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Immuno-modulating properties of saliphenylhalamide, SNS-032, obatoclax, and gemcitabine

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A B S T R A C T

Influenza A viruses (IAVs) impact the public health and global economy by causing yearly epidemics and occasional pandemics. Several anti-IAV drugs are available and many are in development. However, the question remains which of these antiviral agents may allow activation of immune responses and protect patients against co- and re-infections. To answer to this question, we analysed immuno-modulating properties of the antivirals saliphenylhalamide (SaliPhe), SNS-032, obatoclax, and gemcitabine, and found that only gemcitabine did not impair immune responses in infected cells. It also allowed activation of innate immune responses in lipopolysaccharide (LPS)- and interferon alpha (IFNα)-stimulated macrophages. Moreover, immuno-mediators produced by gemcitabine-treated IAV-infected macrophages were able to prime immune responses in non-infected cells. Thus, we identified an antiviral agent which might be beneficial for treatment of patients with severe viral infections.

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1. Introduction

IAVs mutate rapidly. Emerging viruses overcome the prevailing immunity in the human population and cause global epidemics and pandemics (Ferdinands et al., 2011). Antivirals remain an important option for combating influenza outbreaks (Edinger et al., 2014; Loregian et al., 2014; Muller et al., 2012).

The existing antiviral drugs and emerging antiviral agents inhibit different steps of virus replication cycle (Fig. 1). For example, monoclonal antibodies that recognize the hemagglutinin (HA) globular head, as well as compounds containing sialic acids interfere with attachment of IAV to host cells (Colpitts and Schang, 2014; Nicol et al., 2012; Zanin et al., 2015). Inhibitors of cellular signalling pathways attenuate virus endocytic uptake (Denisova et al., 2014). Small molecules that interfere with endosomal acidification and viral M2 function, monoclonal antibodies which inhibit HA-mediated fusion of viral and endosomal membranes, as well as inhibitors of viral M1 degradation and ribonucleoprotein particle (vRNP) uncoating block virus entry into host cells (Banerjee et al., 2014; Cao et al., 2012; Denisova et al., 2012; Fujioka et al., 2011; Muller et al., 2011; Wu et al., 2014). Small molecules directed towards viral nucleoprotein (NP), the viral RNA-directed RNA polymerase (RdRP) complex (consisting of PB1, PB2 and PA) as well as

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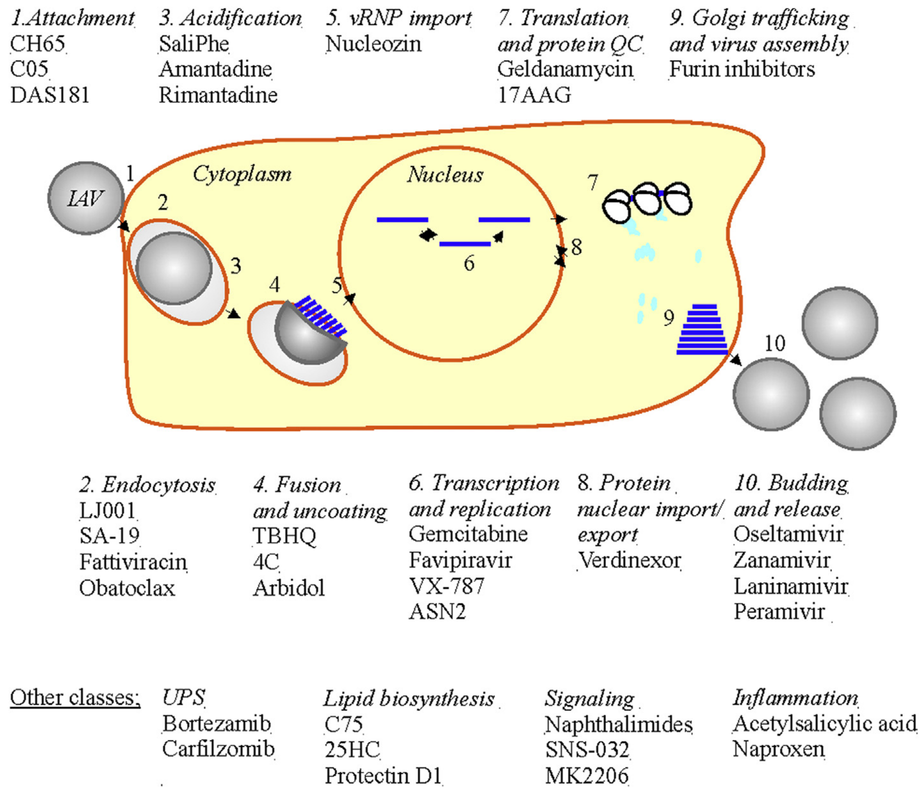


Fig. 1. Steps in the influenza virus replication cycle that are amenable to antiviral interventions. Influenza A virus (IAV) replication begins as the viral hemagglutinin (HA) binds to sialic acid-containing cell-surface receptors (1) and the virus particle is engulfed by the host cell (2). The virus is transported in an endosome to the perinuclear cytoplasm. During transportation, acidification of the endosome triggers HA-mediated fusion of viral and endosomal membranes (3) and the release of viral ribonucleoprotein complexes (vRNPs) into the perinuclear cytoplasm. The vRNPs, which contain the viral genetic information, migrate into the nucleus via nuclear pores (5). In the nucleus, the viral polymerase complex transcribes the eight viral RNAs (6). The resulting viral mRNAs are transported into the cytoplasm and translated into 10–12 viral proteins (depending on the virus strain) (7). Subsequently, eight of these proteins (including non-structural protein NS1) are transported to the nucleus (8). In the nucleus, NS1 inhibits transcription, as well as pre-mRNA processing and mRNA nuclear export, whereas PB1, PB2, PA and NP replicate viral RNAs via complementary RNAs intermediates. Newly synthetized vRNPs leave the nucleus and assemble at the plasma membrane to form new virus particles (9). The new virus particles bud from the cell and are released by a process that requires neuraminidase activity, to infect other cells (10). Almost every step of the virus replication circle could be inhibited with antiviral agents. Examples of such agents are shown. UPS-ubiquitin proteasome system, QC-quality control system.

those altering cellular purine and pyrimidine biosynthesis pathways, or the DNA-directed RNA polymerase II and its regulators inhibit vRNP nuclear import and transcription/replication of viral RNA (Cianci et al., 2013; Clark et al., 2014; Furuta et al., 2009; Ortigoza et al., 2012; Ortiz-Riano et al., 2014; Pautus et al., 2013; Perwitasari et al., 2015; Smee et al., 2012). In addition, there are dozens of small molecules that disturb translation of viral mRNA, protein quality control, as well as maturation, trafficking and assembly of vRNPs (Chairat et al., 2013; Chase et al., 2008; Clark et al., 2014; Das et al., 2013; Furuta et al., 2009; Gold et al., 2014; Lu et al., 2015; Mata et al., 2011; Morita et al., 2013; Ortigoza et al., 2012; Pautus et al., 2013; Perwitasari et al., 2014; Walkiewicz et al., 2011; Zhang et al., 2012). There are also small molecules that alter lipid metabolism and inhibit viral assembly and budding, as well as inhibitors of viral neuraminidase (NA) which block virus release from the cell surface (Chairat et al., 2013; Morita et al., 2013).

Some of these agents are used as anti-IAV drugs in humans (such as amantadine, rimantadine, arbidol, favipiravir, oseltamivir, zanamivir, laninamivir, peramivir, and acetylsalicylic acid), whereas others are in clinical development (such as DAS181, VX-787, ribavirin and verdinexor) or in pre-clinical investigations (such as CH65, C05, SaliPhe, nucleozin, geldanamycin, 17-AAG, LJ001, SA-19, fattiviracin, TBHQ, 4C, gemcitabine, ASN2, bortezamib, carfilzomib, C75, 25HC, SNS-032, MK2206) (Loregian et al., 2014; Muller et al., 2012; Vanderlinden and Naesens, 2014). So far, only oseltamivir has received a blockbuster status; however, oseltamivir-resistant IAV strains emerge and reduce the efficacy of the treatment (Hurt, 2014; Muthuri et al., 2014). The question remains which antiviral agents might be the most beneficial for patients infected with IAVs.

We hypothesised, that antiviral compounds, that efficiently inhibit IAV replication with minimal impairment of host antiviral responses, might be beneficial for patients, because such therapeutics could allow development of innate and adaptive immune responses, which would protect patients from virus co- and re-infections. To test this hypothesis we established a model system comprising human monocyte-derived macrophages and IAV virus which lacks the capacity to antagonize antiviral responses. In this study we used SaliPhe, SNS-032 and obatoclax, which inhibit IAV entry in the cytoplasm, as well as gemcitabine, which attenuates transcription and replication of viral RNA in the nucleus of infected cells (Denisova et al., 2012; Perwitasari et al., 2015). Interestingly, these compounds have been previously approved (gemcitabine) or are in clinical (SNS-032, obatoclax) or pre-clinical (SaliPhe) development for treatment of cancer or other diseases (Bajwa et al., 2012; Gesto et al., 2012; Lebreton et al., 2008; Tong et al., 2010), however, the antiviral effect of these agents *in vitro* could be achieved at much lower concentrations than that needed to mediate cancer or normal cell death (Denisova et al., 2012).

We first tested the effect of anticancer/antiviral SaliPhe, SNS-032, obatoclax and gemcitabine on activation of immune

responses in the macrophages infected with mutant IAV strain. We next studied the immuno-modulatory effect of these compounds in LPS-, IFNα-, dsRNA-, and histamine-stimulated macrophages. Finally, we analysed the effect of immuno-modulators produced by IAV-infected compound-treated macrophages on immune responses in non-treated non-infected macrophages or lung epithelial A549 cells. Our results suggested that treatment with gemcitabine, but not with SaliPhe, SNS-032 or obatoclax, allowed for the development of primary immune responses in IAV-infected cells and secondary responses in non-infected cells. Thus, we identified an antiviral/anticancer agent which could be further developed for treatment of severe IAV infections. Furthermore, our strategy can be exploited in different drug development programmes aiming to discover immuno-modulating properties or to optimize side-effects of therapeutics.

2. Materials and methods

2.1. Antiviral and non-infectious agents

Saliphenylhalamide (SaliPhe) was synthesized as described in Lebreton et al. (2008). Obatoclax, gemcitabine and SNS-032 were from Selleck Chemicals, USA. Compounds were dissolved in 100% dimethyl sulfoxide (Sigma–Aldrich) to obtain 10 mM stock solutions. DsRNA molecules of 2948, 4063 and 6374 base pairs in size were isolated from purified *Pseudomonas* phage phi6 nucleocapsids as described (Romanovskaya et al., 2013). Lyophilised LPS from *Escherichia coli* O55:B5 was from Sigma Aldrich (St. Louis, MO, USA). LPS was dissolved to 10 mg/ml in deionized water. Lyophilized IFNα was from Biomed, Russia. IFNα was dissolved to 500 RU/ml in deionized water. Histamine (SigmaAldrich, St. Louis, MO, USA) was dissolved to 10 mg/ml in deionized water. All agents were stored at −80 °C.

2.2. Cells and viruses

Human primary macrophages were derived from leukocyte-rich buffy coats from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Monocytes were isolated and differentiated into macrophages as described previously (Pirhonen et al., 1999). Monocytes were seeded in 96- or 6-well plates and cultured in serum free macrophage media (Gibco) supplemented with 10 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF; Biosource International) and 50 U/mL penicillin–streptomycin (Lonza) at 37 °C and 5% CO2 for 7 days, polarizing the monocytes into macrophages of the acute pro-inflammatory M1-phenotype. Before stimulation, the media was replaced with fresh GM-CSF free macrophage media and macrophages were infected with IAVs or stimulated with LPS, dsRNA, IFNa, or histamine.

Human influenza A/WSN/33(H1N1) viruses expressing wild type (WSN^{WT}) or R38A, K41A mutant NS1 ($WSN^{RK/AA}$) were generated using the WSN eight-plasmid-based reverse genetics system in HEK293T and Vero cells as described previously (Anastasina et al., 2015; Hoffmann et al., 2000). Human influenza A/Udorn/307/1972(H3N2) (Udorn) was cultured in embryonated hen eggs and the virus stock, with a titer of 256 hemagglutination U/ml, was stored at −80 °C. A virus dose of 2.56 hemagglutination U/ml was used in the infection experiments unless stated otherwise. The virus experiments were carried out under BSL-2 conditions and in compliance with regulations of the University of Helsinki (permit No 21/M/09). Viruses were titered in MDCK cells using a plaque assay as described previously (Denisova et al., 2012, 2014).

2.3. Cell viability assay

The compound efficacy testing was performed in 96-well plates. The compounds were added to the medium and 5 min later the cells were infected with IAVs (moi 3) or stimulated with dsRNA, LPS, IFNα, histamine or mock. The cell viability was analyzed with the CellTiter -Glo assay (CTG; Promega) at 24 h post infection/treatment/stimulation as described (Denisova et al., 2012). The luminescence was read with a PHERAstar FS plate reader (BMG Labtech). A sigmoidal dose-response curve was fitted to the data using SigmaPlot software. The effective concentrations that enhanced the cell viability by 50% (EC50) and the cytotoxic concentrations that reduced cell growth by 50% (CC50), and selectivity indexes were calculated from the sigmoidal functions as described (Muller et al., 2011).

2.4. Immunoblotting

WSN^{WT}-, $WSN^{RK/AA}$- or mock-infected macrophages were lysed in buffer containing kinase and phosphatase inhibitors. Protein aliquots of whole-cell lysates (30 μg) were separated by electrophoresis in 10% SDS polyacrylamide gels. Proteins were transferred onto Immobilon-P membranes, followed by blocking with 5% milk in PBS (or 5% BSA in TBS for anti-phospho-protein antibodies). Primary anti-phospho-IRF3 (Ser396; 4D4G; Cell Signaling), anti-IκBα (44D4; Cell Signaling), guinea pig anti-NS1 (1:2000), rabbit anti-NP (1:500), mouse anti-β-actin (1:5000; Sigma–Aldrich) and anti-GAPDH (Santa Cruz, sc-47724) antibodies were used, as described previously (Anastasina et al., 2015; Kakkola et al., 2013; Veckman et al., 2006). Secondary HRP-conjugated goat anti-rabbit, rabbit anti-guinea pig or rabbit anti-mouse antibodies (DakoCytomation, Carpinteria, CA, USA) were used in secondary staining. Antibody binding was visualized by the ECL system on HyperMax films (GE Healthcare).

2.5. Phosphoprotein profiling

WSN^{WT}-, $WSN^{RK/AA}$- or mock-infected macrophages was collected at 8 h post infection, cells were lysed and phosphorylation profiles of 43 kinases and 2 kinase substrates were analyzed using the human phosphokinase arrays according to the manufacturer's instructions (R&D Systems).

2.6. Cytokine profiling

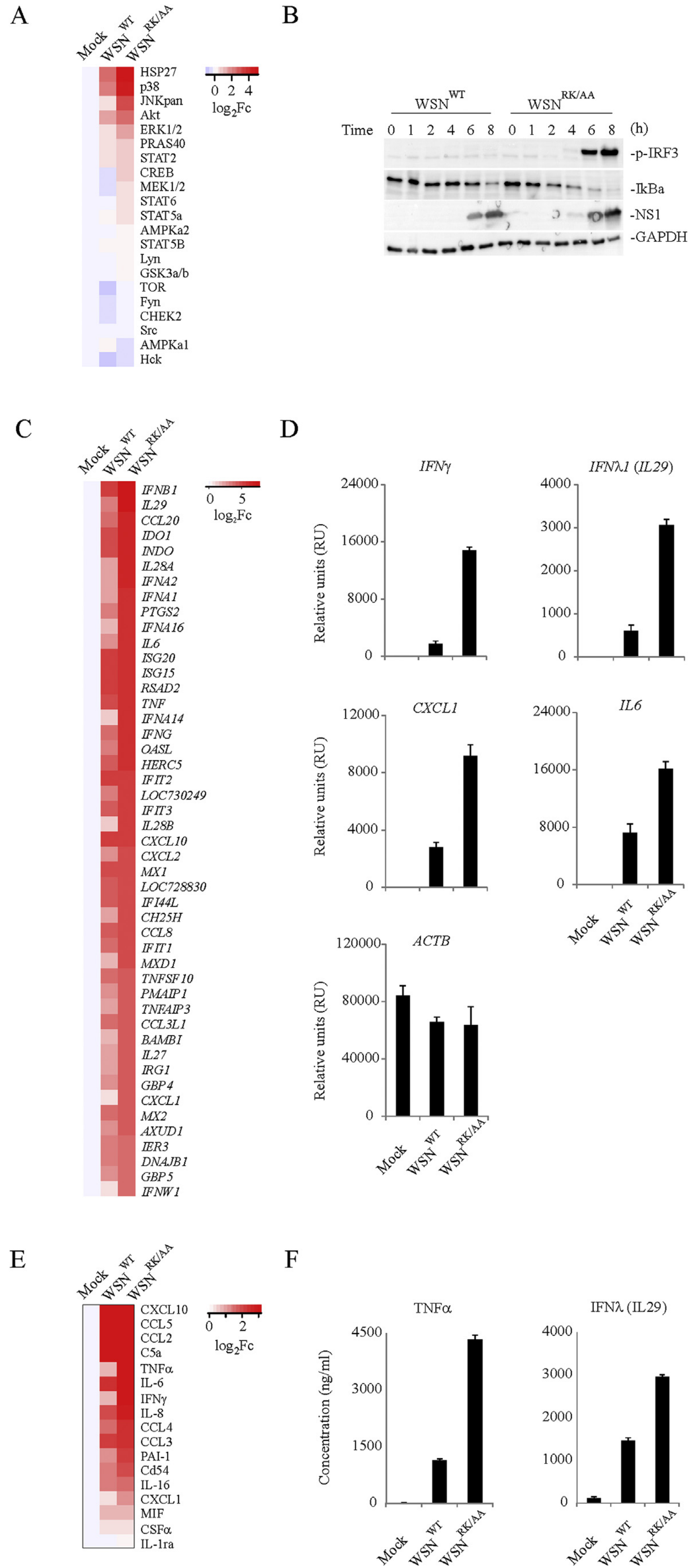
The medium from WSN^{WT}-, $WSN^{RK/AA}$- or mock-infected macrophages was collected at 24 h post infection and clarified by centrifugation for 5 min at 14,000 rpm. Cytokines were analysed using Proteome Profiler Human Cytokine Array panel A kit or Proteome Profiler Human XL Cytokine Array Kit (R&D Systems) according to manufacturer's recommendations. The results were analysed using ImageJ software.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels of TNFα and IFNλ1 from macrophage culture supernatants were analyzed using Ab pairs and standards from BD Pharmingen (San Diego, CA, USA) and a VeriKine-DIY Human Interferon Lambda ELISA kit supplied by PBL Interferon Source (Piscataway, NJ, USA).

2.8. Gene expression profiling

RNA was extracted from WSN^{WT}-, $WSN^{RK/AA}$- or mock- infected macrophages at 8 h post infection using RNeasy Plus mini kit


A
Mock
WSN WT
WSN RK/AA
HSP27
p38
JNKpan
Akt
ERK1/2
PRAS40
STAT2
CREB
MEK1/2
STAT6
STAT5a
AMPKa2
STAT5B
Lyn
GSK3a/b
TOR
Fyn
CHEK2
Src
AMPKa1
Hck
0 2 4
log2Fc
B
WSN WT
WSN RK/AA
Time 0 1 2 4 6 8 0 1 2 4 6 8 (h)
-p-IRF3
-IkBa
-NS1
-GAPDH
C
Mock
WSN WT
WSN RK/AA
IFNB1
IL29
CCL20
IDO1
INDO
IL28A
IFNA2
IFNA1
PTGS2
IFNA16
IL6
ISG20
ISG15
RSAD2
TNF
IFNA14
IFNG
OASL
HERC5
IFIT2
LOC730249
IFIT3
IL28B
CXCL10
CXCL2
MX1
LOC728830
IFI44L
CH25H
CCL8
IFIT1
MXD1
TNFSF10
PMAIP1
TNFAIP3
CCL3L1
BAMBI
IL27
IRG1
GBP4
CXCL1
MX2
AXUD1
IER3
DNAJB1
GBP5
IFNW1
0 5
log2Fc
D
IFNγ
IFNλ1 (IL29)
CXCL1
IL6
ACTB
Relative units (RU)
24000
16000
8000
0
4000
3000
2000
1000
12000
4000
120000
80000
40000
Mock
WSN WT
WSN RK/AA
E
Mock
WSN WT
WSN RK/AA
CXCL10
CCL5
CCL2
C5a
TNFα
IL-6
IFNγ
IL-8
CCL4
CCL3
PAI-1
Cd54
IL-16
CXCL1
MIF
CSFα
IL-1ra
0 2
log2Fc
F
TNFα
IFNλ (IL29)
Concentration (ng/ml)
4500
3000
1500
0
4000
3000
2000
1000
Mock
WSN WT
WSN RK/AA

(Qiagen). Gene expression profiling was performed as described previously (Anastasina et al., 2015). The data was deposited to the GEO database (accession numbers: GSE66015 and GSE65699).

2.9. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Quantitative PCR was performed on the Lightcycler 480 (Roche) using Fast SYBR Green Master Mix (Roche) or TaqMan probes (Denisova et al., 2014). The following sets of primers were used for detection of specific genes or cDNA: IFNB1 (forward: 5′-GCCGCATTGACCACTATGA, reverse: 5′-GCCAGGAGGTTCTCAACAATAG), IFNA1 (forward: 5′-ATGGCAACCAGTTCCAGAAG, reverse: 5′-CATCCCAAGCAGCAGATGAA), IFNA16 (forward: 5′-GACTCACTTCTATAACCACCACAA, reverse: 5′-TAGTGCCTGCACAGGTAAAC), NFKB1 (forward: 5′-TGGAGGCCCTGAGACAAA, reverse 5′-CCTGAGAGGTGGTCTTCACT), IFNG (forward: 5′-AAACAGGGAAGCGAAAA, reverse: 5′-GCAGGCAGGACAACCAT), IL6 (forward: 5′-TCATCACTGGTCTTTTGG, reverse: 5′-CTCTGGCTTGTTCCTCAC), CXCL1 (forward: 5′- TGAGCATCGCTTAGGAGA, reverse: 5′- AGGACAGTGTGCAGGTAG), IL29 (forward: 5′- AGGCTGAGCTGGCCCTGA, reverse: 5′- GGTGTGAAGGGGCTGGTC), IFNγ (forward: 5′-AAACAGGGAAGCGAAAA, reverse: 5′-GCAGGCAGGACAACCAT), and NS1 (forward: 5′- GAAATGTCAMGAGACT, reverse: 5′-AGAAAGCTCTTATCTCTTG).

2.10. ^{35}S protein labeling and autoradiography

Synthesis of viral and cellular proteins in infected macrophages was monitored by incorporation of ^{35}S-labeled methionine. Briefly, 1 μl of ^{35}S-labeled methionine (1200 Ci/mmol, 10 mCi/ml; Perkin–Elmer, USA) was added to 100 μl of cell culture media 30 min before the cells were harvested. Cells were lysed and proteins were resolved by SDS-PAGE. ^{35}S-labeled proteins were visualized by radio autography using a Typhoon 9400 scanner (Amersham, USA).

3. Results

3.1. Human monocyte-derived macrophages and $WSN^{RK/AA}$ virus represent an excellent model system for studying immunomodulatory effects of anti-IAV agents

In this study we utilized human monocyte-derived macrophages isolated from healthy individuals. These cells resemble alveolar macrophages, which represent natural targets for IAV (Anastasina et al., 2015; Short et al., 2012). As a model virus we used the influenza A/WSN/33(H1N1) variant ($WSN^{RK/AA}$), which expresses NS1 protein with R38A and K31A mutations (Fig. A1). These mutations alter the NS1 ability to sequester viral RNA from recognition by cellular pattern recognition receptors, which sense viral RNA and trigger development of interferon responses (Donelan et al., 2003; Min and Krug, 2006; Newby et al., 2007).

To further characterize this system we analysed the effect of $WSN^{RK/AA}$ infection on antiviral responses at transcriptional, translational and post-translational levels. In particular, we infected macrophages with $WSN^{RK/AA}$, WSN^{WT} or mock. After 8 h of infection we prepared cell lysates and analysed phosphorylation status of interferon regulatory factor 3 (IRF3), heat shock protein 27 (HSP27), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinases (Jnk), protein kinase B (Akt), extracellular signal-regulated kinases (ERK) and other phospho-proteins, which modulate the expression of antiviral genes (Borgeling et al., 2014; James et al., 2015; Jiang et al., 2015). We also analysed production of viral NS1 protein and integrity of IkBa, which is an inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which also plays a key role in activation of antiviral responses (Krug, 2015). Phosphoprotein profiling and Western blot analysis revealed that $WSN^{RK/AA}$ infection enhanced phosphorylation of HSP27, p38, Jnk, Akt and ERK proteins in comparison with WSN^{WT} and mock infections (Fig. 2A and B, Fig. A1). These results indicate that cellular MAPK, ERK, NFκB and IRF3 signalling cascades are activated more strongly by $WSN^{RK/AA}$, then by WSN^{WT} infections.

We also analysed the effect of $WSN^{RK/AA}$ infection on expression of antiviral genes. For this, we infected macrophages with $WSN^{RK/AA}$, WSN^{WT} or mock. After 8 h of infection we extracted total RNA from the cells and profiled gene expression using microarrays and qRT-PCRs (Fig. 2C and D). Infection with $WSN^{RK/AA}$ induced expression of 93 genes over 8 fold, whereas infection with WSN^{WT} virus activated expression of only 57 genes over 8 fold. Interestingly, 32 of these genes were induced in both WSN^{WT}- and $WSN^{RK/AA}$-infected cells, however $WSN^{RK/AA}$ infection promoted stronger expression of these genes. These results indicate that $WSN^{RK/AA}$ virus was a better enhancer of transcription of antiviral genes in human macrophages in comparison with the WSN^{WT} virus.

We next analysed the effect of $WSN^{RK/AA}$ on the production of cytokines in macrophages. In agreement with our transcriptomics results, cytokine profiling and ELISA experiments revealed that the secretion of cytokines was increased in $WSN^{RK/AA}$-infected macrophages in comparison with WSN^{WT}-infected cells (Fig. 2E and F, Fig. A1). Altogether, these results suggest that human monocyte-derived macrophages and $WSN^{RK/AA}$ virus could represent an excellent model system for studying the effect of anti-IAV agents on immune responses at transcriptional, translational and post-translational levels.

3.2. Antiviral gemcitabine, but not SaliPhe, SNS-032, or obatoclax allowed the activation of innate immune responses in human macrophages infected with influenza $WSN^{RK/AA}$ virus

We utilized our model system to evaluate the effect of the antiviral/anticancer SaliPhe, obatoclax, SNS-032 and gemcitabine on the cellular antiviral responses. First, we assayed the effect of these compounds on the viability of $WSN^{RK/AA}$- and mock-infected

Fig. 2. Characterisation of the model system for studying immune-modulating properties of anti-IAV agents. (A) Monocyte-derived macrophages were infected with WSN^{WT} (multiplicity of infection, moi 3), $WSN^{RK/AA}$ (moi 3) or mock, the cells were collected at 8 h after infection and total cell lysates were prepared. Phosphorylation levels of kinases and their substrates were profiled using a phosphokinase array. The relative intensities of spots were calculated. A heat map of selected phospho-proteins is shown (cut-off for $WSN^{RK/AA}$, $log_2Fc > 0.3$ and < -0.3). The heat map represents normalized expression data on the logarithmic scale as compared to mock-infected cells. (B) Macrophages were infected as for panel A. The phosphorylation of IRF3 and integrity of IκBα were analysed at different time points by Western blot. GAPDH was stained as a loading control. Viral NS1 was stained to monitor virus replication. (C) Macrophages were infected as for panel A. Eight hours post infection cells were collected, total RNA was isolated and genome-wide gene expression profiling was carried out. A heat map of genes is shown (cut-off for $WSN^{RK/AA}$, $log_2Fc > 4$ and < -4). The heat map represents normalized expression data on the logarithmic scale as compared to mock-infected cells. (D) Macrophages were infected as for panel A. Cells were collected at 8 h after infection, total RNA was isolated, and the expression of four cellular antiviral and one housekeeping genes was analysed using RT-qPCRs. The points are mean values, the number of observations used to derive the values is 3 and error bars represent the standard deviation (SD). (E) Macrophages were infected as for panel A, cell culture supernatants were collected at 24 h post-infection, and cytokine levels were determined. The relative intensities of spots were calculated. A heat map of selected cytokines is shown (cut-off for $WSN^{RK/AA}$, $log_2Fc > 0.1$ and < -0.1). The heat map represents normalized expression data on the logarithmic scale as compared to mock-infected cells. (F) Cells were infected as for panel A. After 24 h cell culture supernatants were collected, and cytokine levels were determined by ELISA. The points are a mean values, the number of observations used to derive the values is 3 and error bars represent the standard deviation.

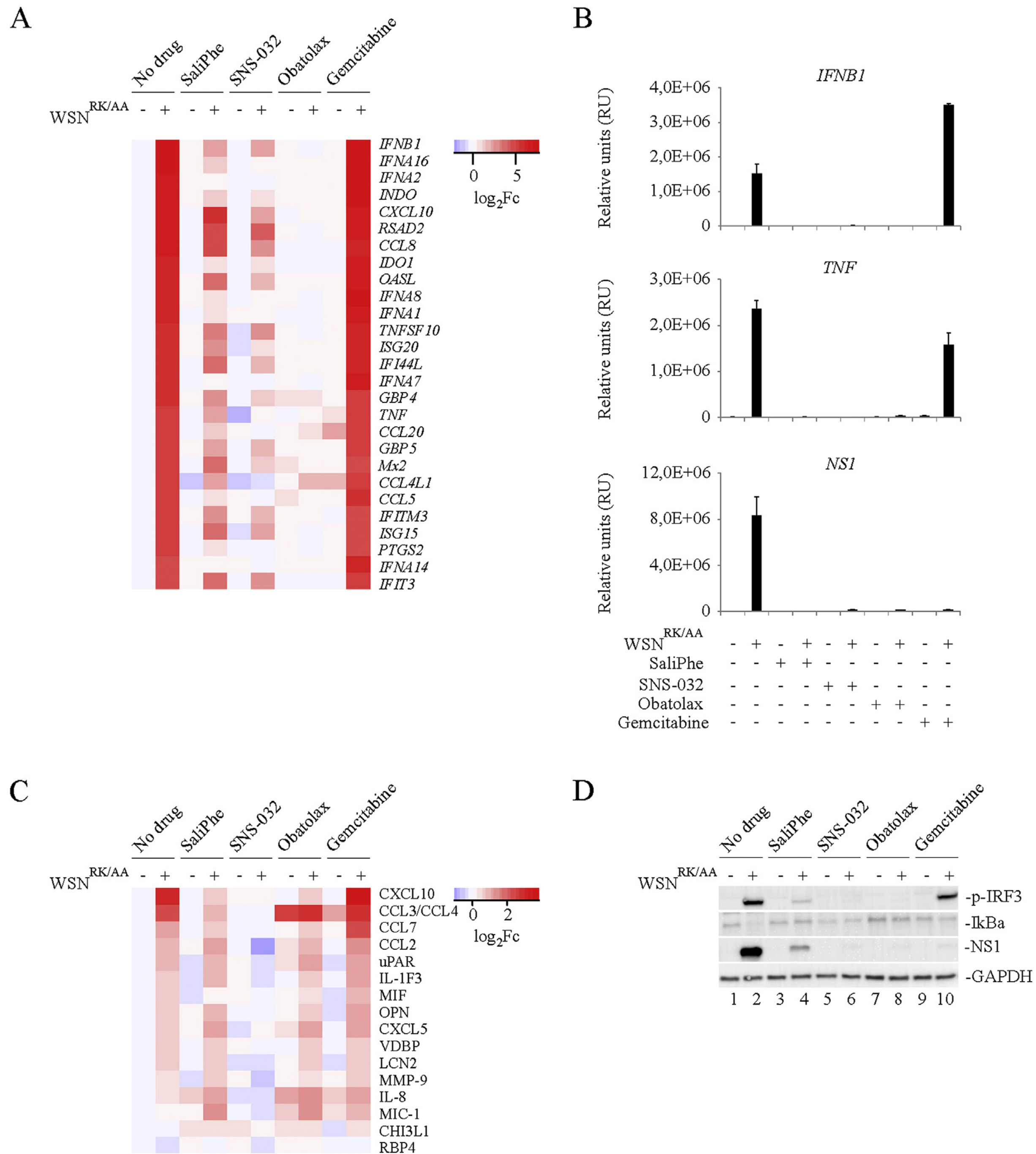


Fig. 3. Effect of SaliPhe, SNS-032, obatoclax, and gemcitabine on transcription of antiviral genes and cytokine secretion in WSN$^{RK/AA}$-infected macrophages. (A) Macrophages were treated with 3 μM SaliPhe, 0.1 μM SNS-032, 2 μM obatoclax, 1 μM gemcitabine or remained non-treated, and infected with WSN$^{RK/AA}$ or mock. At 8 h after infection the cells were collected, total RNA was extracted and gene expression profiling was carried out. A heat map of selected genes is shown (cut-off for WSN$^{RK/AA}$, $\log_2 Fc > 5$ and < -5). (B) Macrophages were treated with different antiviral reagents and total RNA was isolated. The RNA was subjected to quantitative PCRs to detect IFNβ1, TNFα and NS1 mRNA levels. The points are mean values, the number of observations used to derive the values is 3 and error bars represent the standard deviation. (C) Macrophages were treated and infected as for panel B. After 24 h cell culture supernatants were collected, and cytokine levels were determined. The relative intensities of spots were calculated. A heat map of selected cytokines is shown (cut-off for WSN$^{RK/AA}$, $\log_2 Fc > 0.1$ and < -0.1). (D) Macrophages were treated and infected as for panel B. At 8 h after infection cells were collected and the phosphorylation of IRF3 and expression of IκBα were analysed by Western blotting. GAPDH was stained to control equal sample loading. Viral NS1 was used to monitor virus replication.

human macrophages. We found that macrophages treated with 3 μM SaliPhe, 0.1 μM SNS-032, 2 μM obatoclax or 1 μM gemcitabine remained viable after 24 h of infection (Fig. A2). Importantly, the antiviral effect of these agents was independent on the macrophage preparations and IAV subtype (Fig. A2).

Next, we evaluated the effect of antiviral agents on host transcriptional responses in WSN$^{RK/AA}$- and mock-infected macrophages. We treated macrophages with selected concentrations of these compounds and infected them with WSN$^{RK/AA}$ or mock. Eight hours post infection we profiled the expression of cellular genes using microarrays (Fig. 3A). We found that SaliPhe, SNS-032 and obatoclax suppressed the expression of dozens of antiviral genes

including *CXCL10*, *IFNB1* and *TNF*. By contrast, gemcitabine treatment did not attenuate and even enhanced the expression of some of these genes including *IFNB*- and *IFNA*-family genes. RT-qPCR analysis of *IFNB1* and *TNF*, as well as viral *NS1* (control) RNAs, confirmed our transcriptomics results (Fig. 3B). Thus, in contrast to SaliPhe, SNS-032, and obatoclax treatments, treatment with gemcitabine did not interfere with the transcription of cellular genes in $WSN^{RK/AA}$-infected human macrophages.

We next tested whether treatment with SaliPhe, SNS-032, obatoclax or gemcitabine allowed production of cytokines by $WSN^{RK/AA}$-infected macrophages. In agreement with our transcriptomics results, the cytokine profiling experiment revealed that treatment with gemcitabine, but not SaliPhe, SNS-032, or obatoclax allowed production of immuno-modulators by macrophages to similar or even higher levels as compared to non-treated $WSN^{RK/AA}$- infected cells (Fig. 3C; Fig. A3).

We also tested whether the treatment with SaliPhe, SNS-032, obatoclax or gemcitabine allowed the activation of antiviral signalling cascades in $WSN^{RK/AA}$-infected macrophages. Western blot analysis showed that IRF3 was phosphorylated and IkBa was degraded in gemcitabine-, but not in SaliPhe-, SNS-032- or obatoclax-treated $WSN^{RK/AA}$-infected cells (Fig. 3D). These findings are in agreement with our transcriptomics and cytokine profiling results. Thus, we demonstrated that gemcitabine, but not SaliPhe, SNS-032 or obatoclax, allowed development of efficient antiviral responses at transcriptional, translational and posttranslational level in infected macrophages.

3.3. SaliPhe, SNS-032, obatoclax and gemcitabine allowed activation of immune responses in macrophages stimulated with LPS and IFNα, but not with dsRNA or histamine

Anti-influenza drugs are used mainly for treatment of severe infections (Zambon, 2014) which are often associated with viral and bacterial co-infections (Joseph et al., 2013). Therefore, we next studied immuno-modulatory properties of SaliPhe, SNS-032, obatoclax and gemcitabine in human macrophages stimulated with LPS, IFNα, dsRNA or histamine. DsRNA and LPS represent viral and bacterial PAMPs, respectively, whereas IFNα and histamine are immune-mediators which are produced by virus or bacteria-infected cells (Figs. 2C and 3B) (Graham et al., 2015; Katze et al., 2002; Matsumoto and Seya, 2008; Miller et al., 2005; O'Mahony et al., 2011; Randall and Goodbourn, 2008; Sadler and Williams, 2008). For this, we first analysed toxicity of dsRNA, LPS, IFNα and histamine for macrophages (Fig. 4A). We found that 1 μg/ml dsRNA, 1 μg/ml LPS, 1 U/ml IFNα and 1 μg/ml histamine were not toxic for the cells. We then treated macrophages with selected concentrations of SaliPhe, SNS-032, obatoclax, gemcitabine, and stimulated the cells with dsRNA, LPS, IFNα, or histamine. We measured the transcriptional responses (Fig. 4B–E). We found that none of the four antiviral agents affected transcriptional responses in LPS- or IFNα-stimulated macrophages, whereas all four compounds imbalanced the transcription in dsRNA- and histamine-stimulated cells. Thus, our results indicate that different anti-IAV agents can differentially affect immune responses under different stress conditions.

3.4. Immune mediators produced by gemcitabine-, but not by SaliPhe-, SNS-032-, or obatoclax-treated $WSN^{RK/AA}$-infected macrophages primed immune responses in non-infected cells

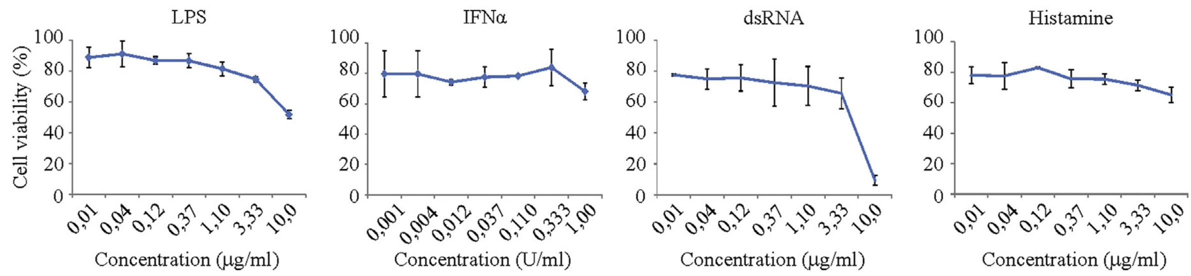
We next evaluated whether immune mediators produced by compound-treated $WSN^{RK/AA}$-infected macrophages can prime the development of innate immune responses in non-treated, non-infected cells. For this, we obtained media from $WSN^{RK/AA}$- or mock-infected, compound-treated or non-treated macrophages (M1). We diluted the media 1:20 and applied it to fresh macrophages (M2) from the same donor (Fig. 5A). After 8 h post treatment we profiled the gene expression of M2 cells. We found that the media from gemcitabine-treated $WSN^{RK/AA}$-infected M1 macrophages primed the transcription of antiviral genes in M2 cells to the levels observed in macrophages treated with the media from non-treated $WSN^{RK/AA}$-infected M1 macrophages, whereas media from SaliPhe-, SNS-032-, or obatoclax-treated $WSN^{RK/AA}$-infected M1 macrophages did not prime transcriptional responses in non-infected M2 cells (Fig. 5B). Interestingly, *IFN* genes were strongly up-regulated in virus-infected non-treated and gemcitabine-treated M1 cells, but not in M2 cells stimulated with media from corresponding M1 cells. RT-qPCR analysis of *IFNB1* RNA confirmed our transcriptomics results, and RT-qPCR analysis of viral NS1 showed that there was no virus replication in M2 cells (Fig. 5C).

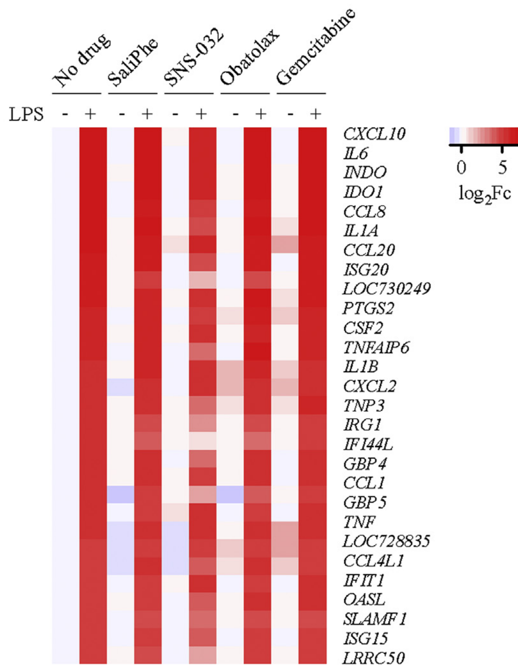
Next, we questioned whether M2 macrophages treated with media from M1 cells produced cytokines. In agreement with our transcriptomics results, cytokine profiling experiment revealed that media from $WSN^{RK/AA}$-infected gemcitabine-treated M1 macrophages similarly to media from $WSN^{RK/AA}$- infected non-treated M1 cells triggered production of cytokines in M2 cells (Fig. 5D, Fig. A4). By contrast, media from SaliPhe-, SNS-032-, or obatoclax-treated M1 macrophages did not prime immune responses in non-infected M2 cells. We also used media from M1 cells and applied it to non-infected human lung epithelial A549 cells, and analysed the produced cytokines after 24 h. We found that the media from gemcitabine-treated virus-infected M1 macrophages, similarly to media from non-treated virus-infected M1 macrophages, primed the production of cytokines in A549 cells (Fig. A4). Media from SaliPhe-, SNS-032-, or obatoclax- treated infected M1 macrophages was unable to trigger production of the cytokines. These results suggest that treatment with gemcitabine, but not with SaliPhe, SNS-032 or obatoclax, activated primary antiviral responses in infected cells and secondary immune responses in non-infected cells.

4. Discussion

In the present study we established a system for testing immuno-modulating properties of drugs and drug candidates. We utilized this system to demonstrate that gemcitabine, but not SaliPhe, obatoclax or SNS-032 allowed the development of innate immune responses in IAV-infected macrophages. Mechanistically, treatment with gemcitabine, which targets cellular RNR and inhibits transcription and replication of vRNA, did not impair antiviral cascades that could be initiated by cellular pattern recognition molecules, such as TLR3, MDA5, and RIG-I upon recognition of viral RNA. It also allowed activation of IRF3- and NFκB-pathways, which cumulated in transcription and translation, as well as post-translational modifications and secretion of immune mediators from infected cells. Furthermore, gemcitabine did not interfere with secondary immune responses in non-infected cells which were triggered by immune-mediators produced by gemcitabine-treated infected cells (Fig. 6). Moreover, gemcitabine was unable to impair macrophage responses to other immune stimuli, such as IFNα and LPS, which could be associated with viral and bacterial co-infections and viral re-infections. Thus, gemcitabine treatment limited viral replication and allowed development of immune responses in infected and non-infected cells, which could be necessary for cell-mediated and humoral protection of patients with severe influenza infections.

Our results pave the way towards further development of the anticancer gemcitabine and its analogues as broad-spectrum antiviral agents. In particular, gemcitabine could be further developed


A
LPS
IFNα
dsRNA
Histamine
Cell viability (%)
100
80
60
40
20
0
0,01 0,04 0,12 0,37 1,10 3,33 10,0
0,001 0,004 0,012 0,037 0,110 0,333 1,00
Concentration (μg/ml)
Concentration (U/ml)


B
No drug
SaliPhe
SNS-032
Obatolax
Gemcitabine
LPS - + - + - + - + - +
CXCL10
IL6
INDO
IDO1
CCL8
IL1A
CCL20
ISG20
LOC730249
PTGS2
CSF2
TNFAIP6
IL1B
CXCL2
TNP3
IRG1
IFI44L
GBP4
CCL1
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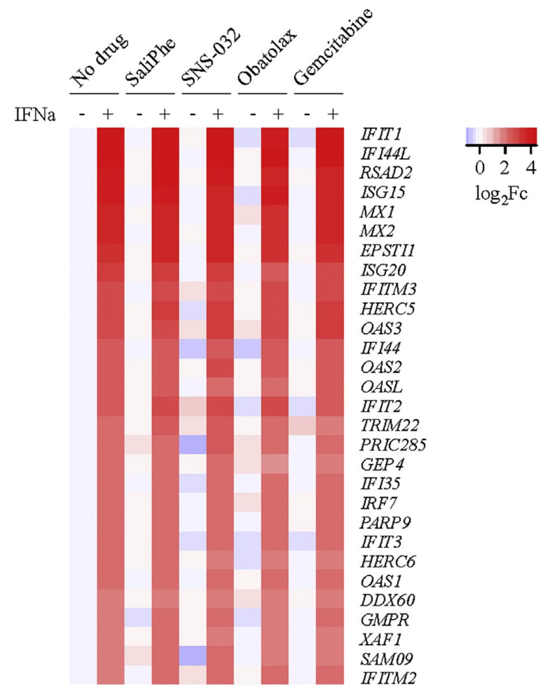
C
No drug
SaliPhe
SNS-032
Obatolax
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IFNa - + - + - + - + - +
IFIT1
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EPSTI1
ISG20
IFITM3
HERC5
OAS3
IFI44
OAS2
OASL
IFIT2
TRIM22
PRIC285
GEP4
IFI35
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DDX60
GMPR
XAF1
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IFITM2
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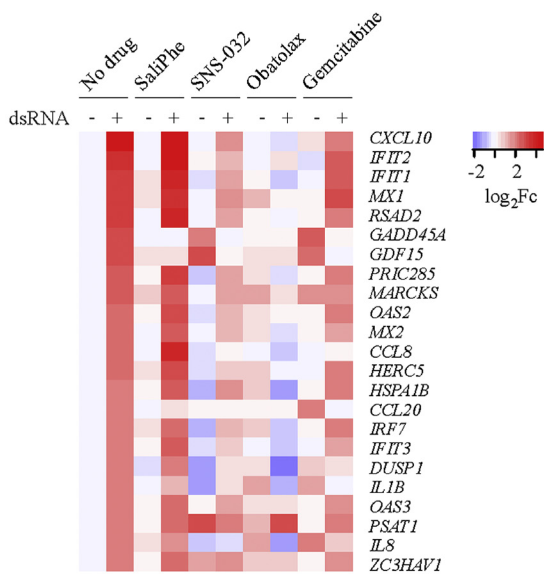
log2Fc


D
No drug
SaliPhe
SNS-032
Obatolax
Gemcitabine
dsRNA - + - + - + - + - +
CXCL10
IFIT2
IFIT1
MX1
RSAD2
GADD45A
GDF15
PRIC285
MARCKS
OAS2
MX2
CCL8
HERC5
HSPA1B
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IL1B
OAS3
PSAT1
IL8
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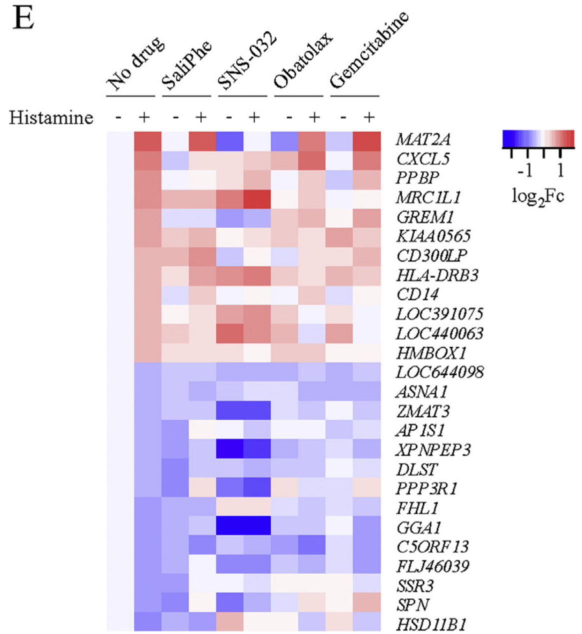
log2Fc


E
No drug
SaliPhe
SNS-032
Obatolax
Gemcitabine
Histamine - + - + - + - + - +
MAT2A
CXCL5
PPBP
MRC1L1
GREM1
KIAA0565
CD300LP
HLA-DRB3
CD14
LOC391075
LOC440063
HMBOX1
LOC644098
ASNA1
ZMAT3
AP1S1
XPNPEP3
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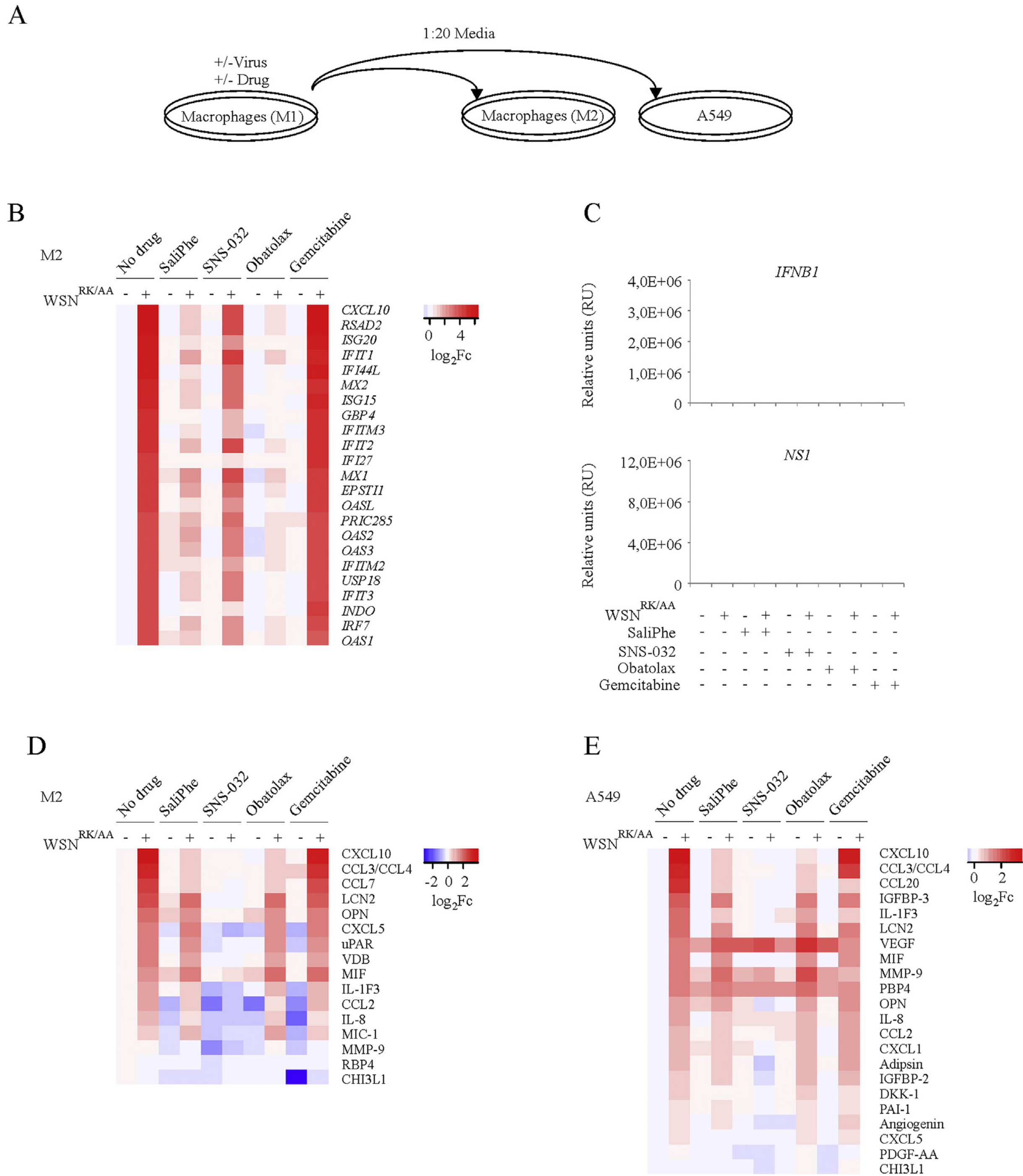

Fig. 5. Immune responses of non-infected macrophages (M2) and A549 cells to immuno-modulators produced by IAV-infected, SaliPhe-, SNS-032-, obatoclax-, and gemcitabine -treated macrophages (M1). (A) Schematics depicting experimental setup. Macrophages (M1) were treated with 3 μM SaliPhe, 0.1 μM SNS-032, 2 μM obatoclax, 1 μM gemcitabine or remained non-treated, and infected with $WSN^{RK/AA}$ or mock. After 1 h the media were changed. After 12 h the media were collected and diluted 1:20 with fresh medium. The media was applied to macrophages (M2) from the same donor or A549 cells. (B) M2 macrophages were stimulated as for panel A. After 8 h M2 cells were collected, total RNA was extracted and subjected to gene expression analysis. A heat map of differentially expressed M2 genes is shown (cut-off for $WSN^{RK/AA}$, $\log_2 Fc > 4$ and <-4). (C) Total cellular RNA was analysed also by quantitative PCRs to detect IFNB1 and NS1 mRNA levels. The points are mean values, the number of observations used to derive the values is 3 and error bars represent the standard deviation. (D) M2 macrophages were stimulated as for panel A. After 24 h cell culture supernatants were collected, and cytokine protein levels were determined. The relative intensities of spots were calculated. A heat map of differentially produced cytokines is shown (cut-off for $WSN^{RK/AA}$, $\log_2 Fc > 0.1$ and <-0.1). (E) A549 cells were stimulated as for panel A. After 24 h cell culture supernatants were collected, and cytokine protein levels were determined. The relative intensities of spots were calculated. A heat map of cytokines produced by A549 cells stimulated with media from M1 macrophages is shown (log2Fc > 0.1 and <−0.1).

Fig. 4. Effect of SaliPhe, SNS-032, obatoclax, and gemcitabine on cellular responses to different immune stimuli. (A) Macrophages were treated with increasing concentrations of dsRNA, IFNα, LPS or histamine. After 24 h cell viability was measured by CTG and results were plotted. (B–E) Macrophages were treated with 3 μM SaliPhe, 0.1 μM SNS-032, 2 μM obatoclax, 1 μM gemcitabine or remained non-treated and stimulated with 1 μg/ml dsRNA, 1 μg/ml LPS, 1 U/ml IFNα, 1 μg/ml histamine or remained non-stimulated. After 8 h, cells were collected; total RNA was extracted and subjected to gene expression analysis. Heat maps of selected genes are shown (cut-offs: $\log_2 Fc^{dsRNA} > 2$ and <-2; $\log_2 Fc^{LPS} > 5$ and <-5; $\log_2 Fc^{IFNa} > 2$ and <-2; $\log_2 Fc^{Histamine} > 0.6$ and <-0.6).

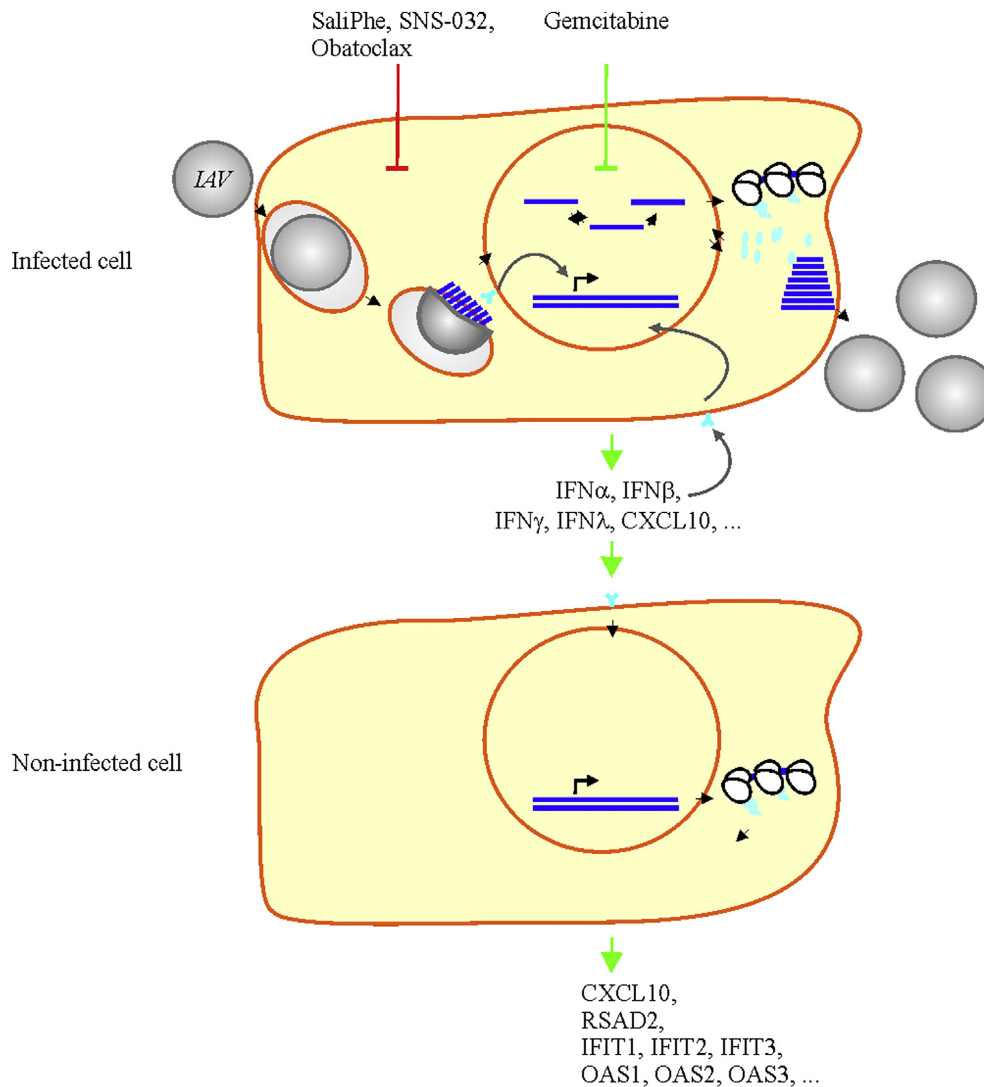

Fig. 6. Schematics showing effects of SaliPhe, SNS-032, obatoclax and gemcitabine on activation of innate immune responses in IAV-infected and non-infected cells. Upon IAV infection of host cell viral ribonucleoprotein complexes (vRNPs) are released from virions into the perinuclear cytoplasm. Specific features on viral RNAs are recognised by cellular pattern recognition receptors (PRRs). PRRs migrate to the nucleus where they mediate chromatin remodelling and activate transcription of antiviral genes. The transcripts are translated on the ribosomes. Some of the translational products are cytokines which are secreted by the infected cells. These immuno-modulators activate immune responses in non-infected cells. Gemcitabine, which inhibits transcription and replication of viral RNA, but not SaliPhe, SNS-032 or obatoclax, which block virus entry, allows activation of immune responses in infected and non-infected cells.

as anti-IAV, Sindbis virus, Semliki Forest virus, Echovirus 6, Herpes Simplex virus-1, Human Immunodeficiency virus type 2, and Rift Valley fever virus agent (Beach et al., 2014; Benedict et al., 2015; Denisova et al., 2012). However, it should be noted that there are some viruses, e.g. H5N1 viruses, which induce a cytokine storm and, therefore, treatment with gemcitabine could harm infected patients (Droebner et al., 2008). In this case, compounds that inhibit cytokine expression (such as SaliPhe, SNS-032, or obatoclax) may be useful for treatment of these viral infections. Thus, immuno-modulatory properties of both virus and antiviral drugs should be carefully considered for treatment of infectious disease.

5. Conclusions

Here we developed a robust method for analysis of immuno-modulating properties of available and prospective anti-influenza agents. We utilized this method to demonstrate that gemcitabine that targets transcription and replication of viral RNA, and not SaliPhe, SNS-032 and obatoclax, which inhibit IAV entry, allows activation of primary antiviral responses in infected cells and secondary responses in non-infected cells. Gemcitabine and its analogues might be beneficial for treatment of patients with severe seasonal IAV infections because treatment with such agents would allow activation of immune responses and thereby could protect patients against co- and re-infections. Thus, our study paves the way towards further development of gemcitabine and its analogues as antiviral drugs. Moreover, our strategy can be utilized in other drug development programmes which aim to discover and exploit immuno-modulating properties or to minimize side-effects of therapeutics.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2015.12.011.

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