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Pharmacokinetics of thalidomide in dogs: can feeding affect it? A preliminary study

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ABSTRACT

Background: Tumor-associated neoangiogenesis is a crucial target for antitumor therapies. Thalidomide (TAL) is a promising anti-neoangiogenetic drug that has recently been used in the treatment of several malignancies in dogs.
Objectives: The aim of the study was to assess the pharmacokinetics of TAL after single oral administration in dogs. Additionally, the influence of feeding on the pharmacokinetic profile of TAL in dogs has been preliminarily investigated.
Methods: Six healthy adult female Labradors were enrolled according to a randomized single-dose, 2-treatment, 2-phase, paired 2 × 2 cross-over study design. The dogs were administered a single 400 mg capsule of TAL in fasted and fed conditions. Blood was collected from 15 min to 48 h after dosing, and TAL quantified in plasma by a validated high-performance liquid chromatography method. The pharmacokinetics of TAL were analyzed using a non-compartmental approach.
Results: TAL concentration was quantifiable up to 10 h and 24 h after fasted and fed conditions, respectively. C_{max} (fasted, 1.34 ± 0.12 μg/mL; fed, 2.47 ± 0.19 μg/mL) and T_{max} (fasted, 3 h; fed, 10 h) differed substantially between the 2 groups. AUC and $t_{1/2}\lambda z$ were significantly higher in fed (42.46 ± 6.64 mg × h/L; 17.14 ± 4.68 h) compared to fasted (12.38 ± 1.13 mg × h/L; 6.55 ± 1.25 h) dogs. The relative oral bioavailability of TAL for the fasted group was low (36.92% ± 3.28%).
Conclusions: Feeding affects the pharmacokinetics of oral TAL in dogs, showing a delayed, but higher absorption with different rate of elimination. These findings are of importance in clinical veterinary settings, and represent a starting point for further related studies.

Keywords: Dogs; fasting; meals; pharmacokinetics; thalidomide

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Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
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INTRODUCTION

Thalidomide (TAL) was first synthesised in 1954, and was used clinically in Europe as a non-barbiturate hypno-sedative and antiemetic drug for morning sickness. It was thought that the sedative effect of TAL was generated by a different mechanism of action than that of barbiturates. This led to the belief that TAL was a 'safe' drug, with little CNS and respiratory depression or muscle incoordination [1], and no deaths from overdose or attempted suicide have ever been recorded [2]. However, in 1961 TAL was found to have a teratogenic effect in humans, and so was withdrawn from market. Despite its known teratogenicity, by 1965 TAL was the drug of choice for erythema nodosum leprosum [3]. The safety profile of TAL was not completely determined until 1998 [4], and since then, several trials in inflammatory and oncologic conditions have been run. TAL has shown promising antitumour activity in several malignancies and has been proposed as a drug of choice in multiple myeloma [5-9].

Neoangiogenesis is a well recognized hallmark of cancer [10]. Today, tumoral-associated neoangiogenesis is a crucial target for antitumoral therapy. Several studies have shown that the tumour microenvironment is able to induce and promote neoangiogenesis [10,11]. The potential anti-angiogenic effects of TAL were suspected in the early 1960s but were only confirmed in the 1990s [12,13]. To date, the precise mechanisms responsible for the clinical activity of TAL have not yet been estabilished. However, TAL has been shown to inhibit angiogenesis induced by basic fibroblast growth factor in rabbit cornea or by vascular endothelial growth factor in a murine model of corneal vascularization [12,14]. TAL also reduced interleukin-6 (IL-6), 1b (IL-1b), 10 (IL-10) and tumour necrosis factor-α production in an *in vitro* model [15,16].

TAL has been used in canine chemotherapy for the treatment of hemangiosarcoma [17,18], pulmonary [19] and mammary carcinoma [20]. Equivalent or even longer survival times have been reported compared to traditional intensive-dose chemotherapy. Unlike many other chemotherapeutic drugs, TAL is relatively well tolerated by dogs. Experimental trials have not found significant toxicity in Beagles treated for up 53 weeks with a dose of up to 1,000 mg/kg/day [21]. To date, the dose of TAL proposed for the treatment of tumours in canine patients has been empirically selected, with studies using a wide range of doses. Indeed, dose in the range of 2 to 26 mg/kg/day or 100–400 mg/dog per day have been reported [17,19,22,23]. A dose regimen selected based on scientific data is thus necessary in order to optimise TAL therapy in canine patients.

To the best of the author's knowledge, no studies on the pharmacokinetics of TAL in dogs have been reported in the literature. The aim of this study was therefore to assess the pharmacokinetics of TAL after single oral administration in dogs. Additionally, the likely influence of feeding on the pharmacokinetic profile of TAL in dogs has been preliminarily investigated.

MATERIALS AND METHODS

Drugs and chemicals

TAL for analytical testing (purity ≥ 99%) and phthalimide (purity ≥ 99%), used as internal standard (IS), were provided by Sigma-Aldrich (USA). Ammonium acetate, methanol (CH_3OH), acetonitrile (CH_3CN) and trifluoroacetic acid (98%) were purchased from VWR International (USA). Acetic acid 99–100% (CH_3COOH) was obtained from J.T. Baker (USA).

The water used was ultrapure grade, purified using a Milli- Q UV Purification System (Millipore Corporation, USA).

Animals and experimental design

Six adult female (2–7 years) Labradors with an average body weight of 34.6 ± 1.69 kg (median, 34.25 kg; range, 28.5–42.4 kg) were used. The experiment was approved by the University of Life Sciences, (Lublin, Poland) welfare ethics committee and carried out in accordance with the European law (2010/63/UE). The dogs were determined to be clinically healthy based on physical examination, serum chemistry and haematological analyses performed 48 h before the beginning of the study and were not treated with other therapeutic agents.

The dogs were randomly divided in to 2 groups (each containing 3 animals) using Research Randomizer software, and participated in a single-dose, 2-treatment, 2-phase, paired 2 × 2 cross-over study.

The drug was prepared by a compounding pharmacy, and administered as capsules containing 400 mg of pure TAL. Since animals had different body weights, the dose administered was an average of 11.74 ± 0.56 mg/kg (median, 11.76 mg/kg; range, 9.4–14.0 mg/kg).

In the first phase, group 1 (n = 3) was administered with 400 mg/dog (one capsule) after over-night fasting and group 2 (n = 3) was fed prior to and after administration of the same dose. The capsule was placed on the back of the tongue and 5 mL of water was administered to ensure that the capsule was swallowed. Canned dog food (Nature's Logic Canine Feast, USA) was provided as half the total amount 15 min before dosing, with the rest provided immediately after TAL administration. On each study day, in order to avoid the possibility of coprophagia impacting on the study, the dogs were kept in individual boxes for 48 h and observed closely during this period. A 2-week wash-out period was observed between the phases, then the treatment groups were inverted, and the experiment was repeated.

The dogs were checked daily for visible adverse effects for 7 days following completion of the study. To facilitate blood sampling, 1 h before the commencement of the study, an 18-gauge soft cannula (Delta Med, Italy) was inserted in the right medial saphenous vein. Blood samples (3 mL) were withdrawn into lithium heparin tubes (Aptaca Spa, Italy) at 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10, 16, 24, 34 and 48 h after administration of TAL. Blood samples were immediately centrifuged for 10 min at 1,500 × g at 4°C and the plasma was harvested. Since TAL is not stable in plasma [24], 2.0 mL of a stabilizer-solution (CH_3OH/CH_3CN, 1/1 (v/v) + CH_3COOH 2%) [25] was immediately added to each mL of plasma sample as soon as it was harvested. Samples were transferred into cryo-vials and immediately frozen and stored at −80°C. Samples were analysed within 2 weeks of collection.

Sample extraction procedure

Analysis was performed according to Saccomanni et al. [25], and slightly modified. In brief, an aliquot of 1.5 mL of sample (containing 0.5 mL of plasma and 1 mL of stabilizer-solution) was added to a 2.0 mL micro-centrifuge tube. After the addition of 100 µL of IS (50 µg/mL) and 2.0 µL of trifluoroacetic acid (deproteinizing agent), samples were vortexed for 30 sec, then sonicated and shaken at 60 oscillation/min for 10 min. Samples were then centrifuged (5,000 × g) for 10 min, and 1 mL of the organic layer was transferred into a clean tube and dried under a gentle stream of nitrogen at 30°C. The residue obtained was reconstituted with

100 μL of CH_3OH/CH_3CN, 1/1 (v/v) and after centrifugation (5,000 × g, 5 min) 20 μL of the upper layer was injected onto the high-performance liquid chromatography (HPLC).

HPLC conditions

TAL in dog plasma was determined using an HPLC coupled with diode array detector (Series 2000; Jasco Europe, Italy) according to a slightly modified version of the method described by Saccomanni et al. [25]. A Gemini C18 analytical column (250 × 4.6 mm, 5 μm particle size; Phenomenex, USA) maintained at 25°C by a Peltier System (LC-4000; Jasco Europe) was used for the chromatographic analysis. The mobile phase consisted of CH_3CN/10 mM acetate ammonium (pH 5.5) solution (25/75, v/v), which was freshly prepared each day before the analysis. The flow rate of the mobile phase was set at 1 mL/min. The wavelength was set at 220 nm.

Method validation and quantification

The analytical method was fully revalidated for dog plasma according to the European Medicines Agency guidelines [26] by examining the within-run precision, calculated from similar responses for 6 repeats of 3 control samples (0.1, 0.5, and 1 μg/mL) in one run. The between-run precision was determined by comparing the calculated response of the low (0.05 μg/mL), middle (1 μg/mL), and high (10 μg/mL) control samples over 3 consecutive daily runs (a total of 6 runs). The assay accuracy for within-run and between-runs was established by determining the ratio of calculated response to expected response for low (0.05 μg/mL), middle (1 μg/mL), and high (10 μg/mL) control samples over 6 runs. The limit of quantification (LOQ) was determined as signal-to-noise ratio of 10, and the limit of detection (LOD) as the signal-to-noise ratio of 3.

TAL and IS stock solutions were prepared in a mixture of CH_3OH/CH_3CN, 1/1 (v/v) and in water, respectively, at a concentration of 1,000 μg/mL and stored at −80°C. These solutions were freshly prepared every 2 weeks. TAL stock solution was then diluted to reach concentrations of 0.25, 2.5, 5, 25 and 50 μg/mL and stored at −20°C. These last concentrations were then diluted immediately prior to use to reach the final concentrations of 0.05, 0.5, 1, 5, 10 μg/mL. These final dilutions were then used in preparation of a 5-point calibration curve of TAL in plasma matrices.

Standard curves were constructed with standard TAL concentrations *vs* ratio of TAL/IS peak areas. The linearity of the regression curve was assessed based on the residual plot, the fit test and the back-calculation. Extraction recovery was evaluated by comparing the response (in area) of high, middle, and low standards and the IS, spiked into blank canine plasma (control), with the response of equivalent standards.

Pharmacokinetic and statistical analysis

The concentration of TAL vs. time was pharmacokinetically analyzed using a non-compartmental approach (ThothPro 4.3; ThothPro LLC, Poland). C_{max} was the peak plasma concentration, and T_{max} was the time at the peak plasma concentration. The elimination half-life ($t_{1/2}\lambda z$) was calculated using linear least squares regression analysis of the concentration-time curve, and the area under the curve (AUC) was calculated by the linear-up log-down rule to the final concentration-time point (Ct). From these values, the apparent volume of distribution (V = dose × area under the first moment curve [AUMC]/AUC^2), mean residence time (MRT = AUMC/AUC) and clearance (Cl = dose/AUC) were determined. The relative bioavailability (F) was calculated for each dog using the following equation:

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$$(\%)F_{(fasted)} = AUC_{(fasted)}/AUC_{(fed)} \times 100$$

Data were found to be normally distributed (Shapiro-Wilk test). Paired *t*-tests were used to investigate statistically significant changes in pharmacokinetic estimates between groups (GraphPad Software; GraphPad, USA). The pharmacokinetic parameters are presented as means ± SE and T_{max} (categorical variable) is expressed as median and range. In all the experiments, differences were considered significant if $p < 0.05$.

RESULTS

The analytical method showed a good linearity in the range between 0.05 and 10 μg/mL with a determination coefficient (R^2) above 0.994 (y = 0.0976x − 0.0456). The intra- and inter-day precision resulted in coefficient of variation < 20%. The mean extraction recovery of TAL was 72.09% ± 5.04%; the LOD and LOQ were 0.05 μg/mL and 0.5 μg/mL, respectively.

In the first phase of the study one dog in group 2 (fed) showed some adverse effects 12 h after TAL administration. These included shaking, stiff walk, staggering and whining. However, the blood samples were still collected at each timepoint, and the dog completely recovered after a few hours. It was replaced in phase 2 with another dog. In all the other experimental animals no adverse effects and no behavioural or health alterations were observed during or after the study.

Plasma TAL concentration was quantifiable up to 10 h and 24 h after oral administration of 400 mg/dog in fasted and in fed conditions, respectively (**Fig. 1**). The main pharmacokinetic estimates are reported in **Table 1**. One fed dog in phase 2 showed a short T_{max} and a higher C_{max} compared with other dogs in the same group, as well as a more similar pharmacokinetic profile to the fasting group. This individual data set was considered as an outlier, and was excluded from the pharmacokinetic analyses.

C_{max}, normalized for the dose expressed in mg/kg, differed substantially between the 2 groups (fasted, 1.34 ± 0.12 μg/mL; fed, 2.47 ± 0.19 μg/mL). T_{max} differed considerably between the fasted (3 h) and the fed (10 h) animals.

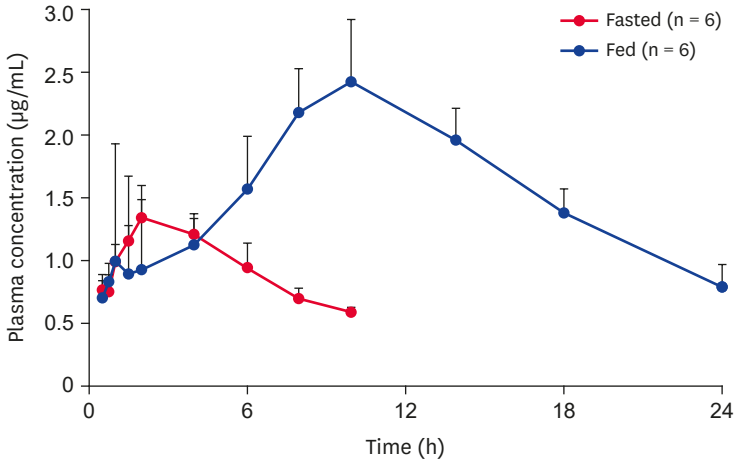

Fig. 1. Mean TAL plasma concentration vs. time curve following single oral administration of 400 mg/dog in fasted (n = 6) and fed (n = 6) conditions.
TAL, thalidomide.

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Table 1. Mean ± SE value of the pharmacokinetic parameters of TAL following a single oral administration at a dosage of 400 mg/dog in fasted (n = 6) and fed conditions (n = 6)

Pharmacokinetic parameter (unit)	Fasted	Fed†
λz (1/h)	0.12 ± 0.02	0.05* ± 0.01
$t_{1/2}$λz (h)	6.55 ± 1.25	17.14 ± 4.68
C_{max}§ (μg/mL)	1.34 ± 0.12	2.47* ± 0.19
T_{max} (h)	3 (1–4)	10* (6–10)
Cl/F (mL/g*h)	1.11 ± 0.08	0.25* ± 0.04
V/F (mL/g)	4.89 ± 0.34	3.05* ± 0.26
$AUC_{0\text{-}last}$§ (mg*h/L)	7.94 ± 0.87	35.28* ± 5.47
$AUC_{0\text{-}\infty}$§ (mg*h/L)	12.38 ± 1.13	42.46* ± 6.64
$MRT_{0\text{-}\infty}$ (h)	10.06 ± 1.56	28.57 ± 6.64
F‡ (%)	36.92 ± 3.28	0.05* ± 0.01

Values are presented as mean ± SE or median value (range).
TAL, thalidomide; λz, terminal phase rate constant; $t_{1/2}$λz, terminal half-life; C_{max}, peak plasma concentration; T_{max}, time of peak concentration; Cl/F, plasma clearance normalized for F; V/F, volume of distribution normalized for F; $AUC_{0\text{-}last}$, area under the curve from 0 to last time collected samples; $AUC_{0\text{-}\infty}$, area under the curve from 0 h to infinity; $MRT_{0\text{-}\infty}$, mean residence time; F, bioavailability.
*$p < 0.05$; †Values computed on 5 dogs; ‡Value computed on 4 dogs; §Values normalized for the dose expressed in mg/kg.

The $t_{1/2}$λz values were variable but significantly different between the groups (fasted, 6.55 ± 1.25 h; fed, 17.14 ± 4.68 h), in-line with a different λz (fasted, 0.12 ± 0.02 1/h; fed, 0.05 ± 0.01 1/h).

The AUC value was significantly higher in the fed group (normalized for the dose expressed in mg/kg: fasted, 12.38 ± 1.13 mg × h/L; fed, 42.46 ± 6.64 mg × h/L). As a result, the relative oral bioavailability of TAL for the fasted group was low (36.92% ± 3.28%).

DISCUSSION

The main aim of the present study was to evaluate the pharmacokinetic profile of TAL after oral administration in dogs and to determine whether this profile is affected by feeding.

The dose of TAL administered in the present study (400 mg/dog, average 11.7 mg/kg) was selected based on clinical efficacy/adverse effects previously reported in dogs. A dose of 8.7 mg/kg/day and a 3-month daily-dose of 20 mg/kg followed by a 3-month daily-dose of 10 mg/kg were successfully used in the management of stage II–III splenic hemangiosarcomas [18] and canine mammary carcinomas [20], respectively, in dogs. This latter study showed adverse sedative effects in some dogs when given the higher dose (20 mg/kg), with symptom improvement when the dose was reduced to 10 mg/kg. The dose administered in our study was found to be safe with no visible signs of toxicity in animals. This concurs with the findings of a previous study [21], which also reported no visible signs of toxicity associated with this dose in 56 dogs. However, a multiple-dose study is needed to confirm this finding.

The toxic signs showed by the subject in the fed group during the first phase of the animal study were transient (around 4 h). The causes of these signs are not clear but are unlikely to be due to TAL. A study into the effects of chronic TAL administration in dogs [21] found that TAL administered up to 1,000 mg/kg/day for 53 weeks did not to induce any major systemic toxicity or tumours in dogs. There were no TAL-related changes in body weights, food consumption, electrocardiography, ophthalmoscopy, neurological function, or endocrine function. Some slight and/or transient variations observed in some hematology and blood chemistry values of dosed dogs were considered to be toxicologically insignificant, with these

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conclusions being supported by the lack of histopathologic changes. The only gross finding attributable to TAL was a yellow-green discoloration of the femur, rib, and/or calvarium. This aspect was not assessed in the present study since animals were not euthanized. The estimated non observed adverse effect level in dogs was 200 mg/kg/day [21] which almost 20 times higher than the dose administered in the present study. However, adverse events such as sedation, dizziness, constipation, and headache have been reported in humans after multiple clinical doses [27-29] that do not match with the signs observed in the dog used in the present study.

Plasma concentrations of TAL after fasted and fed conditions varied widely in our study. Statistical analysis and inspection of the plasma concentration *vs* time curves indicated that feeding considerably affects both the pharmacokinetic parameters and profiles. This information could be of paramount importance in clinical settings. Food intake delayed (T_{max}) but increased TAL absorption (C_{max} and AUC), in line with the negligible hydrophilicity of the active compound [30,31]. Interestingly, the effect of food on TAL pharmacokinetics in humans are conflicting: some studies report no influences while others report minor effects on C_{max} and AUC, with a significant delay to T_{max} [2,28].

The type of food consumed can impact on the quality and quantity of the food effect. For example, fatty foods generally delay gastric emptying, thereby providing ample time for greater dissolution and absorption of drugs. This was seen with griseofulvin, a sparingly water-soluble drug, where coadministration with a fatty meal doubled its absorption relative to the fasted state. High-protein or carbohydrate-rich food had no effect on griseofulvin absorption [32]. The feed administered to dogs in our study was a fatty meal. TAL, which like griseofulvin is sparingly soluble in water, showed significantly higher absorption in 5 of the 6 fed dogs. However, some drugs' bioavailability is increased with a high fat diet, while dietary fiber may reduce drug availability, thus diverse feed types may have different impacts on the pharmacokinetics of TAL [33-35]. Further studies investigating the impact of different types of feeds on TAL pharmacokinetics are warranted to investigate this issue.

One dog in the fed group was found to be a statistical outlier with a reduced T_{max} similar to that reported for the fasting group. This could be explained by the contractile mechanism of the gallbladder emptying and filling in dogs [36]. In fact, the gallbladder alternates filling and emptying excursions even in fasted dogs. Alternatively, the dog may have had a reflux of duodenal fluid (containing bile) in the gastric lumen. Consequently, the production of an earlier emulsion may have led a higher C_{max} and faster T_{max} [37]. A statistical outlier was also described in a previous study that examined the effect of food on TAL pharmacokinetics in humans [28].

Half-life is a pharmacokinetic parameter used to compute the dose interval and the time to achieve the steady state concentration [38]. The half-life of TAL was statistically increased by feeding. This may be due to the feed acting as a drug reservoir, slowly releasing TAL during intestinal transit. If administered once-daily in fed dogs, TAL has an accumulation ratio ($AUC_{steady\ state}/AUC_{1st\ adm}$) of around 2.5, while the steady state plasma concentration would be attained in around 4 days [38]. Half-life is a hybrid parameter that incorporates both clearance and volume of distribution. The low water solubility of TAL has prevented the development of a commercial intravenous formulation [2], and consequently it is impossible to calculate absolute clearance and volume of distribution, making extensive discussion of this estimate too speculative.

Two uncontrolled multiple-dose studies in breast cancer and glioma have attempted to correlate TAL concentration with tumour response in humans. Steady-state plasma concentrations ranging from 5 to 7 μg/mL resulted in stable disease for up to 74 weeks in 12/31 glioma patients, however, similar concentrations (6.2 μg/mL) for 8 weeks in metastatic breast cancer patients showed no tumour response [39,40]. In a recent study in dogs, a similar TAL dose to that reported in the present study, was associated with equal or even longer survival times compared to intensive-dose chemotherapy in splenic hemangiosarcoma and mammary carcinomas [18,20]. The average plasma concentration computed at the steady state at 11.7 mg/kg TAL administration once-daily in fed dogs resulted in 3.7 μg/mL. Even though this concentration is theoretical it might be used as a target for the treatment of several malignancies in dogs, especially for splenic hemangiosarcoma and pulmonary/mammary carcinomas. Although it has been reported that the pharmacokinetics of TAL do not change significantly between healthy and cancer patients, further studies on canine patients are warranted to verify whether TAL pharmacokinetics are unchanged in healthy dogs versus dogs diagnosed with cancer [27,40,41].

The findings of this study should be interpreted while considering some limitations. Namely: the study used only female dogs of a single breed. It is well known that breed or sex-specific differences can lead to variances in drug absorption, distribution, metabolism, or elimination. In particular, differences in body weight and composition, animal size, P-450 enzyme isoforms or in plasma protein binding might occur in different breeds or in animals of different sex of the same breed [42-50]. For instance, Labradors have a higher percentage of body fat compared to other breed such as Greyhounds or Beagles, and this might lead to a larger volume of distribution of certain liphophilic compounds [43,51].

In conclusion, this is the first study to report the pharmacokinetics of TAL in dogs. Feeding significantly affects the pharmacokinetics, and this should be considered by veterinarians when using this drug in a clinical setting.

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