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Synthesized flavanoid-derived ligand reduced dengue virus type-2 replication *in vitro*

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ABSTRACT

Objective: To investigate the antiviral property of a lead ligand, YK51 that was synthesized based on the flavanoid of a natural product toward dengue virus type-2 (DENV2) replication.

Methods: cRNA was isolated from HepG2 cells inoculated with 1 000 median tissue culture infective dose of DENV2 and treated with different doses of the ligand followed by RT-PCR to quantify the virus gene copies. Confocal microscopy of actin and tubulin redistribution was also performed.

Results: The quantitative RT-PCR result showed reduction of the DENV2 gene copies as the ligand concentration was increased. The confocal microscopy result showed increase in the tubulin intensity (79.6%) of infected BHK21 cells treated with the ligand, compared with the non-treated cells (54.8%). The 1.5-fold increase in the intensity of tubulin suggested that the ligand inhibitory effect stabilized the cellular microtubule structure.

Conclusions: The synthesized ligand YK51 reduced DENV2 viral load by inhibiting virus replication thus is highly potential to be developed as antiviral agent.

1. Introduction

Dengue is one of the most important mosquito-borne viral diseases imposing serious health concerns throughout both the tropical and subtropical regions worldwide [1]. Its incidences have increased drastically in recent years with approximately 3 900 million people at risk of transmission [2]. Susceptible human hosts are infected following a bite from an infectious mosquito vector of the *Aedes* species from the subgenus *Stegomyia* [3]. There are four antigenically related but genetically distinct serotypes of the virus that causes dengue, established as dengue virus types 1–4 (DENV1–DENV4). This virus belongs to the Flaviviridae family having a single strand positive-sense RNA genome that codes for three structural proteins and seven non-structural viral proteins [4].

Even though dengue diseases represented significant economic and health burden in endemic countries, neither an effective antiviral agent nor a licensed vaccine was yet available for dengue [5]. Nevertheless, there are many discovery efforts currently to develop intervention agents toward dengue, mostly targeting the replication process, which involved the activity of NS2B3 viral protease [6,7]. Furthermore, advances in computational molecular modelling techniques have enabled the understanding of protein-ligand binding [8]. Through studies using computational dockings of competitive and non-competitive ligands to the DENV2 serine protease [9,10], a group of small molecules were designed and synthesised with reference to a natural product template. A following study has reported on the inhibitory activities of these synthesized flavanoid-derived ligands toward dengue virus activity *in vitro* [11].

The current study aims to investigate the effect of the lead ligand YK51 inhibitory activity at gene level. It was reported previously that this ligand was highly potent against DENV2

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indicated by the 10-fold reduction in the viral load with 92% inhibition against 1 000 median tissue culture infective dose ($TCID_{50}$) of DENV2 *in vitro* [11]. These results suggested intervention at the intracellular level, most possibly during virus replication. Therefore, quantitative RT-PCR was carried out on cRNA isolated at post *in vitro* inhibition assay to quantify the dengue virus protease gene copies. Confocal microscopy was also performed to test the effect of the ligand on cytoskeletal redistribution of the infected cells.

2. Materials and methods

2.1. Inhibitor

The design and synthesis of the flavanoid-derived ligand abbreviated as YK51 used in this have been reported elsewhere (data not shown). The ligand was designed and synthesized at the Drug Design and Development Research Group facility in University of Malaya, Malaysia. The confocal laser scanning microscopy was carried out at the Institute for Molecular Medical Biotechnology in the Faculty of Medicine, Universiti Teknologi MARA, Malaysia. Its molecular structure was stated as ethyl 1-tert-butoxycarbonyl-2-butyl-4-phenyl)-piperidinyl-3-carboxylate. Briefly, the ligand was designed based on the flavonoid panduratin A that was purified from the root of *Boesenbergia rotunda*, a common spice belonging to the ginger family.

2.2. Cells and viruses

HepG2 hepatocellular carcinoma cells and BHK21 cells derived from kidney of baby hamster were sub-cultivated regularly in 1 × concentrated growth medium of Dulbecco's modified Eagle medium (GIBCO, Thermo Fisher Scientific, MA, USA) supplemented with 2 mmol/L L-glutamine, 10% foetal bovine serum (FBS), 35 mmol/L of sodium bicarbonate, 20 mmol/L of HEPES buffer, 100 μg/mL of penicillin G and 100 μg/mL of streptomycin at 37 °C in a humidified incubator with presence of CO_2 at 5%.

DENV2 used in this study was a prototype of the New Guinea C strain and was a kind gift from the Medical Microbiology Department in the Faculty of Medicine, University of Malaya, Malaysia. Virus stock was prepared by inoculating monolayer of C6/36 cells (derived from larvae of *Aedes albopictus*) in a 25 cm^2 culture flask with virus diluted 1:5 to 1:10 in 1 mL of Leibovitz's L-15 (GIBCO, Thermo Fisher Scientific, MA, USA) containing 2% FBS. The flask was incubated at 28 °C for an hour before addition of 4 mL of Leibovitz's L-15 supplemented with 2% FBS. The inoculated cells were allowed to propagate for 6–7 days until the cytophatic effect was confirmed. Cell debris was removed by centrifugation at 3 500 r/min for 5 min and the viral supernatant was collected in aliquots of 1 mL each and stored at −80 °C until further use.

2.3. cRNA extraction and synthesis of DENV2 RNA standard

The *in vitro* inhibition assay was carried out as described previously [11]. Briefly, monolayer of HepG2 cells was inoculated with 1000 $TCID_{50}$ of DENV2 followed by treatment with different doses of the YK51 ligand not exceeding its maximum non-toxic dose. cRNA was isolated at 96-h post-inhibition using the RNeasy Mini Kit (Qiagen, Germany). The post-inhibition HepG2 cells were washed with phosphate buffer saline (PBS) and trypsinised with TrypLE™ Express (Invitrogen, Thermo Fisher Scientific, MA, USA) before centrifugation at 3 000 r/min for 5 min. The harvested cell pellet was resuspended in lysis buffer and cRNA was extracted according to the protocol of the RNeasy Mini Kit (Qiagen, Germany) and finally eluted in 30–50 μL RNase-free water.

The DENV2 RNA standard was prepared by *in vitro* synthesis of RNA transcript from DNA template of DENV2 protease as described previously [11,12]. The *in vitro* transcription was carried out using the Ampliscribe™ T7-Flash Transcription Kit (EPICENTRE® Biotechnologies, Madison, USA) utilising the PCR product of DENV2 amplification. Firstly, RT-PCR was performed using a T7 promoter sequence incorporated into the forward primer (IVT NS2B3 primer) and reverse primer NS2B3 (Table 1). The incorporated T7 promoter sequence would give an extra length of about 25 bp to the RNA standard, thus separating it from the target gene. The PCR product (1 μg) was then subjected to *in vitro* transcription (IVT) at 37 °C for 1 h with a final reaction volume of 20 μL containing 1 × Ampliscribe™ T7-Flash reaction buffer, 9 mmol/L each of adenosine triphosphate, cytidine triphosphate, guanosine triphosphate and uridine triphosphate, 10 mmol/L dithiothreitol and 2 units of Ampliscribe™ T7-Flash enzyme.

The IVT product was then treated with 1 IU of DNase I and incubated at 37 °C for 15 min and purified by ammonium acetate/ethanol precipitation before reconstituted in RNase-free water. The amount of IVT generated DENV2 RNA standard fragment was determined spectrophotometrically with GeneQuantpro (GE Life Sciences, Marlborough, MA, USA) and the reading was converted to molecular copies using the following formula [13]:

$$Y \text{ molecules}/\mu L = [(X \text{ g}/\mu L \text{ RNA})/(\text{transcript length (bp)} \times 340)] \times 6.02 \times 10^{23}$$

Table 1
Oligonucleotide primers used for the *in vitro* transcription synthesis of DENV2 RNA standard.

Primer	Sequence of primer
IVT DENV2 (NS2B/NS3) forward primer	5′-**TAA TAC GAC TCA CTA TAG GGC** GCG GAT CCT CGG CCG ATT TGG AAC TGG AG-3′
DENV2 (NS2B/NS3) reverse primer	5′-CCC AAG CTT CTT TCG AAA AAT GTC ATC-3′

Nucleotide sequences written in bold represent the T7 promoter sequence that was incorporated into DENV2 (NS2B/NS3) forward primer.

2.4. Quantitation of DENV2 protease gene expression level

Quantitative RT-PCR was used to quantify the DENV2 gene copies whereby the target gene was co-amplified with the synthesized DENV2 RNA standard under the same amplification efficiency. Prior to the quantitative RT-PCR, the isolated cRNA was diluted 10-fold serially with RNase-Free water into four micro tubes containing 0.1, 1.0, 10.0 and 100.0 ng of RNA. The DENV2 RNA standard was also diluted in the similar manner into four micro tubes containing 1.5×10^{13} to 1.5×10^{16} copies of standard RNA. The cDNA synthesis and amplification was performed using

SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, Thermo Fisher Scientific, MA, USA) in a single tube consisted of gene target specific primer (oligonucleotides), serially diluted template RNA and DENV2 RNA standard, reaction buffers and PCR enzyme. The oligonucleotides used for amplification were synthesized to span the DENV2 protease (NS2B3) region. A standard RT-PCR mixture of a final volume of 50 μL consisted of 1× reaction mix buffer, 1–100 ng of template RNA and standard RNA, 0.5–1.0 μmol/L each of sense (5′-CGC GGA TCC TCG GCC GAT TTG GAA CTG GAG-3′) and anti-sense (5′-CCC AAG CTT CTT TCG AAA AAT GTC ATC-3′) primers, 1 unit of SuperScript™ III RT/Platinum® Taq Mix and RNase/DNase free water (made up to final volume). The thermal cycler (Biometra®, Germany) was programmed as such the cDNA synthesis was followed immediately by PCR amplification. The thermal profile involved cDNA synthesis at 55 °C for 30 min followed by initial denaturation of dsDNA at 94 °C for 2 min and subsequent 40 cycles of denaturation at 94 °C (15 s), annealing at 60 °C (30 s) and extension at 68 °C (1 min and 30 s). The final extension was carried out at 68 °C for 5 min. The amplified products were separated on 1.5% agarose gel electrophoresis. A series of control RT-PCR was carried out simultaneously using a pair of primers complimentary to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase for the normalization of the target expression data.

The electrophoresed agarose gel of the PCR products were then visualized using the Gel Doc 1000/2000, a unit equipped with UV trans-illuminator and a Canon CCD camera. The Gel Doc 1000/2000 was linked to a computer installed with Quantity One® Software (Bio-Rad Laboratories, California, USA). Data of the volume analysis was transferred to Excel software to generate a linear double log plot of volume (*y*-axis) against the amount of target RNA and RNA standard (*x*-axis) of the DENV2 gene. The amount of copies of DENV2 protease (NS2B3) gene per ng cRNA was determined from the graph by extrapolating two parallel lines between the generated plots. The gene expression level was calculated from the internal standard concentration that produced standard and DENV2 protease bands of equal intensity and was expressed as copies per ng cRNA.

2.5. Confocal microscopy of the cytoskeleton redistribution

The BHK21 cells were seeded (0.5×10^3 cells/well) in 2 wells Lab-Tek II Chambers Slides (Thermo Fisher Scientific Inc., USA) before inoculation with 1000 $TCID_{50}$ of DENV2 and allowed for an hour of virus adsorption. Then the infected cells were treated with 20 μg/mL of ligand YK51. At 96-h post-incubation, the medium was discarded and the cells were washed several times with PBS before being fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Later, the fixed cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (300 nmol/L in PBS) for 20 min to stain the nucleus and then permeabilised with 0.1% Triton X-100 in PBS for 10 min. Subsequently, the cells were incubated with Alexa Fluor® 635 Phalloidin (6.6 μmol/L in PBS) together with anti-α-tubulin conjugated with fluorescein isothiocyanate diluted 1:50 in blocking solution (10% bovine serum albumin, and 1% Triton X-100 in PBS) for 1 h to stain both the actin and tubulin fibres. After that, the cells were washed twice with PBS, and were counterstained with DAPI (300 nmol/L in PBS) for 30 min. Then the chambers were removed from the slides and cover slips were mounted with Prolong® Gold Antifade reagent (Thermo Fisher Scientific Inc., USA). The slides were stored at 4 °C in the dark until being scanned with a Confocal Laser Scanning Microscope (Leica TCS SP5, Germany). The fluorescence intensity of actin and tubulin fibres was measured in a total of 30 cells ($n = 30$) using the Leica QWin software (Leica Microsystems, Germany).

2.6. Statistical analysis

Statistical analysis was done using the Kruskal–Wallis test of SPSS version 17.0 software, a non-parametric test between group analysis of variance for the comparison of scores on continuous variables for three or more groups. The *P* value of less than 0.05 indicated a significant difference across the test groups.

3. Results

3.1. Absolute quantitation of DENV2 gene expression level affected by inhibitor

The IVT synthesized DENV2 RNA standard fragment contained 1.5×10^{18} copies of DENV2 RNA. As this RNA standard was incorporated with a T7 promoter sequence, it produced an amplified product of 845 bp in size that was 25 bp longer than the target gene. Subsequently the PCR bands for both the DENV2 protease and the RNA standard appeared as 820 bp and 845 bp respectively after separation with gel electrophoresis. The quantitation of DENV2 protease gene copies was carried out for four sets of sample; each sample was treated with different doses of ligand YK51 (15, 20, 25 and 30 μg/mL). It was observed that in all samples, the intensity of the PCR target bands was decreased exponentially with the dilution factor relative to the intensity of the RNA standard. The quantitative reduction values for each band were determined using the Quantity One® Software. Volume analysis was carried out on the optimized image of the gel electrophoresis profile and the intensity of the band was expressed as adjusted volume in the final analysis report.

The linear double log plot of volume (*y*-axis) against the amount of cRNA and standard (*x*-axis) was plotted for each set of test sample. An example of the RT-PCR quantitative values obtained from treatment of ligand YK51 at 15 μg/mL was presented in Figure 1. In Figure 1, the target gene and the RNA standard were presented as two parallel lines whereby each line was plotted from four values of the adjusted volume obtained at different dilution factor. The number of DENV2 protease copies in the target RNA was then calculated from the equation of each trend line and it showed that 1 ng of target RNA contained 1.8 copies of DENV2.

The absolute values of DENV2 copies were determined for treatment with ligand YK51 at four doses not exceeding its maximum non-toxic dose. The DENV2 protease gene copy was reduced from 2.7 copies/ng RNA to 2.5 copies/ng RNA after treatment with 15 and 20 μg/mL of the ligand, respectively. Further reduction to 1.8 copies/ng RNA was observed at 25 μg/mL of the ligand used. However, the DENV2 copy was maintained at 1.8 copies/ng RNA after treatment with ligand YK51 at its maximum non-toxic dose of 30 μg/mL.

3.2. Confocal microscopy of the cytoskeleton redistribution

In the mock-infected BHK21 cells shown in Figure 2A, the nucleus were intact and clearly apparent as indicated by the blue

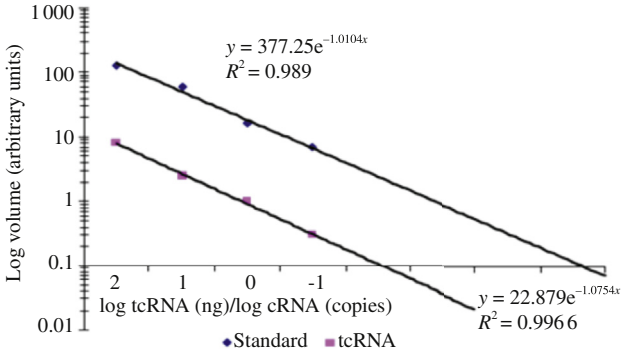

Figure 1. A linear double log plot of volume (arbitrary units) (*y*-axis) plotted against the amount of target RNA (ng) and RNA standard (copies) of quantitative RT-PCR for assessment of the synthesised flavanoid-derived ligand YK 51 inhibitory activity.
tcRNA: Target cRNA.

stain while both the actin and tubulin appeared as wide sheet-like expansion, indicated by the red and green stains respectively. Changes in the morphology of the cytoskeleton due to DENV2 infection were presented in Figure 2B, shown as apparent alteration in the cell shapes, whereby the fibroblast-like cells appeared elongated and highly diffused toward each other compared to the actin and tubulin seen in the mock-infected BHK21 cells. In addition, the nucleus were not present indicated by the absence of the blue stain. The fibroblast-like cell of DENV2 inoculated BHK21 cells treated with ligand YK51 was presented in Figure 2C; it showed similar morphology with the mock-infected cells. The nucleus were intact while the actin and tubulin fibres appeared preserved and well distributed. The fluorescence intensity of tubulin (79.6%) was higher in the infected-treated BHK21 cells compared to the tubulin intensity in the infected BHK21 cells (54.8%). However the infected-treated BHK21 cells showed a lower fluorescence intensity of actin compared to the actin intensity of 30.0% in the infected BHK21 cells.

4. Discussion

Many studies have taken the approach of quantitative RT-PCR in detecting presence of viral genome as well as measuring the level of gene expression. A study that reported inhibition of influenza A virus infection by the Chinese quince extract used RT-PCR to quantify the nucleoprotein, mRNA, cRNA and viral RNA of the virus [14]. Another study employed the quantitative RT-PCR approach for the detection of HIV-2 to establish the importance of glutamine 294 toward ribonuclease H activity of the HIV-2 reverse transcriptase [15]. A more recent study reported that the compound known as DXKK of a Chinese medicine reduced the expression of pro-inflammatory cytokines, both at gene and protein levels. The gene expression level was measured by RT-PCR assay [16]. A previous study has reported a ligand abbreviated as YK51 that showed potent inhibitory activity towards DENV2 *in vitro*. The ligand that was synthesized based on a template of a compound purified from the ginger species (*Boesenbergia rotunda*) showed total elimination of the virus virulence *in vitro*, which suggested intervention at intracellular level, most possibly on the virus replication.

Therefore this current study investigated the inhibitory effect of the synthesized ligand on the replication of the dengue virus. The cRNA was extracted from infected HepG2 cells treated with the synthetic ligand YK51 at four different doses. The cRNA was prepared from the harvested whole cell pellet to represent the cell-virus and cell-inhibitor interactions. High quality cRNA was obtained based on the A260:A280 ratio of 1.8–2.0 and the presence of two distinct bands, 18S and 28S rRNA in the gel electrophoresis profile (data not shown). All reactions in the RT-PCR were normalized against glyceraldehyde-3-phosphate dehydrogenase gene expression, a housekeeping transcript [17], to confirm that the activity of the inhibitors was only toward DENV2 but not affecting the metabolic activity of the cells (data not shown).

This study adapted an absolute quantitative RT-PCR method to quantify the definite values of DENV2 protease gene copies that was affected by the inhibitor. The quantitative measurement of the extent of effect brought by the inhibitor towards DENV2 gene expression level was of relevance to the absolute value of inhibition. The RT-PCR profile showed that treatment with increasing amount of the ligand YK51 resulted in a gradual decrease of intensity of the 820 bp DENV2 protease. At 20 μg/mL, ligand YK51 reduced the intensity of the amplified product by 10-fold. Similar reduction change was observed at 25 μg/mL of ligand YK51. However, when the dose was elevated to 30 μg/mL, the expected 10-fold reduction was not observed but remained comparable to the intensity at 20 μg/mL. Presumably, the saturation concentration for ligand YK51 was reached at 20 μg/mL.

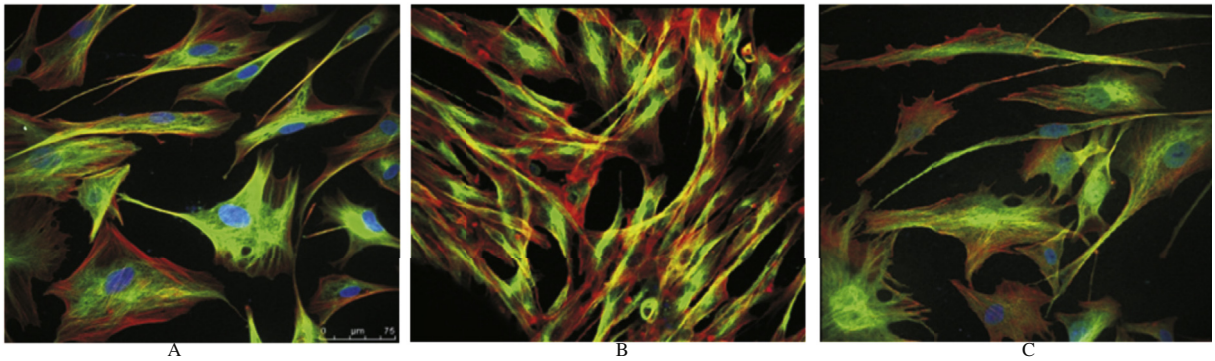

Figure 2. The morphological changes of the BHK21 cytoskeleton cells showed after the fluorescence staining and confocal laser scanning microscopy. A: Confocal image of the BHK21 cells; B: Confocal image of the DENV2 infected BHK21 cells, the fibroblast-like cells were elongated and diffused with no stained nucleus; C: Confocal image of the DENV2 infected BHK21 cells treated with ligand YK51 at 20 μg/mL, the fibroblast-like cells appeared similar with the normal control, reflecting the inhibitory effect of the ligand presented by the presence of intact nucleus and preserved structure of actin and tubulin. [DAPI: blue (nucleus)] [Alexa Fluor® 635 phalliodin: red (actin)] [Anti-α-tubulin-conjugated with FITC: green (tubulin)].

The target RNA was reverse transcribed and co-amplified with known amounts of an internal standard, synthesized *in vitro* from a DNA template of DENV2 protease, incorporated with a T7 promoter sequence. The internal standard RNA virus ensured correction for variation in efficiency of the RT-PCR. A standard curve was generated to deduce the concentration of the target sequence in the test sample. An accurate value of the target RNA was only obtainable when the amplification efficacy of the internal standard and the target was identical, which was indicated by the two parallel lines of the linear double log plot. The graph was plotted, as the known amount of the serially diluted standard RNA virus and target RNA expressed in ng against the 'volume' of each band as measured by a highly sensitive detection system consisted of the Gel Doc 1000/2000 and Quantity One® software. The amount of DENV2 gene copy was obtained from the point on the curve where the amount of target and standard was equal.

Results of the absolute quantitation showed that the reduced copy of DENV2 per ng RNA was strongly associated with the elevated doses of ligand administered during inhibition assay. Subsequently, the prominent reduction in number of the gene copies was presented as prominent decrease of the virus load. Since the gene copies were exclusively reduced when the infected HepG2 cells were treated with the test ligand, the cascade of the down-regulation effect could be attributed mainly to the inhibitory activity of the ligand. Although this study only amplified the protease region, since DENV2 does not have a multipartite genome, RT-PCR performed on any region of the viral RNA coding sequence would have shown reduction of the viral load or replication due to the effect exhibited by the ligand.

The inhibitory effect of the synthesized ligand YK51 was also clearly reflected in the preservation of the actin and tubulin fibres in the infected-treated BHK21 cells. It was clearly showed that in the infected cells, the actin and tubulin were bundled together as they depolymerized and reassembled at high mobility toward each other due to the cytophatic effect. This cytoskeleton redistribution was stabilized upon treatment with the ligand whereby the elongated and diffused microtubules were preserved and appeared well expanded. This indicated the protective effect of the ligand YK51 indicated by the similar morphology of the nucleus, actin and tubulin fibres seen in the mock-infected BHK21 cells, subsequently presenting total cell recovery from the cytoskeletal changes induced by DENV2 activity. The higher percentage of fluorescence intensity in the tubulin fibres compared to that of the actin suggested that the ligand was more effective toward tubulin fibres rather than the actin.

In conclusion, results of the quantitative RT-PCR showed reduction of the viral gene copies in a dose-dependent manner. Consequently, the total virus load was reduced prominently. This result strongly suggested that the synthesized flavanoid-derived ligand YK51 implicated its antiviral property by inhibiting DENV2 replication. This study has also determined the absolute value of inhibition, expressed as copies of DENV2 protease per ng RNA that was decreased by a specific dose of ligand. The inhibitory effect of the ligand YK51 was further presented in the stabilization of the microtubule structure of the cells. Nevertheless, further study has yet to be performed to elucidate the cellular pathway underlying the inhibitory activity of this ligand.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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