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SP600125 inhibits Orthopoxviruses replication in a JNK1/2 -independent manner: Implication as a potential antipoxviral

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ABSTRACT

The pharmacological inhibitor SP600125 [anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone] has been largely employed as a c-JUN N-terminal kinase (JNK1/2) inhibitor. In this study, we evaluated whether pretreatment with SP600125 was able to prevent Orthopoxviruses Vaccinia virus (VACV), Cowpox virus (CPXV) and modified Vaccinia virus Ankara (MVA) replication. We found that incubation with SP600125 not only blocked virus-stimulated JNK phosphorylation, but also, significantly reduced virus production. We observed 1–3 log decline in viral yield depending on the cell line infected (A31, BSC-40 or BHK-21). The reduction in viral yield correlated with a dramatic impact on virus morphogenesis progress, intracellular mature viruses (IMV) were barely detected. Despite the fact that SP600125 can act as an efficient anti-orthopoxviral compound, we also provide evidence that this antiviral effect is not specifically exerted through JNK1/2 inhibition. This conclusion is supported by the fact that viral titers measured after infections of JNK1/2 knockout cells were not altered as compared to those of wild-type cells. In contrast, a decline in viral titers was verified when the infection of KO cells was carried out in the presence of the pharmacological inhibitor. SP600125 has been the focus of recent studies that have evaluated its action on diverse viral infections including DNA viruses. Our data support the notion that SP600125 can be regarded as a potential antipoxviral compound.

1. Introduction

The Orthopoxviruses encompass a family of large, double-stranded DNA viruses, approximately 200 kbp in size, whose replication is entirely carried out in the cytoplasm of infected cells (Condit et al., 2006; Moss, 2007). In 1980, the World Health Organization (WHO) declared that smallpox (Variola) – a devastating human disease caused by Variola virus (VARV) – was eradicated (Fenner et al., 1988; Barquet and Domingo, 1997; Smith and McFadden, 2002). With its eradication, vaccination was discontinued. As a consequence, much of the world's population has either never been immunized or has not been immunized for more than 30 years. Either scenario results in a population that is extremely susceptible to variola or other poxviruses.

Our laboratory is interested in dissecting poxvirus-host cell interactions. We have observed that pharmacological inhibition of the MEK/ERK pathway with UO126 or PD98059 decreased virus yield by at least one order of magnitude (de Magalhães et al., 2001; Andrade et al., 2004). Moreover, pretreatment of cells with LY294002, a pharmacological inhibitor of the PI3K/Akt pathway, decreased Vaccinia virus (VACV) or Cowpox virus (CPXV) replication by 99% (Soares et al., 2009). Here we show that SP600125, an anthrapyrazolone inhibitor of the c-JUN N-terminal kinases 1/2 (JNK1/2) (Bennett et al., 2001), caused a significant decrease in viral yield of VACV, CPXV and modified Vaccinia virus Ankara (MVA). Although SP600125 is regarded as a specific JNK inhibitor (Bennett et al. 2001), our findings demonstrate that its antipoxviral

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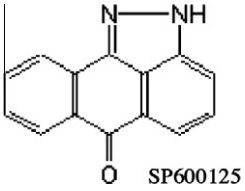
effect is mediated through the target of a yet undefined kinase(s) other than JNK1/2. Since SP600125 has proved to be efficient in vitro against diverse viral infections such as influenza (Mehrotra et al., 2007), rotavirus (Holloway et al., 2006) and herpesvirus (Zapata et al., 2007; Hamza et al., 2004; Perkins et al., 2003; Chen et al., 2002), we propose a potential use of this compound to treat poxviruses infection or complications associated with vaccination.

2. Materials and methods

2.1. Cell culture, antibodies and chemicals

A31 cells (a clone derived from mouse Balb/c 3T3), BSC-40, BHK-21 and mouse embryonic fibroblasts (MEFs) from WT and double knockout (KO) JNK1/2$^{-/-}$ cells (Tournier et al., 2000), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal bovine serum (FBS), (% v/v), as follows: BSC-40 (6%); BHK-21 (10%) and JNK (5%), and antibiotics in 5% CO_2 at 37 °C. FBS was purchased from Cultilab, Campinas, SP, Brazil. A31 cells were kindly provided by Sogayar (Department of Biochemistry, University of São Paulo, Brazil). Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA) gently provided us with WT and JNK1/2 KO cells. The following rabbit polyclonal antibodies were purchased from Sigma–Aldrich (São Paulo, Brazil): anti β-Tubulin or Cell Signaling Technology (Beverly, MA): anti-phospho JNK1/2 (Thr183/Tyr185), anti-c-JUN (Ser73), anti-total ERK1/2, as were the horse radish peroxidase (HRP) conjugated anti-rabbit and anti-mouse secondary antibodies. Both SP600125 [anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone] (structural formula below) and the JNK Inhibitor VIII (JNKi VIII) - (*N*-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenylacetamide), were purchased from Calbiochem (São Paulo, Brazil); inhibitors were diluted in DMSO to a final concentration of 25 mM (SP600125) and 4 mM (JNKi VIII) and stored at −20 °C.



SP600125

2.2. Viruses

(A) *Viral stocks*: Wild-type VACV (strain WR) and CPXV (strain BR) were propagated in Vero or BSC-40 cells. MVA was propagated in BHK-21 cells. Viruses were then highly purified by sucrose gradient sedimentation as described (Joklik, 1962). The experiments presented in this study were carried out using the intracellular mature virus (IMV) form of the virus. (B) *Viral infection*: Cells were allowed to reach 80–90% confluence and starved by changing the media to 1% FBS for 12 h. Cells were infected at the indicated multiplicity of infection (MOI) for the times shown. When needed, cells were treated with the indicated compound for 30 min prior to viral infection and incubated in the continued presence of the drug.

2.3. Multi-step viral growth curves

Thirty five millimeter dishes of A31, BSC-40, BHK-21 and JNK1/2 KO cells (density 5×10^5 cells/dish) were starved and infected at an MOI of 10 for the indicated times 3, 6, 12, 24, 36 and 48 h either in the absence or in the presence of SP600125 (40 μM) or JNKi VIII (4 μM). At each time point, cultures were washed with cold PBS, and cells were disrupted by freeze/thawing. Supernatant were collected and the viral yield was quantified by viral plaque assay as described (da Silva et. al., 2006). Data were confirmed by at least three independent experiments with similar results.

2.4. Electron microscopy

BSC-40 cells were infected with VACV (MOI of 2) either in the absence or in the presence of SP600125 (40 μM) and incubated at 37 °C for 18 h. Cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature, scraped gently, and collected by centrifugation. The cells were washed with cacodylate buffer, postfixed with 1% osmium tetroxide, dehydrated in acetone and processed for conventional transmission electron microscopy. Thin sections were examined with a Morgagni transmission electron microscope operating at 80 kV.

2.5. Cytotoxicity assays

Confluent 35 mm dishes of A31 or BSC-40 cells were treated with increasing concentrations (10, 20, 40 and 50 μM) of SP600125. At 48 h, an equal volume of Trypan Blue stain was added to each well. Cells were stained for 10 min at room temperature after which time the stain was removed and cells were observed for any evidence of stain absorption (an indication of cellular membrane permeability and death). We found that $\geqslant$90% of the cells pretreated with SP600125 at 40 μM were not stained. This concentration was used throughout the experiments. A dose response including 0.4, 4 and 40 μM of JNKi VIII was also performed for cytotoxicity assays and 4 μM was employed in our experiments.

2.6. Western blotting

(A) *Lysate preparation* – A31 and BSC-40 cells were starved and infected with VACV or CPXV (MOI = 10) in the presence or absence of SP600125. At the indicated times, cells were washed with cold PBS and disrupted on ice with lysis buffer [100 mM Tris–HCl (pH 8,0), 1% Triton X-100, 0.2 mM EDTA, 20% glycerol (v/v), 200 mM NaCl, 1 mM $NaVO_3$ (sodium orthovanadate), 1 mM PMSF (phenylmethanesulfonyl fluoride), 5 μg/mL aprotinin, 2.5 μg/mL leupeptin, 1 mM DTT]. Whole cell lysates were collected by centrifugation at 13,500 rpm for 15 min at 4 °C. Protein concentration was determined by the Bio-Rad assay. (B) *Electrophoresis and immunoblotting* – Forty microgram of protein per sample were separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to nitrocellulose membranes (de Magalhães et al., 2001). Briefly, membranes were blocked at room temperature for 1 h with PBS containing 0.1% Tween-20 and 5% (w/v) non-fat milk. The membranes were washed three times with PBS containing 0.1% Tween-20, incubated with specific polyclonal or monoclonal antibody (1:1000–1:3000) in PBS containing 0.1% Tween-20 and 5% (w/v) BSA, followed by incubation with the HRP-conjugated secondary anti-rabbit Ab (1:3000) or anti-mouse Ab (1:1000). Immunoreactive bands were visualized by the ECL detection system as described in the Manufacturer's instructions (GE Healthcare, UK).

3. Results

3.1. VACV and CPXV infection stimulate JNK1/2 phosphorylation

In order to investigate whether the cellular stress associated with orthopoxvirus infection led to the activation of the