A new CYP3A5 variant, CYP3A5*11, is shown to be defective in nifedipine metabolism in a recombinant cDNA expression system

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Running Title Page

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d) Abbreviations: CYP, cytochrome P450; Reductase, NADPH-cytochrome P450 oxidoreductase, PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; HPLC, high performance liquid chromatography; cDNA, complementary deoxyribonucleic acid; ALA, δ-aminolevulinic acid; and Ni-NTA, Nickel-nitrilotriacetic acid.
Abstract

A new CYP3A5 variant, CYP3A5*11, was found in a single white European by DNA sequencing. The CYP3A5*11 allele contains a single nucleotide polymorphism (SNP) (g.3775 A>G) in exon 2 which results in a Tyr53Cys substitution and a g.6986A>G splice change, the latter SNP previously reported in the defective CYP3A5*3 allele. However, the CYP3A5*3 is not a null allele because this variant is associated with leaky splicing, resulting in small amounts of functional protein still being produced. We therefore constructed a cDNA coding for the newly identified CYP3A5.11 protein by site-directed mutagenesis, expressed it in Escherichia coli and partially purified it. While bacteria transformed with wild-type CYP3A5*1 cDNA expressed predominantly cytochrome P450, those transfected with CYP3A5*11 expressed a significant amount of denatured cytochrome P420 in addition to cytochrome P450, suggesting the protein to be unstable. CYP3A5.11 exhibited a 38% decrease in the Vmax for nifedipine metabolism, a 2.7-fold increase in the Km, and a 4.4-fold decrease in the CLint of nifedipine compared with CYP3A5.1. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping procedure was developed, and used to genotyping DNA of 500
white individuals for *CYP3A5*<sup>11</sup>. No additional examples of this allele were identified. In summary, individuals carrying the rare *CYP3A5*<sup>11</sup> allele are predicted to have lower metabolism of CYP3A5 substrates than individuals expressing *CYP3A5*<sup>3</sup>. 
Introduction

Cytochrome P450s (CYPs) metabolize endogenous and xenobiotic compounds to more water-soluble products that are easily eliminated from the body. CYP3A is the most abundant cytochrome P450 subfamily in human liver and intestine (Watkins et al., 1987; Kolars et al., 1992; Kolars et al., 1994; Shimada et al., 1994), accounting for 30% of total P450 in the human liver, and metabolizes approximately 50% of currently used clinical drugs (Shimada et al., 1994; Guengerich, 1999; Rendic, 2002). There are four human CYP3A genes: CYP3A4, CYP3A5, CYP3A7 and CYP3A43. In postnatal human liver, CYP3A4 and CYP3A5 are the two major CYP3A enzymes, which have overlapping substrate specificities (Guengerich, 1999; Lamba et al., 2002; Lee and Goldstein, 2005). The in vivo observed variability in clearance of CYP3A-drugs can be caused by inhibition, induction, co-medication and/or the inheritance of CYP3A variant alleles encoding enzymes with altered activity (Thummel and Wilkinson, 1998). The CYP3A5 protein expression is highly variable in human liver due largely to the high frequency of an intronic SNP, CYP3A5*3 (g.6986A>G), which creates a cryptic splice site which results in aberrant splicing (Kuehl et al., 2001). Although CYP3A5 may represent 50% of the total
CYP3A content in livers of individuals homozygous for CYP3A5*1, CYP3A5 protein is detected at appreciable levels in only 10-30% of Caucasian and Asian livers due to the high frequency of CYP3A5*3 (Kuehl et al., 2001). In contrast, CYP3A5 is highly expressed in livers of 50% of African-Americans, who have a high incidence of the CYP3A5*1 allele (Kuehl et al., 2001). CYP3A5 is also expressed in extra hepatic tissues, such as lung (Kivisto et al., 1996), kidney (Schuetz et al., 1992; Haehner et al., 1996), breast and leukocytes (Huang et al., 1996; Janardan et al., 1996). The extend to which CYP3A5 contributes to total CYP3A activity has been a matter of debate, and ranges from accounting for up to 50% of total CYP3A protein in the liver (Kuehl et al., 2001) to being only a minor contributor (Westlind-Johnsson et al., 2003). However, the fact that plasma concentration and dose requirement of the CYP3A-metabolized immunosuppressive drug tacrolimus was clearly demonstrated to be correlated to the presence of the CYP3A5*3 allele (Hesselink et al., 2003; Thervet et al., 2003, Hauford et al., 2004), this demonstrates that CYP3A5 activity is important for the metabolism of specific drugs. Many additional CYP3A5 SNPs have been described in recent reports (Lee et al., 2003; Saeki et al., 2003; Solus et al., 2004). Although CYP3A5*3/*3 individuals have low expression of CYP3A5
protein (Kuehl et al., 2001; Lin et al., 2002), livers of these individuals contain also normally spliced RNA next to abnormally spliced RNA due to leaky splicing (Kuehl et al., 2001; Lin et al., 2002). This results in expression of some CYP3A5 protein, although at much lower amounts than in individuals containing the CYP3A5*1 allele (Hustert et al., 2001). The CYP3A5*3 allele has been associated with reduced clearance of lipid-lowering drugs such as simvastatin and lovastatin, as well as midazolam and tacrolimus (Hesselink et al., 2003; Thervet et al., 2003, Haufrroid et al., 2004; Goto et al., 2004; Kivisto et al., 2004; Wong et al., 2004). Although several other CYP3A4 and CYP3A5 alleles are predicted to be defective in vitro, a clear association between CYP3A genotypes and clinical phenotype remains unclear, largely because of the overlap in substrate specificity of CYP3A4 and CYP3A5. To date, the association between CYP3A4 and CYP3A5 genotype and variability in metabolism of CYP3A substrates remains an area of active study. The addition of a new allele, CYP3A5*11 (characterized by the g.3775 A>G SNP), to the total known CYP3A4 and CYP3A5 alleles may be helpful in understanding these individual variations in future clinical studies.
Materials and Methods

Materials

Reduced form β-nicotinamide adenine dinucleotide phosphate (NADPH) nifedipine, δ-aminolevulinic acid (ALA), sodium cholate, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, lysozyme, ampicillin, L-α-dilauroyl-sn-glycero-3-phosphocholine, L-α-dioleoyl-sn-glycero-3-phosphocholine, and bovine brain phosphatidylserine were purchased from Sigma Chemical Co. (St. Louis, USA). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Bevery, MA, USA). Oligonucleotide primers were obtained from Sigma Genosys (Woodlands, TX, USA). QuickChange mutagenesis kit and a proofreading Pfu DNA polymerase were from Stratagene (La Jolla, CA, USA). 

E. coli DH5α competent cells and isopropyl β-D-thiogalactopyranoside (IPTG) were from Invitrogen (Boston, MA, USA). Ni-NTA affinity resin was from Qiagen (Valencia, CA, USA). Imidazole was purchased from Calbiochem (La Jolla, CA, USA). Human NADPH-cytochrome P450 oxidoreductase (P450 reductase) and cytochrome b₅ were from Oxford Biomedical Research (Oxford, MI, USA). All other chemicals and organic solvents for HPLC were of the highest grade from commercial sources.
Identification of \textit{CYP3A5*11}

DNA samples were from 500 healthy white Dutch individuals isolated as reported previously (van Schaik et al., 2002). The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam.

\textbf{Construction, Expression, and Purification of CYP3A5.11}

Wild type CYP3A5 cDNA was kindly provided by Dr. Frank Gonzales (National Cancer Institute, National Institutes of Health). The first eight amino acids in the N-terminus of \textit{CYP3A5*1} cDNA were modified to MALLLAVF as described for the 17-alpha hydroxylase (Barnes et al., 1991) and a 5XHis-tag was added to the C-terminal region. Primers for the N- and C-terminal modifications were described previously (Lee et al., 2003). The modified CYP3A5 cDNA was cloned into the pCW vector via \textit{NdeI} and \textit{HindIII} sites and then utilized as the template for mutagenesis using a QuickChange kit (Stratagene, La Jolla, CA, USA). Mutagenesis primers for the construction of \textit{CYP3A5*11} cDNA were 5’-ggaaatgttttgtctgtcaggtgagttg-3’ (forward) and 5’-caactcacctgacgacaggacaaacacatttcc-3’ (reverse). After construction, the entire \textit{CYP3A5*11} cDNA construct was analyzed by sequencing in both directions.
Expression of CYP3A5 wild type and CYP3A5.11 proteins in E. coli DH5α competent cells was induced with 0.5 mM IPTG and 0.5 mM δ-ALA and incubation proceeded at room temperature for 96 hr. To determine an optimal condition for maximal cytochrome P450 expression, CO difference spectra were monitored from 24 hr to 96 hr using a DW-2000/OLIS Spectrophotometer.

Purification of P450 proteins was performed as previously described (Lee et al., 2003; Lee et al., 2005). To minimize inter-experimental variations in expression and purification, CYP3A5 wild-type and CYP3A5*11 cDNA constructs were simultaneously expressed, solubilized by sonication with detergent, and purified under the same conditions twice. Two sets of purified recombinant P450s were used to verify results. To increase the purity and the recovery of cytochrome P450, solubilized membrane extract was purified on Ni-NTA affinity columns utilizing a histidine-tag. The eluted P450 was dialyzed for 48 hrs in dialysis buffer (100 mM potassium phosphate, pH 7.4 and 20% glycerol) and cytochrome P450 content was measured on a DW-2000/OLIS Spectrophotometer.

Reconstitution and Enzyme Assays
The ratio of P450:P450 reductase:cytochrome b$_5$ was 1:4:2 as optimized previously (Lee et al., 2003). Enzyme activity was reconstituted with incubation mixtures containing 4 pmol of P450 (determined spectrally), human NADPH-cytochrome P450 oxidoreductase (16 pmol), cytochrome b$_5$ (8 pmol), 1:1:1 ratio of lipid mix (2 µg/reaction), sodium cholate (0.05 µmoles/reaction), and MgCl$_2$ (30 mM in final) in a total reaction volume of 0.1 ml in 50 mM potassium buffer, pH 7.7. Nifedipine was protected from light during the experiment. Nifedipine concentrations for the kinetic analyses were 10, 20, 40, 60, 120, 240, and 480 µM. These substrate concentrations were above the lower limit of HPLC detection (6.25 µM) and were within the linear range for enzyme activity. Reactions were pre-incubated for 3 min at 37 °C and initiated with NADPH (1 mM in final) at 37 °C for 10 min. All assays were performed in triplicate. The reactions were stopped with the addition of 50 µl methanol and vigorously vortexed for 2 min followed by centrifugation at 10,000 x g for 15 min. The oxidized metabolite in the supernatant was analyzed by HPLC as described earlier (Lee et al., 2003). There was no catalytic activity in the absence of NADPH.
Development of a Genotyping Test

A specific PCR-RFLP test was developed to detect this novel mutation. For a 50 µl PCR, 10 ng of genomic DNA was incubated in a PCR mixture containing 1X buffer [10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 10 mg/L gelatin (Perkin-Elmer)], 0.2 mM each of the deoxynucleotide triphosphates (Roche), 1.25 U of AmpliTag Gold (Perkin-Elmer), and 40 pmol of primers P1 (5’-CATATTACCTCCCTCTtGAC-3’) and P2 (5’-CTCAAGCAACTCACCTG(T/C)G). The underlined, lower case nucleotides are mismatches with the CYP3A5 sequence, creating restriction sites in the PCR product for the detection of the CYP3A5*11 SNP, but also creating an internal control for the digestion. PCR conditions were: 7 min at 94 ºC; 30 cycles of 1 min at 94 ºC, 1 min at 55 ºC and 1 min at 72 ºC; and finally 7 min at 72 ºC. The size of the amplified product was 213 bp. The PCR product (10 µl) was digested with SalI (New England Biolabs) in a total volume of 15 µl for 2 h at 37ºC and subsequently analyzed on a 3% agarose/Tris-borate-EDTA gel with ethidium bromide staining. The fragments obtained for the wild type allele were 196 and 17 bp; for the CYP3A5*11 variant allele the expected fragments are 175, 21 and 17 bp. The sample identified as a heterozygote CYP3A5*11 by sequencing was
Analysis of Enzyme kinetics and statistics

Nifedipine oxidation was analyzed by nonlinear regression using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). The goodness of fit was determined by the degrees of freedom, absolute sum of squares, and the standard error of the parameter estimates. Kinetics for nifedipine metabolism followed the Michaelis-Menten equation for hyperbolic kinetics as the best fit of the data as previously described (Williams et al., 2002; Lee et al., 2003). Values represent the mean ± S.E of at least two independent experiments, each in triplicate. Statistical significance (p<0.001) was determined using a two-tailed Student’s t-test.
Results

We found a novel SNP while validating a wild type sample for our CYP3A5*8 PCR-RFLP assay by sequencing DNA samples from healthy Caucasians. One sample showed a heterozygous peak at a different site than expected for the CYP3A5*8 polymorphism. The sample was resequenced (in both directions) to confirm the presence of the newly discovered g.3775 A>G SNP (Fig.1). Analysis of this sample for the intronic CYP3A5*3 SNP (van Schaik et al., 2002) revealed that this individual was homozygous for a g. 6986A>G SNP associated with the CYP3A5*3/*3, allele. Subsequent sequencing of all CYP3A5 exons in both directions indicated that no other CYP3A5 SNPs were present (results not shown).

Maximal expression obtained for wild type CYP3A5.1 was 100-150 nmol/liter in E. coli. at 72 hr. CYP3A5.11 exhibited approximately 50% lower P450 expression of 40-70 nmol/liter at 72 hr with an additional peak at 420nm, suggesting that the protein may be unstable. CYP3A5.1 protein did not contain a 420 nm peak at 72 hr of expression in E. coli. Purification of P450s by Ni-NTA affinity column through the histidine-tag resulted in approximately 40% recovery for wild type and ~10% recovery for CYP3A5.11. Purified CYP3A5.1 protein
contained only a small peak at 420nm, but CYP3A5.11 protein exhibited comparable amounts of denatured cytochrome P420 and cytochrome P450 despite two different rounds of expression and purification (Fig.2). For enzyme assays, the reaction was normalized to equal amounts of cytochrome P450, not cytochrome P420. Since kinetic analysis requires large amounts of enzyme, we initially compared the statistical differences in turnover number by two aliquots of CYP3A5.1 and CYP3A5.11 proteins purified on separate days at two different concentrations of substrate. The first concentration of nifedipine, 60 µM, approximates the Km value of wild-type CYP3A5 as determined in our laboratory, and the second higher concentration of 240 µM approaches the Vmax of the enzyme. Fig. 3 shows the results of two independent sets of assays. CYP3A5.11 had significantly lower (67% and 70% (P<0.001) turnover numbers for oxidation of nifedipine at the low substrate concentration and 44% and 50% (P<0.001) lower activity at the high concentration of nifedipine for the two preparations. There was no statistically significant difference in nifedipine oxidation by two preparations of CYP3A5.11 or CYP3A5.1, suggesting low inter-experimental variation in expression and purification. Fig. 4 and Table 1 compare kinetic parameters for nifedipine oxidation by CYP3A5.11 with that of
wild type. Kinetic parameters for nifedipine metabolism by CYP3A5.11 protein exhibited a 39% decrease in the Vmax, a 2.7-fold increase in the Km, and a 4.3-fold decrease in the intrinsic clearance compared to wild type.

We screened 500 healthy white Dutch individuals for this novel CYP3A5*11 polymorphism, but could not identify additional individuals with this single nucleotide polymorphism, thus resulting in an allelic frequency of 0.1% in this population.
Discussion

Genetic variation in the CYP3A genes may influence the metabolism and elimination of CYP3A substrates in humans. Since CYP3A4 and CYP3A5 have overlapping substrates, it has been difficult to determine the effect of each CYP3A genotype on \textit{in vivo} phenotype. The variant g.6986A>G (\textit{CYP3A5}∗3) is responsible for dramatic decreases in hepatic expression of CYP3A5 protein (Kuehl et al., 2001) and is a major determinant in low CYP3A5 protein expression in the liver due to its high frequency in many populations. However, individuals having the \textit{CYP3A5}∗3/∗3 genotype still express low amounts of CYP3A5 protein (Hustert et al., 2001; Westlind-Johnsson et al., 2003). Several studies have identified protein coding variants of CYP3A5, as well as splice variants (Jounaidi et al., 1996; Chou et al., 2001; Hustert et al., 2001; Kuehl et al., 2001; Lee et al., 2003; Solus et al., 2004). One such allele, \textit{CYP3A5}∗10, contains a F446S change in the heme-binding region in addition to the g.6986A>G change associated with the major splice variant (Lee et al., 2003), for which the recombinant CYP3A5.10 (F446S) protein exhibited a greater than 95% decrease in the intrinsic clearance for testosterone and nifedipine compared to wild type CYP3A5.1 (Lee et al., 2003).
In the present study, we identified a new CYP3A5 variant, CYP3A5*11, in white Europeans containing a single base pair change coding for Tyr53Cys. We investigated metabolism of the anti-hypertensive drug nifedipine by recombinant CYP3A5.11 protein, showing a defective variant with a 38% decrease in the Vmax, a 2.7-fold increase in the Km and a 4.3-fold decrease in the intrinsic clearance compared to wild-type CYP3A5.1. Because this allele also contained the g.6986A>G SNP, already lower amounts of functional CYP3A5 protein are being produced (Hustert et al., 2001; Westlind-Johnsson et al., 2003). The presence of the Tyr53Cys variant therefore affects this residual CYP3A5 activity. The exact importance of this new SNP, in the context of the *3 splice variant, needs further studies. Although we have assumed that the *3 and *11 SNPs function independently, one affecting mRNA splicing, the other decreasing the activity of residual protein formed, expression of the CYP3A5*11 allele in a mammalian expression system might be a better way to study this combination of SNPs. However, the Tyr53Cys variant may also occur without the g.6986A>G SNP, and would then be expected to have a more profound effect on total CYP3A5 activity. It remains to be studied whether the observed decrease in activity on nifedipine also hold true for other CYP3A5 substrates.
CYP3A4 and CYP3A5 have 84% amino acid sequence identity: alignment of CYP3A5 Cys-53 with CYP3A4 revealed that the CYP3A5 Cys-53 was not located in the known substrate recognition sites (SRSs) or in the active site cavity of CYP3A4 (Harlow and Halpert, 1997; Domanski et al., 1998; Domanski et al., 2000; Yano et al., 2004). The region containing Cys-53, however, is well conserved in the CYP3A4 and CYP3A5 primary amino acid sequences. In the crystal structure of CYP3A4 protein, Cys-53 appears to be located around the N-terminal “A” helix (Williams et al., 2004). A notable hydrophobic region was found around the loop following helix “A” in CYP3A4, and this hydrophobic region was suggested to be mediated with the microsomal membrane interaction (Williams et al., 2004; Scott and Halpert, 2005). The change from the tyrosine, which is a hydrophobic and aromatic amino acid, to the sulphur containing hydrophilic amino acid cysteine could cause structural changes in the membrane binding regions. The Tyr-53 in CYP3A4 was reported to be one of the amino acids involved in a hydrogen-bonding network with Arg-106, Glu-374, Asp-76, Arg-372, and Asp-61 (Yano et al., 2004). CYP3A4 and CYP3A5 have identical amino acids at all these locations except that CYP3A5 contains a Glu-76. This hydrogen-bonding network has been suggested to have
an important role in the solvent access channel in an X-ray crystallography study (Yano et al., 2004). Therefore, the increased P420 and decreased catalytic activity for nifedipine by CYP3A5.11 protein is likely to result from conformational changes caused by membrane interaction and an altered hydrogen-bonding network. However, although we even used 4x reductase, we cannot exclude that the presence of P420 somehow interferes with reductase requirement of the P450 protein, thereby lowering catalytic activity in our system.

A PCR-RFLP genotyping test, developed for the detection of the g.3775 A>G SNP found in CYP3A5*11, detected no additional examples of this SNP in 500 genomic DNA samples from healthy Caucasians, suggesting that CYP3A5*11 is a very rare allele in whites. One of our previous studies detected CYP3A5*10 only in certain white ethnic groups (Adeyi) (Lee et al., 2005), and it is possible that also the CYP3A5*11 allele is more frequent in other ethnic groups. The present genetic test with functional information will be useful for future clinical studies to link its association with other CYP3A SNPs, particularly in individuals who show defective metabolism of CYP3A substrates.
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Footnotes

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Legends for figures

Fig. 1. Sequencing results of the sample heterozygous for g.3775 A>G SNP (CYP3A5*11), shown as the reverse sequence (T>C) (A) and the PCR-RFLP result of that sample (lane 1) and of 2 wild type controls as confirmed by sequencing (lanes 2 and 3) (B).

Fig. 2. Comparison of CO difference spectrum between CYP3A5.1 (A) and CYP3A5.11 (B). The recombinant cDNA containing CYP3A5*1 wild-type and CYP3A5*11 was constructed in pCW vector. The recombinant plasmid was transformed into E. coli DH5α competent cells. After 72 hr expression, the P450 was purified using a Ni-NTA affinity column utilizing the histidine-tag and reduced CO-spectrum was recorded between 400 and 500 nm using a DW-2000 spectrophotometer. The arrow indicates the P450 peak. CYP3A5.1 exhibited a major peak at 450 nm and a small peak at 420nm. The CYP3A5.11 protein showed two major peaks at 450 and at 420 nm.

Fig. 3. Nifedipine metabolism by CYP3A5.1 and CYP3A5.11 proteins. Reactions include 4 pmol P450 protein, 16 pmol reductase, and 8 pmol
cytochrome b5 (the ratio of P450:reductase:cytochrome b5 = 1:4:2) in 0.1 ml reaction buffer. All values are means of triplicate experiments ± S.E. The preparation of proteins and details of reaction mixtures are described under Materials and Methods. Two different concentrations were used, 60 µM which is near the Km value and 240 µM which is near the Vmax. *, significantly lower (p<0.001) than CYP3A5.1 as analyzed by two-tailed Student's t-test.

Fig. 4. Catalytic profiles of nifedipine metabolism by CYP3A5.1 (closed circles) and CYP3A5.11 (open circles). The preparation of proteins and details of reaction mixtures and kinetics are described under Materials and Methods. All reactions were performed with spectrally determined P450 (4 pmol), 16 pmol reductase, and 8 pmol cytochrome b5 (the ratio of P450:reductase:cytochrome b5 = 1:4:2) in 0.1 ml reaction buffer for 10 min. All values plotted are means of triplicate experiments ± S.E.
**Table 1.** Kinetic properties of nifedipine metabolism between CYP3A5.1 and CYP3A5.11<sup>a</sup>

<table>
<thead>
<tr>
<th>P450s</th>
<th>Vmax (nmol/min/nmol P450)</th>
<th>Km (µM)</th>
<th>CLint (ml/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A5.1</td>
<td>144.1±2.5</td>
<td>44.1±2.6</td>
<td>3.23</td>
</tr>
<tr>
<td>CYP3A5.11</td>
<td>88.2±2.6 (↓39%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>118.7±8.8 (↑2.7x)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.74 (↓4.3x)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The best fit of data was determined by the required parameters using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego CA, 2003). All data were from triplicate measurements ± S. E. Purified P450s were determined by the Soret band at 450 nm. The amount of P450 (not P420 form) was considered in the ratio of mixture (P450:reductase:b5=1:4:2). Data were obtained from 4 pmol P450 per reaction with nifedipine concentrations of 10, 20, 40, 60, 120, 240, 480 µM.  

<sup>b</sup> Fold and % difference compared to values of wild type.