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Inhibition of CRM1-mediated nuclear export of influenza A nucleoprotein and nuclear export protein as a novel target for antiviral drug development

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ABSTRACT

An anti-influenza compound, DP2392-E10 based on inhibition of the nuclear export function of the viral nucleoprotein-nuclear export signal 3 (NP-NES3) domain was successfully identified by our previous high-throughput screening system. Here, we demonstrated that DP2392-E10 exerts its antiviral effect by inhibiting replication of a broad range of influenza A subtypes. In regard to the molecular mechanism, we revealed that DP2392-E10 inhibits nuclear export of both viral NP and nuclear export protein (NEP). More specifically, *in vitro* pull-down assays revealed that DP2392-E10 directly binds cellular CRM1, which mediates nuclear export of NP and NEP. *In silico* docking suggested that DP2392-E10 binds at a region close to the HEAT9 and HEAT10 domains of CRM1. Together, these results indicate that the CRM1-mediated nuclear export function of influenza virus represents a new potential target for antiviral drug development, and also provide a core structure for a novel class of inhibitors that target this function.

1. Introduction

Influenza virus, which belongs to family *Orthomyxoviridae*, replicates its single-stranded negative-sense genomic RNAs in the host nucleus. The viral genomic RNAs are then packed into viral ribonucleoproteins (vRNPs) with viral RNA-dependent RNA polymerase and nucleoproteins (NPs) and exported into cytoplasm for further viral particle assembly and budding at the plasma membrane (Eisfeld et al., 2015). vRNP nuclear export is mediated by the cellular chromosome region maintenance 1 (CRM1) pathway, with assistance from viral matrix protein 1 (M1) and nuclear export proteins (NEP or NS2) (Akarsu et al., 2003; Huang et al., 2013; O'Neill et al., 1998). The vRNP–M1–NEP–CRM1–RanGTP complex is then exported through the nuclear pore to the cytoplasm, where the viral cargo is spontaneously released upon hydrolysis of RanGTP to RanGDP (Eisfeld et al., 2015).

Several studies have described the role of NP in vRNP nuclear export. For example, leptomycin B (LMB), an inhibitor of the CRM1 nuclear export signal (NES) binding domain (Kudo et al., 1999), inhibits nuclear export of vRNP and NP, but not NEP or M1, in virally infected or transfected cells (Elton et al., 2001; Watanabe et al., 2001). In addition, CRM1 overexpression promotes the nuclear export of NP, but not NEP or M1 (Elton et al., 2001), and the interaction of NP with viral genomic RNA, and M1 is sufficient for nuclear export of vRNP (Huang et al., 2001). Moreover, NP directly binds to CRM1 (Chutiwitoonchai et al., 2014; Elton et al., 2001; Kakisaka et al., 2015), and the interaction between NP/CRM1 and a recent identified host factor, nuclear transport factor 2-like export protein 1 (NXT1), promotes nuclear export of NP and vRNA (Chutiwitoonchai and Aida, 2016). NP must also interact with cellular nucleolin, a major component of the nucleolar compartment, to facilitate the interaction between vRNP and the cellular nuclear export machinery (Terrier et al., 2016). In addition to its role in vRNP nuclear export, NP plays other important roles in the viral life cycle, including vRNP nuclear import

Abbreviations: NP, nucleoprotein; NEP, nuclear export protein; NES, nuclear export signal; vRNP, viral ribonucleoprotein; CRM1, chromosome region maintenance 1; AcGFP, *Aequorea coerulescens* green fluorescent protein; LMB, leptomycin B; IC_{50}, half-maximal inhibitory concentration; CC_{50}, half-maximal cytotoxic concentration; EC_{50}, half-maximal effective concentration; MOE, Molecular Operating Environment

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and transcription/replication of the viral genome (Aida et al., 2012; Cianci et al., 2013; Portela and Digard, 2002; Sasaki et al., 2013). These functional studies support NP as a promising novel target for antiviral drug development.

Inhibition of vRNP nuclear export is an effective strategy for counteracting influenza replication. Treatment with LMB, an inhibitor that irreversibly covalently binds with the C258 of the CRM1 NES binding domain (Kudo et al., 1999), results in retention of vRNPs in the nuclei of infected cells (Elton et al., 2001; Ma et al., 2001; Watanabe et al., 2001). Similarly, Verdinixor, a representative of a new class of CRM1 inhibitor, forms a reversible covalent bond with C258 of CRM1 and thereby inhibits replications of influenza A subtypes H1N1, H5N1, and H7N9 (Perwitasari et al., 2014). Inhibition of vRNP nuclear export by an inhibitor with an unknown target, 14-deoxy-11,12-dehydroandrographolide (Cai et al., 2015), or NP-target inhibitors such as pyrimido-pyrrolo-quinoxalinedione analog (Lin et al., 2015) and 1,3,4,6-tetra-O-galloyl-β-D-glucopyranoside (Chang et al., 2016) can also potentially diminish replication of influenza viruses. Moreover, targeting of the third NES domain of NP (NP-NES3) by an inhibitor we discovered, RK424, results in antiviral effects *in vitro* and *in vivo* (Kakisaka et al., 2015). Recently, using our high-throughput CELAVIEW screening system, we identified another potential inhibitor, DP2392-E10, which targets the nuclear export function of the NP-NES3 domain (Kakisaka et al., 2016). Although the molecular mechanism of inhibition by DP2392-E10 was not validated at that time, our preliminary study demonstrated that the compound exerts an antiviral effect against influenza A/WSN/1933 (H1N1) replication (Kakisaka et al., 2016).

Here, we extended our study of DP2392-E10 to characterize its broad range of anti-influenza effects, cytotoxicity, and efficiency of nuclear export inhibition. In addition, we investigated the molecular mechanism of inhibition by DP2392-E10 and found that it targets the CRM1 protein to block CRM1-mediated nuclear export of NP and NEP, thereby preventing vRNP nuclear export and viral replication.

2. Results

2.1. DP2392-E10 reduces replication of a broad range of influenza A subtypes

We previously succeeded in identifying a chemical compound, DP2392-E10 (Fig. 1A), that inhibits the nuclear export function of NP-NES3 domain and decreased replication of influenza A/WSN/1933 (Kakisaka et al., 2016). To further characterize the broad-range inhibitory effect of DP2392-E10, we performed plaque assays to monitor the replications of seasonal influenza A H1N1 and H3N2 including other subtypes, H6N2, H8N4, H9N2, H14N5, and H15N8 in the presence ,,,,,,of the compound. The results revealed that DP2392-E10 decreased replication of all tested subtypes in a dose-dependent manner at the half-maximal inhibitory concentration (IC_{50}) range between 12.63 and 34.57 μM, depending on the viral strains (Fig. 1B and C). Cytotoxicity tests were evaluated in Madin-Darby canine kidney (MDCK) and A549 (a human lung adenocarcinoma epithelial) cells indicating that the compound modestly affected cell viability at the half-maximal cytotoxic concentration (CC_{50}) of 61.25 ± 2.67 and 94.49 ± 7.56 μM, respectively (Fig. 1C). Viral replication kinetic in the presence of DP2392-E10 showed that the compound decreased replication throughout the assay course (Fig. 1D). These results demonstrate that DP2392-E10 inhibits replication of a broad range of influenza A subtypes.

2.2. DP2392-E10 decreases virus replication by inhibiting nuclear export of viral NP and NEP

In a high-throughput screen using MDCK cells stably expressing AcGFP-NP-NES3, we identified DP2392-E10 as a compound that could inhibit the CRM1- dependent export of the fusion protein (Kakisaka et al., 2016). To validate the mechanism of inhibition by DP2392-E10, we monitored the intracellular localization of intact NP and another CRM1-dependent nuclear export protein, NEP (O'Neill et al., 1998), in infected cells treated with the compound for 2, 4, or 6 h. In the dimethyl sulfoxide (DMSO) control cells, NP and NEP were localized in the nucleus at 2 h post-infection (hpi), but most of the NP and some NEP had been observed in the cytoplasm at 4 and 6 hpi (Fig. 2, left panel), reflecting nuclear export observation of NP and NEP in the infected cells. In the DP2392-E10–treated cells, NP and NEP were still localized in the nucleus at 4 and 6 hpi, indicating that the compound prevented nuclear export of both proteins (Fig. 2, right panel). Together with the results of the plaque assays described above, these data suggested that DP2392-E10 reduced virus replication by inhibition of NP and NEP nuclear export.

2.3. DP2392-E10 inhibits CRM1-dependent nuclear exports of the NP-NES3 and NEP-NES2 domains

Influenza virus hijacks the cellular CRM1 nuclear export machinery to facilitate nuclear export of NP and NEP via the NP-NES3 and NEP-NES2 domains, respectively (Eisfeld et al., 2015; Huang et al., 2013; Yu et al., 2012). To further confirm that DP2392-E10 inhibits nuclear export of NP-NES3 and NEP-NES2 via the CRM1 pathway, well-known CRM1-dependent nuclear export marker, HIV-1 Rev (Dong et al., 2009; Fornerod et al., 1997), and a specific inhibitor of CRM1 nuclear export, LMB (Kudo et al., 1999), were used. MDCK cells stably expressing *Aequorea coerulescens* green fluorescent protein (AcGFP)-NP-NES3, AcGFP-NEP-NES2, or AcGFP-Rev-NES domain (Fig. 3A) (Kakisaka et al., 2016) were treated for 8 h with DMSO, LMB, or DP2392-E10, and the nuclei were stained with Hoechst 33342. CELAVIEW quantification of the green fluorescence intensity from AcGFP-tagged NES domain protein in the nuclear region revealed that DP2392-E10 inhibited cytoplasmic localization of the NP-NES3 and NEP-NES2 domain proteins in a similar manner to LMB (Fig. 3B and C). As expected, DP2392-E10 also inhibited nuclear export of the positive control, the Rev-NES domain protein (Fig. 3D). The inhibition efficiency of DP2392-E10 against nuclear export of NP-NES3, NEP-NES2, and Rev-NES were at the half-maximal effective concentration (EC_{50}) of 7.42 ± 0.01, 12.33 ± 2.74, and 9.93 ± 0.92 μM, respectively. The comparable EC_{50} values suggest that DP2392-E10 targets the CRM1 protein to inhibit nuclear export of different viral NES domains. This result confirmed that DP2392-E10 inhibits CRM1-dependent nuclear export of NP and NEP via the NP-NES3 and NEP-NES2 domains, respectively, thereby decreasing virus replication.

2.4. DP2392-E10 prevents the NP/CRM1 interaction by directly targeting the CRM1 protein

The nuclear export function of the NP-NES3 domain is CRM1-dependent (Yu et al., 2012), and NP directly binds CRM1 (Chutiwitoonchai et al., 2014; Elton et al., 2001; Kakisaka et al., 2016). To verify that DP2392-E10 inhibits nuclear export by blocking the NP/CRM1 (and NEP/CRM1) interaction, we performed *in vitro* pull-downs of NP and CRM1 proteins in the presence of the compound. For this purpose, NP-FLAG protein was immobilized on anti-FLAG monoclonal antibody (mAb)-conjugated agarose beads and incubated with purified CRM1-HA protein in the presence of DMSO or DP2392-E10 at 3, 10, 30, or 100 μM. Western blot analysis of the pull-down samples indicated that DP2392-E10 inhibited NP/CRM1 binding in a dose-dependent manner (Fig. 4A).

We next investigated whether CRM1 is the target of DP2392-E10 by generating DP2392-E10 photo-crosslinked Sepharose beads and subjecting them to the pull-down assay. Specifically, the beads were incubated with purified NP-FLAG or FLAG-CRM1 protein overnight, and then pulled down. Western blots revealed that DP2392-E10