PHARMACOKINETICS OF A NOVEL CYTOSINE ARABINOSIDE SUBCUTANEOUS PROTOCOL IN DOGS WITH MENINGOENCEPHALOMYELITIS OF UNKNOWN ETIOLOGY

BY

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THESIS

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ABSTRACT

Meningoencephalomyelitis of unknown etiology (MUE) is an umbrella term used to describe a group of noninfectious inflammatory central nervous system (CNS) diseases in dogs. There are many subtypes of MUE identified in the canine population, including granulomatous meningoencephalomyelitis, necrotizing meningoencephalitis, necrotizing leukoencephalitis, steroid responsive meningitis arteritis, eosinophilic meningoencephalomyelitis, and greyhounds non-suppurative meningoencephalitis. The etiology of MUE remains unknown, but it is suspected to have an autoimmune pathogenesis. Emerging research points to genetic and environmental factors that are likely to play a role as well. Because histopathology from brain biopsy or necropsy is required for definitive diagnosis, antemortem diagnostic guidelines have been published in the literature. Treatment with glucocorticoids remain the standard of care for these patients. The prognosis is fair to guarded, with improved survival times when more than one immunomodulatory drug (IMD) is utilized. One IMD, cytosine arabinoside (CA), has been applied to dogs with MUE due to its ability to cross the blood brain barrier, low incidence of adverse effects, and prolonged survival times reported.

In spite of its increasing application for dogs with MUE, there is no standard administration protocol for CA. Various protocols have been proposed, all requiring either 24-48 hours (h) of hospitalization or one prolonged day of hospitalization lasting longer than 12h. Thus, a study was designed to investigate the pharmacokinetic (PK) properties of CA when administered as repeated subcutaneous injections over an 8h period to dogs with MUE in order to determine if this amenable administration protocol is a viable alternative to more laborious CRI administration. The primary objective was to describe PK parameters of CA when administered SC over 8h and when administered as a CRI over 24h. The secondary objective was
to determine if CA concentrations reach its therapeutic target (> 1 µg/mL) with both routes of administration. The final objective was to report the short term outcome of each patient 3 months following initiation of treatment. We hypothesized that both routes of administration would result in CA concentrations that exceeded its therapeutic target concentration at 1h and 8h following initiation of treatment, indicative of the proposed SC protocol providing sustained plasma CA concentrations throughout administration. Additionally, we hypothesize that most dogs receiving this protocol would have a favorable outcome, with the majority being alive 3 months after initiating treatment receiving only 1 oral IMD (either prednisone or prednisolone).

Dogs with MRI and CSF findings compatible with an antemortem diagnosis of MUE weighing greater than 2.5 kilograms were recruited for this study, and 8 dogs met the inclusion criteria. Dogs received CA as a CRI (200 mg/m² IV over 24h) initially, followed by a SC protocol (50 mg/m² every 2h for 4 treatments) four weeks later. Plasma CA concentrations were measured by high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS). For both the IV and SC protocols, PK parameters were calculated including maximum plasma concentration (C_{max}), observed area under the curve (AUC_{0-24}) using the trapezoidal method, plasma concentrations at 1h and 8h after initiating treatment (C_1 and C_8, respectively), and duration of time plasma concentration exceeded 1 µg/mL (T_{>1}). Time to C_{max} (T_{max}) was calculated for the SC protocol only. Wilcoxon sign rank tests were performed to compare C_{max}, C_1, C_8, and T_{>1} between the CRI and SC protocols. McNemar's tests were used to compare the number of dogs that achieved plasma concentrations greater than 1 µg/mL at 1h and 8h following CA administration between the two administration protocols.

Median peak CA concentration for the SC protocol (3.40 µg/mL, range 1.60-9.70 µg/mL) were significantly higher than the CRI (1.09 µg/mL, range 0.77-1.67 µg/mL; P = 0.02). C_{max}
following SC administration were significantly higher than when administered as a CRI, with a median of 3.4 µg/mL (range: 1.6-9.7 µg/mL) and 1.09 (range: 0.77-1.69 µg/mL; \( P = 0.0156 \)), respectively. Median concentration at 1h and 8h following initiation of treatment was significantly higher for the SC protocol (CA\textsubscript{1} 2.28 µg/mL, range 0.97-2.67; CA\textsubscript{8} 1.83 µg/mL, range 0.77-2.84) compared to the CRI (CA\textsubscript{1} 0.01 µg/mL, range 0-0.45; CA\textsubscript{8} 0.74 µg/mL, range 0.67-1.11; \( P = 0.01 \)). \( T_{\geq 1} \) was also significantly higher for the SC protocol (9.25h, range: 4.5-10.5) than for the CRI protocol (3.13h, range: 0-9.75; \( P = 0.039 \)). Five of the 8 dogs remained alive at the time of follow-up 3 months later, and only 1 of these 5 dogs was receiving more than one oral IMD at that time (aside from prednisone or prednisolone).

This study demonstrated that CA when administered as repeated SC injections over an 8h period achieved consistent and prolonged exposure in the plasma of dogs with MUE. Cytosine arabinoside (CA) exceeded its target concentration of 1 µg/mL at 1h and 8h following initiation of treatment via the proposed SC protocol, rarely meeting this target with the CRI protocol administered. These findings indicate further research investigating the therapeutic target of CA in dogs with MUE. Additionally, further research is warranted to determine if there is a significant difference in prognosis for dogs receiving the proposed SC protocol at the time of diagnosis when compared to more laborious CRI administration.

Based on currently available data, the authors recommend the following protocol for CA in dogs with MUE: CA 100-200 mg/m\(^2\) intravenously as a CRI over 24h at the time of diagnosis, followed by 50 mg/m\(^2\) every 2 hours for 4 doses every 4 weeks. This SC protocol can be administered on an outpatient basis, reducing prolonged hospitalization or repeated hospital visits for treatment, reducing cost to the client, and most importantly, reducing pain to the patient by eliminated the indication for repeated intravenous catheterization.
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2. Lampe R, **Levitin HA,** Hecht S, *et al.* MRI of CNS lymphoma with choroid plexus involvement in 5 dogs and 1 cat. *Accepted to J Small Anim Pract 11/26/20 pending revisions.*


CHAPTER 1: LITERATURE REVIEW MENINGOENCEPHALOMYELITIS OF UNKNOWN ETIOLOGY

1.1 PROPOSED ETIOLOGY

Meningoencephalomyelitis of unknown etiology (MUE) is an umbrella term used to describe a group of noninfectious inflammatory central nervous system (CNS) diseases in dogs, including predominantly granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME), and necrotizing leukoencephalitis (NLE) (Vitale 2018). All three of these disease entities require histopathology for definitive diagnosis, however, there are recommendations present in the literature for assignment of an antemortem diagnosis of MUE. Other noninfectious inflammatory CNS diseases exist, such as steroid responsive meningitis arteritis (SRMA), eosinophilic meningoencephalomyelitis (EME), and greyhounds non-suppurative meningoencephalitis (Vitale 2018). The noninfectious inflammatory CNS diseases in dogs are collectively termed MUE when a histopathological diagnosis is lacking and are suspected to be immune mediated in origin.

An autoimmune pathogenesis has been suspected based upon the histopathological features of the CNS in dogs with MUE. This primary theory has been derived from several studies which were performed to elucidate the cell types present within these inflammatory lesions in dogs (Kipar 1998, Park 2012). More specifically, a delayed type hypersensitivity reaction has been suggested by Kippar et al. due to the presence of macrophages and T lymphocytes within inflammatory lesions of dogs with GME (Kipar 1998). Park et al. demonstrated similar findings present in a broader population of dogs with inflammatory CNS disease, including diagnoses of NME, NLE, and GME (Park 2012). In this population of dogs, lesions predominantly consisted of IgG-, CD20-, and CD79a positive cells (B-cells), CD3
positive cells (T-cells), and CD163 positive cells (macrophages). The distribution of these cells differed anatomically between NME, NLE, and GME. In those dogs diagnosed with NME, malacic changes were predominantly localized to the cerebral cortex. Dogs diagnosed with NLE predominantly had malacic changes present in the cerebral white matter and thalamus. Lastly, dogs with GME seemed to have the widest distribution, with lesions being found in the cerebral white matter, cerebellum, and brainstem. There was no significant difference in the proportion of B-cells and macrophages between the groups, but dogs with GME had a higher proportion of T-cells when compared to dogs with NLE and NME. In turn, Park et al. concluded that the predominant cell types found in dogs with NME, NLE, and GME were lymphocytes and macrophages, with T-cells being the predominating cell type in GME.

Genetic risk factors have also been identified to support MUE’s immune-mediated pathogenesis. Two genetic risk loci have been identified in Pug dogs with NME, particularly a region within dog leukocyte antigen (DLA) class II complex on chromosome 12 (Barber 2011). Dog leukocyte antigen class II haplotypes have been associated with development of non-suppurative meningoencephalitis in population of greyhounds in Ireland as well (Shiel 2014). Similarly, genetic risk loci have been identified in Maltese and Chihuahua dogs (Schrauwen 2014). In Maltese dogs, regions of significance were found on chromosomes 4 and 15. These chromosomes contain information pertinent for genes which control regulation of immune system function based on haplotype analysis and fine-mapping performed, namely the ILR7 and FBXW7 genes. Additionally, a shared genetic risk between Pug, Maltese, and Chihuahua dogs was discovered, associated with chromosome 15 and DLA class II complex. These investigations have begun to elucidate the genetic risk factors that play a role in development of MUE in toy breed dogs and in greyhounds with non-suppurative meningoencephalitis.
In addition to genetic risk factors, various infectious etiologies have been excluded as the inciting cause of MUE. A metagenomic investigation was performed in 22 dogs, utilizing 11 dogs with MUE and 11 dogs with noninflammatory central nervous system disease inconsistent with MUE as negative controls (Hoon-Hanks 2018). Cerebrospinal fluid or brain tissue samples were collected, and their RNA and DNA extracted for shotgun metagenomic sequencing, which revealed no candidate etiologic agents for dogs with MUE. Due to the fact that viruses (particularly arbovirus and bornavirus) have precipitated non-suppurative encephalitides in other species, they have also been investigated as etiologic agents (Barber 2012, Collinet 2020). Dogs diagnosed with GME and NME were evaluated by broadly reactive polymerase chain reaction for adenoviruses, bunya viruses, coronaviruses, enteroviruses, flaviviruses, herpesviruses, paramyxoviruses, and parechoviruses, in addition to thorough testing for mycoplasmas by means of polymerase chain reaction (PCR), culture, and immunohistochemistry (IHC) (Barber 2012). No viral nucleic acids were found. Cerebrospinal fluid of dogs with MUE have also been tested via reverse-transcriptase PCR for the presence of astrovirus and bornavirus specifically, and neither were identified. Five of said cases were positive for Mycoplasma genus (1/5 GME and 4/25 NME) via PCR and *Mycoplasma canis* was cultured from 8 cases (4/5 GME and 4/8 NME) but ultimately *M. canis* was not identified on IHC staining of any of these cases. The overall negative results of broad viral testing makes a viral etiologic agent unlikely in dogs, but the possibility of a viral etiology that has yet to be discovered cannot be entirely eliminated (Barber 2012, Collinet 2020). Additionally, given that MUE encompasses a heterogenous collection of diseases, it is possible that the inciting factor of each is slightly varied.

Other factors have begun to be investigated in dogs with MUE as well. One study evaluated dogs with GME for any association between human population density (based upon
home address), signalment, body weight, body condition score, and vaccination status (Barnes Heller 2019). None of the factors investigated were associated with an increased risk of diagnosis. A different investigation, however, found that *Prevotellaceae* is significantly less abundant in the gut of dogs with MUE when evaluating their fecal microbiome, possibly suggestive of difference in the gut microbiota contributing to the development of MUE (Jeffrey 2017). It is overall apparent that genetic risk factors likely play a role in development of MUE, while the effects of other environmental factors require more investigation in a larger population of affected dogs.

1.2 PREVALENCE AND INCIDENCE

Prevalence data is imperative to better understand the impact that MUE has on the canine population as a whole; however, there are no data available pertaining to its prevalence in the general canine population. While a 2010 metanalysis on MUE identified 457 cases reported in the literature between 1962-2008, the canine population within each individual institution was not reported (Granger 2010). Therefore, prevalence could not be determined within the canine population as a whole. The only population in which the prevalence of MUE has been investigated is in the French Bulldog (Mayousse 2017). A retrospective review of all French Bulldogs evaluated for neurological disease between 2001-2016 discovered that 25% of all encephalopathies were attributed to MUE. The only encephalopathic etiology more prevalent in this population was neoplasia, accounting for 36.8% of encephalopathies in neurological French Bulldogs. Further investigation is warranted to determine the prevalence of MUE in the general canine population being treated for encephalopathic and/or myelopathic signs at referral institutions.
In the absence of additional information pertaining to its prevalence, valuable information can still be extracted from those studies that have been able to investigate the incidence of MUE in dogs presenting for neurological disease. In the few studies available, the incidence of inflammatory brain disease diagnosed in neurological dogs has ranged from 12.6-14% (Fluehmann 2006 and Tipold 1995). Upon retrospective review of dogs presenting to a referral hospital in Switzerland between 1989-2000, 14% of dogs presenting with neurological signs of either the brain, spinal cord, or peripheral nervous system were diagnosed with inflammatory/infectious disease (Fluehmann 2006). Unfortunately, MUE (nor any of its associated histopathological diagnoses) was not included in the list of assigned diagnoses. Rather, the diagnoses included within the inflammatory/infectious category included a space-occupying lesion, canine distemper, otitis media-interna, and SRMA. Given that diagnoses of neoplasia and metastatic meningitis were amongst the other disease categories listed, it is suspected that dogs with MUE would have fallen into the category of space-occupying inflammatory/infectious disease. This category could theoretically also include intracranial abscess or granuloma, therefore, more precise data pertaining to MUE cannot be fully extrapolated from this study.

One previous study provided more specific clinical diagnoses of inflammatory disease in dogs, including a multitude of infectious and noninfectious etiology of inflammatory diseases (Tipold 1995). Tipold found that MUE accounted for 12.6% of all histopathologically diagnosed inflammatory CNS disease in dogs between 1988-1993. While this percentage may not appear remarkably high, the only noninfectious disease process which surpassed MUE in this population of 220 dogs was steroid responsive meningitis arteritis (SRMA) (incidence of 14.5%), which has also been categorized as a subtype of MUE in the more recent literature (Vitale 2018). Certainly,
more research is warranted to better define both the incidence and prevalence of MUE, but the data available suggest that it is a fairly common cause of CNS disease in dogs.

1.3 ANTEMORTEM DIAGNOSIS

An antemortem diagnosis of MUE requires systemic testing, including infectious disease testing, followed by magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis. Recommended systemic testing includes screening for both metabolic and neoplastic disease via complete blood counts, chemistry panel, urinalysis, total thyroid level, baseline cortisol, and thoracic and abdominal imaging (Granger 2010, Vitale 2018). Pertinent infectious testing is also recommended, and ultimately depends on the geographical region and patient history. Pertinent infectious agents that can affect the CNS in dogs include protozoa (*Toxoplasma gondii* and *Neospora caninum*), viruses (rabies and canine distemper virus), tick-borne diseases (*Ehrlichia canis* and *Anaplasma phagocytophilum*), and fungi (*Blastomyces dermatitidis*, *Cryptococcus neoformans*, and *Coccidioides immitis*).

Should an inciting cause of neurological signs not be found, advanced imaging is recommended to evaluate the neuroanatomical region of interest. Brain and spinal cord MRI are the gold standard imaging modality. Cerebrospinal fluid (CSF) analysis is subsequently performed to better characterize the type of inflammatory population present. The three largest subtypes of MUE (being GME, NME, and NLE) have neurolocalization predilections that can be appreciated on MRI. Granulomatous meningoencephalomyelitis affects the cerebrum, cerebellum, brainstem, or spinal cord, and can be present as a focal, disseminated, or ocular form of the disease (Vitale 2018). On histopathologic examination, these lesions consist of a mixed lymphoid population and perivascular cuffing. The other two subtypes are not as widely
distributed in the CNS, with NME having a predilection for the cerebrum and NLE having a predilection for the cerebrum and brainstem (Vitale 2018). The histopathologic characteristics associated with NME and NLE include inflammation and necrosis affecting the junction of the gray and white matter or solely the white, respectively (Vitale 2018). These histopathologic findings are required to make a definitive diagnosis postmortem, but are often lacking when assigning an antemortem diagnosis due possible adverse outcomes from brain biopsy, including seizure, stupor, paresis, ataxia, loss of consciousness, or other post-procedure complication such as aspiration pneumonia (Flegel 2012, Granger 2010). With brain biopsy having a morbidity rate of 27-29% (Flegel 2012, Shinn 2020), it is understandable why many owners elect to forgo this procedure and rely upon antemortem diagnostic criteria.

A large metanalysis performed by Granger and colleagues recommends three major antemortem diagnostic criteria: 1) single or diffuse intra-axial hyperintense lesion(s) on T2-weighted MRI; 2) CSF hypercellularity with >50% mononuclear cells (lymphocyte and/or monocytes); 3) negative infectious disease testing. These major criteria were corroborated by a more recent review of noninfectious inflammatory diseases in dogs; however, this review applied a broader description of the disease process than did Granger et al. Vitale and Foss described CSF findings reported in dogs with MUE in addition to other inflammatory CNS disorders (Vitale 2018). These findings included predominantly a mononuclear pleocytosis, in addition to albuminocytologic dissociation, neutrophilic pleocytosis (specifically SRMA), and eosinophilic pleocytosis (specifically EME) (Vitale 2018). Granger and colleagues also suggested excluding several noninfectious inflammatory CNS diseases in dogs from the category of MUE, which were encompassed in Vitale and Foss’s review, such as SRMA, EME, spinal cord only MUE, necrotizing disease (NME and NLE), and optic neuritis (which can be a focal form of GME). It
would seem prudent to include all forms of noninfectious inflammatory CNS disease that are suspected to have an autoimmune pathogenesis under the category of MUE. Subsequently, other factors such as signalment, lesion localization on neurological examination and MRI, and CSF cell type predominance can be used clinically to help prioritize which subtype(s) may be most likely.

1.4 TREATMENT AND PROGNOSIS

There is no standardized treatment for MUE and the prognosis is variable. Glucocorticoids are the mainstay therapy, and at one time were the sole therapy used for MUE. Prednisone and prednisolone at dosages of 1-2 mg/kg/day orally when used as a monotherapy has been associated with median survival times (MST) of 28-602 days (Flegel 2011, Jung 2007, Mercier 2015, Pakzody 2009). Improved outcomes have been documented when additional immunomodulatory agents are used, such as azathioprine (MST 1824 days for MUE; Wong 2010), cytosine arabinoside (MST 26 to >1095 days; Lowrie 2013, Lowrie 2016, Smith 2009, Stee 2020, de Stefani 2007, Menaut 2008, Zarfoss 2006), cyclosporine (MST 240-930 days for GME and NME; Adamo 2007, Gnirs 2006, Jung 2007, Pakozdy 2008), lomustine (MST 329 days for GME and 329 days for NME; Flegel 2011), and mycophenolate (MST 250-731 days for MUE; Baroon 2016, Woolcock 2015). The studies investigating treatment of MUE all have a fairly small sample size with an average of 10 dogs (range: 7-16) for those pertaining to glucocorticoids alone and 22 dogs (range: 3-83) for those pertaining to use of multiple immunomodulatory drugs. Taking these data into account, it is evident that treatment with more than one immunomodulatory drug results in longer survival times.
Studies have found surviving the initial phase of diagnosis is likely a positive prognostic indicator. More specifically, 1/3 of dogs diagnosed with MUE are known to succumb to their disease within 72 hours of diagnosis (Lowrie 2018). Another study found that 1/4 of dogs diagnosed with MUE die or are euthanized within 7 days of diagnosis (Cornelis 2016). Indicators associated with a positive prognosis include younger age at diagnosis (Oliphant 2017), presenting with focal forebrain signs (Muanana 1998), presenting within 7 days of the onset of clinical signs (Baroon 2016), resolution of MRI abnormalities 3 months after initiating treatment (Lowrie 2013), and surviving 3 months (Lowrie 2013, Smith 2009) and 6 months following initiation of treatment (Stee 2020). Conversely, presenting with seizures or an altered mentation (Cornelis 2016), discontinuing treatment prior to resolution of lesions appreciated on MRI (Lowrie 2013), and the appearance of midline shift on MRI (Oliphant 2017) have all been identified as negative prognostic indicators. With 15% of cases dying or being euthanized prior to receiving treatment, it is clear that this is a severe disease process in dogs warranting further investigation of potential treatment protocols (Granger 2010).
CHAPTER 2: LITERATURE REVIEW OF CYTOSINE ARABINOSIDE

2.1 MECHANISM OF ACTION

Cytosine arabinoside (CA) is a synthetic nucleoside analogue of cytidine with antineoplastic and anti-inflammatory properties. It is S-phase specific and causes competitive inhibition of DNA polymerase in mitotically active cells, thus preventing DNA replication and halting the cell cycle (Mulder 1975, Scott-Moncrief 1991, Gmeiner 2003, Withrow 2012, Zarfoss 2006). It also inhibits DNA repair and membrane protein glycosynthesis by altering topoisomerase function (Griffin 1982, Withrow 2012). It is a water-soluble compound that is rapidly transported intracellularly by means of facilitated diffusion via nucleoside transporters within the cell membrane, similar to organic nucleosides uptake (Groothius 2000, Mulder 1975). Once transported intracellularly, CA undergoes repeated phosphorylation via various kinases as it is metabolized to its biologically active form, CA-triphosphate (DeAngelis 1992, Groothius 2000, Mulder 1975, Wiley 1984). Throughout this process cytidine deaminase and deoxycytidylate deaminase metabolize CA into its biologically inactive forms for excretion (Mulder 1975, DeAngelis 1992, Wiley 1984). There is known species variation in deaminase activity that is thought to contribute to the degree of its penetration into the central nervous system. Subsequently, variations in indicated dosage and agent efficacy would be expected across species (Groothius 2000). With that being said, patients with inflammatory CNS disease have alteration in the function of their blood-brain barrier due to changes in architecture of endothelial tight junctions, disruption of the basement membrane, and increased vascular permeability due to upregulation of cytokines, metalloproteinases, and nitrous oxide, respectively (Webb 2000). Changes in the blood brain barrier under such inflammatory
conditions could therefore affect CA penetration into CSF and subsequently the brain and spinal cord parenchyma. Due to its ability to halt the cell cycle, CA has both antineoplastic and anti-inflammatory properties.

2.2 METABOLISM AND EXCRETION

Data regarding CA metabolism and excretion is available in human literature and minimally in veterinary literature (rodents and dogs). In humans and rats, CA undergoes facilitated diffusion to gain intracellular access via nucleoside transporters within the cell membrane (Mulder 1975, Groothuis 2000). It is then phosphorylated to CA-monophosphate via deoxycytidine kinase, undergoes further phosphorylation to CA-diphosphate via monophosphate kinase, and then CA-triphosphate via nucleoside diphosphate kinase, which is the biologically active and predominant intracellular form (Mulder 1975, DeAngelis 1992). The active form is then incorporated into DNA, causing inhibition of DNA polymerase. It is metabolized primarily in the liver via deamination by deoxycytosine deaminase to its biologically inactive form, uracil arabinoside (UA) (Mulder 1975, DeAngelis 1992). UA undergoes renal excretion over the following 24 hours, which occurs more rapidly from the plasma than from CSF (DeAngelis 1992). The mean plasma and CSF elimination half-life reported in healthy dogs is 64 and 165 minutes, respectively (Scott-Moncrieff 1991). These data available regarding elimination of CA in dogs is in agreement with previously published data in humans, indicating that CA is eliminated more rapidly from the plasma than CSF (Mulder 1975). Whether CA is time or concentration dependent in dogs remains unknown.
2.3 INVESTIGATION OF CYTOTOXIC EFFECTS

There are no studies available in the literature evaluating pharmacodynamic end points of CA in dogs with inflammatory brain disease. An in vitro assessment of CA’s cytotoxicity has been performed utilizing healthy mouse and human bone marrow colony forming cells in addition to human leukemic colony forming cells obtained from the marrow and peripheral blood cells of patients with chronic myeloid leukemia (Greenberg 1976). This study demonstrated both time- and concentration dependency of CA in rodent cell lines, and concentration-dependency in leukemic human cell lines. When mouse bone marrow granulocyte progenitor cells (GPC) were exposed to CA for a shorter duration of time (1 and 4 hours), cytotoxicity increased with increasing dosages. Ultimately GPC survival plateaued once CA concentrations were 10 µg/mL. These data suggested that CA activity is concentration dependent in the mouse. These cells were then exposed to 10 µg/mL of CA for a longer duration of time (8 hours), and a statistically significant increase in GPC cytotoxicity was found. The mouse GPC were also exposed to a relatively low concentration of CA (0.01-0.1 µg/mL) over an extended duration of time (10-day period) and markedly decreased cell survival was noted beyond what was seen with other exposure periods. This demonstrates some time-dependent properties of the drug in this species as well. When evaluating healthy human marrow, a dose-related decreases in GPC was found when incubated with 0.01, 0.1, 1.0 and 10 µg/mL CA for both 1 and 4-hour periods. There was no significant difference between the duration of CA exposure and GPC death. When evaluating human leukemic colony forming cells similar dose-related cytotoxic effects were seen, but increased cytotoxicity was found in the leukemic cell population compared to the healthy cells when a shorter exposure period (1-hour) to a higher concentration of CA (10 µg/mL) was applied. Overall, higher dosages were needed to achieve cytocidal activity when shorter periods
of exposure were utilized, whereas lower dosages were needed to achieve the same cytotoxic
activity when longer drug exposure was implemented. The authors thus recommended
administration of CA via a continuous rate infusion at a low-dose or as intermittent high dose
therapy.

Interestingly, a different study found that CA was predominantly time-dependent rather
than concentration dependent in healthy human marrow (Raijmakers 1985). When normal human
bone marrow cells were incubated with increasing concentrations of CA (up to 10^{-5} M) for one
hour, granulocyte-macrophage colony forming unit (CFU-GM) growth was not even decreased
by 50\% when compared to control cultures. As the time of incubation was increased to 24 hours,
there was a significant reduction in the number of CFU-GM when compared to control cultures.
Furthermore, the inhibitory concentration of CA reducing the number of colonies to 50\% of the
control culture (IC_{50}) became greater than 2 logs lower after 5 days of incubation when
compared to 24 hours of incubation. The rate of CA deamination at different cell concentrations
was also evaluated. After a 5-day incubation of 10^6 cells/mL and 10^5 cells/mL, 0\% and 90\% of
CA remained detectable in the supernatant, respectively. The reduced deamination of CA with 5-
day incubation of 10^6 cells/mL was suspected to be related to an increased rate of deamination at
this concentration, resulting decrease in efficacy (reduced exposure). Based on these data, CA
was found to have time dependent cytotoxicity in human bone marrow cells.

Very few reports have been published evaluating the efficacy of CA at a cellular level in
dogs. In 2014, Pawlak et al. evaluated the effect of common anticancer agents on induction of
apoptosis in canine lymphoma and leukemia cell lines, including CA (Pawlak 2014). In the
tested B-cell lymphoma (CLBL-1) and B-cell (GL-1) and T-cell leukemia (Jurkat) cell lines, CA
demonstrated a high efficacy as determined by the percentage of apoptotic/dead cells following
72 hours of incubation with CA (1 µg/mL). This was most notable in CLBL-1. This research group performed another *in vitro* study, which more specifically evaluated the sensitivity of canine lymphoma cells to CA (Pawlak 2016). They found that CA had moderate efficacy (decrease in viable lymphocytes) when incubating canine lymphoma cells in vitro with 1 µg/mL of CA over 72 hours. It is important to bear in mind that neoplastic cells can exhibit altered chemosensitivity and pharmacodynamic properties (Pawlak 2016). Thus, these results may not accurately predict CA activity against the non-neoplastic inflammatory cells that mediate MUE, which has yet to be investigated.

Based on the data available, it appears that CA exhibits both time- and concentration-dependent cytotoxic properties with significant species variation. Further *in vitro* and *in vivo* studies are indicated to better evaluation pharmacodynamic end points in dogs with inflammatory brain disease.

2.4 APPLICATION TO CANINE PATIENTS

CA is a frequently used antineoplastic agent indicated in human patients for treatment of lymphoma and acute myeloid leukemia. It has been utilized in canine patients as treatment for various forms of lymphoma (including those with bone marrow or CNS involvement or cases of relapse), leukemia, and optic neuritis of non-infectious origin (Alvarez 2006, Bedos 2020, Gillem 2015, LaRue MK 2018, Marconato 2008, Pawlak 2014, Pawlak 2016). It has become an emerging treatment option for meningoencephalomyelitis of unknown etiology (MUE) in dogs due to favorable survival data. The earliest report of CA being used for treatment of MUE was published in 2002. Nuhsbaum *et al.* described CA being administered subcutaneously (50 mg/m²) every 12 hours for 48 hours to an 8-year-old female spayed Shih Tzu with
granulomatous meningoencephalitis (Nuhsbaum 2002). The dog was sent home with prednisone (2 mg/kg every 24 hours tapered over 4 weeks) and continued to receive CA in this fashion every 3 weeks. The dog was still alive 12 months after her diagnosis.

CA was also associated with a favorable prognosis in a prospective study utilizing it as treatment in combination with prednisone in 10 dogs (Zarfoss 2006). In this prospective study, partial to complete remission was found in all dogs upon initiation of prednisone (1-2 mg/kg every 12 hours tapered over 3 months) and CA (50-100 mg/m² subcutaneously every 12 hours for 48 hours). CA was administered in this fashion every 3 weeks for 4 months, subsequently increasing the interval between treatments by 1 week every 4 months. The median survival time reported was 531 days (range 46-1025 days) (Zarfoss 2006). These findings are suggestive of prolonged survival times being associated with this combination of drugs.

Prolonged survival was also reported when retrospectively evaluating a population of dogs that were treated with a similar protocol after being diagnosed with MUE (Menaut 2008). All 11 dogs received prednisolone (1-2mg/kg every 12 hours tapered over 3 months) and CA (50 mg/m² subcutaneously every 12 hours for 48 hours). CA was initially administered every 4 weeks, increasing the interval between treatments by 1 week every 4 weeks if possible (Menaut 2008). In this population of dogs, 58% were still alive 2 years after their initial diagnosis, with survival times ranging from 78-603 days (Menaut 2008). Subsequently, another study aimed to retrospectively evaluate an intravenous protocol. In this 2009 study, 7 dogs received prednisolone (40 mg/m² every 24 hours tapered over 8 weeks) and CA (100 mg/m² intravenously over 24 hours) (Smith 2009). This protocol was also associated with a positive outcome for many patients, with a mean survival time of 1,063 days. Furthermore, 71% of dogs were alive 12
months following diagnosis. In Zarfoss’s, Menaut’s, and Smith’s study populations, no signs of
myelosuppression nor other adverse effects were noted when administering CA to dogs.

Due to its lack of adverse effects and reported favorable outcomes, CA continued to be
used in dogs for treatment of MUE. In 2016, Lowrie and colleagues performed the first
investigation of the effect of a CRI of CA on mortality in dogs with MUE (Lowrie 2016). It was
discovered that CA administered as a CRI (100 mg/m$^2$ over 24 hours once) following initial
diagnosis of MUE provided a survival advantage over initial treatment of CA as repeated SC
injections (50 mg/m$^2$ every 12 hours over 48 hours). Survival data was obtained 3 months
following diagnosis, and 90% of dogs receiving the CRI protocol remained alive at 3 months
while only 44% of dogs receiving the SC protocol had survived. A more recent study was able to
provide a significantly longer follow-up period following administration of CA upon initial
diagnosis. When CA was administered as a CRI (100 mg/m$^2$ over 24 hours) to dogs newly
diagnosed with MUE, prolonged survival times were noted (Stee 2020). Due to the number of
dogs still alive at the time of writing, the median survival time was found to be $>1095$ days. In
this patient population, survival was not significantly different between those dogs that received
a single CRI of CA or those that received the CRI in addition to a continued SC protocol (50
mg/m$^2$ every 12 hours over 48 hours). With that being said, the relapse rate was high in both
groups, with 60% of the dogs requiring a low dose of prednisone long term (Stee 2020). This
begs to question whether a different CA protocol of repeated SC injections could provide
increased efficacy compared to the protocol implemented at that time. While CA continues to be
a commonly utilized treatment option for MUE survival times well surpassing prednisone or
prednisolone alone, its protocol of administration has yet to be standardized.
2.5 ADVERSE EFFECTS

While CA is typically well tolerated, adverse effects have been reported in veterinary patients receiving this medication. In one of the first studies investigating CA’s ability to cross the blood-brain barrier in dogs, it was administered at a dose of 600 mg/m² intravenously (Scott-Moncreiff 1991). While this dosage is quite high compared to what is currently recommended for dogs being treated for MUE, the only hematologic abnormality reported was mild thrombocytopenia in 3 of the 6 dogs when administered as an IV bolus. No hematologic abnormalities were noted in dogs who received this dose as a CRI. A more recent study investigating the use of CA in dogs with refractory lymphoma found that dogs treated with a combination of carboplatin and CA were significantly more likely to develop neutropenia and thrombocytopenia than those treated with carboplatin alone (Gillem 2015). While CA certainly has the propensity to cause cytopenias, it is also possible that the myelosuppressive effects were compounded given that none of the dogs received solely CA. In both of studies, the cytopenias reported were mild and not life-threatening (Scott-M 1991, Gillem 2015).

Other reports have noted other rare adverse effects starting to be reported in dogs. Some of these adverse effects were related to the route of administration, including severe calcinosis cutis and deep pyoderma present at the interscapular injection site and seizures following intrathecal administration (Volk 2012 and Genoni 2016, respectively). They proposed etiology of calcinosis cutis in these cases was suspected to be related to the trauma of repeated injections or chemical injury to the skin in that region that might favor local mineral deposition, supporting rotation of the administration site in future protocols (Volk 2012). Seizures have been seen following intrathecal injection of CA; however, this cannot be conclusively linked to CA given that seizures are a known complication of administration of other agents intrathecally, such as
Finally, there has been a single case report of drug-induced infiltrative lung disease was diagnosed in a dog being treated for MUE with prednisone and CA (Hart 2016). Ultimately, this patient developed respiratory failure indicating ventilatory support, and was euthanized 24 hours following initiation of mechanical ventilation due to his static condition. The cause of pulmonary toxicosis was not identified in the case, but given the temporal association between CA exposure and the development of respiratory distress and pulmonary infiltrations (while excluding other causes of the dog’s clinical signs), drug-induced infiltrative lung disease was highly suspected (Hart 2016).

Overall, the most common adverse effects are cytopenias with rare reports of complications associated with the route of administration. With careful monitoring of laboratory work and attention to route of administration, CA can be administered safely to dogs.

2.6 PHARMACOKINETIC STUDIES AVAILABLE IN VETERINARY LITERATURE

Prior to utilizing CA in our canine population for treatment of intracranial disease, it would seem vital to verify that this medication can in fact cross the blood brain barrier to enter the central nervous system. Scott-Moncrieff and colleagues executed a study in 1991 that evaluated the PK properties of CA in the plasma and cerebrospinal fluid of dogs. In this study, a dose of 600 mg/m² was administered to 10 healthy beagles intravenously in the form of intravenous bolus dosing (6 dogs) or as a 12 hours intravenous infusion (4 dogs). It was found the CA crosses the blood brain barrier in dogs when given as an intravenous bolus or as a constant rate infusion with biphasic elimination (Scott-Moncrieff 1991).

Further studies on the PK properties of CA and various routes of administration have been emerging in the canine literature given its low side effect profile and application for
intracranial disease (inflammatory and neoplastic). One study evaluating a single SC injection (50 mg/ m²) and a CRI administered over 8 hours (200 mg/m²) found that the CRI administration produced steady state plasma concentrations of CA, however, this could not be determined following the SC protocol applied as only a single dose was administered (Crook 2013). The mean peak concentration achieved (C_{max}) was not significantly different with either administration route (SC C_{max} of 2.88 µg/mL and CRI C_{max} of 2.80 µg/mL) (Crook 2013). With similar C_{max} being achieved with both administration routes, further studies regarding alternative SC dosing protocols are warranted to determine if sustained plasma levels can be achieved in dogs via repeated SC injections.

After determining PK parameters of CA in healthy dogs, further studies were indicated in clinical patients. A prospective study enrolling 19 client owned dogs with MUE was performed to evaluate plasma and serum concentration of CA when administered as a CRI to dogs diagnosed with MUE. While the C_{max} of plasma reported was lower than that reported in healthy dogs (being 1.7 µg/mL as opposed to 2.8 µg/mL in Crook et al.’s 2013 study), all dogs exceeded suggested target plasma concentrations 1 µg/mL at time points 1 hour and 8 hours after initiation of treatment, indicative of consistent and prolonged exposure in the bloodstream (Early 2016).

While it is valuable to have verified that CA achieves its proposed target concentration when administered as a CRI, understanding the PK properties when administered SC to clinical MUE patients is equally as important when considering the benefits associated with receiving a SC injection as opposed to a CRI. In turn, a single SC dose of CA (50 mg/m²) was administered to 12 dogs with recently diagnosed with MUE, and plasma concentrations were measured over the course of 6 hours. In this study, as in other studies pertaining to MUE, all dogs were receiving varying doses of prednisone (0.5-2 mg/kg/day) as this continues to be the gold
standard. In all dogs, plasma CA measured above 1.0 µg/mL from 30 minutes (first time point) up until 120 minutes after administration, having a rapid absorption followed by immediate decrease in concentration (Pastina 2017). Knowing that CA reaches its target concentration for 120 minutes (2 hours) following administration subcutaneously, a dosing protocol involving every 2-hour administration to achieve the total dose typically administered as CRI may feasible. This conjecture, however, requires appropriate investigation in the PK properties of repeated SC injections of CA in clinical MUE patients.

A more recent investigation was performed evaluating repeated SC dosing protocols of CA, which was published after the current study had been designed and patient recruitment accomplished. In this 2019 publication, a standard 2-day protocol (50 mg/m² SC every 12 hour) was compared to two novel 1-day protocols (a single 200 mg/m² SC dose and two 100 mg/m² SC dose every 12 hours) in 4 client owned dogs previously diagnosed with MUE. All four dogs received each SC protocol, allowing a 21-day washout period in between (Jones 2019). The overall drug exposure for each of the three protocols was similar based on their area under the curve calculation. The authors proposed a recommended administration of CA as two doses of 100–200 mg/m², 12 hours apart. While this dosing protocol is more convenient than previously recommended protocols, it still requires a prolonged day in the hospital for the patient and potentially multiple hospital visits depending on the hours of operation of the hospital. Additionally, it would be prudent to evaluate these protocols and/or others with a larger sample population. Jones et al were the first to investigate the PK properties of SC administration of CA and paved the way for future investigation.
CHAPTER 3: PHARMACOKINETICS OF A NOVEL CYTOSINE ARABINOSIDE SUBCUTANEOUS PROTOCOL IN DOGS WITH MENINGOENCEPHALOMYELITIS OF UNKNOWN ETIOLOGY

3.1 INTRODUCTION

Meningoencephalomyelitis of unknown etiology (MUE) is a common cause of noninfectious inflammatory disease of the central nervous system (CNS) in dogs (Granger 2010, Mayousse 2017, Tiploid 1985). The term MUE is an umbrella term used to describe immune-mediated encephalitides and myelitis, including granulomatous meningoencephalitis (GME), necrotizing meningoencephalitis (NME), and necrotizing leukoencephalitis (NLE), SRMA, eosinophilic meningoencephalomyelitis (EME), greyhound nonsuppurative meningoencephalitis, and spinal-cord only meningo(myelo)litis. Meningoencephalomyelitis of unknown etiology (MUE) is an important disease in the field of veterinary medicine given the severity of neurological symptoms that can be seen at the time of diagnosis, its guarded prognosis, and the large financial investment required by owners for diagnostic testing and treatment. This disease is diagnosed based on history, clinical signs, advanced imaging findings, cerebrospinal fluid (CSF) analysis, and negative infectious disease testing in the absence of histopathology, which is required for definitive diagnosis. Neuroanatomic localization can be multifocal or focal with variable clinical signs such as changes in mentation, seizures, paresis, ataxia, vestibular signs, and cranial nerve deficits (Baroon 2015, Cornelis 2016, Granger 2010, Nuhsbaum 2002, Tiploid 1995, Vitale 2019).

While the underlying etiology of MUE remains unknown, a T-cell immune-mediated etiology is highly suspected (Vitale 2019). Immunosuppression has therefore been the target of treatment. Various immunomodulatory medications have been used in dogs with MUE, with the mainstay of treatment involving corticosteroids (Pakozdy 2008, Flegel 2008, Granger 2010). The
mean survival times reported for treatment with corticosteroids alone are reported in Table 1. Emerging studies have demonstrated improved survival when multimodal treatment is implemented. This includes a corticosteroid with one or more of the following adjunctive medications: cyclosporine (Jung 2013, Behr 2009, Pakozdy 2009, Gnirs 2006, Adamo 2004), mycophenolate mofetil (Woolcock 2016, Baroon 2016), cytosine arabinoside (Lowrie 2016, Lowrie 2013, Granger 2010, Behr 2009, Smith 2009, Menaut 2008, Zarfoss 2006, Nuhsbaum 2002), lomustine (Flegl 2011), vincristine in combination with cyclophosphamide (Smith 2009), azathioprine (Wong 2010), procarbazine (Coates 2007), leflunomide (Sato 2017), along with radiation therapy (Beckmann 2015, Granger 2010). The mean survival times associated with multimodal treatment are on average longer than those reported with corticosteroids alone, ranging from 118-1834 days to 14-323 days, respectively. As these ranges imply, mean survival times are extremely variable. Additionally, these data are extrapolated from a limited number of studies, all of which have fairly small sample sizes. While a wide variety of treatment options exists, there is yet to be a standardized protocol recommended for these patients.

Given that cytosine arabinoside (CA) has been associated with favorable survival times and little to no systemic side effects in dogs with MUE (Arnold 2017, Lowrie 2016, Menaut 2008, Zarfoss 2006, Nuhsbaum 2002), this medication has been used more frequently in practice. Many different dosing protocols using CA exist including administration via subcutaneous (SC) injection or a constant rate infusion (CRI). A previous study demonstrated a significantly higher 3-month survival rate in dogs that had received CA via a CRI (100 mg/m² over 24 hours) for their initial treatment when compared to SC administration (Lowrie 2016). For this reason, administration of CA via CRI for initial dosing has been widely used amongst veterinary neurologists for treatment of MUE in dogs. Administering a CRI, however, comes with
additional financial cost to the client, longer duration of hospitalization for the patient, and repeated intravenous catheterization, making subsequent treatments more uncomfortable and physically challenging to administer. Subsequent treatments with CA are often administered via SC injections with no standardized protocol established. While there have been studies evaluating the pharmacokinetic properties of CA when administered as a CRI in healthy dogs and those with MUE (Crook 2012, Early 2016), the pharmacokinetic properties of SC administration of CA has only recently begun to be evaluated (Pastina 2018, Jones 2019). Additionally, there is very limited data pertaining to the pharmacokinetic properties of repeated SC dosing (Jones 2019).

The objective of this prospective study was to investigate the PK properties of CA when administered as repeated SC injections over 8 hours to dogs with MUE in order to determine if this amenable administration protocol is a viable alternative to more laborious CRI administration. The primary objective was to describe the PK parameters of CA when administered SC over 8 hours and when administered as a CRI over 24 hours. The secondary objectives were to determine if CA’s proposed therapeutic target of 1 µg/mL is achieved with both routes of administration, and if plasma levels exceed this target throughout the time course administered. Finally, short-term outcome of each patient receiving this protocol were reported (survivor or death/euthanasia) and classified based upon how many immunomodulatory medications they are receiving concurrently with CA. We hypothesize that both routes of administration would result in CA concentrations that meet or exceed its proposed therapeutic target concentration at 1 hour and 8 hours following initiating of treatment, making this novel SC protocol a viable alternative to the CRI protocol. Furthermore, we hypothesize that most dogs receiving this protocol would have a favorable short-term outcome.
3.2 MATERIALS AND METHODS

3.2.1 Animals

To be considered for enrollment, dogs had to have either magnetic resonance imaging (MRI) and/or cerebrospinal fluid (CSF) analysis compatible with inflammatory disease, pertinent negative infectious disease testing, and weigh at least 2.5 kilograms. Magnetic resonance imaging findings compatible with MUE include multiple, single, or diffuse intra-axial hyperintense lesions on T2-weighted (T2W) MR images based on proposed guidelines for diagnosis of MUE when histopathology is absent (Granger, 2010). Cerebrospinal fluid analysis compatible with MUE included hypercellularity with >50% mononuclear cells based on proposed guidelines for diagnosis of MUE when histopathology is absent (Granger, 2010). Infectious diseases testing was performed on a case-by-case basis depending upon the patient’s history and risk of exposure. This included testing for Blastomyces dermatitidis, Neospora canis, Toxoplasma gondii, Canine Distemper Virus, and Rickettsial disease (Ehrlichia, Anaplasma, Bartonella, Borrelia, and Rickettsia).

All patients underwent a physical and neurological exam performed by a board-certified veterinary neurologist or a resident in veterinary neurology supervised by a boarded-certified neurologist. Weight, age, breed, gender status, duration of clinical signs prior to diagnosis, and current medications were recorded in the medical record, along with the findings from the neurological exam and the associated anatomic neurolocalization. All physical examination findings were recorded, and patients were excluded from the study if abnormalities present contraindicated sampling line placement or frequent handling. All patients enrolled had a CBC and serum chemistry performed to exclude concurrent metabolic disease.
3.2.2 Experimental design

A non-randomized crossover study was performed over a one-month period. Dogs diagnosed with MUE all initially received a standard CRI protocol of CA intravenously over 24 hours (200 mg/m²) following diagnosis and were discharged to their owners with instructions to receive prednisone or prednisolone (1-2 mg/kg/day orally). Following initial treatment with the CRI, dogs returned 4 weeks later for a novel SC protocol of CA administration over 8 hours (50 mg/m² every 2 hours). Each dog underwent plasma drug monitoring for the 24 hours following initiation of each CA administration protocol (CRI and SC).

3.2.3 Drug administration and sample collection.

For the CRI administration, a triple-lumen centrally inserted venous catheter (CVC) or peripherally inserted venous catheter (PICC) was placed via the previously described modified Seldinger technique under sedation for the purpose of both CA administration and sample collection, utilizing different ports for drug administration and sample collection (Portillo, 2006). Ports utilized for sample collection were flushed with 1 mL of saline to eliminate the possibility of any residual CA being present within the port. Then, 3 mL of blood was withdrawn prior to sample collection and replaced following sample collection. Finally, the sampling port was flushed with 1 mL of heparinized saline and occluded to prevent any retrograde flow between sampling time points. Dogs were sedated with up to two of the following medications for CVC or PICC placement: dexmedetomidine 3-5 mcg/kg intravenously (IV) (4/8), butorphanol 0.20-0.40 mg/kg IV (2/8), methadone 0.20-0.25 mg/kg IV (3/8), hydromorphone 0.10 mg/kg IV (2/8), and fentanyl 3 mcg/kg IV (1/8). All dogs were then administered CA at 200 mg/m². Blood samples (1 mL/sample) were obtained at time 0 (before), 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20,
and 24 hours after initiating treatment the CRI and SC protocols. Immediately following collections, samples were placed into lithium heparin tubes and stored in a standard refrigeration unit (4°C). Within 1 hour of collection, samples were centrifuged at 20,000g for 15 minutes, and plasma stored at -80°C until analysis.

For the SC administration, the same protocol was used to place a CVC or PICC in each patient. Dogs were sedated with up to two of the following medications for CVC or PICC placement: dexmedetomidine 3-7 mcg/kg IV (8/8), midazolam 0.20 mg/k IV (1/8), butorphanol 0.30-0.40 mg/kg IV (2/8), methadone 0.20-0.25 mg/kg IV, (4/8), hydromorphone 0.1 mg/kg IV (2/8), and alfaxalone 2 mg/kg IV (1/8). All dogs received CA 50 mg/m² SC every 2 hours for a total of 4 doses. Sample collection was the same as for the CRI administration.

3.2.4 Plasma analysis

Canine plasma samples were analyzed via high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS) in a similar fashion as previous CA pharmacokinetic studies (Crook, 2012; Early, 2016; Pastina, 2018). Samples were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Framingham, MA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Software Analyst 1.7.1 was used for data acquisition and analysis. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent SB-Aq column (4.6 x 50mm, 5μm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.3 mL/min. The linear gradient was as follows: 0-3 min, 100% A; 10-12 min, 2% A; 12.1-17 min, 100% A. The autosampler was set at 10°C. To prepare samples for injection, 30 μL plasma
samples were rigorously mixed with 10 µL 1.2 µg/mL 2-Cl-2'-deoxyadenosine and 60 µL methanol. The resulting supernatant after centrifugation (1,500 g x 10 min) was subject to the instrument analysis injection. The injection volume was 5 µL. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage was +5500 V. The source temperature was 450°C. The curtain gas, ion source gas 1, and ion source gas 2 were 33, 65, and 55 psi, respectively. Multiple reaction monitoring (MRM) was used for quantitation: Cytarabine m/z 244.1 → m/z 112.0; internal standard 2-Cl-2'-deoxyadenosine m/z 286.0 → m/z 117.0. The limit of quantitation was 2.5 ng/mL. The method of validation and stability is provided in Supplemental Materials.

3.2.5 Outcome assessment

For each dog, outcome was assigned 3-months following initiation of treatment with CA, as outcome at this time-point has been associated with favorable long-term survival in previous studies (Lowrie, 2013; Smith, 2009). Outcome was assessed via conversation with the owner in person or over the phone, phone conversation with the referring veterinarian, or upon review of the medical record. Possible outcomes included: survived-in remission, survived-currently receiving 1 oral immunomodulatory medication (prednisone or prednisolone), survived-currently receiving 2 or more oral immunomodulatory medications, or died/euthanized.

3.2.6 Statistical analysis

Descriptive statistics were used to report age, breed, sex, and weight. Continuous data are reported as median (range). For both the IV and SC protocols, pharmacokinetic parameters were calculated including maximum plasma concentration (C_{max}), observed area under the curve
(AUC₀-₂⁴) using the trapezoidal method, plasma concentrations at 1 h and 8 h after initiating treatment (C₁ and C₈, respectively), and duration of time plasma concentration exceeded 1 µg/mL (T>₁). Time to Cₘₐₓ (Tₘₐₓ) was calculated for the SC protocol only. Wilcoxon sign rank tests were performed to compare Cₘₐₓ, C₁, C₈, and T>₁ between the CRI and SC protocols. McNemar's tests were used to compare the number of dogs that achieved plasma concentrations greater than 1 µg/mL at 1 and 8 h following CA administration between the two administration protocols. Because the sampling period for the CRI protocol did not include the terminal phase of the time-concentration curve, comparison of AUC between IV and SC protocols were not performed. Statistical comparisons were performed using commercial software (Prism 8; GraphPad Software) with significance set at \( P < 0.05 \).

### 3.3 RESULTS

A total of 10 dogs were recruited for enrollment in the study between February 2019 and December 2019 based on history, presenting complaint, unremarkable blood work, MRI findings, and CSF analysis suggestive of antemortem diagnosis of MUE. Two patients were excluded for the following reasons: in one patient, the finalized CSF analysis was not consistent with inflammatory disease and another patient was euthanized prior to completion of the study protocol due to clinical decline requiring mechanical ventilation. Therefore, a total of 8 dogs met the inclusion criteria for this study.

The median age and weight at presentation was 4.90 (range: 1-10) and 13.20 kilograms (range: 2.50-35.10), respectively. The median duration of clinical signs prior to presentation was 45.80 days (range: 1-257). Demographic and 3-month follow-up information are reported in Table 3. Three of the 8 dogs had previously received glucocorticoids (prednisone or
prednisolone) prior to presentation. Upon antemortem diagnosis of MUE, 2/8 were treated with clindamycin while awaiting negative infectious disease testing results. All patients received oral glucocorticoids (prednisone or prednisolone) at a dosage of 1-2 mg/kg/day. No other antimicrobials or antifungals were used. Various medications aside from glucocorticoids were utilized on a patient-by-patient basis given the clinical nature of this study, including levetiracetam (3/8), phenobarbital (1/8), clindamycin (2/8), methadone (1/8), gabapentin (4/8), pantoprazole (3/8), omeprazole (1/8), meclizine (1/8), and maropitant (1/8).

Time-concentration curves for CRI and SC CA administration are presented in Figure 1, and calculated PK parameters are present in Table 4 for both routes of administration. A summary of plasma concentrations at various time points in each dog for CRI and SC routes of administration in addition to other descriptive statistics may be found in the Supplemental Materials (Supplemental Table 1 and Supplemental Table 2, respectively). The $C_{\text{max}}$ for the SC protocol (3.40 µg/mL, range: 1.60-9.70 µg/mL) was significantly higher than for the CRI protocol (1.09 µg/mL, range: 0.77-1.67 µg/mL, $P = 0.02$), as demonstrated in Figure 2. Plasma CA concentration at 1 hour and 8 hours following initiation of treatment are demonstrated in Figure 3A and 3B, and duration above 1 µg/mL in Figure 3C. Plasma $C_1$ for the SC protocol was significantly higher (2.28 µg/mL, range: 0.97-2.67 µg/mL) than for the CRI protocol (0.01 µg/mL, range: 0-0.45 µg/mL, $P = 0.01$). Plasma $C_8$ for the SC protocol (1.83 µg/mL, range: 0.77-2.84 µg/mL) was also significantly higher for the CRI (0.74 µg/mL, range: 0.57-1.11 µg/mL, $P = 0.01$). $T_{\geq 1}$ was significantly higher for the SC protocol (9.25 hours, range: 4.50-10.50 hours) than for the CRI protocol (3.13 hours, 0-9.75 hours, $P = 0.03$). Significantly more dogs achieved plasma concentrations greater than 1 µg/mL one hour after administration of the SC protocol (7/8) than the CRI protocol (0/8, $P = 0.02$). Similarly, significantly more dogs
achieved plasma concentrations greater than 1 µg/mL eight hours after administration of the SC protocol (7/8) than the CRI protocol (1/8, \( P = 0.04 \)).

Five of 8 dogs were alive 3 months following their diagnosis. All five surviving dogs (5/8) were receiving the CA SC protocol every 4 weeks. Four surviving dogs (4/5) were receiving 1 oral immunomodulatory medication (IMM), and one was receiving more than 2 oral IMM (1/5). Two dogs were euthanized after completion of the study period due to lack of improvement in their clinical signs (2/8), and one was lost to follow-up (1/8). No adverse effects of CA previously reported in dogs receiving CA intravenously or subcutaneously, such as thrombocytopenia, neutropenia, calcinosis cutis, or drug-induced infiltrative lung disease, were noted in this study population (Gillem, 2015; Hart, 2016; Volk, 2012; Scott-Moncrieff, 1991).

3.4 DISCUSSION

This study demonstrates that when CA is administered via repeated subcutaneous injections over an 8-hour period, it achieves consistent and prolonged exposure in the plasma of dogs with MUE, exceeding 1 µg/mL at 1 hour and 8 hours after initiation of treatment. While there are many different protocols proposed for CA treatment in dogs with MUE, the investigated protocol was established based on the previous research documenting a single SC dose of CA (50 mg/m²) achieving sustained plasma levels for 2 hours following administration in dogs with MUE (Patina, 2017). Another study evaluating PK parameters between the different routes of administration in healthy dogs found that the SC route did not achieve steady-state concentrations, however, the time to reach its maximum plasma concentration was 1 hour with an elimination half-life of 1.15 hours (Crook, 2013). Similarly, plasma CA levels following CRI administration in MUE affected dogs display rapid absorption, exceeding 1 µg/mL within 30
minutes (Early 2016). Knowing that CA should theoretically reach its target concentration within 1 hour of administration and that plasma levels should be sustained for 2 hours following SC administration, a new dosing protocol involving every 2-hour administration to achieve the total dose typically administered as a CRI was conceptualized. In order to administer the recommended CRI dose of 200 mg/m² in 2-hour increments on an outpatient bases, a dog would indicate four 50 mg/m² SC injections, to total an 8-hour duration of treatment. Keeping these data in mind, a protocol of 50 mg/m² SC every 2 hours was proposed and investigated.

Previous pharmacokinetic studies have found that when administered as a CRI, CA achieves sustained plasma levels throughout administration, indicative of prolonged exposure in the blood of both healthy dogs and dogs with MUE (Crook, 2013; Early, 2016). In both of these reports, a minimum target concentration of 1 µg/mL CA was applied and has continued to be utilized as a threshold concentration for more recent investigations (Patina, 2018). Based on the duration of treatment prescribed by this protocol, we investigated plasma CA levels at 1 hour and 8 hours following initiation of treatment to ensure that the therapeutic target was met throughout the duration of treatment, as has been done in previous studies (Early 2016). In the present study, this minimum target concentration was achieved when using the SC protocol, with median $C_{\text{max}}$, $C_1$, and $C_8$ being 3.4 µg/mL (range: 1.60-9.70 µg/mL), 2.28 µg/mL (range: 0.97-2.67 µg/mL), and 1.83 µg/mL (range: 0.77-2.84 µg/mL), respectively. The median $C_{\text{max}}$ following CRI administration was significantly lower, measuring 1.09 µg/mL (range: 0.77-1.67 µg/mL). In fact, plasma CA levels in dogs receiving the CRI protocol often failed to reach the therapeutic target at 1 hour and 8 hours following initiation of treatment, measuring 0.01 µg/mL (range: 0-0.45 µg/mL) and 0.74 µg/mL (range: 0.57-1.11 µg/mL), respectively. When receiving the CRI protocol, none of the dogs achieved CA concentrations greater than 1 µg/mL at $C_1$ and only one
dog (1/8) did so at C₈, whereas nearly all dogs (7/8) achieved CA concentrations greater than 1 µg/mL at C₁ and C₈ for the SC protocol. The CRI protocol did, however, provide a longer duration of detectable CA in the plasma. It is unknown whether CA is time or concentration dependent in dogs, so the significance of this finding is unknown. If, for example, a longer duration of CA administration is associated with a prognostic benefit, this may make the investigated SC protocol less advantageous. Studies continue to use a minimum therapeutic target concentration that has been extrapolated from the human literature in which passive diffusion of CA becomes significant only at concentrations great than 1 µg/mL in sarcoma cells in vitro, and true therapeutic drug levels are unknown in dogs (Mulder, 1975). Based on previously used therapeutic targets, however, the SC protocol appears to reach superior concentrations for a longer duration of time when compared to the CRI protocol.

To the author’s knowledge, this is the first proposed CA protocol utilizing repeated SC injections that can be fully administered on an outpatient basis. A recently published SC protocol recommends a higher dose of CA (100-200 mg/m²) requiring 12 hours between treatments (Jones, 2019). This study also found that plasma CA concentrations are known to rise in a near-linear fashion with increasing doses of CA, although the significance of this is unknown as CA may be concentration or time dependent in dogs and requires further investigation. A dose of 50 mg/m² of CA over multiple treatment days (50 mg/m2 every 12 hours for 2 days) has been implemented anecdotally and in a previous study, which can present logistical and financial challenges from an owner perspective in addition to increased stress incurred to the patient (Lowrie, 2013). It is unknown if this type of prolonged drug exposure is associated with any degree of a prognostic benefit. It has been demonstrated that dogs with MUE receiving CA via a CRI over 24 (100 mg/m²) hours at the time of diagnosis have a survival advantage at 3 months.
compared to those receiving CA via a SC protocol (50 mg/m² every 12 hours) over 2 days (Lowrie, 2013). These historical findings support initial treatment with a CRI (as was performed in the present study at a dose of 200 mg/m²), however, subsequent treatments with an alternative protocol could be considered provided that minimum therapeutic targets continue to be achieved. We have demonstrated that repeated SC injections of 50 mg/m² administered every 2 hours over an 8-hour period maintains minimum therapeutic target of CA throughout its course of treatment. Repeated SC injections also have the benefit of being less invasive than a CRI, in that they do not require intravenous catheterization. Additionally, administration of treatment over an 8-hour period reduces stress to the patient and to the owner by decreasing the duration and number of hospital visits required in a short period of time. Finally, five dogs (5/8) remained alive 3 months following diagnosis and only one patient (1/8) required more than 1 oral immunomodulatory medication during the follow-up period, indicating that this protocol may be associated with a positive outcome. A larger study population, however, would be required to establish statistical significance. Considering previously published data and the data provided by the present study, the authors recommend the following CA administration protocol for dogs with MUE: CA 100-200 mg/m² administered via CRI at the time of diagnosis, followed by 50 mg/m² SC every 2 hours for 4 doses (to total 200 mg/m²) every 4 weeks.

3.5 LIMITATIONS

3.5.1 Lack of histopathologic diagnosis of MUE

This study is not without limitations. As is the case for many studies pertaining to MUE, his study is lacking histopathologic diagnoses. The authors applied previously established guidelines for diagnosis of MUE when histopathology is absent in order to maximize the
likelihood of a true positive diagnosis. In addition, so as to mirror a clinical setting, infectious
disease testing was performed as deemed necessary by individual clinicians based on the
patient’s history and the possibility of exposure to infectious agents. Infectious disease testing
was therefore not standardized, but determined on a case-by-case basis. While surgical biopsy
can be considered in these cases as well, doing so would increase morbidity and possibly
mortality of our patient population and likely would not provide a survival benefit. For this
reason, surgical biopsy was not recommended in any dogs in our study population. Necropsy was
performed on the one dog that was euthanized during the study period, however, confirming a
diagnosis of nonsuppurative (granulomatous) meningitis involving the brain and spinal cord.

3.5.2 Inclusion of myelitis and optic neuritis under the umbrella of MUE

Another potential limitation of this study is the inclusion of dogs with myelitis and optic
neuritis. All patients in our study met the following proposed guidelines as suggested by Granger
et al: older than 6 months of age with evidence of multifocal or diffuse CNS disease,
hypercellular CSF analysis with >50% mononuclear (monocytes/lymphocytes) cells, and
negative pertinent infectious testing. Notably, Granger et al. does not recommend inclusion of
patients with spinal cord MUE given the current lack of objective diagnostic criteria to
distinguish these cases from other myelopathies. In our cases diagnosed with spinal cord MUE
(3/9), antemortem diagnosis of MUE was extrapolated from Granger et al.’s aforementioned
proposed guidelines for noninfectious inflammatory meningoencephalitis given that this is how
this disease continues to be diagnosed in practice. Inclusion of patients with optic neuritis is also
not recommended when brain MRI and CSF analysis is normal (Granger 2010). However, the
one patient diagnosed with immune-mediated optic neuritis in this study had abnormalities noted
in the optic nerves bilaterally on their MRI in addition to a mononuclear pleocytosis upon CSF
analysis, suggestive of diffuse meningeal involvement. Certainly, there remains a strong
indication to continue investigating for an underlying etiology noninfectious inflammatory
immune-mediated diseases of the meninges, brain, spinal cord, and optic nerves.

3.5.3 Treatment order not randomized

The treatment order was not randomized due to the prognostic benefit documented in
dogs with MUE receiving the CRI protocol as their initial treatment (Lowrie, 2016). Based on
this and our institution’s current practices, it was therefore decided that all patients would receive
CA as a CRI first.

3.5.4 Other treatment modalities not standardized

Treatment modalities outside of CA and glucocorticoid dosages were not standardized in
the present study. Other treatment modalities would be nearly impossible to standardize as dogs
with MUE are treated on an individual basis dependent upon response to care, possibly having to
consider other systemic comorbidities, and often times having been started on some form of
treatment prior to presentation at a referral institution. It is therefore possible that antimicrobial
therapy, variable glucocorticoid dosages, anticonvulsants, analgesia, gastrointestinal support, and
individualized sedation protocols for central venous catheter placement could have altered the
hemodynamics and ultimately metabolism of CA in individual patients. This theoretically could
have altered the PK properties described, although previous studies have not documented any
significant difference in the PK properties of CA in clinically affected dogs receiving CA alone
in comparison to those receiving other treatments similar to those received in the present study
(Early, 2016).

3.5.5 Lack of collection of time points in the terminal phases of the time-concentration curve for the CRI protocol

One final limitation was a lack of collection of time points in the terminal phases of the time-concentration curve for the CRI protocol, impeding our ability to compare AUC between both protocols. This would have provided additional information pertaining to drug exposure and clearance but would not have altered the conclusions drawn in relation to maintenance of a minimum therapeutic target concentration with the designated times (1 and 8 hours).

3.6 PROJECT CONCLUSIONS

In conclusion, the investigated CA SC protocol achieved its proposed therapeutic target concentration in dogs with MUE. Interestingly, the therapeutic target was not reached when implementing the CRI protocol, warranting further investigation of minimum therapeutic targets in dogs with MUE as they may be different following SC and CRI administration. Despite the limitations present in this study, it demonstrates that repeated SC injections when given in 2-hour increments can maintain plasma concentration of CA exceeding its minimum therapeutic target throughout the duration of treatment. Additionally, the investigated protocol may also be given on an outpatient basis, thus reducing hospitalization time, the iatrogenic trauma associated with repeated catheterization, and client cost when compared to other administration protocols. Further study of a larger population of dogs with MUE is needed to assess effects on outcome, ideally with more standardized medication protocols, and to determine if a different target concentration or duration of treatment would provide therapeutic benefit.
3.7 FUTURE DIRECTIONS

3.7.1 Effect of proposed protocol on outcome

Further study of a larger population of clinically affected dogs is needed to assess effects on outcome. While 5 of the 8 dogs treated in this study were alive 3 months following initiation of the investigated CA protocol, this is a fairly small sample size.

3.7.2 Effect of higher target concentrations on outcome

It has now been documented that plasma CA concentrations rise in a nearly linear fashion as dosage increases; however, drug exposure nor incidence of adverse effects seem affected (Jones, 2019). Additionally, even receiving dosages 3 times what is typically used in practice, dogs have had no clinically significant adverse effects reported (Scott-Moncrieff, 1991). Further study investigating the effect on outcome when implementing various dosages of CA when administered as a CRI (for example 100 mg/m2 compared to 200 mg/m2 at the time of diagnosis) and when subsequently administered as intermittent SC injections (for example 50 and 100 mg/m2 every 2 hours for 4 total doses) would be indicated to provide more precise recommendations for administration to dogs with MUE.
**Table 1**: Mean survival time (MST) in dogs with MUE receiving glucocorticoids (GCC) alone.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diagnosis</th>
<th>MST (Days)</th>
<th>Sample Size (n)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone or prednisolone 1 mg/kg PO q12h</td>
<td>MUE</td>
<td>602</td>
<td>16</td>
<td>Mercier &amp; Barnes Heller 2015</td>
</tr>
<tr>
<td>Prednisolone 0.6-1.4 mg/kg/day PO (GME) 1-2.1 mg/kg/day PO (NE)</td>
<td>GME NE</td>
<td>323 91</td>
<td>11</td>
<td>Flegel et al 2011</td>
</tr>
<tr>
<td>Prednisolone 5-30 mg/kg/day PO (6/7 dogs) Dexamethasone 1 mg/kg/day PO (1/7 dogs)</td>
<td>Suspect GME*</td>
<td>28</td>
<td>7</td>
<td>Pakozdy 2009</td>
</tr>
<tr>
<td>Prednisolone 1 mg/kg PO q12h</td>
<td>NME</td>
<td>58</td>
<td>7</td>
<td>Jung 2007</td>
</tr>
</tbody>
</table>


* No histopathology available.
### Table 2: Mean survival time (MST) in dogs with MUE treated receiving multimodal therapy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diagnosis</th>
<th>MST (Days)</th>
<th>Sample Size (n)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCC + azathioprine</td>
<td>MUE</td>
<td>1834</td>
<td>40</td>
<td>Wong <em>et al</em> 2010</td>
</tr>
<tr>
<td>GCC + cyclosporine</td>
<td>GME, NME</td>
<td>240-930</td>
<td>7</td>
<td>Pakozdy <em>et al</em> 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>Gnirs 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>Jung 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>Adamo &amp; O’Brien 2004</td>
</tr>
<tr>
<td>GCC + cytosine arabinoside</td>
<td>MUE</td>
<td>26-531</td>
<td>80</td>
<td>Lowrie 2016†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>39</td>
<td>Lowrie <em>et al</em> 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>Smith <em>et al</em> 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td>de Stefani <em>et al</em> 2007†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>Menaut <em>et al</em> 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zarfoss <em>et al</em> 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCC + leflunomide</td>
<td>MUE</td>
<td>Unavailable</td>
<td>5</td>
<td>Gregory <em>et al</em> 1998†</td>
</tr>
<tr>
<td>GCC + lomustine</td>
<td>GME, NME</td>
<td>457, 329</td>
<td>14</td>
<td>Flegel <em>et al</em> 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25</td>
<td>Woolcock <em>et al</em> 2015</td>
</tr>
<tr>
<td>GCC + procarbazine</td>
<td>Suspect GME</td>
<td>420</td>
<td>21</td>
<td>Coats <em>et al</em> 2007</td>
</tr>
<tr>
<td>GCC + vincristine + cyclophosphamide</td>
<td>MUE</td>
<td>Unavailable</td>
<td>8</td>
<td>Smith <em>et al</em> 2009*</td>
</tr>
<tr>
<td>GCC + radiation therapy</td>
<td>MUE</td>
<td>476</td>
<td>6</td>
<td>Beckmann <em>et al</em> 2015</td>
</tr>
<tr>
<td></td>
<td>GME</td>
<td>14</td>
<td>30</td>
<td>Muñana &amp; Luttgen 1998</td>
</tr>
</tbody>
</table>


† 59/80 dogs alive 3 months following diagnosis; 100% (59/59) survived to 12 months.

1 6/9 alive at follow-up (range: 4-30 months).

‡ All alive at follow-up (range: 4-11 months).

* 4/10 dogs survived >365 days.
Table 3: Signalment, presenting complaint, duration of clinical signs, neuroanatomical localization, and diagnostic findings (MRI and CSF analysis) of the study population.

<table>
<thead>
<tr>
<th>Signalment</th>
<th>Presenting Complaint</th>
<th>Duration of Signs (days)</th>
<th>Neuroanatomic Localization</th>
<th>3-month Follow Up</th>
</tr>
</thead>
<tbody>
<tr>
<td>5y FS Schnauzer</td>
<td>Nonambulatory tetraparesis, neck pain</td>
<td>45</td>
<td>C1-T2 myelopathy</td>
<td>Euthanized</td>
</tr>
<tr>
<td>2y MC Schnauzer</td>
<td>Nonambulatory tetraparesis, obtunded</td>
<td>45</td>
<td>Cerebrothalamic</td>
<td>Survived, 1 oral IMD*</td>
</tr>
<tr>
<td>5y FI Shih Tzu</td>
<td>Status epilepticus, obtunded</td>
<td>4</td>
<td>Cerebrothalamic</td>
<td>Survived, ≥2 oral IMD*</td>
</tr>
<tr>
<td>5y MC Toy Fox Terrier</td>
<td>Blindness</td>
<td>50</td>
<td>Pre-chiasmatic optic nerve OD±OS vs. central blindness</td>
<td>Survived, 1 oral IMD*</td>
</tr>
<tr>
<td>3y MC Boxer</td>
<td>Nonambulatory paraparesis</td>
<td>257</td>
<td>T3-S3 myelopathy</td>
<td>Euthanized</td>
</tr>
<tr>
<td>10y FS Mixed Breed</td>
<td>Nonambulatory tetraparesis, facial droop</td>
<td>3</td>
<td>Brainstem and cerebellum</td>
<td>Information unavailable</td>
</tr>
<tr>
<td>1y FI Yorkshire Terrier</td>
<td>Nonambulatory tetraparesis</td>
<td>1</td>
<td>C1-T2 myelopathy</td>
<td>Survived, 1 oral IMD*</td>
</tr>
<tr>
<td>6y FS Maltese</td>
<td>Tetraparesis, seizure-like activity</td>
<td>150</td>
<td>Cerebrothalamic, brainstem, C1-T2 myelopathy</td>
<td>Survived, 1 oral IMD*</td>
</tr>
</tbody>
</table>

*IMD = immunomodulatory drug
Table 4: Calculated pharmacokinetic parameters for cytosine arabinoside when administered via novel SC protocol and when administered as a CRI, presented as median values with associated range.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>SC protocol</th>
<th>CRI protocol</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>$\mu g/mL$</td>
<td>3.40</td>
<td>1.60-9.70</td>
<td>1.09</td>
</tr>
<tr>
<td>AUC$_{0-24}$</td>
<td>h·$\mu g/mL$</td>
<td>23.54</td>
<td>9.26-31.13</td>
<td>15.74</td>
</tr>
<tr>
<td>$C_1$</td>
<td>$\mu g/mL$</td>
<td>2.28</td>
<td>0.97-2.67</td>
<td>0.014</td>
</tr>
<tr>
<td>$C_8$</td>
<td>$\mu g/mL$</td>
<td>1.83</td>
<td>0.77-2.84</td>
<td>0.74</td>
</tr>
<tr>
<td>$T_{&gt;1}$</td>
<td>h</td>
<td>9.25</td>
<td>4.50-10.5</td>
<td>3.13</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>h</td>
<td>7.00</td>
<td>5.00-7.00</td>
<td>‡</td>
</tr>
</tbody>
</table>

$C_{\text{max}}$, peak plasma concentration; $C_1$, plasma concentration 1 hour following initiation of treatment; $C_8$, plasma concentration 8 hours following initiation of treatment; $T_{>1}$, time plasma concentration was above 1 $\mu g/mL$; N/A, not applicable.

† Did not compare AUC between protocols due to lack of terminal phase of time-concentration curve for the CRI protocol. P-value not applicable.
‡ Did not calculate $T_{\text{max}}$ for the CRI protocol, making P-value not applicable.
**Figure 1:** Plot of mean plasma cytosine arabinoside concentration (CA) time course in dogs with MUE (n=8) for 24 hours following initiation of a standard CRI protocol and a novel SC protocol. Error bars represent standard deviation.
**Figure 2:** Plot of peak cytosine arabinoside (CA) concentrations in dogs with MUE (n = 8) during administration of a standard CRI protocol and a novel SC protocol. Horizontal bars represent the mean.
Figure 3: Plot of plasma CA concentration at 1 hour (a) and 8 hour (b) following initiation of treatment, and time that plasma concentration was measured to be > 1 µg/mL (c) in dogs with MUE (n = 8) during administration of a standard CRI protocol and a novel SC protocol.

Horizontal bars represent the mean.
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APPENDIX A: ASSAY VALIDATION

The quantitative LC/MS/MS method was validated with 3 control samples (15 ng/mL, 300 ng/mL, and 3000 ng/mL) for consistency of results and extraction efficiency of the assay. Stability of cytosine arabinoside in plasma during storage was assessed by use of control plasma with spiked standards (cytosine arabinoside at 15 ng/mL, 300 ng/mL, and 3000 ng/mL). The spiked samples were stored at 10°C for 72 hours. These samples were processed on the day of testing and run together with samples extracted from control plasma spiked with freshly prepared standards. Between-run accuracy (expressed as % nominal value ± standard deviation) was within 9% of the expected concentrations, measuring 92.4 ± 6.1, 91.0 ± 3.0, and 93.8 ± 2.0 for 15 ng/mL, 300 ng/mL, and 3000 ng/mL control samples, respectively. Within-run precision (expressed as relative standard deviation over the expected concentrations) was 6.7, 0.3, and 0.1 for 15 ng/mL, 300 ng/mL, and 3000 ng/mL control samples, respectively. Between-run precision (expressed as relative standard deviation over the expected concentrations) was 1.8, 0.1, and 0.1 for 15 ng/mL, 300 ng/mL, and 3000 ng/mL control samples, respectively.
### APPENDIX B: RAW DATA TABLES

**Raw Data Table 1.** CRI plasma [CA] in dogs with MUE, including mean, standard deviation (SD), standard error (SE), and 95% confidence interval (CI) for each sampling time point.

<table>
<thead>
<tr>
<th>t(h)</th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Dog 3</th>
<th>Dog 4</th>
<th>Dog 5</th>
<th>Dog 6</th>
<th>Dog 7</th>
<th>Dog 8</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.14</td>
<td>0.09</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.05</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>1</td>
<td>0.45</td>
<td>0.02</td>
<td>0.00</td>
<td>0.35</td>
<td>0.01</td>
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<td>0.00</td>
<td>0.00</td>
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<td>0.46</td>
<td>0.00</td>
<td>0.49</td>
<td>0.11</td>
<td>0.61</td>
<td>0.00</td>
<td>0.13</td>
<td>0.26</td>
<td>0.24</td>
<td>0.08</td>
<td>0.20</td>
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<td>0.68</td>
<td>0.00</td>
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<td>0.54</td>
<td>0.54</td>
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<td>0.69</td>
<td>0.02</td>
<td>1.16</td>
<td>0.65</td>
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<td>0.73</td>
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<td>0.74</td>
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<td>0.92</td>
<td>0.62</td>
<td>0.99</td>
<td>0.79</td>
<td>0.18</td>
<td>0.06</td>
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<td>0.72</td>
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<td>0.77</td>
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<td>0.89</td>
<td>0.89</td>
<td>0.60</td>
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† Data unavailable due to lack of samples at these time points
Raw Data Table 2. SC plasma [CA] in dogs with MUE, including mean, standard deviation (SD),
standard error (SE), and 95% confidence interval (CI) for each sampling time point.

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† Data unavailable due to lack of samples at these timepoints
‡ Data unavailable due to technical error during sample processing