Antiviral Drug Ganciclovir Is a Potent Inhibitor of the Proliferation of Müller Glia–Derived Progenitors During Zebrafish Retinal Regeneration

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PURPOSE. The purpose of this study was to investigate the effect of the antiviral drug ganciclovir (GCV) on Müller glia dedifferentiation and proliferation and the underlying cellular and molecular mechanisms in adult zebrafish.

METHODS. A Tg(1016tuba1a:GFP) transgenic line was generated to identify injury-induced dedifferentiation of Müller glia. Mechanical retinal damage was induced by a needle-poke injury on the back of the eyes in adult zebrafish. Phosphate-buffered saline or GCV was injected into the vitreous of the eye at the time of injury or through the cornea. The GCV clearance rate from the eye was determined by a reversed-phase HPLC method. Green fluorescent protein (GFP) and bromodeoxyuridine (BrdU) immunofluorescence were used to determine the effect of GCV on retinal regeneration. Cell apoptosis was evaluated by TUNEL staining. Microglia were labeled by vitreous injection of isoelectin IB4 conjugates. Quantitative (q)PCR and Western blot analysis were used to determine gene expression in the retina.

RESULTS. Ganciclovir treatment significantly reduced the number of BrdU+ Müller glia–derived progenitor cells (MGPCs) at 4 days post injury. Further analysis showed that GCV had no impact on Müller glia dedifferentiation and the initial formation of MGPCs. Our data indicate that GCV reversibly inhibited MGPC proliferation likely through a p53-p21WAF1-dependent pathway. Interestingly, unlike control cells, GCV-treated Müller glia cells were “locked” in a prolonged dedifferentiated state.

CONCLUSIONS. Our study uncovered a novel inhibitory effect of GCV on MGPC proliferation and suggests its potential use as a tool to uncover molecular mechanisms underlying retinal regeneration in zebrafish.

Keywords: ganciclovir, zebrafish, retinal regeneration, Müller glia, cell proliferation
exception is the transcriptional factors Pax6a and Pax6b, which are reported to be required only for MGPC proliferation.18

Ganciclovir (GCV), a synthetic analogue of 2’-deoxyguanosine, was developed in the 1970s as an antiviral drug and is currently used to treat cytomegalovirus (CMV) and herpes simplex virus (HSV) infections.19–21 Ganciclovir itself is a nontoxic prodrug, which can be phosphorylated into GCV monophosphate by thymidine kinase (TK) from the Herpesviridae family, including CMV, HSV, or Epstein-Barr virus.22,23 Ganciclovir monophosphate is further converted by normal cellular enzymes into GCV triphosphate, which is a toxic compound that inhibits viral DNA polymerase and DNA chain elongation.22,23 Besides its canonical antiviral activity, GCV has also been used in research studies to ablate cells genetically modified to express HSV-TK.24–26 Recently it was reported that GCV itself is a potent inhibitor of microglia activation and proliferation in a mouse model of CNS inflammation, though its underlying mechanism was unclear.27

We previously found that intravitreous administration of GCV inhibited retinal regeneration in zebrafish (Xu H, Goldman D, unpublished, 2012). Here we report an unexpected finding that GCV irreversibly inhibited the expansion of MGPCs, but did not inhibit Müller glia dedifferentiation and cell division. Furthermore, we show that GCV treatment resulted in elevated expression of p55 and p21/19, which may underlie the cell cycle arrest of MGPCs. Overall, our study revealed a novel inhibitory effect of the classic antiviral drug GCV on retinal regeneration in adult zebrafish. Our study also suggests GCV as a tool to uncover the mechanisms controlling the transition of dedifferentiation to rapid progenitor proliferation during retinal regeneration.

**Materials and Methods**

**Animals and Eye Injury**

The animals used in this study were treated in accordance with the guidelines for animal use and care at Nantong University, as well as the Guide for the Care and Use of Laboratory Animals. Animal treatment was in adherence to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Zebrafish were kept at 28.5°C in a 14-hour/10-hour light/dark cycle. Retinal injuries were performed as described previously.4 Briefly, fish were anesthetized and the right retina was poked four times, once in each quadrant, with a 30-gauge needle. The needle was inserted through the sclera to the length of the bevel (~0.5 mm).

**Generation of the Tg(1016tuba1a:GFP) Transgenic Lines**

The plasmid for making the Tg(1016tuba1a:GFP) transgenic line was generated using the MultiSite Gateway cloning system (Life Technologies, Carlsbad, CA, USA). A 1016-bp goldfish tuba1a regulatory element9 was subcloned into the pSE-MCS vector to generate the pSE-tuba1a 5’ entry vector. The pSE-tuba1a plasmid and the middle-entry plasmid (containing the coding sequence of green fluorescent protein [GFP]) were then cloned into a destination vector (pDestTo2p2a2) using the Tol2-based Gateway system. This transgene plasmid DNA (30 pg) and transposase RNA (20 pg) were coinjected into 1-cell stage zebrafish embryos. Injected embryos with GFP expression were selected and raised, and stable transgenic lines with retinal GFP expression at the injury site were generated and validated.

**Drug Delivery, Microglia Labeling, and BrdU Incorporation**

Ganciclovir sodium (Santa Cruz Biotechnology, Dallas, TX, USA) was dissolved in PBS at indicated concentrations; 1 μL PBS or GCV was then delivered at the time of injury using the same needle to poke the retina or was injected intravitreally at the indicated time. Intravitreous injection was performed through the front of the eye with a 30-gauge beveled needle attached to a Hamilton syringe (Hamilton Robotics, Ren, NV, USA), and care was taken not to damage the retina or the lens. To label microglia, 1 μL 1 mg/ml isoleucin GS-BI4 (isoleucin GS-BI4 from Griffonia simplicifolia, Alexa Fluor 568 conjugate; Thermo Fisher Scientific, Waltham, MA, USA) was injected intravitreally through the front of the eye 1 day before the fish were killed. For bromodeoxyuridine (BrdU) incorporation, 20 μL 20 mM BrdU solution was injected intraperitoneally into the anesthetized fish.

**HPLC Detection of the GCV Levels in the Eye**

To determine the clearance rate of injected GCV in the eye, an extraction and reversed-phase high-performance liquid chromatography (HPLC) method was used as previously described.28 Briefly, eyes were homogenized in 100 μL PBS after PBS or GCV injection, and 100 μL 50% trichloroacetic acid was then added to the homogenate. After shaking for 30 seconds, the deproteinized samples were centrifuged at 2000g for 10 minutes. The supernatant was transferred to a new tube and neutralized with 50 μL 2 M NaOH. The tube was vortexed for 10 seconds and then extraction was performed with 5 mL chloroform. Aliquots of the aqueous phase (400 μL) were mixed with 40 μL 1 M NaH2PO4 and 0.4 M triethylamine solution, and 30 μL per sample was used for HPLC analysis. High-performance liquid chromatography analyses were performed on a Waters 2695 HPLC system (Milford, MA, USA) equipped with photodiode array detector, auto-sampler, a quaternary pump, online degasser, and column oven. Separation was performed on a Waters Symmetry300 C 8 column (5.0 μm, 4.6 × 250 mm) maintained at 25 ± 2°C at a flow rate of 1 mL/min and a 10-μL sample injection. The detector wavelength was set at 254 nm. The eluent consisted of 95% (vol/vol) water, and 5% (vol/vol) methanol was used in the isocratic elution program.

**RT-PCR and Quantitative PCR**

Retinas were dissected and total RNA was extracted using the TRIzol reagent (Invitrogen). RNA (1 μg) was reverse transcribed into cDNA by the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany) according to the manufacturer’s instructions. Primers for quantitative PCR (qPCR) are listed in the Table. Quantitative PCR was carried out in triplicate using the FastStart Universal SYBR Green Master Mix (Roche Applied Science) on a real-time PCR detection system (CFX96TM Real-Time System; Bio-Rad, Hercules, CA, USA).

**Tissue Preparation and Immunofluorescence**

Fish were overdosed with tricaine. The eyes were dissected and fixed in 4% paraformaldehyde at 4°C overnight. Fixed samples were prepared for immunofluorescence as previously described.10 Primary antibodies used for immunofluorescence included rabbit anti-GFP (1:1000, Life Technologies), rat anti-BrdU (1:500; Abcam, Cambridge, MA, USA), and rabbit anti-
PCNA (1: 500; GeneTex, Irvine, CA, USA). For BrdU staining, sections were first treated with 2 N HCl at 37°C for 25 minutes, rinsed in 0.1 M sodium borate (pH 8.5) for 10 minutes, and then processed using standard procedures.

**Quantification of the Number of BrdU+ Cells at the Injury Site**

Green fluorescent protein and BrdU immunofluorescence were performed on retinal cryosections, and each injury-responsive zone (4 injuries per retina in total) could be clearly distinguished from the others with the help of GFP and BrdU responsive zone (4 injuries per retina in total) could be clearly visible in the INL near the injury site after GCV injection (Fig. 1A). Importantly, the number of TUNEL+ cells at the injury site after GCV treatment was comparable to that of the PBS-treated control, suggesting that GCV did not stimulate cell apoptosis at the site of injury (Fig. 1A). These results indicate that GCV significantly decreased the number of proliferating MGPCs and therefore inhibited retinal regeneration in adult zebrafish.

### GCV Treatment Significantly Decreased the Number of Proliferating MGPCs After Retinal Injury

To evaluate the effects of GCV on zebrafish retinal regeneration, GCV (12–50 μg/eye) or PBS was injected once daily into the vitreous of the eye after retinal injury. Because almost all of the BrdU-labeled cells in the INL after retinal injury are derived from Müller glia,4 BrdU-positive cells in the INL represent injury-induced MGPCs. Incorporation of BrdU could thus be used as an excellent indicator of retinal regeneration. It has been shown that the proliferation of MGPCs reaches a peak at 4 dpi in this mechanical injury model.4 We therefore gave the fish a pulse of BrdU 3 hours before they were killed at 4 dpi, and examined the status of regeneration on retinal cryosections. Immunofluorescence showed that GCV treatment significantly decreased the number of BrdU+ cells in the INL after retinal injury in a dose-dependent manner (Figs. 1A, 1B). In the control retina at 4 dpi, clusters of BrdU+ nuclei with an elongated morphology were clearly visible in the INL near the site of injury (Fig. 1A). In contrast, many fewer BrdU+ cells could be seen in the GCV-injected retina (Fig. 1A). Importantly, the number of TUNEL+ cells at the injury site after GCV treatment was comparable to that of the PBS-treated control, suggesting that GCV did not stimulate cell apoptosis at the site of injury (Fig. 1A). These results indicate that GCV significantly decreased the number of proliferating MGPCs and therefore inhibited retinal regeneration in adult zebrafish.

### GCV Had No Effect on Müller Glia Dedifferentiation

The number of proliferating MGPCs at the injury site observed at 4 dpi could be affected by several cellular events during retinal regeneration, including the activation and dedifferentiation of Müller glia, their asymmetric cell division, and the formation of MGPCs, as well as their later expansion. To investigate whether the observed effect of GCV is due to inhibition of Müller glia activation and dedifferentiation, we generated a transgenic line Tg(1016tuba1a:GFP) with the same promoter as described previously to identify these dedifferentiated Müller glia.5 In this line, a fragment of the α1 tubulin promoter drove GFP expression specifically in dedifferentiated Müller glia and MGPCs at the injury site (Supplementary Figs. S1C, S1D, Fig. 1A), indicating this line as a valuable tool for analyzing Müller glia dedifferentiation and retinal regeneration.

In the injured retina of the Tg(1016tuba1a:GFP) fish, each dedifferentiated Müller glia and its daughter MGPCs displayed a green column-like shape and are referred as a GFP+ column.29
The GFP transgene expression and the number of GFP\(^+\) columns per injury are thus good indicators of Müller glia activation and dedifferentiation. At 4 dpi, confocal microscopy showed that although GCV-treated retina exhibited many fewer BrdU\(^+\) MGPCs than PBS-treated control, GFP\(^+\) Müller glia identified by their radial morphology were still present at the site of injury (Fig. 2A). Importantly, the number of dedifferentiated Müller glia at the injury sites in GCV-injected eyes determined by the number of GFP\(^+\) columns was comparable to that of control (Figs. 2A, 2B; arrows indicate GFP\(^+\) columns). We also examined these Müller glia cells at 2 dpi, when they were dedifferentiated and began to go through the first division.\(^4\) Immunofluorescence showed the presence of GFP\(^+\) Müller glia at the site of injury in both control and GCV-injected retina (Fig. 2C). Together, these data indicate that GCV treatment had no effect on Müller glia activation and dedifferentiation.

**GCV Inhibited the Proliferation of MGPCs**

The above results suggest that GCV may inhibit retinal regeneration at a later stage, such as the division of Müller glia that generates the first group of MGPCs, or subsequent MGPC expansion. We therefore first investigated whether GCV inhibited the initial formation of MGPCs by examining the number of BrdU\(^+\) cells in the INL at 2 dpi when MGPC formation was just beginning.\(^4\) Surprisingly, GCV treatment had no effect on the number of BrdU\(^+\) cells in the INL at 2 dpi (Figs. 2C, 2D), suggesting that GCV did not inhibit Müller glia division and the initial MGPC formation.

We next investigated the effect of GCV on MGPC proliferation. For this purpose, we labeled a population of proliferating MGPCs with a pulse of BrdU at 45 to 48 hours post injury (hpi) in wild-type zebrafish. Fish then received intravitreous PBS or GCV injection once daily until 4 dpi. To trace the progeny of these labeled MGPCs, retinas were collected and examined at 2, 3, and 4 dpi (Fig. 3A). In the control retina, a rapid expansion of the BrdU-labeled MGPCs could be observed from 2 to 4 dpi (Figs. 3B, 3C). In contrast, the expansion of MGPCs in the GCV-treated group occurred at a significantly lower rate (Figs. 3B, 3C). These data indicate that GCV blocked retinal regeneration by inhibiting MGPC proliferation.

The Inhibitory Effect of GCV on MGPC Proliferation Was Irreversible

As we observed a strong inhibitory effect of GCV on MGPC proliferation at 4 dpi, we asked whether MGPCs could recover from this inhibition when GCV was removed from the eye. To determine the clearance rate of GCV from the eye of adult zebrafish, 1 \(\mu\)L GCV was injected into the vitreous. At 0, 1, and 2 hours after the injection, injected eyes were collected and homogenized in PBS. Ganciclovir was then extracted and its concentration was determined by HPLC as described previously.\(^2\) Our results showed that GCV was rapidly removed from the eye after vitreous injection (Supplementary Figs. S2A–D), and its half-life in the eye is between 1 and 2 hours (Supplementary Fig. S2E).

We next performed a washout experiment to determine if MGPCs could recover from GCV treatment. Fish received intravitreous PBS or GCV injection once daily for 4 days, and then received PBS injection once daily to further wash out GCV for another 4 days (Fig. 4A). Because the half-life of GCV in the...
eye is 1 to 2 hours, the 4-day washout experiment is sufficient to eliminate the drug from the eye and also provides enough time for MGPCs to recover. The number of proliferating MGPCs was determined by BrdU incorporation at 4, 6, and 8 dpi. Immunofluorescence of BrdU showed that the number of BrdU-positive cells in the INL at 6 and 8 dpi was not significantly different from that at 4 dpi, suggesting that the inhibitory effect of GCV on MGPC proliferation was irreversible (Fig. 4A). Indeed, a single intravitreal GCV injection at 1 or 2 dpi was sufficient to inhibit retinal regeneration, determined by the number of BrdU-positive cells in the INL at 4 dpi (Fig. 4B). Together, these data indicate that the impact of GCV on MGPC proliferation was strong and irreversible.

Microglia Activation and Accumulation at the Injury Site Were Not Affected by GCV

Previous studies have shown that after retinal injury, phagocytic microglia accumulate at the site of injury in adult zebrafish. Interestingly, a recent study reported that GCV itself is a potent inhibitor of microglia proliferation and activation in a mouse model of CNS neuroinflammation, although the underlying mechanism remains unknown. As microglia accumulation and neuroinflammation may play a role in retinal regeneration in zebrafish, we investigated the effect of GCV on microglia accumulation and proliferation after retinal injury at 2 to 3 dpi. Consistent with the previous study, no proliferating microglia were observed in the retina (Figs. 5A–D). Importantly, the number of microglia at the injury site in GCV-treated retina was comparable to that of control (Figs. 5A–E), suggesting that the inhibitory effect of GCV on retinal regeneration was independent of microglia.

Induction of p53 and p21<sup>cip1</sup> by GCV

The inhibitory effect of GCV on MGPC proliferation prompted us to examine the expression of cell cycle–related genes after GCV injection. Quantitative PCR analysis showed that GCV significantly decreased the expression of cyclin A2, cyclin B1, and cyclin D1 but had no effect on cyclin E1 at 3 dpi (Fig. 6A). Ganciclovir also significantly decreased the expression of Cdk1 and Cdk2 (Fig. 6A). Strikingly, high induction of a Cdk-dependent inhibitor p21<sup>cip1</sup> (gene CDKN1A, Fig. 6B) was found after GCV treatment at 3 dpi. Ganciclovir had no effect on the mRNA level of p27<sup>kip1</sup> and p57<sup>kip2</sup> (Supplementary Fig. S3A). Since it is well known that p21<sup>cip1</sup> can be directly regulated by the tumor suppressor p53, we next examined the expression of p53 by qPCR and Western blot analysis. Indeed, GCV induced a small but significant increase of p53 at both the mRNA and protein level at 3 dpi (Figs. 6C, 6D). We also examined the expression of cell cycle genes at 2 dpi, when...
GCV has not yet had any observable effect on MGPC proliferation. At this time point, we observed a significant induction of p21cip1 and p53 (Figs. 6B, 6C), whereas the expression of most other cell cycle–related genes was comparable to that of control except for cyclin A2 (Supplementary Fig. S3B). Therefore, induction of p53 and p21cip1 was an early event after GCV treatment. Since p21cip1 is a well-known cell cycle inhibitor, our data are consistent with the idea that the induction of p53 and p21cip1 by GCV caused a cell cycle arrest and thus inhibited MGPC proliferation.

It has been shown that the transcription factors Pax6a and Pax6b are required only for MGPC proliferation. At this time point, we observed a significant induction of p21cip1 and p53 (Figs. 6B, 6C), whereas the expression of most other cell cycle–related genes was comparable to that of control except for cyclin A2 (Supplementary Fig. S3B). Therefore, induction of p53 and p21cip1 was an early event after GCV treatment. Since p21cip1 is a well-known cell cycle inhibitor, our data are consistent with the idea that the induction of p53 and p21cip1 by GCV caused a cell cycle arrest and thus inhibited MGPC proliferation.

Postmitotic Müller Glia Remained in a Dedifferentiated State After GCV Treatment

After the proliferation of MGPCs reaches its highest level at 4 dpi, dedifferentiated Müller glia downregulate the expression of regeneration-associated genes (RAGs) and gradually return to their preinjury status after 7 dpi.10 However, the exact mechanism regulating this transition remains unknown. Examining PBS- or GCV-treated retina at 8 and 12 dpi, we had an unexpected finding—that in contrast to control Müller glia, which had almost completely turned off GFP transgene expression, Müller glia cells in GCV-treated retina still exhibited strong GFP fluorescence at these late time points (Fig. 7A). Immunofluorescence of BrdU showed that these GFPþ Müller glia were not proliferating (Fig. 7A). Because the GFP transgene is a marker for dedifferentiated Müller glia, this...
suggests that Müller glia in GCV-treated retina at 8 and 12 dpi were still dedifferentiated. Consistent with this notion, qPCR analysis showed that the expression of gfp and several RAGs in GCV-treated retina was significantly higher than that of PBS-treated control at 12 dpi (Fig. 7B). Importantly, Müller glia in GCV-treated retina maintained a relatively stable and high level of expression of gfp and several RAGs from 4 to 12 dpi (Fig. 7C). These data indicate that instead of returning to a preinjury status, Müller glia in GCV-treated retina were “locked” in a prolonged dedifferentiated state.

DISCUSSION

Ganciclovir, an antiviral nucleoside analogue, was traditionally used in therapies against CMV and HSV infections. In this study, we investigated the novel effect of GCV on retinal regeneration
FIGURE 6. Induction of p53 and p21<sup>cip1</sup> after GCV administration. (A) qPCR shows that GCV treatment inhibits the expression of <i>ccna2</i>, <i>ccnb1</i>, <i>ccnd1</i>, <i>cdk1</i>, and <i>cdk2</i> significantly at 3 dpi. (B) Ganciclovir treatment increases the expression of p21<sup>cip1</sup> at 2 and 3 dpi significantly. (C) Ganciclovir significantly increases the expression level of p53 mRNA at 2 and 3 dpi. (D) Western blot analysis shows elevated expression level of the p53 protein in GCV-treated retina compared to that of control at 3 dpi. <i>n</i> = 3 for each group. *<i>P</i> < 0.05 compared to PBS control; **<i>P</i> < 0.01 compared to PBS control.

FIGURE 7. Müller glia remained in a dedifferentiated state after GCV treatment. (A) Green fluorescent protein and BrdU immunofluorescence showing the presence of strong GFP⁺ Müller glia at the injury site in GCV-treated retina at 8 and 12 dpi. Only very weakly GFP-expressing cells were found in the PBS-treated control. <i>Tg(1016tuba1a:GFP)</i> fish were given a pulse of BrdU 3 hours before they were killed at 8 or 12 dpi. (B) qPCR analysis of the expression of <i>gfp</i>, <i>ascl1a</i>, <i>hbegfa</i>, and <i>socs3a</i> in PBS- or GCV-treated retinas at 12 dpi. (C) qPCR analysis of the same genes in GCV-treated retinas at 0, 4, 8, and 12 dpi. <i>n</i> = 4 for each group. **<i>P</i> < 0.01; ***<i>P</i> < 0.001 compared to PBS control. Scale bars: 100 µm. The asterisks mark the injury site (needle poke).
GCV Inhibits MGPC Proliferation

References


