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Optimization of a Class of Tryptophan Dendrimers That Inhibit HIV Replication Leads to a Selective, Specific, and Low-Nanomolar Inhibitor of Clinical Isolates of Enterovirus A71

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Tryptophan dendrimers that inhibit HIV replication by binding to the HIV envelope glycoproteins gp120 and gp41 have unexpectedly also proven to be potent, specific, and selective inhibitors of the replication of the unrelated enterovirus A71. Dendrimer 12, a consensus compound that was synthesized on the basis of the structure-activity relationship analysis of this series, is 3-fold more potent against the BrCr lab strain and, surprisingly, inhibits a large panel of clinical isolates in the low-nanomolar/high-picomolar range.

Enterovirus A71 (EV71) is a small virus (~30 nm) with a single-stranded positive-sense RNA genome of ~7.4 kb that belongs to the genus *Enterovirus* of the family *Picornaviridae* (1, 2). EV71 is the etiologic agent of hand, foot, and mouth disease (HFMD), a mild syndrome that most frequently affects children younger than 6 years and that is characterized by the development of fever with skin vesicles on the palms and feet, as well as ulcers on the oral mucosa (3). Unlike other HFMD-associated enteroviruses, EV71 can also cause severe neurological problems, such as aseptic meningitis and brain stem encephalitis, which can lead to cardiopulmonary failure and death (4–6). After having suffered from such neurological complications, survivors often have permanent neurological sequelae, such as delayed neurodevelopment, reduced cognitive function, and polio-like paralysis (7). Similar to other human enteroviruses, such as poliovirus, transmission of EV71 occurs through the fecal-oral route (8).

In recent years, large outbreaks of EV71 have been reported throughout the world, and they have been particularly severe in the Pacific region of Asia, with a high number of fatal cases among children (9–11). So far, there is no drug on the market to treat or prevent this infection. An inactivated EV71 vaccine was recently approved in China (12), but it may induce only limited cross-neutralization between EV71 genogroups, which does not make it suitable for widespread use.

Recently, we reported on the anti-HIV activity of a dendrimer family containing different central scaffolds and multiple (9 to 18) peripheral tryptophan (Trp) groups (Fig. 1, compounds 1 to 11) that are linked to the dendrimer branches through an amino group. These compounds were shown to inhibit an early step in the replication cycle of HIV by interacting with glycoproteins gp120 and gp41 of the HIV envelope (13). Further exploration in virus-cell-based assays for broad-spectrum antiviral activity against other viruses (herpes simplex viruses 1 and 2, vaccinia virus, varicella-zoster virus, vesicular stomatitis virus, respiratory syncytial virus, reovirus 1, Sindbis virus, Punta Toro virus, cytomegalovirus, influenza virus A [subtypes H1N1 and H3N2], influenza virus B, feline coronavirus, and feline herpes virus) did not reveal any inhibitory activity, except when evaluated against EV71, a virus whose structure and mechanism of replication are completely different than those of HIV. This unexpected and intriguing observation prompted us to investigate in more detail the anti-EV71 activity of this dendrimer family.

Dendrimers 1 through 11 (Fig. 1) were first evaluated for selective antiviral activity (50% effective concentration [EC_{50}]) against the BrCr lab strain of EV71 in a virus-cell-based assay on rhabdomyosarcoma (RD) cells, which are known for their high susceptibility to EV71-induced cell death (14). Toxicity (50% cytotoxic concentration [CC_{50}]) was also assessed in a similar assay setup with treated uninfected cells. Table 1 summarizes the results of these evaluations. The capsid binder pirodavir was included as a reference (15, 16). While the antiviral activity against HIV was in the 2.2 to 16 μM range, slightly better activity was observed (0.8 to 14 μM) for EV71.

Study of the structure-activity relationship (SAR) led to the following conclusions: (i) the absence of Trp on the periphery is detrimental for anti-EV71 activity ("nude acids" 10 and 11 were inactive at EC_{50}s of >98 and >48 μM, respectively); (ii) multivalent presentation of Trp is important for anti-EV71 activity (compound 9, with only three Trp residues, is inactive, while its respective superior analog, 5, with nine Trp residues is active); (iii) the

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This article is dedicated to the memory of Prof. Chris McGuigan.

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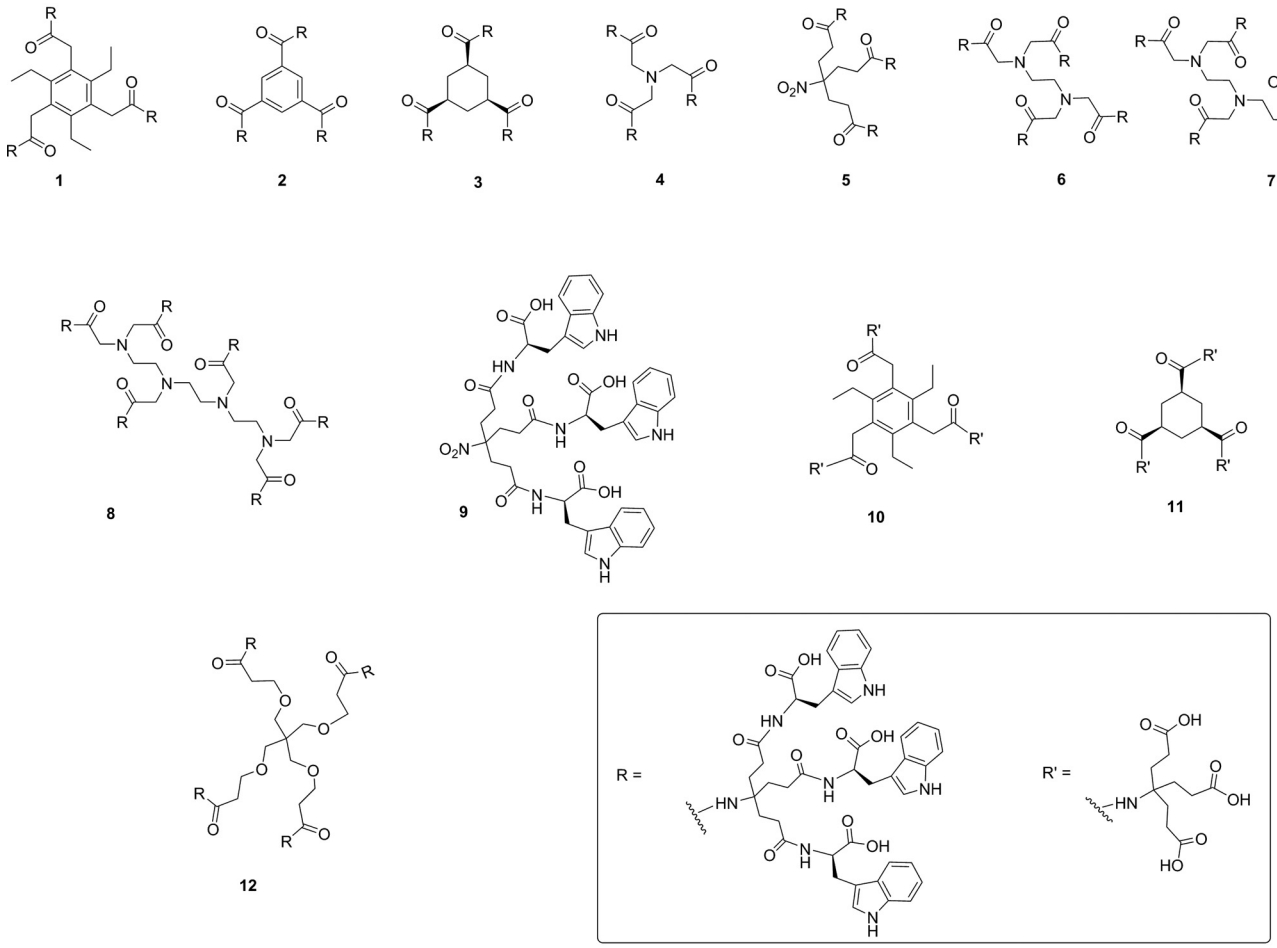

FIG 1 Structures of dendrimers 1 through 12.

TABLE 1 Antiviral activity of dendrimers against the BrCr lab strain of EV71 in RD cells[a]

Compound	EC_{50} (μM)	CC_{50} (μM)	SI
1	10 ± 1[b]	>37	>3.7
2	3.5 ± 0.3[b]	45 ± 2[b]	13
3	3.9 ± 0.2[b]	67 ± 1[b]	17
4	>39	90 ± 2[b]	ND
5	0.9 ± 0.1[b]	>38	>42
6	0.8 ± 0.1[b]	28 ± 13[b]	35
7	1.9 ± 0.1[b]	32 ± 1[b]	17
8	14 ± 1[b]	22 ± 1[b]	1.5
9	>120	>120	ND
10	>98	>98	ND
11	>48	48.0 ± 0.2[b]	ND
12	0.29 ± 0.07[b]	30.0 ± 2.5[b]	103
14	42 ± 7[b]	117 ± 2[b]	2.8
Pirodavir	0.3 ± 0.1[b]	>54	>180

[a] All values are a summary of multiple dose-response curves (>2) from 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}); ND, not determined.

[b] 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.

presence of 9 or 12 Trp residues on the periphery is sufficient for anti-EV71 activity (compound 6, with 12 Trp residues, was more active than compounds 7 and 8, with 15 and 18 Trp residues, respectively); and (iv) the flexibility of the central scaffold does not appear to have a pronounced impact on antiviral activity (compounds 1 and 2, with triethylbenzene and benzene rigid cores, respectively, showed activity similar to that of compound 3, with a more flexible cyclohexane core). Interestingly, the SAR that is described here for EV71 is very similar to that previously reported for HIV (13).

To take a next step in the exploration of the structure-activity relationship of this class of compounds, compound 12, which has a central pentaerythritol scaffold and 12 Trp residues on the periphery, was synthesized (Fig. 1). This compound was prepared using a strategy similar to that described by Rivero-Buceta et al. (13).

Compound 12 proved to be 3-fold more active (EC_{50}, 0.29 μM) than compounds 5 and 6 (EC_{50}s, 0.9 and 0.8 μM, respectively), the most active compounds among dendrimers 1 through 11, and equally as active as pirodavir, the reference compound (Table 1). Compound 12 also has the highest selectivity index (SI of 103) of the dendrimer series.

Subsequently, the antiviral activity of compound 12 was evaluated in virus-cell-based assays against a panel of representative

TABLE 2 Evaluation of the broad-spectrum antiviral effect of compound 12 against a representative panel of enteroviruses

Species	Virus	Host cell	EC_{50} (μM)[a]
Enterovirus A	Enterovirus A71 BrCr strain	RD	0.29 ± 0.07[b]
Enterovirus B	Coxsackievirus B3 Nancy strain	Vero A[c]	>28
	Echovirus 11 Gregory strain	BGM[d]	1.3 ± 0.2[b]
Enterovirus C	Poliovirus 1 Sabin strain	BGM	>28
Enterovirus D	Enterovirus D68 CU70 strain	HeLa Rh	6.0 ± 2.1[b]
Rhinovirus A	Rhinovirus 2	HeLa Rh	>28
Rhinovirus B	Rhinovirus 14	HeLa Rh	>28

[a] All values were obtained from multiple (>2) independent (>1) experiments.
[b] 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.
[c] Vero cells, African green monkey kidney cells (18).
[d] BGM, Buffalo green monkey cells (19).

enteroviruses (Table 2). Some, but less pronounced, antiviral activity was detected against echovirus 11 (ECHO11) and enterovirus D68 (EV-D68), which indicates that this compound is a quite specific inhibitor of the replication of EV71.

Finally, the antiviral potency of compound 12 was also evaluated in virus-cell-based assays against a large panel of clinical EV71 isolates with strains that belong to different clusters (A, B5, C2, and C4). Strikingly, compound 12 proved to be a 250- to 3,800-fold more potent inhibitor of the replication of these viruses than the BrCr lab strain (Table 3), with EC_{50}s in the low-nanomolar/high-picomolar range. To determine at which stage of the virus replication cycle the compound acts, a time-of-drug-addition study was performed in which the capsid binder pirodavir and the 3C protease inhibitor rupintrivir were indicated on reference compounds (Fig. 2) (15, 17). Similar to results for pirodavir, clear inhibition of virus replication was observed only when the drug was added during or before infection. Both compound 12 and pirodavir lost their moderate activity when added after the infection period. This is in line with the observations 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.

TABLE 3 Evaluation of the broad-spectrum antiviral effects of compound 12 against a representative panel of clinical EV71 isolates in RD cells

EV71 genogroup	Virus strain	EC_{50} (nM)[a]
A	BrCr	285 ± 70
B5	TW/70811/08	0.181 ± 0.020
	TW/70886/08	0.097 ± 0.039
	TW/70902/08	0.233 ± 0.081
	TW/96002/08	0.362 ± 0.081
	TW/96016/08	0.166 ± 0.055
	TW/96022/08	0.126 ± 0.040
	11316	0.385 ± 0.016
C2	H08300 461	1.121 ± 0.335
	H08300 496	0.191 ± 0.040
	H08326 456	0.181 ± 0.032
C4	TW/2728/04	0.075 ± 0.003
	TW/2429/04	0.301 ± 0.216
	TW/2639/04	0.079 ± 0.011
	TW/2815/04	0.158 ± 0.017
	TW/2824/04	0.400 ± 0.053
	TW/2871/04	0.226 ± 0.134
	TW/71595/04	0.666 ± 0.193
	TW/72232/04	0.199 ± 0.004
	TW/1956/05	0.229 ± 0.175
	TW/71428/05	0.301 ± 0.166

[a] All values were obtained from 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.

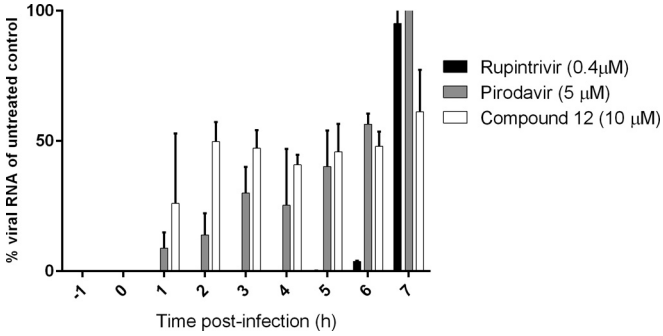

FIG 2 Effects of pirodavir (capsid binder), rupintrivir (3C protease inhibitor), and compound 12 in a time-of-drug-addition assay.

It can be hypothesized that the mechanism of action in the context of EV71 will most involve a glycosylated protein on the host cell surface because of the following: (i) the structures of the two viruses (HIV and EV71) are very different, (ii) the difference in potencies against the clinical isolates versus the laboratory strain is striking, and (iii) the EV71 virion lacks (in contrast to HIV) glycosylated proteins on its surface This is currently being explored further.

In summary, a novel class of potent, selective, specific, and early-stage inhibitors of enterovirus A71 replication has been discovered. To consolidate the structure-activity relationship observations, a new dendrimer with a central pentaerythritol scaffold and 12 Trp residues on the periphery was synthesized. This compound proved to be exquisitely active against clinical isolates of EV71 (belonging to each of the genogroups).

Because enterovirus A71 is a virus that is transmitted through the fecal-oral route and because the molecular weight of the molecules probably would prevent them from being transferred from the gut lumen to the bloodstream following oral administration, one could envisage using this type of compound prophylactically to create a chemotherapeutical barrier in the gut to prevent infection with EV71 or using it as a topical cream to treat virus-induced lesions. However, whether additional modifications can further increase the potency of this compound to obtain a suitable candidate for proof-of-concept studies should be explored first.

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