



WASHINGTON BIOTECHNOLOGY, INC.

Mailing Address:
P.O. Box 211
Simpsonville, MD 21150

Laboratories & Shipping Address:
6200 Seaforth Street.
Baltimore, MD 21224
Phone: 410.633.9210
Fax: 410.633.9213
E-mail: yyang@washingtonbiotech.com

Final Report: Inflammatory Efficacy of Test Compound in an In Vitro Whole Blood Assay (human)

Project Title: WBA-FLD-1

Project: Anti-inflammatory Efficacy of Test Compound in an In Vitro Whole Blood Assay (human)

Sponsor: Stephen Leivers
Fullhold Ltd.
Topshell Park
Western Cape
South Africa 7603

TEL: 0027 881 3074
Email: Stephen@fullhold.com

Summary of Procedures:

1. June 6, 2016: Received a bottle of 50 ml, 4% test CHD-FA solution from Fulhold Ltd at ambient temperature. The test material was stored at room temperature.

2. June 14, 2016: 7.94 mg of dexamethasone-21 phosphate (Sigma, Cat # D1150) was dissolved in 6.6 ml PBS, resulting in 2.5 mM store solution. Dilution of this store solution in PBS resulted in 250 μ M (10 X) working solution.

2.95 mg LPS from E.coli strain 055:B5 (Sigma, L2880, lot #044m4004 V) was dissolved in 29.5 ml PBS to make 100 μ g/ml solution (100X).

1.25 ml CHD solution was diluted to 8.75 ml PBS to make 1000 μ M, and further dilute to 200 μ M, 100 μ M, 10 μ M, and 1 μ M solution (10X).

11:00 am: 10 ml heparinized blood from a healthy donor was delivered. The blood was diluted 1:9 in RPMI 1640 media. 1 ml of diluted blood was dispensed into each well of a 24 well microliter plate. Blood was incubated at 37°C and 5% CO₂.

12:00 pm: 100 μ l of PBS, dexamethasone, and different concentration CHD were added to corresponding wells (table 1).

1:00 pm: 10 μ l of LPS was added to each well except group 1.

5:00 pm: all blood cultures were harvested and spin down the cells. The supernatants were aspirated into Eppendorf tubes, and stored at -80°C.

3. June 17, 2016: The samples were equilibrated to room temperature, diluted, assayed for TNF α , IL-6, IL- β , and IL-17 (RESULTS) by ELISA.

#: TABLE 1: GROUP TREATMENTS

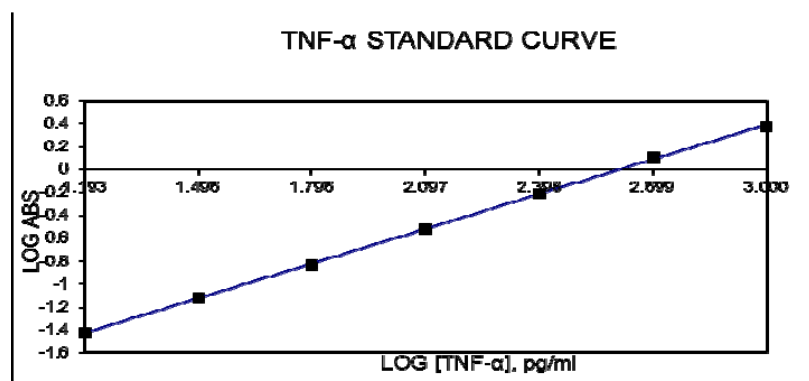
<u>Group</u>	<u>wells</u>	<u>Test Material</u>	<u>Dose (μM)</u>	<u>LPS (μg/ml)</u>
1	3	Vehicle	na	0
2	3	Vehicle	na	1
3	3	Dexamethasone	25	1
4	3	CHD-FA	0.1	1
5	3	CHD-FA	1.0	1
6	3	CHD-FA	10	1
7	3	CHD-FA	20	1
8	3	CHD-FA	100	1

Results:

1. Dose response effects of CHD-FA on LPS-induced TNF- α release

1. TNF- α Standard Curve

TNF CON	LOG TNF	RAW ABS	ABS-BLANK	LOG OF g	LINE		
0		0.034	0				
15.6	1.193	0.072	0.038	-1.42022	-1.42151	-2.61764	= b
31.3	1.496	0.111	0.077	-1.11351	-1.11833	1.002517	=m
62.5	1.796	0.182	0.148	-0.82974	-0.81724		
125.0	2.097	0.334	0.3	-0.52288	-0.51545		y=mx+b
250.0	2.398	0.657	0.623	-0.20551	-0.21366		x=y-b/m
500.0	2.699	1.329	1.295	0.11227	0.088126		
1000.0	3.000	2.386	2.352	0.371437	0.389913		



2. Effect of Treatment on LPS-Induced TNF- α Release (Abs):

groups	mice	abs.	groups	mice	Con.	groups	mice	abs.	groups	mice	abs.
Grp 1	1	0.040	Grp 2	1	0.538	Grp 3	1	0.146	Grp 4	1	0.417
Vehicle	2	0.041	Vehicle	2	0.598	Dex 25 μ m	2	0.139	CHD 0.1 μ m	2	0.510
no LPS	3	0.041		3	0.571		3	0.178		3	0.499
Grp 5	1	0.472	Grp 6	1	0.340	Grp 7	1	0.149	Grp 8	1	0.058
CHD 1 μ m	2	0.460	CHD 10 μ m	2	0.364	CHD 20 μ m	2	0.174	CHD 100 μ m	2	0.049
	3	0.365		3	0.383		3	0.131		3	0.060

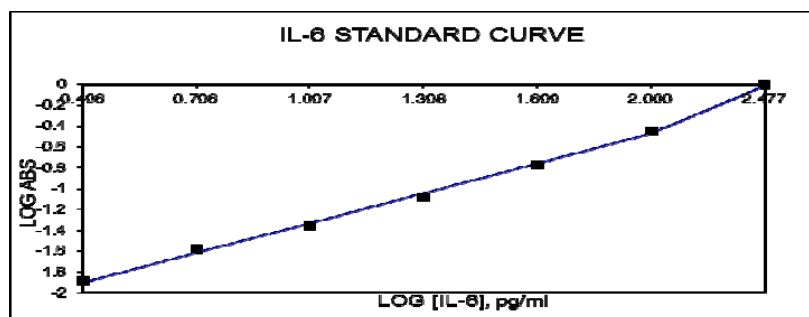
3. Effect of Treatment on LPS-Induced TNF- α Release (pg/ml):

groups	mice	Con.	groups	mice	Con.	groups	mice	Con.	groups	mice	Con.
Grp 1	1	24.82	Grp 2	1	2061.78	Grp 3	1	459.91	Grp 4	1	1567.87
Vehicle	2	28.94	Vehicle	2	2306.58	Dex 25 μ m	2	431.23	CHD 0.1 μ m	2	1947.52
no LPS	3	28.94		3	2196.43		3	590.94		3	1902.63
Grp 5	1	1792.42	Grp 6	1	1253.37	Grp 7	1	472.20	Grp 8	1	98.93
CHD 1 μ m	2	1743.43	CHD 10 μ m	2	1351.41	CHD 20 μ m	2	574.56	CHD 100 μ m	2	61.91
	3	1355.50		3	1429.02		3	398.46		3	107.16

2. Dose response effects of CHD-FA on LPS-induced IL-6 release

1. IL-6 Standard Curve

IL-6 CONC	LOG IL-6	RAW ABS	ABS-BLANK	LOG OF g	LINE	
0		0.018	0			
3.1	0.496	0.031	0.013	-1.88606	-1.90133	-2.37224 = b
6.3	0.796	0.044	0.026	-1.58503	-1.61592	0.950293 =m
12.5	1.097	0.062	0.044	-1.35655	-1.32985	
25.0	1.398	0.1	0.082	-1.08619	-1.04379	y=mx+b
50.0	1.699	0.187	0.169	-0.77211	-0.75772	x=y-b/m
100.0	2.000	0.375	0.357	-0.44733	-0.47165	
300.0	2.477	1.006	0.988	-0.00524	-0.01825	



2. Effect of Treatment on LPS-Induced IL-6 Release (Abs):

groups	mice	abs.	groups	mice	Con.	groups	mice	abs.	groups	mice	abs.
Grp 1	1	0.015	Grp 2	1	1.037	Grp 3	1	0.519	Grp 4	1	0.816
Vehicle	2	0.015	Vehicle	2	1.051	Dex 25 µm	2	0.531	CHD 0.1 µm	2	1.003
no LPS	3	0.015		3	1.077		3	0.609		3	1.008
Grp 5	1	0.941	Grp 6	1	0.754	Grp 7	1	0.485	Grp 8	1	0.120
CHD 1 µm	2	0.870	CHD 10 µm	2	0.810	CHD 20 µm	2	0.474	CHD 100 µm	2	0.069
	3	0.819		3	0.894		3	0.439		3	0.111

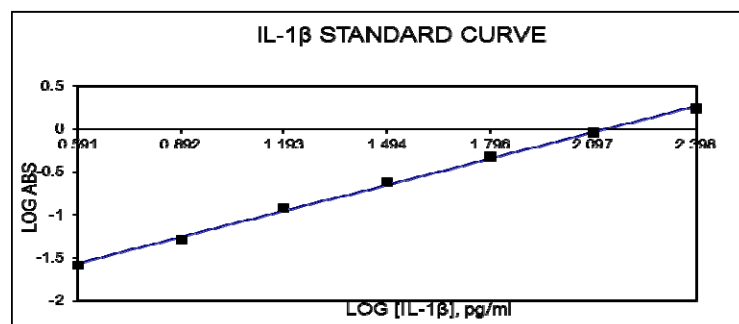
3. Effect of Treatment on LPS-Induced IL-6 Release (pg/ml):

groups	mice	Con.	groups	mice	Con.	groups	mice	Con.	groups	mice	Con.
Grp 1	1	0.00	Grp 2	1	3198.34	Grp 3	1	1515.17	Grp 4	1	2472.86
Vehicle	2	0.00	Vehicle	2	3244.60	Dex 25 µm	2	1553.38	CHD 0.1 µm	2	3086.14
no LPS	3	0.00		3	3330.59		3	1802.86		3	3102.63
Grp 5	1	2882.07	Grp 6	1	2271.11	Grp 7	1	1407.16	Grp 8	1	283.84
CHD 1 µm	2	2649.26	CHD 10 µm	2	2453.30	CHD 20 µm	2	1372.30	CHD 100 µm	2	136.86
	3	2482.65		3	2727.85		3	1261.69		3	257.54

3. Dose response effects of CHD-FA on LPS-induced IL-1 β release

1. IL-1 β Standard Curve

IL-1 β CON	LOG IL-1 β	RAW ABS	ABS-BLANK	LOG OF g	LINE		
0		0.034	0				
3.9	0.591	0.06	0.026	-1.58503	-1.56312	-2.16235	= b
7.8	0.892	0.086	0.052	-1.284	-1.25793	1.01382	=m
15.6	1.193	0.155	0.121	-0.91721	-0.95274		
31.2	1.494	0.274	0.24	-0.61979	-0.64755		y=mx+b
62.5	1.796	0.510	0.476	-0.32239	-0.34165		x=y-b/m
125.0	2.097	0.949	0.915	-0.03858	-0.03646		
250.0	2.398	1.757	1.723	0.236285	0.268729		



2. Effect of Treatment on LPS-Induced IL-1 β Release (Abs):

groups	mice	abs.	groups	mice	Con.	groups	mice	abs.	groups	mice	abs.
Grp 1	1	0.088	Grp 2	1	0.598	Grp 3	1	0.583	Grp 4	1	0.582
Vehicle	2	0.046	Vehicle	2	0.796	Dex 25 μ m	2	0.527	CHD 0.1 μ m	2	0.554
no LPS	3	0.049		3	0.690		3	0.649		3	0.701
Grp 5	1	1.032	Grp 6	1	0.723	Grp 7	1	0.827	Grp 8	1	0.179
CHD 1 μ m	2	0.776	CHD 10 μ m	2	0.698	CHD 20 μ m	2	0.645	CHD 100 μ m	2	0.215
	3	0.588		3	1.011		3	0.623		3	0.238

3. Effect of Treatment on LPS-Induced IL-1 β Release (pg/ml):

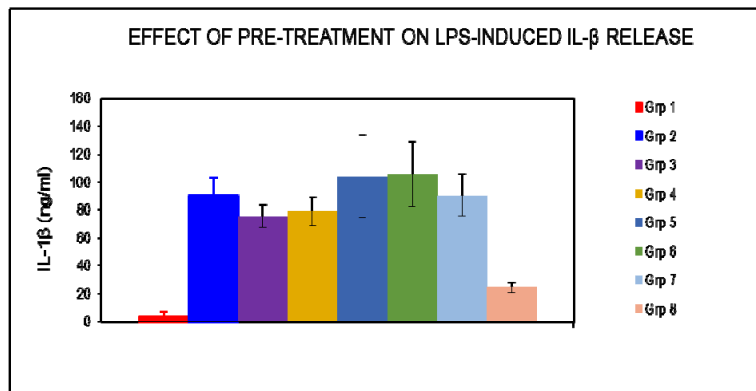
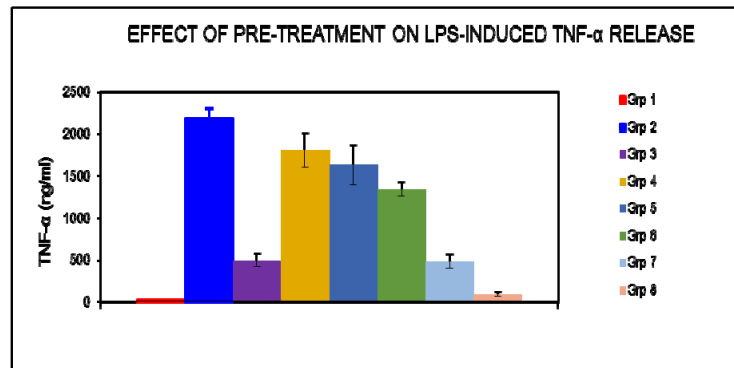
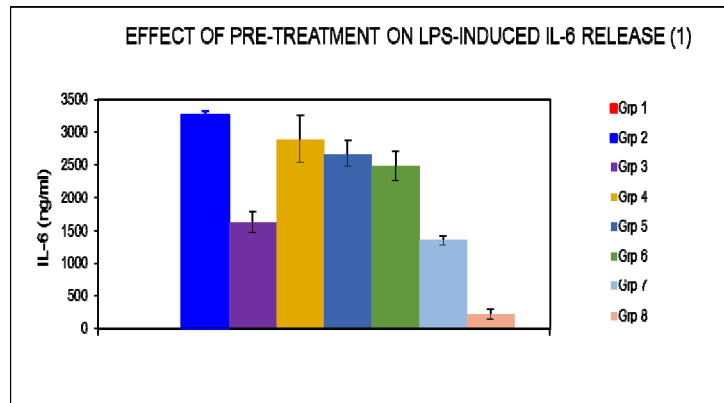
groups	mice	Con.	groups	mice	Con.	groups	mice	Con.	groups	mice	Con.
Grp 1	1	7.63	Grp 2	1	77.19	Grp 3	1	75.16	Grp 4	1	75.03
Vehicle	2	1.73	Vehicle	2	103.86	Dex 25 μ m	2	67.59	CHD 0.1 μ m	2	71.24
no LPS	3	2.16		3	89.59		3	84.07		3	91.07
Grp 5	1	135.52	Grp 6	1	94.04	Grp 7	1	108.02	Grp 8	1	20.22
CHD 1 μ m	2	101.17	CHD 10 μ m	2	90.67	CHD 20 μ m	2	83.53	CHD 100 μ m	2	25.16
	3	75.84		3	132.71		3	80.56		3	28.31

4. Statistical Analysis:

	TNF- α		IL-6		IL- β	
grp1	Mean	27.57	Mean	0.00	Mean	3.84
	SD	2.38	SD	0.00	SD	3.29
	p vs grp2	0.0011	p vs grp2	0.0000	p vs grp2	0.0055
grp2	Mean	2188.27	Mean	3257.84	Mean	90.21
	SD	122.60	SD	67.11	SD	13.35
grp3	Mean	494.03	Mean	1623.80	Mean	75.61
	SD	85.14	SD	156.24	SD	8.25
	p vs grp2	0.0001	p vs grp2	0.0008	p vs grp2	0.1962
grp4	Mean	1806.01	Mean	2887.21	Mean	79.12
	SD	207.45	SD	358.93	SD	10.53
	p vs grp2	0.0649	p vs grp2	0.2126	p vs grp2	0.3245
grp5	Mean	1630.45	Mean	2671.32	Mean	104.18
	SD	239.37	SD	200.62	SD	29.96
	p vs grp2	0.0373	p vs grp2	0.0271	p vs grp2	0.5184
grp6	Mean	1344.60	Mean	2484.08	Mean	105.81
	SD	88.03	SD	229.92	SD	23.36
	p vs grp2	0.0010	p vs grp2	0.0212	p vs grp2	0.3856
grp7	Mean	481.74	Mean	1347.05	Mean	90.70
	SD	88.44	SD	75.95	SD	15.07
	p vs grp2	0.0001	p vs grp2	0.0000	p vs grp2	0.9683
grp8	Mean	89.33	Mean	226.08	Mean	24.56
	SD	24.10	SD	78.37	SD	4.08
	p vs grp2	0.0008	p vs grp2	0.0000	p vs grp2	0.0087

Significance (p-value) was calculated by Student's *t*-Test, compared to group2

Figures:



Conclusions:

1. As expected, LPS dramatically elevated inflammatory cytokines TNF α , IL-1 β , and IL-6 secretion in the whole blood culture.
2. Dexamethasone (25 μ M) significantly inhibited TNF- α , IL-6 release, but had no effect on IL-1 β production at this dose and time point.
3. CHD-FA significantly suppressed TNF- α release at concentration of 1, 10, 20, 100 μ M by 26%, 38%, 78%, and 96%, respectively.
4. CHD-FA significantly inhibited IL-6 release at concentration of 1, 10, 20, 100 μ M by 18%, 24%, 57%, and 93%, respectively.
5. CHD-FA significantly decreased IL-1 β release at concentration of 100 μ M by 73%.
6. LPS has no effect on IL-17 production, the baseline level of IL-17 was not detected.
7. CHD-FA is a potent ant-inflammatory agent, comparable to dexamethasone.
8. We suggest further in vivo toxicity and efficacy testing in our general inflammatory model (LPS induced inflammatory model, air pouch model) and /or inflammatory disease models such as arthritis model and psoriasis model.

Report Submitted By:

Yinghua Yang, PhD, Senior Scientist

Report Audited By:

Sean O'Neill, PhD, Operations Director

Number of pages in this report (not including this page): 9