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Gemcitabine Degradation in Whole Blood from Humans, Dogs, Cats, and Horses

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Abstract

The purpose of this *in vitro* study was to compare the degradation of gemcitabine (2', 2'-difluorodeoxycytidine, dFdC), in Fresh Whole Blood (FWB) from humans, dogs, cats, and horses. A better understanding of the comparative degradation of gemcitabine may aid in the optimal design of therapeutic regimens in veterinary species. Fresh whole blood from humans, dogs, cats, and horses was spiked with dFdC and plasma was analyzed for dFdC and 2', 2'-difluorodeoxyuridine (dFdU) by high performance liquid chromatography. In these species, there was an initial rapid degradation of dFdC with a concomitant proportional increase in dFdU. Degradation of gemcitabine appeared similar in humans, dogs, and horses ($p>0.05$) whereas metabolism was slower in the cat than human ($p=0.014$), dog ($p=0.010$), or horse ($p=0.0015$). Based on these in vitro findings, dosing schemes for humans, dogs, and horses may be similar. In contrast, gemcitabine degradation occurred more slowly in the cat; this difference may dictate a different dosing scheme for optimal response in this species.

Keywords: Gemcitabine; Equinel; Canine; Feline; Plasma catabolism

Abbreviations: CDA: Cytidine Deaminase; dFdC: 2',2'-difluorodeoxycytidine, gemcitabine; dFdCTP: Gemcitabine triphosphate; dFdU: 2',2'-difluorodeoxyuridine; FWB: Fresh Whole Blood; HPLC: High-Performance Liquid Chromatography; PCV: Packed Cell Volume(S)

Introduction

Gemcitabine (2', 2'-difluorodeoxycytidine, dFdC) is a synthetic deoxycytidine nucleoside analog of the pyrimidine antimetabolite cytosine arabinoside. [1] It structurally differs from deoxycytidine due to the geminal fluorine molecules on the 2' position of the furanose ring.[2] The main mechanism of action of gemcitabine is inhibition of DNA synthesis, which blocks the progression of cells through the G_1-S phase of the cell cycle.[1,3] There is influx of gemcitabine through the cell membrane by active nucleoside transporters and metabolism of gemcitabine occurs intracellularly with rapid conversion to the active metabolite gemcitabine triphosphate (dFdCTP) [1,3,4]. Accumulation of the triphosphate is balanced by metabolism of native gemcitabine [1,5,6].

In animals, gemcitabine pharmacokinetics is largely determined by deamination, which represents the main catabolic pathway. [7] Catabolism of gemcitabine occurs through deamination by cytidine deaminase (CDA) to difluorodeoxyuridine (dFdU), which is then excreted in the urine [5,7-9]. Cytidine deaminase is responsible for the rapid metabolic clearance of gemcitabine during clinical use [3] and CDA is known to be found in visceral organs and whole blood [5,10,11]. While gemcitabine has demonstrated efficacy in human cancers including non-small cell lung cancer [12] pancreatic cancer [13], breast cancer [14], and bladder cancer, [15] reports of the use of gemcitabine in veterinary oncology have been limited and veterinary species have not enjoyed the same efficacy that has been demonstrated in humans. [16-22].

Specifically, single agent gemcitabine has shown no efficacy for canine lymphoma, [16] for canine mammary tumors, [17] or multiple other canine tumor types [18]. When used in combination with piroxicam for the treatment of canine transitional cell carcinoma of the urinary bladder [21] or in combination with carboplatin for canine Osteosarcoma , [22] gemcitabine failed to provide a longer survival time in either disease. Additionally, when used in combination with carboplatin for multiple carcinomas, there was only a 13% response rate [19]. In the cancer-bearing cat, this same carboplatin-gemcitabine couplet demonstrated minimal patient benefit [23]. Finally, when used as a radiosensitizer in both dogs and cats with head and neck cancer, one study of 8 cats concluded a possible therapeutic benefit, [22] whereas a larger study did not support the use of this couplet and the authors concluded that pharmacokinetic data were necessary [20] .To date, the use of gemcitabine in the horse has not been reported.

Currently, it remains unknown why some human cancers show response to gemcitabine while veterinary species experience less of a benefit from this drug [24], As suggested by other investigators, [20] complete pharmacokinetic data may provide an explanation for this clinical problem. While some pharmacokinetics parameters such as half-life of gemcitabine have been described in dogs [7,9,25-27] and humans, [28] no such reports exists for the cat or the horse. As it is known that the intracellular accumulation of the active gemcitabine triphosphate moiety is balance between activation and metabolism, [1,5, 6] an understanding of metabolism of this chemotherapeutic may be a first step in developing optimal dosing schedules in clinically relevant veterinary species and may lead to enhanced efficacy of this schedule-dependent chemotherapeutic agent. The purpose of this study is to compare the metabolism of gemcitabine in fresh whole blood from humans, dogs, cats, and horses.

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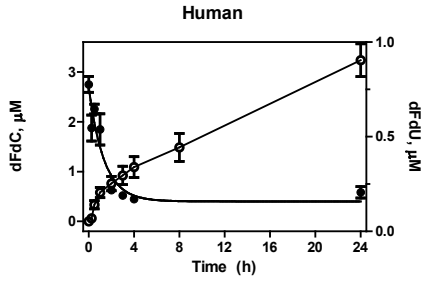
Sample	PCV (%)
Human A	44
Human D	45
Human K	45
Dog 1	50
Dog 2	61
Dog 3	50
Cat 1	45
Cat 2	44
Cat 3	45
Horse 1	40
Horse 2	35
Horse 3	33

Table 1: Packed cell volumes (PCV) of subjects blood samples.



Graph 1: Gemcitabine degradation in fresh whole blood for the human, dog, cat, and horse. Plasma from each species was spiked with gemcitabine, extracted by acid precipitation, and assayed for gemcitabine (dFdC, closed symbols) and 2',2'-difluorodeoxyuridine (dFdU, open symbols) as described in materials and methods.

Materials and Methods

Fresh whole blood (FWB) from humans was used as a positive control and was obtained from three healthy adult volunteers. Canine, feline, and equine FWB was obtained from three each healthy investigator-owned animal after informed consent. Gemcitabine[a] was a gift from Dr. Varsha Gandhi, University of Texas MD Anderson Cancer Center, Houston TX. All other chemicals were reagent grade.

Cultures (10 cc) of FWB were placed in 50 mL conical tubes. The tubes were maintained at 37°C throughout the experiment. Gemcitabine was added to a final concentration of 10 µM, the optimal in vitro and in vivo concentration for the accumulation of the active metabolite [3]. The 37°C cultures were swirled gently to mix and duplicate aliquots (500 µl) were removed at desired time points (time 0, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 8 hours, 12 hours, and 24 hours). The plasma was separated from red blood cells by centrifugation (16.1 RCF x 3 minutes).[b] Plasma was placed in a fresh microfuge tube and mixed with an equal volume of 6% (w:v) sulfasalicylic acid[c] in order to remove plasma proteins. Plasma water was cleared by centrifugation as described above and then frozen at -20°C until analysis by high performance liquid chromatography (HPLC).[d] The analyzed samples were maintained at 10°C and were then applied to an Atlantis 46×100mm µC18 3µm column[e] and eluted at a rate of 1.7 ml/min using a concave gradient (curve #6)[d] as follows: Buffer A (10mM NaH_2PO_4, pH 3[c]) and Buffer B (100% Acetonitrile[c]). 100% Buffer A for 7 minutes, 97% Buffer A: 3% Buffer B between 7 to 14 minutes. 100% Buffer A between 14 to 18 minutes. The column eluate was monitored by photodiode array[d] (200λ and 320λ and a sampling rate of 1 and a resolution of 1.2) and the dFdC and dFdU analytes were identified by comparisons of their retention profiles and absorption spectra with those of the standards[a,c] and quantitated by electronic integration with reference to external standards. The lower limit of detection of this assay is 0.05 nanomoles in 100 µl of acid deproteinated plasma. Empower 2 version 6.0[e] was used to integrate peaks.

Statistical analysis

Elimination curves were generated using GraphPad Software Prism version 5.0[f]. Initially, one-way analysis of variance (ANOVA) was performed. Then, post-hoc analysis was performed using a student's two-tailed paired *t* test to determine the significance between metabolism curves of the various species.

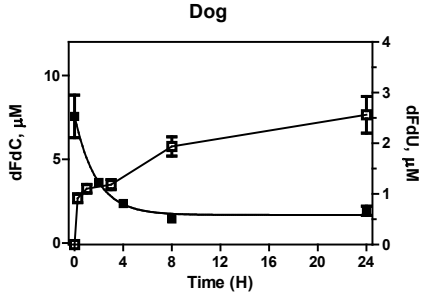
Results

Metabolism of dFdC by CDA occurs rapidly in whole blood [5,10,11]. Therefore, packed cell volumes (PCVs) were obtained to ensure similarity in red blood cell count within groups of species and between species (Table 1). Overall, the PCVs within the groups of species were similar.

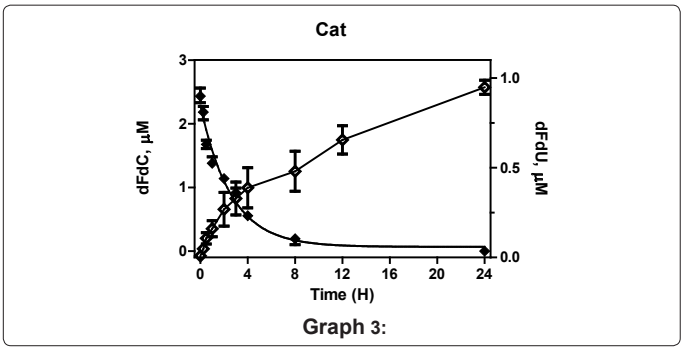
Gemcitabine degradation was found to occur in FWB of humans, dogs, cats, and horses (Figure 4). In these four species, there is an initial rapid degradation of dFdC with a concomitant proportional increase in dFdU. The best fit curve was a one-phase exponential decay. Specifically, for humans (Graph 1, Table 2), the mean $t_{1/2}$ was 57 minutes (range 33 to 69 minutes; r^2 0.8059); for dogs (Graph 2, Table 2), the mean $t_{1/2}$ was 70 minutes (range 56 to 85 minutes; r2 0.9447); for cats (Graph 3, Table 2), the mean $t_{1/2}$ was 108 minutes (range 107 to 110 minutes; r^2 0.9729);

Subject	$t_{1/2}$ (minutes)	r^2
Human A	70	0.5217
Human D	33	0.9861
Human K	69	0.9100
Human (mean)	57	0.8059
Dog 1	56	0.9891
Dog 2	85	0.8554
Dog 3	68	0.9897
Dog (mean)	70	0.9447
Cat 1	108	0.9711
Cat 2	107	0.9735
Cat 3	110	0.9742
Cat (mean)	108	0.9729
Horse 1	77	0.9830
Horse 2	68	0.9532
Horse 3	82	0.9626
Horse (mean)	76	0.9663

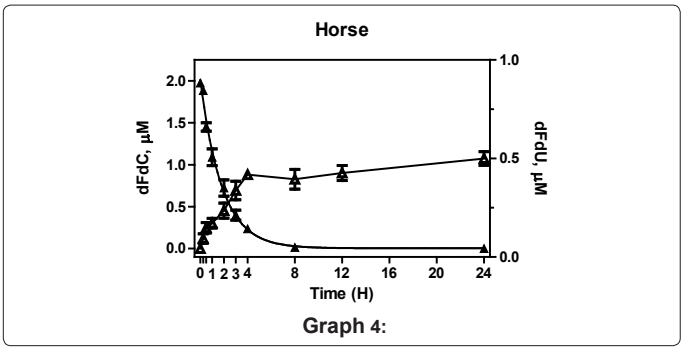
Table 2: One-phase exponential decay of gemcitabine in fresh whole blood.



Graph 2:



Graph 3:



Graph 4:

	p-value			
	Human	Dog	Cat	Horse
Human	---	0.45	0.014	0.23
Dog		---	0.010	0.55
Cat			---	0.0015
Horse				---

Table 3: Post-hoc analysis using 2-group *t* test.

for horses (Figure 4 horse, Table 2), the mean $t_{1/2}$ was 76 minutes (range 68 to 82 minutes; r^2 0.9663).

The initial one-way ANOVA was performed and the differences between curves was significant (p = 0.0085). Gemcitabine metabolism in fresh whole blood was found to be similar between the human, dog, and horse (Table 3) while the metabolism appeared slowest in the cat (Figure 3 and Table 2). This difference was found to be statistically significant (Table 3) when comparing human to cat (p=0.014), dog to cat (p=0.010), and horse to cat (p=0.0015).

Discussion

Gemcitabine is a nucleoside analog, which when administered intravenously undergoes one of two fates: activation or metabolism [8,29]. In the activation pathway, gemcitabine enters the cell by nucleoside transporters and is then rapidly phosphorylated to its cytotoxic metabolite, dFdCTP, in three sequential steps by deoxycytidine kinase and two nucleoside kinases [1,4,29]. In the elimination pathway, plasma gemcitabine undergoes deamination to dFdU by CDA, which is found in abundance in whole blood and visceral organs. [5,10,11]. The fate of plasma gemcitabine has been studied in humans [1,3,6,8,30,31] and dogs, [7,9,25-27] but to date, plasma pharmacokinetics remain unknown in the cat or horse. Additionally, the direct degradation of gemcitabine has not been reported for the dog, cat or horse.

While gemcitabine has shown utility in human oncology for various neoplasms, especially carcinomas, [12-15] the results in tumors of diverse histology in veterinary medicine have been less encouraging [16-24]. Because the anti-tumor efficacy of gemcitabine is directly related to the accumulation of intracellular dFdCTP and because accumulation is a balance between the two opposing fates (activation or metabolism), [1,5,30] we chose to evaluate the metabolism of gemcitabine in whole blood of various species.

Whole blood was selected for use in this evaluation of gemcitabine metabolism due to its high CDA levels and its ease of collection. In the study conducted here, the intra- and inter-PCV variability was low. This likely eliminated inter-subject gemcitabine metabolism variability-associated differences in specific CDA activities. It is possible that anemia may affect gemcitabine metabolism, but this remains to be determined in clinical patients.

In our study, gemcitabine metabolism in FWB was appeared be similar in the human, dog, and horse. These findings suggest that a similar dosing scheme may be used amongst these species, though further investigations including more in vivo experiments are necessary. In humans, historically, the most commonly used schedule in clinical practice was 1000 mg/m^2 intravenously administered weekly for 3 total weeks, followed by 1 week of rest [32]. However, more recent evidence suggests a prolonged infusion schedule may be more clinically efficacious [1,32-35] and Tempero and colleagues demonstrated a twofold increase in intracellular dFdCTP, the active metabolite, in peripheral blood mononuclear cells when patients received a constant infusion of gemcitabine compared to the standard administration over 30 minutes [24]. Additionally, when higher doses of gemcitabine are administered over 30 minutes, there is no improvement in efficacy, [36] yet, prolonged infusion schedules suggest enhanced efficacy. [34] This improved efficacy is thought to be related to the enzyme deoxycytidine kinase, which catalyzes the first rate-limiting step in the accumulation of the cytotoxic metabolite of gemcitabine, dFdCTP [35,37]. Studies show that deoxycytidine kinase-mediated phosphorylation of gemcitabine is saturated at infusion rates of approximately 10 mg/m^2/min IV [38]. It is suggested that the optimal schedule in humans is 1500 mg/m^2/150 minutes [33,34]. The veterinary literature suggests a minimal efficacy of the 30 minute infusion in dogs and cats. It may be that the prolonged infusion schedule used in humans should also be used in the veterinary species. Our study is aimed at understanding one aspect of gemcitabine metabolism (degradation) in multiple species. Because metabolism was found to be slower in the cat, the optimal infusion schedule may be a different than that needed in the human, dog, and horse. Further in vivo studies are necessary to confirm these hypotheses.

In evaluation of the $t_{1/2}$, Human D had the most rapid metabolism of gemcitabine (33 minutes) compared 70 and 69 minutes for humans A and K, respectively. This difference may be attributed to genetic polymorphisms of genes encoding for drug-metabolizing enzymes, which are often the factor involved in the interindividual differences in both therapeutic and toxic responses in humans [39, 40]. We did not identify such variation of $t_{1/2}$ within the studied dogs, cats, or horses. To date, such differences in these drug-metabolizing enzymes have not been evaluated for veterinary species.

Limitations of this study are attributed to evaluation of only one tissue, FWB, using an in vitro technique. Metabolism of gemcitabine is known to occur rapidly in abdominal organs such as the kidney, liver, and intestine, and therefore, the results reported here may differ when gemcitabine metabolism is evaluated in these tissues or translated into the whole animal.

In conclusion, we have shown that metabolism of gemcitabine in FWB from humans, dogs, and horses are similar between these

species. The information from this study suggests that dosing schemes for optimal tumor benefit and reduced toxicity may be similar for the human, dog, and horse. In contrast, the metabolism of gemcitabine in FWB in cats is slower compared to humans, dogs, and horses. Therefore, in the cat, the dosing schemes may need to reflect this difference. We are currently investigating various infusion schedules in the dog and cat.

a. Eli Lily Corporation, Indianapolis Indiana

b. Eppendorf 5415D, Hamburg, Germany

c. Fisher Scientific, Houston, TX

d. Alliance System with autosampler and photodiode array detector, Waters Corporation, Boston, MA

e. Waters Corporation, Boston, MA

f. GraphPad Prism Software, La Jolla, CA

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