A Novel CDK7 Inhibitor of the Pyrazolotriazine Class Exerts Broad-Spectrum Antiviral Activity at Nanomolar Concentrations

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Protein kinases represent central and multifunctional regulators of a balanced virus-host interaction. Cyclin-dependent protein kinase 7 (CDK7) plays crucial regulatory roles in cell cycle and transcription, both connected with the replication of many viruses. Previously, we developed a CDK7 inhibitor, LDC4297, that inhibits CDK7 *in vitro* in the nano-picomolar range. Novel data from a kinome-wide evaluation (>330 kinases profiled *in vitro*) demonstrate a kinase selectivity. Importantly, we provide first evidence for the antiviral potential of the CDK7 inhibitor LDC4297, i.e., in exerting a block of the replication of human cytomegalovirus (HCMV) in primary human fibroblasts at nanomolar concentrations (50% effective concentration, 24.5 ± 1.3 nM). As a unique feature compared to approved antiherpesviral drugs, inhibition occurred already at the immediate-early level of HCMV gene expression. The mode of antiviral action was considered multifaceted since CDK7-regulated cellular factors that are supportive of HCMV replication were substantially affected by the inhibitors. An effect of LDC4297 was identified in the interference with HCMV-driven inactivation of retinoblastoma protein (Rb), a regulatory step generally considered a hallmark of herpesviral replication. In line with this finding, a broad inhibitory activity of the drug could be demonstrated against a selection of human and animal herpesviruses and adenoviruses, whereas other viruses only showed intermediate drug sensitivity. Summarized, the CDK7 inhibitor LDC4297 is a promising candidate for further antiviral drug development, possibly offering new options for a comprehensive approach to antiviral therapy.

Cyclin-dependent kinases (CDKs) are characterized as regulators of two major transitions in the cell cycle, namely, the initiation of the DNA synthesis (S) phase and the entry into mitosis (M) phase. CDKs are generally coregulated in activity by their interaction with distinct types or a selection of cyclins. In addition to cell cycle control, CDK/cyclin complexes have also been identified as conserved components of the RNA polymerase II (RNAP II) transcriptional machinery (1). CDK7 is both a CDK-activating kinase (CAK), which phosphorylates CDKs within the activation segment (T-loop), and a component of the transcription factor TFIIH, which phosphorylates the C-terminal domain (CTD) of RNAP II (2–5). This central importance of CDK7 has long been asserted as an essential role in cellular metabolism and viability. Recently, a study by Ganuza et al. (6) demonstrated that depletion of CDK7 *in vivo* had no phenotypic consequences in adult tissues with low proliferative indexes and that CDK7 is mostly dispensable for transcriptional regulation. In contrast, CDK7 activity appears basically essential for cell cycle activation via phosphorylation of CDKs, primarily CDK1 and CDK2, and genetic inactivation of CDK7 leads to cell cycle arrest in tissues with elevated cellular turnover. Thus, CDK7-related defects may not be universally expressed, but can be restricted to highly proliferating tissues (i.e., age-related or developmental defects seen in animal models). On this basis, the importance of CDK7 has been reconsidered and thus has been experimentally reevaluated with regard to its cell cycle-specific function rather than to its role in transcription (6). This finding supported previous postulates that CDK7 might be a valuable target for drugs directed toward the treatment of malignancies and cell cycle-associated diseases (7). In this context, it was noted that CDK7 and other CDKs are involved in the regulation of the productive replication of a number of viruses (8, 9). Previous studies particularly stressed the relevance of CDK7-driven regulatory pathways for the replication of herpesviruses,

such as human cytomegalovirus (HCMV) (10, 11). HCMV shows a dependence on the activities of CDK7 and CDK9 during the immediate-early and early phases of viral replication (12). Our present investigations with novel selective inhibitors of CDK7 supported this causative link between CDK7 function and the efficiency of HCMV replication. The findings validated CDK7 as an antiviral target and underlined the potential of the CDK7 inhibitor LDC4297 as a candidate for a novel cell-directed antiviral strategy.

MATERIALS AND METHODS

Cultured cells and viruses. Primary cultures of human (i.e., human foreskin fibroblast [HFF]), guinea pig, or murine fibroblast tissues were grown and passaged (splitting ratio, 1:3) in a 5% CO₂ atmosphere at 37°C in minimal essential medium (MEM; Gibco) supplemented with 7.5% (vol/vol) fetal bovine serum (FCS; Sigma-Aldrich), 10 µg/ml gentamicin, and 350 µg/ml glutamine. Immortalized cell lines cultured as adherent

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monolayers, i.e., 293T, A549, ARPE19, and Vero cells, were maintained in Dulbecco minimal essential medium (Gibco); cell lines growing in suspension, i.e., J-Jhan and CEMx174 cells, were maintained in RPMI 1640 medium (Gibco), both supplemented with 10% FCS, gentamicin, and glutamine. Viruses were used as follows: human cytomegaloviruses (HCMVs), strains AD169-GFP and TB40-UL32-EGFP (13, 14); guinea pig cytomegalovirus (GPCMV), strain v403-GFP (15); murine cytomegalovirus (MCMV), strain Smith (16); human herpesvirus 6A (HHV-6A), strain U1102-GFP (17); herpes simplex virus 1 and 2 (HSV-1 and HSV-2), strain 166v VP22-GFP and isolate 01-6332, respectively (18); varicella-zoster virus (VZV), strain Oka (19); Epstein-Barr virus (EBV), strain B95-8 (20); human adenovirus type 2 (HAdV-2) (21); vaccinia virus, strain IHD-5 (obtained from the American Type Culture Collection); human immunodeficiency virus 1 (HIV-1), strains NL4-3 and 4LIG7 (repository of the Institute of Clinical Molecular Virology, University of Erlangen-Nuremberg) (22); influenza A virus, strain A/WSN/33 (repository of laboratory M.M., University of Erlangen-Nuremberg). For virus infections, cells were seeded in 6-well, 12-well, or 24-well plates and infected at multiplicities of infection (MOIs) of 0.01 to 3 under standard conditions (13, 23).

Antiviral assays. Antiviral assays were established for a selection of human and animal viruses used for the infection of a set of different primary and immortalized cells types as described earlier (13, 20, 22, 24–27). Specifically, a green fluorescent protein (GFP)-based viral replication assay was performed with HCMV AD169-GFP in HFFs as previously described (13). In brief, HFFs were cultivated in 12-well plates (2.25×10^5 cells/well), infected with HCMV AD169-GFP (MOI of 0.1 to 0.25, i.e., $\leq 25\%$ GFP-positive cells at 7 days postinfection [p.i.]), and treated with antiviral drugs by onetime addition of the drug immediately after virus infection. At 7 days p.i., the cells were lysed, and the lysates were subjected to automated GFP quantitation using a Victor 1420 multilabel counter (Perkin-Elmer, Germany). All infections were performed in duplicate; GFP quantifications were performed in quadruplicate. Similarly, this assay system was also applied to other GFP-expressing recombinant viruses. For viruses lacking GFP reporter expression, a standard plaque reduction assay was performed under previously established conditions (13). Moreover, a virus yield assay was performed with HFFs grown in 12-well plates used for infection with HCMV AD169-GFP (MOI of 0.3). Antiviral compounds were applied to the culture media, samples were collected at the time points indicated, and the virus yield was determined by plaque reduction assay or GFP fluorimetry. Note that 293T and other epithelial cells have been used for the determination of EBV and influenza virus replication as described previously (20, 24, 28).

Drugs. The antiviral drugs and kinase inhibitors used in the present study were obtained from various sources: ganciclovir (GCV; Sigma-Aldrich), roscovitine (Calbiochem), staurosporine (Calbiochem), G66976 (Sigma-Aldrich), SNS-032 (Selleckchem), and Flavopiridol (Selleckchem). LDC4297 was synthesized in-house by Lead Discovery Center, GmbH, Dortmund, Germany; the chemical structure is shown in Fig. 1A.

Kinome-wide selectivity profiling of LDC4297. In brief, a radiometric protein kinase assay (PanKinase activity assay; performed by ProQinase, GmbH, Freiburg, Germany) was used for measuring the kinase activity of the 333 human protein kinases. All kinase assays were performed in 96-well FlashPlates from Perkin-Elmer (Boston, MA) in a 50- μ l reaction volume. The reaction cocktail was pipetted in four steps in the following order: 10 μ l of nonradioactive ATP solution (in H_2O), 25 μ l of assay buffer–[γ - ^{32}P]ATP mixture, 5 μ l of test sample in 10% dimethyl sulfoxide (DMSO), and 10 μ l of enzyme-substrate mixture. The assay for all enzymes contained 70 mM HEPES-NaOH (pH 7.5), 3 mM MgCl₂, 3 μ M sodium orthovanadate, 1.2 mM dithiothreitol, ATP–[γ - ^{32}P]ATP (variable amounts, corresponding to the apparent ATP K_m of the respective kinase; approximately 8×10^5 cpm per well), and purified protein kinase and substrate (both variable amounts). The chemicals that were used in specific assays were as follows: all protein kinase C (PKC) assays (except the PKC- μ and PKC- ν assays) also contained 1 mM CaCl₂, 4 mM

EDTA, 5 μ g/ml phosphatidylserine, and 1 μ g/ml 1,2-dioleoyl-glycerol; the CAMK1D, CAMK2A, CAMK2B, CAMK2D, CAMK4, CAMKK1, CAMKK2, DAPK2, EEF2K, MYLK, MYLK2, and MYLK3 assays included 1 μ g/ml calmodulin and 0.5 mM CaCl₂; the PRKG1 and PRKG2 assays contained 1 μ M cGMP; and the DNA pharmacokinetic (PK) assay contained 2.5 μ g/ml DNA. All protein kinases provided by ProQinase were expressed in Sf9 insect cells or in *Escherichia coli* as recombinant glutathione S-transferase (GST) fusion proteins or His-tagged proteins. All kinases were expressed from human cDNAs. Kinases were purified by affinity chromatography using either glutathione-agarose or Ni-nitrilotriacetic acid-agarose. The purity and identity of protein kinases was examined by SDS-PAGE/Coomassie blue staining and mass spectroscopy, respectively. Kinases obtained from external vendors (Carna Biosciences, Inc.; Invitrogen Corp.; and Millipore Corp.) were expressed, purified, and quality controlled based on vendor-supplied information.

Cytotoxicity and cell proliferation assays. Assays measuring distinct parameters of cytotoxicity and/or cell proliferation were performed as described earlier (25, 27). In brief, a trypan blue exclusion assay was performed with cultured cells seeded in 24-well plates and incubated with increasing concentrations of antiviral compounds (range, 0.1 to 50 μ M) for the durations indicated. Cell staining was achieved with 0.1% trypan blue for 10 min at room temperature before the percentage of viable cells was determined by microscopic counting (determination at least in quadruplicate). LDH release assays were performed using the CytoTox 96 nonradioactive cytotoxicity assay (Promega) with medium samples of cells cultured for 1 day in the presence of antiviral compounds. The release of LDH activity was determined according to the protocol of the manufacturer. The cell proliferation assay (CellTiter 96 AQueous One Solution cell proliferation assay; Promega) was performed in a 96-well plate format under standard conditions (27).

In vitro kinase assays. For CDK-specific analyses, a fluorescence resonance energy transfer (FRET)-based Lance Ultra KinaSelect Ser/Thr assay (Perkin-Elmer) was used to determine 50% inhibitory concentration (IC₅₀) values for the enzymatic activity of CDK7 under conditions recommended by the manufacturer. In brief, a specific ULight MBP peptide substrate (50 nM) was incubated for phosphorylation by a CDK/CycH/MAT1 complex in 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, and 2 mM dithiothreitol containing ATP at the concentration of the ATP K_m value (35 μ M), or $\times 10$ or $\times 100$ above the ATP K_m value, for 1 h at room temperature. Subsequently, phosphorylation was detected by the addition of an europium-labeled anti-phospho-antibody (2 nM). Antibody binding produced a FRET signal that was recorded in a time-resolved manner using a Perkin-Elmer EnVision reader. For pUL97-specific analyses, *in vitro* kinase assays measuring the activity of viral kinase pUL97 were performed as described earlier (27–32).

Western blot analysis. Detection of cellular and viral proteins by Western blotting was performed according to standard procedures (27–32).

RESULTS

LDC4297, a compound of the chemical class pyrazolotriazines, selectively inhibits CDK7 *in vitro*. A chemical library designed to target kinases was screened against human CDK7/CycH/MAT1 (ProQinase, Freiburg, Germany), using a commercially available *in vitro* kinase assay. After medicinal chemistry optimization of the hit series, distinct lead compounds were identified possessing IC₅₀ values for CDK7 below 5 nM, among them the primary hit LDC4297 (Fig. 1A; for further compound information, see Kelso et al. [33]; patent WO2013128028A1). A kinome-wide analysis with 333 individual kinases was performed to assess compound selectivity on the basis of the inhibition of *in vitro* standard kinase activity (PanKinase activity assay). The results revealed that LDC4297-sensitive kinases were restricted to members of the CDK family (Fig. 1C and see Fig. S1 in the supplemental material;

FIG 1 Chemical structure and selectivity profile of LDC4297. (A) Chemical structure of compound LDC4297, an (*R*)-*N*-(2-(1*H*-pyrazol-1-yl)benzyl)-8-isopropyl-2-(piperidin-3-yloxy)pyrazolo[1,5-*a*][1,3,5]triazin-4-amine, belonging to the chemical class of pyrazolotriazines. (B) Enzymatic activity of CDK7 in the presence of LDC4297 and reference compounds. Kinase activity was measured in a LANCE time-resolved fluorescence resonance energy transfer (TR-FRET) assay using ULight-labeled peptide substrates with their corresponding europium (Eu)-labeled anti-phospho-antibodies. The EC_{50} values for LDC4297 and two reference compounds were calculated in the presence of increasing ATP concentrations (30 μM , 300 μM , and 3 mM; single measurements). (C) Selectivity profiling of LDC4297. Radiometric protein kinase assays (^{33}P ATP-based *in vitro* kinase assay [ProQinase]) were applied to measure the kinase activity of 333 individual protein kinases in the presence of LDC4297 at a concentration of 100 nM. The kinase inhibition profile of LDC4297 was determined by measuring residual activity values (% no inhibition; single measurements; for details, see Fig. S1 in the supplemental material). Residual activity of the most sensitive CDK complexes (and the inactive mutant MET_Y1230A, hepatocyte growth factor receptor) are shown in an enlarged view that includes percentage values.

with the exception of LDC4297-sensitive MET kinase mutant Y1230A, but not the insensitive wild-type MET kinase). Importantly, among the selected CDK complexes, CDK7/CycH/MAT1 showed the lowest residual activity (2%) in the presence of LDC4297.

Furthermore, individual *in vitro* kinase assays (FRET-based LANCE Ultra KinaSelect Ser/Thr assays) were performed for CDK1, CDK2, CDK4, CDK6, CDK7, and CDK9, confirming the selective inhibitory activity of LDC4297 for CDK7 in the nanopicomolar range (IC_{50} , 0.13 ± 0.06 nM for CDK7 versus IC_{50} s between 10 nM and 10,000 nM for all other analyzed CDKs [data not shown]). According to chemical structures and fitting of the compounds to the target kinase, the mode of inhibition was predicted to occur in an ATP-competitive manner of “hinge binders” belonging to the type 1 group of protein kinase inhibitors (34). Indeed, ATP-site directed binding of the inhibitors was confirmed by direct ATP competition (Fig. 1B) and by competition with an ATP-directed chemical probe. Thereby, the affinities of hit com-

pounds for binding to the ATP-binding pocket of CDKs were determined and binding affinities to CDK7 or CDK2 were quantitated as the ability to compete or displace the CDK binding of a labeled tracer (33). The affinity of LDC4297 for CDK7 proved to be extremely high, so that the exact CDK7 affinities could not be resolved by tracer competition approaches under conventional assay conditions. In these kinase activity assays, ATP levels had to be increased to 3 mM (approximately physiological conditions) to attain a measurable range of dose-dependent CDK7 inhibition by LDC4297 (Fig. 1B). It should be stressed that LDC4297 showed an extreme selectivity for CDK7 compared to CDK2 (>280-fold) under physiologically relevant concentrations of ATP (i.e., 3.5 mM, also representing levels of ATP contained in our cell culture systems). This CDK7 selectivity was much more pronounced for LDC4297 than for other compounds of this chemical class (e.g., LDC3140, ~38-fold) (33).

LDC4297 exerts anticytomegaloviral activity. The antiviral potency of the drug was initially analyzed against HCMV in cul-

FIG 2 Inhibitory activity of LDC4297 regarding HCMV replication and cell proliferation. (A) Compounds were analyzed by an HCMV GFP-based replication assay using the recombinant virus AD169-GFP for the infection of HFFs (MOI 0.01; uninfected control, Mock). Antiviral compounds were added immediately postinfection at the concentrations indicated (reference drug GCV, 10 μM). Microscopically detectable cytotoxicity was determined at 6 days posttreatment (arrows). Cells were lysed at 7 days p.i. to perform quantitative GFP fluorimetry ($n = 4$, mean $\pm$ the standard deviation [SD]). The statistical significance was determined by applying an unpaired Student t test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. EC₅₀ values are given above bars. (B) Effect of LDC4297, roscovitine, and GCV on release of infectious virus (HCMV yield

tured primary human fibroblasts (HFFs) using an established GFP-based reporter assay (recombinant virus AD169-GFP) (13, 35). HCMV replication was inhibited by LDC4297 in a concentration-dependent manner with a 50% effective concentration (EC₅₀) value of 24.5 ± 1.3 nM (Fig. 2A). Inhibition was statistically significant (asterisks) and morphological signs of cytotoxicity only occurred at concentrations of 3.3 μM or higher (arrows). In a virus yield assay, efficacy of the compound in inhibiting the production and release of infectious HCMV AD169-GFP was additionally demonstrated (Fig. 2B). A low LDC4297 concentration of 0.37 μM produced a similar efficacy as a 10 μM concentration of the anti-HCMV reference drug ganciclovir (GCV). An entire block of virus production was measurable up to 96 h p.i. This antiviral activity could be dissected from cellular antiproliferative activity (mean growth inhibition concentration [GI₅₀] of primary human fibroblasts, 4.5 ± 2.5 μM; Fig. 2C). Notably, CDK7 inhibition by LDC4297 was not associated with general cytotoxicity at submicromolar concentrations, as shown for primary HFFs (Fig. 2C and Table 1) or peripheral blood mononuclear cells (50% cytotoxic concentration [CC₅₀] = 6.25 ± 3.34 μM, $n = 6$; determined in an accompanying study using a CellTiter-Glo luminescent cell viability assay [Promega]). In contrast, LDC4297 induced cytotoxicity in a set of tumor cell lines, i.e., already at extremely low, nanomolar concentrations in specific cases (data not shown). Specifically for the evaluation of antiviral drug activity, cytotoxicity was determined by the use of two different methods. The data derived from the trypan blue exclusion assay or the LDH release assay indicated that the level of LDC4297-induced cytotoxicity differed between cell types (i.e., CC₅₀ = 5.22 ± 0.50 in the case of primary HFFs used for HCMV). Overall, the CC₅₀ values of eight cell types used for the analysis of 13 viruses remained in a ranged between 17.42 ± 5.99 μM and 2.85 ± 0.14 μM, which was distinguishable from the antiviral activity (i.e., a therapeutic index [CC₅₀/EC₅₀] of 261 was determined for HCMV).

Moreover, the anti-HCMV activity of LDC4297 was confirmed in a second system using HCMV strain TB40 (i.e., recombinant TB40-UL32-EGFP virus expressing pUL32/pp150 fused to enhanced GFP) (14). In primary human fibroblasts, an EC₅₀ value of 0.085 ± 0.001 μM was determined under identical culture and readout conditions applied for strain AD169-GFP. Since dissemination of HCMV in cultured cells is either possible through cell-to-cell transmission or through release of infectious virus into the culture media, the effect of LDC4297 on the release of HCMV strain TB40 was further analyzed. The inhibition of virus release was measured by the staining of IE1-positive cells after transfer of culture medium from drug-treated (0.37 μM), virus-infected HFFs (MOI of 0.3), and the results indicated an almost complete antiviral block (data not shown). Also for the retinal pigment epithelial cell line ARPE19 infected with HCMV TB40-UL32-EGFP, the antiviral activity of LDC4297 could be confirmed in a similar

assay). HFFs were infected with HCMV AD169-GFP (MOI of 0.3) and incubated with inhibitors at the concentrations indicated. Supernatants were harvested 48, 72, and 96 h p.i. and subsequently used for plaque titration on fresh HFFs ($n = 2$; mean $\pm$ the SD). (C) Measurement of putative antiproliferative effects. HFFs were treated for 3 days with LDC4297, GCV, or staurosporine (STP) at the concentrations indicated. The release of NADPH/NADH from proliferating cells was measured by the use of a CellTiter 96 Aqueous One solution cell proliferation assay (Promega; $n = 4$, the means $\pm$ the SD, P values, and GI₅₀ values are indicated).

TABLE 1 Analysis of antiviral activities of LDC4297^a

Family	Virus	Host cell	No. of replicates	Mean concn (μ M) $\pm$ SD ^b	
				EC ₅₀	CC ₅₀
<i>Herpesviridae</i>	HCMV (AD169-GFP)	HFF	4	0.02 $\pm$ 0.00	5.22 $\pm$ 0.50
	GPCMV (v403-GFP)	GPEF	12	0.05 $\pm$ 0.01	3.59 $\pm$ 0.30
	MCMV (Smith)	MEF	8	0.07 $\pm$ 0.02	2.85 $\pm$ 0.14
	HHV-6A (U1102-GFP)	J-Jhan	4	0.04 $\pm$ 0.01	3.33 $\pm$ 0.17
	HSV-1 (166v VP22-GFP)	Vero	4	0.02 $\pm$ 0.07	3.00 $\pm$ 0.26
	HSV-2 (01-6332)	Vero	4	0.27 $\pm$ 0.31	3.00 $\pm$ 0.26
	VZV (Oka)	HFF	4	0.06 $\pm$ 0.01	5.22 $\pm$ 0.50
	EBV (B95-8)	293T	4	1.21 $\pm$ 1.03	3.52 $\pm$ 0.97
<i>Adenoviridae</i>	HAdV-2 (type 2)	A549	4	0.25 $\pm$ 0.01	5.69 $\pm$ 0.71
<i>Poxviridae</i>	Vaccinia virus (IHD-5)	HFF	4	0.77 $\pm$ 0.19	5.22 $\pm$ 0.50
<i>Retroviridae</i>	HIV-1 (NL4-3)	CEMx174	9	1.04 $\pm$ 1.12	17.42 $\pm$ 5.99 ^c
	HIV-1 (4LIG7)	CEMx174	9	1.13 $\pm$ 0.55	17.42 $\pm$ 5.99 ^c
<i>Orthomyxoviridae</i>	Influenza A virus (A/WSN/33)	293T	4	0.99 $\pm$ 0.45	3.52 $\pm$ 0.97

^a Viruses and cells used: HCMV, human cytomegalovirus, strain AD169-GFP, primary human fibroblasts; GPCMV, guinea pig cytomegalovirus, strain v403-GFP, guinea pig embryo fibroblasts; MCMV, murine cytomegalovirus, strain Smith, primary murine fibroblasts; HHV-6A, human herpesvirus 6A, strain U1102-GFP, J-JHAN; HSV-1 and HSV-2, herpes simplex virus types 1 and 2, strain 166v VP22-GFP and isolate 01-6332, respectively, Vero; VZV, varicella-zoster virus, strain Oka, primary human fibroblasts; EBV, Epstein-Barr virus, strain B95-8, 293T (T81GFP); HAdV-2, human adenovirus type 2, A549; vaccinia virus, strain IHD-5, primary human fibroblasts; HIV-1, human immunodeficiency virus 1, strains NL4-3 and 4LIG7, CEMx174; influenza A virus, strain A/WSN/33, 293T.

^b The EC₅₀ values of virus replication were determined by GFP-based reporter assay (HCMV and GPCMV), plaque reduction assay (MCMV, HSV-1, HSV-2, VZV, EBV, HAdV-2, and vaccinia virus), SEAP-based reporter assay (HIV-1), and polymerase activity-based reporter assay (influenza A virus). Mean values derived from n-fold measurements are given. The CC₅₀ values obtained from trypan blue exclusion assay of cell viability ($n \geq 4$). The determination of cell viability was carried out simultaneously with the measurement of antiviral activity: HFF, 7 days; GPEF, 8 days; MEF, 6 days; J-Jhan, 3 days; Vero, 5 days; 293T, 6 days; A549, 6 days; and CEMx174, 5 days (i.e., the period of drug incubation in the case of different virus infections performed with identical cell types was adjusted to the longest duration of the respective assays).

^c CC₅₀ values obtained from measurements of the LDH release at 1 day posttreatment ($n = 2$).

concentration range (data not shown). Taken together, these findings demonstrate for the first time the anti-HCMV activity of a CDK7 inhibitor at extremely low concentrations.

Anti-HCMV activity of LDC4297 is exerted through a multifaceted mode of action that involves an interference with virus-induced Rb phosphorylation. Next, we investigated the effects of CDK7 inhibition on the individual stages of HCMV replication (12 to 96 h p.i.) by monitoring the expression of viral proteins on Western blots. For this purpose, LDC4297 was applied to cell culture media immediately after virus infection (Fig. 3). Although the levels of CDK7 expression varied to some extent in HCMV-infected cells (possibly due to the temporal expression of transcriptionally coregulatory viral proteins), CDK7 was not markedly changed by the addition of LDC4297 or control drugs (i.e., no LDC4297 concentration-dependent decrease of CDK7 levels was detectable; Fig. 3, lanes 5 to 8). Importantly, an inhibitory effect of LDC4297 toward viral protein synthesis was already detected at the stage of immediate-early (IE) gene expression as measured with samples taken at 12 h p.i. (Fig. 3, upper left panel, IE1p72). This immediate-early block very probably translated into the inhibition of viral protein synthesis at the early (pUL44) or early-late (gB) level, which was most clearly demonstrated at 48 and 96 h p.i. (Fig. 3, right panels, lanes 9 to 12; β -actin served as a loading control). These findings indicate an antiviral effect already manifested at the immediate-early stage of HCMV replication. It should be mentioned that the drug-mediated reduction of IE1p72 levels partially recovered over time (Fig. 3, 96 h p.i.; also compare to Fig. 4B and C). This may indicate that the drug's *in vitro* efficacy weakened over time (i.e., one round of HCMV replication enduring $\sim$ 72 h) when added onetime after virus infection. However,

viral protein patterns did not recover entirely (e.g., constant reduction of the essential early-phase regulatory protein pUL44), which is consistent with the finding of an antiviral block using an identical drug concentration of 0.37 μ M in the HCMV yield assay (Fig. 2B). Next, we addressed the question of whether the CDK7 inhibitor may alter virus-induced modulation of cellular proteins. HCMV replication was monitored by the expression of IE1p72 and pUL44 at 72 h p.i. (Fig. 4). The expression levels of the cellular proteins RNA polymerase II (RNAP II), CDK2, cyclins B, H, and E and tumor suppressor retinoblastoma protein (Rb) were analyzed on Western blots in the absence or presence of LDC4297. In all cases, HCMV infection induced an upregulation of protein expression or protein phosphorylation, such as an activating phosphorylation of serine 5 or serine 7 in the C-terminal domain (CTD) of RNAP II or inactivating phosphorylation of serine 807 and serine 811 of Rb (Fig. 4A, lanes 1 and 2). Notably, LDC4297 exerted an inhibitory effect on these HCMV-induced upregulatory events, at least to some extent (lanes 5 and 6). In contrast, the anti-HCMV reference drug GCV, which acts in a CDK7-independent fashion, did not show any comparable effect (lanes 3 and 4). A substantial response to LDC4297 treatment was seen in the level of upregulation of Rb. Previously, it had been demonstrated that HCMV infection drives an upregulation of Rb expression in a first thrust, which subsequently initiates site-specific Rb phosphorylation as a second thrust, leading to inactivation of Rb thus depriving it of its cell cycle suppressor function. This inactivating phosphorylation of Rb is considered a prerequisite for the onset of viral DNA replication during the transition of HCMV-infected cells from G₁ into S phase of the cell cycle (36, 37). Of note, the viral protein kinase pUL97 is highly effective in phosphorylating Rb in

FIG 3 Inhibitory effect of LDC4297 on individual stages of viral gene expression. HFFs were infected with HCMV AD169-GFP at an MOI of 0.3 and kinase inhibitors (roscovitine, Rosco, 10 μ M; LDC4297, 1.1, 0.37, 0.12, and 0.04 μ M), reference compound GCV (10 μ M), or DMSO alone (Inf.) were added immediately after infection. Cells were harvested 12, 24, 48, and 96 h p.i. to perform SDS-PAGE and Western blot analysis with the following antibodies: PAb-CDK7 (N-19, sc-723; Santa Cruz Biotechnology), MAb-IE1p72 (63-27; kindly provided by W. J. Britt, University of Alabama, Birmingham, AL), MAb-UL44 (BS510; B. Plachter, University of Mainz, Mainz, Germany), MAb-gB (27-287; W. J. Britt), and MAb- β -actin (AC-15; Sigma).

HCMV-infected cells (37–39). Thus, we speculated whether LDC4297 might possess any inhibitory activity against pUL97 and addressed this point by performing an *in vitro* kinase assay with active and inactive versions (K355M) of transiently expressed pUL97-FLAG. Neither autophosphorylation nor histone substrate phosphorylation of pUL97 was inhibited by LDC4297, whereas a previously described inhibitor of pUL97, Gö6976 (40, 41), exerted an inhibition of both activities (Fig. 5). Thus, we concluded that viral protein kinase pUL97 is not targeted by LDC4297. Moreover, LDC4297 reduced Rb expression in the uninfected control cells at 24 h (Fig. 4B, lane 3, Mock), supporting the drug's cell-directed effect. As another reference compound, a prominent CDK4/6-specific inhibitor, PD0332991 (42, 43), was applied which similarly exerted a blocking effect on HCMV-induced Rb phosphorylation (Fig. 4B). It should be noted that Rb phosphorylation by CDKs or pUL97 can be stimulated through prior phosphorylation by CDK4/6, so that the finding in Fig. 4B is consistent with this concept. Thus, the block of Rb phosphorylation by CDK7 or CDK4/6 inhibitors may represent one of several mechanistic modes of antiviral activity that also includes other inhibitory activities on HCMV-modulated cellular proteins (e.g., RNAP II and/or CDK/cyclin complexes). This idea was consistent with our experiment demonstrating an additive antiviral effect produced by coadministration of LDC4297 with GCV (see Fig. S2 in the supplemental material; Fig. 4C). Treatment with either drug alone moderately reduced viral protein levels, whereas coadministration showed a much stronger reduction of IE1p72 and pUL44 (Fig. 4C). In contrast to LDC4297, GCV did not interfere with HCMV-induced Rb phosphorylation (although the inhibitory effect of GCV on viral early protein expression, as shown in lane 7,

produces some secondary effect also onto Rb). Thus, the measured additive antiviral effect appears to involve two independent mechanisms.

Broad antiviral activity of LDC4297. We experimentally addressed the question of an assumed broader range of antiviral activity by analyzing LDC4297 sensitivity of further members of the *Betaherpesvirinae* subfamily, i.e., guinea pig and murine cytomegaloviruses (GPCMV and MCMV) and human herpesvirus 6A (HHV-6A). In all cases, EC_{50} values were measured in the submicromolar range ($EC_{50}s \leq 0.07 \mu$ M; Table 1). Moreover, we tested a selection of human-pathogenic *Alpha*- and *Gammaherpesvirinae*, such as HSV-1 and HSV-2, VZV, and EBV. Virus replication was broadly blocked by LDC4297, whereby the antiviral efficacies varied between the viruses used, i.e., strong efficacy for HSV-1 and VZV ($EC_{50}s = 0.02$ and 0.06μ M, respectively; Table 1) and intermediate to low efficacy for HSV-2 and EBV ($EC_{50}s = 0.27$ and 1.21μ M, respectively; Table 1). We also included human viruses belonging to four other families, i.e., HAdV-2, vaccinia virus (strain IHD-5), HIV-1 (strains NL4-3 and 4LIG7), and human influenza A virus (A/WSN/33). LDC4292 exerted intermediate inhibitory activity toward these viruses with EC_{50} values in the low micromolar range ($EC_{50}s = 0.25$ to 1.13μ M; Table 1). It should be noted that for EBV and influenza A virus analyzed in 293T cells, the therapeutic index (CC_{50}/EC_{50}) was relatively low (i.e., 2.9 for EBV and 3.6 for influenza virus), whereas in all other cases the therapeutic index was substantially higher (i.e., between 11.1 and 261.0). Combined, these data indicate a broad antiviral activity and suggest that, for the various viruses analyzed, slightly different CDK7-associated mechanisms of antiviral activity were responsible for the individual levels in drug sensitivity. It should be stressed

FIG 4 LDC4297 exerts differential impact on the expression levels of several cellular and viral proteins. (A) HFFs were infected with HCMV AD169-GFP (MOI of 0.3) and LDC4297 (0.37 μ M), reference compound GCV (20 μ M), or DMSO alone (Inf.) were added immediately after infection. Cells were harvested at 72 h p.i., and cell lysates were subjected to Western blot analysis. The antibodies used were as follows (see also Fig. 3): MAb-RNAP II (8WG16; Covance), MAb-p-S5-RNAP II (AK4H8; Covance), MAb-p-S7-RNAP II (4E12; Chromotek), PAb-CDK2 (M2; Santa Cruz Biotechnology), PAb-Cyclin B1 (H-433; Santa Cruz Biotechnology), MAb-Cyclin H (D-10; Santa Cruz Biotechnology), MAb-Cyclin E (E-4; Santa Cruz Biotechnology), PAb-p-S807/811-Rb (Cell Signaling), and MAb-Rb (4H1; Cell Signaling). (B) Rb phosphorylation during individual stages of replication (24, 48, and 72 h p.i.). Kinase inhibitors: LDC4297, 0.37 μ M; PD0332991, 10 μ M. Signal intensities were quantitated with an AIDA image analyzer. (C) Additive effect of dual antiviral treatment with LDC4297 and GCV. The reference inhibitor PD0332991 (10 μ M) was added alone; LDC4297 (0.37 μ M) and GCV (20 μ M) were added either alone or in combination (the addition occurred immediately after HCMV infection).

that besides the two branches of the CDK7-dependent cellular regulatory pathways (i.e., CDK/cyclin-mediated cell cycle regulation and RNAP II-driven transcription), a third aspect might be involved in this context, namely, the putative regulatory phosphorylation of viral proteins by CDK7. The latter possibility has not been experimentally addressed so far. However, these three regulatory aspects might possess differentially strong importance for the replication of the analyzed viruses. Thus, the levels of viral drug sensitivity may be explained by individual roles of CDK7-regulated processes for viral replication (e.g., low importance of the inhibition of RNAP II-driven transcription for those viruses, such as influenza virus and vaccinia virus, not utilizing cellular RNAP II-driven transcription). As far as herpesviruses are concerned, a general and stringent importance of CDK7 for viral replication was illustrated by the experimentation with CDK7 inhibitors in the present study.

DISCUSSION

Herpesviral infections, facing well-established but numerically limited options of antiviral therapy, represent major concerns in public health. Conventional antiherpesviral drugs are targeted to viral proteins so that novel cell-directed strategies have increasingly been emphasized. In the present study, a novel and selective

CDK7 inhibitor was characterized, and the first evidence for its antiviral potential could be provided. In particular, a broad and efficacious antiherpesviral activity, in addition to inhibitory activity toward viruses from other families, could be demonstrated. We describe a so-far-unknown, crucial role for CDK7 in herpesviral replication and present a CDK7-targeted drug candidate displaying the following advantages: (i) a selectivity for CDK7, (ii) an antiviral efficacy at nanomolar concentrations in cell culture systems, and (iii) a broad-spectrum of antiviral activities.

As far as CDK7 function in metazoan cell cycle regulation is concerned, the trimeric complex CDK7/CycH/MAT1 fulfills a CDK-activating step, commonly called the CDK-activating kinase (CAK) complex (44). Moreover, CDK7 represents a component of the general transcription factor TFIIF and is involved in phosphorylation of serine residues of RNAP II-CTD (C-terminal domain). Interestingly, we and others showed that phosphorylation levels within the RNAP II-CTD are unaffected in fibroblasts when CDK7 activity is inhibited (Fig. 4A) or in cells lacking detectable levels of CDK7 (6). This finding strongly suggests that CDK7 is not essential for global RNAP II-driven transcription but that its functional deficiency in RNAP II regulation may be compensated for by other kinase activities. In contrast, the loss of CDK7 activity is

FIG 5 *In vitro* kinase activity of pUL97 is not inhibited by LDC4297. The kinase activity of HCMV pUL97 was transiently expressed in 293T cells and immunoprecipitated from cell lysates for a radiometric determination of kinase activity *in vitro* (catalytically inactive mutant K355M and empty vector served as controls). Kinase inhibitors were incubated during cell lysis and all subsequent steps at the following concentrations: G6976, 2 μ M; LDC4297 (lane I), 0.02 μ M; LDC4297 (lane II), 0.2 μ M; and LDC4297 (lane III), 2 μ M. Reactions were stopped with SDS loading buffer, and samples were analyzed by SDS-PAGE and Western blot procedures. Radioactive signals of histone phosphorylation and pUL97 autophosphorylation were detected by exposure to phosphorimager plates (upper panels). The levels of expression and immunoprecipitation were monitored by the use of tag-specific MAb-FLAG (M2, Sigma; lower panels).

critical for cell cycle control since it produces a lack of normal CAK function and induces cell cycle arrest by decreasing the E2F-controlled transcription levels (e.g., suppressed transcription of CDK1 and CycA2 genes). Furthermore, Ganuza et al. (6) showed that reactivation of the E2F transcriptional program by inactivation of the Rb family diminishes the proliferative defects caused by elimination of CDK7. These results suggest that cells have mechanisms other than CDK7-mediated CAK activity to phosphorylate CDK1 and CDK2 and resume cell proliferation. Our findings underline this observation since proliferation of fibroblasts almost remained unchanged when treated with CDK7 inhibitors at non-toxic concentrations relevant for antiherpesviral activity (Fig. 2C). In this context, it should be mentioned that an extensive loss of CDK7 expression in young adult mice surprisingly did not lead to a generalized or severe phenotype but that CDK7 depletion could be fully compensated, at least in tissues where the proliferation is low.

The present study provides evidence that HCMV replication is sensitive to CDK7 inhibitors and that the HCMV-driven modulation of Rb was mainly affected. As one of the major targets of distinct CDKs, Rb controls progression through the G_1 phase of the cell cycle. In an unphosphorylated state, Rb is able to bind E2F transcription factors, thus preventing expression of genes that are required for DNA replication, eventually leading to a cell cycle arrest in phase G_0 or G_1 . In its hyperphosphorylated state, however, Rb dissociates from E2F complexes, resulting in cell cycle

progression to S phase. Although Rb phosphorylation can be mediated by a number of CDKs (45), its initial phosphorylation is typically catalyzed by CDK4/CycD or CDK6/CycD, followed by additional phosphorylation by CDK2/CycE. Thereby, Rb remains functionally inactivated throughout the S, G_2 , and M phases. During HCMV infection, the viral kinase pUL97 is involved in the regulatory phosphorylation and inactivation of Rb in HCMV-infected cells (37–39, 46–48). The ability to phosphorylate Rb is shared by herpesviral UL-type (HvUL) protein kinases from the beta- and gammaherpesvirus subfamilies but not by their alpha-herpesviral homologs, supporting the idea that β - and γ -HvUL protein kinases maintain a conserved function as viral CDK orthologs (37, 38, 49, 50). In our analysis, high levels of phospho-Rb were suppressed by the CDK7 inhibitor LDC4297. It appears unlikely that this drug directly interferes with pUL97-mediated Rb phosphorylation, since we demonstrated *in vitro* that no inhibition of pUL97 activity was detectable. Interestingly, the mode of antiviral activity of LDC4297 is already manifested at the immediate-early phase of HCMV replication. Since the progression of lytic HCMV replication is strictly dependent on IE gene expression, this specific drug activity is translated into a drastic limitation of all downstream viral replication events. Moreover, in HCMV-infected cells, the CDK7 inhibitor showed an effect on phospho-Rb levels that was similar to that seen for the CDK4/6 inhibitor PD0332991 (palbociclibin [Pfizer]; this compound is currently being analyzed in a phase III clinical trial on estrogen receptor-positive breast cancer) (51). PD0332991 inhibits Rb phosphorylation in the early G_1 phase by inhibiting CDK4/CDK6, consequently inducing a G_1 cell cycle arrest.

To our knowledge, LDC4297 is the first CDK7 inhibitor that exerts antiviral activity at very low concentrations (i.e., ~ 100 -fold lower than GCV, the reference drug for anticytomegaloviral therapy). According to our data, these antiviral compounds may have the potential to be developed as a novel class of broad-spectrum antiviral drugs. The main advantages of this concept are a reduced risk of drug resistance, the exploitation of a cellular target, and an option for cotreatment with approved antiviral compounds. Current investigations with LDC4297 include the detailed investigation of the CDK7-mediated mode of antiviral action and selection for putative resistant viruses. Since it is difficult to select for resistance to inhibitors that target cellular functions, this approach may improve the therapeutic quality of a novel drug candidate. Moreover, the PK analyses of LDC4297 performed thus far have also been highly promising. eADME parameters (e.g., drug solubility, cell permeability, and microsome stability) were determined both *in vitro* and *in vivo*. An analysis of the PK parameters in CD1 mice revealed positive characteristics after oral administration, as demonstrated for a single-dose treatment (100 mg/kg of LDC4297; data not shown). The half-life ($t_{1/2}$) was determined to be 1.6 h, and a time (T_{max}) to a mean peak plasma concentration of 1,297.6 ng/ml was reached 0.5 h after administration, with a continued presence of LDC4297 plasma levels for at least 8 h and a bioavailability of 97.7% (data not shown). These preliminary findings strongly encourage further preclinical testing and medicinal development. At present, LDC4297 and related compounds are included within a detailed screening program for further *in vitro* and *in vivo* antiviral activities being performed at the National Institute for Allergy and Infectious Diseases (NIAID; HHSN272201100016I, Assessment for Antimicrobial Activity). This analysis should substantiate the qualification of CDK7 inhib-

itors for clinical investigations. This strategy may lead to improved options for a novel type of comprehensive antiviral therapy in the near future.

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