Short Communication

Natural compounds inhibiting the replication of Porcine reproductive and respiratory syndrome virus

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important pathogenic virus in the swine production. Current vaccines against PRRSV do not induce sterile immunity and the virus evolves at a rapid rate with frequent appearances of new strains. In this study, we screened a library of 502 highly purified natural product compounds to identify specific inhibitors of PRRSV replication cycle. Our observations showed that many of the inhibitory compounds identified have activity on the cellular ion transport mechanisms. We identified for the first time, four compounds which inhibit the PRRSV replication cycle at micro molar concentration or less, namely, 12-deoxyphorbol 13-phenylacetate 20-acetate, ouabain, bufalin and valinomycin. Further, we have identified 15 other compounds which can inhibit the PRRSV replication at the concentration of 8 μM. This study provides a basis for further development of pharmacological agents to inhibit PRRSV replication.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an important pathogen of Porcine species which causes an estimated annual loss of more than 600 million dollars to the swine production industry in the United States alone (http://nationalhogfarmer.com August 17, 2011). The emergence of PRRSV as a pathogen was first observed in the 1980s and is currently a global epizootic (Murtaugh and Genzow, 2011). The virus belongs to the order Nidovirales, family Arteriviridae and is characterized by positive strand RNA genome of approximately 15 kb (kilobases) and is enveloped. The PRRSV viral genomic RNA is capped and polyadenylated. The virus has been classified into two major strains, the Type I (European) and Type II (North American) (Shi et al., 2010). The structural proteins of the virus are encoded from the distal third of the viral genome and are transcribed as sub-genomic mRNAs. The non-structural proteins take up the proximal two thirds of the viral genome and are synthesized as a shorter and a longer polyprotein, which are then processed into smaller proteins consisting of a variety of enzymes, such as proteases, RNA polymerases and RNA helicases. The non-structural proteins are shown to suppress the induction of host type I interferon genes and other cytokines (Beura et al., 2010; Patel et al., 2010). The PRRSV is estimated to have a high rate of evolution of 3.29×10^{-3} substitutions per nucleotide site per year and new strains frequently appear in the field (Song et al., 2010).

The currently available inactivated and modified live vaccines are inadequate to eradicate the virus and do not provide complete immunity from infection (Murtaugh and Genzow, 2011). Modified live vaccine PRRSV strains are also characterized by the shedding of the virus from vaccinated animals, which often revert to virulent strains (Murtaugh and Genzow, 2011; Storgaard et al., 1999). Since the global PRRSV epizootic in pigs is not completely amenable to immunological intervention, a pharmacological intervention might be a valuable alternative or additional control measure. For PRRSV, past studies in cell culture models have revealed only few compounds displaying antiviral activity (see Table 1). In general, natural compounds constitute a major source of effective pharmacological agents including, artemisinin, anti tumor alkaloids, salicylates, opiates, etc. (Mishra and Tiwari, 2011). Therefore, we evaluated a commercially available library (Screen-Well™ Natural Product Library, Enzo life sciences) of 502 purified natural compounds for their activity against PRRSV infection and replication. To this end, we established a high-throughput screening method.

An immunofluorescence assay (IFA) based high-throughput screening platform in a 384 well format to detect viral replication was established utilizing MARC-145 cells permissive to PRRSV infection. The viral proteins were detected using a highly specific monoclonal antibody against the nucleocapsid protein (ORF7) of the PRRSV (Clone 5H2; in-house produced). After empir-

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Table 1
A list of compounds in the published literature known to inhibit PRRSV replication and IC_{50} values for those estimated. NP – not published.

Compound	IC_{50}	Reference
Macrolide antibiotic		
Bafilomycin	∼0.2 μM	Kreutz and Ackermann (1996)
Cellular ion transport targeting agents		
Verapamil	NP	Kreutz and Ackermann (1996)
Amantadine	<0.2 μM	
Pyrithione	NP	te Velthuis et al. (2010)
Endosomal acidification blockers		
Ammonium chloride	<0.5 mM	
Chloroquinone	>6 μM	
Monensin	NP	
Plant derived lectins		
Indigowoad root polysaccharide	NP	van der Meer et al. (2007)
Hippeastrum hybrid agglutinin	>2 μM	
Galanthus nivalis agglutinin	>2 μM	
Cymbidium sp. agglutinin	NP	
Urtica dioica agglutinin	4.8 ± 3.2 μM	
Non-plant derived lectins		
Pradimicin-A	>120 μM	van der Meer et al. (2007)
Cyanovirin-N	NP	
Chelator		
N-acetylpenicillamine	NP	Jiang et al. (2010)

Table 2
The list of the 19 compounds found to inhibit the PRRSV replication at 10 μM concentration to less than 50% as observed by IFA signal intensity without decreasing of the DAPI intensity of treated cells to less than 70%. Con – control inhibitor MPA.

No.	Compound	Percent viral signal compared to 0.1% DMSO treated infected cells Concentration (μM)			Percent DAPI signal (nuclei) compared to 0.1% DMSO treated infected cells Concentration (μM)		
		10	1	0.1	10	1	0.1
1	Antimycin A1	23	–	–	81	–	–
2	Bufalin[b]	0	2	17	78	94	96
3	Catechin hydrate (+)-	18	92	96	91	95	96
4	Cepharanthine[b]	21	86	88	89	95	95
5	Cinobufagin[a]	34	–	–	86	–	–
6	Coumermycin A1	19	74	81	82	94	95
7	Cryptotanshinone	16	85	88	86	96	96
8	Cycloheximide	7	–	–	72	–	–
9	Cyclopiazonic acid[b]	14	78	86	83	94	96
10	12-Deoxyphorbol 13-phenylacetate 20-acetate	0	4	36	89	94	95
11	Dicoumarol	36	–	–	89	–	–
12	Digitoxin[a]	12	–	–	84	–	–
13	Gitoxigenin[a]	11	–	–	85	–	–
14	Harmaline	41	–	–	86	–	–
15	Hydrastine, D-β-	24	94	98	79	97	97
16	Hypocrellin B	31	–	–	73	–	–
17	Ouabain (−)-[a]	0	0	2	81	93	96
18	Strophanthidin acetate[a]	27	–	–	77	–	–
19	Valinomycin	0	0	1	83	95	96
Con	Mycophenolic acid	0	12	76	38	96	96

[a] Cardiac glycosides.
[b] Compounds known to act on ion channels.

Table 3
The list of 10 compounds selected for secondary assays, to estimate their efficacy to inhibit PRRSV at doses of 0.1, 1 and 8 μM concentration.

No.	Compound	Known pharmacological action
1	Bufalin	Cardiac glycoside with antiviral activity (Cui et al., 2010).
2	Catechin hydrate, (+)-	Flavonoid, anti-oxidant, cyclo-oxygenase inhibitor, anti inflammatory (Kuzuhara et al., 2009).
3	Cepharanthine	An alkaloid with antiviral activity against SARS CoV; anti-inflammatory, inhibits lipooxygenase (Baba et al., 2001; Zhang et al., 2005).
4	Coumermycin A1	Novobiocin related antibiotic with antiviral activity (Palù et al., 1986).
5	Cryptotanshinone	A herb derived tanshinone that blocks Signal transducer and activator of transcription 3 (STAT3) dimerization and inhibits cytokine production by immune cells (Kang et al., 2000; Shin et al., 2009).
6	Cyclopiazonic acid	A fungal metabolite that acts on endoplasmic reticulum resident Ca^{2+} ATPase and mediates activation of NFkB, a key component of immune cell signaling (Xia et al., 2006).
7	12-Deoxyphorbol 13-phenylacetate 20-acetate	A phorbol ester that activates PKCβ, and mimics IFN mediated signaling (Saraiva et al., 2004).
8	Hydrastine, D-β-	An alkaloid anti-inflammatory agent; inhibits cytochrome P450 mediated oxidation (Raner et al., 2007).
9	Ouabain (−)-	Cardiac glycoside with antiviral activity (Su et al., 2008).
10	Valinomycin	A dodeca-depsipeptide antibiotic, anti inflammatory, lipo-oxygenase inhibitor. Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004).

Table 4
The estimated IC_{50} of the four compounds with the highest inhibitory activity.

| Compound	IC_{50} (±standard deviation)
Bufalin	52 nM (±3.8)
12-Deoxyphorbol 13-phenylacetate 20-acetate	118 nM (±8.4)
Ouabain (−)-	41 nM (±3.2)
Valinomycin	24 nM (+4.6)

Table 5
Effect of time of addition after infection on the inhibition of virus replication of the compounds at 1 μM. hpi – hours post infection.

| Compound	Percent viral signal compared to 0.1% DMSO treated infected cells when compounds were added at indicated hpi		
	0	2	8
Bufalin	0	3	7
12-Deoxyphorbol 13-phenylacetate 20-acetate	0	2	68
Ouabain (−)-	0	0	4
Valinomycin	0	1	5
Mycophenolic acid	0	3	16

ical optimization, the assay was performed by seeding 1000 cells per well, infected at a multiplicity of infection (MOI) of 10 at the time of seeding. The antiviral effect was evaluated at 48 h post-infection by fixing the cells with 4% paraformaldehyde. We included mock-infected cells and PRRSV-infected cells with additional 1 μM of mycophenolic acid (MPA), a de novo purine synthesis inhibitor (Sievers et al., 1997), as negative and positive controls, respectively, for virus inhibition of replication. For the
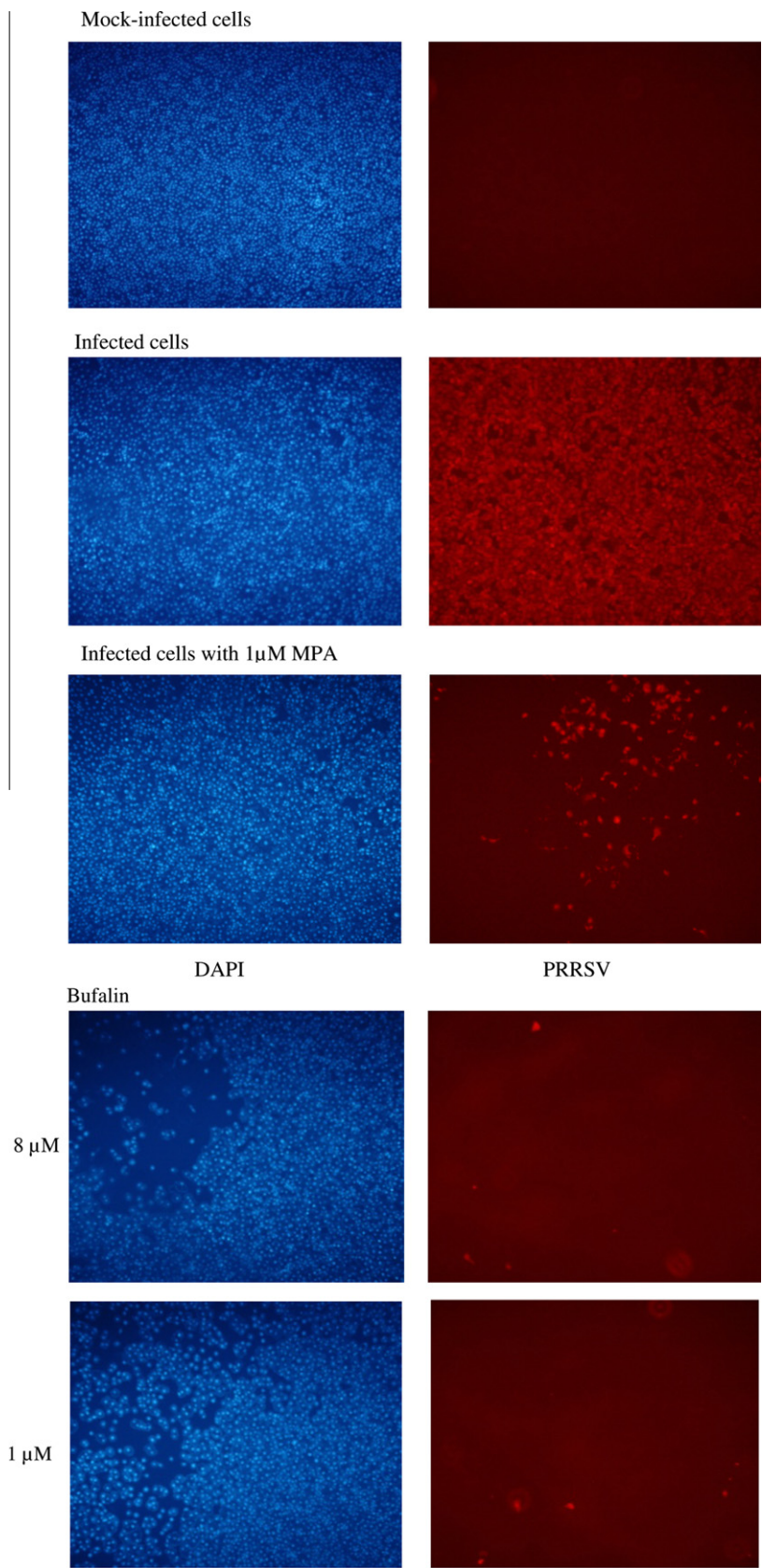
Fig. 1. 12-Deoxyphorbol 13 phenylacetate 20 acetate, ouabain, valinomycin and bufalin exhibited inhibition of the PRRSV replication at micro-molar or sub-micro-molar concentrations. Viral replication in the presence of 8, 1, 0.1 μM of the above compounds was examined by IFA. Nuclei – DAPI staining (blue); PRRSV protein ORF7 staining (red). MPA – mycophenolic acid as a positive control inhibitor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
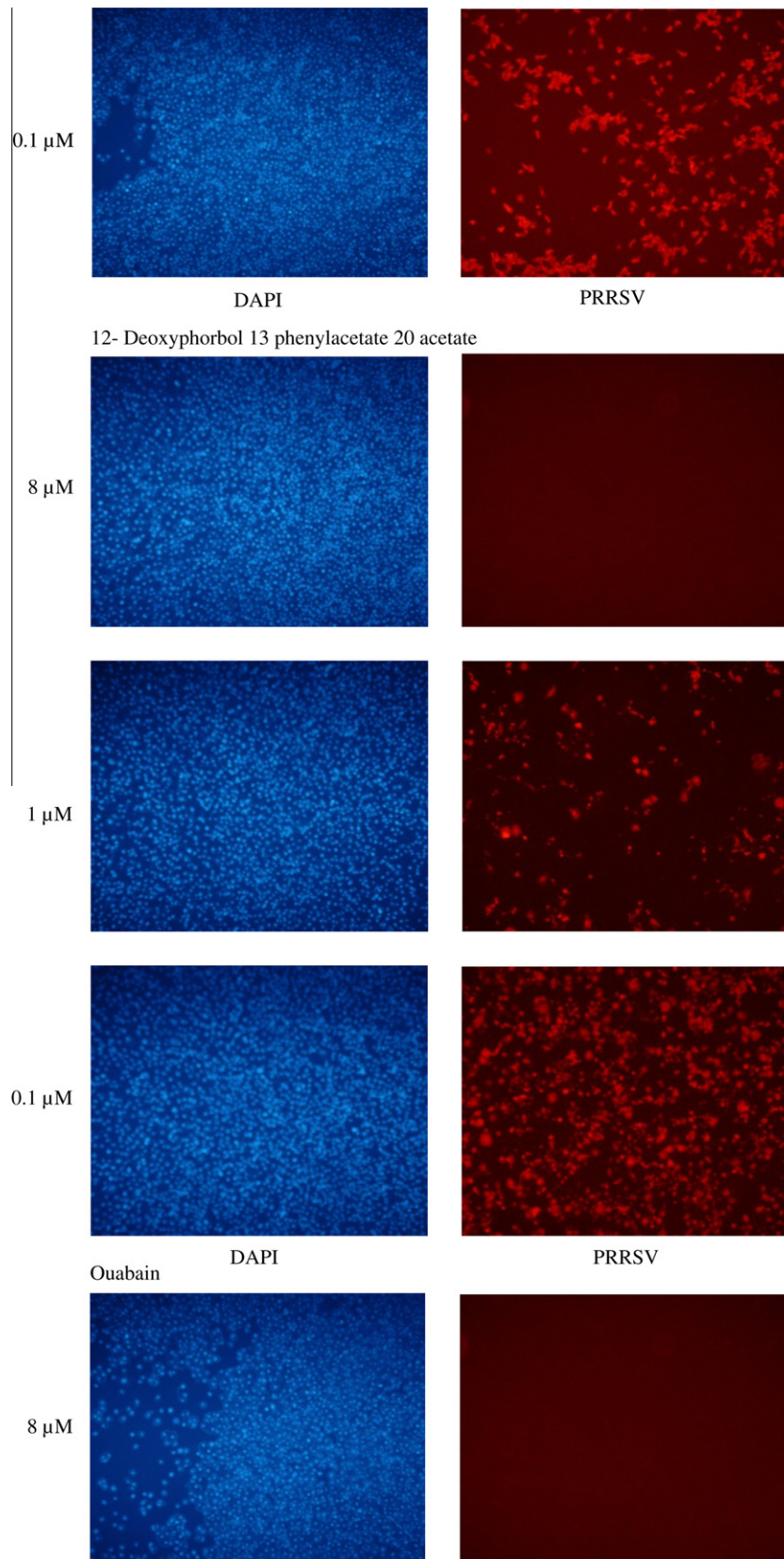
Fig. 1 (*continued*)

immunofluorescence assay, the primary mouse monoclonal antibody from hybridoma supernatant was used undiluted and the secondary donkey anti-mouse immunoglobulins antibody labeled with Alexa Fluor 546 (stock 2 μg/μL) was used at a dilution of 1:500 in PBS–Tween (0.1%). The primary and secondary antibodies were stocked in bulk quantities and aliquots of these stocks
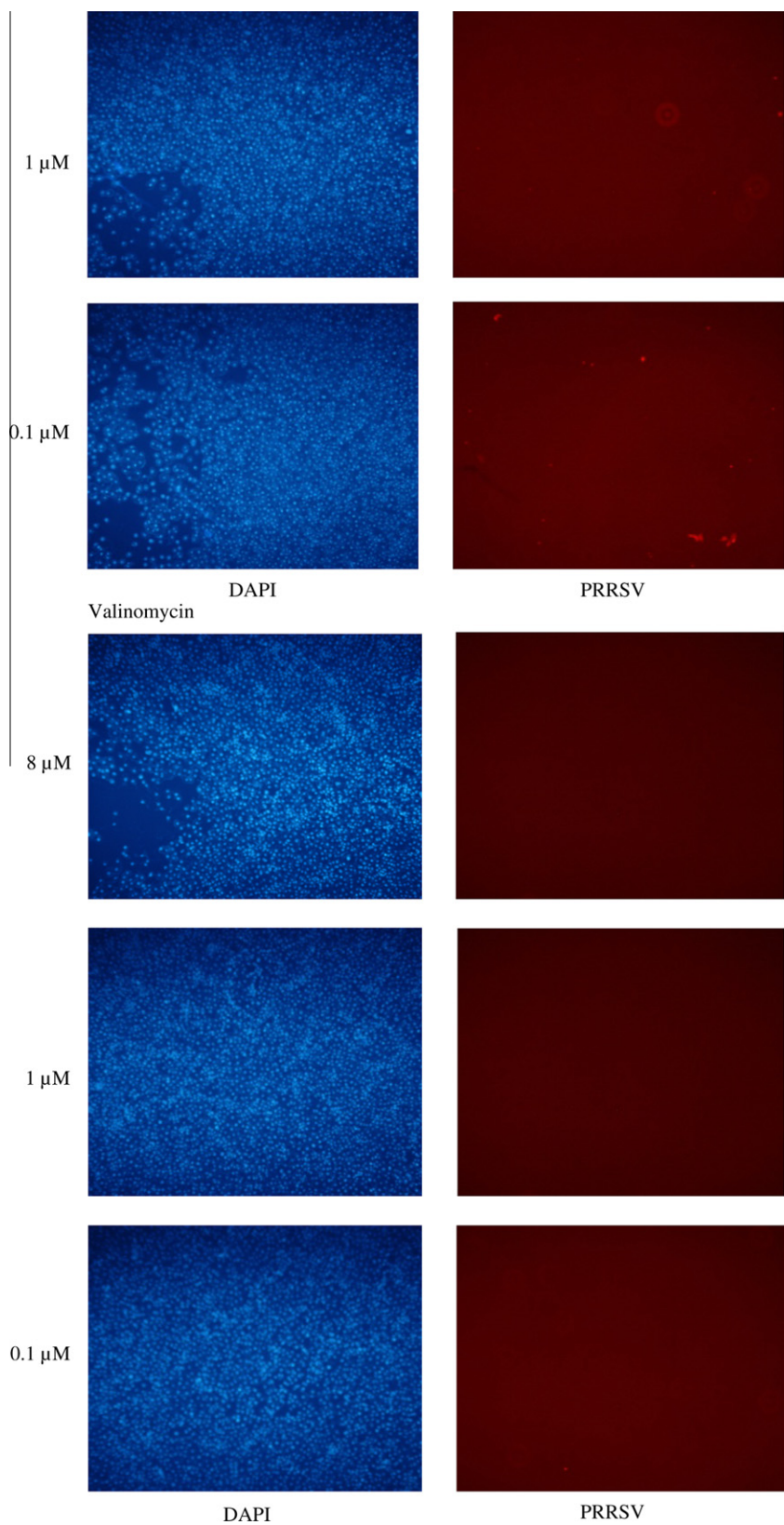
Fig. 1 (*continued*)

were used consistently throughout the studies. Following the antibody staining, the cell nuclei were stained with 4′,6-diamidi-no-2-phenylindole (DAPI). The assay results were machine read with Cellomics® scanner, measuring the intensities of DAPI (indi- cating the nuclei of cells) and Alexa Fluor 543 (indicating the virus infection of cells). The percentage of infected cells was determined from this assay. The assay showed a Z-score of 0.53, when the PRRSV infected and infected cells with 1 μM MPA were compared,

which validates the assay as highly reliable. Utilizing the assay, the compounds in the natural product library were screened for their ability to inhibit viral entry or/and replication by adding 10 μM the compound in DMSO (final concentration of 0.1%) to the cell plus virus mixture at the time of seeding. Mock-infected cells, infected cells, infected cells with 1 μM MPA and infected cells with 0.1% DMSO were used as controls. The compounds which caused a decrease of the DAPI intensity of treated cells to less than 70% of that of infected cells with only 0.1% DMSO were considered toxic to the cells and excluded from further analysis. The screening was repeated twice and we identified 19 compounds which inhibited the virus replication to less than 50% at a 10 μM concentration, as observed by IFA signal intensity, without adversely affecting the host cells (Table 2).

The compound library used for screening comprises of several classes of natural compounds; glycosides, terpenoids, coumarins, isoflavones, peptolides, alkaloids, flavones, macrolides, etc. The compounds belonging to the glycosides (known for their action on cardiac system) and cardio-active steroids were found in higher frequency to inhibit the viral replication in the assay. Of the identified 19 inhibitors, five were the glycosides and two were cardio-active steroids. These compounds share a similar mode of action on the ion channels in cells. Whether these compounds act on the cellular mechanisms and indirectly affect the virus or act directly on a viral component or mechanism is to be determined. The most likely viral target for these compounds could be the viral porin on the PRRSV envelope formed by the multimerization of the small envelope protein (ORF2b) (Lee and Yoo, 2006). Amantadine, known to block the M2 ion channel of the Influenza A, also blocks the PRRSV ORF2b porin (Lee and Yoo, 2006). Other compounds that inhibited the PRRSV included fungal metabolites and flavones. Subsequently, we selected ten compounds (Table 3), considering presence of known related pharmacological compounds and their biological effects. Compounds such as Cycloheximide, Dicoumarol and Harmaline, were not selected from the panel of 19 due to known undesirable biological activity profile. The toxicity effect of the selected ten compounds on MARC-145 cells was analyzed using alamar blue test (Invitrogen) by following the manufacturer's instructions. The alamar blue fluorescence of the infected cells treated with the compounds was not less than 70% of the untreated infected cells and correlated with the DAPI fluorescence intensities estimated previously (data not shown). The inhibitory effect of these compounds were evaluated using the signal intensity in IFA assay, as described earlier, at 0.1, 1 and 8 μM concentrations of each compound (Table 2), by adding the compounds to the virus and cells at the time of seeding. The treatments were performed in triplicates and the experiment was performed twice.

All the ten compounds inhibited the virus replication at 8 μM concentration. Only four of the compounds were inhibitory at 1 μM or sub-micro molar concentrations; 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA), ouabain, valinomycin and bufalin. The representative images of infection are shown in Fig. 1. Interestingly, the dPPA is known to activate the PKCβ and stimulate interferon signaling. Ouabain and bufalin are known to act on the cardiac system by affecting the ion channels. Further, bufalin perturbs signaling mediated by STAT3. Valinomycin, a dodecadepsipeptide antibiotic, is a potassium ionophore. Previous studies have shown that valinomycin has a potent antiviral activity against severe acute respiratory syndrome corona virus (SARS CoV), another important Nidovirus (Wu et al., 2004). We estimated the 50% inhibitory concentrations (IC$_{50}$) for the inhibition of viral replication of the four compounds as described earlier (Wu et al., 2004). The IC$_{50}$ values derived by curve fitting of mean of percent infected cells in triplicates observed by IFA at 8, 1, 0.1, 0.01, 0.001 and 0.0001 μM concentrations of the inhibitors, from three independent experiments. The IC$_{50}$ were well below 1 μM (Table 4) for all the four compounds.

We next evaluated if these four compounds acted on the binding and entry steps of the virus or during the replication process. MARC-145 cells were incubated with each of the compounds (1 μM) for 1 h prior to the addition of the virus at an MOI of 10 and subsequently the cells were incubated at 37 °C for 2 h. After which, the cells were washed thrice with phosphate buffered saline (PBS) and the cells were incubated in fresh media and fixed after 48 h. The results showed that identified four compounds do not block the binding and entry of the virus, but act during the subsequent replication process. Next, the cells were infected with the virus at an MOI of 10 and incubated with compounds (1 μM) from 0, 2 or 8 h post infection (hpi) and fixed at 48 hpi. The IFA signal quantification results (Table 5) showed that virus replication was almost completely inhibited by all the four compounds when added 2 hpi. However, when added at 8 hpi, dPPA decreased the viral signal only by 30% while the other three compounds almost completely inhibited the signal. The results show that dPPA inhibits the virus replication by acting on the early event in virus–cell interaction. The dPPA is known to mimic the cellular interferon signaling (Saraiva et al., 2004), and possibly acts by augmenting the interferon mediated virus suppression. The other three compounds are known act on ion channels and could have a different mode of action. These compounds which are inhibitory even when added in the later stages of infection could have a better value in the in vivo inhibition of the virus. Further detailed analysis needs to be performed to identify their exact mode of action. Next the inhibitory activities of the compounds were tested in a rapid cytopathic effect (CPE) inhibition assay adapted from Cotarelo et al., 1999. The MARC145 cells were infected with PRRS virus at an MOI of 10 with or without the above four compounds (at 8, 1 and 0.1 μM concentration) and examined for CPE at 72 h post infection. The untreated infected cells showed extensive CPE at 72 hpi. At 8 and 1 μM concentrations, all of the four compounds completely inhibited observable CPE at 72 hpi. However, at 0.1 μM concentration dPPA showed mild CPE. The CPE inhibition assay showed that compounds were effective in inhibiting the virus induced cellular pathology.

Apart from these four compounds, the other compounds from the original 19 identified to inhibit PRRSV at 10 μM concentration or the analogs of these compounds could have a better profile of activity in other cell culture models for PRRSV replication or in in vivo studies. However, as PRRSV infection could not be successfully modeled in a laboratory animal, the in vivo studies will have to be performed in pigs or piglets. Considering the rapid evolution rate of PRRSV (Murtaugh and Genzow, 2011; Song et al., 2010), the eradication of the virus cannot be achieved only by immunological intervention, which is the trend observed in the field so far. Therefore, a therapeutic intervention with pharmacological agents inhibiting virus replication could represent a valuable alternative or additional tool against PRRSV. For the latter, a combined immunological and pharmacological intervention could decrease the risk of the emergence of resistant strains as well. In conclusion, we here identified several natural compounds displaying potent antiviral activity, providing a basis for further research for pharmacological agents against PRRSV.

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