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RESEARCH ARTICLE

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Antiviral activity of A771726, the active metabolite of leflunomide, against Junín virus

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The aim of this study was to investigate the effect of A771726, the active metabolite of leflunomide, against the infection with Junín virus (JUNV), agent of Argentine hemorrhagic fever (AHF). The treatment with non-cytotoxic concentrations of A771726 of Vero and A549 cells infected with JUNV inhibited virus replication in a dose-dependent manner, as determined by virus yield reduction assay. The antiviral effectiveness of A771726 was not importantly affected by the multiplicity of infection and the virus strain. Moreover, the combination of A771726 and ribavirin had a significantly more potent antiviral activity than each single drug treatment. Mechanistic studies showed that the main action of A771726 is exerted before 6 h of JUNV infection. Accordingly, inhibition of viral RNA synthesis was detected in treated infected cells by real time RT-PCR. The exogenous addition of uridine or orotic acid produced a partial reversal of the inhibitory effect of A771726 on infective virus production whereas a total reversion was detected on JUNV RNA synthesis, probably by restoration of the enzymatic activity of dihydroorotate dehydrogenase (DHODH) and the intracellular pyrimidine pools. In conclusion, these results suggest that the antiviral target would be viral RNA synthesis through pyrimidine depletion, but any other effect of the compound on JUNV infection cannot be excluded. This study opens the possibility of the therapeutic application of a wide spectrum host-targeted compound alone or in combination with ribavirin to combat AHF as well as other human pathogenic arenaviruses.

KEYWORDS
A771726, antiviral activity, arenavirus, host target, leflunomide, pyrimidine inhibitor

1 | INTRODUCTION

Arenaviruses are enveloped viruses containing a bisegmented single-stranded RNA genome with an ambisense coding strategy that express four primary viral proteins: the RNA-dependent RNA polymerase L, the matrix protein Z, the nucleoprotein NP, and the glycoprotein precursor GPC, post-translationally cleaved to yield the mature virion glycoproteins. Several arenaviruses cause hemorrhagic fevers (HF) with significant level of morbidity and mortality. Among pathogenic arenaviruses, Lassa virus (LASV) and Junín virus (JUNV) are at present the main threat for public health since they generate annual outbreaks of Lassa fever and AHF in endemic areas of West Africa and Argentina, respectively.[1,2]

No specific and safe chemotherapy for any arenavirus is currently available. Treatment is limited to the use of immune convalescent plasma with defined doses of JUNV neutralizing antibodies for AHF[2] or the guanosine analog ribavirin, effective against several RNA viruses including LASV.[3] However, several drawbacks are associated to both treatments, such as failure in advanced infections and undesirable side effects.[2,3] Then, there is a real need of novel therapeutic options against arenaviruses.

In the search of effective and safe antiviral agents, the consideration of host factors involved in virus replication that are known or predicted

to bind to a drug as therapeutic targets has expanded in recent years.[4–6] This strategy provides a potential target for the development of broad spectrum antivirals as well as the possibility of repurposing for viral infections drugs recently licensed for other human diseases with defined safety profiles in clinical use. Furthermore, the focus on already licensed or at an advanced development drug candidates will significantly lower the costs and time frame required for the disclosure of new drugs, an important advantage for antiviral research against emerging viruses in regions with limited resources.

In particular, molecules that inhibit enzymes involved in cellular nucleoside metabolism pathways have been exploited for arenavirus antiviral development. As example, ribavirin is an inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH) that converts IMP to xanthosine monophosphate, a limiting step for de novo intracellular synthesis of guanosine nucleotides.[7] To overcome the shortcomings reported by the treatment of humans with ribavirin, other inhibitors of the biosynthetic pathway of purine and pyrimidine nucleotides were also studied with variable results.[8–13]

In this line of host-targeted nucleotide biosynthesis inhibitors, leflunomide, an isoxazol derivative with potent immunomodulatory and anti-inflammatory activities currently approved by the United States Food and Drug Administration (FDA) for treatment of patients with rheumatoid arthritis and autoimmune disorders,[14] is an interesting lead candidate. Upon oral ingestion, leflunomide is rapidly metabolized to its active form, designated teriflunomide or A771726 [N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotoamide]. Since, A771726 is able to avoid the initial metabolism step in comparison to leflunomide, it has turned a more interesting focus for clinical therapy, and has been recently approved by FDA for treatment of relapsing forms of multiple sclerosis.[15] This metabolite is a potent inhibitor of mitochondrial dihydroorotate dehydrogenase (DHODH), a key enzyme in the de novo biosynthesis of pyrimidine nucleoside triphosphates.[16] Other cellular targets such as protein tyrosine kinases,[17,18] cyclooxygenase,[19] and NF-κB signaling[20] may also be affected by A771726 at higher concentrations. Moreover, leflunomide/A771726 has demonstrated antiviral activity toward diverse DNA and RNA viruses, such as herpes simplex virus (HSV),[21] human cytomegalovirus (HCMV),[22,23] polyoma BK virus (BKV),[24,25] human immunodeficiency virus (HIV),[26] and respiratory syncytial virus (RSV).[27] The efficacy of this compound was also observed in individual clinical trials to control multiresistant HCMV and HSV strains in transplant recipients or HIV-infected patients[28–31] as well as BKV reactivation in kidney or stem cell transplantation recipients,[32–33] but the real beneficial effects of leflunomide/A771726 in antiviral therapy is not still fully corroborated.

In the present study we report the evaluation of antiviral activity and mode of action of A771726 against the arenavirus JUNV in Vero and A549 cells.

2 | MATERIALS AND METHODS

2.1 | Compounds

The leflunomide metabolite A771726 (Figure 1A) and ribavirin were purchased from Sigma-Aldrich, St. Louis, MO.

2.2 | Cells and viruses

The cell lines Vero (African green monkey kidney, ATCC CCL-81) and A549 (lung carcinoma human cells, ATCC CCL-185) were grown in Eagle's minimum essential medium (MEM) (Gibco™ Thermo Fisher Scientific, Carlsbad, CA) supplemented with 5% inactivated bovine serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. The JUNV strains used were as follows: the naturally attenuated IV4454 strain obtained from a mild human case,[34] the XJCl3 strain, attenuated by serial passage from the prototype XJ strain,[35] and the vaccine Candid-1 strain.[36] Virus stocks were propagated in Vero cells and titrated by PFU in the same cell line.

2.3 | Virus yield reduction assay

Antiviral activity was determined by a virus yield inhibition assay. Vero or A549 cells grown in 24-well plates were infected at a multiplicity of infection (m.o.i.) of 0.1 PFU/cell. After 1 h adsorption at 4°C, cells were washed and refed with MM containing or not serial two-fold dilutions of each compound. Ribavirin was used as a reference anti-arenavirus substance. After 48 h of incubation at 37°C, supernatant cultures were harvested and extracellular virus yields were determined by a plaque assay. The effective concentration 50% (EC_{50}) was calculated as the concentration required to reduce virus yield by 50% in the compound-treated cultures compared with untreated ones. All determinations were performed in triplicate.

2.4 | Cytotoxicity test

Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) method using conditions equivalent to those used in antiviral assays, as previously described (Talarico and Damonte, 2007).[37] The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce the MTT signal by 50% compared to untreated controls. The maximal noncytotoxic concentration (MNCC) was determined as the maximal concentration tested that did not affect the MTT signal at 72 h, indicative of 100% viability. All determinations were performed in triplicate.

2.5 | Time of addition assay

Cells grown in 24-well microplates were infected with JUNV (m.o.i. 0.1 PFU/cell) and adsorption was allowed for 1 h at 4°C. After removal of the inocula, the cells were washed with phosphate-buffered saline (PBS), refed with MM and incubated at 37°C. Duplicate wells were treated with 50 μM A771726 at various times after adsorption. An infected culture without drug treatment was performed as control. In all cases, extracellular virus yields at 24 h p.i. were determined by plaque formation; this time was chosen to minimize the accumulation of virus replication cycles and obtain a better approximation to the time period affected by the compound in the JUNV replication cycle.

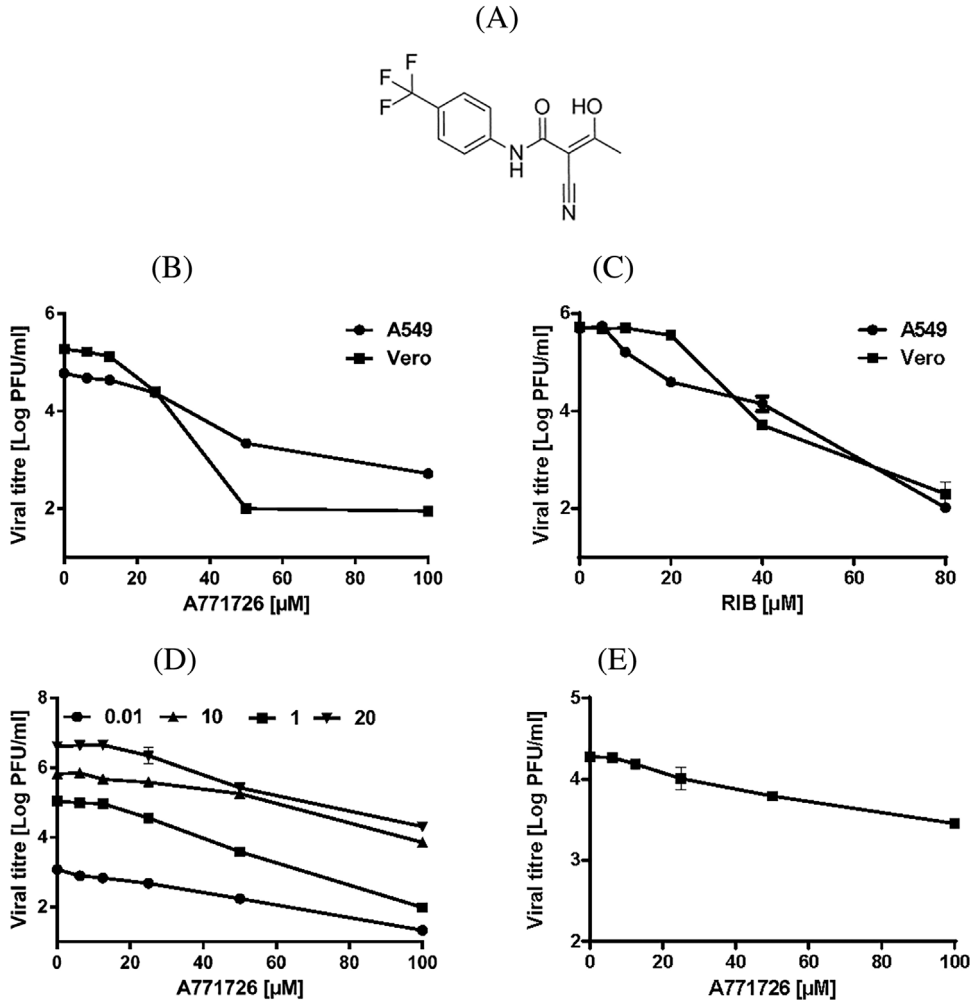

FIGURE 1 Dose-dependent inhibition of JUNV replication by A771726. A, Chemical structure of A771726. (B-C) Vero or A549 cells infected with JUNV IV4454 strain (m.o.i. 0.1 PFU/cell) during 1 h at 4°C were then incubated with serial two-fold concentrations of A771726 or ribavirin. At 48 h p.i. extracellular virus yields were quantified by PFU in Vero cells. D, Vero cells were infected with JUNV IV4454 strain at different m.o.i. in the presence of increasing concentrations of A771726. Virus yields were determined at 48 h p.i. by PFU. E, Vero cells were infected with JUNV IV4454 strain (m.o.i. 0.1 PFU/cell) during 1 h at 4°C and then further incubated at 37°C with MM for 24 h. Thereafter, supernatants were discarded, MM containing or not different A771726 doses was added and incubation was prolonged for 24 h. At 48 h p.i., virus yields were determined by PFU. In (B-E) viral titres represent mean values of triplicate assays ± standard deviation (SD)

2.6 | Virus RNA synthesis

Vero and A549 cells were infected with JUNV (m.o.i. 0.1 PFU/cell) and, after adsorption, cells were refed with MM alone or MM containing 25 or 50 μM A771726. At 48 h after infection, total RNA was extracted by using TRIzol® (Invitrogen™ Thermo Fisher Scientific, Carlsbad CA) according to the manufacturer's instructions. To monitor RNA replication, cDNA was generated by using M-MLV reverse transcriptase (200 U/L, Invitrogen™ Thermo Fisher Scientific) and random primers. This cDNA was amplified by real time PCR using LightCycler® 480 SYBR Green I Master Mix (Roche, Mannheim, Germany) detection. The mix reaction volume was 25 μL including 2 μL of cDNA and specific primers to amplify the viral N gene (Forward 5′-GGCATCCTTCAGAACATC-3′, Reverse 5′-CGCA-CAGTGGATCCTAGGC-3′) as previously described.[12] The cellular *actin* gene was used for reference using forward 5′-GAGACCTTCAACACCC-CAGCC-3′, and reverse 5′-GGCCATCTCTTGCTCGAAGTC-3′ primers. Amplification plots were expressed as Ct values to be analyzed with iQ5® (BioRad, Hercules, CA) software.

To quantify viral RNA in cell supernatants, Vero cells infected with JUNV (m.o.i. 0.1 PFU/cell) were incubated for 48 h in the absence or presence of 50 μM A771726. Then, supernatants from untreated and treated cells were clarified by centrifugation at 5000×*g* for 10 min and concentrated by ultrafiltration using cellulose membranes (Vivacon 500, 100 000 MWCO, Sartorius AG, Göttingen, Germany). Thereafter, RNA was extracted from virus samples with TRIzol® and quantified by real time RT-PCR as above.

2.7 | Reversal by uridine and orotic acid

A549 and Vero cells were infected with JUNV (m.o.i. 0.1 PFU/cell) and treated with MM (VC), 50 μM A771726 alone or A771726 and the indicated concentrations of uridine or orotic acid. After 48 h,

extracellular virus yields were determined by plaque formation and viral RNA was measured by real time PCR as detailed above.

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Comparison of means was tested by one-way analysis of variance (ANOVA) with Dunnett's posttest. Statistical significance was defined as $P < 0.05$ (95% confidence interval).

3 | RESULTS

3.1 | Anti-JUNV activity of A771726

The antiviral activity of A771726 against JUNV strain IV4454 was evaluated by a virus yield reduction assay in two cell systems susceptible to JUNV infection: Vero and A549 cells. Cells were infected at a m.o.i. of 0.1 PFU/cell and further incubated in the presence of different compound concentrations during 48 h. Virus replication was inhibited by A771726 in a dose-dependent manner attaining a reduction in virus titres higher than 90% in the range of concentrations 50-100 μM (Figure 1B). The anti-JUNV efficacy was similar in both cells and the EC_{50} values calculated from data in Figure 1B were 16.6 ± 0.2 and 20.3 ± 1.3 μM for Vero and A549 cells, respectively. Furthermore, no important effects in cell viability were detected by microscopic observation and MTT method in both cell cultures after treatment with this compound. Cell treatment with a range of A771726 concentrations from 300 to 75 μM showed that 75 μM was the maximal non-cytotoxic concentration (MNCC) for both Vero and A549 cells. The CC_{50} values for A771726 in Vero and A549 cells were 223.1 and 259.3 μM, respectively. Based on these data, the selectivity index values (CC_{50}/EC_{50}) were calculated to be 13.4 and 12.7 in Vero and A549 cells, respectively. The compound ribavirin was also tested as positive control against JUNV in virus yield reduction assays performed in Vero and A549 cells, showing an inhibitory response comparable to A771726 (Figure 1C).

In addition, the effectiveness of A771726 to inhibit JUNV infection was not importantly affected by the initial virus inoculum. When a virus yield inhibition assay was performed in Vero cells infected at different m.o.i., the profile of the dose-response curves showed very slight variations in the range 0.01–20.0 PFU/cell (Figure 1D). In fact, when the EC_{50}s were calculated from data presented in Figure 1D, the values obtained were 14.9 ± 0.9, 20.7 ± 0.5, 28.8 ± 0.4, and 28.3 ± 1.3 μM for m.o.i. of 0.01, 1.0, 10.0, and 20.0, respectively, whereas the EC_{50} determined from data in Figure 1B for the m.o.i. of 0.1 was 16.6 ± 0.2 μM. Thus, within a 2000-fold variation in virus inocula, the EC_{50} showed a less than 2-fold change.

In the above mentioned experiments, A771726 was added to cells immediately after virus infection, at 1 h p.i. when virus inoculum is eliminated and cells are incubated at 37°C with MM containing the compound. To determine the effectiveness of A771726 during the course of an established infection, different compound concentrations were added to infected Vero cells after 24 h of infection with JUNV strain IV4454, the incubation in the presence of A771726 was further prolonged during 24 h and then virus yields were titrated in comparison to untreated infected cells. Under these conditions, the drug exerted a dose-dependent inhibition of virus production as observed when drug was added immediately after infection (Figure 1E), although the maximum level of inhibition achieved was lower and the EC_{50} increased 1.8-fold (30.3 vs 16.6 μM for A771726 treatment at 24 or 1 h p.i., respectively). These data suggest that this compound may be used not only to prevent the beginning of JUNV infection but also to reduce the spread of infection in a therapeutical treatment after more than one cycle of virus replication.

The spectrum of antiviral activity of A771726 was also evaluated against other JUNV strains, such as the XJCl3 strain, obtained by serial passage from the prototype XJ strain, and the vaccine strain Candid-1. As shown in Table 1, these two viruses were also susceptible to the inhibitory effect of A771726 as shown for IV4454 strain.

3.2 | Combination of ribavirin and A771726

At present, ribavirin is the only drug in clinical use against arenaviruses, but with some limitations in its effectiveness.[2,3] To improve the therapeutic perspectives of A771726 as antiarenaviral drug, we tested the combination of both compounds to determine if the combined treatment may offer benefits in comparison to each treatment alone. To this end, Vero cells infected with JUNV were then treated with each compound alone or with different combinations of A771726 and ribavirin. It was observed that combination of A771726 and ribavirin had a significant more potent activity than the single drug treatment, attaining higher reduction in virus titre with lower drug concentrations (Figure 2). For example, the reduction in JUNV yields when 10 μM ribavirin was added in combination with 6.25-25 μM A771726 was 1-2 log greater than virus production after each single treatment with A771726 alone (Figure 2).

3.3 | Mode of action

As a first approach to characterize the action of A771726 on JUNV replication, the dependence of the inhibitory effect on the time of drug addition during one cycle of replication was next examined. Cells were treated with 50 μM A771726 for 4 or 24 h prior to infection with JUNV strain IV4454 or at different times after virus adsorption at 4°C, in

TABLE 1 Antiviral activity of A771726 against JUNV strains

	EC_{50} (μM)[a]	
Virus strain	**Vero**	**A549**
IV4454	16.6 ± 0.2	20.3 ± 1.3
Candid 1	45.3 ± 2.4	34.6 ± 0.6
XJCl3	23.3 ± 9.1	26.8 ± 8.0

[a]EC_{50} (effective concentration 50%): compound concentration required to reduce virus yield by 50%.

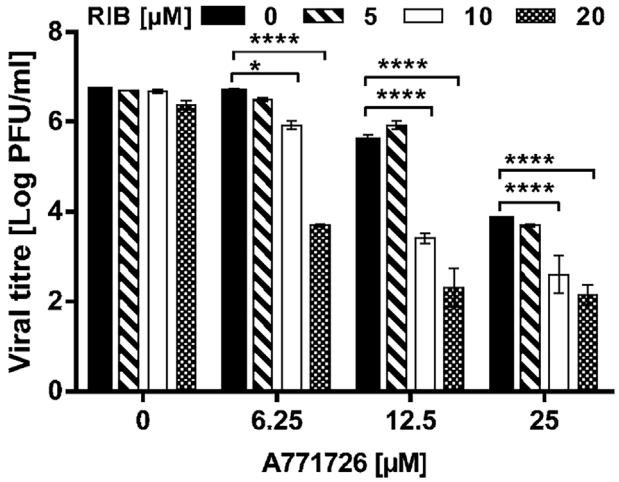

FIGURE 2 Effect of the combination of A771726 and ribavirin on JUNV replication. Vero cells were infected with JUNV (m.o.i. 0.1 PFU/cell) and treated with the indicated concentrations of A771716 (0, 6.25, 12.5, or 25 μM) in the absence (0) or presence of 5, 10, or 20 μM ribavirin (RIB). At 48 h p.i., virus yields were determined by plaque assay. Each value is the mean of triplicate assays ± standard deviation (SD). Asterisks indicate a significant difference (*$P < 0.05$; **** $P < 0.0001$)

order to synchronize the process of infection after internalization into the cell. In all cases, virus titres in cell supernatants were determined at 24 h p.i. Treatment with A771726 before infection did not affect virus production indicating that the drug does not induce an antiviral state in the cell culture (data not shown). On the contrary, a variable but significant inhibition was observed when drug was added from 0 up to 5-6 h post-adsorption whereas no reduction in virus titres was detected at later times (Figure 3). These data suggest that the action of A771726 is mainly exerted during early and intermediate stages of virus replication, a time period in JUNV replication cycle compatible with an effect on viral RNA transcription and replication according to previous studies on the time course of JUNV RNA synthesis in Vero cells.[12,38]

To corroborate the mode of antiviral action of A771726 against JUNV infection, its effect on viral RNA synthesis was next examined by a quantitative RT-PCR assay. Cells infected with JUNV were incubated in MM containing or not compound, and at 48 h p.i. total RNA was extracted. A real-time RT-PCR was performed with RNA samples from treated and untreated cells which were amplified with primers corresponding to *n* gene and with *actin* as cellular gene control. Quantification of viral RNA synthesized under these conditions showed a significant level of inhibition in both A549 and Vero cells after treatment with 25-50 μM A771726 (Figure 4), confirming the hypothesis of a potential drug target in JUNV RNA synthesis.

From data in Figure 1B and Figure 4, it can be observed that the reduction in viral RNA appeared to be lower than the reduction in infective virus yields. These results suggest that A771726 may inhibit RNA synthesis as well as result in the production of non-infectious viral genomes, a similar situation as reported for the guanosine analog ribavirin.[39] To test this possibility, the amount of viral RNA present in supernatants of JUNV infected Vero cells treated or not with 50 μM A771726 at 48 h p.i. was determined by real time RT-PCR simultaneously with the titration of released infectious virions measured by plaque assay. A 1000-fold reduction was detected in the infective JUNV production in A771726 treated cultures in comparison to untreated infected cells whereas the amount of released viral RNA in treated cells respect to untreated ones was 100-fold diminished. Thus, it can be concluded that A771726 inhibited

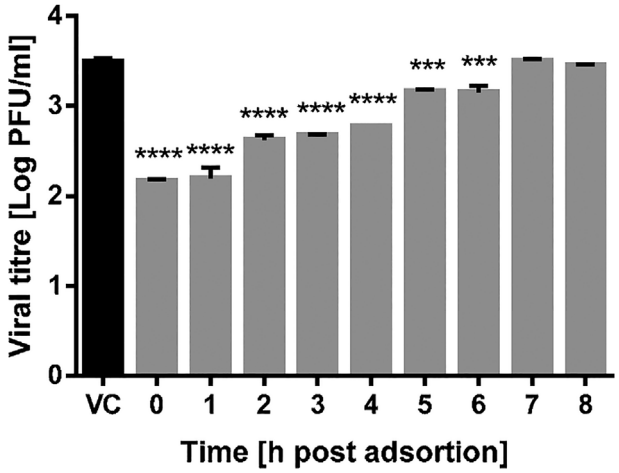

FIGURE 3 Effect of time of addition of A771726 on antiviral activity. Vero cells were incubated with MM containing 50 μM A771726 from different times after infection. In all cases, extracellular virus yields were determined at 24 h p.i. by plaque assay. VC, untreated virus control. Each value is the mean of triplicate assays ± standard deviation (SD). Asterisks indicate a significant difference (*** $P < 0.001$; **** $P < 0.0001$)

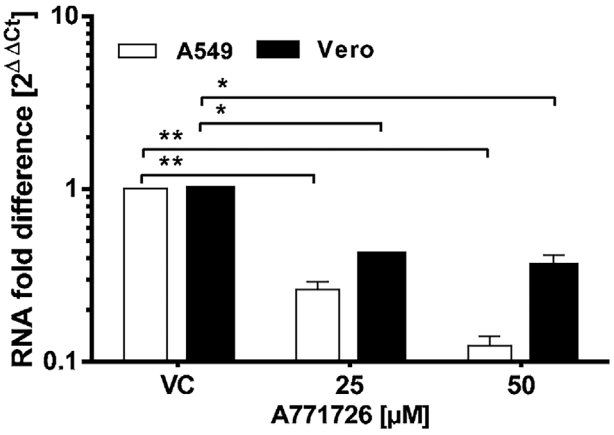

FIGURE 4 Effect of A771726 on virus RNA synthesis. A549 or Vero cells were infected with JUNV IV4454 strain and incubated during 48 h with MM containing or not 50 μM A771726. Then, total RNA was extracted, cDNA was synthesized, and then amplified by real time PCR using JUNV N gene and *actin* specific primers. Results are expressed as fold difference viral RNA level respect to viral control without drug treatment. Each value is the mean of triplicate assays ± standard deviation (SD). Asterisks indicate a significant difference (*$P < 0.05$; **$P < 0.01$)

intracellular viral RNA synthesis and also appeared to induce the production of non-infective viral RNA.

3.3 | Reversal of antiviral activity

Given the known activity of leflunomide/A771726 as inhibitor of DHODH, the enzyme catalyzing the fourth step in the de novo synthesis of pyrimidines,[16] it was considered that the in vitro anti-JUNV activity of A771726 could be mainly due to the interference of the compound with this cellular enzyme. Pyrimidines may be acquired by the cells either through de novo synthesis starting from ammonia, bicarbonate, and aspartic acid in a sequential process involving five enzymatic steps or by salvaging preformed pyrimidine bases or nucleosides.[40] Then, the exogenous addition of the precursors of the biosynthetic or salvage pathway might overcome the effects of inhibition of DHODH and restore normal pyrimidine pools. To investigate this possibility, JUNV infected cells were co-treated with A771726 and exogenous uridine immediately after virus adsorption to generate pyrimidines through the salvage pathway, thus bypassing any possible effect of A771726 on de novo biosynthesis, and JUNV yields were determined at 48 h p.i. As seen in Figures 5A and 5B, treatment of cells with uridine in the range 50-400 μM reversed the antiviral activity of A771726, with a rescue of 82-95% of infectivity, depending on the uridine concentration and the host cell. These findings suggest that pyrimidine depletion may be a main cause of JUNV inhibition by A771726 and uridine shifts the supply of pyrimidine from the de novo to the salvage pathways. To distinguish between de novo biosynthesis and salvage pathway, we tested the ability of orotic acid, the intermediate in the biosynthesis cycle produced from dihydroorotic acid by DHODH enzymatic activity, to restore viral production. Orotic acid showed a moderate reversion effect on the activity of A771726 against JUNV, with significant restoration of viral replication at the highest concentration tested of 400 μM and a higher recovery in A549 cells than in Vero cells (Figures 5A and 5B).

The effect of exogenous uridine and orotic acid addition on the inhibitory activity of A771726 was also corroborated by determination of viral RNA synthesis in infected co-treated cells. A total reversion of JUNV RNA inhibition was observed in the presence of both precursors

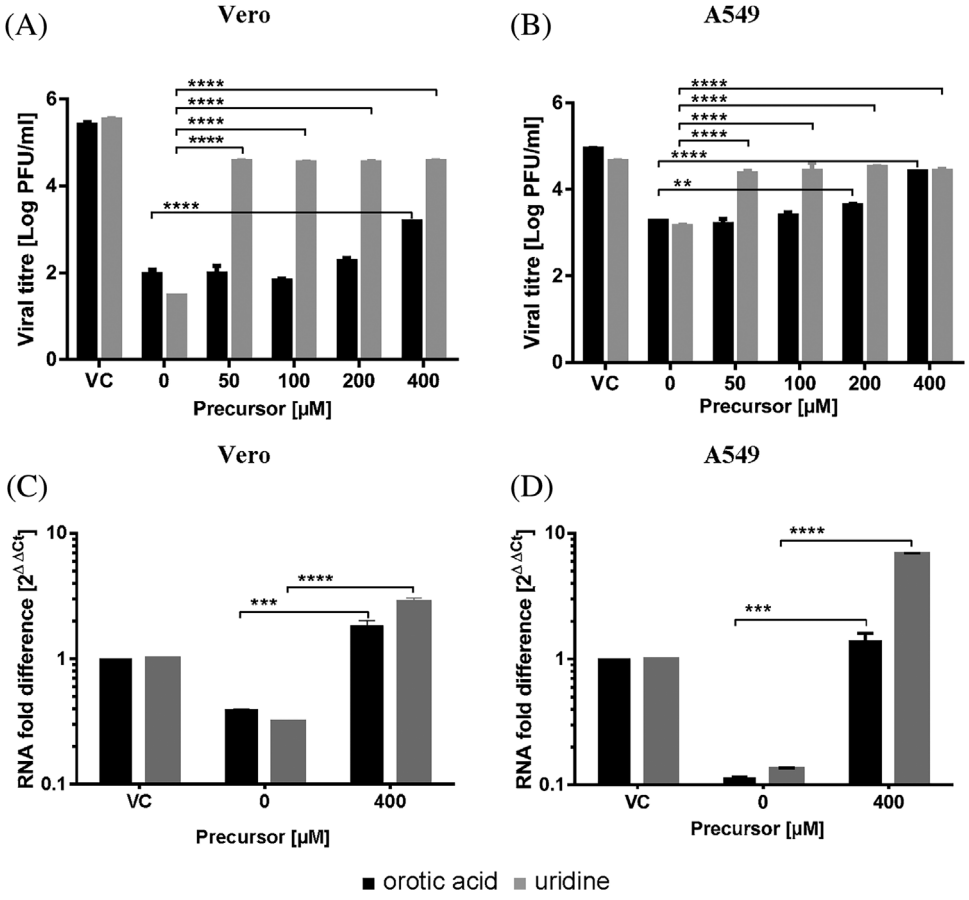

FIGURE 5 Reversal of inhibition by exogenous addition of nucleotide precursors. Vero (A,C) or A549 (B,D) cells were infected with JUNV IV4454 strain and incubated with MM (VC), MM containing 50 μM A771726 alone (0) or 50 μM A771726 and the indicated concentrations of uridine or orotic acid. After 48 h of infection, extracellular virus yields were determined by plaque formation (A,B) and viral RNA was measured by real time PCR using JUNV N gene and *actin* specific primers, expressing the results as fold difference of viral RNA level compared to viral control (C,D). In all cases, each value is the mean of triplicate assays ± standard deviation (SD). Asterisks indicate a significant difference (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$)

(Figures 5C and 5D), in fact an increase in the amount of viral RNA was observed when the precursors were added compared to the control, perhaps related to the formation of defective particles by the excess of precursor. These results are indicative that DHODH may be responsible of the reduction of JUNV replication by A771726, but do not exclude the possibility that besides inhibiting DHODH, the compound may exert its antiviral activities through another mechanism.

4 | DISCUSSION

This study has shown the effective in vitro antiviral activity of A771726 against JUNV, the arenavirus causing AHF. This compound showed a good spectrum of antiviral action since it was inhibitory of several strains of JUNV, including the IV4454 strain obtained from a mild clinical case as well as the laboratory-adapted XJCl3 and Candid-1 strains genetically related to the highly pathogenic XJ strain, in both human A549 and monkey Vero cells. In addition, the antiviral activity of A771726 against JUNV was not significantly affected by the input m.o.i. used, in the range 0.01-20.0 PFU/cell. The level of reduction of viral titres observed between the sample corresponding to the virus control (0 μM) and that one corresponding to the maximum A771726 concentration (100 μM) was about 99% for each m.o.i. tested (Figure 1D), assessing the similar effectiveness of the compound to block JUNV infection with low or high initial virus doses. Furthermore, the combination of A771726 and ribavirin exhibited more potent inhibitory effect than each single drug treatment, suggesting the potential perspectives of A771726 to be used against HF-causing arenaviruses alone or in combination with the existing ribavirin chemotherapy.

Leflunomide/A771726 has demonstrated antiviral activity against several DNA and RNA viruses, but the mode of action is still in discussion and has been very variable depending on the virus and the host cell. It inhibited BKV in Vero and in human renal tubular epithelial cells. In the first case there was no reversion with uridine, but in renal cells treatment with uridine reversed the effect in virus production suggesting a mechanism dependent on pyrimidine depletion.[24] The herpesviruses HSV and HCMV were also inhibited by this compound through a mechanism independent from DNA synthesis and due to interference with the cytoplasmic phase of virion assembly, probably affecting the phosphorylation of the tegument viral proteins.[21,22] FK778, a synthetic analog of A771726, also inhibited HCMV but with a different mode of action since the antiviral activity was reversed by uridine.[23]

In our study, time of addition assays suggested that the target of A771726 during JUNV infection may be early or intermediate steps of the viral replication cycle, located before 6 h of infection. In agreement with this time course, a reduction in viral RNA synthesis was detected in JUNV infected cells treated with A771726. However, the level of inhibition produced by the compound on viral RNA synthesis was lower than the reduction observed in infective virus production at the concentration of 50 μM tested in both assays (see for example Figures 1 and 3 in comparison to Figure 4). Accordingly, the reversal in JUNV RNA inhibition by the exogenous addition of uridine or orotic acid is total, whereas the reversal in PFU is highly significant but not complete (see Figure 5). These data suggest that JUNV RNA synthesis through DHODH inhibition is not the only target affected by A771726, but it could also exert any other effect on virus replication leading to the higher decrease in infectivity. This possibility is not surprising since similar dual mode of action has also been reported for leflunomide against HIV,[26] BKV,[24] and RSV[27] as well as for other DHODH inhibitors such as brequinar against dengue virus (DENV), that affected viral RNA synthesis through depletion of intracellular pyrimidine pools and also inhibited virion assembly/release,[41] and the compound A3 which was effective against lymphocytic choriomeningitis virus (LCMV), another arenavirus, due to its anti-DHODH activity together with other unknown mechanism.[13] Our studies about the release of viral RNA and PFU in A771726 treated infected cells in comparison to untreated ones have detected a decrease of specific infectivity in cell supernatants, suggesting the presence of JUNV non-infectious particles. Then, this secondary effect may contribute together with the intracellular inhibition of viral RNA synthesis to the anti-JUNV activity of A771726.

Moreover, it cannot be discarded the participation of other potential targets in the antiviral activity against JUNV. Besides the inhibitory action of A771726 on DHODH, it has been reported that this drug is capable of preventing tyrosine kinase activity.[17,18] Then, the inhibition of a cellular kinase activity required for viral productive infection may be also involved in the effect on JUNV infectivity here described. In fact, the inhibition of the arenaviruses LASV, Pichinde (PICV) and Pirital by treatment with genistein, a well known general tyrosine kinase inhibitor, has been reported in in vitro and in vivo systems.[42,43] Other possible target for A771726 on JUNV infection may be mediated by the activation of the cell innate immune response. Diverse novel compounds with demonstrable pyrimidine inhibition activity were found to be also able to stimulate innate immunity and both properties contribute to their antiviral activity.[44–46] Interestingly, a recent study demonstrated that a tetrahydro benzothiazole derivative effective against a broad range of RNA viruses inhibited host pyrimidine synthesis and also induced an antiviral status by activating genes involved in innate immunity independently of the production of type I interferons.[44] Since Vero cells are deficient in type I interferon,[47] the involvement of interferon could be excluded in the anti-arenaviral activity of A771726 here shown, but the possibility of an activation of components of the cellular immune response independent of interferon contributing to JUNV inhibition is also an interesting perspective to be considered.

The restorative ability of orotic acid suggests a role for DHODH in the inhibitory activity of A771726 against JUNV in Vero and A549 cells. The comparison of the reversal assays of JUNV inhibition by A771726 either with uridine or with orotic acid has shown consistently a more marked viral infectivity rescue in the presence of uridine. Similar findings were observed with other DHODH inhibitors.[44,48] It is known that the relative contribution of the de novo and salvage pathways in mammalian cells depends on cell type and developmental stage: the activity of the de novo synthesis, in general, is low in resting

cells where the pyrimidine pool is largely provided by the salvage route.[39] Then, in the confluent cell cultures used for virus infection the exogenous supply of excess of uridine may probably overcome the inhibition of DHODH with higher efficacy than the addition of the product of the enzyme reaction.

As previously mentioned, translation from in vitro to in vivo efficacy has been shown in clinical tests for pyrimidine inhibitors with antiviral properties of wide spectrum such as leflunomide/A771726. Then, the elucidation of the complete details of the mechanism/s by which A771726 exerts anti-JUNV activity as well as the evaluation of its in vivo efficacy merits further investigation, particularly considering the potential effectiveness of this compound to combat AHF and other human pathogenic arenaviruses.

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AUTHORS' CONTRIBUTION

CSS, CCG, and EBD conceived and designed the experiments. CSS performed the experiments. CSS, CCG, and EBD analyzed the data. CSS and EBD wrote the manuscript.

CONFLICTS OF INTEREST

The authors have declared no competing interests.

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