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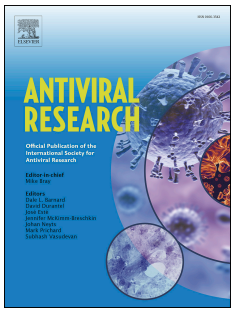

Structure-activity relationship studies on a Trp dendrimer with dual activities against HIV and enterovirus A71. Modifications on the amino acid

Belén Martínez-Gualda, Liang Sun, Eva Rivero-Buceta, Aida Flores, Ernesto Quesada, Jan Balzarini, Sam Noppen, Sandra Liekens, Dominique Schols, Johan Neyts, Pieter Leyssen, Carmen Mirabelli, María-José Camarasa, Ana San-Félix

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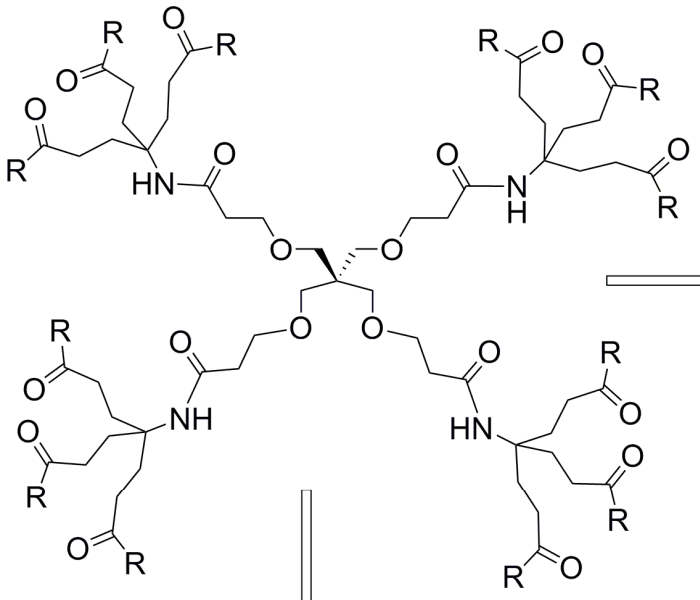
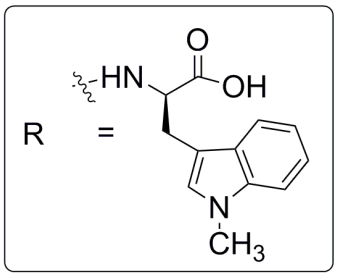
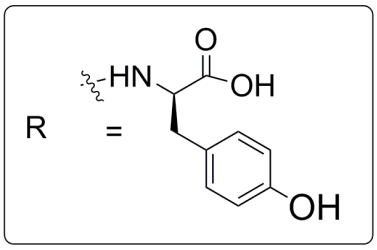




HIV-1 { EC_{50} = 1.7 ± 0.0 μM; CC_{50} > 100 μM

EV71 { EC_{50} = 3.6 ± 1.1 μM; CC_{50} > 27 μM



HIV-1 { EC_{50} = 6.4 ± 1.4 μM; CC_{50} > 100 μM

EV71 { EC_{50} = 0.4 ± 0.1 μM; CC_{50} > 27 μM

EV71 clinical isolates
EC_{50} = 0.2 - 1.1 nM

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STRUCTURE-ACTIVITY RELATIONSHIP STUDIES ON A TRP DENDRIMER WITH DUAL ACTIVITIES AGAINST HIV AND ENTEROVIRUS A71. MODIFICATIONS ON THE AMINO ACID

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Abstract

We have recently described a new class of dendrimers with tryptophan (Trp) on the surface that show dual antiviral activities against HIV and EV71 enterovirus. The prototype compound of this family is a pentaerythritol derivative with 12 Trps on the periphery. Here we complete the structure-activity relationship studies of this family to identify key features that might be significant for the antiviral activity. With this aim, novel dendrimers containing different amino acids (aromatic and non-aromatic), tryptamine (a "decarboxylated" analogue of Trp) and *N*-methyl Trp on the periphery have been prepared. Dendrimer with *N*-Methyl Trp was the most active against HIV-1 and HIV-2 while dendrimer with tyrosine was endowed with the most potent antiviral activity against EV71. This tyrosine dendrimer proved to inhibit a large panel of EV71 clinical isolates (belonging to different clusters) in the low nanomolar/high picomolar range. In addition, a new synthetic procedure (convergent approach) has been developed for the synthesis of the prototype and some other dendrimers. This convergent approach proved more efficient (higher yields, easier purification) than the divergent approach previously reported.

Keywords: Antiviral agents; AIDS; HFMD; HIV; EV71; Tryptophan

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1), the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS), is an enveloped retrovirus with glycoproteins gp120 and gp41 on its surface (Dumas et al., 2014; Terwilliger et al., 1990) while enterovirus A71 (EV71), the etiologic agent of hand-, foot- and mouth-disease (HFMD), is a (+) RNA virus (genus *Enterovirus*, family *Picornaviridae)* without envelope (Ng and Kwang, 2015; Pourianfar and Grollo, 2015).

At present, more than 30 approved drugs or regimens exist for the treatment of AIDS. These compounds, which are administrated in the so-called Highly Active Anti-Retroviral Therapy (HAART) (Butera, 2005; De Clercq, 2009; Pomerantz and Horn, 2003; Volderding and Deeks, 2010), are primarily focused on the viral enzymes integrase, reverse transcriptase and protease. HAART keeps the disease under control, however is often associated with the emergence of cross-resistant HIV strains and side-effects (Dube and Sattler, 2010; Gaardbo et al., 2012; Hawkins, 2010; Margolis et al., 2014; Rojas and Holguín, 2014). These limitations highlight the need for new lead compounds and/or novel therapeutic approaches to fight against HIV transmission and infection (Barré-Sinoussi et al., 2013; Flexner, 2007; Kumari and Singh, 2013; Tintori et al., 2014). In this respect, the entry of HIV into its target cells represents an attractive target for the development of anti-AIDS therapy (Esté, 2003; Haqqani and Tilton, 2013; Hertje et al., 2010; Kuritzkes, 2009; Singh and Chauthe, 2011; Tilton and Doms, 2010; Wilen et al., 2012). In fact, drugs that interfere with this early event may represent an advantage over other existing therapeutic approaches that target the viral enzymes such as reverse transcriptase or protease, as they may show remarkable efficacy against viruses resistant to these anti-HIV inhibitors and prevent the uptake of the virus by uninfected CD4-positive cells. To date, only two drugs among this category have been approved by the FDA, enfuvirtide (T20) (Fletcher, 2003; Williams, 2003), a 36-amino acid peptide that binds viral glycoprotein gp41, and maraviroc (Gulick et al., 2008), a small-molecule CCR5-antagonist.

On the other hand, the therapeutic or prophylactic panorama for EV71 infection is more limited as no approved drugs are available for EV71 treatment to date. As already mentioned, this virus is the etiologic agent of hand-, foot- and mouth-disease (HFMD), a mild syndrome that affects mostly children below 6 years old (Chan, 2011). Unlike other HFMD-related enteroviruses, EV71 also may cause severe neurological problems like aseptic meningitis and brainstem encephalitis, which may lead to cardiopulmonary failure and death (Alexander, 1994; Luan Yin et al., 1999; McMinn, 2002). Patients with neurological syndromes often have permanent neurological sequelae, with delayed neurodevelopment, reduced cognitive function and polio-like paralysis. Large EV71 outbreaks have been reported throughout the world but have been especially severe in the Pacific region of Asia (Chang et al., 2007; Chen et al., 2001). A very recent outbreak in Catalonia, Spain,

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with 73 cases of enterovirus infection with neurological complications suggests that the epidemiological pattern of EV71 is going through a change also in Europe (ProMED, 2016; ECDC, 2016). Therefore, effective antivirals are urgently needed for prophylaxis or treatment of patients with severe EV71 infections.

We have recently reported that a new family of dendrimers containing different central scaffolds and multiple (9 to 18) tryptophans (Trp) on the periphery, whose prototype is the pentaerythritol derivative **1** (Fig. 1), inhibit HIV infection (Rivero-Buceta et al., 2015) and also proved to be potent, specific and selective inhibitors of EV71 (Rivero-Buceta et al., 2016). These compounds may inhibit an early step of the replicative cycles of both HIV and EV71, presumably virus entry into its respective target cells. In the case of HIV, the inhibition is probably due to the interaction with the glycoproteins gp120 and gp41 of the viral surface while for EV71, the mechanism of action is still under investigation (Rivero-Buceta et al., 2015, 2016).

Preliminary structure-activity relationship studies for EV71 and HIV on this family of dendrimers are very similar and revealed that the absence of Trps on the periphery is detrimental for antiviral activity and that a multivalent presentation of Trp is important for both activities, being 9-12 Trps sufficient for activity.

Here we decided to extend the structure-activity relationship studies of this class of compounds. In particular, three aspects have now been studied. The first one is the nature of the amino acid residues on the periphery. This was assessed by replacing the Trp residue of the prototype by aromatic (phenylalanine and tyrosine) and non-aromatic (alanine) amino acids. The second one is the importance of the carboxylic acid on the periphery. This was elucidated by preparing the corresponding tryptamine derivative, a “decarboxylated” analogue of the prototype. The third one is the importance of the NH of the indol moiety on the activity. This was explored by using a *N*-Me analogue of Trp.

Finally, we were also interested in the development of an optimized synthetic procedure that would allow an efficient multi-gram synthesis of the prototype **1,** needed for additional biological studies or mass production. With this aim, an alternative synthetic procedure, based on a convergent approach, has been explored.

2. Materials and methods

2.1. Synthesis

Commercial reagents and solvents were used as received from the suppliers without further purification unless otherwise stated. Dichloromethane was dried prior to use by distillation from CaH_2 and stored over Linde type activated 4Å molecular sieves.

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Analytical thin-layer chromatography (TLC) was performed on aluminum plates pre-coated with silica gel 60 (F_{254}, 0.25 mm). Products were visualized using an ultraviolet lamp (254 nm) or by heating on a hot plate (approx. 200 ºC), directly or after treatment with a 5% solution of phosphomolybdic acid or vanillin in ethanol.

For reversed phase purification, a Biotage HPFC (High Performance Flash Chromatography) purification system using water/acetonitrile (100:0 to 0:100) as eluent was used.

For HPLC analysis an Alliance 2695 (Waters) equipped with a PDA (Photo Diode Array) detector Waters 2996 was used. Acetonitrile was used as mobile phase A with 0.08% of formic acid, and water was used as mobile phase B with 0.1% of formic acid at a flow rate of 1 $mL \cdot min^{-1}$. Two different methods were used, one on a XBridge C_{18} (2.1 x 100 mm, 3.5 µm) column with 5-80% of A, that will be noted as $t_{R(X)}$, and the other on a SunFire C_{18} (4.6 x 50 mm, 3.5 µm) column with 0-100% of A, that will be noted as $t_{R(S)}$. All retention times are quoted in minutes.

Melting points were measured on a Mettler Toledo and are uncorrected.

NMR spectra (^{1}H, ^{13}C NMR) were recorded on a 300 MHz (Varian Inova 300) and 500MHz (Varian System 500) spectrometers, using $(CD_3)_2SO$ and CD_3OD as solvents. Chemical shift (δ) values are reported in parts per million (ppm) relative to tetramethylsilane (TMS) in ^{1}H and $CDCl_3$ (δ = 77.0) in ^{13}C NMR. Coupling constant (*J* values) are reported in hertz (Hz), and multiplicities of signals are indicated by the following symbol: s (singlet), d (doublet), m (multiplet), bs (broad singlet).

Mass spectra (MS) were registered in a quadrupole mass spectrometer 1100 Hewlett Packard equipped with an electrospray source. HRMS was registered in an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler, and a column oven) coupled to a 6520 quadrupole-time of flight (QTOF) mass spectrometer using an ESI interface working in the positive-ion and negative-ion mode. The instrument was from Agilent Technologies (Santa Clara, CA). The electrospray voltage was set at 4.5 kV, the fragmentor voltage at 150 V and the drying gas temperature at 300 °C. Nitrogen (99.5% purity) was used as nebulizer (207 kPa) and drying gas (6 L min−1), while nitrogen of higher purity (99.999%) was used as the collision gas. Data acquisition and processing were performed using Agilent Mass Hunter Workstation Acquisition Rev. B.02.00 software.

Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument.

The purity of final compounds was at least 95%. The purity has been determined by high resolution mass spectrometry (HRMS, “exact mass”) and reverse phase-HPLC.

Details regarding the synthesis and characterization of the compounds can be found in the Supplementary material.

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2.2. Biological methods

2.2.1. Anti-HIV Activity Assay

The compounds were evaluated for their inhibitory activity against HIV-1 (NL4.3) and HIV-2 (ROD) infection in MT-4 cell cultures. Briefly, MT-4 cells (1×10^6 cells/mL) were pre-incubated for 30 min at 37°C with the test compounds in a 200 µl-96-well plate. Next, NL4.3 virus was added at 100 $CCID_{50}$ of the viral stock. The cytopathic effect was scored microscopically 5 days post-infection, and the 50% effective concentration (EC_{50}) values were determined using the MTS method.

2.2.2. Surface Plasmon Resonance Experiments for evaluation of the binding of the compounds to gp120 and gp41

Recombinant gp120 protein from the HIV-1 IIIB strain (ImmunoDiagnostics Inc., Woburn, MA) (produced in cell cultures of chinese hamster ovary cell cultures) and recombinant gp41 HIV-1(HxB2) (Acris Antibodies GmbH, Herford, Germany) were covalently immobilized on the carboxymethylated dextran matrix of a CM5 sensor chip in 10 mM sodium acetate, pH 4.0, using standard amine coupling chemistry up to a final density of, respectively, 700 RUs and 580 RUs. All interaction studies were performed at 25 °C on a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden). The compounds **10** and **24** were diluted in HBS-P (10 mM HEPES, 150 mM NaCl and 0,05% surfactant P20; pH 7.4), and supplemented with 5% dimethyl sulfoxide (DMSO, Merck) and 10 mM Ca^{2+} at a concentration of 50 µM. Samples were injected for 2 minutes at a flow rate of 30 µl/min followed by a dissociation phase of 2 minutes. The sensor chip surface was regenerated with an injection of 50 mM NaOH. A reference flow cell was used as a control for non-specific binding and refractive index changes. Several buffer blanks were used for double referencing. A DMSO concentration series was included to eliminate the contribution of DMSO to the measured response. The binding affinity (K_D) was calculated for **24** based on the data obtained in the sensorgram.

2.2.3. Antiviral Activity against EV71

EV71 BrCr laboratory adapted strain and clinical isolates representative of B genogroup (B2 sub-genogroup: 11316; B5 sub-genogroup: TW/96016/08 and TW/70902/08) and C genogroup (C2 sub-genogroup: H08300 461#812; C4 subegenogroup: TW/1956/05 and TW/2429/04) were used at a low multiplicity of infection (MOI) in a standardized antiviral assay. Briefly, a serial dilution of the compounds was prepared in assay medium that was added to an empty microtiter 96 well-plate, after which the virus inoculum was added first, followed by a suspension of freshly harvested rhabdosarcoma (RD) cells (2*10^4 cells/well). While the

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cells were settling to the bottom, the virus was allowed to infect them in the presence of compound. The assay plates were incubated at 37 ºC, 5% CO_2 with virus inoculum and compounds until full virus –induced cell death was observed in the untreated, infected controls (3-4 days post-infection). Subsequently, the antiviral effect was quantified using a colorimetric readout with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS method) and the concentration of compound at which 50% inhibition of virus-induced cell death would be observed (EC_{50}) was calculated from the antiviral dose-response curves. A similar assay setup was used to determine the adverse effect of the compound on uninfected, treated cells for calculation of the CC_{50} (concentration of compound that reduces overall cell health with 50% as determined by the MTS/PMS method). Selectivity index (SI) was calculated as the ration of CC_{50} to EC_{50}.

3. Results and discussion

3.1. Chemistry

Firstly, the novel amino acid dendrimers were obtained following the divergent approach previously described for **1** (Rivero-Buceta et al , 2016). In this approach, dendrimers were built starting from the central core and growing towards the periphery. The commercially available pentaerythritol scaffold **2**, that incorporates four aminotriester (Behera's amine) branched arms, has been chosen as central core (Scheme 1). Deprotection of the *t*-butyl protecting groups of **2**, followed by condensation of the resulting poly acid **3** with the corresponding (OMe or OBn) protected amino acids gave intermediates **4** (25%), **6** (15%) and **8** (23%). Saponification (OMe esters) or hydrogenation (OBn esters) of the protecting ester moieties of these intermediates afforded the final compounds **5**, **7** and **10**, bearing twelve amino acids on the periphery, in quantitative yields. This divergent approach represents a short route in terms of synthetic steps. However, the high number of covalent bonds that should be simultaneously formed in the final coupling step (12) leads to incomplete reactions and mixtures of dendrimers with very similar chromatographic properties that were very difficult to purify. Particularly arduous was the synthesis and purification of the triptamine derivative **11** that was obtained in a very low yield (5%). In addition, the *N*-Me analogue of Trp could not be obtained by this method. An alternative convergent approach was then explored. In this convergent approach, building blocks, called dendrons, are constructed first and then attached to the central core in the final step of the synthesis.

For the dendron construction, the commercially available aminotriester **12** (Behera's amine) was selected as the starting material (Scheme 2). First, the amino group in **12** was protected with the fluorenylmethyl oxycarbonyl group (Fmoc) to afford intermediate **13** (Carpino and Han, 1972; Karasugi et al., 2012) (73%).

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Deprotection of the *t*-butyl protecting groups of **13** afforded dendron **14** (Carpino and Han, 1972; Karasugi et al., 2012) in quantitative yield. Reaction of **14** with H-Trp-OMe·HCl in the presence of HATU as coupling reagent and DIPEA as base gave intermediate **15** (95%) whose subsequent Fmoc deprotection, using piperidine, afforded dendron **18** (95%), with a reactive amino group as focal point. This free amino group enables further attachment of **18**, through the formation of amide bonds, to the central pentaerythritol scaffold **21** (Flores et al., 2014; Newkome and Weis, 1996) to afford intermediate **22**. Finally, methyl ester deprotection ($LiOH/H_2O$) of **22** afforded compound **1** in quantitative yield. Compared to the divergent approach, the convergent methodology considerably improves the global yield of dendrimer **1** (20% versus 4%).

Next, and based on the good results of the convergent approach, the same synthetic methodology was applied for the synthesis of the tryptamine **11** and (NMe)Trp **24** dendrimers, for which the divergent approach gave poor results (in the case of **11**) or even failed (in the case of **24**). In both cases the key dendron intermediate **14** was reacted with Trp(NMe)-OMe or triptamine to give dendrons **16** (53%) and **17** (67%), respectively. Fmoc deprotection, followed by coupling of the resulting intermediates **19** and **20** with the pentaerytritol derivative **21** gave compounds **23 (**27%) and **11** (16%). Methyl ester deprotection of **23**, as described above ($LiOH/H_2O$) gave **24** in quantitative yield.

Thus, compared to the divergent approach, the convergent methodology improved considerably the global yield of the tryptamine dendrimer **11** (16% versus 5%) and allowed the synthesis of **24** that could not be obtained by the divergent approach.

All the synthesized compounds were characterized by ^{1}H and ^{13}C NMR spectroscopies and HRMS.

3.2. Biological results

3.2.1. Anti-HIV activity of the test compounds

Dendrimers **5**, **7**, **10**, **11** and **24** were first evaluated for their inhibitory effects against HIV-1 and HIV-2 replication in CD4 cell culture. Table 1 summarizes the results of this evaluation. The antiviral data of the prototype **1**, with Trps on the periphery, is also included as a reference, as well as dextran sulfate-5000 (DS-5000), and pradimicin A. DS-5000 is a negatively charged HIV adsorption inhibitor (Baba et al., 1988) and pradimicin A represents a gp120 carbohydrate-binding entry inhibitor (Balzarini et al., 2007). These control compounds showed antiviral activities in the range as previously reported (Baba et al., 1988; Balzarini et al., 2007).

Alanine (**5)** and phenylalanine (**7)** dendrimers, with methyl and phenyl side chains, respectively, were inactive. However, tyrosine derivative **10**, with a phenolic OH side chain, showed significant anti-HIV activity.

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It was three-fold less active against HIV-1, and equally active against HIV-2 than the prototype **1**, with an indol side chain. This result suggests that the nature of the side chain of the amino acid is very important for the activity and in particular, the indol moiety was preferred over the phenolic moiety. Tryptamine derivative **11** was 19-fold less active than the prototype **1** against HIV-1 showing the importance of the concomitant presence of the free COOHs for optimal activity.

Interestingly, the replacement of the indole moiety by an *N*-methyl indole moiety improved the activity of compound **24** versus the prototype **1**. This result suggests that the free indole-NH is not crucial for activity. Dendrimer **24** was also active against HIV-2 at subtoxic concentrations, but the inhibitory potential proved inferior to HIV-1 inhibition, as also observed for dendrimer **10**.

It should be emphasized that the activity showed by our compounds (EC_{50} = 1.7 - 6.4 µM), in the low micromolar range, is very similar to that of pradimicin A (EC_{50} = 3.3 µM), the gp120 carbohydrate-binding entry inhibitor (Balzarini et al., 2007) used as control (Table 1). However, pradimicin A is a structurally complex molecule difficult to synthesize that has to be obtained by fermentation. By contrast, our dendrimers are synthetic molecules easy to prepare and suitable to modification by conventional synthetic procedures.

In addition, our compounds have a precise structure and a well-defined composition because they have been synthesized with complete control over the size, shape and surface functionalization. This is in clear contrast with those happen with other negatively charged HIV entry inhibitors such as sulfated polysaccharides of which dextran sulfate-5000, that has been used as control, is a representative example (Baba et al., 1988). The antiviral activities of these compounds vary depending on the size (molecular weight) and density (charge distribution) of the anionic groups ($-OSO_3$) present on the molecules. For this reason, it is very difficult to obtain standarized preparations of dextran sulfates or other sulfated polymers (De Clercq, 1995). In addition, in certain systems, the sulfate groups ($-OSO_3$) present in these compounds can be subjected to hydrolysis (release) by sulfatases (Balzarini and Van Damme, 2007) resulting in inactivation of the compounds.

For all of these reasons and based on the synthetic accessibility, well defined molecular structure, presence of COOH instead of $–OSO_3$ and relatively moderate Molecular Weight (~3000 Da *vs* 5000 to 500,000 Da of dextran sulfates) the therapeutic possibilities of our compounds might be broader than those of dextran sulfate or pradimicin, that have been used as control compounds in the present study.

*3.2.2. SPR interaction experiments for **10** and **24** with HIV-1 gp120 and gp41*

SPR experiments performed with the most active compounds, **10** and **24**, revealed that they dose-dependently interact with the glycoproteins gp120 and gp41 of the HIV envelope (Fig. 2). In this respect, the 4-fold better

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anti-HIV-1 activity of compound **24** *versus* **10** correlated with the ~ 7-fold higher binding amplitude of **24** to HIV-1 gp120 and gp41 than **10**.

The K_D value (affinity constant) of the most potent HIV-1 inhibitor **24** (EC_{50}: 1.7 µM) was determined by SPR analysis and compared with the K_D value of the prototype compound **1** (EC_{50}: 2.2 µM) that had earlier been determined (Rivero-Buceta, personal communication). Whereas **1** had a K_D of 6.7 µM for HIV-1 gp120 and 18 µM for HIV-1 gp41, **24** proved somewhat superior (K_D: 1.53 µM for HIV-1 gp120 *versus* 3.40 µM for HIV-1 gp41) (Fig. 3). Thus, both **1** and **24** were endowed with comparable anti-HIV-1 activity in cell culture, and with pronounced affinities against glycosylated HIV-1 gp120 and gp41 proteins. In this respect, they were equally inhibitory against HIV-1 as the non-peptidic carbohydrate-binding antibiotic pradimicin S, although pradimicin S has a ~ 5-fold higher affinity for HIV-1 gp120 than **24** (Balzarini et al., 2007). It should be kept in mind that different affinities of **1** and **24** for HIV-1 gp120 can be explained by differential interactions of these compounds with the different glycan types that are present on HIV-1 gp120.

3.2.3. *Anti-EV71activity of the test compounds*

The new synthesized compounds were also evaluated for their inhibitory effects against EV71 *in vitro*. Tryptophan prototype **1** was included in the assay together with pirodavir, a potent EV71 capsid binder, as reference compounds (Tijsma et al., 2014; Andries et al., 1992).

In particular, dendrimers **5**, **7**, **10**, **11** and **24** were evaluated for selective antiviral activity (EC_{50}) against the BrCr laboratory-adapted strain of EV71 in a cell-based assay on rhabdomyosarcoma (RD) cells, which are known for their high susceptibility to EV71-induced cell death (Yamayoshi et al., 2009). Cell viability (CC_{50}) was also assessed in a similar setup with compound-treated, uninfected cells. Overall, with a range of activity 0.4-7.2 µM, the dendrimers showed a better antiviral activity against EV71 BrCr than HIV (range of activity: 1.7-45 µM) (Table 2).

Interestingly, the SAR for EV71 revealed a similar profile to that observed for HIV. The tyrosine derivative **10** and the *N*-Me indole **24** showed the best antiviral activity against EV71. However, compound **24** was three-fold less active than the prototype whereas it was as active as the prototype against HIV. The tryptamine derivative **11** proved inactive similarly to HIV, indicating the importance of the negative (carboxyl) charges in the molecule as a concomitant prerequisite for antiviral activity.

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The most active compounds, **10** and **24,** were evaluated in virus-cell-based assays against a panel of EV71 clinical isolates representative for the different (sub)genogroups (A, B2, B5, C2 and C4) (Table 3). Compound **1** was included as reference.

Similar to the results found for the Trp prototype **1** (EC_{50} values ranging between 0.2 nM and 1.1 nM) (Rivero-Buceta el al., 2016), the tyrosine analogue **10** inhibited the replication of all EV71 strains, with 50% effective concentrations (EC_{50}s) in the low-nanomolar/high-picomolar range (EC_{50}s ranging between 0.7 nM and 6.7 nM). A remarkably lower activity was observed for the *N*-Me indole compound **24.** Nonetheless, it achieved full inhibition of all three genogroups with EC_{50} values ranging between 51 nM and 180 nM. This result clearly demonstrated that the presence of hydrophilic groups (NH, OH) on the aromatic ring of the side chain of the amino acid is beneficial for anti-EV71 activity.

Interestingly, as previously reported (Rivero-Buceta et al., 2016), the selected dendrimer compounds were more potent inhibitor of clinical EV71 isolates than the BrCr laboratory strain (Table 3). Such a difference was not observed when cells were treated with Pirodavir (data not shown). This finding suggests that dendrimers may act with a different mechanism than Pirodavir to block EV71 entry (Tijsma et al., 2014). Further investigations are currently ongoing to determine the mode of action of dendrimers.

To determine at which stage of the virus replication cycle the compounds act, a time-of-drug-addition study was performed with the most active, **10**. In this experiment the capsid binder pirodavir and the 3C protease inhibitor rupintrivir were used as reference compounds (Fig. 4) (Thibaut et al., 2011; Tijsma et al., 2014). Similar to the results found for pirodavir and the prototype **1** (Rivero-Buceta el al., 2016), clear inhibition of virus replication was observed only when the drug was added during or before infection. Compound **10**, like **1** and pirodavir, lost its activity when added after the infection period. This correlates with the observation that the compounds inhibit HIV entry. The viral 3C protease inhibitor, rupintrivir, lost its activity when added 6 or 7 h after infection, which is in line with its mechanism of action.

4. Conclusions

Starting from **1** as prototype, novel dendrimers containing different aromatic and non-aromatic amino acids, tryptamine (a “decarboxylated” analogue of Trp) and *N*-Methyl Trp on the periphery have been synthesized. A new synthetic procedure (convergent approach) has been developed for the obtention of these compounds and the prototype **1**. This convergent approach proved to be more efficient (higher yields, easier purifications) than the divergent approach and was crucial for the synthesis of dendrimers whose synthesis either proved inefficient (triptamine) or failed (*N*-Methyl Trp) when the divergent approach was used.

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Aromatic amino acids Trp (present on the prototype, **1**) and Tyr (present on **10**), with their respective indol and phenol moieties and free COOHs, are required for anti-HIV and anti-EV71 activity. Tryptamine derivative **11** was 19-fold less active against HIV than the prototype **1** and inactive against EV71, thus showing the importance of the COOHs for activity. Dendrimer **24**, with *N*- Methyl Trp on the periphery, was slightly more active against HIV-1 and HIV-2 than the prototype, which suggests that the NH of the indole moiety is not crucial for HIV activity. However, dendrimer **24** was three-fold less active than the prototype against EV71, suggesting that for the anti-enteroviral activity, this NH is important since it may probably be involved in a hydrogen bond with its corresponding binding site. Dendrimer **10**, with tyrosine, was endowed with the most potent antiviral activity against EV71. This compound represents a novel low-nanomolar inhibitor of the clinical isolates of enterovirus A71 while dendrimer **24**, with *N*-Methyl Trp, was the most active against HIV-1.

In conclusion we report herein a family of dendrimers with dual action against HIV and EV71 that deserve further studies and development. The ability of these compounds to act early in the HIV replicative cycle, before HIV enters the host cell, makes them ideal candidates for use as microbicides, compounds in which exist a great interest, especially in developing countries, where live more than 90% of HIV-infected people, whose access to the conventional anti-HIV therapies of the developed world are very limited (WHO, 2016; UNAIDS, 2016). On the other hand, and because this class of molecules show extremely high potency against clinical isolates of EV71, they may be of significant interest for future therapeutic/prophylactic strategies against this virus.

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Appendix. Supplementary data

Details regarding the synthesis and characterization of the compounds are included.

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LIST OF CAPTIONS

Fig. 1. Structure of the prototype pentaerythritol derivative **1**

Fig. 2. SPR analysis of the interaction of **10** and **24** with HIV-1 gp120 and gp41

Fig. 3. Apparent KD for **10**-gp120 and **10**-gp41

Fig. 4. Effects of pirodavir (capsid binder), rupintrivir (3C protease inhibitor), and compound **10** in a time-of-drug-addition assay

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Scheme 1. Synthesis of dendrimers **4-11** by the divergent approach. *Reagents and conditions*: (i) HOOCH, 40ºC, 48 h, (ii) HATU/DIPEA (iii) H_2, Pd/C (iv) LiOH.H_2O

Scheme 2. Synthesis of compounds **1**, **11** and **24** by the divergent approach. *Reagents and conditions:* (i) FmocCl, Na_2CO_3 (ii) HOOCH, 40ºC, 48 h (iii) H-aa, HATU/DIPEA (iv) piperidine (v) HATU/DIPEA (vi) LiOH·H_2O

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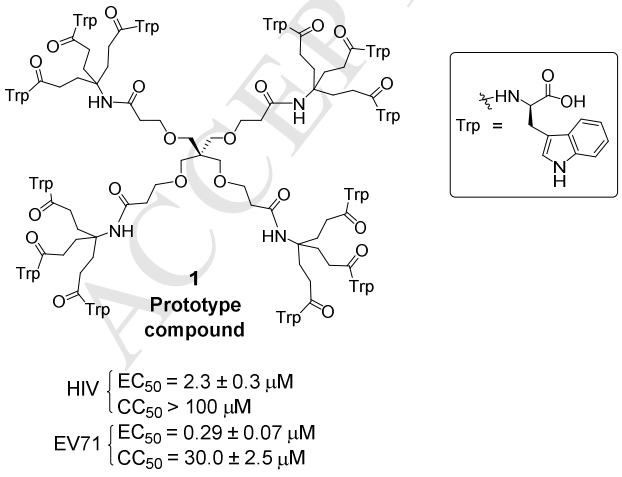
Table 2. Antiviral activity of dendrimers against the BrCr lab strain of EV71 in RD cells

Table 3. Evaluation of the broad-spectrum antiviral effect of compounds **10** and **24** against a representative panel of clinical EV71 isolates in RD cells

LIST OF FIGURES



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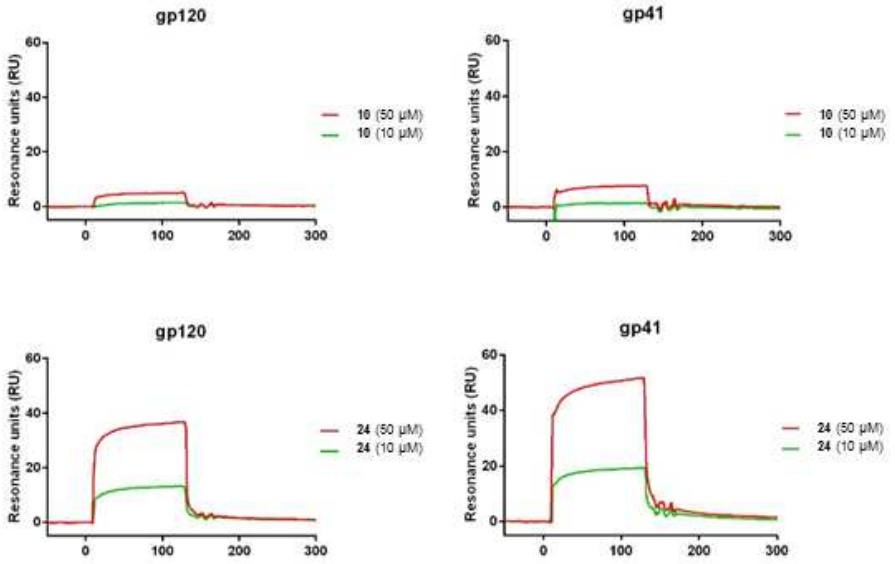

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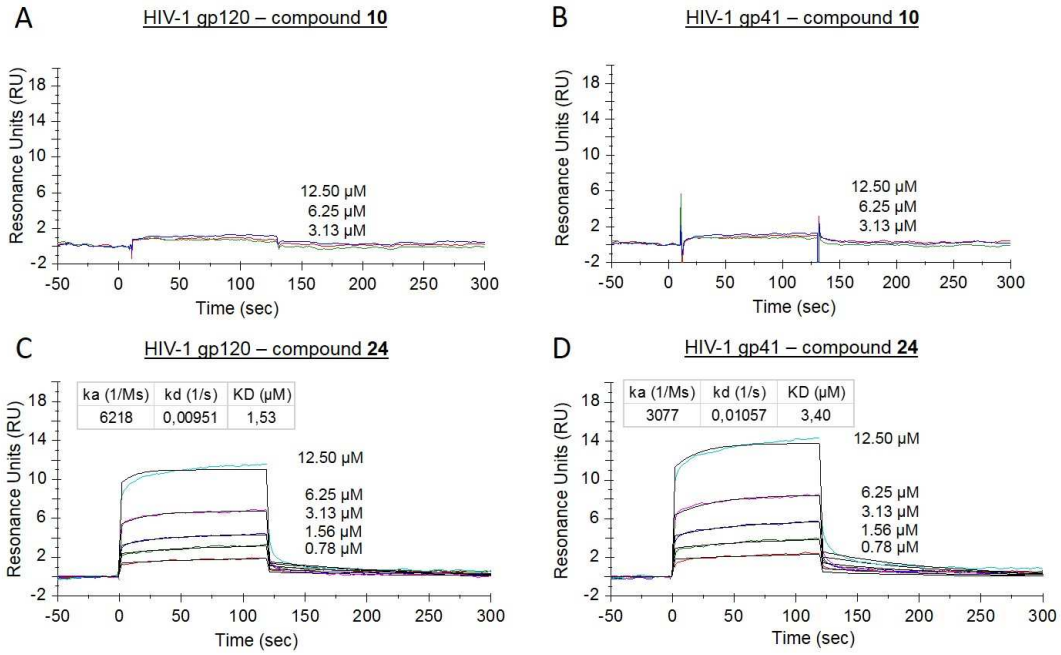

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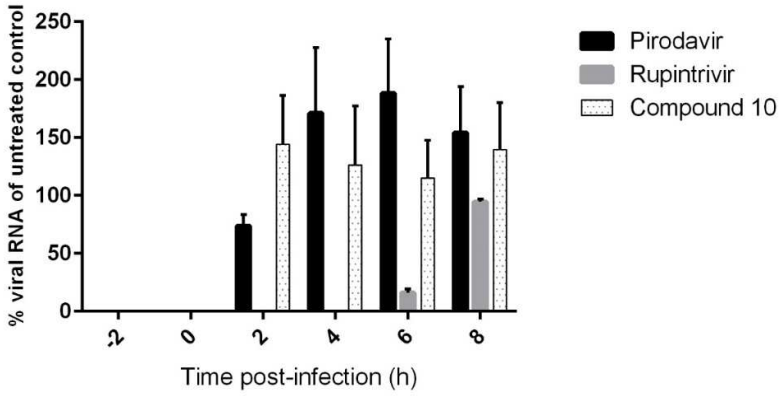

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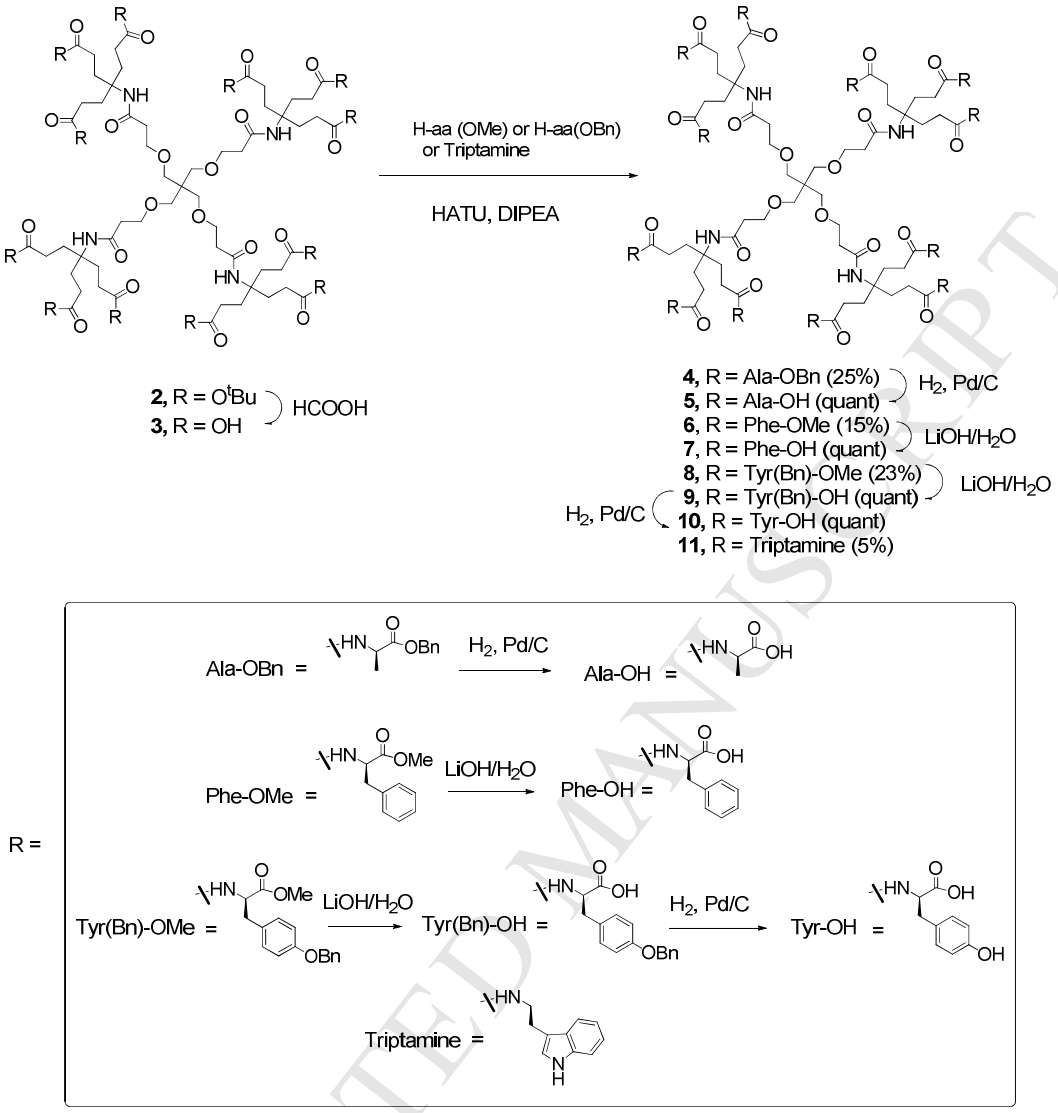

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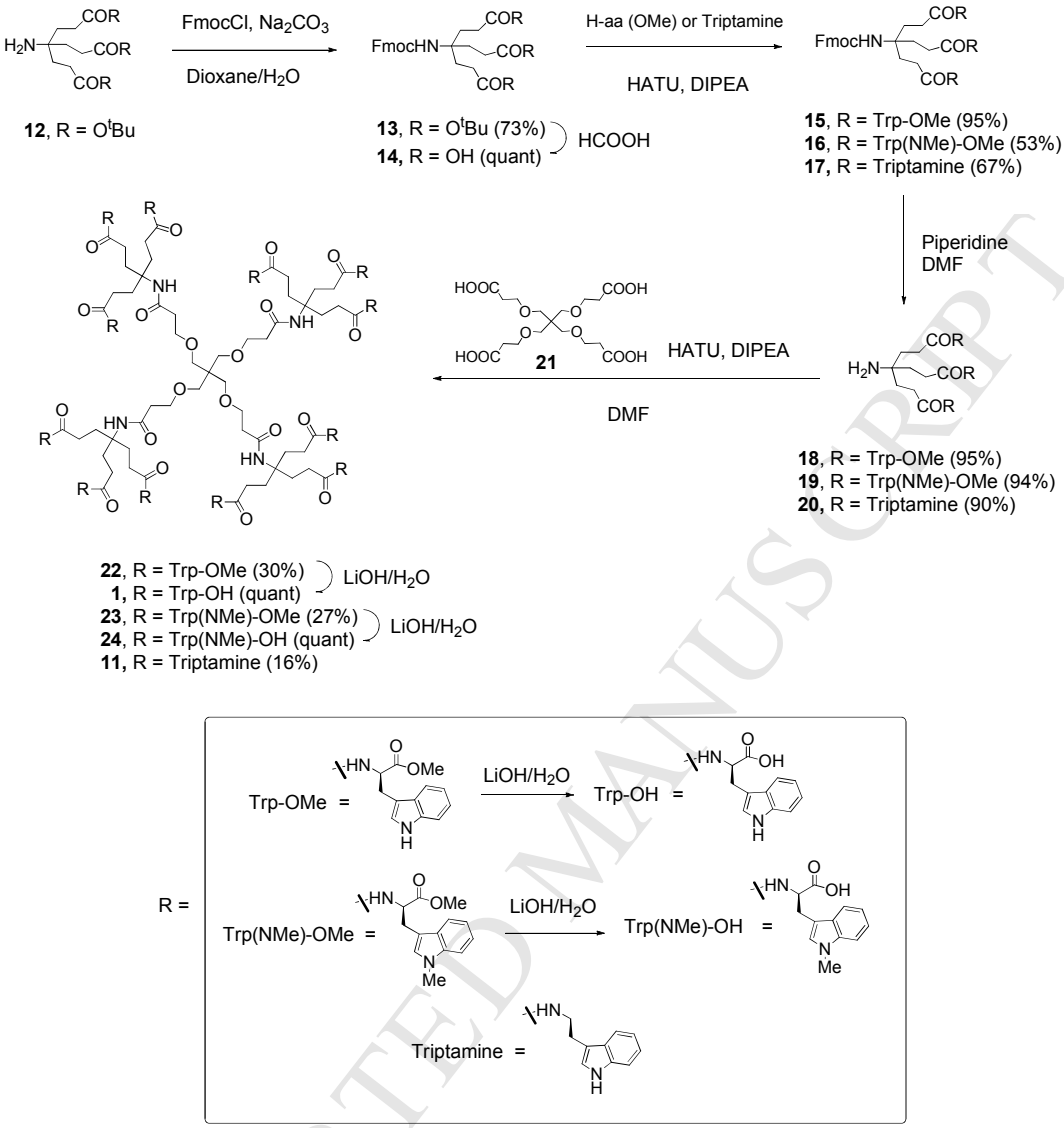

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Table 1. Anti-HIV activity of the selected group of compounds

Compound	EC_{50}[a] (μM) HIV-1	EC_{50}[a] (μM) HIV-2	CC_{50}[b] (μM)	MW[c] (g/mol)
5	>100	>100	>100	2194.25
7	>28	>28	28	3287.58
10	6.4 ± 1.4	20 ± 15	>100	3299.40
11	45	77	>100	3047.72
24	1.7 ± 0.0	7.3 ± 2.8	>100	3744.15
1	2.3 ± 0.3	22 ± 2.8	>100	3575.84
DS-5000	0.07 ± 0.02	0.03 ± 0.01	>20	~ 5000
Pradimicin A	3.3 ± 1.2	5.9 ± 3.7	>100	842.80

Data are the mean ± S.D. of at least 2 to 3 independent experiments
[a] 50% Effective concentration, or the concentration required to inhibit HIV-induced cytopathicity by 50%
[b] 50% Cytostatic concentration, or the compound concentration required to inhibit CD4 T cell proliferation by 50%
[c] Molecular weight of the molecules expressed as g/mol

Table 2. Antiviral activity of dendrimers against the BrCr lab strain of EV71 in RD cells

Compound	EC_{50}[a] (μM) EV71	EC_{90}[a] (μM) EV71	CC_{50}[b] (μM)	SI
5	>46	>46	ND	ND
7	7.2 ± 0.2	>10	>32	>4
10	0.4 ± 0.1	0.7 ± 0.4	>30	78
11	>33	>33	ND	ND
24	3.6 ± 1.1	5.3 ± 0.9	>27	7.5
1	0.3 ± 0.1	0.5 ± 0.1	>100	
Pirodavir	0.3 ± 0.1	0.6 ± 0.2	>54	

ND = Not Determined
All values are in micromolar (μM) and are a summary of multiple dose-response curves (>2) in multiple independent (>1) experiments.
CC_{50} = concentration of compound at which a 50% reduction in cell viability is observed; EC_{50} = concentration of compound at which the virus-induced cytopathic effect is reduced by 50%; SI = selectivity index (CC_{50}/EC_{50}).

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Table 3. Evaluation of the broad-spectrum antiviral effect of compounds **10** and **24** against a representative panel of clinical EV71 isolates in RD cells

EV71 Genogroup	Virus strain	EC_{50} (nM)[a]		
		1	**10**	**24**
A	BrCr	285 ± 70	390 ± 140	3560 ± 1620
B2	11316	0.4 ± 0.0	6.7 ± 3.2	160 ± 10
B5	TW/96016/08	0.2 ± 0.0	0.7 ± 0.1	51 ± 18
	TW/70902/08	0.2 ± 0.1	1.0 ± 0.3	66 ± 11
C2	H08300 461#812	1.1 ± 0.3	2.0 ± 0.4	180 ± 10
C4	TW/1956/05	0.2 ± 0.2	0.3 ± 0.2	58 ± 6
	TW/2429/04	0.3 ± 0.2	0.4 ± 0.1	59 ± 18

[a] All values are in nanomolar (nM) and are obtained in multiple (>2) independent (>1) experiments.
Following microscopic quality control, at least at one concentration of compound, no virus-induced cell death was observed and the compound did not cause an adverse effect on the host cell or monolayer morphology.

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SUPPLEMENTARY MATERIAL

Supplementary material related to the article

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- Novel dendrimers containing different amino acids on the periphery have been synthesized.
- These compounds have dual action against HIV and EV71.
- Tyrosine dendrimer is the most potent against EV71 while dendrimer with *N*-methyl Trp is the most potent against HIV-1.
- Dendrimer with tyrosine is an extremely highly potent inhibitor of clinical EV71 isolates (nanoMolar-picoMolar potency).
- Presence of hydrophilic groups (NH, OH) on the aromatic ring of the amino acid is beneficial for anti-EV71 activity.

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