ORIGINAL ARTICLE

STAT3 inhibition reduces toxicity of oncolytic VSV and provides a potentially synergistic combination therapy for hepatocellular carcinoma

S Marozin¹, J Atomonete¹, KA Muñoz-Alvarez¹, A Rizzani², EN De Toni², WE Thasler¹, RM Schmid¹ and O Ebert¹

Hepatocellular carcinoma (HCC) is a refractory malignancy with a high mortality and increasing worldwide incidence rates, including the United States and central Europe. In this study, we demonstrate that a specific inhibitor of signal transducer and activator of transcription 3 (STAT3), NSC74859, efficiently reduces HCC cell proliferation and can be successfully combined with oncolytic virotherapy using vesicular stomatitis virus (VSV). The potential benefits of this combination treatment are strengthened by the ability of NSC74859 to protect primary hepatocytes and nervous system cells against virus-induced cytoxicity, with an elevation of the VSV maximum tolerated dose in mice. Hereby we propose a strategy for improving the current regimen for HCC treatment and seek to further explore the molecular mechanisms underlying selective oncolytic specificity of VSV.

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INTRODUCTION

Signal transducer and activator of transcription 3 (STAT3) is an important member of the family of STAT. Its signaling pathway is closely associated with the proliferation, differentiation, and apoptosis of cells, and constant activation of STAT3 can promote cell proliferation and carcinogenesis. At present, STAT3 is defined as an oncogene; constitutive activation of STAT3 was first described in transformed cells as a consequence of the oncogenic tyrosine kinases, including v-src, NPM-ALK and RET oncogenes.¹⁻³ In normal cells, activation of STAT3 is a transient process and undergoes strict control to prevent unscheduled gene regulation. However, persistently activated STAT3 has been reported in numerous human cancers (colon and gastric cancer, ovarian cancer, lymphomas, adult T-cell leukemia, gliomas), also including many hepatocellular carcinoma (HCC) cell lines and primary tissues.⁴⁻⁶ STAT3 is involved in oncogenesis through the upregulation of genes encoding apoptosis inhibitors,⁷⁻⁹ cell-cycle regulators,¹⁰⁻¹¹ and inducers of angiogenesis.¹²⁻¹³ The ability of STAT3 to prevent apoptosis supports the potential use of inhibitors of STAT3 as chemo-sensitizers and indicates STAT3 as a novel target for cancer therapy. Small-molecule inhibitors of STAT3 have been shown to reduce tumor cell survival and to induce apoptosis in cells harboring constitutively active STAT3 both in vitro and in vivo.¹⁴⁻¹⁷

HCC is the third leading cause of cancer deaths worldwide, with few effective therapeutic options for advanced disease. HCC is highly resistant to conventional systemic therapies; therefore, further improvement in the management of advanced disease is needed. In recent years, identification of signaling pathways responsible for HCC growth and progression has uncovered crucial molecular targets and led to the development of novel targeted approaches for treatment of this malignancy. It has been shown that inhibition of STAT3 signaling in cancer is usually sufficient to induce tumor cell apoptosis and cause tumor growth inhibition and regression in vivo.¹⁴,¹⁸⁻²¹ Additionally, blocking STAT3 activation also triggers antitumor immune responses by increasing natural killer-mediated cell cytotoxicity.²² For this reason, STAT3 is emerging more and more as a potential molecular target for HCC treatment.

The use of vesicular stomatitis virus (VSV) as an oncolytic agent is particularly promising as it selectively destroys a wide variety of different tumors, including HCC.²³⁻²⁶ As in the case of other oncolytic agents, VSV preferentially kills tumor cells but can replicate, even if to a lesser degree, in normal cells. As a consequence, the therapeutic doses are often significantly restricted due to cytopathic events in healthy tissues.²⁷ Therefore, various efforts have been made in the attempt to broaden the therapeutic index of VSV, mainly by genetically engineering the virus to target specific molecules or signal transduction pathways in cancer cells.²⁸⁻³¹

Here we tested a new therapeutic modality to potentiate antitumor activity by selective blockade of STAT3 signaling with inhibitor NSC74859 (named also S31-201) combined with VSV infection. Antitumor activity of NSC74859 is mediated by inhibition of STAT3 dimer formation, DNA binding and block of transcriptional activity.²⁸ NSC74859 powerfully reduced tumour-cell growth in vitro, and STAT3 inhibition did not interfere with tumor selectivity of VSV, allowing the virus to replicate to high titers in HCC cell lines. Importantly, human primary hepatocytes were protected from VSV cytotoxicity in the presence of NSC74859, and the attenuation of viral growth was not linked to the interferon (IFN) response. Similar results were obtained in

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primary rodent neurons and glia cells and correlated to reduced virus toxicity in vivo, with a significant increase of the maximum tolerated dose (MTD) of VSV in the presence of STAT3 inhibitor NSC74859 in mice.

MATERIALS AND METHODS

Cell lines and primary cells and viruses

Two human HCC cell lines (HepG2, Huh-7) were obtained from Dr. Ulrich Lauer (University Hospital Tübingen, Tübingen, Germany) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), 1% Penicillin/streptomycin, 1% non-essential amino acids and 1% sodium pyruvate as previously described. All cell cultures were regularly tested for mycoplasma contamination.

Primary differentiated rat neurons and glia cells were kindly provided by Dr. Frank Bradke (Max Planck Institute, Munich, Germany) as previously described. Frozen primary HCC samples and short-term cultures of primary human hepatocytes (PHH) were derived from patients (negative for hepatitis B virus, hepatitis C virus and HIV) who underwent surgical resection of hepatocellular tumors and gave informed consent, in accordance with the guidelines of the charitable state-controlled foundation Human Tissue and Cell Research (Regensburg, Germany). PHHs were maintained in culture with Hepatocyte-SFM medium (GIBCO, Darmstadt, Germany) containing 1% L-glutamine.

Wild-type VSV-GFP (green fluorescent protein) was generated as previously described. Virus stocks were produced on BHK-21 cells and stored at −80°C. Titers were determined by plaque assay on BHK-21 cells.

Western blotting

Whole-cell extracts were run on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Total cell lysates were prepared using lysis buffer (Cell lysis buffer, Cell Signaling Technology Inc, Danvers, MA, USA) containing a protease and phosphatase inhibitor cocktail. Protein concentration in the samples was determined using the BCA Protein Assay Kit (Pierce, Rockville, IL, USA). After blocking for 1 h with 5% skim milk/TBS-Tween, the membranes were blotted with the following primary antibodies overnight at 4°C: antibodies against STAT3 (Cell Signaling, no. 12640), the phosphorylated form of STAT3 (Tyrosine 705/Ser 727 Cell Signaling, nos. 9131 and 9134) and STAT1 and phosphorylated STAT1 (Cell Signaling, nos. 9175 and 8826); anti-VSV mouse antibody (kindly provided by Dr. Doug Lyles, Wake Forest Baptist Medical Center, NC, USA), cleaved pro-ADP ribose polymerase (PARP; Cell Signaling, no. 9541), and Actin (Sigma-Aldrich, St Louis, MO, USA, no. A3853). After secondary staining with anti-rabbit or anti-mouse peroxidase-conjugated Abs (Jackson ImmunoResearch Laboratory Inc., West Grove, PA, USA), nos. 111-035-003 and 115-035-003; blots were washed three times with TBS-Tween. Protein bands were visualized on Amersham Hyper-Map film by the ECL chemiluminescence system as recommended by the manufacturer (Amersham, Buckinghamshire, UK).

Treatment with chemicals and biochemical reagents

Cells were seeded at 80−90% confluency in 24-well plates overnight. The morning after, the medium was replaced with Dulbecco’s modified Eagle’s medium/10% fetal calf serum containing dimethyl sulfoxide (DMSO) or STAT3 inhibitor NSC74859 at the indicated concentration. Cultures were pretreated for 16 h, and virus infections were carried out in the presence of freshly added inhibitors. Alternatively, inhibitor NSC74859 was added after viral absorption. Viral titers were determined by TCID50. Similar experiments were also performed using sorafenib (LC Laboratories, Woburn, MA, USA), the extracellular signal-regulated (ERK) inhibitor U0126 (Merck Chemicals, Hissen, Germany), and the IκB inhibitor LY294002 (Merck Chemicals). IFN treatment was performed using universal type I IFN (Human Interferon Alpha A/D [BglII]) from PBL Interferon Source (Piscataway, NJ, USA) at the concentration of 100 IU·ml−1.

Induction of apoptosis in human primary hepatocytes was achieved using a Fas human monoclonal antibody (APO1-3) from Alexis (ALX-805-020; Enzo Life Science, Lörrach, Germany) with cross-linking using recombiant Protein A (BioVision, Milpitas, CA, USA). The APO1-3 antibody was used at a concentration of 2 μg·ml−1 together with Protein A (0.02 μg·μl−1).

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants of mock-treated (DMSO) and NSC74859-treated (STAT3) primary hepatocytes stimulated with Poly I:C or infected with VSV were analyzed for soluble IFN-gamma-inducible protein (IP-10) by ELISA. Poly I:C was purchased from Invivogen (San Diego, CA, USA) and used at a concentration of 1 μg·ml−1. Human IP-10 ELISA was performed following the instructions of the manufacturer (RayBiotech Inc., Norcross, GA, USA).

siRNA assay

For siRNA experiments, reverse transfection was performed using Lipofectamine RNAiMax (Invitrogen). Cells in 24-well plates were transfected either without siRNA or with 100 nM of scrambled siRNA or specific siRNA (100 nM) according to the manufacturer’s instructions using Lipofectamine RNAiMAX (Invitrogen, Karlsruhe, Germany). siRNAs were purchased from Cell Signaling. After 60−72 h of transfection, cultures were infected with wild-type VSV at an multiplicity of infection (MOI) of 0.1 for 16 h. Titers were determined by TCID50. Cell lysates from non-infected duplicates were analyzed by western blotting to assess the efficiency of the RNA silencing against STAT3 expression.

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay

Cell viability, as an indicator of cytotoxicity, was determined by the capacity of cells to reduce MTS into a formazan. The assay was performed according to the manufacturer’s instructions (Promega, Madison, WI, USA).

Quantitative real-time PCR

PHH and HCC cells were grown in six-well plates and were mock-treated (DMSO) or treated with 100 μM of NSC74859 for 16 h. Infection with VSV-GFP was carried out at an MOI of 1, and cell lysates were collected at the indicated time points. Cell debris was eliminated by centrifugation, and total RNA was extracted according to the manufacturer’s instructions by using the High Pure Viral RNA Kit (Roche, Mannheim, Germany). The reverse transcriptase reaction was performed using the QuantiTect Probe Reverse Transcription Kit (Qiagen) and Real-Time PCR was carried out with the KAPA SYBR FAST qPCR Fast LightCycler 480 kit (PeqLab, Erlangen, Germany), using 15 ng of the template. The relative values for mRNA extracted from cell lysates were quantified by normalizing the expression level of the gene of interest to the internal housekeeping control (glyceraldehyde 3-phosphate dehydrogenase).

In vivo experiments

All animal procedures were approved and performed according to the guidelines of the institution’s animal care and use committee, as well as the local government of Bavaria, Germany. Six-week-old male C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed under standard conditions. A dose-escalation study to determine the MTD of VSV was performed in healthy C57BL/6 mice via tail-vein infusion of wild-type VSV. The animals were randomized in groups of five and were infused with VSV at the indicated vector doses in combination with STAT3 inhibitor treatment or with control buffer. Although no statistical analysis was applied, the sample size was chosen based on previous knowledge of variability in toxicity experiments. Randomization was performed by arbitrarily assigning animals to a dosage group prior to the onset of the experiment. The investigators involved were not blinded to the group allocation. Animals were given NSC74859 intraperitoneally at 5 mg·kg−1·day−1 before VSV administration and every other day thereafter for the remainder of the experiment. Animals were monitored daily for the occurrence of toxic events and survival over a 14-day period and euthanized at humane end points by cervical dislocation.

Statistical analysis

For in vitro experiments with cell lines, pilot studies were performed in order to estimate the effect size. Based on this, statistical power calculations were applied, and it was determined that experiments should be performed with biological duplicates or triplicates and experiments performed in triplicate. Variance was similar between groups that were statistically compared. Because of greater variability among preparations of...
PHH, more experiments were performed in order to ensure adequate power. Data were analyzed for statistical significance using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Individual data points were compared by applying a two-sided Student’s t-test, and P values of < 0.05 were considered statistically significant.

RESULTS

STAT3 inhibitor NSC74859 has antiviral effects in human primary hepatocytes but not in HCC cells

We first determined whether inhibition of STAT3 activation could interfere with VSV replication in HCC cells compared with PHH. Ten human HCC cell lines, HepG2 and Huh-7, were pretreated for 16 h with escalating concentrations of STAT3 inhibitor NSC74859 (STAT3i) and infected with rVSV-GFP at an MOI of 0.1 for 24 h. Overall, NSC74859 did not affect viral growth; a small but significant drop in viral titer was observed when the maximum concentration (200 μM) was applied to Huh-7 cells (Figure 1a). As NSC74859 just marginally reduced the activated form of STAT3 (Figure 1b), we tested whether viral attenuation was attributable to cytotoxic effects. At the concentration of 200 μM, NSC74859 indeed compromised cell viability as shown in Figure 1c, which correlated to the lower viral titers. The reported antiproliferative activity of the STAT3 inhibitor in HCC cells7,21 was confirmed by MTS assay over a period of 72 h. Already at the concentration of 50 μM cells grew markedly less than controls and at 100 μM a cytostatic effect was evident (Figure 1d).

In contrast to HCC cells, reduced VSV replication in response to the STAT3 inhibitor was appreciable in PHH in a dose-dependent manner (Figure 2a). Furthermore, in PHH, administration of the inhibitor after viral absorption attenuated VSV replication even if to a lesser extent (Supplementary Figure S1). Additionally, inhibitory activity of NSC74859 was not influenced by the MOI used for infection; comparable fold reduction of viral titers was observed irrespective of infection MOIs of 0.1 and 10 (Supplementary Figure S2). In untreated PHH, STAT3 was phosphorylated similarly to HCC cells (Figure 2b). We also tested samples from liver resections derived from HCC patients; two out of the three samples displayed activation of STAT3 in healthy hepatic tissues (Figure 2c). Phosphorylation state of STAT3 Tyr-705 was monitored in uninfected cells by western blotting analysis, as well as the expression of inactive STAT3 and actin. Levels of phosphorylated STAT3 (pTyr-705) were significantly diminished in PHH when NSC74859 was added, whereas basal STAT3 and actin levels remained unchanged. In contrast, western blotting analysis performed on cell lysates of HCC cells revealed that NSC74859 treatment had less effect on the phosphorylation of STAT3 (Figure 2b). Further, we transfected PHH with scramble and STAT3-specific siRNA. Fifty-six hours posttransfection, infection with VSV-GFP was performed at an MOI of 0.1 for 16 h. Knockdown of STAT3 protein expression was assessed by western blotting analysis on uninfected cells in parallel. As shown in Figure 2d, blocking STAT3 expression significantly inhibited the growth of VSV in PHH, similarly to NSC74859.

To exclude the possibility that attenuation of VSV growth was due to NSC74859 toxicity, we evaluated the effects of increasing concentrations of NSC74859 on the viability of PHH by MTS assay. Treatment with NSC74859 for 24 h did not cause a significant decrease in cell survival (Figure 2e). NSC74859 treatment did not induce cleavage of PARP in PHH, as opposed to treatment with Apo-1 monoclonal antibody, which was used as a positive control. Moreover, pretreatment with NSC74859 abrogated cleavage of PARP upon VSV infection (Figure 2f).

We then investigated the antiviral molecular mechanisms of NSC74859 in PHH. Analysis by real-time PCR showed comparable amounts of viral RNA (mRNA and genomic RNA) in VSV-infected PHH in the presence or absence of NSC74859 (Figure 3a). In contrast, expression of viral proteins was significantly reduced by western blotting analysis in PHH treated with the STAT3 inhibitor (Figure 3b). The inhibitory activity of NSC74859 was reversible. In fact, when PHH underwent only pretreatment with STAT3 inhibitor, and viral infection was allowed to proceed in the absence of the inhibitor, VSV growth was rescued to the level of that in mock-treated cells (Figure 3c).

STAT3 activation is not necessary for VSV growth in HCC cells

As NSC74859 did not consistently alter STAT3 phosphorylation in HCC cells, we further investigated whether deactivation of STAT3 in HCCs could result in attenuation of viral growth, as observed in PHH. To this end, we transfected HepG2 and Huh7 cells with siRNA against STAT3. Although reduction of STAT3 protein expression was confirmed by western blotting, there was no significant change in viral titer as a result (Figure 4), indicating that STAT3 expression does not influence VSV replication in HCC cells. Sorafenib, the only clinically approved systemic therapy for HCC, also has an inhibitory function on STAT3. We therefore investigated the effect of sorafenib treatment on VSV replication in HCC cells. Interestingly, treatment with sorafenib caused a significant attenuation of VSV in both HepG2 and Huh7 cells (Supplementary Figure S3); however, as sorafenib is a multikinase inhibitor, there are multiple targets that could have been responsible for inhibiting virus replication. For example, we treated the same cells with inhibitors of ERK and AKT kinases, both of which are targets of sorafenib, and observed a similar degree of VSV attenuation as that observed for sorafenib-treated cells (Supplementary Figure S4).

Inhibition of STAT3 activation blocks IFN response in primary hepatocytes

PHH treated with DMSO or with 100 μM of NSC74859 (STAT3i) were infected with wild-type VSV-GFP for 16 h. Lysates were collected and subjected to western blotting analysis for STAT1 and STAT3 activation. Membranes were probed with an antibody against VSF matrix (M) protein and with actin, respectively, as infection and loading controls (Figure 5a). VSV infection induced phosphorylation of STAT1 but did not alter the levels of phosphorylated STAT3. When cells were pretreated with the STAT3 inhibitor, VSV infection did not lead to activation of STAT1 (Figure 5a). Similar results were obtained when PHH were stimulated with 100 IU of universal type I IFN (IFNα/β) for 15 min. IFN strongly induced phosphorylation of STAT1 in mock-treated cells. In the presence of NSC74859, the expression of phosphorylated STAT1 was drastically diminished (Figure 5b).

To confirm the inhibitory activity of NSC74859 on IFN response, we assessed the IP-10 concentration in cell supernatants using a solid-phase sandwich ELISA (Figure 5c). PHH were treated with 100 μM of NSC74859 and either infected with wild-type VSV at an MOI of 1 for 24 h or induced with 1 μg ml⁻¹ of poly I:C. The presence of NSC74859 significantly inhibited IP-10 induction by poly I:C. Likewise, inhibition of STAT3 activation hindered IP-10 expression in VSV-infected PHH, though it was just close to statistical significance (P = 0.059).

NSC74859 treatment, despite hampering the IFN signaling pathway, did not counteract viral protection by IFN; co-administration of NSC74859 and IFN did not rescue VSV growth nor display a synergistic effect in attenuating viral replication (Figure 5d).

The MTD of VSV administered intravenously combined with inhibitor NSC74859 in healthy mice is significantly elevated.

To evaluate the toxicity profiles and MTDs of wild-type VSV in vivo, normal C37BL/6 mice were randomized into two groups: a control group that received vehicle alone (phosphate-buffered saline (PBS)) and the NSC74859 group treated with the STAT3 inhibitor at

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Figure 1. Signal transducer and activator of transcription 3 (STAT3) inhibitor NSC74859 does not affect vesicular stomatitis virus (VSV) oncolysis in hepatocellular carcinoma (HCC) but inhibits cell proliferation. (a) HCC cell lines, HepG2 and Huh-7, were treated overnight with increasing concentrations of NSC74859. Control cultures were treated with vehicle alone (dimethyl sulfoxide (DMSO)). After changing the medium containing fresh inhibitor, cells were infected with wild-type VSV-GFP at an multiplicity of infection of 0.1 for 24 h. Viral titers were determined in the cell supernatants by TCID50. Results are the mean of at least three independent experiments and error bars indicate mean ± s.d. *p < 0.05. (b) Cell lysates of infected cells in the presence of increasing concentrations of NSC74859 were subjected to western blotting analysis for detection of the phosphorylated form of STAT3 (pSTAT3-Y705) using a specific antibody. Actin was probed as a loading control. (c) Cell viability in the presence of NSC74859 was examined by MTS [3(4,5-dimethylthiazol-2-yl)-5-(3-carboxy méthoxy phenyl)-2(4-sulfophenyl)-2H-tetrazolium] assay. HCC cells were exposed to the indicated concentrations of STAT3 inhibitors for 24 h. Results are the mean of at least three independent experiments in triplicate; error bars indicate mean ± s.d. ***p ≤ 0.001. (d) HCC proliferation was analyzed over a period of 72 h in the presence or absence of NSC74859 by trypan blue exclusion assay. STAT3 inhibitor was refreshed daily. (h.p.t. = hours posttreatment). Data represent the mean ± s.d. of three independent experiments.

In this study, a dose of 5 mg kg⁻¹ was administered intraperitoneally every other day for the duration of the experiment (14 days). One day after the beginning of the treatment, escalating doses of wild-type VSV were administered intravenously in half-log increments that ranged from 10⁰ to 10⁵ PFU (plaque-forming units). Five animals per group were treated as follows: (a) PBS with no virus inoculation, (b) PBS and infusion with escalating doses of VSV, (c) NSC74859 alone without VSV, and (d) NSC74859 with escalating doses of VSV. The MTD was calculated as the highest dose in which no viral-associated toxicities were observed. The MTD of wild-type VSV was elevated by an order of 1-log when administered in combination with the STAT3 inhibitor NSC74859 as compared with VSV alone (10⁵ PFU for combination-treated animals versus 10⁴ PFU for VSV alone, Table 1).

To examine whether NSC74859 could provide protection of the CNS from VSV infection, we infected primary rat neurons and glial cells in the presence of NSC74859. As observed in PHH, neurons and glial cells were significantly protected during infection by the STAT3 inhibitor in a dose-dependent manner (Figure 6).
DISCUSSION

VSV is an effective oncolytic agent and has the potential to improve the clinical outcome for a spectrum of human tumors, including HCC. Although VSV effectively prolongs survival in HCC animal models, several challenges arise from limited intratumoral virus spread and toxicity at elevated doses, highlighting the need for an improved strategy. In our study, we sought to determine the utility of oncolytic VSV viral therapy when delivered in combination with novel molecularly targeted therapy. Herein, we report the potential to enhance oncolytic VSV therapy by co-administration with the STAT3 signaling inhibitor, NSC74859 (also named S31-201).

Constitutive activation of STAT3 is observed in a large number of tumors and may contribute to carcinogenesis by stimulating cell proliferation and preventing apoptosis. For this reason, strategies to modulate STAT3 activity are emerging as promising anticancer therapies. As already reported, we observed constitutive activation of STAT3 in human HCC cell lines, HepG2 and Huh-7. Upon treatment with the inhibitor NSC74859, a chemical reagent that disrupts the dimerization of STAT3, growth of HCC cells was effectively suppressed in a dose-dependent manner. STAT3 phosphorylation was not significantly abrogated, in contrast to previous findings. As our goal is to enhance virotherapy efficiency in tumor cells, a critical point in the development of a co-treatment regime is to avoid adverse effects caused by the small-molecule inhibitors on viral replication in target cells. Notably, VSV replication was not limited by NSC74859 treatment in HCC cells. In this respect, we further explored the role of STAT3 activation in supporting viral growth in HCC cells. We achieved STAT3 inhibition by RNA silencing, and we analyzed their effects on VSV oncology. Reduced expression levels of STAT3 were observed upon siRNA interference, but viral growth was not significantly affected (Figure 4).

Sorafenib, which is the only approved drug for clinical treatment of HCC, was also included in the screening due to its ability to block STAT3 signaling as a part of its antitumor activity. Sorafenib had an adverse effect on VSV oncology, indicating the unsuitability of a combination treatment (Supplementary Figure S3). It is likely that the inhibitory activity on VSV is referable to its properties as a multikinase inhibitor. In addition to a broad spectrum of targets, sorafenib also hampers the ERK and AKT kinases. When we treated HCC cells with ERK inhibitor U0126 together with PI3K inhibitor LY294002, we achieved a similar reduction in viral titers as in sorafenib-treated cells (Supplementary Figure S4).
Figure 3. Signal transducer and activator of transcription 3 (STAT3) inhibitor NSC74859 impairs viral protein expression in infected PHH. (a) PH-H were treated with 100 μM of NSC74859 (STAT3i) or mock-treated with dimethyl sulfoxide (DMSO) and infected with vesicular stomatitis virus (VSV) at a multiplicity of infection (MOI) of 1. At the indicated time points after infection, total RNA was extracted and analyzed by real-time PCR. Viral mRNA was determined using primers for the VSV–nucleocapsid (N) gene. For viral genome (gRNA), primers designed to amplify the inter-genic region between VSV nucleocapsid and phosphoprotein (N/P) were used. RNA expression levels were expressed as those normalized to the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene. Mean values and s.d. of experiments performed in triplicates are shown. (b) Viral protein expression was analyzed at different time points postinfection (hpi) in the whole-cell lysates of PHH infected after addition of NSC74859 (STAT3i) by western blotting. A specific anti-VSV mouse serum was used together with actin as a loading control. (c) Infection of PH-H was carried out at an MOI of 0.1 in cells incubated with 100 μM of NSC74859 (STAT3i) or mock-treated. After adsorption of the viral inoculum for 1 h, infection was allowed to proceed in the presence of medium where the inhibitor was refreshed or, alternatively, in medium without NSC74859. Viral titers are shown as the mean ± s.d. of three independent experiments. *p ≤ 0.05.

Figure 4. Signal transducer and activator of transcription 3 (STAT3) activation is dispensable for vesicular stomatitis virus (VSV) life cycle in hepatocellular carcinoma (HCC). HCC cells were transfected with scramble siRNA (Scr) or STAT3-specific siRNA (STAT3i). At 72 h posttransfection, cells were infected with VSV-GFP (green fluorescent protein) at a multiplicity of infection of 0.1 for 24 h, and viral titers in the culture supernatants were determined by TCID50 analysis. To confirm siRNA knockdown of STAT3 expression, cell lysates of parallel uninfected cultures was assessed by western blotting. (HepG2 are shown as representative).

Based on the fact that STAT3 siRNA silencing did not impair viral replication, we have concluded that activation of STAT3 is dispensable in the life cycle of VSV in HCC cells. We have also excluded apoptosis as the reason of reduced oncolysis in primary hepatocytes. NSC74859 did not display cytotoxicity in PHH (Figure 2e) nor lead to cleavage of PARP; on the contrary it abrogated PARP activation upon VSV infection (Figure 2f).

In conclusion, although more work has to be done to characterize the molecular mechanisms of NSC74859, our results demonstrated the practical feasibility of combining this STAT3 inhibitor with oncolytic VSV therapy, at least in vitro. Regardless of the mechanism of action of NSC74859, this molecule has significant advantages: (1) it exerts antiproliferative activity in HCC; (2) it supports VSV replication and oncolysis in HCC; and (3) it protects primary hepatocytes from viral infection. STAT3 inhibition in tumor-bearing mice has resulted in a reduction of HCC growth; NSC74859 treatment of Huh7 xenografts in nude mice significantly retarded tumor proliferation. This, coupled with our own data demonstrating the efficacy of VSV in HCC-bearing rats, allowed us to speculate that combination of VSV and NSC74859 will be more effective in improving survival than with either treatment alone. Although we did not observe any significant improvement of VSV replication or oncolysis when applied in combination with STAT3 inhibition in vitro, we hypothesize that combination therapy will lead to significant antitumoral effects by means of two discreet but synergistic mechanisms: VSV acting mainly through direct oncolysis and NSC74859 acting through inhibition of cell proliferation and tumor spread. Furthermore, the improved safety of oncolytic VSV that was observed in response to STAT3 inhibition affords us the opportunity to apply higher viral doses in vivo, which we speculate will result in further enhancement of the VSV-mediated oncolysis.

The utility of VSV and NSC74859 combination therapy is further corroborated in the new finding that primary hepatocytes, as well as primary neurons and glial cells, are protected against VSV cytotoxicity upon treatment with the STAT3 inhibitor. In vitro cultured primary hepatocytes showed activation of STAT3, which was efficiently abrogated by treatment with NSC74859. In two out of the three resections of liver tissue from HCC patients, STAT3 was also phosphorylated. This observation suggests the possibility that, in HCC patients, normal liver could become the target of an active VSV infection with severe toxic implications. Therefore, abolishing aberrant STAT3 signaling becomes a key to revert malignant transformation of hepatocytes while simultaneously protecting them from viral oncolysis.

VSV pathogenicity in normal tissues is a significant challenge in the clinical translation of oncolytic VSV therapy. VSV neurovirulence remains the greatest safety concern, and as viral pathogenicity is observed at high viral loads, it limits the VSV therapeutic window.\(^{30,31}\) In this study, attenuation of VSV growth in normal cells was dose-dependent and could be achieved whether the treatment with NSC74859 was performed before or in parallel with virus infection. This represents an advantage in the design of experimental procedures in vivo, enabling a certain grade of flexibility in treatment protocols. Additionally, we proved that NSC74859 alone does not induce cell toxicity or induces apoptosis in treated primary hepatocytes (Figure 2e and f), which nicely fits to previous reports indicating a lack of significant adverse effects in treated animals.\(^{19,20}\) Quite significantly, NSC74859 protected against apoptosis in infected hepatocytes, as highlighted by inhibition of PARP cleavage (Figure 2f). This indicates a reduction of VSV pathogenicity in normal cells and provides the possibility to use higher viral doses during therapy. In support of this concept, we evaluated the toxicity profile and the MTD of VSV in healthy mice in the presence of NSC74859. The MTD of VSV after intravenous infusion in normal mice exposed to NSC74859 was elevated.

### Table 1. Maximum tolerated dose of VSV in combination with NSC74859

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<th>Virus dose (total PFU)</th>
<th>Toxicity (no. of adverse events)</th>
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<tr>
<td>PBS</td>
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</table>

Abbreviations: PBS, phosphate-buffered saline; PFU, plaque-forming units; VSV, vesicular stomatitis virus.

### Figure 5. NSC74859 activity in primary human hepatocytes (PHH) is interferon (IFN) independent. (a) PHH were cultured in the presence of 100 μM of NSC74859 (signal transducer and activator of transcription 3i (STAT3i)) and infected with vesicular stomatitis virus-green fluorescent protein (VSV-GFP) at a multiplicity of infection (MOI) of 1 overnight. For controls, cultures were incubated with vehicle only (dimethyl sulfoxide (DMSO)). Western blotting analysis of whole-cell extracts was performed to detect phosphorylation levels of STAT1 (pSTAT1) and STAT3-Y705 (pSTAT3). Expression of viral matrix protein (VSV-M) was used as an infection control, while actin was used as a loading control. (b) PHH were mock or NSC74859 treated followed by 15-min stimulation with 1000 IU ml⁻¹ of universal IFN type I (IFNα/β). Levels of phosphorylated STAT1 and STAT3 were assayed with specific antibodies by western blotting. (c) PHH were incubated with DMSO or with 100 μM of NSC74859 (STAT3i) and stimulated with 1 μg ml⁻¹ of Poly I:C (pI:C) or viral infected (VSV). Concentration of IP-10 (pg ml⁻¹) was measured in cell supernatants by enzyme-linked immunosorbent assay. Results indicate the mean ± s.d. of at least three independent experiments performed in duplicates. ***P<0.001 (d) VSV infection in PHH was performed at an MOI of 0.1 after incubation overnight with DMSO, NSC74859 (STAT3i) and IFN alone or with a combination of STAT3 inhibitor and IFN (IFN+STAT3i). Viral titers were determined by TCID50, and error bars represent the s.d. of three independent experiments. Significance was calculated with respect to the DMSO control **P<0.01.
and, at the same time, allows efficient replication of oncolytic VSV in HCC cells. In the presence of the STAT3 inhibitor NSC74859, VSV not only fully retained its strong oncolytic effects in target tumor cells but also its pathogenic potential was significantly reduced in healthy hepatocytes and neurons. If we sum together the increase in safety of VSV virotherapy with the suppression of the cancer-promoting activity ascribed to persistent STAT3 signaling (for example, cell proliferation, metastasis, angiogenesis, host immune evasion and resistance to apoptosis), the benefits of the proposed combination therapy could go beyond our encouraging results. Our preliminary findings may serve as a starting point for further optimization of VSV as a cancer therapeutic through the use of STAT3 inhibitors and, therefore, to improve the survival outcome for HCC patients in the future.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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