Study Design Modifications for the Follow-Up to the Pilot Study of Oral Bioavailability of Dioxins/Furans in Midland Soil

This document describes a proposed study design for a follow-up to the pilot study of the oral bioavailability of dioxins and furans from Midland and Tittabawassee River flood plain soils. The pilot study results showed statistically significant differences in hepatic EROD activity (a marker for cytochrome P450 1A1 induction) between the rats dosed with soils and their respective reference groups, with higher enzyme activity observed in the reference group rats compared to the rats in the respective soil groups. This follow-up study is designed to repeat the pilot rat study with study design modifications intended to attempt to prevent differential enzyme induction.

The observed differences in EROD activity were likely due to a difference in absorbed dose of dioxin and furan (PCDD/F) compounds (Figure 1). Rats in the corn oil reference groups received greater administered doses of PCDD/Fs than the soil/feed mixture groups due to lower than expected consumption of feed by all rat groups (Table 1). In addition, the fraction of administered dose absorbed in the soil groups may have been ¼ to ½ of the fraction absorbed from the corn oil gavage administration. The difference in EROD activity between the soil and reference groups is likely due to higher liver concentrations achieved due to higher absorbed doses of PCDD/Fs in the reference groups compared to the soil groups and resulting hepatic EROD activity.

CYP1A1 is involved in the metabolism of several of the key TEQ-contributing compounds in the Midland and Tittabawassee River flood plain soils, and induction of this enzyme can result in an increased rate of metabolism for these compounds. Because the method used to estimate relative bioavailability in this study relies upon an assumption that the elimination rate (and therefore the metabolism) for each compound is the same in the soil and reference oil dose groups, demonstrated statistically significant differences in EROD activity among the groups may result in invalid estimates of relative bioavailability for any congener with metabolism mediated by CYP1A1. In the pilot study, estimates of relative bioavailability for many of the

compounds in the study were statistically significantly different between the rats and swine. Since the rats displayed different EROD activities in the soil and reference groups (while the swine did not), this factor may account for some of the observed differences in apparent relative bioavailability between the two species.

Table 1: Comparison of administered doses and hepatic TEQ concentrations in rat study groups.

Dose Group	Admin. Dose (ng TEQ/kg-d)	Hepatic TEQ (pg/g)	Fold difference compared to soil group	
			Admin. dose	Hepatic TEQ
Midland Soil/Feed	0.6	41	--	--
Ref. Feed	0.7	104	1.2	2.5
Ref. Oil Gavage	1.0	201	1.7	4.9
T-River Soil/Feed	2.6	684	--	--
Ref. Oil Gavage	2.9	1556	1.1	2.3

This follow-up to the pilot study is designed to repeat the rat study of the Midland and Tittabawassee River flood plain soils used in the pilot study. The pilot study design will be used, with two key modifications designed to ensure that differential EROD or MROD induction does not occur:

1. In the pilot study, the reference corn oil materials were prepared with concentrations of the key contaminants designed to result in a match to the *administered* dose of these compounds in the soil/feed mixture. In this follow-up study, the reference oil will be formulated in order to more closely match the anticipated *absorbed* dose of compounds from the soil/feed mixture. Based on the results of the swine component of the pilot study (in which no differential enzyme induction was observed between soil and reference groups), the mean relative biovailabilities of the five tested furan compounds ranged from a low of 0.22 for 2,3,7,8-TCDF to a high of 0.37 for 1,2,3,6,7,8-HxCDF, with a TEQ-weighted mean of 0.27. Based on this, the reference corn oil material will be formulated to match the anticipated absorbed doses from the soil/feed mixture assuming that the relative bioavailability of the compounds in soil is about 0.3 compared

[6]

to the reference oil. EROD and MROD activity will again be measured in the soil and reference oil groups in order to evaluate whether this adjustment results in equivalent EROD and MROD activities and hepatic TEQ concentration between the soil and reference groups.

Four additional modifications unrelated to the differential EROD activity will be made based on the results of the pilot study in order to streamline the study and respond to animal care issues raised in the first study:

1. In the pilot study, both liver and adipose tissues were analyzed, the fraction of administered dose retained in each tissue was calculated, and the relative bioavailability was estimated based both on the individual tissues and on the total fraction retained in the two tissues combined. However, in the pilot rat study, the majority of each retained compound was found in the liver tissue (Figure 3 from Draft Pilot Study report), concentrations were higher in hepatic tissue than in adipose tissue, and the estimates of relative bioavailability based on the liver compared to estimates based on adipose or on the combined tissues were nearly identical (Figure 4 from Draft Pilot Study report). Thus, in this follow-up study, only liver tissues will be analyzed for the study compounds. The rat carcasses from the study will be frozen and stored in case additional analyses are needed based on the initial hepatic tissue results, but every indication from the pilot study is that analyses of liver tissue alone will provide results comparable to those based on analysis of liver and adipose tissue.

2. In the pilot study, tissues were collected and homogenized from pairs of rats in order to collect large enough fat samples to achieve sufficiently low detection limits to ensure detection of the administered compounds. As discussed above, the follow-up study will focus on liver tissue only. The results of the pilot study demonstrated that the liver tissue concentrations in these animals easily exceeded detection limits for all congeners of relevance for both soils. For that reason, the follow-up study will analyze tissues from five single animals per dose group, rather than five pairs of animals.

3. For the corn oil gavage groups, the volume of the daily gavage vehicle will be halved based on recommendations from the researchers at the University of Missouri-Columbia College of Veterinary Medicine who are handling the animals. This will result in corn oil gavage volumes of ½ ml. Compound concentrations in the corn oil reference material will be increased accordingly in order to match the anticipated absorbed soil doses.

4. Finally, based on gavage-related mortality observed in the pilot study, ten rather than five rats will be included in the each of the reference oil gavage groups during the compound administration phase of the study in order to ensure that at least five animals reach the conclusion of the 30-day dosing period. At the end of the administration period, five rats will be selected at random from all surviving rats in each gavage group for liver tissue collection. Remaining rat carcasses will be frozen and stored in case additional follow-up analyses are deemed necessary.

Tables 2 and 3 present a summary of the dose groups, dosing material analysis, and tissue analysis for the follow-up study.

Table 2: Summary of dose groups for follow-up study

Dose group description	Number of animals on test	Number of liver tissue analyses
1. Tittabawassee River flood plain soil/feed mixture (soil sample Imerman Park 2 THT02769)	5	5
2. Reference corn oil gavage matched to the concentrations of the T-River soil/feed mixture, 1/2 ml, 1 x per day	10	5[a]
3. Midland soil/feed mixture (soil sample CC-S-27)	5	5
4. Reference corn oil gavage matched to the concentrations of the Midland soil/feed mixture, 1/2 ml, 1 x per day	10	5[a]

[a] Five animals randomly selected from all remaining group animals at the end of the 30-day dosing period

Table 3: Summary of samples for HR-GC/MS analysis

Sample description	Number of analyses
Soil/feed mixture, pre-test characterization, triplicate split sample for analysis	6[a]
Soil/feed mixture, post-administration for confirmation of stability	2
Reference corn oil solutions, pre-test characterization for confirmation of compound concentrations	2[a]
Reference corn oil solution, post-administration for confirmation of stability	2
Liver tissue samples, 5 each from four dose groups	20

[a] This analysis will be requested on a "rush" basis in order to prepare dosing solutions and feed mixtures in a compressed time frame.

As in the pilot study, the soil/feed mixture will be prepared at WIL Research. All analytical work, and the preparation of the reference corn oil dosing material, will be conducted at Alta Analytical. Analysis of hepatic tissue samples for EROD and MROD activity will be conducted by Entrix. Animal husbandry and dosing will be conducted at the College of Veterinary Medicine at the University of Missouri-Columbia under the direction of Dr. Stan Casteel. Other details of animal husbandry, diet, etc., will be conducted as described in the pilot study report.

EROD and MROD activity will be compared between the soil group and each of the two gavage reference groups using standard t-tests. If the EROD and MROD activities do not differ between soil and reference groups, relative bioavailability will be estimated based on a comparison of the fraction of administered dose retained in liver tissue in the soil/feed group compared to each of the corn oil gavage reference groups.

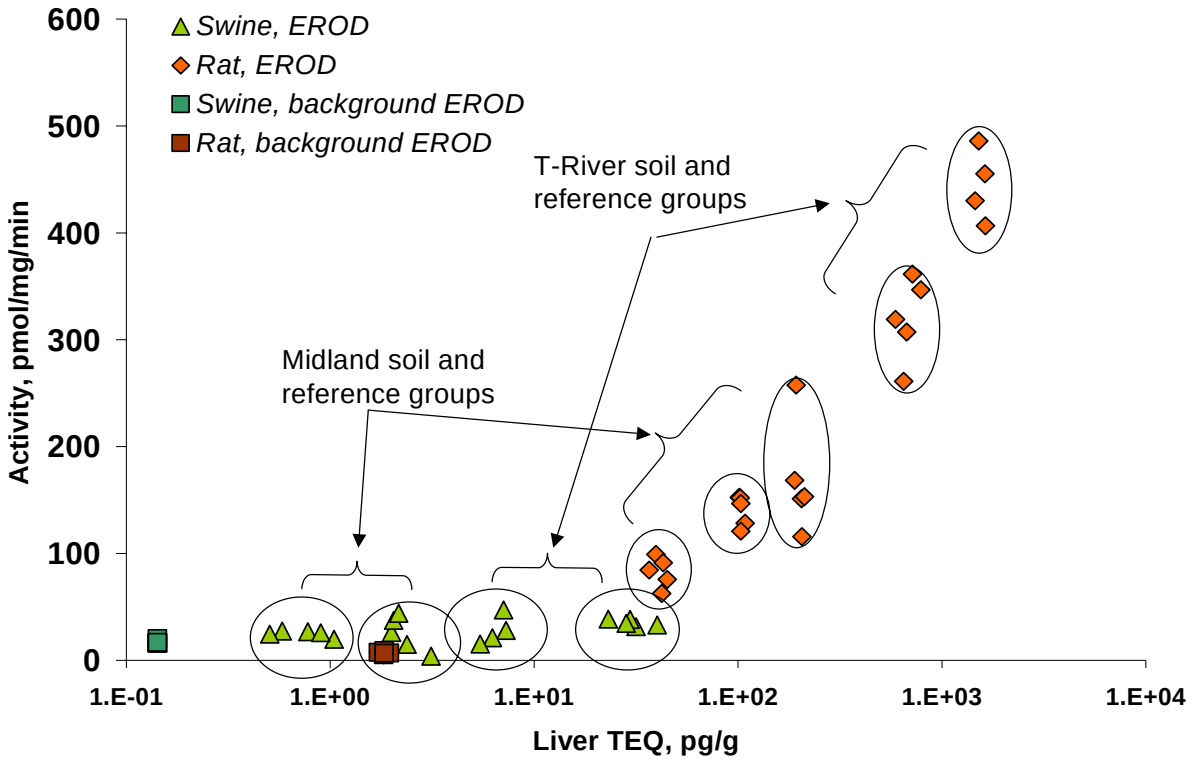

Figure 1: EROD activity as a function of liver TEQ concentration for the rat and swine experimental groups in the oral bioavailability pilot study. While the swine demonstrated no statistically significant differences in hepatic EROD activity between reference oil and soil groups, such statistically significant differences were observed in the rat groups, with reference oil and feed groups demonstrating elevated liver TEQ and EROD activity compared to soil groups for both soils. There was no overlap in the EROD activity or hepatic TEQ concentrations between soil and reference oil groups for either soil.