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FDA-approved antivirals ledipasvir and daclatasvir downregulate the Src-EPHA2-Akt oncogenic pathway in colorectal and triple-negative breast cancer cells

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ABSTRACT

Direct-acting antivirals ledipasvir (LDV) and daclatasvir (DCV) are widely used as part of combination therapies to treat Hepatitis C infections. Here we show that these compounds inhibit the proliferation, invasion, and colony formation of triple-negative MDA-MB-231 breast cancer cells, SRC-transduced SW620 colon cancer cells and SRC- transduced NIH3T3 fibroblasts. DCV also inhibits the expression of PDL-1, which is responsible for resistance to immunotherapy in breast cancer cells. The demonstrated low toxicity in many Hepatitis C patients suggests LDV and DCV could be used in combination therapies for cancer patients. At the molecular level, these direct-acting antivirals inhibit the phosphorylation of Akt and the ephrin type A receptor 2 (EPHA2) by destabilizing a Src-EPHA2 complex, although they do not affect the general kinase activity of Src. Thus, LDV and DCV could be effective drugs for Src-associated cancers without the inherent toxicity of classical Src inhibitors.

1. Introduction

Cancer remains the second leading cause of death globally, accounting for nearly 10 million deaths in 2020, or nearly one in six deaths [1]. Newly diagnosed cases in 2022 in Spain correspond to a rate of 690 cases for 100k residents [2]. Despite the tremendous success of chemotherapy, multidrug resistance remains a major problem causing 90 % of deaths of patients treated with traditional chemotherapy [3]. An alternative to classical chemotherapy is immunotherapy. Immune checkpoint inhibitors have become the first line of treatment for non-small cell lung cancer or melanoma, although they are also affected by acquired resistance. Approximately one-fourth of melanoma patients relapse after treatment [4]. It is for this reason that the medical community works hard in the search and development of new drugs for the treatment of this disease.

Among the cancers with the worldwide highest incidence are lung (13.2 %) female breast (12.2 %), colorectal (10.3 %), prostate (7.8 %), and stomach cancer (5,2 %) [5]. Recently, a high throughput screening helped to identify the capacity of the FDA-approved antiviral DCV to counter enzalutamide resistance in prostate cancer by targeting the pseudokinase TRIB2, while the related antiviral, LDV, showed no effect on TRIB2-mediated enzalutamide resistance [6]. TRIB2 promotes Akt phosphorylation at Ser 473 and mediates the ubiquitination and degradation of transcription factors or other signaling proteins [7–9]. The antiviral target of LDV and DCV is the hepatitis C protein NS5A [10] with no similarity to TRIB2 except for the fact that both contain intrinsically disordered regions and are overexpressed in the respective environments. Overexpression of Src, whose intrinsically disordered region is directly involved in its transforming activity in NIH3T3 cells and human SW620 colon cancer cells [11], is found in many human cancers and participates in the development of cancer and progression to distant metastases [12]. Uncontrollable activation of Src has been reported in colorectal [13], breast [14], lung [15], prostate [16], and pancreatic cancer [17]. Src is also involved in the chemoresistance of cancer cells [18,19].

We wondered if LDV or DCV could counteract the transforming activity induced by overexpression of SRC in cancer cell lines. Thus, we tested these drugs on triple-negative MDA-MB-231 breast cancer cells, SW620 colon cancer cells (SRC transduced), and NIH3T3 fibroblasts (SRC transduced).

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We report that DCV and LDV can decrease colony formation in soft agar and cell invasion in all used cancer cells. Additionally, in SW620 (SRC transduced), we observed, upon treatment with LDV or DCV, a decrease in the activity of the ephrin receptor EPHA2, and a reduction in Akt activity. Similar effects were observed in triple-negative MDA-MB-231 breast cancer cells, including a marked decrease in the oncogenic and immunomodulatory membrane protein PDL-1.

Considering that LDV and DCV have very low toxicity as demonstrated in clinical trials and are widely used in the clinic for hepatitis C therapy, our results demonstrate that they could become part of new therapeutic strategies for the treatment of Src-dependent breast and colorectal cancers.

2. RESULTS

2.1. Ledipasvir and daclatasvir reduce viability, invasion, and colony formation of SRC-transduced NIH3T3 fibroblasts, SRC-transduced SW620 colon cancer cells, and triple-negative MDA-MB-231 breast cancer cells

We have explored the role of LDV and DCV on SRC oncogenic activity in SRC-transduced NIH3T3 fibroblasts, SRC-transduced SW620 colon cancer cells, and triple-negative MDA-MB-231 breast cancer cells. The SRC oncogenic activity of these cells, upon treatment with LDV and DCV, was assessed by measuring the effect of these molecules on cell viability, on their invasive properties (Boyden chambers coated with Matrigel), and on their anchorage-independent growth, determined by the number of colonies in soft agar.

LDV and DCV, in the range 0.5–20 μM, decreased cell viability (Fig. 1) and produced a marked reduction of both cell invasion (Fig. 2) and anchorage-independent cell growth (Fig. 3) in SRC transduced NIH3T3 fibroblasts, SRC transduced SW620 colon cancer cells and triple-negative MDA-MB-231 breast cancer cells. The IC50, that corresponds to the concentration of the drug that reduced the viability of the cells by 50 % is 16.5 μM, 16 μM and 13.3 μM for NIH3T3-SRC, SW620-SRC, and MDA-MB-231 cells respectively in the case of DCV. In the case of LDV the viability remained constant above 5 μM. The plasmatic concentration of DCV and LDV when used as antivirals are around 5 μM and 0.5 μM, respectively [20,21].

2.2. The antivirals ledipasvir and daclatasvir decrease Akt activity in SRC-transduced NIH3T3 fibroblasts, SRC-transduced SW620 colon cancer cells, and triple-negative MDA-MB-231 breast cancer cells

Akt is a serine/threonine protein kinase, and its activation, by phosphorylation of Ser 473, controls cell growth, transformation, differentiation, motility, and survival [22]. Increasing evidence has demonstrated that critical epigenetic modifiers are directly or indirectly modulated by PI3K/AKT signaling and participate in the oncogenicity of the PI3K cascade in cancers [23,24]. An Akt inhibitor reversed the increase in transforming properties induced in colorectal SW620 cancer cells [25]. We wanted to assess whether the oncogenic Akt pathway can be inhibited by the antivirals LDV and DCV.

SRC-transduced fibroblasts, SRC-transduced SW620 colon cancer cells, and triple-negative MDA-MB-231 breast cancer cells were incubated for two days in the absence or the presence of different concentrations of LDV and DCV in the range 0.5–5 μM. Immunoblots of cytosol protein extracts showed that LDV and DCV decrease AKT phosphorylation (Ser 473) in SRC-transduced NIH3T3 fibroblasts (Fig. 4 A-B), in SRC-transduced SW620 colon cancer cells (Fig. 4 C-D), and in MDA-MB-231 triple-negative breast cancer cells (Fig. 4 E-F).

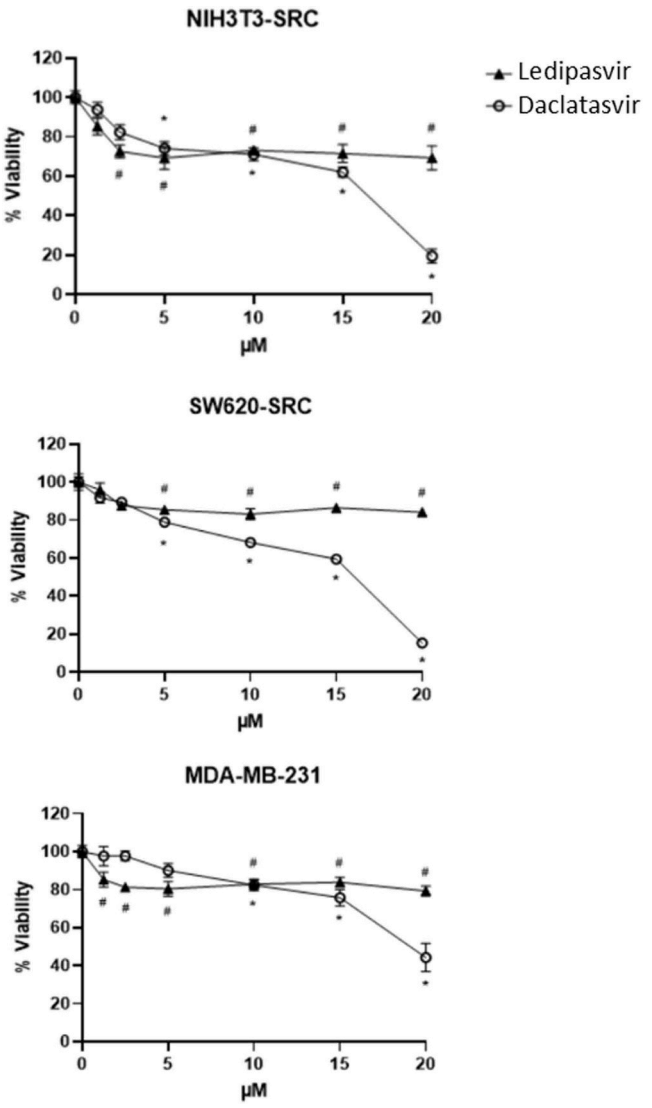

Fig. 1. ***Effect of ledipasvir and daclatasvir on the viability of NIH3T3-SRC, SW620-SRC, and MDA-MB-231 cells.*** NIH3T3-SRC, SW620-SRC, and MDA-MB-231 cells were seeded and treated with increasing doses of LDV or DCV and viability was evaluated using an MTT assay. Data is representative of two independent experiments. Data represents mean ± SEM (n=4–6/group in each experiment). Data were analyzed using two-way ANOVA followed by Dunnett's pos-hoc to compare with the non-treated group. *$p<0.05$ for LDV treatment, # $p<0.05$ for DCV treatment, both compared with the non-treated group.

2.3. Daclatasvir decreases the activity of the ephrin receptor EPHA2 in SRC-transduced colorectal SW620 cancer cells and MDA-MB-231 breast cancer cells

EPHA2 binds to ephrins and regulates cell-cell contacts through bidirectional signaling in neighboring cells. Deregulation of the EPHA2/Ephrin system is frequently observed in human cancer and might participate in malignancy progression [26,27]. In SW260 colorectal cancer cells the activation of the ephrin receptor EPHA2 is essential for the promotion of tumor cell growth and invasion. Src activates the downstream receptor tyrosine kinase EPHA2 through the phosphorylation of Tyr594 and EPHA2 activates the oncogenic Akt signaling pathway through phosphorylation of Ser473 [25].

We show here that upon treatment with 5 μM DCV Y594 EPHA2 phosphorylation decreases in Src transduced SW620 colon cancer cells and in MDA-MB-231 triple negative breast cancer cells (Fig. 5).

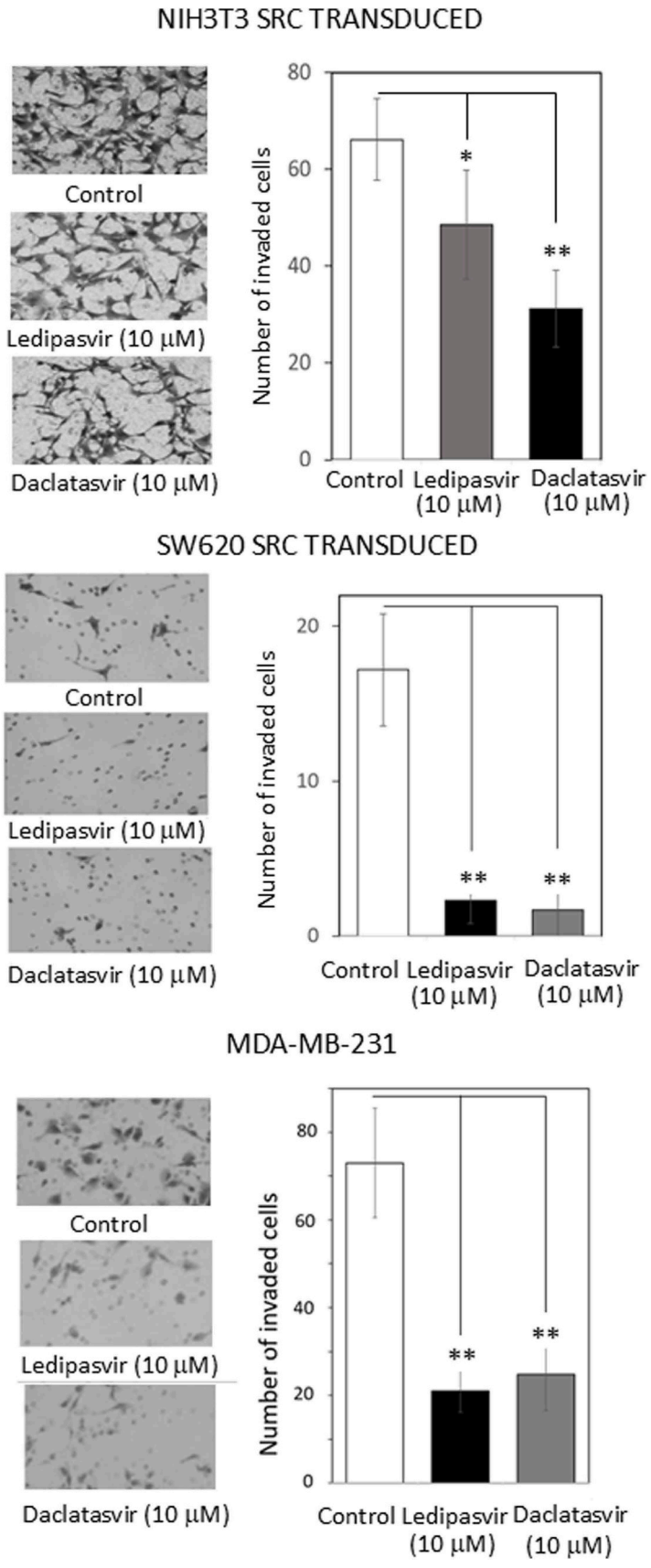

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Fig. 2. ***Effect of ledipasvir and daclatasvir treatment on cell invasion in SRC-transduced NIH3T3 fibroblasts, SRC-transduced SW620 colon cancer cells, and triple negative MDA-MB-231 breast cancer cells.*** Cells were treated with either DMSO (control), LDV, or DCV and seeded on polycarbonate filters coated with Matrigel, as described in the Materials and Methods Section, and incubated for 24 h. Quantification of invaded cells represents the mean number of cells per field counting seven random fields at 40x or 20x magnification. Treatment with LDV and DCV markedly decreased cell invasion in SRC-transduced NIH3T3 fibroblasts, SRC-transduced SW620 colon cancer cells, and triple negative MDA-MB-231 breast cancer cells. Statistical analyses were performed using unpaired t-test. Data from three independent experiments are expressed as means plus or minus SD. *, $p<0.05$, compared with the control¸**, $p<0.01$, compared with the control.

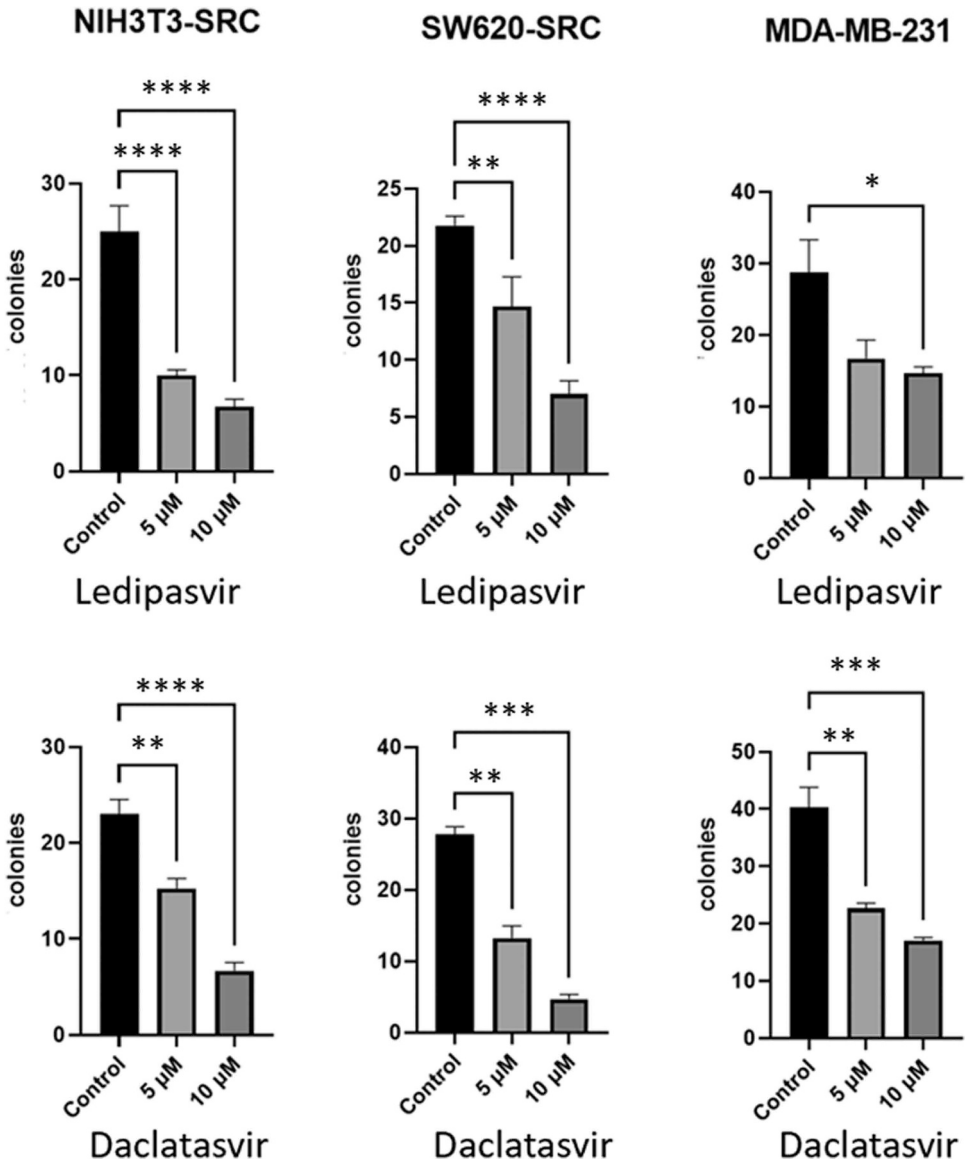

Fig. 3. ***Effect of ledipasvir and daclatasvir treatment on colony formation of SRC transduced NIH3T3 fibroblasts, Src transduced SW620 colon cancer cells, and triple negative MDA-MB-231 breast cancer cells.*** Cells were seeded on a 24-well plate at a density of 10000 cells/well. The cells were treated with 5 μM and 10 μM of LDV or DCV and the number of colonies were compared with the control group (without treatment). Data were analyzed by ordinary one-way ANOVA followed by Dunnett's pos-hoc to compare with the control group, *$p<0.05$, **<0.01, ***$p<0.001$, ****$p<0.0001$.

2.4. Daclatasvir and ledipasvir inhibit the formation of an EPHA2-Src complex in SRC-transduced SW620 cells

In SW620 colon cancer cells, Akt activity was strongly reduced by PP2, an inhibitor of Src, suggesting that this signaling pathway is under the control of Src activity [25]. However, treatment with DCV in a range between 0.5 and 10 μM decreases Akt activity without affecting Src activity (Fig. 6A). This observation indicates that the downregulation by DCV of EPHA2 and Akt activity in the SRC/EPHA2/AKT oncogenic pathway is not dependent on the inhibition of Src. As shown in Fig. 6B, we were able to detect the co-precipitation of endogenous EPHA2 with Src in SRC-transduced SW620 colorectal cancer cells. Immunoprecipitations were performed using an anti-pSrc Y416 antibody followed by Western blotting with an anti-EPHA2 antibody. After treatment with LDV or DCV 10 μM, the amount of EPHA2 co-precipitated with pSrc from the cell membrane of SW620 cells decreased markedly.

These data suggest that LDV and DCV can decrease the interaction of Src with the downstream substrate EPHA2, interfering with the oncogenic pathway SRC-EPHA2-AKT without changing Src activity.

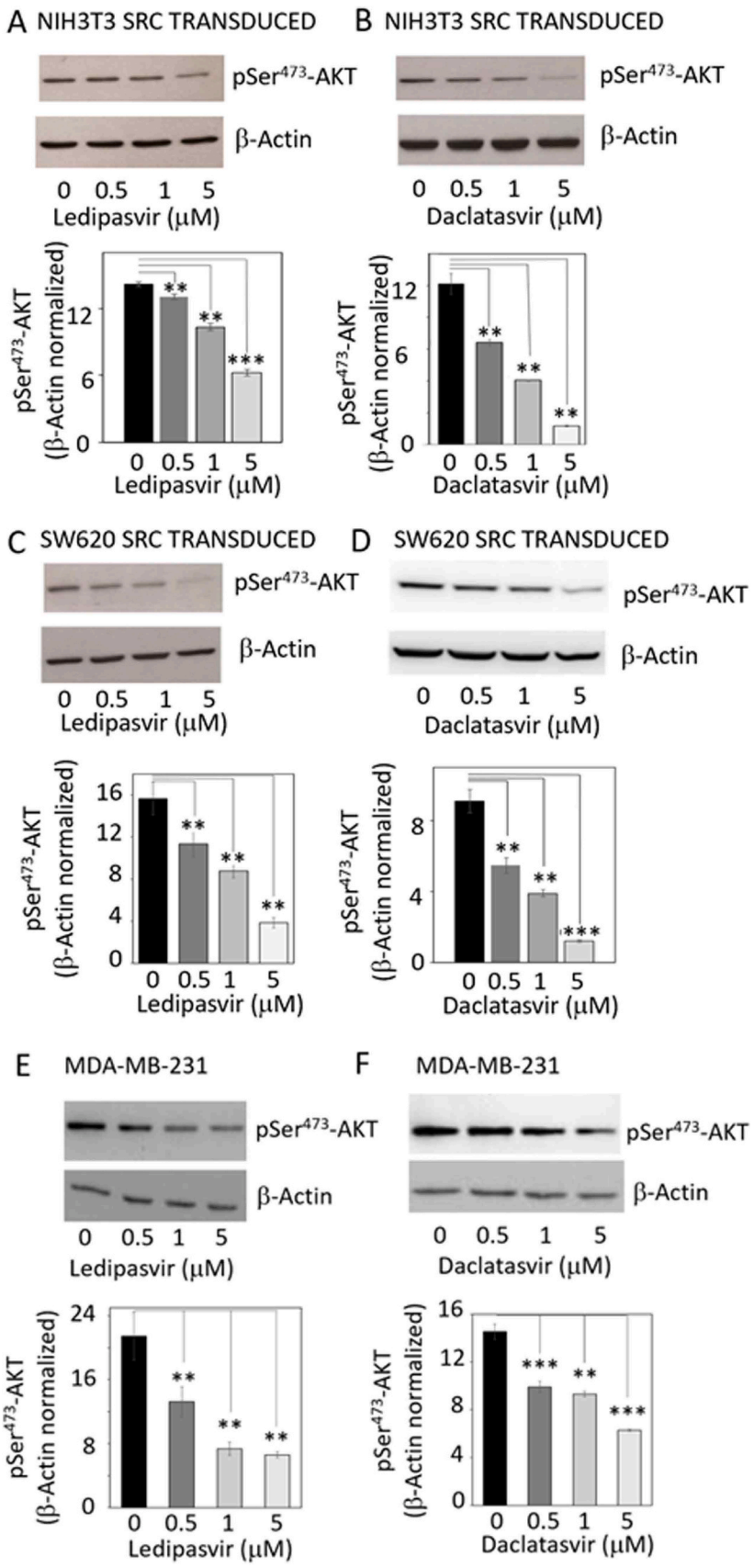

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Fig. 4. ***Ledipasvir and daclatasvir decrease AKT phosphorylation (Ser 473) in SRC-transduced fibroblasts, SRC-transduced colon cancer cells, and triple negative MDA-MB-231 breast cancer cells.*** SRC-transduced fibroblasts, SRC-transduced SW620 colon cancer cells, and triple negative MDA-MB-231 breast cancer cells were incubated for two days in the absence or the presence of different concentrations of LDV and DCV in the range 0–5 μM. Immunoblots of cytosol protein extracts show the decrease of AKT phosphorylation (Ser 473) in SRC-transduced fibroblasts treated with LDV (A) and DCV (B), in SRC-transduced SW620 colon cancer cells treated with LDV (C) and DCV (D), and in triple negative MDA-MB-231 breast cancer cells treated with LDV (E) and DCV (F). Cells incubated with DMSO were utilized as controls. Statistical analyses were performed using unpaired t-test. Data from three independent experiments are expressed as means plus or minus SD. **, $p<0.01$, compared with the control; ***, $p< 0.001$, compared with the control. Actin was used as a loading control.

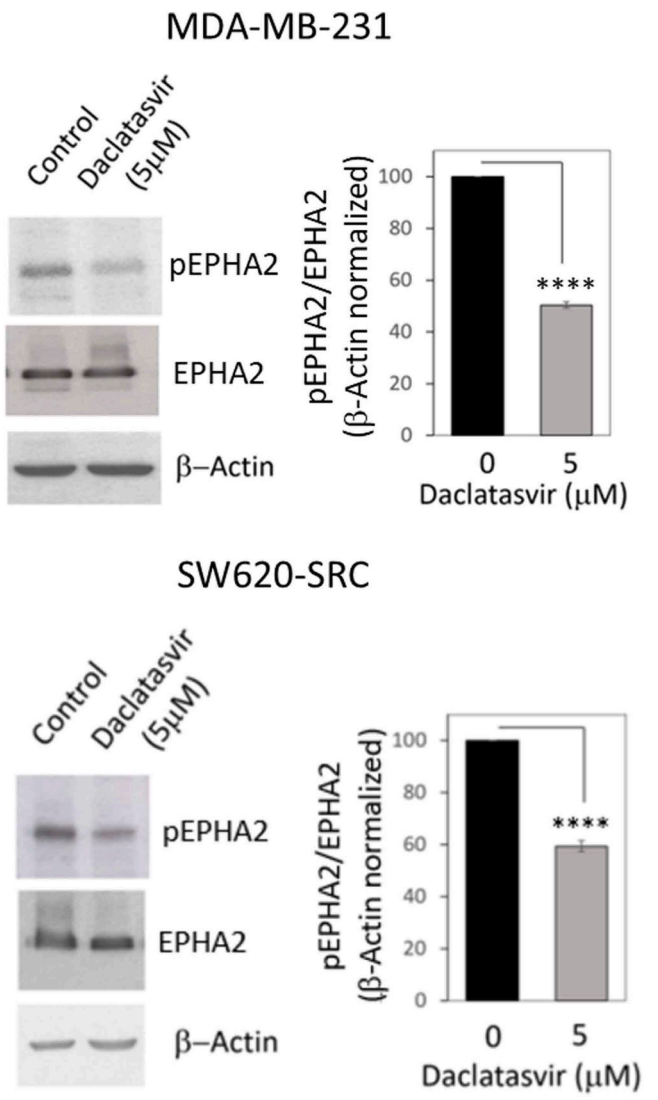

Fig. 5. ***Daclatasvir decreases phosphorylation of the ephrin receptor EPHA2 in MDA-MB-231 breast cancer cells and in SRC-transduced SW620 colon cancer cells.*** MDA-MB-231 breast cancer cells and SRC-transduced SW620 colorectal cancer cells were incubated for two days in the absence or the presence of DCV. Immunoblots of membrane protein extracts show the decrease of EPHA2 phosphorylation in MDA-MB-231 breast cancer cells treated with DCV (A) and in SRC-transduced SW620 colon cancer cells treated with DCV (B). Cells incubated with DMSO were utilized as controls. Statistical analyses were performed using unpaired t-test. Data from three independent experiments are expressed as means plus or minus SD. ****, $p<0.0001$, compared with the control. Actin was used as a loading control.

2.5. Daclatasvir reduces the expression of PDL-1 in triple-negative breast cancer cells

Akt signaling promotes not only breast cancer cell invasion but also PDL-1 expression which leads to the destruction of the effector T cells. We wondered whether DCV was able to decrease PDL-1 expression in MDA-MB-231 breast cancer cells. Indeed, we found that 5 μM DCV downregulates PDL-1 expression along with Akt activity (Fig. 7).

3. Discussion

Our study reveals that the antiviral molecules LDV and DCV downregulate the oncogenic signaling pathway SRC/EPHA2/AKT, decreasing cell invasion and colony formation in SRC transduced fibroblasts, SRC transduced SW620 colon cancer cells and triple-negative MDA-MB-231 breast cancer cells. DCV also reduces the expression of PDL-1 in MDA-MB-231 cells, thus potentially enhancing T-cell-mediated anticancer immunity.

A previous report had shown that DCV counters enzalutamide resistance in prostate cancer by targeting the pseudokinase TRIB2 and decreasing Akt activity [6]. While this first report uncovered the potential of DCV in oncology, the present work suggests a wider applicability to Src-dependent colorectal and breast cancers through a different mechanism. While TRIB2-dependent enzalutamide resistance was only affected by DCV and not by LDV, both DCV and LDV showed similar effects on SRC-transduced SW620 and MDA-MB-231 cells. Furthermore, these cells have low levels of TRIB2 (supplementary figure 1), consistent with a TRIB2-independent mechanism.

Akt is a serine/threonine protein kinase, and its activation controls cell growth, transformation, differentiation, motility, and survival [22]. Combined targeting of Akt and Src resulted in a synergistic efficacy against human pancreatic cancer growth and metastasis [28]. Increasing evidence has demonstrated that critical epigenetic modifiers are directly or indirectly modulated by PI3K/AKT signaling and participate in the oncogenicity of the PI3K cascade in cancers [23,24].

EPHA2 is a receptor tyrosine kinase overexpressed in human cancers and is often linked to poor patient prognosis [27]. Accumulating evidence demonstrates that EPHA2 plays important roles in several critical processes associated with malignant cancer progression, such as proliferation, survival, migration, invasion, drug resistance, metastasis, and angiogenesis. EPHA2 has become a promising therapeutic target for cancer treatment [25–27]. Src phosphorylates EPHA2 on Tyr594, and phosphorylated EPHA2 is an upstream activator of Akt [25]. In addition to Src activity, a decrease in the amount of phosphorylated EPHA2 downregulates the SRC/EPHA2/AKT oncogenic signaling pathway [25].

PDL-1 is expressed in 20 % of triple-negative breast cancers and it is downregulated by Akt inhibition [29]. PDL-1, expressed on the surface of cancer cells, interacts with PD-1 expressed on the surface of T-cells and suppresses T-cell-mediated anticancer immunity [30]. In addition, interactions between PDL-1 and PD-1 not only suppressed anticancer T-cell immunity but also induced chemoresistance in breast cancer cells sustaining activation of Akt [31,32].

Our results show that DCV decreases the amount of phosphorylated Akt and phosphorylated EPHA2 in triple-negative MDA-MB-231 breast cancer cells and SW620 colorectal cancer cells. Similar results are obtained with LDV. However, these drugs are not general Src kinase inhibitors. This apparent contradiction can be solved by the observation that both DCV and LDV inhibit the formation of a specific complex between Src and EPHA2 in SRC-transduced SW620.

Although Src inhibitors such as PP2 can reduce Akt activity, promiscuous inhibition of other kinases as well as unselective inhibition of the kinase activity of Src results in toxicity in healthy cells. Inhibiting Akt phosphorylation without affecting Src kinase activity using DCV or LDV provides a selective way to inhibit the oncogenic SRC/EPHA2/Akt

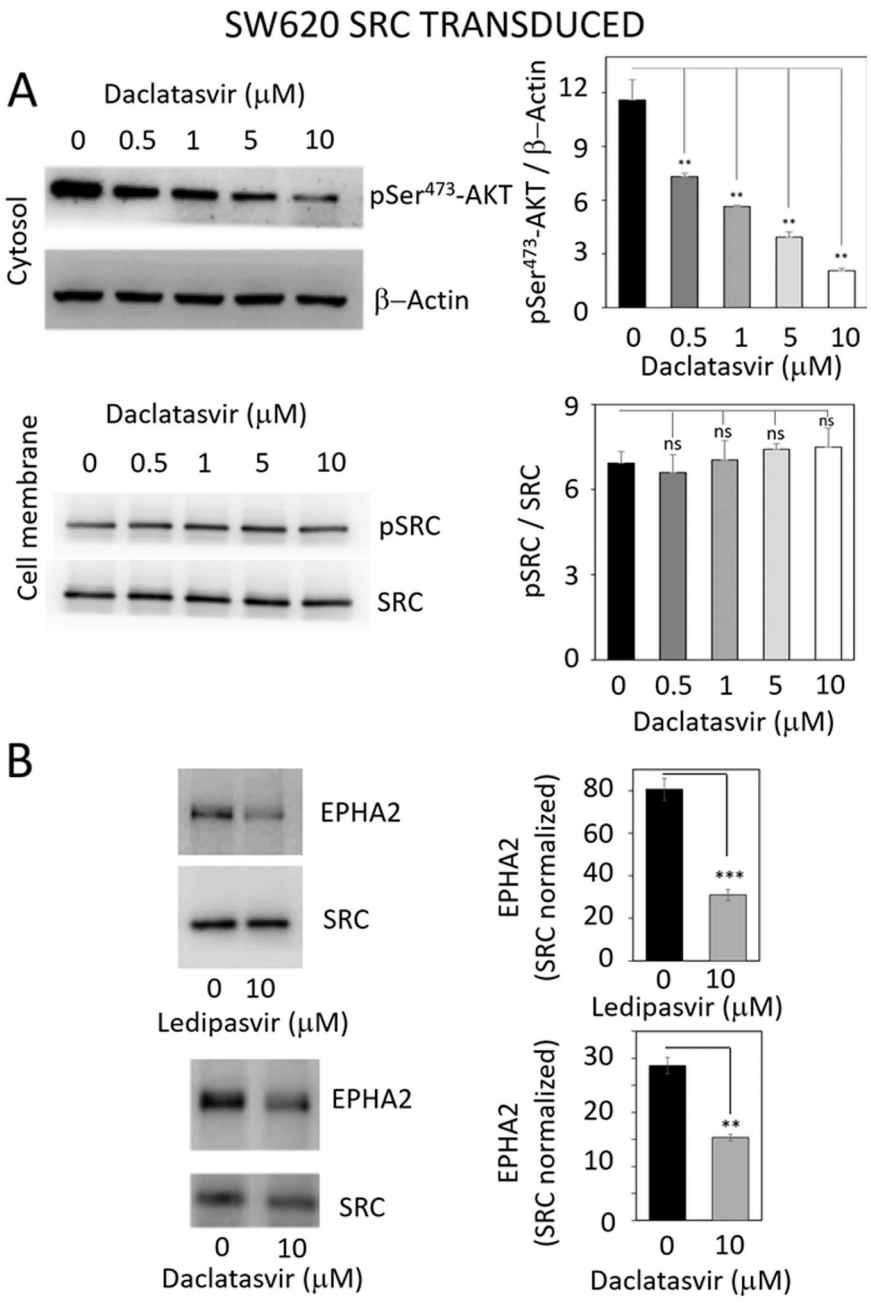

Fig. 6. ***Daclatasvir and ledipasvir decrease AKT activity in SW620 colon cancer cells cytosol without affecting the membrane Src activity but decreasing the co-immunoprecipitation of endogenous EPHA2 with SRC.*** *A) The relative band intensity quantification of pSer 473 -AKT in SRC-transduced SW620 colon cancer cells show a decrease in the activity of AKT with increasing concentrations of DCV without any change in the ratio pSrc/Src in the cell membrane. The effect of additional DCV concentration increases above 0.5 μM* were also statistically significant. β-Actin was used as a loading control. B) SW620 Src transduced cells were lysed after treatment, and immunoprecipitations from the cell membrane were performed with anti-P-Src Y416 antibody followed by immunoblotting with anti-EPHA2. The membrane was then stripped and reproved with anti-SRC antibody. Cells incubated with DMSO were utilized as controls. Statistical analyses were performed using unpaired t-test. Data from three independent experiments are expressed as means plus or minus SD. **, $p<0.01$, compared with the control. ***, $p<0.001$ compared with the control, ns (non-significant difference) compared with the control. Actin was used as a loading control.

oncogenic pathway. Interestingly, mutations in a conserved small region of the N-terminal IDR of Src [33] caused a similar inhibition of the same axis, without affecting Src kinase activity, in NIH3T3 cells and human SW620 colon cancer cells.

While Src is rarely the primary cause of cancer, Src overexpression is associated with poor prognosis in many cancers, and Src is recognized as an important cancer target. However, Src-directed drugs have failed in solid tumors, such as colorectal or breast cancer partially because of unacceptable side effects. LDV and DCV are FDA-approved and used to treat a very large population of chronically infected Hepatitis C patients [34] and show very low toxicity. While in most clinical trials of antivirals cancer patients were excluded, in the observational study NCT03423641 (33,808 patients, including cancer patients) lower cancer incidence was observed in patients treated with direct-acting antivirals [35,36] although the possible direct implication of antivirals as anti-cancer drugs was not discussed.

The anticancer activity by direct-acting, low toxicity, antivirals LDV and DCV in several cancer cell lines that share high levels of Src expression represents a potentially important addition to cancer therapy.

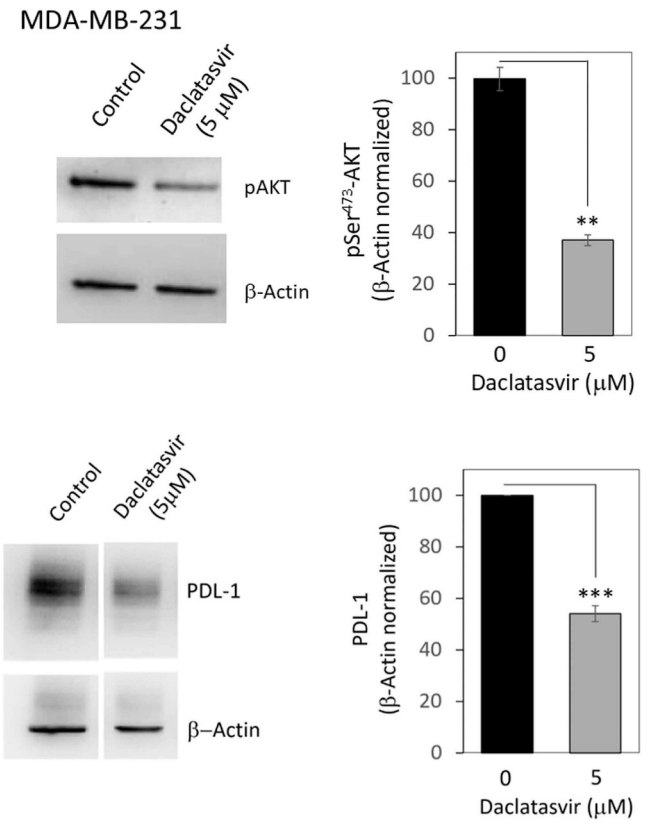

Fig. 7. ***Daclatasvir reduces Akt activity and the expression of PDL-1 in triple-negative MDA-MB-231 breast cancer cells.*** MDA-MB-231 breast cancer cells were incubated for two days in the absence or the presence of 5 μM DCV. Immunoblots of membrane proteins show a decrease of Akt activity (A) and PDL-1 expression (B). Cells incubated with DMSO were utilized as controls. Statistical analyses were performed using unpaired t-test. Data from three independent experiments are expressed as means plus or minus SD. **, $p<0.01$, compared with the control, ***, $p<0.001$, compared with the control. Actin was used as a loading control.

4. MATERIALS AND METHODS

4.1. Chemical compounds

Ledipasvir and daclatasvir were purchased from MedChemExpress (NJ) and used without further purification from aliquoted 20 mM stock DMSO solutions. To treat cells, we prepared the treatment media diluting the drug stocks into complete DMEM media, mixing with a vortex mixer and sonicating using an ultrasound bath at 37°C to allow the complete dissolution of drugs.

4.2. Cell cultures, retroviral infections, and transfections

NIH3T3 and SW620 SRC transduced cell lines cultured, transfected, and infected as described in [25], were a gift from Dr Serge Roche, University of Montpellier, FR. MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle medium/Ham's F12 (1:1) supplemented with 10 % fetal bovine serum (FBS), 2 mM glutamine, and 1 % Penicillin/Streptomycin solution (10,000 units each). Cells were maintained in a 5 % CO_2 incubator at 37 °C. All cell lines were used at low passage (<20) and regularly assessed against mycoplasma.

4.3. Cell viability assay

Cell viability was measured by an MTT assay. Cells were seeded into 96-well plates and cultured for one day. The following day, the medium was changed to a treatment medium in the absence or presence of varying concentrations of LDV and DCV and incubated for 72 h. After the incubation period, MTT solution was added to each well and an incubation for 2 h was performed. Then, the medium was removed from the plates, and the MTT crystals were homogenized by adding acidified isopropyl alcohol. To homogenize the pellets more efficiently, the plates were shaken for 5 min on a shaker. Afterward, the plates were read under 570/690 nm wavelengths by a plate reader (Synergy HTX, Biotek).

4.4. Soft agar colony formation assay

Cells were plated at a density of 10,000 per well in 24-well plates in 0.3 % soft agar on top of a base layer of 0.7 % agar. The cells were treated with 5 μM and 10 μM of LDV or DCV, and the plates were incubated for 3 weeks, replacing the media every week. At the end of the incubation period, colonies were stained by adding 200 μL of nitroblue tetrazolium chloride solution (1 mg/mL) per well and incubating plates overnight at 37°C. Once colonies were stained, photographs were taken with an optical stereomicroscope (MZ216F, Leica) and colonies were counted using ImageJ software. These experiments were repeated twice with 4 replicates per group, and only colonies with >50 cells were counted.

4.5. Cell invasion assay

In vitro, invasion assay was done using 8-μm pore size transwell insert (Sarstedt) coated with 100 μL of 1 mg/mL Matrigel solution (Corning). Coated inserts were left at the cell incubator overnight to allow the polymerization of the Matrigel. Then, 50000 cells (in DMEM without FBS) with or without treatment were placed into the upper chambers. These chambers were then placed in a 24-well plate with 750 μL of DMEM 10 % FBS as a chemoattractant. Cells were incubated at 37ºC in the CO_2 incubator for 24 hours. Non-invaded cells were scraped with a cotton swab and membranes were fixed in 4 % PFA, stained with 0.5 % crystal violet, and observed under a Leica microscope at ×200.

4.6. Immunoblotting

Cells were lysed on ice in NP40 lysis buffer: 150 mM NaCl, 20 mM HEPES (pH 7.5), 0.5 % NP-40, a cocktail of protease inhibitors (Complete, Roche, Basel, Switzerland), and phosphatase inhibitors (Calbiochem, Merck Darmstadt, Germany). Membrane and cytosol extracts were obtained using Abcam's Fraction-PREP Cell Fractionation Kit (ab28808). Protein extracts were separated on SDS-PAGE (10 % acrylamide), transferred to PVDF membranes, and probed with antibodies. Antibodies for Src (#2123), Phospho-Src (Tyr416) (#2101 L), anti-Akt (#9272S), anti-Akt pS473 (#4060S), anti-EPHA2 pY594 (#3970S), anti-EPHA2 (#6997S) were obtained from Cell Signaling Technology (Beverly, MA). The antibody for β-Actin was from Sigma (A5716).

4.7. Statistical analysis

One-way ANOVA was performed when more than two groups were compared, followed by Dunnet's post-hoc. Unpaired Student's t-test statistical analysis was used when two groups were compared. Statistically significant differences were considered when the level of confidence was above 95 % (p-value < 0.05). The number of samples per group used and the statistical analysis used are specified in each figure legend. All statistical analyses and figures have been generated using GraphPad Prism 9.5.1 (GraphPad Software) or Excel (Microsoft, Redmond, WA).

CRediT authorship contribution statement

Betlem Mezquita: Writing – review & editing, Writing – original draft, Investigation. **Marjorie Reyes-Farias:** Writing – review & editing, Visualization, Investigation. **Miquel Pons:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117325.

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