were typically discrete aggregations of cells present in 1–3 sections of the 28–31 ocular sections examined from the left eye of each animal (data not shown).

DISCUSSION

It is noteworthy that all animals that exhibited mixed cell infiltrates in the iris following six bilateral 0.50 mg/eye doses had received their initial dose from a single lot of NX1838 that had a higher endotoxin level (0.135–0.156 endotoxin units/mg NX1838) than the other lots utilized for all subsequent injections in this study (<0.05 endotoxin units/mg NX1838). The histologic alterations observed were consistent with effects due to endotoxin and were not accompanied by abnormalities in ophthalmic examinations, changes in ocular pressure, or ERG alterations. It is also noteworthy that animals that received two biweekly bilateral doses of 1.0 mg/eye with drug substance containing <0.05 endotoxin units/mg NX1838 did not develop lesions. Thus, the lesions observed were attributed to endotoxin and the no observable effect level for NX1838 in this study was greater than six biweekly bilateral doses of 0.50 mg/eye or two biweekly bilateral doses of 1.0 mg/eye.

As determined by direct measurement, NX1838 was eliminated from the vitreous humor with a half-life of approximately 94 h (3.9 days). This estimate is similar to the estimate obtained for the half-life in NZW rabbits (83 h, S.C.G., unpublished results) and is significantly greater than

Table 5. NX1838 Repeated Dosing Intravitreal Toxicity Study in Primates; Electroretinography Summary Table Scotopic White, Single Flash Analysis

		Prestudy		Week 6		Week 10		Week 12	
Group		Amplitude (μV)	Latency (ms)	Amplitude (μV)	Latency (ms)	Amplitude (μV)	Latency (ms)	Amplitude (μV)	Latency (ms)
Group 1,	Median	310.1	39.5	296.1	38.0	292.1	38.75	313.8	39.3
Control	Min, max	254.4, 445.1	29.5, 47.5	195.1, 430.0	29.5, 42.0	211.7, 375.9	37.0, 45.5	223.7, 425.5	35.5, 46.5
	N	12	12	12	12	12	12	12	12
	Significant								
Group 2,	Median	304.3	39.8	285.0	32.8	281.7	40.5	281.4	40.0
0.1 mg/eye doses 1–4,	Min, max	192.4, 337.6	34.0, 43.5	256.3, 376.8	28.0, 40.5	200.5, 362.2	28.5, 42.5	237.3, 376.3	35.5, 43.0
1.0 mg/eye doses 5–6	*N*	12	12	12	12	12	12	12	12
	Significant				T*				
Group 3,	Median	275.3	37.0	306.3	36.3	270.1	39.0	310.4	40.0
0.25 mg/eye,	Min, max	184.4, 384.9	30.0, 42.5	177.1, 445.9	28.5, 56.0	210.0, 420.2	35.0, 44.0	162.4, 383.9	36.5, 44.0
NX1838	*N*	12	12	12	12	12	12	12	12
	Significant								
Group 4,	Median	337.9	37.5	247.8	33.5	299.6	38.8	295.5	39.5
0.5 mg/eye,	Min, max	235.4, 479.3	29.0, 44.0	180.5, 376.6	28.5, 40.5	206.3, 509.5	28.0, 42.0	229.5, 448.1	34.5, 43.0
NX1838	*N*	12	12	12	12	12	12	12	12
	Significant			T					

Comparisons declared significant when $p \leq 0.05$.

T: Significant different from prestudy baseline, parametric test.

T*: Significantly different from prestudy baseline, nonparametric test.

the approximately 22–24 h half-life observed for a nonconjugated antisense phosphorothioate oligonucleotide in cynomolgus monkeys (22). Estimates of vitreous half-life in rabbits obtained with a nonconjugated (no polyethylene glycol [PEG]) version of NX1838 were similar (data not shown) to the vitreous humor phosphorothioate oligonucleotide half-life in monkeys. However, no significant difference in vitreous humor half-life for NX1838 was detected between rabbits and monkeys, as has been reported for the antisense compound (22,23). The 3.9-day vitreous half-life for the 48.8 kD NX1838 was similar to the 3.2-day vitreous half-life observed for a 48.3 kD ^{125}I-labeled humanized anti-VEGF Fab antibody in rhesus monkeys (19).

Following injection into the vitreous humor, NX1838 is absorbed intact into the plasma compartment ($F = 1.2$). Furthermore, the plasma terminal half-life mimicked the vitreous humor half-life, indicative of flip-flop kinetics. These two observations permit the estimation of the vitreous humor half-life, via the plasma terminal half-life, in ongoing clinical studies.

A key finding of this study was that after residing for 28 days in the vitreous humor, NX1838 remained fully capable of binding to $VEGF_{165}$. Thus, for the 0.50 mg/eye dose group, the active concentration of NX1838 in vitreous humor after 28 days was approximately 170 ± 73 nM. This concentration is significantly greater than the ~200 pM equilibrium dissociation constant of NX1838 for $VEGF_{165}$ (1), and much greater than the reported concentration for VEGF (<2 nM) in the vitreous humor of patients with non-age-related subretinal neovasculation (18,24).

The monkeys in this study had approximately 2 ml/eye of vitreous humor. The human eye contains 3–4 ml of vitreous humor. It is therefore possible that a monthly 1.0-2.0 mg/eye dosing regimen in humans would give vitreous humor concentrations similar to those observed in this study for the single 0.50 mg/eye dose group. If vitreous humor NX1838 concentrations are indicative of the NX1838 concentration in the choroid, then a monthly 1–2 mg/eye dose may be efficacious.

ACKNOWLEDGMENTS

We gratefully acknowledge Tom Paquette, Liz Kellogg, and Lauri Wiegand for developing the dual hybridization assay, Jerry Bill for the developing the antiserum assay, and Frank Richardson for review of this manuscript.

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