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A Novel Chemical Delivery System Comprising an Ocular Sustained Release Formulation of a 3α, 17α, 21-trihydroxy-5β-pregnan-20-one-BIS-5-Flourouracil Codrug

Michelle Howard-Sparks, Abeer M. Al-Ghananeem and Peter A. Crooks
College of Pharmacy, Chandler Medical Center, University of Kentucky, Lexington, Kentucky

Andrew P. Pearson
Department of Ophthalmology, Chandler Medical Center, University of Kentucky, Lexington, Kentucky

ABSTRACT Directly compressed sustained release pellets were prepared from material consisting of a molecule of 3α, 17α, 21-trihydroxy-5β-pregnan-20-one (trihydroxy steroid, THS) covalently linked via carbonate moieties to two molecules of 5-flourouracil (5FU) to form a novel THS-BIS-5FU codrug for the treatment of angiogenisis. Dissolution and drug release was tested in vitro in 0.1M phosphate buffer (pH 7.4), human serum, and vitreous humor. The results suggest that neat THS-BIS-5FU codrug pellets are useful for sustained release ocular delivery of the parent compounds, and that the unique physicochemical properties of the codrug allow slow dissolution and rapid release of the two parent drugs. This codrug formulation is regarded as a "chemical delivery" system that involves dissolution of the codrug as the rate-limiting step followed by rapid hydrolysis of the carbonate ester linkages to release the parent drugs via sustained delivery.

KEYWORDS Codrug, Chemical delivery, Sustained release, 5-Fluorouracil, Trihydroxy steroid, Angiogenesis

INTRODUCTION

Retinal neovascularization is a complication in a variety of eye diseases, and is a leading cause of irreversible vision loss in developed countries (Bora et al., 2005; Lee et al., 1998). Although the present treatment, i.e. retinal laser photocoagulation, is partially effective, this procedure can destroy postmitotic retinal neurons and permanently affect visual function (Speicher et al., 2003). During the last several years, a number of therapeutic agents have been developed aimed at inhibiting of the mechanism of retinal angiogenesis.

3α, 17α, 21-trihydroxy-5β-pregnan-20-one (trihydroxy steroid, THS) is an angiostatic steroid that has been shown to possess angiostatic properties similar to cortisone without the glucocorticoid or mineralocorticoid activity and thus, has the capacity of inhibiting angiogenesis without the usual side effects associated with corticosteroids. THS acts through suppressing the protease activity of the

Address correspondence to Peter A. Crooks, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082. E-mail: pcrooks@email.uky.edu

angiogenic protein, basic fibroblast growth factor (bFGF) (Blei et al., 1993). Unfortunetly, the aqueous solubility of THS is only 12 μg/mL; thus, affording poor in vivo bioavailability. Because of this low aqueous solubility, THS is a strong candidate for prodrug formulation.

Codrugs or mutual prodrugs are bipartate or tripartate compounds that contain a covalent linker moiety tethering two or more synergistic compounds together in order to improve the drug delivery properties of one or both drugs. Like prodrugs, codrugs are bioreversible derivatives of the component active parent compounds. This unique concept of codrugs has recently been utilized in our lab where codrugs of ethacrynic acid (ECA) covalently linked to either atenolol (ATL) or timolol (TML) via ester bond linkages were designed and synthesized to improve ocular delivery, and in addition, to take advantage of the apparent synergistic mechanism of ECA and the β-adrenergic receptor antagonists (Cynkowska et al., 2005). Further, Cardillo and coworkers, reported a naproxen and 5-flourouracil (5FU) codrug system for the treatment of experimental post-traumatic proliferative vitreoretinopathy. Their results suggested that this codrug system effectively inhibits the progression of PVR in a rabbit trauma model that closely resembles PVR in humans (Cardillo et al., 2004). 5-flourouracil (5FU) is a known cytostatic, antineoplastic agent; it is effective in the management of proliferative vitreoretinopathy (PVR). However, due to the high aqueous solubility (11 mg/mL) and rapid clearance of 5FU from the vitreous ($t_{1/2}$=2.45 hr) (Del Nozal et al., 1992), multiple intravitreal injections of this drug are needed to maintain therapeutic levels of 5FU.

In previous published work we designed and synthesized a novel codrug of the antiangiostatic steroid, THS with the cytotoxic agent 5-fluorouracil (5FU) (Howard-Sparks et al., 2005). This codrug incorporates two molecules of 5FU attached through carbonate ester linkages at positions O^3, and O^{21} of the THS molecule (Fig. 1). The overall goal of such a codrug strategy was to improve drug delivery of both compounds by overcoming their individual solubility problems and unfavorable physico-chemical properties, and to thus further enhance their bioavailability. The THS-BIS-5FU codrug was found to have superior angiostatic activity compared to that of the parent compounds alone or a physical mixture of THS and 5FU, in the chick chorioallantoic membrane (CAM) assay (Howard-Sparks et al., 2005).

Ocular drug delivery is commonly achieved through topical administration, which is effective for

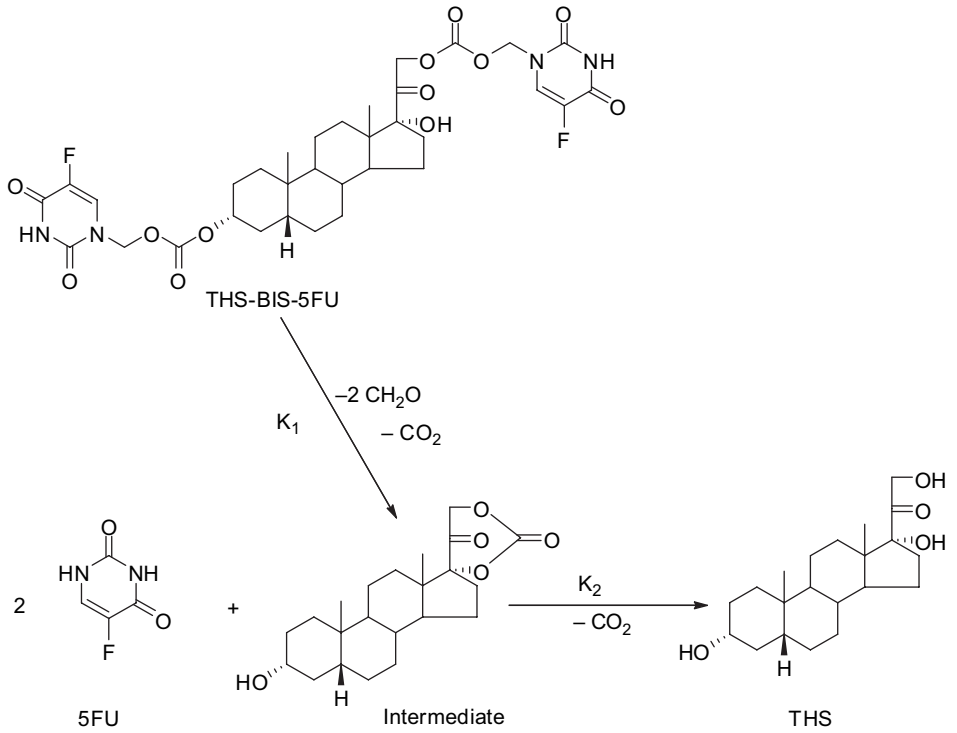

FIGURE 1 **A Schematic Diagram Illustrating the Structure of the THS-BIS-5FU Codrug and the Hydrolytic Pathway for the Release of THS and 5FU.**

the administration of locally acting pharmacological agents and for the treatment of ocular diseases. However, drug molecules applied topically on the surface of the eye do not readily reach the internal ocular tissue, such as the lens and the vitreous body, because of transmucosal diffusion barriers, or because of clearance mechanisms, which rapidly deplete the concentration of the drug in the eye (Fialho et al., 2004).

Systemic administration is another major route of drug delivery for ophthalmic drugs. However, the outer and inner blood-retinal barriers impose limits on the influx of drugs into retinal tissue and the vitreous cavity. Therefore, a large amount of drug, and frequent drug administration are both required to maintain therapeutic concentrations, which may often result in interrupted drug use because of toxicity, and possible serious side effects on nontarget tissues (Frishman et al., 2001).

Although it is not the preferred route of administration by clinicians or patients, intravitreal injections of ophthalmic drugs do provide therapeutic drug concentrations in the vitreous (Peyman et al., 1995; Jonas, 2004). However, rapid clearance of drugs from the vitreous, increased risk of toxic reactions, increased risk of infection, or permanent damage to the retina, and associated pain, render intravitreal injections impractical for chronic treatment of posterior segment disease (Myles et al., 2005). A more desirable drug delivery system, such as an implantable, biodegradable drug delivery device, has the potential to provide local and sustained therapeutic concentrations, fewer adverse side effects, and can be designed not to interfere with vision.

The formulation of small pellets through direct compression of the above THS-BIS-5FU codrug has the potential to afford a device that can be used as a chemical drug delivery system suitable for ocular implantation. In order for the THS and 5FU in the pellet to be available for angiostatic activity after direct compression, the THS-BIS-5FU codrug must first dissolve from the surface of the pellet. Based on the design of the carbonate linkers it is expected that once the THS-BIS-5FU codrug dissolves from the surface of the pellet it will be immediately hydrolyzed by endogenous esterases, and should afford sustained release of THS and 5FU in concomitant and quantitative ratios.

The present study investigates the in vitro release characteristics of a sustained release formulation for intraocular delivery of the novel THS-BIS-5FU codrug as an attractive chemical delivery system.

MATERIALS AND METHODS

Materials

5-Flourouracil (5FU), 5-fluorocytosine (5FC) and Reichstein's Substance S (RSS) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sodium phosphate (monohydrate, monobasic) and acetonitrile were purchased from Fisher Scientific. The THS-BIS-5FU codrug was synthesized in our laboratory via previously reported procedures (Howard-Sparks et al., 2005). Bovine vitreous was obtained from the Bourbon County Meat Locker, and human serum was obtained from the Clinical Research Center at the University of Kentucky.

Formulation of the Pelleted Delivery Devices

Fully bioerodible implantable pellets consisting of 2 mg of either THS alone, 5FU alone, or the THS-BIS-5FU codrug were prepared by direct compression of the powder at 100 psi with a customized press (Parr Instruments, Moline, IL) to afford pellets of approximately 1 mm in thickness and 2 mm in diameter (Fig. 2).

In Vitro Release Kinetics of the Pelleted Delivery Devices

Pellets containing 2 mg of drug or codrug were immersed in 1 mL of human serum, bovine vitreous humor, or 0.1*M* phosphate buffer (pH 7.4). The test

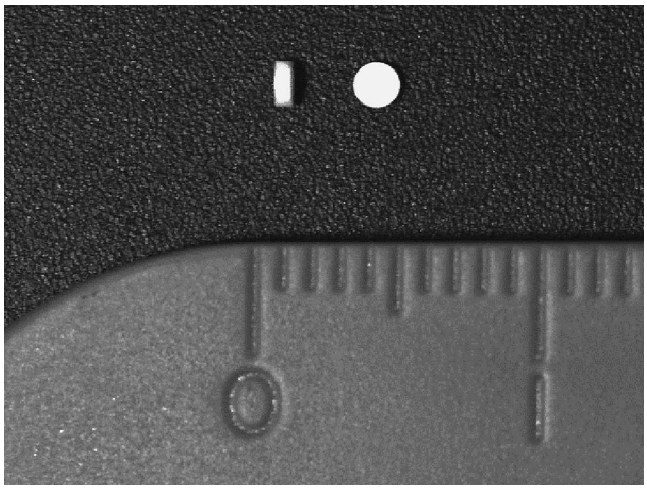

FIGURE 2 **Pelleted Delivery Device of the THS-BIS-5FU Codrug.**

media was maintained at 37°C for the duration of the experiment. The complete 1 mL of test media was removed from the dissolution flask at appropriate time points and replaced with a fresh 1 mL aliquot part of test media. A 200 µL volume of each 1 mL sample was prepared for HPLC analysis.

Sample Preparation

A 200 µL aliquot of each sample was placed in an individual microcentrifuge tube containing a 200 µL solution consisting of 50 µg of Reichstein's Substance S (RSS) and 50 µg 5-fluorocytosine (5FC) in acetonitrile (as internal standards). The samples were vortexed and centrifuged at 14,000 rpm for 5 min. Two 200 µL aliquot parts of each supernatant were removed and prepared for extraction. THS and the THS-BIS-5FU codrug were extracted by placing a 200 µL aliquot part of the supernatant in a microcentrifuge tube containing 1 mL of ethyl acetate. The samples were vortexed and centrifuged at 14,000 rpm for 5 min. For the extraction of 5FU, a 200 µL aliquot part of the supernatant was transferred to a microcentrifuge tube containing 1 mL of a 3:1 v/v mixture of ethyl acetate: acetonitrile. The samples were vortexed and centrifuged at 14,000 rpm for 5 min. Finally, from all samples, a volume of 800 µL of the organic layer was transferred to a separate microcentrifuge tube, evaporated to dryness, and reconstituted in 200 µL phosphate buffer (pH 3.5, 1.0*M*). The resulting solutions were analyzed by high-performance liquid chromatography (HPLC).

HPLC Analysis

The HPLC analytical system used for the codrug kinetic studies consisted of an L-4000 UV detector, an L-6000 intelligent pump, an AS-2000 autosampler, and a D-2500 chromato-integrator, Hitachi (Tokyo, Japan).

For the analysis of THS and THS-BIS-5FU, the column used was an Axxion C-18 reverse phase ODS column (25 cm x 4 mm x 5 µm) preceeded by a Rainin C-18 pre-column (Hamilton, NV). The mobile phase consisted of 50% acetonitrile and 50% water (pH 6.0) at a flow rate of 1 mL/min and UV detection at 208 nm. For the analysis of 5FU, the column used was a Sphereclone (25 cm x 0.46 cm x 5 µm) purchased from Phenomenex (Torrence, CA) preceded by a Hitachi C-18 guard column (Tokyo, Japan). The mobile phase consisted of phosphate buffer (pH 3.5, 1.0*M*) at a flow rate of 1 mL/min and UV detection at 270 nm.

RESULTS AND DISCUSSION

The pellets containing 2 mg of THS demonstrated pseudo-first order dissolution kinetics in phosphate buffer, serum and vitreous humor; in each of the test media, the rate of release of THS from the neat pellets was 3.4 µg/mL/day, 13.5 µg/mL/day, 1.7 µg/mL/day, respectively, over the 28-day duration of the in vitro release test. The dissolution profiles of THS in phosphate buffer, serum and vitreous are illustrated in Fig. 3.

The pellets containing the 2 mg of the more polar 5FU had completely dissolved after 1.5 hr in phosphate buffer, with a release rate of 23.3 µg/mL/min. In vitreous humor and human serum, the 5FU pellets were completely dissolved after 2 hr; release rates of approximately 16.5 µg/mL/min were observed over that period in both media. The pseudo-first rate order dissolution profiles of 5FU in phosphate buffer, serum, and vitreous are shown in Fig. 4.

The pellets containing the THS-BIS-5FU codrug gave a simultaneous, sustained release of both THS and 5FU in each of the media studied (Figs. 5, 6 and 7). In 0.1*M* phosphate buffer, THS was released at a rate of < 1 µg/mL/day (below quantification limits, but not below detection limits) for the first 6 days. After this time, THS was released at a rate of 1.21 µg/mL/day. The release rate for 5FU was 1.46 µg/mL/day over the whole period of the experiment (28 days).

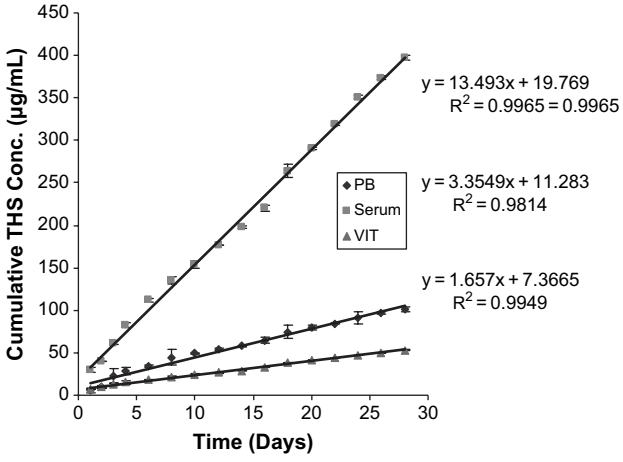

FIGURE 3 **Cumulative Release of THS in Either Phosphate Buffer (PB) (0.1*M*, pH 7.4) or Human Serum or Bovine Vitreous Humor (VIT) from Neat Pellets Containing 2 mg of THS.**

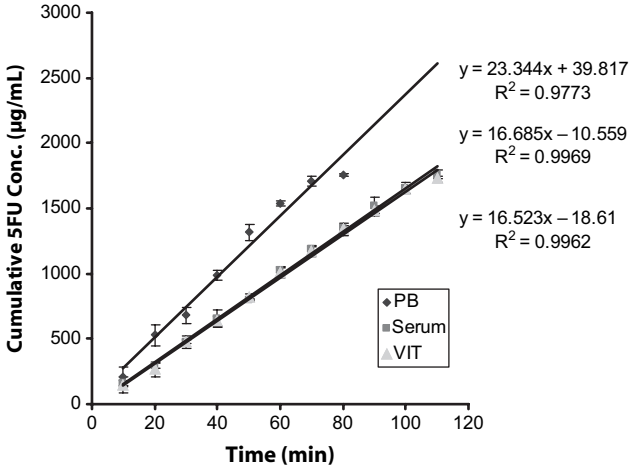

FIGURE 4 **Cumulative Release of 5FU in Phosphate Buffer (PB) (0.1*M*, pH 7.4), Human Serum and Bovine Vitreous Humor (VIT) from Neat Pellets Containing 2 mg of 5FU.**

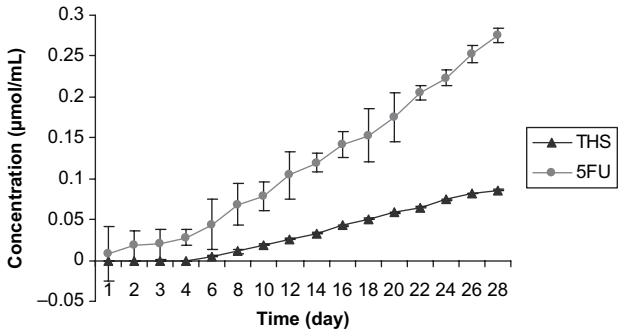

FIGURE 5 **Cumulative Release of THS and 5FU from Neat Pellets Containing 2 mg of the THS-BIS-5FU Codrug in 0.1*M* Phosphate Buffer pH 7.4.**

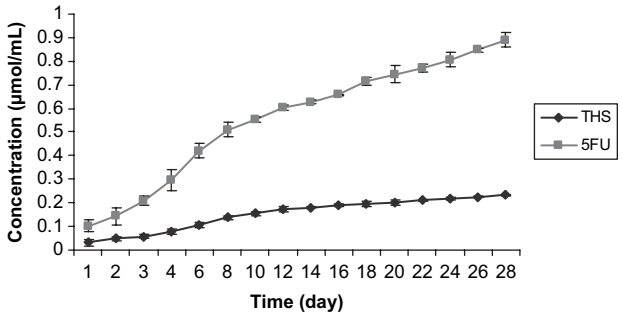

FIGURE 6 **Cumulative Release of THS and 5FU from Neat Pellets Containing 2 mg of the THS-BIS-5FU Codrug in Human Serum.**

In human plasma THS was released at a rate of 5.27 µg/mL/day for the first 8 days, slowing thereafter, to a rate of 1.53 µg/mL/day. 5FU was released at a rate of 9.15 µg/mL/day for the first 8 days, slowing thereafter, to a rate of 2.77 µg/mL/day. In vitreous humor THS

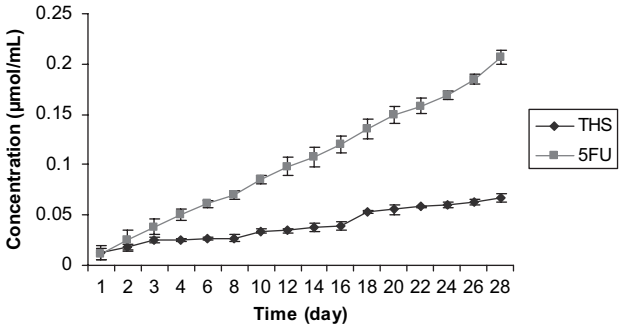

FIGURE 7 **Cumulative Release of THS and 5FU from Neat Pellets Containing 2 mg of the THS-BIS-5FU Codrug in Bovine Vitreous Humor.**

was released at a rate of 0.67 µg/mL/day, while 5FU was released at a rate of 0.99 µg/mL/day over the whole period of the experiment. Interestingly, because of the short half-life of the intact, solubilized codrug conjugate, only trace amounts of codrug were detected in the release medium.

The results of this study demonstrate that neat pellets of the THS-BIS-5FU codrug constitutes a suitable chemical delivery device for delivery of the parent drugs THS and 5FU in a sustained release manner, compared to the release profiles of each pelleted parent drug alone. A pelletized delivery device can be implanted into the vitreous during vitreo-retinal surgery so that it does not require a separate surgical procedure. Also, a pellet usually provided long-term drug delivery for the treatment of chronic vitreo-retinal disease, especially when the pellet consists of sparingly soluble neat drug (i.e., no polymer matrix involved). Direct compression forces particles against one another, causing them to undergo elastic and plastic deformation, thereby increasing interparticle contact.

As expected, the neat formulation pellets containing THS alone gave a sustained release of THS based on the very low solubility of THS in the various media examined. THS dissolution rate constants from the neat pellet in phosphate buffer, human serum and bovine vitreous humor were 4.98/day, 0.61/day, and 12.45/day, respectively. However, the formulation of a pellet containing only 5FU gave an immediate release of 5FU, due to the high solubility of 5FU in the various media examined. The 5FU neat pellet dissolution profile for vitreous humor was similar to the dissolution profile observed in serum with a rate constant of 0.135/min. Furthermore, the dissolution rate constant in phosphate buffer was 0.26/min.

Pellets containing a neat formulation of THS-BIS-5FU codrug gave a sustained release of both THS and 5FU over the 28-day time period of the experiment, chromatograms demonstrated no detectable levels of the THS-BIS-5FU codrug which illustrated that the rate limiting step is the dissolution of the codrug from the pellet, then immediate hydrolysis to regenerate 5FU and a cyclic carbonate intermediate that subsequently degrades to THS.

The release rate of THS was greater in the codrug formulation, than in the neat formulation of THS. This enhancement in release rate is expected, due to the improved dissolution properties of the THS-BIS-5FU codrug when compared to THS alone. It is expected that the water solubility of the THS-BIS-5FU codrug would be somewhere between the water solubility of THS and 5FU.

In all media studied, the release of 5FU from the THS-BIS-5FU codrug pellets was greatly reduced over the release of 5FU from a pellet containing 5FU alone. Although sustained release of 5FU is achieved, THS and 5FU do not appear to be immediately released in 1:2 stoichiometric ratio. This is attributed to mixed hydrolysis kinetic profiles and the observation that cyclic carbonate intermediate is generated from the hydrolysis of the THS-BIS-5FU codrug that then subsequently degrades to generate THS.

CONCLUSION

A formulation of directly compressed pellets containing the novel codrug THS-BIS-5FU can be considered as an ocular chemical delivery system for delivery of THS and 5FU upon hydrolysis over extended periods. The codrug hydrolysis profile in different media illustrated the successful regeneration of the parent drugs. However, the release of THS was slower than the release of 5FU, since THS generation proceeded through a cyclic carbonate intermediate. Results from these studies provide valuable information for optimization of the codrug design and pelleted formulation of codrugs in order to achieve sustained release for ocular angiogenesis therapy.

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