Global Impact of Heparin on Gene Expression Profiles in Neural Cells Infected by Enterovirus 71

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Key Words

Antiviral activity · DNA microarray · Enterovirus 71 · Heparin · Molecular mechanism

Abstract

Objectives: Heparan sulphate mimetics, particularly heparin (Hep), have previously been shown to considerably inhibit infection of enterovirus 71 (EV71) in Vero cells. Therefore, in this study, a genome-wide DNA microarray was performed to gain insight into the mechanism(s) of action of Hep against infection of a human neural cell line, SK-N-SH, with a clinical strain of EV71. **Methods:** This study focused on a selection of EV71-induced genes whose expression profiling was exclusively affected by the antiviral activity of Hep. The selection procedure was performed through a statistical multi-level comparison with the following controls: negative control cells, compound control (cells treated with Hep only), virus control (cells treated with virus only) and treatment control (EV71-infected cells treated with Hep). **Results:** Overall, of more than 30,000 genes studied, 14 well-known annotated genes were selected that may be targets for the antiviral activity of Hep against EV71 infection in neural cells. For most of these genes, Hep appeared to modulate the impact of EV71 infection on the expression pattern of the genes. **Conclusions:** The findings of this research may provide initial assistance in new directions for studies to design molecular drug targets against EV71 infection.

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Introduction

Enterovirus 71 (EV71) infection remains a public health problem at a global level, particularly in the Asia-Pacific region. The infection normally manifests as hand-foot-mouth disease; however, it is capable of causing potentially fatal neurological complications [1]. Despite several compounds having been reported to exhibit antiviral potencies against EV71 infection, there is still currently no approved vaccine or antiviral substance for prophylaxis or treatment of the infection [2]. Therefore, research studies need to continue to explore potential compounds that exhibit excellent potencies against EV71 infection.

In a previous study, we showed that soluble heparan sulphate (HS)-like substances, including heparin (Hep), pentosan polysulphate and HS, exhibited potent antiviral activities against a cloned strain of EV71 in Vero cells [3]. It was further demonstrated that Hep and pentosan polysulphate significantly prevented attachment of a low-passage clinical strain of EV71 to Vero cells [unpubl. data], results that were further supported with the proved role of cell surface HS in serving as an attachment receptor for EV71 [4]. These observations warranted further investigations in order to explore the molecular mechanisms through which soluble Hep, as an HS-mimetic compound, may inhibit EV71 infection in vitro. Thus, in this study we attempted to gain global

insight into the molecular mechanisms of action of Hep in EV71-infected neural cells using a genome-wide microarray analysis.

Materials and Methods

Cell Lines and Viruses

A human neuroblastoma cell line, SK-N-SH, from the European Collection of Cell Cultures (catalogue number 86012802) was purchased from CellBank Australia (Westmead, N.S.W., Australia). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Mulgrave, Vic., Australia) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen).

A clinical isolate of EV71 (number 99018233) was supplied from Victorian Infectious Disease Reference Laboratories (North Melbourne, Vic., Australia). The virus was propagated and titrated in 80% confluent Vero cells as described elsewhere [5].

Reagents

Heparin sodium salt (Hep) from bovine intestinal mucosa (≥ 140 United States Pharmacopeia (USP) units/mg; catalogue number H0777), purchased from Sigma-Aldrich (Castle Hill, N.S.W., Australia), was dissolved in sterile distilled water (10 mg/ml) as described previously [6].

Cell Seeding and Controls

Cells were seeded at 1.3×10^5 cells/well in 12-well plates (Becton Dickinson, North Ryde, N.S.W., Australia) followed by incubation at 37° in 5% CO₂ until 80% confluent. The following conditions were prepared: (1) negative control, i.e. not infected with the virus or treated with Hep; (2) virus control, i.e. infected with 100 median tissue culture infective dose EV71 but not treated with Hep; (3) compound control, i.e. treated with Hep at 125 µg/ml only, and (4) treatment, i.e. treated with Hep and simultaneously infected with EV71.

Three wells were allocated to each of the above-mentioned conditions; 2 wells were used to assess cytotoxicity and antiviral activity and the third well was allocated to total RNA isolation for that condition. Assessment of cytotoxicity and antiviral activity and RNA isolation were performed at the same time, namely after 48 h, and each was independently replicated 3 times.

Cytotoxicity and Antiviral Activity Assays

The cytotoxicity and anti-EV71 activity of Hep were assessed using MTT reagent (Invitrogen), followed by incubation for 2–3 h, after which the formazan was dissolved with DMSO. The colour change was recorded using a microplate reader at 540 nm. Cytotoxicity was measured based on the amount of living cells in the compound control relative to the negative control. Antiviral activity was expressed as a percentage of virus inhibition using the following equation [7]: (treatment – virus control)/(negative control – virus control).

RNA Isolation and Quantification

Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Pty. Ltd., Vic., Australia; catalogue number 74104). For each of the 4 conditions described above, 3 independent biological replicates were prepared so that 12 samples went to the hybridization assays.

In order to assess RNA purity and integrity, the RNA samples were initially monitored by agarose (1%) gel electrophoresis, followed by measurement using the Eukaryote Total RNA Nano assay, version 2.6 (Agilent 2100 Bioanalyzer, Agilent Technologies Inc.). Electropherograms of the total RNA samples with an integrity number over 8.5 were further analysed, and the following criteria were taken into account: at least 20% of fragmented RNA was located between 28S and 18S rRNA, the 28S and 18S peaks account for at least 5 and 10% of the overall RNA, respectively, and there was no more than 60% of RNA with fragment sizes smaller than 18S rRNA [8]. The total RNA samples that passed these criteria were selected for further labelling.

Arrays

GeneChip® Human Gene 1.0 ST arrays offering whole-transcript coverage were purchased from Affymetrix (catalogue number 901086). Each microarray chip used in this study contained 33,297 distinct probe sets (transcript cluster IDs), each represented by approximately 26 probes (25-mer) spread across the full length of the gene. The Gene 1.0 ST arrays require sense orientation of labelled cDNA targets.

Target Preparation and Hybridization Process

The target preparation and hybridization process was performed at the Peter MacCallum Cancer Centre (East Melbourne, Vic., Australia). The Ambion® WT Expression Kit was used to generate sense strand cDNA targets. In brief, 3 µl of starting total RNA (300 ng) was mixed with 2 µl of the mix of T7-(N)6 Primers/Poly-A RNA control solution followed by incubation at 70° for 5 min and 4° for a minimum of 2 min. Following synthesis of the first strand of cDNA, DNA polymerase synthesized the second strand, which served as a template for in vitro transcription, during which anti-sense cRNA was synthesized using T7 RNA polymerase. Then, the second-cycle cDNA (sense strand) was synthesized followed by fragmentation and labelling of sense-stranded cDNA using the Affymetrix GeneChip WT Terminal Labeling Kit. Hybridization was then performed in a hybridization oven at 45° and 60 rpm for 17 ± 1 h followed by washing and scanning of the arrays.

Statistical Analysis

Since Affymetrix does not add mismatch probes in the newer arrays such as the GeneChip Human Gene 1.0 ST array, background noise was handled by a background correction step during the normalization. Partek Genomics Suite version 6.5 was utilized to import and analyse raw data from the array cells. Using a one-step Tukey's bi-weight method, normalized values of multiple probes for the same gene (at each replication) were summarized into a single value representing the consensus level of expression for that gene. The following statistical contrasts were established: (1) three basic comparisons, i.e. gene expression profiling of the negative control condition was contrasted with those of treatment, virus control and compound control, and (2) two complementary comparisons, i.e. gene expression profiling of the treatment condition was statistically compared with those of virus control and compound control.

For the aforementioned statistical comparisons, a single-factor analysis of variance inside Partek was applied to generate p values. These p values were also adjusted by the false discovery rate (FDR) to correct for multiple testing [9].

Table 1. Cytotoxicity and antiviral potentiality of Hep in EV71 infection of human neural cells

	Condition		
	Cyto	Hep	VC
Initial virus titre ^a , TCID ₅₀	–	100	100
Survival 48 h after inoculation ^b , %	94.4±0.23	86.1±1.8	57±2.4
Virus titre inhibition 48 h after inoculation ^c , %	NA	95.37±1.3	0.00

Values represent the average of at least 2 independent experiments from which the standard deviations were calculated. The conditions are detailed in Materials and Methods. Cyto = Compound control; Hep = treatment; VC = virus control; TCID₅₀ = median tissue culture infective dose; NA = not applicable because the compound control condition had no virus, while the virus titre inhibition was 0 in the virus control condition due to lack of the virus inhibitor.

^a The clinical EV71 isolate was propagated and titrated in 80%

confluent Vero cells as stated in Materials and Methods. The Vero cell-based titrated virus was then used to infect neural cells in both treatment control and virus control conditions.

^b Survival of the neural cells was calculated based on the amount of living cells in each condition relative to negative control 48 h after inoculation.

^c Virus titre inhibition caused by Hep was determined 48 h after viral inoculation using the following equation: (treatment – virus control)/(negative control – virus control).

Results

Cytotoxicity and Antiviral Activity

Hep at 125 µg/ml significantly prevented in vitro EV71 infection without having a cytotoxic effect on the neural cells (table 1). These results were in agreement with the previous findings, where Hep along with other HS mimetics demonstrated significant anti-EV71 activity with minimal cytotoxicity in Vero cells [3].

Global Profiling of Gene Expression

Changes in gene expression intensities of the 12 RNA samples of neural cells were independently normalized. Overall, results showed that the histograms of gene expression values of the samples followed the same trend, suggesting the arrays were technically acceptable. The microarray data were then deposited in the National Center for Biotechnology Information's Gene Expression Omnibus, accessible through the accession number GSE34234 (online at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34234>).

Impact of Antiviral Activity of Hep on Gene Expression of EV71-Infected Cells

A multi-level selection procedure was employed in order to attribute changes at the gene transcription level to the antiviral activity of Hep. In order to simplify this procedure, we began with statistical comparisons between two conditions, namely treatment and virus control. In this way, it was possible to statistically extract the effect of the virus on neural cells from the antiviral effect of Hep on EV71-infected neural cells. Choosing a cut-off p value

of less than 0.05 with fold changes equal to or more than 1.5 (up- or down-regulation) resulted in selection of 144 genes that were differentially expressed between the conditions. These 144 selected genes were further monitored in the condition of virus control to assess how significantly their expression was affected by EV71 infection. As a result, 14 genes (of 144 genes) were found to be significantly induced by EV71 infection at a p value less than 0.05 and an FDR-adjusted p value lower than 0.48 with a fold change of at least 1.5 (up- or down-regulation; table 2).

In the next step, we examined whether gene expression changes attributed to the antiviral activity of Hep might have been due to the general effect of Hep as a substance. Hence, the expression patterns were assessed in the condition of compound control, showing that Hep alone had no significant inductive effect on the 14 selected genes ($p > 0.05$ and/or a fold change less than 1.5), except for the basic leucine zipper transcription factor, ATF-like gene, which was thus excluded from further bioinformatics analysis (table 2).

A further selection approach revealed 5 other genes that were not significantly induced by EV71 infection (virus control) or Hep alone (compound control) but demonstrated considerable induction in the treatment condition as compared to negative control (table 3). Consequently, there were significant differences in the expression pattern of these genes between the treatment condition and virus control or compound control ($p < 0.05$, with a fold change more than 1.5). Thus, these genes were also chosen for further bioinformatics characterization.

Table 2. Antiviral effect of Hep on altering gene expression patterns induced by EV71 infection

Ref Seq No. / gene name	Gene description	Hep vs. VC		VC vs. CC		Cyto vs. CC	
		fold change	p value	fold change	p value	fold change	p value
NM_024094 / DSCC1	defective in sister chromatid cohesion 1 homolog (<i>S. cerevisiae</i>)	+2.26	3.1×10^{-3}	-1.52	0.03	+1.01	n.s.
NM_001001522 / TAGLN	transgelin	+2.14	0.024	-2.17	0.042	-1.46	n.s.
NM_004541 / NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7	+2.08	0.046	-1.96	0.038	+1.41	n.s.
NM_006520 / DYNLT3	dynein, light chain, Tctex-type 3	+2.03	0.01	-2.08	7.8×10^{-3}	-1.90	n.s.
NM_181786 / HKR1	HKR1, GLL-Kruppel zinc finger family member	+2.00	4.49×10^{-3}	-2.19	2.06×10^{-3}	-1.21	n.s.
NM_020200 / PRTFDC1	phosphoribosyltransferase domain containing 1	+1.57	3.73×10^{-3}	-2.12	9.22×10^{-4}	-1.10	n.s.
NM_016328 / GTF2IRD1	GTF2I repeat domain containing 1	+1.53	0.026	-1.55	0.043	+1.04	n.s.
NM_014729 / TOX	thymocyte selection-associated high mobility group box	+1.50	0.038	-5.81	4.68×10^{-5}	+1.08	n.s.
NM_030968 / C1QTNF1	C1q and tumor necrosis factor related protein 1	-1.510	0.02	+3.35	4.87×10^{-4}	-1.17	n.s.
NM_007170 / TESK2	testis-specific kinase 2	-1.62	4.16×10^{-3}	+1.71	5.71×10^{-3}	-1.24	n.s.
NM_006399 / BATF	basic leucine zipper transcription factor, ATF-like	-1.62	0.03	+1.67	0.02	+1.54	0.026
NM_052862 / RCSD1	RCSD domain containing 1	-1.63	3.22×10^{-3}	+1.55	4.92×10^{-3}	+1.22	9.44×10^{-3}
NM_001008540 / CXCR4	chemokine (C-X-C motif) receptor 4	-1.63	0.028	+1.55	0.019	+1.14	n.s.
NM_175617 / MTIE	metallothionein 1E	-2.92	7.18×10^{-3}	+2.49	0.012	+1.19	n.s.

For fold change values, the + and - symbols indicate the direction of change (+ for up-regulation, - for down-regulation). For each pair of contrasts, the direction of fold change is toward the left-hand condition. The fold change values in the Hep vs. VC column are sorted in descending order. CC = Negative control; VC = virus control; Hep = treatment; Cyto = compound control; n.s. = not significant ($p > 0.05$).

Table 3. Induction of genes in Hep-treated cells infected by EV71 which were not induced by EV71 infection or Hep alone

Ref Seq No. / gene name	Gene description	Hep vs. CC		VC vs. CC		Cyto vs. CC		Hep vs. VC		Hep vs. Cyto	
		fold change	p value	fold change	p value	fold change	p value	fold change	p value	fold change	p value
NM_016090 / RBM7	RNA binding motif protein 7	+1.63	0.047	-1.15	n.s.	-1.17	n.s.	+1.87	0.047	+1.96	0.03
NM_024329 / EFHD2	EF-hand domain family, member D2	+1.88	1.67×10^{-3}	1	n.s.	-1.05	n.s.	+1.87	0.036	+1.98	1.25×10^{-3}
NM_018039 / KDM4D	lysine (K)-specific demethylase 4D	+1.52	0.027	-1.22	n.s.	-1.29	n.s.	+1.85	0.01	+1.95	7.39×10^{-3}
NM_001005340 / GPNMB	glycoprotein (transmembrane) NMB	-2.27	4.6×10^{-3}	-1.45	n.s.	-1.38	n.s.	-1.57	0.01	-1.65	0.013
NM_006287 / TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	-1.68	0.048	-1.06	n.s.	1	n.s.	-1.58	0.033	-1.69	0.019

For fold change values, the + and - symbols indicate the direction of change (+ for up-regulation, - for down-regulation). CC = Negative control; VC = virus control; Hep = treatment; Cyto = compound control; n.s. = not significant ($p > 0.05$).

Table 4. Antiviral effect of Hep on altering expression patterns of un-annotated genes induced by EV71 infection

Cluster transcript ID	Hep vs. VC		VC vs. CC		Cyto vs. CC	
	fold change	p value	fold change	p value	fold change	p value
7892904	+2.34	5.27×10^{-3}	-2.12	7.93×10^{-3}	+1.26	n.s.
7893771	+1.86	0.039	-2.15	8.49×10^{-3}	-1.03	n.s.
7895000	+1.81	0.033	-1.84	0.031	-1.09	n.s.
7892628	+1.72	0.037	-2.29	2.45×10^{-3}	-1.29	n.s.
7896113	+1.67	2.87×10^{-3}	-1.75	0.019	-1.35	n.s.
7894973	+1.64	2.98×10^{-3}	-1.64	0.034	+1.48	n.s.
7896426	+1.57	0.039	-2.02	0.018	-1.30	n.s.
7895836	+1.56	0.025	-1.50	0.041	+1.24	n.s.
7893253	-1.51	0.031	+1.55	0.019	+1.40	5.88×10^{-3}
7894742	-1.61	0.019	+1.65	7.56×10^{-3}	+1.37	n.s.
7896256	-1.62	0.037	+1.65	0.025	+1.26	n.s.
7894614	-1.76	8.48×10^{-3}	+1.78	0.015	+1.16	n.s.
7893180	-2.83	2.02×10^{-3}	+1.82	0.011	-1.05	n.s.

For fold change values, the + and - symbols indicate the direction of change (+ for up-regulation, - for down-regulation). CC = Negative control; VC = virus control; Hep = treatment; Cyto = compound control; n.s. = not significant ($p > 0.05$).

In addition to the well-annotated genes listed in tables 2 and 3, the same selection procedure was also employed to select genes for which no annotation was found using the NetAffx annotation pipeline (table 4).

The results of this study also revealed that there were more than 1,000 other genes induced by EV71 infection in the condition of virus control with expression changes that ranged from +46.5- to -10.7-fold at significance levels lower than 0.01 and an FDR-adjusted p value lower than 0.33. However, since these changes were not related to the antiviral activity of Hep, they were not further analysed in the current study but will be investigated in future studies.

In addition to the quantifiable comparisons stated above, figure 1 graphically visualizes comparisons across the 4 conditions, i.e. treatment, virus control, compound control and negative control. These illustrative comparisons were drawn based on the same positions (features) on the chips belonging to one of the replicons.

Discussion

There are numerous studies using DNA microarrays to explore the mechanisms of action of commercial antiviral drugs such as interferons in human cell lines [10, 11]. However, there has been minimal investigation into

the global impact of a purported antiviral compound on gene expression of cells that have been infected with the desired virus. Among the few examples, microarray analysis was used to reveal changes to the gene expression pattern of Alexander cells secreting hepatitis B virus surface antigen that were treated with an ethanolic extract of *Phyllanthus nanus* [12]. In addition, microarray analysis of a monoclonal antibody showed that it prevented hepatitis C virus infection through the betaine/GABA transporter-1 gene [13]. Such studies with EV71 are even more limited, as the only reported large-scale microarray analysis of EV71 detailed expression patterns of a human glioblastoma cell line following virus infection [14]. To this effect, there has been no study reported in the literature to assess the mechanisms of action of antiviral compounds against EV71 infection using DNA microarray analysis.

Overall, in this study 14 well-annotated genes were found whose EV71-induced expression profiles were modified by Hep, leading to a substantial difference (>1.5 -fold change, $p < 0.05$) between the conditions of treatment and virus control. In fact, Hep lessened the intensities of EV71-mediated down-regulation or up-regulation of these genes. In the following, we discuss the selected genes for which we were able to make speculations, while we could not draw conclusions for the rest.

Fig. 1. Images of microarray analysis of 4 conditions. The images illustrate small parts of each chip and represent how the genes are differentially expressed across different conditions. The colour intensity (right) shows different gene expression values, such that dark-green and white represent up-regulated and down-regulated genes, respectively (colours refer to the online version

only). To make comparisons simpler, the same features are compared between treatment and virus control, and other (same) features are compared between negative control and compound control. The same expression intensity is indicated with straight arrows, while differentially expressed features are indicated with elbow arrows.

Group 1: EV71-Suppressed Genes Whose Expression Was Altered by Hep

DSCC1

In eukaryotes, DSCC1, along with chromosome transmission fidelity factor 18 homolog (*S. cerevisiae*) (CHTF18) and chromosome transmission fidelity factor 8 homolog (*S. cerevisiae*) (CHTF8), plays an essential role in cell proliferation [15]. As such, mutations in any one of these genes are believed to result in growth arrest and ultimately cell death [16–18]. In this study, EV71-mediated down-regulation of DSCC1 may thus have resulted in increased cell death, while this effect was counteracted by the addition of Hep.

TAGLN

Disruption of the cytoplasmic organization of microfilaments during virus infection has been linked to a down-regulation of TAGLN along with other genes involved in the assembly and disassembly of actin fibers, leading to observation of a viral cytopathic effect [19].

Therefore, Hep could possibly negate an EV71-mediated cytopathic effect by modifying the negative impact of EV71 on expression of TAGLN.

NDUFA1

Recently, evidence was provided for a role of NDUFA1 and other genes of respiratory chain complexes in regulating coxsackievirus B3-mediated myocarditis in vivo. Decreased expression of respiratory chain complexes led to suppression of both oxidative stress and mitochondrial apoptosis, increasing viral replication in the heart [20]. In the context of this in vitro study, it could be suggested that Hep might have assisted a mitochondrial intracellular antiviral state by neutralization of EV71-induced down-regulation of NDUFA1.

GTF2IRD1

GTF2IRD1 has been shown to play an important role in cellular processes and cell cycle regulation [21]. As such, it could be proposed that EV71-mediated down-

regulation of GTF2IRD1 may have affected cellular processes such as signaling and the cell cycle, which would benefit progression of the infection; this affect appeared to be neutralized by Hep.

Group 2: EV71-Up-Regulated Genes Whose Expression Was Altered by Hep
TESK2

It has been demonstrated that expression of TESK2 in HeLa cells was involved in actin cytoskeletal re-organization by phosphorylating and regulating cofilin/actin-depolymerizing factor. In addition, a TESK2 mutant with excess phosphorylation activity caused condensation and fragmentation of nuclei followed by apoptosis in HeLa cells [22]. Thus, these findings may have an implication for the current investigation in that the EV71-mediated increase of TESK2 would ultimately lead to the death of neural cells. Again, Hep might have modulated these events through a decrease in the up-regulation of the protein.

CXCR4

Hep suppressed EV71-induced expression of CXCR4 receptor, which is 1 of the 7 known CXC chemokine receptors in many mammalian cells [23]. In addition, this receptor together with CCR5 is utilized by HIV-1 to gain entry into target cells [24]. Up-regulation of CCR5 following EV71 infection in glioblastoma cells was reported [14]. However, use of CXCR4 receptor by EV71 has not been reported to date. It remains to investigate whether EV71 penetration would be hindered by the effect of Hep on EV71-induced up-regulation of CXCR4.

Apart from the genes discussed above, we found that heparan sulphate 2-O-sulfotransferase 1 (HS2ST1) was significantly up-regulated by EV71 (+1.81-fold). This protein is responsible for the biosynthesis of cell surface HS. Addition of Hep to EV71-infected neural cells did not significantly change the expression of HS2ST1 compared to virus control. This initially meant that Hep did not have any effect on the induction of HS2ST1. However, these findings provide evidence that clinical EV71 needs cell surface HS to infect or bind neural cells, in agreement with previous results from cell culture [3, 4]. Considering this, it would then be reasonable to suppose that an increase in the level of HS2ST1 by EV71 may be masked by competitive electrostatic interaction between soluble Hep and the EV71 capsid that ultimately leads to prevention of viral infection or attachment.

In this study, we also found genes that were not significantly induced by EV71 infection or Hep alone but

were seen to be changed in the treatment condition. Thus, we proposed that Hep in the presence of EV71 might have altered the expression pattern of these genes in order to prevent viral infection. We limited our analysis to GPNMB, for which there was adequate information in the literature. Hep suppressed EV71-induced expression of GPNMB significantly in comparison with both virus control and compound control. GPNMB encodes the glycoprotein (transmembrane) NMB, which serves as a feedback regulator of pro-inflammatory responses [25]. A lymphatic tissue microarray analysis showed an increase in several immunosuppressive genes including GPNMB during HIV-1 infection [26]. Similar results were gained with pigs infected by porcine circovirus type 2 [27]. These findings might suggest a possible molecular target for antiviral activity of Hep in vivo, where lymph nodes would be infected with EV71.

One of the possibilities suggested by the previous findings [3, 4] was that electrostatic interaction between the negatively charged Hep and the positively charged viral capsid of EV71 could interfere with EV71 attachment or entry. Hence, it should not be ruled out that changes to EV71-induced genes seen in this microarray analysis might be partially attributable to a significant inhibitory effect of Hep on EV71 entry into the cells. However, the EV71-induced genes whose expression was seen to be changed by Hep might still suggest molecular targets for further studies to design antiviral drugs against EV71. Furthermore, EV71 attachment was not completely blocked by Hep in Vero cells [3] or human neural cells (data not shown). As a result, EV71-mediated induction of gene expression in the host cells could have been counteracted by the presence of Hep. To the best of our knowledge, this is the first microarray analysis of an anti-EV71 compound through a multi-level comparison such that the microarray data were directly linked to the findings of antiviral activity and cytotoxicity tests.

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