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Award Number: W81XWH-08-2-0024

TITLE: Development and Validation of a High Throughput Screen for compounds with antiviral activity against encephalitic alphaviruses

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REPORT DATE: September 2010

TYPE OF REPORT: final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			<i>Form Approved</i> OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 15-SEP-2010		2. REPORT TYPE final		3. DATES COVERED (From - To) 1 MAR 2008 - 15 AUG 2010
4. TITLE AND SUBTITLE Development and Validation of a High Throughput Screen for compounds with antiviral activity against encephalitic alphaviruses			5a. CONTRACT NUMBER W81XWH-08-2-0024	
			5b. GRANT NUMBER	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Colleen B. Jonsson, Ph.D Dong Hoon Chung, Ph.D cjonsson@nmsu.edu			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Southern Research Institute 2000 9 th Ave S Birmingham AL 35205			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)	
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT A high throughput assay using a luciferase tagged VEEV has been established to generate effective probes against encephalitic alphaviruses. The virus was rescued from in vitro transcribed RNA in BHK cells and initial validation has been done. The assay was adapted in an HTS automated form with 384-well system after it has been tested in various conditions including cell number, MOI optimization. Z' value which indicates the robustness of an assay was above 0.6 indicating suitable for a single dose HTS. The Prestwick library, 1120 compounds and Arbovirus hit library, 658 compounds have been tested in duplicate mode. In combination with cytotoxicity of the compounds, 63 compounds were screened out as active hits with > 50% efficacy and <30% cytotoxicity. Currently those 63 hits were further characterized in a dose response manner. A diverse library consisting of 8960 compounds was tested in the system twice. 16 compounds meeting the criteria were selected and tested further in a dose response format for cytotoxicity and efficacy. A total of 39 compounds which have structural similarity of the selected candidates were tested in a CPE based assay using TC-83.				
15. SUBJECT TERMS				
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	d. THIS PAGE U	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION (Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.)
: Southern Research Institute (SRI) developed a high throughput screen (HTS) using a novel luciferase-tagged VEEV that was developed at USAMRIID and then screened a 10,000 compound small molecule diversity library and lead compounds were optimized by chemi-informatic research.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative. This approval must be obtained prior to initiating any change to the original Statement of Work.

Major task 1: SRI will adapt a HTS CPE assay to VEEV virus in a 384-well plate format

1. Procedure

SRI has established a three step protocol for adapting a specific viral infection to their platform.

Step 1. Determine culture conditions to yield optimal dynamic range:

The difference in the luminescence values produced from virally infected cells and viable cells gives the assay its dynamic range. Generally, a HTS screen will be effective if the signal generated by the virus is 5- times greater than viable cells given minimum deviation in the signal produced. The greater the difference between these two values, the more sensitive the assay and the more likely to capture compounds that have lower potency.

The first step in the protocol determines the optimal cell and virus culturing conditions to obtain the optimal dynamic range. In this phase, various different medium conditions, MOIs, and harvesting times are compared to determine the optimal experimental conditions. This includes the determination of the cell density per well that provides the maximum number of targets with minimal amounts of background luminescence and determination of the optimal virus growth conditions, kinetics of infection, and input MOI. Briefly, Vero and BHK cells will be grown in at least two different media, such as DMEM and Opti-MEM, to determine optimal growth and virus producing conditions. Cells will be seeded into 384-well plates and the following morning infected with VEEV at varying MOIs. After 24 and 48 hours of infection, a plate will be removed and end point reagent (Bright-Glo Luciferase Assay System) will be added. Luminescence values will be detected using an EnVision plate reader (Perkin – Elmer).

The culturing conditions yielding the greatest dynamic range, which is the greatest difference between infected and uninfected cells, will be utilized in the experiment. Based upon preliminary data, we expect this luciferase assay will have a dynamic range of 400 times above background or greater. The extreme dynamic range of this assay will allow us to identify a large number of lead compounds, including those with lower activity that might be missed in screens with lower dynamic ranges.

Step 2 Determination of compound addition parameters: The second step in the protocol is to determine the parameters for the addition of compound. During this phase, the effect of a control drug and at least one drug solvent will be assayed to determine the maximum concentration of each compound that can be used with the minimal toxic effects and maximal antiviral activity. Briefly, virus (quantities sufficient to result in greater than 95% cell death) will be added to wells. Following virus addition, SRI will add varying amounts of DMSO (dimethyl sulfoxide), a commonly used solvent, and control drug, ribavirin, and a combination of drug and solvent to each well. The experimental results will be analyzed to determine the maximal amount of solvent and drug that can be added to each well that allows cells to remain viable while inhibiting viral infection.

Step 3 Determine assay robustness: The final step of the protocol involves determining assay robustness, which is defined as the consistency of signal for each of the experimental conditions, and the consistency of the dynamic range. To measure assay consistency, we will determine the standard deviation observed for all of the experimental conditions. Individual 384-well plates will be prepared that contain a single condition including, but not limited to cells alone, virus alone, cells + DMSO, virus + control drug. An assay is considered successful when the variation for the conditions is less than 10% from well to well. To confirm the consistency of the dynamic range, 384-well plates will be prepared containing uninfected cells and infected cells, and the luciferase levels determined. The experimental results will provide data on the variability of the two conditions. These dynamic range data will be subjected to Z-test statistical analysis to confirm that the standard deviation of the uninfected wells is statistically significantly different from the infected wells.

2. Result

1) Assay implementation and validation

The plasmid V3526-Luc clone (pV3526-luc) was acquired from USAMRIID then amplified for the experiment downstream.

Rescue of V3526-luc virus. The stock virus was prepared in a process of rescuing virus from infectious RNAs as directed by Dr. Brett Beitzel USAMRIID. Total a 60 mL of stock virus was generated and then 1.5-mL of aliquots were stored at -80°C as a stock virus.

Characterization of V3526-luc stock

Luciferase activity: The initial virus stock was characterized in Vero 76 cells which have been well known for VEEV replication study. The luciferase activities from the infected cells were confirmed and we validated the virus stock (Figure 1).

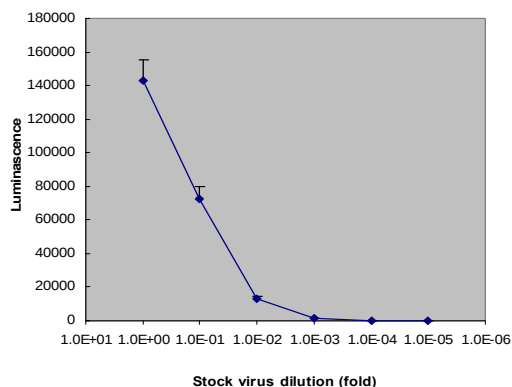


Figure 1. Luminescence reading from Vero 76 cells infected with serially diluted V3526-luc virus

Virus titers: The stock virus titers were determined in two ways, 1) a standard agarose plaque assay and 2) TCID 50 assay using endogenous luciferase as a marker of infection and the titers are shown in Table 1 showing that the stock virus titer was enough to infect cells up to 0.5 PFU per cell.

Table 1

	PFU/ml	TCID 50 /mL
V3526-luc stock	1.90×10^5 /mL	4.37×10^6 /mL

2) Assay optimization/validation for HTS

We have developed and validated a 384-well cell based assay that measures luciferase activity from V3526-luc virus infection. A number of parameters which are critical for HTS such as the mode of infection (M.O.I), cell density and determination of endpoint were investigated as proposed in the approved proposal.

Determination of the development time after infection: We have determined the best endpoint timing by developing plates at different days post infection. The cell plates were infected with different amount of virus and developed 1, 2 and 3 days post infection. As shown in Figure 2, the highest luminescence was achieved one day post infection with higher amount of virus infection. The studies indicated that the optimum incubation time is 1 day after infection, which is well short assay within cell based assay.

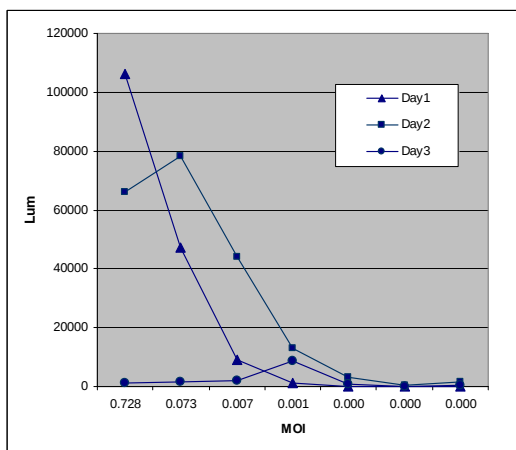


Figure 2. The optimal incubation time and MOI determination. Vero 76 cells were infected with various MOIs then developed day 1,2 and 3 post infection.

Determination of the optimal cell density Z' factor also was calculated in order to determine an optimal cell density as well as the virus amount for infection (Figure 3). Vero 76 cells were seeded in 384-well plate at either 3000 or 6000 cells per well and cells were infected with a range from 1000 - 6000 TCID₅₀ of the stock virus per well. The results showed that the luminescence reading is dependent on the cell numbers so the bigger dynamic range was obtained at a cell density of 6000 cells per well. Regarding to the robustness, Z', was also higher at the higher cell density, however there was no significant difference between 3000 cells and 6000 cells per well when 6000 TCID₅₀ per well was infected in.

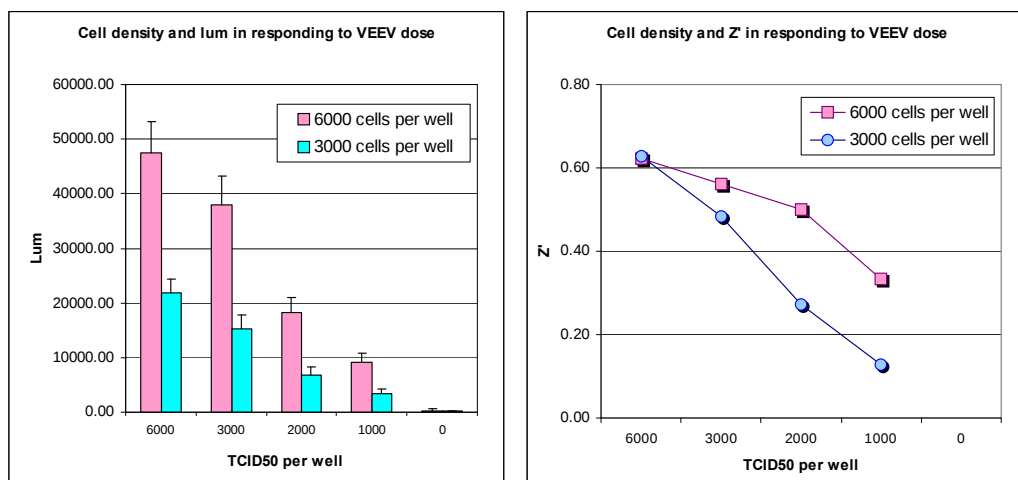


Figure 3. Optimum cell density and MOI determination based on luminescence (A) and Z' analysis (B). 3000 or 6000 cells per well was seeded in 384-well plates and then various amount of virus was infected.

Dimethylsulphoxide (DMSO) tolerance. The effect of DMSO in the assay system was tested within a range of 0.1 to 1%. One day post cell seeding, 5 μ L of media containing various amounts of DMSO were added to the plate. Diluted virus at a concentration of a 6000 TCID₅₀ per 15 μ L or cell culture media was then added to the wells and the plates were incubated for 3 days. The experiment revealed that cell viability in cells only and virus-infected wells were decreased as the DMSO concentration increased. However, the effect was significant only above 0.6% (Fig 4). Therefore these data suggest that the final DMSO for the assay should be kept at less than 0.6% in final.

3) Robustness and Performance of the assay

With the optimized condition, finally we have evaluated the assay robustness for high-throughput screening in a 384-well format. We designed an experiment to determine the Signal to Noise ratio (S/N), the Signal to background ratio (S/B), CV% and the Z factor. The experiment composed with duplicated set of plates for cell infection only and for virus infected and two consecutive runs.

Table 2. Assay Robustness

	Z' S/N	S/B	
V3526-luc assay	0.714 $\pm$ 0.025	10.6 $\pm$ 0.94	1635 $\pm$ 52

Conclusion : We successfully adapted the task assay into HTS screening format. All variable parameters were optimized for the assay and the assay metrics were over the requirement for an HTS.

MAJOR TASK 2: PRIMARY SCREENING OF 10,000 COMPOUND DIVERSITY LIBRARY.

Major task 2: Primary screening of 10,000 compound diversity library. The screening was done with the assay conditions determined during the optimization process. The SRI HTS group and cell biology core prepared reagents sufficient to screen a 10,000 compound diversity set for inhibitory activity.

Briefly, cells were plated in 384-well plates at a density of 4500 cells per well. The following morning, dilute concentrations of compound (10 μ M) was added. V3526-luc was added, 6000 TCID₅₀ per well, to one of the compound containing wells

The plates will be incubated at 37°C incubator containing 5% CO₂ for 24 hours. Luciferase detection reagent (Bright-Glo™, Promega) was added, and then the amount of luminescence was determined using an Envision microplate reader. We also performed a cytotoxicity test to rule of false positives. For the cytotoxicity test, we incubated the cells in the presence of compounds for 72 hours without virus infection. We determined the compounds as cytotoxic if the viability is less than 85% in the screening.

1. Single dose study

A. Small library screening

We have run two sets of pre-selected compound libraries to statically verify the screening before main screening. We selected a total of 1778 compounds for this purpose 1) PrestWick (1120 compounds, PW) and 2) Arbovirus library (658 compounds, Arbo). The Prestwick library is a set of compounds representing diverse chemical structure classes. Arbo virus library is a collection of compounds that has shown inhibitory activity against Arbo virus replication from MLSCN compounds library. Compounds were tested at 10 uM concentration for Arbo library or 10 ug/mL for PrestWick library in 0.3 % DMSO in culture media. To test reproducibility of the assay, we ran two consecutive experiments independently. The cytotoxicity was performed as a counter screening to get rid of false positives.

B. Validation of assay performance using the PW pilot library

(1) Correlation study: A successful HTS should meet criteria in assay performance variation. We tested the variation by executing two experiments independently and then used a statistical analysis to verify the metrics.

Correlations of PW compounds, 1120 compounds

We analyze performance of correlation with the Pearson's and the Spearman's equation and the results are shown in Table 3. As the table indicated (the closer to 1, the better), two independent experiments showed very close results. Also we report the performance in **Scatter Plots** format (Fig 4).

Table 3. Correlation analysis using Pearson and Spearman relation.

	Pearson's Correlation	Spearman's Correlation
Luminescence1 vs. Luminescence2	0.92	0.77
%Inhibition1 vs. %Inhibition2	0.92	0.76

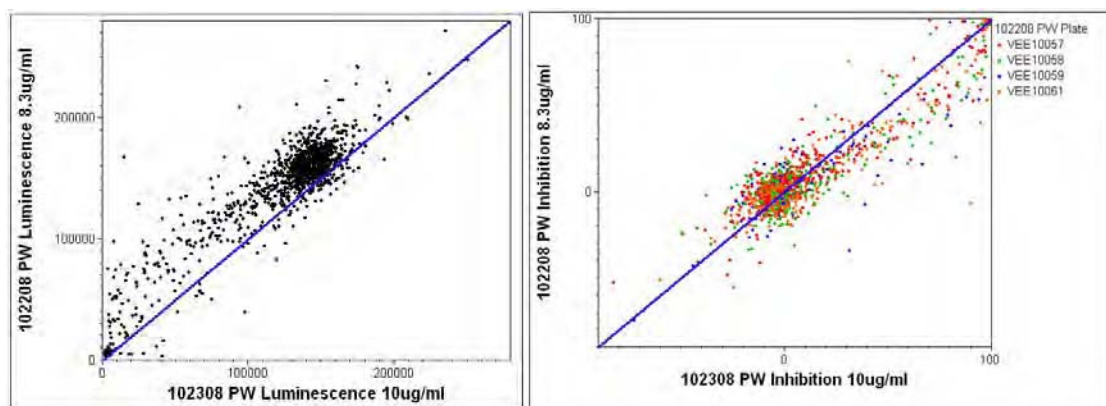


Figure 4. V3526-luc HTS assay performance in two independent experiments with Prestwick library. Correlation between two experiment were shown very tight.

C. Determination of a cut-off value

Southern Research has developed and used a cut-off for selection of active compounds from an HTS. In most of the cases, we have used Mean $\pm$ 3 times standard of whole screened compounds as a cut off value. To verify if the cut-off can be used for the screen we plotted the results in a Sequence plot.

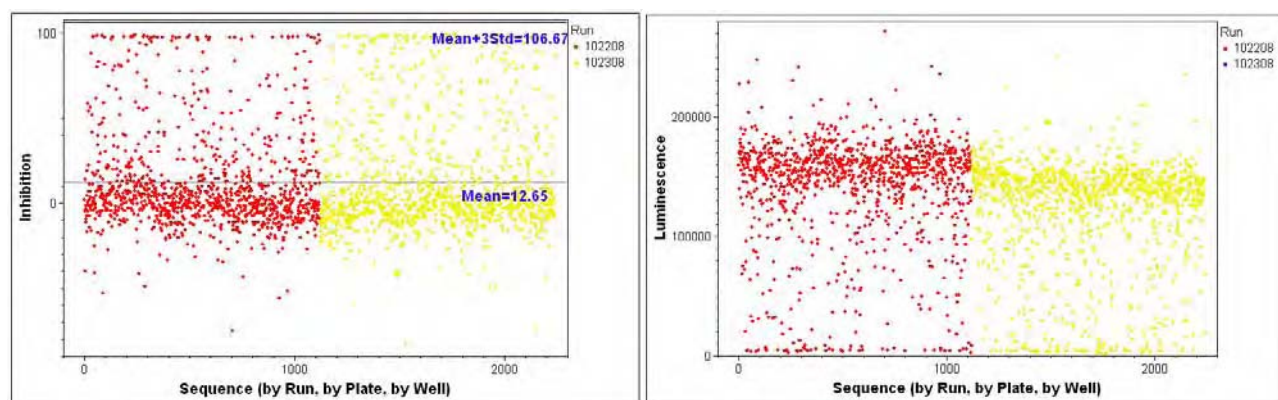


Figure 5. V3526-luc HTS assay performance in a sequence plot. Even though the correlation between two experiments was tight, the standard deviation from the library was too high to use Mean $\pm$ 3Std as a cut-off value for the experiment.

As shown in Fig 5, mean inhibition % and the threshold from the PW library was 12.65 and 106.67% which is high compared to other CPE based assays such as West Nile virus assay with a threshold value of 3.42%.

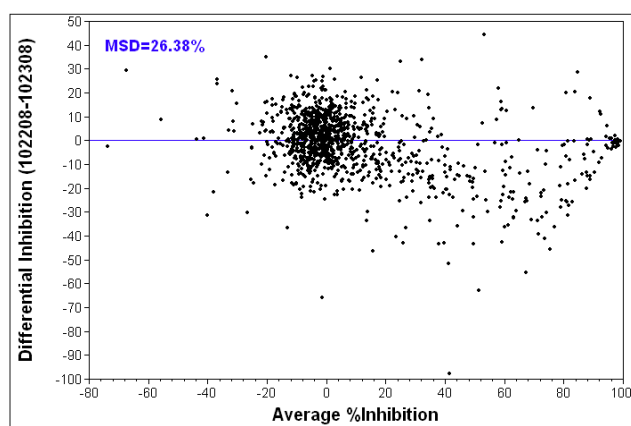


Figure 6. Bland-Altman Plot of PW library in the VEEV-luc HTS

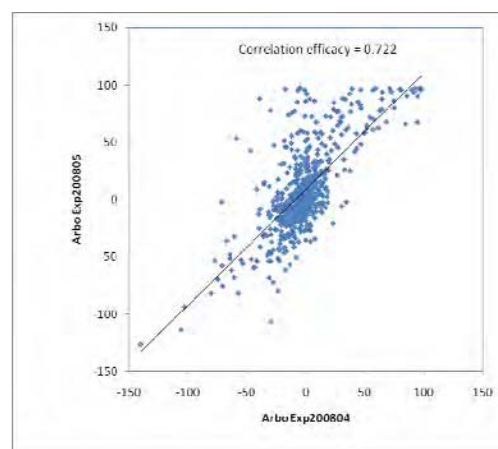


Figure 7. V3526-luc HTS assay performance in two independent experiments with Arbovirus library.

D. Single Dose experiment with Arbo virus library. The Arbovirus library was also tested in a single dose HTS at concentration of 10uM. We found almost similar result as that of the PW library (Fig 7.). From the two single dose experiments we could conclude that *the VEEV HTS using luciferase is HTS-ready however the general cutoff methods using mean $\pm$ SD should be changed.*

E. Initial putative probes

Based on two repetitive experiments for activity we screened compounds showing more than 50% inhibition activities and less than 30 % of cytotoxicity.

- (1) PrestWick Library : **14 compounds were selected as actives** from the both runs.
- (2) Arbo virus library : **18 compounds were selected as actives** from the both runs. 63 compounds were selected of which the activity was confirmed either run.

2. Dose Response Study

We tested a chemical library (Arbo virus library) in a dose response study to obtain EC₅₀, CC₅₀ and SI₅₀ for each compound. The Arbo virus library consists of 658 compounds that were active against Blue Tongue virus (PubChem: AID1250 & 1251). The compounds were prepared in a 2 fold dilution series with 50 μ M serving as the highest concentration. Cytotoxicity tests were also conducted in parallel by incubating the compounds with cells without virus infection.

A. Results : We identified 56 compounds that were active in anti-VEEV assay with IC₅₀ values of less than 50 μ M and an SI₅₀ > 1 (Fig. 8) from the library. Detailed analysis revealed that four of these compounds have sub-micromolar IC₅₀ values and an SI₅₀ > 50 (Fig. 8 and Table 4). Twelve compounds showed SI₅₀ > 2 which indicates possible hits.

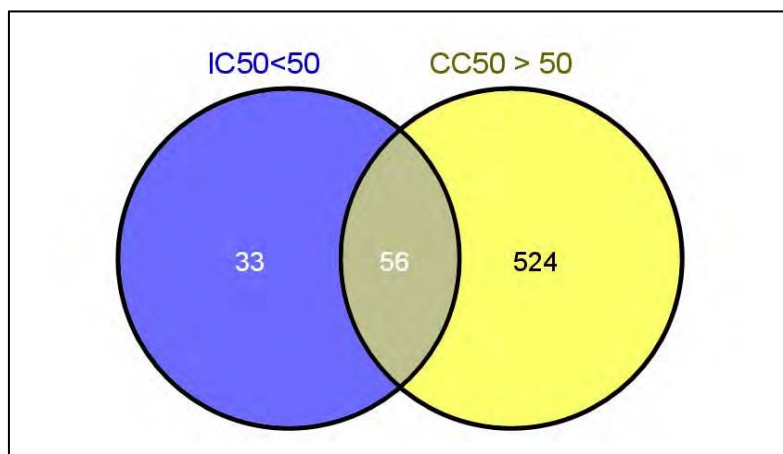


Figure 7. 50 compounds which have CC50 > 50 μ M and IC50 < 50 were depicted in the Venn diagram.

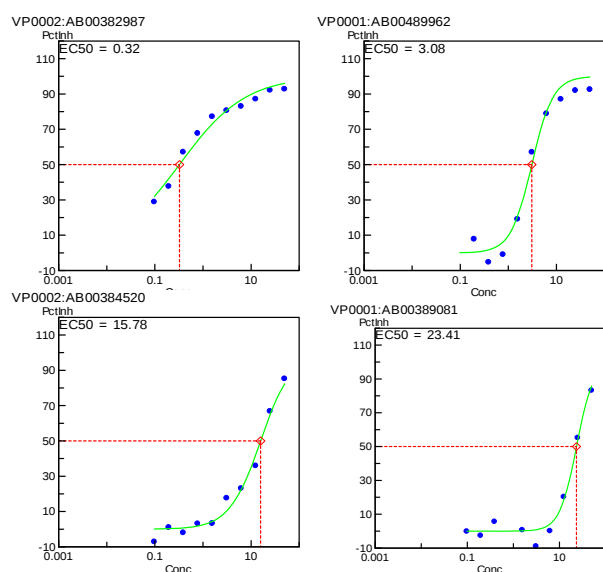


Figure 8. Examples of dose response curves showing broad range of EC50 between 0.32 and 23.41. Most of patterns have fitted in a sigmoid pattern.

Supplier ID	EC50 (μ M)	CC50 (μ M)	SI50
SMR000058373	0.32	> 50	>156.25
SMR000372439	0.33		>151.52
SMR000394098	0.55		>90.91
SMR000242567	0.83		>60.24
SMR000027739	1.55		>32.26
SMR000059052	3.08		>16.23
SMR000278122	10.62		>4.71
SMR000299933	12.22		>4.09
SMR000038207	15.78		>3.17
SMR000071291	17.23		>2.9
SMR000193314	23.11		>2.16
SMR000071286	23.41		>2.14

Table 4. Selected compounds from Arbovirus library which showed promising anti-VEEV-luc activity in a dose response study.

B. Discussion : Based on the $SI_{50} > 2$, the hit rate was calculated as 1.8% (56 compounds out of 658 compounds). This rate is higher than in our prior experience in HTS which showed around 0.01 ~ 0.2% of hit rate. *The higher rate is most likely due to the approach in that the compounds were from an enriched library* of known actives. In retrospect, this proves that the high throughput screening strategy of V3526-luc can generate valuable potential probes against Venezuelan Equine Encephalitis virus.

3. High Throughput screening of 10,000 compound diversity library.

We select a diversity compound library from Enamine (Ltd). Because the assay can select cytotoxic compounds as a false positive, we have screened the compounds at a concentration of 10 μ M. The screening was conducted in two steps; 1) Screen1 of 8320 compounds and Screen2 of 8960 compounds independently and 2) cytotoxicity test of 9600 compounds which cover Screen 1 and Screen2.

A. Results

(1) Experiment 1 and 2.

Screen1 : 8320 compounds were screened with the optimized condition. As shown Fig.9 and 10, the antiviral activity ranged between 99.5% and -248.00%. Usually an assay does not make negative inhibition that indicates the signal

readout has been increased by the addition of compounds. In this assay that can be interpreted either 1) viral replication was increased or 2) luciferase-readout was increased by unknown reason (noise). Out previous experience with this type of assay suggests that the negative inhibition might be unrelated phenomena.

Screen 2 ; A total of 8960 compounds were screened and the results were depicted in Figure 10. Same phenomena was observed in this experiment as the Screen1.

Comparison of Screen 1 vs. Screen 2; The screen metrics meets the criteria for a succesful high throughput screen; Z' values were above >0.6 for all screen. And the **correlation coefficiency was 0.849** that is considered as medium-high correlation between two runs. However the criteria problem, high cut-off, Mean $\pm$ 3 SD, which is driven from high SD from all screened compounds was repeated in the main screen as we have seen in the pilot screening.

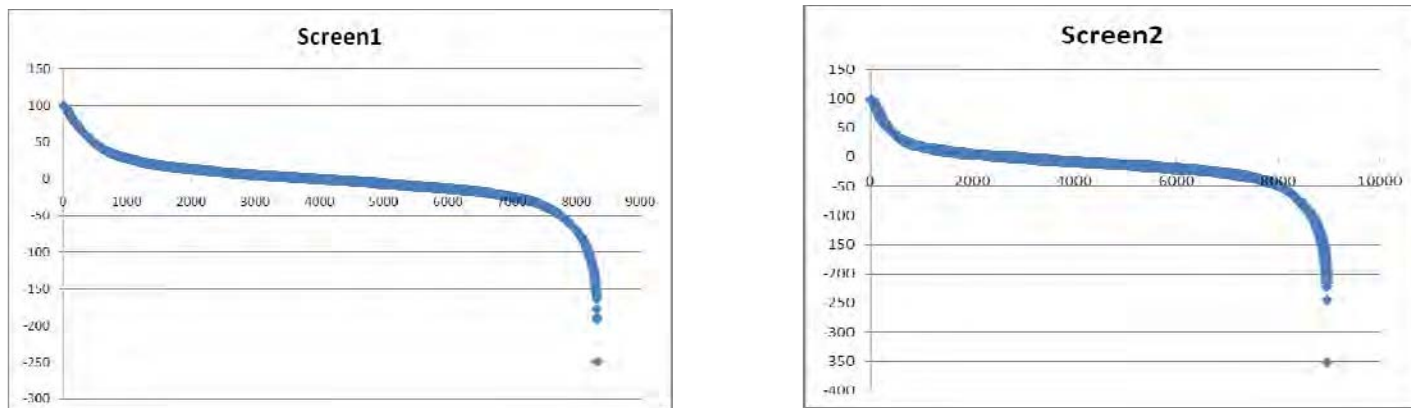


Figure 9. Primary screening of Enamine diversity library for V3526-luc. Screen was done twice (Screen1 and 2) and both runs showed various level of efficacy from the library.

(2) Cytotoxicity

We performed a cytotoxicity test as a coutner screening for the assay. Because the assay is dependent on expression of viral protein synthesized in the host cells, a cytotoxic compound could be selected as a positive (false). Therefore it is vital to screen out the compounds which has high cytotoxicity.

Cytotoxicity test was done by employing a cell viability using Promega's CellTiter-Glo®, which produces a luminescent signal in relation to the quantity of ATP in host cells (directly related to cell viability). In contrast to dye formation or dye-uptake methods, which have a low dynamic range, the CellTiter-Glo® assay shows a higher dynamic range with less background. The test was carried out in the same format, 384-well black plates. The volume of virus suspension was replaced with the same amount of the cell culture media.

We used a 72-hours exposure time for the cytotoxicity test. The Vero 76 cells were seeded in a volume of 15 μ L per well in 384-well plates and then incubated 37°C with 5% CO₂ for 24 hours. Drug-containing media was added to the plate in a volume of 10 μ L with 0.6% of DMSO concentration. 72-hours after compound treatment in the cellsThough reading of the primary screen is done at 24-hours post exposure, cytotoxicity from a compound is not evident within 24 hours after treatment. Hence we have chosen a 72-hours exposure time that is a general procedure for a cytotoxicity test.

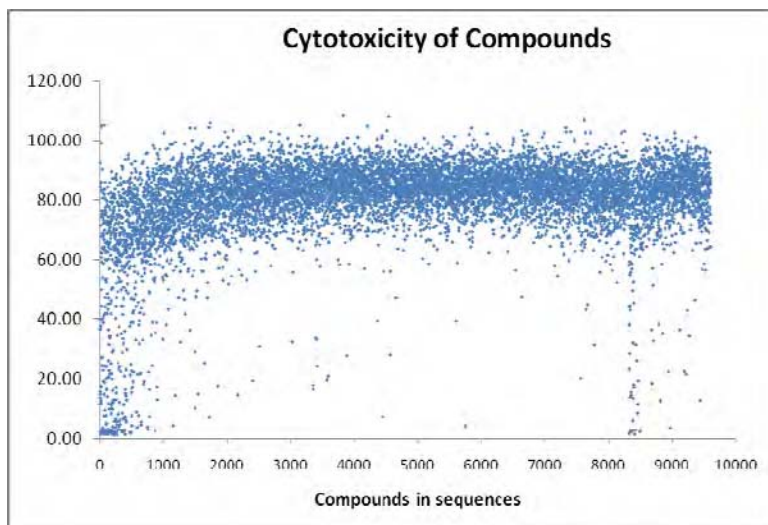


Figure 10. Cytotoxicity profile of the Enamine diversity library. Average cytotoxicity was 80.7% with a standard deviation of 13.7%. Despite of high standard deviation, assay control showed very tight assay performance.

C. Conclusion and Discussion

Selected Hit compounds

Based on the data from efficacy assays (Screening1 and 2) and cytotoxicity study, we finally selected hit compounds for the second round of experiments. The compounds were selected by using criteria based on % activities. As we discussed above, the conventional method to select active compounds from a primary high throughput screen was not helpful for this project. Instead, we set up a criteria to select compounds 1) higher than 50% inhibitory efficacy in two assays (Screen1 and 2) and 2) cytotoxicity less than 15% (viability >85%) in the cytotoxicity. Using this cut-off value, 16 compounds which meet the criteria were selected from the library (Figure 11 and Table 5).

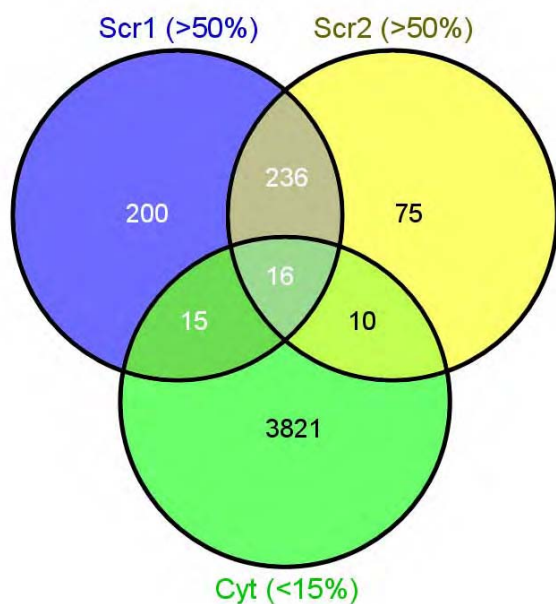


Figure 11. Our cut-off revealed 16 active hit compounds from the library.

Supplier ID	Efficacy% inhibition		Cytotoxicity (% viability)
	Screen1	Screen2	
T0508-6443	55.52	56.49	85.91
T0505-5368	75.34	53.74	87.41
T0507-8041	60.72	72.80	85.15
T0508-6439	58.81	86.32	91.29
T0519-9671	79.91	78.42	85.82
T0520-6049	57.30	64.76	86.32
T5210727	77.21	88.04	89.73
T0400-0402	50.10	63.73	85.98
T5358361	55.37	54.58	93.69
T5405145	91.30	71.89	86.05
T5776969	93.29	95.23	105.26
T5954145	97.86	97.31	104.86
T5971958	97.90	64.56	86.13
T5966139	59.65	56.92	91.56
T5966163	59.13	62.72	90.60
T5932913	70.45	60.65	87.84

Table 5. List of compounds which shows higher than 50% efficacy in two assays and cytotoxicity lower than 15%.

Conclusion : We successfully performed the task assays, the efficacy and cytotoxicity assays in an HTS format. The assay discovered 16 active hits of which the activity exceeds our criteria .

MAJOR TASK 3: SRI WILL CONFIRM THE EFFICACY OF HIT COMPOUNDS

As a result of the primary screening, we have discovered 16 compounds which showed activity in the assay repeatedly with low cytotoxicity. To confirm and validate the hit compounds as Task 3 indicated, we have performed a dose response study with re-supplied compounds.

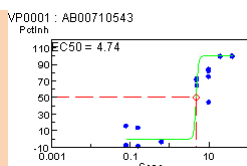
Fifteen compounds were available in powder form from the supplier. The compounds, 5mg each were dissolved in DMSO at a concentration of 20mM. The compounds were serially diluted in DMSO. A 5 µl aliquot of each of the serial dilution in two-fold was transferred to the assay plates along with an equal volume. For each dilution, quadruplicates were used. The effective concentration at which the drug inhibited cell death at 50% (EC50) in the presence or absence of virus and the cytotoxicity of the drug alone at 50% (CC50) were calculated using ActivityBase software (IDBS, Inc, Guildford, UK). CPE inhibition (%) and cell viability were calculated as described in elsewhere¹.

Table 6. Confirmation of ant-V3526-luc activities of selected compounds in a dose response format.

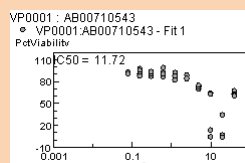
Supplier ID	Efficacy IC50 (uM)	Toxicity CC50 (uM)	SI50	Structure
T0508-6443	11.43	22.6	2.0	

T0505-5368

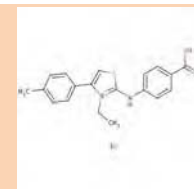
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11.72

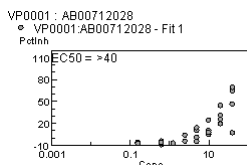


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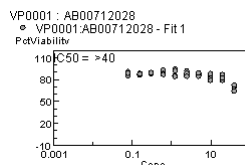


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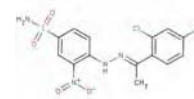
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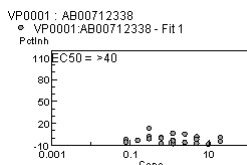


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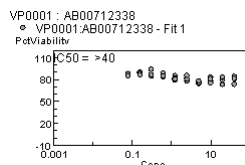


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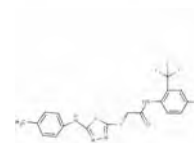
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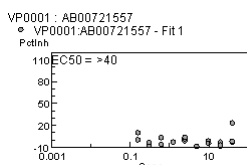


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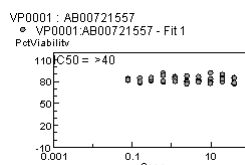


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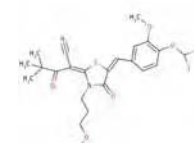
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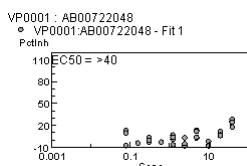


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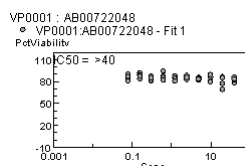


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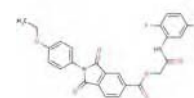
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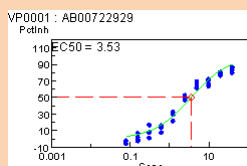


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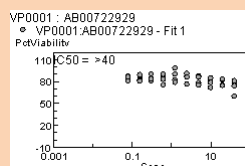


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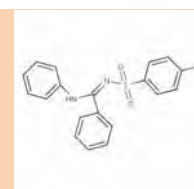
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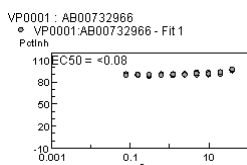


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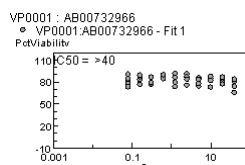


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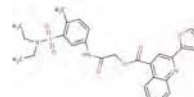
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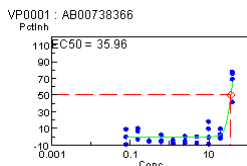


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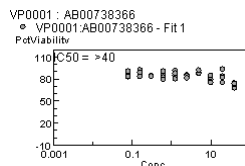


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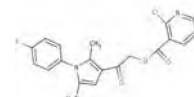
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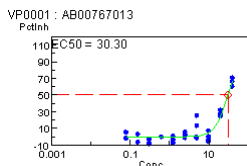


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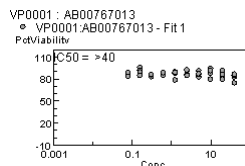


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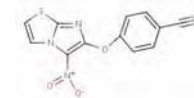
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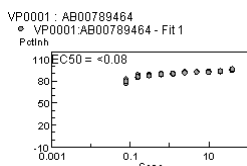


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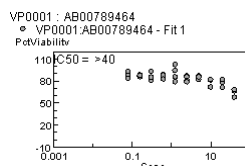


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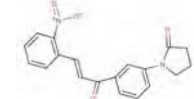
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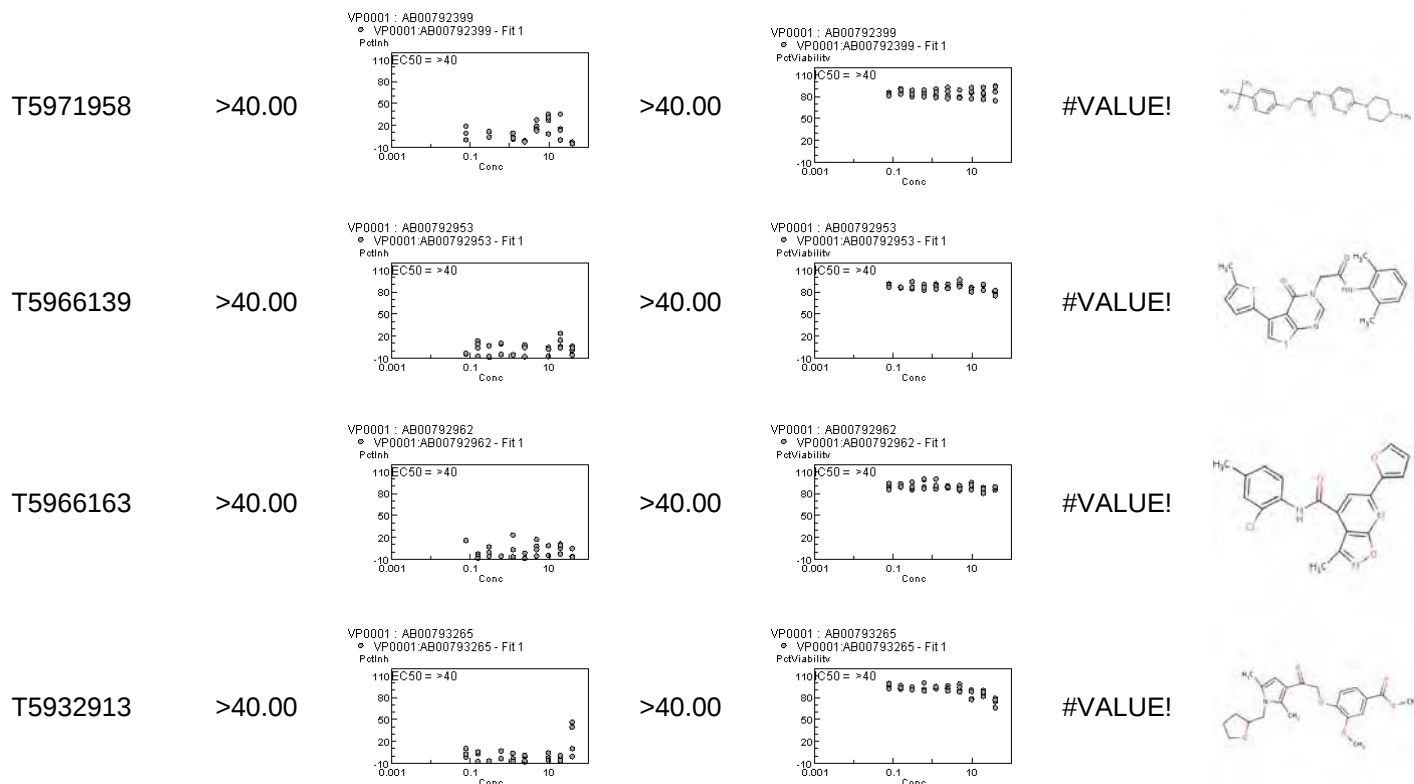


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The dose response experiment discovered that 6 compounds; T0508-6443, T0505-5368, T0507-8041, T0400-0402, T5405145 and T5776969, have Selective Index 50 (CC50/EC50) greater than 1. Among these, three compounds with SI> 2.0 was identified (Table 4 rows in tan).

MAJOR TASK 4: HIT COMPOUND OPTIMIZATION

SRI chemists analyzed hit compounds by cheminformatics and virtual screening/modeling. Commercially available compounds was purchased for structure-activity relationship analyses using the aforementioned approaches.

SAR Analysis and Hit Follow-up

Objectives The major aims of this proposed work are to (a) analyze the activity data from the screening results (b) perform structure- and activity-based clustering of the hits (c) identify scaffolds of promise (d) identify structure-activity relationships (SARs) among the members of the chosen scaffolds, and (e) acquire and assemble small focused set of analogues of the promising scaffolds from in-house collection of compounds or for acquiring from commercial vendors of libraries of compounds.

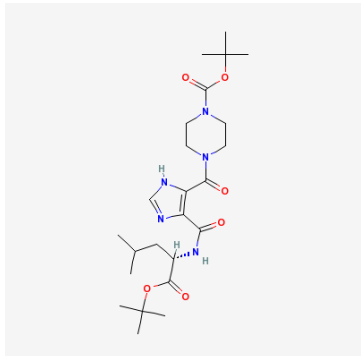
RESULT

1. Hit compounds nomination

A. Arbo virus hits

(1) SMR000372439 and SMR000058373 : Informatics analysis discovered that SMR000372439 and SMR000058373 are “Anti-luciferase” compounds. The assay uses a luciferase as a read-out, therefore compounds inhibiting luciferase activities are expected to be selected. These compounds were discarded in the SAR analysis.

(2) SMR000394098 was selected as a “hit” compound after chemi-informatics analysis.



SMR000394098; $C_{24}H_{39}N_5O_6$ EC50 = 0.55 μ M and CC50 > 50 μ M

B. Enamine Library : SRI chemist nominated Thiazole compounds as hit compounds (T0505-5368; EC50 =4.74 μ M).

2. Analogues by Catalogueing

To explore the chemical space around the hit compounds, SRI chemists analyzed the primary and the secondary results carefully.

- (1) SMR000394098 : We found 9 compounds which are available commercially. (Table 7)
- (2) Thiazole : 34 compounds were secured through commercial sources.

We were able to secure the 39 compounds as in the Table 7 and then the compounds were solublized in DMSO at a concentration of 20 mM.

Table 7. List of chemical analogues of SMR000394098 and Thiazole compounds.

Supplier	Supplier ID	Note	SAR comment
ChemDiv	D340-0900	SAR for VEEV	SMR000394098 analogue
ChemDiv	D340-0930	SAR for VEEV	SMR000394098 analogue
ChemDiv	D340-1533	SAR for VEEV	SMR000394098 analogue
ChemDiv	D340-1545	SAR for VEEV	SMR000394098 analogue
ChemDiv	D340-1556	SAR for VEEV	SMR000394098 analogue
ChemDiv	D340-1557	SAR for VEEV	SMR000394098 analogue
ChemDiv	D340-1560	SAR for VEEV	SMR000394098 analogue
ChemDiv	D340-1561	SAR for VEEV	SMR000394098 analogue
ChemDiv	6712-0929	SAR for VEEV	Thiazole analogue
ChemDiv	6712-1013	SAR for VEEV	Thiazole analogue
ChemDiv	D340-1530	SAR for VEEV	Thiazole analogue
Enamine	T5595019	SAR for VEEV	Thiazole analogue
Enamine	T5211770	SAR for VEEV	Thiazole analogue
Enamine	T5818454	SAR for VEEV	Thiazole analogue
Enamine	T5505960	SAR for VEEV	Thiazole analogue
Enamine	T5427962	SAR for VEEV	Thiazole analogue
Enamine	T5444758	SAR for VEEV	Thiazole analogue
Enamine	T5395939	SAR for VEEV	Thiazole analogue
Enamine	T5249361	SAR for VEEV	Thiazole analogue
Enamine	T5423011	SAR for VEEV	Thiazole analogue
Enamine	T6490641	SAR for VEEV	Thiazole analogue
Enamine	T0520-0720	SAR for VEEV	Thiazole analogue
Enamine	T5395696	SAR for VEEV	Thiazole analogue
Enamine	T5623848	SAR for VEEV	Thiazole analogue
Enamine	T6525773	SAR for VEEV	Thiazole analogue
Enamine	T5416735	SAR for VEEV	Thiazole analogue
Enamine	T5394250	SAR for VEEV	Thiazole analogue
Enamine	T5392891	SAR for VEEV	Thiazole analogue
Enamine	T5588159	SAR for VEEV	Thiazole analogue
Enamine	T5236830	SAR for VEEV	Thiazole analogue
Enamine	T5567934	SAR for VEEV	Thiazole analogue
Enamine	T6134289	SAR for VEEV	Thiazole analogue
Enamine	T0507-3969	SAR for VEEV	Thiazole analogue
Enamine	T5248395	SAR for VEEV	Thiazole analogue
Enamine	T5577306	SAR for VEEV	Thiazole analogue
LifeChemicals	F0866-0344	SAR for VEEV	Thiazole analogue
LifeChemicals	F0298-0167	SAR for VEEV	Thiazole analogue
LifeChemicals	F0174-0087	SAR for VEEV	Thiazole analogue
ChemBridge	8915256	SAR for VEEV	Thiazole analogue

The compounds were supplied to USAMRIID, Dr. Brett Beitzel for testing them for the wild type VEEV and other alphaviruses.

3. Activity confirmation in an alternative format.

Dr. Chung has developed a cell based CPE assay for VEEV using TC-83 strain. The assay, v3526-luc provided from the USAMRIID is to screen inhibitors of early viral replication steps. Therefore, active compounds in the assay are supposed to show antiviral activities for actual virus replication in other format of assay. To complement the original assay, we devised an assay using a vaccine strain, TC-83.

A. Description of an alternate assay.

A CPE-based phenotypic assay has been developed to measure the extent of VEEV replication in Vero 76 cell lines. VEEV infection of vertebrate cells usually results in cell death by apoptosis⁵. The cell death caused by the VEEV infection is evident enough to make a clear plaque followed by the neutral red staining after two days infection of a cell culture (Fig. 12). The assay proposed here employs a CPE caused by an attenuated VEEV strain, TC-83. The strain was attenuated from a wild type VEEV, subtype IAB Trinidad donkey strain (equivalent with V3000) by serial passages in a tissue culture⁶. TC-83 has only 11 genetic mutations compared to the wild type. Seven mutations result in amino acid changes in nsP3, E1 and E2 genes, with the remainder being silent mutations. The proposed HTS assay measures cell viability after a challenge of TC-83 virus in Vero 76 cells grown in 384-well plates. Cell viability will be measured with CellTiter-Glo™ (Promega), which produces measurable luminescence that is proportional to cell viability. CellTiter-Glo™ measures the amount of adenosine tri-phosphate (ATP) inside cells; the method has been verified for HTS assays with many CPE-causing viruses, including West Nile virus (WNV)⁷ (PubChem AID: 1635), respiratory syncytial virus (PubChem AID: 2440), influenza viruses⁸ or dengue virus (PubChem AID: 1251).

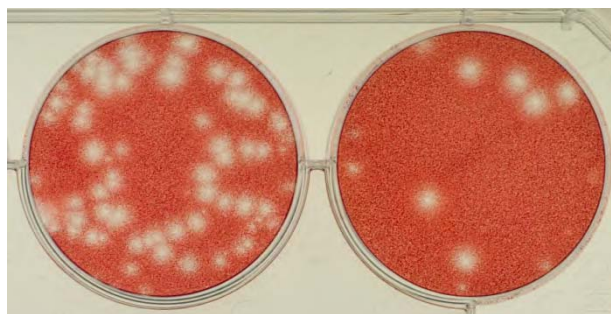


Figure 12. VEEV, strain TC-83, induces viral CPE detected by vital staining. Dead cells from viral replication are present in the clear zone while uninfected cells are stained with neutral red. (Dr. Chung, preliminary experiment)

B. Description of the assay conditions.

Cell and Virus. Vero 76 cells (ATCC No. CRL-1587™, derived from African green monkey epithelial cells) were cultivated in a complete cell culture media; minimum essential media with Eagle salt (MEM-E) with 10% of fetal bovine serum (FBS) and 2 mM of glutamic acid as a supplement in a 37 °C incubator with 5% CO₂. Cells were passed every 3 or 4 days by detaching the cells with a 0.05% Trypsin-ethylenediaminetetraacetic acid (EDTA) solution. The TC-83 virus was obtained from the USAMRIID and amplified in BHK-21 [C-13] cell lines cultured in T-150 cell culture flasks at the PI's lab. The titer of the amplified stock was determined by TCID₅₀ methods employing 384-well plate. It was found to be 1.1×10^{10} TCID₅₀ per mL.

Optimization. The following key assay conditions have been optimized that can affect the robustness of the assay during the primary screening.

Optimal concentration of virus. Various amounts of virus per well was tested to obtain optimized results. In our preliminary experiment, the amount of virus was tested between 1 and 5,000 TCID₅₀ per well. The results showed a narrower range of the virus amount between 100 and 2,000 TCID₅₀ per well. It was tested further to find an optimal concentration of the virus within the range. The 384-well cell culture plates infected with different amount of virus were developed 3 days post infection. The Z' indicated that the assay was so robust that any amount within the range gives Z' above 0.6. It was determined that 500 TCID₅₀ per well is the optimized virus amount for this assay.

Incubation time. Incubation time was also optimized. Two identical plates were set with half the plates area prepared for cells only and the other half for infection with a 500 TCID₅₀ of TC-83 per well. Each plate was developed on days 2 and 3 (Fig 14). While variation among wells was high on day 2 (Z' = 0.42), results on day 3 showed lower a variation with a higher Z' value of 0.634 in the experiment. It was concluded that the incubation time for the assay is three days post infection.

Cell conditions. Cell concentration was titrated and it was found that 4,500 cells per well for a 384-well plate permitted a maximum number of plates per preparatory flask without compromising assay performance (data not shown). The maximum-allowable passage number for the assay was not tested. However the preliminary assays have

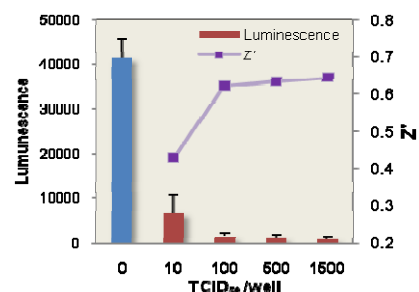


Figure 13. Optimization for virus amount per well

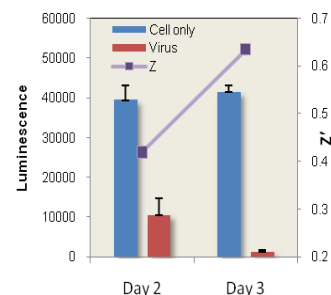


Figure 14. Optimization for incubation time

performed well between passages 33 and 50.

Dimethylsulphoxide (DMSO) tolerance. The effect of DMSO in the assay system was tested within a range of 0.1 to 1%. One day post cell seeding, 5 µL of media containing various amounts of DMSO were added to the plate. Diluted TC-83 virus at a concentration of a 500 TCID₅₀ per 15 µL was then added to the wells and the plates were incubated for 3 days. The experiment revealed that cell viability in cells only and virus-infected wells were decreased as the DMSO concentration increased. However, the effect was significant only above 0.5% (Fig 15). Therefore these data suggest that the final DMSO for the assay should be kept at less than 0.5% in final.

Assay plate format. The assay has been developed in 384-well, flat clear bottom, black polystyrene microplate (e.g., Corning, Cat No. 3712).

Description of the assay performance

Assay evaluation. The assay performance has been examined to determine whether the assay is adequate for HTS. Triplicate assay sets of 384-well plates were run on two independent days and the results are shown below (Table 8). The assay metrics meet the requirement for a successful HTS assay for a library screening. The assay performance was ascertained by testing plate-to-plate variation (data not shown).

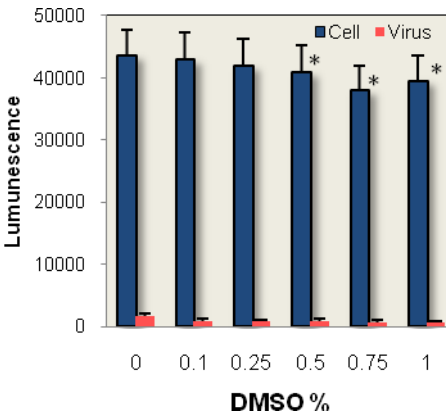


Figure 15. DMSO tolerance in the HTS assay. * indicate $p < 0.05$ (Student's t test)

Table 8. Assay performance of the assay using TC-83 CPE.

Assay metrics	Z'	S/B	S/N	CV
Results	0.59 ± 0.03	12.75 ± 1.0	9.60 ± 0.24	Cell control: 1.16% Virus control: 7.2%

Dose dependent response of known agents, mycophenolic acid (MPA). To my knowledge, there is no commercially available antiviral drug for VEEV. Using our assay format, MPA was evaluated in a dose-response manner, which is a broad spectrum antiviral drug reducing the amount of guanosine-5'- tri-phosphate (GTP) in host cells through inosine-5'- monophosphate dehydrogenase (IMPDH) inhibition. MPA has been used as a positive control compound for anti-WNV screening assay, (PubChem AID: 1635), when effective antivirals were available. A 10-point dose-response in concentrations was used that were two-fold serially diluted from 50 µM and ended at 0.19 µM. Serial dilution was performed in DMSO first and added to cell culture media to make a 6 X final concentration (3% in DMSO concentration). The diluted compounds were then added into cell plates to make the final concentrations indicated in Figure 6. The data in Figure 16 demonstrate that MPA showed a good dose response in efficacy (with infection) up to 6.5 µM. However, the results showed a decrease in cell viability above 12.5 µM. It is believed that the adverse effect at higher concentrations is due to cytotoxicity. Nevertheless, the concentration of 5 µM MPA was chosen, which inhibits VEEV replication by ~ 50% without compromising cell viability in the assay. Interestingly the concentration is the same as used for anti-WNV HTS. Considering that the cell line for the WNV assay (Vero E6) is very close with Vero76 and the mechanism of action of MPA is based on inhibition of cell metabolism, the

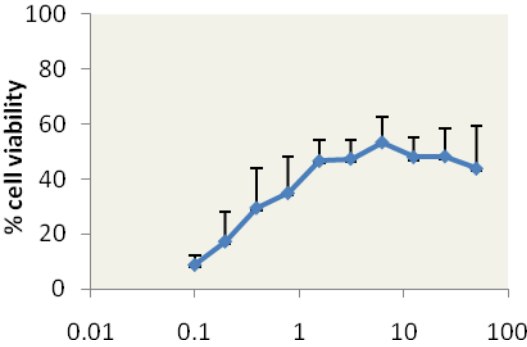


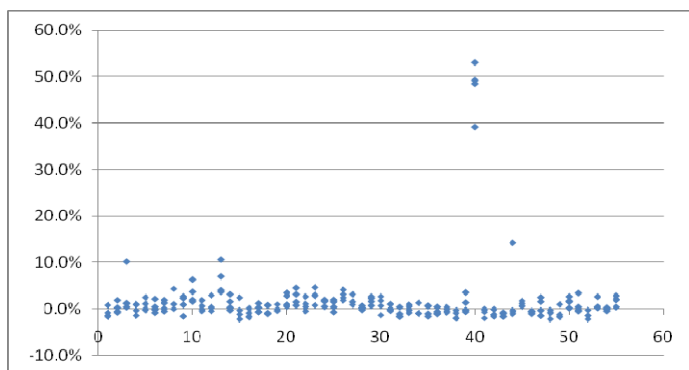
Figure 16. A dose dependent response of MPA in anti-VEEV assay.

antiviral activity of MPA against VEEV implies that the CPE-based anti-VEEV assay developed and proposed is robust enough to screen possible hits from a library.

C. Activities of compounds in the CPE based assay

We tested the purchased compounds in the CPE based TC-83 assay to confirm anti-VEEV activities. The compounds are consisted of 1) 15 compounds selected for the dose response study from the primary screening, 2) 39 compounds purchased by chemical catalogueing approached and 3) MPA, a positive control. The compounds were treated in the cells grown in 384-well plates and then the cells were challenged with virus. Three days later the cells were developed by CellTiter-Glo™ to measure a cell viability which is inversely proportional to the extent of virus replication. For a single dose experiment, was a 12.5 uM of concentration used. DMSO was kept at 0.5% for the assay.

A single dose experiment at showed that only MPA, a positive control compound, has measurable anti TC-83 activity. Unfortunately none of chemical compounds showed anti-VEEV activities in this assay (Figure 17).



To verify the results from the single dose experiment, we also performed a dose response assays treatment concentration range between 100 μ M and 0.2 μ M in the same assay format. As congruent with the single dose experiment, we did not find any anti-VEEV activities in this assay with the selected compounds.

Table 9. A list and result of compounds tested in CPE based VEEV assay using TC-83 strain.

Supplier ID	Supplier	SAR comment	EC50 in CPE assay (uM)	
D340-0900	ChemDiv	SMR000394098 analogues	>100	
D340-0930			>100	
D340-1533			>100	
D340-1545			>100	
D340-1556			>100	
D340-1557			>100	
D340-1560			>100	
D340-1561			>100	
6712-0929			Thiazole analogues	>100
6712-1013				>100
D340-1530	>100			
T5595019	>100			
T5211770	>100			
T5818454	>100			
T5505960	>100			
T5427962	>100			
T5444758	>100			
T5395939	>100			
T5249361	>100			
T5423011	>100			
T6490641	Enamine	>100		
T0520-0720		>100		
T5395696		>100		
T5623848		>100		
T6525773		>100		
T5416735		>100		
T5394250		>100		
T5392891		>100		
T5588159		>100		
T5236830		>100		
T5567934	>100			
T6134289	>100			
T0507-3969	LifeChemicals	>100		
T5248395		>100		
T5577306		>100		
F0866-0344			>100	

F0298-0167			>100
F0174-0087			>100
8915256			>100
380-015-G001	ChemBridge	MPA, Positive control	11.2
T0400-0402	Alexis		>100
T0505-5368	Enamine	Enamine Hits for V3526-luc	>100
T0507-8041			>100
T0508-6439			>100
T0508-6443			>100
T0519-9671			>100
T0520-6049			>100
T5358361			>100
T5405145			>100
T5776969			>100
T5932913			>100
T5954145			>100
T5966139			>100
T5966163			>100
T5971958			>100

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- An HTS using v3625-luc was successfully developed and validated.
- 10,000 compounds from a chemically diverse and defined library were screened with the HTS
- 56 compounds from a pre-screened library (Arbo) and 16 compounds from a diverse library were selected from the primary screen.
- Secondary screening effort elucidated 18 compounds which showed a dose response in the assay with low cytotoxicity.
- Chemi-informatic approach identified two potential hits for the assay.
- A total of 39 compounds around the hit compounds were tested in an SAR effort.
- A cell based CPE assay employing TC-83 was developed as a part of confirmation assay.
- Unfortunately, none of the 39 analogues and 15 original hits compound showed anti-viral activity in TC-83 assay.
- Activities for a titer reduction assay with a wild type virus, V3000 is in progress in the USAMRIID.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- Activity database : All results were managed and achieved in a database integrated by ActivityBase™, Microsoft Access (Microsoft) and Instant JChem (ChemAxon).
- A grant proposal : Dr. Chung has submitted an R3 grant proposal to NIH. The grant was solicited by the MLPCN seeking for an HTS-ready assay. The assay employing TC-83 with its CPE was submitted in 2010 May.
- A manuscripts to report the outcomes and procedure is being prepared.

CONCLUSION:

This proposed research is relevant to two of the listed gaps in the FY08 solicitation: development and proof-of-concept evaluation of novel therapeutic technologies against viral threat agents of interest, and the development of multi-agent therapeutics. This proposal would address the first gap by identifying lead compounds that have antiviral efficacy against VEEV, and that could form the basis for the design of drugs that inhibit VEEV. This proposal addresses the second gap by testing the compounds identified in the original VEEV screen against EEEV and WEEV. VEEV, EEEV, and WEEV are closely related, so it is likely that compounds active against one virus may also be active against the others.

Through the tasks we performed for the project, two key compound motives were identified. The activities of two compounds in the V3526-luc were verified. However an alternative assay to measure anti-VEEV activity in a CPE read-out did not confirm the activities. We hypothesize that difference in genotypes and/or in the mechanism of early replication requiring NS proteins may result in the difference in activities. Therefore activity confirmation with other types of virus such

as V3000 in a titer reduction assay (plaqueing or TCID50) would assess a usefulness of of the selected chemical scaffolds in a therapeutic measures for anti-VEEV or other alphaviruses.

1. Severson, W.E., N. Shindo, M. Sosa, T. Fletcher, 3rd, E.L. White, S. Ananthan, and C.B. Jonsson, *Development and validation of a high-throughput screen for inhibitors of SARS CoV and its application in screening of a 100,000-compound library*. J Biomol Screen, 2007. **12**(1): p. 33-40.