Influence of Dosing Time on the Efficacy and Safety of Finasteride in Rats

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ABSTRACT

Finasteride (FIN), a widely used medication for the treatment of androgen-dependent diseases, blocks the conversion of testosterone to a more potent androgen, dihydrotestosterone (DHT). In this study, we investigated a dosing time-dependent effect and safety of FIN in rats. Androgen receptor (AR) mRNA and nuclear protein levels exhibited clear daily rhythms with the peak during the dark period in the prostate and during the light period in the liver. Repeated oral administration of FIN (5 or 100 mg/kg) at 3 h after lights on (HALO) for 2 weeks decreased serum DHT concentration throughout a 24-h period, whereas the dosing of the agent at 15 HALO decreased its level only transiently even in the higher dose group. FIN caused laboratory abnormalities in the 3 HALO group but not in the 15 HALO group. However, the effect of FIN on the prostate weight was not influenced by the dosing time. These results suggest that the safety, but not effect, of FIN depends on its dosing time in rats. The dosing of FIN in the active period might be a rational dosage regimen, which is needed to be confirmed in human subjects.

Introduction

The clinical usefulness of medications generally depends on their efficacy and safety. Chronotherapy is one of the approaches to enhance the therapeutic and diminish the adverse effects by optimizing a dosing time. We have previously demonstrated the merits of chronotherapies with various drugs in animals and human subjects (Tsuruoka et al., 2003; Ushijima et al., 2005; Nozawa et al., 2006; Takahashi et al., 2010). Dosing time-dependent changes in the pharmacokinetics and pharmacodynamics are involved in the chronopharmacological phenomenon (Lemmer, 2005).

Finasteride (FIN), a competitive and specific inhibitor of 5α-reductase, blocks the conversion of testosterone to a more potent androgen, dihydrotestosterone (DHT), in the prostate, hair follicles, and other androgen-sensitive tissues, leading to the suppression of serum and intraprostatic DHT concentrations (Steers, 2001). FIN is widely used in the treatment of androgen-dependent diseases, specifically male pattern baldness, benign prostatic hyperplasia (BPH), and prostate cancer (De Nunzio et al., 2008). Many clinical trials have demonstrated that FIN is well tolerated in most patients with BPH, and adverse effects other than the decreased libido and ejaculatory and erectile dysfunctions are rare (Gormley et al., 1992; Rittmaster, 1994; Stoner, 1994; Oesterling, 1995; Boyle et al., 1996). However, hepatic toxicity is reported in a small subset of patients under clinical conditions, according to the manufacturer’s product information.

Androgen plays a crucial role in the growth, maintenance, and function of the normal prostate gland, and the biological function in the prostate is mediated through the androgen receptor (AR) (Steers, 2001). Previous studies have shown that AR expression in the liver (Francavilla et al., 1986) and prostate tissue (Cao et al., 2009) exhibits daily rhythm. Therefore, it is hypothesized that the effects of FIN on androgen-sensitive tissues depend on its dosing time. To address this possibility, we investigated the dosing time-dependent influences of FIN on the efficacy and safety in rats.

ABBREVIATIONS: FIN, finasteride; DHT, dihydrotestosterone; BPH, benign prostatic hyperplasia; AR, androgen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HALO, hours after light on; ALT, alanine aminotransferase; IS, internal standard; HSP90, heat shock protein 90; PCR, polymerase chain reaction.
Materials and Methods

Chemicals and Antibodies

Finasteride [N-(2-methyl-2-propyl)-3-oxo-4-aza-5a-androst-1-ene-17β-carboxamide] was kindly provided by Merck & Co. (Whitehouse Station, NJ). Antibodies against lamin A/C and β-actin were purchased from Cell Signaling Technology (Danvers, MA). AR, glycerolaldehyde-3-phosphate dehydrogenase (GAPDH), and donkey anti-goat IgG-horseradish peroxidase antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ECL anti-rabbit IgG, horseradish peroxidase-linked F(ab’)2 fragment donkey antibody was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Animals

Male Wistar rats were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan) at 8 weeks of age and maintained under a specific pathogen-free condition with controlled temperature and humidity and a 12-h light/dark cycle. They were given standard laboratory diet and water ad libitum. After 2 weeks of acclimation, the rats were used for each experiment. All animal procedures were performed in accordance with the Guidelines for Animal Research at Jichi Medical University (Tochigi, Japan) and approved by the Use and Care of Experimental Animals Committee.

Treatments

Experiment 1: Daily Rhythms of AR Expression in the Prostate and Liver in Normal Rats. At 10 weeks of age, rats (n = 20) were sequentially killed at 0, 4, 8, 12, 16, and 20 h after light on (HALO) (n = 3–4 for each time point), and the prostate gland and liver samples were collected for the measurements of AR mRNA and protein levels.

Experiment 2: Drug Dosing and Sample Collection. Rats (n = 48) were divided into six groups (n = 8 in each) and FIN (5 or 100 mg/kg) or vehicle (0.5% tragacanth gum solution) alone was given once daily by a gastric gavage at each of the two different time points (3 or 15 HALO) for 2 weeks: group 1, vehicle at 3 HALO; group 2, 5 mg/kg FIN at 3 HALO; group 3, 100 mg/kg FIN at 3 HALO; group 4, vehicle at 15 HALO; group 5, 5 mg/kg FIN at 15 HALO; and group 6, 100 mg/kg FIN at 15 HALO. Thereafter, blood, prostate, and liver samples were obtained. Blood sampling for the measurements of serum DHT and testosterone concentrations (n = 3–4 in each group) were initiated 1 h after the final FIN dosing, with a 4-h interval during a 24-h period. Prostate and liver samples were obtained immediately after the final blood sampling at 0 or 12 HALO. Biochemical parameters were also measured in the last samples. Because the levels of laboratory parameters may exhibit circadian rhythms (Morrison et al., 1979), we set the vehicle in each dosing time group and compared the data with those in the respective vehicle group. Drug dosing and blood sampling in the dark room were conducted under dim red light to avoid the effects of lights on circadian rhythms.

Experiment 3: A Single Dosing of FIN for Measuring Its Plasma Concentrations. Rats (n = 20) were divided into four groups (n = 5 in each), and a single dose of FIN (5 or 100 mg/kg) was given by a gastric gavage at each of two different time points (3 or 15 HALO): group 1, 5 mg/kg FIN at 3 HALO; group 2, 100 mg/kg FIN at 3 HALO; group 3, 5 mg/kg FIN at 15 HALO; and group 4, 100 mg/kg FIN at 15 HALO. Blood samples for the measurements of plasma FIN concentrations were collected at 1, 2, 4, 8, and 24 h after FIN administration.

Biochemical Measurements

Serum DHT and testosterone concentrations were determined using the following commercial kits according to the manufacturer’s instructions: ACTIVE Dihydrotestosterone Radioimmunoassay Kit (Diagnostic Systems Laboratories, Inc., Webster, TX) and Rodent Testosterone Enzyme-Linked Immunosorbent Assay Kit (Endocrine Technologies, Inc., Newark, CA), respectively. Serum concentrations of aspartate aminotransferase, alanine aminotransferase (ALT), γ-glutamyl transpeptidase, total bilirubin, lactate dehydrogenase, total cholesterol, total protein, creatinine, and blood urea nitrogen were measured using commercial reagents (Wako Pure Chemicals, Osaka, Japan). Intra-assay and interassay coefficients of variation were all <10%.

RNA Extraction and Real-Time Quantitative PCR Analysis

Isolation of total RNA was carried out using the RNeasy Mini Kit according to the manufacturer’s instructions (QIAGEN, Valencia, CA). Reverse transcription was done by a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). The real-time quantitative PCR was performed with the Applied Biosystems StepOnePlus Real-Time PCR System (Invitrogen, Carlsbad, CA). All specific sets of primers and TaqMan probes (Applied Biosystems TaqMan Gene Expression Assays) were obtained from Invitrogen. To control the variation in the amount of cDNA available for PCR in the different samples, the gene expression levels of the target sequences were normalized to the expression of an endogenous control, Gapdh. The GenBank accession numbers, assay ID, and target exons were as follows: NM_012502.1, Rn00560747_m1, and 4-5 for Ar and NM_017008.3, Rn99999916_s1, and 1-1 for Gapdh, respectively. Data were analyzed using the comparative threshold cycle method.

Western Blot Analysis

Tissue extractions were performed using a ProteoExtract Subcellular Proteome Extraction Kit (EMD Biosciences, San Diego, CA) on 25 to 50 mg of frozen rat prostate and liver tissues following the manufacturer’s protocol. Western blot analysis was performed as described previously (Ando et al., 2004). In brief, after the measurement of protein concentrations, proteins were separated by SDS-polyacrylamide gel electrophoresis (10% NuPAGE gel; Invitrogen) and then transferred to 0.45-μm polyvinylidene difluoride membranes (Invitrogen). Non-specific binding sites were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 and incubated overnight at 4°C in the following antibodies: 1:1000 AR, lamin A/C, β-actin, and GAPDH. Membranes were washed three times in Tris-buffered saline containing 0.1% Tween 20 and incubated for 1 h at room temperature in appropriate dilutions of secondary antibodies labeled with horseradish peroxidase. The blots were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare). The bands for lamin A/C, β-actin, and GAPDH were used as internal controls of nucleus, cytoplasm, and whole cell, respectively.

Plasma FIN Measurement

Rat plasma was mixed with 10 μl of internal standard (IS) (10 ppm beclometasone) and 250 μl of acetonitrile. After vortexing and centrifugation (5000 rpm, 10 min), the supernatant was separated, and 10 ml of distilled water was added. The mixture was loaded onto an Oasis HLB solid-phase extraction column (30 mg; Waters, Milford, MA) pretreated with 1 ml of acetonitrile, followed by 1 ml of distilled water. Solid-phase extraction columns were washed with 2 ml of distilled water. The analytes were eluted with 2 ml of acetonitrile. The eluate was collected, evaporated to dryness under a stream of nitrogen at 40°C, and reconstituted with 0.5 ml of 50% acetonitrile. The calibration curve was prepared by adding IS and FIN in acetonitrile to control rat plasma to obtain final concentrations of 1.0 to 10,000 ng/ml FIN. Linear regression analysis of the ratio of the area of the analyte to that of IS versus concentration was used for calibration. Good linearity was obtained (coefficients of determination in the range of 1–2000 ng/ml r² > 0.9999 and in the range of 2000–10,000 ng/ml r² > 0.99). The detection limit of FIN, at a signal/noise ratio of 3, was 0.67 ng/ml. The quantification limits of FIN, at a signal/noise ratio of 10, was 2.22 ng/ml.
An Agilent high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) was used, and 10-μl samples were injected automatically. A reverse-phase; Imtact, Kyoto, Japan Cadenza HS-C18 (50 × 2.0 mm i.d., particle size 3.0 μm) was used. Solvent A (0.1% formic acid in distilled water) and solvent B (0.1% formic acid in methanol) were used as mobile phases for gradient elution (gradient curve: 0 min, 40%; 0–5 min, linear change from 40 to 95%; B; 5–8.5 min, 95%; B; 8.5–8.51 min, linear change from 95 to 40%; B; run time, 12.5 min). The flow rate was set at 0.5 ml/min. A time-of-flight mass spectrometer with an electrospray ionization interface (JEOL, Tokyo, Japan) was used. Detection was performed by monitoring the positive ions. The theoretical m/z values of the [M + H]+ ion were 373.3 for FIN and 409.2 for beclometasone.

### Statistical Analysis

All data are presented as the mean ± S.E.M. One-way analysis of variance was used to compare means among groups. The Bonferroni/Dunn procedure was used as a post hoc test. All statistical analyses were performed with StatView 5.0 software (SAS Institute, Cary, NC). Differences were considered to be significant at $p < 0.05$.

### Results

#### Daily Rhythmic Expression of AR in Normal Rat Prostate

To determine the optimal dosing time points of FIN in this study, we first examined the daily expression profile of AR in the prostate tissue of normal rats. As shown in Fig. 1A, the mRNA expression of AR in the prostate clearly showed the daily rhythm with a peak at 20 HALO, consistent with a previous report using BALB/c mice (Cao et al., 2009). Moreover, the nuclear protein level, but not the cytosolic or total level, of AR also exhibited a daily rhythm with a peak in the middle of the dark period (Fig. 1B). Because DHT binding to AR dimerizes, translocates into the nucleus, and thereafter acts as a transcription factor by binding to the hormone response element on DNA (Steers, 2001), these results indicate that sensitivity to DHT has daily rhythmicity with a peak in the dark period. Therefore, we decided to compare the effects of FIN dosing at 15 HALO with those at 3 HALO in this study.

#### Effects of FIN Dosing Time on the Serum Androgen Concentrations

Compared with vehicle, FIN did not affect the food intake and body weight change in all groups (Supplemental Fig. 1). As shown in Fig. 2A, both doses of FIN administered at 3 HALO significantly decreased DHT concentrations throughout a 24-h period. On the other hand, the suppressive effects were transient in 15 HALO groups, and the DHT concentration returned to almost the control level at 12 HALO even in the high-dose group. As for testosterone, only a high dose of FIN in 15 HALO group significantly increased serum concentrations (Supplemental Fig. 2).

#### Effects of FIN Dosing Time on the Prostate Weight and Biochemical Parameters

Contrary to our expectation, plasma FIN concentrations after dosing at 3 HALO were higher than those at 15 HALO. Therefore, we next compared the pharmacokinetics of FIN between the two different time points. Compared with vehicle, FIN did not affect total protein and cholesterol. On the other hand, FIN administered at 15 HALO did not cause abnormalities, even in the high-dose group.

#### Determination of the Pharmacokinetics after a Single Dose of FIN

The above findings concerning DHT concentrations and biochemical parameters led us to speculate that plasma FIN concentrations after dosing at 3 HALO were higher than those at 15 HALO. Therefore, we next compared the pharmacokinetics of FIN between the two different time points. Contrary to our expectation, plasma FIN concentrations were higher in 15 HALO groups than in 3 HALO groups until 4 h after dosing (Fig. 4). The values of maximum concentration and area under the plasma concentration-time curve of FIN in the 15 HALO group were similar or higher than those in the 3 HALO group (data not shown). However, compared with the 15 HALO groups, plasma FIN concentrations were higher, although not significantly, at 8 h after dosing of 5 mg/kg and 24 h after dosing of 100 mg/kg in the 3 HALO groups.

#### mRNA and Protein Expression Levels of AR in the Liver of Normal Rat

Previous studies have shown that androgens promote the synthesis of certain proteins via AR in the liver (Eagon et al., 1985; Francavilla et al., 1986) and the treatment with DHT increases total cholesterol in orchidectomized mice (Movar-Skrtic et al., 2006). Therefore, the alterations in these parameters after FIN dosing at 3
HALO appears to be the expected effect of FIN in the liver. Then we further investigated the daily rhythms of AR in the liver. As shown in Fig. 5, mRNA and nuclear protein levels of AR in the liver had their peaks in the light period. Thus, the sensitivity of liver to DHT may also have daily rhythmicity with a peak in the light period in contrast with that of prostate.

**Discussion**

The present study provides the first evidence that the effects of FIN on DHT concentration and its safety are influenced by dosing time. A high-dose FIN treatment for 2 weeks significantly reduced the prostate weight regardless of the dosing time. On the other hand, mild abnormalities of hepatic biochemical parameters were detected only after dosing of FIN during the first half of the light period.

Androgen plays a crucial role in the growth, maintenance, and function of the normal prostate gland, and the biological function in the prostate is mediated through the AR (Steers, 2001). AR transcriptional regulatory functions are dependent on the proper subcellular localization of the receptor (Shank et al., 2008). Nuclear transfer of AR has been shown to be hormone concentration- and heat shock protein 90 (HSP90)-dependent (Prescott and Coetzee, 2006). AR associates with a HSP90-based chaperone complex in the cytoplasm until the binding of cognate ligand induces a conformational change in AR, chaperone dissociation, and subsequent AR nuclear import (Tyagi et al., 2000; Marcelli et al., 2006). Because cytosolic HSP90 concentrations exhibit daily rhythms and correlate with glucocorticoid receptor-HSP90 association levels in rat brain (Furay et al., 2006), the daily profiles of nuclear AR might also be influenced at least partly by the rhythmic expression of HSP90. In this study, we demonstrated that the mRNA and nuclear protein expression of AR in the prostate was up-regulated during the dark (active) period. Moreover, the suppression of serum DHT concentration during the light period was not associated with the effect of FIN on the prostate. Taken together, these results suggest that the suppression of DHT concentration during the dark period is necessary to decrease the prostate weight. Further studies are needed to determine whether FIN treatment alters the nuclear AR expression rhythm and the necessity for the chronotherapy.

AR expression in the liver was up-regulated during the light period, which was in contrast to that in the prostate. The mechanism by which AR expression rhythms in the liver and prostate are oppositely regulated is unknown, but the rhythms of nuclear glucocorticoid receptor and cytosolic glucocorticoid receptor-HSP90 association levels in the hippocampus are reported to be opposite in phase to those in the hypothalamus (Furay et al., 2006). DHT is known to be responsible for physiological functions in the liver, including production of proteins (Eagon et al., 1985; Francavilla et al., 1986) and cholesterol (Movéare-Skrptic et al., 2006). The present study showed that high-dose FIN administered at 3 HALO caused not only the decreases in total protein and total cholesterol concentrations but also the increase in the ALT level. Therefore, although whether the blockade of androgen action is involved in FIN-induced liver injury remains to be elucidated, the suppression of DHT during the dark period may have less effect on hepatic functions than that during the light period.

It is well known that the absorption of several orally administered drugs exhibits circadian variability, and morning...
drug administration frequently induces higher peak plasma drug concentrations, shorter time to peak plasma concentrations, and a faster drug disappearance rate in humans (Reinberg and Smolensky, 1982; Goo et al., 1987). Consistent with these findings, dosing of FIN at 15 HALO (active period) showed higher plasma drug concentrations in rats. On the other hand, FIN concentrations at 8 h after the low-dose administration and at 24 h after the high-dose dosing were lower in the 15 HALO groups than in the 3 HALO groups. In addition, by one-compartment model analysis, the elimina-

TABLE 1
Effects of the FIN dosing time on various biochemical parameters

<table>
<thead>
<tr>
<th>Dosing Time</th>
<th>3 HALO</th>
<th>15 HALO</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FIN, 5 mg/kg</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.7 ± 0.1</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>71.7 ± 5.0</td>
<td>57.9 ± 2.0*</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>137 ± 11</td>
<td>145 ± 16</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>35 ± 3</td>
<td>44 ± 6</td>
</tr>
<tr>
<td>γ-GTP (IU/l)</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>T-bil (mg/dl)</td>
<td>0.051 ± 0.003</td>
<td>0.046 ± 0.003</td>
</tr>
<tr>
<td>LDH (IU/l)</td>
<td>1480 ± 441</td>
<td>1474 ± 304</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.25 ± 0.01</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>26.1 ± 1.0</td>
<td>25.8 ± 0.9</td>
</tr>
</tbody>
</table>

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; T-bil, total bilirubin; LDH, lactate dehydrogenase; BUN, blood urea nitrogen.

* p < 0.05, compared with the control at the same dosing time.

** p < 0.01.
tion half-life of the high-dose FIN was significantly shorter in the 15 HALO group (2.4 ± 0.3 h) than in the 3 HALO group (4.2 ± 0.6 h). Thus, drug disappearance was faster in the 15 HALO group than in the 3 HALO group. These chronopharmacological differences may be involved in the dosing time-dependent safety of FIN.

The doses used in this study (5 or 100 mg/kg per day) were high compared with those in clinical use (5 mg once a day for the treatment of BPH or prostate cancer prevention and 1 mg/day for male pattern baldness). However, such a high dosage is necessary for decreasing serum DHT concentration and prostate weight within 14 days, at least in rats (Marty et al., 2001). Further studies are needed to determine whether chronotherapy with FIN is also useful in humans under clinical conditions.

In summary, the dosing time of FIN did not influence its efficacy on prostate weight but affected hepatic laboratory abnormalities. Further clinical studies are needed to determine whether the dosing of FIN in the morning is a rational dosage regimen to obtain sufficient efficacy and avoid the adverse effects.

Authorship Contributions
Participated in research design: Kumazaki, Ando, Ushijima, and Fujimura.
Conducted experiments: Kumazaki, Ando, Ushijima, Maekawa, Motosugi, Takada, Tateishi, and Fujimura.
Performed data analysis: Kumazaki, Ando, Ushijima, and Fujimura.
Wrote or contributed to the writing of the manuscript: Kumazaki, Ando, Ushijima, Tateishi, and Fujimura.

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