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Determining anti-betanodavirus compounds through a GF-1 cell-based screening platform

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ABSTRACT

Betanodavirus is a highly contagious pathogen, responsible for severe losses incurred in the aquaculture industry. Currently, there are no commercially available antivirals against the virulence observed during very early stages of fish larvae development. Therefore, we developed a novel GF-1 (grouper fin cell) cell viability-based screening assay to facilitate the discovery of an anti-betanodavirus agent. The assay conditions were optimized and the robustness of the assay was confirmed by a Z' factor value ranging from 0.7 to 0.94. After screening a library of 2000 small molecule compounds, 43 compounds with a virus inhibition capacity of $\geqslant$55% were identified. A cytochrome P450 inhibitor, proadifen hydrochloride, was validated with an EC_{50} value of 6.48 μM and a CC_{50} value of 20.63 μM. This compound inhibited the amplification of viral RNA by 99.68% 5 days post-infection. Surprisingly, we found that 18 of 43 compounds act as neurotransmitter agents. These findings indicate a novel way of investigating the infection mechanism of betanodavirus, and suggest potential candidates for an anti-betanodavirus drug.

1. Introduction

Viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER), is a contagious disease affecting both marine and freshwater fish. In the 1990s, the disease was shown to be caused by the betanodavirus, also known as the nervous necrosis virus (NNV). It is a non-enveloped, icosahedral virus with 2 positive-sense single-stranded RNA molecules (Yoshikoshi and Inoue, 1990). This virus, with worldwide distribution, has caused high mortality rates among many aquaculture fishes, including Barramundi, European sea bass, striped jack, Japanese parrotfish, and grouper (Munday et al., 2002). In 1997, VNN was observed in hatchery-reared juvenile groupers in southern Taiwan. The infected juvenile fish showed uncoordinated swimming patterns and high mortality rates (Chi et al., 1997). Although the viral infection is observed throughout the life cycle of the host fish, the virus-induced mass mortalities mainly occur during the larval and juvenile stages of the host fish. The death rate often reaches 50–100% (Munday et al., 2002). Such losses greatly damage the aquaculture industry, especially in fish larva production.

The NNV is transmitted both vertically and horizontally (Arimoto et al., 1993, 1992; Le Breton et al., 1997). Currently, there are many methods and technologies for prevention of NNV infections. The vertical NNV transmission can be controlled by selecting NNV-free brood fish through PCR detection of the viral gene from the gonads before spawning (Mushiake et al., 1994). The virus, which survives on the surface of fish eggs, can also be inactivated by ozonated seawater (Arimoto et al., 1996; Grotmol and Totland, 2000). To intervene in horizontal transmission, the regular screening of NNV from fresh feed and stockfish can reduce contamination rates. Improving field management methods, thereby decreasing stress levels imposed on the cultured fish, can reduce the rate of virus infection (Munday et al., 2002; Samuelsen et al., 2006; Shetty et al., 2012). However, NNV has been found to be carried by various aquatic invertebrates, including the charybdid crab, Southern humpback shrimp, and Mediterranean mussel (Gomez et al., 2008). It is, therefore, difficult to completely block viral infections. Antimicrobial peptides (AMPs) have been widely studied for the prevention of bacterial, fungal, parasitic, and viral infections. Grouper epinecidin-1 has been found to have an antiviral activity against the NNV in both cBB cells and freshwater medaka (Chia et al., 2010; Wang et al., 2010). AMPs may inhibit NNV infections

Abbreviations: AMPs, antimicrobial peptides; GF-1, grouper fin-1; MTT, methylthiazol tetrazolium; NNV, nervous necrosis virus; ROS, reactive oxygen species; VER, encephalopathy and retinopathy; VNN, viral nervous necrosis.

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through agglutination of virus particles. However, because of the complexity of the aquatic environment, the mechanism of agglutination is limited due to the lack of a specific target. Vaccination of host fish by virus-like particles and recombinant capsid proteins has proven to induce host-specific immunity in adult and juvenile fish (Husgard et al., 2001; Liu et al., 2006). For fish larvae older than 40 days post-hatch (dph), a bath immunization strategy using chemically inactivated betanodavirus has been shown to protect grouper larvae from VNN (Kai and Chi, 2008). However, a previous study on *Epinephelus bruneus* found that the primordial primary lymphoid organs, the thymus and kidney, are present at 12 and 1 dph respectively; nevertheless, the lymphocytes in these organs are not distinguishable until 21 and 30 dph, respectively (Kato et al., 2004). This indicates that the development of the immune system during the larval stages before 30 dph is incomplete. Therefore, this immunization method is ineffective during the very early stage of fish larval development. Because of the lack of an effective antiviral system during the very early stage of fish larva and the low availability of commercial vaccine, the development of new antiviral methods is needed.

Synthetic compounds or extracts from natural products with antiviral properties are potential antiviral candidates, which may be used in the aquaculture field. Since these compounds are stable at room temperature and are membrane permeable, they are easily preserved and can be applied by injection, immersion, or oral administration. Previous research has shown that inhibitors of endosomal acidification (e.g., NH_4Cl, chloroquine, and bafilomycin A1) can inhibit RGNNV-induced CPE 6 days post-infection (dpi) and beyond in E-11 cells (Adachi et al., 2007). In 2010, Dasyscyphin C, an extract from *Eclipta prostrata*, was found to have anti-NNV properties at a concentration of 20 $\mu g\,mL^{-1}$ in the SIGE cell line where the cell viability was 22% 6 dpi (Krishnan et al., 2010). Furthermore, by treating with gymnemagenol and furan-2-yl acetate, the NNV titer ($TCID_{50}\,ml^{-1}$) reduced to log 2.8 and log 2.45 and the SIGE cell viability was 47% and 90%, respectively. These extracts are considered as antiviral agents (Gopiesh Khanna et al., 2011; Suthindhiran et al., 2011). Although there are several confirmed anti-NNV agents, the development of an anti-NNV drug is still delayed because of the lack of a large-scale, systemic compound screening process, which can focus on compounds with both known biological activities and commercial availability.

In this study, by developing a cell-based, virus-induced CPE-dependent assay, we constructed a platform for screening small molecule compounds as potential inhibitors of the NNV. Some novel antiviral agents that protect host cells from NNV infection have been identified.

2. Materials and methods

2.1. Cells, viruses, and compounds

Grouper-fin cells (GF-1) (Chi et al., 1999) and GNNV (grouper nervous necrosis virus) (Chi et al., 2001) were obtained as kind gifts from Prof. Shau-Chi Chi at the National Taiwan University, Taiwan. GF-1 cells were cultured as a monolayer at 28 °C in Leibovitz L-15 medium (Gibco) supplemented with 10% fetal bovine serum (Invitrogen). The GNNV was amplified by infecting GF-1 cells with an M.O.I. (multiplicity of infection) of 10. The spectrum collection (MicroSource Discovery System, Inc. http://www.msdiscovery.com) used in the screen consisted of 1000 known drugs, 600 natural products, and 400 bioactive components. These compounds are featured in a wide range of biological activities and structural diversity. Each compound was dissolved in 100% DMSO and stored at −20 °C.

2.2. Cell preparation

For antiviral screening and the dose-dependent assay, GF-1 cells were seeded in 96-wells tissue culture plates at a cell density of 20,000 cells/well in L-15 medium with 10% FBS and incubated at 28 °C for 12 h. For the RT-qPCR assay, the cells were seeded in 12-well tissue culture plates at a cell density of 100,000 cells/well.

2.3. Antiviral screen

2.3.1. Screening optimization

In order to obtain the highest dynamic range between the mock infection group and the virus infection group, the cells were infected with a GNNV dosage at an M.O.I. of 1, 10, and 100, and incubated at 28 °C for 5 days. The cells were sampled every day and analyzed by MTT assay.

2.3.2. Screening procedure

Each test compound was diluted in PBS to a final concentration of 10 μM and incubated with the betanodavirus suspension (M.O.I. = 10 in L-15 medium containing 1% FBS) at 28 °C for 1 h. The final DMSO concentration was 0.1%. Each plate consisted of 8 wells representing the cell-only control, 8 wells for the virus-only control, and 80 wells for testing individual compounds. The old medium in the cell plates was replaced with medium containing the compound-virus mix and incubated at 28 °C for 5 days. The inhibition percentage of GNNV-induced CPE was determined by an MTT cell viability assay.

2.3.3. MTT cell viability assay

Methylthiazol tetrazolium (MTT; Invitrogen)-based cell viability test was performed according to the manufacturer's instruction. In brief, when cells were ready for testing, the medium was replaced with fresh L-15 medium containing 1% FBS supplemented with 10 μl MTT (5 gm/ml) and stored at 28 °C for 4 h. 100 μl SDS–HCl (10% SDS/0.01 M HCl) was then added to each well and the plate was incubated at 28 °C for 12 h. The OD_{570} absorption was measured using an uQUANT microplate reader (BioTek).

The OD_{570} absorbance data collected from each MTT assay plate were converted to inhibition percentages of NNV-induced CPE by using the following equation:

$$[A_{OD570} - Mean_V]/[Mean_C - Mean_V] \times 100\%$$

Here, A_{OD570} is the OD_{570} absorbance signal of each tested well, $Mean_V$ is the mean signal of virus-only control wells, and $Mean_C$ is the mean signal of cell-only control wells. The Z' factor for each screen for the evaluation of the quality of assays was calculated as follows:

$1 - [3 \times (\text{standard deviation of cell control} + \text{standard deviation of virus control})/(Mean_C - Mean_V)]$ (Zhang et al., 1999). The signal-to-background noise ratio was determined by $[Mean_C/Mean_V]$.

2.4. Hits validation

2.4.1. Dose-dependent assay

Compounds showing antiviral activity in the primary screen were subjected to further tests in a dose-dependent assay. Each test compound was serially diluted to a final concentration ranging from 0.20 to 100 μM and transferred to another plate. After incubation with betanodavirus suspension (M.O.I. = 10 in L-15 medium containing 1% FBS) for 1 h, each solution was transferred to a cell plate and incubated at 28 °C for 5 days. The results were determined by MTT assay, as described above.

2.4.2. RNA extraction, reverse-transcription, and real-time PCR

To further understand the dynamic change of viral RNA following treatment with the hit compounds, the hit compound was diluted to a final concentration of 1 and 10 μM and mixed with NNV (M.O.I. = 100) in L-15 medium (containing 1% FBS). The solution was incubated at 28 °C for 1 h, and then transferred to a cell plate and incubated at 28 °C. For each well, 100 μl of medium was sampled 3 and 5 dpi and stored at −80 °C until RNA extraction.

RNA extraction was conducted according to the following protocol: 100 μl of each sample was homogenized in 200 μl of TRI reagent (Bioman). Samples were left to stand at room temperature for 5 min after which 50 μl of chloroform was added. The sample was then mixed gently and left to stand for 3 min at RT. It was then centrifuged at 13,600 rpm for 15 min at 4 °C. The aqueous phase (150 μl) was transferred to a fresh tube, precipitated by adding 500 μl of isopropanol, and incubated at 4 °C for 20 min. The samples were then centrifuged at 4 °C for 10 min. The isopropanol was removed and the tube was washed with 500 μl of 70% EtOH (13,500 rpm for 5 min). The RNA pellet was air-dried briefly and dissolved in 20 μl of DEPC-treated water.

The first strand of cDNA was synthesized with HiScript Reverse Transcriptase (Bionova) and a random hexamer according to the manufacturer's instructions. Real-time PCR was performed in a 25-μl mixture containing 12.5 μl of SYBR green supermix (Bionova) and 1 μl of each primer (0.2 μM), namely, qNODAfor1 and qNODArev2 (Ciulli et al., 2006). After adding 5.5 μl of DEPC treated water and 5 μl of cDNA template, the mixture was transferred to a Bio-Rad MyIQ realtime PCR system (Bio-Rad, Hercules, CA, USA). The following amplification conditions were used: 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, 20 s of annealing at 58 °C, and 30 s of extension at 72 °C for 40 cycles. Fluorescence was acquired during each extension step. A standard curve was constructed by serially diluting an NNV stock (10^9 $TCID_{50}$ ml^{-1}) in L-15 medium before RNA extraction (Mazelet et al., 2010). An MTT cell viability assay was conducted at 5 dpi for the confirmation of GF-1 cell survival.

3. Results

3.1. Optimization of the cell-based screen

The cytotoxicity of DMSO to GF-1 cells has previously been investigated (data not shown), and the results indicated that a DMSO concentration lower than 0.125% has no significant effect on cell viability in an MTT assay. The results of a time-course assay are shown in terms of absorbance values, measured as OD_{570} (Fig. 1). The mock infection group grew rapidly throughout the test, while the growth of other cells peaked at 3 dpi and gradually decreased until 5 dpi. This indicates that GF-1 cells have a similar survival rate when treated with each viral dosage in the test and that there is an approximately 3-fold difference between the mock and infection groups 5 dpi.

3.2. Antiviral screen

A primary screen of 2000 compounds was conducted in a 96-well format. *Z′* factors ranged from 0.7 to 0.94, with a median of 0.82 (Fig. 2a) and an average signal-to-background noise ratio of 6.05 ± 1.63 (Fig. 2b). These results indicate that the assay is a robust technique of high quality. In this assay, the average inhibition of all compounds was 4%, with a standard deviation of 17%. The threshold value (55%) was determined by the average inhibition percentage of all compounds plus 3-times the standard deviation. Forty-three compounds had an antiviral activity higher than the

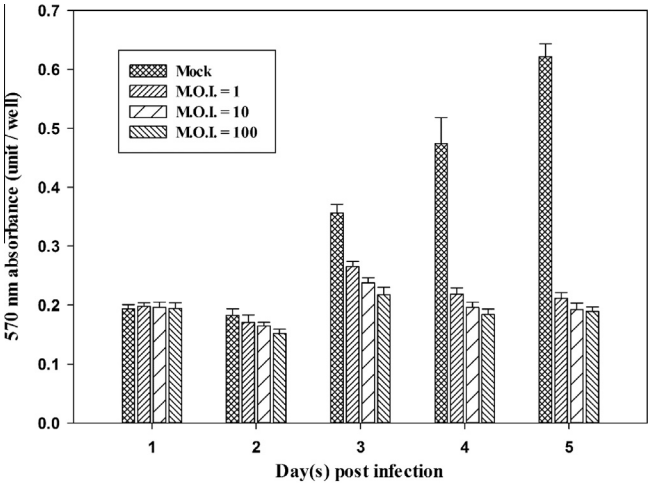

Fig. 1. Time-course assay of GNNV infection. GF-1 cells, which were infected with GNNV at an M.O.I. ranging from 1 to 100, were sampled every day, and the cell viability is expressed as the absorbance at 570 nm.

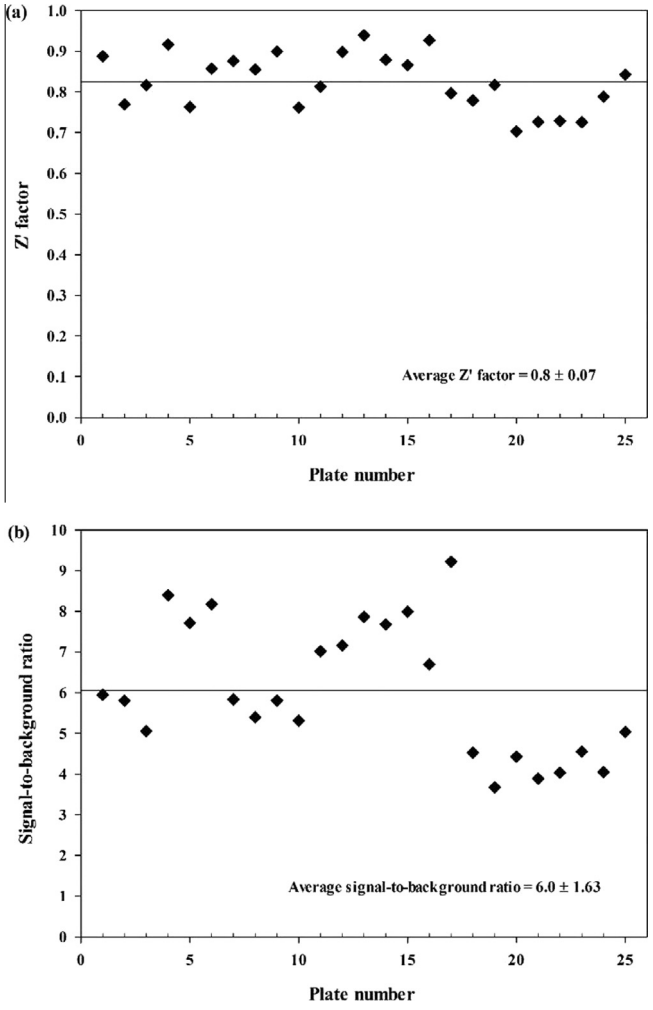

Fig. 2. Validation of antiviral screen assay robustness. (a) The average *Z′* factor. Each dot represents the average *Z′* factor for each plate. (b) Signal-to-background noise ratio for each plate. Each dot represents the average signal-to-background noise ratio for each plate. The horizontal line indicates the average value of the *Z′* factor and the signal-to-background noise ratio of 25 plates.

threshold value. These compounds were considered potential candidates for further evaluation (Fig. 3).

3.3. Hits validation

3.3.1. Dose-dependent assay

One of the selected hit compounds, proadifen hydrochloride (Fig. 4), was analyzed in a dose-dependent assay. The results showed that the inhibition percentage of GNNV-induced CPE in GF-1 cells gradually increased as the concentration of proadifen hydrochloride increased (Fig. 5). The inhibition percentage peaked at a concentration of 12.5 μM (80.79% inhibition), and dropped rapidly to a inhibition percentage under 0% at a concentration above 25 μM. The EC_{50} and CC_{50} values for proadifen hydrochloride were 6.48 and 20.63 μM, respectively.

3.3.2. RT-qPCR confirmation of viral RNA amplification

To further investigate the viral RNA amplification changes when treated with a hit compound, the betanodavirus was mixed with proadifen hydrochloride at concentrations of 1 and 10 μM for 1 h and then used to infect the GF-1 cells. The relative quantity of viral RNA2 was detected 3 and 5 dpi. As a result, the inhibition percentage was higher at 10 μM (125.81%) than at 1 μM (23.96%) in the MTT cell viability assay 5 dpi (Fig. 6a). The NNV-infected GF-1 cells treated with 1 μM proadifen hydrochloride showed a viral RNA2 percentage of 4.13% and 54.89% 3 and 5 dpi, respectively (Fig. 6b). The viral RNA2 percentage was 1.33% and 0.32% for cells treated with 10 μM proadifen hydrochloride (Fig. 6b). These data indicate that the compound significantly inhibited viral RNA2 amplification at 10 μM 5 dpi.

4. Discussion

For prevention of virus infection, one of the most concerning issues is the adaptability of certain viruses to selective evolutionary pressure. This is especially the case for RNA viruses, which have high mutation rates, high yields, and short replication times (Domingo and Holland, 1997). Therefore, a long-term antiviral treatment with a specific mechanism will be at a higher risk of generating resistance. In this study, we developed a cell-based antiviral screen against the NNV. Such screening has the potential to

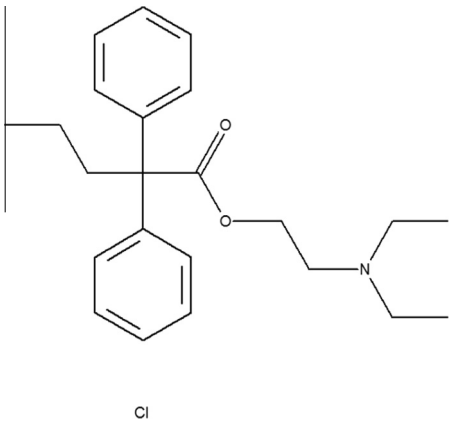

Fig. 4. Chemical structure of proadifen hydrochloride.

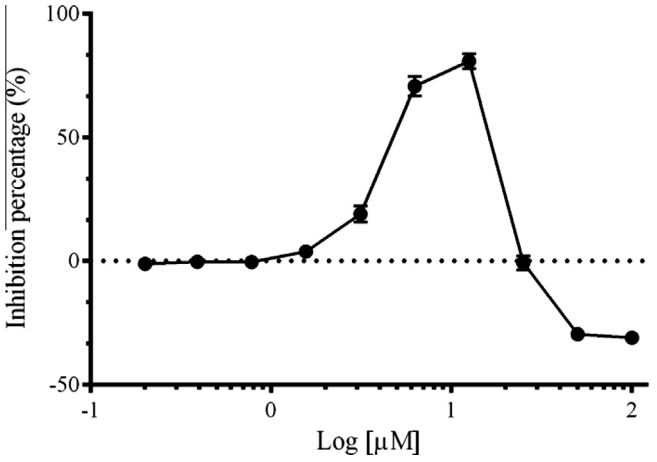

Fig. 5. Dose dependency assay of proadifen hydrochloride. The GF-1 cells were infected by GNNV (M.O.I. = 10) and incubated with serial-diluted concentrations of proadifen hydrochloride, the inhibition of GNNV-induced CPE was determined by an MTT assay and expressed in terms of inhibition percentage.

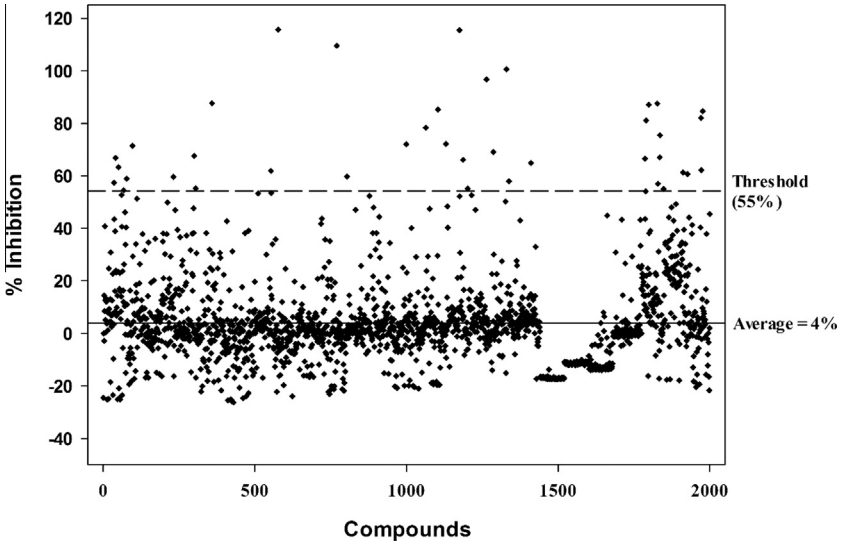

Fig. 3. The inhibition percentage of GNNV-induced cell death obtained when screening 2000 compounds. Each dot represents the inhibition percentage of one compound. The dashed line indicates the threshold value for the selection of the hit compounds.

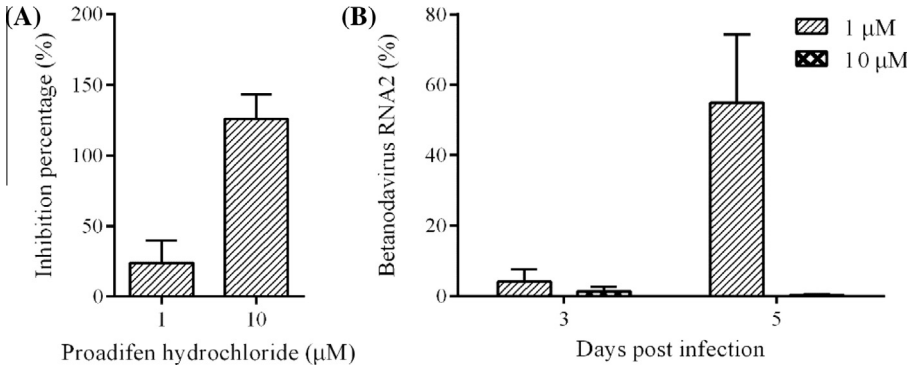

Fig. 6. Validation of antiviral effect. (A) Inhibition percentage of betanodavirus-induced CPE at 5 dpi after treatment with proadifen hydrochloride. (B) Real-time RT-PCR against GNNV RNA2 after treatment with proadifen hydrochloride 3 and 5 dpi. GF-1 cells infected with GNNV were incubated with proadifen hydrochloride at 1 and 10 μM. The culture medium was sampled 3 and 5 dpi for real-time RT-PCR and the cells were harvested 5 dpi for the MTT assay.

identified compounds with various antiviral properties at the cellular level. The results may not only be used as antiviral therapies, but will also help us to further understand the interactions between viruses and host cells.

We present the results of primary screening of a 2000-compound library and measure the reproducibility of the assay robustness. The robustness and quality of the assay were estimated by determining the Z' factor and signal-to-background noise ratio of each screen. As a characteristic parameter, the Z' factor reflected the quality of the assay itself. Therefore, an excellent assay should have a Z' factor ranging from 0.5 to 1 (Zhang et al., 1999). In our system, the Z' factor of each screen ranged from 0.7 to 0.94, with a median of 0.82. This indicates that our screening platform is suitable for screening larger numbers of compounds.

For the majority of the hit compounds, it is important to investigate their antiviral effects as well as their bioactivities within host cells. Fortunately, many of these compounds already have bioactivities that have been identified in previous research. These known bioactivities were collected from the PubChem database and are summarized in Table 1. As expected, there were 4 hit compounds with ion regulation relative bioactivities (e.g., calcium channel blocker and proton pump inhibitor). Calcium has been found to be required during the assembly of DGNNV virus-like particles (Wu et al., 2008). Therefore, blocking calcium channels may result in the interruption of virus assembly.

Neurotransmitter agents are mostly categorized as antipsychotics in the spectrum collection. Though the category accounts for only 3.3% in the collection, the neurotransmitter agents were significantly enriched in the list of hit compounds (41.9%). Moreover, a monoamine oxidase inhibitor (MAOI), which prevents the breakdown of monoamine neurotransmitters, was identified. Since the neurotransmitter agents that identified in the screen are all biologically related to monoamine neurotransmitters. It implies a potential involvement of the betanodavirus infection in the monoamine neurotransmitter system. However, negative results were also generated by neurotransmitter agents with similar activities (e.g., dopamine antagonist). This may due to the structural or functional variation between human and fish version of target protein. Also, since GF-1 cells are not neuron cells but fibroblast-like cells. The antiviral effect of neurotransmitter agents may due to the structural similarity between agents and functional protein of virus or host cell rather than alteration in the synaptic transmission between neuron cells. However, since the betanodavirus causes major damage to the nervous system of fish, this finding provides a novel way for better understanding the amplification mechanism used by the betanodavirus.

In this study, one of the hit compounds, proadifen hydrochloride (SKF-525A), was found to be a nonspecific cytochrome P450 (CYP) inhibitor (Franklin and Hathaway, 2008), which shows inhibitory effect on CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A. It has been widely used for studying cancer therapies and drug metabolism. In the present study, proadifen hydrochloride was shown to possess anti-betanodavirus activity (70.04% inhibition). Such activity includes inhibition of both virus-induced CPE and viral RNA amplification in GNNV-infected GF-1 cells.

Previous research indicates that betanodavirus induces reactive oxygen species (ROS) production in GF-1 cells. Such phenomenon is correlated with the opening of mitochondrial permeability transition pore (MPTP) and leads to the loss of mitochondrial membrane potential (MMP) (Chen et al., 2006). The ROS production can be inhibited through treatment with anti-oxidants such as N-acetylcysteine (NAC) and diphenyliodonium (DPI, Complex I inhibitor) within 48 h post-infection. Due to the insufficient of the reduction in oxidative stress with the treatment. The mitochondrial-mediated cell death occurs in the late replication stage of virus (48–72 h post-infection) (Chang et al., 2011). In this study, the NAC in the spectrum collection were shown to be negative in the screen. This may due to the relatively low dosage (10 vs. 1000 μM) and longer incubation time (5 d vs. 48 h).

CYP has been identified as a source of ROS (Park et al., 1996; Zangar et al., 2004). Recently, a research demonstrated that ROS production and mitochondrial permeability transition (MPT) induced by tetrandrine, a compound extracted from the root of *Stephania tetrandra*, can be inhibited by proadifen in rat hepatocyte (Qi et al., 2013). Since betanodavirus infection also triggers the ROS production and MPT, the mechanism of the anti-betanodavirus activity of proadifen might be the inhibitory effect of cytochrome P450, which would, therefore, reduce GF-1 cell death induced by cellular ROS production. Moreover, morin, a specific CYP3A inhibitor (Hodek et al., 2002) in the spectrum collection showed no effects on GNNV-induced CPE in the screen. These might indicates that only the nonspecific CYP inhibitor like proadifen could block ROS production more efficiently. Indeed, it is possible that proadifen blocks virus infection via a specific enzyme inhibition, however, due to the lack of the knowledge of the cellular events during virus infection, this possibility needs further investigation.

In summary, our system can fulfill the requirements of a screening platform for anti-betanodavirus inhibitors. The system may be a useful tool to investigate the possible mechanism of virus-host interactions. Future research should focus on further validation of the antiviral mechanism of hit compounds and the possible application of fish antiviral therapeutics.

Table 1
Summary of the known bioactivities of the hit compounds.

Category	Bioactivity	Compound*
Anti-infective agents	Antibacterials	Azithromycin
	Antifungals	Hexetidine
	Antimalarials	Quinidine gluconate
Coloring agents	Fluoresceins	Erythrosine sodium
Enzyme inhibitor	Cytochrome P450 inhibitor	Lansoprazole
		Proadifen hydrochloride
	Monoamine oxidase inhibitors	Harmaline
Hormones	Adrenal cortex hormones	5alpha-androstan-3,17-dione
	Androsterone	Epiandrosterone
	Estrogen antagonists	Tamoxifen citrate
Membrane transport modulators	Calcium channel blockers	Amlodipine besylate
		Bepridil hydrochloride
		Diltiazem hydrochloride
	Proton pump inhibitor	Lansoprazole
Neurotransmitter agents	Adrenergic antagonists	Carvedilol
		Dexpropranolol hydrochloride
		Mianserin hydrochloride
		Propranolol hydrochloride (+/−)
	Adrenergic uptake inhibitors	Maprotiline hydrochloride
		Trimipramine maleate
	Dopamine agonists	Metergoline
	Dopamine antagonists	Prochlorperazine edisylate
		Promazine hydrochloride
		Spiperone
		Thioridazine hydrochloride
		Thiothixene
		Trifluoperazine hydrochloride
		Triflupromazine hydrochloride
	Histamine h1 antagonists	Mebhydrolin naphthalenesulfonate
		Mianserin hydrochloride
	Serotonin antagonists	6-Methoxyharmalan
		Metergoline
		Mianserin hydrochloride
		Nafronyl oxalate
	Serotonin uptake inhibitors	Alaproclate
Peripheral nervous system agents	Anesthetics	Bupivacaine hydrochloride
		Tetracaine hydrochloride
	Anti-inflammatory agents	Cepharanthine
		Nimesulide
	Parasympatholytics	Hydroquinidine
		Mebeverine hydrochloride
Phytochemicals	Unknown	Pregnenolone succinate
Sterols	Unknown	Lanosterol acetate
		Rockogenin
Unknown	Unknown	18-Aminoabieta-8,11,13-triene sulfate
		6-Hydroxyangolensic acid methyl ester
		Hydroquinine hydrobromide hydrate

* One compound may possess multiple bioactivities.

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