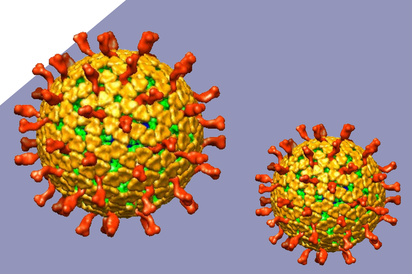
The Addition of Arachidin 1 or Arachidin 3 to Human Rotavirus-infected Cells Inhibits Viral Replication and Alters the Apoptotic Cell Death Pathway

Macie N. Mattila[1], Caleb M. Witcher[1], Rebekah Napier-Jameson[1], Hannah N. Lockwood[1], Josephine Taylor[1], Beatrice A. Clack[1], Judith M. Ball[2], Fabricio Medina Bolivar[3], Rebecca D. Parr[1*]

1) Department of Biology, Stephen F. Austin State University, Nacogdoches, TX 75962. 2) Department of Pathobiology, Texas A&M University Commerce, Commerce, TX 75428.
3) Department of Biological Sciences and Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR 72401. *email: parrr1@sfasu.edu

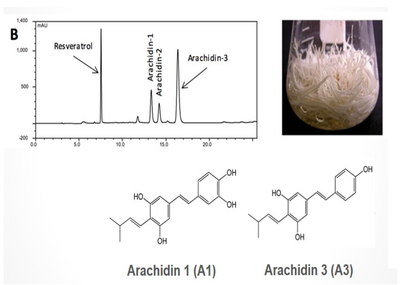


Jayaram, H. 2004. *Virus Research* 101: 67-81.

Introduction

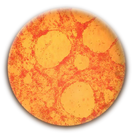
Rotavirus (RV) infections are a leading cause of severe gastroenteritis in infants and children under the age of five. There are two vaccines available in the United States and one in India that can be administered early in childhood, however they only protect against specific strains[1]. From our previous work, both arachidin-1 (A1) and arachidin-3 (A3) from peanut (*Arachis hypogaea*) hairy root cultures significantly inhibit simian RV replication[2,3,4]. The purpose of this study was to determine if a human intestinal cell line, HT29.f8, infected with a human RV, Wa, was affected by A1 and A3. Cell viability assays were utilized to determine if A1 and A3 affect the HT29.f8 cells with/without RV infections. At eighteen hours post infection (hpi), supernatants from the RV-infected HT29.f8 cells with/without the arachidins were used in plaque forming assays to quantify and compare the amount of infectious RV particles that are produced during an infection. Transmission electron microscopy (TEM) was used to visualize cell ultrastructure and individual RV particles. Additionally, tunable resistive pulse sensing technology (TRPS) using the qNano system by IZON was employed to quantify and measure virus particle sizes, and display the size distribution of RV particles. Likewise, quantitative real time polymerase chain reactions (qRT-PCR) were performed to determine if A1 and A3 regulated cell death pathways in the HT29.f8 cell line. This data will guide our future studies to determine the antiviral mechanism(s) of action of A1 and A3.

Materials and Methods



1. **Uninfected + no treatment**
2. **RV (Wa) + Worthington Trypsin (1µg/ml)**
3. **RV (Wa) + Worthington Trypsin (1µg/ml) + 20µM A1/3**
4. **Uninfected + 20µM A1/3**

Plaque Forming Unit Assays (PFU/ml)



RV plaques in HT29.f8 cells

- Incubate log-fold dilutions of infected cell supernatants with/without Arachidins on MA104 cells
- Add medium and Agarose
- Stain cells with neutral red and count plaques

Transmission Electron Microscopy (TEM)

- **Cells were fixed in a resin mold**
- **Ultra-microtome**
- **Load to grid**
- **Stain**
  - **Uranyl acetate**
  - **Lead citrate**

Presentation at the Stephen F. Austin State University Undergraduate Research Conference, April 18, 2017

Results

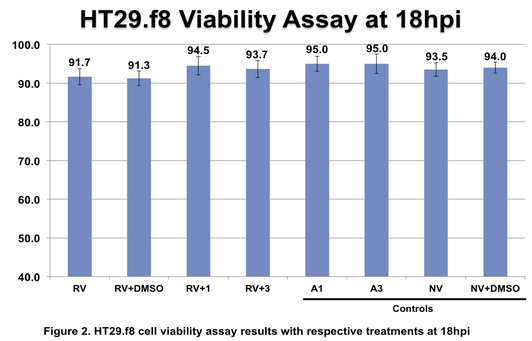


Figure 2. HT29.f8 cell viability assay results with respective treatments at 18hpi

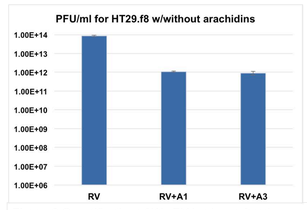
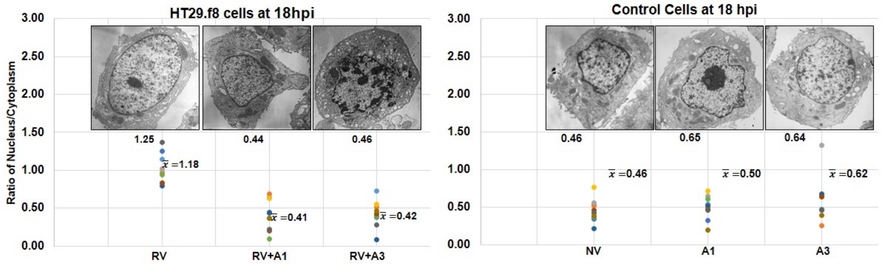
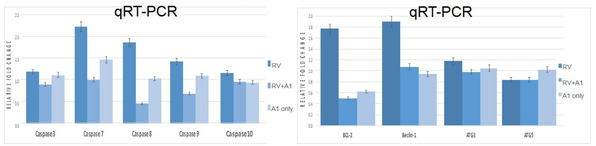


Figure 3 Quantification of Wa RV in plaque forming units/mL (PFU/mL) at 18 hpi.
HT29.f8 cells were infected with RV, RV and 20 µM A1, or RV +20 µM of A3

p values	
2.3773E-05	RV/RV+20uM A3
2.39272E-05	RV/RV+20uM A1
0.254288038	RV+20uM A1/RV+20uM A3



Quantification of Transcripts for Caspase Genes HT29.f8 treated with A1



Real-time quantitative PCR (qRT-PCR) analyses of key genes in signaling pathways involving the caspase and autophagy that are regulated by A1 during an RV infection. Total RNA was extracted from HT29.f8 cells with rotavirus, rotavirus+20µM A1, or 20µM A1 at 8 hpi, and synthesized to cDNA that was used for qRT-PCR analyses in triplicate experiments. Fold change in signals of Expression of the genes of interest relative to GAPDH and β-actin were determined using the $2^{\Delta\Delta CT}$ method. The results are expressed as mean ± SD from three separate experiments.

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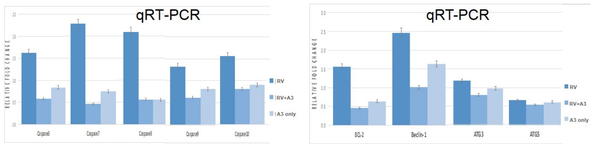
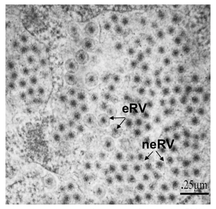
Quantification of Transcripts for Caspase Genes in HT29.f8 cells treated with A3

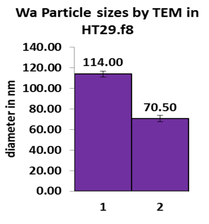


Real-time quantitative PCR (qRT-PCR) analyses of key genes in signaling pathways involving the caspase and autophagy that are regulated by A3 during an RV infection. Total RNA was extracted from HT29.f8 cells with rotavirus, rotavirus+20µM A3, or 20µM A3 at 8 hpi, and synthesized to cDNA that was used for qRT-PCR analyses in triplicate experiments. Fold change in signals of Expression of the genes of interest relative to GAPDH and β-actin were determined using the $2^{\Delta\Delta CT}$ method. The results are expressed as mean ± SD from three separate experiments.

Real-time quantitative PCR (qRT-PCR) analyses of key genes in signaling pathways involving the caspase and autophagy that are regulated by A3 during an RV infection. Total RNA was extracted from HT29.f8 cells with rotavirus, rotavirus+20µM A3, or 20µM A3 at 8 hpi, and synthesized to cDNA that was used for qRT-PCR analyses in triplicate experiments. Fold change in signals of Expression of the genes of interest relative to GAPDH and β-actin were determined using the $2^{\Delta\Delta CT}$ method. The results are expressed as mean ± SD from three separate experiments.

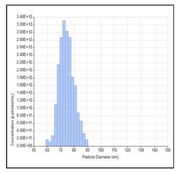


Non-enveloped rotavirus particles (neRV) and enveloped rotavirus particles (eRV) in RV-infected HT29.f8 cells with 20µM A3. Caleb Witcher, SFASU.

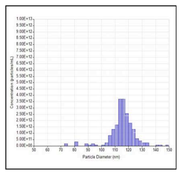


Genes	Primer name	Primer sequence (5'-3')*	Size (bp)
GAPDH	GAPDHfor	GAGTCCACTGGCGTCTTCA	190
	GAPDHrev	GGGGTGCTAAGCAGTTGGT	
β-Actin	β-Actin for	ATCCTCACCCTGAAGTACCC	183
	β-Actin rev	TAGAAGGTGTGGTGCCAGAT	
Caspase 3	Casp3for	GAGTCCATTGATTGGCTTCC	209
	Casp3rev	TCTGGTTTTCGGTTGGGTG	
Caspase 4	Casp4for	ATGGCAGGACAAATGCTTCT	205
	Casp4rev	TGCGGGTTGTTTCTCTTT	
Caspase 6	Casp6for	AAGAGGAGGGCAAGGTGTCT	179
	Casp6rev	GCAATTCTCTCCCTCTGTG	
Caspase 7	Cas7for2	TCAGTGGATGCTAAGCCAGA	230
	Cas7rev2	GAACGCCCATACCTGTCACT	
Caspase 8	NCIcasp8for	GGTCGACTTGAACCTTGGGAA	146
	NCIcasp8rev	CGGAATGTAGTCCAGGCTCA	
Caspase 9	NCIcasp9for	CACGGCAGAAGTTCACATTG	148
	NCIcasp9rev	ACACCCAGAACCACAGGACT	
Caspase 10	NCIcasp10for	GGGAGGTAAAGCTGTGGTTG	182
	NCIcasp10rev	GCCGAGTCGTATCCAAGGAGA	
Caspase 12	Casp12for	CCATCCAACGGTGTTCTGGT	195
	Casp12rev	GCCTGCAATTTGAGCTGTCT	
Bcl-2	BCL2 For	CAGTTGGGCAACAGAGAACCAT	171
	BCL2 Rev	AGCCCTTGTCCCCAATTTGGAA	
ATG 3	atg3 For	ACATCAGTCAGGATCATGTGAAGA	206
	atg3 Rev	CGTTAACAGCCATTTTGCCA	
ATG 5	atg5 For	ATGTGCTTCGAGATGTGTGGT	220
	atg5 Rev	CATTTCGAGTGGTGTGGTTCA	
Beclin-1	beclin-1 For	AGCCAGACGCTGTTTGGAG	189
	beclin-1 Rev	TGATCCAGTCTCTCAGCCTCA	

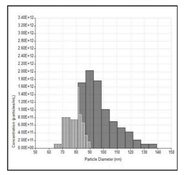
HT29.f8 RV ONLY
18hpi
Concentration: 2.13E13



HT29.f8 RV+A3
18hpi
Concentration: 2.01E13



HT29.f8 RV+A1
18hpi
Concentration: 1.05E13



Conclusion

From the data collected, the viability of the cells was not impacted with the introduction of A1 or A3, therefore the arachidins do not adversely affect the cells. However, the amount of infectious virus particles produced when treated with A1 and A3 was decreased by approximately one hundred fold and were both statistically significant (p=2.4E-5). Also, TRPS analysis showed a size pattern of distribution of particles that was consistent with the size range of virus particles measured by TEM of the more mature noneveloped RV (neRV 67-73.5nm) and more immature enveloped RV (eRV 111-117nm). In addition, TRPS analysis of RV only supernatants demonstrated a size population that is consistent with infectious RV, but with a few larger immature RV particles. On the other hand, the arachidin treated cell supernatants show a population of more immature RV sizes. This suggests that the arachidins do impact RV maturation. The TEM images depicted changes of the ultrastructures of RV-infected HT29.f8 cells, and indicates apoptosis while RV-infected cells with the addition of A1 or A3 showed signs of the autophagy pathway. The nucleus to cytoplasm ratios of TEM micrographs (n=12 per treatment) of the cells from each of the treatment groups demonstrated that the increase in the nucleus observed with RV alone was decreased with the addition of the arachidins to a similar size of the untreated or arachidin alone cells. qRT-PCR analysis of gene transcripts, that are important in regulations of the apoptosis and autophagy pathways, validated the TEM observation. The up regulation of the apoptosis transcripts were down regulated with the addition of the arachidins. Whereas only beclin-1 and bcl-2 where regulated like the apoptosis transcripts. This pattern of regulation implies a cross talk between the two pathways. Our data suggest a mechanism of action of two natural small molecules showing anti-RV activity that implies potential therapeutic applications.

References

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Funding

This work was supported by: 1) the Animal Formula Health Grant # AH-9240 (USDA Cooperative State Research, Education, and Extension Service); 2) The Office of Research and Sponsored Programs at Stephen F. Austin State University (RPS # 107552-26112-150); and 3) The NSF-EPSCoR (grant# EPS- 0701890; Center for Plant-Powered Production-P3).