

Contract NO1-AI-05415

**Specialized *In Vitro* Virological Evaluations
Of Strategies To Combat HIV/AIDS**

**Report on Special Toxicity Assays Performed for
NIAID 11039**

Submitted: June 21, 2005

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Summary of Results

Table 1. Results of Toxicity Assays Performed on MAGI-R5 Cells

Compound	MTS TC₅₀	LIVE/DEAD TC₅₀	Vybrant TC₅₀	Comments
Triton X-100 (%)	0.02	0.02	0.006	Toxic Control Compound
NIAID 11039 (μM)	>100	>100	>100	Not Toxic

Summary

1. NIAID compound 11039 was received January 10, 2005 as a powder, solubilized in DMSO at 10mM, and stored at -80°C until tested in the standard panel of topical microbicide evaluation assays supplied by the NIAID under contract N01-A1-05415 (*Specialized In Vitro Virological Evaluations Of Strategies To Combat HIV/AIDS*). The compound is light brown and not very soluble. It was mixed well before each use.
2. For the toxicity assays, the compound was tested under the same conditions as the standard R5 attachment assay with the exception that no virus was added. Thus, the plates were incubated at 37°C for 3 hours after drug addition and then washed extensively after the incubation. The MTS dye reduction assay is the standard toxicity endpoint for the R5 attachment assay. The LIVE/DEAD cytotoxicity endpoint and the Vybrant cytotoxicity assays were included to provide alternate mechanisms for detection of cytotoxicity.
3. Triton X-100 was used as a positive toxicity control and therefore demonstrated toxicity in all three of the cytotoxicity assays with reproducible TC₅₀ values between assays.
4. NIAID 11039 demonstrated no toxicity in the 3 cytotoxicity assays.

Results

The individual graphical results for the evaluations of NIAID 11039 for toxicity in the 3 cytotoxicity assays appear in the Appendix. The summary table for the data appears at the beginning of this report. Each data sheet uses the same general format of one table representing the individual values for each well of the triplicate determinations and a graphical representation of the data. For all evaluations, the table represents the cell viability as determined by either MTS dye reduction (OD 490/650), LIVE/DEAD cytotoxicity (Fluorescence Intensity), or Vybrant cytotoxicity (Fluorescence Intensity) in replicate plates. The TC_{50} is used to determine relative toxicity of the compounds. The graphical representation presents the compound toxicity (%CC) expressed as a percent of the cell control for each concentration tested.

The assays performed met internal validation and standardization criteria for performance of each of the assays. These criteria include triplicate variability, total fluorescence intensity produced in the LIVE/DEAD and Vybrant assays, OD readings in the MTS dye reduction assay, and the toxicity of the control compounds. Thus, the assays performed meet specific criteria set for these parameters and represent an experimentally valid result.

Methods

Description of the HIV-1 CCR5-tropic Attachment Assay for Cytotoxicity

Twenty-four hours prior to initiation of the assay, the cells were plated to triplicate 96-well-flat bottom plates (1 clear plate and 2 black plates). On the day of the assay, working solutions were prepared for each compound. Then, 50 μ L of media was removed from the wells on all plates where drug was to be added and 50 μ L of a 2x solution of diluted compounds was added in triplicate to the 50 μ L of media remaining in these wells. Wells not containing drug were designated as the cell control. The plates were incubated for 3 hours at 37°C. At the end of incubation, the wells were washed extensively by removing all media from all wells and adding 200 μ L of fresh DMEM media (without phenol red). Then, all media was removed a 2nd time and 150 μ L of fresh DMEM media (without phenol red) was added. All media was removed a 3rd and final time, and 200 μ L of fresh DMEM complete media (1X DMEM media with final concentrations of 10% FBS, 100 μ g/mL Pen/Strep, 0.3mg/mL L-glutamine, and 1x Nonessential Amino Acids) was added and incubation continued for 48 hours. After 48 hours, the media was removed from all wells and toxicity was determined using the MTS Cytotoxicity assay (CellTiter 96 Reagent, Promega), the LIVE/DEAD Viability/Cytotoxicity assay (L-3224, Molecular Probes), and the Vybrant Cytotoxicity assay (V-23111, Molecular Probes).

LIVE/DEAD[®] Cytotoxicity Assay

The LIVE/DEAD cytotoxicity assay provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. Preparation of Reagent Solutions: LIVE/DEAD reagent stock solutions were allowed to warm to room temperature before use. The appropriate volume of the supplied 2mM EthD-1 stock solution was added (Component B) to sterile, tissue culture-grade D-PBS, and vortexed to ensure thorough mixing, to yield a 5 μ M EthD-1 solution. The appropriate volume of the supplied 4mM calcein AM solution in DMSO (Component A) was transferred to the 5 μ M EthD-1 solution and vortexed to ensure thorough mixing. The final working solution of components was 2.5 μ M calcein AM and 5 μ M EthD-1. Assay Procedure: All media was removed from the wells and

100µL of fresh DMEM complete media (without phenol red) was added to each well followed by the addition of 100µL of the LIVE/DEAD working solution to provide final concentrations of 1.25µM calcein AM and 2.5µM EthD-1. The plates were incubated at room temperature for 30-45 minutes. Fluorescence intensity was measured in the wells using the appropriate excitation and emission filters: Calcein excitation/emission: Fluorescein 485 nm/Fluorescein 530nm; EthD-1 excitation/emission: Rhodamine B/Alamar 535-05/Europium 620nm

Vybrant™ Cytotoxicity Assay

The Vybrant Cytotoxicity kit provides an alternative method that monitors the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) from damaged cells into the surrounding medium. Preparation of Stock Solutions: Vybrant reagents were allowed to warm to room temperature. A 4mM stock solution of resazurin was prepared by dissolving the contents of the vial (Component A) in 75µL of DMSO (Component B). A 1X reaction buffer was prepared by diluting 2mL of the 5X reaction buffer (Component D) fivefold in 8mL of deionized water. The reaction mixture solution was prepared by dissolving the contents of one vial of the lyophilized reaction mixture (Component C) in 400µL of 1X reaction buffer. Then, the components were mixed gently without vortexing. A 2X resazurin/reaction mixture was made by combining 75µL of the 4mM resazurin stock solution, 400µL of the reaction mixture solution, and 9.52mL of the 1X reaction buffer. The components were mixed gently without vortexing. Assay Procedure: All media was removed from the wells and 50µL of fresh DMEM complete media (without phenol red) was added to each well followed by the addition of 50µL of the 2X resazurin/reaction mixture to each well. Then, 1µL of 100X cell-lysis buffer (Component E) was added to the lysed-cell control wells ONLY. The plates were incubated at 37°C for 30 minutes. Fluorescence intensity was measured in the wells using the appropriate excitation and emission filters: excitation/emission: Rhodamine B/Alamar 535-05/ Rhodamine B/Alamar 580-10.

MTS (Cell Titer) Assay

The MTS (Cell Titer) Assay contains an MTS tetrazolium compound that is bio-reduced by cells into a colored formazan product that is soluble in tissue culture medium. Assay Procedure: All media was removed from the wells and 100 μ L of fresh DMEM complete media (without phenol red) was added followed by the addition of 15 μ L of Cell Titer. The plates were then incubated at 37°C for 1 ½ to 2 hours. After the incubation, the OD readings at 490nm and 650nm were taken.

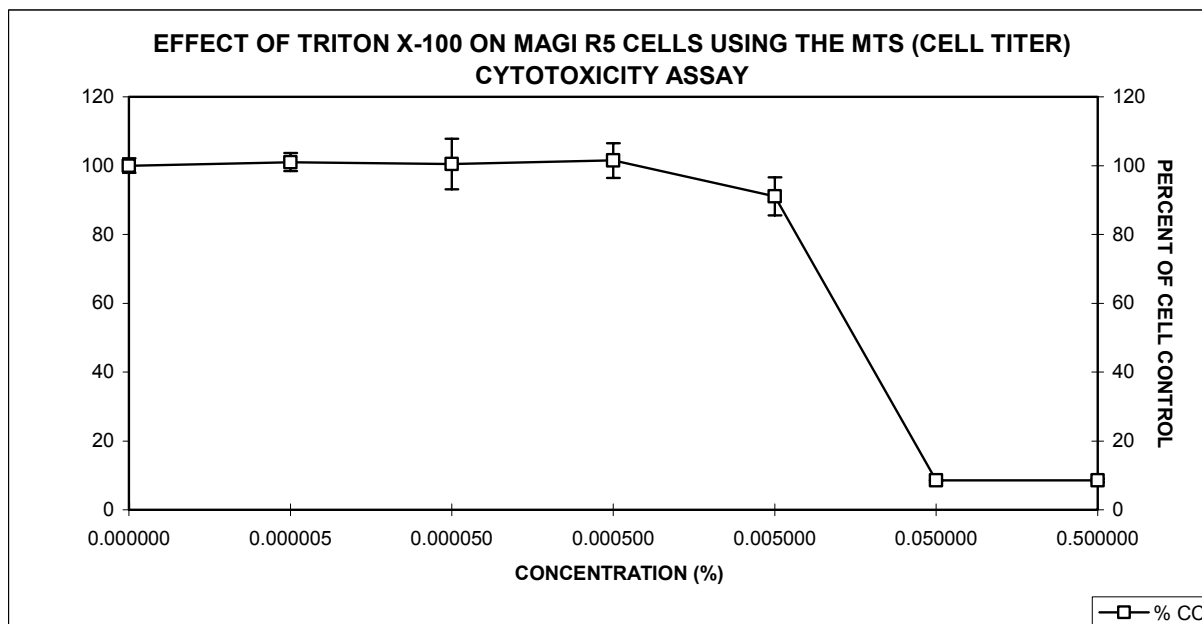
APPENDIX I

Results of Toxicity Assays Performed on MAGI-R5 Cells

EFFECT OF TRITON X-100 ON MAGI R5 CELLS USING THE MTS (CELL TITER) CYTOTOXICITY ASSAY

TOXICITY VALUES (CellTiter96 - O. D. @ 490/650 nm)							
CONC (%)	0.00	0.000005	0.00005	0.0005	0.005	0.05	0.5
SAMPLE 1	0.967	0.999	1.055	1.047	0.849	0.083	0.082
SAMPLE 2	0.962	1.003	0.912	0.969	0.950	0.084	0.080
SAMPLE 3	1.000	0.957	0.976	0.957	0.869	0.084	0.088
MEAN	0.976	0.986	0.981	0.991	0.889	0.084	0.083
% CC	100.0	101.0	100.5	101.5	91.1	8.6	8.5
STD DEV	2.1	2.6	7.3	5.0	5.5	0.1	0.5

TC50 (%) = 0.02



June 1, 2005
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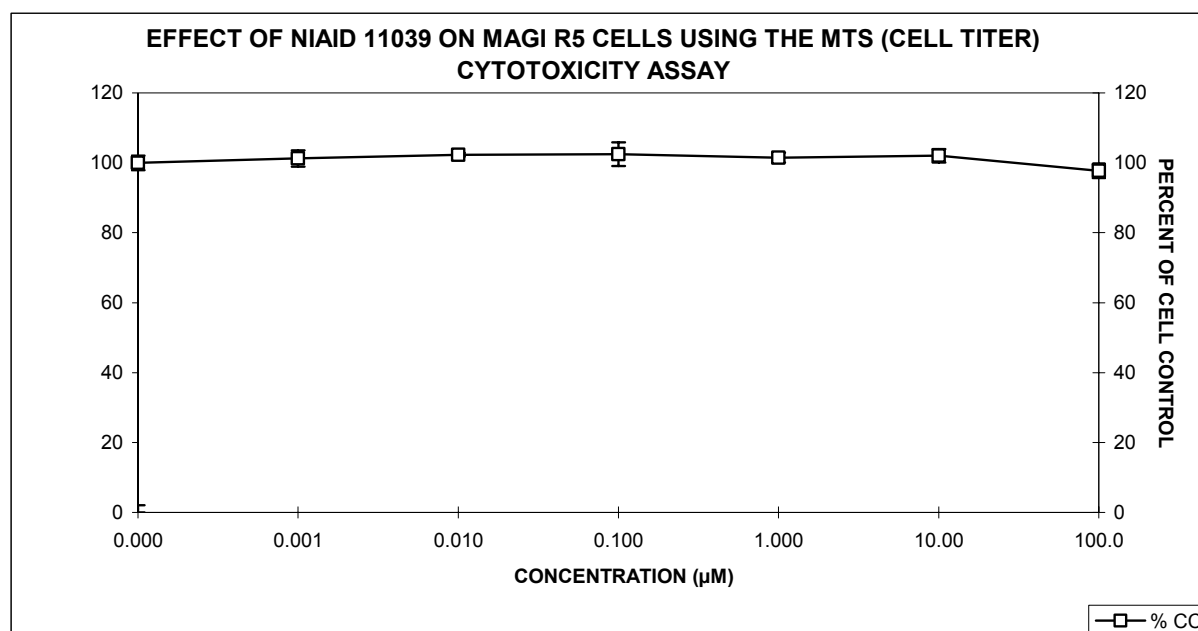
Cytotoxicity Assay in MAGI R5 cells
with 3 hour exposure and 48 hour endpoint

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EFFECT OF NIAID 11039 ON MAGI R5 CELLS USING THE MTS (CELL TITER) CYTOTOXICITY ASSAY

TOXICITY VALUES (CellTiter96 - O. D. @ 490/650 nm)							
CONC (μM)	0.00	0.001	0.010	0.1	1.0	10.0	100.0
SAMPLE 1	0.967	0.987	1.000	1.020	0.989	1.010	0.976
SAMPLE 2	0.962	0.967	0.993	0.963	0.978	0.975	0.940
SAMPLE 3	1.000	1.013	1.003	1.020	1.005	1.004	0.946
MEAN	0.976	0.989	0.999	1.001	0.991	0.996	0.954
% CC	100.0	101.3	102.3	102.5	101.5	102.1	97.7
STD DEV	2.1	2.4	0.5	3.4	1.4	1.9	2.0

TC50 (μM) = >100.0



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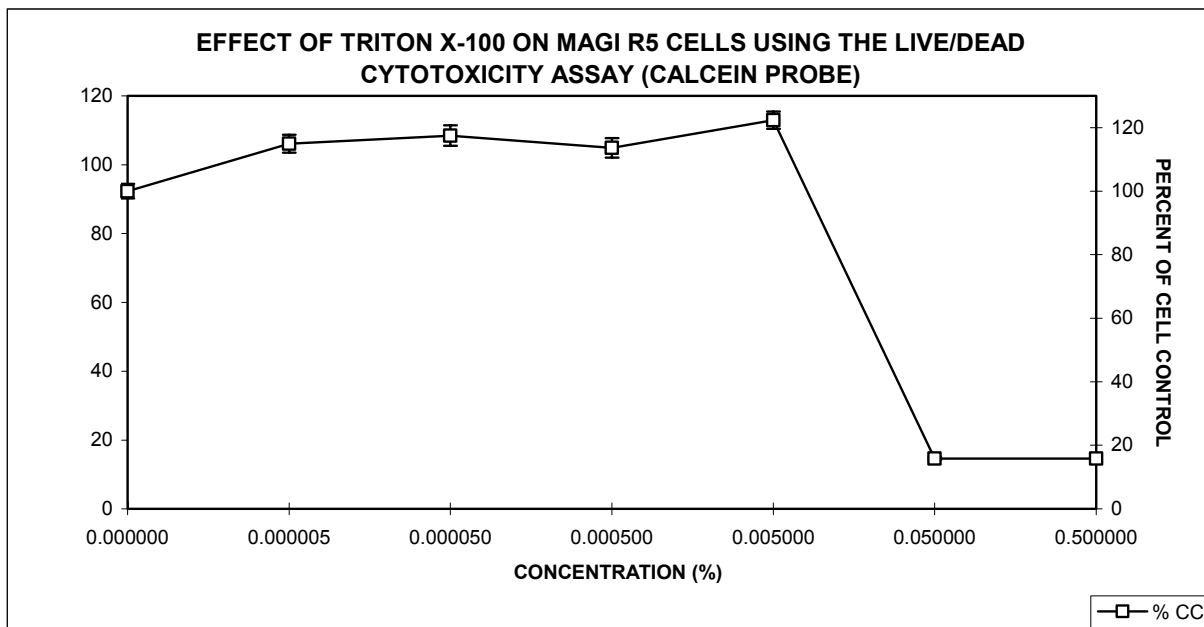
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EFFECT OF TRITON X-100 ON MAGI R5 CELLS USING THE LIVE/DEAD CYTOTOXICITY ASSAY (CALCEIN PROBE)

TOXICITY VALUES (CellTiter96 - O. D. @ 490/650 nm)							
CONC (%)	0.00	0.000005	0.00005	0.0005	0.005	0.05	0.5
SAMPLE 1	318748.4	361175.0	353605.0	363730.0	370353.0	48257.0	49109.0
SAMPLE 2	308126.8	363162.0	369312.0	350577.0	385020.0	50433.0	49866.0
SAMPLE 3	305299.9	347076.0	372151.0	344900.0	384925.0	48919.0	48636.0
MEAN	310725.0	357137.7	365022.7	353069.0	380099.3	49203.0	49203.7
% CC	100.0	114.9	117.5	113.6	122.3	15.8	15.8
STD DEV	2.3	2.8	3.2	3.1	2.7	0.4	0.2

TC50 (%) = 0.02



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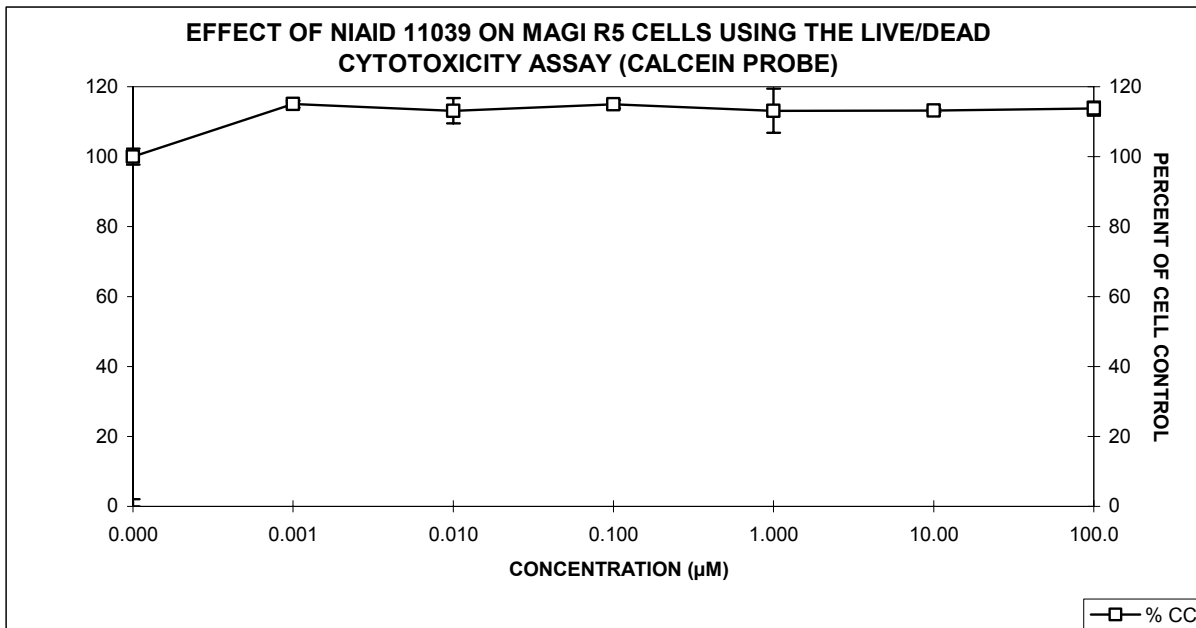
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**EFFECT OF NIAID 11039 ON MAGI R5 CELLS
USING THE LIVE/DEAD CYTOTOXICITY ASSAY
(CALCEIN PROBE)**

TOXICITY VALUES (CellTiter96 - O. D. @ 490/650 nm)							
CONC (μ M)	0.00	0.001	0.010	0.1	1.0	10.0	100.0
SAMPLE 1	318748.4	360229.0	343764.0	353226.0	356254.0	351334.0	359755.0
SAMPLE 2	308126.8	354078.0	364392.0	358336.0	368461.0	350482.0	353132.0
SAMPLE 3	305299.9	358052.0	346603.0	360229.0	330044.0	353605.0	347454.0
MEAN	310725.0	357453.0	351586.3	357263.7	351586.3	351807.0	353447.0
% CC	100.0	115.0	113.2	115.0	113.2	113.2	113.7
STD DEV	2.3	1.0	3.6	1.2	6.3	0.5	2.0

TC50 (μ M) = >100.0



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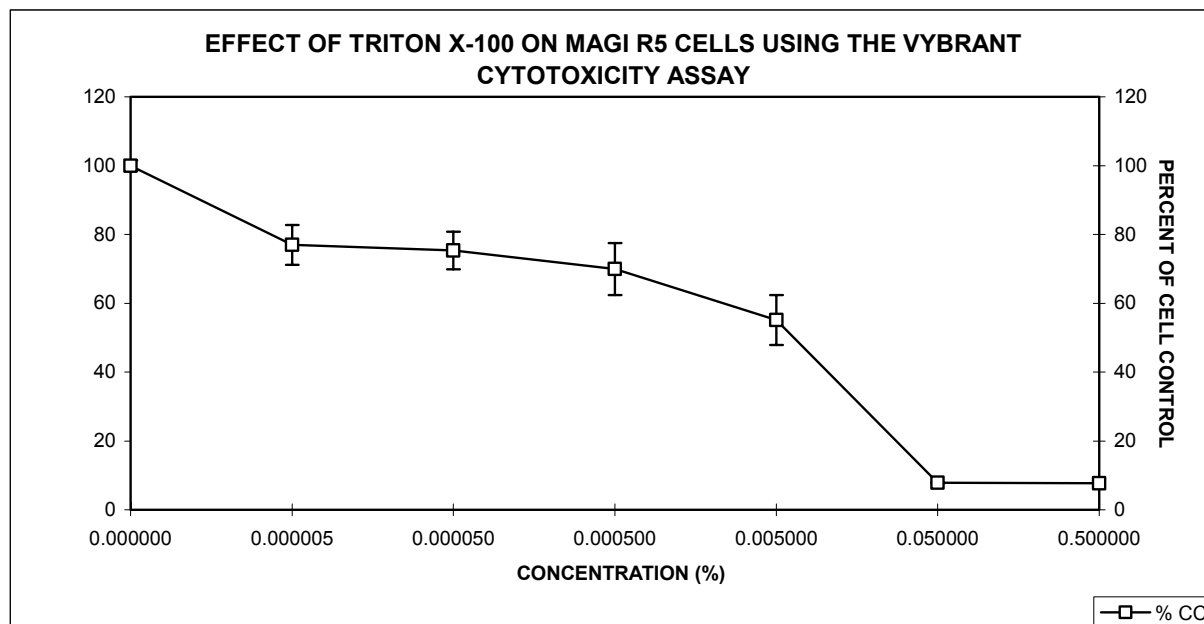
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EFFECT OF TRITON X-100 ON MAGI R5 CELLS USING THE VYBRANT CYTOTOXICITY ASSAY

TOXICITY VALUES (CellTiter96 - O. D. @ 490/650 nm)							
CONC (%)	0.00	0.000005	0.00005	0.0005	0.005	0.05	0.5
SAMPLE 1	1357121.1	1043878.0	952851.0	856147.0	699641.0	106261.0	106261.0
SAMPLE 2	1401129.4	1148531.0	1088351.0	973952.0	874977.0	109005.0	105315.0
SAMPLE 3	1377513.3	990795.0	1074347.0	1063938.0	703709.0	108153.0	104652.0
MEAN	1378587.9	1061068.0	1038516.3	964679.0	759442.3	107806.3	105409.3
% CC	100.0	77.0	75.3	70.0	55.1	7.8	7.6
STD DEV	1.6	5.8	5.4	7.6	7.3	0.1	0.1

TC50 (%) = 0.006



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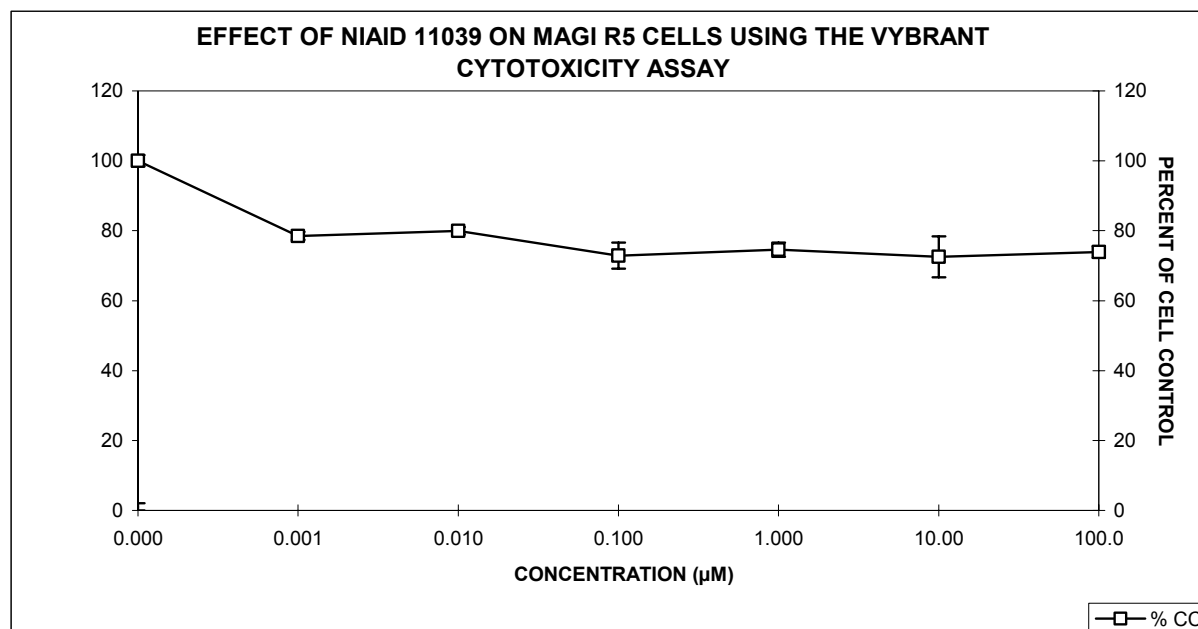
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EFFECT OF NIAID 11039 ON MAGI R5 CELLS USING THE VYBRANT CYTOTOXICITY ASSAY

TOXICITY VALUES (CellTiter96 - O. D. @ 490/650 nm)							
CONC (μM)	0.00	0.001	0.010	0.1	1.0	10.0	100.0
SAMPLE 1	1357121.1	1091095.0	1113142.0	1018141.0	1003758.0	1065736.0	1029779.0
SAMPLE 2	1401129.4	1064033.0	1085512.0	947931.0	1022872.0	909419.0	1032902.0
SAMPLE 3	1377513.3	1092798.0	1109263.0	1047663.0	1059302.0	1024291.0	993728.0
MEAN	1378587.9	1082642.0	1102639.0	1004578.3	1028644.0	999815.3	1018803.0
% CC	100.0	78.5	80.0	72.9	74.6	72.5	73.9
STD DEV	1.6	1.2	1.1	3.7	2.0	5.9	1.6

TC50 (μM) = >100.0



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