

In vitro Inhibition of Zika Virus by Metadichol[®], A Novel Nano Emulsion Lipid

Raghavan PR*

Abstract

Objective: To show the antiviral activity of Metadichol[®] against Zika virus by an *in-vitro* assay.

Methods: The primary Anti-Zika virus assay was performed using real-time RT-qPCR (TaqMan) to measure extracellular Zika virus RNA copy number associated with virions released from vero cells. The 'vero' cell line (kidney epithelial cells extracted from an African green monkey) treated with antiviral test articles is infected with Zika virus followed by Zika virus associated RNA measurement in the cell culture supernatant. Antiviral compounds blocking any step of viral replication such as transcription, translation, encapsidation, the particle assembly and release were identified and characterized using this sensitive assay system.

Findings: Metadichol[®] (1-2) *in vitro* assays, (inhibited the Zika Virus with a EC50 of 1.48 µg/ml.

Conclusion: Metadichol is a safe and effective inhibitor for enveloped viruses in humans. Since it is known to bind to the vitamin D receptor (VDR), its action mechanism likely involves the competitive displacement of virus particles from VDR's on host cell membranes. Metabolism studies of long chain alcohol in fibroblasts suggest that very long chain fatty alcohols, fatty aldehydes, and fatty acids are reversibly interconverted in a fatty alcohol cycle [3]. Metadichol consists of natural components of common foods (classified as GRAS), Metadichol has no known negative side effects. The inhibition of Zika virus by Metadichol is not surprising, given that we have recently published the results of Metadichol which showed broad-spectrum antiviral activity against Dengue, Ebola, H1N1, SARS, Chikungunya and other enveloped viruses.(4)

Keywords

Zika; Ebola; Dengue; Chikungunya; H1N1; Respiratory viruses; Metadichol; VDR; Inverse agonist; Protean agonist

Introduction

So often, news about a viral outbreak goes viral that makes news headline. Human immunodeficiency virus (HIV), West Nile virus, avian influenza (bird flu), Ebola, Middle East respiratory virus, and Zika virus, each of these have become the focus of the media's spotlight. Their views leave the public with a new health concern to worry about but little knowledge about the actual factors is involved in the problem.

*Corresponding author: PR Raghavan, Founder and CEO, Nanorx Inc., PO Box 131, Chappaqua, NY 10514, USA, E-mail: raghavan@nanorxinc.com

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In February 2016, the WHO [5] asserted that the potential association between Zika and microcephaly constitutes an international public health emergency.

Mosquito-born Zika virus and complications associated with the transmission of the virus has been at the forefront of much public discussion. The epidemic of Zika virus (ZIKV) infection was reported in 2015 in South and Central America and the Caribbean. A major concern associated with this infection is the apparent increased incidence of microcephaly in fetuses born to the mothers infected with ZIKV. The potential congenital problems associated with the contracting Zika during pregnancy as well as the risk for serious autoimmune and neurological problems in affected subjects is being highlighted [6].

The family Flaviviridae contains some of the most clinically important arboviruses. This genus includes viruses like the West Nile, Dengue, tick borne encephalitis, Yellow fever, Zika and several other viruses which may cause encephalitis. Flaviviruses share several common aspects, such as common size (40-65 nm), symmetry (enveloped, icosahedral nucleocapsid), nucleic acid (positive-sense, single-stranded RNA around 10,000-11,000 bases). Most of these viruses are transmitted by an infected mosquito bite or tick and hence, classified as arboviruses [7].

Enveloped viruses are then free to begin a new cycle of infection by fusing their cell-derived envelope with the cellular membrane of an uninfected cell. Some types of enveloped virus fuse directly to the cell's outer (plasma) membrane, whereas others are engulfed wholly by endocytosis or similar process and then fuse their envelope with the membrane of the engulfed internal organelle (e.g., endosome) to gain access to the interior of the cell. In either case, the genetic material of the virus invades the cell through the barrier of its membrane, and infection follows inevitably. The most reliable way to prevent infection caused by any virus is to eliminate its entry in the first place. All available antiviral therapeutic compounds block replication processes shared by the virus and infected target cells [8]. Such compounds are thus potentially toxic, mutagenic, and teratogenic for the host and can induce drug-resistant for viral mutant sub-strains. Consequently, identification of efficacious new antiviral compounds that lack such deleterious effects is very important.

Metadichol is a nanoemulsion of long-chain alcohols found in many foods. Its active ingredients are commonly called Policosanols. It is present in many foods such as rice, sugar cane, wheat, peanuts [3]. Metadichol acts on Nuclear Vitamin D receptor (VDR) [2] in cells throughout the body to stimulate the immune system and inhibit a variety of disease processes, including those resulting from viral infections [9].

We had previously documented two patients diagnosed with dengue fever in SE Asia [4] who were volunteered to be treated with Metadichol. Based on the positive outcome, we then tested for antiviral activity of Metadichol in vero and MDCK cells infected with Dengue, Ebola, Marburg, Influenza A (H1N1), Chikungunya and Human Respiratory Syncytial viruses. In addition, we tested the efficacy of Metadichol in preventing the cell death caused by Adenovirus, Tacaribe Mammarenavirus, Rift Valley Fever virus, SARS coronavirus, Japanese Encephalitis virus, West Nile virus, and Yellow

Fever virus (Figure 1). At that time, there was no assay available for Zika. Recently, it became widely available and Metadichol was tested against the Zika Virus.

As shown previously [4], Metadichol exhibit potent, broad spectrum viral inhibitory activity and its lack of toxic, mutagenic, or teratogenic properties has been well documented [10,11,12].

Experimental

The in-vitro assay was outsourced on commercial contract basis to Southern Research and the assay was carried out at their Infectious Disease Research Facility in Frederick, Maryland, USA. Vero cells were plated in 96-well micro titer plates at 1×10^4 cells/well in Dulbecco's Modified Eagle's Medium supplemented with 2% FBS, 2.0 mM L-Glutamine, 100 units/mL Penicillin, 100 µg/mL Streptomycin, and 0.1 mM non-essential amino acids. The interior was utilized to reduce "edge effects" observed during cell culture; the exterior wells were filled with complete medium to help minimize sample evaporation. After 16-24 hours the confluent monolayer of vero cells were washed and the medium was replaced with complete medium containing various concentrations of the test compound in triplicate (Table 1) for a representative plate layout for testing compounds at six half-log concentration), followed by infection with Zika virus. Interferon alpha (IFNα) was used as the positive control, while media alone was added to cells as a negative control (virus control, VC). Three days followed by the initial administration of the test compound, the cell culture supernatant was used in a real-time quantitative TaqMan qPCR assay. The RT-PCR-amplified Zika viral RNA was detected in real-time by

monitoring the increase in fluorescent signal that resulted from the Exonucleolytic degradation of a quenched fluorescent probe molecule that hybridized to the amplified Zika RNA. For each PCR amplification, a standard curve is simultaneously generated using dilutions of purified Zika viral RNA. Antiviral activity was calculated from the reduction in Zika viral RNA levels (EC_{50} & EC_{90} values determined). A tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Cell Titer'96 Reagent, Promega) uptake assay was then employed to measure cell viability using the same assay plate, and the viability data was used to calculate compound cytotoxicity (CC_{50}). The Selectivity Index (SI_{50}) was calculated as CC_{50}/EC_{50} .

Results of In-Vitro Antiviral Activity

Shown in Tables 1-4 and Figures 1-3.

Discussion

Metadichol against Zika virus showed an EC_{50} of 1.48 µg/ml and EC_{90} of 5.01 µml. These results were in line with the EC_{50} of 8.75 µg/mL and 3.96 µg/mL as we previously reported [4] against Ebola (Mayinga) and Marburg virus (Musoke), respectively. The EC_{50} of Metadichol against DENV-2 (New Guinea C), CHIKV (181/25), and HRSV (A2) was 2.91 µg/mL, 3.54 µg/mL, and 0.41 µg/mL, respectively. The EC_{50} of Metadichol against Influenza A (CA/07/09) was 5.44 µg/mL.

Previous studies have demonstrated the antiviral activities of moderate-length saturated and unsaturated alcohols at mM concentrations [13,14]. Optimal antiviral activity was observed with saturated alcohols 10 to 12 carbons long; however, those compounds

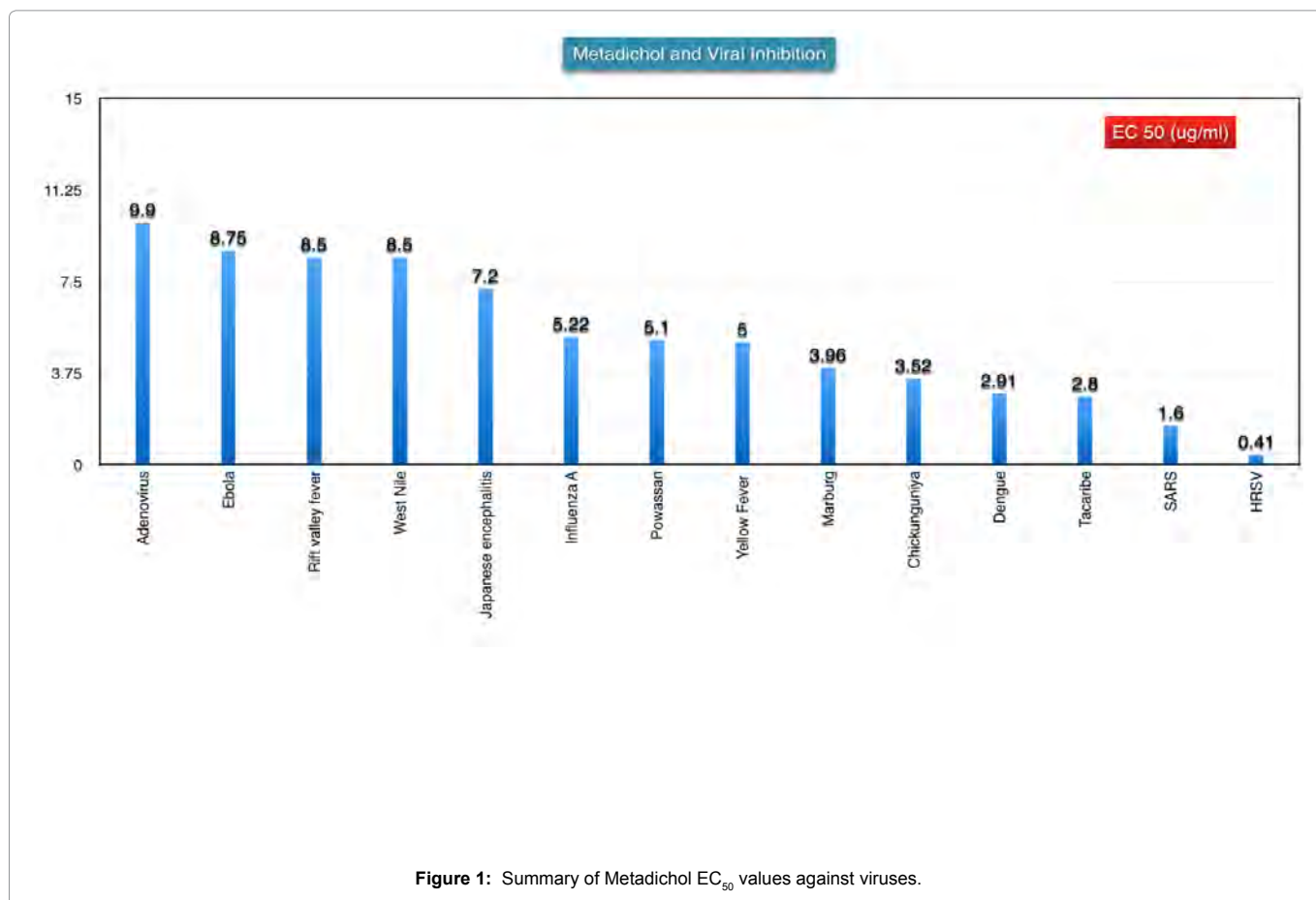


Table 1: Illustrative example of a plate layout, 96-Well Tissue Culture Plate Format for Zika virus Drug Screening; VC = Virus control, which is the Vero cells alone without compound.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media
B	Media	Cells + Drug 1 100 uM	Cells + Drug 1 100 uM	Cells + Drug 1 100uM	Cells + Drug 2 100 uM	Cells Drug 100uM	+ 2 Cells + Drug 2 100uM	Cells + Drug 3 100uM	Cells + Drug 3 100 uM	Cells + Drug 3 100 uM	VC	Media
C	Media	Cells + Drug 1 32 uM	Cells + Drug 1 32 uM	Cells + Drug 1 32 uM	Cells + Drug 2 32 uM	Cells Drug 2 32 uM	+ 2 Cells + Drug 2 32 uM	Cells + Drug 3 32 uM	Cells + Drug 3 32 uM	Cells + Drug 3 32 uM	VC	Media
D	Media	Cells + Drug 1 10 uM	Cells + Drug 1 10 uM	Cells + Drug 1 10 uM	Cells + Drug 2 10 uM	Cells Drug 2 10 uM	+ 2 Cells + Drug 2 10 uM	Cells + Drug 3 10 uM	Cells + Drug 3 10 uM	Cells + Drug 3 10 uM	VC	Media
E	Media	Cells + Drug 1 3.2 uM	Cells + Drug 1 3.2 uM	Cells + Drug 1 3.2 uM	Cells + Drug 2 3.2 uM	Cells Drug 2 3.2 uM	+ 2 Cells + Drug 2 3.2 uM	Cells + Drug 3 3.2 uM	Cells + Drug 3 3.2 uM	Cells + Drug 3 3.2 uM	VC	Media
F	Media	Cells + Drug 1 1 uM	Cells + Drug 1 1 uM	Cells + Drug 1 1 uM	Cells + Drug 2 1 uM	Cells Drug 2 1 uM	+ 2 Cells + Drug 2 1 uM	Cells + Drug 3 1 uM	Cells + Drug 3 1 uM	Cells + Drug 3 1 uM	VC	Media
G	Media	Cells + Drug 1 320nM	Cells + Drug 1 320nM	Cells + Drug 1 320nM	Cells + Drug 2 320nM	Cells Drug 2 320nM	+ 2 Cells + Drug 2 320nM	Cells + Drug 3 320nM	Cells + Drug 3 320nM	Cells + Drug 3 320nM	VC	Media
H	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media

Table 2: *In-vitro* Anti-Zika Evaluation: Raw Data and Summary Graph for Metadichol vs Zika Virus.

Compound	Concentration Units	EC ₉₀	EC ₅₀	CC ₅₀	Selectivity Index (CC ₅₀ /EC ₅₀)
Metadichol	µg/mL	5.01	1.48	4.25	2.87
IFN-alpha	IU/mL	27.2	<3.16	>1000	>316

also exhibited cytotoxic and hemolytic effects. Less antiviral activity was observed with alcohols 14 to 18 carbons long; alcohols with longer chain lengths were not tested. Katz [15] showed that compositions of one or more aliphatic alcohols containing 27 to 32 carbons were suitable for intravenous or intramuscular injection into humans or mammals.

Nutritional status might exert a profound effect on immune system functions. Hence, several parameters of immune system are modified by dietary lipid administration, such as lymphocyte proliferation, cytokine production, natural killer activity, antigen presentation, etc. Thus, numerous studies have indicated the key role of lipids as immune response modulators. These properties have been applied in the treatment of autoimmune and inflammatory diseases [16].

Metadichol is a nanoemulsion of long-chain lipid alcohols (C-26, C-28 and C-30), which are commonly known as Policosanols. Metabolism studies in fibroblasts suggest that very long chain fatty alcohols, fatty aldehydes, and fatty acids are reversibly interconverted in a fatty alcohol cycle [17,18]. Since the metabolites of long chain alcohols are interconverted, a single dosage even at low doses can theoretically have lasting effects. Metadichol has a particle size of less than 60 nm. We have shown that it binds to the vitamin D receptor (VDR) as an inverse agonist. It is the only known inverse agonist of VDR found in medical literature [2].

Calcitriol (1,25-Dihydroxy Vitamin D) is the natural ligand for the VDR and act as an agonist. Metadichol likely behaves more like a protean agonist. Protean agonists act as both positive and negative agonists on the same receptor, depending on the degree of presence of constitutive activity. If there is no constitutive activity, the agonist would be a positive agonist. When constitutive activity is present, the protean agonist would be an inverse agonist [19].

Vitamin D is essential to the skeletal system [9,20,21] and recent evidence suggested that it also play a major role in regulating the immune system, perhaps through the involvement in immune responses to viral infections [22]. Cell culture experiments supported the hypothesis that vitamin D has direct antiviral effects, particularly against enveloped viruses. The antiviral mechanism of vitamin D may be due to the ability of vitamin D to up regulate the antimicrobial peptides LL-37 (cathelicidin) and human beta-defensin. Human cathelicidin has been shown to affect several viruses including VV, RSV, influenza virus, HIV, HSV, DENV and Adenovirus via virus envelope disruption, and polymerase or protease inhibition [23].

Viruses have evolved strategies to exploit the VDR and other receptors to regulate the expression of their genes and to optimize the cellular processes intrinsic to the viral life cycle. Persistent Epstein-Barr virus infection down regulates VDR >10 fold [24,25]. While the specific receptors targeted by viruses vary and involve processes that directly or indirectly modulate receptor function. The specific receptor(s) targeted by a particular virus are likely to reflect the tissue tropism of the virus. By binding to the VDR (which is a key receptor for innate immunity and is present in all cells), Metadichol can displace viruses bound to it and block viral entry into the host cells. The fact that Metadichol has inhibitory effects against many viruses suggests that viral binding to the VDR does occur and that Metadichol competitively disrupts this process. In addition to VDR binding, Metadichol shares cross-reactivity with other nuclear receptors [26], which may explain its activity against a wide range of viruses.

Conclusion

Metadichol constituents are long-chain lipid alcohols which are classified as GRAS, and present in foods that are consumed on a daily basis. Metadichol has demonstrated no toxicity at doses of up to 5000 mg/kg [10,11,12] and is a renewable resource.

Table 3: Raw Data: Metadichol Vs. Zika Virus.

LOCATION ON PLATE	CONC(µG/ML)	ANTIVIRAL TEST VALUES				CYTOTOXICITY TEST VALUES			
		MEAN ZIKA RNA	%CONTROL ZIKA RNA	SD ZIKA RNA	% CV	MEAN Cytotox	% CONTROL VI-ABILITY	SD Cytotox	% CV
B2-G2	CONTROL	1118459	100%	206630	18.5%	0.9212	100%	0.0589	6.4%
B3-B5	0.079	1029493	92%	344895	30.8%	0.8927	97%	0.0107	1.2%
C3-C5	0.25	10966952	98%	899504	80.4%	0.8580	93%	0.353	3.8%
D3-D5	0.79	905626	81%	95733	8.6%	0.8642	94%	0.0770	8.4%
E3-E5	2.50	273262	24%	225308	20.1%	0.8229	89%	0.0369	4.0%
F3-F5	7.91	5546	0%	1658	0.1%	0.0366	4%	0.0361	3.9%
G3-G5	25.0	4226	0%	664	0.1%	0.0175	2%	0.0029	0.3%

Metadichol and its Cytotoxicity data (O.D.) and efficacy data (copies of Zika RNA)

RAW DATA

CYTOTOXICITY DATA (O.D.):

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Media	Media											
		CC	drug experimental										
B		0.8623	0.9003		0.8852								
C		1.0058	0.8330		0.8829								
D		0.9743	0.8098		0.9187								Media
E		0.9293	0.7968		0.8490								
F		0.8837	0.0622		0.0111								
G		0.8719	0.0154		0.0195								
H	Media												

CC=Cell control

BOLD-highest drug concentration

EFFICACY DATA (COPIES OF ZIKA RNA):

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Media	Media											
		VC	drug experimental										
B		906836	785616	1273371									
C		1083625	460907	1732998									
D		1364918	973320	837933									Media
E		906836	113945	432579									
F		1083623	4373	6718									
G		1364918	3756	4695									
H	Media												

VC=Virus control

BOLD-highest drug concentration

Table 4: Raw Data IFN-alpha vs Zika Virus.

LOCATION ON PLATE	CONC(µG/ML)	ANTIVIRAL TEST VALUES				CYTOTOXICITY TEST VALUES			
		MEAN ZIKA RNA	%CONTROL ZIKA RNA	SD ZIKA RNA	%CV	MEAN Cytotox	% CONTROL VI-ABILITY	SD Cytotox	% CV
B2-G2	CONTROL	1118459	100%	206630	18.5%	0.9212	100%	0.0589	6.4%
B6-B8	3.16	371155	33%	101695	9.1%	0.9037	98%	0.0021	0.2%
C6-C8	9.99	227176	20%	91634	8.2%	0.8885	96%	0.0116	1.3%
D6-D8	31.6	94763	8%	77641	6.9%	0.9277	101%	0.0206	2.2%
E6-E8	99.9	86443	8%	26033	2.3%	0.9478	103%	0.0216	2.3%
F6-F8	316	4793	0%	1881	0.2%	0.9485	103%	0.0607	6.6%
G6-G8	1,000	1421	0%	1009	0.1%	1.0556	115%	0.0168	1.8%

IFN-alpha and its Cytotoxicity data (O.D.) and efficacy data (copies of Zika RNA)

RAW DATA

CYTOTOXICITY DATA (O.D.):

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Media	Media											
		CC	drug experimental										
B		0.8623	0.9023		0.9052								
C		1.0058	0.8803		0.8067								
D		0.9743	0.9131		0.9423								Media
E		0.9293	0.9326		0.9631								
F		0.8837	0.9055		0.9914								
G		0.8719	1.0675		1.0437								
H	Media												

VC=Virus control

BOLD-highest drug concentration

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media	Media										Media
		VC	drug experimental									
B		906836	443064	299245								
C		1083625	162381	291791								
D		1364918	149664	39362								
E		906836	68035	104852								
F		1083623	6123	3463								
G		1364918	707	2134								
H	Media											

VC=Virus control

BOLD-highest drug concentration

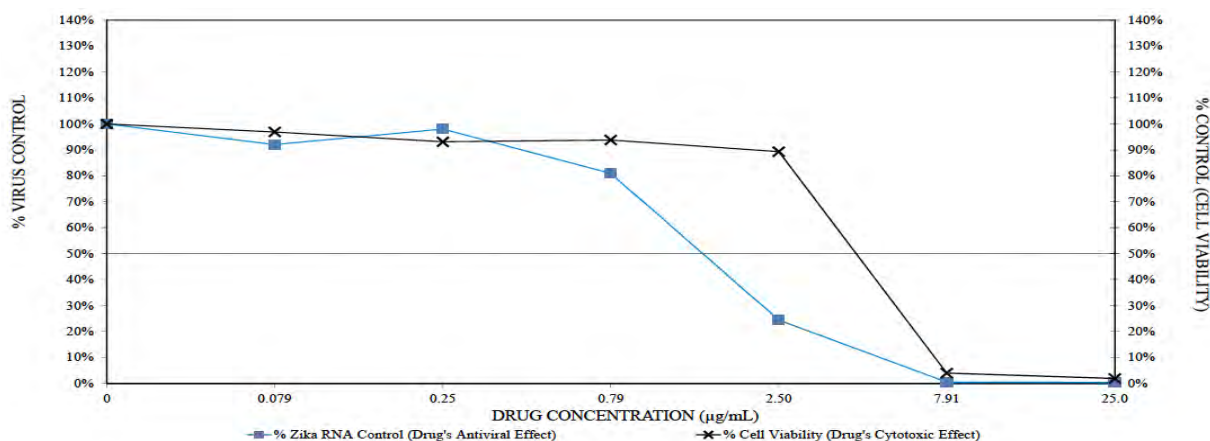


Figure 2: Summary graph for Metadichol versus Zika Virus.
Drug: Metadichol
Virus: Zika MR 766
Cells: VERO

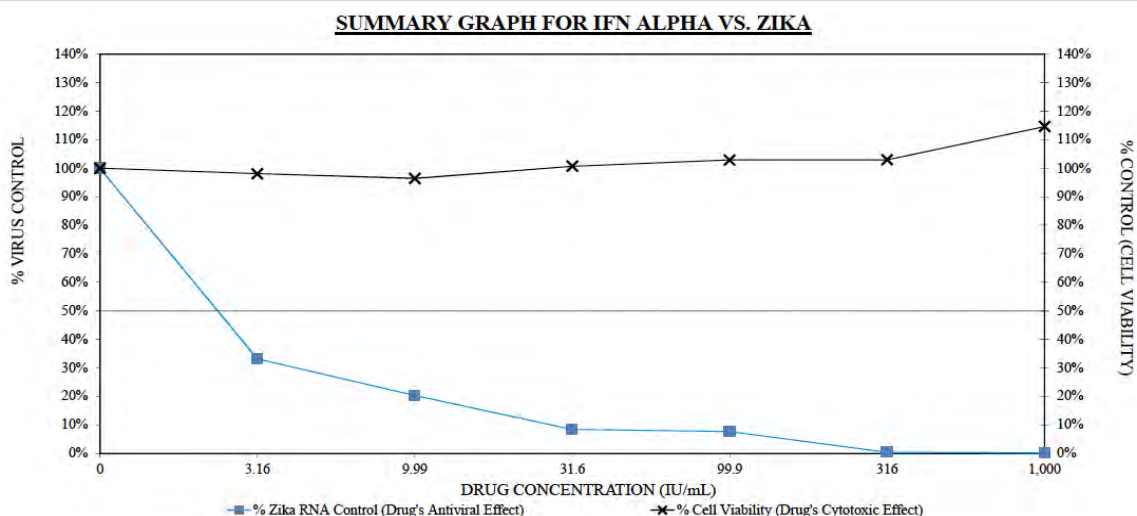


Figure 3: In-vitro Anti-Zika Evaluation:
Drug: IFN-alpha
Virus: Zika MR 766
Cells:VERO

Metadichol could serve as a preventive agent for Zika given that it strengthens the innate immunity through VDR binding, and represent the first key step in preventing diseases. Metadichol is ready for large scale testing in areas which are ravaged by viruses. Once proven on large populations, Metadichol could be used as a preventive nutritional supplement in countries where viral fevers are widely prevalent. Metadichol is being sold as a nutritional supplement in a few Asian countries for the last two years and is extremely well tolerated. So far, there have been no reports of any adverse side effects.

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Author Affiliations

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