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Mini-review

Metabolic interactions of agrochemicals in humans

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Abstract: Agrochemicals and other xenobiotics are metabolized by xenobiotic-metabolizing enzymes (XMEs) to products that may be more or less toxic than the parent chemical. In this regard, phase-I XMEs such as cytochrome P450s (CYPs) are of primary importance. Interactions at the level of metabolism may take place via either inhibition or induction of XMEs. Such interactions have often been investigated, *in vitro*, in experimental animals, using subcellular fractions such as liver microsomes, but seldom in humans or at the level of individual XME isoforms. The authors have been investigating the metabolism of a number of agrochemicals by human liver microsomes and recombinant CYP isoforms and have recently embarked on studies of the induction of XMEs in human hepatocytes. The insecticides chlorpyrifos, carbaryl, carbofuran and fipronil, as well as the repellant DEET, are all extensively metabolized by human liver microsomes and, although a number of CYP isoforms may be involved, CYP2B6 and CYP3A4 are usually the most important. Permethrin is hydrolyzed by esterase(s) present in both human liver microsomes and cytosol. A number of metabolic interactions have been observed. Chlorpyrifos and other phosphorothioates are potent inhibitors of the CYP-dependent metabolism of both endogenous substrates, such as testosterone and estradiol, and exogenous substrates, such as carbaryl, presumably as a result of the interaction of highly reactive sulfur, released during the oxidative desulfuration reaction, with the heme iron of CYP. The hydrolysis of permethrin in human liver can be inhibited by chlorpyrifos oxon and by carbaryl. Fipronil can inhibit testosterone metabolism by CYP3A4 and is an effective inducer of CYP isoforms in human hepatocytes.

Keywords: cytochrome P450; environmental chemicals; hepatocytes; human metabolism; organophosphorus chemicals; risk assessment

1 INTRODUCTION

Even though studies of the human metabolism of agrochemicals may yield information invaluable in risk analysis, few such studies have been reported, risk analysis being almost exclusively dependent on studies on surrogate animals.

Risk assessment is generally held to consist of four interrelated components:[1] hazard definition, dose–response assessment, exposure assessment and risk characterization. The result of this process is a numerical estimate of the probability of a deleterious effect following a specific chemical exposure. The more general term, risk analysis, includes risk assessment as well as risk communication and risk management. Since much, if not all, of the experimental data on hazard and dose–response assessment is derived from studies on surrogate animals, extrapolations are necessary to produce a risk assessment for humans. Because it is usually unclear how to extrapolate from high to low dose or between species, uncertainty factors are included. The numerical values of these uncertainty factors are themselves uncertain and default values of 10 are usually used for species differences, high to low dose extrapolation and variation within the human population. Since three uncertainty factors of 10 would increase a risk assessment by 1000-fold, it is clear that risk assessments are often overestimates of the actual risk.

Toxicity is seldom due to a single, defining, molecular event. Rather, it is due to a cascade of events initiated by exposure and ending with the expression of a toxic endpoint (Chapter 6 and Fig. 5.11 in Ref. 2). Two levels in this cascade, metabolism and interaction with endogenous macromolecules, are, however, of particular importance. One of these, metabolism, is the subject of this review. Metabolism of toxicants, including agrochemicals, may decrease toxicity (detoxification) or, by the generation of reactive intermediates, increase toxicity (activation).

While agrochemicals may, at appropriate doses, give rise to any of several toxic endpoints such as neurotoxicity, reproductive and developmental toxicity or cancer, it should be borne in mind

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that metabolism is always a step in the progression of events between exposure and the expression of the endpoint in question. At the same time, it is important to understand that *in vitro* studies can only suggest the potential for effects *in vivo*. In the case of metabolic interactions based on inhibition or induction of xenobiotic-metabolizing enzymes (XMEs), the concentrations at the active site(s) become critical and are not always known. In exposure to a single chemical, the dose necessary to inhibit an XME may be higher than that necessary to cause a particular toxic endpoint. Interactions, by definition, involve more than one toxicant, and this raises the possibility of synergistic effects or effects qualitatively different to those of exposure to a single toxicant.

In order to study interactions in humans involving agrochemicals, it is first necessary to study the human metabolism of each agrochemical alone. This involves the development of an analytical method for the substrate and its metabolites and the measurement of metabolic activity with human cell preparations such as liver microsomes and/or cytosol, followed by identification of the enzymes involved, as well as their isoforms and polymorphic variants. The study of variation between individuals is of critical importance. Some of the chemicals studied from one or more of these aspects are listed in Table 1.

Many XMEs are involved in the metabolism of agrochemicals.[2] In phase-I metabolism a reactive substituent is introduced into the molecule. In phase II this substituent is conjugated with an endogenous metabolite to produce a water-soluble product that is readily excreted in bile or urine. Although any phase-I or phase-II XME can be involved in either detoxification or activation reactions, activation is more likely to occur as a result of phase-I oxidation reactions. This is particular true of the cytochrome P450s (CYPs), a large family of iron porphyrin-containing heme proteins, found in essentially all living species, that catalyze the monooxygenation of a wide range of chemicals of many chemical classes. It is clear from the studies referenced that, while many CYP isoforms may be involved, CYP2B6 and CYP3A4 are often the most important, and that hydrolases and dehydrogenases may also be involved.

Table 1. *In vitro* human metabolism of environmental chemicals: examples of chemicals studied

Chemical use class	Reference
Agrochemicals	
Aldicarb	22
Azinphos-methyl	23, 24
Chloroacetamide herbicides	25, 26, 27
Chlorpyrifos	4, 5, 23, 24, 28, 29
Carbaryl	11
Carbofuran	30
Cyfluthrin	31
Disulfoton	32
Diazinon	23, 24, 28, 33, 34
Endosulfan	3, 35
Fenthion	29
Fipronil	36
Imidacloprid	37
Malathion	29, 38
Methoxychlor	39
Parathion	10, 23, 25, 29, 33, 40, 41
Permethrin	15, 17
Phorate	25, 32, 42
Triazine herbicides	43, 44
Repellants	
DEET	14

A study[3] of endosulfan-α is an excellent example of what can be learned from the study of the human metabolism of a single agrochemical, a study that provides leads to potential interactions and further studies. Endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, in pooled human liver microsomes. Using recombinant cytochrome P450 isoforms, the authors identified CYP2B6 and CYP3A4 as the primary enzymes catalyzing the metabolism of endosulfan-α, although CYP2B6 had an eight-fold higher intrinsic clearance rate than CYP3A4. Using individual human liver microsomes from 16 donors, a strong correlation was observed between endosulfan sulfate formation and *S*-mephenytoin *N*-demethylase activity of CYP2B6 ($r^2 = 0.79$), while a moderate correlation with testosterone 6β-hydroxylase activity of CYP3A4 ($r^2 = 0.54$) was observed. Ticlopidine, a potent CYP2B6 inhibitor, and ketoconazole, a selective CYP3A4 inhibitor, together inhibited approximately 90% of endosulfan-α metabolism by human liver microsomes. Using six human liver microsome samples, the percentage total normalized rate (%TNR) was calculated to estimate the contribution of each CYP in the total metabolism of endosulfan-α. In five of the six human liver microsome samples used, the percentage inhibition with ticlopidine and ketoconazole in the same incubation correlated with the combined %TNRs for CYP2B6 and CYP3A4, showing that endosulfan-α has potential use, in combination with inhibitors, as a simultaneous *in vitro* probe for CYP2B6 and CYP3A4 catalytic activities.

2 IMPORTANCE OF HUMAN METABOLISM STUDIES IN HUMAN HEALTH RISK ANALYSIS

In vitro human metabolism studies can have significance relative to both risk assessment and risk analysis. Table 2 summarizes those aspects likely to be impacted by human studies. It may be noted that most of these aspects cannot be investigated using surrogate animals. Of those that can only be done through human studies, some (exposure assessment and epidemiology) have been done for some time, while others (*in vitro* metabolism and genotyping) have become possible only in the last decade or less.

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Table 2. Human studies relevant to human health risk analysis[a]

1. Defining exposure.*
2. Showing associations between exposure and toxic endpoints.*
3. Defining human variation.*
4. Determining the most appropriate animal model.
5. Providing insight into uncertainty factors.
6. Demonstrating the potential for human-specific interactions.*
7. Defining human populations or individuals at increased or decreased risk.*

[a] Those marked * cannot be determined using surrogate animals.

3 INTERACTIONS BASED ON INHIBITION OF XENOBIOTIC SUBSTRATE METABOLISM

Investigations carried out in the authors' laboratories[4,5] demonstrated that human liver microsomes metabolize chlorpyrifos (CPS) to chlorpyrifos oxon (CPO) and to 3,5,6-trichloro-2-pyridinol (TCP). This metabolism is due primarily to CYP2B6, CYP2C19 and CYP3A4, CYP2B6 producing predominantly the oxon, CYP2C19 producing primarily the detoxication product, TCP, and CYP3A4 producing approximately equal amounts of CPO and TCP. A tenfold difference was found between microsomes from different donors in the desulfuration to dearylation ratio (i.e. activation to detoxification ratio), and polymorphic variants of CYP3A4 varied widely in their ability to metabolize CPS.[4,5]

CYP-dependent activation of organophosphorus cholinesterase inhibitors was shown earlier,[6–10] using rabbit microsomes and rabbit CYP isoforms, to produce not only the corresponding oxon but also a potent CYP inhibitor. This inhibitor was believed to be a reactive sulfur species produced during the desulfuration reaction. This inhibition of CYP isoforms has not previously been examined in human preparations.

Subsequently, it has been shown[11] that CPS inhibits the metabolism of carbaryl by human microsomes, production of the carbaryl methylol metabolite being inhibited more effectively than that of other metabolites. This is important from a mechanistic point of view, as CYP2B6 is the most effective human isoform not only for the activation of chlorpyrifos but also for the production of the methylol metabolite of carbaryl. Other exogenous CYP substrates inhibited by chlorpyrifos include fipronil,[12] nonane,[12] naphthalene[13] and DEET.[14]

A metabolic interaction in humans, not involving CYP, is that of the inhibition of permethrin metabolism by chlorpyrifos oxon and carbaryl. Earlier studies[15] had shown that the pyrethroid permethrin is first hydrolyzed by esterases in human microsomes and cytosol, and that the phenoxybenzyl alcohol produced is metabolized first to phenoxybenzaldehyde by alcohol dehydrogenase and then to phenoxybenzoic acid by aldehyde dehydrogenase.

Subsequently, the authors showed[16] that chlorpyrifos oxon and carbaryl both effectively inhibit the initial hydrolysis of permethrin. Chlorpyrifos oxon completely inhibits permethrin hydrolysis in human cytosol at concentrations as low as 60 nM (Ki = 20 nM). This inhibition is non-competitive and irreversible, reflective of the known mechanism of inhibition of esterases by OP oxons. Carbaryl is a potent, although less effective, inhibitor of permethrin hydrolysis than chlorpyrifos oxon, which does not, however, completely inhibit permethrin hydrolysis even at high doses. Thus, multiple esterases are probably involved in permethrin hydrolysis in human liver cytosol, all of which are inhibited by chlorpyrifos oxon and some of which are inhibited by carbaryl. Ross *et al.*[17] have demonstrated the importance of the human carboxylases in the hydrolysis of pyrethroids, including permethrin.

Recently, Di Consiglio *et al.*[18] have shown that three organophosphorothionates, azinphos-methyl, CPS and parathion, inhibit the CYP-dependent metabolism of the antidepressant imipramine to its active form, desipramine.

4 INTERACTIONS BASED ON INHIBITION OF STEROID HORMONE METABOLISM

Chlorpyrifos and other organophosphorus chemicals have been shown to be potent inhibitors, *in vitro*, of the human metabolism of both testosterone[19] and estradiol.[20] It has been known for some time that 6β-hydroxytesterone accounts for approximately 86% of all testosterone metabolites produced by human liver microsomes, and that most of this activity is catalyzed by CYP3A4. Preincubation of CYP3A4[19] with chlorpyrifos (2 μM), followed by testosterone (100 μM), resulted in 98% inhibition of testosterone metabolism. Although chlorpyrifos was the most potent inhibitor of testosterone metabolism, other organophosphorus chemicals such as fonofos and phorate were also active. Estradiol is metabolized in humans primarily by CYP3A4 and CYP1A2. Estradiol metabolism by these two isoforms is inhibited by both chlorpyrifos and fonofos.[20]

While inhibition of steroid hormone metabolism by organophosphorus chemicals containing the P = S moiety is assumed to be due to the generation of reactive sulfur (i.e. is irreversible and mechanism based), inhibition by such chemicals as deltamethrin and permethrin[19,20] is more likely to be competitive.

5 INTERACTIONS BASED ON INDUCTION OF XENOBIOTIC-METABOLIZING ENZYMES

Preliminary studies (unpublished) in the authors' laboratories demonstrated that a number of agrochemicals are active in the induction of CYP isoforms in human hepatocytes. Fipronil, the most active, has been studied further.[21] The rather complex results showed differences between the induction of CYP1A1 and CYP3A4, the two principal isoforms induced. For CYP1A1 mRNA induction there appears to be a threshold for effect between 0.5 and 1.0 μM, with

over fivefold induction at 25 μM. CYP3A4, on the other hand, shows a threshold for induction at or below 0.1 μM, peak induction at 1.0 μM, followed by a decline, almost to control values, by 25 μM. Induction of CYP3A4 protein and metabolism of testosterone showed the same pattern, i.e. an increase to 1.0 μM, followed by a decline. Since fipronil is a hepatotoxicant at doses somewhat above those required for induction, the decline may represent one of the initial toxic effects, with CYP3A4 induction being more sensitive than CYP1A1 induction.

Recently, endosulfan-α (Casabar RCT *et al*., private communication) has been shown to induce CYP3A4, this induction being mediated via the SXR nuclear receptor.

6 HEPATOTOXICITY

The relevance of hepatotoxicity, whether evidenced by necrosis or apoptosis in studies utilizing human hepatocytes, is not yet clear, but the authors have shown that fipronil[21] and other agrochemicals (Das PC *et al*., private communication) have this effect.

7 SUMMARY AND CONCLUSIONS

Metabolic interactions of agrochemicals based on inhibition, induction and/or cytotoxicity can be demonstrated, *in vitro*, using human liver microsomes, recombinant human enzymes or human hepatocytes. These interactions may be between two or more agrochemicals or between agrochemicals and endogenous substrates. Potency varies, but some effects are dramatic and occur at very low substrate concentrations. It is clear that the groundwork has been laid for studies involving microarray techniques and genotyping of exposed individuals expressing particular toxic endpoints. Such studies are currently being initiated.

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