FINAL REPORT

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ADME-TOX

Half-life (plasma, mouse, CD-1) - Study of Several Compounds

STUDY ID: US034-0024476

STUDY NUMBER
100072972

June 26, 2024

CONFIDENTIAL

1. STUDY REFERENCES

Study title	**ADME-TOX Half-life (plasma, mouse, CD-1) - Study of Several Compounds**	
Study number	**100072972**	**FINAL REPORT June 26, 2024**
Study ID	**US034-0024476**	
Experimental period	June 25, 2024 - June 25, 2024	
PO number	EUROMYT-060624	

2. PERSONS INVOLVED IN THE STUDY

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3. APPROVAL

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Kevin THOMAS
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I certify that this report accurately reflects all relevant data collected in this study.

Date

Signature

Quality Control Review

Our quality Control Designee assures that data results presented in this report were generated using appropriate materials and controlled methods and that the final results accurately reflect the raw data.

Date

Signature

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5. PURPOSE OF THE STUDY

The purpose of this study was to test 10 compounds in the Half-life (plasma, mouse, CD-1) assay.

6. COMPOUNDS

6.1. Test Compounds

Client Compound ID	Compound ID	Reference Number	Batch Number	FW	MW	Purity	Received Form	Stock solution	Flag
MYT-251	100072972-1	-	1	231.2	231.2	100.0	Oil	-	-
ML-116	100072972-2	-	1	428.35	428.35	100.0	Powder	-	-
MYT-204	100072972-3	-	1	372.33	372.33	100.0	Powder	-	-
"MAR-4"	100072972-4	-	1	307.3	307.3	100.0	Powder	-	-
"MAR-5"	100072972-5	-	1	243.22	243.22	100.0	Powder	-	-
MYT-253	100072972-6	-	1	293.27	293.27	100.0	Powder	-	-
ML-110	100072972-7	-	1	288.21	288.21	100.0	Powder	-	-
ML-111a	100072972-8	-	1	400.29	400.29	100.0	Powder	-	-
DMF	100072972-9	-	1	144.13	144.13	100.0	Powder	-	-
MMF	100072972-10	-	1	130.1	130.1	100.0	Powder	-	-

FW: Formula Weight - MW: Molecular Weight

6.2. Reference Compounds

In each experiment and if applicable, the respective reference compound was tested concurrently with the test compounds, and the data were compared with historical values determined at Eurofins. The experiment was accepted in accordance with Eurofins validation Standard Operating Procedure.

7. RESULTS

7.1. ADME-Tox: *In Vitro* Metabolism

7.1.1. Test Compound Results

Compound I.D.	Client Compound I.D.	Test Concentration	Incubation (minutes)	% Compound Remaining (% remaining)			Half-Life (minutes)			Flags
				1st	2nd	Mean	1st	2nd	Mean	
Half-life (plasma, mouse, CD-1)										
100072972-1	MYT-251	1.0E-06 M	0	100.00	100.00	100.00	138.5	101.4	119.9	
100072972-1	MYT-251	1.0E-06 M	30	89.81	96.05	92.93				
100072972-1	MYT-251	1.0E-06 M	60	78.30	88.72	83.51				
100072972-1	MYT-251	1.0E-06 M	90	{41.7}	60.20	60.20				
100072972-1	MYT-251	1.0E-06 M	120	55.28	45.32	50.30				
100072972-2	ML-116	1.0E-06 M	0	100.00	100.00	100.00	82.6	139.7	111.1	
100072972-2	ML-116	1.0E-06 M	30	78.56	59.94	69.25				
100072972-2	ML-116	1.0E-06 M	60	50.23	{37.7}	50.23				
100072972-2	ML-116	1.0E-06 M	90	43.01	{62.5}	43.01				
100072972-2	ML-116	1.0E-06 M	120	38.38	49.70	44.04				
100072972-3	MYT-204	1.0E-06 M	0	100.00	100.00	100.00	30.5	30.1	30.3	
100072972-3	MYT-204	1.0E-06 M	30	50.58	50.15	50.36				
100072972-3	MYT-204	1.0E-06 M	60	17.39	17.68	17.53				
100072972-3	MYT-204	1.0E-06 M	90	1.13	1.09	1.11				
100072972-3	MYT-204	1.0E-06 M	120	0.59	0.70	0.64				
100072972-4	"MAR-4"	1.0E-06 M	0	100.00	100.00	100.00	13.7	15.0	14.4	
100072972-4	"MAR-4"	1.0E-06 M	30	21.99	25.01	23.50				
100072972-4	"MAR-4"	1.0E-06 M	60	8.89	9.15	9.02				
100072972-4	"MAR-4"	1.0E-06 M	90	4.32	4.39	4.36				
100072972-4	"MAR-4"	1.0E-06 M	120	2.56	2.25	2.40				
100072972-5	"MAR-5"	1.0E-06 M	0	100.00	100.00	100.00	4.2	4.1	4.2	
100072972-5	"MAR-5"	1.0E-06 M	30	0.70	0.64	0.67				
100072972-5	"MAR-5"	1.0E-06 M	60	1.22	0.48	0.85				
100072972-5	"MAR-5"	1.0E-06 M	90	0.37	0.31	0.34				
100072972-5	"MAR-5"	1.0E-06 M	120	0.20	0.97	0.59				
100072972-6	MYT-253	1.0E-06 M	0	100.00	100.00	100.00	5.5	5.3	5.4	
100072972-6	MYT-253	1.0E-06 M	30	2.22	1.94	2.08				
100072972-6	MYT-253	1.0E-06 M	60	1.75	1.53	1.64				
100072972-6	MYT-253	1.0E-06 M	90	1.02	1.52	1.27				
100072972-6	MYT-253	1.0E-06 M	120	0.45	0.30	0.38				
100072972-7	ML-110	1.0E-06 M	0	100.00	100.00	100.00	417.2	1070.7	>240	
100072972-7	ML-110	1.0E-06 M	30	112.36	91.10	101.73				
100072972-7	ML-110	1.0E-06 M	60	97.90	{252.0}	97.90				
100072972-7	ML-110	1.0E-06 M	90	89.01	98.35	93.68				
100072972-7	ML-110	1.0E-06 M	120	87.57	87.34	87.45				
100072972-8	ML-111a	1.0E-06 M	0	100.00	100.00	100.00	8.5	8.8	8.7	
100072972-8	ML-111a	1.0E-06 M	30	8.69	9.40	9.04				
100072972-8	ML-111a	1.0E-06 M	60	1.39	1.95	1.67				
100072972-8	ML-111a	1.0E-06 M	90	0.93	1.07	1.00				
100072972-8	ML-111a	1.0E-06 M	120	0.50	0.62	0.56				
100072972-9	DMF	1.0E-06 M	0	-	-	-	-	-	-	ND
100072972-9	DMF	1.0E-06 M	30	-	-	-				ND
100072972-9	DMF	1.0E-06 M	60	-	-	-				ND
100072972-9	DMF	1.0E-06 M	90	-	-	-				ND
100072972-9	DMF	1.0E-06 M	120	-	-	-				ND

Compound I.D.	Client Compound I.D.	Test Concentration	Incubation (minutes)	% Compound Remaining (% remaining)			Half-Life (minutes)			Flags
				1st	2nd	Mean	1st	2nd	Mean	
100072972-10	MMF	1.0E-06 M	0	-	-	-	-	-	-	ND
100072972-10	MMF	1.0E-06 M	30	-	-	-				ND
100072972-10	MMF	1.0E-06 M	60	-	-	-				ND
100072972-10	MMF	1.0E-06 M	90	-	-	-				ND
100072972-10	MMF	1.0E-06 M	120	-	-	-				ND

Note: Unit of Clint is µL/min/mg for microsomes, S9 and UGT assays; µL/min/pmol for CYP assays; µL/min/Million cells for hepatocyte assays

ND: Not Detected. Test compound was not reliably detected in the assay matrix.

7.1.2. Reference Compound Results

Compound I.D.	Test Concentration	Half-Life (minutes)		
		1st	2nd	Mean
Half-life (plasma, mouse, CD-1)				
Propantheline	1.0E-06 M	125.1	82.8	103.9
Propoxycaine	1.0E-06 M	2.4	2.4	2.4

Note: Unit of Clint is µL/min/mg for microsomes, S9 and UGT assays; µL/min/pmol for CYP assays; µL/min/Million cells for hepatocyte assays

8. MATERIALS AND METHODS

8.1. Experimental Conditions

Minor variations to the experimental protocol described below may have occurred during the testing, they have no impact on the quality of the results obtained.

8.1.1. ADME-Tox: *In Vitro* Metabolism

Assay	Source	Substrate	Incubation	Measured Component	Detection Method	Bibl.
ADME						
Half-life (plasma, mouse, CD-1)	Mouse plasma	Test compound	0, 0.5, 1, 1.5, 2 hr 37°C	Test compound	HPLC-MS/MS	1127

8.2. Analysis and expression of results

8.2.1. ADME-Tox: *In Vitro* Metabolism

Half-Life Determination (plasma or blood)

At the end of incubation at each of the time points, acetonitrile was added to the incubation mixture followed by centrifugation. Samples were analyzed by HPLC-MS/MS and peak areas were recorded for each analyte. The area of precursor compound remaining after each of the time points relative to the amount remaining at time zero, expressed as percent, is calculated. Subsequently, the half-life ($T_{1/2}$) is estimated from the slope of the initial linear range of the logarithmic curve of compound remaining (%) *versus* time, assuming first order kinetics.

9. BIBLIOGRAPHY

1127. Di, L. et al. (2005), *Int. J. Pharm.,* 297 *(1-2): 110-9.*

MytoLyfe Biotherapeutics Mouse Pharmacokinetic Results

Mark Olsen

(09/06/2024)

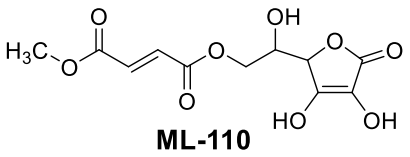
Following synthesis of the MMF and MEF prodrugs, the compounds were tested for CD-1 mouse plasma stability. The compounds were not tested in CD-1 Mouse Liver Microsome tests to save money. Each assay costs about $512/compound, and testing all 8 compounds including MMF and DMF would be $5120. Briefly, MMF is primarily metabolized by the Tri-Carboxylic Acid (TCA) cycle, and not by microsomal processes. The primary site of metabolism is most likely to be the ester bond, which has already been evaluated for stability using CD-1 mouse plasma $t_{1/2}$. CACO-2 layer permeability and P-gp assessment cost $1985/compound and was also bypassed, due to cost.

A mouse pharmacokinetic experiment was designed and executed to incorporate both drug plasma and drug brain concentration measurements, and both the prodrug and MMF concentrations were measured in all samples. If the drugs are rapidly taken up by the brain, then simply monitoring the drug plasma level would not be particularly informative. For this experiment, a dose of 1 mg/kg was selected. Briefly, the capacity of the SVCT2 and LAT1 transporters is not known for these compounds, and administration of a higher dose such as 10 mg/kg might result in saturation of the active transport mechanisms, and show lower brain transport. For this reason, 1 mg/kg was selected.

For the experimental design, 3 compounds were evaluated: MMF, ML-110 and MYT-251. ML-110 and ML-251 were selected because of the plasma half-life, and ML-110 represented the ascorbic acid class compounds, while MYT-251 represented the amino acid class compounds. The compounds were all injected IV. Animals, three per timepoint, were sacrificed at 30 minutes, 1, 4, 8 and 24 hours. Blood was collected by cardiac puncture, and brain material was collected. Blood would be processed to plasma, and plasma and brain concentrations would be quantified by LC/MS. The total cost for the mouse plasma and brain pharmacokinetics was $12,112.50.

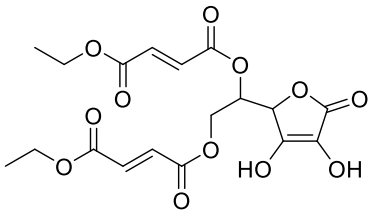
Results showed that 1mg/kg MMF resulted in detectable concentrations in plasma, but did not show reliable brain penetration. Of the 3 mice dosed with MMF at the 24 hour timepoint, one was Below Level of Quantification (BLOQ), one was at 10ng/g or brain material, and one was aberrantly high at 72 ng/g despite plasma concentrations being consistent. Neither ML-110 nor MYT-251 showed any consistent concentrations in the plasma or brain, but low levels of MMF were detected in the plasma with roughly similar pharmacokinetics to MMF.

MytoLyfe Biotherapeutics CD-1 Mouse Plasma Half-Life Results



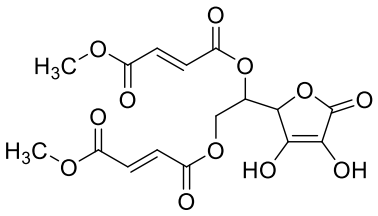
ML-110

CD-1 Mouse Plasma $t_{1/2}$ >240 minutes



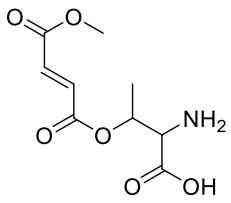
ML-116

CD-1 Mouse Plasma $t_{1/2}$ = 111.1 minutes



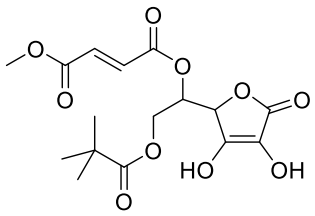
ML-111a

CD-1 Mouse Plasma $t_{1/2}$ = 8.7 minutes



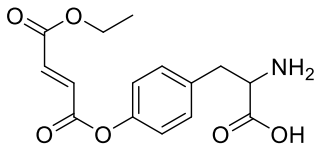
MYT-251

CD-1 Mouse Plasma $t_{1/2}$ = 119.9 minutes



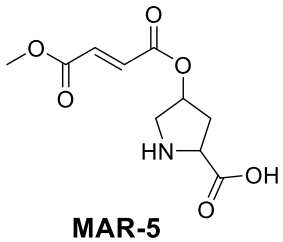
MYT-204

CD-1 Mouse Plasma $t_{1/2}$ = 30.3 minutes



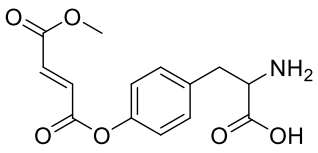
MAR-4

CD-1 Mouse Plasma $t_{1/2}$ = 14.4 minutes



MAR-5

CD-1 Mouse Plasma $t_{1/2}$ = 4.2 minutes



MYT-253

CD-1 Mouse Plasma $t_{1/2}$ = 5.4 minutes

Table 1-1 . The exposure levels of MMF in mouse plasma samples – MMF

Time (h)	Sample concentration (ng/mL)			Mean
0.5	46	41	32	40
1	9	6	6	7
4	8	7	5	7
8	BLOQ	7	11	9
24	6	5	4	5

Table 2-1 . The exposure levels of ML-110 in mouse plasma samples – M

Time (h)	Sample concentration (ng/mL)			Mean
0.5	BLOQ	BLOQ	6	6
1	BLOQ	BLOQ	BLOQ	BLOQ
4	BLOQ	BLOQ	5	5
8	BLOQ	BLOQ	4	4
24	BLOQ	BLOQ	BLOQ	BLOQ

Table 3-1 . The exposure levels of MMF in mouse plasma samples – ML-

Time (h)	Sample concentration (ng/mL)			Mean
0.5	16	11	19	15
1	4	7	6	6
4	7	6	7	7
8	5	11	11	9
24	6	6	6	6

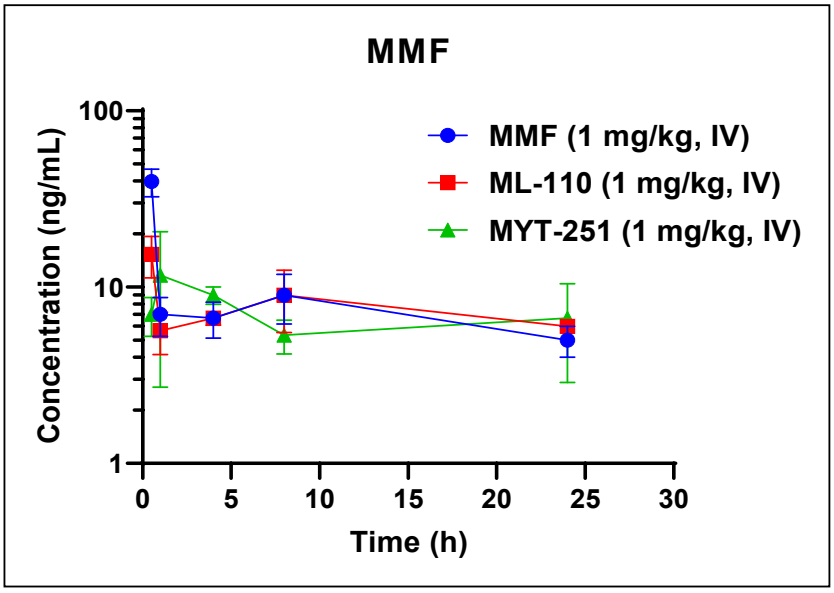
Table 4-1 . The exposure levels of MYT-251 in mouse plasma samples –

Time (h)	Sample concentration (ng/mL)			Mean
0.5	24	4	BLOQ	14
1	BLOQ	BLOQ	BLOQ	BLOQ
4	BLOQ	BLOQ	BLOQ	BLOQ
8	BLOQ	BLOQ	BLOQ	BLOQ
24	BLOQ	BLOQ	BLOQ	BLOQ

Table 5-1 . The exposure levels of MMF in mouse plasma samples – MYT

Time (h)	Sample concentration (ng/mL)			Mean
0.5	8	5	8	7
1	6	7	22	12
4	9	10	8	9

	8 24	4 11	6 5	6 4	5 7



F (1 mg/kg, IV) group :

SD
7
2
2
3
1

Table 2-1 . The PK param

MMF
1 mg/kg, IV

L-110 (1 mg/kg, IV) group :

SD
NA
NA
NA
NA
NA

Table 2-2 . The PK param

ML-110
1 mg/kg, IV

110 (1 mg/kg, IV) group :

SD
4
2
1
3
0

Table 3-2 . The PK param

MMF
1 mg/kg, IV

MYT-251 (1 mg/kg, IV) group :

SD
14
NA
NA
NA
NA

Table 4-2 . The PK param

MYT-251
1 mg/kg, IV

T-251 (1 mg/kg, IV) group :

SD
2
9
1

Table 5-2 . The PK param

MMF
1 mg/kg, IV

1
4

eters of MMF in mouse samples – MMF (1 mg/kg, IV) group :

$t_{1/2}$	C_0	AUC_{last}	AUC_{Inf}	AUC/D
(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)
30.79	229	244	466	465.99

eters of ML-110 in mouse samples – ML-110 (1 mg/kg, IV) group :

$t_{1/2}$	C_0	AUC_{last}	AUC_{Inf}	AUC/D
(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)
12.81	6	40	114	114.22

eters of MMF in mouse samples – ML-110 (1 mg/kg, IV) group :

$t_{1/2}$	C_0	AUC_{last}	AUC_{Inf}	AUC/D
(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)
38.61	38	190	524	524.06

eters of MYT-251 in mouse samples – MYT-251 (1 mg/kg, IV) group :

$t_{1/2}$	C_0	AUC_{last}	AUC_{Inf}	AUC/D
(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)
NA	NA	NA	NA	NA

eters of MMF in mouse samples – MYT-251 (1 mg/kg, IV) group :

$t_{1/2}$	C_0	AUC_{last}	AUC_{Inf}	AUC/D
(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)
40.81	7	164	576	575.92

AUC Extr	MRT	Vss	CL
(%)	(h)	(L/kg)	(mL/min/kg)
47.66	36.47	78.27	35.77

AUC Extr	MRT	Vss	CL
(%)	(h)	(L/kg)	(mL/min/kg)
64.73	18.41	161.19	145.91

AUC Extr	MRT	Vss	CL
(%)	(h)	(L/kg)	(mL/min/kg)
63.77	54.61	104.20	31.80

AUC Extr	MRT	Vss	CL
(%)	(h)	(L/kg)	(mL/min/kg)
NA	NA	NA	NA

AUC Extr	MRT	Vss	CL
(%)	(h)	(L/kg)	(mL/min/kg)
71.57	62.60	108.70	28.94

Group	Analyte	Treatment	Route	Dose
1	MMF	MMF (API)	IV	1 mg/kg, 5 mL/kg, QD x 1
	ML-110	ML-110 (Prodrug-1)	IV	1 mg/kg, 5 mL/kg, QD x 1

2				
	MMF	**ML-110 (Prodrug-1)**	**IV**	**1 mg/kg, 5 mL/kg, QD x 1**
	MYT-251	**MYT-251 (Prodrug-2)**	**IV**	**1 mg/kg, 5 mL/kg, QD x 1**

3				
	MMF	**MYT-251 (Prodrug-2)**	**IV**	**1 mg/kg, 5 mL/kg, QD x 1**

Collection Time (h)	N	Brain Concentration (ng/g)	Plasma Concentration (ng/mL)	Brain/Plasma Ratio
0.5	1	BLOQ	46	NA
	2	BLOQ	41	NA
	3	BLOQ	32	NA
	Mean	BLOQ	39.7	NA
	SD	NA	7.1	NA
1	4	BLOQ	9	NA
	5	BLOQ	6	NA
	6	BLOQ	6	NA
	Mean	BLOQ	7.0	NA
	SD	NA	1.7	NA
4	7	BLOQ	8	NA
	8	BLOQ	7	NA
	9	BLOQ	5	NA
	Mean	BLOQ	6.7	NA
	SD	NA	1.5	NA
8	10	BLOQ	BLOQ	NA
	11	13	7	1.86
	12	BLOQ	11	NA
	Mean	13.0	9.0	1.86
	SD	NA	2.8	NA
24	13	BLOQ	6	NA
	14	10	5	2.00
	15	72	4	18.00
	Mean	41.0	5.0	10.00
	SD	43.8	1.0	11.31
0.5	1	14	BLOQ	NA
	2	BLOQ	BLOQ	NA
	3	BLOQ	6	NA
	Mean	14.0	6.0	NA
	SD	NA	NA	NA
1	4	BLOQ	BLOQ	NA
	5	8	BLOQ	NA
	6	BLOQ	BLOQ	NA
	Mean	8.0	BLOQ	NA
	SD	NA	NA	NA
4	7	BLOQ	BLOQ	NA
	8	BLOQ	BLOQ	NA
	9	BLOQ	5	NA
	Mean	BLOQ	5.0	NA
	SD	NA	NA	NA
8	10	BLOQ	BLOQ	NA
	11	BLOQ	BLOQ	NA
	12	BLOQ	4	NA
	Mean	BLOQ	4.0	NA
	SD	NA	NA	NA
	13	BLOQ	BLOQ	NA

24	14	BLOQ	BLOQ	NA
	15	BLOQ	BLOQ	NA
	Mean	BLOQ	BLOQ	NA
	SD	NA	NA	NA
0.5	1	BLOQ	16	NA
	2	BLOQ	11	NA
	3	BLOQ	19	NA
	Mean	BLOQ	15.3	NA
	SD	NA	4.0	NA
1	4	BLOQ	4	NA
	5	BLOQ	7	NA
	6	BLOQ	6	NA
	Mean	BLOQ	5.7	NA
	SD	NA	1.5	NA
4	7	BLOQ	7	NA
	8	BLOQ	6	NA
	9	BLOQ	7	NA
	Mean	BLOQ	6.7	NA
	SD	NA	0.6	NA
8	10	BLOQ	5	NA
	11	BLOQ	11	NA
	12	BLOQ	11	NA
	Mean	BLOQ	9.0	NA
	SD	NA	3.5	NA
24	13	BLOQ	6	NA
	14	BLOQ	6	NA
	15	BLOQ	6	NA
	Mean	BLOQ	6.0	NA
	SD	NA	0.0	NA
0.5	1	4	24	0.17
	2	BLOQ	4	BLOQ
	3	BLOQ	BLOQ	BLOQ
	Mean	4.0	14.0	0.17
	SD	NA	14.1	NA
1	4	BLOQ	BLOQ	NA
	5	BLOQ	BLOQ	NA
	6	BLOQ	BLOQ	NA
	Mean	BLOQ	BLOQ	NA
	SD	NA	NA	NA
4	7	BLOQ	BLOQ	NA
	8	7	BLOQ	NA
	9	BLOQ	BLOQ	NA
	Mean	7.0	BLOQ	NA
	SD	NA	NA	NA
8	10	BLOQ	BLOQ	NA
	11	BLOQ	BLOQ	NA
	12	BLOQ	BLOQ	NA
	Mean	BLOQ	BLOQ	NA
	SD	NA	NA	NA
	13	BLOQ	BLOQ	NA

24	14	7	BLOQ	NA
	15	BLOQ	BLOQ	NA
	Mean	7.0	BLOQ	NA
	SD	NA	NA	NA
0.5	1	BLOQ	8	BLOQ
	2	14	5	2.80
	3	BLOQ	8	BLOQ
	Mean	14.0	7.0	2.80
	SD	NA	1.7	NA
1	4	BLOQ	6	NA
	5	BLOQ	7	NA
	6	BLOQ	22	NA
	Mean	BLOQ	11.7	NA
	SD	NA	9.0	NA
4	7	9	9	1.00
	8	BLOQ	10	NA
	9	BLOQ	8	NA
	Mean	9.0	9.0	1.00
	SD	NA	1.0	NA
8	10	BLOQ	4	NA
	11	BLOQ	6	NA
	12	BLOQ	6	NA
	Mean	BLOQ	5.3	NA
	SD	NA	1.2	NA
24	13	BLOQ	11	NA
	14	BLOQ	5	NA
	15	BLOQ	4	NA
	Mean	BLOQ	6.7	NA
	SD	NA	3.8	NA