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Serinol-Based Benzoic Acid Esters as New Scaffolds for the Development of Adenovirus Infection Inhibitors: Design, Synthesis, and *In Vitro* Biological Evaluation

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progressively been recognized as a significant viral pathogen. Traditionally associated with self-limited respiratory, gastrointestinal, and conjunctival infections, mainly in immunocompromised patients, HAdV is currently considered to be a pathogen presenting significant morbidity and mortality in both immunosuppressed and otherwise healthy individuals. Currently available therapeutic options are limited because of their lack of effectivity and related side effects. In this context, there is an urgent need to develop effective anti-HAdV drugs with suitable therapeutic indexes. In this work, we identified new serinol-derived benzoic acid esters as novel scaffolds for the inhibition of HAdV infections. A set of 38 compounds were designed and synthesized, and their antiviral activity and cytotoxicity were evaluated. Four compounds (13, 14,



27, and 32) inhibited HAdV infection at low micromolar concentrations (2.82–5.35 μ M). Their half maximal inhibitory concentration (IC₅₀) values were lower compared to that of cidofovir, the current drug of choice. All compounds significantly reduced the HAdV DNA replication process, while they did not block any step of the viral entry. Our results showed that compounds 13, 14, and 32 seem to be targeting the expression of the E1A early gene. Moreover, all four derivatives demonstrated a significant inhibition of human cytomegalovirus (HCMV) DNA replication. This new scaffold may represent a potential tool useful for the development of effective anti-HAdV drugs.

KEYWORDS: serinol derivatives, benzoic acid esters, adenovirus, antivirals agents, urea derivatives, chemical synthesis

H uman adenoviruses (HAdVs) belong to the family Adenoviridae that includes more than 100 types associated with human infections and are classified into 7 species (HAdV A–G). They are nonenveloped and icosahedral viruses with a linear double-stranded DNA (dsDNA) genome of 34–36 kb.^{1–3} HAdVs are responsible for a wide range of global clinical diseases, mostly in immunocompromised patients,^{4,5} although a number of cases in healthy individuals with severe community-acquired pneumonia have been described.^{6,7}

Young children and immunosuppressed adult patients are more susceptible to serious HAdV infections.^{4,8} HAdVs usually infect the epithelium of the respiratory tract^{9,10} but also cause conjunctivitis,¹¹ urinary tract infections,¹² gastrointestinal diseases,¹³ and multiple organ failures.¹⁴ Among them, pneumonia and hemorrhagic cystitis are the most common complications.¹⁵ Respiratory infections are traditionally associated with types 1–3, 5, and 7. Types 8, 19, and 37 are commonly associated with ocular diseases, while 40 and 41 are frequently involved in enteric infections.¹⁶ Immunosuppressive therapies used during transplantations can cause an increase in the frequency of HAdV infections that can be acquired from the transplanted organ or by the reactivation of a latent infection in the recipient.^{17,18} In pediatric hematopoietic stem cell transplantation (HSCT) patients, the incidence of HAdV infections ranges from 15% to 44% with high mortality rates for those with disseminated disease.^{8,19,20}

At this time, there is no formally approved antiviral therapy for the treatment of HAdV infections. Nowadays, antiviral

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drugs such as nucleoside analogues cidofovir (CDV; 1, Figure 1), ganciclovir (2, Figure 1), and ribavirin provide no



Figure 1. Some reported antiviral and anti-HAdV agents (1-8) and the design of the general backbone of serinol derivatives.

satisfactory results in terms of efficacy or safety.^{21–23} CDV is a cytosine analogue (acyclic nucleoside phosphonate) that represents the most common antiviral agent used for HAdVrelated diseases. It showed broad spectrum antiviral properties, acting as a false substrate for the viral DNA polymerase blocking DNA replication.²⁴

Unfortunately, the clinical use of CDV is limited because of its low oral bioavailability, nephrotoxicity, and myelotoxicity.^{25,26} Over the years, several lipid conjugates of CDV have been developed, like brincidofovir (CMX001, BCV), which displayed greater *in vitro* antiviral activity and a good inhibition of viral replication in animal models. Furthermore, it demonstrated an increase in oral bioavailability and reduced nephrotoxicity compared to CDV.^{27,28} Phases II and III of clinical trials (NCT01231344, NCT02087306) confirmed the anti-HAdV activity of BCV with reductions in HAdV-related symptoms; nevertheless, it induced some gastrointestinal disorders, limiting its use in therapy.^{29,30}

These features support the need for the development of new antiviral agents with activity against HAdV and a good therapeutic index. Different new nucleoside derivatives or nitrogen base-based analogues have been described. A series of octadecyloxyethyl esters of (S)-[(3-hydroxy-2-phosphonomethoxy)propyl] nucleoside (ODE nucleoside) showed antiviral activity against HAdV 14, an emerging type associated with a high rate of serious complications.³¹ S-Aminouracil derivatives were tested against HAdV 5, and one of them, the morpholino-substituted compound (3, Figure 1), displayed a significant *in vitro* inhibition, achieving a half maximal inhibitory concentration (IC₅₀) value of 0.5 μ M.³²

In the past few years, also, non-nucleoside and structural diversified compounds have been reported as new potential antiviral candidates; many of them presented amide/urea functions and aromatic moieties as common features in their structures. Pyrimidine–nitrile derivatives with amide moieties were prepared as new potential adenain inhibitors, an important viral protease involved in the virus propagation.³³ Kang et al. reported the antiviral activity of 3-hydroxy-

quinazoline-2,4(1*H*,3*H*)-diones against Vaccinia virus and HAdV 2 (4, Figure 1). Regarding the HAdV type, compounds with fluorine or methoxy groups on the aromatic ring were considered the most active.³⁴

A range of benzoyl esters and amides have been identified as interesting scaffolds due to their ability to block HAdV infection. Salicylamides with electron withdrawing substituents on the phenyl ring represented excellent inhibitors of HAdV 5 replication; some of them showed IC_{50} values in the nanomolar range (5, Figure 1).^{35,36} Benzoic acid esters were also developed as potential antiviral agents against HAdV 7 (6, Figure 1).³⁷ Our previous research was focused on the preparation of new piperazine-derived ureas/thioureas with inhibitory activity against HAdV type 5 (7, Figure 1). Optimized compounds blocked HAdV infection at nanomolar or low micromolar ranges, showing low cytotoxicity and acting in several phases of its life cycle. $^{38-40}$ On the basis of our previous work and experience in the synthesis of new small libraries of compounds with other potential biological activity^{41,42} and focused on searching novel scaffolds for the preparation of potential anti-HAdV agents, the major aim of this report was to present the design, synthesis, and in vitro biological evaluation of a small library (38 compounds) of different serinol derivatives. Biological screening and SAR (structure activity relationships) studies were developed in order to identify novel lead compounds.

RESULTS AND DISCUSSION

As the source for our central backbone, the cheap and commercially available reactive serinol was employed. Three structural considerations were behind this choice (Figure 1). (i) The aminoalcohol skeleton: Small molecules based on an isopropanolamine core have been prepared and evaluated as HIV inhibitors. They possessed simple nonpeptidic heteroaryl structures, where the benzenesulfonamide group presented a suitable moiety to be linked to the isopropanolic core to obtain biological activity (8, Figure 1).^{43,44} In this work, we have employed an isomeric core, 2-amino-propane-1,3-diol. (ii) The question of the symmetry: In the field of the development of different scaffolds with antiviral activities, different types of symmetric compounds have been described, such as novel symmetrical phenylenediamines targeting the viral NS3 helicase being potential anti-HCV agents,⁴⁵ symmetrically disposed stilbenes as potent inhibitors of NS5A,⁴⁶ and complex homodimeric structures with large molecular weights (daclastavir and other related symmetric compounds).47,48 To the best of our knowledge, no symmetric compounds have been described as potential anti-HAdV agents. (iii) The functionalization: The serinol scaffold allowed us to develop new molecules keeping many important features present in the reported anti-HAdV agents mentioned above, mainly urea^{32,34,38} and ester functions,³⁷ that have been identified as important for the antiviral activity (Figure 1). In our general backbone, the urea function was introduced at the nitrogen (this moiety could be considered an acyclic modification of the nitrogen base present in some anti-HAdV agents) (1, 3), while aromatic esters at the hydroxyls as an isosteric modification with respect to the aromatic amide function, which is present in niclosamide (5) and its derivatives, and also because of their presence in effective anti-HAdV agents (6).

As it is shown in Figure 1, our original framework has three points for different structural modifications to generate chemical diversity. Two of them are related to the substituents

Table 1. Diester (13-37), Monoester (38-40), Dicarbamate (41-49), and Monocarbamate (50) Serinol Derivatives^a



entry	comp.	\mathbb{R}^1	\mathbb{R}^2	R ³	\mathbb{R}^4	R ⁵	\mathbb{R}^{6}	percentage of plaque-formation inhibition b	structure
1	13	Н	CF_3	Н	Н	CH ₃	Н	66.33 ± 2.49	А
2	14	Н	CF_3	CH_3	Н	Н	Н	89.69 ± 7.13	А
3	15	Н	CF_3	Н	Н	OCH ₃	Н	97.17 ± 0.77	А
4	16	Н	CF_3	Н	Н	CN	Н	7.66 ± 10.83	А
5	17	Н	CF_3	Н	Н	NO ₂	Н	0.00 ± 0.00	А
6	18	Н	CF ₃	OCH ₃	Н	OCH ₃	Н	55.83 ± 4.31	А
7	19	Н	CF_3	Н	OCH ₃	OCH ₃	OCH ₃	47.33 ± 38.86	А
8	20	Н	CH_3	Н	Н	CH ₃	Н	9.40 ± 13.29	А
9	21	Н	CH_3	CH_3	Н	Н	Н	0.00 ± 0.00	А
10	22	Н	CH_3	Н	Н	OCH ₃	Н	6.78 ± 13.90	А
11	23	Н	CH_3	Н	Н	NO ₂	Н	0.00 ± 0.00	А
12	24	Н	CH_3	OCH ₃	Н	OCH ₃	Н	41.48 ± 1.23	А
13	25	Н	CH_3	Н	OCH ₃	OCH ₃	OCH ₃	34.58 ± 6.23	А
14	26	CF_3	Cl	Н	Н	CH ₃	Н	25.68 ± 36.31	А
15	27	CF_3	Cl	CH_3	Н	Н	Н	98.25 ± 2.48	Α
16	28	CF_3	Cl	Н	Н	OCH ₃	Н	91.51 ± 11.9	Α
17	29	CF_3	Cl	Н	Н	CN	Н	6.25 ± 8.84	Α
18	30	CF_3	Cl	Н	Н	NO ₂	Н	5.81 ± 8.22	А
19	31	CF_3	Cl	OCH ₃	Н	OCH ₃	Н	0.00 ± 0.00	А
20	32	CF_3	Cl	Н	OCH ₃	OCH ₃	OCH ₃	97.58 ± 4.73	Α
21	33	CF_3	Cl	Н	Н	$N(CH_3)_2$	Н	44.76 ± 2.69	А
22	34	Н	Cl	Н	Н	OCH ₃	Н	43.29 ± 11.86	А
23	35	CF_3	Cl					58.79 ± 3.43	В
24	36	Н	CF_3					0.00 ± 0.00	В
25	37	Н	CH_3					0.00 ± 0.00	В
26	38	CF_3	Cl	Н	Н	OCH ₃	Н	30.0 ± 27.85	D
27	39	Н	CH_3	OCH ₃	Н	OCH ₃	Н	34.93 ± 16.67	D
28	40	Н	CH_3	Н	OCH ₃	OCH ₃	OCH ₃	27.51 ± 59.28	D
29	41	Н	CF_3	Н	Н	CH ₃	Н	0.00 ± 0.00	С
30	42	Н	CF_3	CH_3	Н	Н	Н	99.74 ± 0.36	С
31	43	Н	CF_3	Н	Н	OCH ₃	Н	0.00 ± 0.00	С
32	44	Н	CF ₃	Η	Н	CN	Н	98.59 ± 2.00	С
33	45	Н	CF_3	Η	Н	NO ₂	Н	99.54 ± 0.87	С
34	46	CF_3	Cl	Н	Н	CH ₃	Н	0.00 ± 0.00	С
35	47	CF_3	Cl	CH_3	Н	Н	Н	100 ± 0.00	С
37	48	CF_3	Cl	Η	Н	NO ₂	Н	37.43 ± 3.12	С
37	49	Н	CH_3	Н	Н	CH ₃	Н	0.00 ± 0.00	С
38	50	Н	CF_3	Н	Н	NO ₂	Н	99.45 ± 0.77	E
41	cidofovir ^c							3.51 + 4.97	

^{*a*}Inhibition of HAdV infection in the plaque assay. ^{*b*}Percentage of control HAdV5-GFP inhibition in a plaque assay at 10 μ M using the 293 β 5 cell line. See the Methods section for details. ^{*c*}The data of cidofovir, as a positive clinical drug candidate, have been listed.

of the aromatic rings of both urea and ester functions. The third consists of the replacement of the ester function by a carbamate one.

Chemistry. General synthetic routes for the preparation of new derivatives (Table 1) are summarized in Schemes 1 and 2

and included two reactions starting from commercial 2-amino-1,3-propanediol. As it is shown in Scheme 1, in the first step, the urea function was introduced by the reaction with appropriate substituted phenyl isocyanate in dichloromethane at room temperature (9-12). Three different substituted Scheme 1. Synthetic Pathways for the Preparation of Urea Derivative Precursors (9-12) and Serinol Derivative Esters (13-40)



Scheme 2. Synthetic Pathway for the Preparation of Serinol Derivative Dicarbamates (41–49)



phenyl isocyanates (p-CF₃ and p-CH₃ as models of electronwithdrawing and electron-releasing groups and disubstituted 3-CF₃-4-Cl) were employed. In the second reaction, ester or carbamate derivatives were generated from compounds 9-12. Diester derivatives of serinol (13-34) were synthesized by an acylation reaction of both primary hydroxyl groups, using acyl chloride and DMAP in dichloromethane at rt. At this step, the pursued chemical diversity was introduced by the different types of substituent on the aromatic ester. It was decorated with electron-withdrawing or -donating groups to assess the effect of electronic properties, with a special focus on the methoxy group (mono-, di-, and trimethoxy substituted), since these benzoyl derivatives (amides and esters) are privileged structural scaffolds, which are widely distributed in different anticancer and antiviral compounds.^{34,44,49-52} Other relevant scaffolds broadly distributed in natural products with diverse and important biological functions (among them antiviral activities) are trimethoxycynnamic derivatives (esters and amides).⁵³ For the preparation of these serinol derivative esters (35-37), the condensation took place using carboxylic acid as acylating agent under Steglich conditions (EDCI, DMAP).

Monoester derivatives (38-40) were obtained through a selective *O*-acylation reaction of serinol ureas with acyl

chloride by strictly controlling the time (1 h), the stoichiometry (1 equiv), and the temperature (-15 °C).

For the third structural diversification, dicarbamate derivatives (41–50) were prepared by the reaction of the urea derivative with the isocyanate. When commercial isocyanates were employed, the reaction was performed at 110 °C in toluene.⁵⁴ In the case of compounds 42 and 47, the corresponding isocyanate was previously prepared from *o*toluidine by the reaction with triphosgene in basic conditions (Na₂CO₃).⁵⁵ In a second step, dicarbamate was generated in milder conditions, using DMAP in dichloromethane at rt (Table 1). These compounds were prepared with the aim to increase their potential ability to form hydrogen interactions through both urethane functions (Scheme 2).

Biological Evaluation. Compounds 13–50 were screened for their potential anti-HAdV activity through the plaque assay, quantifying HAdV plaque formation in the presence of candidate molecules (Table 1). As CDV remains the drug of choice for the treatment of these infections, it was also evaluated in parallel to our compounds, following our own methodologies.^{38,39,56} In our protocol for the plaque assay, we used the HAdV5-GFP vector in combination with the cell line $293\beta5$ to generate the infection plaques to be quantified. This may be a limitation in our initial screening since this vector itself does not express the E1A gene, which is expressed by the cells. Thus, there is a possibility that some potential molecules were missed in our screening.

Among the serinol-derived diesters, the subfamily with a methyl group at the phenylurea function (20-25) showed very little plaque-formation inhibition. Only two of them. 24 and 25 (dimethoxy and trimethoxy benzoyl derivatives) displayed an appreciable inhibitory activity (41.48% and 34.58%, respectively). On the other hand, trifluoromethyl and disubstituted (3-CF₃-4-Cl) urea analogues (13-19 and 26-33) presented significantly higher levels of inhibition. Depending on the substituent at the benzoyl moiety, different behaviors were observed. In particular, the presence of electron-withdrawing groups (p-CN, p-NO₂) resulted in very low or no active compounds (16, 17, 23, 29, and 30) with percentages of plaque formation inhibition ranging from 0% to 7.66%. Higher inhibition was detected among those compounds with electron donating groups (13-15, 18, 19, 24-28, and 33). p-CH₃, o-CH₃, p-OCH₃, 2,4-dimethoxy, and 3,4,5-trimethoxy derivatives displayed plaque formation inhibition values ranging from 66% to 97%. o-CH₃ and p-OCH₃ analogues with p-CF₃ and 3-CF₃-4-Cl ureas (14, 15, 27, and 28) were the most active compounds with percentages of inhibition >90%, together with compound 32, the trimethoxy derivative with a disubstituted urea that showed an almost complete inhibition (97.58% of inhibition; Table 1).

As compounds 15 and 28, both *p*-methoxy diesters with p-CF₃ and 3-CF₃-4-Cl substituted urea, showed 90% of inhibition, their analogue *p*-Cl substituted on the urea function (34) was prepared to diversify the structure. It reached a moderate level of inhibition (43.29%).

Similarly, the presence of a *p*-dimethylamino group, a different electron donor moiety (33), did not improve the activity (44.76% of inhibition).

The introduction of the trimethoxycinnamic esters in our skeleton did not offer active compounds, 0% of inhibition for compounds **36** and **37** and 58.79% of inhibition for **35**. This group failed to provide any improvement over their

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entry	comp.	$IC_{50} (\mu M)^{b}$	$CC_{50} (\mu M)^c$	selectivity index (SI) ^d	yield reduction $(fold-reduction)^e$
1	13	5.35 ± 0.66	93.24 ± 7.7	17.42	7.23 ± 5.38
2	14	4.53 ± 0.64	200.00 ± 33.87	44.15	92.28 ± 33.77
3	15	2.63 ± 0.26	11.73 ± 0.26	4.46	
4	27	2.82 ± 0.31	25.10 ± 0.16	8.90	42.83 ± 15.68
5	28	8.96 ± 1.12	11.24 ± 4.48	1.25	
6	32	3.67 ± 1.46	63.73 ± 0.50	17.37	1.22 ± 0.63
7	42	3.76 ± 0.23	19.8 ± 0.16	5.27	
8	44	7.05 ± 2.65	14.4 ± 0.63	2.04	
9	45	2.05 ± 0.02	11.56 ± 4.30	5.64	
10	47	7.78 ± 2.02	18.4 ± 0.60	2.37	
11	50	9.74 ± 0.90	41.16 ± 3.17	4.23	
12	cidofovir	24.06 ± 5.9	179.09 ± 35.10	7.5	82.5 ± 21.4

Table 2. IC₅₀, CC₅₀, SI, and Virus Yield Reduction Values for Selected Compounds and for Cidofovir as a Positive Clinical Drug Candidate^a

^{*a*}The results represent means \pm SD of triplicate samples from three independent experiments. See the Methods section for details. ^{*b*}Inhibitory concentration 50 at a low MOI in a plaque assay. ^{*c*}Cytotoxic concentration 50. ^{*d*}The selectivity index value was determined as the ratio of 50% cytotoxic concentration (CC₅₀) to half maximal inhibitory concentration (IC₅₀) in a plaque assay for each compound. ^{*e*}Fold-reduction in virus yield as the ratio of particles produced in the presence of DMSO divided by the yield in the presence of each compound (50 μ M).

trimethoxybenzoyl esters analogues **19** (47.33%), **25** (34.58%), and **32** (97.58%), respectively.

By strict control of the acylation reaction, monoester derivatives were prepared (38-40). The rationale behind this structural modification was the evaluation of the presence of a free primary hydroxyl group, as in CDV, to provide active compounds (high% of inhibition) that could act through a different mechanism. As depicted in Table 1, the plaque formation inhibitions observed for monoacylated compounds **38–40** were 30.0%, 34.93%, and 27.51%, respectively.

For the preparation of dicarbamate derivatives (41-49), both *p*-CF₃ and 3-CF₃-4-Cl urea derivatives were employed as they led to most active diesters. The dicarbamate derivatives provided different results in terms of the structure–activity relationship. Compounds with the *p*-CF₃ substituted urea and electron-withdrawing substituents on the aromatic carbamate function (44 *p*-CN and 45 *p*-NO₂) showed a high percentage of inhibition (98.59% and 99.45%, respectively). Moreover, both *ortho* methyl derivatives from *p*-CF₃ and disubstituted phenyl urea (42 and 47) inhibited 99.54% and 100% of plaque formation, respectively. Finally, the monocarbamate derivative **50**, isolated during the synthetic preparation of compound 45, was also evaluated for its antiviral activity and demonstrated to be the only active monoacyl compound (99.45% of inhibition; Table 1).

Interestingly, these results suggest that the presence of a carbamate function seems to be crucial for the inhibition of HAdV infection when electron-withdrawing groups are present.

It is important to note that precursors 9-11 were also assessed in the plaque assay, resulting in plaque formation inhibitions of 6%, 0%, and 58%, respectively, which suggests that the presence of acyl functions on primary hydroxyl groups could be important for the antiviral activity.

Those compounds showing a HAdV inhibition >60% in the plaque assay (a total of 11 out of 38, 28%) were selected to evaluate their specificity, measured as the quotient between the 50% cytotoxic concentration (CC_{50}) and their IC_{50} values. All 11 compounds inhibited HAdV5 infection in a dose-dependent manner showing IC_{50} values from 2.05 μ M (compound 45) to 9.74 μ M (compound 50). These values were significantly lower than those observed for the drug cidofovir (24.06 μ M),

the current drug of choice for the treatment of HAdV infections.⁵⁷ Only compounds **13**, **14**, **27**, and **32** showed a CC_{50} value at least 10 times over their IC_{50} values, presenting selectivity indexes ranging from 8.9 to 44.15, in all cases higher than that obtained for cidofovir (Table 2). Thus, they were selected for further biological analysis to gain some knowledge regarding their potential mechanism of action.

First, we evaluated their anti-HAdV potency using a virus burst assay to measure their efficacy in blocking the production of new HAdV viral particles. The treatment with these compounds was associated with overall reductions in virus yield between 1.22-fold and 92.3-fold (Table 2). These values lie in the same range as those for cidofovir (82.5-fold).

Next, we evaluated the capacity of these compounds to block the HAdV DNA replication process. For this purpose, we carried out a real-time PCR assay to evaluate the HAdV DNA replication efficiency in the presence of these compounds in a single round of infection for 24 h. The presence of compound 14 blocked almost completely (99.2%) the synthesis of new copies of the HAdV DNA, while compounds 13 and 27 showed lightly lower inhibitions, 95.1% and 92.1%, respectively. Compound 32, although to a lesser extent, also showed a significant inhibition of HAdV DNA replication (73.5%) (Figure 2)

To elucidate if any of these compounds was interfering with some steps between HAdV entry and HAdV DNA import into the nucleus, we carried out a nuclear association assay to quantitatively measure the HAdV genome accessibility to the host nucleus. After binding to the cellular receptors, internalization, and endosomal escape mediated by protein VI, the partially uncoated HAdV capsids are transported to the nuclear membrane via the microtubule network and their genome is imported into the nucleus via the nuclear pore complex.^{58,59} So, if the mechanism of action of these molecules would involve interference with any of these steps of the HAdV entry, this would be reflected in the amount of HAdV genomes that reach the nucleus after a synchronized infection. As depicted in Figure 3, the treatment with any of these four molecules did not show a significant difference in the number of HAdV genomes that reached the nucleus compared with those treated with DMSO. The DNA copy number of the cellular housekeeping gene GAPDH in both the nucleus and the



Figure 2. Effect of the selected compounds on HAdV DNA replication. All four compounds significantly reduced the HAdV DNA replication process. Compounds **13** and **14** were evaluated at a 50 μ M concentration; compound **27** was evaluated at a 15 μ M concentration and compound **32**, at a 37 μ M concentration. CDV was used as a positive control for the inhibition of HAdV DNA replication (48 μ M). Results are expressed as the relative copy number of HAdV DNA normalized to GAPDH copy number, and they are presented as the mean \pm SD of duplicate assays. ** $p \leq 0.01$; *** $p \leq 0.001$.



Figure 3. Effect of the selected compounds on HAdV genome nuclear association. Compounds **13**, **14**, **27**, and **32** did not block any of the steps during the HAdV entry phase. Compounds **13** and **14** were evaluated at a 50 μ M concentration; compound **27** was evaluated at a 15 μ M concentration and compound **32**, at a 37 μ M concentration. Results are expressed as the mean \pm SD of duplicate assays.

cytoplasm was also measured as a control for the purity of nuclear isolation, indicating that we specifically quantified the HAdV genomes that reached the nuclear membrane. On the basis of this assay, we could confirm that any of the studied compounds was interfering with the early steps that span the entry phase of the HAdV viral particles.

Since the mechanism of action of these four compounds seems to be related to the early steps after HAdV genome entry into the nucleus, we decided to go a step forward in our mechanistic studies. Once HAdV genomes enter the nucleus of the host cell, the transcription of the immediate, early gene E1A takes place first, and this product will be essential for the transcription of other early proteins and the subsequent replication of the HAdV DNA. We quantified the mRNA copy number of the E1A gene using quantitative reverse transcription PCR (RT-PCR). As shown in Figure 4, only compounds 13, 14, and 32 significantly blocked the expression of the E1A gene in a 6 h assay. Compound 27 did not show any decrease in the expression of the E1A gene compared with a control treated with DMSO. The ability of compounds 13, 14, and 32 to interfere with HAdV early gene transcription



Figure 4. Effect of the selected compounds on HAdV E1A gene expression. Compounds **13**, **14**, and **32** significantly blocked the expression of the E1A gene. Compounds **13** and **14** were evaluated at 50 μ M concentration; compound **27** was evaluated at a 15 μ M concentration and compound **32**, at a 37 μ M concentration. Results are expressed as the relative copy number of HAdV E1A mRNA normalized to GAPDH copy number, and they are presented as the mean \pm SD of duplicate assays. * $p \leq 0.05$; ** $p \leq 0.01$.

may be the cause of their DNA replication inhibition. On the other hand, since compound **27** did not display inhibition of the early gene transcription, it may suggest that this compound could interact with viral proteins essential for HAdV DNA replication, including the HAdV DNA polymerase, the precursor of the terminal protein (pTP), or the DNA-binding protein (DBP).

As other compounds have been demonstrated to inhibit both HAdV and human cytomegalovirus (HCMV) DNA replication,^{38,39} we decided to perform an inhibitory assay against HCMV with these four anti-HAdV compounds. As shown in Figure 5, all of them were able to significantly block the HCMV DNA replication in a 72 h assay. Compounds 13 and 27 showed a total inhibition of HCMV replication, while compounds 14 and 32 gave percentages of inhibition of 91.8% and 90%, respectively. This result suggests a broad antiviral mechanism of action for these molecules and, since they seem to act at different stages of the HAdV replicative cycle, it supports high similarities in HAdV and HCMV routes of infection. However, further studies will be needed to clarify their specific mechanism for antiviral activity.

CONCLUSIONS

In summary, a set of 38 novel serinol derivatives were designed and synthesized as new potential anti-HAdV agents. According to the biological evaluation data, serinol-based aromatic diesters emerged as promising derivatives. Those compounds having electron-withdrawing groups at the phenyl urea function and electron-donor groups at the benzoic moiety were the most active. In particular, we have identified four compounds (13, 14, 27, and 32) that significantly inhibited HAdV infection in a dose-dependent manner. These compounds showed IC₅₀ values at low micromolar concentration (from 2.82 to 5.35 μ M), being more active than cidofovir in our in vitro assays (24.06 $\mu M).$ Our mechanistic studies suggest that all four compounds interfered with HAdV DNA replication. Specifically, 13, 14, and 32 seemed to be targeting the E1A early gene expression. The antiviral activity of these derivatives was also evaluated against HCMV, demonstrating a significant inhibition of HCMV DNA replication, which suggests an antiviral broad-spectrum activity for these compounds. Our work did not include additional approaches such as genomics or proteomics methods that





Figure 5. Effect of the selected compounds on HCMV DNA replication. All four compounds significantly inhibited HCMV DNA replication. Compounds 13 and 14 were evaluated at 50 μ M concentration; compound 27 was evaluated at a 15 μ M concentration and compound 32, at a 37 μ M concentration. Results are expressed as the relative copy number of HCMV DNA and normalized to GAPDH copy number, and they are presented as the mean \pm SD of duplicate assays. ** $p \leq 0.01$.

could give us more solid information regarding the specific mechanism of action of these derivatives. Thus, alternative mechanisms involving interactions with specific proteins involved in the HAdV DNA replication and transcription processes should be considered. Further investigations will be necessary in order to fully clarify the specific mode of action for their antiviral activity and to confirm their potential utility as antiviral drugs after the evaluation of their stability in serum and efficacy and safety in an animal model of infection. It will also be necessary to prepare additional monoacylated derivatives, following both structural aspects (analogues of interest to be compared to the diacylated derivatives) and stereochemical considerations (the racemic form and both pure enantiomers) in order to thoroughly study structure– activity relationships.

When one takes into consideration all the results collected so far and the novelty of the scaffold proposed, it is possible to consider these four serinol-derived benzoic acid esters as promising hits for the development of a new class of anti-HAdV agents.

METHODS

Chemistry. General Methods. All reagents, solvents, and starting materials were obtained from commercial suppliers and used without further purification. The crude reaction mixtures were concentrated under reduced pressure by removing the organic solvents in a rotary evaporator. Reactions were monitored by thin layer chromatography (TLC) using Kieselgel 60 F254 (E. Merck) plates and a UV detector for visualization. Flash column chromatography was performed on Silica Gel 60 (E. Merck). All reported yields are of purified products. Melting points were obtained on a Stuart Melting Point Apparatus SMP 10 and are uncorrected. Mass spectra were recorded on a Micromass AUTOSPECQ mass spectrometer: EI at 70 eV and CI at 150 eV and HR mass measurements with resolutions of 10 000. FAB mass spectra were recorded using a thioglycerol matrix. NMR spectra were recorded at 25 °C on a Bruker AV500 spectrometer at 500 MHz for ¹H and 125 MHz for ¹³C. COSY, DEPT, HSQC, and NOESY experiments were performed to assign the signals in the NMR spectra. The chemical shifts (d) reported are given in parts per million (ppm) on the δ scale relative to TMS, and the coupling constants (J) are in hertz (Hz). ¹H chemical shift values (δ) are referenced to the residual nondeuterated components of the NMR solvents (δ 2.54 ppm for DMSO). The spin multiplicities are reported as s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet), or br s (broad singlet). The purity of final compounds was evaluated by elemental analysis (C, H, and N). The purity of all the final compounds was confirmed to be \geq 95% by combustion.

General Procedure 1: Synthesis of Urea Derivatives of Serinol (9-12). To a solution of the corresponding isocyanate (3.6 mmol) in dry dichloromethane (20 mL) was added dropwise a solution of the aminoalcohol (3 mmol) in methanol (1 mL). A white precipitate appeared, and it was isolated by filtration *in vacuum*.

General Procedure 2: Synthesis of the Diester Derivatives (13–37). Method A. To a solution of the urea derivative 9–12 (0.54 mmol) in dry dichloromethane (20 mL) and DMAP (1.35 mmol), the appropriate acylating agent (1.1 mmol) in dry dichloromethane (5 mL) was added. The reaction mixture was stirred at rt until TLC showed that all the starting material had reacted; then, it was evaporated to dryness. The resulting residue was treated with a 1 N HCl aqueous solution and extracted with ethyl acetate (3 × 50 mL). The combined organic phases were washed with saturated NaHCO₃ aqueous solution and brine. The organic layer was dried (Na₂SO₄), filtered, and evaporated *in vacuum*. The compound was further purified through flash column chromatography on silica gel using the appropriate eluent.

Method B.⁶⁰ Carboxylic acid (2.87 mmol) was dissolved in dichloromethane, and EDCI (3.55 mmol) was added. The mixture was stirred for 1 h at rt (mixture 1). At the same time, to a suspension of urea derivative (9-11) (0.78 mmol) in dichloromethane was added DMAP (2.13 mmol), and the mixture was stirred for 1 h at rt (mixture 2); then, mixture 2 was added dropwise into mixture 1. The reaction was stirred for 24 h at rt. The organic layer was washed with saturated NaHCO₃ aqueous solution and brine; then, it was dried over Na₂SO₄, filtered, and evaporated *in vacuum*. The compound was further purified through flash column chromatography using the appropriate mixture of hexane–ethyl acetate as eluent.

General Procedure 3: Selective Mono-O-acylation Reaction of Serinol Ureas (38–40). To a cooled solution (–15 °C) of urea derivative (10 or 11, 1 mmol) and DMAP (0.9 mmol) in dry dichloromethane (20 mL) and dimethylformammide (1 mL) was added dropwise a solution of acyl chloride (0.9 mmol) in dry dichloromethane (15 mL). The reaction mixture was stirred at –15 °C for 1 h; then, it was washed with a 1 N HCl aqueous solution (2 × 15 mL), saturated NaHCO₃ (2 × 15 mL), and brine. The organic layer was dried (Na₂SO₄), filtered, and evaporated to dryness. The compound was purified through flash column chromatography using the appropriate mixture of hexane–ethyl acetate as eluent.

General Procedure 4: Synthesis of Dicarbamate Derivatives (41–49). Method A.⁵⁴ Urea derivatives (9–11) (0.64 mmol) and isocyanate (1.47 mmol) were mixed in toluene (10 mL). The resulting suspension was refluxed (110 °C) for 24 h; then, the reaction mixture was filtered and washed with fresh toluene. The obtained solid was dried at rt and analyzed as pure compound or further purified through flash column chromatography using hexane–ethyl acetate as eluent. In the case of 4-nitrophenyl isocyanate, after column chromatography of the mixture, the monoderivative was also isolated as a side compound.

*Method B.*⁵⁵ First, 2-methylphenyl isocyanate was prepared. To a solution of *o*-toluidine (1.87 mmol) in dichloromethane (20 mL) was added an aqueous solution of Na_2CO_3 (3 mmol, 20 mL), and the heterogeneous mixture was vigorously stirred for 5 min at rt. Triphosgene (0.62 mmol) was added to the flask and stirred for 30 min. The phases were manually separated, and the organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The isocyanate was used without further purification in the next reaction.

2-Methylphenyl isocyanate (1 mmol) was dissolved in dichloromethane (20 mL); then, DMAP (1 mmol) and the appropriate urea derivative (9 or 11) (0.42 mmol) were added. The reaction mixture was stirred for 48 h and then concentrated under reduced pressure. The crude product was purified through flash column chromatography using hexane–ethyl acetate as eluent.

Biological Methods. *Cell Lines and Virus Strain.* Human A549 (ATCC CCL-185), HEK-293 (ATCC CRL-1573), and HFF (ATCC SCRC-1041) cell lines were from the American Type Culture Collection (ATCC, Manassas, VA). The 293 β 5 stable cell line overexpressing the human β 5 integrin subunit was kindly provided by Dr. Glen Nemerow.⁶¹ The cell lines were propagated in Dulbecco's modified Eagle medium (DMEM, Life Technologies/Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA), 10 mM HEPES, 4 mM L-glutamine, 100

units/mL penicillin, 100 μ g/mL streptomycin, and 0.1 mM nonessential amino acids (complete DMEM).

Wild-type HAdV-5 and HCMV (AD169) were obtained from the ATCC. The HAdV-5-GFP used in this study is a replication-defective virus in the noncomplementing A549 cell line containing a CMV promoter-driven enhanced green fluorescent protein (eGFP) reporter gene cassette in place of the E1/E3 regions.⁶² HAdV was propagated in 293 β 5 cells and isolated from the cellular lysate by cesium chloride density centrifugation. Virus concentration, in mg/mL, was calculated with the Bio-Rad Protein Assay (Bio-Rad Laboratories) and converted to virus particles/mL (vp/mL) using 4 × 10¹² vp/ mg.

HAdV Plaque Assay. Derivatives were tested in a doseresponse assay using an MOI of 0.06 vp/cell and drug concentrations ranging from 10 to 0.3125 μ M in a plaque assay. Briefly, $293\beta5$ cells were seeded in 6-well plates at a density of 4×10^5 cells per well in duplicates for each condition. At 80-90% confluency, they were infected with HAdV5-GFP and rocked for 2 h at 37 °C. After this incubation, wells were washed once with PBS. Then, cells were carefully overlaid with 4 mL/well of equal parts of 1.6% (water/vol) bacteriological agar (EULABOR S.L.) and 2× EMEM (Minimum Essential Medium Eagle, BioWhittaker) supplemented with $2 \times \text{ penicillin/streptomycin}$, $2 \times \text{ L-gluta-}$ mine, and 10% FBS. The mixture also contained the drugs in concentrations ranging from 10 to 0.3125 μ M. After incubation for 7 days at 37 °C, virus plaques were scanned with a Typhoon 9410 imager (GE Healthcare Life Sciences) and quantified with ImageJ.⁶

Cytotoxicity Assay. The cytotoxicity of the cidofovir derivatives was measured using the AlamarBlue (Invitrogen) according to the manufacturer's instructions. A549 cells at a density of 5×10^3 cells per well in 96-well plates were seeded. Decreasing concentrations of each compound (200, 150, 100, 80, 60, 40, 30, 20, 10, 5, 2.5, and 0 μ M) were diluted in 100 μ L of DMEM, and cells were then incubated at 37 °C for the following 48 h. After the incubation, the AlamarBlue reagent was added to the cells (1/10th Alamar Blue reagent in culture medium) for an extra 4 h. The CC₅₀ value was obtained using the statistical package GraphPad Prism. This assay was performed in duplicate. The selectivity index (SI) was evaluated as the ratio of CC₅₀ to IC₅₀, where the IC₅₀ is defined as the concentration of compound that inhibits HAdV infection by 50%.

HAdV DNA and mRNA E1A Quantification by Real-Time *PCR*. For DNA quantification, A549 cells $(1.5 \times 10^5 \text{ cells/well})$ in a 24-well plate) were incubated for 24 h in 500 μ L of complete DMEM, and they were infected with wild-type HAdV5 (100 vp/cell) when more than 90% of confluency was observed. Infected cells were incubated for 24 h at 37 °C in 500 μ L of complete DMEM containing a 50 μ M concentration of either compounds 13 or 14 and 15 and 37 μ M for compounds 27 and 32, respectively, or the same volume of DMSO (positive control). All samples were done in duplicate. After 24 h of incubation at 37 °C and 5% CO2, DNA was purified from the cell lysate with the E.Z.N.A. Tissue DNA Kit (Omega Biotek, Norcross, GA) following the manufacturer's instructions. TaqMan primers and probes for a common region of the HAdV5 were designed with the GenScript Real-Time PCR (TaqMan) Primer Design software (GenScript). Oligonucleotide sequences were as follows: AQ1: 5'-GCC ACG GTG GGG TTT CTA AAC TT-3'; AQ2: 5'-GCC CCA

GTG GTC TTA CAT GCA CAT-3'; Probe: 6-FAM-5'-TGC ACC AGA CCC GGG CTC AGG TAC TCC GA-3'-TAMRA'. Real-time PCR mixtures consisted of 9.5 μ L of the purified DNA, AQ1 and AQ2 at a concentration of 200 nM each, and probe at a concentration of 50 nM in a total volume of 12.5 μ L mixed with 12.5 μ L of FastGene 2× PROBE Universal (NIPPON Genetics). The PCR cycling protocol was 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s.

For the evaluation of RNA expression, the same conditions of infection applied for the DNA quantification were used. Six hours after infection, RNA was purified with the RNeasy Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. Quantification of RNA copy numbers was performed using primers and conditions previously reported for E1A.⁶⁴

The internal control used was the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Oligonucleotide sequences for GAPDH and the conditions were those previously reported.⁶⁴

For quantification, gene fragments from hexon and GAPDH were cloned into the pGEM-T Easy vector (Promega), and known concentrations of template were used to generate a standard curve in parallel for each experiment. All assays were performed in a thermal cycler LightCycler 96 System (Roche).

Virus Yield Reduction. A burst assay was used to assay the effect of selected compounds on virus production. A549 cells $(1.5 \times 10^5 \text{ cells/well} \text{ in a 24-well plate})$ were infected with wild-type HAdV5 (100 vp/cell) when more than 90% of confluency was observed. Infected cells were incubated for 48 h at 37 °C in 500 μ L of complete DMEM at a 50 μ M concentration of either compounds 13 or 14 and 15 and 37 μ M for compounds 27 and 32, respectively, or the same volume of DMSO (positive control). After 48 h, cells were harvested and subjected to three rounds of freezing/thawing. Serial dilutions of clarified lysates were titrated on A549 cells (3 × 10⁴ cells/well), and TCID50 values were calculated using an end-point dilution method.⁶⁵

Nuclear-Associated HAdV Genomes. The nuclear delivery of the HAdV genomes was assessed with real-time PCR following nuclear isolation from infected cells. A549 cells (1 \times 10⁶ cells/well) in 6-well plates were infected with HAdV5 wildtype at an MOI of 2000 vp/cell in the presence of compounds at a 50 μ M concentration of either compounds 13 or 14 and 15 and 37 μ M for compounds 27 and 32, respectively, or the same volume of DMSO (positive control). Forty-five minutes after the infection, A549 cells were trypsinized and collected and then washed twice with PBS. Then, cytoplasmic and nuclear fractions were separated using a hypotonic buffer solution and NP-40 detergent. The cell pellet was resuspended in 500 μ L of 1× hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂) and incubated for 15 min at 4 °C. Then, 25 μ L of NP-40 was added, and the samples were vortexed. The homogenates were centrifuged for 10 min at 835g at 4 °C. Following the removal of the cytoplasmic fraction (supernatant), HAdV DNA was isolated from the nuclear fraction (pellet) using the E.Z.N.A. Tissue DNA Kit (Omega Biotek, Norcross, GA) following the manufacturer's instructions.

CMV DNA Quantification by Real-Time PCR. To test the anti-HCMV activity of these compounds, HFF cells were seeded in a 24-well plate (1×10^6 cells/plate) and infected with HCMV (MOI of 0.05 vp/cell). Infected cells were

incubated in complete DMEM at a 50 μ M concentration of either compounds 13 or 14 and 15 and 37 μ M for compounds 27 and 32, respectively, or the same volume of DMSO (positive control) in duplicate. Then, cells were incubated for 72 h at 37 °C and 5% CO₂, and HCMV DNA was purified from the cell lysate using the E.Z.N.A. Tissue DNA Kit (Omega Biotek, Norcross, GA) following the manufacturer's instructions. Real-time PCR primers, mixtures, and protocols were the same as previously reported,³⁸ and the assay was performed in a thermal cycler LightCycler 96 System (Roche).

Statistical Analyses. One-way ANOVA tests (Dunnet method) were carried out using the GraphPad Prism 6. We considered statistical significance with a p value under 0.05. This statistical significance was pointed out with asterisks in the graphs, and the numbers of them indicate the level of significance (* $p \le 0.05$, ** $p \le 0.01$, and *** $p \le 0.001$).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00515.

Full description of the chemical characterization of compounds 9-50; copies of NMR spectra of compounds 9-50, Figures S1-S39 (PDF)

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The authors declare no competing financial interest.

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ABBREVIATIONS

HAdVs, human adenoviruses; dsDNA, double-stranded DNA; HSCT, hematopoietic stem cell transplantation; CDV, cidofovir; BCV, brincidofovir; SAR, structure–activity relationship; HIV, human immunodeficiency virus; HCV, Hepatitis C virus; DMAP, 4-(dimethylamino)-pyridine; EDCI, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide; CC_{50} , 50% cytotoxic concentration; IC_{50} , half maximal inhibitory concentration; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; pTP, precursor of the terminal protein; DBP, DNA-binding protein; HCMV, human cytomegalovirus; TLC, thin layer chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance spectroscopy; COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; HSQC, heteronuclear single quantum correlation; NOESY, nuclear Overhauser effect spectroscopy; TMS, TMS, tetramethylsilane; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EMEM, minimum essential medium Eagle; TCID50, 50% tissue culture infective dose

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