Pharmacogenetic analysis of human steroid 5 α reductase type II: comparison of finasteride and dutasteride

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Abstract

Human steroid 5 α -reductase type II is a prostate-specific, membrane-associated enzyme that catalyzes the conversion of testosterone to dihydrotestosterone, the most potent androgen in the prostate gland. Genetic variants of this enzyme have been associated with both the development and the progression of prostate cancer. Both finasteride and dutasteride are competitive inhibitors of the type II steroid 5 α -reductase that have been effectively used for the treatment of benign prostatic hyperplasia. Finasteride has also been successfully utilized for prostate cancer chemoprevention. We here investigate 5 α -reductase inhibition assays *in vitro* to measure the effect of incubation time on the apparent inhibition constant (K_i) for both constitutional and somatic (prostate cancer) enzyme variants. Our systematic pharmacogenetic analysis shows that both finasteride and dutasteride are slow, time-dependent inhibitors of steroid 5 α -reductase type II, and that the inhibition kinetics depend on the 5 α -reductase genotype. We also show that, overall, dutasteride is a more efficient steroid 5 α -reductase inhibitor than finasteride. Based on our data, we are able to map areas of the enzyme that are responsible for this time-dependent inhibition for either (or both) enzyme inhibitor(s). This comprehensive pharmacogenetic analysis of steroid 5 α -reductase variants unveiled significant pharmacogenetic variation for both finasteride and dutasteride and thus should be taken into account when designing protocols for treatment and/or chemoprevention of prostatic diseases with either one of these 5 α -reductase inhibitors since there is considerable pharmacogenetic variation for both drugs.

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Introduction

The prostate gland depends on androgens for both its growth and development (Cheng *et al.* 1993). We have reported evidence that increased intraprostatic androgen metabolism, particularly through genetic variants of the enzyme steroid 5 α -reductase, may play an important role in both predisposition to prostate cancer (Makridakis *et al.* 1999) as well as prostate cancer progression (Makridakis *et al.* 2004). Human prostatic (type II) steroid 5 α -reductase is encoded by the SRD5A2 gene located in chromosome band 2p23 (Russell & Wilson 1994). Steroid 5 α -reductase is a membrane-associated enzyme that catalyzes the conversion of testosterone to dihydrotestosterone (DHT), the most potent androgen in the prostate (e.g. Cheng *et al.* 1993). Thus, genetic variants encoded by the SRD5A2 gene may play an important role in both the development and the progression of prostate cancer (Ross *et al.* 1998).

Finasteride is a specific competitive inhibitor of the prostatic (type II) steroid 5 α -reductase, both *in vivo* and *in vitro* (Stoner 1996, Span *et al.* 1999). These properties

have made finasteride an attractive drug for the treatment of benign prostatic hyperplasia (BPH) (McConnell *et al.* 1998, Lowe *et al.* 2003) and prostate cancer chemoprevention (Thompson *et al.* 2003), with successful results. Specifically, the prostate cancer prevention trial (PCPT) showed that treatment of men 55 years of age or older with finasteride resulted in a significant decrease in prostate cancer incidence measured over a seven-year period (Thompson *et al.* 2003), suggesting that finasteride prevents or delays the appearance of prostate cancer. However, finasteride treatment also resulted in a significant increase in the incidence of prostate tumors of high grade (7 or higher) Gleason score (Thompson *et al.* 2003). Dutasteride (or GG745), a competitive inhibitor of both steroid 5 α -reductase isozymes (type I and type II; Bramson *et al.* 1997), has also been shown to improve the symptoms associated with BPH without any significant increase in adverse side effects (Brown & Nuttall 2003, Clark *et al.* 2004).

We have reported significant genetic and pharmacogenetic variation for both finasteride and dutasteride at

the SRD5A2 locus in both somatic prostate cancer tissue and constitutional DNA of prostate cancer patients (Makridakis *et al.* 2000, 2004). Some of these SRD5A2 mutations resulted in increased enzyme activity and lowered inhibition by finasteride (Makridakis *et al.* 2000, 2004). These findings suggest that the presence of specific SRD5A2 mutants that are not efficiently inhibited by finasteride may, at least partially, explain the unexpected finding of an increase in high grade prostate cancer rate in the PCPT (reported by Thompson *et al.* 2003) since these mutants might result in more aggressive growth of tumors.

The kinetics of steroid 5 α -reductase inhibition that we reported previously (Makridakis *et al.* 2000, 2004) were based on 10-min incubations for all enzyme variants. Finasteride, however, dissociates from the enzyme very slowly, and therefore is a time-dependent inhibitor of steroid 5 α -reductase (Faller *et al.* 1993, Tian *et al.* 1994, 1995). In addition, dutasteride (Frye *et al.* 1998), like finasteride, retains the $\Delta^{1,2}$ bond that is critical for time-dependent inhibition in 4-azasteroids (Russell & Wilson 1994). Thus we decided to compare the kinetics of steroid 5 α -reductase inhibition using 10- and 30-min incubation of the enzyme with either finasteride or dutasteride.

We report here that, like finasteride, dutasteride is a time-dependent inhibitor of steroid 5 α -reductase type II. We also show that dutasteride is a more efficient steroid 5 α -reductase inhibitor than finasteride for most of the enzyme variants that we studied. Based on our data, we are able to map areas of the enzyme that when mutated result in time-independent inhibition for either (or both) enzyme inhibitor(s). This comprehensive pharmacogenetic analysis of steroid 5 α -reductase variants unveiled significant pharmacogenetic variation for both finasteride and dutasteride and this should be taken into account when designing protocols for treatment and/or chemoprevention of prostatic diseases with either one of these drugs.

Materials and methods

Steroid inhibitors

Finasteride was obtained from Merck (Rahway, NJ, USA) and dutasteride was synthesized by Pharmacia & Upjohn (Nerviano, Italy) (Makridakis *et al.* 2000).

Pharmacogenetic analysis

Individual missense substitutions were reconstructed in the SRD5A2 cDNA mammalian expression vector pS303–5 α R2 (originally obtained from Dr D Russell, UT Southwestern Medical College, Dallas, TX, USA), by site-directed mutagenesis using the QuickChange kit (Stratagene, San Diego, CA, USA) with custom primers

(Invitrogen, Carlsbad, CA, USA) (Makridakis *et al.* 2000). The various SRD5A2 cDNAs were electroporated into COS 7 cells together with a β -galactosidase control plasmid (pCMV β , obtained originally from Dr Larry Kedes, University of Southern California, Los Angeles, CA, USA), and the respective protein extracts were collected 48 h later, by sonication. Total protein was quantified with a BioRad protein assay (Hercules, CA, USA) and β -galactosidase assay was then performed to normalize the extracts for differences in transfection efficiency (Makridakis *et al.* 2000). Normalized extract amounts were then used for 5 α -reductase activity assay, as described (Makridakis *et al.* 2000). Briefly, a mixture containing 5 mM NADPH (Sigma, St Louis, MO, USA) and normalized protein extracts was added to specific amounts of each inhibitor at room temperature. Then, 0.8 or 1.6 μ M [14 C]testosterone (NEN, Boston, MA, USA) and 0.1 M Tris–citrate buffer of optimum pH for each variant (Makridakis *et al.* 2000, 2004) were added to the mix, and the reactions were placed at 37 °C for either 10 or 30 min. Initially, the concentration of the inhibitor was varied in 10-fold increments between 10^{-10} and 10^{-5} M, to approximate the apparent inhibition constant (K_i). After the initial approximation, the concentration was varied in five increments around the approximate K_i in triplicate experiments. Following incubation at 37 °C, the reactions were stopped with methylene chloride, and after extraction of the organic phase, steroids were dried, redissolved in ethanol and separated by thin-layer chromatography (TLC) using K6 silica TLC plates (Whatman, Clifton, NJ, USA) (Makridakis *et al.* 1999, 2000). Dried TLC plates were then exposed on phosphorimager screens (Molecular Dynamics, Mountain View, CA, USA) and quantified on a Storm phosphorimager (Molecular Dynamics). Then, substrate-to-product conversion values were translated into velocity values and plotted using Cricket Graph 1.3 (Cricket Software, Malvern, PA, USA). Linear regression analysis of the respective Lineweaver-Burk plots was used to calculate K_i values. The apparent K_i for each variant was determined by averaging the K_i values from three independent experiments. Further experimental details can be found in Makridakis *et al.* (2000).

Results

We previously reported significant genetic and pharmacogenetic variation at the SRD5A2 locus for both finasteride and dutasteride in both somatic prostate cancer tissue and constitutional DNA of prostate cancer patients (Makridakis *et al.* 2000, 2004). Some of the SRD5A2 mutations we characterized are common and/or recurrent, including the A49T (alanine-49 to threonine) mutation, which has been found to be

Table 1 Apparent inhibition constant (K_i) values for finasteride (the wild-type protein and enzyme variants) at the standard reaction time of 10 mins and at the extended reaction time of 30 mins

	Reaction time		Change (10 min vs 30 min)
	10 min	30 min	
Wild type (normal variation)	60 (52–72)	23 (17–29)	2.6
Constitutional variants			
C5R	63	22	2.9
P30L	420	120	3.5
P48R	22	8	2.8
A49T	180	270	0.7
A51T	49	8	6.1
V89L	113	64	1.8
T187M	70	19	3.7
F194L	7	1.9	3.7
R227Q	260	55	4.7
F234L	200	38	5.3
Range	60	142	
Somatic variants			
V3I	27	11	2.5
A49T	180	270	0.7
V63M	38	6	89.7
F118L	5	3.2	1.6
G183D	45	15	3
V189A	90	35	2.6
G191E	40	13	3.1
L221P	57	22	2.6
L226P	105	5.3	19.8
A248V	85	2.6	32.7
Range	36	104	

Range=fold variation between the lowest and highest values.

involved in prostate cancer predisposition and progression (Makridakis *et al.* 1999, 2004). The apparent K_i for both finasteride and dutasteride in those experiments was calculated after transient expression of the reconstructed variant clones in mammalian cells followed by 10-min *in vitro* enzyme assays (Makridakis *et al.* 2000, 2004). However, finasteride is a slow, time-dependent inhibitor of steroid 5 α -reductase (Faller *et al.* 1993, Tian *et al.* 1994, 1995). Therefore, longer finasteride incubations with the wild-type enzyme are expected to result in better inhibition, or lower apparent K_i (see e.g. Moss *et al.* 1996). We decided to test this hypothesis for both finasteride and dutasteride and all the steroid 5 α -reductase type II variants, using 30-min incubations.

As expected, the apparent K_i for finasteride dropped significantly, from 60 nM for the 10-min enzyme assays to 23 nM for the 30-min enzyme assays for the normal (aka wild-type) protein (Table 1). The apparent K_i for dutasteride also dropped significantly, from 17 nM to 4.3 nM (Table 2), suggesting that, like finasteride, the 4-azasteroid dutasteride is also a slow, time-dependent inhibitor of steroid 5 α -reductase type II. The majority of

the steroid 5 α -reductase enzyme variants resulted in a reduction of the apparent K_i for both inhibitors from the 10-min to the 30-min reaction, and thus behave like the wild-type enzyme (Tables 1 and 2). The significance of these findings is emphasized by the small variability observed in the values measured from three independent experiments (Tables 1 and 2 and data not shown). However, several variants resulted in an increase of the apparent K_i for each inhibitor or no significant change between the 10- and 30-min reactions (Fig. 1 and Tables 1 and 2), and thus they exhibit time-independent inhibition. The steroid 5 α -reductase variants that resulted in time-independent inhibition affect enzyme residues in both the amino-terminus and the carboxy-terminus of the protein (residues 49, 118, 226, 227 and 234; Fig. 1), and include the A49T, a gain-of-function mutation in the enzyme (Makridakis *et al.* 1999) that has been associated with both predisposition and progression of prostate cancer (Makridakis *et al.* 1999, Jaffe *et al.* 2000, Makridakis *et al.* 2004).

Comparison of the apparent K_i values for each inhibitor suggests that dutasteride may be a more efficient steroid 5 α -reductase inhibitor (due to its lower

Table 2 Apparent inhibition constant (K_i) values for dutasteride (the wild-type protein and enzyme variants) at the standard reaction time of 10 mins and at the extended reaction time of 30 mins

	Reaction time		Change (10 min vs 30 min)
	10 min	30 min	
Wild type (normal variation)	17 (15–20)	4.3 (3.9–4.9)	4.0
Constitutional variants			
C5R	18	4.5	4.0
P30L	12	6	2.0
P48R	27	3.8	7.1
A49T	1.1	2.3	0.5
A51T	29	2.2	13.2
V89L	9	2.1	4.3
T187M	21	5	4.2
F194L	21	12	1.8
R227Q	3.2	7	0.5
F234L	6	11	0.5
Range	25	6	
Somatic variants			
V3I	93	15	6.2
A49T	1.1	2.3	0.5
V63M	36	5	7.2
F118L	4	0.5	8
G183D	13	3.1	4.2
V189A	23	5	4.6
G191E	25	5	5
L221P	14	4.2	3.3
L226P	9	7	1.3
A248V	13	2.9	4.5
Range	85	30	

Range=fold variation between the lowest and highest values.

apparent K_i) than finasteride for most of the enzyme variants, including the wild-type enzyme and the A49T mutant (Tables 1 and 2). The P30L and A49T mutants, in particular, display a more than 100-fold lower apparent K_i for dutasteride than for finasteride, independently of reaction time (Tables 1 and 2). However, two of the enzyme variants do not follow this trend: the F194L variant (independently of reaction

time) and the P48R variant (but only in the 10-min reaction) display lower apparent K_i (higher affinity) for finasteride than dutasteride (Tables 1 and 2).

Overall, we report a significant pharmacogenetic variation for both somatic and constitutional SRD5A2 variants and for both finasteride and dutasteride (Tables 1 and 2). The distribution of the apparent K_i for finasteride for the distinct SRD5A2 missense substitutions

Figure 1 Mapping of enzyme residues that do **not** show time-dependent inhibition for finasteride (regular font) and dutasteride (*italics*). Black blocks under the sequence indicate 5α-reductase enzyme binding regions for both finasteride and dutasteride (deduced from the effect of specific enzyme variants on the apparent K_i).

varied 84-fold (5–420 nM) in the 10-min reactions and 142-fold (1.9–270 nM) in the 30-min reactions (Table 1). The distribution of the apparent K_i for dutasteride varied 85-fold (1.1–93 nM) in the 10-min reactions and 30-fold (0.5–15 nM) in the 30-min reactions (Table 2). Thus, 30-min incubations with dutasteride resulted in the lowest apparent K_i values overall and the lowest variation (Tables 1 and 2).

Discussion

Both finasteride and dutasteride are important 4-azasteroid inhibitors of steroid 5 α -reductase that have been successfully utilized *in vivo* for the treatment and prevention of prostatic diseases. We previously reported significant pharmacogenetic variation of steroid 5 α -reductase for both finasteride and dutasteride in both somatic prostate cancer tissue and constitutional DNA (Makridakis *et al.* 2000, 2004). Those results, however, were based on 10-min reactions, while finasteride is a slow, time-dependent inhibitor of steroid 5 α -reductase (e.g. Faller *et al.* 1993, Tian *et al.* 1994). Thus, we reasoned that longer incubations with either inhibitor would be better approximations of the true inhibition *in vivo*. Due to the lability of the enzyme *in vitro* and the loss of linearity in the DHT production versus time curve after 40 min for the overactive A49T mutant (data not shown), we decided to use 30-min incubations for all the enzyme variants as the longer reaction time.

Comparison of the apparent K_i values between our standard (10 min) reactions and the extended (30 min) reactions for finasteride (Table 1) indicates that, as reported previously, finasteride is a time-dependent inhibitor of steroid 5 α -reductase. This fact is not only true for the wild-type protein, but also for most of the enzyme variants, whether constitutional or somatic (Table 1). Dutasteride is a dual (i.e. type I and type II) 5 α -reductase inhibitor (Bramson *et al.* 1997) and thus may be more efficacious in reducing DHT levels in the blood, *in vivo*. In addition, most of the 5 α -reductase enzyme variants resulted in significantly lower apparent K_i values for dutasteride in the 30-min reactions compared with the 10-min reactions (Table 2). Therefore, dutasteride is, like finasteride, a slow, time-dependent inhibitor of steroid 5 α -reductase. This finding is not surprising since both dutasteride and finasteride retain the $\Delta^{1,2}$ bond that is critical for time-dependent inhibition in 4-azasteroids (Russell & Wilson 1994). These results also suggest a similar mechanism of time-dependent steroid 5 α -reductase inhibition for both finasteride and dutasteride.

Comparison of the apparent K_i values for each inhibitor suggests that dutasteride was the steroid 5 α -reductase inhibitor that exhibited the lowest appar-

ent K_i for most steroid 5 α -reductase variants, including the wild-type, in both the 10-min and the 30-min reactions (Tables 1 and 2). The only exceptions to this observation were the F194 L variant (independently of reaction time) and the P48R variant (but only in the 10-min reaction). The disease-relevant A49T variant is of particular interest, since it is inhibited much more efficiently by dutasteride than finasteride, irrespective of the reaction time (Tables 1 and 2). Overall, the dual 5 α -reductase inhibitor, dutasteride, has higher affinity for steroid 5 α -reductase type II than finasteride, irrespective of genotype. Dutasteride is also expected to result in lower pharmacogenetic variation than finasteride *in vivo*, since it displays significantly lower pharmacogenetic variation of the apparent K_i than finasteride in the 30-min assays (see below; Tables 1 and 2). Thus, this compound may also be a better choice *in vivo*.

Thirty-minute 5 α -reductase inhibition reactions are expected to be more representative of the *in vivo* 5 α -reductase inhibition than the 10-min reactions we previously reported (Makridakis *et al.* 2000, 2004). The distribution of the apparent K_i for the distinct SRD5A2 variants in the 30-min reactions varied 142-fold (1.9–270 nM) for finasteride (Table 1) and 30-fold (0.5 nM–15 nM) for dutasteride (Table 2). Thus the apparent K_i values for the distinct SRD5A2 variants vary widely, irrespective of reaction time. This significant SRD5A2 pharmacogenetic variation may be important *in vivo* and should be taken into account when using finasteride or dutasteride for the treatment or prevention of prostate cancer. The PCPT trial reported that finasteride treatment in men followed over seven years resulted in a significant decrease in the overall rates of prostate cancer, but also in a significant increase in the rates of high-grade prostate tumors (Thompson *et al.* 2003). This unexpected finding of an increase in high grade prostate cancer rate in the PCPT may, at least partially, be explained by the presence of specific SRD5A2 variants (like A49T) in a subset of the study population that are not efficiently inhibited by finasteride. For individuals carrying the A49T allele, dutasteride might have been a better choice (Table 2 versus Table 1). Thus, future trials such as the PCPT and treatment protocols using 5 α -reductase inhibitors should consider genotyping men for SRD5A2 variants. In fact, we note that the pharmacogenetic inhibition increases for finasteride with time (Table 1) while it decreases for dutasteride (Table 2). This may suggest that dutasteride has a 'tighter' range for allelic and somatic variants, which may lessen the still considerable impact of pharmacogenetic variation *in vitro*. Thus, this compound may be generally better tolerated *in vivo* as well.

The inclusion of a high number of naturally occurring constitutional or somatic (prostate cancer) SRD5A2 variants in our pharmacogenetic analysis allowed us to identify areas of the protein that are important for the

slow, time-dependent inhibition, for both 4-azasteroid inhibitors (Fig. 1). The wild-type as well as most of the steroid 5 α -reductase variants resulted in a reduction of the apparent K_i for both inhibitors from the 10-min to the 30-min reaction, and thus display time-dependent inhibition (Tables 1 and 2). However, several 5 α -reductase variants resulted in time-independent inhibition: (a) the A49T and F118 L variants for finasteride, and (b) the A49T, L226P, R227Q and F234 L variants for dutasteride (Fig. 1, Tables 1 and 2). These enzyme variants affect residues in both the amino-terminus and the carboxy-terminus of the protein (Fig. 1). Comparison of the wild-type versus mutant residue at positions 49, 118, 226, 227 and 234 indicates that the mutant residues are less hydrophobic. Indeed, the average hydropathy index (Kyte & Doolittle 1982) for residues 49 and 118 (for finasteride) is +2.3 for the wild-type residues and +1.5 for the mutant residues. Similarly, for dutasteride, the average hydropathy index (Kyte & Doolittle 1982) for residues 49, 226, 227 and 234 is +1.0 for the wild-type residues and +0.3 for the mutant residues. Thus, hydrophobic interactions between the 4-azasteroid (finasteride/dutasteride) and specific wild-type 5 α -reductase residues may be important for the slow, time-dependent inhibition displayed by these inhibitors. Pharmacogenetic analysis of these 5 α -reductase variants with another 4-azasteroid, such as PNU157706 (di Salle *et al.* 1998) may be instrumental in confirming this observation.

In summary, our systematic analysis of both constitutional and somatic (prostate cancer) variants of steroid 5 α -reductase type II indicates that dutasteride is a more efficient steroid 5 α -reductase type II inhibitor than finasteride *in vitro* for most of the enzyme variants, and that dutasteride treatment is also expected to result in lower pharmacogenetic variation *in vivo* than finasteride treatment. However, the pharmacogenetic variation we uncovered for both finasteride and dutasteride is still very significant and this should be taken into account when designing protocols for treatment and/or chemoprevention of prostatic diseases with either one of these drugs. Our data also suggest that both finasteride and dutasteride are slow, time-dependent inhibitors of steroid 5 α -reductase type II and allow us to map areas of the wild-type enzyme that are (at least partially) responsible for this time-dependent inhibition for either (or both) enzyme inhibitor(s). Pharmacogenetic analyses such as the one presented here may one day help in achieving individualized prostate cancer treatment (and perhaps even prevention).

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