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

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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.

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Table 1
A list of compounds in the published literature known to inhibit PRRSV replication and IC50 values for those estimated. NP – not published.

| 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 lipoxygenase (Baba et al., 2001; Zhang et al., 2005). |
| 4   | Coumarin A1 | Novobiocin related antibiotic with antiviral activity (Pali 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   | Cycloheximide | An dodeca-depsipeptide antibiotic, anti-inflammatory, lipo-oxygenase inhibitor. |
| 7   | 12-Deoxyphorbol 13-phenylacetate 20-acetate | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |

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 the DAPI intensity of treated cells to less than 70%. Con – control inhibitor MPA.

| No. | Compound | Known pharmacological action |
|-----|----------|-------------------------------|
| 1   | Antimycin A1 | A phorbol ester that activates PKCζ, and mimics IFN mediated signaling (Saraiva et al., 2004). |
| 2   | Bufalinb | An alkaloid anti-inflammatory agent; inhibits cytochrome P450 mediated oxidation (Raner et al., 2007). |
| 3   | Catechin hydrate (+)- | A phorbol ester that activates PKCζ, and mimics IFN mediated signaling (Saraiva et al., 2004). |
| 4   | Cepharanthineb | An alkaloid antiviral agent; inhibits cytokine production by immune cells (Kang et al., 2000; Shin et al., 2009). |
| 5   | Cinobufagina | A fungal metabolite that acts on endoplasmic reticulum resident Ca2+ ATPase and mediates activation of NFkB, a key component of immune cell signaling (Xia et al., 2006). |
| 6   | Cyclodextrin | A phorbol ester that activates PKCζ, and mimics IFN mediated signaling (Saraiva et al., 2004). |
| 7   | Cryptotanshinone | An alkaloid anti-inflammatory agent; inhibits cytochrome P450 mediated oxidation (Raner et al., 2007). |
| 8   | Hydrastine, D- | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| 9   | Cycloheximide | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| 10  | 12-Deoxyphorbol 13-phenylacetate 20-acetate | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |

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 6 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 lipoxygenase (Baba et al., 2001; Zhang et al., 2005). |
| 4   | Coumarin A1 | Novobiocin related antibiotic with antiviral activity (Pali 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   | Cycloheximide | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| 7   | 12-Deoxyphorbol 13-phenylacetate 20-acetate | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| 8   | Hydrastine, D- | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| 9   | Cycloheximide | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| 10  | 12-Deoxyphorbol 13-phenylacetate 20-acetate | 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 IC50 of the four compounds with the highest inhibitory activity. NP – not published.

| Compound | Known pharmacological action |
|----------|-------------------------------|
| Bufalin | Cardiac glycoside with antiviral activity (Cui et al., 2010). |
| 12-Deoxyphorbol 13-phenylacetate 20-acetate | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| Ouabain | Cardiac glycoside with antiviral activity (Cui et al., 2010). |
| Valinomycin | A dodeca-depsipeptide antibiotic, anti-inflammatory, lipo-oxygenase inhibitor. |

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 | Known pharmacological action |
|----------|-------------------------------|
| Bufalin | Cardiac glycoside with antiviral activity (Cui et al., 2010). |
| 12-Deoxyphorbol 13-phenylacetate 20-acetate | Known to be a potent antiviral agent against Severe Acute Respiratory Syndrome Corona virus (SARS CoV) (De Clercq, 2006; Wu et al., 2004). |
| Ouabain | Cardiac glycoside with antiviral activity (Cui et al., 2010). |
| Valinomycin | A dodeca-depsipeptide antibiotic, anti-inflammatory, lipo-oxygenase inhibitor. |

* Cardiac glycosides.

b Compounds known to act on ion channels.

Furnish 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 \( \mu \text{M} \) 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.)
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.
were used consistently throughout the studies. Following the antibody staining, the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The assay results were machine read with Cellomics® scanner, measuring the intensities of DAPI (indicating 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.

Fig. 1 (continued)
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 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, iso flavones, peptides, alkaloids, flavons, 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 PKC IFA assay, as described earlier, at 0.1, 1 and 8 μM concentrations of each 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 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.

References
Baba, M., Okamoto, M., Kashiwaha, N., Ono, M., 2001. Anti-HIV-1 activity and structure–activity relationship of cepharanoline derivatives in chronically infected cells. Antivir. Chem. Chemother. 12, 307–312.
Beura, L.K., Sarkar, S.N., Kwon, B., Subramaniam, S., Jones, C., Pattinak, A.K., Osorio, F.A., 2010. Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IF3 activation. J. Virol. 84, 1574–1584.
Cotarelo, M., Catalán, P., Sánchez-Carrillo, C., Menasalvas, A., Cercenado, E., Tenorio, A.K. Karuppannan et al. / Antiviral Research 94 (2012) 188–194 193
A., Bouza, E., Cytopathic effect inhibition assay for determining the in-vitro susceptibility of herpes simplex virus to antiviral agents. J. Antimicrob. Chemother. 44, 705–708.

Cui, X., Inagaki, Y., Xu, H., Wang, D., Qi, F., Kokudo, N., Fang, D., Tang, W., 2010. Anti-hepatitis B virus activities of cinobufacini and its active components bufalin and cinobufolin in HepG2.2.15 cells. Biol. Pharm. Bull. 33, 1728–1732.

De Clercq, E., 2006. Potential antivirals and antiviral strategies against SARS coronavirus infections. Expert. Rev. Ant. Infect. Ther. 4, 291–302.

Jiang, Y., Fang, L., Luo, R., Xiao, S., Chen, H., 2010. N-Acetylpencillamine inhibits the replication of porcine reproductive and respiratory syndrome virus in vitro. Vet. Res. Commun. 34, 607–617.

Kang, B.Y., Chung, S.W., Kim, S.H., Ryu, S.Y., Kim, T.S., 2000. Inhibition of interleukin-12 and interferon-gamma production in immune cells by tanshinones from Salvia miltiorrhiza. Immunopharmacology 49, 355–361.

Kreutz, L.C., Ackermann, M.R., 1996. Porcine reproductive and respiratory syndrome virus enters cells through a low pH-dependent endocytic pathway. Virus Res. 42, 137–147.

Kuzuhashira, T., Isawa, Y., Takahashi, H., Hatakeyama, D., Echigo, N., 2009. Green tea catechins inhibit the endonuclease activity of influenza A virus RNA polymerase. PLoS Curr. 1, RRN1052.

Lee, C., Yoo, D., 2006. The small envelope protein of porcine reproductive and respiratory syndrome virus possesses ion channel protein-like properties. Virology 10, 30–43.

Murgaht, M.P., Cenzow, M., 2011. Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). Vaccine 29, 8192–8204.

Mishra, B.B., Tiwari, V.K., 2011. Natural products: an evolving role in future drug discovery. Eur. J. Med. Chem. 46, 4769–4807.

Patel, D., Nan, Y., Shen, M., Ritthipichai, K., Zhu, X., Zhang, Y.J., 2010. Porcine reproductive and respiratory syndrome virus inhibits type I interferon signaling by blocking STAT1/STAT2 nuclear translocation. J Virol. 84, 11045–11055.

Raner, C.M., Cornelious, S., Moullick, K., Wang, Y., Mortenson, A., Coch, N.B., 2007. Effects of herbal products and their constituents on human cytochrome P450(2E1) activity. Food Chem. Toxicol. 45, 2359–2365.

Saraiva, L., Fresco, P., Pinto, E., Gonçalves, J., 2004. Characterization of phorbol esters activity on individual mammalian protein kinase C isoforms, using the yeast phenotypic assay. Eur. J. Pharmacol. 491, 101–110.

Shi, M., Lam, T.T., Hon, C.C., Hui, R.K., Faahbeg, K.S., Wenhblom, T., Murtaugh, M.P., Stadnejek, T., Leung, F.C., 2010. Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res. 154, 7–17.

Shin, D.S., Kim, H.N., Shin, K.D., Yoon, Y.J., Kim, S.J., Han, D.C., Kwon, B.M., 2009. Cryptotanshinone inhibits constitutive signal transducer and activator of transcription 3 function through blocking the dimerization in DU145 prostate cancer cells. Cancer Res. 69, 193–202.

Sievers, T.M., Rossi, S.J., Ghobrial, R.M., Arriola, E., Nishimura, P., Kawan, M., Holt, C.D., 1997. Mycophenolate mofetil. Pharmacotherapy 17, 1178–1197.

Shin, D., Cui, D., Cui, J., Zhao, B., 2010. Accelerated evolution of PRRSV during recent outbreaks in China. Virus Genes 41, 241–245.

Song, J., Chen, H., Shi, M., Lam, T.T., Hon, C.C., Hui, R.K., Faahbeg, K.S., Wenhblom, T., Murtaugh, M.P., Stadnejek, T., Leung, F.C., 2010. Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res. 154, 7–17.

Su, C.T., Hsu, J.T., Hsieh, H.P., Lin, P.H., Chen, T.C., Kao, C.L., Lee, C.N., Chang, S.Y., 2008. Anti-HSV activity of digitoxin and its possible mechanisms. Antiviral Res. 79, 62–70.

van der Meer, F.J., de Haan, C.A., Schuurman, N.M., Hjartmen, B.J., Peumans, W.J., Van Denme, E.J., Delport, P.L., Balzarini, J., Egberink, H.F., 2007. Antiviral activity of carbohydrate-binding agents against Nidovirales in cell culture. Antiviral Res. 76, 21–29.

Wu, C.Y., Jan, J.T., Ma, S.H., Kuo, C.J., Juan, H.F., Cheng, Y.S., Hsu, H.H., Huang, H.C., Wu, D., Brik, A., Liang, F.S., Liu, R.S., Fang, J.M., Chen, S.T., Liang, P.H., Wong, C.H., 2004. Small molecules targeting severe acute respiratory syndrome human coronavirus. Proc. Natl. Acad. Sci. USA 6, 10012–10017.

Xia, W., Shen, Y., Xie, H., Zheng, S., 2006. Involvement of endoplasmic reticulum in hepatitis B virus replication. Virus Res. 121, 116–121

Zhang, C.H., Wang, Y.F., Liu, X.J., Lu, J.H., Qian, C.W., Wan, Z.Y., Yan, X.G., Zheng, H.Y., Zhang, M.Y., Xiong, S., Li, J.X., Qi, S.Y., 2008. Antiviral activity of cepharanthine against severe acute respiratory syndrome coronavirus in vitro. Chin. Med. J. (Engl.) 20, 493–496.