

Synthesis of three triterpene series and their activity against respiratory syncytial virus

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Abstract

The human respiratory syncytial virus (hRSV) is a leading cause of hospitalization due to acute lower respiratory infection especially in infants and young children, sometimes causing fatal cases. The monoclonal antibody palivizumab is one of the available options for preventing this virus, and at the moment there are several hRSV vaccine trials underway. Unfortunately, the only drug option to treat hRSV infection is ribavirin, which can be used in severe high-risk cases. For this reason, new medicines are needed and, in this context, the triterpenes and their derivatives are promising alternatives, since many of them have shown important antiviral activity, such as bevirimat. Therefore, we report three series of triterpene (betulin (BE), betulinic acid (BA), and ursolic acid (UA)) derivatives tested against hRSV. The derivatives were synthesized by using commercial anhydrides in an easy and inexpensive step reaction. For the antiviral assay, A549 cells were infected by hRSV and after 96 h of compound or ribavirin (positive control) treatment, the cell viability was tested by MTT assay. DMSO, non-infected cells and infected cells without treatment were used as negative control. The triterpene esterification at the hydroxyl group resulted in 17 derivatives. The 3,28-di-O-acetylbetulin derivative (**1a**) showed the best results for cell viability, and real-time PCR amplification was performed for **1a** treatment. Remarkably, one new anti-hRSV prototype was obtained through an easy synthesis of BE, which shall represent an alternative for a new lead compound for anti-hRSV therapy.

KEYWORDS

A549 cells, betulin, betulinic acid, respiratory syncytial virus, ursolic acid

1 | INTRODUCTION

Several viral diseases are a global public health burden, such as dengue,^[1] Zika,^[2] Ebola,^[3] viral hepatitis, and acquired immunodeficiency syndrome (AIDS).^[4] Another virus causing a global impact on human health is the pathogen named human respiratory syncytial virus

(hRSV), which is a member of the *Paramyxoviridae* family and subfamily *Pneumovirinae*.^[5] Despite considerable efforts toward the development of effective treatments for hRSV disease, this seasonal infection is still a major cause of pneumonia and bronchiolitis in infants, in young children, and even in adults.^[6–8] A United States study estimated that hRSV is the most common cause of viral mortality in children aged

under 5 years.^[9] hRSV is highly contagious and is transmitted by direct contact with nasal secretions or by fomite spread on hands, cots, and fluffy toys. Preventing the spread of the virus is possible by promoting cough etiquette, hand washing, and cohorting hospitalized hRSV patients.^[10] The incubation period is typically 2–8 days, with virus replication spreading from the upper respiratory tract epithelial cells to the lower respiratory tract.^[11]

The available options for preventing and treating hRSV are limited to select populations in high-resource settings.^[12] One of this is the monoclonal antibody palivizumab for prophylaxis.^[13] The aerosolized antiviral agent ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is the approved drug for the treatment of hRSV infections. Ribavirin is a synthetic guanosine nucleoside analogue with *in vitro* antiviral activity against a range of RNA and DNA viruses.^[14] There is a concern regarding the efficacy of ribavirin and based on the available data it is not recommended in the current guidelines for management of bronchiolitis.^[15]

An example of therapeutic strategies that have been developed for the treatment of hRSV infection is GS-5806, which is an hRSV fusion and entry inhibitor that successfully completed a phase I safety trial and a phase II hRSV challenge trial in adults.^[16] Other studies have been developing but the majority of the compounds are still in the preliminary stages of research, such as emodin.^[17]

Although some agents have been reported to have anti-hRSV activity,^[16–19] new strategies for therapeutic intervention during moderate to severe hRSV infection are evidently needed. It is clearly seen that the progress made in hRSV vaccines and therapeutics since the discovery of the virus in 1950 has been poor, particularly compared to other viruses like influenza virus, hepatitis C virus, and HIV.^[12] In this context, natural compounds, as triterpenes, might be an alternative. The triterpenes, betulin (BE), betulinic acid (BA), and ursolic acid (UA) (Figure 1), have shown a variety of biological activity.

BE is a pentacyclic triterpene alcohol with a lupane skeleton. BE can be used as such, or after chemical modification as a starting compound for other useful materials and compounds, such as BA which possess various interesting pharmacological properties.^[20] For instance, BA has described its anti-HIV, antibacterial, antimalarial, anti-inflammatory, and anthelmintic activities.^[21] Furthermore, a promising BA derivative called bevirimat, the first-in-class HIV-1 maturation inhibitor, has been modified at C-28 position and resulted in even more

active compound.^[22] Consequently, BE and BA are of interest for medicine as the basis for the development of new antiviral agents.

The therapeutic potential of UA, a pentacyclic triterpene found in several traditional plants, has also attracted attention. The functional properties have been described for UA, such as antibacterial, antiprotozoal, anti-inflammatory, anti-HIV, and antitumor.^[23,24]

However, the ability of these triterpenes for the inhibition of hRSV was not evaluated. Since there is not a variety of medicine for hRSV treatment, this current study shows efforts to find a new and effective compound as an alternative. Thus, we assayed 20 compounds divided in the three triterpenes series; BE, BA, and UA and their carbon 3 (C-3) and carbon 28 (C-28 for BE) derivatives to verify their anti-hRSV potential.

2 | RESULTS AND DISCUSSION

Through an easy and inexpensive step reaction, it was possible to obtain 17 triterpenes derivatives with satisfactory yield (range of 70–93%). The structures of all derivatives are depicted in Table 1. After the synthesis of these compounds, their structural elucidation could be deduced from ¹H, ¹³C NMR, and HR-ESI-MS data. The data of BA and UA series were compared with those previously described in our prior studies,^[25,26] and 3,28-di-O-succinylbetulin (**1d**) was compared with data published by Tian et al.^[27] In addition, we have described for the first time four new BE derivatives. The BE was modified at C-3 and C-28 to determine if ester substituents influence anti-hRSV activity, whereas the anti-HIV derivative from BA, bevirimat, is the successful result of C-3 modification and in our previous studies against another microorganism, malaria parasite, also displayed improvement of activity when C-3 and C-28 were chemically modified.^[25,26] The ¹H NMR spectra of the BE derivatives have two singlets around δ 4.50 ppm suggesting the presence of an isoprenyl group at position H-29. The loss of *dd* at δ 3.05 ppm suggests that in the presence of H-3 the carbon binding at hydroxyl was substituted for an ester group. Also, after the change at C-3 the presence of $-\text{CHOCO}-$ around δ 2.50 ppm suggested that esterification had occurred. The signals of ester groups were observed in the ¹³C NMR spectrum at the range of δ 157–177 ppm. The isoprenyl was evidenced in signals around δ 109 (C-29) and δ 150 ppm (C-20).

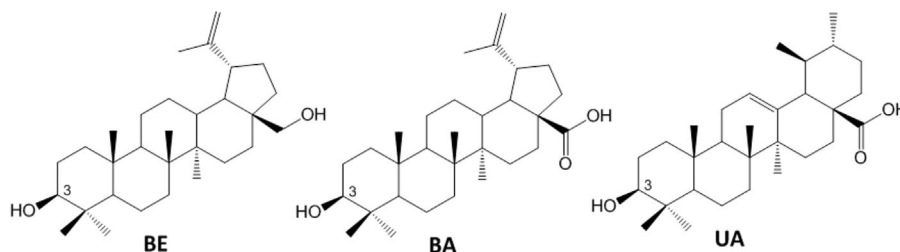


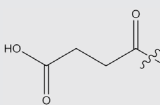
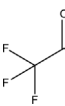
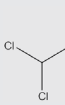


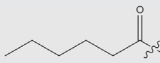
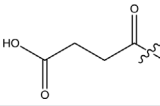
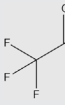
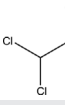




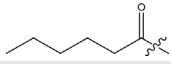
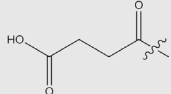


FIGURE 1 Chemical structures of betulin (BE), betulinic acid (BA), and ursolic acid (UA)

TABLE 1 Library of the three series of triterpene derivatives, cytotoxicity and anti-hRSV activity by MTT assay

Code	R	CC ₅₀ (μM)	EC ₅₀ (μM)	TI
BE	H	7.48	69.72	0.11
1a		2245.38	104.66	21.45
1b		268.27	196.81	1.36
1d		11.197	120.15	0.093
1e		624.95	98.87	6.32
1f		244.96	73.05	3.35
BA	H	30.31	***	***
2a		13.74	27.77	0.49
2b		33.14	38.51	0.86
2c		120.48	33.81	3.56
2d		30.79	93.77	0.33
2e		16.02	22.19	0.72
2f		140.08	84.57	1.66
UA	H	23.73	18.67	1.27
3a		26.80	48.75	0.54

(Continues)

TABLE 1 (Continued)

Code	R	CC ₅₀ (μM)	EC ₅₀ (μM)	TI
3b		14.61	71.00	0.20
3c		685.00	86.85	7.89
3d		46.57	***	***
3e		12.77	28.09	0.45
3f		12.22	76.44	0.16
Ribavirin	-	7.02 ^a	1.96 ^a	3.57

CC₅₀, 50% cytotoxic concentration; EC₅₀, 50% inhibitory concentration of the viral effect; TI, therapeutic index defined as CC₅₀ over EC₅₀; ***, beyond the highest tested concentration (100 μM).

^aμg.

Regarding the biological assay, Table 1 presents the antiviral activity expressed as the EC₅₀ for all compounds against hRSV in A549 cells. EC₅₀ is the compound concentration required to reduce virus-induced cytopathogenicity or viral plaque formation by 50%. The 50% cytotoxic concentration (CC₅₀), causing a 50% decrease in cell viability was determined using a colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay system. Ribavirin was the reference compound included. Among the precursor triterpenes tested, UA proved to be more active with EC₅₀ = 18.67 μM, although in the UA series the 3c derivative was more selective with TI = 7.89. Furthermore, the BE derivative (1a) displayed the higher value for TI = 21.45, showing the importance of lupane for the acetyl modification. Based on the result of TI, we selected the BE derivative (1a) to perform an experiment to evaluate antiviral activity associated with virus replication. Real-time PCR amplification was performed to hRSV protein F gene and β-actin from cells infected with hRSV or infected and treated with 1a (12.5 and 50 μM) or ribavirin (0.3 and 1.2 μg) during 96 h. We found that BE derivative (1a) was able to significantly reduce the hRSV RNA gene expression suggesting a reduction in virus replication (Figure 2). Thus, our results show that the derivative more

active is not selective. It is relevant research since few triterpene derivatives have been evaluated against hRSV, such as described by Li et al.^[28] The planning of new derivatives from 1a may be a promising alternative for obtaining a future drug, as alternative therapy against a virus with high morbidity and mortality range.

3 | CONCLUSION

In summary, three series of triterpene derivatives (BE, BA, and UA) were prepared in high yield and in a total of 17 derivatives. All compounds were evaluated against hRSV and BE derivative 1a was the most selective. Then real-time PCR amplification was performed for hRSV protein F gene and β-actin of infected cells treated with 1a, demonstrating an effect of this compound in reducing virus RNA gene expression. Further, structure modifications of 1a might afford a novel series of anti-hRSV compounds that hold promise for treating a burden pathogen. These modifications will be planned in order to find a compound activity and selective. However, here we display information that may base further investigations, in the scenario where the

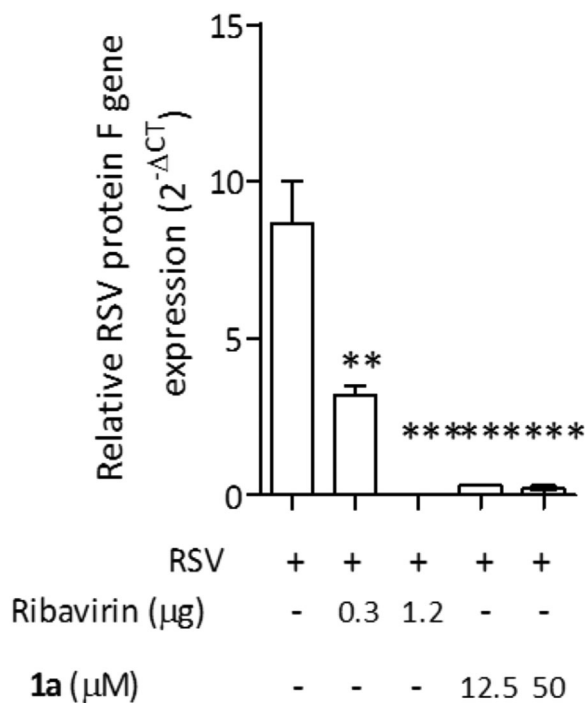


FIGURE 2 Antiviral activity of **1a**. Real-time PCR amplification was performed for hRSV protein F gene and β -actin gene from cells infected with hRSV or infected and treated with **1a** (12.5 and 50 μ M) or ribavirin (0.3 and 1.2 μ g) during 96 h. Relative expression was obtained by subtracting the CT value of β -actin from CT value of hRSV protein F gene. Statistical significance between the groups and hRSV was determined with one-way ANOVA followed by Bonferroni *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$

only drug available, ribavirin, is not selective and for this reason is not safe to use.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | Obtaining of BE, BA, and UA

BA was isolated from *Platanus acerifolia* bark (voucher specimen: ICN 171329) and UA was isolated from waste apple (*Malus domestica*) peels obtained from a local juice factory, as described by Silva et al.^[26] Both compounds were identified using full spectroscopy data, which are consistent with those previously described.^[25,26] BE was purchased from Sigma.

4.1.2 | Semisynthesis of BE, BA, and UA derivatives

BE, BA, and UA derivatives were synthesized according to the general procedure described in the literature.^[25,26] In brief, for ester derivatives the commercial anhydride (1.1 mmol, 5 Eq) and DMAP (0.22 mmol, 1 Eq) were added to BA or UA (0.22 mmol) in pyridine or CH_2Cl_2 (2 mL) and refluxed for 24 h (for cyclic anhydrides) or

processed without refluxing for 1 h (for acyclic anhydrides). The crude residue was purified in column chromatography to give the expected pure compounds. Column chromatography was carried out using silica gel 60 (Merck). Analytical thin layer chromatography was performed on silica gel 60 plates (Merck) and spots visualized by spraying with anisaldehyde/sulfuric. All compounds obtained are illustrated in Table 1.

The NMR spectra of compounds **1a**, **1b**, **1e**, and **1f** are provided as Supporting Information. The InChI codes of the investigated compounds together with some biological activity data are also provided as Supporting Information.

3,28-Di-O-acetylbetulin (**1a**)

Compound **1a** was prepared by using acetic anhydride. White powder, yield = 90%. ¹H NMR (400 MHz, CDCl_3), δ (ppm): 1.03 (s, 15H, CH_3 -23; CH_3 -24; CH_3 -25; CH_3 -26 and CH_3 -27); 1.06 (m, 1H, CH-13); 1.09 (t, 1H, CH-18); 1.12 (t, 2H, CH_2 -1); 1.24 (t, 2H, CH_2 -16); 1.36 (t, 2H, CH_2 -15); 1.39 (m, 4H, CH_2 -2 and CH_2 -21); 1.43 (t, 4H, CH_2 -11 and CH_2 -22); 1.50 (t, 2H, CH_2 -6); 1.51 (m, 2H, CH_2 -12); 1.56 (t, 2H, CH_2 -7); 1.62 (t, 1H, CH-5); 1.79 (br s, 3H, CH_3 -30); 1.84 (m, 2H, CH_2 -2); 1.86 (t, 1H, CH-9); 2.07 (s, 6H, CH_3 -32 and CH_3 -34); 2.44 (ddd, 1H, CH-19); 4.24 (dd, 2H, CH_2 -28); 4.46 (dd, 1H, CH-3); 4.58 (s, 1H, CH-29b); 4.68 (s, 1H, CH-29a). ¹³C NMR (75 MHz, CDCl_3), δ (ppm): 16.15 (C-25); 16.48 (C-26 and C-27); 18.15 (C-6); 19.09 (C-34); 20.78 (C-32); 21.06 (C-30); 21.32 (C-11); 25.12 (C-2); 26.90 (C-12); 23.68 (C-23 and C-24); 27.93 (C-15); 29.55 (C-21); 29.71 (C-16 and C-22); 35.53 (C-7); 37.03 (C-10); 37.53 (C-13); 37.77 (C-4); 38.36 (C-1); 40.86 (C-8); 42.66 (C-14); 48.74 (C-19); 46.28 (C-18); 47.69 (C-17); 50.25 (C-9); 55.35 (C-5); 62.78 (C-28); 80.89 (C-3); 109.88 (C-29); 150.09 (C-20); 170.99 (C-31); 171.60 (C-33). HRMS (ESI-MS, m/z): $[\text{M}+\text{Na}]^+$ calcd. for $\text{C}_{34}\text{H}_{54}\text{O}_4\text{Na}$: 549.3915. Found: 549.2620, mp: 210–214°C.

3,28-Di-O-isobutylbetulin (**1b**)

The compound **1b** was prepared by using isobutyric anhydride. White powder, yield = 93%. ¹H NMR (400 MHz, CDCl_3), δ (ppm): 0.96 (t, 2H, CH_2 -16); 1.02 (s, 15H, CH_3 -23, CH_3 -24, CH_3 -25, CH_3 -26 and CH_3 -27); 1.06 (t, 1H, CH-18); 1.13 (m, 3H, CH_2 -1 and CH-13); 1.17 (d, 12H, CH_3 -33, CH_3 -34, CH_3 -37 and CH_3 -38); 1.38 (t, 4H, CH_2 -15 and CH_2 -21); 1.41 (m, 4H, CH_2 -2 and CH_2 -11); 1.49 (m, 4H, CH_2 -6 and CH_2 -12); 1.57 (t, 2H, CH_2 -7); 1.60 (t, 1H, CH-5); 1.67 (t, 2H, CH_2 -22); 1.79 (br s, 3H, CH_3 -30); 2.42 (m, 1H, CH-36); 2.53 (m, 1H, CH-32); 1.82 (t, 1H, CH-9); 2.03 (ddd, 1H, CH-19); 2.26 (t, 2H, CH_2 -36); 2.38 (t, 2H, CH_2 -32); 4.26 (dd, 2H, CH_2 -28); 4.44 (dd, 1H, CH-3); 4.57 (s, 1H, CH-29b); 4.67 (s, 1H, CH-29a). ¹³C NMR (75 MHz, CDCl_3), δ (ppm): 16.05 (C-25); 16.07 (C-26); 16.59 (C-27); 19.12 (C-33, C-34, C-37 and C-38); 19.25 (C-6); 20.82 (C-11 and C-30); 23.68 (C-23 and C-24); 25.18 (C-2); 26.95 (C-12); 27.96 (C-15); 29.61 (C-21); 29.82 (C-16); 34.12 (C-22); 34.26 (C-36); 34.49 (C-32); 34.57 (C-7); 37.10 (C-10); 37.57 (C-13); 37.97 (C-1 and C-4); 38.36 (C-8); 40.91 (C-14); 42.73 (C-18); 46.57 (C-17); 48.83 (C-19); 50.29 (C-9); 55.39 (C-5); 62.42 (C-28); 80.35 (C-3); 109.88 (C-29); 150.19 (C-20); 176.82 (C-35); 177.52 (C-31). HRMS (ESI-MS, m/z): $[\text{M}+\text{H}+\text{K}]^+$ calcd. for $\text{C}_{38}\text{H}_{61}\text{O}_4\text{K}$: 619.4124. Found: 619.3745, mp: 110–112°C.

3,28-Di-O-trifluoroacetylbetulin (1e)

The compound **1e** was prepared by using trifluoroacetic anhydride. White powder, yield = 80%. ^1H NMR (400 MHz, CDCl_3), δ (ppm): 0.83 (t, 2H, CH_2 -16); 0.99 (m, 1H, CH-13); 1.06 (s, 15H, CH_3 -23, CH_3 -24, CH_3 -25, CH_3 -26 and CH_3 -27); 1.08 (t, 1H, CH-18); 1.15 (t, 2H, CH_2 -1); 1.30 (m, 2H, CH_2 -12); 1.33 (t, 2H, CH_2 -15); 1.42 (m, 2H, CH_2 -21); 1.43 (t, 4H, CH_2 -11 and CH_2 -22); 1.45 (t, 2H, CH_2 -6); 1.62 (t, 1H, CH-5); 1.64 (t, 2H, CH_2 -7); 1.78 (br s, 3H, CH_3 -30); 1.80 (m, 2H, CH_2 -2); 1.84 (t, 1H, CH-9); 1.99 (ddd, 1H, CH-19); 4.15 (dd, 2H, CH_2 -28); 4.58 (dd, 1H, CH-3); 4.62 (s, 1H, CH-29b); 4.71 (s, 1H, CH-29a). ^{13}C NMR (75 MHz, CDCl_3), δ (ppm): 15.98 (C-26); 16.14 (C-27); 16.24 (C-25); 18.07 (C-6 and C-30); 19.08 (C-11); 20.77 (C-23 and C-24); 23.25 (C-2); 25.04 (C-12); 26.92 (C-15); 27.77 (C-21); 29.33 (C-16); 34.00 (C-22); 34.21 (C-7); 37.04 (C-10); 37.74 (C-13); 38.07 (C-4); 38.20 (C-1); 40.88 (C-8); 42.74 (C-14); 46.61 (C-18); 47.53 (C-17); 48.76 (C-19); 50.19 (C-9); 55.23 (C-5); 66.84 (C-28); 86.23 (C-3); 110.30 (C-29); 116.05 (C-32); 116.11 (C-34); 149.50 (C-20); 157.74 (C-31); 158.16 (C-33). HRMS (ESI-MS, m/z); $[\text{M}-\text{H}+3\text{OH}]^+$ calcd. for $\text{C}_{34}\text{H}_{50}\text{F}_6\text{O}_7$: 683.3383. Found: 683.3650, mp: 175–176°C.

3,28-Di-O-dichloroacetylbetulin (1f)

The compound **1f** was prepared by using chloroacetic anhydride. White powder, yield = 85%. ^1H NMR (400 MHz, CDCl_3), δ (ppm): 0.99 (s, 15H, CH_3 -23, CH_3 -24, CH_3 -25, CH_3 -26 and CH_3 -27); 1.06 (m, 3H, CH-13 and CH_2 -16); 1.06 (t, 1H, CH-18); 1.13 (t, 2H, CH_2 -1); 1.26 (m, 2H, CH_2 -21); 1.30 (t, 2H, CH_2 -15); 1.34 (m, 2H, CH_2 -11); 1.42 (t, 2H, CH_2 -7); 1.45 (m, 4H, CH_2 -2 and CH_2 -12); 1.51 (t, 2H, CH_2 -6); 1.64 (t, 1H, CH-5); 1.74 (t, 2H, CH_2 -22); 1.79 (br s, 3H, CH_3 -30); 1.81 (t, 1H, CH-9); 2.00 (ddd, 1H, CH-19); 4.07 (dd, 2H, CH_2 -28); 4.48 (dd, 1H, CH-3); 4.61 (s, 1H, CH-29b); 4.70 (s, 1H, CH-29a); 5.92 (s, 1H, CH-34); 5.96 (s, 1H, CH-32). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 15.96 (C-25); 16.11 (C-26); 16.32 (C-27); 18.01 (C-6); 19.08 (C-30); 20.74 (C-11); 23.19 (C-23 and C-24); 25.06 (C-2); 26.95 (C-12); 27.80 (C-15); 29.41 (C-21); 29.48 (C-16); 33.98 (C-7 and C-22); 34.29 (C-10); 37.01 (C-13); 37.66 (C-4); 38.21 (C-1); 40.84 (C-8); 42.70 (C-14); 46.75 (C-18); 47.66 (C-17); 48.80 (C-19); 50.17 (C-9); 55.28 (C-5); 64.43 (C-34); 64.78 (C-32); 66.16 (C-28); 80.90 (C-3); 109.08 (C-29); 149.70 (C-20); 164.30 (C-31); 164.96 (C-33). HRMS (ESI-MS, m/z); $[\text{M}-\text{H}+\text{Na}]^+$ calcd. for $\text{C}_{34}\text{H}_{50}\text{Cl}_4\text{O}_4\text{Na}$: 683.2198. Found: 683.3828, mp: 166–170°C.

4.2 | Antiviral activity and cytotoxicity assay

4.2.1 | Cell lines and virus

Human adenocarcinomic alveolar epithelial cells (A549) and Vero cells were cultured in DMEM low glucose (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil). RSV A2 strain was kindly provided by Dr. Fernando Polack, Fundación Infant, Argentina. The virus was grown on Vero cells in Opti-MEM medium (Gibco™, Thermo Fisher Scientific) supplemented with 2% of FBS and was then purified from cell culture supernatant. Viral plaque-forming units (PFU) were identified using

an anti-hRSV antibody (Millipore, Billerica, MA, USA). Viral aliquots were stored in -80°C .

4.2.2 | Cytotoxicity assay

A549 cells were seeded in a 96-well flat-bottom plate at 5×10^3 cells per well. After 24 h incubation, each compound at 100, 50, 25, 12.5, and 6.25 μM was added to the plates which were then incubated for a further 96 h at 37°C in a humidified incubator with 5% CO_2 . Cell viability was assessed by a colorimetric assay based on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2-diphenyltetrazolium bromide (MTT) (Molecular Probes™, Thermo Fisher Scientific) by mitochondrial enzymes. Briefly, 100 μL of medium was removed and 40 μL of MTT reagent from a 5 mg/mL stock solution was added in each well. Cells were incubated for 4 h and the precipitated formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Finally, the optic density (OD) was analyzed at 570/620 nm using micro-plate reader (EZ Read 400, Biochrom). DMSO was added as a control in the same volume used to dilute the compounds. The results were obtained from triplicate assays. The percentage of cytotoxicity of each compound was calculated considering 100% the OD of untreated cells and using as reference the OD of cells treated with DMSO.

4.2.3 | Antiviral assay

To assess the effect of the compound in inhibiting the cell death caused by the virus, A549 cells were seeded in 96-well flat-bottom plate (5×10^3 per well) and after 24 h infected with 5×10^4 PFU of RSV. After 2 h each compound at 100, 50, 25, 12.5, and 6.25 μM was added to the plates which were then incubated for a further 96 h at 37°C in a humidified incubator with 5% CO_2 . Cell viability was assessed by MTT colorimetric assay as described above. Ribavirin was used as positive control (TCI America) at 2.4, 1.2, 0.6, 0.3, 0.15 μg per well. The results were obtained from triplicate assays. The percent protection of each compound was calculated as $[(A - B)/C - B] \times 100$, where A, B, and C are the OD values of treated infected, untreated infected, and untreated uninfected cells, respectively.

4.2.4 | Data analysis

The 50% cytotoxic concentration (CC_{50}) and the 50% antiviral effective concentration (EC_{50}) for each compound were obtained from dose-effect curves and determined by linear regression analysis (not shown). The therapeutic index (TI) (i.e., selective index) is defined as $\text{CC}_{50}/\text{EC}_{50}$.

4.2.5 | Viral load quantification by real-time PCR

Total RNA was extracted from cells infected with RSV or infected and treated with **1a** (12.5 and 50 μM) or ribavirin (0.3 and 1.2 μg). Complementary DNA (cDNA) was synthesized using transcriptase kit GoScrip™ (Promega™, Madison, WI, USA). Real-time PCR was performed for the amplification of the RSV F protein gene using

specific primers and probes: forward-5'-AACAGATGTAAGCAGCTCCGTTATC-3', reverse-5'-GATTTTTATTGGATGCTGTACATTT-3', and probe 5'-FAM/TGCCATAGCATGACACAATGGCTCCT-TAMRA/-3' and for amplification of human β -actin as endogenous control gene using TaqMan assay (Hs00174103_m1 ACTB). The reaction was performed in a StepOne[®] (Applied Biosystems[®]) under the following conditions: 50°C for 2 min and 95°C for 10 min followed by for 40 cycles of 95°C for 15 s and 60°C for 1 min. The Delta cycle-threshold (Δ Ct) was obtained by subtracting the endogenous control gene Ct value from the Ct value of the RSV protein F gene.^[29]

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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