



**The American Academy of Veterinary  
Pharmacology and Therapeutics**

**Proceedings of the  
Thirteenth Biennial Symposium  
June 3<sup>rd</sup> - 5<sup>th</sup>, 2003  
Charlotte, North Carolina**

**“THE CUTTING EDGE”**







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**“THE CUTTING EDGE”**

**Edited by Dr. Ted Whitem**

**The American Academy of Veterinary  
Pharmacology and Therapeutics**

Thirteenth Biennial Symposium

“THE CUTTING EDGE”

Program and Organizing Committee

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## PRECLINICAL PROGRAM

**Tuesday, June 3, 2003**

8:00 -10:00 am	Registration at the Adam's Mark Hotel
10:00 – 10:50 am	Drug Discovery: computer aided drug design. Kathy E. Mitchell, Kansas State University
11:00 – 11:50 am	Drug Discovery: antibacterial peptides. Frank Blecha, Kansas State University
12:00 pm	Lunch break
1:00 – 1:50 pm	Drug Discovery: targets for CNS disorders. Geoffrey Varty, Schering Plough Research Institute
2:00 – 2:50 pm	Pharmacokinetics in drug development: beyond simple models. Pierre-Louis Toutain, ENV Toulouse France
3:00 – 3:50 pm	Evaluating variability in drug response: pharmacogenetics. Hervé Lefebvre, ENV Toulouse France
4:00 – 4:50 pm	Monitoring of responses; pharmacovigilance. Mark Novotny. Pfizer Animal Health, Groton, CT
5:30 – 6:30 pm	Social hour
6:30 pm	Banquet, awards, business session

## **PRECLINICAL PROGRAM**

**Wednesday, June 4, 2003**

8:00 – 9:50 am	Metabolism: the Cytochrome p450's of the dogs. Lauren Trepanier, University of Wisconsin-Madison, and Alistair Cribb, University of Prince Edward Island, Canada
10:00 – 10:50 am	Metabolism: In vitro techniques to investigate small animal drug metabolism. Jane Owens-Clark, Elanco Animal Health, Greenfield, IN
11:00 – 11:50 am	The role of genomics in drug discovery. Carla Chieffo, Pfizer Global R&D, Groton, CT.
12:00 pm	Lunch break

## CLINICAL PROGRAM

Note: The clinical portion of the program of the American Academy of Veterinary Pharmacology and Therapeutics 13<sup>th</sup> Biennial Symposium is co-sponsored by the American College of Veterinary Clinical Pharmacology and the American College of Veterinary Internal Medicine.

### Wednesday, June 4, 2003

2:00 – 2:45 pm	Implications drug-protein binding and the occurrence of drug-drug interactions. Betty-Ann Hoener, University of California - San Francisco
3:00 – 3:45 pm	COX1, COX2, COX3: relevance to pain management. Steve Budzburg, University of Georgia
3:45 pm	Break
4:15 – 5:00 pm	New Therapeutic Horizons: Transdermal Drug Delivery. Katrina Mealey Washington State University
5:15 – 6:00 pm	New Therapeutic Horizons: Novel drug delivery methods. Gijsbert Van Der Wijdeven, Injectiles, Netherlands

## **CLINICAL PROGRAM**

### **Thursday, June 5, 2003**

- 8:00 – 8:45 am      New Therapeutic Horizons: Peptide drug delivery.  
Mark Jones, University of Western Sydney, Australia
- 9:00 – 9:45 am      New Therapeutic Horizons: Erythropoietin.  
Mark Walker, Applied Genetic Technologies Corporation
- 9:45 am              Break
- 10:45 – 11:30 am    New Therapeutic Horizons: Choosing a New Drug for Inducing Anaesthesia: Propofol or Alfaxalone?  
Martin Pearson, South Tamworth Animal Hospital, Australia
- 11:45 – 12:30 am    New Therapeutic Horizons: Fluoxetine Pharmacology, Safety and Use in Cats and Dogs and its Role in Behavior Modification.  
Kirby Pasloske, Elanco Animal Health, Greenfield, IN

### **Thursday afternoon, June 5, 2003**

- 2:00 – 3:45 pm      Pharmacology Research Abstracts

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# DRUG DISCOVERY: COMPUTER-AIDED DRUG DESIGN

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Manhattan, KS

## INTRODUCTION

Drug discovery in the 21<sup>st</sup> century has been greatly enhanced by the additional tools made available through information technology and the increase in computer processor efficiency. In the information-rich post-genomic era, expectations are high for identifying new targets and the rapid development of effective treatments with low side effects.

## Animal Health Care Market

The animal health care market could benefit substantially from more streamlined and economic drug discovery processes. There are important challenges in veterinary medicine including maintaining the health of 3.3 billion livestock animals and 16 billion poultry worldwide, which is critical for human health in this age of antibiotic-resistance and emerging diseases that could pose threats to the food supply (1). In addition, there are 1 billion companion animals that require traditional veterinary treatments such as for parasites but also increasingly are being treated for diseases associated with aging and more recently for behavioral disorders. As a result, the companion animal market has grown strongly over the past decade as pet owners are more willing to spend money on veterinary health care and the availability of therapeutics for heartworm, flea and tick control, non-steroidal anti-inflammatory agents for canine arthritis as well as behavioral drugs (1). Despite this growth, the animal health care market is still a small percentage of the human market and can not support its own primary research.

## Stages of Drug Discovery Process

Drug discovery is a process that includes identification of a target, development of an assay of target function, screening of compounds and natural products, lead identifications, and lead optimizations. This is followed by animal studies to measure absorption, distribution, metabolism and excretion (ADME) properties as well as toxicity. The time from the target identification to approval of a new drug is typically 10 to 15 years. The overall estimated cost to bring a drug to market is now \$800 million (2). One of the factors contributing to this high cost is the large number of lead compounds that fail late in the drug discovery process due to either poor ADME/Tox or adverse side effects such as induction of long QT interval. Increasing efficiency of drug discovery by making the overall time for drug development shorter so that the patent life of a compound is extended and elimination of compounds from further development early on in the discovery process that will have poor ADME/Tox properties or undesirable side effects are the major challenges that could enhance profitability of drug development.

## HISTORY OF COMPUTER-AIDED DRUG DESIGN

Computational chemistry is a relatively new discipline and is the foundation of computer-aided drug design. One of the first major advances that led to the development of many of the most powerful techniques in computer-aided drug design today was the development of the quantitative structure activity relationship (QSAR) analysis by Hansch and Fujita which described a new method for analyzing drug actions (3). This was followed by the development of molecular mechanics by Allinger in 1971 which is the major foundation for energy-based minimizations of molecules (4). In 1977, Garland Marshall described the active analog approach, another breakthrough in computer-aided drug design and shortly thereafter established the computational chemistry/drug discovery software company, Tripos (5). Peter Kohlman developed the AMBER force field in 1981 which allowed for energy minimizations of large protein molecules (6). An algorithm for docking

small molecules to receptors that later became the powerful DOCK program was developed by Kuntz in 1982 (7). In 1984, partial least squares analysis was introduced. This method is commonly employed in QSAR studies today as it allows for the derivation of linear equations from data tables that have more columns than rows (8). Robert Pearlman published the first description of CONCORD, a program that allowed for the rapid approximation of 3D structures of molecules (9). The first description of comparative molecular field analysis (CoMFA), a QSAR technique that explicitly incorporates 3D geometries of small molecules and relates them to activity was published by Richard Cramer in 1988 (10). These are only a few of the breakthroughs that have contributed to modern computer-aided drug design. In addition to innovations in the way we think about drug design, availability of high-resolution structural information from X-ray crystallography and NMR, the vast amount of information available from the genomic databases and the related discipline of bioinformatics and the enhancements of computer processors and graphical interfaces have also been key in advancing computer-aided drug design. A multidisciplinary approach to drug design that truly integrates all of these facets is required to address the challenges of drug discovery in the 21<sup>st</sup> century.

## STRUCTURE-BASED DESIGN

Most drugs on the market today were found either serendipitously or by screening large numbers of natural products and synthetic substances. These novel compounds were then improved by synthesizing analogs in hopes of enhancing efficacy or reducing unfavorable side effects. In the post-genomic era, where specific drug targets can be identified and their three-dimensional structures determined either by X-ray crystallography or NMR, structure-based design of drugs based on the principles of molecular recognition has become a new paradigm in drug discovery (11). Ondetti and Cushman were the first to successfully utilize X-ray crystallographic data in drug design. While they didn't know the structure of their intended target, human angiotensin-converting enzyme (ACE), they used the structure of a related protein as a model to develop the first ACE inhibitor, Captopril (12). Similar strategies were used to develop inhibitors of HIV protease (13).

The key requirement for structure-based design is having a high resolution structure for the target protein or of a closely related protein, preferably with a bound ligand, to identify the drug receptor site. Once known, the structure of the receptor site can be used to define a pharmacophore for virtual screening of libraries and in docking studies which can be used to design improvements in lead compounds. Finding the active site of the target protein is necessary for structure-based design of drugs. Homology-modeling of related proteins where the active site is known is the preferred method. Other methods for predicting active sites include algorithms that predict solvent accessible surfaces or pockets of proteins and those that evaluate the solvent accessibility, hydrophilicity, lipophilicity and clustering algorithms to define potential binding sites (14).

## Virtual screening

Virtual screening of library compounds is a complementary approach to high throughput screening in the process of lead identification. Defining the pharmacophore, the steric and electrostatic features and their arrangement in space that are required for high affinity binding, is a key element for virtual or in silico screening of compounds. The pharmacophore is used as a template for searching virtual libraries of compounds, often using successive "filters" to continue to reduce the number of compounds that will be actually used in a high throughput screening. This can reduce the cost of the actual high throughput screen by reducing a library of 100,000 compounds to 3,000 that meet the pharmacophore criteria (15).

Recently, the Kuntz research group showed how structure-based design could start with calculating free energies of binding of a combinatorial library with cathepsin D, an aspartyl protease responsible for cleavage of  $\beta$ -amyloid peptide, using the molecular dynamics-based continuum solvent method (MM-PBSA) (16). They were able to predict binding affinities for a set of seven inhibitors within 1 kcal/mol. The molecular dynamics simulations predict a binding conformation of



the inhibitors that is in close agreement with the X-ray crystal structure of a peptide inhibitor-cathepsin D complex. In addition, they were able to identify substitutions that improved inhibitor binding. This work demonstrates the utility of virtual screening in a multi-step structure-based drug design process.

## **LIGAND-BASED DESIGN**

### **Three-dimensional quantitative structure-activity relationship techniques**

QSAR techniques have been important in the design of pharmaceuticals since they were first proposed by Hansch and Fujita in 1964 (3). More recently, QSAR analyses of ligand receptor interactions have included three dimensional properties of molecules such as comparative molecular field analysis (CoMFA) (10), comparative molecular similarity indices analyses (CoMSIA) (17) and comparative molecular surface analysis (COMSA) (18). CoMFA is based on the premise that steric and electrostatic fields around an aligned set of molecules can be used to predict biological activity using partial least squares analysis. This technique has been successfully employed to develop predictive models of activity for a wide range of compounds and, although not always successful, it has become a standard tool in computer-aided drug discovery. In CoMSIA, similarity is expressed in terms of different physicochemical properties like steric occupancy, H-bond donor-acceptor properties, local hydrophobicity and partial atomic charges and uses a Gaussian – type distance dependent function as opposed to the grid approach taken in CoMFA. COMSA is based on the mean electrostatic potential along with a neural network approach and partial least squares analysis. These methods vary in their success and are often used in combination with other techniques to help establish their validity. Recent studies which combined CoMFA, CoMSIA and docking studies to design selective COX-2 inhibitors demonstrate how using multiple approaches in computer aided drug design are particularly effective (19). Another novel use of CoMFA published recently showed how 3D QSAR can be used to identify a pharmacophore for LQT-inducing effects from a set of chemically diverse compounds (20).

### **PREDICTIVE MODELS OF ADME/TOX**

Drug-like properties include aqueous solubility, ability to cross membranes, metabolic stability, and safety. These properties are described by the absorption, distribution, metabolism, excretion and toxicity (ADME/TOX) parameters. The primary reason for failure of drugs late in the drug discovery process is due to poor ADME/TOX at which point there has already been a substantial financial investment in its development. It is thus desirable to discover early on in the drug discovery process which compounds have poor ADME/TOX properties. Recently, advances have been made in modeling ADME/TOX characteristics, so that compounds can be eliminated from screening (21). One particularly successful method is VolSurf, which correlates 3D structures with physicochemical properties and pharmacokinetics (22). More recently, this technique has been applied in an integrated framework that predicts both activity and ADME/TOX simultaneously, a strategy that would guide lead optimization to increase efficacy while designing in favorable ADMET/TOX properties as well. (23). Making reliable predictive models of ADME/TOX will reduce development time and will avoid investment in leads that would make poor drugs. This will be a major breakthrough that would also facilitate the development of companion animal drugs from leads by developing species-specific models of ADMET/TOX based on known differences in CYT P450 structure (24).

### **CONCLUDING COMMENTS**

In the post-genomic era, we are faced with new opportunities based on the wealth of information about drug targets that is available. We are also faced with new challenges that include antibiotic resistance, emerging diseases that require novel treatments and strategies for developing drugs for companion animals that are economically feasible. Computer-aided drug design is a tool that can help us to meet these challenges.

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**Keywords**

Virtual screening, ADME/Tox predictions, structure-based design, ligand-based design

# DRUG DISCOVERY: ANTIBACTERIAL PEPTIDES

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## INTRODUCTION

The discovery and development of antibiotics has led to dramatic improvements in the ability to treat infectious diseases and significant increases in food-animal production. Unquestionably, they represent one of the major scientific and medical advances of the 20<sup>th</sup> century. Unfortunately, widespread and sometimes indiscriminate use of antibiotics has been accompanied by the emergence of microorganisms that are resistant to these agents. To address the important health issue of antibiotic resistance and to maintain consumer confidence in a safe food supply, health specialists and food-animal producers are searching for alternatives to conventional antibiotics.

Antibacterial or antimicrobial peptides constitute a ubiquitous and broadly effective component of innate immunity. Unlike conventional antibiotics, which are synthesized enzymatically by microorganisms, each antimicrobial peptide is encoded by a distinct gene and made from an mRNA template. All antimicrobial peptides share common features, such as small size (12-100 amino acid residues), polycationic charge, and amphipathic structure. Based on structural similarities, they are often classified into two broad groups, cyclic and linear peptides. The first group consists of peptides containing one or more disulfide bridges with loop or  $\beta$ -sheet structures, and the second group comprises linear peptides with amphipathic  $\alpha$ -helical structures and linear peptides adopting extended helices with a high proportion of certain residues. Many cells of the immune system or on mucosal surfaces have the potential to produce antimicrobial peptides. For example, granules of polymorphonuclear neutrophils, macrophages, eosinophils, T lymphocytes, and natural killer (NK) cells are equipped with an impressive array of antimicrobial peptides. Upon cell activation and degranulation, these granule-associated peptides are either fused intracellularly with pathogen-containing vacuoles or secreted extracellularly and exert their effects through non-oxidative killing mechanisms. Mucosal epithelial cells, which do not have granules, also express and secrete antimicrobial peptides. This brief review, using porcine antimicrobial peptides as a model system, will describe the diversity and multifunctional activities of antimicrobial peptides, and will discuss their therapeutic drug potential.

## PORCINE ANTIMICROBIAL PEPTIDES: A MODEL SYSTEM

More than a dozen distinct antimicrobial peptides have been identified in pigs (1). All of these peptides adopt diverse spatial structures and are relatively small with a molecular weight of less than 10 kDa, but broadly effective against various species of microorganisms. They were either isolated as mature peptides from neutrophils, lymphocytes, and the small intestine, or their amino acid sequences were deduced from cDNA or gene sequences. Cecropin P1 was the first porcine antimicrobial peptide isolated from the upper part of the small intestine in 1989 by Hans Boman's group. Two years later, this group also discovered PR-39 from the small intestine. Protegrins are a group of cysteine-rich, broad-spectrum antimicrobial peptides of porcine myeloid origin identified by Robert Lehrer's group. This research group also has isolated two proline-phenylalanine-rich antimicrobial peptides, prophenins 1 and 2, from porcine neutrophils. Three porcine myeloid antimicrobial peptides (PMAP)-23, -36, and -37 also have been identified by cDNA cloning. A novel antimicrobial peptide termed NK-lysin was isolated from the porcine small intestine and has been shown to be a new effector molecule of cytotoxic T and NK cells. Recently, we cloned porcine  $\beta$ -defensin-1, which is expressed throughout epithelia of respiratory and gastrointestinal tracts (2). All of these antimicrobial peptides, except cecropin P1 and NK-lysin, belong to either the defensin or cathelicidin family, which are the two major groups of antimicrobial peptides found in most

mammalian species. Although  $\alpha$ -defensins are the most abundant antimicrobial peptides in granules of neutrophils or intestinal paneth cells in many mammalian species, these peptides have not been found in pigs. Conversely, as indicated above, pigs do possess at least one  $\beta$ -defensin, which was found to be most prominent in tongue epithelial cells. Cathelicidins represent the majority of antimicrobial peptides identified in pigs.

### **b-Defensins**

To date, porcine  $\beta$ -defensin-1 is the only member of the defensin family identified in pigs (2). Porcine  $\beta$ -defensin-1 mRNA is expressed abundantly in tongue epithelia and to a lesser extent throughout the respiratory and digestive tracts. The porcine  $\beta$ -defensin-1 gene spans about 1.9 kb and, like its mammalian congeners, consists of two short exons separated by a 1.5-kb intron. Exon 1 encodes the 5'-untranslated region (UTR) and signal sequence of the 64-amino acid prepro-porcine  $\beta$ -defensin-1 and exon 2 encodes the pro-sequence, mature peptide, and the 3'-UTR. Despite its resemblance to many inducible  $\beta$ -defensins in amino acid sequence, gene structure, and sites of expression, the porcine  $\beta$ -defensin-1 gene is not inducible. Expression of the gene was not upregulated by in vitro stimulation of tongue epithelial cells with lipopolysaccharide (LPS), tumor necrosis factor (TNF)- $\alpha$  or interleukin (IL)-1 $\beta$  and an in vivo infection of pigs with *Salmonella enterica* serovar Typhimurium or *Actinobacillus pleuropneumoniae*. In addition, direct transfection of the porcine  $\beta$ -defensin-1 gene promoter into NIH/3T3 cells showed no difference in reporter gene activity upon stimulation with LPS and IL-1 $\beta$ . Thus, porcine  $\beta$ -defensin-1 appears to be the only  $\beta$ -defensin that can be classified structurally into the inducible group but exhibits a constitutive expression pattern. The constitutive expression of porcine  $\beta$ -defensin-1 in airway and oral mucosa is also consistent with a lack of consensus binding sites for nuclear factor-kappa B (NF- $\kappa$ B) or NF-IL-6 in its promoter region, suggesting that it may play a surveillance role in maintaining the steady state of microflora on mucosal surfaces. Fluorescence *in situ* hybridization mapped the porcine  $\beta$ -defensin-1 gene to porcine chromosome 15q14-q15.1 within a region of conserved synteny to the chromosomal locations of human  $\alpha$ - and  $\beta$ -defensins, supporting the notion that defensins are highly conserved innate defense molecules with a common ancestry.

### **Cathelicidins**

Cathelicidins are a group of antimicrobial peptides sharing a conserved N-terminal pro-sequence followed by highly heterogeneous 12-79-amino acid C-terminal mature peptides (3). The C-terminal peptides of cathelicidins in various mammalian species have extremely diverse amino acid sequences and subsequent spatial structures ranging from  $\alpha$ -helix to  $\beta$ -sheet. They are named cathelicidins for the high homology of their pro-sequences to cathelin, a 96-amino acid polypeptide originally purified from porcine neutrophils. These peptides are synthesized as prepro-peptides by bone-marrow myeloid cells, then constitutively stored in peripheral neutrophil granules as pro-peptides, from which mature active peptides are cleaved by endogenous elastase upon neutrophil activation and degranulation. In some cases, the mature molecules are further modified by C-terminal amidation.

Porcine cathelicidins include PR-39, protegrins 1-5, prophenins 1-2, and PMAP-23, -36, and -37. They are all derived from bone-marrow myeloid cells and constitutively stored as pro-peptides in peripheral neutrophil granules, where little or no transcript is expressed. However, gene expression of the porcine cathelicidins, PR-39 and protegrin, and the human cathelicidin, LL-37/hCAP-18, has been detected outside of the bone marrow. Both PR-39 and protegrin gene expression was detected in peripheral neutrophils in young pigs and expression of PR-39 mRNA was detected in the kidney and liver, and several lymphoid organs, including the thymus, spleen, and mesenteric lymph nodes. Similarly, skin keratinocytes and airway epithelial cells synthesize LL-37 inducibly. These findings suggest that cathelicidin gene expression is more extensive than originally thought and raises the intriguing possibility that porcine cathelicidins could participate in the critical early

stages of developmental maturation of the porcine immune system. Moreover, they suggest the possibility of a complex interaction between this aspect of the porcine immune system and adaptive immunity. Cathelicidin genes, as exemplified by PR-39, protegrins, and prophenins, are all rather compact and organized in the same manner, comprised of four exons and three introns. Exons 1-3 encode the prepro-sequence, and exon 4 encodes several terminal residues of the pro-sequence followed by the mature peptide sequence. Promoter regions of cathelicidin genes contain several binding sites for NF- $\kappa$ B, NF-IL-6, and acute phase response factor (APRF), suggesting that cytokines generated early in infections may upregulate cathelicidin gene expression, similar to inducible  $\beta$ -defensins. All porcine cathelicidin genes are clustered densely on chromosome 13. Their homology and nearby chromosomal locations indicate that this family may have evolved through gene duplications. To date, nearly 30 cathelicidins have been identified in at least eight mammalian species, including humans, pigs, cattle, sheep, rabbits, mice, guinea pigs, and horses, either by cDNA cloning of bone-marrow cells or by direct purification from peripheral neutrophils. Although members of the cathelicidin family share a highly conserved gene structure in the prepro-sequence, little similarity exists in the promoter region of protegrin, PR-39, and the human peptide antibiotic LL-37/FALL-39.

### **PR-39 is a multifunctional porcine cathelicidin**

Much of our research has been focused on PR-39, a linear peptide of 39 amino acid residues with a high content of proline (49%) and arginine (26%). It was isolated originally from bulk homogenates of porcine small intestines, but later cloning of PR-39 cDNA from porcine bone-marrow cells suggested that enteric PR-39 might be derived from resident leukocytes in the intestine rather than from intestinal epithelia (1). Indeed, we isolated PR-39 peptide from porcine neutrophils, but PR-39 mRNA could not be detected by reverse-transcriptase-polymerase chain reaction (RT-PCR) in small intestines of pigs at any age. Conversely, gene expression of PR-39 was detected in several lymphoid organs of young pigs, including the thymus and spleen, suggesting that it may be involved in the development of adaptive immunity in neonates.

PR-39 is a potent natural antibiotic active mainly against gram-negative bacteria. We have shown that concentrations of mature peptide are increased significantly in sera of pigs during the onset of salmonellosis, which further suggests an important *in vivo* role for this peptide in host defense. We also have examined the functional interactions of porcine cathelicidins with porcine  $\beta$ -defensin-1 and found that a synergism exists between these porcine antimicrobial peptides. Against *E. coli* and the multidrug-resistant strain of *S. enterica* serotype Typhimurium known as definitive phage type 104 (DT104), the combination of PR-39 and porcine  $\beta$ -defensin-1 led to a 1000-fold reduction in colony forming units per milliliter after 20 hr of incubation in comparison with either antimicrobial peptide alone. Clearly, the secretion and activation of porcine cathelicidins will allow these peptides to interact with epithelial antimicrobial peptides, such as porcine  $\beta$ -defensin-1, which may further amplify the microbicidal defenses of porcine mucosal surfaces.

In addition to its antibacterial activity, PR-39 has several other important functions. It is a specific neutrophil chemoattractant (3) and accumulates in wound fluid where it induces the expression of syndecans-1 and -4, which are important cell surface heparan sulfate proteoglycans involved in wound repair. Recently, PR-39 has been further implicated as an important mediator in wound repair and inflammation as a potent inducer of angiogenesis. It also has the ability to inhibit the assembly of the phagocyte NADPH oxidase complex by binding to Src homology 3 (SH3) domains of the oxidative subunit p47<sup>phox</sup>, thereby limiting the production of reactive oxygen species (ROS). Consistent with its function as a potent NADPH oxidase inhibitor, PR-39 has been shown to block ischemia- and high K<sup>+</sup>-induced ROS production in isolated perfused rat lungs. *In vivo* studies showed that a single intravenous injection of PR-39 completely abolishes postischemic ROS production, neutrophil adhesion, and transvascular emigration in rat mesenteric venules subjected to ischemia-reperfusion [76]. Furthermore, pretreatment with PR-39

significantly increases survival rate and abrogates liver injury of galactosamine-sensitized mice following lethal endotoxic shock. These findings suggest that PR-39 may be therapeutically useful as a potent anti-inflammatory drug to prevent neutrophil adhesion and activation as well as excessive tissue injury during postischemic and other inflammatory responses. Although the complex in vivo role of PR-39 has not been fully elucidated, it is tempting to speculate that all of the above activities may be tightly integrated and finely tuned in pigs during injury, infection, and wound healing.

### SUMMARY

Antimicrobial peptides are an ancient but effective mechanism of host defense and are being evaluated as possible alternatives to conventional antibiotics. They have been investigated in detail with respect to structure, spectrum of activity and mechanism of action. Although it is difficult to conclusively demonstrate the contribution of any single antimicrobial peptide to disease resistance, the broad antimicrobial spectrum and strategic locations of these effector molecules provide the necessary requirements to combat disease. The diversity of antimicrobial peptide structures found in a variety of biological settings provides optimism that some of these compounds will prove useful as therapeutic antibiotics. Further studies evaluating these peptides for clinical purposes will undoubtedly lead to progress in the treatment of infectious diseases.

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### Keywords

Antimicrobial peptides, Defensins, Cathelicidins

# DRUG DISCOVERY: TARGETS FOR CNS DISORDERS

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## INTRODUCTION

In recent years, there has been an increased interest into research of the central nervous system (CNS) within the pharmaceutical industry, with increased research budgets and focused recruitment of scientists specializing in neuroscience. There is a very high level of unmet medical need in neurological and psychiatric diseases and therefore a large potential to discover and develop novel, billion dollar CNS therapeutics. However, the area of CNS research is renowned for a significant number of clinical failures and an array of challenges to the scientists. These challenges include the requirement of chemists to synthesized low molecular weight molecules that will penetrate the blood-brain barrier at sufficient levels to hit the target protein, to selectively target CNS receptors while producing minimal effects on peripheral systems and thus limiting potential side-effects, and most importantly, to identify potential targets for neurological and psychiatric diseases when the underlying neurobiology of these diseases is still not fully understood. This presentation will discuss a number of the key diseases being investigated by both academic and industrial neuroscientists and the challenges that they face in developing novel therapeutics.

## NEUROLOGICAL DISEASES

### Alzheimer's Disease

Alzheimer's Disease (AD) is characterized by distinct pathological changes in the brain, specifically, the deposition of  $\beta$ -amyloid protein neuritic plaques and neurofibrillary tangles. The major symptoms commonly associated with AD include deficits in aspects of memory including short-term working memory and spatial memory and reduced attention. While the neurobiology and neurochemistry changes underlying AD remain unclear, deficits in the brain cholinergic system have been implicated in the symptomology. However, approaches aimed at increasing acetylcholine (ACh) transmission have had mixed success. Acetylcholinesterase (AChE) inhibitors such as Cognex (tacrine) and Aricept (donepezil) have been shown clinically to improve some of the symptoms of AD, yet are burdened with troublesome side effects. Antagonists of the muscarinic M2 autoreceptor were found to potently increase the levels of synaptic ACh, but ultimately failed in the clinic due to cardiovascular side-effects. Direct agonists at the muscarinic M1 receptor are currently under clinical evaluation. Current research efforts have re-focused on the pathological changes and attempts are underway to develop therapies that prevent the deposition of plaques and tangles. Examples of this include inhibiting the  $\beta$ - and  $\gamma$ -secretase enzymes implicated in the generation of the amyloid proteins contained in plaques.

### Parkinson's Disease

Parkinson's Disease (AD) is characterized by significant changes to the motor system with patients showing marked behaviors such as tremor, muscle rigidity, postural instability and bradykinesia (slowness and poverty of movement). PD has received a significant amount of media coverage from the affliction of a number of public figures, including the boxing legend, Muhammad Ali, the former Attorney General, Janet Reno, and the actor, Michael J. Fox. The neurochemistry of PD has focused for over 50 years on the breakdown of the dopaminergic system, particularly in areas such as the basal ganglia that are implicated in the control of movement. One of the successful initial treatments was the use of levodopa (L-DOPA), a precursor to dopamine that was used to enhance production of the neurotransmitter. However, this approach was beset by side-effects, particularly dyskinesia, that resulted in discontinuation of treatment in PD patients. Newer



approaches have aimed at developing subtype specific dopamine receptor agonists, specifically agonists at D<sub>1</sub> and D<sub>2</sub> receptors, and a number of these compounds are either in use or are under evaluation in the clinic. Approaches to indirectly modulate the dopaminergic system are also under evaluation including the development of adenosine A<sub>2A</sub> receptor antagonists.

## **PSYCHIATRIC DISEASES**

### **Schizophrenia**

Psychosis, specifically schizophrenia, is characterized by a specific set of symptoms that resulting in debilitation of the patient. Most notably, patients experience sensory hallucinations, most often auditory or olfactory, and delusions of grandeur, paranoia or persecution. However, there are a number of other severe symptoms including social withdrawal, poverty of speech, thought disorders and cognitive deficits. Although there are some effective treatments available including clozapine, olanzapine, risperidone, and ziprasidone, a significant number of patients either do not make a sufficient recovery or are treatment resistant, and the treatments themselves are associated with some significant side-effects including agranulocytosis, weight gain, Parkinson-like syndrome, and QTc prolongation. New approaches to treatment include focusing on subtype specific ligands for the dopamine, serotonin or glutamate systems, as well as new target systems such neurotensin, cannabinoid and neurokinin.

### **Anxiety**

Anxiety results from maladaptive responses to stressful/threatening situations and is associated with symptoms such as excessive worrying, irritability, muscle tension, restlessness and sleep disturbance. Recently, anxiety disorder was subdivided into five distinct subtypes: Generalized Anxiety Disorder (GAD), Social Phobia, Panic Disorder, Obsessive Compulsive Disorder (OCD) and Post Traumatic Stress Disorder (PTSD). Although there are treatments for these disorders there are issues with the benzodiazepines such as diazepam including sedation, dependence/abuse potential, interaction with alcohol and tolerance to the beneficial effects, and a general lack of efficacy and delayed onset of action with the 5-HT<sub>1A</sub> partial agonist, Buspar (buspirone). In recent years there has been considerable interest in the use of the selective serotonin reuptake inhibitor (SSRI) class of antidepressants in anxiety disorders. Paxil (paroxetine) was approved for use in social phobia, and a number of SSRIs, as well as the serotonin/norepinephrine reuptake inhibitor (SNRI), Effexor (venlafaxine), are being evaluated in the treatment of GAD. New approaches for the treatment of anxiety disorders include a refocus on the pivotal role of the hypothalamic-pituitary-adrenal (HPA) axis, which controls the synthesis and release of circulating cortisol (or corticosterone in many mammals). The HPA axis is being targeted in the brain at the level of the hypothalamus and pituitary with approaches aimed at reducing the hormonal signal sent to the adrenals. Antagonists of the corticotrophin releasing factor 1 (CRF1) receptor are being actively pursued by a number of pharmaceutical companies, while Sanofi-Synthelabo have a vasopressin V<sub>1b</sub> receptor antagonist that produced promising anxiolytic effects in preclinical studies and is currently in clinical trials. Antagonists of the glucocorticoid receptors are also under development by a number of companies. Other approaches include selective ligands for subunits of the GABA-A receptor (to produce the beneficial effects of the benzodiazepines without the side-effects), receptor specific ligands for serotonin or metabotropic glutamate receptors, as well as agonists at the newest opioid receptor, NOP1.

### **Depression**

Depression can be thought of simply as a state of chronic anxiety. Symptoms are similar to anxiety and include depressed mood, anhedonia (inability to experience pleasure), sleep disturbances, fatigue, feelings of worthlessness or inappropriate guilt, and diminished ability to concentrate, and patients often resort to suicide. Of all the CNS disorders, depression has probably had the most success in terms of discovering treatments, although many of these treatments were found by

accident. The classical tricyclic antidepressants (TCAs) were initially developed as antihistamines and antipsychotics when clinicians noted that the mood of patients was drastically improved. The discovery of the first SSRI, zimelidine (failed in clinic due to liver issues) then led to the eventual development of Prozac (fluoxetine), Paxil (paroxetine), Zoloft (sertraline), Luvox (fluvoxamine) and Celexa (citalopram). The discovery of the SSRI antidepressants, along with the newer SNRIs such as Effexor (venlafaxine), has been a major breakthrough in the treatment of depression due to their improved safety profile compared to the TCAs. However, the SSRIs and SNRIs are associated with side-effects including sexual dysfunction, sleep disorders and nausea. Furthermore, these treatments require at least two weeks of treatment before the onset of efficacy. A recent area of development was the finding from Merck that a selective neurokinin NK1 receptor antagonist, MK-869, had the same efficacy as SSRI antidepressants in human depression trials, but without the side-effects. A number of pharmaceutical companies have active NK1 antagonists programs and results from on-going clinical trials will determine the value of this approach. As with anxiety, the HPA axis has become a major target for depression and many of the targets discussed earlier are also efficacious in preclinical depression models. As compounds are developed that target the HPA axis, companies will no doubt test the efficacy of compounds in depression trials alongside trials for anxiety disorders. Finally, while the research effort has focused largely on the role of serotonin and norepinephrine in depression, there is increasing interest in the role of dopamine, particularly as the well documented role of dopamine in rewarding situations may contribute to the anhedonia associated with depression.

As a final section to this presentation, some of the new techniques being employed in CNS research will be discussed including the use of genetically-altered mice, data from the human and mammalian genomics efforts, and the use of pharmacogenomics in drug discovery and development. Also, the potential use of psychiatric drugs in veterinary medicine to treat conditions such as separation anxiety in household dogs will be discussed.

**Keywords**

CNS Disorders, Affective Disorders, Psychosis, Alzheimer's Disease, Parkinson's Disease

# PHARMACOKINETICS IN DRUG DEVELOPMENT: BEYOND SINGLE MODELS

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## INTRODUCTION

Thanks to its different modeling approaches, pharmacokinetics (PK) can no longer be reduced to the status of a regulatory requirement. PK is a major scientific tool, able to assist veterinary drug companies with their program of drug discovery and drug development. Conditions for that are two-fold : (i) advanced PK approaches should not get lost in a fog of mathematical complexity and (ii) developers must have a clear understanding of the physiological and physiopathological meaning and potential usefulness of PK, pharmacodynamics (PD) and statistical parameters obtained from the different modeling approaches.

## A SIMPLE MODELING APPROACH

For veterinary drugs, PK is still too often performed only following guidelines recommendations (FDA, EMEA) *i.e.* as a set of standardized studies aimed at meeting regulatory requirements. It should be remembered that PK guidelines were written by and for regulatory authorities to provide them with the minimal information needed to make a judgement on a submitted dossier. In any case, guidelines were not written to suggest an optimal use of PK for drug discovery and drug development.

The goal of regulatory PK is to report the basic PK parameters describing absorption, distribution, metabolism and excretion (ADME) of drugs in generally healthy animals *i.e.* to give a PK fingerprint of the drug (1). For this purpose a very simple modeling approach is generally selected, which is inappropriately termed a “model independent” or “non-compartmental” approach. Actually, this approach is founded on a definite structured model in which exit (irreversible removal of drug) and measurement (plasma concentration) are explicitly associated with one central compartment. In addition, it allows any number of recirculations or exchanges with any number of non-central pools none of which is identified with any physiological structure (2). The advantage of this recirculatory (stochastic) model is that it embodies the concept of statistical moments and mean transit time. Thus, very simple and user-friendly software can be used to compute area under the curve (AUC), area under the first moment curve (AUMC) etc.

This so-called non-compartmental approach allows us to compute basic (but of a major clinical interest) PK parameters namely plasma (body, systemic) clearance (Cl), volume of distribution at equilibrium (V<sub>ss</sub>) and mean residence time (MRT), which is the mean time an individual molecule resides in the body. In addition, by comparing AUCs obtained after intravenous and extravascular routes, the extent of bioavailability (F%) can easily be assessed.

The relevance of this set of parameters (especially Cl) is often overlooked because some drug companies have generated these parameters with a checklist for regulatory authorities in mind rather than a scientific approach to drug efficacy and safety. It should be realized that this very simple modeling approach allows us to capture the two most important PK parameters namely Cl and F%. Indeed, the two key questions in drug development are “Has the right drug been selected?” and “Has the optimal dosage regimen been established?”. This second question can be addressed by the following relationship:

$$\text{Dose (maintenance) / per dosing interval} = \frac{Cl \times C_{ss}}{F}$$

where C<sub>ss</sub> is the targeted [optimal] steady state plasma concentration. Inspection of equation 1 shows that the required maintenance dose has both PK and PD determinants. It is an hybrid PK/PD variable influenced by two PK parameters (Cl, F%) and a PD parameter (effective plasma concentration) which is a measure of drug

potency.  $Cl/F$  is the only determinant controlling overall drug exposure (measured by AUC) and is the ratio on which dosage regimen adaptations are based. Discussion of this relationship and its use for different purposes including interspecies or *in vitro* to *in vivo* extrapolation (e.g. for antibiotics) are extensively discussed elsewhere (3, 4).

For a few drugs, a loading dose (LD) is required, especially for those having a long half-life that accumulate progressively during a multiple dosage regimen and for which a full effect (*i.e.*  $C_{ss}$ ) is immediately required. Here also, the so-called "non-compartmental" approach provides useful information because (equation 2):

$$\text{Loading dose} = \frac{V_{ss} \times C_{ss}}{F} \quad \text{Eq. 2}$$

where  $V_{ss}$  and  $C_{ss}$  are as previously defined. On the other hand, it is rather unfortunate that  $V_{ss}$  is too often and inappropriately used to discuss the "extent of drug distribution" by an illicit rearrangement of equation 2 (*i.e.* by assuming that  $C_{ss}$  is controlled by  $V_{ss}$  whereas  $C_{ss}$  is only controlled by plasma clearance).

Finally, the simple so-called "non-compartmental" approach allows us to compute the two doses of therapeutic interest (maintenance and loading dose).

### THE CLASSICAL AND LESS CLASSICAL COMPARTMENTAL MODELS

"The data were fitted to a two-compartment open model ..." is likely to be the most frequent sentence encountered in veterinary PK publications. Not only is the statement inaccurate (exponential models are usually fitted to data and then interpreted in terms of a compartmental model) but classical compartmental models provide little supplementary information beyond that captured from the single "non-compartmental" approach, because the minimal physiological interpretation represented by these models is nearly never explored for PK parameter interpretation. What is actually done when fitting an exponential model to data is an empirical modeling which considers the body as a black box. However, unlike with "non-compartmental models" compartmental connectivity should be qualified, whether or not the physiological or anatomical identity of the various compartments is known (2). The compartment model most often selected in veterinary publications corresponds to a simplistic view of the parallel organization of the mammalian circulation, whereas an alternative interpretation involves considering that the different compartments correspond to the catenary organization of the three hydric sectors of the organism. The two concurrent views of the three-open compartmental models are identifiable from parameters of a triexponential equation but are indistinguishable *i.e.* selection of one of the two interpretations requires *a priori* assessment of the connectivity of the model.

Using this anatomical or physiological interpretations of compartmental models, parameter interpretation could be done; e.g., the product of  $k_{12}$  (the first order rate constant of transfer from compartment 1 to compartment 2) and  $V_c$  (the volume of the central compartment) can be compared to a regional blood flow in the first interpretation whereas the same product could be compared to the rate of water exchange between plasma and extracellular fluid in the second interpretation.

These compartmental models are generally used to evaluate and interpret terminal half-life. In a classical compartmental model, terminal half-life (*i.e.* the time required to divide plasma concentration by two after reaching pseudo-equilibrium distribution) is a hybrid parameter influenced by both clearance and extent of distribution. This is not the case in some other classes of "compartmental model"; for example, disposition of angiotension converting enzyme (ACE) inhibitors (benazeprilat, enalaprilat...) looks like a classical bicompartamental model. Actually, the physiological interpretation of the terminal half-life involves the saturable binding of ACE inhibitors to circulating and non-circulating ACE, whereas the process of elimination is reflected by the phase that in a classical interpretation represents the distributional phase (5). This physiological interpretation of terminal half-life explains why despite a long terminal half-life, ACE inhibitors do not accumulate during a multiple dose regimen (5).

For a classical compartmental model, the terminal half-life [or the relevant terminal half-life] is the parameter of interest which defines the dosing interval.

Drugs with short half-life are problematic in maintaining  $C_{ss}$  and will require a specific dosage form, allowing a slow [or a controlled] release, *i.e.* a flip-flop process, the terminal half-life reflecting now the half-time of absorption (or liberation) rather than the half-time of elimination. Drugs with a long half-life should be investigated for drug accumulation (if multiple dosage regimen is used) and can require a loading dose.

### PHYSIOLOGICALLY BASED MODELS

Full physiological based models are developed *a priori* before the experimental response is available (6). They are built on compartments that represent the different anatomical and physiological structures of the body. They require independent experimental data such as tissue blood flow and volume, blood-tissue partition coefficient, drug protein binding, metabolic clearance... They use the principle of mass balance to describe regional drug distribution. They are mainly used for simulation for human risk assessment as pollutant and toxicant. They have been more rarely used in pharmacology because they are complicated, need much information and their validity needs to be established. As they predict tissue concentration, a possible application of this class of models could be the study of residue depletion in tissues among different species including orphan species for which experimental data are not available.

### PHARMACOKINETIC/PHARMACODYNAMIC INTEGRATION (PK/PD MODELING)

The PK/PD modeling approach integrates the PK model (describing the relationship between dose and plasma concentration vs. time), the PD model (describing the relationship between concentration and effect), a link model (bridging the PK and PD models) and ideally, a statistical model (describing intra- and inter-individual variability).

The ultimate aim of a PK/PD model is to forecast drug efficacy and if possible clinical outcome.

Different methodological approaches can be used for PK/PD analysis. In PK/PD models for direct effects, concentrations are directly related to drug effects (actually throughout a hypothetical effect compartment). For most drugs, the measured response is not a primary action resulting from drug-target interaction. Instead, there is a cascade of time-consuming biological events that entails an indirect relationship between plasma drug concentrations and the final observed response. The observed delay between the kinetics of the plasma concentrations and the time development of effect reflects the intrinsic temporal responsiveness of the system. For these drugs, indirect response models are selected. Both models were recently reviewed in the context of veterinary medicine (3).

PK/PD modeling allows *in vivo* estimation of the two most important PD parameters namely  $EC_{50}$  (the plasma concentration that produces 50% of the maximal response *i.e.*  $E_{max}$ ) which is a measure of drug potency and  $E_{max}$  itself. PK/PD modeling also estimates the slope of the concentration-effect curve which can be used as an index for drug selectivity.

The advantages of a PK/PD study are to separate the two main sources of drug response variability (PK and PD) thus opening the way to optimizing individual drug dosages based not only on PK parameters but also on PD covariables. PK/PD offers the opportunity of simultaneously determining the two components of a dosage regimen *i.e.* both the dose and the dosage interval. In addition, PK/PD precludes the need for multiple dose titration studies. Indeed, an  $EC_{50}$  is a parameter and its value is independent of the formulation or route of administration. Also if the company decides to develop another drug formulation, it will not be necessary to perform a new dose titration but only a new PK study to quantify the bioavailability factor. Another advantage of the PK/PD approach is to offer a sound framework for interspecies extrapolation (for veterinary examples of PK/PD applications see ref. 3).

One of the limits of PK/PD modeling is that very often the drug response of interest is difficult to obtain (e.g.: bactericidal action of an antibiotic), difficult to quantify (e.g. mood for an antidepressant) or delayed in time (survival time for

cancer therapy). Therefore, the effect of ultimate interest is replaced by a surrogate endpoint (e.g. a biomarker which has been validated for its clinical relevance). Examples of surrogates used in veterinary medicine include the PK/PD indices that have been proposed for predicting clinical success and bacteriological cure of antibiotics, such as the inhibitory AUC (AUIC), peak concentration vs. MIC ratio ( $C_{max}/MIC$ ), and time above MIC ( $t > MIC$ ). Prospective and retrospective trials in human medicine have demonstrated statistical correlation between these surrogate markers and either clinical success or prevention of resistance emergence. These indices are mechanistically related to clinical outcomes since they are all constructed using the MIC value (7). For ACE inhibitors that help prevent heart failure (such as benazepril and enalapril), PK/PD relationships have been investigated using plasma and tissue ACE inhibition. Based upon these relationships, canine dosage regimens have been determined for doses that totally inhibit ACE activity (5). As with the case of AUC/MIC or  $C_{max}/MIC$ , ACE inhibition is only a surrogate endpoint. Nevertheless, its utility has been documented, given that it is a more rapid method for estimating effect than is the traditional approach of estimating survival time and is easier to quantify than improvement in quality of life, the latter two points being the ultimate goals of ACE inhibition therapy.

To date, unique opportunities exist for the development of new biomarkers on the basis of genomics and proteomics.

### **POPULATION KINETIC MODELING**

PK studies performed in a limited number of healthy animals, generally in a good laboratory practice (GLP) environment, are valuable in obtaining the order of magnitude of the different basic PK parameters. However, parameters of great importance such as clearance and bioavailability (exposure) should be assessed in relevant target populations (e.g.: diseased animals). More importantly, in a conventional GLP environment, some major kinetic determinant of drug disposition can totally be missed. This is the case in the social herd behavior in cattle for ivermectin pour-on disposition where, allo- and hetero-licking is responsible for oral rather than skin absorption (8).

Similarly, experimental GLP studies, with the classical two-stage data analysis, cannot document properly inter-animal variability which can be of a crucial importance in designing a proper dosage regimen in individual animals (companion animals) or to promote good veterinary practice. For instance, for a mass antibiotic treatment (pig, poultry) it can be hypothesized that selection (or emergence) of resistance can be promoted by underexposure of a subpopulation due to interindividual competition for access to the medicated food (hierarchy and dominance influence compliance) or to relative weakness of some diseased (pyretic) animals. By contrast, metaphylaxis in homogenous groups of animals can be a practice consistent with the concept of the prudent use of antibiotic. The only way to test such hypothesis is to perform PK studies in field conditions on the relevant target population.

Population kinetics is by essence observational, not experimental. It consists of obtaining in a large collection of individuals from the representative population, a limited number of samples (sparse data). By means of some specific modeling approaches (e.g. the nonlinear mixed effect model) typical PK parameters, their interindividual variability, their possible association with different covariables (age, sex, weight...) and their unexplained variability, can be estimated (4). Population kinetics supports flexible labeling policies and extralabel use or is able to document the question of withdrawal time variability (4).

Limitations of population kinetic studies in drug development are the absence of user-friendly software, lack of clear understanding of its interest by industry and, especially, absence of encouragement from some regulatory agencies that seem to prefer very precise but possibly misleading GLP PK studies.

### **CONCLUSION**

PK studies do not consist just of injecting a drug, measuring the plasma drug concentration and reporting (or publishing) parameters given by a computer

program. PK is a truly scientific tool which, when mastered, is of great value to speed up drug discovery and to contribute to rational drug development.

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#### KEY WORDS

Non-compartmental approach ; compartmental interpretation ; PK/PD modeling ; population kinetics.

## **EVALUATING VARIABILITY IN DRUG RESPONSE: PHARMACOGENETICS**

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### **INTRODUCTION**

Drug responses to a fixed dose vary to differing extents among patients. The standard dosage regimen of a drug may prove to be therapeutically effective in most patients, ineffective or toxic in others. At present, the lack of efficacy is discovered by trial and errors for most medications. In case of poor efficacy, the treatment may be changed but sometimes the desired therapeutic benefit is difficult or impossible to assess (eg survival prolongation) or evaluation of efficacy is made well into therapy, at a point when treatment failure reduces the likelihood of other therapies being successful (cancer chemotherapy) (1). The consequences of adverse reactions are also quite important for the patient. In humans, it has been estimated that 2.2 million cases (i.e., 6.7% of inpatients) of severe adverse drug therapy occur per year in US hospitals from correctly applied drug therapy, causing about 100,000 deaths (2). Therefore, interindividual variability in drug response is a critical issue in humans, and deserves more and more attention in veterinary medicine.

### **WHAT DETERMINES THE INDIVIDUAL'S RISK OF THERAPEUTIC FAILURE OR DRUG ADVERSE REACTIONS ?**

Drug response is a complex phenotype to which it is probable that genetics, age, disease and environmental factors will contribute.

Breed differences in drug pharmacokinetics or response have been described in veterinary medicine: for example, a slower cutaneous absorption of moxidectin after topical administration in Aberdeen Angus compared to Holstein calves (3), longer anaesthetic effects of thiobarbiturates in Greyhound dogs than in mixed-breed dogs (4), and higher intestinal permeability (assessed by urinary lactulose to rhamnose recovery ratios) in Greyhounds than in Golden Retrievers (5). Nevertheless, such differences may be explained also by non-genetic factors like a different environment, exercise regimen, stress level, or some other things.

A key issue in interindividual variation in drug response is indeed the differentiation between genetic and environmental factors. However, drug response depends on successive events, controlled by different gene products, which may moreover interact with environmental factors. A single trait associated with an adverse drug reaction may be a risk factor, but may not be necessary nor sufficient to produce the adverse reaction by itself (6). A simple approach to differentiate hereditary from environmental factors of variability is the comparison of small series of monozygotic and dizygotic twins, or comparison of the inter- and intraindividual variability after repeated administrations of the same drug (7).

Most of the information available about genetically associated variability in drug response involves pharmacokinetic studies. For example, interindividual differences in drug binding may be genetically determined. More than 30 variants of human serum albumin have been identified and it was shown that for some variants the association constants may be decreased by 4-10 fold for some test compounds (8). The genetic factors represent an important source of interindividual variation in drug metabolism. The major polymorphisms have been described for CYP2D6 and CYP2C19, N-acetyltransferase, methyltransferase and butyrylcholinesterase. The main pharmacodynamic studies in humans have focused on malignant hyperthermia, long QT syndrome, response to beta-agonists in asthmatics, sensitivity to ACE inhibitors, and responsiveness to sulfonylurea hypoglycemic drugs.



### WHAT IS PHARMACOGENETICS ?

The term “Pharmacogenetics” has been initially defined as the science of pharmacological response and its modification by hereditary influences (9), after incidental observations of adverse effects associated with the use of different drugs (primaquine, succinylcholine, isoniazid) in human patients.

Molecular genetic tools have considerably transformed pharmacogenetics in the 1990s. The two alleles carried by an individual at a given gene locus (referred as the genotype) can now be identified at the DNA level . Pharmacogenetics is nowadays the way to characterize an individual with respect to disease susceptibility, severe drug adverse events, or whether the drug is effective. It aims to select the ‘right drug for the right patient at the right time’ (10). A pharmacogenetic test is intended to predict differential drug response through analysis of DNA sequence variations (polymorphism)(10).

Pharmacogenomics can best be defined as the description of drug effects using whole-genome technologies (e.g. gene and protein expression data)

### WHAT ARE THE GENETIC BASES OF PHARMACOGENETICS ?

The relationship between genetics and its pharmacological consequences is explained by differences in proteins (e.g., enzyme for drug metabolism, structure of receptors, carrier proteins and ion channels for drug effect) between patients with different response to a given treatment. For a given species, pharmacodynamic variability is probably most often greater than pharmacokinetic variability (11). This may be explained by the fact that genetic control of an enzyme is most often via a single locus, while the complexity of receptor structure, often involving multiple units and proteins, will involve multiple genes and increase the potential for polymorphism (12).

DNA mutations may lead to production of functionally altered proteins or altered amounts of a normal gene product (more often a decrease). If mutant or variant genes exist at a frequency >1% in the normal population, they are called genetic polymorphisms. Single nucleotide polymorphism (SNP) is the simple change of one base pair at any point in the DNA molecule and is therefore the most common form of genetic variation. SNPs occur approximately once every 300-3000 base pairs if one compares genomes of 2 unrelated individuals. Any 2 individuals thus differ by approximately 3 million base pairs, i.e. only 0.1% of the approximately 3.2 billion base pairs of the human haploid genome. Informative SNPs are those that occur at frequencies of greater than 20% in large populations (13). An SNP may have clinical relevance when it involves one which is at the active site for example of an enzyme involved in drug metabolism. On the other hand, most of the mutations do not lead to clinically or therapeutically relevant effects. They remain silent because mutations may not change the corresponding aminoacids in the protein or because they affect neither the binding site nor a functionally important part of the protein structure. Identification of SNPs will be a critical step in knowledge in pharmacogenetics.

High density maps of SNPs will allow their use as markers of drug responses even if the target remains unknown, providing a ‘drug profile’ associated with contributions from multiple genes to a response phenotype. The SNP Consortium Ltd. (14) for example has been formed to advance the field of medicine and the development of genetic based diagnostics and therapeutics, through the creation of such a high density SNP map of the human genome. A major obstacle however in pharmacogenetics is the actual collection of patients of interest (for example, who have had an adverse event or therapeutic failure), and proper controls (i.e., with no adverse reaction or therapeutic failure) treated with comparable doses.

Polymorphism has been described in dogs for the metabolism of the COX-2 inhibitor, celecoxib. There are at least two populations of dogs, differing by their ability to clear celecoxib from plasma at either a fast or a slow rate after intravenous administration. In 242 animals, 45% dogs eliminated celecoxib from plasma at a rapid rate (phenotype EM, mean plasma clearance: 18.2 mL/min/kg) and 54% at a slower rate (phenotype PM, plasma clearance: 7.2 mL/kg/min). Hepatic microsomes from EM dogs metabolized celecoxib at a higher rate than microsomes from PM dogs (15)

## PHENOTYPING OR GENOTYPING ?

Individuals can be screened for genetic polymorphism via phenotyping or genotyping. For example, phenotyping the polymorphism of a drug-metabolising enzyme is the indirect analysis of genetic variation by examining an individual's metabolic capacity. Measurements of metabolites are performed after administration of a drug probe and an individual can then be classified as a poor, intermediate, extensive or ultrarapid metaboliser. The disadvantages of phenotyping include: i) limited specificity of probes, ii) potential adverse effects from drug administration, iii) the fact that the phenotype may be influenced by a variety of factors such as concurrent medications, hormonal status, and concomitant diseases; environmental factors moreover, are continually changing (16).

Genotyping involves the direct analysis of genetic variation by examining an individual's DNA. The advantages of genotyping include: i) direct determination of an individual's genetic information; genotyping may be therefore specific for a mutation, but it is nowadays possible to assess simultaneously a large number of mutations for several genes of interest, ii) less invasive than phenotyping because DNA may be isolated from buccal swabs, hair roots and saliva, or at most requires collection of only one blood sample, iii) the information has long-life validity, and iv) no influence from other factors (coadministered medications, clinical status). However, limitations of the genotyping approach are that i) the functional significance of many of the specific genotypes remains unknown at the present time, ii) genotyping tests are designed for identifying the most current variants, and iii) the cost remains high for a screening test (16), until there are simple, non expensive, high throughput methods for the routine genotyping of large-scale clinical samples.

## Impact on drug development

Historically, the treatment of a disease was empirical and the only way to determine whether or not a drug would work was to try it. By a process of trial and error, the best drug and the best dose were selected. The future hope of pharmacogenetics is that by understanding the molecular basis of individual variation in drug response, knowledge will be gained on how to focus on the patient as an individual, defining the medicine and dose most suited to that patient before prescription.

Pharmacogenetic departments should be developed in pharmaceutical industries, not only to identify drug targets but also to achieve the goal of faster development of more drugs with greater efficacy, while simultaneously ensuring inherently better market definition of such drugs (17). For example, a promising approach will be to detect, at an early stage of the development, SNP in patients to select only patients with adequate susceptibility polymorphism before launching the clinical trial. Consequently, trials will provide sharper results with fewer people.

The following areas relating to the use of clinical genetics within drug development have already received mention from the FDA: understanding trans-racial metabolic heterogeneity as it relates to pharmacokinetics and pharmacodynamics, and predicting drug safety and efficacy against the background of inter-individual heterogeneity of drug metabolism (18).

Pharmacogenetics is therefore a wonderful challenge for this new century, but will involve more and more collaborations between academia, industry and regulatory affairs. 'The right drug for the right patient' concept will be difficult to develop especially in food-animal pharmacology for obvious practical reasons and will be replaced more probably by 'The right drug for the right population', population being defined by any relevant variable (such as breed or polymorphism).

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## **MONITORING OF RESPONSES: PHARMACOVIGILANCE**

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### **INTRODUCTION**

Pharmacovigilance refers to the collection, investigation, maintenance, and evaluation of spontaneous reports of suspected adverse events associated with the use of marketed veterinary medicinal products (1,2). Veterinary medicinal products include therapeutic agents, biologics, vaccines, agents used in disease diagnosis, or agents otherwise administered or applied to an animal for protective, therapeutic, or diagnostic effects or to alter physiological functions (2,3). An adverse event is any observation in an animal, whether or not considered to be product-related, that is unfavorable and unintended and that occurs after any use of a veterinary medicinal product (2). The term adverse experience is generally, but not universally, used synonymously with the term adverse event.

A suspected adverse event is associated with a veterinary medicinal product when there is a reasonable possibility that the adverse event may have been caused by the product (4). Determination of whether there is a reasonable possibility that the product is etiologically related to the adverse event should include factors such as temporal relationships, dechallenge/rechallenge information, association with (or lack of association with) underlying disease, presence (or absence) of a more likely cause, and physiologic plausibility (4). Dechallenge pertains to the withdrawal of the suspect product, while rechallenge pertains to reintroduction of a product suspected of having caused an adverse event after partial or complete disappearance of the event following dechallenge. The pharmacovigilance process begins with the detection of a clinical event by a veterinarian or an animal owner, with attribution of the event to the use of a particular veterinary medicinal product (5). It continues with spontaneous reporting of the event to the product manufacturer or regulatory authority. In most cases a cause-and-effect relationship between the event and product use cannot be definitively established, hence the importance of characterizing the event as a "suspected" adverse product event. Taken in isolation, a suspected adverse event may be associated with a veterinary medicinal product, however there is no certainty that the suspect product caused the adverse event (5). Accumulated adverse event reports within the scope of veterinary pharmacovigilance may indeed provide pertinent safety and efficacy information.

Suspected adverse events include suspected adverse reactions in animals, suspected adverse reactions in humans administering or otherwise handling veterinary medicinal products, suspected lack of product effectiveness, suspected violative residues in products for human consumption following administration of veterinary medicinal products to food-producing animals, and suspected ectotoxicity or environmental events associated with use of the veterinary medicinal product (6). Some regulatory authorities consider suspected product defects to be adverse events (3). Suspected adverse events within the scope of pharmacovigilance do not include adverse events detected during planned, pre-approval field trials or clinical studies, or directed target animal safety studies (1).

Adverse events can be characterized as serious or non-serious, and expected or unexpected. A serious adverse event is any adverse event which results in death, is life-threatening, results in persistent or significant disability/incapacity, or a congenital anomaly or birth defect (2). A non-serious adverse event is one that does not meet any criteria for a serious adverse event. An unexpected adverse event is an adverse event of which the nature, severity or outcome is not consistent with approved labeling or approved documents describing expected adverse events for the veterinary medicinal product (2).

## **IMPORTANCE OF PHARMACOVIGILANCE**

It is generally believed that testing of veterinary medicinal products during pre-marketing development programs, and review of data by regulatory authorities in licensing these products, does not guarantee absolute safety and effectiveness due in part to the inherent limitations of pre-marketing development programs (7). Due to the limited size and controlled nature of pre-marketing clinical trials, only the most common adverse events will be observed and included in product labeling at the time of product approval (1). Following marketing of a new product, the number and variety of animals exposed to the product increase greatly. In addition, patients with multiple medical conditions or that are receiving multiple concomitant veterinary medical products are exposed to the new product (8). Thus, the patient experience base will be much broader than that from development studies.

### **Strengths and Weaknesses of Spontaneous Reports of Suspected Adverse Events**

The limitations and strengths of a voluntary, spontaneous reporting process were recently reviewed (8). The limitations include: 1) the subjective and imprecise recognition of the adverse event; 2) underreporting by consumers or health care professionals; 3) existence of biases; 4) inability to adequately estimate incidence rates; and 5) frequently, low quality of reports. Placebo or sham treatment situations can be associated with adverse events. Biases relate to the uncontrolled conditions under which an adverse event may have occurred, the length of time a product has been on the market (reports generally peak during the first 2 years post approval; 9), the country in which the report originated, and the reporting environment. The patient population exposed to the drug needed to calculate the incidence rate of the adverse event is, at best, an estimate. The quality of a report is dependent on the quality of the information provided by the reporter. Strengths of the spontaneous reporting process include the larger scale and cost-effectiveness of collecting safety and effectiveness data, and the ability to detect signals of potential problems that warrant further investigation (8).

### **Data Evaluation and Signal Detection**

Causality assessment is an important step in the pharmacovigilance process. The outcome of a causality assessment of an adverse event report is the gauge of the degree of certainty that the adverse event is in fact product-related (6). There are many methods of causality assessment ranging from fuzzy reasoning to Bayesian methods. The FDA/CVM uses an algorithm based on published work by Kramer et al. (10). A commonly used informal guide is the ABON system of Probable, Possible, Unclassifiable, or Unlikely (6). Recently, the Committee for Veterinary Medicinal Products of the European Agency for the Evaluation of Medicinal Products proposed a six-factor approach to causality assessment (11). These factors are: 1) associative connection in time and the location or distribution of the signs or symptoms; 2) pharmacological explanation; 3) presence of characteristic clinical or pathological phenomena; 4) previous knowledge of similar reports; 5) exclusion of other causes; and 6) completeness and reliability of the data in the case reports (11). The cases in which the adverse event is at least probably related to the product are best for signal detection.

A calculation of incidence rates is inherently inaccurate and can be misleading, in part, because a denominator representing the population exposed to the product cannot be determined with accuracy (8). Sales or distribution data are frequently used as the denominator, however even if accurate data are obtainable, the data are affected by sales incentives, time of the year, or product guarantee programs. It is difficult to ascertain the quantity of doses reaching the end user and actually administered to veterinary patients. An alternative signal detection approach that has been proposed as being accurate for detecting a safety signal is the creation of an adverse event profile for the product that reflects the percentage of cases that contain a specific adverse event associated with a specific body system or clinical sign within that system (12). For example, the proportion of cases with an adverse event referable to the cardiovascular system or to a cardiovascular clinical sign

during a specified period can be determined from the total number of case reports for the period (i.e. report rate = number of case reports involving a particular system or sign/total number of case reports). The report rate can be compared to the percentage for the same period 1 year ago, or between similar products. The extent of the change in the percentage can be the trigger for further investigation of the reason for differences between the two periods. This approach has the advantages of: 1) correcting for potential seasonal fluctuations in adverse event rates; and 2) not requiring sales or distribution data (12).

### ***Signal Detection: Potential Outcomes***

Signal detection may result in a high degree of suspicion that an adverse event is associated with a veterinary medicinal product. Actions stemming from signal detection may include further investigation by the manufacturer or lead to regulatory decisions (8). Manufacturers may voluntarily or under the direction of a regulatory authority initiate a variety of actions including: 1) sending safety alert (“Dear Doctor”) letters to veterinarians; 2) changing product labels by adding warnings, contraindications, or human safety information; 3) conducting post-marketing research; 4) recalling specific product lots; 5) inspecting of manufacturing facilities and records; or 6) withdrawing the veterinary medicinal product from the market (1,8).

## **REGULATIONS AND GUIDELINES GOVERNING ADVERSE EVENT REPORTING TO REGULATORY AUTHORITIES AND MECHANISMS FOR REPORTING**

Regulatory reporting requirements vary between countries, and even within a specific country reporting requirements may differ depending on the licensed product or class of products. Efforts have been made to standardize the management of adverse event reports between the European Union, Japan, and the USA through the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH; 2). Although it is beyond the scope of this paper to consider specific reporting obligations of manufactures in all regions, the efforts to harmonize pharmacovigilance through VICH and reporting regulations in the USA will be briefly reviewed.

A draft guidance document of the VICH Expert Working Group on pharmacovigilance was issued in June 2000 in which the importance of developing harmonized and common systems, common definitions, and standard terminology was described (2). Harmonization of these elements across regions will facilitate reporting responsibilities and inter-regional comparison of data and exchange of information. The VICH document provides definitions of terms used in veterinary pharmacovigilance, an outline of the reporting process, and detailed listing of the data elements useful to assess an adverse event report. Collection of these data elements represents one of the more challenging, but essential, aspects of pharmacovigilance. Among the data elements are details on the persons involved in the adverse event report (e.g. veterinarian, animal owner), detailed description of the adverse event (including animal data), product data and usage, information on dechallenge-rechallenge, and assessment of the adverse event by the attending veterinarian and the manufacturer. The data collected should be sufficiently comprehensive; however, it is acknowledged that substantial pieces of data will not be known. None-the-less, reporters of adverse events (veterinarians, animal owners) and collectors of adverse event data (product manufacturers, regulatory authorities) should strive to provide and record, respectively, as comprehensive set of information as possible so that the suspected adverse event can be properly assessed. Although anyone directly involved with a suspected adverse event may report the event to a regulatory authority or manufacturer, reporting by the attending veterinarian is encouraged.

Contained in the VICH draft document is an outline for assigning a likelihood of association between the veterinary medicinal product and the adverse event. The reporting veterinarian, the manufacturer, or both can make this assessment. A “probable” assessment should be given if all of the following criteria

are met: 1) there is a reasonable association in time between the administration of the product and the onset and duration of the adverse event; 2) the description of the clinical signs should be consistent with, or at least plausible, given the known pharmacology and toxicology of the product; and 3) there is not other equally plausible explanations for the adverse event. A “possible” association should be given if the association of the adverse event with administration of the product is one of other possible and equally plausible explanations for the described event. An “unlikely” association should be given where sufficient information exists to establish that the described event was not likely to have been associated with the administration of the veterinary medicinal product, or other more plausible explanations exist. An “unknown” association applies to all events where reliable data is either unavailable or is insufficient to make an assessment (2).

Although the VICH draft guidance was issued almost 3 years ago, it has yet to be adopted by regulatory authorities in the three regions. One of the major obstacles for adoption appears to be lack of agreement on data elements required for reporting adverse events for the purpose of electronic transfer of data (13).

In the United States animal vaccines and most biologics are regulated by the United States Department of Agriculture under the Virus-Serum-Toxin Act. Federal regulations require the manufacturer, licensee, or permittee to notify the USDA Animal and Plant Health Inspection Service (APHIS) of circumstances and action taken pertaining to questions regarding the purity, safety, potency, or efficacy of a product, or if it appears that there may be a problem regarding the preparation, testing, or distribution of a product (14). At present, routine reporting of suspected adverse events to USDA is not required. However, APHIS has proposed amending the Virus-Serum-Toxin Act to require veterinary biologic licensees and permittees to record and submit reports to APHIS concerning adverse events associated with the use of biological products they produce or distribute (15).

Most products used topically for the control of ectoparasites and insects on animals are regulated by the US Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide and Rodenticide Act (3,16). For purposes of reporting to the EPA, adverse events in domestic animals must be placed in one of five categories of decreasing severity. The first category (D-A) includes death or euthanasia, while the fifth category (D-E) is for suspected adverse events for which the symptoms are unknown or not specified (16). Registrants of pesticide products are required to submit to the EPA reports of adverse events occurring in domestic animals which are accumulated over 90 day periods within 60 days after the end of each 90-day accumulation period.

Adverse drug events associated with animal drugs and medicated feeds are reported to the FDA/CVM under the Federal Food, Drug, and Cosmetic Act. Section 510.300 of the Code of Federal Regulations requires drug manufacturers (new animal drug applicants) to maintain full reports of information pertinent to the safety or effectiveness of new animal drugs, including unpublished clinical or other animal experiences (17). Copies of these reports concerning unexpected side effects, injury, toxicity, or sensitivity reaction or any unexpected incidence or severity associated with the clinical use, studies, investigations, or tests, whether or not determined to be attributable to the new animal drug must be submitted by the manufacturer to FDA/CVM on Form FDA-1932 within 15 working days of receipt by the manufacturer. Reports not submitted as 15-day alert reports are required to be submitted at 6 month intervals during the first year following approval by FDA/CVM, and then yearly thereafter (periodic reports).

On February 4, 2002, FDA/CVM published in the Federal Register an interim final rule that more clearly defines the kinds of information to be maintained and submitted by manufacturers for a new animal drug application or an abbreviated new animal drug application (7,18). Further, the interim final rule revises the timing and content of certain reports (7,18). For example, in the interim final rule an adverse drug experience is defined as any adverse event associated with the use of a new animal drug, whether or not considered to be drug related, and whether or not the new animal drug was used in accordance with approved labeling (18). FDA/CVM includes in the definition of serious adverse drug events those that cause

an abortion, stillbirth, or infertility, and those that require professional (veterinary) intervention. The interim final requires submission of periodic reports every 6 months for the first 2 years following approval, then yearly thereafter. The interim final rule was originally scheduled to take effect on August 5, 2002. However, on July 31, 2002, FDA/CVM delayed indefinitely the effective date of the interim final rule while it seeks approval on information collection provisions of the rule and addresses comments received on the rule (19).

While manufacturers of veterinary medicinal products are required to submit reports of suspected adverse events to regulatory authorities, the reporting of suspected adverse events by veterinarians and animal owners in the United States is voluntary. Veterinarians and animal owners are encouraged to report suspected adverse events. In doing so, reporters should be prepared to provide as comprehensive set of information as possible so that the suspected adverse event can be properly assessed. In the United States adverse events can be reported to the regulatory authority (i.e. FDA/CVM, EPA, or USDA) that licensed the veterinary medicinal product, or to the product manufacturer. Form FDA-1932a can be used to file reports with FDA/CVM, or the suspected adverse event may be reported by telephoning the Center for Veterinary Medicine at 1-888-332-8387 (7). The telephone number for the EPA is 1-800-858-7378; that for the USDA is 1-800-752-6255 (20). Many veterinary medicinal product labels include instructions for contacting the manufacturer (20). In the past the United States Pharmacopeia provided an adverse event reporting service entitled the USP Veterinary Practitioner's Reporting Network (USP PRN), however this service was discontinued in December 2002.

## **SUMMARY**

Testing during pre-marketing development programs may not guarantee absolute safety and effectiveness of a veterinary medicinal product. For this reason accumulated spontaneous adverse event reports collected during post-marketing pharmacovigilance are important to the understanding of safety and efficacy profiles of veterinary medicinal products. The future of veterinary pharmacovigilance may include routine reporting of adverse events associated with USDA-registered vaccines and biologics in the USA, international harmonization of pharmacovigilance processes among regulatory authorities, and electronic exchange of data (6).

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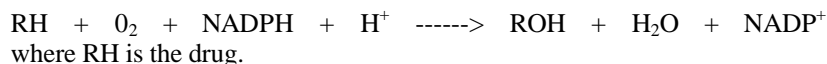
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## METABOLISM : THE CYTOCHROME P450s OF THE DOG

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The cytochrome P450 (CYP) enzyme system is responsible for the metabolism and hence clearance of a wide array of drugs, toxins, and endogenous substrates. Metabolism by CYP leads to a variety of metabolic transformations, all of which have at their foundation the insertion of a single molecule of oxygen into the substrate. From a clinical standpoint, biotransformation by CYP can lead to the loss of pharmacological activity, the maintenance or production (from a prodrug) of a pharmacological activity, or the production of toxic and/or reactive metabolites. Thus, any factors that alter the ability of the CYP system to metabolize a given compound may have clinical implications for drugs, toxins, or endogenous substrates. In recent years, more information regarding the CYP system in dogs has been forthcoming and veterinary clinical pharmacologists are gaining a better understanding of the clinical implications of variability in CYP expression and function in dogs. The purpose of this presentation is to review the fundamental principles of CYP regulation and activity, with a particular emphasis on understanding species differences in CYP (ie. when is a CYP study in another species clinically applicable to the dog) and the application of *in vitro* techniques to the study of CYP-based metabolic interactions in the dog.

The cytochrome P450 proteins form a super-family of heme-containing enzymes. It is a membrane-bound enzyme that is primarily located in the endoplasmic reticulum and has its active site located on the cytosolic face. Quantitatively, the liver is the most important site of CYP-dependent biotransformation, although clinically significant CYP expression can also be found in many other organs. CYP activity requires NADPH as a cofactor and NADPH-cytochrome P450 reductase as a coenzyme. The cytochrome P450 reaction involves transfer of electrons from NADPH to NADPH-cytochrome P450 reductase and then to cytochrome P450. This leads to the reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate. Virtually all subsequent chemical changes (eg demethylation) for this initial step. The basic reaction can be written as follows:



All the CYP have not been identified to date in any species. However, there are at least two dozen different forms in each species. The CYP enzymes can catalyse a wide variety of drug biotransformations. The enzymes have over-lapping but distinct substrate specificities. The CYP system is readily inducible by a variety of environmental contaminants and drugs. Induction can involve several or a single family of enzymes. Induction means the amount of the enzymes is increased and in general this leads to increased metabolism of some drugs. Some drugs will directly inhibit a CYP enzyme or compete for metabolism with another drug. As a result, the metabolism of one drug may be decreased by another drug. It is these properties that lead to the important effects of CYP on the clinical pharmacology of many drugs.

Naming of CYP enzymes now follows a system based on DNA sequencing. This system has removed the multiple naming of the enzymes that used to be common and has recognizes the distinct properties of each individual enzyme from different species, but for those unfamiliar with the system it can lead to misunderstandings. The old names of mixed function oxidases, microsomal monooxygenases, and naming based on activities or protein purification profiles should be avoided once the gene has been cloned. There are a multitude of CYP gene families, however only a few are generally involved in the metabolism of

clinically relevant drugs and xenobiotics. The CYP1, CYP2, and CYP3 families are those generally considered important for drug metabolism. The figure below illustrates the general principles of naming CYP:

<i>CYP3A12</i>	<b>3</b>	<b>A</b>	<b>12</b>
<i>Full name of CYP</i>	<i>Family</i>	<i>Subfamily</i>	<i>Individual enzyme</i>
<i>Sequence homology</i>	40%	40-80%	100% (number assigned on order of identification)

When the name is presented in italics, it refers to the gene; without italics, it refers to the protein. Following this system, except for a few hold-overs from the early days of naming CYP enzymes such as *CYP1A1*, a completed named CYP enzyme can only be found in one species. Thus, *CYP2C9* is only found in humans, while *CYP2C21* is only found in dogs. Why is this system followed and why is it important to understand? CYP families share general sequence properties but there are considerable differences within families in terms of regulation and substrate specificity. Within subfamilies (eg *CYP2C*), the similarities at the level of regulation within a species increases but substrate specificity (ie biotransformation) can still differ markedly. Each CYP enzyme has a broad substrate specificity, but in many instances a xenobiotic is metabolised *in vivo* predominantly by one or two CYP enzymes. A single amino acid change resulting from a single nucleotide change can markedly change the expression and/or substrate specificity of the enzyme. Thus, between species, individual enzymes within a subfamily display markedly different substrate specificities across species even if they have very similar DNA sequences. Similarly, within subfamilies, the extent and specificity of regulation (eg inducibility) by specific chemicals can be markedly different between species. Thus, if a drug is shown to be metabolised by *CYP3A4* in humans, it is important first to recognize that *CYP3A4* does not exist in the dogs and that second, while there is an increased probability that the drug is metabolised by a member of the *CYP3A* subfamily in the dog, the drug could be metabolised by an enzyme from another subfamily and will almost certainly display different kinetic properties. One of several species differences in CYP metabolism that illustrate this point can be found with tolbutamide and phenytoin. In man, both tolbutamide and phenytoin oxidation are primarily catalyzed by *CYP2C9* and their metabolism is similarly regulated between individuals. In the dog, CYP-dependent oxidation of tolbutamide can barely be measured, while phenytoin is metabolised so rapidly that its use as a therapeutic agent in dogs is limited. The enzyme specificity for the metabolism of these compounds in dogs has not been characterized but they illustrate the point that compounds with similar metabolism/enzyme specificity in one species do not necessarily have similar profiles in another species. Nevertheless, there are often similarities within CYP subfamilies and families between species that can help direct us in investigating or understanding the clinical relevance of xenobiotic-CYP combinations. To utilize this information, a basic understanding of CYP regulation and inhibition is required.

CYP expression can be regulated at multiple levels: transcription, translation, mRNA processing and stability, and protein stability. Regulation can be general throughout the body, or can be tissue-specific. Most of the CYP are constitutively expressed, but many of them can be further induced by exposure to exogenous substances. The constitutive expression is under a relatively complex control and is dependent on the subfamily considered. In rodents, there are considerable sex and developmental differences in constitutive CYP expression. Such differences are less pronounced in humans and very little is known about developmental, sex, and breed differences in dogs. From a clinical standpoint, transcriptional regulation of CYP expression by xenobiotics is probably the most important. Very little work on the molecular mechanisms of CYP regulation in dogs has been conducted, therefore the following summary of transcriptional regulation is based on work in the mouse, rat, and humans. While we believe that the general

principles hold, it is important to recognize that the specifics differ. Examples to illustrate this will be presented. For the *CYP1A*, *1B*, *2B*, *2C*, *3A*, and *4A* subfamilies, specific nuclear receptors that can be activated by both endogenous and exogenous compounds regulate their expression through specific binding to xenobiotic response elements. For the *CYP1* family, the well-known AhR (aryl-hydrocarbon receptor) is primarily responsible for the transcriptional induction observed after exposure to a wide variety of aromatic hydrocarbons and some therapeutic agents (eg omeprazole). For the *CYP2B*, *CYP2C* and *CYP3A* subfamilies, recent studies in rodents and humans suggest that their regulation by phenobarbital-like and glucocorticoid-like inducers are mediated primarily by the CAR (constitutive androstane receptor) and the PXR (pregnane X receptor), respectively, in conjunction with additional nuclear receptors (particularly the retinoic acid receptor RXR and the glucocorticoid receptor GR). The PPAR (peroxisome proliferator activated receptor) mediates induction of the *CYP4A* genes. It is important to note that while induction of *CYP* genes in the dog shares many characteristics with rodents and humans, these receptors have not been directly characterized in dogs and there are known to be significant species differences in the activators of the receptors, both in the qualitative nature of the activators and quantitative extent of the response. The *CYP2B11*, *CYP1A*, and *CYP3A* genes in the dog appear to be readily inducible in the dog. Of particular note, there is evidence to suggest that, in contrast to humans, dexamethasone does not significantly induce CYP activities in the dog while several NSAIDs appear to cause a marked induction CYP3A-related metabolic activities.

In addition to increased activity of *CYP* through increased protein expression, decreased activity can occur through loss of protein or through inhibition of activity by other compounds. The most thoroughly documented initiators of down-regulation of *CYP* gene and protein expression are the cytokines and interferons. However, this has not been documented in dogs and its clinical relevance is uncertain for this species. Probably the most important cause of loss of activity in dogs, therefore, is inhibition by concomitant administration of medications or interactions with food components. There are two major mechanisms of inhibition: non-competitive, mechanism-based (or suicide) inhibition and competitive inhibition. A number of specific instances of clinically relevant drug interactions, either demonstrated or presumed to occur at the level of CYP activity have been demonstrated in dogs, however the vast majority of interactions that are considered in dogs are based on extrapolation from humans. However, it is clear that direct extrapolation (quantitative and qualitative) is not possible, although it can provide some clinical guidance in the absence of better information. *In vivo* controlled clinical studies are the most reliable method of documenting drug interactions. However, they are expensive. Hence, we often rely on spontaneous reporting, something that is notoriously poor, to identify potential interactions or we extrapolate from the human literature. Therefore, alternative methods based on *in vitro* studies are being used more frequently. To be valuable, however, the limitations of *in vitro* studies must be appreciated and the appropriate experimental conditions must be employed. Liver slices, cultured hepatocytes, and microsomal studies can all be used. Each has its own advantages and disadvantages, however microsomal studies are the most convenient and versatile. They are however limited to assessing *CYP*-based interactions and other interactions will be missed. Nevertheless, in identifying potentially clinically relevant interactions, assessing the relevance of interactions reported in other species, and investigating the mechanism of interactions, *in vitro* microsomal studies are perhaps the most valuable. Once again, the characteristics of dog *CYP* differ from those of other species so that the conditions employed must be adjusted appropriately and one can not simply assume that identical conditions to those employed in other species are appropriate. We will present data showing how the effects of solvents and classical CYP inhibitors on dog CYP differs from that observed in humans and rodents. Further, clinically relevant conditions must be employed or extrapolated from the generated to data. There are two main approaches that can be used. The first is to test specific combinations of drugs of interest. The second approach is more general and consists of identifying

groups of compounds that are metabolised by specific CYP and then using this information to predict likely interactions.

A third source of variation in metabolism is genetic variation. As our knowledge of *CYP* genes and other metabolic pathways in dogs (and other species of veterinary interest) increases, the impact of genetic variation on therapeutic outcome will become clearer. This topic will be addressed in detail in the following talk by Dr. Lauren Trepanier.

To take advantage of our increasing knowledge of CYP in the dog, knowledge of the involvement of CYP and of specific CYP enzymes in the metabolism of compounds is required. A series of steps must be followed to ensure the correct assignment of metabolic reactions to specific CYP and these studies must be conducted at clinically relevant concentrations or the results will be misleading. The molecular tools available to investigate the specificity of CYP-mediated metabolism in dogs are increasing. Examples of identifying specificity of CYP-metabolism will be provided. There are generally five steps that are involved, once a reaction has been shown to be CYP-dependent. The latter is accomplished by demonstrating that a microsomally-mediated reaction is dependent on NADPH, is inhibited carbon monoxide, and is thermally stable. Once this has been demonstrated, the following steps are used to identify the specific enzyme involved. It is essentially that clinically relevant concentrations be considered when conducting these experiments. Correlation with marker activities

- (1) Correlation with immunoquantitated P450 levels
- (2) In vitro chemical inhibition by form-specific inhibitors
- (3) Induction experiments
- (4) Immunoinhibition experiments, with specific inhibitory antibodies
- (5) Recombinant or purified P450 activity

We will present examples from our own work and results from the literature to illustrate the principles above and to demonstrate the role and importance of *CYP* metabolism in dogs to veterinary clinical pharmacology. Although not covered in this talk, we must also be cognisant of the relationship between *CYP* expression and function and those of the drug transporters that can also significantly influence the clinical pharmacological properties of drugs.

*See Table on next page*

\*Very little is actually known about the specificity of substrates, inhibitors, and inducers of dog CYP and much of the work has been conducted without the availability of the molecular tools required to conclusively verify this specificity. Therefore, the majority of compounds listed should still be considered preliminary, particularly in terms of clinical relevance. For example, while it is clear the CYP2D15 can readily metabolize celecoxib *in vitro*, the experimental studies still suggest that another CYP is predominantly responsible for metabolism *in vivo*. Confirmed *in vivo* interactions include: enrofloxacin/theophylline; chloramphenicol/phenobarbital; chloramphenicol/propofol; ketoconazole/cyclosporine; ketoconazole/midazolam; ketoconazole/nifedipine; cimetidine/verapamil (several other studies with cimetidine have produced equivocal or relative insignificant changes in pharmacokinetic parameters).

**Current knowledge of the cytochrome P450 enzyme system in dogs**

<b>CYP sub-family</b>	<b>Constitutive Expression</b>	<b>Individual Enzymes</b>	<b>Inducibility</b>	<b>Probable substrates*</b>	<b>Probable inhibitors*</b>	<b>Inducers</b>	<b>Genetic variability</b>
1A	moderate	1A1, 1A2	yes	theophylline? ethoxy-resorufin	enrofloxacin?	PCBs omeprazole	?
2A	probably		?				?
2B	may be approximately 10% of total hepatic CYP.	2B11	yes	phenobarbital benzyloxy-resorufin progesterone testosterone propofol	chloramphenicol	Phenobarbital	?
2C	low	2C21, 2C41	?	Testosterone			Yes (2C41)
2D	may be approximately 20% of total hepatic CYP.	2D15	-	β-blockers dextromethorphan celecoxib			Yes
2E	yes		?	Chlorzoxazone?			Yes
3A	may be approximately 10% of total hepatic CYP.	3A12, 3A26	yes	macrolides steroids quinine cyclosporine midazolam nifedipine?	troleandomycin tetracyclines ketoconazole	Phenobarbital NSAIDs Rifampin	yes (3A12)

**Keywords:** dog, metabolism, cytochrome P450, drug interactions, induction, pharmacokinetics

# CYTOCHROME P450'S IN DOGS: FAMILIES AND PHARMACOGENETICS

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## CYTOCHROME P450 FAMILIES

Cytochrome P450's (CYP's) are heme-containing proteins found in many tissues, which catalyze oxidation and reduction reactions of endogenous products, drugs, and other foreign chemicals. There are hundreds of P450's recognized; these enzymes are categorized within and between different species using a family/subfamily/individual enzyme nomenclature. For example, CYP3A4, an abundant P450 in human liver, is a member of the CYP3 family, CYP3A subfamily, and represents a unique enzyme (3A4) in humans. The ortholog (comparable enzyme) in dogs is CYP3A12. Orthologs between dogs and humans often metabolize the same drug substrates, but there can also be marked unexpected differences in substrate specificity between these and other species.

## WHAT IS PHARMACOGENETICS?

Pharmacogenetics is the study of genetic variability in drug absorption, metabolism, and/or response. Much of the initial work done in humans has focused on variability in drug metabolizing enzymes, especially cytochrome P450's. However, recent studies have also characterized variability in Phase II conjugating enzymes, drug transporters, and drug receptors, each of which can influence response to drug therapy. Genetic variability in these proteins is determined by heritable differences in the nucleotide sequences of their respective genes, often at single base pairs (single nucleotide polymorphisms, or SNP's). A polymorphism, by definition, is present in the population in at least two allelic forms, with the least common allele maintained in the population with a frequency of at least 1%. SNP's are therefore distinguished from independent spontaneous mutations by their consistency and higher frequency. SNP's can occur in the coding sequence of the gene (which may lead to a change in amino acid sequence), the promoter region of the gene (leading to a change in the level of expression), or at splice junctions in the gene (leading to altered RNA transcription). Changes in the amino acid sequence can result in decreased enzyme activity due to altered  $K_m$  (affinity for the substrate), altered  $V_{max}$  (catalytic activity of the enzyme), or altered enzyme stability. Thus, a single nucleotide change can have marked effects on enzyme function, metabolic capacity, and the outcome of efficacy or toxicity in a patient.

## CYTOCHROME P450 PHARMACOGENETICS IN HUMANS

There are a number of examples of polymorphisms in human cytochrome P450 enzymes that directly affect clinical outcome in patients. For example, CYP2D6 is a highly variable P450 pathway in humans, with individuals ranging from undetectable activity (found in 6-10% of Caucasians), to "ultrarapid" activity (due to a unique gene duplication, and found in 3-10% of Europeans and up to 30% of black Ethiopians).<sup>1</sup> A large number of clinically important drug are metabolized by CYP2D6, to include beta-blockers (propranolol, timolol, metoprolol), antiarrhythmics (quinidine, flecainide), antidepressants (amitriptyline, clomipramine, fluoxetine, imipramine), neuroleptics, and opioid derivatives such as codeine and dextromethorphan. CYP2D6 status can markedly affect drug dosage requirements; for example, poor metabolizers need to be given 1/10 of the standard dosage of nortriptyline to avoid side effects, while ultrarapid metabolizers require 5 times the normal dosage for clinical effect.<sup>1</sup> Interestingly, poor CYP2D6 activity (which is inefficient at converting codeine to its more potent morphine metabolite) may be a protective factor against opiate addiction in humans.<sup>2</sup> Another P450 that is subject to genetic variation in humans is CYP2C9, which metabolizes warfarin, phenytoin, fluconazole, glipizide, piroxicam, and ibuprofen. Although "slow" metabolism by

CYP2C9 is relatively uncommon (less than 1% of subjects), the clinical consequences of this defect can be severe, such as profound bleeding from warfarin unless the dosage is reduced by a factor of ten.<sup>3,4</sup> Although the CYP3A4 pathway predominates in the clearance of the largest number of drugs compared to other P450's in humans, no patients have yet been identified that lack this CYP.<sup>5</sup> However, level of CYP3A expression (in the liver and the intestine) can vary up to 40-fold among individuals, and clearance of therapeutic drugs can differ markedly (e.g. midazolam, 18-fold<sup>5</sup>). Drug interactions are a more common cause of drug toxicity for CYP3A substrates, such as those between ketoconazole and cisapride, diltiazem and quinidine, fluoxetine and midazolam, and many others.<sup>6</sup>

### CYTOCHROME P450 PHARMACOGENETICS IN DOGS

Cytochrome P450's in dogs are not as completely characterized as they are in humans, but there is recent interest (because of the use of dogs for pre-clinical drug testing) in learning more about the differences between human and canine CYP's. Most of the major CYP subfamilies have been identified in dogs, but substrate specificities (from which we derive the most clinical information) are still lacking. The canine P450's characterized to date are shown in Table 1, along with known substrates in humans and in dogs.

A few CYP pathways have been shown to be polymorphic in dogs, and more work is ongoing. For example, CYP2B11, which metabolizes propofol, varies at least 14-fold in activity in mixed breed dogs.<sup>7</sup> Greyhounds have particularly low activity,<sup>8</sup> which corresponds to reduced clearance of propofol *in vivo*, higher blood propofol concentrations for a given dosage, and delayed propofol recovery compared to mixed breeds.<sup>9,10</sup> The genetic basis for this variability in CYP2B11 has not yet been characterized, and other purebreds have yet to be evaluated. CYP2B11 activity is induced by phenobarbital,<sup>11,12</sup> and inhibited selectively by chloramphenicol.<sup>7,13</sup> This is consistent with the *in vivo* finding that chloramphenicol delays propofol clearance, and dramatically prolongs recovery times, in propofol-anesthetized dogs.<sup>14</sup>

A second CYP pathway that is polymorphic in dogs is CYP2C. Two isoforms have been identified to date in dogs; CYP2C21 is present in all dogs evaluated so far (25/25), while CYP2C41 is present in only 16% (4/25) of dogs tested.<sup>15</sup> Unfortunately, the substrate ranges of these two enzymes are not yet known, although it is known that CYP2C21 is modestly induced by phenobarbital.<sup>16</sup> The variable presence of CYP2C41 may well have implications for clinically used drugs.

CYP2D15 also appears to be polymorphic in dogs, at least with regard to the metabolism of the COX-2 selective NSAID, celecoxib. Celecoxib is primarily a CYP2D15 substrate in dogs, and its clearance is polymorphic in beagles; with extensive metabolizer dogs (EM; about 50% of those tested) having an elimination half-life of about 1.5-2 hours, and poor metabolizer (PM) dogs having a half-life of about 5 hours.<sup>17</sup> One of six allelic variants of CYP2D15 that has been characterized has a deletion of exon 3 (CYP2D15 $\delta$ ) with essentially undetectable celecoxib metabolism, and an almost 80-fold lower intrinsic clearance for bufarolol, compared to wild type.<sup>17</sup> Although the frequency and breed distribution of this allele is not yet known, it is likely to have clinical significance for other CYP2D15 substrates, such as metoprolol, dextromethorphan, and imipramine. As an example of the dangers of cross-species extrapolations among CYP substrates, celecoxib clearance is mediated by the CYP2C family in humans, not by CYP2D.

Relatively little is known about CYP3A polymorphisms in dogs. This pathway is induced by phenobarbital and rifampin,<sup>11,18,19</sup> and is inhibited by ketoconazole.<sup>20</sup> Two alleles of CYP3A12 are recognized so far (CYP3A12\*1 and CYP3A12\*2), which differ by 5 amino acids.<sup>17</sup> In addition, another isoform, CYP3A26, is also present, which has lower activity for steroid substrates compared to CYP3A12.<sup>21,22</sup> The biochemical and clinical implications of these 3A variants are not yet known.



**Table 1:** Human and canine cytochrome P450 substrates, and known P450 polymorphisms in the dog.

Cytochrome P450 in humans	Human substrates	Cytochrome P450 in dogs	Known dog substrates	Known dog polymorphisms
CYP1A1	Dioxin, other environmental chemicals	CYP1A	Induced by environmental toxins (e.g. polychlorinated biphenyls)	None yet reported
CYP1A2	Caffeine, theophylline  Inhibited by ciprofloxacin		Enrofloxacin (?) Theophylline (?)  Induced by omeprazole	None yet reported
CYP2B6	Propofol	CYP2B11  Second gene?	Propofol, progesterone, testosterone Induced by phenobarbital  Inhibited by chloramphenicol	Propofol is poorly metabolized in Greyhounds; 14-fold variability in mixed breeds
CYP2E1	Ethanol, chlorzoxazone  Bioactivation of acetaminophen  Inhibited by cimetidine	CYP2E ortholog	Chlorzoxazone	Two alleles recognized in 100 mixed breed dogs, but same catalytic activity
CYP2C9	Phenytoin, fluconazole, warfarin, flubiprofen, piroxicam, ibuprofen, glipizide, celecoxib  Bioactivation of cyclophosphamide  Inhibited by cimetidine	CYP2C21    CYP2C41	Testosterone  Modest induction by phenobarbital	    CYP2C41 present in < 20% of dogs
CYP2D6	Codeine, propranolol, phenothiazines, quinidine, dextromethorphan, chlorpheniramine	CYP2D15	Celecoxib, dextromethorphan, imipramine, metoprolol  Inhibited by quinidine	50% of beagles are slow metabolizers of celecoxib; another genetic variant with undetectable celecoxib metabolism
CYP3A4	Ketoconazole, itraconazole, cyclosporine, tacrolimus, erythromycin, clarithromycin, cisapride, diazepam, diltiazem, digoxin, quinidine, verapamil, aflatoxin	CYP3A12	Erythromycin, progesterone, testosterone, tacrolimus, cyclosporine, midazolam  Induced by phenobarbital, rifampin  Inhibited by ketoconazole	Two CYP3A12 alleles recognized so far, differing in 5 amino acids  Second isoform, CYP3A26, also present in dogs

## FUTURE DIRECTIONS

The field of pharmacogenetics in veterinary medicine is still in its infancy. However, we now have many of the molecular tools needed to explore the relationships between genetic polymorphisms, biochemical activity, *in vivo* pharmacokinetics, and efficacy / toxicity in patients. We have a unique opportunity in that our purebred patients represent distinct gene pools, and breed specific differences in drug response are clinically recognized. Further work is needed to characterize individual, breed, and species (dog vs. cat) differences in cytochrome P450 and other metabolic pathways.

## Key words

Drug metabolism, drug toxicity, polymorphism, variability

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# IN VITRO TECHNIQUES TO INVESTIGATE SMALL ANIMAL DRUG METABOLISM

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## Introduction

Drug metabolism influences several key drug properties including metabolic stability, drug-drug interactions, and safety. Drugs that are rapidly metabolized have low stability i.e., short half lives, and require frequent dosing to remain within a therapeutic window. In contrast, drugs that are poorly metabolized are highly stable and require less frequent dosing intervals. Drug metabolism is a major cause of drug-drug interactions in that some drugs inhibit the metabolism of a concomitantly administered drug. Drug safety may be affected by metabolism because the resultant metabolites may be safer or less safe than the parent compound. Further, persistent drug levels from long half-life compounds may influence their safety profile.

There are two phases of drug metabolism. Phase I metabolism is associated with chemical modification of the parent drug that includes oxidation, reduction and hydrolysis. Phase II metabolism is associated with chemical conjugation of the parent drug or metabolite with a more polar moiety by the process of glucuronidation, sulfation, or acetylation. The liver is the major organ responsible for drug metabolism and it has been utilized as an investigative *in vitro* tool. Within the liver, metabolic enzymes reside bound to endoplasmic reticulum or free in the cytosol. The liver is not the sole source of drug metabolizing enzymes and several enzymes reside in the blood. Table 1 summarizes the location and action of the Phase I enzymes responsible for metabolism. Table 2 summarizes the Phase II enzymes that are involved in drug conjugation.

**Table 1. Common enzymes involved in phase I metabolism and their location**

<b>Microsomal enzymes</b>	<b>Cytosolic enzymes</b>
Cytochrome P-450 (CYP)	Alcohol dehydrogenase
Flavin monooxygenase (FMO)	Esterases e.g., carboxyesterase
Epoxide hydrolase	Aldehyde dehydrogenase
Esterases e.g., carboxyesterase	Aldehyde oxidase
Prostaglandin H synthase	Carboxylesterases
<b>Blood enzymes</b>	Xanthine oxidase
Peptidases	Epoxide hydrolase
Esterases e.g., acetylcholinesterase	Reductases e.g., quinine reductase
Carbonyl reductases	<b>Mitochondrial enzymes</b>
	Monoamine oxidase
	Aldehyde dehydrogenase

**Table 2. Common enzymes involved in phase II metabolism and their location**

<b>Microsomal enzymes</b>	<b>Cytosolic enzymes</b>
UDP-glucuronosyl transferase (UGT)	Glutathione transferases
Glutathione transferases	N-acetyltransferases
Amino acid conjugation	Sulfotransferases
Methyl transferases	Methyl transferases
<b>Blood enzymes</b>	<b>Mitochondrial enzymes</b>
Methyl transferases	Amino acid conjugation
	N-acetyltransferases

Several *in vitro* test systems, derived from liver and other types of metabolically active tissues are listed in Table 3. In the pharmaceutical industry, these tools are implemented in a high-throughput fashion to screen for new human and veterinary drugs with metabolic profiles that support effectiveness and safety.

These tools are particularly suited for high-throughput studies and to compare metabolism between species because a small amount of hepatic tissue from one animal may supply enough material for hundreds of *in vitro* experiments. In veterinary species, where the biotransformation pathways for drugs are not fully understood and where *in vivo* metabolism studies using radio labeled material may be costly to conduct, it is logical that these techniques have great utility for studying xenobiotic metabolism.

**Table 3. *In vitro* tools to assess drug clearance or metabolite formation**

Plasma
Liver microsomes
Augmented Microsomes (Detergents + UDPGA)
Hepatocytes
Liver Slices
Liver Beads
S9 Fraction
Cytosol Fractions
Intestinal Microsomes
Rumen Fluid

### **MICROSOMES**

Of the tools listed in Table 3, microsomes and hepatocytes are the most commonly used. Liver microsomes are made by homogenization of a freshly isolated liver, followed by centrifugation at ~670g to remove nuclei and cellular debris, centrifugation of the supernatant at ~9,000 g to remove mitochondria, isolation of the resultant supernatant fraction, and further centrifugation at ~100,000 g. This 'microsomal pellet' is made up of smooth endoplasmic reticulum where the drug metabolism enzymes, particularly the CYP family of enzymes are found in high abundance. Microsomes are typically used to evaluate phase I oxidation reactions by supplementing the microsomal incubate with CYP cofactors. In addition, some phase II metabolic reactions may also be studied in microsomes. The endoplasmic reticulum-located enzyme, UGT, is responsible for glucuronidation but studying this in microsomes requires supplementation with uridine diphosphate glucuronic acid (UDPGA) and detergents to reduce enzyme constraint in the microsomal membrane.

### **HEPATOCYTES**

Unlike microsomes, which contain primarily phase I oxidative enzymes, hepatocytes contain the full complement of liver drug metabolizing enzymes, including those responsible for phase II conjugation reactions. Hepatocytes are derived from freshly isolated livers by a two-step collagenase digestion process. First the liver is perfused with isotonic buffer solution containing a calcium chelating agent to remove blood and loosen tight junctions. Then it is perfused with a collagenase solution to dissociate the hepatocytes from the liver parenchyma. Freshly isolated hepatocytes may be used as cell suspensions or as primary cell cultures. Freshly isolated hepatocytes are typically only viable for a few hours. However, efficient cryopreservation techniques have been developed to store hepatocytes at <-150°C, allowing for more convenient use. Dog cryopreserved hepatocytes are commercially available and cat cryopreserved hepatocytes are available by custom order from In Vitro Technologies, Baltimore, MD.

### **OTHER TOOLS**

The supernatant that results from the initial centrifugation in the microsomal preparation process is called the S9 fraction. This fraction is useful as it contains several of the phase II enzymes that microsomes lack. Another *in vitro* metabolism tool are cryopreserved liver beads, which are hepatocytes adhered to alginate gel (colloidal salt) beads. Rat, dog, mouse and monkey liver beads, commercially available from Gentest, are stored in liquid nitrogen and are thawed in a 37°C water bath prior to use. Early data on the rat liver beads indicate that both Phase I and II enzyme systems appear functional. However, it is reported that the

addition of 3% BSA is needed to obtain accurate results for low clearance, highly protein bound drugs. Another tool that has value in identifying metabolites is precision cut liver slices. A limitation of liver slices is that they are not as accurate at estimating kinetic variables as other methods. The clearance rates calculated from liver slices are typically less than hepatocytes. This difference is thought to result from the lack of a proper distribution equilibrium which may be an artifact resulting from the slice thickness.

### UTILITY OF *IN VITRO* TOOLS

Many of the aforementioned *in vitro* techniques may be used in experiments to examine *in vitro* drug clearance over time. In the case of microsomes, drugs are typically incubated for 45 to 60 minutes at 37°C in a shaking water bath. Samples for drug analysis are withdrawn over time and the parent drug is quantified, typically by LC-MS. The *in vitro* half-life is calculated from the elimination rate, which is obtained by plotting the parent compound disappearance over time. These results may then be used to calculate an *in vitro* clearance value that may correlate to *in vivo* clearance. A potential shortcoming to this approach is that results may be affected by non-specific binding of some drugs to microsomal protein. The resulting clearance values in these situations are difficult to interpret because non-specific binding may artificially limit the metabolic rate. For hepatocytes the process of determining *in vitro* half-lives and clearance are similar but the incubations are typically 4 hours. Unlike microsomes, the enzymes and proteins in intact hepatocytes are present under more physiological conditions. Cultured hepatocytes may also be used to detect drug-induced metabolic enzyme induction by quantifying CYP activity in treated cultures versus a control.

Another utilization of *in vitro* techniques is to generate and identify metabolites. In order to do this, relatively high drug concentrations e.g., 20 µM are incubated over time and a single sample is collected for analysis. The identification of all drug-related metabolites formed *in vitro* is typically done by LC-MS-MS and requires an analyst experienced in biotransformation. The detection of metabolites utilizing *in vitro* systems is often easier than *in vivo* experiments because of the complex biological matrices and low metabolite concentrations innate to live animal experiments. Furthermore, fully characterizing formed metabolites in live animals usually requires radio labeled material, which makes these experiments costly and time consuming. Thus, biotransformation studies utilizing *in vitro* systems, particularly preparations that contain the full complement of drug metabolizing enzymes, offer significant experimental advantages over *in vivo* studies.

Microsomes and hepatocytes are also used as screening tools to predict drug-drug interactions that result from CYP inhibition or induction. Induced microsomes may be prepared from animals that have been pre-treated with specific enzyme inducers prior to liver collection. For example, microsomes collected from dogs administered phenobarbital will have greater CYP2B11 activity. These tools are useful for examining specific CYP isoforms and for drug interaction studies. Recombinantly expressed human CYPs are also finding utility for this same purpose. Recombinant canine CYP isoforms are just recently becoming commercially available.

### IN VITRO TO IN VIVO CORRELATES OF METABOLISM

The metabolic profile obtained from most *in vitro* tools generally correlates well with *in vivo* results. In many situations, clearance rates determined from *in vitro* experiments may correlate well to *in vivo* plasma clearance values. However, for some drugs, *in vitro* clearance estimates poorly correlate to results obtained in pharmacokinetic experiments conducted in the whole animal. As a consequence, *in vitro* clearance estimates, as predictors of *in vivo* results are considered difficult and controversial. Discordant results may be due to one or more causes including nonspecific *in vitro* binding to microsomal proteins, significant *in vivo* extra hepatic metabolism (e.g. renal clearance), enterohepatic circulation and/or active transport in the liver.

**COMPARATIVE DRUG METABOLISM**

Recent significant advances in canine drug metabolism have been driven in large part by the use of *in vitro* technologies by the pharmaceutical industry as dogs are used in pre-clinical studies for human drug development. However, very little is known about feline CYPs and Phase II metabolism. To date, no feline hepatic CYP has been identified or fully characterized. Further, our knowledge of the inter-species differences in metabolism is limited. Table 4 outlines several known species differences in Phase II metabolism.

**Table 4. Phase II Species Differences**

<u>Species</u>	<u>Conjugation</u>	<u>Functional group</u>	<u>State</u>
Cat	Glucuronide	OH, -COOH, amines	Very slow
Dog, fox	Acetylation	Aryl-NH <sub>2</sub>	Absent
Pig	Sulfate	Phenols, Aryl-NH <sub>2</sub>	Low extent

Some cases of inter-species metabolism differences have profound toxicological implications, e.g. the classic example of acetaminophen toxicosis in cats is related to deficient glucuronidation. Utilizing *in vitro* techniques may advance the state-of-the-art of drug metabolism in veterinary species as well as increase the efficiency of identifying safe and effective drugs intended for veterinary use.

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**KEY WORDS:** Cytochrome P-450, drug metabolism, clearance, microsomes, hepatocytes, *in vitro*, glucuronidation.

# APPLICATION OF GENETICS AND GENOMICS IN DRUG DEVELOPMENT

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## INTRODUCTION

Genomics has been defined as the study of the function and interactions of all the genes in the genome. Genomics has promised to change how drugs are discovered, developed and prescribed. With the completion of the human genomic sequence and near completion of the mouse sequence, institutions are now concentrating their efforts on sequencing other genomes including the dog. This will create a huge database of information for use in both veterinary and human medicine. In depth sequencing of the dog genome will begin this year.

Several related technologies have been used in genomics. I will try to highlight what goals have been achieved from each technology and how they have impacted on drug discovery and development. Since these technologies have been utilized more widely in human medicine, most examples presented will be from human medicine. I will give examples of how these technologies are being used in veterinary medicine as well.

## TECHNOLOGIES USED IN GENOMICS

### Expressed sequenced tag (EST)

One of the first applications of genomics to drug discovery was the development of EST sequences and the creation of large gene sequenced databases. EST's are random cDNA sequences from one or both ends of genes. The fragments are usually 300-500 basepairs (bp) of nucleotides in length creating partial cDNA sequenced. EST databases have allowed pharmaceutical companies access to potentially new drug targets related to previously known ones such as G protein coupled receptors, steroid hormone receptors and ion channels.

### Gene Mapping and Positional Cloning

Positional cloning refers to the identification of disease-causing genes by using genetic markers to study the inheritance of a disease within a family. The gene is first localized to a specific region of a chromosome by genetic mapping. Once the gene is located, it is sequenced to find the mutation. One of the objectives of both the human and dog genome projects is to advance genetic mapping using microsatellite markers. Microsatellites markers are small repeated sequences of DNA, for example: (GATA)<sub>n</sub>, that are distributed randomly throughout the genome. The accent now is on biallelic marker systems using Single Nucleotide Polymorphisms (SNP's). These are highly abundant, stable sequences within the genome that vary by a single basepair within populations or individuals. They have allowed the cloning of many single genes both in humans and animals. Positional cloning has been one of the major techniques used to isolate several genes in dogs. The dog is unique in that there are breed predispositions to genetically inherited diseases. Thus, large families with inherited diseases can be studied readily. An example of how positional cloning has been used in drug development is exemplified by the discovery of the gene encoding leptin. Leptin was discovered using positional cloning in mice that were genetically obese referred to as ob/ob mice. These mice are severely obese and have a mutated form of the Leptin gene. Leptin has now become a target for drug development in the treatment of obesity.

### Gene Expression Assays

This technology looks at patterns of expression of thousands of genes utilizing microarrays. Affymetrix, a company who make microchips, pioneered the approach of using overlapping oligonucleotides, representing sequences from thousand of genes. These chips can be used for detection of SNPs, to examine pattern of expression under different conditions and to profile the effects of various



drugs or compounds on gene expression to assess efficacy and safety. Pfizer has developed a dog “gene chip” containing multiple tissues. This chip is being used in human and veterinary medicine to assess safety of compounds as well as to identify new targets. We have been using the dog gene chip to identify new target for osteoarthritis in dogs. It is also possible to use gene expression profiling to compare the profile of genes expressed in a deletion mutant compared to wild type and to drug induced mutants. New technologies such as Laser Capture allow scientist to examine gene expression patterns from homogeneous populations of cells taken from histological sections of heterogeneous tissues. This will allow for a more targeted approach to drug development.

### **Pharmacogenetics**

The concept of pharmacogenetics is concerned with individual variation in response to drugs caused by heritable differences. The application of pharmacogenetics in drug development will be to develop drugs targeted to specific populations or even individuals. Most of the work has centered on polymorphisms in drug-metabolizing enzymes including CYP1A, CYP2D6, CYP3A4, CYP2C9 and CYP2C19. Less is currently known about genetic factors involved in drug absorption, transport, kinetics and adverse effects. One area of active research is in understanding the genetic factors involved in drug-induced QT syndrome.

Pharmacogenetics has been used to understand the genetic factors involved in diseases and how they correlate to response to treatment. Susceptibility genes or genetic risk factors are being identified. Identifying genetic variation in response to particular drugs can help the pharmaceutical industry design more efficient, less costly clinical trials. Only those patients with genotypes predicted to respond to a given therapy would be enrolled, therefore reducing the number of patients required to see an effect.

Finally pharmacogenetics has application in examining genetic variation at the drug targets. Genetic variation at these sites can lead to better understanding of drug targets and clinical trial outcomes.

### **SUMMARY**

With the sequencing of the human genome complete and the sequencing of other species including the dog in progress, veterinary medicine will be entering an era in which the individual animals genome will help determine the optimal approach to care both preventive, diagnostic or therapeutic. New technologies will allow for a more targeted approach to treatment and better understandings of disease as well as the effects therapies have on these diseases.

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# IMPLICATIONS OF DRUG-PROTEIN BINDING AND THE OCCURRENCE OF DRUG-DRUG INTERACTIONS

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## INTRODUCTION

Most drugs bind to plasma proteins. However, only unbound drug has access to the tissues where drug effect occurs. This unbound concentration is:

$$C_u = f_u C,$$

where  $C_u$  is the unbound concentration,  $f_u$  is the fraction bound to plasma proteins and  $C$  is the total concentration in the blood.

It would seem obvious that if  $f_u$  doubles, then  $C_u$  must double and, therefore, concentration of drug at the site of action would double and so too would the effect! However, for most drugs this seemingly obvious conclusion is incorrect and exposure of the body to the drug is not changed. Thus, other drug- or disease-induced changes in protein binding will have no therapeutic consequences and clinicians will not need to adjust their patients dosing regimens. (1- 6)

## DISCUSSION

### Pharmacokinetic parameters

There is clear evidence that plasma protein binding is relevant in the pharmacokinetic modeling of drugs.

The volume of distribution depends on the fraction unbound in plasma, the fraction unbound in tissue ( $f_{uT}$ ), the volume of tissue ( $V_T$ ) and the volume of plasma ( $V_p$ ).

$$V = \frac{f_u}{f_{uT}} V_T + V_p$$

Thus, for all drugs with  $V \geq 30L$  (when  $V_p$  has only a minor effect on  $V$ ), changes in  $f_u$  translate directly into changes in  $V$ .

Similarly, clearance,

$$CL = \frac{f_u CL_{int} \cdot Q_{organ}}{f_u CL_{int} + Q_{organ}}$$

depends on  $f_u$ , the intrinsic ability of the clearing organ,  $CL_{int}$ , and blood flow to the organ,  $Q_{organ}$ . High extraction ratio drugs ( $Q_{organ} \ll f_u CL_{int}$ ) exhibit organ clearance independent of  $f_u$  (i.e.  $CL \approx Q_{organ}$ ), but for low extraction ratio drugs ( $Q_{organ} \gg f_u \cdot CL_{int}$ ),  $CL \approx f_u CL_{int}$ , clearance depends on  $f_u$  and  $CL_{int}$ .

The oral availability of a drug will exhibit a hepatic first pass effect,  $F_H$ , in animals whose mesenteric blood supply empties into the portal vein.

$$F_H = \frac{Q_H}{Q_H + f_u \cdot CL_{int}}.$$

Low extraction ratio drugs have  $F_H \approx 1$ . But, for a high extraction ratio

$$\text{drug } F_H \approx \frac{Q_H}{f_u \cdot CL_{int}}.$$

Moreover, because the half life,  $t_{1/2}$ , of a drug depends on  $V$  and  $CL$ , it may also depend on,

$$f_u, t_{1/2} = \frac{0.693V}{CL}$$

For high extraction ratio drugs,

$$\text{when } V \geq 30 \text{ L, } t_{1/2} \approx \frac{0.693 \frac{f_u}{f_{uT}} V_T}{Q_{\text{organ}}}$$

But half life is independent of  $f_u$  for low extraction ratio drugs,

$$\text{for which } V \geq 30 \text{ L } t_{1/2} \approx \frac{0.693 \frac{V_T}{f_{uT}}}{CL_{\text{int}}} \text{ and is independent of } f_u.$$

Thus, it is correct that depending on the pharmacokinetic parameters measured, and the extraction ratio of the drug, certain pharmacokinetic parameters will change with protein binding but others will not. However, the belief that the effective concentration of all drugs depends on protein binding is not correct.

### Exposure

Exposure is a term that reflects the drug levels to which a patient is exposed following a dose or a series of doses. It is a measure of concentration integrated over time commonly referred to as area under the curve, AUC.

$$AUC = \frac{F \cdot D}{CL}$$

where F is bioavailability. When given intravenously,  $F = 1$  by definition and when given orally  $F = F_{\text{abs}} \cdot F_G \cdot F_H$ , with  $F_{\text{abs}}$  equal to the fraction of drug that reaches the gut wall intact,  $F_G$  equals the fraction of drug that crosses the gut wall intact and  $F_H$  equals the hepatic first pass availability. For low extraction ratio drugs given orally and cleared hepatically or nonhepatically

$$AUC_{\text{oral}} = \frac{F_{\text{abs}} \cdot F_G \cdot \text{Dose}}{f_u \cdot CL_{\text{int}}}$$

However, the area under the unbound C vs t curve is independent of  $f_u$  for low ER drugs given orally

$$AUC_{\text{oral}}^u = f_u \cdot AUC_{\text{oral}} = \frac{F_{\text{abs}} \cdot F_G \cdot \text{Dose}}{CL_{\text{int}}}$$

When low extraction ratio drugs are given intravenously (or any nonoral route when corrected for incomplete absorption))

$$AUC_{\text{iv}}^u = f_u \cdot AUC_{\text{iv}} \approx \frac{\text{Dose}}{CL_{\text{int}}}$$

Exposure to high extraction ratio drugs given intravenously does depend on  $f_u$

$$AUC_{\text{iv}}^u = f_u \cdot AUC_{\text{iv}} \approx \frac{f_u \cdot \text{Dose}}{Q_{\text{organ}}}$$

as does exposure to high extraction ratio drugs given orally but cleared nonhepatically

$$AUC_{\text{oral,nonhepatic}}^u = \frac{F_{\text{abs}} \cdot F_G \cdot f_u \cdot \text{Dose}_{\text{oral}}}{Q_{\text{organ}}}$$

but not to those given orally and cleared hepatically

$$AUC_{\text{oral,hepatic}}^u = \frac{F_{\text{abs}} \cdot F_G \cdot D}{CL_{\text{int}}}$$

The question then becomes how many drugs given intravenously are high extraction ratio drugs or are given orally and cleared nonhepatically. If we set the cutoff for high extraction ratio as  $ER > 0.3$ , then the answer in humans is 26/456 or about 6%(Table 1)(7, 8)

**Table 1. High Extraction Ratio drugs for which changes in protein binding may affect exposure.**

Nonoral administration		Given orally and cleared hepatically
Alfentanil	Idarubicin	none
Amitriptyline	Itraconazole	
Buprenorphine	Lidocaine	
Butorphanol	Methylprednisolone	
Chlorpromazine	Midazolam	
Cocaine	Milrinone	
Diphenhydramine	Nicardipine	
Diltiazem	Pentamidine	
Doxorubicin	Propofol	
Erythromycin	Propranolol	
Fentanyl	Remifentanyl	
Gold sodium thiomalate	Sufentanyl	
Haloperidol	Verapamil	

There is no reason to assume that this percentage will be significantly different in animals. The percentage would be even smaller if we were to consider each drug's therapeutic index, because if a drug has a wide therapeutic index, changes in free drug concentrations resulting from protein binding changes will have negligible clinical effects.

### When is protein binding important?

In drug development, in the scale-up of pharmacokinetic and pharmacodynamic parameters from model animals, it is essential to consider interspecies differences in binding in predicting volumes and clearances. When the first dose of a new molecular entity is calculated from in vitro measures of target concentrations, fraction unbound must be factored in to the estimated size of the dose. In the clinic, for narrow therapeutic index drugs when therapeutic drug monitoring of plasma or blood concentrations is routinely used to adjust dosing it is essential to factor in any changes in protein binding. This is due to the fact that many routine therapeutic drug-monitoring techniques measure total drug concentrations rather than unbound concentrations.

### SUMMARY

While changes (induced by other drugs or by disease) in plasma protein binding can have an impact on individual pharmacokinetic parameters it is rare for such changes to translate into clinically relevant changes in drug exposure.

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**Keywords**

Exposure, clearance, volume, extraction ratio

# COX-1, COX-2, COX-3: RELEVANCE TO PAIN MANAGEMENT

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## INTRODUCTION

The mechanisms of how the different cyclooxygenase isoenzymes are involved in the generation of a painful sensation are not completely understood and new information is appearing almost every month. Thus this presentation is designed to ask as many questions as it hopes to answer. Pain is a complex experience involving not only the transduction of noxious stimuli from the periphery to the central nervous system (CNS) but also the processing of the stimuli by the higher centers in the CNS. Pain can be classified in several ways. Two of the more commonly used classifications are clinical pain defined as acute or chronic and biological pain defined as nociceptive (somatic and visceral) or neuropathic.

To discuss the participation of COX isoenzymes in the perception of pain, we need to briefly review the peripheral and central mechanisms of pain. The perception of pain involves sensitization of both nociceptors and secondary central sensitization. Peripheral sensitization is defined as enhanced sensitivity of nociceptive nerve endings. Central sensitization is defined as enhanced sensitivity of nociceptive spinal dorsal horn neurons to sensory stimulation.<sup>1,2</sup> Nociceptive pain is evoked by activation of peripheral nociceptors. These sensory receptors are classified according to their responses to mechanical, thermal, and chemical stimuli. During inflammation, a high proportion of somatic and visceral peripheral nociceptors can be sensitized by various mediators including bradykinin, prostaglandin (PG), various leukotrienes, serotonin, histamine and perhaps free radicals. Central sensitization is triggered by impulses in nociceptive C-fibers. The neural mechanisms that underlie central sensitization are still being explored. Central sensitization is also evoked by several mediators in the dorsal horn of the spinal cord including PG, nitric oxide (NO), glutamate and other excitatory amino acids and substance P.<sup>2</sup>

## Peripheral inflammation and pain

Peripheral inflammation is characterized by hyperalgesia. This hyperalgesia is caused by release of a variety of inflammatory mediators. In the periphery, only COX-1 is constitutively expressed, while COX-2 is up-regulated during inflammation. PGs play a significant role in nociception. PGs themselves are not important mediators of pain, but they increase the sensitivity of peripheral nociceptor terminals to other stimuli and mediators to produce localized pain hypersensitivity. Peripheral inflammation also generates secondary hyperalgesia (hypersensitivity in local uninjured tissues) and increases neuronal excitability in the spinal cord. The expression of COX-2 is mediated primarily by IL-1 $\beta$  and TNF $\alpha$ . PGs also contribute to peripheral sensitization through protein kinase A (PKA) mediated phosphorylation of sodium channels in nociceptor terminals increasing excitability and decreasing the pain threshold.<sup>2,4</sup> Thus in the continuum of pain perception, the initiation of the spinal component of the inflammatory cascade is the persistent activity of small primary afferents.

## Central inflammation and pain

In contrast to the periphery, both COX-1 and COX-2 mRNA and protein are constitutively expressed in the dorsal root ganglia (DRG). In DRG COX-1 is found in small and medium sized neuronal cell bodies. In the neurons of the spinal cord however, no COX-1 has been reported. COX-1 has been found in astrocytes along with COX-2.<sup>5,6</sup> COX-1 appears to play a very small role in spinal PG-mediated cascade and likewise selective COX-1 inhibitors have minimal abilities to block evoked spinal PGE<sub>2</sub> release. However this statement is based on limited studies and should not be used to reject a role of COX-1 in hyperalgesia.<sup>7</sup> In contrast,

COX-2 is present in neurons of all lamina, particularly laminae I and II.<sup>2,7</sup> The discovery of COX-2 in the spinal cord suggests it is responsible for spinal PG release in nociceptive processes following peripheral stimuli. As in peripheral inflamed tissue, the expression of central COX-2 is mediated primarily by IL-1 $\beta$ .<sup>3</sup> In contrast to peripheral inflammation, COX-2 alone appears to be pivotal in PGE<sub>2</sub> production as the increased expression of COX-2 in the neuronal and nonneuronal cells is paralleled by increases in basal and evoked PG release. No induction or activation of phospholipase A<sub>2</sub> has been noted in the CNS with peripheral inflammation.<sup>3,8</sup>

### **COX-1 and pain**

Most of the data puts all the emphasis on COX-2 for the induction and initiation of pain perception. However the COX-1 enzyme may have a role in pain and fever production. Certainly the COX-1 variant COX-3 and PCOX-1 proteins promote this concept.<sup>9</sup> While it is true that COX-1 may not be a significant player in the CNS, it is important in inhibition of peripheral pain production in a variety of models. Furthermore, the nonselective NSAIDs may affect COX-3 (COX-1 variant) in the brain and thus have more wide ranging effects.

### **COX-2 and pain**

COX-2 is a significant factor as outlined above. Certainly understanding the data suggesting that PG production in the DRG is likely to contribute to the establishment and maintenance of peripheral inflammatory hypersensitivity by facilitating transmitter release and direct activation of receptors on dorsal horn neurons is paramount to our changing treatment methods.<sup>3</sup> Furthermore, the rapid, substantial changes in COX-2 throughout the CNS and the subsequent PG production may also significantly contribute to the generalized signs of fever and lethargy, etc. we see in our patients.

### **COX-3 and pain**

The COX-1/-2 model has provided some much needed information in our understanding of the inflammatory process. However it does have some areas in which it is lacking. One of the most glaring is the inability to account for the characteristics of acetaminophen. Recent evidence of a variant of COX-1 that is especially sensitive to acetaminophen and related compounds may solve part of this problem.<sup>9</sup> Also if this variant is tissue specific in expression (primarily the brain) could this explain more about the actions of acetaminophen? There are puzzling differences in this area of research. This is especially true when discussing pain. As previously described, PGs are produced by COX-2 induced local inflammatory processes. Yet then how does acetaminophen produce analgesic effects if it is not a peripheral anti-inflammatory agent? Does acetaminophen act on the CNS enzyme that up-regulates COX-2 during pain perception? Thus one would expect that due to the success of COX-2 inhibitors in pain that a variant of COX-2 may be a key player. Or does this COX-1 variant target other enzymes not yet elucidated?

These data produced by several authors strongly suggests that multiple COX isoenzymes can be derived from just two distinct genes. This concept has been termed the "COX continuum of enzymes."<sup>10</sup> This continuum theory opens several questions including the idea that potentially different NSAIDs may have varying effects on the multiple isoforms and that may explain the range in benefits to different patients. Also does that alteration/inhibition of one isoform allow the expression or up-regulation of expression of a different isoform?

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# New Therapeutic Horizons: Transdermal Drug Delivery

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Chronic administration of medications, particularly to cats, can be problematic for several reasons. Since relatively few drugs have received FDA approval for use in cats, drug formulations appropriate for use in cats (i.e., small tablet or capsule size and strength, flavor of liquid formulations) are limited. Cats generally resist oral drug administration, and even the most docile of cats may resort to the use of claws and teeth to avoid being “pilled” on a daily basis. Liquid medications are also difficult to administer because the presence of an unpleasant taste in a cat’s mouth may induce profuse salivation. If drugs are added to a cat’s food, one risks the development of food aversions. Consequently, veterinarians and cat owners continue to seek alternative drug formulations. In response, veterinary compounding pharmacies have recently been advertising topical gels as an alternative route of administration for many drugs that are commercially available as oral formulations. While this appears to be an ideal solution to many of the problems associated with chronic administration of drugs to cats, there is limited information regarding the safety and efficacy of these topical drug formulations in feline (and canine) patients. In order to make knowledgeable decisions about prescribing transdermal gels, it is crucial that veterinarians understand the factors involved in transdermal drug delivery. This discussion will briefly summarize factors affecting transdermal absorption of drugs, currently available experimental data, and potential adverse effects.

## Factors affecting transdermal absorption

In human medicine, a number of FDA-approved transdermal drug products are available, primarily as transdermal patch formulations (e.g., scopolamine, nitroglycerin, clonidine, fentanyl, nicotine, and others). In veterinary medicine, FDA-approved ‘pour-on’ and ‘spot-on’ formulations of antiparasitic agents have been used successfully. Research and development of these FDA-approved topical drug products intended for systemic delivery has greatly enhanced our understanding of the factors affecting transdermal drug delivery (Table 1).

**Table 1. Some factors affecting transdermal drug absorption**

<b>Patient Factors</b>	<b>Comments</b>
Species	Skin thickness, lipid content, density of hair follicles; presence and/or density of sweat glands; skin pH
Anatomic area	Blood flow; thickness of stratum corneum; density of hair follicles and sweat glands
Integrity of Stratum Corneum	Denuded, hydrated, or inflamed stratum corneum enhances absorption
<b>Drug Factors</b>	<b>Comments</b>
Partition coefficient	Optimal log octanol/water partition coefficient has been reported to be two
Molecular weight	Large drugs (MW > 400 Da) have limited diffusion across stratum corneum
Degree of ionization	Only unionized fraction diffuses across stratum corneum
Vehicle effects	Very complex

## RESULTS OF EXPERIMENTAL INVESTIGATIONS

Based on the number and apparent financial success of veterinary compounding pharmacies, it appears that topical gels are prescribed frequently for small animal veterinary patients. While this seems to be an ideal solution to many of

the problems associated with chronic administration of drugs to cats, there are several questions that must be addressed to ensure that these topical drug formulations are safe and effective for feline (and canine) patients. Questions that veterinarians should consider when using these drugs are:

1. Are topically administered drugs bioavailable (does the drug actually get absorbed)?
2. Is the bioavailability of topically administered drugs consistent between patients or is there a wide range of bioavailability (i.e., can the same topical dose be expected to produce equivalent plasma concentrations of drug in most cats, or do drug doses need to be individualized by therapeutic drug monitoring)?
3. Since topically administered drugs bypass the liver, would a drug that undergoes a significant degree of 1<sup>st</sup> pass hepatic metabolism after oral administration be likely to induce toxicity after topical administration (particularly if a dosage reduction is not made)?

Currently, there is very limited data regarding the safety and efficacy of any of these drugs for veterinary patients. Several veterinary researchers (Dr. Trepanier at the University of Wisconsin, Dr. Boothe at Texas A&M University, the author, and others) are investigating the bioavailability of drugs administered by the transdermal route. Preliminary results of some of these studies (methimazole, buspirone, amitriptyline, amikacin, morphine, enrofloxacin, and others) suggest that the bioavailability of transdermally administered drugs in cats is substantially lower than the oral bioavailability of the same drug. For example, a single oral dose of amitriptyline to cats yielded plasma concentrations that reached the therapeutic range, while amitriptyline concentrations after transdermal administration were approximately 10-fold lower and well below the therapeutic range. Studies involving buspirone, a drug known to undergo substantial (> 90%) first-pass hepatic metabolism in people, yielded similar results.

One potential problem with most of these studies is the fact that they were single-dose studies. Diffusion of drug through skin is generally delayed, owing to accumulation of drug in the stratum corneum and dermis. A depot of drug is then formed, which slowly delivers drug to the systemic circulation. In the single-dose studies, it is possible that the dose was not sufficient to saturate the stratum corneum and dermis (i.e., a depot was not formed). Multiple-dose studies may yield more favorable results. For example, in single-dose studies of methimazole, bioavailability after topical administration was poor and variable, with only 2 of 6 cats achieving detectable serum methimazole concentrations. In a long-term efficacy study, however, transdermal methimazole in PLO gel was effective in rendering cats euthyroid, but it generally took longer to do so than with oral methimazole (personal communication, Dr. Lauren Trepanier, University of Wisconsin).

### **Potential Adverse Effects**

Adverse effects from topical gels can be local (cutaneous) or systemic. Local inflammation from topical gels, particularly PLO gels, is fairly common. Inflammation is generally mild and subsides when the drug is discontinued. Systemic reactions may also occur. One component of the vehicle for most topical formulations is soy lecithin. Soy lecithin is a common allergen in people and has been reported to cause asthma and food allergies. A suspected adverse (allergic?) reaction to a topical formulation of methimazole was observed in a cat. Within 20 minutes of application, the cat experienced protracted vomiting that continued for approximately 8 hours. The owner reported that the cat had vomited approximately 50 times and required hospitalization. The veterinarian and compounding pharmacist suspected the cat was allergic to methimazole and referred the cat to Washington State University Veterinary Teaching Hospital for radioactive iodine treatment of its hyperthyroidism. Since adverse reactions of this type have not been previously reported in cats treated with oral methimazole, we suspected that the

reaction may have been caused by the vehicle (lecithin) in the topical formulation. In order to test this hypothesis, the owner agreed to admit the cat to the intensive care unit for observation while the cat was treated with an oral methimazole tablet. No adverse reactions were observed and the cat was discharged. The owner elected to continue treatment with oral methimazole, and the cat has not experienced adverse effects after several months of treatment.

## CONCLUSION

While the transdermal route of administration may ultimately prove to provide safe, effective, and consistent delivery of some drugs, its use should be limited until scientifically based evidence becomes available. There are some transdermal drug formulations that have received rigorous safety and efficacy studies (i.e., the pesticides TopSpot®; Frontline®; Advantage®; Revolution®), but the pharmacological characteristics of these drugs and the nature of the disease processes they counteract are somewhat unique compared with many of the drugs advertised for transdermal use in veterinary medicine. The transdermal pesticides generally have a wide therapeutic window and are effective if plasma drug concentrations reach the nanogram per milliliter range intermittently. For most drugs, therapeutic efficacy depends upon maintaining plasma drug concentrations within a narrow therapeutic window for a sustained period of time. There is a documented limit to the amount of drug that can be absorbed transdermally in people using current technologies. Under *optimal* conditions, a maximum of 1 mg of a favorable drug can be delivered across the skin (1 square cm) per 24-hour period. It is unreasonable, therefore, to assume that adequate systemic concentrations of all drugs can be achieved using current transdermal gel delivery techniques. A rational approach to using these compounded drugs would be to reserve their use for treating conditions with a measurable endpoint. For example, it is quite simple to measure thyroid hormone levels to determine efficacy if using methimazole topically. Drugs to avoid, until supporting evidence is available, include those drugs used to treat serious conditions that require immediate efficacy (i.e., hypertension, heart failure, cardiac arrhythmias, bronchospasm, seizures) and antimicrobials (low levels may promote resistance).

Veterinarians should also consider public safety when prescribing topical formulations. When owners apply topical formulations to their pets, they may also be absorbing drug through their own skin. To avoid this possibility, non-permeable gloves should be dispensed to owners. Children in contact with pets may also be at risk for drug absorption. Appropriate precautions should be followed if a topical drug is dispensed to households with small children.

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# **NEW THERAPEUTIC HORIZONS: NOVEL DRUG DELIVERY METHODS. A NOVEL INJECTION TECHNIQUE: PREFILLED BIODEGRADABLE INJECTION NEEDLES**

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## **INTRODUCTION**

The term "drug delivery" refers to two different systems: the physical delivery of a pharmaceutical product to the patient and the release of the bioactive ingredient from the pharmaceutical product within the patient. Examples of novel drug delivery methods that deliver pharmaceutical products to the patient are the intranasal application of powders with positive pressure (blowing: OptiNose®), the intrapulmonary delivery of powders with negative pressure (inhalers such as Taifun®) and the needlefree injection of powders (Powderject®). Much Research and Development is being conducted with regards to the release of bioactive ingredients from the pharmaceutical product; examples include the incorporation of bioactive substances into microspheres (e.g. ProHeart®, Fort Dodge, US) and of antigens or DNA vaccines in liposomes (e.g. Lipoxen Ltd, UK), whereby both microspheres and liposomes have to be injected.

In this presentation, special attention will be paid to existing and novel injection techniques.

Our own Research Group is working on a novel drug delivery method by injection of solid dose pharmaceutical preparations. The core technology consists of prefilled biodegradable injection needles (Injectiles®), which are physical delivery vehicle and pharmaceutical product in one.

## **INJECTION TECHNIQUES.**

### **Injection with needles**

In 1665 Dr. Christopher Wren (UK) gave the first registered injection ever, using a sow's bladder with a goose's quill, for the intravenous injection of different liquids (including red wine and milk) in dogs. In 1853 Charles Gabriel Pravaz (a French surgeon) and Alexander Wood (a Scottish physician) independently invented the hypodermic glass syringe with a hollow, pointed, metal needle. In 1956 Colin Murdoch (New Zealand) invented the plastic, disposable syringe.

Today, it is standard veterinary and farming practice to use one single needle on multidose syringes for the injection of several animals in large herds. According to the World Health Organization, "unsafe injections" can be defined as "the reuse of a syringe or needle between patients without sterilization". Using the WHO definition, virtually no injection given in livestock is "safe". Today, an estimated 12 billion injections are given worldwide in human medicine and 6 billion in veterinary medicine (livestock only) on a yearly basis.

Although many injection needles and syringes are disposable items, they can be reused, thereby transmitting diseases such as Bovine Leukosis, Bovine Virus Diarrhea, Classical Swine Fever, Aujeszky's disease and possibly prion diseases. To prevent such reuse, autodisable syringes have been designed, disabling the retraction of the plunger after injection. For use in humans in Third World countries, PATH (Program for Appropriate Technology in Health, Seattle, WA, US) has developed the UniJect, which is a prefilled single-use pouch with a needle fixed to it. UniJect has been in use since 1991 for acceptability studies, and has been on the marketplace since 1997.

### **Needle free injection of liquids**

In 1936 the needle free injection technique for the injection of liquids (until 1 cc) was invented by ML Lockhart for high work load mass injection campaigns. It

has been proven (CDC, US), however, that in 1 to 10% of injections given with needle free injection devices transmission of blood born pathogens can take place by the nozzle's contamination with body fluid in quantities as low as picoliters. Several companies are developing variations on this concept, for use in humans, in order to prevent these iatrogenic (or "technogenic") transmissions. Examples of such companies are Felton International (1 protection cap per injection) and DCI Lecraject (on-site single-use ampule filling).

### **Remote injections**

A number of remote syringes with a needle on top are in use, mainly in wild animals (PneumoDart, Chapchur, Paxarms, Telinject and more). Different propelling systems have been developed.

Vladil Afanasievich Komarov and James Drake filed each for a patent (1970 respectively 1974), claiming a ballistic animal implant ("shell"), e.g. for the immobilization of animals. The company Ballistivet introduced Biobullets for the remote injection of drugs; these are propelled by means of CO<sub>2</sub> and the projectiles are spin stabilized.

### **Needle free powder injection**

In 1992 Brian Bellhouse, of the Oxford University, UK, invented the injection of powder particles by accelerating them with compressed helium to above the speed of sound. Each shot delivers thousands of particles over a surface of several square cm; so the skin is being pierced in thousands of spots. In order to give each particle sufficient kinetic energy to pierce the skin, they have a core of tungsten or gold, coated with the bioactive substance (e.g. a protein). After subcutaneous application, the bioactive substance is released, while the tungsten and gold particles stay in place during the rest of the lifetime. The concept has been marketed since 1993 by Powderject for use in humans.

### **Microneedles**

In 1998, silicon based micro needles were developed by Dr. Mark Prausnitz. Microneedle patches (1 cm x 1 cm) are produced by the same technologies as computer chips; one microneedle patch has 400 microneedles (length 200 - 500 micrometers, diameter 20 to 50 micrometers) and so engenders 400 skinpiercings. One microneedle patch allows the injection of 1 cc in 15 minutes. The microneedles allow parenteral injection, without the needles reaching the innervated subcutis, so injections are said to be painless.

### **A NOVEL INJECTION TECHNIQUE: PREFILLED BIODEGRADABLE INJECTION NEEDLES.**

It is current veterinary and farming practice to use a single needle for the injection of larger numbers of animals. The concept of biodegradable prefilled injection needles (Injectile®) is the result of development efforts to provide a safe and cost-effective alternative to this practice.

### **The concept**

The prefilled biodegradable injection needle (Injectile®) concept consists of a biodegradable carbohydrate needle, which can be prefilled with bioactive ingredients and which has to be injected subcutaneously or intramuscularly. After injection the Injectile® immediately absorbs body fluid and rapidly dissolves / degrades, releasing the bioactive ingredient. So the Injectile® is a delivery vehicle and pharmaceutical product in one. This concept avoids the use of syringes with metal needles, as well as of vials with liquids. It also avoids blood-to-blood contact between consecutive injections.

### **Injection of prefilled biodegradable injection needles (Injectiles®) into animals**

There are three main methods of injecting Injectiles®:

1. by finger / hand pressure (e.g. at the ear base of piglets, on the ear of cattle or on the shoulder of pets), most comparable to conventional injection needles. Simple finger pressure can be performed from a sterile blister packaging. A single use hand applicator, filled from a sterile blister packaging, allows deeper injection of the Injectile®. We already have applied a large number of implants and electronic identification devices subcutaneously on ears.
2. by shooting through a barrel (e.g. in the neck of pigs or cattle) [1]; extensions around the nozzle of the barrel prevent the barrel from touching the skin. Kinetic application of the Injectile® (or “mini projectile”) allows extreme fast injection: less than 1 millisecond. The physiological speed of action potentials along nerves is 2 to 5 cm per millisecond. This implies, that the injection has already taken place long before it is “realized” by the animal. Kinetic applicators are being developed for both single shot and multi shot applications for high workload, mass vaccinations. A fully automated, compressed gas powered applicator is envisaged to allow 1,500 injections per hour in e.g. pigs. The air, that propells the Injectile®, also cleans the skin by firmly blowing hair aside and dust and dirt away. While withdrawing the applicator, a liquid disinfectant is sprayed upon the site of injection and a colorant added to mark the animal.
3. by remote injection, e.g. in livestock and wildlife (under development).

### **Release of bioactive ingredients from the prefilled biodegradable injection needle (Injectile®) after injection**

The release of the bioactive substance depends on the carrier material of the Injectile®, the pharmaceutical formulation and the bioactive substance.

Carrier material: Injectiles® can be made of different carrier biomaterials, such as Poly Lactic Acid (PLA), Poly Glycolic Acid (PGA), Poly Hydroxy Butyrate (PHB) and similar substances. By choosing specific compositions of the biomaterial, specific degradation rates can be achieved, thus influencing bioavailability of the bioactive ingredient.

Pharmaceutical formulations: Injectiles® can be prefilled with a range of different solid dosage pharmaceutical preparations such as powders, pastes, emulsions, pellets, compressed tablets, spray-dried pellets and freeze dried pellets (meaning that reconstitution is no longer required). By choosing specific pharmaceutical formulations it is possible to determine bioavailability of the bioactive ingredients. By combining two or more different pharmaceutical formulations with the same active ingredient (e.g. a vaccine) in one Injectile®, both primer and several booster vaccinations could be given in one shot, thus extra saving labour cost and animal stress.

Bioactive substances: the Injectile® concept allows the injection of broad ranges of low volume active substances, such as peptides, oligopeptides, proteins, hormones, vaccines / biologicals, including live viruses and live bacterial. Also electronic identification devices can be injected by means of Injectiles®.

### **IN VIVO TRIAL WITH PREFILLED BIODEGRADABLE INJECTION NEEDLES**

Biodegradable injection needles ( Injectiles®) were made of rapidly degradable carbohydrates, measuring 17 mm in length and 3.00 mm in diameter; they were prefilled with the model antigen BSA (Bovine Serum Albumine), formulated in three different ways (freeze dried, spray dried and emulsion); no adjuvans was added; subsequently, they were kinetically injected as mini projectiles into the neck of pigs (n = 6); each animal got two Injectiles®, with an interval of 22 days. During and after injection of the Injectiles®, the animals did not show any sign

of discomfort. Blood samples were taken for antibody determination (indirect ELISA) [1].

**Table 1. Seroconversion to BSA (n = 6) following administration of three different formulations contained within Injectiles® (sd = standard deviation)**

Formulation tested	Titre 1 (1 day prior to admin)	Titre 2 (22 days post admin)	Titre 3 (14 days post 2 <sup>nd</sup> admin)
Freeze dried	53 (sd 24)	3525 (sd 8924)	3866 (sd 8832)
Spray dried	88 (sd 104)	1062 (sd 1352)	1538 (sd 2240)
emulsion	43 (sd 25)	162 (sd 106)	1650 (sd 1057)

These results show that all three formulations release the model antigen, inducing seroconversion. They do not show the release profiles of the model antigen from the three different formulations. It cannot be conclusively determined that the emulsion released the antigen slower than the freeze dried and spray dried formulations. Blood samples, taken after more than 36 days, may have shown higher titres. However, it can be concluded that Injectiles® allow formulations with modified release patterns. This trial proves that it is possible to make biodegradable injection needles, to prefill them with an antigen and to inject them into animals for inducing seroconversion.

#### SUMMARY

Prefilled biodegradable injection needles (Injectiles®) are a novel injection technique which can potentially replace conventional needles with syringe in many applications, including vaccinations. Injectiles® significantly improve logistics, because no liquids and no reconstitution are needed. Injectiles® are easy to use and allow high workload, high speed injections in livestock, saving labour and improving animal welfare. This injection technique avoids blood-to-blood contact between consecutive injections, thus minimizing or excluding the possibility of transmitting contagious blood-borne diseases. Injectiles® can contribute to a safer injection practice.

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# NEW THERAPEUTIC HORIZONS: PEPTIDE DRUG DELIVERY

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## INTRODUCTION

Delivering therapeutic peptides is increasingly becoming a carefully orchestrated strategy in which the aim is to achieve optimal patient responses through 'personalized or designer molecular medicine'. The exponential increase in genome database information and the development of sophisticated molecular tools, to better understand cell activity, have enhanced opportunities to discover new peptide targets at the same time adding to the complexity of drug delivery. Differentiating existing and future therapeutic peptides by drug delivery mechanisms is likely to be a key factor in the contribution this growing sector makes to the pharmaceutical industry as a whole.

## PEPTIDE TARGETS

Peptide molecules have been useful targets throughout their history with numerous examples of effective transition from basic science to pharmaceutical benefit. However, the molecular revolution has spawned new interest in identifying potential peptide targets from genome databases. Bio-informatics (software developed to assist with identification of potential therapeutic peptides from nucleotide and amino acid sequence databases), has added predictive dimensions to the development of peptide derivatives.

### Modification of peptide targets

Therapeutic protein molecules are currently restricted composition forms in which peptide chemistry synthesis or bioreactors with genetically modified cells, produce peptide mixtures from precursor molecules. Purification is a significant step in the preparation of peptides manufactured *ex-vivo*. Peptides produced in this manner frequently require modification in order to survive the manufacturing, purification and delivery processes. A number of authors have comprehensively reviewed this area over the years with the realization that post translational modification of peptides by addition of signature molecules, like carbohydrate moieties, may significantly alter bioavailability and efficacy.

## POLYMERIC DELIVERY OF PEPTIDES

Administration of therapeutic peptide and protein drugs has historically relied upon on their parenteral injection in order to achieve effective bioactivity. Nonparenteral routes, including oral and nasal administration, often require significant derivatization of the therapeutic peptide or formulation. In order to be a successful peptide delivery technology the challenges of ever increasing stringency with regard to efficacy and safety are the first requirement to meet.

There are numerous strategies to deliver therapeutic peptides, all of which have a few common elements: Therapeutic peptides manufactured by recombinant or chemical means are generally formulated into delivery vehicles and presented to the patient in such a manner as to achieve maximum bio-availability. The commercial delivery systems that are available include vaccination, emulsions, biodegradable polymers, gels, nanocrystals, membranes, aerosols and viral particles.

### Transport enhancement of peptides across absorptive barriers

The development of delivery systems to carry therapeutic peptides across absorptive barriers is currently the most desirable formulation but a significant challenge for the pharmaceutical industry. The dynamic nature of mucosal microenvironments contributes to reduce efficacy even within the same host species. Delivery vehicle formulations developed for absorptive barrier transport of peptides and proteins are often peptide and host specific.



## TARGETING PEPTIDES

Peptides with unique activity may require modifications that enable them to survive the journey to the target cell. This process may require protective arming of the peptide molecules with cell-seeking devices, including antibodies or receptor recognition molecules. Many of the exquisite recognition properties of cell surface receptors and internalization molecules are a result of peptide and carbohydrate moieties associated in three-dimensional structures.

## PEPTIDE PHARMACOKINETICS/PHARMACODYNAMICS

With Real-Time molecular analysis of gene expression of complex cell behaviors, such as differentiation, proliferation, and migration that occur during disease states, comes the comprehensive monitoring of peptide pharmacokinetics and pharmacodynamics. These new molecular tools have also enabled the pharmaceutical industry regulators to expect comprehensive patient response profiles of gene activity. As a consequence we are likely to see the demand for increased orchestration of peptide delivery and greater monitoring of the molecular responses of patients. This in itself will increase the demands on the pharmaceutical industry but also increase the opportunities for new strategies to affect disease outcomes.

## HORIZON PEPTIDE DELIVERY SYSTEMS

Emerging areas of interest can be seen in technologies which combine, biomolecular structure and function, nanomaterials with intelligent structures, and molecular and tissue engineering.

### Biomolecular structure and function

The complex behavior of cells is a consequence of cell signaling events that regulate the activity of simple molecules and enzymes. Cell signaling is not only an active target for therapeutic peptides but also a potential mechanism to orchestrate and monitor peptide bioactivity. Cell signaling molecules are being used to activate or regulate pro-drugs by: activating cascade mechanisms, responding to changes in the hormonal and/or cytokine milieu and reporting changes to cell activity with key indicators (eg, Green Fluorescence Protein or melanophores)

### Nanomaterials and intelligent structures

Bio-compatible materials are also under active investigation with an ever increasing library of information with regard to host specific compatibility and longevity. These emerging nano-materials have the capability to actively respond and report molecular changes that may require intelligent response activation. To date this is occurring when molecular targets reach threshold levels, however, they have the capability to adopt complex interfaces that monitor a number of parameters at the target interface.

### Molecular/tissue engineering

The current focus on stem cells to identify alternative strategies to manipulate gene expression and supercede simple recombinant molecular technologies is likely to contribute to the development of cells that act as peptide factories. Stem cells donated by the host may be directed to produce therapeutic peptides following the engineering of gene elements that enable the production of a mature, bioactive peptide using the cells own synthesis and secretion machinery. More complex scenarios are emerging in which multiplexing genes are introduced so that combinatorial or sequential peptide delivery can occur.

## PATIENT MANUFACTURE OF PEPTIDES

The manufacture and delivery of peptides *ex-vivo* is a challenging task. The manufacture of complex peptides for cell proliferation, differentiation and migration is a normal event in any host. Exploiting the endogenous cell systems that generate complex interactive peptides is the focus of gene therapy. Gene therapy offers the patient the opportunity to manufacture the target peptide or protein, through gene

activation events within host cells, and present the molecule in its active form. This technology, although in its relative infancy is expected to contribute to new developments in orchestrated peptide delivery.

#### **INTERACTIVE PEPTIDE DELIVERY SYSTEMS**

Combinatorial peptide delivery systems are expected to have the capabilities to sense, report and respond to disease status. This is likely to be manifested in biomolecular or electronic. All of which contribute to an increasingly orchestrated drug delivery system that interacts with and reacts to the micro-environment.

Many peptide agents are potent and multifunctional therefore control of tissue concentration and spatial localization of delivery is essential for safety and effectiveness.

#### **DESIGNER MEDICINE**

With better understanding of the molecular mechanisms that regulate cellular activity comes the potential to design effective therapeutic strategies for individual patients.

#### **REFERENCES**

1. References can be obtained upon request from the author.

#### **Keywords**

Gene therapy, Cell signalling, Nanomaterials, Molecular medicine, Molecular Engineering, Somatic Cell Therapy.

# NEW THERAPEUTIC HORIZONS: ERYTHROPOIETIN

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## BACKGROUND & BIOLOGY

Life is sustained by a steady flow of oxygen from air to mitochondria. This is made possible by a chain of oxygen carriers adjusted to secure the cells of an adequate supply to meet their metabolic demands. In anemia patients, the total amount of oxygen carried by the arterial blood is usually adequate for such demands. However, unless it is present in the capillaries at sufficiently high pressures, it cannot diffuse far into the tissues. Consequently, the aim of most adaptive mechanisms is to sustain a capillary pressure to prevent cellular hypoxia. Certainly, some degree of hypoxia must be retained to initiate needed adjustments; stimulating the production of erythrocytes, altering oxygen affinity of hemoglobin, and changing cardiopulmonary indices.

## Hypoxic Signals

Studies of molecular responses have determined that the major mediator denoting cellular hypoxia is a complex protein, named hypoxia inducible factor (HIF-1). It is composed of two subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ . The  $\beta$ -subunit is constitutively and stably expressed in tissues in normal and hypoxic conditions. HIF-1 $\alpha$ , on the other hand, is synthesized under normoxic conditions but is rapidly degraded. However, under hypoxic conditions this subunit is stabilized, and along with HIF-1 $\alpha$ , forms the functional HIF-1 protein. HIF-1 belongs to a class of transcription factors with an affinity for specific DNA sequences named hypoxic response elements (HRE). These sequences are enhancers found in a number of genes involved in the defense against hypoxia. Among these are genes for vascular endothelial growth factor, glucose transport, glycolytic enzymes and erythropoietin (EPO)<sup>1,2,3</sup>.

## Erythropoietin Production and Biological Effect

The most appropriate adaptive measure in the defense against anemic hypoxia is a compensatory increase in the rate for erythrocyte production. Transcription of the EPO gene is enhanced by HRE transcribed by HIF-1. Although the gene is present in all cells, its promoter is only active in certain interstitial cells in the kidney, and to a much lesser extent in hepatocytes and Kupffer cells of the liver. The hypoxic enhancement of its transcription will cause an increased release of the short-lived EPO, with titers in circulating blood logarithmically proportional to the degree of hypoxia. EPO regulates the proliferation and differentiation of erythroid progenitor cells to mature erythrocytes. The major target cells of EPO have been identified as colony-forming progenitor cells committed to the erythroid lineage (CFU-E, colony-forming unit-erythroid) and to a minor extent as more immature erythroid progenitor cells, the BFU-E (burst-forming unit-erythroid). Although *in vitro* data indicate that EPO induces the proliferation of megakaryocyte and macrophage/granulocyte progenitor cells, no clinically significant changes in circulating leucocyte or platelet numbers are seen with standard dosing<sup>4,5,6</sup>.

## Erythropoietin Molecular Biology

Human EPO is an acidic glycoprotein with a molecular mass of 34 kD. Lee-Huang (1984) cloned human EPO cDNA in *E. coli*. EPO is highly conserved between species. The human EPO gene has 5 exons that code for a 193-amino acid polypeptide. A 27-amino acid leader sequence is cleaved from the amino terminus of the propeptide, yielding the functional 166-amino acid protein. Recombinant human EPO (epoetin) was approved for marketing in France in 1988 for the treatment of anemia in patients undergoing dialysis for chronic renal failure

(CRF). Endogenous EPO and epoetin have different patterns of glycosylation, which involve primarily the sialic acid composition of oligosaccharide groups. Epoetin- $\alpha$  (Johnson and Johnson) and epoetin- $\beta$  (Roche) are produced by recombinant methods in Chinese-hamster-ovary (CHO) cells. They have slight differences in glycosylation; epoetin- $\alpha$  has more sialic acid residues than epoetin- $\beta$ . Furthermore, epoetin expressed in CHO cells contains only 165 amino acids, having lost arg166<sup>7,8,9</sup>. The EPO gene has been mapped to chromosome 7 in humans (7q21) and chromosome 5 in mice<sup>10</sup>.

### **Epoetin as a Therapeutic Agent in Human and Veterinary Medicine**

Clinical studies have documented the effectiveness of epoetin in the correction of anemia in humans and cats with hypoproliferative anemia secondary to CRF. Anemia is primarily responsible for the weakness, fatigue, increased somnolence, mental depression and poor appetite observed in cats with CRF. In these patients, concentrations of serum EPO are reduced. In contrast, chronic anemia in cancer patients, although not characterized by EPO deficiency, often responds to exogenously administered epoetin. One reason for this may be that many cancer patients have inappropriately low EPO concentrations for the degree of their anemia. Finally, epoetin administration can be beneficial in the treatment of anemia of malignancy due to neoplastic bone marrow infiltration.

Epoetin therapy has become important in human patients undergoing chemotherapy and radiotherapy because it has been demonstrated that higher concentrations of hemoglobin correlate with higher treatment efficacy. Better oxygen delivery to target tissues, allowing for enhanced production of cytotoxic oxygen radicals is the proposed mechanism for the improved effects.

The initial enthusiasm for epoetin administration in cats has been tempered as anti-epoetin antibodies were detected in 80% of cats treated for > 180 days. Seventy percent of these cats developed anemia refractory to epoetin treatment. A subset of these cats became transfusion dependent until anti-epoetin antibody concentrations decreased 2-4 months after discontinuation of therapy. Although side effects during epoetin therapy are uncommon in cancer patients, those diagnosed with CRF, may suffer with elevated blood pressure and high blood viscosity. This is explained by a predisposition of patients with renal disease to hypertension, poor regulation of fluid balance, hence, increased blood viscosity due to increased hematocrit percentage<sup>11</sup>.

### **ADVANCES IN ERYTHROPOIETIN THERAPY**

Novel erythropoiesis-stimulating protein (NESP) stimulates erythropoiesis in the same manner a epoetin. NESP is distinct from EPO in that it has additional sialic acid residues which confer an increased terminal half life in animal models, human patients with CRF and cancer.

Studies have also been performed examining whether the secretion of EPO from genetically modified cells could represent an alternative to repeated injections of epoetin. In these rodent studies, cells (skin fibroblasts or vascular smooth muscle cells) were harvested and transduced by retrovirus vectors containing EPO cDNA before being re-introduced to the host. Although, long term expression of EPO was measured this approach is expensive and labor intensive.

Currently, administration of EPO cDNA by viral vectors holds the most promise for a prolonged, controlled EPO delivery system that does not require repeated injections. Adeno-associated viral constructs appear to have an advantage over adenovirus and retrovirus vectors which have caused morbidity and mortality in recent human clinical trials.

### **Adeno-associated Viral Vectors**

Wild-type human adeno-associated virus (AAV) is a non-pathogenic parvovirus that only productively replicates in cells co-infected by a helper virus, usually adenovirus or herpes virus. The virus has a wide host range, and can productively infect many cell types from a variety of animal species. Sero-epidemiologic studies have shown that most people (50-96%) in the U.S.A. have

been exposed, probably as a passenger during a productive adenovirus infection. Nevertheless, AAV has not been implicated in any human or animal disease.

Recombinant AAV vectors (rAAV) are typically produced by replacing the viral coding sequences with transgenes of interest. These vectors have been shown to be highly efficient for gene transfer and expression at a number of different sites *in vitro* and *in vivo*. Skeletal muscle is often chosen as the target tissue because it is accessible, efficiently transduced by rAAV vectors, well vascularized, and is able to express and process secreted proteins.

Recombinant AAV vectors containing feline EPO cDNA under the control of constitutive promoters (chicken beta actin and cytomegalovirus) have been described in cats. These vectors have limited therapeutic application because expression of feline EPO cannot be regulated. Furthermore, there is marked individual variation in transgene expression in animals treated with rAAV vectors. Hence, poor expression of feline EPO results in no therapeutic effect and strong expression results in iatrogenic erythrocytosis and attendant hyperviscosity. We have developed a rAAV construct which includes a tetracycline regulatory element that only allows the expression of feline EPO when a tetracycline compound is administered to the animal. Expression of feline EPO is negligible in the absence of a tetracycline compound. Furthermore, control of feline EPO expression is regulated by a tetracycline-controlled transcriptional silencer which serves to diminish “leaky expression” of feline EPO when tetracycline compounds are not present.

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# NEW THERAPEUTIC HORIZONS: CHOOSING A NEW DRUG FOR INDUCING ANAESTHESIA: PROPOFOL OR ALFAXALONE

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## INTRODUCTION

The injectable anaesthetic agents propofol and alfaxalone have significant advantages over the ultra short acting barbiturates such as thiopental due to their rapid metabolism which permits a rapid and "clear headed" recovery from anaesthesia even when anaesthesia has been maintained by intravenous increments or infusion. Both propofol and alfaxalone, however, are poorly soluble in water. In most commercial preparations, propofol is solubilized in soybean oil, glycerol and egg phosphatide with some generic preparations also containing bisulfate as a preservative. Alfaxalone, a steroid, has been combined with alphadolone (alfaxalone 9 mg/ml, alphadolone 3 mg/ml) and 20% w/v Cremaphor EL to increase its water solubility. Alphadolone is also a steroidal anaesthetic, but much less potent than alfaxalone. Cremaphor EL is a polyoxyethylated castor oil that has been associated with histamine release on first exposure, resulting in hypotension, swollen paws and ears and occasional pulmonary oedema in cats and severe hypotension in dogs. Histamine release may be avoided by the use of 2-hydroxypropyl-beta cyclodextrin as the solubilizing agent for alfaxalone (1) and such a formulation has been recently registered for clinical use in dogs and cats in Australia (2). The therapeutic index of the Cremaphor formulation of alfaxalone is three to four times higher than that of propofol or thiopental (3,4).

## CARDIOVASCULAR EFFECTS

Induction of anaesthesia with propofol causes more hypotension than is seen with thiopentone (5). Propofol causes vasodilation by a direct effect on the blood vessels. This is thought to be mediated by the release of nitric oxide from the endothelial cells with veins being affected to greater extent than arterioles (6). In humans an induction dose of propofol has been reported to reduce peripheral vascular resistance by approximately 28%. (7). The hypotension produced by propofol is not always accompanied by a compensatory increase in heart rate or cardiac output (8) due to a re-setting of the baroreflex threshold (9).

It has been shown in cats that the hypotension produced by alfaxalone is caused mostly by myocardial depression although there is some contribution from peripheral vasodilation. (10). In a study using rabbits, in which propofol was compared with the cremaphor formulation of alfaxalone, both drugs produced a similar fall in blood pressure but propofol produced a significant fall in total peripheral resistance and an increase in cardiac output. (11).

Afaxalone (in the cremaphor preparation) has been shown to have a small protective effect against arrhythmias induced by epinephrine (12,10) whereas propofol enhances epinephrine-induced arrhythmias in a dose-dependent manner in dogs (13) although in cats, the arrhythmia threshold to epinephrine was greater than with halothane anaesthesia. (14). Propofol has been reported to suppress or terminate supraventricular and ventricular tachycardia in humans (15,16)

## POTENTIAL PROBLEMS

There are, however, a number of potential problems that may be associated with the use of these drugs. Intralipid (the soybean emulsion used as a vehicle for propofol) and cyclodextrin both have the potential to support bacterial growth. There have been a number of clinical reports concerning bacterial contamination of propofol (17), Intralipid has been shown to impair cell mediated immune function

and there have been reports of an increase in the incidence of wound infections following anaesthesia with propofol (18). In a study of 863 dogs and cats undergoing aseptic surgery there was a 13% incidence of wound infections in patients who had been given propofol compared with a 4% incidence in patients not given propofol (19).

Anaesthesia with propofol has been associated with pancreatitis in humans. Whilst most of these reports have been in patients receiving infusions of propofol (20), pancreatitis has been reportedly associated with a single administration of propofol (21). There is, however, some controversy about whether propofol (or its vehicle) can be the cause of the pancreatitis and there have been no reports of an association between propofol and pancreatitis in the veterinary literature to date.

Propofol may cause pain on intravenous injection, particularly if it is injected into small veins. The reported incidence of this is as high as 50 % in humans but considerably lower in dogs and cats. Pain on injection can be minimised by the addition of a small amount of lidocaine to the propofol (0.05 –0.1%) or by injecting a small dose (0.5 mg/ml) of lidocaine through the cannula immediately before the propofol. The use of cold (refrigerated) propofol will also help in this regard. There have been no reports of pain associated with the injection of alfaxalone, either in the cremaphor or cyclodextrin formulations. None of the formulations of propofol or alfaxalone have caused tissue injury after perivascular injection.

## **EXPERIMENTAL STUDIES**

### **Introduction**

A pre-registration safety studies were carried out in the alfaxalone solubilized in 2-hydroxypropyl-beta cyclodextrin in cats and dogs. Both studies used a Latin square design with doses of 5mg/kg, 10mg/kg, 20mg/kg and 30 mg/kg used for cats and 5mg/kg, 9 mg/kg, 16 mg/kg and 30 mg/kg used for dogs. Each dose was given by intravenous injection over a period of one minute. All of these doses were well in excess of the 0.6-2.0 mg/kg needed to induce anaesthesia in clinical patients. The cats were allowed to breathe air throughout the study but the dogs were breathing 100% oxygen, commencing five minutes before the control measurements were made.

### **Induction of Anaesthesia**

Induction of anaesthesia was smooth in both dogs and cats, although fine tremors of the fore limbs were seen in about 20% of the subjects.

### **Respiratory Effects**

Apnoea on induction was seen in most animals, the duration of the apnoea increasing with increasing dose rates of alfaxalone. Dose related respiratory depression occurred, moderate at the lower doses and severe in most animals at the high doses. There was marked individual variation in susceptibility to respiratory depression.

### **Cardiovascular Effects**

Cardiovascular effects of the drug were well tolerated in both species. Dose related hypotension occurred, with a small reduction in mean arterial pressure at the two lower dose rates. In the dogs, mean arterial pressure decreased to a little below 60 mmHg only at the 30 mg/kg dose. Arterial pressures in the cats tended to be higher than in the dogs however, this was probably due to sympathetic nervous system stimulation induced by hypoxemia in the cats. Heart rates increased with induction of anaesthesia and in the dogs (where thermodilution cardiac output catheters were used) it was found that this was accompanied by a significant reduction in systemic vascular resistance and a corresponding increase in cardiac output.

### **Toe Pinch Response**

Response to a toe pinch was abolished with induction of anesthesia. During the recovery period the toe pinch response became exaggerated in both species at all dose rates

### **Histamine Release**

We were unable to demonstrate an increase in blood histamine or urinary 1-methyl histamine in either species.

### **Recovery**

Time to first "head lift" increased with increasing dose rate. A six-fold increase in dose rate resulted in a three-fold increase in the time to first head lift in dogs and a four-fold increase in cats. From the time of first head lift, all animals progressed rapidly to sternal recumbency and standing regardless of the dose rate of alfaxalone used. All of the dogs recovered smoothly, however, between the time of first head lift and the time of first standing, about one quarter of the cats went through a period where they displayed exaggerated responses to being handled.

### **CLINICAL EXPERIENCE WITH ALFAXALONE IN CYCLODEXTRIN**

Respiratory depression is the major side effect of alfaxalone anaesthesia and, as with all other general anesthetic agents, supplemental oxygen should be given to the anaesthetised patient in order to prevent hypoxemia. The quality of induction and recovery has been similar to that seen with propofol. Recovery from anaesthesia, especially in cats, is much smoother when premedication (eg acepromazine & opioid) is used. The incidence of clinically significant apnoea is minimal if the drug is given slowly to effect, however, as with all injectable anesthetics, pre-oxygenation is recommended. Episodes of face rubbing frequently seen after propofol anaesthesia in cats are rare after alfaxalone. Vomiting has not been seen in the recovery period when anaesthesia has been maintained with incremental doses of alfaxalone. After premedication with acepromazine & methadone the dose rate of alfaxalone needed to induce anaesthesia is a little less than 1 mg/kg in dogs and slightly more than this in cats. Excellent results have been obtained in Caesarean sections when alfaxalone has been used to induce anaesthesia prior to maintenance with isoflurane.

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#### **KEY WORDS**

Alfaxalone, cyclodextrin, propofol, Saffan, Althesin

# NEW THERAPEUTIC HORIZONS: FLUOXETINE PHARMACOLOGY AND SAFETY IN DOGS AND CATS AND ITS ROLE IN BEHAVIOR MODIFICATION

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## INTRODUCTION

Fluoxetine hydrochloride is a selective serotonin reuptake inhibitor (SSRI) and approved for use in the treatment of depression. It is also approved for the treatment of obsessive compulsive disorder (OCD), bulimia nervosa, and for the treatment of premenstrual dysphoric disorder (PMDD)<sup>1</sup>.

Although fluoxetine is not approved for use in animals, veterinarians have been recommending its use in an off-label manner to treat a variety of behavioral problems in companion animals<sup>2</sup>. The use of psychotropic medications has become increasingly more common in veterinary medicine and combined with behavior and environmental modification, the use of various types of anxiolytics and antidepressants has made the treatment of various disorders increasingly more successful<sup>3</sup>. Dogs with problem behaviors such as compulsive disorder<sup>4</sup>, separation anxiety<sup>5</sup>, aggression<sup>6</sup> and various phobic conditions<sup>7</sup> have been helped through the conscientious use of fluoxetine. In cats, fluoxetine has been recommended for the treatment of hyperesthesia, intermale aggression, wool sucking/fabric chewing, feline psychogenic alopecia, and territorial aggression<sup>3</sup>. In a recent study, fluoxetine treatment significantly reduced the rate of urine marking in cats when administered at a daily dose 1 mg/kg of body weight<sup>8</sup>.

## THE 5-HT HYPOTHESIS AND GENERAL PHARMACOLOGY

In the early 1970s the oxalate salt of fluoxetine was tested along with a series of molecules called aryloxyphenylpropylamines and fluoxetine was found to be the most potent and selective inhibitor of 5-HT uptake<sup>9,10,11</sup>. For example, when fluoxetine is compared to the TCA, amitriptyline, it is comparable in potency for serotonin uptake inhibition ( $K_i < 100$  nM), however, it does not have affinity for other putative neuronal receptors including adrenergic, histaminic, muscarinic, opiate or dopaminergic<sup>12</sup>. Fluoxetine's lack of affinity for these receptors is what prevents the sedation and anticholinergic properties seen with the TCAs<sup>13</sup>. Fluoxetine is a racemic mixture containing 50:50 R and S enantiomers. R and S fluoxetine display comparable potencies for 5-HT uptake processes ( $K_i \sim 20$  nM)<sup>14,15</sup> whereas S-norfluoxetine is 14X more potent than R-norfluoxetine with  $K_i$  values of 20 and 268 nM, respectively<sup>16</sup>.

## SAFETY, PHARMACOKINETICS AND ADME IN THE DOG

### Safety

Beagle dogs have tolerated single oral doses of 100 mg/kg of fluoxetine. Adverse events observed at this dose were emesis, mydriasis, tremors, and anorexia<sup>17</sup>.

Beagle dogs, 5 per sex per treatment group, have received oral doses of 1.0, 4.5, or 20.0 mg/kg/day of fluoxetine for 1 year with a 2-month reversibility phase (2 per sex per group)<sup>18</sup>. The study design was altered after 6 months due to intolerance of dogs at the highest dose of 20 mg/kg/day. For the last 6 months of the study the high dose was reduced to 10 mg/kg/day. The primary physical signs of toxicity attributable to fluoxetine treatment at 20/10 mg/kg/day were tremors, anorexia, slow and/or incomplete pupillary response, mydriasis, aggressive behavior, nystagmus, emesis, hypoactivity, and ataxia. Six of the ten dogs in the high dose group were removed from treatment for periods of 1-17 days during the first six months of treatment due to severe occurrences of either aggressive behavior (perhaps associated with hypersensitivity to touch) or ataxia and anorexia. All of the physical

signs of toxicity were reversible within the two-month recovery period. Most dogs at 4.5 mg/kg/day exhibited tremors, slow and/or incomplete pupillary response, and occasional anorexia. Effects in dogs at 1.0 mg/kg were limited to tremors, mydriasis, and slow and/or incomplete pupillary response. The occasional physical signs of toxicity observed at 1.0 and 4.5 mg/kg/day were minimal, reversible and consistent with the pharmacologic activity of fluoxetine.

Weekly body weight determinations indicated that dogs in the control, 1.0 and 4.5 mg/kg groups, despite occasional anorexia in the latter group, generally gained weight throughout the treatment period. Phospholipidosis was identified as a major toxicologic effect of fluoxetine after chronic administration to dogs and was observed in the lung, liver, adrenals, lymph nodes, spleen, and peripheral leukocytes in animals receiving the high dose. Phospholipidosis was only observed in the lung and leukocytes in a few of the dogs in the 1.0 mg/kg dose group. Systemic phospholipidosis is common to a number of other clinically useful drugs, including TCAs, which share similar physical-chemical characteristics (i.e., cationic, amphiphilic). No cardiovascular effects were seen in dogs apart from a slight decrease in basal heart rate. All effects observed in this study were reversible following a 2-month reversibility phase.

### Pharmacokinetics (PK)

In a single 1 mg/kg dose, cross-over, IV versus oral PK study performed at Lilly the absolute oral bioavailability of fluoxetine in male and female beagle dogs averaged 72%<sup>19</sup>. The remaining 28% was either metabolized (first pass-effect) or was not absorbed. The  $T_{max}$  after oral dosing averaged 1.8 hr for fluoxetine while norfluoxetine averaged 9.3 hr. The  $C_{max}$  in plasma for fluoxetine averaged 48.8 ng/mL while norfluoxetine averaged 70.1 ng/mL. The volume of distribution ( $V_D$ ) for fluoxetine averaged 38.9 L/kg while norfluoxetine averaged 10.9 L/kg. Clearance of fluoxetine from plasma (Cl) averaged approximately 6 mL/kg/min while norfluoxetine clearance averaged approximately 2 mL/kg/min. Two more recent and separate single dose PK studies of fluoxetine in dogs showed the half-lives of fluoxetine to average 6 (six)<sup>20</sup> and 10 (ten)<sup>21</sup> hr while the elimination half-lives for norfluoxetine were 48 and 57 hr, respectively.

Fluoxetine and norfluoxetine enantiomers have also been evaluated pharmacokinetically<sup>22</sup>. Beagle dogs dosed at 12 mg/kg/day showed a difference in the exposure of R-fluoxetine compared to S-fluoxetine; however, no differences in the exposure to norfluoxetine enantiomers were observed. The AUC values for S-fluoxetine were approximately 3-fold those observed for R-fluoxetine. There were no sex differences in the pharmacokinetics of fluoxetine or norfluoxetine in this study.

### ADME (Absorption, Distribution, Metabolism, Excretion)

In male and female dogs treated at doses of 5 to 20 mg/kg of fluoxetine for 95 days the highest concentration of fluoxetine was in the liver followed in decreasing order of concentration by lung, kidney, various areas of the brain and plasma<sup>23</sup>. The heart had the lowest levels. Concentrations of fluoxetine in these tissues were 50 to 100 times greater than the plasma concentrations, and norfluoxetine concentrations were 2 to 3 times greater than those of fluoxetine in the plasma.

In male and female beagle dogs treated with fluoxetine at doses of 1 to 10 mg/kg for 1 year, dose-dependent increases in fluoxetine and norfluoxetine concentrations were observed in liver, adrenal gland, and lung (in descending order)<sup>17</sup>. Norfluoxetine concentrations greatly exceeded fluoxetine tissue concentrations. Similarly to the plasma compartment, norfluoxetine (but not fluoxetine) was detectable 2 months after terminating fluoxetine administration; levels were approximately 1% of those observed at treatment termination.

In people, N-demethylation of fluoxetine takes place in the liver producing norfluoxetine. In addition, the CYP1D6 isoenzyme that is at least partially responsible for demethylation is inhibited by both fluoxetine and norfluoxetine<sup>24</sup>. In canine liver slice work conducted at Lilly, norfluoxetine was the major metabolite

present, accounting for >56% of the total peak area<sup>25</sup>. Fluoxetine, and an alcohol and acid metabolites of fluoxetine accounted for 6, 14, and 7%, respectively of the total peak area.

<sup>14</sup>C-fluoxetine has been dosed to dogs in a drug excretion study<sup>26</sup>. The mean percent of total radioactivity excreted in the urine and feces collected over 4 days was 17.8 and 22.7%, respectively. A mean of 29.8 and 44% of the dose was excreted in urine and feces by 14 days following dosing. The urinary HPLC profile demonstrated 5 major peaks including both fluoxetine and norfluoxetine.

## **SAFETY, PHARMACOKINETICS AND ADME IN THE CAT**

### **Safety**

Cats have tolerated single oral doses of 50 mg/kg of fluoxetine<sup>27</sup>. Adverse events observed at this dose were emesis, mydriasis, tremors, and anorexia.

Cats, 4 per sex per treatment group, have received oral doses of 1.0, 3.0, or 5.0 mg/kg/day of fluoxetine for 89 days<sup>28</sup>. Oral administration of fluoxetine-HCl to domestic shorthair cats by capsule once daily was well tolerated at doses of 1 and 3 mg/kg. At 3 mg/kg/day sporadic observations of low food consumption and vomiting were observed, and decreased pupillary light reflexes were observed in males. Daily oral administration of fluoxetine-HCl at 5 mg/kg/day produced clinical signs of toxicity in some animals beginning at approximately day 56, including whole body tremors, hypoactivity, convulsions, low food consumption, and dehydration. These findings appeared to resolve after discontinuation of dose administration and a recovery period. Electrocardiographic examination found non dose-dependent decreased heart rates in treated males.

Histopathologic findings included increased incidence/severity of alveolar macrophage infiltration in animals given 3 or 5 mg/kg/day, slightly increased histiocytes in the mesenteric lymph nodes at 5 mg/kg/day, centrilobular hepatocellular degeneration/swelling at an increased incidence and severity in animals given 5 mg/kg/day, and an increased incidence of thymic lymphocyte depletion in females given 5 mg/kg/day. The increased incidence/severity of alveolar macrophage infiltration and histiocytes in the mesenteric lymph nodes are consistent with phospholipidosis.

### **Pharmacokinetics**

In a pilot IV versus oral study in cats the bioavailability (F) of orally dosed fluoxetine at 1 mg/kg was approximately 100%<sup>29</sup>. In the same study, the elimination half-life ( $t_{1/2}$ ) of a 0.5 mg/kg IV dose of fluoxetine was 34 hours, the volume of distribution ( $V_D$ ) was 18.7 L/kg and the clearance (Cl) was 6.4 ml/kg/min. For norfluoxetine the  $t_{1/2}$  was 50.8 hours, the  $V_D$  was 32.6 L/kg and the Cl was 7.4 mL/kg/min. For oral fluoxetine the  $T_{max}$  averaged 2.3 hours and the  $C_{max}$  82.6 ng/mL while for norfluoxetine the  $T_{max}$  averaged 40.0 hours and the  $C_{max}$  25.9 ng/mL. In toxicokinetic studies<sup>28</sup>, no apparent gender differences in the disposition of fluoxetine or norfluoxetine were found in the cat. In addition, there was accumulation of both fluoxetine and its metabolite, norfluoxetine, due to the long half-lives of both molecules. Steady state was reached before or at day 14 after dosing. The  $C_{max}$  and AUC values for fluoxetine appeared to increase proportionally with the fluoxetine dose, and increased in less than a dose-proportional manner for norfluoxetine.

### **SUMMARY**

In conclusion, the clinical success of fluoxetine in people appears transferable to companion animals. Like humans, fluoxetine is well absorbed in dogs and cats, forms the active metabolite, norfluoxetine, and the elimination half-lives of the parent and metabolite in companion animals are long. Fluoxetine is metabolized through oxidation and conjugation and is excreted both in the feces and urine in the dog. In both dogs and cats the no-observed-adverse-effect level for toxicity is approximately 1 mg/kg. The more common clinical signs of toxicity include inappetance, mydriasis, slow and/or incomplete pupillary response, body tremors

and change in behavior. Histologically, phospholipidosis is the major reversible toxicity observed at doses greater than 1 mg/kg.

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# AAVPT POSITION PAPER: POST-EXPOSURE MANAGEMENT AND TREATMENT OF ANTHRAX IN DOGS

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As anthrax is not communicable, pets are likely to be incidental victims of a bioterrorism attack with this organism. Also, carnivores seem quite resistant to infection and therefore large scale preventative treatment of pets would seldom be warranted. Dogs have nevertheless been known to contract the disease, usually through ingestion of meat from animals having died of anthrax. It is noteworthy that the respiratory tract does not appear to be a primary route of infection in the dog. Though this route of exposure was suggested in one case of naturally occurring canine anthrax, this was in part because no source of carrion could be found to otherwise explain the infection.<sup>i</sup> In a disease model where 14 dogs were exposed to clouds of anthrax spores, only three animals became febrile and none developed the actual disease.<sup>ii</sup> Cutaneous anthrax has not been reported in animals, though entry of the organism through a skin lesion cannot be discounted. The American Academy of Veterinary Pharmacology and Therapeutics (AAVPT) and the American College of Veterinary Clinical Pharmacology (ACVCP) nevertheless realize that concerns exist about proper management of guide dogs, police dogs, and search & rescue dogs that may have become exposed to anthrax. Accordingly, the following information on the disease, its prevention, and treatment in dogs is offered. (For information on anthrax in cats, the reader is referred to <http://web.vet.cornell.edu/public/fhc/anthrax.html>.)

Anthrax appears to enter the body of the dog through the oropharynx and upper GI tract. Accordingly, regional lymph nodes of these areas are most commonly affected and massive swelling of the head, neck, and mediastinal regions are the most frequent signs.<sup>iii, iv</sup> Death often is due to toxemia and shock, though asphyxia can play a role. Hemorrhagic gastroenteritis has also been reported in a dog that also had ptialism and a swollen foreleg.<sup>v</sup>

Antemortem diagnosis of anthrax is based on probable exposure, clinical signs, and demonstration of the organism in blood, lymph node or tissue aspirates, or pharyngeal swabs. It is important to note that anthrax spores survive nearly all cytological staining techniques, including the brief heat fixing used in Gram staining. Definitive diagnosis is based on culture of the organism. Animals that die of suspected anthrax will usually be septicemic such that a blood sample will reveal the organism cytologically and by culture. Necropsy of an anthrax suspect is not advised as exposure to air rapidly causes sporulation of the vegetative bacteria. If an animal has however already been opened for post-mortem evaluation, a sample of spleen, lymph nodes, intestine, lung, liver, bronchial lymph nodes, tonsil and pharynx should be collected. Anthrax is a reportable disease in all species. Contaminated areas should be treated with a sporicidal disinfectant such as a 1:10 dilution of household bleach (final solution containing 0.5% sodium hypochlorite; allow 60 minutes of contact).<sup>vi</sup>

As with any bacterial infection, treatment of anthrax is based on the susceptibility of the organism to available antibiotics. While alteration of the bacteria to resist common antibiotics is a concern for "weaponized" anthrax, the anthrax associated with the fall of 2001 terrorist attack appears to have an antimicrobial susceptibility pattern similar to endemic anthrax found within the U.S.A. As such, if a pet is considered exposed to *Bacillus anthracis* (the causative organism of the disease anthrax), the AAVPT and the ACVCP recommend prophylactic treatment with doxycycline at 5 mg/kg orally every 24 hours. In animals for which doxycycline cannot be administered (e.g., pregnant animals, young animals where teeth staining is an issue), amoxicillin at 20 mg/kg orally every 12 hours may be substituted. The required duration of prophylactic drug

administration is unknown, but should probably mimic that used in humans of 45 to 60 days. Unless new evidence suggests resistance to these antibiotics or introduction of a new strain of unknown susceptibility, the fluoroquinolones should not be used prophylactically. Such use will only promote general bacterial resistance to this valuable family of antibiotics. Furthermore, there is no evidence to suggest that fluoroquinolones are more efficacious than either the tetracyclines or penicillins for susceptible anthrax. If a pet is exposed to anthrax, care to decontaminate the fur to avoid transmission to humans is advised. Since no present disinfectants that kill spores are safe for use on living animals, repeated bathing is recommended to mechanically remove the organism.

Treatment of clinical anthrax must be early and aggressive with parenteral therapy usually warranted initially. Any of three antibiotic regimens may be considered. These include:

1. Oxytetracycline 5 mg/kg iv every 24 hours.
2. Potassium penicillin G at 20,000 u/kg iv every 8 hours (Note: The likelihood of beta-lactamase induction that would increase the MIC of the organism to penicillin is significantly higher for anthrax disease as opposed to post-exposure prophylaxis. Penicillin G should be used only when other agents are contraindicated.)
3. Enrofloxacin 5 mg/kg every 24 hours

There is no evidence to suggest which, if any, regimen provides the best outcome. In addition to antibiotic therapy, supportive therapy is warranted. (Oral therapy may be substituted for parenteral therapy if the animal survives the acute disease.) A tracheostomy may be required if edema in the pharyngeal region is severe. Pleural effusions may also need to be removed. If intestinal anthrax is suspected, oral therapy may be used to supplement parenteral therapy, provided the animal can swallow.

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<sup>i</sup> McGee, ED; Frita, DL; Ezzell, JW; Newcomb, HL; Brown, RL; Jaax, NK. Anthrax in a dog. *Vet Pathol.* 1994, 31(4): 471-473.

<sup>ii</sup> Gleiser, CA; Gochenour, WS; Ward, MK. Pulmonary lesions in dogs and pigs exposed to a cloud of anthrax spores. *J. Comp. Path.* 1968, 78:445-448.

<sup>iii</sup> Davies, ME; Hodgman, SFJ; Skulski, G. An outbreak of anthrax in a hound kennel. *The Veterinary Record.* 1957, August 17:775-776.

<sup>iv</sup> Timoney, JF; et al. The genus *Bacillus*. In: *Hagan and Bruner's Microbiology and Infectious Diseases of Domestic Animals*, Edition 8. Editor(s): Timoney, JF; et al.. Ithaca: Cornell University Press. 1988. p.206-211.

<sup>v</sup> McGee, ED; Frita, DL; Ezzell, JW; Newcomb, HL; Brown, RL; Jaax, NK. Anthrax in a dog. *Vet Pathol.* 1994, 31(4): 471-473.

<sup>vi</sup> EPA Office of Pesticide Programs. Emergency Use of Bleach in Anthrax Decontamination.

<http://www.epa.gov/pesticides/factsheets/bleachfactsheet.htm#bkmrk7>