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Determination of the Tissue Distribution and Excretion by Accelerator Mass Spectrometry of the Nonadecapeptide ^{14}C-Moli1901 in Beagle Dogs after Intratracheal Instillation

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Abstract

1.   Administration of ^{14}C-Moli1901 (duramycin, 2622U90), a 19 amino acid polycyclic peptide by intratracheal instillation (approximately 100μg) into the left cranial lobe of the lung of beagle dogs resulted in retention of 64% of the dose in the left cranial lobe for up to 28 days.

2.   In this study, we used accelerator mass spectrometry (AMS) to quantify Moli901 following administration of only 0.045μCi of ^{14}C-Moli901 per dog. Limits of quantitation of AMS were 0.03 (urine) to 0.3 (feces) ng equiv. Moli1901/g.

3.   Whole blood and plasma concentrations of ^{14}C were <5ng/ml at all times after the dose. Concentrations of ^{14}C in whole blood and plasma declined over the first day after the dose and rose thereafter, with the rise in plasma concentrations lagging behind those in whole blood. During the first 3 days after the dose, plasma accounted for the majority of ^{14}C in whole blood, but after that time, plasma accounted for only 25-30% of the ^{14}C in whole blood.

4.   Tissue (left and right caudal lung lobe, liver, kidney, spleen, brain) and bile concentrations were low, always less than 0.25% the concentrations found in the left cranial lung lobe.

5.   Approximately 13% of the dose was eliminated in urine and feces in 28 days, with fecal elimination accounting for about 10% of the dose.

6.   The data presented here are consistent with that obtained in other species. Moli1901 is slowly absorbed and excreted from the lung, and it does not accumulate in other tissues.

7.   Moli1901 is currently in the clinic and has proven to be safe in single dose studies in human volunteers and cystic fibrosis patients by the inhalation route. No information on the disposition of the compound in humans is available. This study in dogs demonstrates the feasibility of obtaining that information using ^{14}C-Moli1901 and AMS.

Introduction

Moli1901, commonly named duramycin,[1] is a 19 residue polycyclic peptide antibiotic (Shotwell et al., 1958, Hayashi et al., 1990) which increases chloride transport (Cloutier et al., 1990, Cloutier et al., 1993, Molina y Vedia et al., 1996, Henke et al., 1998) and water mobilization (Molina y Vedia et al., 1996, Henke et al., 1998) when applied to the apical surface of the airway epithelium. Because of these properties it may be a useful treatment for cystic fibrosis (CF), a genetic disease characterized by abnormal chloride ion transport. This abnormal chloride transport alters the water content of the airway mucus, impairing its clearance and leading to infection, inflammation, lung disease and death. Moli1901 can increase the chloride permeability in nasal epithelium from healthy volunteers and CF patients (Zeitlin et al., 2004) and it can increase the volume of airway surface liquid in normal dogs (Molina y Vedia et al., 1996, Henke et al. 1998). The therapeutic goal for the treatment of CF and other pulmonary diseases like asthma and chronic bronchitis in which the water content of the mucus is altered, is to remove retained secretions from the lungs.

The known pharmacokinetic profile of Moli1901 is intriguing. The molecule is poorly absorbed after inhalation exposure, intratracheal instillation or oral administration in rats and after oral administration in mice (McNulty et al., 2003). The main route of elimination in rats was the feces. The half-life of

disappearance from the respiratory tract after intratracheal instillation was 64 days and there was no degradation or metabolism of the compound present in this tissue. The half-life of elimination in the feces in these animals was 52 days (McNulty et al., 2003).   Plasma and blood concentrations of Moli1901-related radioactivity were always less than 1ng eq.ml^{-1} and could not be accurately measured. A preliminary study (McNulty et al., 1997) in dogs in which the animals inhaled aerosolized ^{3}H-labeled Moli1901 provided data suggesting that absorption from the lung was slow in this species as well. As in the rat model, accurate measurements of circulating Moli1901-related material were not possible in the dog, and the major route of elimination was the feces.

In preparation for determination of Moli1901 disposition in humans, it was important to determine the systemic exposure to Moli1901-related material in dogs after exposure via the respiratory tract. Carbon-14-labeled Moli1901 was used as a tracer to avoid the possibility of erroneous disposition data due to ^{3}H exchange. At the same time, a methodology that would quantify low amounts of ^{14}C-labeled compound, allowing its application in future human studies was needed. Accelerator Mass Spectrometry (AMS) is an extremely sensitive and precise technique for quantifying certain rare, long-lived isotopes such as carbon-14 and was chosen to quantify the ^{14}C-Moli1901 in this study. AMS quantifies isotopic nuclei, rather than radioactive decay, resulting in 10^3-10^9 fold increase in sensitivity compared to the decay counting methods that are widely

employed in radioisotope tracer studies (reviewed by Turteltaub and Vogel, 2000).

In this paper we describe the kinetics, distribution and elimination of ^{14}C-Moli1901 in beagle dogs after instillation of the dose into the left cranial lobe of the lung. ^{14}C-Moli1901-related radiocarbon is slowly absorbed, achieves only very low concentrations in the circulation and major organs, and is slowly eliminated in the feces.

Materials and Methods

Chemicals. Unlabeled and ^{14}C-labeled Moli1901 were prepared by Apotex Fermentation, Inc., Winnipeg, Manitoba, Canada. The radiolabeled material was produced by fermentation with ^{14}C-Phe. The ^{14}C-Moli1901 thus produced had a radioactive purity of 93% and a specific activity of 3.4mCi/mmol. Unlabeled and ^{14}C-labeled Moli1901 were mixed to allow a dose of approximately 100µg of Moli1901 containing about 0.045µCi of ^{14}C-labeled Moli1901 in 1ml of 0.9% sodium chloride to be given to each dog.

Dose administration. A total of 12 male beagle dogs, aged 9-10 months were used in the study. Anesthetized (isoflurane) dogs were administered the dose via a tube attached to a bronchoscope that was passed into the caudal portion of the left cranial lobe of the lung. Three of the dogs were sacrificed on Day 3

(72 hours after the dose), Day 7 (168 hours after the dose), Day 14 (336 hours after the dose) and Day 28 (672 hours after the dose). At the time of sacrifice, samples of liver, left kidney, spleen, brain, gall bladder bile, left cranial lobe of the lung, right cranial lobe of the lung and left caudal lobe of the lung were taken. Urine and feces were collected daily from the three dogs sacrificed 28 days after the dose. Blood was collected from all dogs at pre-dose, 2, 4, 8, 12, 24, 48 and 72 hours. Thereafter, blood was collected from all dogs remaining at 168, 240, 336, 504, and 672 hours.

Sample collection. Plasma was separated by centrifugation of samples of whole blood (collected from the jugular vein). Blood and plasma samples were frozen until analysis by AMS. The weight of each daily urine collection was recorded, and aliquots were frozen until analysis by AMS.   The weight of each fecal sample was recorded and the total sample was placed in a plastic bag and homogenized manually. Samples were taken and frozen until analysis by AMS.

Gall bladder bile was collected, and the total volume was recorded. Samples were taken and frozen until further analysis by AMS.

The left cranial, left caudal and right cranial lobes of the lung were removed and weighed. They were individually flash frozen in liquid nitrogen and

homogenized in a blender with an approximately equal mass of water. Aliquots of each lung sample were analyzed by liquid scintillation counting (left cranial lobe) or AMS (left caudal and right cranial lobes). For analysis of lung tissue by liquid scintillation counting, aliquots of each homogenate (ca. 1 g) were digested with Soluene-350 (Packard Instrument Company, Inc.; Meriden, CT), neutralized with nitric acid and decolorized with hydrogen peroxide prior to analysis by liquid scintillation counting (Packard Model 2500TR Liquid Scintillation Analyzer). Ultima Gold™ scintillation cocktail (Packard Instrument Company, Inc.) was used in all determinations of radiochemical content.

All other tissues were homogenized in an approximately equal volume of water. Aliquots were taken and frozen until analysis by AMS.

Sample analysis. AMS was used to measure the ratio of ^{14}C to total carbon in aliquots of whole blood, plasma, urine, feces, bile, liver, kidney, spleen, lung and brain. Samples were thawed at room temperature, and an aliquot of each sample (20 μl blood, 50 μl plasma, 100 μl urine, 10-20 mg feces, 25-50 μl of bile and homogenate from liver brain, lung, kidney and spleen) were placed in quartz tubes and dried in a vacuum centrifuge. The samples were converted to CO_2 by combustion, and the CO_2 was quantitatively reduced to graphite in the presence of zinc and titanium hydride, condensing onto cobalt at approximately 500 ºC for 4 hours (Vogel, 1992, Ognibene et al., 2003). The carbon ratios of the graphite samples were then quantified using the 1MV accelerator mass

spectrometer at Lawrence Livermore National Laboratory (Ognibene et al., 2002). Between three and seven replicate measurements were recorded for each sample. All measurements were normalized to similarly prepared standards of known carbon isotope ratios.

The carbon ratios of "Time 0 hours" or "Day 0" whole blood, plasma, urine and fecal samples for each dog were subtracted from the isotope ratios of the other samples. No control samples were available for the tissues and bile; hence a theoretical background carbon ratio of 1.145 Modern was employed. Excess ^{14}C concentrations were converted to Moli1901 equivalents (parent compound and all metabolites, if present) using the specific activity of the dose, the compound molecular weight, and the carbon content of the sample as follows, where one Modern is equivalent to 98 amol ^{14}C/mg carbon, and the % labeling of Moli1901 was 1.45. One amol is 10^{-18} moles; one ag is 10^{-18} g. The molecular weight of Moli1901 is 2013.29. All calculations were performed using Microsoft Excel.

amoles ^{14}C/mg carbon=(Fraction Modern of sample-Fraction Modern of control) X 98 amoles ^{14}C/mg carbon

amoles ^{14}C/mg tissue=(amoles ^{14}C/mg carbon/100) X % w/w carbon

amoles Moli1901/mg tissue=(amoles ^{14}C/mg tissue) X (100/% labeling of Moli1901)

ag Moli1901/mg tissue=(amoles Moli1901/mg tissue) X molecular weight

ng Moli1901/g tissue=(ag Moli1901/mg tissue/10^6)

The carbon content (w/w percentage carbon) was measured using an Exeter 440 CHN Analyzer, using at least 3 replicates for each sample type. The average values found and used in calculations were: plasma--3.3%, whole blood--11.0%, feces--12.8%, bile--11.8%, kidney--6.1%, spleen--8.3%, brain--6.4%, liver--7.9%, left caudal lung lobe--5.0% and right cranial lung lobe--4.6%. As the percentage carbon is more variable in urine (in this study it ranged from 0.2-3.1%), each individual urine sample was analyzed for carbon content.

The limit of quantitation of the AMS analysis is calculated as the mean plus 3 times the SD of the background (i.e., the control samples). Controls from whole blood, plasma, urine and feces were used for this calculation. The limits of quantitation (ng Moli1901 equivalents/g sample) were calculated as: whole blood—0.25, plasma—0.08, feces—0.30, urine—0.03 (based on an average % carbon of 1.2), bile—0.28, liver—0.19, spleen—0.20, kidney—0.15, left caudal

lung lobe—0.12, right cranial lung lobe—0.11 and brain—0.15. All samples contained Moli1901 levels that were well above the limits of quantitation.

Results

Blood and Plasma Concentrations of Moli1901-Related ^{14}C. At all times studied after an intratracheal instillation dose of ^{14}C Moli1901, blood and plasma concentrations of ^{14}C were low (<5ng/ml; table 1). The concentration of ^{14}C in whole blood declined during the first day after the dose and slowly rose thereafter. The concentration of ^{14}C in plasma mirrored the decline and rise seen in whole blood, although the increase in plasma concentration began somewhat later (after about 168 hours). During the first 3 days after the dose, plasma accounted for the majority of ^{14}C in whole blood, but after that time, plasma accounted for only 25-30% of the ^{14}C in whole blood.

[Insert table 1 about here.]

Lung Concentrations of Moli1901-Related ^{14}C. Concentrations of ^{14}C in the lobe into which ^{14}C-Moli1901 was instilled (left cranial lobe) were high enough to measure by liquid scintillation counting throughout the 28-day duration of the experiment (table 2), and the total ng equivalents/g lung remained fairly constant between 72 and 672 hours. The percent dose remaining in the lobe did decrease, however. This is because the animals got slightly different total doses due to body weight differences.

[Insert table 2 about here.]

Bile and Tissue Concentrations of Moli1901-Related ^{14}C. Concentrations of Moli1901-related ^{14}C in the right cranial and left caudal lung lobes and in bile, liver, kidney, spleen and brain were much lower than in the left cranial lung lobe (table 3). In tissues other than the left cranial lung lobe the highest concentrations were found in the left caudal lung lobe, and those concentrations were less than 0.25% of those found in the left cranial lobe.

The next highest concentrations were found in the kidney and right cranial lung lobe. Bile and liver had approximately equivalent concentrations, while spleen had about half the concentration found in liver. Brain contained only about one-tenth the concentrations found in spleen.

[Insert table 3 about here.]

Excretion of Moli1901-Related ^{14}C in Urine and Feces. Approximately 13% of the dose was eliminated in urine and feces in 28 days (table 4), with fecal elimination accounting for about 10% of the dose.

[Insert table 4 about here.]

Excretion of Moli1901-related ^{14}C in urine remained fairly constant for approximately the first 20 days after the dose; between Days 20 and 28 excretion was also constant, but at a higher rate in two of the three dogs (figure 1).

[Insert figure 1 about here.]

Excretion in feces was more rapid during the first four days after the dose than it was thereafter (figure 2). From approximately Day 5 through Day 28 fecal elimination was slow, but constant.

[Insert figure 2 about here.]

Discussion

The data in this paper are consistent with those reported previously for rats, mice and dogs (McNulty et al., 1997, 2003) in that Moli1901-related material disappears slowly from lungs after an inhalation or instillation dose, and it is slowly excreted, primarily in the feces. Based on earlier data (McNulty et al., 1997, 2003) it is probable that little or no metabolism of Moli1901 occurred. It is quite likely that most of the Moli1901-related material appearing in the feces is the result of swallowing the compound subsequent to mucociliary clearance from the lungs.

Of the tissues examined, excluding the site of administration, kidney and lung contained the highest concentrations of Moli1901-related material. In rats given an intravenous dose of the compound (McNulty et al., 2003), the highest concentrations were found in spleen and liver. No explanation for this difference in distribution of absorbed Moli1901-related material is readily apparent from the data gathered.

The concentration of Moli1901-related material in blood and plasma declined at first and then rose toward the end of the experimental period. This suggests that the compound was being removed more slowly from blood than it was being added by absorption from the lung or from the gastrointestinal tract after swallowing (see above). Furthermore, as the time after the dose increased, so did the proportion of Moli1901-related material associated with the cellular fraction of blood. Several studies have shown that Moli1901 interacts with membrane phospholipids (Navarro et al., 1985, Sokolove et al., 1989). The NMR analysis from Wakamatsu et al. (1990) indicates that a biological analog to Moli1901 named Ro90198 differing only in one residue (Lys present in Moli1901 is replaced by Arg in Ro90198) binds to the polar head of phosphatidyl ethanolamine (PE). We have confirmed that Moli1901 possesses this same property (Molina, unpublished data). In addition, Choung et al. (1988) and Aoki et al. (1994) have characterized the binding of Ro90198 to human erythrocytes and demonstrated that the peptide binds to PE and could induce its outward transbilayer movement. Similarly, we anticipate binding of Moli1901 to the PE present in the red blood cells. It is even conceivable that these cells would concentrate compound present in plasma. This may be due to the association of Moli1901 with cell membranes in whole blood.

A similar association with membrane lipids may account for the slow removal of Moli1901-related material from the site of application in the lung. Alternatively, the slow removal of the compound from the lung may be due to the size and

molecular weight of Moli1901 limiting its absorption. While peptides with more amino acid residues such as insulin (51 residues) and growth hormone (192 residues) can be absorbed from the lungs of humans and animals in active form (reviewed in Patton and Platz, 1992), the structure of Moli1901 differs from those molecules in having several intramolecular bonds.   These intramolecular bonds may force the compound into retaining a 3-dimensional structure with a greater molecular radius than insulin or growth hormone. Studies of model peptide transport across rat alveolar cell monolayers have demonstrated that absorption is primarily paracellular and is inversely related to molecular radius (Dodoo et al., 2000).

The data in this study are consistent with data in the previous study of inhaled aerosolized ^{3}H-Moli1901 in dogs. In both studies, a significant portion (50-65%) of the dose of radiolabel remained in lung tissue at 4 weeks after dose administration, although in the earlier study, the radiolabel was more evenly distributed in lung tissue. This is an expected result given that the dose was delivered to all lobes of the lung in that study. In both studies fecal elimination was the primary route of excretion, and the bulk of the fecal excretion occurred over the first couple of days.

Moli1901 is currently in the clinic and has proven to be safe in single dose studies in human volunteers and CF patients by the inhalation route. Although technically challenging, determination of the half-life or residence time in the

lung, as well as the biodistribution and excretion are long term goals of the project. This study in dogs demonstrates the feasibility of the execution of a full clinical protocol using ^{14}C-Moli1901 and AMS to attain those objectives.

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Footnotes

[1] Moli1901 is used throughout to denote the compound studied. It has also been known as 2262U90 and duramycin; those names are used in some of the cited material.

Table 1: Average concentrations of Moli1901-related ^{14}C in blood and plasma

Time (hr)	Concentration (ng equiv/ml)	
	Blood	Plasma
2	3.24±0.85^a	4.98±0.88^a
4	2.15±0.57^a	3.33±0.69^a
8	1.23±0.18^a	2.34±0.34^a
12	1.02±0.20^a	1.96±0.28^a
24	1.02±0.22^a	1.78±0.28^a
48	1.10±0.24^a	1.54±0.27^a
72	1.20±0.26^a	1.42±0.24^a
168	1.59±0.22^b	1.19±0.17^b
240	1.75±0.28^c	1.04±0.07^c
336	1.92±0.29^c	1.03±0.11^c
504	2.28±0.21^d	1.18±0.11^d
672	2.58±0.77^d	1.46±0.07^d

Values are mean±SD.
[a]N=12
[b]N=9
[c]N=6
[d]N=3

Table 2: Percent dose and concentrations of Moli1901-related ^{14}C remaining in the left cranial lung lobe at various times after dose administration

Time (hr)	% dose in lobe	ng equiv/g in lobe
72	73.4±4.4	34911±6076
168	65.3±3.8	46981±1622
336	69.8±4.9	46370±3293
672	64.1±9.4	47659±5232

Values are mean ±SD; N=3.

Table 3: Average concentrations of Moli1901-related ^{14}C in bile and tissues at various times after the dose.

Time (hr)	Concentration (ng equiv/g) in:						
	Bile	Liver	Kidney	Spleen	Brain	Lung, right cranial lobe	Lung, left caudal lobe
72	8.03±0.41	6.67±2.87	20.15±1.55	4.83±3.84	0.43±0.04	10.24±7.34	28.04±26.66
168	9.65±2.37	10.71±4.35	16.74±3.02	4.50±1.71	0.54±0.11	10.68±6.61	42.01±30.17
336	5.30±1.05	9.93±2.96	16.37±0.56	6.25±3.84	0.48±0.02	9.51±7.93	25.05±21.43
672	15.31±3.41	6.46±0.66	18.81±1.92	3.37±0.15	0.64±0.08	4.17±0.78	20.71±12.35

Values are mean ±SD; N=3.

Table 4: Cumulative Excretion of Moli1901-related ^{14}C in urine and feces.

Dog #	% dose in urine	% dose in feces
352	3.7	11.9
353	2.8	10.0
395	2.0	8.6
average[a]	2.8±0.8	10.2±1.6

[a]Average of all three dogs±SD.

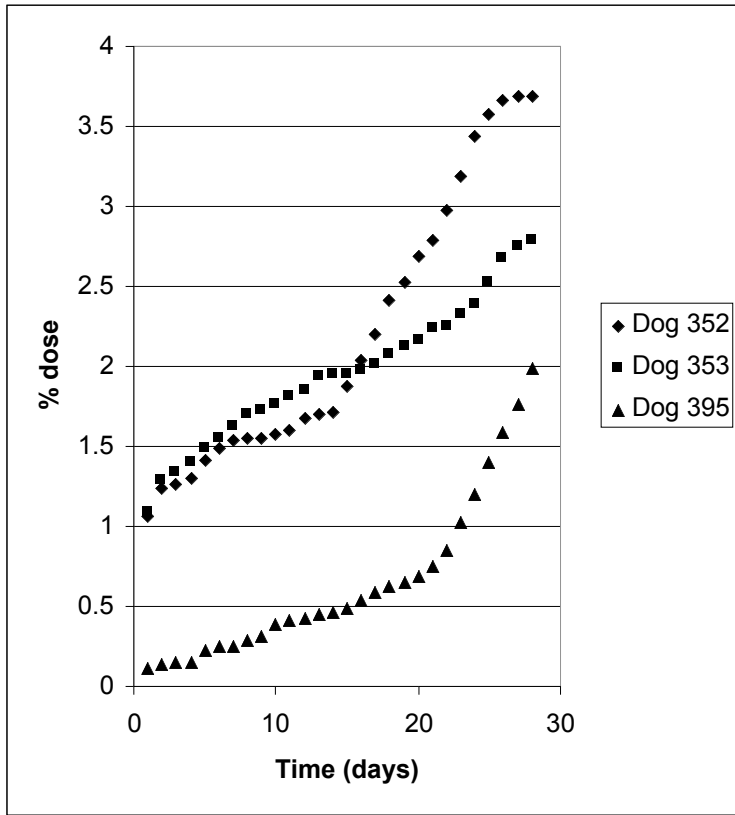**Figure 1: Cumulative excretion of Moli1901-related ^{14}C in urine.**

Figure 2: Cumulative excretion of Moli1901-related ^{14}C in feces.