Report of Work

# Proposal #

LV-33

# Title

Effect of AC-11® on 5 major Cytochrome P450 enzymes (biochemical assays)

# Client

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# Prepared by

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# Background

Optigenex is currently performing due diligence on a Cat’s claw extract, AC-11®. Optigenex wanted to determine the effect of AC-11® alone and in combination with common pharmaceutical agents on the activity of major cytochrome P450 enzymes (enzymes involved in, among other functions, the metabolism of pharmaceutical agents).

# Materials and Methods

**Test Compounds**

Fresh AC-11® was delivered to Wasatch Scientific on 06-15-18 by Optigenex Inc.

Diltiazem was purchased through Selleck Chemicals (Cat. # S1865).

Verapamil was purchased through Sigma-Aldrich (Cat. # V4629).

Erythromycin was purchased through Selleck Chemicals (Cat. # S1635).

**CYP450 inhibition assays**

CYP screening systems and positive control inhibitors were purchased according to **Table 1**.

**Table 1**. Particular substrates, enzymes, and control inhibitors used in the present study.

|  |  |  |  |
| --- | --- | --- | --- |
| **CYP** | **Cat. #** | **Vendor** | **Description** |
| 1A2 | v8772 | Promega | Luciferin-ME; substrate for 1A2  |
| v4770 | Promega | 1A2 enzyme system |
| N3633 | Sigma | naphthoflavone; control inhibitor |
| 2C9 | v8792 | Promega | Luciferin-H; substrate for 2C9  |
| v4790 | Promega | 2C9 enzyme system |
| S0758 | Sigma | sulfaphenazole, control inhibitor |
| 2C19 | v8882 | Promega |  Luciferin-H EGE; substrate for 2C19 |
| v4880 | Promega | 2C19 enzyme system |
| T2573 | Sigma | troglitazone, control inhibitor |
| 2D6 | v8892 | Promega | Luciferin-ME EGE; substrate for 2D6  |
| MTOXCE2D6 | Sigma | 2D6 enzyme  |
| Q3626 | Sigma | quinidine, control inhibitor |
| 3A4 | v8912 | Promega | PPXE DMSO tolerant substrate |
| v4910 | Promega | enzyme system |
| K1003 | Sigma | ketoconazole; control inhibitor |

Assays for five cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) for their sensitivity to the test articles were conducted as biochemical, *in vitro* assays. Briefly, a membrane-enzyme preparation containing one of the CYP450 enzymes was incubated with a substrate metabolized by that enzyme. The substrate, when acted upon by the CYP450 enzyme, converts to a form which luminesces in the presence of luciferase; the substrate does not luminesce without CYP450 conversion. These incubations took place in the presence or absence of the test article. AC-11 was administered in an eight-step half-log dilution series of 100, 30, 10, 3, 1, 0.3, 0.1, and 0.03 µg/mL. Each control inhibitor or pure compound for combination was administered in an eight-step half-log dilution series of 10, 3, 1, 0.3, 0.1, 0.03, 0.01, and 0.003 µg/mL). All assays were carried out according to the manufacturer’s recommendations, with the following exception; the 2D6 enzyme was used at a concentration of 10mg/mL. Assays were run in duplicate in half-volume, white, non-treated, 96-well plates (Costar 3693). Following assay incubation, the CYP450 reaction was halted with a luciferase detection reagent, and activity/luminescence was measured on an Envision plate reader (Perkin Elmer).

**Graph generation and IC50 analysis**

Data was input into Prism 7 (Graphpad), and IC50 values were calculated using log transformed x (concentration) values, using a four-parameter non-linear regression least squares model. Data was also plotted by Prism 7.

**Combination Index Calculations**

Combination Indices were calculated using CompuSyn software, V. 1.0 (Biosoft, Cambridge, UK). Drug interactions were classified by determining a combination index (CI) recognized as the standard measure of combination effect based on the Chou-Talalay method. The CI values were obtained over a range of fractional enzyme inhibition levels (Fa), based on the Chou-Talalay method (Ref. 1,2,3,4), CI < 1 means synergism, CI = 1 means additivity, and CI > 1 is interpreted as antagonism.

1. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984;22:27–55. [PubMed]

2. Chou TC, Talalay P. Analysis of combined drug effects: a new look at a very old problem. Trends Pharmacol Sci. 1983;4:450–454.

3. Flis S, Splawinski J. Inhibitory effects of 5- fluorouracil and oxaliplatin on human colorectal cancer cell survival are synergistically enhanced by sulindac sulfide. Anticancer Res. 2009;29(1):435–441. [PubMed]

4. Flis S, Gnyszka A, Misiewicz-Krzeminska I, Spławinski J. Decytabine enhances cytotoxicity induced by oxaliplatin and 5-fluorouracil in the colorectal cancer cell line Colo-205. Cancer Cell Int. 2009;9:10. doi: 10.1186/1475-2867-9-10. [PMC free article] [PubMed]

# Results and Discussion

CYP assays were performed as described in the materials and methods. The effect of each duplicate for each concentration is recorded in an excel sheet that was sent separately (titled, “LV-33\_CYP\_testing.xlsx”). CYP1A2, 2C9, 2C19, 3A4, and 2D6 enzymatic activity is depicted graphically in **Figures 1-5**, respectively.


**Figure 1**. CYP1A2 activity. X-axis in log scale.


**Figure 2**. CYP2C9 activity. X-axis in log scale.



**Figure 3**. CYP2C19 activity. X-axis in log scale.



**Figure 4**. CYP3A4 activity. X-axis in log scale. \* For this graph, each data point for single agents corresponds to the correct concentration. However, for the combinations the concentration shown on the points in the graph are the concentrations of the compounds being combined with AC-11 (since the single species compounds are typically more potent than blends)(i.e. for the combinations at 10µg/mL, it is 10µg/mL of the drug added for combination, and 100µg/mL of AC-11).



**Figure 5**. CYP2D6 activity. X-axis in log scale.

Curve fitting software was used to identify the concentration of each test article that inhibited the CYP activity by 50% (IC50 value). These IC50 values are recorded in the accompanying spreadsheet, where applicable. Briefly, for the 1A2, 2C9, 2C19, and 2D6 enzymes, AC-11 did not inhibit the enzyme below 50% activity, even at the highest dose of 100µg/mL. For the 3A4 enzyme, the IC50 was calculated at 19.32µg/mL.

Based on these data it appears that AC-11 had little effect on CYP1A2, 2C9, and 2C19. However, AC-11 seemed to increase the activity of CYP3A4 slightly at moderate concentrations, while inhibiting the enzyme at higher concentrations. CYP3A4 is probably the most recognizable CYP and the one that is the focus of many drug interaction studies (since 3A4 metabolizes 45-60% of currently prescribed drugs). Due to this reason, AC-11 was tested in combination with diltiazem, verapamil, and erythromycin (which are known to have some effect on CYP3A4). Using a fixed ratio combination, it can be observed that the combinations with AC-11 are not dramatically different than diltiazem, verapamil, and erythromycin as single agents (**Figure 4**). This is especially evident in comparison to the positive control ketoconazole. It should be noted that for combination graphs in this report, each data point for single test agents corresponds to the correct concentration. However, for the combinations the concentration shown on the points in the graph are the concentrations of the compounds being combined with AC-11 (since the single species compounds are more potent than AC-11 [which is made of multiple constituents]) (i.e. for the combinations at 10µg/mL, it is 10µg/mL of the drug added for combination, and 100ug/mL [always a 10-fold higher concentration] of AC-11).

To investigate the combinations even further, combinatorial indices were calculated using the program Compusyn. At concentrations with a high effect (considered the most relevant to the model), the combinations showed more antagonism than synergism (**Table 2**); antagonism shown as CI values >1.

**Table 2**. CI values calculated for combinations with AC-11. (Effective

|  |  |  |
| --- | --- | --- |
|   | Effective Concentration |   |
| Combination | 80 | 85 | 90 | 95 | 97 |   |
| AC-11 + Diltiazem | 0.67 | 0.72 | 0.82 | 1.05 | 1.29 | CI Values |
| AC-11 + Verapamil | 0.63 | 0.87 | 1.37 | 2.86 | 4.82 |
| AC-11 + Erythromycin | 1.02 | 1.30 | 1.78 | 2.97 | 4.30 |

\*1=additive; <1 = synergism; >1 = antagonism

\*\*(Effective concentration of 90 means compound needed to inhibit the enzyme 90%)

CYP2D6 appeared to be possibly activated by AC-11 at high concentrations in this particular biochemical assay. The positive control of inhibition for 2D6 (quinidine), inhibited 50% of 2D6 activity at a low concentration of ~0.01 ug/mL. To determine if AC-11 enhanced the inhibition of CYP2D6 by Fluoxetine, a combination study was carried out (in a similar fashion to the 3A4 combinations). The curve with AC-11 added to Fluoxetine showed less inhibition than Fluoxetine by itself (**Figure 5**) (underscoring the possibility of AC-11 slightly enhancing the 2D6 biochemical activity).

# Conclusions

Wasatch Scientific has evaluated AC-11 alone, and in select combinations for its ability to impact CYP activity for (5) CYP enzymes in biochemical assays. A separate file with the data for each point of each assay has been attached separately (“LV-33\_CYP\_testing.xlsx”).

It should be noted that there are several systems, substrates, and methods to test CYP inhibition. In this particular assay, a single recombinant enzyme was used and not liver microsomes, which contain all CYPs and have the associated problem of specificity of an enzyme for a particular substrate (most substrates can me metabolized to a certain extent by multiple CYPs). One downside to a biochemical assay is that it does not measure the ability of a substance to change the expression of a particular CYP in a living organism (and thus change the activity of the CYP in a less direct manor). Therefore, caution should be taken when interpreting the results, as overall activity in human subjects could be different.

On behalf of Wasatch Scientific Services, I would like to thank you for the opportunity to work on your project, and look forward to working with you in the future.



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Wasatch Scientific Services