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Breaking resistance of pancreatic cancer cells to an attenuated vesicular stomatitis virus through a novel activity of IKK inhibitor TPCA-1

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ABSTRACT

Vesicular stomatitis virus (VSV) is an effective oncolytic virus against most human pancreatic ductal adenocarcinoma (PDAC) cell lines. However, some PDAC cell lines are highly resistant to oncolytic VSV-ΔM51 infection. To better understand the mechanism of resistance, we tested a panel of 16 small molecule inhibitors of different cellular signaling pathways, and identified TPCA-1 (IKK-β inhibitor) and ruxolitinib (JAK1/2 inhibitor), as strong enhancers of VSV-ΔM51 replication and virus-mediated oncolysis in all VSV-resistant PDAC cell lines. Both TPCA-1 and ruxolitinib similarly inhibited STAT1 and STAT2 phosphorylation and decreased expression of antiviral genes MxA and OAS. Moreover, an in situ kinase assay provided biochemical evidence that TPCA-1 directly inhibits JAK1 kinase activity. Together, our data demonstrate that TPCA-1 is a unique dual inhibitor of IKK-β and JAK1 kinase, and provide a new evidence that upregulated type I interferon signaling plays a major role in resistance of pancreatic cancer cells to oncolytic viruses.

Introduction

The use of oncolytic viruses (OVs) as an anticancer strategy arises from their ability to infect, replicate in and kill cancer cells. Compared to non-malignant cells, cancer cells are generally more susceptible to viral infection due to their defects in type I interferon (IFN)-mediated antiviral responses [reviewed in (Barber, 2005; Hastie et al., 2013; Lichty et al., 2004)]. Vesicular stomatitis virus (VSV, a rhabdovirus) is a promising OV successfully used in preclinical models for the treatment of a variety of cancers, and currently in a phase I clinical trial for treatment of hepatocellular carcinoma (clinical trial NCT01628640). Pancreatic ductal adenocarcinoma (PDAC) comprises about 95% of pancreatic cancers and is highly invasive with aggressive local growth and rapid metastases to surrounding tissues. Standard cancer therapies show little efficacy in treating PDAC (Stathis and Moore, 2010). Our recent studies demonstrated that VSV recombinants are effective against a majority of clinically relevant human PDAC cells lines tested (Murphy et al., 2012). However, out of 11 human PDAC cell lines, 4 were resistant to VSV infection, replication and virus-mediated oncolysis (Murphy et al., 2012). In all VSV-resistant cell lines several interferon stimulated genes (ISGs), including the antiviral genes MxA and OAS, were constitutively expressed at high-level, and inhibition of type I IFN signaling pathway using JAK Inhibitor I (JAK Inh. I, a pan-JAK inhibitor) reduced ISG expression and decreased their resistance to VSV (Moerdyk-Schauwecker et al., 2013).

In the present study, to better understand the mechanism of the resistance and find new approaches to overcome it, we tested a panel of 16 inhibitors of different cellular signaling pathways previously shown to affect replication of VSV and other viruses. Our experiments identified one inhibitor of IkB kinase β (IKK-β), TPCA-1, and one selective JAK1/2 inhibitor, ruxolitinib (trade name Jakafi) that decreased levels of ISGs and increased VSV replication and VSV-mediated oncolysis more efficiently than JAK Inhibitor I. Further studies provided evidence that IKK-β inhibitor TPCA-1 also functions as a direct inhibitor of JAK1 kinase. Together, our data show that TPCA-1 is a unique dual inhibitor of IKK-β and JAK1 kinase, and provide a new evidence that the upregulated type I interferon signaling plays a major role in resistance of pancreatic cancer cells to oncolytic viruses.

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Results

Identification of TPCA-1 and ruxolitinib as effective enhancers of VSV replication in VSV-resistant HPAF-II cells

We have shown previously that 4 out of 11 tested human PDAC cell lines were resistant to VSV infection (Moerdyk-Schauwecker et al., 2013; Murphy et al., 2012), at least in part due to constitutive high-level expression of ISGs (Moerdyk-Schauwecker et al., 2013). Pretreatment of resistant cell lines with JAK Inh. I (a reversible inhibitor of JAK1, JAK2, JAK3 and TYK2) reduced ISG expression and partially overcame resistance to VSV (Moerdyk-Schauwecker et al., 2013), suggesting potential for further improvement by utilizing other inhibitors and/or targeting additional pathways. Therefore, in the present study we tested a panel of 16 inhibitors targeting different pathways, shown to directly or indirectly affect ISG expression and/or replication of VSV or other viruses in other experimental systems. As a positive control we included JAK Inh. I. In addition, we included ruxolitinib (INCB018424, trade name Jakafi), a selective inhibitor of JAK1 and JAK2. We also tested two histone deacetylase (HDAC) inhibitors, SAHA (also known as Vorinostat), and valproic acid (VPA), both previously shown to inhibit ISG expression and enhance VSV replication in other systems (Chang et al., 2004; Nguyên et al., 2008; Shulak et al., 2014). As the NF-κB signaling pathway was reported to affect IFN regulated gene expression (Pfeffer et al., 2004), the following inhibitors affecting different factors/steps in the NF-κB signaling pathway were included: eight IKK inhibitors (TPCA-1, SC-514, IKK-16, IKK Inh. XIII, IMD-0354, BMS-345541, IKK-2 Inh. VIII, and sulfasalazine); a 20S proteosome inhibitor (bortezomib); a MEK1/2 inhibitor (U-0126); a mTOR inhibitor (rapamycin); and a COX-2 inhibitor (celecoxib).

VSV-ΔM51-GFP, which has a deletion of the methionine at amino acid position 51 of the matrix protein, and the green fluorescent protein (GFP) ORF inserted at position 5 of the viral genome (between the VSV G and L genes) (Wollmann et al., 2010) was used in this study. The ΔM51 and other M51 mutations in the VSV matrix protein prevent the ability of wild type (wt) matrix protein to shut down expression of antiviral genes (Ahmed et al., 2003; Kopecky et al., 2001; Stojdl et al., 2003). Therefore, VSV-ΔM51 is unable to successfully replicate in healthy cells with intact type I IFN responses. However, as many cancer cells have defective type I IFN signaling (Obuchi et al., 2003), they remain susceptible to VSV-ΔM51 infection. VSV recombinants with M51 mutation are some of the best performing oncolytic VSVs [reviewed in (Hastie and Grdzelishvili, 2012)], and, compared to wt VSV (Bi et al., 1995; Reiss et al., 1998; van den Pol et al., 2002), they show a significantly improved oncoselectivity and decreased neurotoxicity (Stojdl et al., 2003; Wollmann et al., 2010).

The screening of the inhibitors was conducted on one of the most VSV-resistant human PDAC cell lines, HPAF-II (Moerdyk-Schauwecker et al., 2013; Murphy et al., 2012). Cells were treated with each inhibitor at different concentrations based on previously reported effective doses. Following inhibitor treatment for 48 h, cells were infected with VSV-ΔM51-GFP at MOI of 0.001. VSV-driven GFP fluorescence was measured for 5 days p.i. (Fig. 1A and Supplementary Fig. 1A) and cell viability was determined at 5 days p.i. by MTT assay (Fig. 1B and Supplementary Fig. 1B).

In agreement with our previous study (Moerdyk-Schauwecker et al., 2013), JAK Inh. I treatment increased VSV-driven GFP fluorescence (Fig. 1A). A similar enhancement of VSV replication was shown for ruxolitinib, which was previously shown to break resistance of human head and neck cancer cells to VSV (Escobar-Zarate et al., 2013), but has never been tested in combination with VSV in PDAC cells. It should be noted that at the highest concentration tested, ruxolitinib was highly toxic to the cancer cells (Fig. 1B). The HDAC inhibitor SAHA (8 μM) showed a small effect, which was statistically significant but 25-fold less effective compared to fluorescence values reached by treatment with JAK Inh. I or ruxolitinib (Supplementary Fig. 1A). Surprisingly, among the inhibitors targeting the NF-κB pathway, only one, TPCA-1, increased VSV-driven GFP fluorescence and matched levels achieved with the JAK inhibitors (JAK Inh. I and ruxolitinib) (Supplementary Fig. 1A). The effect of TPCA-1 treatment on VSV replication was confirmed with TPCA-1 purchased from two different providers (data not show).

Importantly, increase in VSV-driven GFP expression in HPAF-II cells treated with TPCA-1, ruxolitinib or JAK Inh. I directly correlated with increases in new viral particle production (Fig. 1C). Percentage of GFP positive cells measured by flow cytometry at 48 h p.i. showed an increase from 1.7% for cells treated with no drug to 99.1%, 98.7% and 89.2% for TPCA-1, ruxolitinib and JAK Inh. I treatment, respectively (Fig. 1D).

When VSV-mediated cell killing was determined by MTT, striking decreases of 83%, 90%, and 86% in cell viability were observed for JAK Inh. I (5 μM), ruxolitinib (8 μM) and TPCA-1 (8 μM) treatments, respectively, compared to uninfected cells (Fig. 1B). Treatment with SAHA (8 μM) caused a decrease in cell viability comparable to TPCA-1 (8 μM), even though its effect on VSV-driven GFP fluorescence was marginal (Supplementary Fig. 1A and B). This suggests epigenetic modifications of chromatin may affect VSV induced cell death independently of viral replication. While treatments with IKK Inh. XIII (0.8 μM), BMS-345541 (4 μM), and rapamycin (80, 8 and 0.8 nM) also showed statistically significant decrease in cell viability of infected compared to uninfected cells (21%, 33%, and up to 26%, respectively), these were not as pronounced as the effect of TPCA-1, ruxolitinib or JAK Inh. I treatment (Supplementary Fig. 1B).

As all inhibitors, except JAK Inh. I, rapamycin, celecoxib and VPA, showed significant toxicity in uninfected cells at the highest tested concentrations compared to uninfected cells treated with no drug, it is unlikely that any of the ineffective inhibitors would enhance VSV replication at even higher concentrations (Supplementary Fig. 1B).

TPCA-1 and ruxolitinib overcome resistance to VSV in all VSV-resistant PDAC cell lines

To determine if the enhancement of VSV replication by TPCA-1 and ruxolitinib was limited only to HPAF-II cells, we tested these inhibitors as well as JAK Inh. I in three additional VSV-resistant PDAC cell lines, Hs766T cells (shows a high resistance to VSV, similar to HPAF-II), CFPAC-1 and HPAC (both show an intermediate resistance to VSV) (Moerdyk-Schauwecker et al., 2013; Murphy et al., 2012). Cells were treated with 4 different concentrations of TPCA-1, ruxolitinib or JAK Inh. I for 48 h prior to infection with VSV-ΔM51-GFP at an MOI of 1.5 (based on titration on BHK-21 cells. Fig. 2 legend indicates cell specific MOIs). TPCA-1, ruxolitinib, and JAK Inh. I, enhanced VSV-ΔM51-GFP replication in all VSV-resistant PDAC cell lines (Fig. 2A). The observed lack of a dose-dependent response in some of the cell lines may be due to the narrow range of drug dilutions used in this experiment. For example, there was no dose-dependent effect for ruxolitinib in HPAF-II cells in Fig. 2A (2-fold dilution), but clearly showed a dose dependency when tested at 10-fold dilution (Fig. 1A). In agreement with GFP fluorescence data, for all VSV-resistant PDAC cell lines, treatment with TPCA-1, ruxolitinib and JAK Inh. I caused strong decrease in cell viability in all VSV infected cells compared to uninfected cells (Fig. 2B). Together, our results show that TPCA-1, ruxolitinib and JAK Inh. I are effective in overcoming resistance to VSV in all identified VSV-resistant PDAC cell lines.

Treatment with TPCA-1, ruxolitinib or JAK Inh. I immediately post infection is sufficient to increase VSV replication

Our previous experiments with JAK Inh. I (Moerdyk-Schauwecker et al., 2013) and those presented above were performed by pre-