

APPLICATION OF A FOOD AZO DYE, BRILLIANT BLACK BN, ON INHIBITION OF INFECTIVITY OF HUMAN ENTEROVIRUSES CAUSING HAND FOOT AND MOUTH DISEASES

5 FIELD OF THE INVENTION

The present invention relates to the use of food dyes to reduce the infectivity of human enteroviruses, more particularly the use of food azo dyes to reduce the infectivity of enteroviruses such as EV71 and coxsackievirus that cause hand, foot and mouth disease.

10 The invention also relates to anti-viral pharmaceutical compositions comprising food azo dyes.

BACKGROUND

15 Hand, foot and mouth disease (HFMD), a common and highly contagious disease in children, is caused by human enteroviruses, including enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Coxsackievirus A6 (CVA6) [Chumakov M, *et al.*, (1979) *Arch Virol* 60: 329-340; Solomon T, *et al.*, (2010) *Lancet Infect Dis* 10: 778-790]. HFMD is usually mild and self-limited; however, the infection of EV71 occasionally leads to fatal pulmonary edema and
20 neurological disorders, such as encephalitis, aseptic meningitis and acute flaccid paralysis [Solomon T, *et al.*, (2010) *Lancet Infect Dis* 10: 778-790; Chang LY, *et al.*, (1999) *Lancet* 354: 1682-1686]. Millions of infected cases and several thousand deaths have been reported during EV71 epidemics in the Asia-Pacific region since 1990s [Shimizu H, *et al.*, (1999) *Jpn J Infect Dis* 52: 12-15; McMinn P, *et al.*, (2001) *Clin Infect Dis* 32: 236-242; Wu JM, *et al.*,
25 (2002) *Pediatrics* 109: E26-; Wu Y, *et al.*, (2010) *Int J Infect Dis* 14: e1076-1081; Yang F, *et al.*, (2009) *J Clin Microbiol* 47: 2351-2352]. Although inactivated EV71 vaccines have been available since 2015 [Zhu F, *et al.*, (2014) *N Engl J Med* 370: 818-828; Zhou Y, *et al.*, (2016) *Expert Rev Vaccines* 15: 803-813], there is still no availability of commercial antiviral drugs to treat HFMD and its associated morbidities and mortalities.

30 A human enterovirus, belonging to the genus *Enterovirus* in the family *Picornaviridae*, has a positive-sense, single-stranded RNA genome encapsidated in a non-enveloped, icosahedral particle with 60 copies of each of the 4 viral structural proteins: VP1 to VP4. VP1 is involved in the cell attachment and entry of enteroviruses. For example, every VP1 pentamer on an EV71 virion forms a prominent, star shaped plateau at the 5-fold axis of
35 symmetry, surrounded by a deep depression called canyon [Plevka P, *et al.*, (2012) *Science* 336: 1274; Wang X, *et al.*, (2012) *Nat Struct Mol Biol* 19: 424-429]. Scavenger receptor class B, member 2 (SCARB2), served as a functional receptor for all tested EV71 viruses, has

been identified to bind the canyon [Yamayoshi S, *et al.*, (2009) *Nat Med* 15: 798-801; Dang M, *et al.*, (2014) *Protein Cell* 5: 692-703]. In contrast, sulfated P-selectin glycoprotein ligand-1 (PSGL-1) and glycosaminoglycans (GAGs) on the cell surface interact with the vertex of the 5-fold axis of viral capsid to facilitate EV71 attachment and infection [Nishimura Y, *et al.*, (2009) *Nat Med* 15: 794-797; Nishimura Y, *et al.*, (2010) *PLoS Pathog* 6: e1001174; Nishimura Y, *et al.*, (2013) *PLoS Pathog* 9: e1003511; Pourianfar HR, *et al.*, (2012) *Virus Res* 169: 22-29; Tan CW, *et al.*, (2013) *J Virol* 87: 611-620; Tan CW, *et al.*, (2017) *Virology* 501: 79-87]. Sulfated/sulfonated heparin, suramin and suramin derivatives have been discovered to interact and bind to the vertex of the 5-fold axis to block EV71 attachment to host cells [Wang Y, *et al.*, (2014) *Antiviral Res* 103: 1-6; Ren P, *et al.*, (2014) *Emerg Microbes Infect* 3: e62; Nishimura Y, *et al.*, (2015) *PLoS Pathog* 11: e1005184]. Cellular protein cyclophilin A has been determined as an EV71 uncoating regulating factor through interacting with and modifying the conformation of H-I loop of VP1 at the vertex of the 5-fold axis [Qing J, *et al.*, (2014) *PLoS Pathog* 10: e1004422]. Antibodies binding to the vertex of the 5-fold axis also prevent EV71 infection [Lee H, *et al.*, (2013) *J Virol* 87: 11363-11370]. Moreover, positively charged amino acids, arginine and lysine, around the canyon and the vertex of the 5-fold axis on the surface of EV71 are important for the production of viable viruses [Yuan S, *et al.*, (2015) *J Virol* 90: 741-752]. Therefore, chemical and biological agents targeting positively charged amino acids around the canyon and the vertex of the 5-fold axis of EV71 could be promising candidates as therapeutics against EV71 infection.

Azo dyes are usually synthesized from petroleum and constitute up to 70% of all organic dyes in the world [Zollinger H (2003) *Color chemistry: syntheses, properties, and applications of organic dyes and pigments* 3rd Edition, Wiley-VCH, Cambridge]. They are characterized by the presence of azo bond (-N=N-) in their structure, conjugated with two, distinct or identical, mono- or polycyclic aromatic groups. They may contain only one azo moiety, but some have two (diaz) or more. Synthesis and production of azo dyes are easy and it is estimated that more than 10,000 kinds of azo dyes have been synthesized, with about 3,000 kinds used globally in various fields [Zollinger H (2003) *Color chemistry: syntheses, properties, and applications of organic dyes and pigments* 3rd Edition, Wiley-VCH, Cambridge; Robinson T, *et al.*, (2001) *Bioresour Technol* 77: 247-255]. Most of the azo dyes are water soluble and have one or more sulfonate groups linked to aromatic rings and exist as sodium salts. Sulfonation of azo dyes increases their solubility and, at the same time, decreases their absorption in humans and animals by reducing their permeability across cell membranes, enhancing their urinary excretion, and metabolism. Some azo dyes are called food azo dyes as they are permitted to be used as additives in food, beverages and pharmaceutical industries. All food azo dyes with E numbers can be found in the list of

food additives from European Food Safety Authority (EFSA). It has been known that some azo dyes interact with proteins and have antimicrobial activities [Baba M, *et al.*, (1993) *Antiviral Chem Chemother* 4: 229-234; Ojala WH, *et al.*, (1995) *Antiviral Chem Chemother* 6: 25-33; Ojala WH, *et al.*, (1996) *J Am Chem Soc* 118: 2131-2142; Ono M., *et al.*, (1997) *Nat Biotechnol* 15: 343-348; Weglarz TE, Gorecki L (2012) *CHEMIK* 66: 1303-1307]. However, biological effects and medical potentials of food azo dyes, especially on enteroviruses, are largely unknown.

There is a need for new therapies to treat enterovirus infections including those that cause HFMD.

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SUMMARY OF THE INVENTION

The present invention relates to the use of food dyes to reduce the infectivity of human enteroviruses. More particularly, the invention relates to the use of food azo dyes, sulfonated food azo dyes to reduce the infectivity of enteroviruses such as EV71 and coxsackievirus that can cause hand, foot and mouth disease.

According to a first aspect, the present invention provides a pharmaceutical composition comprising at least one food dye for use in medicine.

In some embodiments, the food dye is an azo dye.

In some embodiments, the food azo dye is a sulfonated food azo dye.

In some embodiments, the composition is for use in the prophylaxis or treatment of virus infection.

In some embodiments, the composition is for use in the prophylaxis or treatment of virus-related disease. In preferred embodiments the virus is enterovirus, more preferably EV71 and/or coxsackievirus.

In some embodiments the virus-related disease is selected from a group comprising hand, foot and mouth disease (HFMD), polio, aseptic meningitis, encephalitis, acute flaccid paralysis and acute cardiopulmonary dysfunction.

In preferred embodiments, the virus-related disease is hand, foot and mouth disease (HFMD).

In some embodiments, the at least one food dye is selected from a group comprising E102 Tartrazine, E110 Sunset Yellow FCF, E122 Carmoisine, E123 Amaranth, E124 Ponceau 4R, E126 Ponceau 6R, E129 Allura Red and E151 Brilliant Black BN.

In some embodiments, the at least one food dye is selected from a group comprising E122 Carmoisine, E123 Amaranth, E129 Allura Red and E151 Brilliant Black BN.

In preferred embodiments, the at least one food dye is E151 Brilliant Black BN.

In some embodiments, the at least one food dye is a sulfonated food azo dye selected from a group consisting of E122 Carmoisine, E123 Amaranth, E129 Allura Red and E151 Brilliant Black BN, preferably E151 Brilliant Black BN.

5 In some embodiments, the composition of any aspect of the invention comprises pharmaceutically acceptable salts or solvates, or pharmaceutically functional derivatives of said at least one food dye.

In some embodiments, the composition is formulated to be administered orally and/or topically to a subject. In some embodiments, the composition is formulated as a gel, cream,
10 foam, lotion or ointment.

In some embodiments, the composition is formulated to be administered continuously to a subject for a period of 2 to 5 days, preferably a period of 3 to 4 days.

In some embodiments, the composition is formulated to be administered at up to a maximum dose of 4 mg/kg body weight/day ("body weight/day" hereafter abbreviated to
15 "bw/day") (E122); 15 mg/kg bw/day (E123); 7 mg/kg bw/day (E129) or 5 mg/kg bw/day (E151).

According to another aspect, the present invention provides the use of the composition according to any aspect of the invention for the manufacture of a medicament for the treatment or prophylaxis of enterovirus infection or enterovirus-related disease.

20 In some embodiments, the virus infection or virus-related disease is caused by enterovirus.

In preferred embodiments, the virus is EV71 and/or coxsackievirus, more preferably EV71.

In some embodiments, the virus-related disease is selected from a group comprising
25 hand, foot and mouth disease (HFMD), polio, aseptic meningitis, encephalitis, acute flaccid paralysis and acute cardiopulmonary dysfunction.

In some embodiments, the enterovirus infection is manifested in a subject as hand, foot and mouth disease (HFMD).

According to a further aspect, the present invention provides a method of treating or
30 preventing virus infection or virus-related disease, the method comprising administering an effective amount of a composition according to the invention to a subject in need thereof.

In some embodiments, the virus infection or virus-related disease is caused by enterovirus.

In preferred embodiments, the virus is EV71 and/or coxsackievirus, more preferably
35 EV71.

In some embodiments, the enterovirus-related disease is selected from a group comprising hand, foot and mouth disease (HFMD), polio, aseptic meningitis, encephalitis, acute flaccid paralysis and acute cardiopulmonary dysfunction. In preferred embodiments the enterovirus-related disease is hand, foot and mouth disease (HFMD).

5 In some embodiments, the composition is administered for a period of 2 to 5 days, preferably a period of 3 to 4 days.

In some embodiments, the composition is administered at up to a maximum dose of 4 mg/kg bw/day (E122); 15 mg/kg bw/day (E123); 7 mg/kg bw/day (E129) or 5 mg/kg bw/day (E151).

10 According to a further aspect, the present invention provides a kit for treating or preventing enterovirus infection or enterovirus-related disease, the kit comprising the composition according to any aspect of the invention.

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole
15 content of such bibliographic references is herein incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows effects of food dyes on the infection of RD cells by EV71-GFP. EV71-GFP viruses were incubated with different dyes diluted in cell culture medium at 37 °C for 1
20 h, and then inoculated into RD cells in 96-well plates at a multiplicity of infection (MOI) of 1. The GFP signals were observed and recorded at 24 h post-infection. All sulfonated food azo dyes selected under this group; E102, E110, E122, E123, E124, E126, E129 and E151 demonstrated some degree of inhibition of infection of EV71-GFP at a concentration of 300
25 µM as shown by the reduction of GFP signals in comparison to mock control, without exhibiting cytotoxicity. Three additional sulfonated food dyes E131, E132 and E133 were similarly tested but only E132 exhibited inhibition of EV71-GFP, at a concentration of 300 µM. Overall, E122, E123, E129 and E151 were the 4 strongest inhibitors of EV71-GFP infection.

30 **Figs. 2A to 2D** show antiviral and cytotoxic studies of dyes E122, E123, E129 and E151. **(A)** Chemical structure of the 4 anti-EV71 food azo dyes. All of them contain sulfonated aromatic rings. **(B)** Concentration-dependent inhibition of EV71-GFP infection by E151 *in vitro*. RD cells were infected by EV71-GFP at a MOI of 1 for 12 h in the presence of different concentrations of E151. After cell fixation, the nuclei (dark grey) of all cells were
35 visualized by Hoechst 33258 staining and the infected cells were indicated by the GFP signal (light grey) under a fluorescent microscope. The number of GFP positive cells was gradually reduced with the increased concentration of E151. **(C)** Percentage of infected cells

(GFP positive) after treatment of one of 4 azo dyes at indicated concentrations from 0 μ M to 200 μ M. The average value $\pm$ standard error of 3 random spots for each concentration was presented. (D) Cytotoxicity study of E122, E123, E129 and E151 in RD cells. The cells were cultured in different concentrations of each of the dyes in DMEM-10. After 3 days, the cell viability in the presence of each respective dye was measured by an MTT assay and presented as a percentage of that in the absence of the dye. The average $\pm$ standard error of triplicated experiments was shown.

Figs. 3A to 3C show the inhibitory effect of azo dyes on titers of human enteroviruses. Titer reduction assay of (A) Eight RD cell-adapted EV71 strains from different subgenogroups and (B) other 4 clinical isolates of EV71 and 6 coxsackie A viruses, in the presence of E122, E123, E129 or E151. (C) Inhibitory effect of E151 on the virus progeny yields of 5 representative enteroviruses. RD cells were infected with the virus at MOI of 0.1 in the presence of various concentrations of E151 for 48 h. The progeny viruses were titrated in RD cells and their titers were presented as percentage of that without E151 treatment. The average value $\pm$ standard error of triplicated experiments was shown. The brackets indicated p values <0.05 .

Figs. 4A and 4B show that the amino acids at VP1-98 and VP1-145 of EV71 modulated EV71 sensitivity to the dyes. (A) Multiple protein alignment of EV71 VP1. The EV71-B2, EV71-B4, EV71-B5, EV71-C4 and EV71-202 which were highly sensitive to all of 4 dyes E122, E123, E129 and E151 had VP1-98E,145G/Q, while EV71-C1, EV71-C4, EV71-252 and EV71-2242 which were slightly sensitive or resistant to the 4 dyes had VP1-98K,145E (highlighted by boxes). (B) Effects of the 4 dyes on the infectivity of EV71 variants with different amino acid combinations at VP1-98 and VP1-145. The titer reduction assay of the EV71 variants was performed in RD cells in the absence or presence of each respective dye. The average value $\pm$ standard error of triplicated experiments was shown. The brackets indicated p values <0.05 .

Figs. 5A to 5D shows the selection and characterization of E151-resistant EV71 mutants, designated EV71-B5^{res}. (A) EV71-B5 was passaged in RD cells in the presence of E151. The 22nd passage EV71-B5^{res} (i.e. EV71-E151-P22) exhibited complete E151 resistance with 3 amino acid changes: E98K, G145E and P246A in VP1 when compared to the parental wild type EV71-B5. Nucleotide mutations were underlined in double brackets. (B) VP1-P246A conferred total E151 resistance on the EV71 KE (VP1-98K,145E) strains. P246A was introduced into EV71-B5, EV71-C4 and EV71-2242 strains by reverse genetics (RG). The second passaged viruses were titrated in RD cells in the absence or presence of 100 μ M E151. The mean $\pm$ SEM of triplicated experiments is shown. The brackets indicated p values <0.05 . (C) Cartoon image of an EV71 virion (adopted from ViralZone website)

which is a non-enveloped icosahedron with 60 copies of the structural proteins: VP1 to VP4. The black regular pentagon indicates the vertex of the 5-fold axis formed by a VP1 pentamer. (D) Three dimensional surface of the vertex of EV71 5-fold axis. Amino acids at VP1-246 (dark) and VP1-145 (dark) are critical for E151 binding to prevent EV71 infection.

5 VP1-246P is conserved, while VP1-145E is dominant. EV71 strains with VP1-145G/Q are highly sensitive to E151, whereas strains with VP1-145E have low sensitivity to E151. Positively charged VP1-98K, VP1-242K and VP1-244K (dim grey) might also interact with E151 through electrostatic attraction.

Figs. 6A to 6D show the inhibition of EV71 attachment to cells or attachment factors by E151. (A) E151 affected the early stage (cell entry) of EV71-eGFP infection. RD or Vero cells were infected by EV71-eGFP at a MOI of 1. The E151 was administered at -1, 0, 1, 2, 3 or 4 h post-infection at the final concentration of 100 μ M. At 12 h post-infection, the cells were fixed and stained with Hoechst 33258. The images were taken under a fluorescent microscope and merged by using Adobe® Photoshop® software. When E151 was added from 2 h post-infection onwards, it failed to prevent the infection of EV71-eGFP (light grey). The percentage of infected cells (light grey) in 3 random fields for each treatment was presented as mean $\pm$ SEM at the upper left corner of a representative image. (B) E151 prevented the attachment of EV71-B4 (highly sensitive to E151) and EV71-C1 (low sensitivity to E151) onto RD cells in a concentration-dependent manner. Pre-chilled viruses and cells were incubated at 4 °C for attachment, and then attached viruses and cells were analysed by Western blot. Although EV71-B5^{res} is resistant to E151, E151 also partially blocked its attachment to RD cells at a concentration of 300 μ M. (C) E151 disrupted the binding of EV71-B4 to human PSGL-1, but not to human SCARB2 recombinant protein in a pull-down assay. 3 μ g SCARB2-Fc, 1 μ g PSGL-1-Fc and 3 μ g CTLA-4-Fc (control) were bound to Protein A/G plus sepharose and then incubated with purified EV71-B4. The precipitated proteins were analysed by Western blot. (D) E151 prevented the binding of EV71-B4 to heparin sepharose in a dose-dependent manner.

Figs. 7A and 7B show the inhibitory effects of E151 on the attachment of EV71 KE strains abolished by VP1-P246A. (A) E151 prevented the attachment of RG/EV71-B5-EGP (VP1-98E,145G,246P, highly sensitive to E151) and RG/EV71-B5-KEP (VP1-98K,145E,246P, slightly sensitive to E151), but not RG/EV71-B5-KEA (VP1-98K,145E,246A, resistant to E151) onto RD cells and heparin in a dose-dependent manner in Western blot assay. (B) E151 prevented the attachment of RG/EV71-B5-KEP, but not RG/EV71-B5-KEA virions onto Vero cells in immunofluorescence confocal microscopy assay. Virions were allowed to attach to pre-chilled Vero cells at 4 °C for 1 h in the presence or absence of 100 μ M E151. Attached virions (small light grey dots; identified by arrows in enlarged views),

cellular early endosomes (small grey dots) and cellular DNA or cell nuclei (light grey areas) were stained by anti-EV71 sera, anti-EEA1 antibody and Hoechst 33258 after cell fixation, respectively. The number of EV71 puncta per cell (average value $\pm$ standard error) in a representative green EV71 channel image (600X magnification) is at the upper right corner.

- 5 The enlarged view of a small square in the corresponding multiple EV71/EEA1/DNA channel image was at the upper left corner.

Figs. 8A to 8C show that E151 inhibited EV71-eGFP infection following attachment.

- (A) RD or Vero cells seeded in 96-well plates were first pre-chilled at 4 °C for 15 min, and EV71-eGFP in ice-cold medium was added onto the cells at a MOI of 10 for attachment. After incubation at 4 °C for 1 h, the unattached viruses were washed away from cells using ice-cold PBS. Fresh DMEM 10 with or without 100 μ M E151 were added and the cells were transferred into the 37 °C incubator. After 11 h, the cells were fixed and stained with Hoechst 33258. The images were taken under a fluorescent microscope and merged using Adobe® Photoshop® software. The percentage of infected cells (light grey) at 3 random spots in each condition was presented as average value $\pm$ standard error at the upper left corner of a representative image. (B) E151 prevented internalization of RG/EV71-B5-KEP, but not RG/EV71-B5-KEA into Vero cells in the immunofluorescence confocal microscopy assay. Attached virions were allowed to internalize into the cells at 37 °C for 30 min in the presence or absence of 100 μ M E151. Virions (small light grey dots; identified by arrows in enlarged views), cellular early endosomes (small grey dots) and cellular DNA or cell nuclei (light grey areas) were stained by anti-EV71 sera, anti-EEA1 antibody and Hoechst 33258 after cell fixation, respectively. The number of EV71 puncta per cell (average value $\pm$ standard error) in a representative green EV71 channel image (600X magnification) was at the upper right corner. The enlarged view of a small square in the corresponding multiple EV71/EEA1/DNA channel image was at the upper left corner. (C) Attached EV71 virions eluted from RD cells by E151 in a concentration dependent manner. E151 sensitive EV71-B5 or resistant EV71-B5^{res} was incubated with RD cells at 4 °C for 1 h for attachment, and unattached viruses were washed away with ice-cold PBS. The attached viruses-cells were divided into 3 portions and re-suspended in ice-cold PBS with 0, 30 or 100 μ M E151. After incubated at 4 °C for 15 min with gentle agitation, the cell pellets and supernatants were separated for EV71 antigen detection using Western blot. E151 eluted the attached EV71-B5, but not resistant EV71-B5^{res} from RD cells, implying that the virus-cell early interaction was reversible and E151 competed with cells for wild type EV71 binding.

- Fig. 9** shows the prevention of EV71 binding to the uncoating factor cyclophilin A (CypA) by E151. In GST pull down assay, glutathione sepharose bound GST or GST-CypA was incubated with purified EV71-B5 or EV71-E5^{res} in the absence or presence of E151. The

precipitated proteins were analysed by Western blot with the 1D9 antibody and anti-GST sera. EV71-B5 interacted with GST-CypA but not GST, and it failed to bind to GST-CypA in the presence of 100 μ M E151. P246A in EV71-B5^{res} almost abolished the interaction between the virus and GST-CypA as reported before.

5 **Figs. 10A to 10C** show the protection of EV71 challenged AG129 mice by E151. **(A)** The diagram indicates the timeline of the procedure. 10 LD₅₀ of EV71-B4 (highly sensitive to E151) or EV71-C1 (slightly sensitive to E151) were intraperitoneally inoculated into 14-day old AG129 mice with 1 dose of E151 (200 mg/kg bw/day) or PBS, and then 1 dose of E151 administered daily in following 3 days (i.e. on the 15th to 17th day after birth of the mice).
10 The morbidity and mortality of mice were recorded daily until 21 days after virus inoculation. **(B)** The daily survival percentage of mice challenged by EV71-B4 or EV71-C1 with or without the treatment of E151 (8 mice per group) was recorded until 21 days after the challenge. All challenged mice without the treatment of E151 died, while the treated mice survived. **(C)** Titers of progeny viruses in brains (circles) and hind limb muscle (triangles)
15 from EV71-C1 infected mice with PBS or E151 treatment. The mice (5 per group per time point) were euthanized at 4, 8 or 12 days post-challenge, and infectious viruses in the homogenized tissue were titrated in RD cells. Means are shown as solid lines (brain) or dash lines (hind limb muscle), and *p*-values of brains (circles) or hind limb muscle (triangles) between PBS and E151 treated mouse were also presented.

20

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

25 Certain terms employed in the specification, examples and appended claims are collected here for convenience.

As used herein, the term “comprising” or “including” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or
30 components, or groups thereof. However, in context with the present disclosure, the term “comprising” or “including” also includes “consisting of”. The variations of the word “comprising”, such as “comprise” and “comprises”, and “including”, such as “include” and “includes”, have correspondingly varied meanings.

As used herein, the term “food dye” is to be interpreted as including any artificial or
35 natural colorant, pigments or substances that impart color when added to food, drinks or pharmaceuticals that are for consumption.

As used herein, the term "sulfonated azo food dye" or "sulfonated azo dye" is to be interpreted as including synthetic organic compounds of the general formula $R-N=N-R'$, comprising at least one azo group $-N=N-$ and substituted aliphatic, aromatic or heterocyclic systems (R, R') as part of the structure, whereby the substituted aromatic systems include at least one sulfonic acid group.

References herein (in any aspect or embodiment of the invention) to said "food dye", "sulfonated azo food dye" or "sulfonated azo dye" includes references to such compounds per se, to tautomers of such compounds, as well as to pharmaceutically acceptable salts or solvates, or pharmaceutically functional derivatives of such compounds.

Pharmaceutically acceptable salts that may be mentioned include acid addition salts and base addition salts. Such salts may be formed by conventional means, for example by reaction of a free acid or a free base form of a compound of formula I with one or more equivalents of an appropriate acid or base, optionally in a solvent, or in a medium in which the salt is insoluble, followed by removal of said solvent, or said medium, using standard techniques (e.g. in vacuo, by freeze-drying or by filtration). Salts may also be prepared by exchanging a counter-ion of a serotonergic compound in the form of a salt with another counter-ion, for example using a suitable ion exchange resin.

Examples of pharmaceutically acceptable salts include acid addition salts derived from mineral acids and organic acids, and salts derived from metals such as sodium, magnesium, or preferably, potassium and calcium.

Examples of acid addition salts include acid addition salts formed with acetic, 2,2-dichloroacetic, adipic, alginic, aryl sulfonic acids (e.g. benzenesulfonic, naphthalene-2-sulfonic, naphthalene-1,5-disulfonic and p-toluenesulfonic), ascorbic (e.g. L-ascorbic), L-aspartic, benzoic, 4-acetamidobenzoic, butanoic, (+) camphoric, camphor-sulfonic, (+)-(1S)-camphor-10-sulfonic, capric, caproic, caprylic, cinnamic, citric, cyclamic, dodecylsulphuric, ethane-1,2-disulfonic, ethanesulfonic, 2-hydroxyethanesulfonic, formic, fumaric, galactaric, gentisic, glucoheptonic, gluconic (e.g. D-gluconic), glucuronic (e.g. D-glucuronic), glutamic (e.g. L-glutamic), α -oxoglutaric, glycolic, hippuric, hydrobromic, hydrochloric, hydriodic, isethionic, lactic (e.g. (+)-L-lactic and ($\pm$)-DL-lactic), lactobionic, maleic, malic (e.g. (-)-L-malic), malonic, ($\pm$)-DL-mandelic, metaphosphoric, methanesulfonic, 1-hydroxy-2-naphthoic, nicotinic, nitric, oleic, orotic, oxalic, palmitic, pamoic, phosphoric, propionic, L-pyroglutamic, salicylic, 4-amino-salicylic, sebacic, stearic, succinic, sulphuric, tannic, tartaric (e.g. (+)-L-tartaric), thiocyanic, undecylenic and valeric acids.

Particular examples of salts are salts derived from mineral acids such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric and sulphuric acids; from organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, arylsulfonic acids; and from metals such as sodium, magnesium, or preferably, potassium and calcium.

As mentioned above, also encompassed by said "food dye", "sulfonated azo food dye" or "sulfonated azo dye" compounds are any solvates of the compounds and their salts. Preferred solvates are solvates formed by the incorporation into the solid state structure (e.g. crystal structure) of the compounds of the invention of molecules of a non-toxic pharmaceutically acceptable solvent (referred to below as the solvating solvent). Examples of such solvents include water, alcohols (such as ethanol, isopropanol and butanol) and dimethylsulphoxide. Solvates can be prepared by recrystallizing the compounds of the invention with a solvent or mixture of solvents containing the solvating solvent. Whether or not a solvate has been formed in any given instance can be determined by subjecting crystals of the compound to analysis using well known and standard techniques such as thermogravimetric analysis (TGE), differential scanning calorimetry (DSC) and X-ray crystallography.

The solvates can be stoichiometric or non-stoichiometric solvates. Particularly preferred solvates are hydrates, and examples of hydrates include hemihydrates, monohydrates and dihydrates.

Compounds of the present invention will generally be administered as a pharmaceutical formulation in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier, which may be selected with due regard to the intended route of administration and standard pharmaceutical practice. Such pharmaceutically acceptable carriers may be chemically inert to the active compounds and may have no detrimental side effects or toxicity under the conditions of use. Suitable pharmaceutical formulations may be found in, for example, Remington The Science and Practice of Pharmacy, 19th ed., Mack Printing Company, Easton, Pennsylvania (1995). For parenteral administration, a parenterally acceptable aqueous solution may be employed, which is pyrogen free and has requisite pH, isotonicity, and stability. Suitable solutions will be well known to the skilled person, with numerous methods being described in the literature. A brief review of methods of drug delivery may also be found in e.g. Langer, Science (1990) 249, 1527.

Otherwise, the preparation of suitable formulations may be achieved routinely by the skilled person using routine techniques and/or in accordance with standard and/or accepted pharmaceutical practice.

In some embodiments, such as those which include a pharmaceutical composition comprising a sulfonated azo food dye of the presently-disclosed subject matter, the pharmaceutical composition can be administered orally and/or topically to thereby treat a viral infection. In some embodiments, the composition is formulated as a gel, cream, foam, lotion or ointment.

Regardless of the route of administration, the compounds of the present invention are typically administered in amount effective to achieve the desired response. As used herein, the terms "effective amount" and "therapeutically effective amount" refer to an amount of the therapeutic composition (e.g., a composition comprising a sulfonated azo food dye, and a pharmaceutically vehicle, carrier, or excipient) sufficient to produce a measurable biological response (e.g., a decrease in the amount of an enterovirus infection). Actual dosage levels of active ingredients in a therapeutic composition of the present invention can be varied so as to administer an amount of the active sulfonated azo food dye(s) that is effective to achieve the desired therapeutic response for a particular subject and/or application. Of course, the effective amount in any particular case will depend upon a variety of factors including the activity of the therapeutic composition, formulation, route of administration, combination with other drugs or treatments, severity of the condition being treated, and the physical condition and prior medical history of the subject being treated. Preferably, a minimal dose is administered, and the dose is escalated within the mandated maximum daily dose for each respective sulfonated azo food dye to a minimally effective amount. Determination and adjustment of a therapeutically effective dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art.

For additional guidance regarding formulation and dose, see U.S. Pat. Nos. 5,326,902 and 5,234,933; PCT International Publication No. WO 93/25521; Berkow, et al., (1997) The Merck Manual of Medical Information, Home ed. Merck Research Laboratories, Whitehouse Station, N.J.; Goodman, et al., (2006) Goodman & Gilman's the Pharmacological Basis of Therapeutics, 11th ed. McGraw-Hill Health Professions Division, New York; Ebadi. (1998) CRC Desk Reference of Clinical Pharmacology. CRC Press, Boca Raton, Fla.; Katzung, (2007) Basic & Clinical Pharmacology, 10th ed. Lange Medical Books/McGraw-Hill Medical Pub. Division, New York; Remington, et al., (1990) Remington's Pharmaceutical Sciences, 18th ed. Mack Pub. Co., Easton, Pa.; Speight, et al., (1997) Avery's Drug Treatment: A Guide to the Properties, Choice, Therapeutic Use and Economic Value of Drugs in Disease Management, 4th ed. Adis International, Auckland/Philadelphia; and Duch, et al., (1998) Toxicol. Lett. 100-101:255-263, each of which are incorporated herein by reference.

The term "subject" is herein defined as vertebrate, particularly mammal, more particularly human. For purposes of research, the subject may particularly be at least one animal model, e.g., a mouse, rat and the like. In particular, for treatment of enterovirus infection and/or enterovirus-linked diseases, the subject may be a human infected by EV71 and/or coxsackievirus. Examples of enterovirus-related diseases include but are not limited to HFMD, polio, aseptic meningitis, encephalitis, acute flaccid paralysis, and acute cardiopulmonary dysfunction.

The term "treatment", as used in the context of the invention refers to prophylactic, ameliorating, therapeutic or curative treatment.

DETAILED DESCRIPTION OF THE INVENTION

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Green and Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (2012).

Methods

Cells, viruses and food azo dyes

Human rhabdomyosarcoma (RD; ATCC number CCL-136) and monkey Vero (ATCC number CCL-81) cell lines were maintained in Dulbecco's Modified Eagles' Medium (DMEM) (Life technologies) supplemented with 10% fetal bovine serum (FBS) (Biowest) and 1X Antibiotic-Antimycotic (Life Technologies) at 37 °C in 5% CO₂ incubator.

18 clinical isolates of human enteroviruses (Table 1) and 1 synthetic EV71-C4 generated by reverse genetics system [Meng T, et al., (2012) *Viral J* 9: 238] were amplified in RD cells and then stored at -80 °C for future experiments.

Table 1. List of viruses used in this experimental study.

Strain name	Subgenogroup	Location	Year	Major symptoms	GenBank accession no. *
EV71-A (BrCr)	A	USA	1970	Meningitis	U22521
EV71-Viet	A	Vietnam	2010	HFMD	Nil
EV71-B2 (7423)	B2	USA	1987	Encephalitis	U22522
EV71-B4 (#HFMD47)	B4	Singapore	2002	Death	AF316321
EV71-B5 (NUH0083)	B5	Singapore	2008	HFMD	FJ461781
EV71-C1 (Y90)	C1	Japan	Nil	unknown	AB433864
EV71-C2 (NUH0075)	C2	Singapore	2008	HFMD	FJ172159
EV71-202	C2	Japan	2010	HFMD	Nil
EV71-252	C2	Japan	2010	HFMD	Nil
EV71-2242	C2	Japan	2010	HFMD	Nil
EV71-C4 (FY08)	C4	China	2008	HFMD	EU703813
EV71-C5 (3437)	C5	Singapore	2006	HFMD	GU222654
CVA16-G10	A	South Africa	1951	HFMD	U05876
CVA16-MOH1	2B2	Japan	2010	HFMD	Nil
CVA16-OS	2B2	Malaysia	Nil	HFMD	Nil
CVA10**	Nil	Singapore	2008	HFMD	Nil
CVA4**	Nil	Singapore	2008	HFMD	Nil
CVA6**	Nil	Singapore	2008	HFMD	Nil

* The genomic sequence of viruses in our laboratory might be different from ones published in GenBank due to mutations during viral propagation *in vitro*.

5 ** [Wu Y, *et al.*, (2010) *Int J Infect Dis* 14: e1076-1081].

The titration of viruses was determined in RD cells as 50% tissue culture infective dose (TCID₅₀) using Reed and Muench formula. The P1 gene of EV71 and CVA16 was amplified by OneStep RT-PCR kit (Qiagen) using primers seqEV71-P1-F and seqEV71-P1-R, and then inserted into the pJET1.2 vector (Thermo Scientific) for sequencing (Table 2).

10

Table 2. List of primers used in this experimental study.

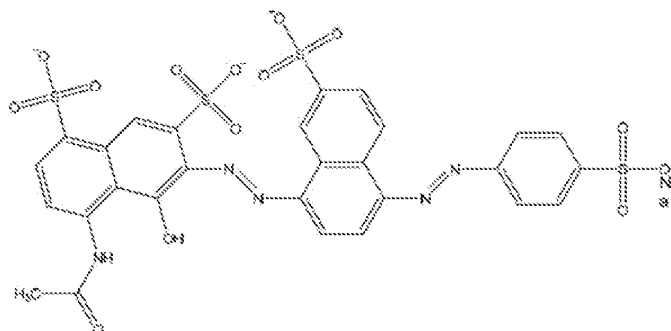
Primer name	Sequence (5' - 3')	SEQ ID NO:
GFP/EV71-B4-F	GCCATTACTACCCTTGGCTCACAGGTGTCTACTC	1
GFP/EV71-B4-R	CTTGGACTGGGCCATGTTTGATTGTATTGAGG	2
GFP-F	ATGGCCCAGTCCAAGCAC	3
GFP-R	AAGGGTAGTAATGGCGGGCAAGGCGGAGCCGGAG	4
GST-CypA-F	GGTCCGCGTGATCCGTCAACCCACCGTGTTTC	5
GST-CypA-R	GGGAATTCGGGGATCTTATTTCGAGTTGTCCACAG	6
B5-VP1-E98K-F	CCCCCTAAAGGGTACCACCAATCC	7
B5-VP1-E98K-R	GTACCCTTTAGGGGGAGATCTATCTC	8
B5-VP1-G145E-F	ACTGGTGAGGTCGTTCCACAATTAC	9
B5-VP1-G145E-R	AACGACCTCACCAGTAGGAGTG	10
B5-VP1-P246A-F	CAAGTACGCTTTGGTTGTCAGAATATAC	11
B5-VP1-P246A-R	ACCAAAGCGTACTTGGACTTTGAAGAC	12
C4-VP1-E98K-F	CCCTCTTAAGGGCACAATAACCC	13
C4-VP1-E98K-R	GTGCCCTTAAGAGGGAGATCTATCTC	14
C4-VP1-E145Q-F	CACCGGGCAAGTTGTCCACAATTG	15
C4-VP1-E145Q-R	ACAACCTGCCCGGTGGGTGTG	16
C4-VP1-P246A-F	AAGTACGCTTTAGTGGTTAGGATTTAC	17
C4-VP1-P246A-R	CACTAAAGCGTACTTGGACTTGGAG	18
2242-VP1-P246A-F	AAGTATGCACTGGTGATCAGGATTTAC	19
2242-VP1-P246A-R	CACCAAGTGCATACTTGGACTTCGAG	20
seqEV71-P1-F	CCTCCGGCCCCCTGAATG	21
seqEV71-P1-R	GCGRGAGCTRTCTTCCCA	22
EV-Genotyping-F	CTTGTRATAACCATGGATYAG	23
EV-Genotyping-R	GCGRGAGCTRTCTTCCCA	24
seqEV71-C-f1188	GGAAGTTCCCGGATGTGTAAAC	25
seqEV71-C-r2909	GTGGCACAAACATATATTGGAG	26
seqEV71-B-f	GTGGGAAAAGTCATCCAAG	27
seqEV71-B-r	CATCGTGTCTCAATCATACTC	28
seqCA16-P1-f2	GGAAATTCCCKGAYGTTTTGAC	29
seqCA16-P1-r2	GTGGAGTGATGGTTCAACAC	30

R = A or G; Y = C or T; K = G or T

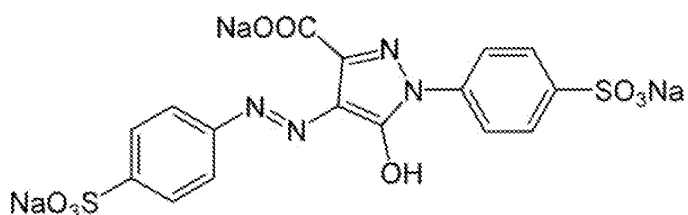
All food dyes tartrazine (03322-25MG), sunset yellow (68775-25MG), Carmoisine (52245-25MG), Amaranth (87612-25MG), Ponceau 4R (18137-25MG), Ponceau 6R (96365-25MG), Allura Red AC (38213-25MG, 458848-100G), Black PN (11220-25MG, 211842-10G), patent blue V (74748-25MG) and brilliant blue FCF (80717-100MG) were purchased

from Sigma-Aldrich. All the dyes were dissolved in sterile water at 10 mM and then diluted to desired concentrations with DMEM.

5 The chemical structure of E151 exemplified in the present invention is as shown below.

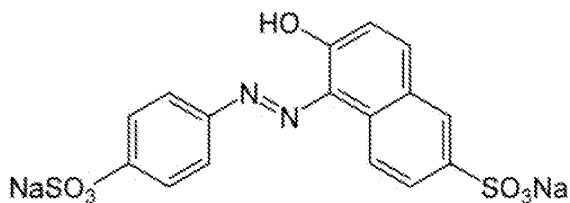


E151 is a sulfonated bis-azo dye with the molecular formula $C_{28}H_{17}N_5Na_4O_{14}S_4$, having a molecular weight of 867.68 g/mol. Its IUPAC chemical name is tetrasodium 4-acetamido-5-hydroxy-6-[7-sulfonato-4-(4-sulfonatophenylazo)-1-naphthylazo]naphthalene-1,7-disulfonate. "E151" may also be referred to herein by one of its several common names
10 such as "B09", Brilliant Black, Brilliant Black BN or Brilliant Black PN.



E102 Tartrazine

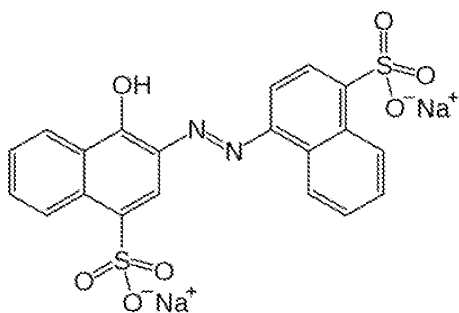
Its IUPAC chemical name is Trisodium (4E)-5-oxo-1-(4-sulfonatophenyl)-4-[(4-sulfonatophenyl)hydrazono]-3-pyrazolecarboxylate; chemical formula $C_{16}H_9N_4Na_3O_9S_2$ and
15 molar mass 534.3 g/mol.



E110 Sunset Yellow FCF

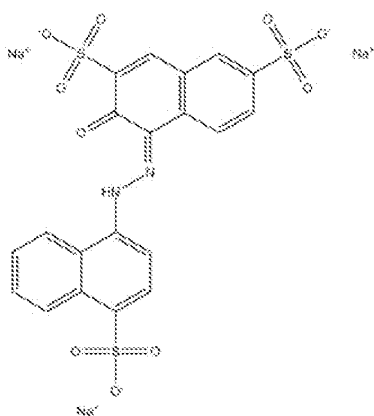
Its IUPAC chemical name is Disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate; chemical formula $C_{16}H_{10}N_2Na_2O_7S_2$ and molar mass 452.36 g/mol.

17



E122 Carmoisine

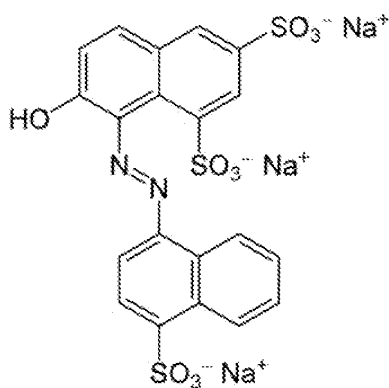
Its IUPAC chemical name is disodium 4-hydroxy-2-[(*E*)-(4-sulfonato-1-naphthyl)diazenyl]naphthalene-1-sulfonate; chemical formula $C_{20}H_{12}N_2Na_2O_7S_2$ and molar mass 502.44 g/mol.



5

E123 Amaranth

Its IUPAC chemical name is Trisodium (4*E*)-3-oxo-4-[(4-sulfonato-1-naphthyl)hydrazono]naphthalene-2,7-disulfonate; chemical formula $C_{20}H_{11}N_2Na_3O_{10}S_3$ and molar mass 604.47 g/mol.

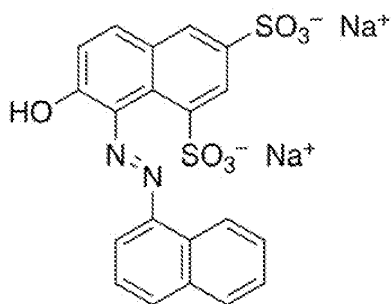


10

E124 Ponceau 4R

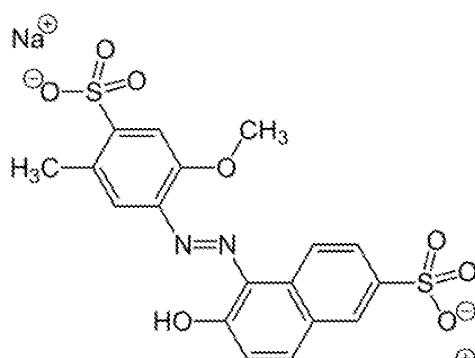
Its IUPAC chemical name is 1-(4-sulfo-1-naphthylazo)- 2-naphthol- 6,8-disulfonic acid, trisodium salt; chemical formula $C_{20}H_{11}N_2Na_3O_{10}S_3$ and molar mass 604.46 g/mol.

18



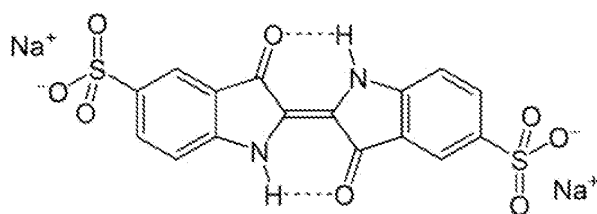
E126 Ponceau 6R

Its IUPAC chemical name is disodium 7-hydroxy-8-(naphthalen-1-diazenyl)naphthalene-1,3-disulfonate; chemical formula $C_{20}H_{12}N_2Na_2O_7S_2$ and molar mass 502.43 g/mol.

Na⁺ E129 Allura Red

Its IUPAC chemical name is Disodium 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]-2-naphthalenesulfonate; chemical formula $C_{18}H_{14}N_2Na_2O_8S_2$ and molar mass 496.42 g/mol.

10



E132 Indigo carmine

Its IUPAC chemical name is 3,3'-dioxo-2,2'-bisindolyden-5,5'-disulfonic acid disodium salt; chemical formula $C_{16}H_8N_2Na_2O_8S_2$ and molar mass 466.36 g/mol.

15 Antibodies and recombinant proteins

Mouse anti-EV71 sera were produced from AG129 pups immunized by purified EV71-B4 at sub-lethal dose. A mouse anti-EV71 monoclonal antibody 1D9 [Tang ML, *et al.*, (2012) *J Med Virol* 84: 1620-1627] was used to detect and quantify VP1 in Western blots.

Mouse anti-GST sera were prepared in-house. Mouse HRP conjugated anti- β -actin antibodies and goat anti-mouse IgG-HRP antibodies were purchased from Dako and Life Technologies, respectively. Horseradish peroxidase (HRP)-conjugated mouse anti- β -actin monoclonal antibody (sc-47778) was purchased from Santa Cruz Biotechnology, and HRP-conjugated goat anti-mouse immunoglobulin antibodies (P0260, Dako) and rabbit anti-human IgG antibodies (P021402-2, Dako) were from Agilent. Alexa Fluor (AF) 594-conjugated rabbit anti-early endosome antigen 1 (EEA1) monoclonal antibody (ab206913) and AF488-conjugated goat anti-mouse IgG antibodies (A32723) were from Abcam and Life technologies, respectively.

EV71 attachment factors human SCARB2 and PSGL-1 fused to the Fc region of human IgG1 (SCARB2-Fc and PSGL-1-Fc) and CTLA-4-Fc (a negative control Fc protein) were purchased from R&D Systems. EV71 uncoating factor human cyclophilin A fused to GST was expressed and purified according to a standard protocol.

Expression and purification of GST tagged cyclophilin A (GST-CypA)

Human Cyclophilin A ORF (NM_203431) was amplified from total RNA of RD cells by Qiagen one-step RT-PCR kit (Qiagen) using primers: GST-CypA-F and GST-CypA-R. It was cloned into BamH I digested pGEX-4T-1 expression vector with a GST tag fused at the N-terminus by In-Fusion HD cloning kit (Clontech). The recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells, and the correctness of the insert was confirmed by sequencing after plasmid purification. To express GST or GST-CypA, the transformed cells were cultured at 37°C in LB media containing 100 mg/L ampicillin. After the OD₆₀₀ reached 0.8, protein expression was induced at 22°C by IPTG at the final concentration of 0.2 mM. After 5 hours induction, the cells were harvested, and soluble GST and GST-CypA were purified according to a standard protocol. The purified proteins were finally dissolved in PBS and their respective concentrations were measured by A₂₈₀.

Generation of EV71-GFP reporter virus

The genome of EV71-B4 with green fluorescent protein (GFP) gene was constructed as described previously [Yamayoshi S, *et al.*, (2009) *Nat Med* 15: 798-801] with modifications. The infectious plasmid pJET-hPoll-B4 [Meng T, *et al.*, (2012) *Virology* 439: 238] was linearized by PCR using primers GFP/EV71-B4-F and GFP/EV71-B4-R (Table 2) so that the 5'UTR and VP4 were separated. The GFP gene from pLVX-IRES-ZsGreen1 vector (Clontech) with an EV71 protease 2A recognition sequence AITTL at the 3'-end was amplified with primers GFP-F and GFP-R (Table 2). All PCR was done using Q5 high fidelity 2X Master Mix (New England Biolabs), and PCR products were extracted using QIAquick gel

extraction kit (Qiagen) after DNA gel electrophoresis. The purified GFP-AITTL gene and linearized pJET-hPoll-B4 plasmid were ligated together by In-Fusion HD cloning kit (Clontech), forming a recombinant plasmid pJET-hPoll-B4/GFP. The infectious EV71-GFP reporter virus was generated by direct transfection of the plasmid pJET-hPoll-B4/GFP into RD cells using Lipofectamine 2000 (Thermo Fisher Scientific) and then further propagated in RD cells for another 2 passages.

Cytotoxicity assay of food azo dyes

A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay kit (TOX-1, Sigma) was used to determine the cytotoxicity of azo dyes in cell culture. 10^4 RD cells in 100 μ l DMEM with 10% FBS (DMEM-10) were seeded into 96-well plates and incubated for 6 h in the incubator. The medium was then replaced by fresh DMEM-10 containing different concentrations of azo dyes. After 3 days of incubation, the medium was removed and extra azo dyes were washed away by PBS. 100 μ l of MTT at the concentration of 0.5 mg/ml in DMEM-10 was added to each well and the incubation was continued for another 3 h in the incubator. Then 100 μ l of MTT solubilisation solution was added, and the plates were gently swirled at room temperature for 15 min. The absorbance of each well at a wavelength of 570 nm was measured by a Tecan's Sunrise microplate reader. The cytotoxicity of azo dyes was estimated by comparing the absorbance of the azo dyes treated cells with that of mock cells.

Antiviral activity of food azo dyes against EV71 in vitro

For screening of anti-EV71 drugs, EV71-GFP, containing GFP reporter gene in EV71-B4 genome, was diluted to 10^4 TCID₅₀/100 μ l in DMEM-10 containing an azo dye at the concentration of 300 μ M, and incubated for 1 h at 37 °C. The treated viruses were inoculated into RD cells in 96-well plates at a density of 10^4 cells/well, and the plates were transferred into the incubator. At 24 h post-infection, the GFP signals in each well were observed and recorded under a UV light microscope (Olympus).

In the azo dye concentration-dependent inhibition of EV71 infection assay, 10^4 TCID₅₀ of EV71-GFP was dissolved in 100 μ l DMEM-10 with 2-fold diluted dyes from 200 μ M to 3.1 μ M, and incubated for 1 h at 37°C. The treated viruses were inoculated into RD cells in 96-well plates at a multiplicity of infection (MOI) of 1 TCID₅₀/cell.

In the time-dependent inhibition of EV71 infection assay, RD cells in 96-well plates at a density of 10^4 cells/well were infected by EV71-GFP at a MOI of 1. E151 was added into cell culture medium at a final concentration of 100 μ M at -1, 0, 1, 2, 3 and 4 h post-infection.

In inhibition of EV71 infection at the post-attachment stage, RD cells in 96-well plates at a density of 10^4 cells/well were cooled for 30 min in a 4°C chiller and incubated with 10^5 TCID₅₀ of EV71-GFP at 4°C for viral attachment. The unattached viruses were removed and washed away with ice-cold PBS twice 1 h later. The cells were topped up with fresh DMEM-10 and incubated in the incubator.

After 12 h incubation, the cells were fixed with 4% paraformaldehyde (PFA) and stained with Hoechst 33258. The percentage of GFP positive cells were calculated using Adobe® Photoshop® software after merging of images recorded by the UV light microscope. The percentage of GFP positive cells in 3 random images of each sample was calculated.

Viral titer reduction assay

The sensitivities of enteroviruses to the food azo dyes were determined in a cell culture assay that measured the degree of protection of the dyes on RD cell monolayers from undergoing cytopathic effect (CPE) caused by viral infection. RD cells were seeded into 96-well plate at 10^4 cells in 100 µl DMEM-10 per well and incubated at the incubator for 6 hours. 100 µl of ten-fold serial dilutions of viruses in DMEM containing azo dyes or none were incubated for 1 hour at room temperature and transferred into the plates. After 4 days, positive infected wells were counted as clear CPE on cell monolayers. TCID₅₀ values were determined by the Reed and Muensch method.

Determination of IC₅₀ of E151 on virus infection

EV71 and CVA16 viruses were diluted to a concentration of 10^4 TCID₅₀/0.5 ml and treated with different concentrations of E151 in DMEM-10 for 1 h inside the incubator. RD cells were seeded into 24-well plates at density of 10^5 cells/well and incubated for 6 h in the incubator, and then the medium was replaced by 0.5 ml of EV71 diluents so that the cells were infected at a multiplicity of infection (MOI) of 0.1 TCID₅₀/cell. After 48 h infection at the incubator, the infected cells were frozen-thawed 3 times and viruses in supernatant were titrated. The incubations for each virus at each concentration treatment were done in triplicate. Two independent experiments were performed.

Selection of E151 resistant EV71s

E151 high sensitive EV71-B4, EV71-B5 and EV71-C5 strains were repeatedly passaged in RD cells in the presence of 10 µM E151 for 10 times and then in the presence of 100 µM E151 for another 12 times, while E151 low sensitive EV71-2242, EV71-C1 and EV71-C4 were directly passaged in RD cells in the presence of 100 µM E151 for 12 times. E151 resistant EV71-B5-P22, also named EV71-B5^{res}, was selected to identify mutations

responsible for E151 resistance. Briefly, their RNAs were extracted from supernatant of infected cells with RNeasy kits (Qiagen), and then their P1 genes were amplified by OneStep RT-PCR kit (Qiagen) using corresponding primers for sequencing.

5 Generation of EV71 mutants by reverse genetics

The genome of EV71-B5 was first amplified by RT-PCR and put under human RNA polymerase I promoter reverse genetics (RG) system as described in a previous paper [Meng T, *et al.*, (2012) *Viral J* 9: 238], and the infectious plasmids were named pJET-B5. The mutations at VP1-98, VP1-145 and VP1-246 were introduced into the pJET-B5 by site-directed mutagenesis with corresponding primers according to the In-Fusion protocol (Clontech). The infectious mutants were generated by direct transfection of their corresponding infectious plasmids into RD cells. Briefly, 1 µg plasmids were mixed with 2.5 µl of Lipofectamine 2000 (Life Technologies) in Opti-MEM (Life technologies) and then transfected into cell monolayers in 6-well plates. All generated viruses were further propagated in RD cells for 2 passages and the correctness of their VP1 genes of the 2nd passage was confirmed by sequencing. All subsequent cell infection experiments used the 2nd passage RG viruses if no special mention was made.

Binding inhibition assay of EV71s and cells

RD cells were detached by PBS with 5 mM EDTA and washed with DMEM twice. Cells were then re-suspended in DMEM with 1% FBS (DMEM-1) and chilled on ice for 15 min. 10⁸ TCID₅₀ of purified EV71-B4 (high sensitive to E151), EV71-C1 (low sensitive to E151) or EV71-B5^{res} (resistant to E151) were respectively mixed with 2 million RD cells in 500 µl of DMEM-1 containing E151 at a final concentration of 0, 30, 100 or 300 µM. After incubation at 4°C for 1 h with gentle agitation, cells were washed with ice-cold PBS 3 times and the bound viruses were analysed and detected by Western blot.

For assay of E151 elution of attached EV71 from RD cells, 3x10⁸ TCID₅₀ of E151 sensitive EV71-B5 or resistant EV71-B5^{res} were separately incubated with 6 million pre-chilled RD cells at 4°C for 1 h for attachment. Unattached viruses were then washed away with ice-cold PBS. The attached viruses-cells were divided into 3 portions and re-suspended in ice-cold PBS with 0, 30 or 100 µM E151. After incubated at 4°C for another 15 min with gentle agitation, the cell pellets and supernatants were separated for EV71 antigen detection as above.

All binding assays were performed in duplicate in two independent experiments.

Binding inhibition assay of EV71 and viral attachment/uncoating factors

All sepharose beads were first blocked by 3% BSA in PBS with gentle agitation at 4°C for at least 3 h because they can non-specifically interact with EV71 particles.

40 µl of BSA pre-treated protein A/G sepharose beads (Santa Cruz) were directly mixed with
5 2 µg of SCARB2-Fc, PSGL-1-Fc or CTLA-4-Fc in 250 µl of PBS+ (pH7.4, 0.1% BSA and 0.2% Igepal CA-630). At the same time, 10⁸ TCID₅₀ of purified EV71-B4 were diluted in 250 µl of PBS+ or PBS+ containing 600 mM of E151. After incubation at 4°C for 30 min, the viruses were added and incubated with beads and recombinant proteins for another 2 h at 4°C with gentle agitation.

10 20-40 µl of BSA treated heparin sepharose (Abcam) were incubated with 10⁸ TCID₅₀ of purified EV71 in 500 µl of PBS+ containing E151 at a concentration of 0, 10, 30, 100, 300 or 1000 µM at 4°C for 2 h with gentle agitation. BSA treated Glutathione-sepharose beads (Thermo Fisher) containing 30 µg of GST or GST-CylcophilinA were incubated with 10⁸ TCID₅₀ of purified EV71-B5 or EV71-B5^{res} particles in 500 µl of PBS+ or PBS+ containing
15 300 µM of E151 at 4°C with gentle agitation. After incubation, the beads were washed 3 times with ice-cold PBS containing 0.01% Tween-20 and the bound proteins and viruses were analysed by Western blot.

All binding assays were performed in duplicate in two independent experiments.

20 Western blot

The samples were dissolved in SDS loading buffer. After heated at 100°C for 5 to 10 min, the proteins in the samples were separated by 12% SDS-PAGE gel and transferred onto 0.2 µm nitrocellulose membrane (Bio-rad). The membrane was first blocked by 5% non-fat milk in PBS with 0.1% Tween-20 (PBST). For detection of EV71 VP1 protein, the
25 membranes were blotted with mouse anti-VP1 mAb 1D9 (supernatant from hybridoma culture) followed by HRP conjugated goat anti-mouse IgG antibodies (1:2000). For detection of GST tagged proteins, the membranes were blotted with mouse anti-GST sera (1:5000) followed by HRP conjugated goat anti-mouse IgG antibodies (1:2000). For detection of human Fc recombinant proteins, the membranes were blotted with HRP conjugated anti-
30 human Fc antibodies (1:2000). For detection of cellular beta-actin, the membranes were blotted with HRP conjugated anti-human beta-actin monoclonal antibodies (1:8000). All blotting buffers were PBST with 3% non-fat milk and blotting time was usually 2h at room temperature or overnight at 4°C.

The membranes were washed 3 times for 5 to 10 min using PBST after each blotting.
35 After the membranes were incubated with Clarity Western ECL substrate (Bio-Rad), the

chemiluminescent signals were detected and recorded by a Gel-Imager ChemiDoc Touch (Bio-Rad) and analysed by the Image Lab software.

Immunofluorescence confocal microscopy assay

5 Vero cells (5×10^4) in 300 μ l of DMEM-10 were seeded onto μ -Slide 8-well glass bottom slides (ibidi), and 24 h later, the slides were pre-chilled at 4°C for 15 min and the medium was removed. For viral attachment, purified EV71 viruses were diluted in ice-cold DMEM-10 with or without 100 μ M of E151 and then added to the cells at an MOI of 100. After 1 h incubation at 4°C, the cells were washed 3 times with ice-cold DMEM to remove
10 the unattached viruses and then fixed with 4% PFA in PBS for 15 min. For viral internalization, the cells with attached viruses were topped up with fresh 300 μ l of DMEM-10 with or without 100 μ M of E151, and further incubated at 37°C incubator for 15 min to 1 h followed by fixation. The fixed cells were permeabilized with 0.5% triton X-100 in PBS for 10 min and blocked overnight with PBS containing 5% BSA at 4°C. After being washed with
15 PBST, the cells were incubated at 4°C with mouse anti-EV71 sera in PBS+ (1:1000) for at least 3 h with gentle agitation. After PBST washing, the cells were then incubated with AR594 conjugated rabbit anti-EEA1 monoclonal antibody (1:1000) and AR448 conjugated anti-mouse IgG antibodies (1:1000) overnight. The cells were then stained by Hoechst 33258 in PBS (0.1 μ g/ml) for 10 min. After another 3 washes with PBST, the cells in PBS
20 were observed under a confocal laser scanning microscope FV3000 (Olympus).

Ethics Statement

AG129 mice from B&K Universal (United Kingdom) were housed in individual ventilated cages inside ABSL2 lab for animal care and use. All animal experiments were
25 carried out in accordance with the Guides for Animal Experiments of the National Institute of Infectious Diseases (NIID), and experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the Temasek Life Sciences Laboratory Ltd, Singapore (IACUC Project Approval No. TLL-16-023: Influence of food colorings on the infection of human enterovirus 71 *in vivo*).

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EV71 infection in AG129 mice

14 days old AG129 neonates were challenged with 10 LD₅₀ of EV71-B4 (3×10^{10} TCID₅₀) or EV71-C1 (1.5×10^8 TCID₅₀) in 0.2 ml of PBS via the intraperitoneal (i.p.) route.

From 0 to 3 post-challenge days, the mice in E151 protection group were
35 administered daily with 1 dose of E151 in PBS at 200 mg/kg body weight through i.p. route, while only PBS was used in the mock group. The clinical scores and survival rates were

recorded daily until 21 days post-challenge. To study viral propagation in the challenged mice, the muscle of hind limbs and the brain were harvested, weighed and stored at - 80°C after euthanasia of the mice with CO₂. The samples were homogenized at 500 mg/ml in DMEM with 10% FBS and 5x Antibiotic-Antimycotic by using a TissueLyser LT homogenizer (Qiagen). The homogenates were frozen-thawed twice and then kept at 4°C for 1 h. The virus progeny in the supernatants of clarified homogenates (10,000g x 10 min at 4°C) were titrated in RD cells.

Statistics

All quantification of viral titer and binding assays were performed in duplicates or triplicates. The mean values were compared using Student's t-test (two-tailed) and *p* values <0.05 were considered statistically significant.

EXAMPLE 1

15

Inhibitory effects of food dyes on the propagation of EV71-GFP

Eleven widely used food dyes were screened for their potency to inhibit the infection and replication of EV71-GFP, producing GFP as a reporter during viral replication, in rhabdomyosarcoma (RD) cells at a multiplicity of infection (MOI) of 1 following 24 h of incubation at 37°C in the presence of 5% CO₂ (Figure 1). By comparing the GFP signals in the presence of each dye to the mock control, all sulfonated azo dyes Tartrazine (E102), Sunset yellow FCF (E110), Carmoisine (E122), Amaranth (E123), Ponceau 4R (E124), Ponceau 6R (E126), Allura red (E129) and Brilliant black BN (E151) inhibited the infection of EV71-GFP at a concentration of 300 µM because of the reduced GFP signals, without causing cytotoxicity. Among the other 3 sulfonated food dyes Patent blue V (E131), Indigo carmine (E132) and Brilliant blue FCF (E133), only E132 exhibited some degree of inhibition of EV71-GFP at a concentration of 300 µM. Overall, E122, E123, E129 and E151 were the 4 strongest inhibitors of EV71-GFP *in vitro* (Fig. 1).

Antiviral activity of dyes E122, E123, E129 and E151 against infection of human enteroviruses *in vitro*

The four azo dyes E122, E123, E129 and E151, all of which contain sulfonated aromatic rings (Fig. 2A), were further tested in antiviral and cytotoxic studies. They exhibited the dose-dependent inhibition of EV71-GFP in infected RD cells because the number of GFP positive cells was noted to be sequentially reduced with their increased concentration (Fig 2B and 2C). The dye E151 was the best drug against EV71-GFP in RD cells. Based on the

percentage of GFP positive cells, the 50% inhibitory concentration (IC_{50}) of E151 was 10.1 μ M, while the IC_{50} of other three dyes E122, E123 and E129 was about 50 μ M (Fig 2C). In contrast, the 50% cellular cytotoxic concentration (CC_{50}) of E151 in RD was 1870 μ M and the CC_{50} of the other three dyes was more than 5000 μ M, which were evaluated by an MTT assay (Fig 2D).

By using a virus titer reduction assay, the inhibitory effects of the four azo dyes on the infectivity of wild type human enteroviruses was investigated in RD cells. E151 at 100 μ M significantly prevented cytopathic effect (CPE) induced by viral infection and reduced the titers of all 8 RD cell adapted EV71 strains, belonging to different subgenogroups (Fig 3A), and 4 clinical EV71 isolates (Fig 3B). However, E122, E123 and E129 failed to reduce the titers of EV71-C1 and EV71-C4 at the concentration 300 μ M. Moreover, E151 also inhibited the infectivity of all 3 CVA16 and 1 CVA6 viruses, while it did not show the inhibitory effect on the infectivity of 1 CVA4 and 1 CVA10 viruses (Fig 3B). The inhibitory effect of E151 on the growth of 5 representative enteroviruses was also evaluated by titrating progeny viruses from RD cells infected at MOI of 0.1 for 2 days in the presence of various concentrations of E151. The IC_{50} of E151 ranged from 2.39 μ M (highly sensitive EV71-B2) to 28.12 μ M (slightly sensitive EV71-C1) for EV71 (Fig 3C).

Interestingly, the inhibitory effects of the dye E151 on human enteroviruses varied. PSGL-1 binding EV71 strains with VP1-98E,145G/Q, such as EV71-B2, EV71-B4, EV71-B5, EV71-C2, EV71-C5 and EV71-202 (Fig 4A), were highly sensitive to E151 with a titer reduction of $>10^5$, while non-PSGL-1 binding strains with VP1-98K,145E, such as EV71-C1, EV71-C4, EV71252 and EV71 2242 (Fig 4A), were slightly sensitive to E151 with a titer reduction of $\sim 10^2$ in the presence of 100 μ M E151 (Fig 3A and 3B). In order to determine whether the amino acids at VP1-98 and/or VP1-145 affect the sensitivity of EV71 to E151 and the 3 dyes E122, E123 and E129, variants of EV71-B5 and EV71-C4 with different amino acid combinations at VP1-98 and VP1-145 were generated using a reverse genetics (RG) system [Meng T, *et al.*, (2012) *Virology* 9: 238]. In the titer reduction assay, the –EE variants (VP1-98E,145E) of RG/EV71-B5 and RG/EV71-C4 were slightly sensitive to E122, E123 and E151, but resistant to E129. The –KE variants were only slightly sensitive to E151, and resistant to E122 and E123. The –EG and –EQ variants were highly sensitive to all 4 dyes, while the –KG and –KQ variants were highly sensitive to only E122 and E151. Surprisingly, E129 enhanced CPE caused by EV71 variants with VP1-98K in RD cells and increased their titers (Fig 4B). A multiple protein alignment of 6585 full EV71 VP1 sequences released in NCBI GenBank from 1969 to 2017, using DNASTAR MegAlign Pro 13 software, indicates that the –EE variants are dominant and their percentage in total EV71 released

VP1 sequences is about 82.4%. And the percentage of -KE variants ranged from 6.5% to 15.6% since 2008 in every two years interval (Table 3).

Table 3. Percentage of EV71 variants with different amino acid combinations at VP1-98 and VP1-145.

	Year	1969-2007	2008-2009	2010-2011	2012-2013	2014-2015	2016-2017	Total
	Number of sequences	607	497	1481	1314	1388	1298	6585
EV71 variants	VP1-98E,145E	64.0%	68.5%	81.9%	88.7%	88.3%	84.3%	82.4%
	VP1-98K,145E	10.7%	15.6%	6.5%	6.5%	6.7%	9.8%	8.3%
	VP1-98E,145G/Q	24.3%	15.1%	10.8%	4.6%	4.6%	5.5%	8.8%
	VP1-98K,145G/Q	0%	0%	0%	0%	0%	0%	0%
	others	1%	0.8%	0.8%	0.2%	0.4%	0.4%	0.5%

Therefore, E151 would appear to be a more suitable antiviral agent against EV71 infection in nature than other tested dyes.

10 Selection and characterization of E151 dye resistant EV71 mutants

E151 resistant EV71 mutants were selected by passaging EV71 viruses in RD cells in the presence of E151 as described in the methods. The mutant EV71-B5 that was passaged 22 times, named B5-E151-P22 or EV71-B5^{res}, was finally found to be completely resistant to E151 at a concentration of 100 μ M in RD cells (Fig 5A). Sequencing of the genome of EV71-B5^{res} revealed three amino acid mutations: E98K, G145E and P246A in its VP1 protein when compared to its parental wild type EV71-B5 (Fig 5A), implying that switching from E151 highly sensitive strain (-EG) to slightly sensitive strain (-KE) is necessary for EV71-B5 to develop E151 resistance. The sequencing result also suggested that one mutation P246A might be enough for -KE strains of EV71 to resist E151. Most importantly, VP1-246P is highly conserved (>99.9%) in EV71 (Fig 4A), which highlighted that E151 has the potential to inhibit all known circulating EV71 strains.

The EV71 mutants were generated by introducing these mutations into a reverse genetics EV71-B5 infectious clone. Mutants from a second passaging were tested in the titer reduction assay in the presence of 100 μ M E151. The results indicated that E98K did not significantly affect the sensitivity of EV71-B5 to E151 (Fig 4B), but it compensated the growth defects caused by P246A, which did not induce CPE after 3 passages in RD cells

(data not shown). Combined with E98K, the mutation G145E or P246A significantly reduced the titer reduction from $>10^5$ in EV71-B5-wt to $\sim 10^2$ in these double mutants, but only the combination of E98K, G145E and P246A confer the total E151 resistance on EV71-B5 (Fig 4B and 5B). Moreover, the mutation P246A in EV71-C4 and EV71-2242 strains rendered them E151 resistant. However, P246A reduced the growth titer of the mutants about 10 to 100 times (Fig 5B). The findings also corroborated with the results shown in Figure 2 that EV71 strains with VP1-145G/Q were highly sensitive to E151, while EV71 strains with VP1-145E had low sensitivity to E151. Based on the crystal structure of EV71 virions, VP1-145 and VP1-246 are located at the vertex of the 5-fold axis and proximate to the VP1-242K and VP1-244K (Figure 5C and 5D). The negatively charged E151 (Figure 5B) might interact with VP1-242K and VP1-244K to affect the viral infection, and mutations VP1-145E and VP1-246A could disrupt the interaction of E151 and EV71.

Inhibition of EV71 entry by E151

To investigate which steps in EV71 replication life cycle could be affected by E151, E151 was added at different time points of post-infection of EV71-GFP in RD or Vero cells at MOI of 1 (Figure 6A). The inhibitory effect of E151 on the infectivity of EV71-GFP occurred only when the addition of E151 was done within 2 h post-infection, as indicated by the significant reduction of infected cells with positive GFP signals at 12 h post-infection. Therefore, E151 disrupted the early stages of EV71 infection, such as viral attachment, internalization and/or uncoating, but not its replication inside the cells.

To further dissect the inhibitory mechanism of E151, attachment of EV71 to cells and attachment factors were performed in the absence or presence of E151 (Figure 7). In a cell-virus binding assay, E151 prevented the viral attachment to RD cells in a concentration dependent manner. Attachment of EV71-B4 (highly sensitive to E151) and EV71-C1 (slightly sensitive to E151) onto RD cells was significantly blocked by E151 at a concentration of 30 μ M and 100 μ M, respectively, detected by specific anti-VP1 monoclonal antibody 1D9 in Western blot. Although EV71-B5^{res} is resistant to E151, E151 also partially blocked its attachment to RD cells at a very high concentration of 300 μ M (Figure 6B). However, 300 μ M of E151 did not reduce the infectivity of EV71-B5^{res} in RD cell culture (data not shown). In a pull-down assay, E151 also blocked the binding of EV71-B4 to the PSGL-1-Fc fusion protein as both E151 and PSGL-1 interacted with the vertex of viral 5-fold axis [Nishimura Y, *et al.*, (2013) *PLoS Pathog* 9: e1003511], while it did not prevent the interaction between EV71-B4 and human SCARB2-Fc recombinant protein which has been identified to bind the viral canyon region [Dang M, *et al.*, (2014) *Protein Cell* 5: 692-703](Figure 6C). Furthermore,

E151 prevented the binding of EV71-B4 to heparin sepharose, which also targets the vertex, in a concentration dependent manner (Figure 6D).

In order to evaluate whether amino acids at VP1-145 and VP1-246 determine the effects of the dye E151 on EV71 attachment, RG/EV71-B5-EGP (VP1-98E,145G,246P, highly sensitive to E151), RG/EV71-B5-KEP (VP1-98K,145E,246P, slightly sensitive to E151) and RG/EV71-B5-KEA (VP1-98K,145E,246A, resistant to E151) mutants were tested in the same cell-virus attachment assay. The results concurred with that of corresponding wild type EV71 variants. Compared to the RG/EV71-B5-EGP whose attachment to RD cells and heparin was inhibited by E151 at a concentration of 30 μ M and 100 μ M, respectively; the attachment of RG/EV71-B5-KEP to both RD cells and heparin was only inhibited at higher concentrations of E151. As expected, P246A in RG/EV71-B5-KEA rendered attachment of the mutant not to be affected by E151 (Fig 7A). In an immunofluorescence confocal microscopy assay, the attached EV71 virions on Vero cells was detected by anti EV71-B4 sera after 1 h incubation at 4 °C. The attachment of RG/EV71-B5-KEP was strongly prevented by 100 μ M of E151 as the viral puncta (green) per cell were significantly reduced from 49 ± 5.9 (average number $\pm$ standard error) in the absence of E151 to 1.8 ± 0.8 in the presence of E151, while the attachment of E151 resistant RG/EV71-B5-KEA was not significantly affected by E151 as no statistical differences in viral puncta per cell between mock and E151 treated cells (Fig 7B).

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Inhibition of EV71 infection at post-attachment stage by the dye E151

At the post-attachment stage, 100 μ M E151 significantly prevented the EV71-GFP infection and reduced the percentage of infected cells from 32.9 ± 0.59 to 0.65 ± 0.22 in RD cells and from 12.5 ± 0.51 to 0.43 ± 0.16 in Vero cells (Fig 8A). The influence of E151 on the internalization of purified EV71 was further examined in the immunofluorescence confocal microscopy assay. RG/EV71-B5-KEP and RG/EV71-B5-KEA were incubated with Vero cells in the absence of E151 at 4 °C for attachment. After unattached viruses were washed away by cold PBS, the attached viruses and cells in the absence or presence of 100 μ M E151 were shifted into 37 °C incubator for 30 min to allow the viral internalization. The cells were then fixed, and internalized EV71 virions and early endosome antigen 1 (EEA1) were observed. In the absence of E151 (mock), the internalization of both RG/EV71-B5-KEP and RG/EV71-B5-KEA was normal, and some of the EV71 puncta (green) were co-localized with EEA1 (red), indicating that the internalized virions fused with early endosomes (Fig 8B). However, in the presence of E151, fewer RG/EV71-B5-KEP puncta per cell (3 ± 1.3) were detected compared to that (44.5 ± 4.9) in mock, while the number of RG/EV71-B5-KEA puncta per cell was not significantly different between mock (38.6 ± 4.5) and E151 treated

cells (39 ± 4.1). Therefore, the internalization of RG/EV71-B5-KEP was inhibited by E151. The viral particles could probably be detached from cells by E151 as the internalized EV71 could not be degraded by cells in the presence of E151 in 30 min.

In order to demonstrate that E151 could elute the attached EV71, RD cells attached by E151 sensitive EV71-B5 or resistant EV71-B5^{res} were re-suspended in ice-cold PBS with 0, 30 or 100 μ M E151. After incubated at 4 °C for 15 min with gentle agitation, the cell pellets and supernatants were separated for EV71 antigen detection by Western blot. The result indicated that the attached EV71-B5 but not EV71-B5^{res} was eluted or released from the cells into the supernatants by E151 at a concentration dependent manner (Fig 8C).

After viral internalization, E151 was further found to prevent the binding of EV71-B5 to an EV71 uncoating factor cyclophilin A (CypA). In the GST pull down assay, EV71-B5 interacted with GST-CypA but not GST, and it failed to bind to GST-CypA in the presence of 100 μ M E151 (Fig 9). The mutation P246A in EV71-B5^{res} significantly weakened the interaction between the virus and GST-CypA as reported before [Qing J, *et al.*, (2014) *PLoS Pathog* 10: e1004422], and this weak interaction was also inhibited by E151 (Fig 9).

***In vivo* protection of EV71 challenged AG129 mice by E151**

The efficacy of E151 was assessed in 14-days old AG129 pups intraperitoneally challenged with 10 LD₅₀ of wild type EV71-B4 (3×10^{10} TCID₅₀) or EV71-C1 (1.5×10^8 TCID₅₀). The mice (8 pups per group) were then treated with E151 200 mg/kg body weight (bw)/day or 200 μ l PBS/day through intraperitoneal inoculation from the fourteenth to seventeenth day (0 to 3 days post challenge). The PBS treated mice in control groups started to exhibit clinical symptoms, such as a hunched back and limb paralysis, from as early as 6-day post-challenge and died from 8-day post-challenge. Eventually they all died with the survival percentage declining to 0. But in the case of E151 treatment, all mice did not show limb paralysis and were completely protected from death throughout the whole experiment (Fig 10A).

To further evaluate the protective efficacy of E151, the EV71-C1 challenged mice with the PBS or E151 treatment were euthanized at 4, 8 or 12 days post-challenge (5 mice per group per time point), and then titers of progeny virus in the brain and hind limb muscle tissues were titrated (Fig 10B). For the PBS treated mice, infectious progeny viruses were detected in the muscle and brain from 4-day post-challenge. The titers of progeny virus in brains continually increased with time, while those in muscle reached the highest at 4-day post-challenge and declined in following time as previously reported [Khong WX, *et al.*, (2012) *J Virol* 86: 2121-2131]. In contrast, significantly lower virus titers in both brain and muscle tissues were detected in the mice treated with E151. Although the titers of progeny

virus in muscle of E151 treated mice increased after ceasing E151 treatment from 4-day post-challenge; those in brains did not increase to a detectable level ($10^{3.5}$ TCID₅₀/g).

Summary

5 Drug development for human infectious diseases is facing obstacles due to its requirement for a prolonged time of evaluation and huge costs. Discovery of novel uses of chemicals with a good safety record and previous approved usage could be an attractive approach. The cell entry of EV71 begins with its attachment to the molecules on the cell membrane and ends with the release of its genome into the cytoplasm [Dimitrov DS, (2004) *Nat Rev Microbiol* 2: 109–122]. Multiple anti-EV71 drugs targeting the EV71 entry have been reported [Shang L, *et al.*, (2013) *Antiviral Res* 97: 183-194; Tan CW, *et al.*, (2014) *J Biomed Sci* 21: 14]. Food azo dyes, approved as food additives, are very stable at room temperature and easy to be produced at a low cost [Zollinger H (2003) *Color chemistry: syntheses, properties, and applications of organic dyes and pigments* 3rd Edition, Wiley-VCH, Cambridge]. They have been used for many years without obvious reported untoward side effects. In this study, they were demonstrated to be able to inhibit the infectivity of EV71, CVA16 and CVA6, which are 3 major causative agents of HFMD.

We discovered that several of the food azo dyes tested have some degree of inhibitory effects on the infectivity of EV71, CVA16 and/or CVA6 *in vitro* through blocking virus entry. Here, Brilliant Black BN (E151) is more fully explored as it has been shown in our study to be able to inhibit all known EV71 strains with the highest efficiency. E151 inhibited the infectivity of EV71 by targeting the vertex of the viral 5-fold axis to prevent the viral attachment to the attachment factors sulfated PSGL-1 and heparin (Fig 6). On the other hand, both E151 and NF449 did not affect the binding of EV71 to the human SCARB2, a functional receptor interacting with the canyon region of EV71. These results suggested that human SCARB2 might not be necessary for EV71 attachment; and coincided with that knockdown of SCARB2 in RD or Vero cells had no influence on the EV71 attachment [Ku Z, *et al.*, (2015) *J Virol* 89: 12084-12095]. Interestingly, E151 also prevented EV71 infection at the post-attachment stage by eluting the attached virions from RD and Vero cells, implying that the EV71 attachment onto the cells was reversible and E151 competed with cellular attachment factors to bind to EV71 (Fig 8). Moreover, E151 disrupted the interaction between EV71 and the viral uncoating factor human cyclophilin A, but not human SCARB2. However, the total effects of E151 on the uncoating and RNA release of EV71 need further study. After the cell entry, the following replication of EV71 seemed not to be inhibited by E151 (Fig 6A). Overall, E151 prevented the entry of EV71 *in vitro*.

The dye E151 exhibited more inhibitory effects on infection of EV71 isolates with VP1-145G/Q than that with VP1-145E (Fig 3 and 4). Interestingly, the substitution of G/Q for E at VP1-145 also decreased the efficacy of suramin in preventing EV71 infection *in vitro* by 30 times [Ren P, *et al.*, (2017) *Sci Rep* 7: 42902], suggesting that negatively charged VP1-145E altered the electrostatic property of the vertex (Fig 5D) and weakened the binding affinity between EV71 and E151/suramin. Besides, VP1-246P was involved in the interaction of E151 and EV71 too. The mutation VP1-P246A conferred E151 resistance on the EV71 strains with VP1-98K,145E, but reduced the growth titer of mutants, implying that the E151 resistant EV71 might be evolutionarily disadvantaged. Although the amino acids at VP1-98 and VP1-145 constantly switch from E to K and E to G/Q, respectively [Tee KK, *et al.*, (2010) *J Virol* 84: 3339-3350], VP1-246P is highly conserved in EV71 (Fig. 4A). Therefore, E151 could potentially inhibit all known circulating EV71 strains.

Although HFMD is mild and self-limited, it is highly contagious, associated with significant morbidities, occasionally severe neurological complications and fatalities especially those due to EV71. Therefore, antiviral drugs, especially against EV71, are urgently needed to combat HFMD in two scenarios: (i) therapeutic treatment of children with HFMD to speed up recovery and reduce morbidity, and (ii) to reduce the infectivity of the shed virus which will indirectly reduce its transmissibility.

E151 and other food azo dyes tested herein have been used for many years as food additives, so they would be considered safe enough to be used as therapeutics. Considering that food azo dye E151 effectively prevented EV71 infection *in vitro* and *in vivo*, it could be a promising candidate drug to treat HFMD caused by EV71, CVA16 and CVA6 in children.

REFERENCES

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Any listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that such document is part of the state of the art or is common general knowledge.

- 30 1. Baba M, Schols D, Mohan P, De Clercq E, Shigeta S (1993) Inhibition of HIV-1-induced cytopathogenicity, syncytium formation, and virus-cell binding by naphthalenedisulfonic acids through interaction with the viral envelope gp120 glycoprotein. *Antiviral Chem Chemother* 4: 229-234.
- 35 2. Chang LY, Lin TY, Hsu KH, Huang YC, Lin KL, et al. (1999) Clinical features and risk factors of pulmonary oedema after enterovirus-71-related hand, foot, and mouth disease. *Lancet* 354: 1682-1686.

3. Chumakov M, Voroshilova M, Shindarov L, Lavrova I, Gracheva L, et al. (1979) Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol 60: 329-340.
4. Dang M, Wang X, Wang Q, Wang Y, Lin J, et al. (2014) Molecular mechanism of SCARB2- mediated attachment and uncoating of EV71. Protein Cell 5: 692-703.
5. Dimitrov DS. (2004) Virus entry: molecular mechanisms and biomedical applications. Nat Rev Microbiol 2:109–122.
6. Khong WX, Yan B, Yeo H, Tan EL, Lee JJ, et al. (2012) A non-mouse-adapted enterovirus 71 (EV71) strain exhibits neurotropism, causing neurological manifestations in a novel mouse model of EV71 infection. J Virol 86: 2121-2131.
10. 7. Ku Z, Ye X, Shi J, Wang X, Liu Q, et al. (2015) Single Neutralizing Monoclonal Antibodies Targeting the VP1 GH Loop of Enterovirus 71 Inhibit both Virus Attachment and Internalization during Viral Entry. J Virol 89: 12084-12095.
8. Lee H, Cifuentes JO, Ashley RE, Conway JF, Makhov AM, et al. (2013) A strain-specific epitope of enterovirus 71 identified by cryo-electron microscopy of the complex with Fab from neutralizing antibody. J Virol 87: 11363-11370.
15. 9. McMinn P, Stratov I, Nagarajan L, Davis S (2001) Neurological manifestations of enterovirus 71 infection in children during an outbreak of hand, foot, and mouth disease in Western Australia. Clin Infect Dis 32: 236-242.
20. 10. Meng T, Kiener TK, Kwang J (2012) RNA polymerase I-driven reverse genetics system for enterovirus 71 and its implications for vaccine production. Virol J 9: 238.
11. Nishimura Y, Shimojima M, Tano Y, Miyamura T, Wakita T, et al. (2009) Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nat Med 15: 794-797.
25. 12. Nishimura Y, Wakita T, Shimizu H (2010) Tyrosine sulfation of the amino terminus of PSGL-1 is critical for enterovirus 71 infection. PLoS Pathog 6: e1001174.
13. Nishimura Y, Lee H, Hafenstein S, Kataoka C, Wakita T, et al. (2013) Enterovirus 71 binding to PSGL-1 on leukocytes: VP1-145 acts as a molecular switch to control receptor interaction. PLoS Pathog 9: e1003511.
30. 14. Nishimura Y, McLaughlin NP, Pan J, Goldstein S, Hafenstein S, et al. (2015) The Suramin Derivative NF449 Interacts with the 5-fold Vertex of the Enterovirus A71 Capsid to Prevent Virus Attachment to PSGL-1 and Heparan Sulfate. PLoS Pathog 11: e1005184.
35. 15. Ojala WH, Ojala CR, Gleason WB. (1995) The X-ray Crystal Structure of the Sulfonated Azo Dye Congo Red, a Non-Peptidic Inhibitor of HIV-1 Protease which

- also Binds to Reverse Transcriptase and Amyloid Proteins. *Antiviral Chem Chemother* 6: 25-33.
16. Ojala WH, Sudbeck EA, Lu LK, Richardson TI, Lovrien RE, et al. (1996) Complexes of Lysine, Histidine, and Arginine with Sulfonated Azo Dyes: Model Systems for Understanding the Biomolecular Recognition of Glycosaminoglycans by Proteins. *J Am Chem Soc* 118: 2131-2142.
17. Ono M., Wada Y., Wu Y., Nemori R., Jinbo Y., et al. (1997) FP-21399 blocks HIV envelope protein-mediated membrane fusion and concentrates in lymph nodes. *Nat Biotechnol* 15: 343-348.
18. Plevka P, Perera R, Cardoso J, Kuhn RJ, Rossmann MG (2012) Crystal structure of human enterovirus 71. *Science* 336: 1274.
19. Pourianfar HR, Poh CL, Fecondo J, Grollo L (2012) In vitro evaluation of the antiviral activity of heparan sulfate mimetic compounds against Enterovirus 71. *Virus Res* 169: 22-29.
20. Qing J, Wang Y, Sun Y, Huang J, Yan W, et al. (2014) Cyclophilin A associates with enterovirus-71 virus capsid and plays an essential role in viral infection as an uncoating regulator. *PLoS Pathog* 10: e1004422.
21. Ren P, Zou G, Bailly B, Xu S, Zeng M, et al. (2014) The approved pediatric drug suramin identified as a clinical candidate for the treatment of EV71 infection-suramin inhibits EV71 infection in vitro and in vivo. *Emerg Microbes Infect* 3: e62.
22. Ren P, Zheng Y, Wang W, Hong L, Delpeyroux F, et al. (2017) Suramin interacts with the positively charged region surrounding the 5-fold axis of the EV-A71 capsid and inhibits multiple enterovirus A. *Sci Rep* 7: 42902.
23. Robinson T, McMullan G, Marchant R, Nigam P (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresour Technol* 77: 247-255.
24. Shang L, Xu M, Yin Z (2013) Antiviral drug discovery for the treatment of enterovirus 71 infections. *Antiviral Res* 97: 183-194.
25. Shimizu H, Utama A, Yoshii K, Yoshida H, Yoneyama T, et al. (1999) Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. *Jpn J Infect Dis* 52: 12-15.
26. Solomon T, Lewthwaite P, Perera D, Cardoso MJ, McMinn P, et al. (2010) Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis* 10: 778-790.
27. Tan CW, Poh CL, Sam IC, Chan YF (2013) Enterovirus 71 uses cell surface heparan sulfate glycosaminoglycan as an attachment receptor. *J Virol* 87: 611-620.

28. Tan CW, Lai JK, Sam IC, Chan YF (2014) Recent developments in antiviral agents against enterovirus 71 infection. *J Biomed Sci* 21: 14.
29. Tan CW, Sam IC, Lee VS, Wong HV, Chan YF (2017) VP1 residues around the five-fold axis of enterovirus A71 mediate heparan sulfate interaction. *Virology* 501: 79-87.
- 5 30. Tang ML, Kiener TK, Lim XF, Kwang J (2012) Identification and characterization of a monoclonal antibody recognizing the linear epitope RVADVI on VP1 protein of enterovirus 71. *J Med Virol* 84: 1620-1627.
31. Tee KK, Lam TT, Chan YF, Bible JM, Kamarulzaman A, et al. (2010) Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene. *J Virol* 84: 3339-3350.
- 10 32. Wang X, Peng W, Ren J, Hu Z, Xu J, et al. (2012) A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. *Nat Struct Mol Biol* 19: 424-429.
33. Wang Y, Qing J, Sun Y, Rao Z (2014) Suramin inhibits EV71 infection. *Antiviral Res* 103: 1-6.
- 15 34. Weglarz TE, Gorecki L (2012) Azo dyes – biological activity and synthetic strategy. *CHEMIK* 66: 1303-1307.
35. Wu JM, Wang JN, Tsai YC, Liu CC, Huang CC, et al. (2002) Cardiopulmonary manifestations of fulminant enterovirus 71 infection. *Pediatrics* 109: E26-.
36. Wu Y, Yeo A, Phoon MC, Tan EL, Poh CL, et al. (2010) The largest outbreak of hand, foot and mouth disease in Singapore in 2008: the role of enterovirus 71 and coxsackievirus A strains. *Int J Infect Dis* 14: e1076-1081.
- 20 37. Yamayoshi S, Yamashita Y, Li J, Hanagata N, Minowa T, et al. (2009) Scavenger receptor B2 is a cellular receptor for enterovirus 71. *Nat Med* 15: 798-801.
38. Yang F, Ren L, Xiong Z, Li J, Xiao Y, et al. (2009) Enterovirus 71 outbreak in the People's Republic of China in 2008. *J Clin Microbiol* 47: 2351-2352.
- 25 39. Yuan S, Li G, Wang Y, Gao Q, Cui R, et al. (2015) Identification of Positively Charged Residues in Enterovirus 71 Capsid Protein VP1 Essential for Production of Infectious Particles. *J Virol* 90: 741-752.
40. Zhu F, Xu W, Xia J, Liang Z, Liu Y, et al. (2014) Efficacy, safety, and immunogenicity of an enterovirus 71 vaccine in China. *N Engl J Med* 370: 818-828.
- 30 41. Zhou Y, Li JX, Jin PF, Wang YX, Zhu FC (2016) Enterovirus 71: a whole virion inactivated enterovirus 71 vaccine. *Expert Rev Vaccines* 15: 803-813.
42. Zollinger H (2003) Color chemistry: syntheses, properties, and applications of organic dyes and pigments 3rd Edition, Wiley-VCH, Cambridge.