Azathioprine

Pharmacodynamic Laboratory, Mississippi State University, 2017

Azathioprine has been used as an immunosuppressive agent in dogs for over 50 years. The drug was initially primarily used in studies that utilized dogs as a model for investigations of organ transplantation and the effects of immunosuppression on various body systems. Within a few years, azathioprine was also being used to treat naturally occurring diseases in dogs. Despite almost half a century of cumulative clinical and research experience on the use of azathioprine in dogs, however, there have been remarkably few studies that actually elucidate the precise effects that azathioprine has on the canine immune system. Most of our understanding of the mechanism of action of azathioprine in dogs is extrapolated from work in other species.

Azathioprine is a prodrug for the active metabolite 6-mercaptopurine, and the primary mechanism of action was long believed to be inhibition of the synthesis of the purines adenine and guanine by blockage of enzymes such as amidophosphoribosyltranserase, with resultant production of nonfunctional nucleic acid strands. Disruption of *de novo* purine synthesis inhibits DNA and RNA synthesis, thereby inhibiting the proliferation of fast-growing cells such as lymphocytes. Lymphocytes are particularly susceptible to the effects of inhibition of *de novo* purine synthesis, because they are relatively lacking in the alternative pathway of purine synthesis, the purine "salvage" pathway, in which nucleotides are re-synthesized from nucleotide degradation products. In the past few decades, however, multiple other mechanisms of action mediated by various azathioprine metabolites have been proposed, including blockage of T cell activation and stimulation of T cell apoptosis. Azathioprine has long been reported to be more effective against T cell function than B cell function, although strong evidence supporting this is lacking, and recent work in our laboratory demonstrated that azathioprine inhibits both B and T cell proliferation.

One of the key enzymes involved in azathioprine metabolism and inactivation is thiopurine methyltransferase (TPMT). Individual human patients (about one in 300 people) inherit a marked deficiency in the TPMT enzyme that renders them highly susceptible to azathioprine toxicity, particularly life-threatening bone marrow suppression. Interestingly, cats have also been shown to have a marked deficiency in TPMT enzyme activity, which may explain why azathioprine causes marked myelosuppression in cats at standard canine doses. Although the use of azathioprine at a very reduced dose rates has previously been published in cats, given the narrow margin for safety it is probably wisest to recommend that azathioprine never be used in cats at any dose, especially considering the availability of other immunosuppressive agents that appear to be much safer in cats, such as chlorambucil and cyclosporine. Although TPMT expression in dogs is widely variable, severe deficiencies in enzyme activity of the magnitude seen in cats and some people have not been commonly reported, and TPMT deficiency does not typically appear to be associated with the severe drug toxicities sometimes seen in dogs. In people, despite known variabilities in drug metabolism, pharmacokinetic monitoring of azathioprine has not become established, although several recent papers have suggested that monitoring of drug metabolites such as 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine (6-MeMP) may help predict drug efficacy and toxicity.

The standard azathioprine starting dose in dogs is 2 mg/kg orally once daily. This dose is usually well-tolerated and, although gastrointestinal side effects such as nausea, anorexia, vomiting and diarrhea are occasionally reported, they are typically mild and self-limiting. Although, in dogs, marked myelosuppression is uncommon, chronic azathioprine usage sometimes causes mild to moderate poorly regenerative anemia. Since anemia is a possible outcome in dogs receiving azathioprine, and is typically very well tolerated (that is, sub-clinical), mild to moderate anemia alone should not be mistaken as evidence of either drug overdose or treatment failure. Azathioprine can also cause profound myelosuppression or severe hepatotoxicity in some dogs. Marked myelosuppression and hepatotoxicity appear to be idiosyncratic non-dose-dependent reactions (Type B reactions), and are typically reversible if the problem is recognized early enough and azathioprine is discontinued. Severe myelosuppression is uncommon, but hepatotoxicity (typically characterized by a rise in reversible rise in ALT in the absence of clinical signs) occurs in about 15% of dogs. Hepatotoxicity may be more common in German Shepherds. Hepatotoxicity usually develops in the first few weeks of therapy and, if the drug is well-tolerated for

the first 2 to 4 weeks of therapy, it tends to be well-tolerated long-term. Myelosuppression, in contrast, can be more delayed. Anecdotally, some veterinarians believe that hepatotoxicity is more common with the generic forms of azathioprine compared to the proprietary product, but this observation may be limited to past reported individual incidents with generic azathioprine from dubious (overseas) sources. Complete blood counts and serum biochemistry panels (especially ALT) should be monitored regularly during initial azathioprine therapy. Several individual case reports have also reported pancreatitis in dogs receiving azathioprine, but cause and effect has not been established.

Azathioprine has, over the years, become well-established as an “add on” immunosuppressive agent to be considered for the treatment of many different immune-mediated and inflammatory conditions when glucocorticoids alone are ineffective or poorly tolerated, including immune-mediated hemolytic anemia, immune-mediated thrombocytopenia, inflammatory bowel disease, chronic hepatitis, glomerulonephritis, immune-mediated polyarthritis, myasthenia gravis, non-infectious meningoencephalitis, immune-mediated skin diseases, and anal furunculosis. Despite decades of azathioprine usage, evidence supporting immunosuppressive efficacy for many of these common diseases is remarkably limited. One recent study in a large number of dogs showed that one year survival in dogs with IMHA on azathioprine and prednisolone was only slightly better than outcomes in dogs on prednisolone alone. Interestingly, because (despite a relative paucity of evidence) azathioprine has commonly been recommended as the standard immunosuppressive drug of choice for many conditions, the efficacy of newer drugs for the treatment of these conditions is sometimes compared to a parallel group receiving azathioprine. One perceived “limitation” of azathioprine compared to other immunosuppressive agents, that it can take many weeks or even months to exert its effects, is based on limited and dated data derived predominantly in humans. In my experience, azathioprine in a clinical setting exerts its immunosuppressive effects in dogs about as rapidly as most other comparable agents, and recent research in our laboratory also demonstrated inhibition of canine lymphocyte proliferation within 2 weeks of commencing azathioprine.

Compared to most other immunosuppressive agents, azathioprine is relatively inexpensive, which is an important consideration with long-term immunosuppressive therapy, especially in large dogs. While the proprietary product (Imuran® or Azasan®) typically still costs over $5 per 50 mg tablet, the generic equivalent can be obtained for less than $1 a tablet. The smallest tablet size is 50 mg (although tablet scoring permits a 25 mg dose), which can present dosing problems in small (under 20 lb) dogs.

Chlorambucil

Pharmacodynamic Laboratory, Mississippi State University, 2017

Chlorambucil is a nitrogen mustard derivative cell-cycle nonspecific alkylating agent that has, for many decades, been used in both human and veterinary medicine predominantly as an antineoplastic agent for the treatment of cancers such as lymphoid leukemia, lymphoma, mast cell tumors, multiple myeloma and polycythemia vera. Antineoplastic cytotoxicity is derived from inappropriate cross-linkage of cellular DNA and RNA by insertion of alkyl radicals on the purine base, guanine. Chlorambucil also has immunosuppressive properties, and has occasionally been used in human medicine to treat immune-mediated and inflammatory conditions such as glomerulonephritis. More than 30 years ago, some veterinary clinicians began suggesting the use of chlorambucil as an immunosuppressive agent for our small animal patients. Since then, the use of chlorambucil for the treatment of a number of feline inflammatory skin conditions, such as pemphigus and eosinophilic granuloma complex, and for treatment of diseases such as immune-mediated thrombocytopenia and refractory inflammatory bowel disease, has become very well established, primarily because of a paucity of viable alternative medications that could be accurately dosed with safety in cats. The use of chlorambucil as immunosuppressive agent in dogs has been slower to evolve, but its use has been described for the treatment of pemphigus, glomerulonephritis and, most recently, protein-losing enteropathy associated with inflammatory bowel disease, with a promisingly high success rate. It is somewhat surprising that chlorambucil has not attained more common usage as an immunosuppressive agent in dogs, since it appears to have much the same mechanism of action as cyclophosphamide with significantly less onerous side effects (specifically, chlorambucil does not cause sterile cystitis), and comes in a more convenient tablet size.

Chlorambucil is metabolized predominantly in the liver, primarily to the active metabolite phenylacetic acid mustard. Compared to other alkylating agents, chlorambucil is relatively well tolerated, especially at immunosuppressive doses, but does occasionally cause gastrointestinal side effects such as vomiting and diarrhea, and/or myelosuppression with neutropenia, thrombocytopenia and non-regenerative anemia (anemia is usually mild). Alopecia and poor hair growth are sometimes reported in susceptible dog breeds, such as poodles. Neurologic side effects are reported with chronic chlorambucil use in people, and chlorambucil-associated neurologic signs (including myoclonus, twitches and seizures) have been reported in cats, and in one dog in a recent case report. Recently, acquired Fanconi syndrome has also been reported in cats on chlorambucil.

Chlorambucil is available as a coated 2 mg tablet that cannot feasibly be divided, and dosing recommendations in smaller patients are therefore typically provided in multiples of two, and/or "pulsed" at infrequent dosing intervals (given at an interval that ensures the overall weekly dose is equivalent to seven times the calculated daily dose) in order to avoid overdose. For immunosuppressive therapy, chlorambucil is almost always given in combination with an oral glucocorticoid. In dogs, recommended starting oral immunosuppressive chlorambucil doses (with a glucocorticoid) range from 0.1 to 0.2 mg/kg (or, alternatively, 4 to 6 mg/m^2) every one to two days, with dosing individualized based on patient size and disease severity. In cats (and small dogs) with inflammatory or immune-mediated disease, a starting oral chlorambucil dose of 2 mg every second day (with a glucocorticoid), tapered to every 3rd or 4th day, is my preferred dosing regime, although a number of other tapered dosing protocols are also available. Lower daily doses of chlorambucil, comparable to dog dosing regimes, can also be used in cats if the drug is compounded, but the compounded product, as with cyclophosphamide, has been shown to be associated with significant dosage variability when obtained from veterinary compounding pharmacies. Complete blood counts must be monitored regularly (weekly at first) and, since myelosuppression tends to be dose-dependent rather than idiosyncratic, doses can be tapered "to effect" rather than discontinued completely. Myelosuppression, provided it is detected promptly, is typically reversible. Anecdotally, myelosuppression can sometimes be delayed, especially in cats, and first appear up to one year into chronic low-dose chlorambucil therapy, and can either affect all cell lines, or particularly impact platelet counts.

Compared to many other immunosuppressive agents, chlorambucil has until recently been moderately priced. Unfortunately, the patent on the only available chlorambucil product, Leukeran®, recently expired, leading to a

change in ownership of the company responsible for distributing the drug, and the US price of chlorambucil has quadrupled as a result, to over $25 for a 2 mg tablet. There are currently no other US generic alternatives, apart from compounded products. The efficacy of compounded chlorambucil in dogs and cats has not been established, although anecdotally veterinarians have reported success when switching from the proprietary to the compounded product.

Cyclophosphamide

Pharmacodynamic Laboratory, Mississippi State University, 2017

Cyclophosphamide, a cell-cycle nonspecific nitrogen mustard derivative alkylating agent, was one of the first major chemotherapeutic agents approved by the FDA over 50 years ago, and has since become very well-established in human medicine as both an antineoplastic drug and as an immunosuppressive agent. Within a few years of FDA approval in the late 1950s, the use of cyclophosphamide for the prevention of transplant rejection in experimental models and for the treatment of both neoplasia and immune-mediated diseases was described in both dogs and cats. Cyclophosphamide has persisted to this day as one of the core drugs used in many small animal cancer chemotherapeutic protocols. In contrast, after many years as one of the most commonly immunosuppressive drugs utilized to treat immune-mediated diseases in cats and dogs, the use of cyclophosphamide as an immunosuppressive agent in small animal patients has in the past two decades essentially faded away. The reasons for the steadily diminishing popularity of cyclophosphamide as an immunosuppressive agent are myriad, and include a high incidence of unacceptable side effects, the development of other immunosuppressive agents that are generally safer and more convenient to administer, and the publication of a number of papers a decade or so ago that suggested that cyclophosphamide was associated with poor outcomes when used to treat conditions such as immune-mediated hemolytic anemia.

Cyclophosphamide is a prodrug that is metabolized by the hepatic cytochrome P450 enzyme system to eventually form active metabolites such as 4-hydroxycyclophosphamide, 4-hydroperoxycyclophosphamide, and aldophosphamide. These metabolites can enter cell cytoplasm, where they are ultimately metabolized to phosphoramide mustard and acrolein. Phosphoramide mustard is an alkylating agent that replaces a hydrogen atom with an alkyl group on the guanine base of DNA, which interferes with nuclear DNA replication and cytoplasmic RNA transcription by forming crosslinks both within and between nucleotide strands. Cyclophosphamide has long been reported to be a potent immunosuppressive that inhibits humoral and cell-mediated immunity, including inhibition of primary and secondary immune responses, reduction of antigen trapping in lymph nodes, and inhibition of local inflammatory responses, although in a number of experimental studies in dogs, cyclophosphamide often appears to be relatively mildly immunosuppressive compared to other drugs.

Cyclophosphamide shares the toxicity profile of most alkylating agents, with common side effects including gastrointestinal signs, myelosuppression, and hair loss. Gastrointestinal signs are relatively common, and include nausea, anorexia, vomiting and diarrhea. Dose reduction and antiemetic agents are often enough to control gastrointestinal signs, but occasionally persistent gastrointestinal side effects will prevent ongoing use of the drug, especially in cats. Myelosuppression appears to be dose dependent, and is associated with both the use of high drug doses and the use of lower doses over a sustained period of time. Moderate to severe neutropenia is the most potentially life-threatening feature of cyclophosphamide myelosuppression, but moderate thrombocytopenia and mild anemia may also occur. Neutropenia is typically reversible with drug dose reduction or discontinuance, but can occasionally persist for weeks or even months, particularly after chronic cyclophosphamide therapy. Recombinant granulocyte colony-stimulating factor can be used to hasten recovery in dogs with severe cyclophosphamide-induced neutropenia. Alopecia is most common is susceptible breeds such as poodles and old English sheepdogs. Interestingly, cyclophosphamide has been used in the past to “chemically shear” sheep.

A major side effect of cyclophosphamide that is (to a large extent) unique to this drug is the development sterile hemorrhagic cystitis (common in dogs, and uncommon but reported in cats). Cystitis is mediated by urinary excretion of the cyclophosphamide metabolite acrolein. Cystitis is often severe and debilitating to the patient, and will not resolve until the drug is discontinued. Unfortunately, distressing signs of cystitis can sometimes persist for days or even weeks after drug discontinuation. Cystitis is more likely to develop after long-term therapy with cyclophosphamide, which can present a particular problem for patients with immune-mediated diseases because such diseases are often persistent, and tend to relapse if immunosuppressive therapy is discontinued prematurely. The incidence of cystitis can be reduced significantly by the concurrent administration of furosemide or sodium 2-

mercaptoethane sulfonate (mesna), a sulfhydryl donor that binds acrolein, by ensuring ready access to water, by giving concurrent low doses of furosemide, and by taking canine patients for regular walks. The chronic local bladder inflammatory effects of cyclophosphamide have also been reported to predispose to the development of irreversible bladder wall fibrosis and even transitional cell carcinoma.

Over the years, a number of immune-mediated and inflammatory diseases in dogs and cats have been treated with cyclophosphamide, including immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia, megakaryocyte hypoplasia; pure red cell aplasia, systemic lupus erythematosus, immune-mediated polyarthritis, inflammatory bowel disease, glomerulonephritis, noninfectious inflammatory meningoencephalitis, immune-mediated vasculitis and pemphigus. For many years, cyclophosphamide was considered a “big gun” to be used in dogs with severe or life-threatening IMHA. However, in the late 1990s and early 2000s, a number of case studies were published that suggested that, at best, cyclophosphamide was not better than glucocorticoids alone for the treatment of IMHA and, at worst, associated with a higher than expected mortality rate. Given the known limitations of retrospective studies, including the associated potential for “case selection bias” (that is, the dogs with the most severe IMHA may have been given cyclophosphamide because it was the drug perceived to be most potent), it is hard to know with any real certainty whether cyclophosphamide actually worsens prognosis in dogs with IMHA. Nevertheless, there is no doubt that, since publication of these papers, the use of cyclophosphamide to treat conditions such as canine IMHA has markedly decreased.

Compared to many immunosuppressive agents, cyclophosphamide has been relatively cheap, with a generic 25 mg tablet costing under $2, and a 50 mg tablet costing under $4, although there has been a recent (hopefully temporary) marked surge in drug prices, with a 50 mg tablet now costing over $10. One of the major problems associated with using cyclophosphamide as an immunosuppressive agent is that it is difficult to dose accurately, and even more difficult to taper, especially in smaller patients. Cyclophosphamide is available as 25 or 50 mg tablets that composed of an active inner tablet surrounded by an inert outer flecked tablet. Because of uneven distribution of the drug through the tablet, cyclophosphamide tablets cannot be split or crushed without a risk of major dosing inaccuracies. Without drug compounding, cyclophosphamide doses must therefore be in multiples of 25 or 50. Compounded cyclophosphamide can solve dosing issues, and is cheaper, but a recent paper showed a worrying variation in dose accuracy with cyclophosphamide from various veterinary compounding pharmacies. Published immunosuppressive doses for cyclophosphamide in dogs include 50 mg/m^2 or 1.5 to 2.5 mg/kg every second day or daily on a “4 days on, 3 days off” weekly protocol. In cats and small dogs, similar total weekly doses can be used, but “pulsed” at infrequent dosing intervals that ensure that the total weekly dose is equivalent to seven times the calculated daily dose. Since myelosuppression can occur at any time during chronic cyclophosphamide therapy, complete blood counts must be performed regularly throughout the course of drug treatment. Cyclophosphamide is available in an intravenous form as well as an oral form, and a recent study in dogs confirmed that equivalent oral and intravenous doses of cyclophosphamide achieved comparable blood levels of the active metabolite 4-hydroxycyclophosphamide. Intravenous cyclophosphamide may therefore be a viable treatment option in vomiting animals that are unable to tolerate oral immunosuppressive agents.

Cyclosporine

Pharmacodynamic Laboratory, Mississippi State University, 2017

Cyclosporine is a potent immunosuppressive drug indicated for the treatment of inflammatory and immune-mediated diseases, and for organ transplantation. Cyclosporins are cyclic polypeptide macrolides originally derived from the soil fungus *Beauveria nivea (Tolypocladium inflatum)*, but are also produced by other fungal organisms. Cyclosporine A is the molecule developed for commercial use as an immunosuppressive agent. Discovered in the 1970s, the use of cyclosporine as an immunosuppressive agent was first described in humans to prevent rejection of renal allografts. Within a decade, cyclosporine had become the cornerstone of immunosuppression for organ transplantation. In veterinary medicine, oral cyclosporine capsules received FDA approval in 2003 for the treatment of canine atopy, and were more recently also approved for allergic skin disease in cats. Cyclosporine has been used in an extra-label fashion for many years for renal transplantation in dogs and cats, and for the treatment of a variety of inflammatory and immune-mediated conditions.

Cyclosporine's primary immunosuppressive mechanism of action is inhibition of T lymphocyte function. Cyclosporine acts to inhibit calcineurin, an intracellular protein phosphatase that activates gene transcription factors through dephosphorylation. In the untreated patient, activation of T cells results in activation of calcineurin, which dephosphorylates inactive nuclear factor (NFAT). NFAT translocates into the nucleus, where it upregulates transcription of genes coding for several important cytokines, including IL-2, IL-4, TNF-α, and INF-γ. Production of IL-2 in particular plays a key role in the activation and proliferation of T cells. Calcineurin inhibitors, including cyclosporine, act by binding to intracellular cyclophilins, which are proteins that facilitate protein folding. Binding of cyclosporine to cyclophilin A creates a complex with high affinity for calcineurin. Through inhibition of calcineurin, cyclosporine specifically inhibits T cell function and thus, cell-mediated immunity, but has little immediate impact on humoral immunity. Decreased IL-2 expression in CD4+ Th1 cells associated with cyclosporine therapy leads to inhibition of proliferation and activation of both T-helper and T-cytotoxic lymphocytes, and blunting of the immune response. Cyclosporine has also been shown to have many other local anti-inflammatory and immunosuppressive effects, especially in the skin.

Cyclosporine is a large lipophilic molecule which must be solubilized prior to intestinal absorption. Commercial cyclosporine is available as two very different types of oral formulations. Cyclosporine was initially approved in humans as a vegetable-oil based preparation (Sandimmune®), but variability in oral bioavailability caused marked variability in blood drug concentrations. A more recent formulation, an ultramicronized ("modified") preparation approved in 1996 (Neoral®), forms a microemulsion upon contact with aqueous fluids, resulting in more consistent and predictable absorption. Oral bioavailability of the microemulsion is improved by up to 50% compared to the oil-based preparation. Because of the marked variability in bioavailability of the non-ultramicronized (Sandimmune®) preparation, it is not recommended for oral use in small animals.

Cyclosporine has a high binding affinity for red blood cells and plasma lipoproteins. Because up to 50% of the drug in blood is located in red cells, whole blood is recommended for therapeutic drug monitoring (TDM). Once in the circulation, cyclosporine distributes widely, accumulating in the skin, liver, kidneys, and fat of dogs, resulting in a large volume of distribution. Tissue levels exceed levels in serum by a factor of 3 to 14. Peak blood concentrations generally occurring approximately 2 hours after oral administration of cyclosporine. Blood concentrations then rapidly decrease over the remainder of the dosing interval, reflecting a relatively rapid half-life as the drug is cleared from plasma.

Extensive metabolism of cyclosporine by the hepatic cytochrome P-450 system yields many different metabolites, some of which may retain therapeutic efficacy. In dogs, several drugs that inhibit P-450 enzymes have been given concurrently with cyclosporine in order to decrease the dose needed to maintain adequate blood drug concentrations. Ketoconazole, in particular, has been used to decrease in oral cyclosporine dosages in dogs by as much as 75 percent, although individual responses are variable. Like vincristine, cyclosporine is a drug that is handled by the P-glycoprotein efflux pump, a pump coded for by the ABCB1-1Δ (MDR1) gene. Dogs with the

MDR1 gene mutation, however, have been shown to have relatively unchanged cyclosporine pharmacokinetics compared to dogs with normal P-glycoprotein function. However, P-glycoprotein, as well as having a potential effect on drug bioavailablity, metabolism, and excretion (functions usually evaluated pharmacokinetically), may also impact the effect that the drug has on target cells. For cyclosporine, in particular, P-glycoprotein is responsible for pumping the drug out of target T cells, and a lack of this pump may lead to very high intracellular T cell drug levels. Certainly, our laboratory has seen individual dogs with the MDR1 gene mutation that had very markedly suppressed T cell function despite blood cyclosporine levels that were within target ranges.

The complexities of cyclosporine disposition in normal animals, coupled with confounding factors associated with disease and differences in drug preparation, may contribute to markedly variable blood drug concentrations both between patients and even within the same patient. Therapeutic management may therefore be facilitated by monitoring blood cyclosporine concentrations. Unfortunately, however, the process of adjusting drug doses based on monitoring cyclosporine blood concentrations is clinically complex, and not necessarily associated with the desired clinical outcome. Currently available methods for TDM include HPLC, a specific monoclonal RIA, and a dimersion cyclosporine immunoassay. HPLC has the advantage that the parent drug can be discriminated from metabolites, although most methods detect only the parent compound. Both RIA and dimersion cyclosporine immunoassay, in contrast, measure metabolites as well as the parent drug, and blood cyclosporine concentrations will therefore be higher by a factor of 1.5 to 1.7 compared to the same sample analyzed using HPLC. Although HPLC is considered the gold standard for cyclosporine assays, HPLC is labor intensive and not routinely offered for patient monitoring. TDx and RIA have been the methods most often employed in clinical situations, with the laboratory performing the assay typically providing recommendations regarding ideal target blood drug concentrations. Some laboratories have adjusted target blood concentrations upward to reflect the fact that TDx and RIA results will be approximately double HPLC assay results. Other laboratories have not made this adjustment, with the rationale that the cyclosporine metabolites measured by the TDx and RIA assays may arguably be pharmacologically active and contribute to overall immunosuppressive effects. Much study has gone into determining the most appropriate sample collection time in patients receiving cyclosporine. In human medicine, trough blood concentrations were the initial basis for adjustment of drug dosages. However, multiple studies in people have since suggested that area under the plasma drug concentration time curve (AUC) or 2 hour peak drug concentrations are preferred. With measurement of peak cyclosporine concentrations requiring only a single sample, adjusting drug doses to attain target peak drug levels has become the single best blood concentration measurement for use during human organ transplantation. In veterinary medicine, measurement of trough cyclosporine concentrations also prevailed for many years based on initial work done in canine and feline renal transplant studies. Recommendations from laboratories offering TDM have often involved measurement of both peak and trough cyclosporine blood levels, although target peak concentrations have not been well established. Individual laboratory recommendations depended on the target ranges determined by each laboratory as well as the assay used to measure cyclosporine concentrations. Currently, the Auburn University Clinical Pharmacology Laboratory is the only veterinary pharmacology laboratory routinely offering cyclosporine blood level assays.

Pharmacodynamic assays investigate a drug's effect on target cells. Several pharmacodynamic biomarkers of the immunosuppressive effects of cyclosporine have been studied in human medicine, including lymphocyte proliferation, calcineurin enzyme activity, lymphocyte surface antigen expression, and intracellular cytokine quantification. Through pharmacodynamic monitoring, human studies have shown individually distinct degrees of calcineurin inhibitor sensitivity in patients. Pharmacodynamic monitoring shows great promise for optimizing cyclosporine therapy and delivering individualized therapy. At Mississippi State University, we have conducted extensive investigations into the pharmacodynamic evaluation of cyclosporine in dogs. We initially measured activated T cell expression of IL-2, IL-4, and IFN-γ via flow cytometry in dogs administered two different oral cyclosporine dosages. The dogs were first administered a high dose of cyclosporine (10 mg/kg orally twice daily), with doses adjusted upwards as needed to attain a target trough drug concentration greater than 600 ng/mL as measured via HPLC, a dosing protocol known to be sufficiently immunosuppressive for canine organ transplantation. With high dose cyclosporine, activated T cell expression of IL-2 and IFN-γ was significantly

suppressed. The dogs were then administered the FDA-approved dose of cyclosporine used to treat canine atopy (5 mg/kg orally once a day), a dose which has been considered to be low enough to avoid predisposing to immunosuppression-associated infection. Even with this low dose of cyclosporine, however, T cell expression of IFN-γ and IL-2 was still markedly suppressed in some dogs. Subsequent studies evaluating activated T cell mRNA IL-2 and IFN-γ expression utilizing molecular methods have demonstrated that results using a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay are comparable to flow cytometry, and that the technique shows promise as a pharmacodynamic assay in dogs. One advantage of the qRT-PCR assay compared to flow cytometry is that it can be performed on blood samples mailed in by practitioners. This assay has now been offered to practitioners for several years through our Mississippi State University Pharmacodynamic Laboratory, and assists veterinarians in adjusting oral cyclosporine doses in dogs to optimize systemic immunosuppressive effects. Cyclosporine has been shown to have much the same effect on T cell cytokine production in cats as it does in dogs. A pharmacodynamic assay based on this effect in cats is not yet commercially available, although MSU is currently working on developing such an assay.

Cyclosporine is FDA-approved for the treatment of canine atopic dermatitis and feline allergic skin disease, and has also been used to prevent transplant rejection and to treat sebaceous adenitis, pemphigus foliaceus, anal furunculosis, feline stomatitis, inflammatory bowel disease (IBD), myasthenia gravis, non-infectious inflammatory meningoencephalitis, pure red cell aplasia, immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia (IMT), and immune-mediated polyarthritis in dogs and cats. Pharmacodynamic research evaluating T cell responses to cyclosporine in dogs has confirmed that canine responses are comparable to the response profile that is well recognized in people: that individual responses to cyclosporine are extremely variable from dog-to-dog, both in dogs receiving the same standard oral dose, and in dogs with oral doses adjusted to attain comparable blood levels. Given that a high degree of variability of individual responsiveness to cyclosporine has been established in dogs, cyclosporine dosing protocols should be tailored to allow for this patient-to-patient variability. In my opinion, recommended dosing protocols in dogs with chronic, non-life-threatening inflammatory skin and gastrointestinal diseases should be quite different from the protocols used in dogs with more acute and life-threatening immune-mediated diseases.

In chronic inflammatory diseases that are typically not immediately life-threatening, such as skin conditions, anal furunculosis, and mild IBD, cyclosporine is often effective at a standard, relatively low starting dose. Cyclosporine therapy is typically delivered long term, with drug doses adjusted upwards if needed “to effect”, based predominantly on clinical signs. Most commonly, however, starting doses do not need to be increased and, in the long-term, the cyclosporine dosage is typically tapered to the lowest effective dosage needed to maintain disease remission. Currently recommended starting cyclosporine doses in dogs are 5 mg/kg once daily for most skin diseases and IBD, and 5 mg/kg once to twice daily for anal furunculosis. In cats with skin conditions such as allergic skin disease, eosinophilic granuloma complex and pemphigus foliaceus, a starting cyclosporine dose of around 5-8 mg/kg daily is recommended. Cyclosporine blood concentrations are usually not necessary for treatment of these conditions, as remission of disease is the main criterion used to decide whether adequate cyclosporine therapy is being delivered. In fact, for many of these conditions, cyclosporine blood concentrations have been shown to have minimal correlation with disease remission, perhaps because the drug is selectively concentrated in tissues such as the skin. Recent pharmacodynamic studies, however, have shown that, even at standard low FDA-approved doses, some dogs can still develop significant suppression of certain T-lymphocyte biomarkers of immunosuppression despite very low trough cyclosporine concentrations. This could explain the phenomenon reported by dermatologists, that individual dogs treated for atopic dermatitis can develop severe secondary infections, although the “atopy” cyclosporine dose was originally not thought to cause clinically significant immunosuppression. Therefore, even in dogs on low cyclosporine doses, clinicians should remain vigilant for potential signs of systemic infection.

In canine patients suffering from more acute and immediately life-threatening diseases such as severe IMHA and IMT, in contrast, cyclosporine must be targeted to attain effective immunosuppression as rapidly as possible. These animals are somewhat comparable to patients that have recently undergone organ transplantation, in that

any delay in attaining effective immunosuppression can lead to a disastrous outcome. In these patients, starting cyclosporine at a low dose and adjusting doses upwards "to effect" is not recommended. Attaining effective oral doses as rapidly and accurately as possible is essential for ensuring adequate immunosuppression whilst avoiding overdosage with associated adverse effects and expense. Currently recommended starting cyclosporine doses for life-threatening diseases range from 5 mg/kg to 10 mg/kg twice daily. Subsequent measurement of blood cyclosporine concentrations and/or assessment of activated T cell mRNA IL-2 expression using qRT-PCR within one week of commencement of treatment, with dose adjustments as needed, are the best methods that are currently routinely available to assess adequacy of therapy, and are strongly recommended in patients with life-threatening diseases.

Side effects are uncommon with cyclosporine therapy in dogs and cats, with the exception of gastrointestinal side effects such as vomiting, diarrhea, anorexia and nausea. Administering the medication frozen and/or with food can reduce gastrointestinal side effects, although there is a risk that such measures will also alter drug absorption profiles. Uncommonly, cyclosporine can cause an idiosyncratic hepatotoxicity, which does not seem to be dose dependent. Gingival hyperplasia and hypertrichosis have also occasionally been reported with cyclosporine therapy. Chronic cyclosporine therapy may also predispose to neoplasia such as lymphoma. One advantage of cyclosporine as an immunosuppressive agent is that it is not myelosuppressive. Experimentally, oral cyclosporine has been shown to increase platelet thromboxane production, which may be a concern in patients with IMHA, where hypercoagulability and resultant pulmonary thromboembolism can be a major contributor to patient mortality. However, to date, it has not been demonstrated whether this phenomenon is clinically relevant in IMHA patients with naturally occurring disease. Furthermore, recent work in our laboratory has shown that, when cyclosporine and low-dose aspirin are given concurrently, the aspirin nullifies the surge in thromboxane seen in dogs that are receiving cyclosporine alone.

Cyclosporine is an expensive drug, particularly at higher immunosuppressive doses, and clinicians are therefore tempted to explore cheaper forms of the drug. In human medicine, there are many approved human generic microemulsion ("modified") preparations similar to the Neoral® formulation, and these generic preparations have been shown to have therapeutic equivalency in people. Studies investigating the pharmacokinetic properties of these generic preparations in dogs have not been performed, and it is not safe to assume that a generic formulation is therapeutically equivalent to the approved canine product (Atopica®). Clinically, there appears to be some variability seen in individual dogs in the oral bioavailability of these generic products. Use of generic products may therefore have the potential place our patients at risk of either therapeutic failure or toxicity although, if blood drug levels or pharmacodynamics assays are used to monitor therapy, this risk is minimized. The proprietary human microemulsified cyclosporine product, Neoral®, currently costs around $2 for a 25 mg capsule and $8 for a 100 mg capsule, while the generic equivalents cost less than $1 for the 25 mg capsule, and around $2 for the 100 mg capsule. The veterinary product, Atopica®, tends to be priced comparably to the human proprietary products, but has the advantage of being FDA-approved and available in a range of capsule sizes that are convenient for dosing accuracy in our small animal patients (10 mg, 25 mg, 50 mg and 100 mg), as well as a 100 mg/ml oral suspension and, currently, is price-supported by a manufacturer's coupon. Non-modified (Sandimmune® or equivalent) cyclosporine has highly unreliable bioavailability, and should not be used. Nor should compounded cyclosporine, because compounders usually do not specify if the product is modified or non-modified. Unfortunately, transdermal cyclosporine has been shown to be inadequately absorbed in cats.

Danazol

Pharmacodynamic Laboratory, Mississippi State University, 2017

Danazol, a synthetic androgen with weak ("impeded") androgenic effects, has in the past been suggested for the treatment of canine immune-mediated hemolytic anemia and immune-mediated thrombocytopenia, in combination with glucocorticoids, in order to reduce the dose of steroid that is needed. Danazol is derived from the synthetic steroid ethisterone, a modified progestogen. Danazol's most important mechanism of action is probably to reduce macrophage Fc receptor/antibody binding affinity. Danazol also competes with glucocorticoids for combination with steroid-binding globulin, consequently increasing the availability of active unbound glucocorticoid. Concurrent danazol therefore enables significant glucocorticoid dose reduction. Danazol may also reduce the degree of binding of antibody and complement to the red blood cell or platelet surface. Side effects are uncommon, and include hepatotoxicity and masculinization of female dogs. However, although some dogs with refractory IMHA and IMT have been reported to benefit from danazol, the drug fell out of favor a few decades ago, probably because it was very expensive at the time, and response to therapy was sluggish and highly unpredictable.

Reported oral danazol doses in dogs with IMHA or IMT, in combination with glucocorticoids, range from 5 to 15 mg/kg daily, either given as a single dose or 2-3 divided doses. Danazol comes in 50 mg, 100 mg, and 200 mg capsules. Danazol currently costs about $3 for a 50 mg capsule, and not much more for the 200 mg capsule.

Mycophenolate

Pharmacodynamic Laboratory, Mississippi State University, 2017

Mycophenolate mofetil is the synthesized prodrug form of mycophenolic acid, a selective and reversible inhibitor of inosine monophosphate dehydrogenase, an enzyme that controls the rate of synthesis of guanine monophosphate in the *de novo* pathway of purine synthesis. Mycophenolate mofetil is a fermentation product derived from fungi in the *Penicillium* group. Mycophenolic acid inhibits B and T cell proliferation, and decreases antibody production. Mycophenolate mofetil is primarily used in human medicine for prevention of rejection of transplanted organs, although it also used to treat immune-mediated diseases such as systemic lupus erythematosus, immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia and pemphigus vulgaris. Mycophenolate mofetil is often used in the place of azathioprine in human medicine and, since they have similar mechanisms of action, the two drugs should not be used together.

The original proprietary mycophenolate mofetil product, CellCept®, and the closely related mycophenolate sodium product, Myfortic®, were expensive, and as a result the products only achieved limited usage in small animal medicine. However, more recently, the availability of much cheaper generic alternatives has led to a greatly increased usage of mycophenolate mofetil in small animal patients. A single 250 mg CellCept® capsule currently costs around $9, whereas the equivalent generic 250 mg capsule costs less than 50c. An oral suspension version of mycophenolate mofetil (200 mg/ml) is available for more convenient dosing in smaller patients. Successful usage of mycophenolate mofetil in a small animal patient with naturally-occuring disease was first described in a dog with acquired myasthenia gravis. Much of the subsequent anecdotal usage of mycophenolate mofetil for a variety of different immune-mediated diseases was similar to the dosing reported in this original paper. Mycophenolate mofetil is also available in an injectable form, and the intravenous use of the drug has been described during the successful initial stabilization of three dogs with acquired myasthenia gravis that could not tolerate oral medications. Ironically, a more recent case report of 15 dogs with acquired myasthenia gravis treated with mycophenolate mofetil reported that the drug was ineffective at attaining clinical remission. Recent papers reporting the use of mycophenolate mofetil in dogs with IMHA or IMT have shown variable but sometimes promising results: while individual dogs appear to respond to therapy, overall response rates in some papers were are no better than those seen with more established drugs, and gastrointestinal side effects can often limit clinical usefulness of the drug. Recently, several retrospective case series reporting the use of mycophenolate mofetil for the treatment of meningitis of unknown origin in dogs showed promising results: interestingly, mycophenolate has also shown some promise in treating multiple sclerosis in people, where it is believed to have a neuroprotective effect. Mycophenolate was also recently shown to be of benefit in the treatment of autoimmune skin diseases in dogs. Mycophenolate has been suggested for the treatment of immune glomerulonephritis in the dog. The clinical effectiveness of mycophenolate for treating most immune disease in dogs has not yet been well-established. The extensive protein binding of the drug, which can vary widely from patient to patient, may explain variable and unpredictable responses to drug, as could variations in drug metabolite profiles produced in individual profiles. Promisingly, recently completed work in our laboratory has established that mycophenolate mofetil at maximally tolerated doses (in individual dogs, this dose varies between 10 mg/kg and 20 mg/kg twice daily) does significantly inhibit lymphocyte proliferation in normal dogs, although this effect is not observed until two weeks into therapy, suggesting a delayed response to the drug.

A recommended starting dose for mycophenolate mofetil in dogs is 10-20 mg/kg twice daily, although often gastrointestinal signs (particularly vomiting and, especially, severe diarrhea) at the higher end of the dose rate will necessitate dose reductions. Recent work by our group has found that, often, diarrhea doesn't develop until after approximately one week of therapy, and so it shouldn't be assumed that higher doses will be well-tolerated until 1-2 weeks of therapy have passed without gastrointestinal side effects. In stable patients, a low end starting dose of 10 mg/kg twice daily is probably advisable. Mycophenolate mofetil appears to have variable oral bioavailability in dogs, so variability in response to therapy should probably be expected. An older pharmacodynamic study in dogs measuring inosine monophosphate dehydrogenase enzyme activity suggested

that mycophenolate mofetil would best be dosed three times daily, but this recommendation has not entered common usage. Mycophenolate mofetil has not been used widely enough in veterinary medicine to establish the frequency of serious side effects but, in people, gastrointestinal signs and, less commonly, marked myelosuppression and a rare and fatal neurologic disease (progressive multifocal leukoencephalopathy) have been reported. Based on the human side effect profile, complete blood counts should probably be regularly monitored in dogs receiving mycophenolate mofetil. In humans, gastrointestinal side effects can be reduced by replacing mycophenolate mofetil with mycophenolate sodium, but this does not appear to help reduce gastrointestinal side effects in dogs. Mycophenolic acid in humans is primarily excreted conjugated to glucuronide and, since cats lack the glucuronyl transferases responsible for glucuronidation of drugs such as mycophenolate mofetil, concern has been expressed that the drug should used with caution, if at all, in this species. However, the use of mycophenolate mofetil has been described at a dose rate of 10 mg/kg twice daily, with no obvious side effects, in two cats with IMHA. Recent work at Washington State University, reassuringly, has found that cats seem to be able to metabolize mycophenolate rapidly, by actually utilizing glucosidation rather than glucuronidation.

Vincristine

Pharmacodynamic Laboratory, Mississippi State University, 2017

The vinca alkaloids are biologically-active dimeric alkaloids derived from the Madagascar (or rosy) periwinkle plant, *Catharanthus roseus*. Vincristine, a naturally-occurring vinca alkaloid, were originally characterized phytochemically more than fifty years ago. The diverse biological effects of vincristine have traditionally been attributed to drug-induced disruption of various intracellular microtubules. Microtubules are elongated, tubular cytoplasmic organelles involved in a broad spectrum of cellular processes including chromosomal migration, conduction of cellular secretions, ciliary and flagellar motility, and maintenance of cell shape. Microtubules are composed predominantly of complex helical polymers of the structural protein tubulin. Vincristine binds directly to tubulin, causing both inhibition of microtubule synthesis and disruption of intact microtubules. Microtubular structures susceptible to the effects of vinca-tubulin binding include the mitotic spindle in dividing cells, the neurotubules in neurons, and the cytoskeletal microtubules in platelets. Vincristine may also exert biological effects that are independent of disruption of intracellular microtubules, such as inhibition of RNA, DNA and protein synthesis, and modification of prostaglandin production.

Vincristine is a cell-cycle-specific cytotoxic agent. Vincristine disrupts microtubules within the mitotic spindle of dividing cells, thereby arresting chromosomal separation in metaphase. Vincristine at standard therapeutic doses is minimally myelotoxic, and is therefore commonly used in combination with more myelosuppressive chemotherapeutic agents. Vincristine is frequently used in veterinary cancer chemotherapy, both as a single agent for the treatment of canine transmissible venereal tumors, and as a component of combination protocols for the treatment of acute leukemia, lymphoreticular neoplasms, mast cell tumors, and various carcinomas and sarcomas.

Vincristine is usually administered intravenously as a sulfate salt, which is chemically more stable than its corresponding free base. Inadvertent subcutaneous or intramuscular administration causes severe local tissue irritation and necrosis. Oral absorption of vincristine is poor. Plasma disappearance of vincristine following intravenous administration is markedly biphasic, with a short initial half-life and a prolonged terminal half-life. The short initial clearance phase reflects extensive extravascular drug redistribution due to a combination of both avid binding to intracellular tubulin and rapid biliary excretion. The prolonged terminal clearance phase is due to the gradual release of vincristine bound to circulating plasma proteins and intracellular tubulin. Platelets demonstrate a remarkable ability to concentrate vincristine from plasma, and are therefore the principal circulating cellular carriers of the drug.

The degree of immunosuppression induced by vincristine at intravenous therapeutic doses is minimal compared to that induced by glucocorticoids, cyclophosphamide or azathioprine, and vincristine therefore is not used as an immunosuppressive agent for the treatment of most immune-mediated or inflammatory diseases in dogs and cats. The one exception is immune-mediated thrombocytopenia (IMT), where vincristine has become a mainstay of treatment.

During early clinical trials in human cancer patients, it was observed that the administration of vincristine was frequently associated significant but transient increases in circulating platelet numbers. A similar phenomenon has since been reported in dogs, both in research animals and in cancer patients. This effect appears to be due to increased megakaryocytopoiesis and thrombopoiesis, although the precise mechanisms of vincristine-associated thrombocytosis are still uncertain. Circulating platelet life-span does not appear to be significantly affected by standard low doses of vincristine in healthy animals.

The serendipitous discovery that vincristine induced thrombocytosis in human cancer patients with normal pre-treatment platelet numbers prompted conjecture that a similar outcome could be obtained in thrombocytopenic patients. Following publication of several anecdotal reports describing prompt, marked increases in circulating platelet numbers after administration of vincristine to people with IMT, vincristine gained favor with some hematologists as the treatment of choice for chronic refractory IMT. Vincristine frequently induces partial or

complete remission of thrombocytopenia within one week of commencing therapy, although such remissions are typically transient. Only a relatively small proportion of human chronic refractory IMT patients achieve complete sustained remission with vincristine therapy.

Rapid drug clearance from plasma reduces the therapeutic efficacy of a standard intravenous bolus of vincristine. Several alternate methods of vincristine administration have therefore been used in human IMT patients in order to sustain therapeutic plasma concentrations. Constant intravenous vincristine infusion (over six to eight hours) effectively maintains therapeutic plasma concentrations the drug throughout the period of administration. Alternatively, the ability of platelets to concentrate vinca alkaloids from plasma has been utilized to enhance therapeutic efficacy via transfusion of vincristine -loaded platelets. Incubation of donor platelets in high concentrations of vincristine (vinca loading) prior to transfusion maximizes intracellular vinca-tubulin binding. Following transfusion, circulating vinca-loaded donor platelets gradually release vincristine into the recipient's plasma, thereby sustaining therapeutic plasma drug concentrations. Both constant rate infusion with vincristine and transfusion with vinca-loaded platelets induce sustained remissions in some human chronic IMT patients previously refractory to single intravenous boluses of the drug.

Vincristine, typically in combination with prednisone, has been reported to similarly facilitate remission of thrombocytopenia in many canine patients with IMT. Original case reports demonstrating an apparent rapid response to vincristine in dogs with IMT have been supported, decades later, by evidence obtained from prospective studies. Circulating platelet numbers increase markedly within three to five days of vincristine administration in responsive dogs, and the addition of vincristine to standard immunosuppressive therapy in dogs with IMT appears to shorten hospitalization time by several days. Most authors currently recommend an intravenous vincristine bolus dose of 0.02 mg/kg for the treatment of canine IMT. Vincristine boluses may subsequently be repeated weekly if thrombocytopenia recurs. Apparent rapid clinical response to vincristine-loaded platelets has been reported in one dog with refractory IMT. Vincristine has been used in cats with IMT, although evidence of clinical efficacy is lacking. One significant advantage of vincristine compared to other therapeutic options for IMT (such as human intravenous globulin) is that vincristine is inexpensive (a 1 ml vial of 1mg/ml vincristine sulfate costs around $20).

The pathogenesis of vincristine-induced remission of thrombocytopenia in IMT patients is uncertain. Clinicians initially assumed that remissions were due to increased megakaryocyte production and release of platelets, the principal mechanism assumed to underlie the vinca-induced thrombocytosis seen in healthy animals and cancer patients. Studies in people, however, suggest that the main therapeutic effect of vincristine in IMT patients is not increased thrombopoiesis. Post-treatment average platelet life-spans are significantly prolonged in human IMT patients that respond to vincristine, suggesting that remission is due to reduced platelet destruction rather than increased platelet production. Since platelets are the major circulating cellular carriers of vincristine, researchers have speculated that antibody-coated platelets selectively deliver vincristine to those phagocytes within the mononuclear phagocytic system that are actively involved in platelet destruction. This proposed mechanism explains why, despite being an ineffective immunosuppressive agent for the treatment of most conditions, vincristine can still be very effective for the treatment of IMT.

During electron microscopic studies of platelet ultrastructure, it was discovered that prolonged incubation of platelets in vincristine solutions caused marked disruption of cytoskeletal microtubules. Laboratory investigations have since demonstrated that as well as disrupting platelet structure, exposure to high concentrations of vincristine also significantly impairs platelet function. Based on the *in vitro* evidence that exposure to vincristine impairs platelet function, hematologists expressed concern that using the drug in patients with IMT could similarly induce platelet dysfunction. Subsequent studies revealed that vincristine affected platelet function (aggregation) in dogs with lymphoma, but not in healthy dogs. Since several recent prospective studies showed no significant increase in bleeding in IMT dogs receiving vincristine, the effect of vincristine on platelet function, if it occurs, does not appear to be severe enough to be clinically significant.

Neurotoxicity, although uncommon, is the most frequent significant side-effect associated with therapeutic doses of vincristine in dogs and cats. Reversible vincristine-induced neurotoxicity in the dog has been reported with chronic cancer chemotherapy, but is not likely to be an issue with the single doses used to treat IMT. Other side-effects such as gastrointestinal disorders (including megaesophagus and gastric hypomotility) and alopecia, occur less frequently and are typically mild and temporary. Vincristine at doses used for IMT typically causes minimal myelosuppression in dogs, although dogs with the ABCB1-1Δ (MDR1) gene mutation and some Border Collies have been reported to be more susceptible than other dog breeds to myelosuppression, especially at antineoplastic vincristine doses. In affected Border Collies, this effect appears to sometimes be independent of the MDR1 gene mutation reported in this breed. Genetic testing prior to vincristine is recommended in breeds at high risk of the MDR1 gene mutation, such as Collies and Australian Shepherds, and drug doses should be reduced by 50% in homozygous affected dogs, and by 25% in heterozygous affected dogs. Temporary erythrodysplasia of erythroid precursors in the bone marrow and peripheral blood smears, featuring bizarre mitotic figures, abnormal nuclear configurations, and Howell-Jolly bodies, can be observed after administration of vincristine in dogs, but is of little clinical significance. An unusual transient pulmonary toxicity has been reported in a cat receiving chemotherapeutic doses of vincristine. Vincristine has no known mutagenic or carcinogenic potential.