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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, 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.

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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 LY29402, 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
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% CO2 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-dimethoxyphenyl)acetamide), 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.

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. Virus stocks were titrated and stored in liquid nitrogen after passage in Vero cells. Cells were 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 NaVO3 (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
stress-associated protein kinases (SAPKs)/c-Jun N-terminal kinase (JNKs), BSC-40 cells were infected with VACV or CPXV. At 3, 6, 12, 24 and 36 h post-infection (h.p.i) whole cell lysates were collected and subjected to western blot to evaluate the phosphorylation status of JNK1/2. Our data (Fig. 1A and B) demonstrated that both CPXV and VACV infections were able to stimulate JNK1/2 phosphorylation (JNK1/2-P) as early as 3 h.p.i., and to a maximal level reached at late times in the infective cycle, 36 h.p.i. As a control, and as expected, we observed no difference in the levels of ERK1/2 during infection. Additionally, viral stimulation of JNK1/2-P was blocked in a dose-dependent manner [10, 20, 40 and 50 μM (Fig. 1C, lanes 4–7)] when VACV infection was performed in the continued presence of SP600125. Similar results were obtained with CPXV infection (data not shown).

3.2. SP600125 inhibits VACV, CPXV and MVA growth

In order to investigate whether the Orthopoxvirus-stimulated JNK1/2-P was biological relevant to the virus, we performed multi-step viral growth curves (MOI = 10) in the presence or absence of SP600125. Cellular extracts were collected at 3, 6, 12, 24, 36 and 48 h.p.i and assayed for viral yield. We observed that the SP600125-mediated inhibition played a relevant role in both VACV and CPXV biology. A significant reduction in the viral titers (>1 log reduction) was observed when VACV (Fig. 2A) or CPXV (Fig. 2B) infections were carried out in the continued presence of SP600125. To verify that the inhibitory effect associated with SP600125 was not restricted to the A31 cells, BSC-40 were infected with VACV or CPXV as described above. As shown in Fig. 2C and D, treatment with SP600125 resulted in a severe decrease in viral production (2–3 log reduction) thereby demonstrating that viral growth inhibition is not cell-type specific. Additionally, we investigated whether SP600125 was able to affect MVA replication. To that end, BHK-21 cells were infected with MVA as described above. Again, our results showed (Fig 2E) that the inhibitor caused a significant decline in virus yield (nearly 3 log reduction); while a more mild decrease (1 log) in infectivity was noted with VACV and CPXV (2F and 2G). The variation in the levels of inhibition caused by SP600125 might be due to the viruses’ tropism within different species such as murine (A31 cells), monkey (BSC-40 cells) and hamster (BHK-21 cells).

3.3. SP600125 severely compromise progress of Orthopoxviruses morphogenesis

In order to investigate at what stage the progression of the viral cycle was affected by SP600125, BSC-40 cells were left untreated (Fig 3A, B and C) or were pretreated with the inhibitor (Fig 3D, E, F and G) and infected with VACV at an MOI of 2. At 18 h.p.i, infected cells were harvested and examined by electron microscopy. While infected cells in the absence of inhibitor (panels A, B and C) contained the full spectrum of virion morphogenesis forms characterized by the identification of crescent, spherical, immature virions (IV), immature virions with nucleoids (IVN) and brick-shaped mature virions (IMV), cells pre-incubated with SP600125 (panels D, E, F and G) showed a severe impairment of morphogenesis progression. Large virosomes surrounded by crescents were repeatedly detected. IVs could be also observed, however IVNs or IMVs were rarely seen. Identical phenotype was also observed when cells were infected with CPXV in the presence of SP600125 (data not shown).

Fig. 1. VACV and CPXV infection stimulate JNK1/2 phosphorylation. BSC-40 cells were left uninfected (MOCK) or were infected with CPXV (A) or VACV (B) for the times shown. (C) Cells were left uninfected (MOCK) or infected (VACV) and treated with SP600125 (10, 20, 40 or 50 μM) prior to and during viral infection (lanes 4–7). Cell lysates (40 μg) were prepared, subjected to western blot and probed with anti-phospho JNK1/2 (Thr183/Tyr185) – upper panels, or probed with anti-ERK1/2 or anti-β-actin antibody as a loading control – lower panels. Data are representative of three distinct experiments with similar results.
3.4. Inhibition of VACV and CPXV growth by SP600125 is independent of JNK1/2

Although SP600125 has been characterized as a specific JNK pharmacological inhibitor (Bennett et al., 2001), a growing body of evidence suggests that SP600125 may be an inhibitor of other kinases as well (Bain et al., 2003, 2007; Bogoyevitch and Arthur, 2008). Thus, to further define whether the reduction in viral yields associated with SP600125 treatment was a direct consequence of JNK1/2 inhibition, WT (Fig. 4A) or JNK1/2 KO MEF cells (Fig. 4B) were infected with VACV or with CPXV. Infections were carried out either in the absence or presence of SP600125 (40 µM) or the pharmacological inhibitor of JNK (JNKi VIII - 4 µM). After 24 h, infected cells were collected and assayed for viral production. As shown in Fig. 4A and B, in the absence of any inhibitor, the viral titers were comparable when produced in either cell line (WT or JNK KO cells lines). This observation strongly suggests that neither VACV nor CPXV require JNK for productive infection. Furthermore, both the WT and JNK KO cells were equally susceptible to SP600125, while being refractory to JNKi VIII treatment.
In order to confirm that JNK does not contribute to the viral replication, we evaluated the phosphorylation levels of its substrate, c-Jun, during viral infection in the presence or absence of either SP600125 or JNKi VIII. Both compounds are known as reversible ATP-competitive JNK inhibitors that ultimately block phosphorylation of JNK substrates such as c-Jun (Bennet et al., 2001; Vivanco et al., 2007). Fig. 4C shows that both SP600125 and JNKi VIII affected VACV- and CPXV-stimulated c-Jun phosphorylation (c-Jun-P). Taken together these findings demonstrated that even though both pharmacological inhibitors targeted the same downstream substrate of JNK (c-Jun), viral replication was only affected in the presence of SP600125. Thus, our data strongly suggest that SP600125 is targeting kinase(s) other than JNK1/2 and, therefore, provide evidence of its JNK-independent inhibitory action.

4. Discussion

Smallpox was announced eradicated by WHO in 1980 and since then, vaccination has been discontinued. As a consequence, much of the world’s population is vulnerable and, therefore, under continuous threat. Moreover, even though the smallpox vaccine (VACV) was successfully used in the WHO’s eradication program, the vaccine has an imperfect safety record and cannot be used with those having immunological deficiency or eczema (Fenner et al., 1988; Barquet and Domingo, 1997; Smith and McFadden, 2002). Furthermore, the re-emergence of CPXV in Europe (Vorou et al., 2008), Monkeypox virus (MPXV) outbreaks in Africa and the United States (Sejvar et al., 2004; Reynolds et al., 2004; Formenty et al., 2010), and the emergence of VACV in Brazil (Fonseca et al., 1998; Damaso et al., 2000; Trindade et al., 2007), emphasizes the need for searching for new antipoxviral compounds with potential use in clinical trials.

Currently, the only antiviral agent currently approved by FDA (Food and Drug Administration) for use against Orthopoxviruses is cidofovir (CDV), an acyclic nucleoside phosphonate analogue, which is known to inhibit not only poxvirus replication but also the replication of a variety of other DNA viruses such as herpesvirus, adenovirus, papillomavirus, and polyomavirus (De Clercq, 2003). In 2009, it was shown that cidofovir impairs Vaccinia DNA encapsidation and, consequently, affects viral morphogenesis (Jesus et al., 2009). In humans, cidofovir has been used successfully
against Molluscum contagiosum virus and ORF virus, however renal toxicity is a known side effect caused by this drug (De Clercq, 2002). Importantly, cidofovir-resistant strains of camelpox, cowpox, monkeypox and vaccinia viruses have also been isolated (Smee et al., 2002). To overcome nephrotoxicity, a derivative form of CDV has been generated and tested. CMX001 is a lipid conjugate of the acyclic nucleotide phosphonate and is currently in Phase II clinical trials for the prophylaxis of human cytomegalovirus infection and under development using the Animal Rule for smallpox infection. CMX001 has demonstrated in vitro and in vivo efficacy against orthopoxvirus infections, and no evidence of nephrotoxicity in either animals or humans was found. Both drugs target the viral DNA polymerase, and VACV strains have been shown to be cross resistant to CMX001 as well.

A new class of anti-poxvirus drugs, which affects both viral spread and dissemination, has also emerged. One of them, ST-246, has been intensely tested against a number of Orthopoxvirus species in animal studies (Yang et al., 2005a,b; Sbrana et al., 2007; Quenelle et al., 2007). ST-246 specifically inhibits the viral protein F13, which is required for the formation of enveloped virus forms. Similar to CDV in which viral resistance is conferred by point mutations in the DNA polymerase gene (Becker et al., 2008), it has also been described that a single point mutation in F13 conferred resistance to ST-246 (Yang et al., 2005a,b). ST-246 was recently tested in a Phase I clinical trial and found to be well tolerated and safe in healthy humans (Jordan et al., 2008, 2010).

An additional approach to inhibit viral multiplication is targeting cellular signaling pathways stimulated and required for successful replication and dissemination. In the past years, we and others have shown the ability of the Orthopoxviruses VACV and CPXV to induce protein kinase pathways to provide an adequate environment to favor their viral replication cycles (de Magalhães et al., 2001; Andrade et al., 2004; da Silva et al., 2006; Mercer and Helenius, 2008; Soares et al., 2009; McNulty et al., 2010). It is also known that poxviruses use the Src and Abl family kinase activities to modulate intracellular spread and release (Frischknecht et al., 1999; Reeves et al., 2005, 2011) but only the Abl family of kinases mediate release of CEV to form EEV (Reeves et al., 2005). Therefore, tyrosine kinase inhibitors originally developed for treating cancers have also been tested against many poxviruses. One of these drugs is imatinib mesylate (STI-571; Gleevec), which is approved for treating human cancers (Tolomeo et al., 2009; Wolf and Rumpold, 2009). Gleevec specifically inhibits the Abl family of kinases that mediate release of CEV to form EEV (Reeves et al., 2005). It has been suggested that cardiotoxicity can be a side-effect caused by this drug; but even targeting cellular kinases may bring attention about unwanted side effects (Kerkelä et al., 2006), it seems that drug resistance cannot readily develop, which is a benefit for antiviral chemotherapy.

The anthrapyrazolone inhibitor of c-JUN N-terminal kinases 1/2 (JNK1/2), SP600125 (Bennett et al., 2001; Bogoyevitch et al., 2004), the focus of this manuscript, has been largely utilized as a potential
therapeutic for the treatment of cancer and diseases caused by inflammation and neurodegeneration (Sharma et al., 2010; Holm et al., 2011; Hu and Liu, 2009; de Borst et al., 2009; Wang et al., 2009; Song et al., 2008). Some derivatives of SP600125 are being tested in diverse clinical trials (Manning and Davis, 2003; Bogoyevitch et al., 2004; Bennett, 2006; Bogoyevitch and Arthur, 2008). In addition, the antiviral effects of SP600125 have been investigated in diverse viral models suggesting that JNK inhibitors may provide new therapeutic interventions (Manning and Davis, 2003; Bogoyevitch and Arthur, 2008). For instance, it has been shown that the viral kinase ORF36 of the Kaposi’s sarcoma-associated herpesvirus activates JNK1/2 and its inhibition by SP600125 blocks viral gene expression at late stages of infection (Hamza et al., 2004). Varicella-zoster virus (VZV) replication was also decreased in a dose-dependent manner by treatment with SP600125 (Zapata et al., 2007). Another report showed that SP600125 inhibited the activation of JNK by the hepatitis C virus protein NS3, which contributes to hepatitis C related hepatocarcinogenesis (Hassan et al., 2007). Furthermore, the use of signal transduction pathways modulators, either singly (Yang et al., 2005a,b; Reeves et al., 2005) or in combination, could be the most appropriate therapeutic strategy. In fact, it has been shown that SP600125 used together with inhibitors of phosphatidylinositol 3-kinase/Akt prevented the establishment of persistent SARS-CoV infection (Mizutani et al., 2005).

While studying the Orthopoxviruses VACV, CPXV, and MVA-cell host- interaction, we found that SP600125 exerted an antiviral effect. Our results showed a dramatic reduction in virus yield when infections were performed in the presence of this inhibitor. Electron microscope images demonstrated that in the presence of SP600125, Orthopoxviruses replication is compromised; normal-looking IVs are frequently seen but IVN are very rare and no IMVs could be detected (Fig 3, Bottom panel). SP600125 is considered as a specific pharmacological inhibitor of JNK1/2, not only in response to cytokine stimulation (Dong et al., 2000), but also with viral infections (Bogoyevitch and Arthur, 2008). Our results show that upon VACV or CPXV infection JNK1/2 is activated during the entire viral cycle and SP600125, indeed, inhibits JNK1/2 phosphorylation in a dose-dependent manner (Fig 1C). However, the block identified in the viral cycle caused by SP600125 is an event that occurs independently of JNK1/2 since no effect on viral yield was observed when infections were performed in JNK1/2 KO MEF cells. Similar results were found with the use of JNKi VIII inhibitor.

Previous reports have shown that SP600125 inhibits cellular kinases in vitro other than JNK1/2 (Bain et al., 2003, 2007), but even in the face of the concerns raised on the specificity of this inhibitor, several studies still rely on this drug for a possible therapeutic application regarding treatment of human diseases. Furthermore, since its discovery in 2001, SP600125 has been extensively studied for treatment of numerous non-viral diseases in murine model (Ikezumi et al., 2004; Gao et al., 2005; Han et al., 2005; Gunawan et al., 2006; Guan et al., 2006; Takamura et al., 2007; Syrkin et al., 2007; Hu and Liu, 2009). However, up to the publication of this work, a search in the literature did not show a single report demonstrating that SP600125 is effective against viral infection in animal studies to support the results observed in cell culture system. Furthermore, studies have shown that viral infection can lead to JNK activation and the inhibition of these cellular kinases by SP600125 affects viral multiplication (Hamza et al., 2004; Hassan et al., 2005; Zapata et al., 2007; Gupta et al., 2011).

Most of these studies make a strict connection between the inhibition of JNK by SP600125 and its impact on viral infection. Because JNK is only one of the kinases targeted by this drug, additional analyses with other inhibitors of JNK1/2 or cell lines knockouts for those kinases or even RNAi approach should be taken into consideration to confirm this direct relationship. Therefore, since animal studies are a cost, time and energy-dependent system, it is possible that researchers are more careful about taking a step further and testing SP600125 in mice, for instance, and do not succeed in correlating the data observed in tissue culture. Additional disadvantages of SP600125 may be considerable off-target activity, or perhaps its poor solubility in aqueous solution or/and possible undesirable side-effects (Bennett et al., 2001; Bain et al., 2003; Begleiter et al., 2006). In effort to get around these complications, a derivative of SP600125 (CC-401) was developed by Celgene has successfully completed a Phase I trial in healthy volunteers as stated by the pharmaceutical company. CC-401 has also been reported in Phase II evaluation for the treatment of acute myelogenous leukemia, and has also been considered for the treatment of respiratory diseases (Roberts and Der, 2007; Bogoyevitch and Arthur, 2008). Nevertheless, a shortest path to evaluate SP600125 in vivo against an orthopoxvirus infection would be a viral challenge in a murine model.

Taken together, questions still remain regarding the potential protein kinase(s) targeted by SP600125 during Orthopoxvirus infection causing the impairment of viral morphogenesis. Poxviruses encode two essential serine/threonine kinases, B1 (Traktman et al., 1989; Lin et al., 1992; Rempel and Traktman, 1992) and F10 (Lin and Broyles, 1994). While B1 plays a function during viral DNA replication (Traktman et al., 1989; Rempel et al., 1990; Domi and Beaud, 2000), F10 plays a role in the very early stages of virion morphogenesis (Wang and Shuman, 1995; Traktman et al., 1995). When B1 or F10 proteins are repressed or inactive, none of the hallmarks of morphogenesis are identified. Therefore, it is doubtful that SP600125 would target one or both viral kinases. In addition, some viral proteins that play a role in morphogenesis are proposed to be also phosphorylated by cellular kinases (Resch et al., 2005; Unger and Traktman, 2004; Wickramasekera and Traktman, 2010). By comparison with electron microscopy images of VACV mutants, under nonpermissive conditions, we observed that some of them phenotypically copy our results when infections are performed in the presence of SP600125. The repression of the phosphoprotein A13L arrests morphogenesis at the stage of IV formation. Essentially, no IMVs are seen and IVNs are rare; DNA crystalloids accumulate in the cytoplasm (Unger and Traktman, 2004). A similar phenotype is also seen when H3L, a major immunodominant protein, is repressed or deleted (da Fonseca et al., 2000). When the muristylated L1R protein is repressed, the transition from IV to IMV is blocked (Ravanello et al., 1994). Thus far, it is hard to predict a putative cellular target for SP600125 that would affect viral morphogenesis. Steps that prior and subsequently lead to the formation and maturation of IMVs are very complex and not fully understood. Protein phosphorylation, protein–protein interactions and proteolytic processing are some of the events involved. Since cellular kinases are likely thought to contribute to phosphorylation of viral proteins, it is plausible that their inhibition by SP600125 could affect those events blocking morphogenesis progress.

In conclusion, our results demonstrate the use of SP600125 inhibits Orthopoxviruses replication in a JNK independent-manner. This suggests that other cellular and/or viral substrates are affected by the action of SP600125. While significant progress has been made in the discovery of novel compounds against Orthopoxviruses, the need for a range of antiviral drugs is imperative since the occurrence of resistance to antiviral drugs is not a rare event. Our data support the notion that SP600125 can be regarded as a potential antipoxviral compound; the combined use of SP600125 with other antipoxviral drugs may enhance their antiviral activity and, perhaps, minimize major side effects with the advantage of reducing drug resistance.
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