Published in final edited form as:

Food Chem Toxicol. 2008 January ; 46(1): 203–211. doi:10.1016/j.fct.2007.07.017.

Toxicology and pharmacokinetics of 1-methyl-[D]-tryptophan: absence of toxicity due to saturating absorption

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Abstract

1-methyl-D-tryptophan (D-1MT) reverses the immunosuppressive effect of indoleamine 2,3-dioxygenase (IDO), and it is currently being developed both as a vaccine adjuvant and as an immunotherapeutic agent for combination with chemotherapy. The present study examined the pharmacokinetics and toxicity of D-1MT in preparation for clinical trials. Incubation of D-1MT in rat plasma for 24 h produced no significant degradation, with <15% of D-1MT being bound to plasma protein. Following oral administration, D-1MT exhibited a larger AUC and V_d , longer elimination $t_{1/2}$, and slower clearance in rats than in dogs. When oral doses of D-1MT exceeded levels of 600 mg/m²/day in rats, or 1200 mg/m²/day in dogs, the C_{max} and AUC values decreased, resulting in a corresponding decrease in oral bioavailability. Thus, the doses were indicative of the lowest saturating doses in dogs and rats corresponding with an elimination $t_{1/2}$ of 6.0 h and 28.7 h, a T_{max} of 1 h and 8 h, and a bioavailability of 47% and 92%, respectively. Tissue concentrations of D-1MT in mice were highest in the kidney, followed by the liver, muscle, heart, lung, and spleen, respectively; 48 h post dosing, D-1MT was excreted in the urine (35.1%) and feces (13.5%). Oral administration of D-1MT in rats from 150 to 3000 mg/m²/day (25 to 500 mg/kg/day) and in dogs from 600 to 1200 mg/m²/day (30 and 60 mg/kg/day) for 28 consecutive days did not lead to mortality, adverse events, histopathological lesions, or significant changes in hematology, clinical chemistry, and body weight. These results suggested that 3000 and 1200 mg/m²/day were the no-observed-adverse-effect levels in rats and dogs, respectively. Mean plasma concentrations of D-1MT (600 and 1200 mg/m²/day) in dogs 1 h post dosing were 54.4 and 69.5 µg/ml on Day 1, respectively, and 53.1 and 66.6 µg/ml on Day 28, respectively; thus, indicating no increase in plasma D-1MT with a change in dose. In conclusion, D-1MT has little toxicity when administered orally to rats and dogs. Exceeding the saturating dose of D-1MT is unlikely to cause systemic toxicity, since any further increase in D-1MT plasma levels would be minimal.

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Keywords

1-methyl-[D]-tryptophan; indoleamine 2,3-dioxygenase; pharmacokinetics; toxicity; saturating absorption

1. Introduction

The process of malignant transformation of normal cells creates aberrant expression of antigens and abnormal growth patterns that are sufficient to activate the innate effector cells with antitumor activity, as well as stimulate the presentation of tumor antigens to T and B lymphocytes (Blattman and Greenberg, 2004). Despite this well-orchestrated surveillance, the presence of a clinically apparent tumor indicates that the developing cancer was able to avoid detection, or it overwhelmed the immune response by creating a state of immunologic unresponsiveness (tolerance) toward its own antigens, which allows tumors to escape the host's immune system. A contributing factor to this tolerance is the immunoregulatory enzyme, indoleamine 2,3-dioxygenase (IDO) (Mellor and Munn, 2004). IDO degrades the essential amino acid tryptophan. Local depletion of tryptophan may be immunosuppressive to T cells due to the activation of the stress-response kinase GCN2 pathway, leading to T-cell arrest and anergy (Munn et al., 2005). IDO may also produce immunomodulatory tryptophan metabolites. Expression of IDO by human and mouse antigen-presenting cells inhibits T-cell mediated immune responses in vitro and in vivo. Tumor cells transfected with IDO become immunosuppressive in vivo (Uyttenhove et al., 2003; Mellor et al., 2002), and expression of IDO has been reported in tumor cells from a variety of human tumors (Uyttenhove et al., 2003). IDO is also expressed by dendritic cells found in tumor-draining lymph nodes of melanoma, breast cancer, and a variety of other tumors (Munn et al., 2004a), which may induce tolerance to tumor antigens.

One challenge for immunotherapy is to develop strategies that can effectively overcome tumor-induced immunosuppression and evasion, while safely augmenting antitumor responses. Based on the above rationale and mechanisms of IDO-induced tolerance, a pharmacologic blockade of IDO could be clinically useful in reversing local immunosuppression. Indeed, in a number of different in vitro immunologic model systems, the addition of the IDO inhibitor, 1-methyl-D-tryptophan (D-1MT, MW 218.25), reversed its suppressive effects on proliferation of either peripheral blood mononuclear cells (Sarkhosh et al., 2004) or CD4⁺ T-cell primary mixed leukocyte reaction cultures (Rutella et al., 2006). Furthermore, in vivo administration of D-1MT or its racemic mixture, 1-methyl-DL-tryptophan, blocks the immunosuppressive effect of IDO (Mellor and Munn 2004; Uyttenhove et al., 2003; Muller et al., 2005; Potula et al., 2005). The DL mixture is synergistic with a number of commonly used chemotherapeutic agents (Muller et al., 2005). This synergistic effect is immune mediated, and it may reflect increased antigen presentation following chemotherapy and/or depletion of regulatory T cells. Compared with the DL mixture, the D isomer appears to be at least as effective in vivo and less inhibitory towards T cells. The D isomer has been used in a number of recent human and mouse studies (Munn et al., 2004a; 2004b; 2005; Mellor et al., 2004; 2005; Baban et al., 2005). D-1MT is currently under development, both, as a vaccine adjuvant and as an immunotherapeutic agent for combination with chemotherapy. The current studies were carried out to examine the safety and pharmacokinetic profile of D-1MT in animals in preparation for clinical trials.

2. Methods and Materials

2.1. Materials and animal use

D-1MT (NSC 721782) was synthesized under contract with the National Cancer Institute. The purity of D-1MT was determined to be 98%–99% by a chiral HPLC method, and lot Nos. F68/L-1 to F68/L-9 were used in the present studies. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), or the Principles of Laboratory Animal Care (<http://history.nih.gov/laws>).

2.2. Analytical method for measuring D-1MT in biomatrices

Separation of D-1MT from various biomatrices was achieved with the use of a Polaris C18-A analytical column (100 × 2 mm, 5 µm) preceded by a Polaris C₁₈ guard column at 25°C. A mobile phase composed of 11% acetonitrile and 0.1% formic acid in water was used to elute D-1MT and α-methyltryptophan (internal standard) from the columns at a flow rate of 0.2 ml/min. The column effluent was diverted to waste from 0 to 4 min after sample injection. The column effluent was then switched to the mass spectrometer for the remainder of the chromatographic run.

The detection system consisted of a Shimadzu LC-10ADVP liquid chromatograph (Wood Dale, IL) coupled to a triple quadrupole Quattro Micro mass spectrometer (Quattro Micro, Micromass, UK) in the positive electrospray mode. The detection of D-1MT was accomplished via a multiple-reaction monitoring scan using the M+H m/z transition 219 → 160 for D-1MT and 219 → 132 for α-methyltryptophan. The dwell time, cone voltage, and collision energy values for D-1MT were 0.2 sec, 16 volts, and 20 eV, respectively.

Calibration standards were prepared by spiking 100 µl of the corresponding biomatrices (e.g., plasma, urine, and tissue homogenates) with 5 µl of known D-1MT amounts over a final concentration range of 31.25 to 10,000 nM. Each standard was also spiked with 5 µl of α-methyltryptophan 50 µM, while the blank was spiked with 5 µl of deionized water. The plasma proteins were precipitated with 200 µl methanol and kept on ice for 15 min, followed by 10 min of centrifugation (12,600 × g) at 4°C. The supernatant was transferred to a glass auto-sampler vial, and 2 µl of formic acid were added to improve the D-1MT ionization and peak shape; 20 µl of the mixed solution were then injected into the LC/MS/MS to measure the D-1MT concentration. The D-1MT and α-methyltryptophan spectra data acquired were processed using the Quanlynx routines in the MassLynx v3.5 software program. The program generated standard curves using the peak area ratio of D-1MT versus α-methyltryptophan at known concentrations. The lower limit of quantitation for D-1MT in plasma was determined to be 31 nM (6.75 ng/ml). The standard curve for D-1MT was linear over the range of 31.25–10,000 nM, with correlation coefficients of >0.99. The D-1MT recovery from plasma was 100%.

2.3. Plasma stability and plasma protein binding of D-1MT

D-1MT at concentrations of 250 and 5000 nM were incubated with rat plasma at 4 and 37°C in silanized amber glass vials. The concentrations were demonstrated active in various in vitro and in vivo experiments (Munn et al., 2004a; Munn et al., 2005; Hou et al., 2007). Aliquots of plasma were removed at 0, 0.5, 1, 2, 4, and 24 h, transferred to silanized brown microcentrifuge tubes, immediately frozen and stored at –20°C until analysis.

Aliquots (1.0 ml) of rat plasma, rat plasma water, and phosphate buffer saline containing 250 and 5000 nM of D-1MT were separately added to the sample reservoir of an Amicon Centrifree Micropartition device (Amicon Co.; Danvers, MA). The mixtures were kept at room temperature for 30 min and then centrifuged (1500× g) for 30 min at 4°C to separate free

D-1MT from the plasma proteins, plasma water, and phosphate buffer saline. The protein-free plasma water was prepared by adding plasma to preparatory centrifugal ultrafilters (Centriprep-10, Amicon) followed by ultrafiltration with the Centricon-10. The D-1MT concentrations were measured in samples before (sample reservoir) and after (filtrate cup) centrifugation. The percentage of D-1MT recovered and the fraction of protein binding were calculated with the use of the following equations: percentage recovered = (filtrate cup concentration/sample reservoir concentration) $\times$ 100%; protein binding = [1 – percentage recovered (plasma)/percentage recovered (ultrafiltration)] $\times$ 100%.

2.4. Pharmacokinetic studies following single administration of D-1MT

Male Fischer 344 rats (256 ± 4 g, 10–11 weeks old, $n = 3$ –4/dose group) were used in the study. For single intravenous administration, D-1MT (7.5 mg) was added to 0.967 ml of sterile saline solution; 16 μ l of 2 N NaOH and 17 μ l of 2 M HCl were then sequentially added to dissolve D-1MT completely. The solution was administered to catheterized rats at doses of 12.5 and 50 mg/kg (75 and 300 mg/m²) via the jugular vein. For single oral administration, various concentrations of D-1MT (1.25–100 mg/ml) were prepared in corn oil to produce a uniform volume of 10 ml/kg, and administered to the rats at doses of 12.5, 50, 100, 500, and 1000 mg/kg (75, 300, 600, 3000, and 6000 mg/m²) via gavage using a ball-tipped needle. Blood samples (1.0 ml) were collected from the retro-orbital sinus of the anesthetized rats by CO₂/O₂ (70%/30%).

Beagle dogs, weighing 7.6 ± 0.6 kg (female) and 9.5 ± 0.2 kg (male), were administered D-1MT intravenously at 15 mg/kg (300 mg/m²), or by gavage at doses of 30, 60, and 100 mg/kg (600, 1200, and 2000 mg/m²) in the dosing volume of 2 ml/kg uniformly. Each dose group included one male and one female dogs. Dose levels in rats and dogs were selected so that the lowest dose was the biological effective dose of D-1MT in animal models (Potula et al., 2005; Hou, et al., 2007), and the highest dose was the highest volume that animals could be administered based on acceptable dosing volume limitations (Diehl, et al., 2001). Blood was withdrawn from the jugular vein prior to dosing, and at 5, 10, 20, 30 min and 1, 2, 4, 6, 8, 12, 24, and 48 h following administration of a single dose. Plasma was separated by centrifugation (9,000x g, 5 min) and stored at -20°C until analysis. D-1MT concentrations in samples were calculated from the standard calibration curves. Pharmacokinetic parameters were determined by a non-compartmental model using WinNonlin (Professional Version 3.0; Pharsight Corp; Mountain View, CA). The program default was used to calculate the parameters based on the goodness-of-fit criteria set by lower condition number, higher precision, lower standard errors of the program. The AUC from 0 to infinity ($\text{AUC}_{0 \rightarrow \infty}$) was calculated by the logarithmic trapezoidal rule, and only the $\text{AUC}_{0 \rightarrow \infty}$ value was expressed because it is often similar to $\text{AUC}_{0 \rightarrow \text{last time}}$ and covers more; C_{max} represented maximum plasma concentration observed following administration; V_{ss} indicated volume of distribution at a steady state. $T_{1/2}$ was defined as the half lives of the test compounds in the elimination phase. CL represented the clearance. Bioavailability was calculated as follows: $(\text{AUC}_{\text{po}}/\text{AUC}_{\text{iv}}) \times (\text{dose}_{\text{iv}}/\text{dose}_{\text{po}}) \times 100\%$.

2.5. Tissue distribution and elimination of D-1MT in mice

For the tissue distribution and elimination studies, D-1MT (100 mg/kg; 10 ml/kg) was suspended in corn oil and administered to male CD mice (19.5 ± 2.5 g) by gavage following an overnight fast. Groups of four mice each were anesthetized with methoxyflurane vapors at 1, 5, 10, 24 and 48 h after dosing, and blood samples (1.0 ml) were collected by cardiac puncture into syringes containing 150 μ l of 10% heparin/citrate-phosphate dextrose solution. Tissues and organs were immediately removed, individually weighed, transferred to silanized microcentrifuge tubes and stored at -20°C until analysis. For D-1MT elimination studies, groups of four male CD mice were housed in metabolism cages, where urine and feces were

separated by a cone-shaped device. Pooled urine and feces were cumulatively collected prior to D-1MT administration, and at 5, 10, 24 and 48 h following a single oral dose and stored at -20°C . On the day of analysis, urine was processed as plasma was. Tissues and feces were homogenized in ice-cold phosphate buffered saline (1:2, v/w) using a PRO200 post-mounted laboratory homogenizer. Aliquots of methanol were added into individual sample homogenates to extract D-1MT into the supernatant layer. After centrifugation ($12,600\times g$, 10 min), the supernatant was transferred to a glass autosampler vial, and mixed with 2 μl of formic acid. Twenty μl of the mixed solution was then injected into the LC/MS/MS for measurement of D-1MT.

2.6. Animals and dose procedures in toxicity studies

The toxicity studies were performed under good laboratory practice (GLP) regulations by following the general guidelines for designing and conducting toxicity studies issued by the US Food and Drug Administration as well as standard protocols used at the National Cancer Institute for preclinical toxicology studies of oncology drugs (www.fda.gov/cber/gdlns/canclin).

Male Lewis rats weighing 325 ± 4 g (11 weeks old) were used in the dose-range-finding study ($n=3/\text{dose group}$), while 40 male (324 ± 7 g) and 40 female (208 ± 5 g) Lewis rats (12–13 weeks old) as well as 6 male (8.4 ± 0.9 kg) and 6 female (6.7 ± 0.6 kg) beagle dogs (12–13 months old) were used in the GLP-compliant toxicity study (10 rats/sex/dose group, or 2 dogs/sex/dose group). The rats were housed individually in solid-bottom polycarbonate cages equipped with stainless steel racks, and the dogs were housed in stainless steel cages. The animal rooms were maintained between $63\text{--}73^{\circ}\text{F}$ with a relative humidity of 35–87% during the study. Room lights were controlled by an automatic timer set to provide 12 h of light and 12 h of dark.

Using a computerized randomization procedure, the rats and dogs were assigned to yield treatment groups with comparable group mean weights after randomization. For rats, the dose groups included a vehicle control and groups at the 150, 300, 600, 1200, 2100, and 3000 $\text{mg}/\text{m}^2/\text{day}$ dose levels (25, 50, 100, 200, 350, and 500 $\text{mg}/\text{kg}/\text{day}$). For dogs, the dose groups included a vehicle control, and groups at the 600 and 1200 $\text{mg}/\text{m}^2/\text{day}$ (30 and 60 $\text{mg}/\text{kg}/\text{day}$) dose levels. Dose formulations containing various concentrations (5–100 mg/ml) of D-1MT were prepared in 1% carboxymethylcellulose to produce a uniform volume of 5 ml/kg and 2 ml/kg for administration of D-1MT to rats and dogs, respectively, thus reducing the possibility of masking drug effects. The mean concentrations of D-1MT in the dose formulations ranged from 94–103% of the theoretical concentrations with relative standard deviations of 0.25–1.8% as measured by an HPLC method, indicating homogeneous formulations. Fresh dose formulations were prepared 3 times during the study and used for dosing within 12 days of preparation. Each dose formulation was stirred prior to and during dosing. Dose formulations containing D-1MT in 1% carboxymethylcellulose were deemed to be stable at refrigerated temperatures for at least 21 days.

2.7. Toxicity examination procedures

Rats and dogs were evaluated for mortality and morbidity twice daily and abnormal behaviors once daily on day 1 through day 57. Body weight was measured prior to dosing and on days 1, 5, 8, 12, 15, 19, 21, 22, 26, 29, 36, 42, 50, and 57. Blood samples were collected from the dogs' peripheral veins, or from the retro-orbital plexuses of rats, under CO_2/O_2 anesthesia, into tubes containing EDTA (hematology samples), no anticoagulant (clinical chemistry samples), or sodium citrate (coagulation samples) before dosing and on days 8, 15, 29, 36, 42, and 57 for clinical pathology determinations. Correlative plasma levels of D-1MT were analyzed in rats on day 22 at 6 h following D-1 MT doses of 1200, 2100, and 3000 mg/m^2 , and in dogs on

days 1 and 28 at 1 h following 600 and 1200 mg/m² doses. Microscopic pathology was performed in both rats and dogs. The rats (5/sex/dose group) and dogs (1/sex/dose group) were euthanized by CO₂ asphyxiation (day 29) and an overdose of barbiturate (day 57), respectively. The collected tissues and organs included bone marrow (femur), brain, cecum, colon, duodenum, epididymides, esophagus, heart, ileum, jejunum, kidneys, liver, lungs (infused with formalin), lymph nodes (mandibular, mesenteric), pancreas, sciatic nerve, skeletal muscle, skin (ventroabdominal), spinal cord (thoracolumbar segment for routine section; and cervical and posterior lumbar sections, if neurological signs indicated cord involvement), spleen, stomach (forestomach and glandular) and thymus. These tissues and organs were fixed in 10% neutral buffered formalin. Testes and epididymides were saved in Bouin's fixative. Eyes were fixed in Davidson's solution. Sections of the fixed tissues to be processed for histopathological examination were embedded in paraffin blocks and placed on glass microscope slides for hematoxylin and eosin staining.

2.8. Statistical analyses

Group means and standard deviations were calculated and subjected to analysis of variance (ANOVA). The Dunnett's test was used to compare group means in the toxicity study. It is specifically designed for situations where all dose groups are to be pitted against one control group after ANOVA has rejected the hypothesis of equality of the means of the distributions. The *t*-test analysis was applied to the pharmacokinetic study to assess whether the means of two dose groups of small samples are statistically different from each other.

3. Results

3.1. Plasma stability and protein binding of D-1MT

Under the validated LC/MS/MS conditions, D-1MT eluted at a retention time of 9.4 min. D-1MT was stable in rat plasma: incubation of D-1MT (250 and 5000 nM) with plasma at 37°C for 24 h produced no significant degradation of D-1MT, and its stability at 37°C was the same as that observed at 4°C during that time frame. The percentage binding of D-1MT at concentrations of 250 nM and 5000 nM to rat plasma was 15% and 2 %, respectively. These results suggest that free D-1MT predominates in blood after it enters the systemic circulation.

3.2. Pharmacokinetics of D-1MT in rats and dogs

Plasma concentration-time courses after single dosing demonstrated a fast decline in D-1MT levels in dogs compared with those observed in rats (Fig. 1). Pharmacokinetic parameters of D-1MT in rats and dogs are presented in Table 1.

The rat AUC was significantly larger than the dog AUC, when the two species were administered equivalent body-surface-area doses of D-1MT via the same administration route (Fig. 1; Table 1). For example, intravenous administration of D-1MT (300 mg/m²) resulted in an AUC value that was 6.2-fold higher in rats (1,941 μM·h) than that observed in dogs (314 μM·h). Likewise, oral administration of D-1MT (600 mg/m²) resulted in an AUC value that was 11-fold higher in rats (3,566 μM·h) than that observed in dogs (321 μM·h; Table 1). This phenomenon could be explained by the remarkably long half-life of D-1MT in rats compared with that of dogs at equivalent body-surface-area doses administered via the same route. The longer half-life of D-1MT in rats may be due to the slower clearance rate (868 mL/h/m²) and larger volume of distribution (27,960 mL/m²) compared with those of dogs (1332 mL/h/m² and 4737 mL/m², respectively) at the same intravenous dose of 300 mg/m².

Oral bioavailability decreased when D-1MT was administered orally to rats (>600 mg/m²) or dogs (>1200 mg/m²). This result prompted the quantitative analysis of the relationship between D-1MT doses and the corresponding D-1MT plasma C_{max} and AUC values. C_{max} and AUC

increased linearly with increasing D-1MT oral doses of up to 600 mg/m² in rats and 1200 mg/m² in dogs (Fig. 2). Thus, D-1MT plasma concentrations and AUC values are directly proportional to the oral doses administered, up to the dose levels noted. Beyond those dose levels, AUC values reach a plateau and C_{max} values begin to decline. Therefore, those values are indicative of the lowest saturating doses in rats and dogs at a maximal gastrointestinal absorption rate. A further increase in dose is unlikely to alter the blood concentration of D-1MT and its consequential systemic efficacy and toxicity.

3.3. D-1MT tissue distribution and elimination of D-1MT in mice

Figure 3 shows D-1MT levels in various tissues of mice at 1, 5, 10, 24 and 48 h following an oral administration of D-1MT at 300 mg/m² (100 mg/kg). A substantial variability in D-1MT concentrations was observed among different tissues in mice. In general, excluding the gastrointestinal tract, the highest concentrations of D-1MT at 1 h after dosing were found in the kidney (188 nmol/g), followed by the liver (71 nmol/g), muscle (50 nmol/g), heart (28 nmol/g), lung (17 nmol/g) and spleen (16 nmol/g). The brain D-1MT tissue concentration was merely 3.6 nmol/g. The heart, lung, and muscle D-1MT tissue concentrations displayed a biphasic, prolonged elimination t_{1/2} (≥15 h), while the liver, spleen, and brain tissues exhibited an elimination t_{1/2} comparable to that of plasma (~6 h). The mean concentrations of D-1MT in the stomach, and small and large intestines were 3220, 1230 and 81.7 nmol/g, respectively, at 1 h, and then fell to ~10 nmol/g at 24 h post dose, kinetically reflecting the gastrointestinal transit and absorption time of D-1MT.

In mice, 35.1% of the administered D-1MT dose was excreted in the urine and 13.5% in the feces 48 h post dose (Fig. 4). The D-1MT excretion rate (μmol/h) via the urine was faster than that via the feces during the elimination period.

3.4. D-1MT plasma levels following 28-day multiple dosing in rats

Figure 5 illustrates D-1MT plasma levels in rats monitored at 5 h after oral dosing on days 1, 14 and 28 during a consecutive 28-day administration period with 75, 150, 300 and 450 mg/m²/day (12.5, 25, 50 and 75 mg/kg/day, b.i.d.). The differences in plasma concentrations among these dose groups were statistically significant on day 1, but not on day 14 or 28. For example, on day 1, the mean plasma concentration of D-1MT in the 75 mg/m²/day group was 62.5 ± 6.2 μM. In contrast, the mean plasma concentration from the 300 and 450 mg/m²/day groups was 153.7 ± 34.9 μM and 168.5 ± 26.9 μM, respectively, indicating a dose-dependent difference in plasma concentrations between the 75 and 300 mg/m²/day groups (*P* = 0.028), as well as between the 75 and 450 mg/m²/day groups (*P* = 0.006) on day 1. However, on days 14 and 28, the differences in D-1MT plasma concentrations among these dose groups became insignificant (*P* > 0.05). Within the same dose group, a trend of accumulation of D-1MT in plasma over the consecutive administration period was observed. All these suggest that consecutive administration of D-1MT make its accumulation in blood, and low-dose of D-1MT accumulates faster in blood than high dose does. The accumulation of D-1MT in plasma during the repeated dosing period suggests that no unusual enzymatic activity is induced following the long period of oral administration of D-1MT in rats, which would otherwise accelerate its normal degradation, resulting in no accumulation or even decreases of D-1MT in the plasma. The long elimination t_{1/2} and good biostability of D-1MT in plasma may contribute to its steady plasma concentrations during the 28-day dosing period.

3.5. Toxicological observations

Clinical signs in dogs treated with D-1MT (600 and 1200 mg/m²/day) were limited to sporadic gastrointestinal events, which were also observed in the vehicle control group. The incidence of these events did not appear to be dose related; therefore, they were considered unrelated to the treatment. In rats treated with D-1MT at doses of 75 to 3000 mg/m²/day, an incidence of

nasal discharge was also observed in some of the animals on one or more days between days 22–52, which was slightly higher than in the vehicle control animals; however, this difference was not statistically significant. D-1MT treatment had no effect on body weight in rats or dogs.

Analysis of hematological data revealed no drug-related changes associated with administration of D-1MT in rats and dogs, although some sporadic changes from baseline that reached statistical significance were observed (Dunnett's test), including changes in hemoglobin, reticulocyte, lymphocyte, and eosinophil counts in the rats. Mild, sporadic decreases in red blood cells and hemoglobin levels were also detected in the dogs. These changes were considered to be secondary to individual variability in response to blood collection and were not considered biologically relevant or related to D-1MT treatment.

In rats, some sporadic significant changes in clinical chemistry data were found (Dunnett's test). During the dose-range finding study, these changes included an increase in globulin (3000 mg/m²) on Day 8, a decrease in alanine aminotransferase (1200 mg/m²) on Day 15, and a decrease in creatinine on Days 36 (1200 mg/m²) and 42 (3000 mg/m²). During the GLP-compliant toxicity study, these changes included increases in blood urea nitrogen (300 mg/m²/day, Day 57), glucose (150 mg/m²/day, Day 29), total protein (600 mg/m²/day, Day 29), globulin (600 mg/m²/day, Day 29), and cholesterol (300 mg/m²/day, Day 15). These changes were not considered biologically relevant or related to D-1MT treatment.

In dogs, mild-to-moderate increases in alkaline phosphatase (ALP) values were observed in males in the control (2.6-fold higher on Day 22), 600 mg/m²/day (2.4–2.7-fold higher on Days 15, 22, and 29), and 1200 mg/m²/day (2.9–5.7-fold higher) groups, compared with baseline.

Gross lesions observed in dogs at necropsy on Days 29 and/or 57 in the 600 and 1200 mg/m²/day dose groups included lesions in the lung, spleen, kidney, and skin. Microscopic lesions observed in rats in the vehicle control and D-1MT-treated groups were found in the renal pelvis, adrenal gland, lymph node, and liver; in dogs, these were found in the kidney, prostate, lung, liver, pancreas, thyroid gland, parathyroid gland, pituitary gland, tonsil, thymus, spleen, and lymph nodes. The lesions were generally minimal in severity and occurred randomly among groups, with no dose-related effects observed. They were considered unrelated to treatment.

4. Discussion

The present study shows that D-1MT, a tryptophan derivative, shares similar characteristics with the parent compound tryptophan in terms of bioavailability and safety: 1) Both molecules exhibit good stability in biomatrices, for example, incubation of D-1MT with plasma at 37°C results in no appreciable degradation. Similarly, only negligible losses of tryptophan were observed during different food processes (Nielsen et al., 1985). 2) Both molecules also exhibit high bioavailability; the bioavailability of D-1MT following oral administration ranged among species, being the highest in rats (>100%), followed by mice (85% [data not shown]) and dogs (51%; Table 1). Likewise, the bioavailability of tryptophan in various formulations varied from 83 to 95% (Sarwar and Botting, 1999). 3) Both molecules also show good safety profiles; in the present study, oral administration of D-1MT in rats at doses up to 3000 mg/m²/day (500 mg/kg/day), or dogs at doses up to 1200 mg/m²/day (60 mg/kg/day), for 28 days did not produce significant adverse events or changes in hematology and clinical chemistry. Similarly, Chung et al. (1991) reported that neither acute mortality nor significant changes in total blood leukocytes, relative eosinophil counts, and plasma activities of aspartate transferase, creatine phosphokinase, and lactate dehydrogenase were observed in pigs following intra-gastric administration of tryptophan at the maximal dose of 5.71 g/kg. These results suggest that addition of a methyl group into tryptophan does not significantly change its biostability, bioavailability and safety profiles. Both compounds can be tolerated at high oral doses.

Eosinophilia-myalgia syndrome is a previously described autoimmune disease (Hertzman et al., 1990; Silver et al., 1990) that has been associated with the use of L-tryptophan; 1,1'-ethylidenbis(L-tryptophan) has been implicated as a possible etiologic agent of the product (Belongia et al., 1990). Lewis rats receiving case-associated L-tryptophan developed fasciitis and perimyositis similar to the conditions observed in the human eosinophilia-myalgia syndrome (Crofford et al., 1990). Additional study showed that L-tryptophan induces some, but not all, of the pathology associated with chronic administration of case-associated L-tryptophan in Lewis rats (Love et al., 1993). Some of the associated pathology included significant myofascial thickening, pancreatic fibrosis, and acinar changes with increased CD8, Ia, and IL-2 receptor-positive cells in the peripheral blood. Thus, in light of these findings, Lewis rats were specifically selected for inclusion in the present toxicity study. However, following careful examination of the histopathological results no apparent signs of the eosinophilia-myalgia syndrome were exhibited in the D-1MT-treated Lewis rats. Additionally, no changes in CD8+ cell counts were observed in these animals after the 28-day treatment regimen.

The lack of dose-escalation toxicity observed with D-1MT raises the question as to whether or not the absorbed amount of D-1MT increases proportionally to the doses administered. If direct proportionality does not exist, it may explain the reason behind the failure to see this trend. For example, if oral absorption reaches saturation at 600 mg/m² in rats or 1200 mg/m² in dogs, then, neither more toxicity nor activity would be observed, and the resultant bioavailability would decrease disproportionately, as in fact was the case in the current study (Table 1); this may perhaps be due to direct elimination of the D-1MT excess into the feces. The meaningful bioavailability of drugs should not be determined beyond the saturating point. The present study identified the saturable D-1MT oral absorption dose in rats and dogs, provided important insights regarding the relationship between the maximally achievable blood concentration of D-1MT and its limited toxicity.

Mice are commonly chosen for tissue distribution study because they are inexpensive and consume less tested drugs (Jia et al., 2003; 2005). Processing tissues from mice is easy and less concerns about cruelty than from large animals. Information about tissue distribution and elimination of a test drug is important in terms of toxicokinetics in identifying target organs of the test drug, determining its disposition and accumulation in body, and at what extent and rate the drug is eliminated. These pieces of information are difficult to obtain from large animals and clinical trials. Moreover, mice are commonly used for disease models to test efficacy of investigational drugs such as D-1MT (Potula et al., 2005; Hou et al., 2007). The present study demonstrated that the kidney contained the highest concentration of D-1MT, followed by the liver, muscle, heart, lung, and spleen, respectively; 48 h post dosing, D-1MT was excreted primarily in the urine (35.1%), and to a less degree, in feces (13.5%). Therefore, kidney function should be monitored in clinical trials of D-1MT.

The ability of plasma to bind to a variety of drugs enables it to perform a considerable role in their transport, distribution, and metabolism (Jia et al., 2003). Binding of drugs to plasma proteins, mostly to serum albumin and α -acid glycoprotein, is one of the many factors that influences drug disposition. It is generally accepted that the effects of a drug are related to the exposure of a patient to the unbound concentration of the drug at the action site rather than its total concentration (Rolan, 1994). In the present protein-binding studies, we determined that the majority of D-1MT was found in an unbound state in plasma, with less than 15% being bound to plasma proteins. It is therefore somewhat surprising that 77–94% of tryptophan has been reported to be bound to human serum albumin or plasma protein (Yang and Hage, 1997; Talbert et al., 2002). The differences in protein binding between the two compounds suggest that the addition of a methyl group to tryptophan reduces its binding capacity.

In general, the period of plasma sample collection from animals should include 4–5 half-lives of the test drug. However, in practice, it is often not implemented for a drug with a long $t_{1/2}$. D-1MT showed its $t_{1/2} > 30$ h in rats (Table 1). Thus, it is difficult to manage blood drawing for a week-long period in order to cover the 4–5 elimination half-lives of D-1MT. On the other hand, the WinNonlin program can automatically extrapolate the theoretical values needed by filling in the other measurements beyond those actually measured within 48 h.

In the present toxicity study, only ALP values in male dogs were increased. The activities of other hepatic enzymes remained unchanged. It is unclear whether the increase in ALP values was related to D-1MT treatment. This is because no corresponding changes in AST and ALT values were observed in any dog in the study, which would otherwise be expected to increase concomitantly, as additional indicators of liver damage induced by D-1MT. Furthermore, the increase in ALP values occurred only in male dogs, and the treated groups showed more increase in ALP values than the control group. Nonetheless, these observations suggest that ALP activity should be carefully monitored when conducting clinical trials with this agent.

In summary, D-1MT shows a good pharmacokinetic profile, with some interspecies differences observed. Oral absorption is saturated at doses that are non-toxic. A further increase in dose over the saturating level is unlikely to alter the blood concentration of D-1MT and its consequential systemic efficacy and toxicity. The present study provides the basis for determining the oral maximum tolerated dose of D-1MT in clinical trials. As our understanding increases of the requirements for immune-cell activation, homing, and accumulation at tumor sites, as well as disruption of the regulatory mechanisms that limit responses, immunotherapy with D-1MT may constitute a meaningful treatment for human malignancy.

Acknowledgments

The studies were supported by NCI Contract funds NO1-CM-07105, NO1-CM-52206, and NO1-CM-42201.

Abbreviations

D-1MT	1-methyl-[D]-tryptophan
IDO	indoleamine 2,3-dioxygenase
GLP	good laboratory practice

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Fig. 1.

Plasma concentration-time course of D-1MT following oral or intravenous administration to dogs (upper panel) and rats (lower panel) at various doses. Note that there are equal AUCs between oral 1200 and 2000 mg/m² groups in dogs, and between oral 3000 and 6000 mg/m² groups in rats.

Fig. 2. Dose-AUC and dose-C_{max} relationships of 1MT following single oral administration to rats and dogs. The dash lines indicate a saturating dose at 600 and 1200 mg/m² for rats and dogs, respectively.

Fig. 3.

Tissue distribution of D-1MT at different intervals following oral administration (300 mg/m²) to mice (n= 4 per time point).

Fig. 4. Percentage of total administered D-1MT (300 mg/m², p.o.) excreted into pooled urine and feces of mice (n= 4).

Fig. 5.

Plasma concentrations of D-1MT at 5 h following oral administration of 12.5, 25, 50 and 75 mg/kg/day (70, 150, 300 and 450 mg/m²/day) to rats on days 1, 14 and 28. Statistical differences in mean plasma concentrations among the dose groups were found on day 1 between dose groups of 12.5 and 50 mg/kg/day (*, $P=0.028$), and between dose groups of 12.5 and 75 mg/kg/day (**, $P=0.006$). The differences in plasma concentrations among these dose groups became insignificant on days 14 and 28 after the consecutive administration.

Table 1
Comparison of major pharmacokinetic parameters of D-IMT administered to rats and dogs.

Species	Dose ^a (mg/kg)	Dose ^a (mg/m ²)	Route	t _{1/2} (h)	CL (ml/h/kg)	CL (ml/h/m ²)	V _{ss} (ml/kg)	V _{ss} (ml/m ²)	C _{max} (μM)	AUC _(0-∞) (μM ² h)	T _{max} (h)	F%
Rat	12.5	75	p.o.	13.7					16	440	5.7	87
	50	300	p.o.	46					37	2118	4.7	109 ^b
	100	600	p.o.	28.7					72	3566	8	92 ^b
	500	3000	p.o.	30					114	5370	6	28 ^b
	1000	6000	p.o.	37.8					99	5396	6	14 ^b
	12.5	75	i.v.	16.8	115	691	2690	16140		498		
Dog	50	300	i.v.	23.5	145	868	4660	27960		1941		
	15	300	i.v.	3.3	222	1332	789	4737		314		
	30	600	p.o.	5.1					51	321	1	51 ^b
	60	1200	p.o.	6.0					84	594	1	47 ^b
	100	2000	p.o.	5.4					78	572	1	27 ^b

^a, The conversion factors used for changing a dose expressed in terms of mg/kg to an equivalent surface area dose mg/m² are 6 (rat), and 20 (dog), respectively, referring to the FDA Guidance <http://www.fda.gov/cber/gdlns/dose.pdf>.

^b, F% (oral bioavailability) for rats and dogs was estimated based on the AUC value obtained after intravenous dosing of 300 mg/m² of IMT to the same species.