Effects of Fluorouracil and Fluorouridine on Protein Synthesis in Rabbit Retina


5-Fluorouracil (5-FU) and its active metabolite 5-fluorouridine (FUR) are currently being evaluated for the treatment of proliferative vitreoretinopathy and the control of scarring after glaucoma filtering procedures. To test for retinal toxicity, the authors examined the effect of intravitreal injections of 5-FU and FUR on protein synthesis in rabbit retinal photoreceptors and ganglion cells. In addition, the toxic effect of subconjunctival 5-FU injections, after a trephine filtering procedure, on ganglion cell protein synthesis was examined. Albino rabbit eyes were given either unilateral intravitreal injections of 1 mg of 5-FU, 2.5 mg of 5-FU, or 0.1 mg of FUR, or subconjunctival injections of 3 mg of 5-FU twice daily after a trephine procedure. Quantitative autoradiography was used to study ganglion cells and photoreceptor outer segment renewal, and scintillation counting was used to quantify newly synthesized protein transported axonally from ganglion cell bodies to the superior colliculus (SC). Marked reduction of labeled protein reaching the SC was noted after either intravitreal 0.1 mg of FUR (41% inhibition after a single injection and 53% after two injections) or 2.5 mg of 5-FU (41% after one injection and 26% after two injections). This reduction was still present after 8 days in eyes receiving 0.1 mg of FUR (32%) and 2.5 mg of 5-FU (22%). Quantitative autoradiography of retinal photoreceptors and ganglion cells corroborated these data, demonstrating inhibition of outer segment renewal after one or two injections of either 0.1 mg of FUR or 2.5 mg of 5-FU. This inhibitory effect was statistically significant using the paired t-test for both drugs. No mean inhibition was observed after intravitreal 1 mg of 5-FU injections or after subconjunctival injections of 5-FU.

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The drug 5-fluorouracil (5-FU) is potent antimetabolite that inhibits intraocular fibroblast and retinal pigment epithelium proliferation and contraction in animal models and in vitro. Currently 5-FU is under evaluation for treatment of proliferative vitreoretinopathy (PVR), and it was found to significantly reduce the incidence of traction retinal detachment (TRD) produced by injection of cultured fibroblasts into the vitreous chamber of animal eyes. A dose of 1 mg of 5-FU injected intravitreally reduced TRD in nonvitrectomized rabbit eyes.1,2,5 Multiple intravitreal injections of 0.5 mg of 5-FU into vitrectomized rabbit eyes have also been efficacious against PVR.3 A pilot study on patients with PVR demonstrated a moderate success rate of 65% for retinal reattachment after intravitreal 5-FU.10 When applied topically or injected subconjunctivally, 5-FU also inhibits postoperative scarring after glaucoma filtration procedures in animal models and in initial clinical studies.12-15 It is currently being evaluated in a multicenter clinical trial.

The 5-FU alters DNA synthesis through inhibition of the enzyme thymidylate synthetase and decreases RNA function secondary to incorporation of 5-FU metabolites into newly synthesized RNA.16 The antiproliferative and anticontractile effects of 5-FU are probably due to production of altered RNA.17 Fluorouridine (FUR) is the key 5-FU metabolite that affects RNA synthesis and is up to 100 times more effective than 5-FU in inhibiting fibroblast proliferation in vitro.18

Toxicity of 5-FU and FUR has been assessed by use of light and electron microscopy and by electrophysiology. We evaluated retinal toxicity of intravitreal and subconjunctival injection of 5-FU and FUR in rabbits. Since ganglion cells and photoreceptors have high rates of protein synthesis, we measured the effects of these drugs on protein synthesis and axonal transport in retinal ganglion cells and on outer segment (OS) renewal in photoreceptors.

Materials and Methods

Fifty-three albino rabbits (2-3 kg) were used for this study. This study was conducted in accordance
with the ARVO Resolution on the Use of Animals in Research. The eyes of 24 rabbits were anesthetized topically with proparacaine 0.5%, and the right eyes (OD) received two intravitreal injections 24 hrs apart of 1.0 mg of 5-FU, 2.5 mg of 5-FU, or 0.1 mg of FUR, diluted in 0.1 ml of 0.9% saline, doses previously thought to be nontoxic to rabbit or primate retinas.\(^\text{10,18,19}\) The needle tip was positioned just anterior to the optic disc in the manner of Orcutt et al.\(^\text{20}\) A second group of seven rabbits underwent a bilateral Elliot trephine filtering procedure after sedation with intramuscular ketamine and Rompun. A 1.0-mm trephine was used, straddling the limbus, followed by conjunctival coverage with a fornix-based flap. These rabbits received subconjunctival injections OD of 3 mg of 5-FU in 0.3 ml of 0.9% saline 180° from the surgery wound. These injections were repeated twice daily for 7 days, according to the method of Gressel et al.,\(^\text{11}\) using intramuscular ketamine and Rompun for anesthesia. Left eyes served as controls in all rabbits and received equivalent volumes of saline injected intravitreally or subconjunctivally. Twenty-four hours after the last drug or saline injection, all eyes were injected intravitreally with 150 \(^\text{3}\)H-leucine (New England Nuclear). A third group of 22 rabbits received only one injection of 2.5 mg of 5-FU or 0.1 mg of FUR into the midvitreous cavity OD under ophthalmoscopic control. Again, the left eye served as a control and received an equivalent volume of saline injected intravitreally. After drug exposure times of 1 day for 10 rabbits and 8 days for 12 rabbits, all eyes were injected intravitreally with \(^\text{3}\)H-leucine. All rabbits showing evidence of leakage from the injection site were excluded from the study.

**Protein Synthesis in Ganglion Cells**

Quantitative light microscopic autoradiography was used to determine the effects of 5-FU and FUR on protein synthesis in ganglion cells of the rabbit retina.\(^\text{21}\) Four hours after intravitreal injection of \(^\text{3}\)H-leucine, nine rabbits from the first group were killed with an overdose of sodium thiopental; the eyes were immediately removed and fixed in 4% paraformaldehyde (New England Nuclear). A third group of 22 rabbits received only one injection of 2.5 mg of 5-FU or 0.1 mg of FUR into the midvitreous cavity OD under ophthalmoscopic control. Again, the left eye served as a control and received an equivalent volume of saline injected intravitreally. After drug exposure times of 1 day for 10 rabbits and 8 days for 12 rabbits, all eyes were injected intravitreally with \(^\text{3}\)H-leucine. All rabbits showing evidence of leakage from the injection site were excluded from the study.

**Outer Segment Renewal in Photoreceptors**

Quantitative autoradiography was also used to assess effects of 5-FU and FUR on protein synthesis in retinal photoreceptors. Rod outer segment (ROS) renewal occurs continuously, reflecting a high level of protein synthesis in the rod inner segment and insertion of newly synthesized protein as a band in the ROS.\(^\text{22}\) Fifteen rabbits from the first group and 22 rabbits from the third group were anesthetized with sodium thiopental and perfused intravascularly with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.13 M phosphate buffer 72 hrs after intravitreal injection of \(^\text{3}\)H-leucine. The eyes were immediately enucleated and immersed in the fixative for 24 hr. The anterior third of each eye was removed, and the retina was sectioned and processed as described above for autoradiography. Autoradiographic grains were counted per unit area of radioactive ROS band in a standardized segment of retina, just temporal to the optic disc, to determine if photoreceptor protein synthesis, as evidenced by OS renewal, had been affected by prior drug treatment.

### Protein Synthesis In

**Ganglion Cells**

Fig. 1. Inhibitory effects of 5-FU and FUR on protein synthesis in ganglion cells of the rabbit retina. Mild inhibition was found after two intravitreal injections of 2.5 mg 5-FU and 0.1 mg FUR. No reduction was found after intravitreal injection of 1.0 mg 5-FU. The vertical bars represent means ± SEM.
Axonal Transport of Newly Synthesized Protein

Liquid scintillation counting was used to quantify $^3$H-leucine-labeled protein transported axonally by ganglion cells to the contralateral superior colliculus (SC). Albino rabbits have nearly total crossing of optic axons at the optic chiasm, so that axons from a drug-treated (right) eye terminate in the contralateral (left) SC. Fifteen rabbits from the first group, 7 rabbits from the second group, and 22 rabbits from the third group were perfused with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.13 M phosphate buffer 72 hr after intravitreal injection of both eyes with $^3$H-leucine. Uniform samples were taken from both SC and the cerebellum, the latter sampled as a control for blood-borne label, using a 3-mm trephine set at a depth of 1.5 mm. The samples were digested overnight in 1 ml of Protosol at 50°C, mixed with 200 ml
of H₂O₂ and 40 ml of glacial acetic acid, followed by 10 ml of Scintiverse, and quantified (cpm/mg) using a Beckman LS 3801 liquid scintillation counter. The percentage of inhibition of axonal transport was determined from the ratio:

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\% \text{ Inhibition} = \frac{(\text{cpm/mg right SC} - C) - (\text{cpm/mg left SC} - C)}{(\text{cpm/mg right SC} - C)}
\]

### Results

#### Protein Synthesis in Ganglion Cells

Intravitreal injections of 2.5 mg of 5-FU and 0.1 mg of FUR produced inhibition of protein synthesis in ganglion cell somata (Figs. 1, 2). A mean of 22% inhibition (range, 19–25%) was found after two daily intravitreal injections of 0.1 mg of FUR, and 17% inhibition of protein synthesis (range, 10–31%) was noted after two injections of 2.5 mg of 5-FU. Only the FUR results were statistically significant using the paired t-test \((P < 0.02)\). Intravitreal injections of 1.0 mg of 5-FU caused no significant inhibition of protein synthesis, with a mean negative inhibitory response of 15% (range, −52–+4%).

#### OS Renewal in Photoreceptors

Marked inhibition of incorporation of newly synthesized protein into the OS of retinal photoreceptors occurred after two intravitreal injections of 0.1 mg of FUR (45%; range, 36–58%) and 2.5 mg of 5-FU (48%; range, 35–55%) (Figs. 3, 4); both were statistically significant \((P < 0.3)\). A similar inhibitory effect was seen after a single intravitreal injection of 0.1 mg of FUR (35%; range, 3–70%) or 2.5 mg of 5-FU (35%; range, −3–+69%), which persisted up to 8 days, for both 0.1 mg of FUR (43%; range, 8–68%; \(P < 0.03\)) and 2.5 mg of 5-FU (25%; range, −19–+50%; \(P < 0.10\); Fig. 5). There was essentially no effect after injections of 1.0 mg of 5-FU (−3% inhibition), but a wide range of values was obtained (−58–+54%).

#### Axonal Transport

Liquid scintillation counting of the SC revealed greatest reduction of axonally transported \(^3\)H-leucine-labeled protein after two intravitreal injections of 0.1 mg of FUR, with an average of 53% inhibition (range, 33–73%; Fig. 6). Less inhibition was found after two intravitreal injections of 2.5 mg of 5-FU (26%; range, 9–41%). When only one intravitreal injection of 0.1 mg of FUR or 2.5 mg of 5-FU was given, the mean inhibitory effect was 41% (range, 18–71%) and 41% (range, 12–59%), respectively, which were both statistically significant \((P < 0.05)\). The mean effect persisted, although somewhat diminished, when remeasured at 8 days, 32% (range, −15–+57%) for 0.1 mg of FUR and 22% (range, −9–+57%) for 2.5 mg of 5-FU, neither of which was statistically significant (Fig. 7). A smaller and not statistically significant mean inhibitory effect of 13% was noted after two intravitreal injections of 1 mg of 5-FU, and this dosage produced a wide range of values (−64–+78% inhibition). Twice-daily subconjunctival injections of 5-FU for 1 week after a trephine filtering procedure did not produce significant reduction of axonally transported protein (−7%; range, −34–+40%).

### Discussion

The drug 5-FU is metabolized to 5-fluorodeoxyuridine monophosphate, a potent and irreversible inhibitor of thymidylate synthetase. The 5-FU and FUR are also converted to FUR triphosphate which, when incorporated into RNA, inhibits the processing of messenger RNA and ribosomal maturation.\(^{17,24}\) Retinal tissue, being of neural origin, is comprised of nonreplicating cells so that drugs which inhibit DNA synthesis would appear less likely to be toxic. How-
ever, high rates of protein synthesis occur in several cell types of the retina, particularly photoreceptor and ganglion cells. Therefore, one would expect that the retinotoxic effects of 5-FU would be secondary to inhibition of RNA maturation and most deleterious to cells with high rates of protein synthesis. This is consistent with previous studies, where toxic doses of 5-FU produced ultrastructural changes suggesting inhibition of retinal protein synthesis, including OS disruption, swelling of inner segment mitochondria, swelling and loss of substructure in axons and mitochondria of the outer plexiform layer, and loss of ribosomes in all neuron types. Since the effectiveness of an anti-PVR drug that acts by altering nucleic acid function is based on a balance between inhibition of intraocular cellular proliferation and toxic effects on the retina, an understanding of drug effects on protein synthesis is essential.

Several studies evaluate the retinotoxicity of 5-FU in animal models using other methods. Blu-
menkranz and associates\textsuperscript{10} found that 5-FU was not
toxic to rabbit retina, as assessed by light and electron
microscopy and electrophysiology after a single in-
travitreal dose of 2.5 mg in nonvitrectomized eyes or
after subconjunctival injections of 10 mg daily for 7
days. Barrad et al\textsuperscript{19} found 1.0 mg of intravitreal 5-FU
to be nontoxic in primate eyes. However, other stud-
ies note retinal toxicity in rabbits using light and
electron microscopy after a single intravitreal injec-
tion of 1.0 mg of 5-FU.\textsuperscript{2,25} The FUR appeared non-
toxic to the retina after an intravitreal injection of 0.1
mg, while doses of 1 mg and 10 mg of FUR were toxic
to the retina and retinal pigment epithelium, as as-
sessed by light microscopy and electroretinography.\textsuperscript{18}

Our data showed marked inhibition of protein syn-
thesis after one or two intravitreal injections of 0.1
mg of FUR and 2.5 mg of 5-FU using three different
methods for evaluating protein synthesis in the rabbit
retina. This effect was statistically significant for 0.1
mg of FUR and 2.5 mg of 5-FU with at least two of
the three methods used, and was persistent for both
drugs when tissue was reevaluated 8 days after the
injection. After two intravitreal injections of 1.0 mg
of 5-FU, no net inhibitory effect on protein synthesis
was found in ganglion cell somata and photoreceptor
OS, and only a small negative effect was noted on
axonal transport. However, the results were widely
scattered after injections of 1.0 mg of 5-FU, especially
compared with the more consistent results obtained
from the other doses and injections. Therefore, al-
though the results could indicate that 1.0 mg is a
nontoxic dose of 5-FU, the variability could also indi-
cate that this concentration of 5-FU is close to the
threshold for drug toxicity of the rabbit retina.

Historically, toxicity studies using intravitreal in-
jections in nonvitrectomized eyes yield results with
significant levels of variability. Injection techniques,
differential transport of tritiated amino acids or phar-
macologic agents across the retina at various ana-
tomic points, occasional leakage of material from the

Fig. 5. Inhibitory effects of 5-FU and FUR on protein synthesis
in retinal photoreceptors after a single intravitreal injection.
Marked inhibition was found on the first day following intravitreal
0.1 mg FUR and 2.5 mg 5-FU. The inhibitory effect was persistent
when measured at 8 days after injection of either drug. The vertical
bars represent means ± SEM.

Fig. 6. Inhibitory effects of 5-FU and FUR on axonal transport of
newly synthesized protein by retinal ganglion cells. Scintillation
counting of superior colliculi revealed the greatest mean reduction
of axonally transported $^3$H-leucine-labeled protein after two intra-
vitreal injections of 0.1 mg FUR with less inhibition noted after 2.5
mg 5-FU. The vertical bars represent means ± SEM.

Fig. 7. Inhibitory effects of 5-FU and FUR on axonal transport of
newly synthesized protein by retinal ganglion cells. Scintillation
counting of superior colliculi revealed a similar reduction 24 hr
after a single intravitreal injection of either 0.1 mg FUR or 2.5 mg
5-FU. The results at 8 days demonstrated persistence of this inhibi-
tory effect after injection of either drug. The vertical bars represent
means ± SEM.
injection site, and interanimal variability all complicate the interpretation of data.

Orcutt et al described a two-person injection technique that produces equal transport of radioactivity to the SCs of rabbits after bilateral intravitreal injections. Their technique of injecting over a standardized region of the retina yielded reproducible results with acceptable variability (1.5% to ± 4.8). Our data and that of Orcutt et al (personal communication) demonstrated significant interanimal variability. However, the reproducibility of results with minimal variability between eyes of the same rabbit argue that the technique is reproducible. Interanimal variability did not affect the statistical outcome since both the treated and control eyes of an animal were affected equally.

Use of 5-FU to reduce postoperative scarring after a glaucoma filtering procedure presently requires numerous subconjunctival injections of the drug over several weeks. A single subconjunctival injection of 6.25 mg of 5-FU in a rabbit produced an intravitreal peak level of 0.5 μg/ml, which appears small when compared with the peak vitreous level of 664 μg/ml after an intravitreal injection of 1 mg of 5-FU. However, toxicity might accrue after multiple injections to produce sustained drug levels, especially after a filtering procedure which may increase intraocular drug levels by breakdown of the blood–retinal barrier and/or by producing a direct route of drug entry into the eye. We found no inhibition of protein synthesis in the rabbit retina after repeated subconjunctival injections of 5-FU following a trephine filtering procedure. This corroborates previous findings in nonoperated rabbit eyes; there was no toxicity to the retina after subconjunctival injections of 10 mg of 5-FU given daily for 7 days.

In summary, 5-FU injected subconjunctivally after a trephine filtering procedure did not inhibit retinal protein synthesis in this animal model. On the other hand, this study documented statistically significant inhibition by intravitreally injected 5-FU and FUR of protein synthesis in photoreceptors and ganglion cells of rabbit retina. This protein synthesis inhibition persisted at least 8 days after injection of 5-FU and FUR, suggesting a long-term effect at dosage levels noted in some studies to be “nontoxic.” Further studies are indicated to determine the long-term significance of this inhibition of rabbit retinal protein synthesis.

Key words: fluorouracil, fluorouridine, glaucoma, proliferative vitreoretinopathy

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