Induced thiacloprid insensitivity in honeybees (*Apis mellifera* L.) is associated with up-regulation of detoxification genes

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Abstract

Honey bees, *Apis mellifera*, are markedly less sensitive to neonicotinoid insecticides containing a cyanoimino pharmacophore than to those with a nitroimino group. Although previous work has suggested this results from enhanced metabolism of the former by detoxification enzymes, the specific enzyme(s) involved remain to be characterised. In this work, a pre-treatment of honey bees with a sub-lethal dose of thiacloprid resulted in induced insensitivity to the same compound immediately following thiacloprid feeding. A longer pre-treatment time resulted in no, or increased, sensitivity. Transcriptome profiling, using microarrays, identified a number of genes encoding detoxification enzymes that were overexpressed significantly in insecticide-treated bees compared to untreated controls. These included five P450s, CYP6BE1, CYP305D1, CYP6AS5, CYP315A1, CYP301A1, and an esterase CCE8. Four of these P450s were functionally expressed in *E. coli* and their ability to metabolise thiacloprid examined by LC-MS analysis.

1. INTRODUCTION

Neonicotinoid insecticides are selective agonists of the invertebrate nicotinic acetylcholine receptor (nAChR), resulting in persistent excitation and ultimately death of the exposed pest insect (Jeschke and Nauen, 2008). Due to their excellent efficacy in crop protection, high specificity for insects and versatility in application methods they have become the market-leading class of synthetic insecticides (Jeschke *et al* 2011). There have been concerns, however, on the possible effects of neonicotinoids on non-target organisms, especially insect pollinators such as honey bees (Goulson *et al* 2013). Seed treatment and soil applications have tended to be the preferred application route for neonicotinioids as they protect young plants due to a long-lasting systemic effect, negating the need for foliar spray (Elbert *et al.,* 2008). Non-target arthropods such as bees may be exposed to sublethal doses of these insecticides through both contact and oral routes when they visit plants during foraging activity and consume the affected food source (nectar and pollen) (Krupke *et al.,* 2012; Blacquière *et al* 2012). The question of whether the sublethal doses, received by pollinators in the field, leads to significant impairment in individual and colony performance is a topic of active research and considerable controversy (for a review see Godfray *et al.* 2014).

Cyano-substituted neonicotinoids (thiacloprid and acetamiprid) have been shown to be orders of magnitude less acutely toxic to honey bees than nitro-substituted compounds (imidacloprid, clothianidin, thiamethoxam, dinotefuran, nitenpyram) (Iwasa *et al*., 2004) and bioassays using inhibitors of detoxification enzymes have provided a strong indication that the differential toxicity observed between the two groups of neonicotinoids is due to increased metabolism of cyano-substituted compounds, rather than intrinsic differences in their affinity for the nAChR (Iwasa *et al*., 2004). The use of synergists has not, however, provided unequivocal evidence as to the primary enzyme system involved in the enhanced metabolism. It is perhaps more likely to be mediated by cytochrome P450s as pretreatment of honey bees with piperonyl butoxide (inhibitor of P450s and esterases) and other chemically distinct P450 inhibitors was shown to dramatically increase the toxicity of

thiacloprid and acetamiprid, whereas no significant differences were observed between bioassays with imidacloprid alone and those pretreated with these inhibitors (Iwasa et al., 2004).

It is well known that neonicotinoids can be metabolised by insecticide-resistant insect pests as a result of enhanced expression of specific cytochrome P450s, for example, CYP6CY3 in *Myzus persicae* and CYP6CM1 in *Bemisia tabaci* (Bass *et al* 2013, Karunker *et al* 2008). Although honey bees have a relatively low number of CYP genes (46) encoding P450s compared to other insect species (Claudianos *et al* 2006), Hardstone et al. concluded " (honey bees)..are not a highly sensitive species to insecticides overall, or even to specific classes of insecticides" (Hardstone *et al* 2010). The precise P450s involved in the metabolism of cyano-substituted neonicotinoids in honey bees and whether their expression is constitutive or induced on exposure to neonicotinoids is unknown. However, P450 induction by xenobiotics including non-neonicotinoid insecticides has been studied previously. Phenobarbital, a well-known general P450 inducer chemical, failed to induce the expression of any CYP genes in a microarray analysis of honey bees (Johnson *et al*., 2012). In contrast, Kezic´ *et al.* (1992) reported that benzo(a)pyrene monooxidase activity was induced after exposure of honey bees to benzo-(a)-pyrene (an inducer of human P450 CYP1A1), the pyrethroid insecticide tau-fluvalinate and the miticide cymiazole. More recently *in vitro* characterisation of eight honey bee P450s of the CYP3 clan revealed that three members of the CYP9Q family have the capacity to metabolise the insecticides tau-fluvalinate and coumaphos. Furthermore, the expression of the P450 CYP9Q3 was induced approximately 1.5-fold by tau-fluvalinate and CYP9Q2 by >1.5-fold by bifenthrin (Mao *et al*., 2011). These findings demonstrate that using xenobiotics, particularly insecticides, as inducing factors might increase metabolic activity and allow identification of specific metabolic enzymes from honey bee that are involved in chemical defence.

The aims of this study were to use an induction strategy, in combination with a range of biological, biochemical and genomic approaches to determine 1) Do honey bees have the

ability to mount a molecular defence (via gene induction) to a neonicotinoid (thiacloprid) after initial exposure to a sub-lethal dose that results in a measurable alteration in phenotype to subsequent exposure? 2) What are the specific detoxification genes, particularly members of the P450 superfamily, induced by exposure to a neonicotinoid (thiacloprid) and 3) Do the enzymes induced have the capacity to metabolise the neonicotinoids and explain the differential toxicity of different members of the class?

2 RESULTS

2.1 Toxicity Bioassays

Full-dose mortality response curves for the oral and contact toxicity bioassays with thiacloprid (Table 1) gave an estimated dose for induction (oral LD_5) of 10 µg/bee and a dose for contact toxicity (LD_{50}) of 61 µg/bee. For the oral toxicity assays the LD_5 was estimated by assuming average consumption (0.055 mg/ml active).

2.2 Induction Experiment

Bee mortality differed between treatments applied topically (acetone or thiacloprid; $F_{1,52}$ = 157.73, $P < 0.001$) and in the timing of topical application following oral pretreatment ($F_{3,52}$ = 3.45, $P = 0.023$). There was also an interaction between oral treatment (thiacloprid or acetone) and time ($F_{3,52} = 4.35$, $P = 0.008$). Immediately following a pre-treatment time of 24 h, topical application of thiacloprid resulted in increased tolerance compared to the controls (t-test, $P = 0.006$). At 48 h and 144 h there were no differences between thiacloprid or acetone pretreatment (t-test, $P > 0.05$). At 96 h there was a significantly increased sensitivity in the bees pre-treated with thiacloprid (t-test, $P = 0.043$). There was no significant difference in mortality in bees topically applied with acetone, regardless of whether oral pretreatment was thiacloprid or acetone (see Table 2 , Figure 1).

2.3 Transcriptome Profiling

Transcriptome profiling using microarrays was used to compare gene expression in bees fed sucrose-insecticide (treated) and those fed sucrose syrup (control) at each time point. An additional array comparison was conducted comprising bees fed sucrose-insecticide that subsequently survived the 0 h topical bioassay versus the non-treated control from the same time point ('survivor' experiment). In the time course experiment 21 probes (11 upregulated and 10 downregulated), 39 probes (21 upregulated and 18 downregulated), 25 probes (18 upregulated and 7 downregulated) and 13 probes (7 upregulated and 6 downregulated) were identified as encoding sequences significantly differentially expressed between control and treated bees at the 0 h, 48 h, 96 h and 144 h time points respectively. In the 'survivor' experiment 95 probes were identified as differentially expressed (57 probes were upregulated and 38 downregulated) between treated bees surviving the topical bioassay at 0 h and non-treated controls from the same time point. The full lists of these probes, the genes to which they correspond and the calculated fold-change is in supplementary table 1. Gene enrichment analysis based on gene ontology revealed the enrichment of a number of GO-terms in the differentially expressed gene sets of each time point (see Supp figures 1-5) with terms related to stress response ('innate immune response', 'defense response to bacterium', 'response to oxidative stress', 'antioxidant activity'), a common theme observed between the time points. A greater number of GO-terms were enriched in the 'survivor' experiment, (Supp figure 5), with several terms suggestive of enhanced oxidative/P450 activity including 'oxidoreductase activity', 'oxidation-reduction process', 'heme binding' and 'monooxygenase activity'.

Among the differentially expressed probes were several corresponding to genes with putative roles in insecticide metabolism that are potential candidates to explain the alterations seen in phenotype in treated bees compared to controls. Thus in the 0 h

comparison two probes representing the P450 gene *CYP315A1* were overexpressed (~1.5-fold) and in the 48 h comparison a single probe for the gene *cytochrome b5* was overexpressed 4.5-fold. At the 96 h time point three probes corresponding to the P450 gene *CYP9Q1* and a single probe representing the carboxylesterase gene *CCE11* were differentially expressed, however in all cases these were down regulated (-1.5 to -1.7). At the 144 h time point no probes encoding detoxification enzymes were differentially expressed. The 'survivor comparison' displayed the greatest number of up-regulated probes encoding detoxification genes. This included four P450 genes, four probes for *CYP6BE1* (1.9-2.2-fold), four probes for *CYP305D1* (1.8-1.9-fold), four probes for *CYP6AS5* (1.6-1.7-fold) and a single probe encoding *CYP301A1* (1.6-fold). For esterases five probes encoding *CCE8* were up-regulated 2.1-2.2-fold and for glutathione-s-transferases a single probe encoding *GSTD1* was over-expressed 1.9-fold. Finally a single probe representing the gene cytochrome b5 was overexpressed (1.5-fold).

A number of probes encoding genes associated with the regulation of transcription/signal transduction, which might be involved in the observed induction, were differentially expressed in multiple array comparisons. This included three G-protein-coupled receptor genes (GPCRs) in the survivor comparison (*GB18244-RA*, *GB18304-RA* and *GB17560-RA*), one GPCR related-gene (*GB15369-RA*) in the 0 h time point and one GPCR in the 144 h time point (*GB18786-RA*). Two genes encoding transcription factors (*GB15791-RA* and *GB10501-RA*) were identified as differentially expressed in the 0 h time point, one in the 48 h time point (*GB14951-RA*), and two in the 96 h time point (*GB18833-RA, GB12301-RA*).

The expression levels of seven of the detoxification candidate genes from the microarray experiment were validated by qPCR with excellent concordance between fold-changes calculated using the qPCR and array data (see Figure 2). The qPCR experiments provided confirmation that six of the seven candidate genes were significantly up-regulated

in treated bees compared to controls with the exception being GSTD1 which was eliminated as a potential candidate.

2.4 Heterologous Expression of Candidate Genes

For the genes (*CYP305D1, CYP315A1, CYP6AS5, CYP6BE1* and *cytochrome b5*) confirmed as being up-regulated by qPCR, heterologous expression focused on exploring the functional role of the P450s in insecticide detoxification. In order to produce catalytically active P450s the candidate genes were co-expressed with an *A. gambiae* CPR in *E. coli* as previously described (McLaughlin et al., 2008). Variation was observed in the yield of recombinant P450 (Table 3), however, reduced CO-difference spectra suggested correctly folded and active enzyme as indicated by significant peaks at 450 nm and lesser 420 nm peaks (Supp Figure 6). The cytochrome b5 was also successfully expressed and purified from *E. coli* membranes.

2.5 Metabolism Assays

The ability of CYP305D1, CYP315A1, CYP6AS5 and CYP6BE1 proteins (in combination with the *A. gambiae* CPR and bee cytochrome b5) to detoxify thiacloprid and imidacloprid in the presence and absence of NADPH was examined in insecticide metabolism assays. Figures 3 and 4 outline the results of monitoring thiacloprid and imidacloprid recovery in the samples using srm methods, with quantification against standard calibration curves. No significant differences were observed in thiacloprid or imidacloprid recoveries between the +/- NADPH samples for any of the four P450s.

3. DISCUSSION

Honey bees display profound differences in their susceptibility to different neonicotionoid insecticides, being considerably less sensitive to cyano-substituted neonicotinoids such as

thiacloprid. Research to date has provided strong indications that this is due to an innate ability of the bees to metabolise neonicotinoids containing a cyano pharmacophore; however, the precise metabolic enzymes involved and whether their expression is constitutive or induced upon exposure to neonicotinoids was unknown. The main aim of the present study was to address these two questions by feeding bees a sublethal dose of thiacloprid and assessing 1) changes in thiacloprid sensitivity in bioassays and 2) changes in gene expression in whole transcriptome microarrays.

The bioassay time course experiment showed that a measurable reduction in thiacloprid sensitivity could be induced in honey bees after exposure to a sub-lethal dose of this neonicotinoid for 24 hours. This effect was time dependent with increased tolerance only observed immediately following the 24 h of thiacloprid feeding, with treated bees becoming more susceptible than controls at the later time point (96 h). To our knowledge this is the first report with honey bees of a sub-lethal dose of an insecticide providing a protective effect to subsequent exposure of the same insecticide. Indeed, in a related study on honey bees, no effect on the toxicity of the pyrethroid insecticide tau-fluvalinate was observed in bees fed phenobarbital, xanthotoxin, salicylic acid, indole-3-carbinol compared to controls fed sucrose, although the effect of tau-fluvalinate feeding on subsequent tau-fluvalinate toxicity was not examined (Johnson et al 2012).

One possible explanation for our finding of an induced decrease in sensitivity to thiacloprid in bees is that the sub-lethal exposure activates the transcription of one or more genes encoding detoxification/defence proteins over the 0-48h time points and that these subsequently return to constitutive levels or lower than constitutive levels at the later time points. To explore this we carried out a series of microarray comparisons of global gene expression levels in treated versus control bees over the time series ('time course experiment') and a second experiment where treated bees surviving the topical bioassay at 0 h were compared with non-treated controls from the same time point ('survivor experiment'). Across all comparisons the number of genes differentially expressed (13-96

probes representing 0.08-0.6% of the 15737 probes on the array), and the fold changes observed (<8-fold), between treated and control bees were low. Nevertheless, the observed changes were subsequently confirmed by qPCR with a number of candidate genes being validated as moderately, but significantly, over-expressed in treated bees. GO-term analysis of these differentially expressed genes revealed enriched ontology terms associated with a general stress response and also terms relating to P450-mediated detoxification, the latter resulting from the enhanced expression of a number of probes encoding several honey bee P450s/cytochrome b5. P450 genes were only identified in the earlier time points (0h, 0h survivors, 48h) where altered thiacloprid toxicity was observed in bioassays and the only gene related to detoxification observed in two separate array experiments was cytochrome b5, which can act as an electron donor to P450s. Among the CYP genes *CYP315A1* was the only P450 identified as overexpressed in the time course experiment (at the 0 h time point) and this is the ortholog of the *Drosophila melanogaster sad* gene encoding the steroid 2-hydroxylase (Claudianos et al 2006). However, in the 'survivor' experiment in which 'treated bees' were fed thiacloprid for 24 h and then survived a subsequent topical application of thiacloprid (LD_{50}) a number of P450s were identified as being over-expressed. This included two members of the CYP3 clade *CYP6BE1* and *CYP6AS5*, whose members have been most commonly involved in detoxification of xenobiotics including pesticides in other insects (Li et al 2007). Two further P450s *CYP305D1* and *CYP301A1*, the latter of which was only represented by a single probe, belong to the CYP2 and mitochondrial clades respectively were also overexpressed. The role of *CYP305D1* is yet to be determined but *CYP301A1* is thought to be involved in ecdysone regulation during adult cuticle formation (Sztal et al 2012). Beyond detoxification genes several genes involved in the regulation of transcription/signal transduction were also identified as differentially expressed in multiple array comparisons including both transcription factors and a number of G-protein-coupled receptors (GPCRs). It is possible that these genes may play a role in triggering/regulating the enhanced transcription of the CYP/detox genes. In the case of GPCRs recent work has suggested they may be involved in regulating overexpressed P450s observed in resistant

moquitoes, *Culex quinquefasciatus*, and housefly, *Musca domestica*, (Li et al 2014, Li et al 2013). For *C. quinquefasciatus* knockdown of four GPCR genes by RNAi both decreased resistance to permethrin and repressed the expression of four insecticide-resistance related P450 genes (Li et al 2014). It would be interesting to examine the role of these receptors in honey bee gene expression responses to xenobiotics in more detail using a similar approach.

The four P450s, *CYP6BE1, CYP6AS5, CYP315A1* and *CYP305D1*, all of which were represented by multiple overexpressed probes in array comparisons and validated by qPCR, were functionally expressed in *E.coli* in combination with the *A. gambiae* P450 reductase to examine their potential to metabolise thiacloprid. Honey bee cytochrome b5 was also expressed and included in metabolism assays as this enzyme has been show to modify the catalytic activity of P450s in other insect systems. Although functional P450 proteins were obtained for all four CYP genes, no metabolism of thiacloprid (as assessed by parent compound depletion after incubation of thiacloprid with recombinant P450 in the presence of NADPH) was observed for any of the four P450s. These findings suggest that the P450s induced in our experiments do not have the ability to detoxify thiacloprid and if innate bee tolerance to this compound is indeed mediated by P450s, their expression may be constitutive and hence would not have been detected in our experiments. Alternatively a different enzyme system, such as esterases may be responsible for thiacloprid metabolism/ sequestration. Indeed, the toxicity of the nitro cyano-substituted neonicotinoid acetamiprid was synergised (synergism ration of 2.96) by the inhibitor S,S,S,-tributylphosphphorotrithioate (DEF) suggesting esterases may play a contributory role in detoxification (Iwasa *et al* 2004). In our 'survivor' array comparison five probes representing the esterase CCE8 were overexpressed and this was confirmed by qPCR. This CCE falls into clade A, classified as intracellular enzymes with dietary/detoxification functions (Claudianos et al 2006). Attempts to functionally express this esterase resulted in non-

functional enzyme (data not shown) so we were unable to confirm any role of this enzyme in thiacloprid metabolism.

In summary a number of genes, including several P450s, are induced in honey bees exposed to a sub-lethal dose of thiacloprid and this is associated with a measurable, temporary, reduction in toxicity on subsequent thiacloprid exposure. However a causative role for these P450s in thiacloprid tolerance could not be demonstrated and the specific enzymes involved in the thiacloprid insensitivity remain to be determined.

4 EXPERIMENTAL PROCEDURES

4.1 Insect Material

Apis mellifera carnica was provided by the AgroEcology Department, Rothamsted Research. All Rothamsted Research hives were treated with Fumidil (an antibiotic to treat Nosema disease) in September, and treated with Apiguard (to reduce Varroa mite) in August and September by the beekeeper. The colonies were checked weekly between April and September, given more boxes/space as required, honey taken off for extraction during the summer, and fed sugar syrup in the autumn to maintain the hive through winter. Frames of sealed brood were collected and incubated at 34°C overnight. Emerged worker bees were used for the bioassays.

4.2 Reagents

Restriction enzymes were supplied by Promega (UK); oligonucleotides, technical insecticides (PESTANAL) and other analytical grade reagents were obtained from Sigma-Aldrich (UK).

4.3 Toxicity Bioassays

4.3.1 Oral toxicity tests

Technical grade thiacloprid was dissolved in acetone and then added to sucrose syrup (50%) in water. Newly emerged worker bees (~10) were transferred to plastic cages after anesthetising with CO_2 where they were treated with a range of thiacloprid concentrations for 24 h through oral feeding; the amount of treated diet consumed by each cage was measured by the difference in weight of sucrose syrup before and after the experiment. All treated bees were maintained at 25^{o}C in the dark. All bioassays were scored after 24 h and bees not walking or flying were counted as dead.

4.3.2 Contact toxicity tests

Samples were also subjected to a range of thiacloprid concentrations by topical application: newly emerged worker bees were transferred to plastic cages after anesthetising with CO_2 (ten bees per cage, at least three replicates per insecticide dosage). For each cage a reservoir of 50% sucrose in water was available for *ad lib* feeding. Prior to treatment bees were anesthetised by low exposure to CO_2. Each bee was topically dosed (1uL) with either thiacloprid in acetone or acetone alone applied to the dorsal thorax using a Burkard microapplicator (Burkard, Rickmansworth, UK). All treated bees were maintained at 25^{o}C in the dark and bioassays were scored after 24 h, bees not walking or flying were counted as dead.

4.4 Induction Experiment

Thiacloprid toxicity was assayed *in vivo* after exposure to a sub-lethal concentration of thiacloprid, to check for measurable alteration in phenotype. A factorial set of 16 treatments was tested in two repeat experiments. Bees (9–15 per cage = one replicate, 72 cages in total) were fed either a sub-lethal dose (LD_5 0.055 mg/ml) of thiacloprid (dissolved in acetone and then sugar solution) or acetone in sugar solution (controls) for 24 h. At each time point, (0 h, 48 h, 96 h and 144 h) immediately prior to topical application, 2 cages of oral fed acetone and 2 cages oral fed thiacloprid were snap frozen for microarray analysis as described in section 4.5 (16 cages total for each of experiment 1 and 2); . At each of 0 h, 48

h, 96 h and 144 h a diagnostic dose of thiacloprid (61 µg equating to LD_{50}) in acetone or acetone alone was then topically applied to at least 4 cages of oral fed acetone and oral fed thiacloprid to give at least 2 cages of each possible combination (acetone-acetone, acetone-thiacloprid, thiacloprid-acetone, thiacloprid-thiacloprid) at least 32 cages total for each of experiment 1 and 2 (Figure 5). Samples of bees surviving the treatment ("survivors") were snap frozen and stored at −80°C for subsequent molecular analyses as described in section 4.5.

4.5 Microarray Analysis

A custom microarray designed using the Agilent eArray platform (Agilent Technologies) contained 60bp oligonucleotide probes for each of the honey bee consensus gene set (~10,000 genes) derived from the annotated honey bee genome. A SurePrint HD (8×15k) expression array was designed using the base composition and the best probe methodologies to design sense orientation 60-mer probes with a 3′ bias. For each contig encoding a detoxification enzyme (P450s, GSTs and CEs) three probes were designed. Additional probe groups for 15 control genes were included.

Groups of four bees per replicate were ground to a fine powder in liquid nitrogen using a pestle and mortar. RNA was extracted from the pooled homogenates using the Bioline Isolate RNA Mini Kit according to the product manual. The quantity of RNA was checked using a nanodrop spectrophotometer and by running an aliquot on a 1.5% agarose gel. For the latter, RNA was mixed with 1x loading buffer (95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM EDTA; 0.025% SDS), heated for 5 minutes at 65 °C and briefly chilled on ice prior to loading. Two micrograms of each RNA was used to generate labelled cRNA, which was hybridised to the arrays, which were then washed and scanned as described in the Agilent Quick Amp Labeling Protocol (Version 5.7). The

experiments consisted of four/five biological replicates and for each of these, hybridisations were done in which the Cy3 and Cy5 labels were swapped between samples.

Microarrays were scanned with an Agilent G2565CA scanner and fluorescence intensities of individual spots were obtained using the Agilent Feature Extraction software with default Agilent parameters. Data normalization, filtering, dye flipping and statistical analysis were performed using the GeneSpring GX suite. For statistical analysis, a t-test with null hypothesis of no difference between treatments was used to detect differentially expressed genes. Genes were considered differentially expressed if they had a *P* value of <0.05 and a fold change (up or down) greater than 1.5.

4.6 Quantitative PCR

Primers were designed to amplify a fragment ~100 bp using the Primer3 program (Table 4). 4 µg of RNA was used for reverse transcription using Superscript II Reverse Transcriptase and random hexamers (Invitrogen). Each PCR reaction consisted of 4 µl of cDNA (10 ng), 5 µl of SensiMix SYBR Kit (Bioline) and 0.5 µl of each forward and reverse primer (0.25mM). PCRs were run on a Rotor-Gene 6000 (Corbett Research) with cycling conditions: 10 minutes at 95°C followed by 40 cycles of 95°C for 15 s, 60 °C for 15 s and 72°C for 20 s. A final melt-curve step was included post-PCR (ramping from 72°C to 95°C by 1°C every 5 s) to confirm the absence of any non-specific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution from 100 ng to 0.01 ng of cDNA. Each qRT-PCR experiment consisted of at least three independent biological replicates with two technical replicates. Data were analysed according to the ΔΔCT method (Pfaffl 2001), using the geometric mean of two selected housekeeping genes (elongation factor and actin) for normalisation according to the strategy described previously (Vandesompele et al. 2002).

4.7 Heterologous Expression of Candidate Genes

4.7.1 Cloning CYPs

The candidate honey bee P450s (CYP305D1, CYP315A1, CYP6AS5, CYP6BE1) were amplified from cDNA using KAPA high-fidelity DNA Polymerase (Kapa Biosystems) following the product manual. As a proofreading DNA polymerase was used for amplification, which leaves blunt ended DNA, A-tailing reactions were carried out prior to cloning. After the product was cleaned, it was ligated into the pSC-A-amp/kan cloning vector. For functional P450 expression in *E. coli* the N-terminal coding region of each P450 cDNA was modified: the ompA leader sequence (21 amino acid residues) and two linker amino acid residues (alanine-proline) were added to the 5' end of P450s (ompA+2 strategy) (Pritchard et al, 1997; 2006, McLaughlin et al, 2008). This was achieved by two fusion PCR reactions, carried out using high-fidelity DNA polymerase according to the manufacturer's instructions. In the first PCR, genomic DNA of the *E. coli* JM109 cell line was used as template to amplify a ~100 bp nucleotide fragment (containing the ompA+2 sequence and the first 21 bases of the target P450 gene) preceded by a *Nde*I restriction site using the primers described in Table 5. This intermediate PCR product was purified and then fused to the P450 plasmid template in a second PCR reaction using the same forward and CYP specific reverse primers (table 5) to generate the full-length ompA-AP-CYP coding sequence flanked by *Nde*I and *Xba*I restriction sites. The final product was digested and ligated into modified pCW-ori+ vector via *Xba*I and *Nde*I restriction sites and the final sequence were confirmed by sequencing prior to expression.

4.7.2 Preparation of membranes

Competent *E. coli* JM109 cells were co-transformed with pCW-Ori+CYPs and pACYC-AgCPR to enable co-expression of each CYP with the *Anopheles gambiae* CPR following the methods described by Stevenson et al. (2012). Plasmids were transformed into JM109 cells and overnight cultures in 200 mL terrific broth incubated at 30°C with shaking. When the cultures reached early log phase growth expression was induced by adding 1 mM IPTG. δ-aminolevulinic acid hydrochloride was added to a final concentration of 0.5 mM at the same time to compensate the low levels of endogenous heme in the bacterial cells. Further

incubation was at 24°C for 23 hours before the cells were harvested by centrifugation and membranes prepared as described by Pritchard *et al.* 1998. Membranes were resuspended in ice-cold 1X TSE buffer in a Dounce tissue homogeniser and aliquots stored at -80^{o}C. P450 content (Omura and Sato, 1964), total protein concentration (Bradford, 1976) and CPR content (Strobel and Dignam, 1978) were analysed.

4.7.3 Cloning cytochrome b5

To simplify the purification of the expressed b5 protein the N-terminal coding region was modified by the addition of six histidine residues (6H) to the 5' end (Holmans et al., 1994; Stevenson et al., 2011). This was achieved using high-fidelity DNA polymerase and the forward primer 5'-GGAATTCCATATG**CACCATCACCATCACCAC**ATGTCGAAAATTTTTACAGCGGA-3' (*Nde*I restriction site underlined and six histidine codons in bold before start codon) and reverse primer 5'-GAATTCTCTAGATTATGAATACCAAAAATAGTAAAAT-3' (*Xba*I restriction site underlined). The final product was digested and ligated into the modified pCW-ori+ vector via the *Xba*I and *Nde*I restriction sites with the final sequences confirmed by sequencing prior to expression.

4.7.4 Expression and purification of cytochrome b5

The 6H-b5 construct was transformed into JM109 cells and after overnight culture was transferred to 500 mL terrific broth (TB) media containing 50 µg/mL of ampicillin in a 1L flask and shaken at 37°C at 200 rpm and expression induced as described above. Cells were harvested as for the P450s. Pelleted cells were resuspended and treated by the Holmans *et al.* (1994) method with Stevenson et al. (2011) modifications. Expression of b5 was checked and quantified by spectrophotometry (Omura and Sato, 1964).

4.8 Insecticide Metabolism

Insecticide (thiacloprid and imidacloprid) metabolism assays of recombinant bee P450s/CPR/b5 standard reactions were carried out using three replicates for each P450 in the presence or absence of NADPH. A 10 mM stock solution of thiacloprid and imidacloprid

were prepared in DMSO and diluted to 100 µM in phosphate buffer (0.1 M, pH 7.6) before adding the reaction to avoid the precipitation of insecticide. Standard reactions consisted of final concentrations of 10 µM insecticide, 100 µl NADPH regeneration system (Promega) (or buffer alone in the case of minus NADPH controls), 0.0117 µM cytochrome b5 and 0.1- 0.4 µM P450 membrane. Reactions (200 µl total) were incubated at 30^0C, shaking at 1200 rpm for 2 h and then stopped by adding 300 µl of acetonitrile. Samples were then spun at 2000 g for 5 min and 250 µl of supernatant was transferred to HPLC vials and stored at -20^0C for LC-MS/MS analysis.

4.9 LC-MS Analysis

Aliquots of each sample were diluted 50:50 in acetonitrile prior to LC-MS/MS analysis. Separation was achieved using Ultra Performance LC® (ACQUITY UPLC-System; Waters, UK) using an ACQUITY UPLC column (HSS T3, 1.8 µm, 100 x 2.1 mm), with a mobile phase consisting of water (+0.2% formic acid), with a flow rate of 0.6 mL/min. The gradient elution conditions of acetonitrile:water were: 0 min 0:100, 0.5 min 0:100, 3.5 min 95:5, 4.5 min 95:5, 4.6 min 0:100, 5 min 0:100. The mass spectrometer was a Finnigan TSQ Quantum Discovery (Thermo Scientific, UK) equipped with an Ion Max source operating in positive ion mode. Analytes were detected using selected-reaction-monitoring (SRM), transitions as outlined in table 6. Quantification was achieved using standard calibration curves constructed in 50:50 acetonitrile:water.

4.10 Statistical analysis

Table 1 shows the results of probit analysis of the data from full dose oral and contact bioassays using the statistical program PC Polo Plus (LeOra Software, Berkeley, CA), and concentrations required to kill 5% of the population (LD_5, oral) and 50% of the population (LD_{50}, contact) were estimated after correcting for control mortality (Abbott 1925, Finney 1971). Data from the induction bioassays were analysed in GenStat (14^{th} edition, VSN International) using logistic regression (i.e. a generalized linear model with binomial error

and logit link), allowing for differences between experiments before testing treatment effects and with adjustment for over-dispersion.

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Tables

Table 1

Full dose response bioassays of honey bee against thiacloprid (µg/bee)

Bioassay	LD_{05}	LD_{50}	CL 95%	Slope ± SE	df	X^2	No.
Oral	10.1		4.45 – 16.2	2.29 ± 0.33	13	12.3	150
Contact		61.3	41.0 – 85.2	2.05 ± 0.32	13	17.3	150

LD_{05} = lethal dose of thiacloprid to kill 5% of the population.

LD_{50} = lethal dose of thiacloprid to kill 50% of the population

CL = Confidence limits; df = degrees of freedom; X^2 = chi-square; No. = number of bees

Table 2 Comparison of mean mortalities (logit scale) for bees fed either thiacloprid or acetone and subsequently topically applied with either acetone or thiacloprid at various times post-feeding

TIME	0 h		48 h		96 h		144 h	
Topical	Ace	Thia	Ace	Thia	Ace	Thia	Ace	Thia
Difference	0.966	-1.351	0.471	-0.628	-0.419	0.978	0.766	0.789
t-statistic	0.628	-2.884	0.384	-1.199	-0.403	2.075	0.470	1.547
P value	0.533	-0.006	0.702	0.236	0.688	0.043	0.640	0.128

Table 3 P450 concentration and CPR activity in expressed CYPs

CYPs	P450 concentration (nmol P450/mg protein)	CPR activity (nmoles cyt c/min/mg protein)
CYP305D1	0.17	8.8
CYP315A1	0.116	6.9
CYP6AS5	0.045	4
CYP6BE1	0.0518	7

Table 4 Oligonucleotide primer sequences used in qPCR

Gene	Accession no	Primer	Sequence (5′- 3′)	Product size (bp)
CYP305D1	GB11943	Forward	GGACGTCCTTGGAACGAAT	108
		Reverse	TCGCATCATCCAATTTCGTA	
CYP315A1	GB16447	Forward	CTGGGTCCCGTTTACAAAGA	101
		Reverse	GGTGTTGACCCTTCAAGTCG	
CYP6AS5	GB17434	Forward	CAGGCTCTCCCCAATATTCA	120
		Reverse	TCGATGGGCTCATTTTTCTC	
CYP6BE1	GB14612	Forward	CGAAAGGAACTTGCATAGCC	120
		Reverse	TCTTCGGAAAATCGTTCTGG	
Cytb5	GB12288	Forward	CAGCGGAAGAAGTAGCGAAA	101
		Reverse	GCCTGGATGTTCGCTTAGAA	
GSTD1	GB18045	Forward	AAAAATGCTTGTTATTTTCTGTCTGA	110
		Reverse	TCAAACGCGTCTTCGAGTATC	
CCE8	GB11064	Forward	TCTGCTTGCGCATTCTATTG	106
		Reverse	CTTTACGCGCTTCTTTGTCC	

Table 5 Primers used for fusion PCRs. All primers are listed in the 5' to 3' direction

CYPs[a]	Reverse Primer[b]	CYP Specific Reverse Primer[c]
ompA-AP-CYP305D1	ACTATTAACATTATAACAAACATX	GAATTCTCTAGATTATCGTTTTTCAACTAATACA
ompA-AP-CYP315A1	AATATTTTGCGCAAGATTCAT X	GAATTCTCTAGACTAATTTCTCTCCATCAATTT
ompA-AP-CYP6AS5	AATTTCGAAACTGCTCGCCAT X	GAATTCTCTAGATCATATTTTTGTTATTTTCAAATA
ompA-AP-CYP6BE1	TAACCACGTAGTTAAAAACAT X	GAATTCTCTAGATTATATTGGCTCAATATTTAGA

[a]The forward primer used for ompA+2 fusion PCR strategy was always 5'-GGAATTCCATATGAAAAAGACAGCTATCGCG -3' with *the Nde*I restriction site underlined.
[b]Reverse complement of the start of CYPs and X represents 5'-CGGAGCGGCCTGCGCTACGGTAGCGAA-3' which corresponds to the reverse complement of proline and alanine codons and the ompa segment sequence.
[c]The region corresponding to the reverse complement of the end of CYPs with *Xba*I restriction site (underlined).

Table 6: SRM transitions and collision energies

Analyte	Molecular Weight (Da)	SRM Transition Parent *m\z* > Product *m\z*	Retention Time (min)
Thiacloprid	254	253 > 126 253 > 186	4.83
Imidacloprid	257	256 > 175 256 > 209	4.47

Figures

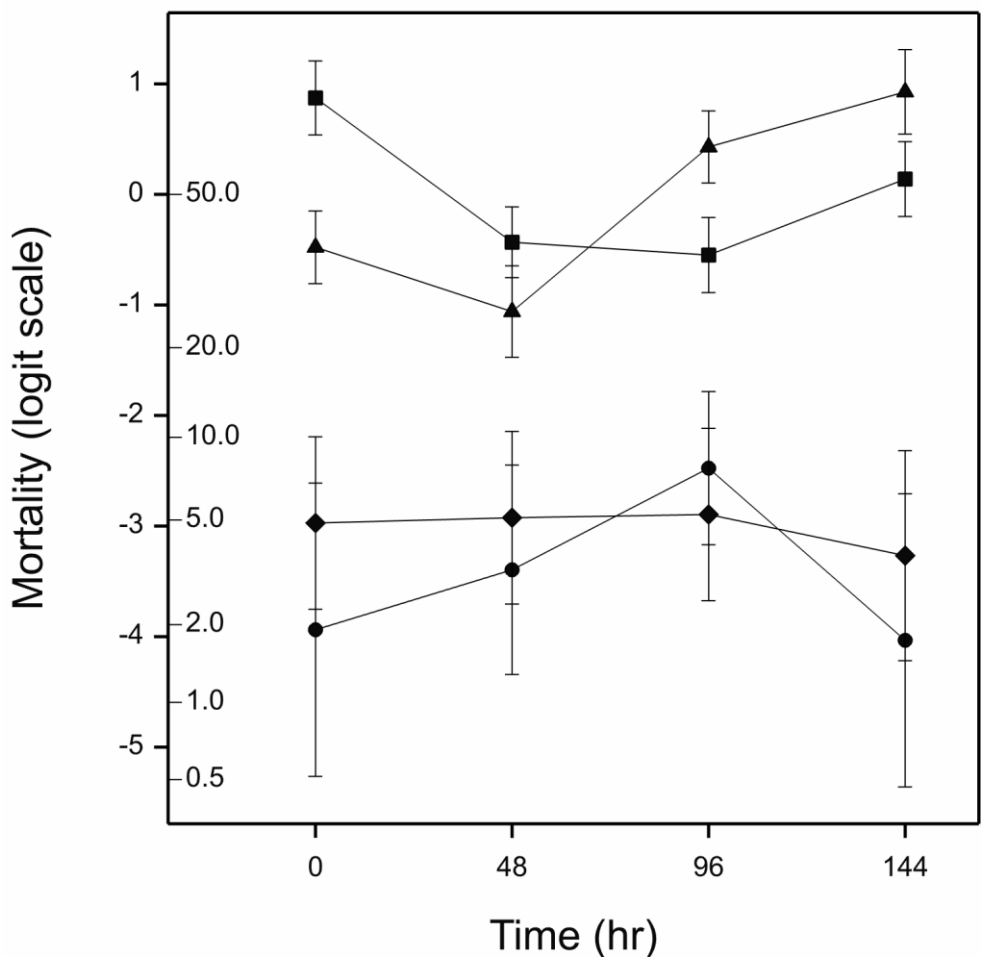

Figure 1 Treatment mean mortalities (logit scale; n = 1–3 cages of 9–15 bees) from logistic regression (± SE). Fed acetone, topical acetone (circles); fed thiacloprid, topical acetone (diamonds); fed acetone, topical thiacloprid (squares); fed thiacloprid, topical thiacloprid (triangles). Time = delay following 24 h oral pre-treatment. Inner Y-scale represents mortality (%). Mortality was assessed 24 h after topical application.

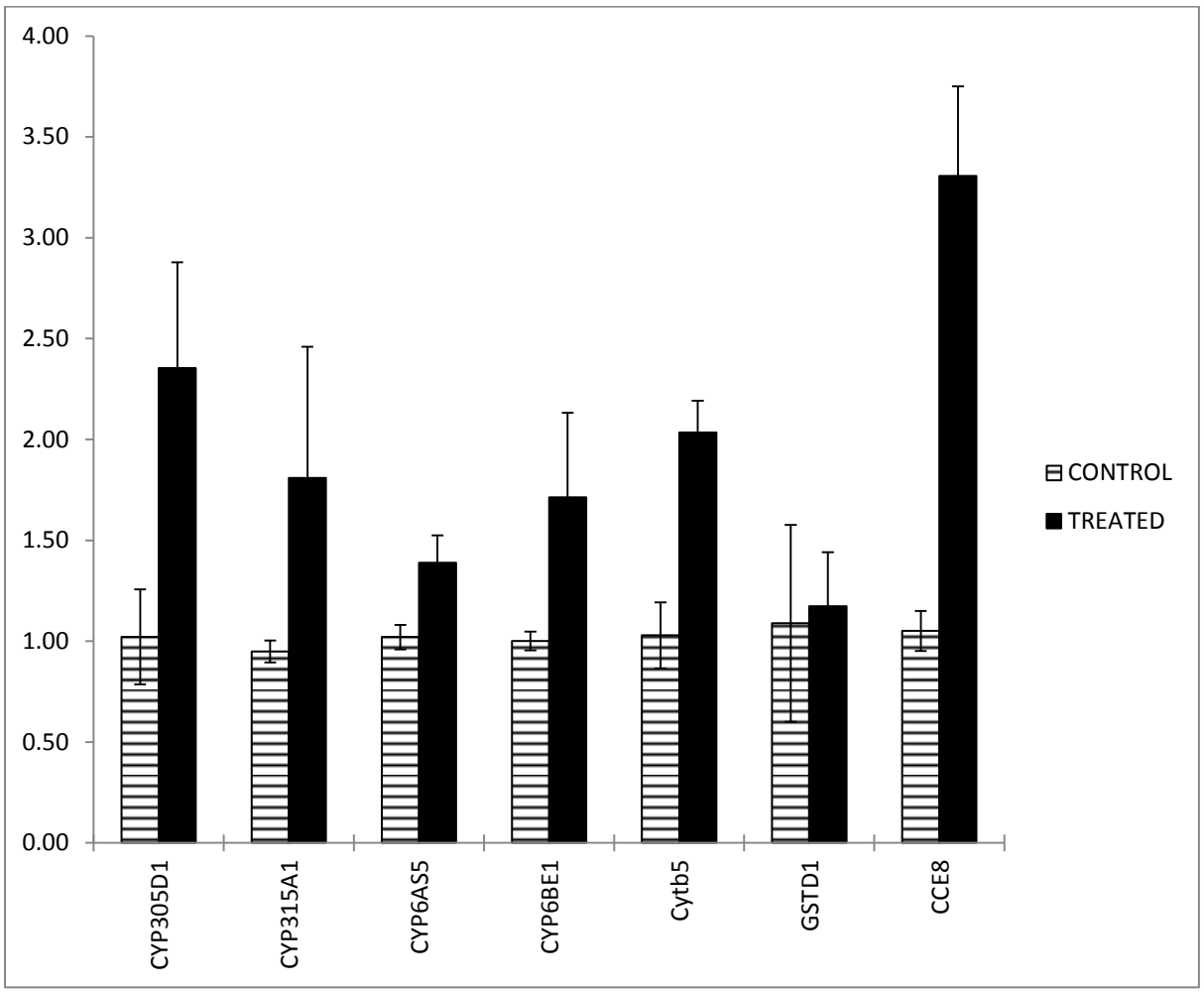

Figure 2 Expression levels determined with qPCR for selected genes using the geometric mean of a selected housekeeping genes (ef1 and actin) (error bars show 95% confidence limits)

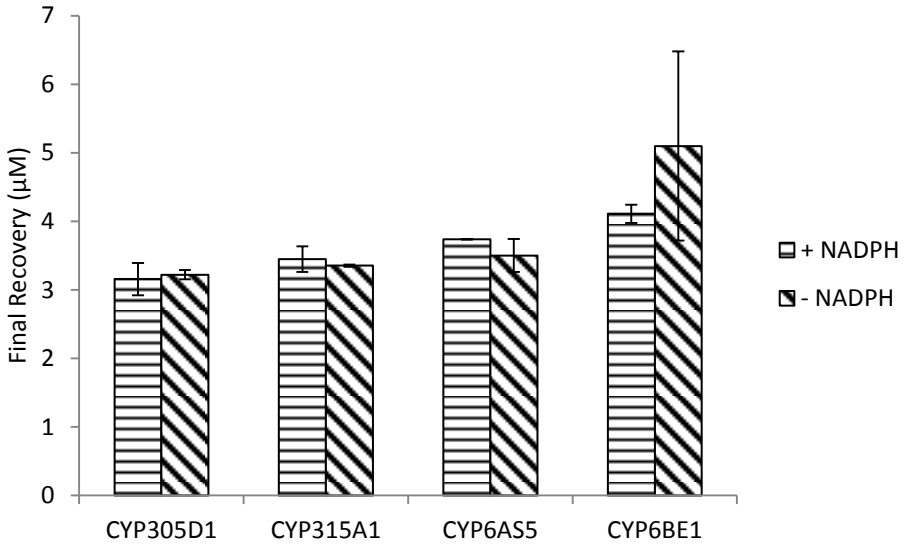

Figure 3 Recovery of thiacloprid after a 2 h incubation with the four honeybee P450s. Graph represents mean final recovery (µM) ± SE (n = 3)

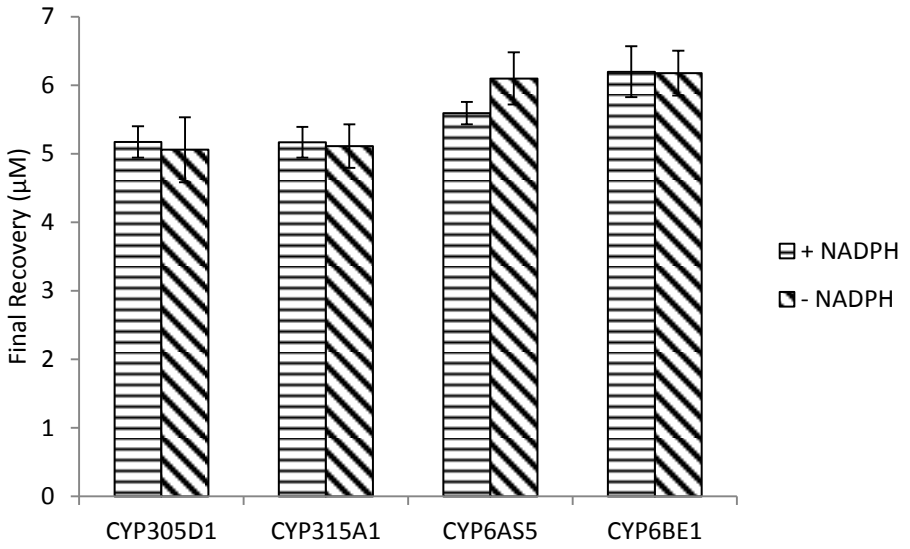

Figure 4 Recoveries of imidacloprid after a 2 h incubation with the four honeybee P450 expression systems. Graph represents mean final recovery (µM) ± SE (n = 3)

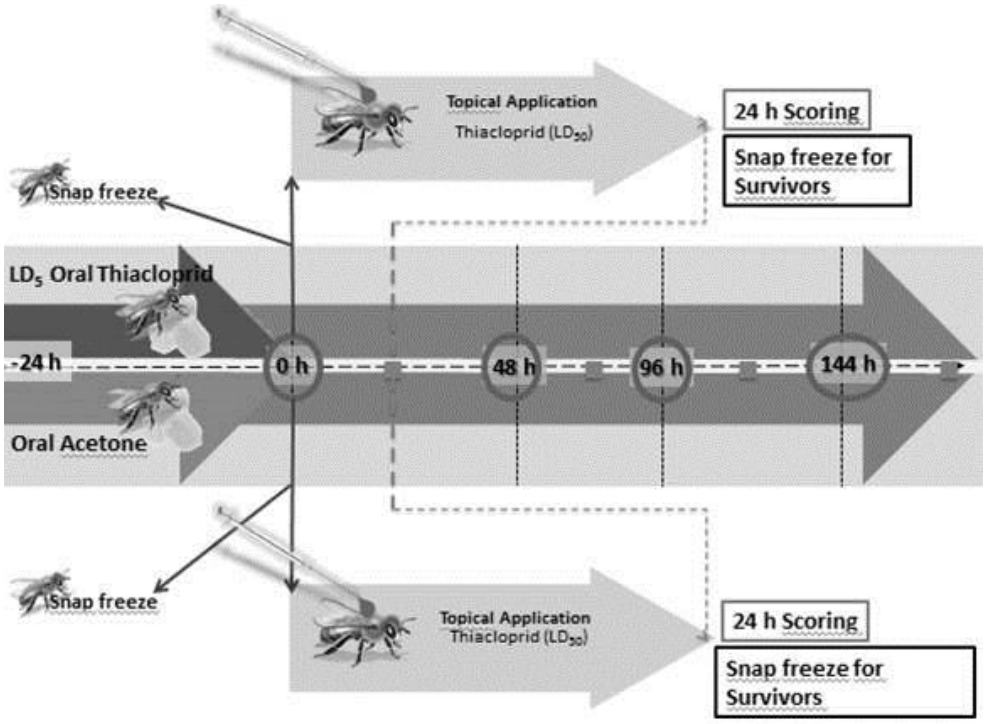

Figure 5 Experimental design

Honey bees were fed either thiacloprid at an estimated LD_5 dosage or acetone (time -24 h to 0 h). At times in circles (0, 48, 96, 144 hours after oral feeding finished) topical application (at estimated LD_{50} dosage or acetone) took place; concurrently, separate cages were taken for microarray analysis. Mortality assessment (scoring) took place 24 h after topical application and survivors were snap frozen.

Supplementary Table 1 Lists of differentially expressed probes in all microarray experiment, the genes to which they correspond, p values and the calculated fold-changes are detailed.

Supplementary Figures 1-5 Enriched Gene Ontology (GO) terms in genes differentially expressed in each microarray comparison.

Supplementary Figure 6 Reduced CO-difference spectra of recombinant *CYP6BE1, CYP6AS5, CYP315A1* and *CYP305D1.*