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Synthesis and antiviral effect of novel fluoxetine analogues as enterovirus 2C inhibitors.

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Abstract:

Enteroviruses (EV) are a group of positive-strand RNA (+RNA) viruses that include many important human pathogens (e.g. poliovirus, coxsackievirus, echovirus, numbered enteroviruses and rhinoviruses). Fluoxetine was identified in drug repurposing screens as potent inhibitor of enterovirus B and enterovirus D replication. In this paper we are reporting the synthesis and the antiviral effect of a series of fluoxetine analogues. The results obtained offer a preliminary insight into the structure-activity relationship of its chemical scaffold and confirm the importance of the chiral configuration. We identified a racemic fluoxetine analogue, **2b**, which showed a similar antiviral activity compared to (*S*)-fluoxetine. Investigating the stereochemistry of **2b** revealed that the *S*-enantiomer exerts potent antiviral activity and increased the antiviral spectrum compared to the racemic mixture of **2b**. In line with the observed antiviral effect, the *S*-enantiomer displayed a dose-dependent shift in the melting temperature in thermal shift assay, indicative for direct binding to the recombinant 2C protein.

Keywords:

Enterovirus; 2C Inhibitors; Fluoxetine; Thermal shift assay.

Enteroviruses (EV) form the largest genus in the *Picornaviridae* family of positive-strand RNA (+RNA) viruses and include many important human pathogens (e.g. poliovirus, coxsackievirus, echovirus, numbered enteroviruses and rhinoviruses). Infections with EV cause a wide variety of clinical manifestations ranging from mild diseases like hand-foot-and-mouth disease, conjunctivitis to severe conditions like aseptic meningitis, severe neonatal sepsis like diseases and acute flaccid paralysis and myelitis. Rhinoviruses (RV) cause the common cold and can trigger exacerbation of asthma and chronic obstructive pulmonary disease (COPD). (1) These diseases are mostly self-limiting but can give rise to life-threatening respiratory and/or neurological complications especially in infants, young children and immunocompromised individuals. The increasing outbreaks of EV-D68 and several other emerging enteroviruses (e.g. EV-A71 and CV-A16) with severe neurological complications worldwide exemplify the public health threat emerging from EVs. (2–4) Despite their huge socioeconomical and medical burden, vaccines only exist against poliovirus and EV-A71, for which vaccines were recently approved in China. (5) Currently, no antiviral therapy to combat EV infections is approved and treatment is limited to supportive care.

Fluoxetine (Prozac®), a selective serotonin reuptake inhibitor (SSRI) licensed for the treatment of major depression and anxiety disorders, was identified in drug repurposing screens as potent inhibitor of enterovirus B and enterovirus D replication. (6,7) Mode-of-action studies revealed that only the *S*-enantiomer of fluoxetine inhibits viral replication by directly binding to the non-structural protein 2C. (8) The ATP^{ase} dependent RNA helicase 2C is a highly conserved non-structural protein among EVs and involved in pleiotropic functions during the viral life cycle (uncoating, RNA replication, encapsidation, membrane rearrangement). (9–17) Fluoxetine was shown to inhibit EV-B replication in mice and additionally has already been successfully used to treat an immunocompromised child with life-threatening chronic enterovirus encephalitis. (18,19) Together this indicates that fluoxetine offers a potential option as antiviral therapy for clinical use. Here, we report an initial investigation of a series of fluoxetine analogues, in which we introduce some basic changes in the original scaffold, to gain an early insight into the structure-activity relationships of fluoxetine.

We previously reported a profiling of several fluoxetine fragments and described that the fragment *N*-Methyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine showed modest antiviral activity against coxsackievirus B3 (CVB3). (20) This result indicated that the structural features of the trifluoro-phenoxy moiety and the amino moiety are essential for the antiviral activity whereas the 3-phenyl moiety seems dispensable. The *para*-trifluoro-phenoxy moiety is crucial for the SSRI activity because changes of the substituent lower the affinity towards the serotonin transporter (SERT) (21). Hence, fluoxetine analogues with modifications on the CF₃-substituent positions on the phenoxy ring were synthesized. Rather than in *para* position, the CF₃ group was placed in *ortho* or in *meta* position on compounds **1a** and **1b**, respectively. In compounds **1c** and **1d**, an additional substituent in *ortho* position was introduced to the parent compound (22).

The second moiety of interest was the methylamine group. The well-characterised pan-enterovirus inhibitor guanidine hydrochloride (GuaHCl) has been shown to target 2C (23). We designed fluoxetine analogues substituting the methylamine group with GuaHCl. Compounds **5a** and **5b** differ in the length of the linker chain accounting for the additional atom in GuaHCl compared to the methylamine. Further, in compound **3** the *N*-methyl group was replaced by an acetyl group, to explore the need of a basic nitrogen in that position.

CVB3 causes an observable cytopathic effect (CPE) apparent as rounding, detachment and eventually dying of cells. The newly synthesized compounds were tested in a multicycle CPE-reduction assay to elucidate whether they were capable of inhibiting virus replication and thereby preventing the formation of CPE similar to fluoxetine. Therefore, subconfluent HeLaR19 cells were treated with a concentration range of compounds and the cells were immediately infected with CVB3 at MOI 0.001 resulting in full CPE in the infected control without compound treatment within 3 days. In parallel, cytotoxicity was determined using a colorimetric method using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) salt (MTS). It should be noted at this point that all compounds were synthesized as racemic mixture and therefore both the racemic mixture as well as the enantiomers of fluoxetine were used as positive controls. Changing the CF₃ substituent from

para to *ortho* or *meta* position or introducing a second substituent on the ring abolished the antiviral activity of compounds **1a-1d** (Table 1). On the contrary, compounds **2a**, **2b** and **4a**, which contained changes on the amino moiety, retained antiviral activity. Changing the *N*-methyl group to the acetamide group resulted in the inactive compound **3** (Table1).

Like fluoxetine, compound **2b** was synthesized and tested as a racemic mixture. It proves to be as potent in inhibiting CVB3 replication as (*S*)-fluoxetine and 10-fold more potent than racemic fluoxetine. To exclude a cell-type specific effect of the antiviral efficacy and to evaluate the cytotoxicity of compounds **2a** and **2b**, multicycle assays using different cell lines was performed. Subconfluent HEK239T cells and HAP1 cells were treated with serial dilution of the compounds **2a** and **2b** and cytotoxicity as well as antiviral activity against CVB3 were evaluated in parallel. Compound **2a** and compound **2b** show the same range of antiviral activity against CVB3 in all cell lines. Importantly, both compounds are 2- to 3-fold less cytotoxic than racemic or (*S*)-fluoxetine in all three cell lines tested (Table 2). For unknown reasons, compounds **2a** and **2b** did not show antiviral activity in the monkey cell lines BGM and Vero (data not shown). Taken together, changes in the trifluoro phenoxy part of the molecule resulted in loss of antiviral activity. Modifications on the amine part were tolerated and increased the antiviral activity and the selectivity index (SI) of the compounds slightly.

To investigate the broad-spectrum anti-enteroviral activity of the compounds **2a** and **2b**, Hela R19 cells were infected with representative virus serotypes of different enteroviruses species in both a multicycle CPE reduction assay (MOI 0.001 or 0.01, depending on virus, see Supplementary Information) and in a single cycle assay (MOI 1) in which virus reproduction was evaluated after 8 h or 10 h of infection (depending on virus, see Supplementary Information). Both, **2a** and **2b** inhibited CVB3 and EV-D68, but not EV-A71 or representatives of the EV-C species (poliovirus and CV-A24) (Table 3 and Figure 1). Compound **2b** showed a slightly higher potency towards CVB3 and EV-D68 compared to compound **2a**. Unlike racemic fluoxetine, **2b** also inhibited HRV-14 replication. Notably, **2b** inhibited HRV-14 even more potently than (*S*)-fluoxetine. However, unlike (*S*)-fluoxetine, **2b** did not inhibit HRV-2 (Table 3 and Figure 1).

Over the last decades several structurally disparate 2C inhibitors were identified but the mode of action is poorly understood. (24) We previously reported the putative binding area of (S)-fluoxetine in a homology model of CVB3 2C, which was based on the published crystal structure of EV-A71 2C, and provided experimental support for that model through mutational analysis of potential interacting residues. (20,25) We demonstrated that the triple mutations A224V-I227V-A229V, which gives cross resistance towards most of the 2C inhibitors, (16) as well as the single mutations I227V, C179F and F190L conferred resistance towards (S)-fluoxetine. (20) To explore if the newly synthesized compounds have a similar resistance profile, and thus potentially occupy the same binding site, we infected Hela R19 cells with viruses carrying mutations which confer resistance to (S)-fluoxetine. Viruses harbouring the 2C triple mutations A224V-I227V-A229V or the single mutations I227V, C179F or F190L were tested for cross-resistance towards the novel analogues **2a** and **2b**. HelaR19 cells were infected with mutant viruses at an MOI of 1 and virus titers were determined by endpoint titration at 8 hours post infection. The triple mutant A224V-I227V-A229V conferred a high level of resistance towards both compound **2a** and **2b** as it does to (S)-fluoxetine. Remarkably, the single mutation I227V showed resistance towards (S)-fluoxetine but not against the new analogues **2a** or **2b**. The residue C179F conferred resistance towards (S)-fluoxetine and **2b**, but not against **2a**. Notably, the mutation F190L did not confer resistance to either **2a** or **2b**. Summarized, the overall resistance profile for the new compounds is very similar to (S)-fluoxetine but not identical. This suggests that the compounds likely occupy the same binding pocket as (S)-fluoxetine, but the exact binding mode could be slightly different. Unfortunately, the lack of an experimental structure of the fluoxetine/2C complex does not yet allow us to generate an accurate binding model for the newly reported compounds.

Given the improved antiviral activity of the racemic mixture of **2b**, we dissected the role of the two **2b**-enantiomers. The antiviral activity of the enantiomers was evaluated in a multicycle assay. The S-enantiomer of **2b** showed a ~3-4 fold increased antiviral activity against CVB3, EV-D68 and HRV-14 compared to the racemic **2b** and (S)-fluoxetine (Figure 3A). Additionally, the S-enantiomer but not the racemic mixture of **2b** also inhibited HRV-2 (Figure 3A).

Remarkably, the *R*-enantiomer showed subtle antiviral activity against CVB3 and EV-D68 (Figure 3A). Both enantiomers did not inhibit EV-A71 or the representative members of the EV-C species (PV-1 and CV-A24, data not shown). Additionally, we investigated the binding of the two enantiomers to a recombinant fragment of CVB3 2C (Δ 116) using a thermal shift assay. As reported previously, (*S*)-fluoxetine shifted the melting temperature of 2C in a dose-dependent manner. Consistent with the antiviral activity, the **2b** *S*-enantiomer caused a dose-dependent shift in the melting temperature of 2C, indicative of direct binding. Unlike (*R*)-fluoxetine, a thermal shift was also observed for the **2b** *R*-enantiomer of **2b** at higher concentrations. This suggests that the *R*-enantiomer of **2b** exerts indeed subtle antiviral activity.

In conclusion, our study established that the introduced changes on the *para*-trifluoro-phenoxy moiety of fluoxetine resulted in the loss of antiviral activity. Although it may not be possible to fully uncouple the SSRI activity from the antiviral activity, it appears that modifications on the amine moiety can increase the antiviral activity and reduce cytotoxicity. Additionally, we confirmed the importance of the chiral configuration in maintaining the antiviral activity. Similar to fluoxetine, the antiviral activity of the **2b** *S*-enantiomer was higher compared to the *R*-enantiomer or the racemic mixture of **2b**. Interestingly, unlike (*R*)-fluoxetine, the **2b** *R*-enantiomer gained subtle antiviral activity against CV-B3 and EV-D68. In line with the antiviral activity, the *S*-enantiomer as well as high concentrations of *R*-enantiomer caused a dose-dependent thermal shift of 2C, suggestive of a direct interaction. Known resistance mutations confer cross-resistance to the analogues **2a** and **2b** and our data indicate that the novel compounds interact with 2C in a similar manner as (*S*)-fluoxetine. However, the observed variations in the resistance profile of the two drugs point to subtle differences in the interaction with the 2C protein.

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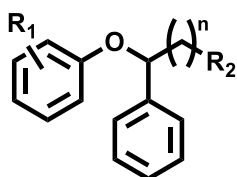
References:

1. Tapparel C, Siegrist F, Petty TJ, Kaiser L. Picornavirus and enterovirus diversity with associated human diseases. *Infect Genet Evol.* 2013;14(1):282–93.
2. Pons-Salort M, Parker EPK, Grassly NC. The epidemiology of non-polio enteroviruses: Recent advances and outstanding questions. *Curr Opin Infect Dis.* 2015;28(5):479–87.
3. Cassidy H, Poelman R, Knoester M, Van Leer-Buter CC, Niesters HGM. Enterovirus D68 – The New Polio? *Front Microbiol. Frontiers*; 2018 Nov;9:2677.
4. Morens DM, Folkers GK, Fauci AS. Acute Flaccid Myelitis: Something Old and Something New. *MBio. American Society for Microbiology (ASM)*; 2019;10(2).
5. Aw-Yong KL, NikNadia NMN, Tan CW, Sam I, Chan YF. Immune responses against enterovirus A71 infection: Implications for vaccine success. *Rev Med Virol. John Wiley & Sons, Ltd*; 2019 Sep;29(5).
6. Ulferts R, Van Der Linden L, Thibaut HJ, Lanke KHW, Leyssen P, Coutard B, et al. Selective serotonin reuptake inhibitor fluoxetine inhibits replication of human enteroviruses B and D by targeting viral protein 2C. *Antimicrob Agents Chemother.* 2013;57(4):1952–6.
7. Zuo J, Quinn KK, Kye S, Cooper P, Damoiseaux R, Krogstad P. Fluoxetine is a potent inhibitor of coxsackievirus replication. *Antimicrob Agents Chemother.* 2012;56(9):4838–44.
8. Bauer L, Manganaro R, Zonsics B, Strating JRP., El Kazzi P, Lorenzo Lopez M, et al. Fluoxetine inhibits enterovirus replication by targeting the viral 2C protein in a stereospecific manner. *ACS Infect Dis. American Chemical Society*; 2019 Jul;acsinfecdis.9b00179.
9. Mirzayan C, Wimmer E. Genetic analysis of an NTP-binding motif in poliovirus polypeptide 2C. *Virology.* 1992;189(2):547–55.
10. Rodriguez PL, Carrasco L. Poliovirus protein 2C has ATPase and GTPase activities. *J Biol Chem.* 1993;268(11):8105–10.
11. Mirzayan C, Wimmer E. Biochemical Studies on Poliovirus Polypeptide 2C: Evidence for ATPase Activity. *Virology. Academic Press*; 1994 Feb;199(1):176–87.
12. Papageorgiou N, Coutard B, Lantéz V, Gautron E, Chauvet O, Baronti C, et al. The 2C putative helicase of echovirus 30 adopts a hexameric ring-shaped structure. *Acta Crystallogr Sect D Biol Crystallogr.* 2010;66(10):1116–20.
13. Xia H, Wang P, Wang GC, Yang J, Sun X, Wu W, et al. Human Enterovirus Nonstructural Protein 2C^{ATPase} Functions as Both an RNA Helicase and ATP-Independent RNA Chaperone. *PLoS Pathog.* 2015;11(7):1–29.
14. Bienz K, Egger D, Pfister T, Troxler M. Structural and functional characterization of the poliovirus replication complex. *J Virol.* 1992;66(5):2740–7.
15. Adams P, Kandiah E, Effantin G, Steven AC, Ehrenfeld E. Poliovirus 2C protein forms homo-oligomeric structures required for ATPase activity. *J Biol Chem.* 2009;284(33):22012–21.
16. De Palma AM, Heggermont W, Lanke K, Coutard B, Bergmann M, Monforte A-M, et al. The Thiazolobenzimidazole TBZE-029 Inhibits Enterovirus Replication by Targeting a Short Region Immediately Downstream from Motif C in the Nonstructural Protein 2C. *J Virol.* 2008;82(10):4720–30.
17. Sweeney TR, Cisnetto V, Bose D, Bailey M, Wilson JR, Zhang X, et al. Foot-and-mouth disease virus 2C is a hexameric AAA+ protein with a coordinated ATP hydrolysis mechanism. *J Biol Chem.* 2010;285(32):24347–59.
18. Benkahla MA, Alidjinou EK, Sane F, Desailoud R, Hober D. Fluoxetine can inhibit coxsackievirus-B4 E2 in vitro and in vivo. *Antiviral Res. Elsevier*; 2018 Nov;159:130–3.
19. Gofshteyn J, Cárdenas AM, Bearden D. Treatment of Chronic Enterovirus Encephalitis With Fluoxetine in a Patient With X-Linked Agammaglobulinemia. *Pediatr Neurol. Elsevier Inc*; 2016;64:94–8.

20. Bauer L, Manganaro R, Zonsics B, Strating JRPM, El Kazzi P, Lorenzo Lopez M, et al. Fluoxetine Inhibits Enterovirus Replication by Targeting the Viral 2C Protein in a Stereospecific Manner. *ACS Infect Dis.* American Chemical Society; 2019 Sep;5(9):1609–23.
21. Wenthur CJ, Bennett MR, Lindsley CW. Classics in chemical neuroscience: Fluoxetine (Prozac). *ACS Chem Neurosci.* 2014;5(1):14–23.
22. Wenthur CJ. Classics in Chemical Neuroscience: Methylphenidate. *ACS Chem Neurosci.* 2016;7(8):1030–40.
23. Pincus SE, Diamond DC, Emini EA, Wimmer E. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. *J Virol.* 1986;57(2):638–46.
24. Bauer L, Lyoo H, van der Schaar HM, Strating JR, van Kuppeveld FJ. Direct-acting antivirals and host-targeting strategies to combat enterovirus infections. *Curr Opin Virol.* Elsevier B.V.; 2017;24:1–8.
25. Guan H, Tian J, Qin B, Wojdyla JA, Wang B, Zhao Z, et al. Crystal structure of 2C helicase from enterovirus 71. *Sci Adv.* 2017;3(4):1–10.

Table 1: Sensitivity of CVB3 to Fluoxetine Analogues

Shown are EC₅₀ and CC₅₀ values in μM. Data represents mean values ± standard deviation calculated from three independent experiments and each experiment was performed in biological triplicates. NA = not active.



Compound	n	R ₁	R ₂	EC ₅₀ (μM)	CC ₅₀ (μM)	SI
(RS)-fluoxetine	2	4-CF ₃		3.03 ± 0.56	18.81 ± 1.36	6.21
(S)-fluoxetine	2	4-CF ₃		0.50 ± 0.14	21.63 ± 1.40	43.26
(R)-fluoxetine	2	4-CF ₃		NA	18.82 ± 1.34	-
1a	2	2-CF ₃		NA	>30	-
1b	2	3-CF ₃		NA	12.56 ± 1.79	-
1c	2	2-Cl, 4-CF ₃		NA	3.14 ± 0.07	-
1d	2	2-OMe, 4-CF ₃		NA	18.41 ± 1.26	-
2a	1	4-CF ₃		1.22 ± 0.15	>30	>24.60
2b	2	4-CF ₃		0.41 ± 0.27	>30	>73.17
3	2	4-CF ₃		NA	>30	-
4a	1	4-CF ₃		4.2 ± 0.927	>30	>7.14

Table 2. Comparison of compound cytotoxicity and antiviral activity (CVB3) in different cell lines.

Shown are EC₅₀ and CC₅₀ values in μM . Data represents mean values \pm standard deviation calculated from two independent experiments. Each experiment was done in biological triplicates.

SI= Selectivity Index (CC₅₀/EC₅₀). Selectivity index was calculated from the averaged EC₅₀ and CC₅₀ values of the independent experiments.

Compound	HelaR19			HEK293T			HAP1		
	EC ₅₀ (μM)	CC ₅₀ (μM)	SI	EC ₅₀ (μM)	CC ₅₀ (μM)	SI	EC ₅₀ (μM)	CC ₅₀ (μM)	SI
(RS)- fluoxetine	2.70 \pm 0.54	29.03 \pm 1.04	11	1.41 \pm 0.89	25.87 \pm 1.45	18	2.40 \pm 0.33	23.74 \pm 1.01	10
(S)- fluoxetine	0.62 \pm 0.01	27.67 \pm 0.87	45	0.7 \pm 0.65	24.65 \pm 2.56	35	0.69 \pm 0.04	24.00 \pm 1.87	35
2a	2.43 \pm 0.32	56.78 \pm 0.03	23	2.30 \pm 0.44	45.87 \pm 0.45	20	2.20 \pm 0.32	40.72 \pm 0.61	19
2b	0.87 \pm 0.98	64.99 \pm 1.34	75	1.05 \pm 0.02	52.98 \pm 0.21	53	0.98 \pm 0.21	49.39 \pm 0.11	50

Table 3. Broad-Spectrum antiviral activity of most potent compounds.

Shown are EC₅₀ values in μM . Data represents mean values \pm standard deviation calculated from three independent experiments. Each experiment was done in biological triplicates
NA = not active.

Virus Species	EV-A71	CVB3	PV-1	CV-A24	EV-D68	HRV-A2	HRV-B14
Strain	EV-A	EV-B	EV-C	EV-C	EV-D	RV-A	RV-B
	BrCr	Nancy	Sabin1	Joseph	Fermon		
(RS)-fluoxetine	NA	2.84 \pm 0.73	NA	NA	2.49 \pm 0.23	NA	NA
(S)-fluoxetine	NA	0.70 \pm 0.14	NA	NA	0.63 \pm 0.17	6.99 \pm 0.84	7.25 \pm 0.69
2a	NA	1.98 \pm 0.68	NA	NA	3.03 \pm 0.48	NA	NA
2b	NA	0.87 \pm 0.33	NA	NA	0.56 \pm 0.21	NA	3.33 \pm 1.05

Table 4. Antiviral activity of the 2b enantiomers.

Shown are EC₅₀ and CC₅₀ values in μM . Data represents mean values \pm standard deviation calculated from three independent experiments and each experiment was performed in biological triplicates.

Compound	EV-A71	CVB3	PV-1	CV-A24	EV-D68	HR-V2	HR-V14	CC ₅₀
(S)- fluoxetine 2b	>30	0.83 \pm 0.29	>30	>30	0.68 \pm 0.95	2.49 \pm 2.5	3.76 \pm 1.45	27.81 \pm 0.95
(R)-2b	>30	0.81 \pm 0.46	>30	>30	0.56 \pm 0.77	>30	4.3 \pm 2.98	60.76 \pm 0.45
(S)-2b	>30	>30*	>30	>30	4.13 \pm 1.4	>30	>30	57.95 \pm 0.65
	>30	0.19 \pm 0.45	>30	>30	0.11 \pm 0.02	7.49 \pm 1.12	0.98 \pm 1.25	59.6 \pm 1.45

*subtle antiviral activity observed

Figure Legends

Figure 1. Antiviral effect of analogue 2a and 2b on a panel of enteroviruses.

In a single cycle assay HeLa R19 cells were infected with different enterovirus species (A) EV-A71 (strain BrCr) (B) CVB3 (strain Nancy) (C) poliovirus (strain Sabin). (D) CV-A24 (Strain Joseph) (E) EV-D68 (strain Fermon) (F) HRV-A2 (G) HRV-B14 at MOI 1 and treated with serial dilutions of (S)-fluoxetine (SFX) and the analogues **2a** and **2b**. As a control, guanidine hydrochloride (GuaHCl) was used as a pan-enterovirus inhibitor targeting 2C. At 8 or 10 hours post infection (depending on the virus, see Supplementary Information), cells were freeze-thawed three times and virus titers of lysates were determined by endpoint titration. (H) In parallel, uninfected cells were treated with compound and cell viability was determined using an MTS assay. Data represent mean values \pm standard deviation from one representative of two independent experiments. Every experiment was performed in biological triplicates.

Figure 2. Mutations in the CVB3-2C protein confer resistance to compound 2a and 2b.

HeLa R19 cells were infected with a selection of CVB3 viruses harbouring previously identified mutations in the non-structural protein 2C, which confer resistance towards (S)-fluoxetine (SFX). (20) HeLa R19 were infected with and MOI 1 of (A) CVB3 wildtype virus. (B) the AVIVAV mutant (A224V-I227V-A229V triple mutant) (C) the I227V single mutant (D) the C179F and (E) the F190L mutant. Eight hours post infection cells were freeze-thawed three times and virus titers were determined with endpoint titration. Data represented show mean values \pm standard deviation from one experiment representative of two independent experiments. Every experiment was performed in biological triplicates.

Figure 3. The S-enantiomer of 2b exerts potent antiviral activity concomitant with 2C binding.

(A) A Multicycle CPE reduction assay to determine the antiviral activity of the 2b-enantiomers was performed. HeLa R19 cells were treated with serial dilutions of racemate, (S)-, or (R)- enantiomer of 2b and infected with CVB3 (strain Nancy), EVD68 (strain Fermon), HRV-2 (G) and HRV-14 at low MOI (depending on the virus, see Supplementary Information) to reach full CPE within three days. As positive control, cells were treated with (S)-fluoxetine. * indicates cytotoxicity of (S)-fluoxetine. Data shown are from one experiment representative of three independent experiments done in biological triplicates (B) The binding of the 2b-enantiomers to a recombinant fragment of CVB3-2C was determined by thermal shift assay. The thermal stabilization of 2C is represented by change in melting temperature. The dashed line represents data from the negative control BF738735, a phosphatidylinositol-4-kinase III beta inhibitor, used at a concentration of 250 μ M. Data shown is representative of two independent experiments, each of which was done in technical triplicates. Error bars depict standard deviation calculated from both experiments.