A Peptide Inhibitor of c-Jun N-Terminal Kinase for the Treatment of Endotoxin-Induced Uveitis

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PURPOSE. To evaluate the effect of XG-102 (formerly D-JNKI1), a TAT-coupled dextrogyre peptide that selectively inhibits the c-Jun N-terminal kinase, in the treatment of endotoxin-induced uveitis (EIU).

METHODS. EIU was induced in Lewis rats by LPS injection. XG-102 was administered at the time of LPS challenge. The ocular biodistribution of XG-102 was evaluated using immunodetection at 24 hours after either 20 μg/kg IV (IV) or 0.2 μg/injection intravitreous (IVT) administrations in healthy or uveitic eyes. The effect of XG-102 on EIU was evaluated using clinical scoring, infiltration cell quantification, inducible nitric oxide synthase (iNOS) expression and immunohistochemistry, and cytokines and chemokines kinetics at 6, 24, and 48 hours using multiplex analysis on ocular media. Control EIU eyes received vehicle injection IV or IVT. The effect of XG-102 on c-Jun phosphorylation in EIU was evaluated by Western blot in eye tissues.

RESULTS. After IVT injection, XG-102 was internalized in epithelial cells from iris/ciliary body and retina and in glial and microglial cells in both healthy and uveitic eyes. After IV injection, XG-102 was concentrated primarily in inflammatory cells of uveitic eyes. Using both routes of administration, XG-102 significantly inhibited clinical signs of EIU, intraocular cell infiltration, and iNOS expression together with reduced phosphorylation of c-Jun. The anti-inflammatory effect of XG-102 was mediated by iNOS, IFN-γ, IL-2, and IL-13.

CONCLUSIONS. This is the first evidence that interfering with the JNK pathway can reduce intraocular inflammation. Local administration of XG-102, a clinically evaluated peptide, may have potential for treating uveitis. (Invest Ophtalmol Vis Sci. 2010;51:4683–4693) DOI:10.1167/iovs.09-4733

The c-Jun NH2-terminal kinases (JNKs) have been identified as stress-activated protein kinases that phosphorylate c-Jun on two sites in its NH2-terminal activation domain.1 The JNK pathway is activated by certain cytokines, mitogens, osmotic stress, and irradiation. The phosphorylation of the c-Jun component of the activator protein AP-1 transcription factor results in proinflammatory cytokine production. During inflammation, leukocyte infiltration and rolling result from the early activation of the vascular endothelium, releasing important chemotactic factors such as RANTES, IL-8, ICAM, and VCAM. Infiltrating cells, in turn, release distinct sets of proinflammatory or anti-inflammatory products that contribute to tissue damage and inflammation. Many of the gene products involved in the inflammatory response are regulated by the transcription factor activator protein-1 (AP-1) and the c-Jun NH2-terminal kinase (JNK) pathway (e.g., cyclooxygenase-2, IFN-γ, inducible nitric oxide synthase [iNOS], TNF-α, membrane cofactor protein-1 [MCP-1], major intrinsic protein-1 [MIP-1], IL-2). In lipopolysaccharide (LPS)–stimulated monocytes and tissue macrophages, TNF-α is produced through the JNK pathway activation and is modulated by its inhibition.1,2

JNK inhibitors therefore have been used in various models of inflammation1 and have been shown to exert anti-inflammatory and beneficial effects in inflammatory diseases such as arthritis and asthma.3–5

XG-102 (formerly D-JNKI1) is a TAT-coupled dextrogyre peptide that selectively inhibits the c-Jun N-terminal kinase in vitro. XG-102 dextrogyre is a peptide containing a 20-aa sequence of the JNK-binding domain of islet-brain-1/JNK–interacting protein-1, a scaffold protein, combined to a 10-aa TAT sequence of the HIV TAT protein, allowing intracellular penetration.1,2

Its D-retro-inverso form (made of D-amino acids in reversed sequence) retains the capacity of JNK inhibition and translocation and has an extended activity because it is resistant to degradation by proteases.6 D-JNKI1 has been shown to protect against cell neuronal death in different models of ischemia even several hours after IV (IV) administration.8,9

In this exploratory study, we evaluated the effect of XG-102 in an endotoxin-induced uveitis (EIU) rat model of ocular inflammation using either IV or intravitreous (IVT) administration. Biodistribution of XG-102 was analyzed by immunolocalization of XG-102 in ocular tissues, and the efficacy of the treatment was evaluated clinically and histologically. The mechanism of action of XG-102 was evaluated by analyzing the phosphorylation of the JNK/JUN pathway proteins in ocular tissues, the production of chemokines/ cytokines at different time points after EIU induction using...
multiplex analysis on ocular media, and the expression of iNOS.

**Materials and Methods**

**Solutions and Products**

XG-102 peptide was produced by Polypeptide Laboratories (Strasbourg, France) and purified by high-performance liquid chromatography (HPLC). It was analyzed by mass spectrometry for identity and reverse phase HPLC for purity (Polypeptide Laboratories, France). Once lyophilized, the powder was stored at 2°C to 8°C. One day before the experiment, XG-102 powder was dissolved under sterile conditions at the concentration of 10 μM in saline (NaCl 0.9%, Versol; Aguettant, Lyon, France) in a deactivated glass vial (NSC-C4015-S1; National Scientific, Rockwood, TN) and stored at 4°C until use.

For each experiment, a fraction of freshly dissolved XG-102 was stored at –20°C, and its concentration was confirmed by HPLC analysis.

Dexamethasone sodium phosphate 4 mg/mL (Soludacrodon; Laboratoire Roussel, Paris, France) was used as positive control for anti-inflammatory activity on EIU.10

**Animals**

Seven-week-old female Lewis rats, each weighing 175 g, (Elevage Janvier, Le Genest Saint Isle, France) were used and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were anesthetized with intramuscular injection of ketamine (88 mg/kg; Virbac, France) and chlorpromazine (Largactil, 0.6 mg/kg; Sanofi-Aventis, Paris, France) before IV or ocular injection.

**Injections**

For IV injection, 100 μL saline (NaCl 0.9%) or XG-102 (20 μg/kg in saline) was injected in a tail vein with a 25-gauge needle connected to a 1-mL syringe (Becton Dickinson, Le Pont de Claix, France). For IVT injection, 5 μL saline or XG-102 (0.2 μg/injection in saline) was injected in both eyes using a 30-gauge disposable needle (BD-microfine; nm Médical, Asnières, Paris, France). The IV dose of 20 μg/kg (i.e., 3.5 μg/rat in rats weighing 175 g) was chosen based on studies showing that XG-102 is active at very low doses in other models.9 For IVT injections, we used the minimal dose used in direct car application after acute noise trauma in patients.11 This corresponds to 5% of the dose injected intravenously. Immediately after IV or IVT treatment, EIU was induced by a single footpad injection of 100 μL sterile pyrogen-free saline containing 200 μg lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma-Aldrich, Saint-Quentin Fallavier, France). At the end the experiments (6, 24, or 48 hours after LPS challenge), rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg; Sanofi-Aventis) before blood was collected by intracardiac puncture. Each rat was then killed with a lethal dose of pentobarbital, and both eyes were enucleated.

**Sample Collection**

Aqueous humor and vitreous were collected and pooled from each enucleated eye. Ocular fluids were immediately centrifuged, and the cell-free fractions were collected and frozen at –20°C before analysis by multiplex assay. Blood samples were collected first at room temperature for 2 hours and then at 4°C overnight. Serum was collected and centrifuged, and the clear supernatant was collected and frozen at –20°C before multiplex analysis. Retinas and RPE/choroid/sclera complexes were carefully dissected from the enucleated eyes, snap frozen, and stored at –80°C until use for RT-PCR and Western blot analyses.

For immunohistochemistry, eyeballs were collected and fixed for 1 hour at room temperature in phosphate-buffered saline (PBS) containing 4% paraformaldehyde before they were rinsed overnight in PBS.

The next day, samples were embedded and frozen in optimal cutting-temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Zoeterwoude, The Netherlands) and stored at –80°C. Frozen anteroposterior sections of eyes (10-μm thick) were performed at the optic nerve level using a cryostat (CM 3050S; Leica, Rueil-Malmaison, France) and mounted on slides (Superfrost; Gerhard Menzel, Braunschweig, Germany) for immunohistochemical analysis.

**Experimental Design**

In the first set of experiments, 70 rats were randomized into 14 experimental groups with five rats per group. Uveitis was induced in each group, and rats were killed 6 hours (four groups), 24 hours (six groups), and 48 hours (four groups) after LPS challenge. For each time point tested (6 hours, 24 hours, and 48 hours), rats treated by IV or IVT injection of vehicle (NaCl 0.9%) or XG-102 were compared with untreated control uveitic rats. Two additional groups treated by IV injection of vehicle and intravitreal injection of dexamethasone were used at 24 hours. Clinical ocular inflammation was recorded only at 24 hours (see Scoring of Endotoxin-Induced Uveitis section). At each time point, intraocular fluids from each eye (n = 10 per group) and serum from each animal (n = 5 per group) were used for chemokine/cytokine multiplex assay.

Retinas and RPE/choroid/sclera complexes were also collected at 24 hours to analyze iNOS mRNA levels by RT-PCR and the c-Jun phosphorylation state by Western blot. Tissues were collected only from eyes treated by IV injection of vehicle, IV injection of XG-102, or IVT injection of vehicle and IVT injection of XG-102 (n = 2 eyes per condition collected from separate rats). Eyes were selected so that their EIU clinical score was representative of the mean of the experimental group to which they belong (i.e., three for eyes treated by IV and IVT injection of vehicle and two for eyes treated by IV or IVT injection of XG-102).

A second set of experiments was designed to evaluate the anti-inflammatory effect of XG-102 at the cellular and tissue level and the biodistribution of this molecule 24 hours after administration. Rats were randomized into 11 experimental groups, six groups of rats with uveitis (untreated uveitic rats, rats injected intravenously with NaCl or XG-102, and rats injected intravitreally with vehicle, XG-102, or dexamethasone) and five groups without uveitis (untreated healthy rats, rats treated by NaCl IV or XG-102 IV, and rats injected IVT with NaCl or XG-102). Three eyes of separate rats were collected per group and used for immunohistochemistry. For clinical and histologic analyses, dexamethasone was used as a reference treatment.

**Scoring of Endotoxin-Induced Uveitis**

Animals were examined by slit lamp at 24 hours, the clinical peak of the disease in our experiments. The intensity of clinical ocular inflammation was scored on a scale from 0 to 5 for each eye, as described previously10: grade 0, no inflammation; grade 1, minimal iris and conjunctival vasodilation; grade 2, moderate inflammation; grade 3, intense iris vessels dilation, flare, and fewer than 10 cells per slit lamp field in the AC; grade 4, more severe clinical signs than grade 3, with >10 cells in the AC with or without the formation of hypopyon; grade 5, intense inflammatory reaction, fibrin formation in the AC, and total seclusion of the pupil. Clinical evaluation was performed in a masked manner.

**Western Blot Analysis**

RPE/choroid/sclera complexes and neuroretinas (two per experimental group) were snap frozen immediately after dissection and stored at –80°C until use. Tissues were homogenized in 500 μL lysis buffer (MOPS SDS Running Buffer; Invitrogen, Cergy-Pontoise, France) supplemented with protease inhibitor cocktail (Roche Diagnostics, Meylan, France) (one tablet for 50 mL). After the addition of LDS sample buffer (NuPAGE; Invitrogen) and heating for 5 minutes at 100°C, equal
amounts of proteins were subjected to electrophoresis (NuPAGE 4%-12% Bis-Tris gel; Invitrogen) using MOPS SDS running buffer. Bands obtained were then electrotransferred onto nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany).

Western blot analyses were carried out to analyze the effect of XG-102 on the three mitogen-activated protein kinase (MAPK) pathways. To analyze the JNK pathway, blots were sequentially incubated with a rabbit Phospho-c-Jun (Ser63) primary antibody (or Phospho-c-Jun (Ser73) antibody) and an anti-rabbit IgG HRP-conjugated secondary antibody according to the manufacturer’s instruction (Cell Signaling Technology, Ozyme, St. Quentin Yvelines, France). Bands were visualized with ECL Western blot analysis detection reagent kits (Amer sham Biosciences, Orsay, France). Blots were then dehybridized and rehybridized successively with a mouse anti-β-tubulin (D-10) (sc-5274) primary antibody (dilution 1:400) and an HRP-conjugated goat anti-mouse secondary antibody (sc-3697) (dilution 1:5000; both purchased from Santa Cruz Biotechnology, Tebu-bio, Le Perray en Yvelines Cedex, France). The relative band intensity for phospho c-Jun (Ser63 or Ser73) was calculated in comparison to that for β-tubulin after densitometry analysis (ImageJ software, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

To analyze the ERK and p38 MAPK pathways, blots were sequentially incubated with a rabbit phospho-p44/p42 MAPK (Erk1/2 [Thr202/Tyr204; 4370]) primary antibody (or a rabbit phospho-p38 MAPK [Thr180/Tyr182; 9215]) antibody) and a horseradish peroxidase-conjugated goat anti–rabbit IgG HRP-linked secondary antibody (H11021; Scleicher & Schuell BioScience, Dassel, Germany). Blots were then dehybridized and rehybridized successively with a mouse anti–iNOS (1/75e; Transduction Laboratories, Lexington, KY) and confirmed by (A) immunoblot (top lane: phospho-c-Jun [Ser63]; bottom lane: β-tubulin reporter protein) and confirmed by (B) densitometric quantitation.

FIGURE 1. Clinical efficacy of XG-102 in LPS-induced uveitis. Clinical scores (expressed in arbitrary units [AU]) were evaluated at the peak of the disease and at 24 hours after (A) IV or (B) IVT injection of XG-102. Comparison was made with untreated uveitic eyes (LPS) and IV/IVT treatment with vehicle (n = 10 eyes per group). Clinical manifestations of uveitis were reduced after (A) IV injection of XG-102 (***P < 0.001 vs. LPS; ***P < 0.001 vs. vehicle) and (B) IVT injection of XG-102 (***P < 0.001 vs. LPS; ###P < 0.01 vs. vehicle) and dexamethasone (*P < 0.05 vs. LPS). No statistical difference (ns) was observed between IVT injection of XG-102 and dexamethasone, which was used as positive control.

Immunohistology

To characterize the cellular infiltrate, sections were double-stained with ED1 and iNOS. Briefly, after permeabilization with 0.1% Triton X-100 in PBS for 30 minutes, specimens were rinsed and saturated for 1 hour at room temperature with ED1 and iNOS. Briefly, after permeabilization with 0.1% Triton X-100 in PBS for 30 minutes, specimens were rinsed and saturated for 1 hour at room temperature with ED1 and iNOS. The relative band intensity for phospho c-Jun (Ser63 or Ser73) was calculated in comparison to that for β-tubulin after densitometry analysis (ImageJ software, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

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FIGURE 2. Inhibition of the JNK pathway by XG-102 in LPS-induced uveitis. Western blot analysis of c-Jun phosphorylation in RPE/choroid/ sclera complexes 24 hours after IV or IVT injection of XG-102 and vehicle (n = 2 eyes per group) in EIU conditions. Inhibition of c-Jun phosphorylation by XG-102 was visualized on (A) immunoblot (top lane: phospho-c-Jun [Ser63]; bottom lane: β-tubulin reporter protein) and confirmed by (B) densitometric quantitation.
administration. Briefly, sections were permeabilized, as described, before they were sequentially incubated with an anti-XG-102 purified rabbit IgG and a secondary Alexa 594 (red)-conjugated goat anti-rabbit IgG (Invitrogen) diluted 1:100 and 1:250 in PBS, respectively. Immunostained untreated healthy and uveitic eyes were used as negative controls. Nuclei were stained with DAPI before mounting and observation.

Immunostaining of p-Erk1/2 was performed to evaluate the effect of XG-102 on the ERK pathway after IV or IVT administration. Sections were permeabilized as described and incubated in blocking solution containing 0.1% Triton X-100 and 10% fetal calf serum in PBS for 1 hour at room temperature. Sections were incubated overnight at 4°C with a rabbit anti–phospho-p44/42 MAPK (Erk1/2) primary antibody (4570) purchased from Cell Signaling Technology (Ozyme) diluted 1:400 in blocking solution. After having been rinsed three times in PBS, sections were incubated with a secondary Alexa Fluor 488 (green)-conjugated goat anti–rabbit mAb (diluted 1:300 in blocking solution) for 2 hours at room temperature. Nuclei were stained with DAPI before mounting and observation.

**Evaluation of iNOS Expression in Ocular Tissues Using Semiquantitative PCR**

Two eyes per group were used for this analysis. Immediately after dissection, retinas extracted from each eye were separately snap frozen and stored at −80°C until use. Total RNA was extracted from tissues (RNeasy Mini-Kit; Qiagen, Courtaboeuf, France) in accordance with the manufacturer’s instructions. Reverse transcription was performed on 1 μg total RNA in a total volume of 20 μL using reverse transcriptase (Superscript II; Invitrogen) in accordance with the manufacturer’s instructions. To amplify GAPDH and iNOS cDNA, PCR was conducted in a total volume of 25 μL containing 2 μL first-strand reaction product, 0.4 μM forward and 0.4 μM reverse primers, 0.4 μM dNTP mix, 1.5 mM MgCl₂, 1× PCR buffer, and 2.5 U Taq DNA polymerase (Invitrogen). Primers specific for GAPDH (forward, 5'-ATGCCCCCATGTGTTGATG-3'; reverse, 5'-ATGGCATGGACTGTGTCAT-3') and iNOS (forward, 5'-TTTTCTCTCAAATGCAAATCTCA-CCA-3'; reverse, 5'-TGTGCTCGAGATGTGTGAAAC-3') were obtained from Invitrogen. After an initial denaturation (3 minutes at 94°C), 30 to 32 PCR cycles of denaturation (30 seconds, 94°C), annealing (1 minute, 58°C [GAPDH], and 52°C [iNOS]), and elongation (1–2 minutes, 72°C) were performed (Crocodile III; Appligene-Oncor, Illkirch, France). The final cycle was completed by 5 minutes of elongation at 72°C. PCR fragments (162 bp for GAPDH and 657 bp for iNOS) were analyzed by 2.5% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The relative band intensity for iNOS was calculated in comparison to that for GAPDH after densitometry analysis (ImageJ software).

**Chemokine/Cytokine Multiplex Assay**

Intraocular fluids (diluted to obtain a final volume of 25 μL) and sera (25 μL of 1:5 dilution) were subjected to multiplex bead analysis. This method uses microspheres as the solid support for immunoas-

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**FIGURE 3.** Effects of XG-102 on the LPS-ERK pathway activation. Immunohistochemistry against phospho-p44/42 MAPK (Erk1/2; green) was carried out on ocular histologic sections of untreated uveitic control eyes and IV or IVT administered animals (vehicle or XG-102; n = 3 eyes analyzed per condition; time point, 24 hours). Nuclei (blue) were stained with DAPI. p-Erk1/2 was strongly expressed in the iris epithelium after IVT administration of vehicle (A, a) and XG-102 (B, b), with no detectable difference between the two. Similar expression was observed in uveitic eyes treated by IV of vehicle and XG-102 (not shown). Erk1/2 was strongly activated in the numerous infiltrating cells after IV (C, c) or IVT (not shown) administration of vehicle but not in the few inflammatory cells observed after IV (D, d) or IVT (not shown) injection of XG-102. Only a faint positive signal could be detected in retinal Müller glial cells in EIU eyes treated by IV injection of either the vehicle (E, e) or XG-102 (F, f) (arrows). Similar results were obtained with IVT of vehicle or XG-102 (not shown). Scale bar, 100 μm. c, cornea; ir, iris; st, stroma; ep, epithelium; ONH, optic nerve head; INL, inner nuclear layer; ONL, outer nuclear layer.
says\textsuperscript{12} and allows the titration of a greater number of cytokines with increased sensitivity than occurs with ELISA.\textsuperscript{13} For each sample, 17 analytes were quantified simultaneously using the rat cytokine/chemokine-17plex kit (Milliplex Map Kit; Millipore, Saint-Quentin-en-Yvelines, France) according to the manufacturer's instructions, as follows: chemokines MCP-1/CCL2, MIP-1\textalpha/CCL3, RANTES/CCL5, IP-10/CXCL10 (IFN-inducible protein-10) and GRO/KC; proinflammatory mediators IL-1\textbeta, IL-18, and TNF-\alpha; Th1/Th2/Th17 cytokines IL-2 and IFN-\gamma/IL-4, IL-5, IL-6, IL-10, and IL-13/IL-17. The assay was performed in a 96-well filter plate, and standard curves for each cytokine were generated with a rat cytokine standard provided in the kit. All incubation steps were performed under medium orbital agitation in the dark to protect the beads from light. Data acquisition and analysis were performed with the manager software version 4.1 (Bio-Plex; Bio-Rad) with four or five logistic parameters for standard curves. Detection thresholds for all the analytes were estimated to be approximately 1 to 10 pg/mL.

Statistical Analysis

Numerical results were expressed as mean ± SEM. Data were compared using the nonparametric Mann-Whitney $U$ test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Reduction of EIU by XG-102

XG-102 significantly reduced EIU clinical scores after 20 \(\mu\)g/kg IV injection (2.0 ± 0.1) compared with untreated uveitic eyes (3.2 ± 0.1; $P < 0.001$) and vehicle IV (3.2 ± 0.1; $P < 0.001$; Fig. 1A). In a similar manner, clinical scores were significantly decreased after 0.2 \(\mu\)g/injection IVT administration of XG-102 (2.2 ± 0.2) in comparison with untreated uveitic eyes (3.2 ± 0.1; $P < 0.001$) and vehicle IVT (3.0 ± 0.1; $P < 0.01$; Fig. 1B). The effect of IVT injection of XG-102 on clinical signs of EIU was not statistically different from that observed after IVT of dexamethasone (1.8 ± 0.4), suggesting that XG-102 was as efficient as dexamethasone in reducing EIU when administered at this time point (Fig. 1B).

XG-102 Efficacy as a Result of JNK Pathway Inhibition

To determine whether the clinical effect of XG-102 was related to its mode of action, i.e., its ability to interfere with JNK signaling,\textsuperscript{6} c-Jun phosphorylation state was analyzed in ocular...
tissues by Western blot analysis. Phosphorylation of c-Jun on Ser63 (Fig. 2A) and Ser73 (not shown) residues was reduced in RPE/choroid extracts 24 hours after XG-102 was injected intravenously or intravitreally. In the neuroretina, phospho-c-Jun could be only faintly detected. An approximately threefold decrease in c-Jun phosphorylation was observed in RPE/choroid either after IV (0.28 ± 0.01 vs. 0.77 ± 0.26 in IV of vehicle) or IVT (0.35 ± 0.08 vs. 0.79 ± 0.25 in IVT of vehicle) administration of XG-102 (Fig. 2B). The ability of XG-102 to block JNK activity in the eye tissues demonstrated the specific intraocular activity of XG-102.

To determine whether XG-102 could have any effect on the other MAPK pathways, the phosphorylation state of Erk1/2 and p38 was evaluated. Whereas Erk1/2 and p38 were detected in RPE/choroid complexes at similar levels among all groups, the phosphorylation form of these two MAPKs could not be detected by Western blot analysis (data not shown). These results demonstrate that JNK is the predominantly activated MAPK pathway in the RPE/choroid during EIU. Using histochemical analysis, performed without any signal amplification, we found an intense p-Erk1/2 signal in inflammatory cells infiltrating the anterior and the posterior eye segments in the control LPS- and saline-treated eyes (Figs. 3C, 3D). The effect of XG-102 administered either intravenously or intravitreally could not be evaluated on those cells because the infiltration was almost absent in the treated eyes. However, in the neuroretina, where p-Erk1/2 could be detected and located in retinal Müller glial cells in the control and saline-treated eyes (Fig. 3E), no effect of XG-102 administered by either route was observed (Fig. 3F). Interestingly, in the iris, an intense p-Erk1/2 signal was observed in the epithelium of the control and saline-injected eyes (Fig. 3A), with no effect of XG-102 treatment on the p-Erk1/2 signal in these cells (Fig. 3B), strongly suggesting that XG-102 does not seem to directly act on p-Erk1/2 phosphorylation during EIU in our model, at least in resident cells.

Differential Distribution of XG-102 in Ocular Tissues after IV and IVT Administration

Immunohistochemistry was carried out on histologic sections to evaluate the biodistribution of XG-102 in ocular tissues 24 hours after systemic (IV) or local (IVT) administration, both in healthy eyes and in uveitic conditions (Fig. 4).

No inflammatory cell infiltration was observed in healthy eyes after IV or IVT of XG-102 or vehicle. No immunoreactivity against XG-102 was detected in untreated control eyes or in eyes treated by vehicle, demonstrating the specificity of the signal observed in XG-102–treated eyes. Whereas no signal was observed in normal eyes after systemic (IV) injection (Figs. 4A–C) at the dose used, XG-102 was distributed in almost all ocular tissues of normal rats after IVT administration (Figs. 4D–L). Interestingly, an accumulation of XG-102 was detected mainly in the iris/ciliary body epithelium (Figs. 4G, 4J) and in the retinal pigment epithelium (Fig. 4L). Penetration of XG-102 was also detected in the iris stroma (Fig. 4G) and in the neural retina in the ganglion cell layer (Fig. 4H), the inner nuclear layer (Fig. 4K) and the inner segment (Fig. 4I) of photoreceptor cells. In all cell types, XG-102 accumulated within the cytoplasm. Occasional staining was found in the corneal endothelium and in the lens capsule (not shown).

In uveitic conditions, no XG-102 staining was detected in ocular tissues or in infiltrating inflammatory cells of untreated eyes (Figs. 4V, 4W). IV or IVT of vehicle gave results similar to those of untreated eyes (not shown). In EIU eyes treated by IV injection, XG-102 was not detected in ocular tissues, but occasional infiltrating inflammatory cells were immunopositive in the iris (Fig. 4O) and in the aqueous humor (Fig. 4P). In uveitic eyes treated by IVT injection, XG-102 was found primarily in ocular tissues, such as in healthy eyes, and in resident cells that are mobilized and participate actively to the inflammatory processes in pathologic conditions, such as microglial cells (Figs. 4S, 4T).

**Significant Reduction in Cells Infiltrating the Ocular Tissues after XG-102 Administration**

To further characterize the effect of XG-102 in uveitis, the infiltrated inflammatory cells were quantified in ocular tissues (Fig. 5) by enumeration on histologic sections immunostained with ED1 and iNOS antibodies (Fig. 6).

Twenty-four hours after LPS challenge, the number of ED1+ cells was significantly reduced in eyes treated with IV injection of XG-102 (157 ± 7; Figs. 5A, 6M, 6P, 6S) compared with untreated uveitic eyes (LPS; 187 ± 13; P < 0.005; Figs. 5A, 6A, 6D, 6G, 6J) or vehicle-injected eyes (not shown). Similarly, IVT of XG-102 significantly reduced ED1+ infiltrating cells (93 ± 8) compared with vehicle IVT-injected eyes (175 ± 15; P < 0.009) and untreated uveitic eyes (P < 0.005; Fig. 5A).

![Figure 5](https://example.com/figure5.png)
reducing effect of XG-102 on ED1$$^+$$ cell infiltration (93 ± 8) did not differ from that induced by dexamethasone (79 ± 15), suggesting that both treatments have a similar efficacy on this parameter.

The number of polymorphonuclear (PMN) cells (Fig. 5B) was also significantly reduced at 24 hours after IV injection of XG-102 (60 ± 6) compared with control eyes (237 ± 15; P < 0.005) and after IVT injection of XG-102 (40 ± 5) compared with IVT injection of the vehicle (152 ± 31; P < 0.009) and control uveitic eyes (P < 0.005). Again, the effect of XG-102 on PMN ocular tissue infiltration did not significantly differ from that of dexamethasone (42 ± 11).

**Downregulation of iNOS Expression by XG-102**

Because iNOS has been described as a key mediator in the pathogenesis of uveitis, the effect of XG-102 on its expression was investigated at both the protein and the mRNA levels.

As shown on Figure 6, the number of iNOS$$^+$$ cells was reduced in eyes treated with IV injection of XG-102 (Figs. 6N, 6Q, 6T) or IVT (not shown) compared with control eyes (Figs. 6B, 6E, 6H, 6K). Among iNOS$$^+$$ cells observed in control eyes, a few were ED1$$^+$$ whereas most were ED1$$^-$$. The effect of XG-102 on iNOS expression was confirmed by RT-PCR on ocular tissues (Fig. 7). Levels of iNOS mRNA were downregulated from 1.02 ± 0.21 to 0.40 ± 0.11 after IV administration of XG-102 and from 1.18 ± 0.05 to 0.27 ± 0.09 in eyes treated by IVT injection. Comparisons were made with IV or IVT of vehicle, respectively.

**Chemokine/Cytokine Profiles in Ocular Media of Eyes Treated with XG-102**

To evaluate the effect of the treatment on the production of proinflammatory and anti-inflammatory mediators, chemokines and cytokines were dosed by multiplex analysis on ocular media (Figs. 8, 9) and sera (not shown).

Among the 17 chemokines/cytokines tested, some (IP-10, IL-5, IL-17) were below detectable levels both in control or treated eyes. Others (IL-18, IL-4, IL-1$$\beta$$) did not differ in treated versus untreated eyes at any of the tested time points. In the serum (not shown), though some cytokines tended to change...
after IV administration of XG-102 (reduction of MIP-1α and IL-2) or after IVT (reduction of IL-2), this was not statistically significant. For the other chemokines/cytokines, their profile was different in ocular fluids from eyes treated with IV administration of XG-102 compared with IVT administration. Indeed, when XG-102 was injected systemically at the time of LPS challenge, it induced a significant reduction of MCP-1, MIP-1α, and RANTES at 6 and 24 hours (Fig. 8A). GRO/KC (not shown) was also significantly reduced at 6 hours. Th1 cytokines such as TNF-α, IL-6, and INF-γ were significantly reduced at different time points, whereas IL-10 tended to increase (but not significantly) at 6 hours in treated eyes, suggesting a switch toward a Th2 profile (Fig. 8B). No statistical differences were noted between eyes from vehicle IV-injected rats (not shown) and untreated uveitic control eyes.

When XG-102 was injected into the vitreous at the time of LPS challenge, the chemokine/cytokine profile was not strikingly different from that of eyes injected with vehicle (Fig. 9). IVT administration of vehicle had a marked effect on the cytokine profile compared with that of untreated uveitic control eyes (not shown). At 6 hours, a trend to a decrease of MCP-1, TNF-α, IL-6, and IL-2, and, at 24 hours, a marked decrease of IL-2 and an increase of IL-13 were detected, suggesting again a switch toward a Th2 profile.

**Discussion**

We have shown for the first time that XG-102 is as effective as dexamethasone in reducing clinical EIU and ocular inflammatory cell infiltration. This effect is linked to the specific activity of XG-102, as demonstrated by the reduced phosphorylation of c-Jun in RPE/choroid extracts, whereas XG-102 particularly accumulates, but not in the neuroretina in which c-Jun could only be faintly detected. These results are in accordance with previous work by Takeda et al., showing that in the neuroretina of EIU eyes, the main MAPK pathway is the ERK pathway, which occurs in retinal Müller glial cells. In our experiments, the p-ERK signal observed in retinal Müller glial cells in LPS or in LPS saline-treated eyes was not reduced by XG-102 administered either intravenously or intravitreally. More interestingly, in the iris, where XG-102 strongly accumulated after IVT injection, the p-ERK signal was intense in both the control and the treated eyes, suggesting that XG-102 did not intervene in the ERK pathway in resident cells in our model. In agreement with Takeda et al., p38 was not found to be activated in the neuroretina or in the RPE/choroid complexes, explaining why the effects of XG-102 on this MAPK pathway could not have been investigated. However, other reports have demonstrated in cell-free conditions that XG-102 does not intervene directly in the p38 or the ERK pathway.

We cannot exclude that XG-102 could intervene in transduction pathways in macrophages and neutrophils and the subsequent iNOS expression in those cells. Indeed, intense ERK phosphorylation was found in infiltrating cells of control and LPS eyes. Because XG-102 was also shown to regulate the activity of MKK4, which activates not only the JNK (thus interrupting upstream in addition to downstream events along the JNK cascade) but also the p38 pathway, we cannot exclude that p38 could also be targeted by XG-102 in infiltrating cells. Further fluorescence-activated cell sorting analysis studies are required to explore these potential effects on infiltrating cells more thoroughly.

XG-102 has interesting properties for ocular use. Its specific D-retro-inverso structure makes it resistant to degradation and is responsible for its long half-life. The TAT sequence allows its cellular translocation, which has been observed after intraocular injection, suggesting that a sustained efficacy with limited need for repeated injections could result from these properties. Both IV and IVT administrations were efficient, but the IVT dose of XG-102 administered was 20 times lower than the IV dose. Both types of administration did not induce significant changes in the cytokines produced in the serum. Changes in serum were more pronounced in the IV-treated group but remained below statistical significance, which does not mean that those changes do not have a clinical significance. After IVT injection, XG-102 distributed homogeneously in the ocular tissues, probably because of its low molecular weight (MWt 3822) and entered the cells because of its TAT sequence. It accumulated primarily in uveal cells (iris and ciliary body) and retinal pigment epithelial cells, which could be of interest in the prospect of uveitis recurrence prevention. In addition, XG-102 was found in astrocytes and microglial cells that play an important role in the ocular barrier and in retinal homeostasis. As shown in normal eyes injected with XG-102, no inflammation or adverse reaction was observed, suggesting that XG-102 is well tolerated in the eye. However, further specific toxicologic studies should be performed before any clinical application. After IV administration, XG-102 could not be detected in healthy ocular tissues or cells but could be found in uveitic eyes in a few infiltrating cells and microglia. The apparent discrepancy between the good clinical efficacy of IV administration of XG-102 and the poor immunolocalization of XG-102 in ocular tissues at 24 hours after IV injection could have resulted from the low dose of XG-102 injected in our experiments, and to the fact that in inflamed tissues, XG-102 concentrates primarily in infiltrating cells. Indeed, in our experiments, XG-102 was administered intravenously at 0.02 mg/kg, at 20 times the dose injected into the vitreous, possibly leading to intraocular levels of XG-102 below the immunodetection threshold. However, in stroke models, XG-102 exerted significant effects at systemic dose as low as 0.0003 mg/kg,2 suggesting that XG-102 crosses the blood brain barrier and retains its activity even at very low dose, below immunodetection threshold. After IV injection, XG-102 could also act directly on circulating inflammatory cells, preventing them from penetrating the eye. Using both routes of administration, we could show that XG-102 specifically decreased p-Jun using Western blot analysis performed on RPE/choroid. These extracts contained both resident and infiltrating cells.

**Figure 7.** Downregulation of LPS-induced iNOS expression by XG-102. RT-PCR analysis of iNOS mRNA levels in neuroretinas 24 hours after IV or IVT injection of XG-102 or vehicle (n = 2 eyes per group) in EIU conditions. Downregulation of iNOS mRNA was visualized on (A) agarose gel under ultraviolet transilluminator (top lane: 657 bp iNOS cDNA amplification product; bottom lane: 162 bp GAPDH cDNA amplification product) and confirmed by (B) densitometric quantitation.

**Figure 8.** ERK phosphorylation was found in infiltrating cells of control and the treated eyes, suggesting that XG-102 did not intervene in the ERK pathway in resident cells in our model.
fore the effect observed after IV injection cannot be directly linked to an action on resident cells. Interestingly, despite very different biodistribution resulting from IV and IVT injection, the subsequent clinical effect was not significantly different for the two routes of administration. However, because IVT injection results in XG-102 accumulation in inflammatory cells and in resident ocular cell, and because degradation of the dextrogyre XG-102 peptide should be very slow, IVT injection should be favored compared with IV administration in the prospect of a longer duration of action. This point will be evaluated in an EAU model that lasts longer than EIU. Because JNK pathways are ubiquitous in the body, local blockade should be preferred when the eye is the only inflamed organ to reduce potential systemic side effects. The mechanisms of action of XG-102 in our experiments primarily involved a reduction of expression of iNOS produced by the macrophages and neutrophils IL-2 and IFN-γ. Using knockout mice, it was shown that JNK1 and JNK2 pathways resulted in opposing effects regarding the expression of IL-2 and IFN-γ in CD8^+ T cells. Although JNK1 null mice had a reduced AP-1 transcription activity and decreased IL-2 and IFN-γ production, JNK2 null mice had an increased expression of both. The inhibition of all isoforms can therefore result in various effects. In our experiments, the reduced IL-2 and IFN-γ expression in the ocular media resulted from XG-102 action on the three JNK isoforms blocking access of MAPK/JNK to their substrates within the nucleus. IL-1 expression was not affected by XG-102 administration, as expected, because it acts upstream of JNK pathway activation. The clinical and histologic effects of XG-102 on EIU cannot be directly related to its effects on IFN-γ because controversial effects of IFN-γ have been described in EIU with a potential inhibitory effect in mice. What probably sounds more relevant to reduced EIU is the iNOS downregulation induced by XG-102 and documented at the mRNA level in ocular tissues and on immunohistochemistry in infiltrating cells. Indeed, the number of inflammatory infiltrating cells (ED1+ and PMNs) was...
decreased in treated animals, with no expression of iNOS in these cells. The pathogenesis of EIU has been shown to be related to the expression of iNOS in resident and inflammatory cells, and specific iNOS inhibitors have been proven to ameliorate cell infiltration and clinical signs of uveitis in different models. The iNOS has been shown to be regulated through the JNK pathway, and iNOS expression was inhibited by JNK inhibitors in macrophages in vitro. The reduced number of infiltrating cells in XG-102–treated eyes with EIU could also have resulted from several other mechanisms. Indeed, JNK inhibitors could act on the blood ocular barrier because it was recently shown that the JNK pathway controls tight-junction assembly in intestine epithelia.

Interestingly, we show also that intravitreal injection of XG-102 induced a trend to an increase of IL-13 at 24 hours in the ocular media, suggesting a switch toward a Th2 profile. We have previously shown that EIU was reduced by systemic and local intraocular injection of IL-13. The effect of IL-13 was dependent on the signaling activity of PKCζ. Indeed, intravitreal injection of IL-13 or a specific PKCζ inhibitory peptide (PKCζi) reduced the permeability of ocular vascular endothelial cells and limited inflammatory cell infiltration, together with an inhibition of PKCζ cleavage, and increased the expression of NF-kB. Treatment with PKCζi also decreased TNF-α, IL-6, and IL-2 at 6 hours; a lower level of IL-2; and a higher level of IL-13 at 24 hours.

XG-102 has been shown to exert beneficial effects, protecting against ischemic damage in animal models. It has also been extensively used locally in the ear to prevent traumatic or toxic hearing loss. Moreover, in a recent phase I/II study, XG-102

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**Figure 9.** Modulation of intraocular LPS-induced chemokine/cytokine profiles after IVT XG-102. Multiplex analysis was performed on ocular fluids collected 6 hours, 24 hours, and 48 hours after EIU induction. Comparison was made between rats treated by IVT injection of vehicle and XG-102 (n = 10 eyes analyzed per time point and per condition). P-values of statistical analysis are indicated on each graph. No significant changes were observed on chemokine expression between vehicle IVT or XG-102 IVT injection except a decrease of MCP-1 (A). Few changes in cytokine expression were induced by IVT injection of XG-102, as follows: lower levels of TNF-α, IL-6, and IL-2 at 6 hours; a lower level of IL-2; and a higher level of IL-13 at 24 hours (B).
was administered in the tympan of patients with acute acoustic trauma, demonstrating the feasibility of local XG-102 therapy.11

This exploratory study was designed to evaluate the potential of XG-102 in a model of intraocular inflammation. Because XG-102 has already been used in patients, is well distributed in ocular tissues after local IVT injection, and seems as effective as dexamethasone in EIU in rats, it can be considered a promising local treatment for intraocular inflammation without the undesirable side effects of glucocorticoids. Further studies are, however, required to dissect more thoroughly the exact mechanisms of XG-102 effects on both resident and infiltrating cells.

References

Treatment of Rat Uveitis by a JNK Inhibitory Peptide 4693