Subconjunctival Injection of XG-102, a c-Jun N-Terminal Kinase Inhibitor Peptide, in the Treatment of Endotoxin-Induced Uveitis in Rats

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Abstract

Purpose: XG-102, a TAT-coupled dextrogyre peptide inhibiting the c-Jun N-terminal kinase, was shown efficient in the treatment of experimental uveitis. Preclinical studies are now performed to determine optimal XG-102 dose and route of administration in endotoxin-induced uveitis (EIU) in rats with the purpose of clinical study design.

Methods: EIU was induced in Lewis rats by lipopolysaccharides (LPS) injection. XG-102 was administered at the time of LPS challenge by intravenous (IV; 3.2, 35 or 355 μg/injection), intravitreal (IVT; 0.08, 0.2 or 2.2 μg/eye), or subconjunctival (SCJ; 0.2, 1.8 or 22 μg/eye) routes. Controls received either the vehicle (saline) or dexamethasone phosphate injections. Efficacy was assessed by clinical scoring, infiltrating cells count, and expression of inflammatory mediators [inducible nitric oxide synthase (iNOS), cytokine-induced neutrophil chemoattractant-1 (CINC-1)]. The effect of XG-102 on phosphorylation of c-Jun was evaluated by Western blot.

Results: XG-102 demonstrated a dose-dependent anti-inflammatory effect in EIU after IV and SCJ administrations. Respective doses of 35 and 1.8 μg were efficient as compared with the vehicle-injected controls, but only the highest doses, respectively 355 and 22 μg, were as efficient as dexamethasone phosphate. After IVT injections, the anti-inflammatory effect of XG-102 was clinically evaluated similar to the corticoid’s effect with all the tested doses. Regardless of the administration route, the lowest efficient doses of XG-102 significantly decreased the ration of phospho c-Jun/total c-Jun, reduced cells infiltration in the treated eyes, and significantly downregulated iNOS and CINC-1 expression in the retina.

Conclusion: These results confirm that XG-102 peptide has potential for treating intraocular inflammation. SCJ injection appears as a good compromise to provide a therapeutic effect while limiting side effects.

Introduction

Uveitis has an annual incidence of 52 cases per 100,000, resulting in a prevalence of about 115 per 100,000.1 Acute anterior uveitis (AAU) is, by far, the most common form of uveitis, representing around 80% of the cases. Its recurrent nature may result in secondary complications such as cataract, cystoid macular edema, glaucoma, and, ultimately, if untreated, it may lead to blindness.2,3 Current treatments for uveitis include corticosteroids,4 immunosuppressive agents such as tumor-necrosis factor-alpha (TNF-alpha) inhibitors and cyclosporine in corticosteroid-resistant cases, or chemotherapeutic agents such as cyclophosphamide in the most severe uveitis cases. For AAU, not associated with systemic diseases, local therapies are preferred. Corticosteroids show clear clinical benefits and are efficient to control most of AAU cases, but their chronic local use is associated with known sight-threatening side effects, such as glaucoma and cataract, while their systemic use similar to other immunosuppressive agents is associated with general side-effects.1,5 Given these restrictions, there is an obvious demand for the development of new local therapeutic strategies.6

Current clinical trials concern the development of sustained-release delivery systems such as corticosteroid intravitreal...
phosphate used as a positive control for anti-inflammatory activity on EIU. Clinical ocular inflammation was scored at 24 h after EIU induction. At this timepoint, retinal pigment epithelium (RPE)-choroid-sclera complexes and neuroretinas were carefully dissected from the enucleated eyes, snap frozen, and stored at −80°C until use, respectively, for the analysis of c-Jun phosphorylation stated by Western blot and for the evaluation of inflammatory mediators (iNOS and cytokine-induced neutrophil chemoattractant-1 (CINC-1)) expression by reverse transcription polymerase chain reaction (RT-PCR). Tissues were collected from eyes treated with XG-102, with the vehicle and noninjected eyes. Three eyes collected from these groups were kept to evaluate inflammatory cell infiltration in ocular tissues.

**Solutions and injections**

XG-102 peptide was produced and purified by Polypeptide Laboratories. Once lyophilized, XG-102 powder was dissolved in the vehicle (NaCl 0.9%, Versol: Aguettant) at the concentration of 5 mg/mL under sterile conditions, and stored at −80°C until use. For each experiment, XG-102 solutions were prepared extemporaneously at the required concentration in the vehicle. A fraction of each diluted solution was stored at −80°C until its assay by HPLC. A commercial solution of dexamethasone phosphate at 4 mg/mL (Soludecadron; Laboratoire Roussel) was used as a positive control.

In our previous study, the anti-inflammatory effect of XG-102 in EIU was demonstrated with an IV dose of 20 μg/kg (3.5 μg/rat weighing 175 mg) and IVT dose of 0.2 μg/eye. Based on these results, 3 doses were chosen as per route of administration. Three solutions were prepared at 32, 350, and 3,550 μg/mL for IV injections. For the local treatments, 3 dilutions per type of injections were prepared: XG-102 solutions at 17.43 and 430 μg/mL for IVT injections and solutions at 30, 360, and 4,400 μg/mL for SCJ injections.

For the systemic treatment evaluation, 100 μL of vehicle, dexamethasone phosphate (400 μg/injection), or XG-102 (3.2, 35 or 355 μg/injection) were injected in the tail vein with a 25-gauge needle connected to a 1 mL syringe (Becton Dickinson). For the local delivery study, 5 μL of vehicle dexamethasone phosphate (20 μg/eye) or XG-102 solutions were injected in the right eye, using a 30-gauge disposable needle (BD-microfine syringes; NM Medical). In the XG-102-treated groups, right eyes received 0.08, 0.2, or 2.2 μg of XG-102 through an IVT injection, and 0.2, 1.8, or 22 μg through an SCJ injection.

**Clinical assessment of EIU**

Clinical examinations were performed by slit-lamp microscopy at 24 h after EIU induction. The severity of uveitis was scored, in a masked manner, on a scale from 0 to 5 for each eye, as previously described. Grade 0, no inflammation; grade 1, minimal iris and conjunctival vasodilation but without the observation of flare or cells in the anterior chamber; grade 2, moderate iris and conjunctival vessel dilation but without evident flare or cells in the anterior chamber; grade 3, intense iris vessels dilation, flare, and fewer than 10 cells per slit-lamp field in the anterior chamber; grade 4, more severe clinical signs than grade 3, with cells in the anterior chamber, with or without the formation of hypopyon; grade 5, intense inflammatory...
reaction, fibrin formation in the anterior chamber, and total seclusion of the pupil.

**Western blot analysis of the phosphorylated form of c-Jun (phospho c-Jun)**

RPE-choroid-sclera complexes were snap frozen immediately after dissection and stored at −80°C until use. Tissues were homogenized in 500 µL of lysis buffer, consisting of MOPS SDS Running Buffer (Invitrogen) supplemented with a cocktail of proteases (Roche Diagnostics) and phosphatases (Sigma-Aldrich) inhibitors. After heating for 5 min at 100°C, equal amounts of proteins were separated by electrophoresis in a NuPAGE 4%–12% Bis-Tris gel (Invitrogen) using MOPS SDS Running Buffer. The bands obtained were then electroblotted onto a nitrocellulose membrane (Schleicher & Schuell BioScience). Blots were sequentially incubated with a rabbit anti-Phospho-c-Jun (Ser 63) primary antibody and an anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody according to the manufacturer’s instructions (Cell Signaling Technology). Bands were visualized using the ECL Western Blotting Detection Reagents Kit (Amersham Biosciences). Blots were then dehybridized and rehybridized successively with a mouse monoclonal anti c-Jun primary antibody (1:400; Santa Cruz Biotech) and an HRP-conjugated goat anti-mouse IgG secondary antibody (1:5,000; Santa Cruz Biotechnology). The relative band intensity for phospho c-Jun/total c-Jun was calculated using densitometry analysis (on ImageJ software).

**Evaluation of inflammatory cells infiltration using immuno-histochemistry**

For immunohistochemical analysis, eyeballs were enucleated, fixed for 1 h at room temperature in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, and rinsed overnight in PBS. After careful orientation, samples were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek) and stored at −80°C. Frozen 10 µm sections were collected on Superfrost slides (Gerhard Menzel) using a cryostat (CM 3050S; Leica). Frozen 10 µm

![Graph](image_url)

**FIG. 1.** Clinical evaluation of XG-102 efficacy in endotoxin-induced uveitis (EIU). Clinical scores [mean ± standard error mean (SEM) arbitrary units, a.u.] were evaluated at peak disease activity, at 24 h after injections of different doses of XG-102, using 3 administration routes: intravenous (IV, A; n ≥ 8 eyes per group), intravitreal (IVT, B; n ≥ 4 eyes per group), and subconjunctival (SCJ, C; n ≥ 5 eyes per group). Dexamethasone phosphate injections, IV (n = 6), IVT (n = 5), or SCJ (n = 5) were used as positive controls. Comparisons were made with vehicle using different routes (n = 26). Clinical signs of uveitis were significantly reduced after XG-102 administration: (A) IV injections of 35 µg (*), 355 µg (**); (B) IVT injections of 0.08 (**), 0.2 (**) and 2.2 µg (**); (C) SCJ injections of 1.8 µg (*) and 22 µg (**). No statistical difference (ns) was observed between the clinical scores of vehicle and all IVT-uninjected eyes and SCJ-uninjected eyes except the higher dose (SCJ 22 µg). No statistical difference (ns) was observed between the clinical scores of the IV-injected 355 µg, the SCJ-injected 22 µg, and all the IVT-injected doses and those of the dexamethasone phosphate groups for the respective routes of administration. *P < 0.05, **P < 0.01, and ***P < 0.001. ns, not significant.
sections collected at the optic nerve level were permeabilized with 0.1% Triton X-100 in PBS for 25 min. Specimens were rinsed and saturated for 1 h with 5% of goat serum. They were incubated overnight at 4°C with primary antibody: a 1:50 mouse monoclonal anti-macrostatin CD68 (clone ED1), directed against a cytoplasmic antigen in rat monocytes, macrophages, and dendritic cells (purchased from Serotec Ltd.). After washing, sections were incubated for 1 h at room temperature with a secondary Alexa Fluor 488 (green)-conjugated goat anti-mouse antibody. Nuclei were stained for 5 min with 4,6-diamidino-2-phenylindole solution (DAPI, 1:5,000; Sigma-Aldrich). Sections were mounted in PBS:glycerol (1:1) and examined with a fluorescence microscope Olympus BX51 (Rungis) coupled with a digital camera Olympus DP70. Polymorphonuclear (PMN) cells were identified by the shape of their nuclei stained with a digital camera Olympus DP70. Sections were stained for 5 min with 4',6-diamidino-2-phenylindole (DAPI, 1:5,000; Sigma-Aldrich). Sections were washed and saturated for 1 h with 5% of goat serum. The specimens were rinsed and saturated for 1 h with 5% of goat serum. The specimens were rinsed and saturated for 1 h with 5% of goat serum.

**Evaluation of inflammatory mediator expression (iNOS, CINC-1) using RT-PCR**

Selected doses of XG-102 were used to evaluate the expression of iNOS mRNA and CINC-1 mRNA in eyes (35 µg for IV injection, 0.2 µg for IVT, and 1.8 µg for SCJ injection). Total RNA was extracted from the neuroretinas stored at −80°C according to the manufacturer’s instructions (RNeasy mini kit; Qiagen). For each sample, 1 µg total RNA was readjusted according to the RNA optic density at 260 nm and transcribed in a total volume of 20 µL using Superscript II Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), iNOS, and CINC-1 cDNA were amplified in a total volume of 25 µL containing 2 µL of first-strand reaction product, 0.4 µM of each primer (sense and antisense), 0.4 µM dNTP Mix, 1.5 mM MgCl2, 1 × PCR buffer, and 2.5 U Taq DNA polymerase (Invitrogen). Specific primers for GAPDH (sense: 5'-ATGCCCCCATGTTTGTAGATG-3'; antisense: 5'-ATGGCATGGACTGTGGTCAT-3'), primers specific for CINC-1 (sense: 5'-GGGTGTCCCCAAAGTAATGA-3'; antisense: 5'-CAGAACGAGCAGTGCAACCA-3'), and specific primers for iNOS (sense: 5'-TTTCTCTCAAATGCCAAATCC-3'; antisense: 5'-TGTGTCTGCAGATGTCGTGAAAC-3') were obtained from Invitrogen. After an initial denaturation (1 min at 94°C), 34 to 37 PCR cycles of denaturation (30s at 94°C), annealing (1 min at 58°C for GAPDH, at 60°C for CINC, and at 52°C for iNOS), and elongation (1–2 min at 72°C) were performed on a Crocodile III (Appligene-Oncom). The final cycle was completed by 5 min of elongation at 72°C. PCR fragments (162 bp for GAPDH, 53 bp for CINC, and 657 bp for iNOS) were analyzed by 2.5% agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light. The relative bands intensity for CINC-1 and iNOS were calculated in comparison to that for GAPDH after densitometry analysis using ImageJ software.

**Statistical analysis**

Numerical results were expressed as mean±SEM and compared using the nonparametric Mann–Whitney U-test and a 1-way ANOVA test followed by Bonferroni multiple-comparisons when appropriate. P < 0.05 was considered significant.

**Results**

**XG-102 clinical effect on EIU using different routes of administration**

Clinical scores were evaluated at 24 h after EIU induction, which is the peak of inflammation in this model. Using either IV or SCJ injections (Fig. 1A, C), a dose-response effect was observed: The lowest doses (3.2 µg for IV and 0.2 µg for SCJ) were not efficient; increasing the dose induced increased anti-inflammatory effects. At the highest tested doses (355 µg IV, 2.2 µg IVT, and 22 µg SCJ), the observed anti-inflammatory effects of XG-102 were better or equivalent to dexamethasone phosphate, used as a positive control. The highest SCJ dose...
(22 µg) also significantly reduced inflammation in the contralateral uninjected eye, suggesting a systemic passage of XG-102 at a high dose. Using IVT injections, all tested doses significantly reduced intraocular inflammation with the same efficacy as dexamethasone phosphate but without dose-response effects, due to the fact that we may not have reached the lowest efficient dose using this route of administration (Fig. 1B).

**Effect of XG-102 on in vivo JNK phosphorylation**

Western blot analysis of the RPE-choroid-sclera complex collected at the peak of the disease demonstrated a reduced phosphorylation of c-Jun on Ser63 residue/total c-Jun without any changes in total c-Jun expression, using IV, SCJ, and IVT (Fig. 2). The densitometry analysis of Western blot showed a significant decrease of the phospho c-Jun/total c-Jun ratio in eyes treated with 35 µg XG-102 IV injection (0.596 ± 0.15 vs. 1.591 ± 0.24 in vehicle, \( P < 0.05 \), Fig. 2A), with 0.2 µg XG-102 IVT injection (0.425 ± 0.13 vs. 0.979 ± 0.02 in the uninjected eye, \( P < 0.05 \) and 1.102 ± 0.59 in the vehicle, \( P < 0.01 \)) and with 1.8 µg XG-102 SCJ injection (0.399 ± 0.10 vs. 0.931 ± 0.04 in the uninjected eye, \( P < 0.05 \) and 1.622 ± 0.09 in the vehicle, \( P < 0.001 \), Fig. 2C).

The significant reduction of phosphorylation of c-Jun/total c-Jun indicates a specific effect of XG-102 by the 3 tested administration routes. Using SCJ injection, the effect of XG-102 on c-Jun phosphorylation was also observed in the contralateral uninjected eye, indicating a systemic passage of XG-102. The effect was, however, significantly higher in the injected eye (\( P < 0.05 \)).

**FIG. 3.** Effect of XG-102 on polymorphonuclear (PMN) cell infiltration in ocular tissues. PMN leukocyte infiltration was evaluated at 24 h after EIU induction in eyes treated with 35 µg XG-102 IV, 0.2 µg XG-102 IVT, and 1.8 µg XG-102 SCJ injections on frozen ocular histological sections. (A) Average count number of PMNs (\( n \geq 5 \) eyes per group, 3 sections per eye). (B) Effect of XG-102 on ED1+ cells, PMNs stained with DAPI expression in lipopolysaccharides-induced uveitis. ED1 and DAPI labeling was analyzed by immuno-histochemistry on untreated (vehicle A–I) and treated (XG-102 J–R) SCJ uveitic rats (IV and IVT immuno-histochemistry stained not shown). The arrowheads (C, F, I) represent the magnification (c, f, i). Scale bar, 20 µm (A–I, M–O) and 50 µm (J–L, P–R). C, cornea; 1, lens; ONH, optic nerve head. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \).
Effect of XG-102 on ocular cell infiltration

Identified by the shape of their nuclei, stained with DAPI, PMN cells were quantified on histological sections of the ocular tissues. One day later, 35 μg XG-102 IV delivery, the PMN cells infiltration decreased by 40% compared with vehicle IV injection (308 ± 27 vs. 515 ± 71, Fig. 3A). Using IVT, 0.2 μg XG-102 delivery decreased by 83% the number of infiltrating cells as compared with the vehicle-injected eyes (87 ± 26 vs. 506 ± 118, Fig. 3A). XG-102 SCJ administration decreased by 75% the PMN infiltration in the eyes treated with 1.8 μg XG-102 as compared with the vehicle delivery (113 ± 34 vs. 445 ± 43, Fig. 3A). Infiltrating monocytes/macrophages cells were also labeled using ED1, and microphotographs were taken on the optic nerve head-level sections. As shown in Fig. 3B, while numerous ED1-positive cells were observed in the anterior segment and at the optic nerve head in the control EIU group, it was greatly...
reduced in eyes receiving an SCJ injection of XG-102 (Fig. 3B). The reduction of ED1-positive cells was also observed in eyes treated by the other routes of XG-102 administration (not shown). In EIU, the majority of infiltrating cells are PMN cells (Fig. 3); we, therefore, did not count ED1-positive cells and only used PMN quantification (Fig. 3A).

Expression of pro-inflammatory mediators

To confirm the effect of XG-102 on the recruitment of inflammatory cells, the expression of CINC-1, a mediator of neutrophil infiltration, was evaluated in the retina. Along with all routes of administration, XG-102 significantly reduced the expression of CINC-1 as compared with the control ($P<0.05$ for all comparisons) and did not significantly reduce its expression in the contralateral eye after IVT or SCJ injection (not shown). As compared with the control group, the IV injection of $35 \mu g$ XG-102 reduced the CINC-1 expression from $0.819\pm0.23$ to $0.205\pm0.06$ (Fig. 4A). The decrease of CINC-1 mRNA was also observed after IVT injection of $0.2 \mu g$ XG-102, from $0.819\pm0.23$ to $0.202\pm0.06$ as compared with the vehicle-injected eyes (Fig. 4B). The SCJ injection of $1.8 \mu g$ XG-102 led to a downregulation of CINC-1, from $0.819\pm0.23$ to $0.18\pm0.03$ as compared with the vehicle (Fig. 4C). These results go hand in hand with the decrease in PMNs infiltration in the eyes treated with $35 \mu g$XG-102 IV, $0.2 \mu g$XG-102 IVT, and $1.8 \mu g$XG-102 SCJ injections (Fig. 3).

A significant reduction of iNOS expression was observed in eyes treated with a local (IVT and SCJ) injection of XG-102, but the reduction was not significant in eyes treated with $35 \mu g$XG-102 IV. In comparison with the vehicle-injected eyes, IV injections of $35 \mu g$XG-102 led to a downregulation of iNOS mRNA from $0.826\pm0.20$ to $0.390\pm0.21$ (53% reduction, $P>0.05$, Fig. 5A), IVT injections of $0.2 \mu g$XG-102 reduced iNOS expression from $0.912\pm0.12$ to $0.023\pm0.01$ (97% reduction, $P<0.01$, Fig. 5B), and SCJ injections of $1.8 \mu g$XG-102 from $0.74\pm0.20$ to $0.100\pm0.06$ (86% reduction, $P<0.05$, Fig. 5C).

Discussion

The JNK pathway is activated in inflammatory reactions in various tissues of the body, particularly in the central nervous and digestive systems. Its specific inhibition by XG-102 blocks the phosphorylation of its nuclear target, c-Jun, which prevents the transcription of pro-inflammatory cytokines/chemokines and the activation of the harmful intracellular processes such as apoptosis. In this study, we confirmed that XG-102 exerts anti-inflammatory effects on EIU using IV and IVT. In this experiment, the uveitis score in the control eyes was very high (4.5), explaining that the lowest IV XG-102 dose (3.2 $\mu g$) was not efficient, in contrast to the previous experiments where uveitis was less severe in the control group. This dose finding will guide appropriate choice for further clinical applications, knowing that intraocular inflammation intensity varies in patients. Interestingly, the SCJ injection of XG-102 also significantly reduced the uveitis clinical score without effect on the contralateral eye at the dose of 1.8 $\mu g$, demonstrating the potential for a local penetration of the peptide. Using the IVT route, the lowest dose, that is, 0.08 $\mu g$ was as effective as higher doses, indicating that the lowest efficient dose has not yet been reached. For all administration routes, the effect of XG-102 was associated with a significant reduction of c-Jun phosphorylation as compared with total c-Jun, demonstrating its specific mode of action.

The clinical anti-inflammatory activity was confirmed by reduced cell infiltration in ocular tissues and decreased expression of iNOS and CINC-1 using a specific dose for each tested route of administration. However, iNOS was not significantly downregulated by IV injection, and seems less efficient than local administration routes. For clinical use, an SCJ injection of a D-peptide is particularly interesting. This route of administration is minimally invasive, with no risk of possible infection associated with IVT injections. Moreover, XG-102 has a long half life in vivo due to its resistance from proteases, limiting the need for re-injections. After an SCJ injection of 22 $\mu g$ XG-102, the uninjected contralateral eye also showed reduced inflammation, suggesting a systemic diffusion of XG-102 after SCJ injection in the treated eye. However, at a lower dose of 1.8 $\mu g$, no effect was observed in the contralateral eye, suggesting a direct transscleral passage of XG-102 after SCJ injection without systemic diffusion. XG-102 is a small peptide with a molecular weight of around 2.2 kDa adapted for trans-scleral diffusion. Indeed, previous works have shown that hydrophilic molecules of approximately 150 kDa were able to cross the sclera, with the scleral permeability being inversely proportional to the molecular weight. SCJ injections are rarely used and no drug is approved for such injections. However, studies comparing the ocula media levels of dexamethasone phosphate after oral administration, latero bulbar, or SCJ injections in humans found that the highest concentrations in aqueous and vitreous humors were achieved after SCJ injections. For a long-lasting drug, such as XG-102, a single SCJ injection could be a simple alternative to repeated instillations, ensuring optimal compliance and surface tolerance.

This is to our knowledge the first study evaluating the effect of SCJ injection of a peptide for the treatment of uveitis. A phase Ib clinical trial (EudraCT No. 2011-000171-14) has been performed to evaluate the safety and tolerance of SCJ XG-102, showing a good tolerance (submitted for publication).

Conclusion

If clinical results validate its efficacy, XG-102 could be considered a promising drug for many ocular diseases associated with intraocular inflammation and/or excitotoxicity.

Author Disclosure Statement

No competing financial interests exist.

References


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