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Discovery of oxochroman-yl-naphthoquinone hybrids as a new chemotype of selective Enterovirus A71 inhibitors

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Abstract

Enterovirus A71 (EV-A71) is a major causative agent of hand, foot, and mouth disease and is associated with severe neurological complications for which effective antiviral therapies remain unavailable. Inspired by the documented antiviral properties of both naphthoquinone and chromone-based scaffolds, we designed and synthesized a focused library of oxochroman-yl-naphthoquinone hybrids through a tandem Michael addition-decarboxylation strategy. The resulting compounds were evaluated for cytotoxicity and antiviral activity against a panel of RNA viruses, including EV-A71, coxsackievirus B5 (CVB5), respiratory syncytial virus (RSV), and SARS-CoV-2. All derivatives exhibited low cytotoxicity in Vero-76 cells ($CC_{50} \geq 100 \mu M$), while showing a remarkable degree of selectivity toward EV-A71. Structure-activity relationship analysis revealed that antiviral activity was retained across multiple substitution patterns, with several analogues displaying submicromolar potency, indicating that inhibition is a general feature of the oxochroman-yl scaffold rather than a feature of a single compound. Several derivatives displayed submicromolar antiviral activity, with compound **3k** emerging as the most potent

analogue, exhibiting an EC₅₀ value of 0.1 ± 0.01 μM and an estimated selectivity index of approximately 1000. Yield-reduction assays confirmed a dose-dependent suppression of viral production, resulting in a reduction of infectious virus titers of approximately two orders of magnitude. Furthermore, virucidal and time-of-addition experiments demonstrated that the antiviral activity of 3k is not associated with direct virion inactivation but is consistent with interference during the earliest stages of the EV-A71 replication cycle. Collectively, these findings identify oxochroman-yl-naphthoquinone hybrids as a previously unexplored class of selective EV-A71 inhibitors and establish the oxochroman-yl scaffold as a promising platform for the development of new anti-enteroviral agents.

Keywords: Enterovirus A71; Antiviral agents; Oxochroman-yl scaffold; Naphthoquinone hybrids; Structure–activity relationship; Early-stage viral replication.

1 Introduction

Enterovirus A71 (EV-A71), a member of the Picornaviridae family, is one of the principal causative agents of hand, foot, and mouth disease (HFMD), a highly contagious infection that primarily affects infants and young children worldwide.[1] Although HFMD is generally a self-limiting illness, EV-A71 infection can progress to severe neurological complications, including aseptic meningitis, encephalitis, pulmonary edema, and acute flaccid paralysis, resulting in significant morbidity and mortality.[1,2] Recurrent outbreaks across Asia and the continuous emergence of genetically distinct viral variants have further highlighted EV-A71 as an important global public health concern.[3,4] While three inactivated vaccines have been approved for use in China, their geographical availability remains limited and their long-term effectiveness against newly emerging viral strains has yet to be fully established.[2-6] Consequently, there is currently no clinically approved antiviral therapy for EV-A71 infection, making the discovery of effective antiviral agents an important unmet medical need. Considerable efforts have therefore focused on identifying small molecules capable of disrupting different stages of the EV-A71 replication cycle. Both viral and host-directed strategies have been explored, leading to inhibitors targeting the capsid protein VP1, the viral protease 3C_{pro}, the RNA-dependent RNA polymerase 3D_{pol}, and several host signaling pathways involved in viral replication.[7-17] Although many of these compounds exhibit promising in vitro activity, only a limited number have advanced beyond the preclinical stage, underscoring the need for structurally distinct antiviral chemotypes capable of

expanding the chemical space currently available for EV-A71 drug discovery.[3,16,17] Natural products and their synthetic derivatives continue to represent one of the richest sources of biologically active small molecules and have inspired numerous antiviral drug discovery programs (Figure 1). Among these, naphthoquinone derivatives have attracted considerable attention owing to their broad spectrum of biological activities, including antibacterial, anticancer, antiparasitic, and antiviral effects.[18] In recent years, members of this family have demonstrated inhibitory activity against several RNA viruses, including dengue virus,[19] SARS-CoV-2,[20–22] Ebola virus,[23] and feline calicivirus,[24] highlighting the versatility of the quinone scaffold as a privileged pharmacophore for antiviral development. Likewise, flavonoids and chromone-derived natural products, including chrysin, luteolin, fisetin, rutin, and quercetin, have been reported to suppress EV-A71 infection through different mechanisms, further identifying oxygen-containing heteroaromatic scaffolds as valuable starting points for anti-enteroviral drug discovery.[25–29]

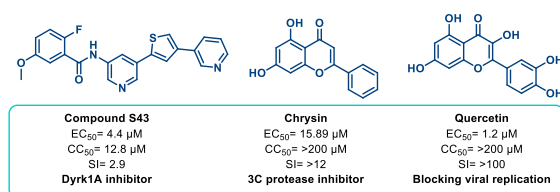


Figure 1.

Despite these advances, the antiviral potential of molecular architectures integrating these complementary structural motifs has remained largely unexplored. Inspired by these observations, we hypothesized that merging the structural features associated with the antiviral activity of naphthoquinones and chromone-derived compounds within a rigid oxochromanyl framework could generate a previously unreported class of anti-EV-A71 agents (Figure 2). Besides combining two privileged bioactive motifs, the resulting molecular architecture offers a conformationally defined scaffold that can be readily diversified through aromatic substitution, thereby enabling systematic structure–activity relationship (SAR) investigations and the identification of molecular features governing antiviral activity

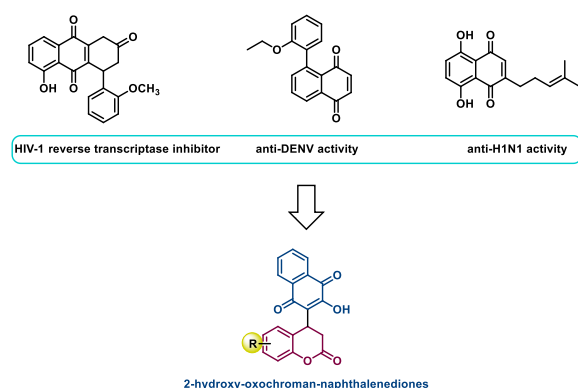


Figure 2.

To test this hypothesis, we synthesized a focused library of oxochroman-*naphthoquinone* hybrids using a tandem Michael addition-decarboxylation strategy recently developed in our laboratories.[30] The resulting compounds were evaluated for cytotoxicity and antiviral activity against a representative panel of RNA viruses comprising EV-A71, coxsackievirus B5 (CVB5), respiratory syncytial virus (RSV), and SARS-CoV-2. Rather than identifying a single active molecule, our study demonstrates that potent anti-EV-A71 activity is conserved across multiple structurally distinct analogues, establishing the oxochroman-*naphthoquinone* scaffold as a genuine antiviral chemotype. Structure-activity relationship analysis identified compound 3k as the lead derivative, displaying nanomolar antiviral potency together with excellent selectivity, while preliminary mechanistic investigations indicate that this new class of compounds interferes with the earliest stages of the EV-A71 replication cycle. Collectively, these findings introduce oxochroman-*naphthoquinone* hybrids as a new platform for anti-enteroviral drug discovery and provide a foundation for the future development of mechanistically distinct antiviral agents.

Results

A focused library of sixteen oxochroman-*naphthoquinone* hybrids (**3a-p**) was synthesized using the tandem Michael addition-decarboxylation strategy recently developed[30] in our laboratories (Figure 3). The synthetic protocol enabled the efficient preparation of analogues bearing diverse substitution patterns on the oxochroman-*naphthoquinone* framework, including halogen, nitro, hydroxy, alkoxy, propargyloxy, benzyloxy, and annulated aromatic functionalities. The target compounds were isolated in moderate to good yields (45–85%), affording a structurally diverse collection that provided a robust platform for systematic structure-activity relationship (SAR) investigations aimed at evaluating the influence of electronic, steric, and topological modifications on antiviral activity.

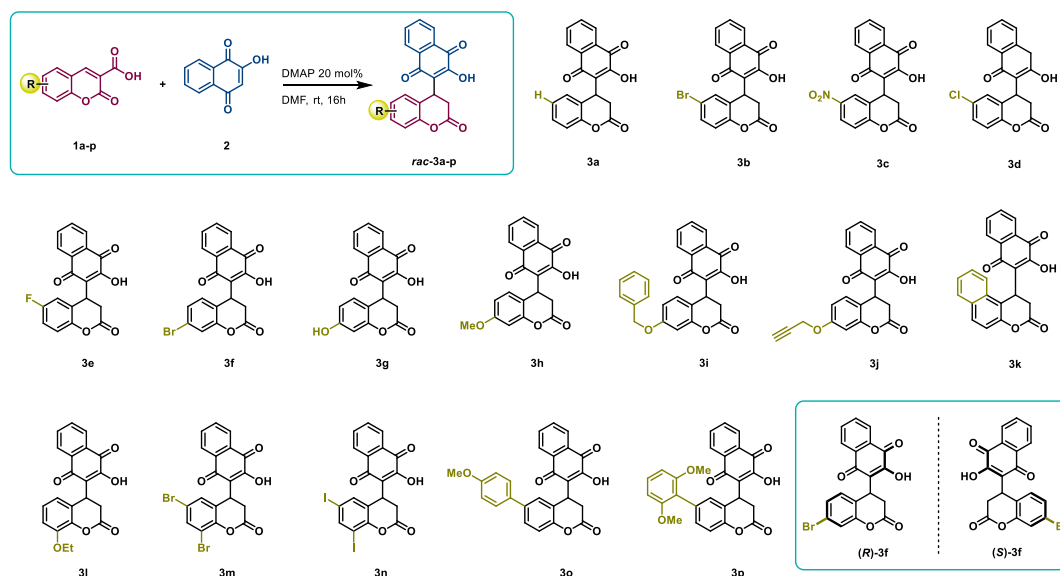


Figure 3.

The antiviral activity of the oxochromanyl-naphthoquinone hybrids was first evaluated against a representative panel of RNA viruses comprising EV-A71, CVB5, RSV, and SARS-CoV-2 (Table 1). The biological evaluation revealed a highly selective antiviral profile, as none of the synthesized compounds exhibited significant activity against SARS-CoV-2 or RSV, while only compounds **3d** and **3k** displayed modest activity against CVB5. In contrast, several derivatives selectively inhibited EV-A71 replication with submicromolar potency. Importantly, all compounds exhibited low cytotoxicity toward Vero-76 cells, with CC_{50} values of ≥ 100 μ M, indicating that the observed antiviral activity was not associated with nonspecific cellular toxicity. This favorable combination of potency and low cytotoxicity resulted in high estimated selectivity indices (*SI*) for the most active analogues, with compounds **3d**, **3i**, and **3l** displaying *SI* values >300 and compound **3k** reaching an estimated *SI* of approximately 1000. Collectively, these findings identify the oxochromanyl scaffold as a selective anti-EV-A71 chemotype rather than a broadly cytotoxic quinone-containing framework.

Notably, the unsubstituted derivative **3a** already exhibited potent activity against EV-A71 ($EC_{50} = 0.8 \pm 0.05$ μ M), indicating that the parent oxochromanyl-naphthoquinone scaffold is intrinsically active and therefore represents a suitable starting point for further structural optimization. To investigate the influence of aromatic substitution on antiviral activity, a series of derivatives bearing different substituents on the oxochromanyl ring was subsequently examined. Within the 6-substituted series, pronounced differences in activity were observed. Introduction of a bromine atom at the 6-position (**3b**) completely abolished antiviral activity, whereas replacement with

chlorine (**3d**) or fluorine (**3e**) restored potent inhibition, affording EC₅₀ values of 0.3 ± 0.02 μM and 0.4 ± 0.2 μM, respectively.

Table 1

Similarly, the nitro-substituted analogue **3c** retained potent antiviral activity (EC₅₀ = 1.0 ± 0.2 μM). Collectively, these observations suggest that antiviral potency cannot be rationalized solely on the basis of the electronic nature of the substituents, but rather arises from a more complex interplay of steric and electronic effects.

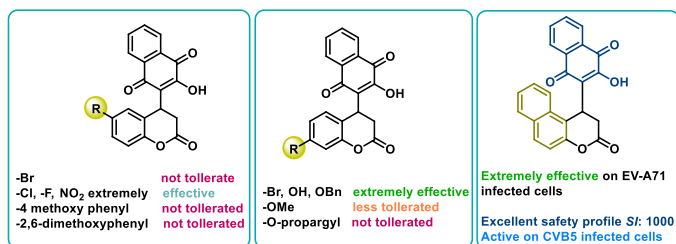


Figure 4.

The importance of substitution topology became even more apparent within the brominated series. While compound **3b** was inactive, relocating the bromine atom from the 6-position to the 7-position (**3f**) restored antiviral activity, yielding an EC₅₀ value of 0.5 ± 0.03 μM. Likewise, the dibrominated analogue **3m** recovered only a fraction of the lost activity, whereas replacement of bromine with iodine afforded compound **3n**, which exhibited improved potency (EC₅₀ = 0.5 ± 0.1 μM). Collectively, these observations indicate that both the identity and the position of the halogen substituent play a critical role in modulating antiviral activity, supporting the existence of a well-defined structure-activity relationship.

Substituents containing oxygen functionalities also had a marked influence on biological activity. The 7-hydroxy analogue **3g** retained submicromolar potency (EC₅₀ = 0.6 ± 0.16 μM), whereas methylation of the hydroxyl group to afford **3h** resulted in a pronounced reduction in activity. Interestingly, replacement with a propargyloxy substituent (**3j**) completely abolished antiviral activity, while introduction of a benzyloxy group restored potency in compound **3i** (EC₅₀ = 0.3 ± 0.07 μM). Similarly, incorporation of an ethoxy substituent at the 8-position was well tolerated, as demonstrated by the high activity of compound **3l** (EC₅₀ = 0.3 ± 0.05 μM). These findings further highlight the sensitivity of antiviral activity to relatively subtle structural modifications within the oxochroman framework.

Finally, to assess whether the stereochemistry associated with the stereogenic axis influenced antiviral activity, the atropisomers (*R*)-**3f** (ee = 91%) and (*S*)-**3f** (ee = 92%) were evaluated against EV-A71-infected cells and compared with the corresponding racemate (*rac*-**3f**). No significant differences in antiviral activity were observed, indicating that the absolute configuration of the stereogenic axis does not play a major

role in determining the biological activity of this scaffold. Among all derivatives examined, compound **3k** emerged as the most potent analogue of the series (Figures 3 and 4). The presence of an additional fused aromatic ring resulted in a marked enhancement of antiviral activity, affording an EC₅₀ value of 0.1 ± 0.01 μ M together with an estimated selectivity index of approximately 1000. These findings identify annulated analogues as particularly promising templates for future lead optimization and suggest that expansion of the aromatic framework can be exploited to further improve antiviral potency. An important outcome of the present SAR study is that potent antiviral activity was maintained across multiple structural classes. Indeed, compounds **3a**, **3c-g**, **3i**, **3k**, **3l**, and **3n** all exhibited submicromolar activity against EV-A71 despite bearing substantially different substitution patterns. Rather than identifying a single active molecule, these results demonstrate that antiviral activity is a general feature of the oxochroman-yl-naphthoquinone scaffold, establishing this molecular architecture as a new chemotype for selective EV-A71 inhibition and a promising platform for the future development of anti-enteroviral agents.

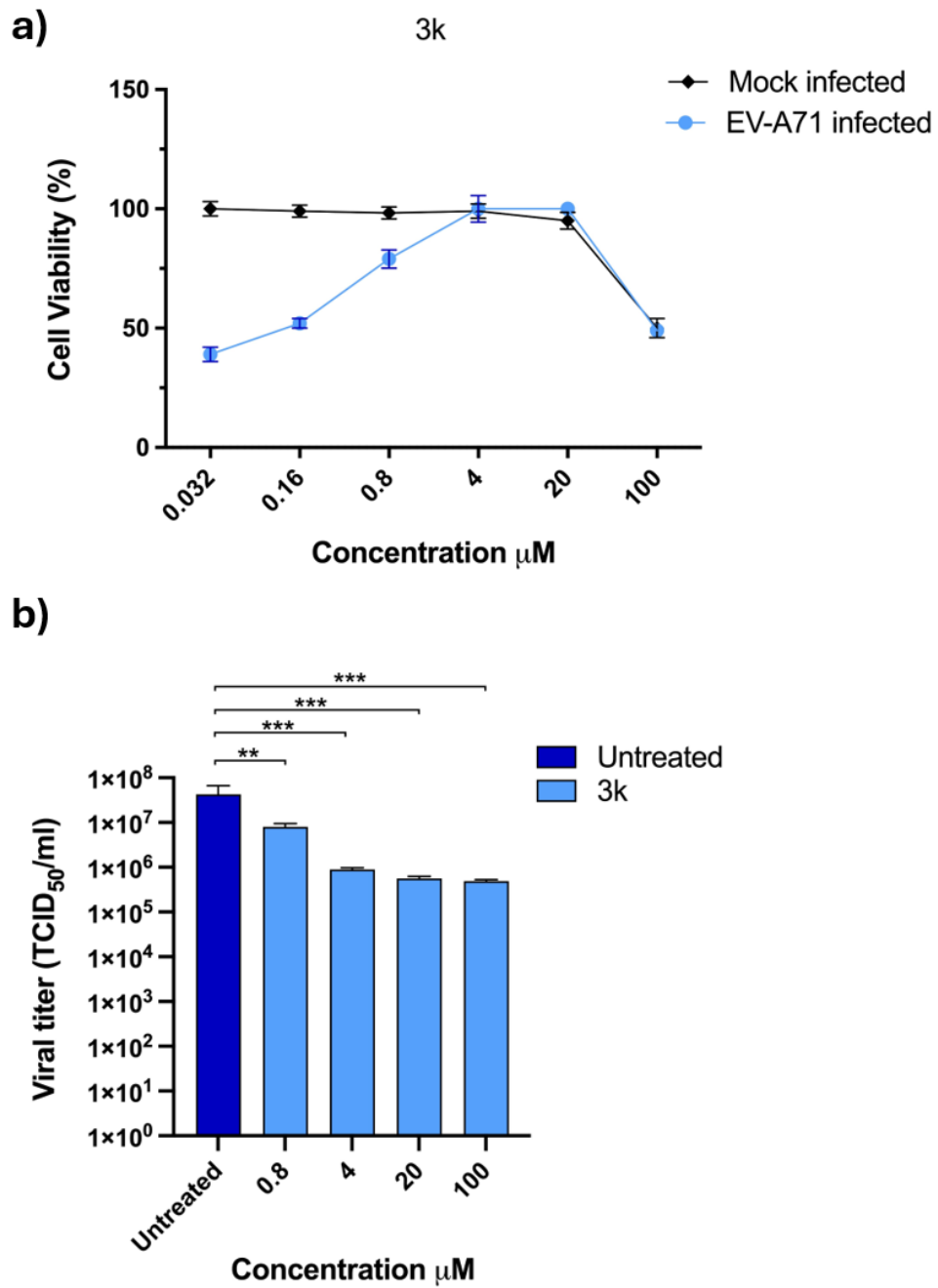


Figure 5.

Based on its superior antiviral potency and selectivity, compound **3k** was selected for further biological characterization. The antiviral activity initially observed in the cytoprotection assay (Figure 5a) was confirmed by a virus yield reduction assay (Figure 5b), which demonstrated a clear dose-dependent suppression of viral production. Treatment with **3k** resulted in an approximately two-log reduction in infectious viral titer, confirming that the compound effectively inhibits productive EV-A71 replication rather than merely preventing virus-induced cytopathic effects.

To obtain preliminary mechanistic insights into the antiviral activity of this new chemotype, virucidal and time-of-addition assays were subsequently performed using compound **3k** (Figure 6). The virucidal assay (Figure 6a) showed that direct exposure of EV-A71 particles to the compound did not significantly affect viral infectivity, indicating that the antiviral effect is not mediated by direct virion inactivation. In contrast, the time-of-addition experiment (Figure 6b) revealed that maximal antiviral activity was achieved when **3k** was present during the earliest stages of infection, whereas progressively delayed administration resulted in a marked loss of efficacy. Collectively, these findings indicate that compound **3k** interferes with an early stage of the EV-A71 replication cycle, although the precise molecular target and mechanism of action remain to be established.

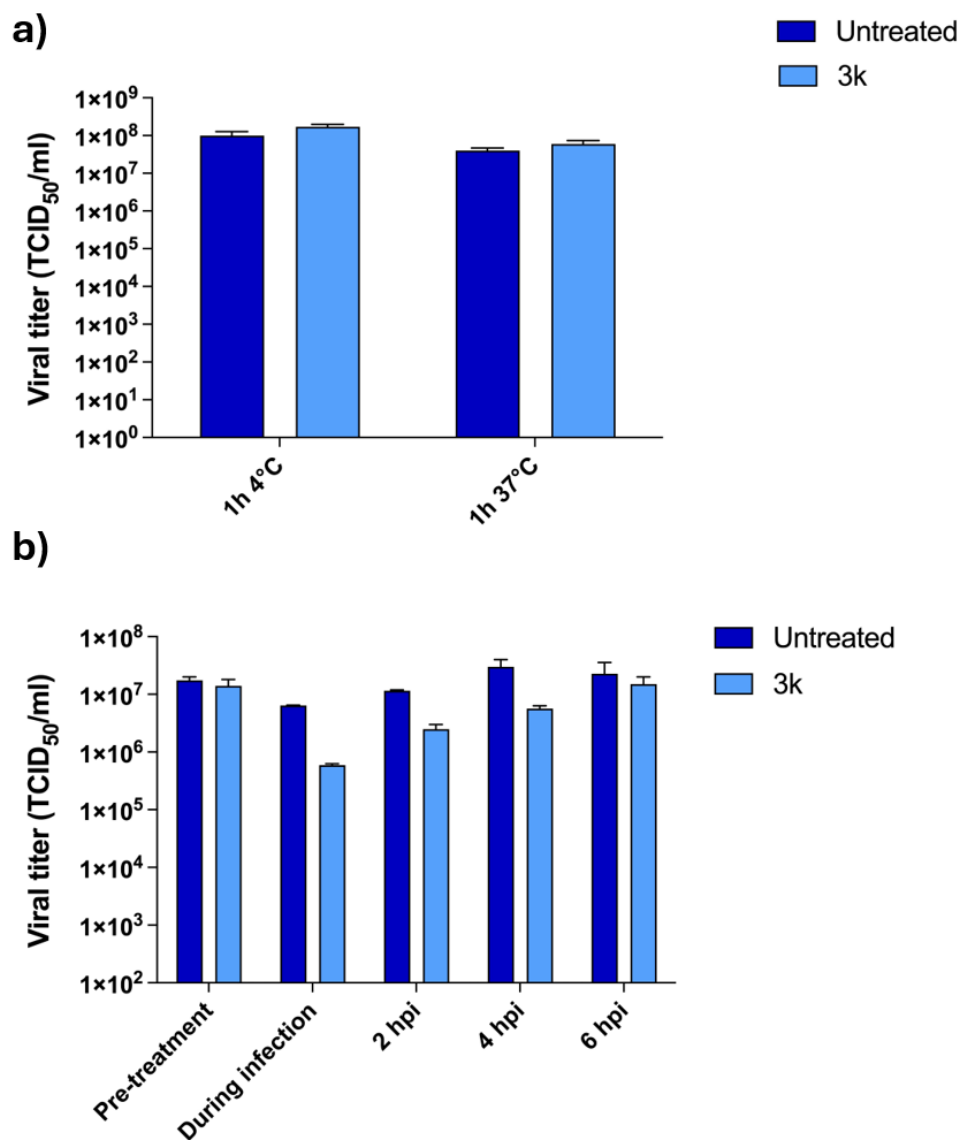


Figure 6.

Conclusions

In this study, we identified oxochroman-yl-naphthoquinone hybrids as a previously unexplored class of selective inhibitors of Enterovirus A71. By integrating two privileged bioactive motifs within a rigid oxochroman-yl framework, we generated a focused library of compounds that consistently displayed potent antiviral activity while maintaining negligible cytotoxicity. The broad distribution of submicromolar activity across

structurally diverse analogues demonstrates that antiviral efficacy is an intrinsic feature of this scaffold rather than an isolated property of a single derivative, establishing the oxochroman-4-yl framework as a promising chemotype for anti-enteroviral drug discovery. Systematic structure-activity relationship studies revealed that subtle modifications to the substitution pattern strongly influence antiviral potency, providing valuable guidelines for future molecular optimization. Among the synthesized compounds, derivative **3k** emerged as the lead candidate, exhibiting nanomolar activity against EV-A71 ($EC_{50} = 0.1 \pm 0.01 \mu M$) together with an excellent selectivity index, highlighting the therapeutic potential of this new chemical class. Mechanistic investigations further indicate that antiviral activity is associated with interference during the early stages of the EV-A71 replication cycle rather than direct virion inactivation. Although the precise molecular target remains to be identified, these findings support the existence of a specific antiviral mode of action. Taken together, our results expand the chemical space available for EV-A71 antiviral discovery by introducing a novel and synthetically accessible scaffold with excellent potency, selectivity, and broad opportunities for structural diversification. Overall, this work establishes oxochroman-4-yl-naphthoquinone hybrids as a new platform for anti-enteroviral drug discovery, laying the foundation for future lead optimization and target-oriented mechanistic studies.

Methods

Chemistry

General information. All reagents and solvents were purchased from commercial suppliers and used without further purification. Coumarin-3-carboxylic acid derivatives **1a-p** were prepared according to previously reported procedures. [1-5] NMR spectra were recorded on Bruker Ascend-600 spectrometers operating at 600 MHz for ^1H , 151 MHz for ^{13}C , and 564 MHz for ^{19}F nuclei. Chemical shifts (δ) are reported in parts per million (ppm) relative to residual solvent signals. High-resolution mass spectra (HRMS) were acquired on a Bruker Daltonics MicroTOF-Q spectrometer equipped with an electrospray ionization source. Infrared spectra were recorded using a Nicolet iS10 spectrometer. Melting points were determined using a Stuart SMP1 apparatus and are uncorrected. All synthesized compounds were characterized by NMR and HRMS analysis and displayed a purity greater than 95% as determined by HPLC. Detailed synthetic procedures and characterization data are provided in the Supporting Information.

General procedure for the synthesis of oxochroman-4-yl derivatives 3a-p. A mixture of coumarin-3-carboxylic acid derivative **1a-p** (1.0 equiv.), 2-hydroxynaphthalene-1,4-dione (2, 1.0 equiv.) and 4-dimethylaminopyridine (DMAP, 20 mol%) was stirred in dry DMF (0.1 M) at room temperature for 4 h. The reaction mixture was diluted with ethyl acetate, washed twice with saturated aqueous ammonium chloride solution, and extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. Purification by flash

chromatography afforded the desired oxochromanyl derivatives **3a–p** in moderate to good yields. Structural characterization data for all compounds are reported in the Supporting Information.

Biology

Cell lines and viruses. Vero-76 cells (ATCC CRL-1587) were obtained from the American Type Culture Collection (ATCC) and routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and kanamycin (25 mg L⁻¹). Cells were periodically tested for mycoplasma contamination using Hoechst staining.

The antiviral activity of the compounds was evaluated against a panel of RNA viruses comprising Enterovirus A71 (EV-A71, strain BrCr, ATCC VR-1775), Coxsackievirus B5 (CVB5, strain Faulkner, ATCC VR-185), respiratory syncytial virus (RSV A2, ATCC VR-1540), and SARS-CoV-2 (Italian strain PV10734, lineage B.1.1). Virus stocks were propagated in appropriate host cells and stored at -80 °C until use.

Cytotoxicity assay. The cytotoxicity of compounds **3a–p** was determined in Vero-76 cells using the MTT colorimetric assay.[31–32] Cells were seeded in 96-well plates at a density of 5×10^5 cells mL⁻¹ and incubated with serial dilutions of test compounds for 72–96 h at 37 °C in a humidified atmosphere containing 5% CO₂. Cell viability was quantified by MTT reduction and expressed relative to untreated controls. The concentration required to reduce cell viability by 50% (CC₅₀) was calculated from dose-response curves.

Antiviral assays. All experiments involving infectious viruses were conducted in BSL-2 or BSL-3 facilities at the Microbiology and Virology Unit, University of Cagliari. Antiviral activity against EV-A71 and RSV was assessed by inhibition of virus-induced cytopathic effect in Vero-76 cells infected at a multiplicity of infection (MOI) of 0.01. Antiviral activity against SARS-CoV-2 and CVB5 was evaluated using plaque reduction assays as previously described.[33] The effective concentration required to inhibit viral replication by 50% (EC₅₀) was determined by nonlinear regression analysis of dose-response curves.

Virus yield reduction assay. To confirm antiviral activity, Vero-76 cells were infected with EV-A71 at an MOI of 0.1 and treated with non-cytotoxic concentrations of compound **3k**. Following a 2 h adsorption period at 37 °C, the inoculum was removed and replaced with fresh medium containing the corresponding concentration of compound. After 96 h, viral yields in culture supernatants were quantified by endpoint titration and expressed as TCID₅₀ mL⁻¹.

Virucidal assay. To investigate whether compound **3k** directly inactivates viral particles, EV-A71 suspensions (1×10^5 PFU mL⁻¹) were incubated with **3k** (20 µM) for 1 h at either 4 °C or 37 °C. Untreated virus suspensions served as controls. Residual infectivity was subsequently measured by endpoint titration and expressed as TCID₅₀ mL⁻¹.

Time-of-addition assay. The effect of compound **3k** on different stages of EV-A71 infection was evaluated using a time-of-addition assay. Confluent Vero-76 monolayers were infected with EV-A71 at an MOI of 1 and treated with **3k** (20 μ M) either during virus adsorption or at selected time points after infection. Following treatment, cells were washed and incubated in fresh medium until 12 h post-infection. Viral production was determined by endpoint titration of cell lysates and culture supernatants, and results were expressed as TCID₅₀ mL⁻¹.

Statistical analysis. All experiments were independently performed at least three times. Results are reported as mean $\pm$ standard deviation (SD). Statistical analyses were carried out using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences between experimental groups were evaluated by one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

Supplementary information.

Supplementary Information accompanies this paper and includes synthetic procedures, compound characterization data, NMR spectra, and additional biological data.

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Conflict of interest

The authors declare no conflicts of interest.

Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files. The Supplementary Information contains detailed experimental procedures, full characterization data for all synthesized

compounds, including ^1H and ^{13}C NMR spectra, and additional biological data supporting the findings of this study.

Author contribution

Conceptualization: G.S., F.S.; Methodology: L.V., M.C., V.M. D.M.,; Investigation: C.M.C., R.P., D.M. R.L.; Writing – Original Draft: L.V., D.M.; V.O.; Writing – Review and Editing: L.V., D.M.; V.O.; G.S., A.M., F.S.; Supervision: G.S., F.S.; Funding acquisition: G.S., F.S. The manuscript was written through contributions of all authors. All authors have given approval to the final ver-sion of the manuscript.

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Figure legends

Figure 1 Representative antiviral scaffolds reported to inhibit EV-A71 replication that served as inspiration for the design of the oxochromanyl-naphthoquinone hybrids.

Figure 2 Design strategy based on the molecular hybridization of naphthoquinone and chromone-derived scaffolds to generate oxochromanyl-naphthoquinone hybrids as potential EV-A71 inhibitors.

Figure 3 Tandem Michael addition–decarboxylation strategy for the synthesis of oxochromanyl-naphthoquinone hybrids **3a-p** and scope of the resulting compound library. Isolated yields after chromatographic purification are indicated.

Figure 4 Structure-activity relationship (SAR) overview highlighting the influence of aromatic substitution on the antiviral activity of the oxochromanyl-naphthoquinone scaffold.

Figure 5 Biological characterization of compound **3k**. (A) Cytoprotection assay showing the concentration-dependent antiviral activity of **3k** against EV-A71 in Vero-76 cells. (B) Virus yield reduction assay confirming dose-dependent inhibition of EV-A71 replication by compound **3k**.

Figure 6 Preliminary mechanistic characterization of compound **3k** against EV-A71. (A) Virucidal assay. EV-A71 particles were incubated with **3k** (20 μ M) for 1 h at either 4 °C or 37 °C before measurement of residual viral infectivity. Dark blue bars represent untreated virus, whereas light blue bars correspond to virus incubated with compound **3k**. (B) Time-of-

addition assay. Vero-76 cells were infected with EV-A71 (MOI = 1), and compound **3k** (20 μ M) was added at the indicated time points after infection. Viral yields were quantified by endpoint titration and expressed as TCID₅₀/mL.

Tables

Table 1

Cytotoxicity and antiviral activity of compounds 3a-p against CVB5, EV-A71, SARS-CoV-2, and RSV viruses in normal monkey kidney (Vero-76) cells.

Compound	Vero-76 ¹	CVB5 ²	EV-A71 ³	SARS-CoV-2 ²	RSV ³
	CC ₅₀ [μ M]	EC ₅₀ [μ M]			
3a	>100	>100	0.8±0.05	>100	>100
3b	>100	>100	>100	>100	>100
3c	>100	>100	1±0.2	>100	>100
3d	>100	75.0	0.3±0.02	>100	>100
3e	>100	>100	0.4±0.2	>100	/
3f	>100	>100	0.5±0.03	>100	>100
3g	>100	>100	0.6±0.16	>100	>100
3h	>100	>100	5.8±1	>100	>100
3i	>100	>100	0.3±0.07	>100	/
3j	>100	>100	>100	>100	/
3k	100	67.5	0.1±0.01	>100	>100
3l	>100	>100	0.3±0.05	>100	>100
3m	>100	>100	6±1	>100	>100
3n	>100	75.0	0.5±0.1	>100	>100
3o	>100	>100	>100	>100	/
3p	>100	>100	>100	>100	/
References					
Pleconaril	>100	0.005±0.001	/	/	/
NM107	>100	/	2±0.5	/	/
Remdesivir	>100	/	/	1.0±0.3	/
6-Azauridine	14	/	/	/	1.2±0.05

¹Compound concentration (μ M) required to reduce by 50% the viability of mock-infected Vero-76 cells (72 h post-treatment), as determined by the MTT method.

²Compound concentration (μ M) required to reduce by 50% the plaque number of SARS-CoV-2 and CVB5

³Compound concentration (μ M) required to achieve 50% protection of Vero-76 cells from EV-A71 and RSV

Selectivity index (SI). / = not tested

Appendix A Supporting Information

Supplementary Files

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- [AppendixASupportingInformation.pdf](#)