J Chem Ecol (2006) 32: 1229–1246
DOI 10.1007/s10886-006-9086-z

REVIEW ARTICLE

Application of Pharmacological Approaches to Plant–Mammal Interactions

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Received: 11 December 2005 / Revised: 23 January 2006 /
Accepted: 10 February 2006 / Published online: 23 May 2006

Abstract The dominant theory in the field of mammalian herbivore–plant interactions is that intake, and therefore tolerance, of plant secondary metabolites (PSMs) is regulated by mechanisms that reduce absorption and increase detoxification of PSMs. Methods designed by pharmacologists to measure detoxification enzyme activity, metabolite excretion, and most recently, drug absorption, have been successfully applied by ecologists to study PSM intake in a variety of mammalian study systems. Here, we describe several pharmacological and molecular techniques used to investigate the fate of drugs in human that have potential to further advance knowledge of mammalian herbivore–plant interactions.

Keywords Absorption · Distribution · Foraging behavior · Mammalian herbivores · Metabolism · Molecular ecological techniques · Pharmacology · Plant secondary metabolites

Introduction

The application of pharmacological techniques has significantly advanced the field of plant–animal interactions. Much of the success stems from the overlap between human–drug interactions and plant–animal secondary metabolite (PSM) interactions. Humans and other animals experience constant and unavoidable exposure to foreign chemicals, or xenobiotics. Xenobiotics include both manufactured and natural chemicals such as drugs, pollutants, and PSMs. Regardless of xenobiotic origin, the mechanisms by which humans and other animals process xenobiotics that enter the body are similar, and this overlap provides the platform from which these

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two fields can be integrated. Pharmacological techniques developed for human-drug studies have been most aggressively applied to insect–PSM interactions, and those reviews are found elsewhere (Rosenthal and Berenbaum, 1992; Berenbaum, 1999; Karban and Agrawal, 2002). Here, we focus on the less-explored aspects of pharmacology in understanding mammalian herbivore–PSM interactions.

In general, pharmacologists define the mechanisms by which animals process ingested xenobiotics by four major parameters: absorption, distribution, metabolism (detoxification), and excretion, or collectively ADME, each of which is described in detail elsewhere (Gibaldi and Perrier, 1982; Neubig, 1990; Boroujerdi, 2001; Hayes, 2001; Klaasen and Watkins, 2003). Tolerance to a particular xenobiotic is maximized in individuals that possess mechanisms to minimize absorption and distribution and maximize metabolism and elimination of xenobiotics. The relationship between ADME parameters and xenobiotic tolerance largely explains the variation in therapeutic efficacy of drugs in humans, and has the potential to account for the variation in the foraging ecology of mammalian herbivores. For humans, high tolerance to drugs afforded by low absorption, low distribution to target organs, and high metabolism and excretion results in lower therapeutic efficacy. For mammalian herbivores, high tolerance to PSMs afforded through these same mechanisms results in potentially greater intake of plants containing PSMs.

The general concepts of ADME have only recently been applied to study PSM tolerance and foraging behavior of mammalian herbivores, despite Freeland and Janzen's (1974) introduction of these concepts over 30 years ago. This delay is likely the result of numerous factors, including the cost of conducting pharmacological studies, the daunting task of searching the extensive pharmacological literature, and the complexity of investigating the complex mixtures of xenobiotics in plants. Despite these challenges, several ecologists have employed pharmacological techniques to investigate certain aspects of ADME in mammalian herbivores, primarily metabolism and excretion (McManus and Ilett, 1977; Bolton and Ahokas, 1997a,b; Boyle et al., 1999a,b; Pass et al., 1999, 2001, 2002; Stupans et al., 1999, 2001; Liapis et al., 2000; Ngo et al., 2000, 2003; Mangione et al., 2001; McLean et al., 2001; Pass and McLean, 2002; Lamb et al., 2004). Excretion of PSM metabolites has been largely used to measure metabolism qualitatively. However, to fully understand how PSMs influence the foraging behavior of mammalian herbivores, absorption and distribution (primarily in the blood) must also be considered. In this review, we provide summaries of several pharmacological techniques of ADME that can be used to understand the fate of PSMs in mammalian herbivores. Although ADME parameters are interconnected, absorption and metabolism receive the majority of attention, as they tend to guide the design of studies on distribution and excretion. Our goal is to introduce ecologists interested in studying interactions of PSMs and mammalian herbivores to pharmacological concepts and techniques that can advance the field. To that end, we outline the basic principles of absorption, metabolism, and distribution (as it relates to absorption and metabolism), and how they apply to several hypotheses important to studies of mammalian herbivores.

Absorption

Absorption is the first parameter of ADME that may significantly affect diet selection in mammalian herbivores. Mammalian herbivores may reduce absorption

of PSMs through various mechanisms, but here we focus on efflux transporters in cells lining the gut. Studies of efflux transporters, such as permeability glycoprotein (Pgp), are revolutionizing our understanding of the absorption of pharmaceuticals and other xenobiotics in humans (Bellamy, 1996; Hunter and Hirst, 1997; Sharom, 1997; Stein, 1997; Watkins, 1997; Benet et al., 1999; Schinkel, 1999; Silverman, 1999; Scheffer et al., 2000; Ayrton and Morgan, 2001; Benet and Cummins, 2001; Saier and Paulsen, 2001; Washington et al., 2001; Zhang and Benet, 2001; Melaine et al., 2002; Ambudkar et al., 2003; Dietrich et al., 2003; Fromm, 2003; Lin and Yamazaki, 2003a,b; Schinkel and Jonker, 2003; Sparreboom et al., 2003; Chan et al., 2004; Fromm, 2004; von Richter et al., 2004). Prior to the discovery of efflux transporters, absorption of compounds across the gut was thought to occur passively. We now know that transporters in the gut actively efflux xenobiotics, thereby preventing absorption. Research in this area is intensely active, with over 200 papers published on Pgp's impact on drug absorption and bioavailability in 2005. Despite the celebrity of efflux transporters in the areas of pharmacology and medicine, they have only recently been recognized in the area of herbivore–PSM interactions (Murray et al., 1994; Lanning et al., 1996a,b; Sorensen and Dearing, 2003; Green et al., 2004). An overview of the mechanisms and role of efflux transporters in effluxing PSMs and influencing plant–herbivore interactions is found in Sorensen and Dearing (2006, this issue). Here, we summarize specific assays that directly or indirectly investigate efflux transporters and their role in regulating absorption of PSMs in mammalian herbivores. Studies on efflux transporters as regulators of PSM absorption should first focus on quantifying these transporters in the gastrointestinal tract and testing PSMs as substrates for efflux transporters in enterocytes. However, efflux transporters are found in a variety of barrier tissues including the blood–brain barrier, liver, kidney, testes, and uterus.

Direct Quantification of Efflux Transporters

A combination of Western blots and quantitative polymerase chain reaction (qPCR) is used to determine the presence and quantify gene expression of efflux proteins in various tissue types. These assays can be used to test hypotheses of mammalian herbivore feeding preferences and tolerances. The exceptional tolerance to PSMs by dietary specialists may be, in part, attributed to reduced absorption afforded by efflux transporters. The most celebrated efflux transporter, Pgp, has been fully quantified by using monoclonal antibodies in a variety of organisms including herbivorous mammals (Dudler and Hertig, 1992; Murray et al., 1994; Sangster, 1994; Lanning et al., 1996a,b; Barnes, 2001; Doi et al., 2001; Keppler and Ringwood, 2001; Lee et al., 2001; Saier and Paulsen, 2001; Yazaki et al., 2001; Bard et al., 2002; Buss et al., 2002; Smith and Prichard, 2002; Sasaki et al., 2002; Green et al., 2004). An additional approach is to quantify mRNA of efflux transporters via qPCR (Wong and Medrano, 2005) by using species-specific primers. These primers are often designed from known sequence homology between species (for sequence information, see UCS Genome Bioinformatics website, http://genome.ucsc.edu). The benefits and constraints of each approach are described elsewhere (Sorensen and Dearing, 2006, this issue), but each has been applied to a variety of distantly related species and, therefore, holds promise for further investigation of efflux transporters in mammalian herbivores. Although the majority of assays designed to quantify efflux transporters focus on Pgp,

several exist, and new transporters are continuously being identified and should be considered as the field progresses.

Indirect Investigation of Efflux Transporters

Investigating the proportion of PSM ingested that is excreted unchanged in the feces is an indirect method to assess the role of efflux transporters. In general, a herbivore is fed a PSM or whole plant tissue, and the absolute amount of each PSM ingested is quantified (Sorensen et al., 2004). Feces are collected from the animal following intake of PSMs, and the quantity of unchanged (unmetabolized) PSM in the feces is determined. The assumption is that unchanged PSM in excreta represents PSMs that were not absorbed. Such data would indirectly indicate that some mechanism regulated the absorption of the ingested PSM. However, lack of unchanged PSM in the feces is not necessarily indicative of a limited role of efflux transporters. The PSM may indeed be a substrate for transporters, but is acted on by metabolizing enzymes or microbes prior to defecation. Another confounding factor associated with fecal studies is that positive results do not reveal the mechanism responsible for regulating absorption. A variety of mechanisms regulate absorption of ingested PSMs, and each is reliant on the physiochemical properties of the PSM. For example, the peritrophic membrane of insects (Lehane, 1997; Barbehenn, 2001) and tannin-binding salivary proteins (TBSPs) in mammals (McArthur et al., 1995; Skopec et al., 2004) act to regulate absorption of large complex PSMs, such as tannins. However, both peritrophic membranes and TBSPs are not effective regulators of the absorption of lipophilic compounds. Therefore, fecal excretion of unchanged lipophilic molecules (i.e., those with high partition coefficients), such as terpenes and alkaloids, may be a result of efflux transporters. Promising evidence that efflux transporters may play a role in regulating absorption of PSMs is that several PSMs (e.g., alkaloid vinblastine from periwinkle, *Vinca rosea*, Sharom, 1997) and diterpenes from spurges (*Euphorbia* spp., Hohmann et al., 2002; Appendino et al., 2003) are substrates for Pgp. A list of Pgp substrates, inhibitors, and inducers from plants is given in Sorensen and Dearing (2006, this issue) and Zhou et al. (2004).

Testing PSMs as Efflux Transporter Substrates

A variety of *in vitro* assays are available to test whether PSMs are substrates for efflux transporters. Monolayer cell systems have been developed to positively identify xenobiotics as substrates, inhibitors, and inducers of efflux transporters (Brayden, 1997; Bjornsson et al., 2003, Weiss et al., 2003; Fröhlich et al., 2004). Although many cell lines are specifically designed to overexpress Pgp, they can be selected to express a variety of different efflux transporters. Results from established cell lines and a particular PSM are compared with known transporter substrates (Sharom, 1997; Seelig and Landwojtowicz, 2000; Polli et al., 2001; Bjornsson et al., 2003). Although efflux monolayer systems are regarded as the standard for identifying transporter substrates, other techniques include ATPase activity assays (Scarborough, 1995; Litman et al., 1997; Schmid et al., 1999) and calcein–AM fluorescence assays (Liminga et al., 1994; Tiberghien and Loor, 1996). Each has advantages and disadvantages compared to efflux monolayer systems that

are reviewed elsewhere (Polli et al., 2001). Although these *in vitro* assays provide a noninvasive approach to screen PSMs as substrates for efflux transports, results from such studies can be confounded. First, results may not necessarily translate to the organism of interest. Identifying which efflux transporter a PSM interacts with in cell-based systems containing multiple transporter proteins is another complication. In addition, *in vitro* studies may not correspond to *in vivo* studies. Therefore, follow-up studies need to isolate cells and tissues from the herbivore of interest (Green et al., 2004, 2005), and/or researchers should conduct *in vivo* experiments (described below in Blood Distribution) to confirm the role of efflux transporters in mammalian herbivores.

Metabolism

Once a PSM enters the blood stream, metabolism becomes the most important factor in ADME, and directly affects distribution and excretion. In general, the faster an herbivore metabolizes a given PSM, the more quickly it clears it from the body and, thus, the greater its tolerance should be for the PSM. In the following paragraphs, we outline the various techniques that can be utilized to study the metabolism or detoxification of PSMs by mammalian herbivores. Before discussing pharmacological techniques, we briefly touch on the overall process of detoxification in mammals and the general groups of enzymes involved.

Detoxification Pathways

Mammalian detoxification is split into a two-phase process. Phase I, or functionalization, contains a suite of enzymes that act on xenobiotics by adding functional groups. Oxidation and reduction are two of the most common types of chemical reactions carried out by functionalization enzymes. The family of enzymes called cytochrome P450s (CYPs) is one of the largest and best studied groups of functionalization enzymes that carries out oxidation reactions in mammals. The functionalization of xenobiotics has three possible outcomes: (1) the metabolite becomes sufficiently water-soluble to be excreted in bile or urine; (2) the metabolite becomes more reactive and more toxic than the parent compound; or (3) the metabolite is further detoxified by phase II enzymes. Phase II, or conjugation, contains a suite of enzymes that add a water-soluble conjugate, often a sugar or amino acid moiety. These conjugates make the xenobiotic more water-soluble so that it can be excreted in urine or bile. The detoxification system of vertebrate herbivores is reviewed in Dearing et al. (2005), and a complete description can be found in Casarett and Doull's Toxicology (Klaasen and Watkins, 2003). Although the overall outcome of the detoxification system is relatively straightforward, i.e., to make xenobiotics more water-soluble for excretion, regulation of the detoxification system is complicated and dynamic. Up- and down-regulation of enzyme content and activity occur frequently and rapidly, on the order of days, in response to the presence of xenobiotics in the blood (Hollenberg, 2002; Bock and Köhle, 2004). Therefore, carefully designed studies are needed to identify the importance and relative capacity of specific detoxification pathways that mammalian herbivores use to metabolize ingested PSMs.

Analysis of Metabolites

Several studies have documented the metabolites produced and excreted when either whole plant material or purified PSMs are fed to mammalian herbivores (Boyle et al., 1999a,b; Mangione et al., 2001; McLean et al., 2001). Depending on the chemical nature of the PSM, gas chromatography, mass spectrometry, or high-performance liquid chromatography can be used to analyze the metabolites produced. Metabolites can be analyzed in blood or urine. Depending on the size and nature of the herbivore, one type of sample may be easier to collect than the other. Metabolite studies are an excellent first step in studying mammal–PSM interactions. Whereas characterizing metabolites gives potential clues as to which pathways may be involved in the detoxification of PSMs, these types of studies are more qualitative than quantitative with respect to rate and route of detoxification.

Enzyme Assays

Another technique frequently used in pharmacology that lends itself well to studying nonmodel species is measuring the amount of liver detoxification enzymes. Total CYP content can be measured in a spectrophotometric assay (Hayes, 2001) that yields an overall view of one of the most important groups of phase I enzymes in mammalian herbivores. To measure the CYP content, fresh liver tissue is required, making this type of study usually a lethal endpoint one. However, if the mammalian herbivore were large enough, a liver biopsy may be possible. The liver tissue is homogenized and differentially centrifuged to separate the microsomal (endoplasmic reticulum) fraction from the cytosolic fraction. The CYP content is measured in the microsomal fraction of the liver. A number of labs studying mammalian herbivores have used total CYP content as an assay, and these results are summarized in Table 1. Total CYP content can be used to determine the detoxification ability of a single species (Bolton and Ahokas, 1997a,b;

Table 1 Total cytochrome P450 contents in the livers of mammalian herbivores

Species	Status	Diet	P450 content	Reference
Koala ($N = 4$) (*Phascolarctos cinereus*)	Wild	Eucalyptus	0.87 ± 0.18	(Stupans et al., 1999)
Tammar wallaby ($N = 6$) (*Macropus eugenii*)	Wild	Grazer	0.24 ± 0.04	(Stupans et al., 1999)
Quoakka ($N = 8$) (*Setonix brachyurus*)	Captive	Lab diet	0.95 ± 0.08	(McManus and Ilett, 1977)
Kangaroo ($N = 10$) (*Macropus fulginosus*)	Wild	Browser	0.47 ± 0.05	(McManus and Ilett, 1977)
Brushtail possum ($N = 4$) (*Trichosurus vulpecula*)	Captive	Control lab diet	0.27 ± 0.04	(Pass et al., 1999)
Brushtail possum ($N = 4$) (*Trichosurus vulpecula*)	Captive	Terpene lab diet	0.41 ± 0.02	(Pass et al., 1999)
Brushtail possum ($N = 20$) (*Trichosurus vulpecula*)	Wild	Eucalyptus	0.90 ± 0.06	(Bolton and Ahokas, 1997a)
Stephens woodrat ($N = 4$) (*Neotoma stephensi*)	Wild	Juniper	1.74 ± 0.25	(Lamb et al., 2004)

Lamb et al., 2004), and also for comparative studies among species on different diets (McManus and Ilett, 1977; Stupans et al., 1999), as well as species on different diets (Pass et al., 1999).

In addition to measuring total CYP content, a number of researchers have measured the activity of specific detoxification enzymes in wild herbivores (Bolton and Ahokas, 1997a,b; Pass et al., 1999; Stupans et al., 1999, 2001; Liapis et al., 2000; Ngo et al., 2000, 2003; Lamb et al., 2004). Enzyme-specific substrates are used to determine the activity of individual enzymes of interest. The disappearance of the substrate or the appearance of metabolites is generally measured spectrophotometrically. Enzyme activity assays are robust methods that lend themselves well to nonmodel species studies and interspecies comparisons. Hayes (2001) is an excellent reference for enzyme activity assays. A table containing liver CYP enzyme activities in selected species and associated references can be obtained from Xenotech, Lenexa, KS, USA (http://xenotechllc.com).

Although enzyme assays are robust in nature, they require expensive equipment (e.g., homogenizers, spectrophotometers for turbid solutions, high-speed centrifuges) that might not be a part of a standard ecology lab. Careful selection of the detoxification enzymes to analyze is important because the activity assays can be time consuming and expensive. Another limitation of enzyme activity assays is that they exist for only a subset of the more than a hundred detoxification enzymes. The most thorough studies typically measure the activities of about 10 detoxification enzymes. A carefully designed study with well-chosen enzymes can lead to useful intraspecific or interspecific comparisons. Studies on urinary or blood metabolites can be used to guide the selection of enzyme assays. Comparing the detoxification enzyme activities of mammalian herbivores on different diets will allow ecologists to understand not only how the PSMs are detoxified, but also potentially how the herbivore regulates detoxification enzymes.

Researchers can also use PSMs directly in microsomal assays. The PSM can be added to the microsomal fraction, and its disappearance or the appearance of metabolites can be measured over time (Pass et al., 2001, 2002; Pass and McLean, 2002). This assay measures the combined activities of all detoxification enzymes present in the microsomal fraction but does not elucidate the particular enzymes by which detoxification occurs. However, this approach will give an idea of the speed and capacity of a herbivore's detoxification system for a particular PSM.

A noninvasive approach to rapidly screen PSMs of interest as substrates for various metabolizing enzymes and guide future enzyme studies is to use commercially available hepatocytes or recombinant enzymes and specific probe substrates (LeCluyse, 2001). The approach can be used to test which metabolic pathways are most likely to metabolize a specific metabolite and also which are produced without the use of live mammalian herbivores. Hepatocytes are advantageous in that they represent the "true" physiological environment of the organism from which they are derived (e.g., human, rat, insect, and frog). Recombinant enzymes, on the other hand, are more readily available, less expensive, and the results are not confounded by metabolic processes other than those from the enzyme of interest. One limitation of using hepatocytes and recombinant enzymes is that they only indicate whether the PSM is a substrate for an enzyme from the organism from which the enzyme was isolated. Enzymes in the herbivore of interest may differ significantly in their interaction with the same PSM. These studies are useful as an initial screen to predict which enzymes may be

responsible for PSM metabolism in the herbivore of interest prior to whole animal studies. Although time and resource intensive, researchers can isolate, culture, develop hepatocytes, and design recombinant enzymes directly from mammalian herbivores (Mankowski et al., 1999; Gardmo et al., 2005; Jung'a et al., 2005).

Gene Expression

An alternative strategy to determine how PSMs are processed by mammals is to quantify expression of detoxification enzyme genes. The three major techniques used to analyze gene expression are Northern blots, qPCR, and microarrays. Northern blots involve running mRNA samples on a gel and using specially designed probes to identify and quantify the mRNA of interest on the gel (Belin, 1998; Sabelli, 1998). Northern blots have been used successfully for the genus *Neotoma* with probes designed for laboratory rats (Lamb et al., 2001, 2004). Ecologists studying herbivores closely related to a model species may also be able to take advantage of predesigned probes. However, for herbivores not closely related to a model species, genes of interest will need to be sequenced to design appropriate probes. Quantitative PCR is a relatively new technique that can also be used to study gene expression of detoxification enzymes. As in quantification of efflux transporters, specific primers are needed for the reactions to ensure that the only double-strand DNA produced is from the gene of interest (Wong and Medrano, 2005). Both Northern blots and qPCR can be used to study how PSMs affect the regulation of the detoxification system by quantifying changes in gene expression of detoxification genes. Depending on quantity of sample, equipment access, budget, etc., ecologists can decide between Northern blots or qPCR.

Microarrays

Microarrays hold promise for investigating detoxification systems on a much larger scale (Gibson, 2002; Meyer and Gut, 2002; Gracey and Cossins, 2003; Klaper and Thomas, 2004; Thomas and Klaper, 2004; Tittiger, 2004). Microarrays are a cutting-edge technology in the field of genomics that allow for the parallel analysis of the expression of thousands of genes. Representative sequences of genes are printed in high density onto a solid surface, often a glass microscope slide. A number of commercial arrays made for model organisms are available, and cross-species hybridization works well between closely related species (Gonzalez and Nebert, 1990; Enard et al., 2002; Moody et al., 2002). Therefore, the commercial rat, mouse, or human arrays may be acceptable for a number of mammalian herbivores. To perform microarray analysis, extracted RNA is reverse-transcribed, fluorescently labeled, and hybridized to the array. The arrays are then scanned by specialized scanners, and the intensity and color of the spot at each gene are related to the abundance of the gene transcript in the sample. The popularity of microarrays as a technique for studying gene expression has led most major research universities to develop core facilities that perform microarray experiments for a fee; therefore, specialized equipment is not needed. Even with a core facility, the cost of microarray experiments is considerable. Arrays cost ~$200–400; a single experiment with 10 individuals and two treatments (i.e., 20 samples) can easily cost between $2000–8000 depending on the experimental design and the array. Although the laboratory methods for running a microarray are straightforward, the data analysis

can be overwhelming because microarrays produce data for thousands of genes. Therefore, careful planning of the experimental design and analysis is essential before starting a microarray experiment (Churchill, 2002; Reiner et al., 2003; Yang et al., 2003; Woo et al., 2004; Khatri and Drăghici, 2005). A well-designed microarray experiment will not only give a fairly complete picture of gene regulation occurring in response to PSMs, but also provide a number of candidate genes for further studies.

Gene Sequencing

Whereas quantitative differences in gene products produce detoxification differences within and between species, qualitative differences in gene products because of sequence differences may also play a large role in the variation of detoxification abilities. The field of pharmacogenomics examines the relationships between polymorphisms in detoxification genes and their effects on drug detoxification and disease susceptibility in humans (Puga et al., 1997; Nebert and Roe 2001; Meyer, 2004). Polymorphisms within human populations can lead to 30- to 40-fold differences in detoxification of drugs and xenobiotics (Nebert and Dieter, 2000). The plant–insect interaction community has also studied the effects of differences in sequences in detoxification genes on herbivore detoxification capacity (e.g., Li et al., 2004). With the advent of automated sequencing facilities at most research universities, comparative sequencing is a relatively straightforward procedure. Sequencing projects will lead to increased understanding of detoxification differences both within and between different species of mammalian herbivores as well as to the discovery of novel detoxification genes. For example, a novel CYP has been reported in koalas, CYP4A15 (Ngo et al., 2000; Stupans et al., 2001).

Hypnotic State Assays

Although the application of the assays described in the previous sections holds promise for exploring the metabolism of PSMs in mammalian herbivores, they often require a lethal endpoint and are, therefore, not feasible for many researchers working on mammals. Several nonlethal assays have recently been borrowed from pharmacology by ecologists that allow for repeated measures of detoxification capacity in mammals. Hypnotic state assays measure the length of time an animal spends in a drug-induced hypnotic state (or loss of righting reflex) as a proxy for detoxification capacity for a specific enzyme. This assay is often used in the investigation of new pharmaceuticals (Sasaki, 1994; Konishi et al., 2002; Kim and Shin, 2005) to determine whether detoxification of a novel compound will cause interactions with other drugs through competition for the same detoxification pathways. In such studies, a dose of the compound is administered prior to the hypnotic agent, and the length of the hypnotic state is measured. Drug interactions are considered possible if administration of the compound increases the length of time the animal spends in a hypnotic state compared to the control treatment, i.e., when only the hypnotic agent is delivered. Because the detoxification pathways of the hypnotic agents have been established, but those of the novel compound typically have not, the results can be used to infer putative detoxification pathways for the novel compound.

Sleep time under hexobarbital and paralysis time under zoxazolamine are two of the more common agents used for hypnotic state assays (Sasaki, 1994; Koizumi et al., 2001). For either assay, the compound is injected intraperitoneally. After losing the ability to right itself, the animal is placed on its back with its head elevated, and the length of time (typically 20–300 min) the animal remains in this state is measured. As there is considerable variation among individuals and sexes, sample sizes greater than 8 for each sex are advisable for comparative studies.

Hypnotic state assays can easily be applied to detoxification of PSMs by mammalian herbivores. The potential safety of PSMs for use as pharmaceuticals has been assessed with hypnotic state assays (Wada et al., 1993; Oliveira et al., 2005). Two significant benefits of this assay compared to other pharmacological approaches (e.g., microsomal assays) are that it is relatively noninvasive and nonlethal. Because different sleep agents are acted on by different detoxification enzymes (e.g., hexobarbital by CYP2B; zoxazolamine by CYP1A), these assays can be used to measure the capacity or role of particular pathways. The assays can also be performed under field conditions, as well as repeatedly to permit comparative studies between animals on different diets, treatments, or from various populations.

Blood Distribution

Pharmacokinetic Studies

Pharmacokinetics (PK) is a nonlethal approach that evaluates the distribution of PSM in the blood (or plasma). In general, PK is the study of the time course of xenobiotic absorption, distribution, metabolism, and excretion and can be used to compare these attributes among mammalian herbivores under various conditions. PK studies require collection of serial samples of blood, tissues, urine, or feces, as well as strong analytical chemistry skills or access to radiolabeled PSMs for quantification. In general, the animal is given an intravenous and/or oral administration of a PSM, and the subsequent increase and decrease of its concentrations in body compartments are measured over time. Blood is the most commonly used and informative compartment investigated, but organs and excreta can also be evaluated to provide information on the time course and fate of PSMs and their metabolites. PK experiments require that the herbivore be catheterized or that researchers have sufficient sample sizes to generate a blood concentration–time curve from single blood collections from the orbital sinus, tail vein, or venous punctures from different animals. Pharmacokinetics in blood provides information on clearance, half-life, bioavailability, total exposure [i.e., area under the curve (AUC)], and apparent volume of distribution of the PSM (Fig. 1). The detailed calculations and relationships among these parameters are available elsewhere (Gibaldi and Perrier, 1982; Neubig, 1990; Boroujerdi, 2001). Recent PK studies have illustrated the link between PSM concentrations in the diet, feeding behavior, foraging strategy, and metabolism and absorption in mammalian herbivores (Sorensen and Dearing, 2003; Boyle and McLean, 2004). Here, we outline additional hypotheses that can be generated and tested by using PK studies.

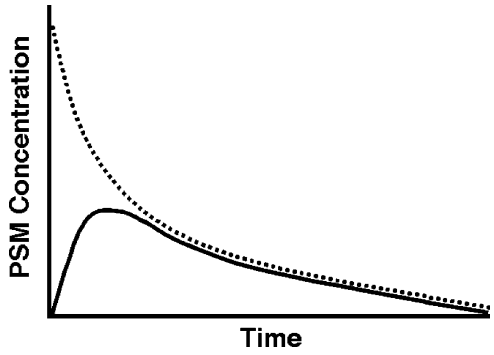

Fig. 1 Schematic representation of possible pharmacokinetic (PK) results following intravenous (IV, dashed line) and oral (solid line) administration of a plant secondary metabolite (PMS) to an animal in a linear scale (see Gibaldi and Perrier 1982; Neubig 1990; Boroujerdi 2001). Data are based on first-order kinetics and a one-compartmental model. Resultant PSM concentrations over time may be derived from blood, plasma, tissue, bile, feces, or urine. The time profile is dependent on feasibility, but reliable PK data should adhere to the following criteria: (1) initial time points should be collected more frequently than latter time points; (2) blood collections should be collected out to at lease five half-lives; and (3) total blood collection should be <10% of total blood volume of the herbivore. PK parameters obtained from IV and oral dosing are defined in Table 2

Absorption

Pharmacokinetic studies can be used to assess absorption. For example, if oral half-life ($t_{1/2}$, hr) is longer than intravenous half-life and/or if bioavailability is low, then the distribution of the PSM may be influenced by the absorption process. Ecologists can take advantage of the established list of substrates for efflux transporters as positive controls, inhibitors, and inducers (Sharom, 1997; Seelig, 1998; Seelig and Landwojtowicz, 2000; Polli et al., 2001; Bjornsson et al., 2003) to investigate regulated absorption of PSMs. Studies can be designed that administer a PSM in the diet and then compare food intake, PSM blood concentrations, and fecal excretion

Table 2 Pharmacokinetic parameters that can be determined from intravenous (IV) and oral dosing of a PSM to a mammalian herbivore (Gibaldi and Perrier, 1982; Neubig, 1990; Boroujerdi, 2001)

PK parameter	Symbol	Definition	Calculation
Clearance (mL/min/kg)	Cl	Efficiency of drug elimination from the blood	$\mathrm{Dose_{IV}}/\mathrm{AUC_0^\infty}$
Half-life (hr)	$t_{1/2}$	Time it takes for concentration of PSM to decline 50%. This is referred to as the "terminal half-life"	1. $0.693 \times \mathrm{V_d/Cl}$
Area under the curve (min*mg/mL)	$\mathrm{A \cup C_0^\infty}$	Area under the plasma concentration–time curve from time 0 to infinity. Considered as an index of exposure to the PSM	$\int_0^\infty \mathrm{C \cdot dt}$, where C is PSM concentration
Apparent volume of distribution (mL/kg)	$\mathrm{V_d}$	Hypothetical volume of body fluid dissolving the xenobiotic at the same concentration as that in the plasma	$\mathrm{Dose_{IV}/C_0}$, where $\mathrm{C_0}$ is the concentration of PSM at time 0
Bioavailability (%)	F	The fraction of the dose absorbed	$(\mathrm{AUC_{oral} \cdot Dose_{IV}})/(\mathrm{AUC_{IV} \cdot Dose_{oral}})$

of the PSM with and without the administration of a known inhibitor or inducer of the efflux transporter. If the PSM is a substrate for a transporter, then coadministration of the metabolite with a transporter inhibitor should result in decreased intake of diet containing the PSM, increased blood concentrations of the PSM, longer half-life, and decreased excretion of unchanged PSM in the feces.

Metabolism

Pharmacokinetic studies can also be used to assess metabolism. Faster clearance (Cl, in ml/min/kg) indicates faster rates of metabolism. Therefore, clearance rates can be evaluated to test whether dietary specialists or animals with evolutionary history with PSMs have higher detoxification capacity than dietary generalists or animals naive to PSMs (Sorensen and Dearing, 2003). The use of inhibitors of detoxification enzymes can also be employed in PK studies to evaluate the role of specific detoxification pathways on PSM clearance. Parameters can be compared between herbivores administered a PSM with an inhibitor (or inducer) of the putative metabolizing enzyme to that of herbivores administered the PSM without the inhibitor. Such studies are currently underway in brushtail possums (McLean, personal communication) and take advantage of the litany of enzyme substrates as positive controls, inhibitors, and/or inducers (http://medicine.iupui.edu/flockhart/table.htm; Newton et al., 1995; Tucker et al., 2001; Bjornsson et al., 2003; Sorensen and Dearing, 2006, this issue). PK studies can also be used to determine if a PSM interacts with a specific metabolizing enzyme that uses known substrates. For example, the CYP3A inhibitory properties of a PSM can be evaluated by comparing *in vivo* blood concentrations of a well-known CYP3A substrate, midazolam, orally administered with and without oral administration of a PSM (Bjornsson et al., 2003). If plasma levels of midazolam are higher with the PSM compared to without it, then the metabolite would be considered a strong inhibitor of CYP3A. Inhibition can be a result of either competitive or noncompetitive interactions with the enzyme of interest, and further studies are then required to elucidate the type of inhibition (Bjornsson et al., 2003). In contrast, if plasma concentrations of midazolam are lower with the PSM than without it, this suggests that it may induce CYP3A and therefore aid in midazolam clearance.

Tissue Affinity

Pharmacokinetic studies can also provide information on PSM affinity for tissues by evaluating the apparent volume of distribution (V_d, in L/kg) following intravenous PSM administration. A V_d that is higher than the water volume of the animal (0.7 L/kg, Davies and Morris, 1993) suggests high tissue affinity and is usually associated with a long half-life of the PSM. Potentially, a higher V_d may be a characteristic of PSMs that bind with tissues by interacting with specific receptors. For example, cannabinoids from *Cannabis sativa* have a high V_d (Samara et al., 1988), are distributed in the brain, and have high affinity and selectivity for G-protein-coupled cannabinoid receptors (Grotenhermen, 2005). In addition, the phenolics from *Eucalyptus jensenii* are thought to mediate the feeding behavior of mammalian herbivores via serotonin action on 5HT3 receptors (Lawler et al., 1998). We predict that these phenolics will also have high brain distribution and a large V_d. Distribution studies could guide research into which receptors are targeted by

PSMs and help identify the mechanisms responsible for PSM-related changes in behavior and physiology in mammalian herbivores.

Summary

Over three decades ago, Freeland and Janzen (1974) proposed that the feeding strategies of mammalian herbivores were governed by their detoxification systems. Since then, surprisingly few researchers have examined metabolism of PSMs in wild herbivores, and even less have examined the role of absorption or distribution. The objective of this review was to provide a brief overview of assays typically used in pharmacology that can be applied to research on mammalian herbivores. We would like to point out that as in pharmacology, no single assay can uncover the complexities of the fate of xenobiotics in mammalian herbivores. Each technique has benefits and limitations. The most informative future studies will integrate several of these *in vitro* and *in vivo* techniques to yield a comprehensive understanding of how mammalian herbivores process PSMs. The particular suite of techniques that will result in the most useful information is dependent on the particular questions asked and the study system. Lastly, because many of the techniques described herein require expensive equipment and/or reagents as well as technical expertise, we encourage novices to visit a laboratory that regularly performs the technique and to establish collaborations with pharmacologists and toxicologists.

Acknowledgments Funding was provided by NSF International Research Fellowship INT-0301898 to J. S. Sorensen and NSF IBN0236402 to M. D. Dearing. We thank K. Smith for assistance with manuscript preparation. Two anonymous reviewers provided insightful comments. Finally, we thank R. Osawa, W. J. Foley, and T. Shimada for organizing a productive symposium at the IX International Mammalogical Congress in Sapporo, Japan, where these ideas were discussed.

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