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1. SUMMARY

The results of this study are presented in Section 4. Conclusions and Discussion of the results are provided in Section 5.

2. INTRODUCTION AND STUDY DESIGN

2.1 AIM OF STUDY

The aim of this study was to evaluate two test compounds for possible agonist activity to human androgen receptor. Test compounds were supplied by the study sponsor, USP Labs, LLC.

2.2 SELECTION OF DOSES

Assay dose ranges for each test compound were designated by the sponsor (see Section 3.3).

2.3 TEST GUIDELINES

As agreed upon in Invoice 080523-1, test compounds were assayed through serial dilution, with each dose being tested in at least triplicate. The sponsor provided us with specific instructions for solvating and clarifying each test compound (see Section 3.3).

3. MATERIAL AND METHODS

3.1 TEST SUBSTANCES

USP Labs provided us with two test compounds for evaluation.

Compound 1 was provided as a solid mass, and had the appearance of hardened tar. This compound is henceforth designated “**Compound 1 (Tar)**”, or “Cmpd1 (Tar)”

Compound 2 was provided as a finely flaked material, and had the appearance of crushed tea leaves. This compound is henceforth designated “**Compound 2 (Tea)**”, or “Cmpd 2 (Tea)”

3.2 ASSAYS PERFORMED

Three different variations of hAR agonism assays were performed in this study:

a. A “positive-control” DHT agonist dose-response assay was performed to validate the functionality and responsiveness of the hAR reporter cells used at the specific time of this study.

b. A solution of Compound 1 (Tar) was prepared and used in a limiting dilution assay to assess potential hAR agonist activity of this test material.

c. A solution of Compound 2 (Tea) was prepared and used in a limiting dilution assay to assess potential hAR agonist activity of this test material.

3.3 TEST GROUPS AND DOSES/CONCENTRATIONS

a. DHT was initially prepared as a 10 mM stock in DMSO. Treatment doses were prepared via serial dilution using cell culture treatment media to obtain the following picoMolar (pM; 10^{-12} Molar) concentrations: 0, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, 100, 200, and 400 pM. The “0” (i.e., media only) treatment was performed using 8 assay replicates. These measurements provided the “0” control values to which all subsequent experimental values were compared. All other DHT treatment conditions were performed in replicates of 4.

500 μ l of treatment media was added into each assay well. Given that the molecular weight of DHT is 290.44 Dalton, the above concentrations correspond to the following picoGram (pGm; 10^{-12} Gram) quantities of DHT per assay well: 0, 0.2269, 0.4538, 0.9077, 1.815, 3.631, 7.261, 14.52, 29.05, and 58.09 pGm.

b. Compound 1 (Tar) was added to 95% methanol at a concentration equivalent to 1 gram (Gm) per 100 ml. This preparation was allowed to swirl overnight, at room temperature, in a tightly capped & foil-wrapped glass bottle. The sponsor advised us that the maximum solubility of cmpd 1 (Tar) is 89.07%. Immediately prior to assay, the solution was clarified via filtration. Based on the provided solubility information, the neat filtrate contained 890.7 μ Gm of compound 1 (Tar) per ml of solution. Serial dilutions were prepared using cell culture treatment media, as follows: 1/100 > 1/200 > 1/400 > 1/800 > 1/1600 > 1/3200. 500 μ l of these treatment media dilutions were added to respective assay wells. Hence, the microGram (μ Gm; 10^{-6} Gm) amount of compound 1 (Tar) added to respective assay wells was: 4.454, 2.227, 1.113, 0.5567, 0.2783, 0.1392, and 0 μ Gm. The various Compound 1 (Tar) treatment conditions were all performed using 3 assay replicates.

c. Compound 2 (Tea) was added to 95% methanol at a concentration equivalent to 1 gram (Gm) per 100 ml. This preparation was allowed to swirl overnight, at room temperature, in a tightly capped & foil-wrapped glass bottle. The sponsor advised us that the maximum solubility of cmpd 1 (Tar) is 89.02%. Immediately prior to assay, the solution was clarified via filtration. Based on the provided solubility information, the neat filtrate contained 890.2 μ Gm of compound 2 (Tea) per ml of solution. Serial dilutions were prepared using cell culture treatment media, as follows: 1/100 > 1/200 > 1/400 > 1/800 > 1/1600 > 1/3200. 500 μ l of treatment media dilutions were added to respective assay wells. Hence, the microGram (μ Gm; 10^{-6} Gm) amount of compound 2 (Tea) added to respective assay wells was: 4.454, 2.225, 1.113, 0.5563, 0.2781, 0.1391, and 0 μ Gm. The various Compound 2 (Tea) treatment conditions were all performed using 4 assay replicates – the extra replicate (relative to Cmpd 1 treatments) was possible due to the availability of extra wells in the assay plate.

3.4 TEST SUBSTANCE PREPARATION

See section 3.3.

3.5 EXPERIMENTAL PROCEDURE AND TIME SCHEDULE

All assays were conducted using hAR Reporter Cells, the composition and preparation of which are proprietary to Indigo Biosciences. In general, the host cell line is of human origin. The native, full-length hAR protein is expressed in these reporter cells. This assay employs firefly (FF) luciferase as the experimental reporter gene, and sea pansy (*Renilla*) luciferase as an internal-control reporter gene. The expression of *Renilla* luciferase provides a quantitative measure to monitor for adverse cytological effects that may arise from exposure to the test compounds, and provides a convenient means of normalizing experimental data within assay sets. Assay measurements for FF and *Renilla* luciferases are quantified using a plate-reading luminometer, and are reported in terms of Relative Light Units (RLU).

All graphical representations of hAR activity are presented as normalized reporter data, calculated by dividing FF luciferase RLU values by *Renilla* luciferase RLU values. Raw data corresponding to independent FF luciferase and *Renilla* luciferase measurements are also provided (see Section 8).

3.6 STATISTICAL ANALYSES

Statistical analyses of Average (Ave.), Normalized Average (Norm Ave.), Standard Deviation (StDev), percent Coefficient of Variation (%CV), Z-Prime (Z') and Fold-Increase were all performed using MicroSoft Excel software.

Non-linear curve-fitting of transformed reporter data, as well as all associated statistical analyses, including EC50 calculations, as well as ANOVA analyses and Dunnetts post-tests (to determine statistical significance of individual data points) were all performed using GraphPad Prism, v.5.0.

3.7 RETENTION OF RECORDS

Unless otherwise requested, Indigo Biosciences will retain electronic versions of all quotes, reports to, and communications with, the study sponsor.

4. RESULTS AND ASSESSMENT OF FINDINGS

Throughout the results sections, when intergroup differences are referred to as “significant” it implies that the differences have attained statistical significance ($p < 0.05$) when compared to the control group.

4.1 TEST SUBSTANCE SOLUBILITY AND STABILITY

See 3.3b and 3.3c for solubility data. Stabilities of the two test compounds are unknown. Solutions of compounds were prepared in sealed, light resistant glass bottles.

4.2 hAR AGONISM ASSAY RESULTS: DHT Dose-Curve Assay

Normalized (FF luc/*Renilla* luc) average RLU values, as well as respective standard deviations, percent coefficients of variation, Z', and fold-increases over the "0" treatment condition, were calculated for each DHT treatment set (**Table 4.2.1**).

Normalized RLU values were curve-fit against Log₁₀-transformed picoMolar (pM) concentrations of DHT, and the EC₅₀ value for the positive-control DHT dosing was determined (**Figure 4.2.1**). Additionally, normalized RLU values were curve-fit against Log₁₀ microGram (μGm) mass of DHT in each treatment condition (**Figure 4.2.2**). This non-standard representation of agonist dose-curve data is provided for comparison to the subsequent graphical representations of Compound 1 (Tar) and Compound 2 (Tea) experimental data, which is presented in this same format.

Table 4.2.1

FF luc / <i>Renilla</i> luc: Normalized DHT Agonist Dose Response										
DHT pGm	[DHT] pM	#1	#2	#3	#4	Norm Ave	StDev	%CV	Z'	Fold Increase
0	0	0.5013	0.4934	0.3800	0.5352	0.5054	0.0596	11.8	na	1.00
		0.5085	0.5714	0.5642	0.4892					
0.2269	1.56	0.5874	0.5989	0.5978	0.5818	0.5915	0.00827	1.40	-	1.17
0.4538	3.13	0.6237	0.5953	0.6359	0.5987	0.6134	0.0196	3.20	-	1.21
0.9077	6.25	0.7239	0.8020	0.7859	0.7502	0.7655	0.0352	4.59	-	1.51
1.815	12.5	1.2711	1.3187	1.1506	1.2309	1.243	0.0712	5.73	0.468	2.46
3.631	25.0	2.9347	2.3861	2.5003	2.1489	2.493	0.329	13.2	0.413	4.93
7.261	50.0	4.9820	4.7561	4.3007	4.1246	4.541	0.397	8.73	0.661	8.98
14.52	100	6.9974	6.5535	6.2214	6.0894	6.465	0.405	6.26	0.766	12.8
29.05	200	7.7395	7.1878	6.5573	6.8202	7.076	0.512	7.24	0.739	14.0
58.09	400	6.804	6.391	6.150	5.361	6.177	0.607	9.82	0.648	12.2

Figure 4.2.1

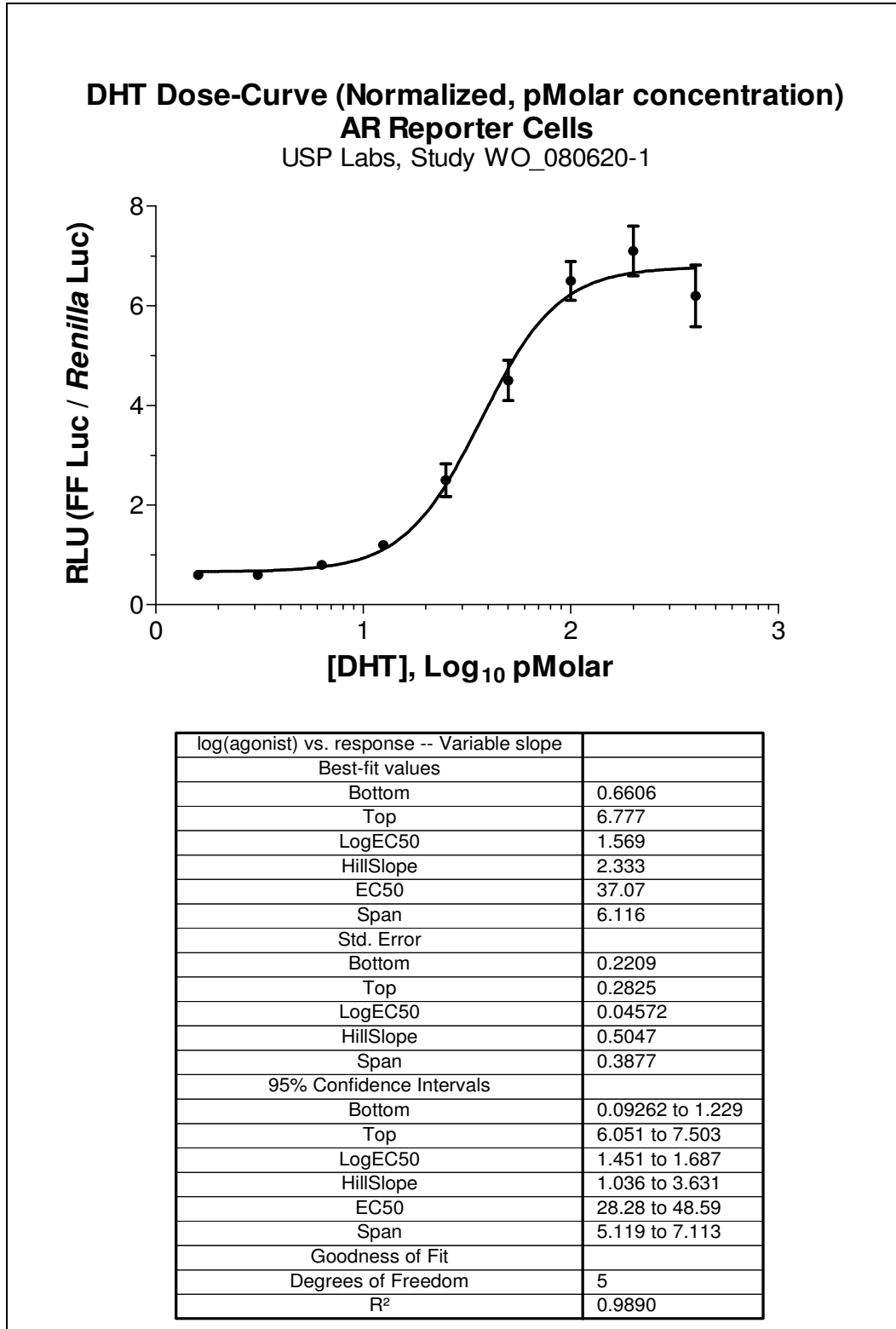
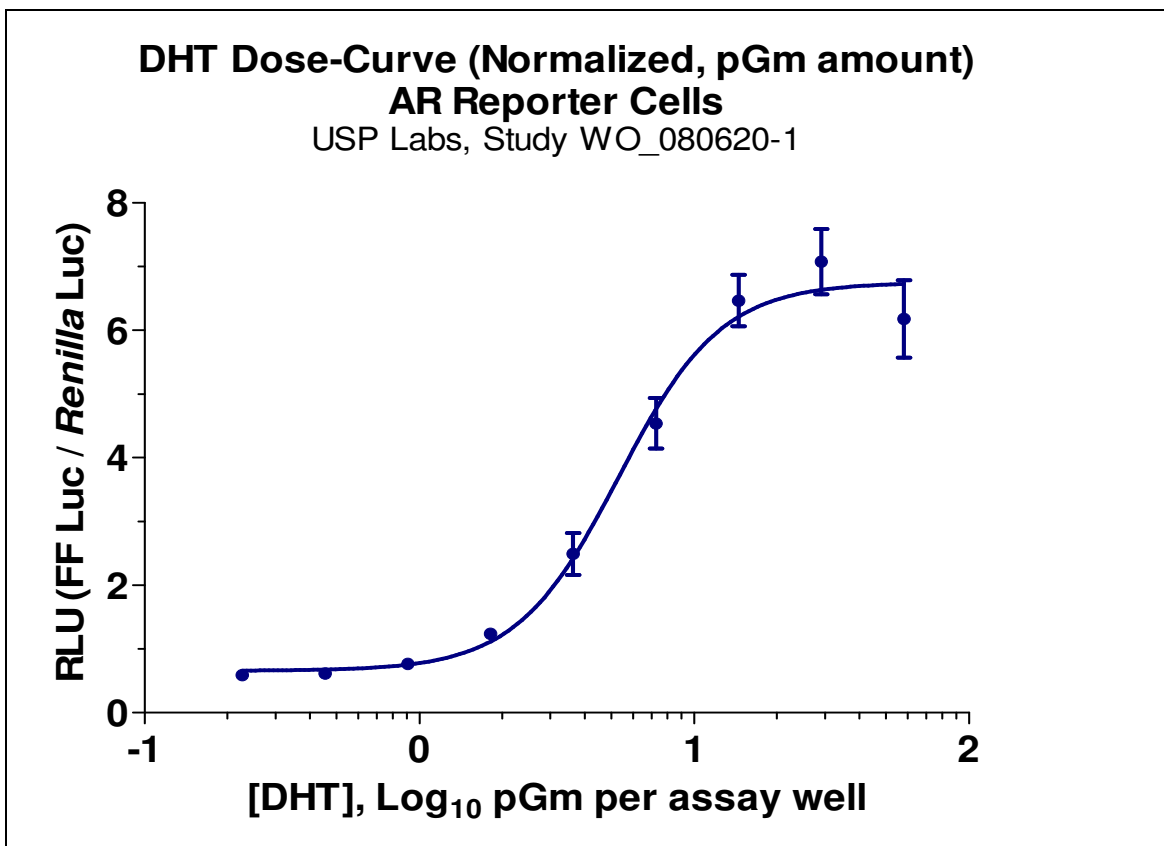


Figure 4.2.2



4.3 hAR AGONISM ASSAY RESULTS: Test Compound 1 (Tar)

Normalized (FF luc/*Renilla* luc) average RLU values, as well as respective standard deviations, percent coefficients of variation, Z', and fold-increases over the "0" treatment condition, were calculated for each Compound 1 (Tar) treatment set (**Table 4.3.1**). For the purposes of comparing to the DHT control dose-curve, normalized average RLU values were curve-fit against Log₁₀ –transformed μGm mass of Compound 1 (Tar) (**Figure 4.3.1**).

Normalized RLU values for each individual measurement within a set of replicates (for each of the 7 dose treatments) were analyzed by 1-way ANOVA followed by Dunnett's post-test to determine the statistical significance, if any, of differences in hAR activation between the "0" treatment and respective Compound 1 (Tar) dose treatments. **Table 4.3.2** presents the findings of these statistical treatments. **Figure 4.3.2** provides a graphical representation of Normalized Average RLU values and respective standard deviation for each of the seven treatment doses. Green bars and asterisks (*) denote values determined to be significantly different from the "0" dose control treatment.

Table 4.3.1

USP Compound 1		FF Luc / <i>Renilla</i> Luc: Compound 1 (Tar)						
Dilution	ug per 500 ul	#1	#2	#3	Ave	Std Dev	%CV	Fold Increase
Neat 89.07%	4,453.5							
1/100	44.5	0.8483	0.9143	0.9994	0.9206	0.0757	8.23	1.82
1/200	22.3	0.6915	0.7568	0.6810	0.7098	0.0411	5.78	1.40
1/400	11.1	0.5989	0.5996	0.6752	0.6246	0.0438	7.01	1.24
1/800	5.57	0.5285	0.5457	0.6254	0.5666	0.0517	9.13	1.12
1/1600	2.78	0.4992	0.5141	0.5651	0.5261	0.0346	6.57	1.04
1/3200	1.39	0.5095	0.5107	0.5174	0.5125	0.00426	0.831	1.01
-	0				0.5054	0.0596	11.8	1.00

Figure 4.3.1

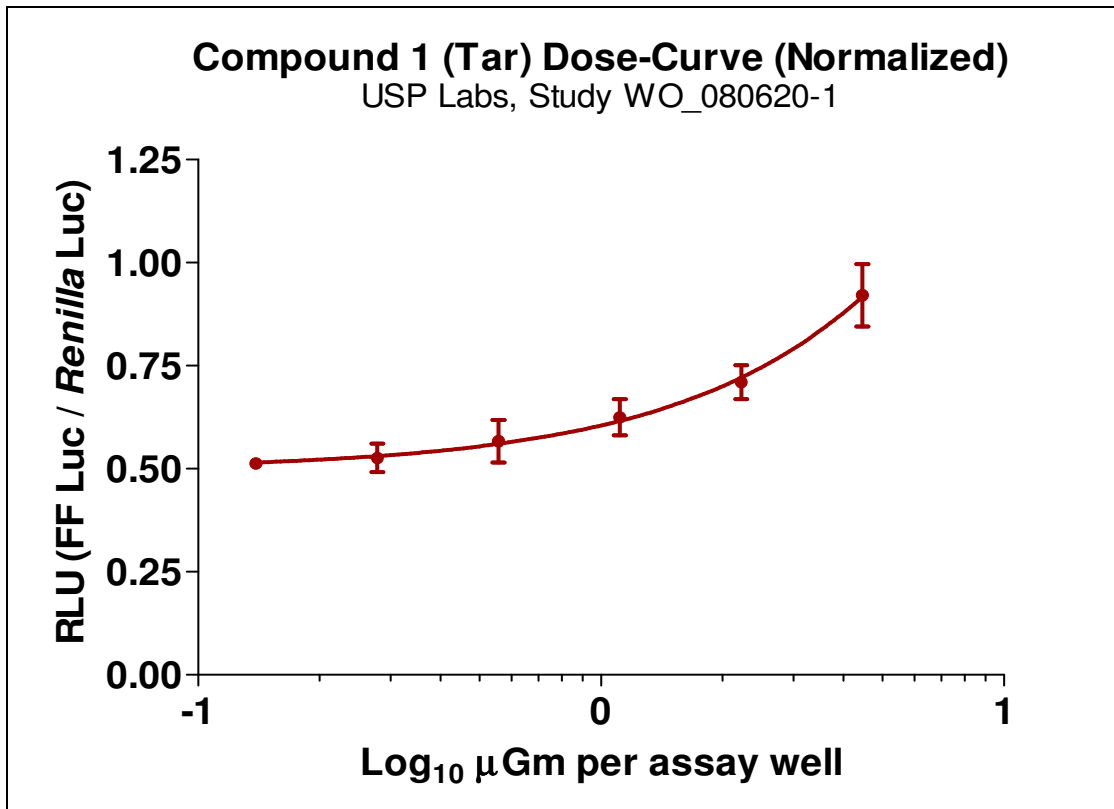


Figure 4.3.2
hAR Agonism Study, Compound 1 (Tar)
 USP Labs, Study WO_080620-1

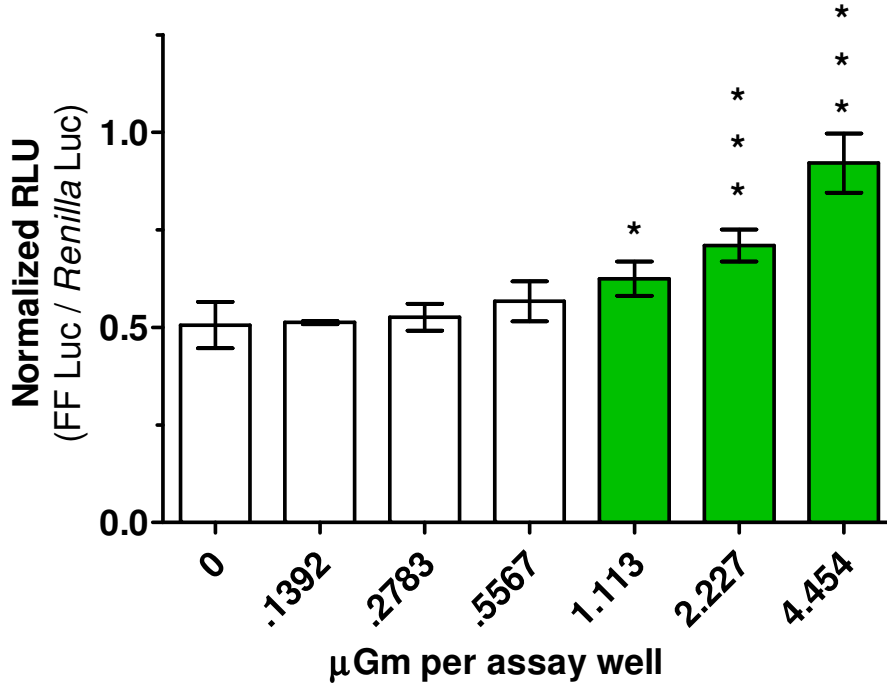


Table 4.3.2: ANOVA Analysis & Dunnett's Multiple Comparison Test

Cmpd 1, microGm	0	0.1392	0.2783	0.5567	1.113	2.227	4.454
Number of values	8	3	3	3	3	3	3

Minimum	0.38	0.5095	0.4992	0.5285	0.5989	0.681	0.8483
25% Percentile	0.4902	0.5095	0.4992	0.5285	0.5989	0.681	0.8483
Median	0.5049	0.5107	0.5141	0.5457	0.5996	0.6915	0.9143
75% Percentile	0.557	0.5174	0.5651	0.6254	0.6752	0.7568	0.9994
Maximum	0.5714	0.5174	0.5651	0.6254	0.6752	0.7568	0.9994

Mean	0.5054	0.5125	0.5261	0.5666	0.6246	0.7098	0.9206
Std. Deviation	0.0596	0.00426	0.03455	0.05171	0.04381	0.04106	0.07574
Std. Error	0.02107	0.00246	0.01995	0.02986	0.02529	0.02371	0.04373
Lower 95% CI	0.4556	0.5019	0.4403	0.4381	0.5157	0.6078	0.7325
Upper 95% CI	0.5552	0.5231	0.6119	0.695	0.7334	0.8118	1.109

Control vs Treatment	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
0 vs 1.392	-0.00711	0.2021	No	ns	-0.1062 to 0.09194
0 vs 2.783	-0.0207	0.5883	No	ns	-0.1197 to 0.07835
0 vs 5.567	-0.06115	1.738	No	ns	-0.1602 to 0.03790
0 vs 11.13	-0.1192	3.387	Yes	*	-0.2182 to -0.02012
0 vs 22.27	-0.2044	5.809	Yes	***	-0.3034 to -0.1053
0 vs 44.54	-0.4152	11.8	Yes	***	-0.5143 to -0.3162

4.4 hAR AGONISM ASSAY RESULTS: Test Compound 2 (Tea)

Normalized (FF luc/*Renilla* luc) average RLU values (derived from raw data provided in Section 8.3), as well as respective standard deviations, percent coefficients of variation, Z', and fold-increases over the "0" treatment condition, were calculated for each Compound 2 (Tea) treatment set (**Table 4.4.1**). For the purpose of comparing with the DHT control dose-curve, normalized average RLU values were curve-fit against Log10 μ Gm mass of Compound 1 (Tar) (**Figure 4.4.1**).

Normalized RLU values for each individual measurement within a set of replicates (for each of the 7 dose treatments) were analyzed by 1-way ANOVA followed by Dunnetts post-test to determine the statistical significance, if any, of differences in hAR activation between the "0" treatment and respective Cmpd 2 dose treatments. **Table 4.4.2** presents the findings of these statistical treatments. **Figure 4.4.2** provides a graphical representation of Normalized Average RLU values and respective standard deviation for each of the seven treatment doses. Green bars and astrics (*) denote values determined to be significantly different from the "0" dose control treatment.

Table 4.4.1

USP Compound 2		FF Luc / <i>Renilla</i> Luc: Compound 2 (Tea)							
Dilution	ug per 500 ul	#1	#2	#3	#4	Ave	Std Dev	%CV	Fold Increase
Neat 89.02%	4,450								
1/100	44.5	0.9483	0.8291	0.8651	0.9029	0.8864	0.0511	5.77	1.75
1/200	22.3	0.6589	0.6350	0.6108	0.6445	0.6373	0.0202	3.17	1.26
1/400	11.1	0.5975	0.6181	0.5956	0.5768	0.5970	0.0169	2.83	1.18
1/800	5.56	0.5582	0.5655	0.5128	0.5580	0.5486	0.0241	4.40	1.09
1/1600	2.78	0.4661	0.5118	0.5899	0.5344	0.5255	0.0515	9.79	1.04
1/3200	1.39	0.5727	0.4327	0.4253	0.6856	0.5291	0.124	23.5	1.05
-	0					0.5054	0.0596	11.8	1.00

Figure 4.4.1

Compound 2 (Tea) Dose-Curve (Normalized)

USP Labs, Study WO_080620-1

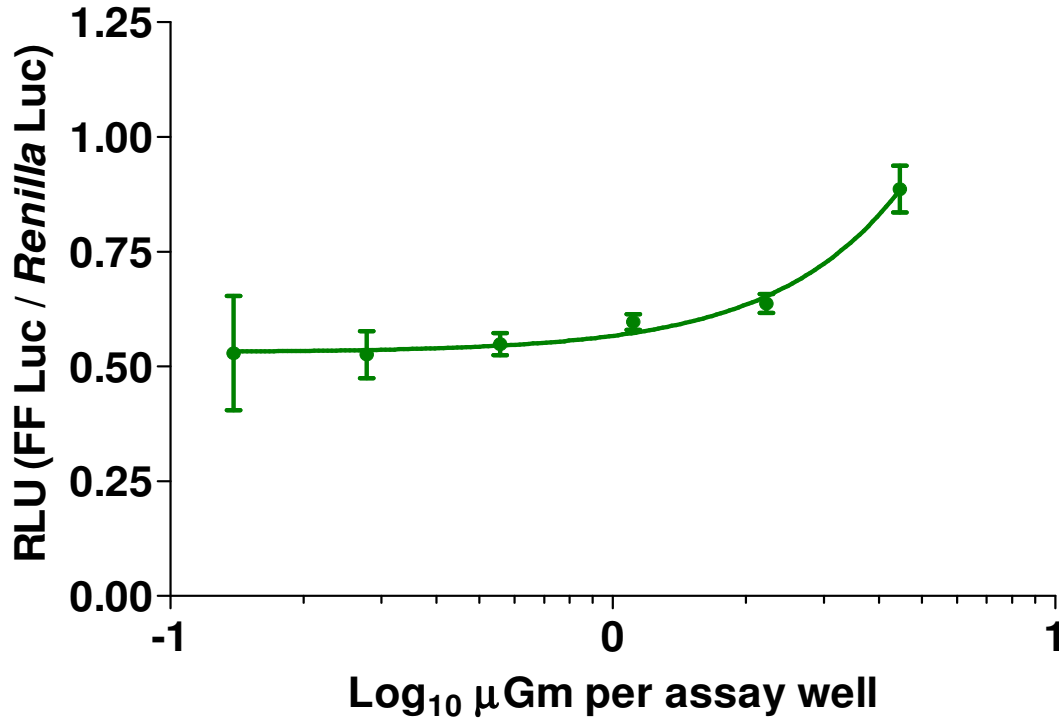


Figure 4.4.2
hAR Agonism Study, Compound 2 (Tea)
 USP Labs, Study WO_080620-1

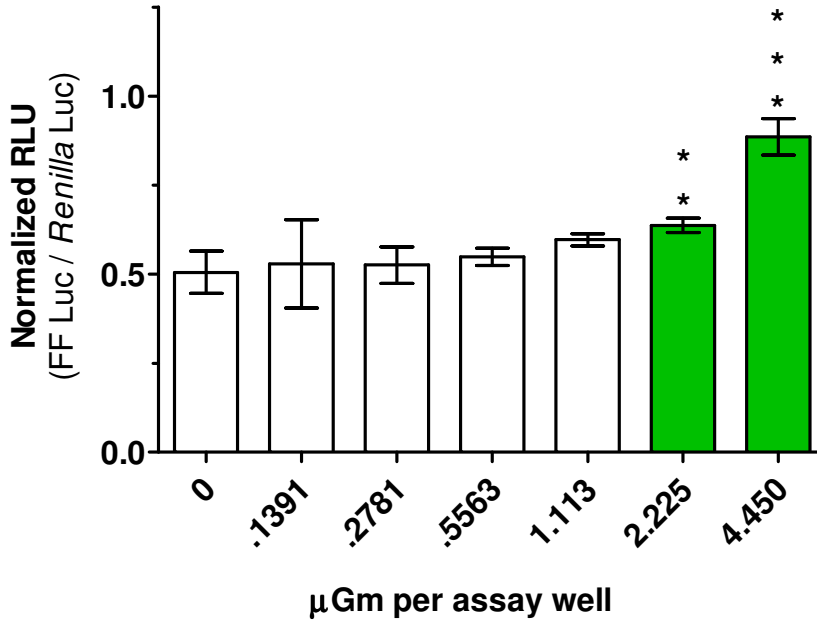


Table 4.4.2: ANOVA Analysis & Dunnett's Multiple Comparison Test

Cmpd 2, microGm	0	0.1391	0.2781	0.5563	1.113	2.225	4.45
Number of values	8	4	4	4	4	4	4
Minimum	0.38	0.4253	0.4661	0.5128	0.5768	0.6108	0.8291
25% Percentile	0.4902	0.4272	0.4775	0.5241	0.5815	0.6169	0.8381
Median	0.5049	0.5027	0.5231	0.5581	0.5965	0.6397	0.884
75% Percentile	0.557	0.6573	0.576	0.5637	0.613	0.6553	0.937
Maximum	0.5714	0.6856	0.5899	0.5655	0.6181	0.6589	0.9483
Mean	0.5054	0.5291	0.5255	0.5486	0.597	0.6373	0.8864
Std. Deviation	0.0596	0.1244	0.05147	0.02412	0.01692	0.02018	0.05113
Std. Error	0.02107	0.06221	0.02574	0.01206	0.00846	0.01009	0.02557
Lower 95% CI	0.4556	0.3311	0.4436	0.5103	0.5701	0.6052	0.805
Upper 95% CI	0.5552	0.7271	0.6074	0.587	0.6239	0.6694	0.9677

Control vs Treatment	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
0 vs 1.391	-0.02366	0.6406	No	ns	-0.1254 to 0.07803
0 vs 2.781	-0.02013	0.545	No	ns	-0.1218 to 0.08157
0 vs 5.563	-0.04323	1.17	No	ns	-0.1449 to 0.05847
0 vs 11.13	-0.09157	2.48	No	ns	-0.1933 to 0.01012
0 vs 22.25	-0.1319	3.571	Yes	**	-0.2336 to -0.03019
0 vs 44.50	-0.381	10.32	Yes	***	-0.4826 to -0.2793

5. CONCLUSIONS & DISCUSSION

5.1 DHT Agonist, Control Dose-Curve Assay: The hAR agonism reporter assay, as determined from analysis of the DHT dose-response control assay, performed very well during the course of this study. The determined EC₅₀ of 37 pM, as well as the fold-increase over the “0” treatment for this DHT agonist assay, are closely similar to historical values obtained by Indigo Biosciences for this hAR agonism dose-response assay. Additionally, the low %CV and moderately high Z’ scores attest to the robust nature of this specific assay run.

5.2 Compound 1 (Tar) Agonism Dose-Response: Careful examination of the raw *Renilla* luciferase data (Section 8.2, Table 8.2.2), specifically the calculation of “Fold Change Relative to “0” Treatment”, suggests that Compound 1 (Tar) exerted a mild, dose-dependent, cytotoxic effect on the reporter cells.

Notwithstanding the mild cytotoxic effects of Compound 1 (Tar), the material was determined to provide *statistically* significant, though low-level, agonist activity at the 1.113, 2.227 and 4.454 µGm per 500 µl concentrations (see Table 4.3.2 and Figure 4.3.2). However, without knowledge of the molecular weight of this compound and, hence, not knowing the respective Molar concentrations of these doses, it is difficult to assess the *physiological* relevance of these doses as agonists of hAR.

5.3 Compound 2 (Tea) Agonism Dose-Response: As with Compound 1, examination of the raw *Renilla* luciferase data (Section 8.3, Table 8.3.2), specifically the calculation of “Fold Change Relative to “0” Treatment”, suggests that Compound 2 (Tea) exhibited a mild, dose-dependent, cytotoxic effect on the reporter cells.

Notwithstanding the mild cytotoxic effects of Compound 2 (Tea), the material was determined to provide *statistically* significant agonist activity at the 2.225 and 4.450 µGm per 500 µl concentrations (see Table 4.4.2 and Figure 4.4.2). However, without knowledge of the molecular weight of this compound and, hence, not knowing the respective Molar concentrations of these doses, it is difficult to assess the *physiological* relevance of these doses as agonists of hAR.

7. REFERENCES

None

8. APPENDICES

8.1 RAW DATA: DHT Agonist, Control Dose-Curve Assay

Table 8.1.1

FF Luc: DHT Agonist Dose-Response									
DHT pGm	[DHT] pM	#1	#2	#3	#4	Ave	StDev	%CV	Fold Increase
0	0	105063	107567	81622	119398	106,750	12853	12.0	1.00
		121887	111728	109874	96862				
0.2269	1.56	120062	115955	122134	127840	121,498	4947	4.07	1.14
0.4538	3.13	136851	142017	154629	133930	141,857	9148	6.45	1.33
0.9077	6.25	161188	167125	178014	184899	172,807	10655	6.17	1.62
1.815	12.5	265204	301572	283880	315285	291,485	21731	7.46	2.73
3.631	25.0	477366	478008	538172	507105	500,163	28887	5.78	4.69
7.261	50.0	949047	1200256	1055116	1054362	1,064,695	103198	9.69	10.0
14.52	100	1422252	1597129	1406446	1543473	1,492,325	92890	6.22	14.0
29.05	200	1842157	1914329	1515737	1927187	1,799,853	193072	10.7	16.9
58.09	400	1798567	1649012	1428871	840765	1,429,304	420716	29.4	13.4

Table 8.1.2

Renilla luc: DHT-Treated Reporter Cells									
DHT pGm	[DHT] pM	#1	#2	#3	#4	Ave	StDev	%CV	Fold Increase
0	0	209593	217995	214784	223084	211,677	5662	2.67	1.00
		239699	195521	194727	198015				
0.2269	1.56	204398	193624	204304	219732	205,515	10743	5.23	0.971
0.4538	3.13	219428	238562	243152	223715	231,214	11426	4.94	1.09
0.9077	6.25	222674	208394	226523	246465	226,014	15707	6.95	1.07
1.815	12.5	208641	228687	246726	256150	235,051	20972	8.92	1.11
3.631	25.0	162660	200331	215239	235982	203,553	30934	15.2	0.962
7.261	50.0	190497	252364	245334	255630	235,956	30609	13.0	1.11
14.52	100	203254	243708	226066	253470	231,625	22053	9.52	1.09
29.05	200	238019	266330	231151	282569	254,517	24115	9.47	1.20
58.09	400	264355	258020	232326	156820	227,880	49356	21.7	1.08

8.2 RAW DATA: Compound 1 (Tar) Agonism Dose-Response:**Table 8.2.1**

Compound 1		FF Luc: Compound 1 (Tar)				
Dilution	ug per 500 ul	#1	#2	#3	Ave	Fold Change Relative to "0" Treatment
Neat 89.07%	4,453.5					
1/100	44.54	157698	148242	153291	153,077	1.48
1/200	22.27	146486	140864	107723	131,691	1.27
1/400	11.13	119156	113838	114897	115,964	1.12
1/800	5.567	121551	122323	111411	118,428	1.15
1/1600	2.783	118088	108445	110239	112,257	1.09
1/3200	1.392	118088	108445	110239	112,257	1.09
-	0				103,413	1.00

Table 8.2.2

Compound 1		Renilla Luc: Compound 1 (Tar)				
Dilution	ug per 500 ul	#1	#2	#3	Ave	Fold Change Relative to "0" Treatment
Neat 89.07%	4,453.5					
1/100	44.54	185899	162142	153387	167,143	0.790
1/200	22.27	211824	186126	158177	185,376	0.876
1/400	11.13	198948	189845	170177	186,323	0.880
1/800	5.567	229981	224155	178131	210,756	0.996
1/1600	2.783	236571	210941	195090	214,201	1.01
1/3200	1.392	231791	212338	213069	219,066	1.03
-	0				211,677	1.00

8.2 RAW DATA: Compound 2 (Tea) Agonism Dose-Response:**Table 8.3.1**

Compound 2		FF Luc: Compound 2 (Tea)					
Dilution	ug per 500 ul					Ave	Fold Change Relative to "0" Treatment
Neat 89.02%	4,450	#1	#2	#3	#4	Ave	
1/100	44.50	121587	124641	128691	152973	131,973	1.28
1/200	22.25	129356	85895	87939	108210	102,850	0.995
1/400	11.13	111980	96760	95956	109247	103,486	1.00
1/800	5.563	131625	91643	87217	110159	105,161	1.02
1/1600	2.781	106346	91951	97556	98204	98,514	0.953
1/3200	1.391	131625	91643	87217	110159	105,161	1.02
-	0					103,413	1.00

Table 8.3.2

Compound 2		Renilla Luc: Compound 2 (Tea)					
Dilution	ug per 500 ul					Ave	Fold Change Relative to "0" Treatment
Neat 89.02%	4,450	#1	#2	#3	#4	Ave	
1/100	44.50	128211	150331	148755	169424	142,432	0.673
1/200	22.25	196334	135276	143963	167895	158,524	0.749
1/400	11.13	187421	156537	161116	189416	168,358	0.795
1/800	5.563	235793	162067	170068	197407	189,309	0.894
1/1600	2.781	228173	179676	165375	183763	191,075	0.903
1/3200	1.391	229835	211799	205056	160683	215,563	1.02
-	0					211,677	1.00

(~ End of Report ~)