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Assessment of drug candidates for broad-spectrum antiviral therapy targeting cellular pyrimidine biosynthesis

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ABSTRACT

Currently available antiviral drugs frequently induce side-effects or selection of drug-resistant viruses. We describe a novel antiviral principle based on targeting the cellular enzyme dihydroorotate dehydrogenase (DHODH). *In silico* drug design and biochemical evaluation identified Compound **1** (Cmp**1**) as a selective inhibitor of human DHODH *in vitro* (IC_{50} 1.5 ± 0.2 nM). Crystallization data specified the mode of drug–target interaction. Importantly, Cmp**1** displayed a very potent antiviral activity that could be reversed by co-application of uridine or other pyrimidine precursors, underlining the postulated DHODH-directed mode of activity. Human and animal cytomegaloviruses as well as adenoviruses showed strong sensitivity towards Cmp**1** in cell culture-based infection systems with IC_{50} values in the low micromolar to nanomolar range. Particularly, broad inhibitory activity was demonstrated for various types of laboratory and clinically relevant adenoviruses. For replication of human cytomegalovirus in primary fibroblasts, antiviral mode of activity was attributed to the early stage of gene expression. A mouse *in vivo* model proved reduced replication of murine cytomegalovirus in various organs upon Cmp**1** treatment. These findings suggested Cmp**1** as drug candidate and validated DHODH as a promising cellular target for antiviral therapy.

1. Introduction

Viruses are leading cause of human disease worldwide. Despite the fact numerous antiviral drugs have been developed during the last 25 years, there is a constant need for optimized drugs that may be highly effective and safe in the treatment of human virus infections. Interest in the development of novel antiviral compounds is mainly based on three considerations: first, novel antiviral drugs for the therapy of highly pathogenic, so far scarcely treatable virus infections; second, novel antiviral strategies achieving a broad-spectrum drug efficacy; and third, novel antiviral targets, including cellular targets, improving current therapy options by reducing the risk of viral drug resistance. In these aspects, our focus has mostly been directed towards human cytomegalovirus (HCMV), which is the type species of the beta-subfamily of *Herpesviridae*, worldwide spread and associated with severe forms of human diseases (Mocarski et al., 2007). In immunocompetent persons, primary HCMV infection is generally asymptomatic, while in some cases a self-limiting, mild mononucleosis syndrome or even more severe manifestations can develop. In immunocompromised persons and neonates, however, HCMV frequently causes systemic disease with typical clinical consequences, like retinitis, pneumonitis, and gastroenteritis, or generalized severe congenital infection, respectively. In addition, congenital infection with HCMV that affects app. 1% of births is an unresolved clinical problem. Currently, antiviral therapy is mainly based on inhibitors of viral DNA synthesis, such as ganciclovir (GCV), its prodrug valganciclovir (VGCV), foscarnet (FOS) and cidofovir (CDV). Clinical treatment with these antivirals is routinely practiced and wide-spread, albeit at times accompanied by severe adverse side effects, such as myelotoxicity for GCV or nephrotoxicity for FOS and CDV, as well as selection of viral drug resistance. Thus, despite prophylactic and preemptive therapy has reduced the number of severe HCMV infections in transplant patients, the development of improved drugs is a major issue of current research (Andrei et al., 2009; Lischka and Zimmermann, 2008; Marschall and Stamminger, 2009; Steininger, 2007). Likewise, infections with human adenoviruses (HAdV) represent

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a field with clinically broad relevance. HAdVs replicate primarily in the upper respiratory tract or in the gut, and symptoms of infection can range from respiratory disease over gastroenteritis to ophthalmic infections (keratoconjunctivitis). Anti-adenoviral therapy options are very limited at the moment, i.e. no specific anti-HAdV drug has been approved so far. To date, viral proteins have represented the main targets of antiviral therapies, but it is increasingly recognized that viral replication can likewise be effectively inhibited by targeting host factors that exert essential functions for viral replication. Hereby, a major advantage is seen in the fact that this host protein-directed strategy may be less prone to select resistance-conferring mutations in the viral genome (Richman, 2006). Furthermore, drugs targeted to host proteins may be ideally suited for combination therapy with specific inhibitors of viral proteins.

We followed a strategy in developing inhibitors of the cellular dihydroorotate dehydrogenase (DHODH), an enzyme that exerts important function in *de novo* biosynthesis of pyrimidine ribonucleotides. Pyrimidines are essential cellular metabolites, representing precursor molecules for DNA and RNA biosynthesis (Hyde, 2007). Cells acquire pyrimidines either through *de novo* synthesis starting from ammonia, bicarbonate, and aspartic acid or by salvaging preformed pyrimidine bases or nucleosides (Vyas et al., 2011; Phillips and Rathod, 2010). Human DHODH is a clinically validated drug target based on the successful development and approval of leflunomide for the treatment of rheumatoid arthritis (Goldenberg, 1999; Herrmann et al., 2000; Olsen and Stein, 2004; Munier-Lehmann et al., 2013). In addition, anti-cytomegaloviral activity of leflunomide *in vitro* and *in vivo* has been studied for more than a decade (Waldman et al., 1999a,b; Chacko and John, 2012; Henao-Martínez et al., 2012). However, the antiviral mode of action proved to be complex (including a pronounced inhibitory effect towards cellular proteins; Chacko and John, 2012; Breedveld and Dayer, 2000), so that inhibition of DHODH appears not to be the sole activity of leflunomide accounting for its anti-cytomegaloviral activity. Despite of these postulated secondary activities of leflunomide, it has been shown that the drug decreases intracellular pyrimidine levels *in vitro* (Rückemann et al., 1998). For brequinar, a DHODH inhibitor tested in clinical studies and distinct disease models in rodents, *in vivo* reduction of plasma levels of uridine in humans (40–85% depletion) and mice (40% depletion within 2 h) could be attributed to its inhibition of the *de novo* pathway of pyrimidine synthesis (Peters et al., 1990). In our approach, the development of novel DHODH inhibitors exerting best possible selectivity was undertaken by utilizing the specified *in silico* technology 4SCan® drug design, followed by various levels of drug analysis *in vitro* and *in vivo*. Our data provide new insight into the previous view that DHODH represents a highly interesting cellular target for antiviral therapy. Moreover, current data exemplify that a highly potent DHODH inhibitor could be experimentally qualified as an antiviral drug candidate.

2. Material and methods

2.1. 4SCan® *in silico* technology

The iterative screening algorithm behind 4SCan® is based on three components: (i) a ligand protein docking engine (ProPose), (ii) a large compound library with pre-calculated ionization states and specific properties, so-called molecular descriptors, and (iii) a classifier that allows to pre-select putative high scoring molecules. The iterative process starts with a randomly generated set of compounds which are docked and scored with ProPose. By means of a descriptor- and score-based forecast model, the classifier routine generates a new equally sized set of library compounds for docking with ProPose. These iteration cycles are continued repeatedly with molecule sets of 4.5×10^3–5×10^3 exemplars. A typical 4SCan® docking run is presented as score finding history in Fig. 1A. Putative hits are selected only among the top scoring compounds (Fig. 1A, below cut-off line) and subjected to biological tests. This *in silico* hit identification has been extensively tested for structure-based approaches as well as molecular alignment and pharmacophore screening as described before (Herz et al., 2006; Tasler et al., 2007, 2009).

2.2. Chemical synthesis of putative antiviral compounds

Compounds were synthesized in-house; for details see Supplementary material and methods.

2.3. Determination of pharmacological parameters of candidate compounds in vitro and in vivo

Pharmacological parameters of compounds (Table 1) were determined by standard procedures (see Supplementary material and methods).

2.4. DHODH in vitro activity assay

Analysis of *in vitro* enzymatic activity was performed by the use of purified human DHODH in an established assay as described before (Davis et al., 1996; Leban et al., 2006). Putative inhibitory compounds were directly applied to the reaction in a concentration range between 100 pM and 10 μM.

2.5. Drug–target cocrystallization

Purified human DHODH (Davis et al., 1996; Leban et al., 2006) was used for cocrystallization with Cmp**1** under standard procedures applying in-house optimized conditions for structural evaluation (see Supplementary material and methods).

2.6. Cells and viruses

Primary human foreskin fibroblasts (HFFs) and murine embryonic fibroblasts (MEFs) were cultivated in MEM with 7.5% FCS, Vero cells and A549 cells in DMEM with 10% FCS under conditions described previously (Marschall et al., 2012; Rechter et al., 2006). For infection experiments, see description of viruses in Supplementary material and methods.

2.7. Antiviral assays in cell culture

Antiviral assays were performed as described before (Marschall et al., 2000, 2012; Kindsmüller et al., 2009; Rechter et al., 2006). For HCMV GFP-based replication assays, HFFs were cultivated in 12-well plates (2.25×10^5 cells/well), infected with HCMV AD169-GFP (MOI 0.1–0.25) and treated with antiviral drugs added immediately after virus infection. At 7 days post-infection, cells were lysed and lysates were subjected to automated GFP quantification using a Victor 1420 Multilabel Counter (Perkin Elmer, Germany). All infections were performed in duplicate; GFP quantifications were performed in quadruplicate. Standard plaque reduction assays and reporter-based antiviral assays for the viruses tested were performed under previously established conditions (Marschall et al., 2000; Rechter et al., 2006; Chou et al., 2011). Adenovirus yield assays were performed as described earlier (Kindsmüller et al., 2009).

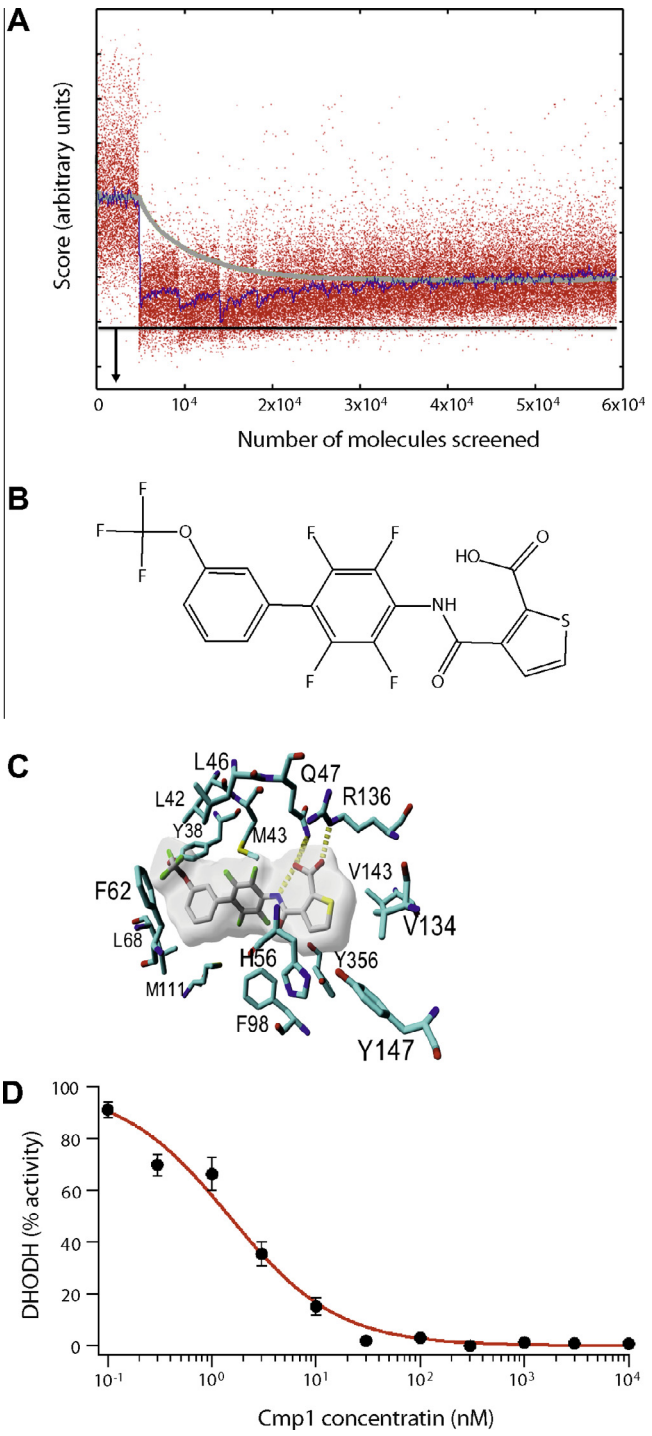

Fig. 1. DHODH inhibitor screening and hit-to-lead nomination of Cmp**1**. (A) History of docking scores generated during a 4SCan® screening run to identify initial hits. Red dots indicate individual scores (app. 4.5×10^3 per round), the blue curve presents the moving average of last 100 scores and the grey curve presents the moving average of all scores. A cut-off line marks the area of top scoring compounds placed below the line. Among top scoring molecules, putative hits were selected for initial testing (see arrow). (B) The chemical structure of hit compound Cmp**1** is 3-(2,3,5,6-tetrafluoro-3′-trifluoromethoxy-biphenyl-4-ylcarbamoyl)-thiophene-2-carboxylic acid. (C) Targeting of Cmp**1** (grey surface plus bright-coloured stick presentation) to specific residues of human DHODH (dark-coloured stick presentation) was based on cocrystallization data. (D) *In vitro* inhibition of human DHODH by Cmp**1**. The DHODH *in vitro* activity assay was adapted to a 96-well format as published previously (28). Determinations were performed in duplicate and the IC_{50} was calculated as 1.5 ± 0.2 nM (mean ± standard error).

Table 1
Biochemical and pharmacological characterization of Cmp**1**.*

Solubility	
50 mM phosphate pH 4.0	0.003 mM
50 mM phosphate pH 7.0	>1.7 mM
50 mM phosphate pH 9.0	>1.7 mM
Inhibitory activity against putative targets in vitro (IC_{50})	
DHODH (human)	1.5 ± 0.2 nM
DHODH (mouse)	3.2 ± 0.8 nM
PI3K-α/-β/-γ/-δ	>1 μM (1.4/4.6/2.14/2.0)
AKT-1/-2/-3	>15 μM (19.2/18.9/23.4)
JAK-1/-2/-3	>10 μM (15.0/13.2/12.5)
Toxicity	
PBMCs (human)	Not toxic ⩽ 50 μM
HepG2 (human)	Not toxic ⩽ 50 μM
NIH3T3 (mouse)	Not toxic ⩽ 50 μM
hERG	No inhibition ⩽ 20 μM
AMES	Negative
Pharmacokinetics in mice: 30 mg/kg/d	
2 h	71 ± 64 μg/ml (148 ± 134 μM)
6 h	129 ± 40 μg/ml (269 ± 83 μM)
8 h	88 ± 51 μg/ml (184 ± 106 μM)
24 h	18 ± 8 μg/ml (38 ± 17 μM)
Pharmacokinetics in mice: 100 mg/kg/d	
2 h	231 ± 82 μg/ml (482 ± 171 μM)
6 h	264 ± 87 μg/ml (551 ± 182 μM)
8 h	250 ± 73 μg/ml (522 ± 152 μM)
24 h	47 ± 5 μg/ml (98 ± 10 μM)

* For description of assays, see Section 2.

2.8. Western blot analysis

HCMV-infected HFFs were treated with antiviral drugs added immediately after infection and lysed at time points indicated. Protein lysates were subjected to standard SDS–PAGE/Western blot procedure as described previously (Thomas et al., 2009; Webel et al., 2011), using the following monoclonal antibodies: MAb-6327 (IE1p72; antibody repository of TS), MAb-UL44 (BS510; kindly provided by Bodo Plachter, Univ. Mainz, Germany) (Becke et al., 2010) and MAb-pp28 (41-18; kindly provided by William Britt, University of Alabama, AL, USA) and MAb-β-actin (Ac-15; Sigma).

2.9. Animal model

Black6 RAG-1−/− mice were purchased from Charles River Laboratories (Sulzfeld, Germany), maintained under specific pathogen-free conditions and utilized between 8 and 14 weeks of age. For infection experiments, animals were infected with MCMV157luc (Klenovsek et al., 2007) with 5×10^5 PFU i.p. For *in vivo* imaging of luciferase signals and quantitative *in vitro* luciferase assays with organ homogenates, see Supplementary material and methods. All *in vivo* experiments were conducted in accordance with national, institutional guidelines for animal care and use. Experimental protocols were reviewed and approved by the *Regierung von Mittelfranken*, Ansbach, Germany (permit 54-2532.1-8/08; Jan 28, 2009).

2.10. Software and data processing

The Propose and 4SCan® software programs were described in detail by Seifert et al. (2004) and Herz et al. (2006), respectively.

3. Results

3.1. Application of the 4SCan® procedure for in silico modeling of DHODH-targeted compounds

An *in silico* modeling procedure, termed 4SCan®, had been developed for the identification of drug and target candidates as

reported recently (Herz et al., 2006; Tasler et al., 2007, 2009). The principle strategy of this procedure is a multi-round optimization of *in silico* docking of compounds into the active site of a putative target (for details see Movies S1-S4). In the present case, the crystal structure of human DHODH (Liu et al., 2000) was used as a template for identifying candidate inhibitors directed to the DHODH ubiquinone (cofactor) binding site. The basis of this approach was provided by a library of approximately 7×10^6 compounds belonging to various chemical classes (mostly commercially available). The docking and scoring procedure was performed with 4SCan® following the algorithm described in Section 2. The direct screening results of thousands of high scoring binders to the DHODH active site were reduced to a highly diverse subset of approximately 300 compounds that were chemically synthesized for biological tests (Fig. 1A). Hit compounds belonging to different chemical classes were identified and used as a starting point for a medicinal chemistry hit-to-lead campaign. Most promising leads were subsequently improved in a lead optimization program. As an important result, Compound **1** (Cmp**1**) was identified as one of the most promising binders and inhibitors of DHODH. Cmp**1** (Fig. 1B; 3-(2,3,5,6-tetrafluoro-3′-trifluoromethoxy-biphenyl-4-ylcarbamoyl)-thiophene-2-carboxylic acid) belongs to the chemical class of biphenyl-thiophene-carboxylic acids. Cmp**1** was chemically synthesized in-house by performing a four-step synthesis procedure as described in Section 2 and shown in Fig. S1. Cocrystallization approaches with a resolution of 2 Å verified the unambiguous placement of Cmp**1** and thus confirmed its specific docking to DHODH (B-factor of 30.5 $Å^2$ consistent with an overall B-factor of 35.1 $Å^2$). Drug–target contact was mainly determined by amino acids R136 and Q47 (Fig. 1C). Binding occurred in a 1:1 stoichiometry.

3.2. Cmp**1** exerts strong inhibition of human and murine DHODH activity

In a series of tests performed to characterize the compound *in vitro* and *in vivo*, Cmp**1** proved to possess valuable chemical and pharmacological properties. Solubility was >1.7 mM in 50 mM phosphate buffer at neutral pH (Table 1; including stock solutions in DMSO ⩾ 100 mM). A strong and selective inhibition of human and murine DHODH could be demonstrated *in vitro* (Fig. 1D), with IC_{50} values of 1.5 ± 0.2 nM and 3.2 ± 0.8 nM, respectively (Table 1). In a comparative setting, the nucleoside analogue GCV (used as anti-cytomegaloviral reference drug) did not exert any inhibition on DHODH *in vitro*. No effects onto human DHODH were measured up to high concentrations of GCV (duplicate testings), i.e. DHODH exerted 100% activity even at 10 μM. This finding underlined the specificity of Cmp**1** and confirmed its strong and concentration-dependent anti-DHODH activity. Other enzymes, not related to DHODH, such as a series of protein kinases including PI3K, AKT and JAK (three to four isoforms each), were not inhibited by Cmp**1** (Table 1; data not shown for an additional panel of cellular kinases tested at a single drug concentration also not showing Cmp**1** susceptibility). Moreover, we addressed the question whether the activity of viral kinase pUL97 could be affected by Cmp**1**. For this purpose, a Western blot analysis was performed to investigate the pUL97-mediated Ser807-specific phosphorylation of retinoblastoma protein (Rb) in HCMV-infected HFFs (Hume et al., 2008); no reduction of Rb phosphorylation was observed by treatment with 3.3 μM or 10 μM of Cmp**1** (data not shown). This profile discriminated Cmp**1** from other DHODH inhibitors, in particular leflunomide, showing marked additional inhibitory effects on tyrosine kinases and their respective signaling pathways (Pytel et al., 2009). Moreover, Cmp**1** showed no sign of cytotoxic signals induced in human PBMCs, human HepG2 and murine NIH3T3 cells (up to 50 μM). Standard tests for potassium ion channel activity and *in vitro* mutagenicity were also negative (Table 1). An *in vivo* determination of Cmp**1** plasma levels in mice indicated high concentrations up to 8 h post-treatment (184 ± 106 μM or 522 ± 152 μM after the last of four daily i.p. treatments with 30 mg/kg or 100 mg/kg, respectively). Peak values were measured at 6 h post-treatment (269 ± 83 μM or 551 ± 182 μM, respectively). Terminal half-life (t_z) in rats was 14.5 h after single i.v. treatment.

3.3. Cmp**1** possesses pronounced antiviral activity in cell culture

Compounds were analyzed in an antiviral assay determining the replication efficiency of a GFP-expressing recombinant of HCMV laboratory strain AD169 in primary human fibroblasts (Marschall et al., 2000, 2012). Importantly, Cmp**1** exerted strong anti-HCMV activity with an IC_{50} value of 0.78 ± 0.14 μM, thus being slightly more effective than the reference drug GCV (IC_{50} 1.49 ± 0.58 μM; Fig. 2A, upper panel and Table 2). In a rescue experiment, the antiviral activity of Cmp**1** (3.3 μM) could be reversed in part or completely by the co-application of uridine at concentrations of 10–1000 μM (Fig. 3). In addition, orotate was likewise effective in reversing the anti-HCMV activity of Cmp**1** (uracil showed a limited reversing effect). A similar profile of rescue could also be produced when using a higher concentration of 10 μM Cmp**1** (data not shown). Importantly, in a control these precursors of pyrimidine biosynthesis did not affect the anti-HCMV activity of the reference drug GCV (Fig. 3). This finding provides a proof-of-concept for the postulated DHODH-based mode of antiviral activity of Cmp**1** and clearly demonstrates a mechanistic difference towards GCV (that acts as an inhibitor of viral genome synthesis by blocking chain termination through the viral DNA polymerase). Concerning the question of a broad range of antiviral activities of Cmp**1**, we also demonstrated an inhibition of plaque formation of murine cytomegalovirus replication (MCMV) in the micromolar range (IC_{50} 3.69 ± 0.49 μM; Fig. 2A, lower panel and Table 2). When testing further human herpesviruses, human herpesvirus 6 type A (HHV-6A) and varicella zoster virus (VZV) likewise showed a marked sensitivity to Cmp**1** (IC_{50} 1.55 ± 0.31 and 3.55 ± 0.26 μM, respectively), while herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) as well as Epstein-Barr virus (EBV) were almost insensitive (IC_{50} > 10 μM). The lack of sensitivity of herpes simplex viruses towards Cmp**1** was in contrast to the strong sensitivity of the two selected virus strains (i.e. HSV-1 recombinant reporter virus 166v VP22-GFP and HSV-2 clinical isolate 01-6332) towards reference drugs GCV (0.70 ± 0.01 and 2.70 ± 0.70 μM, respectively; Table 2) and ACV (2.18 ± 0.19 and 2.79 ± 1.31; data not shown). Concerning DNA viruses belonging to other families, vaccinia virus, strain IHD-5, only showed a low level of sensitivity (IC_{50} 9.85 ± 6.61 μM; Table 2). Notably, however, human adenovirus type 2 (HAdV-2) showed high level of sensitivity (IC_{50} 0.56 ± 0.02 μM; Table 2) as measured in a standard plaque reduction assay. This result was confirmed and substantiated by performing a previously established virus yield assay (Kindsmüller et al., 2009) with a series of HAdVs spanning virus species A to E (one to three types each, including clinical isolates B03k and E04k). This selection of HAdVs represented the natural variety of adenovirus-induced pathogenic infections, i.e. association with respiratory, gastrointestinal, ophthalmic or other types of symptoms. Importantly, all HAdVs were subject to inhibition at a drug concentration of 1.11 μM, in an efficacy range between 71% and 100% (Table 3). Highest sensitivity was seen for virus species A, C and D, lower sensitivity for B and E. The drug concentration of 0.37 μM showed poor efficacy for most viruses used, with exception of D08 that behaved strongly sensitive (94% inhibition). Thus, adenoviruses, besides cytomegaloviruses, are broadly covered by

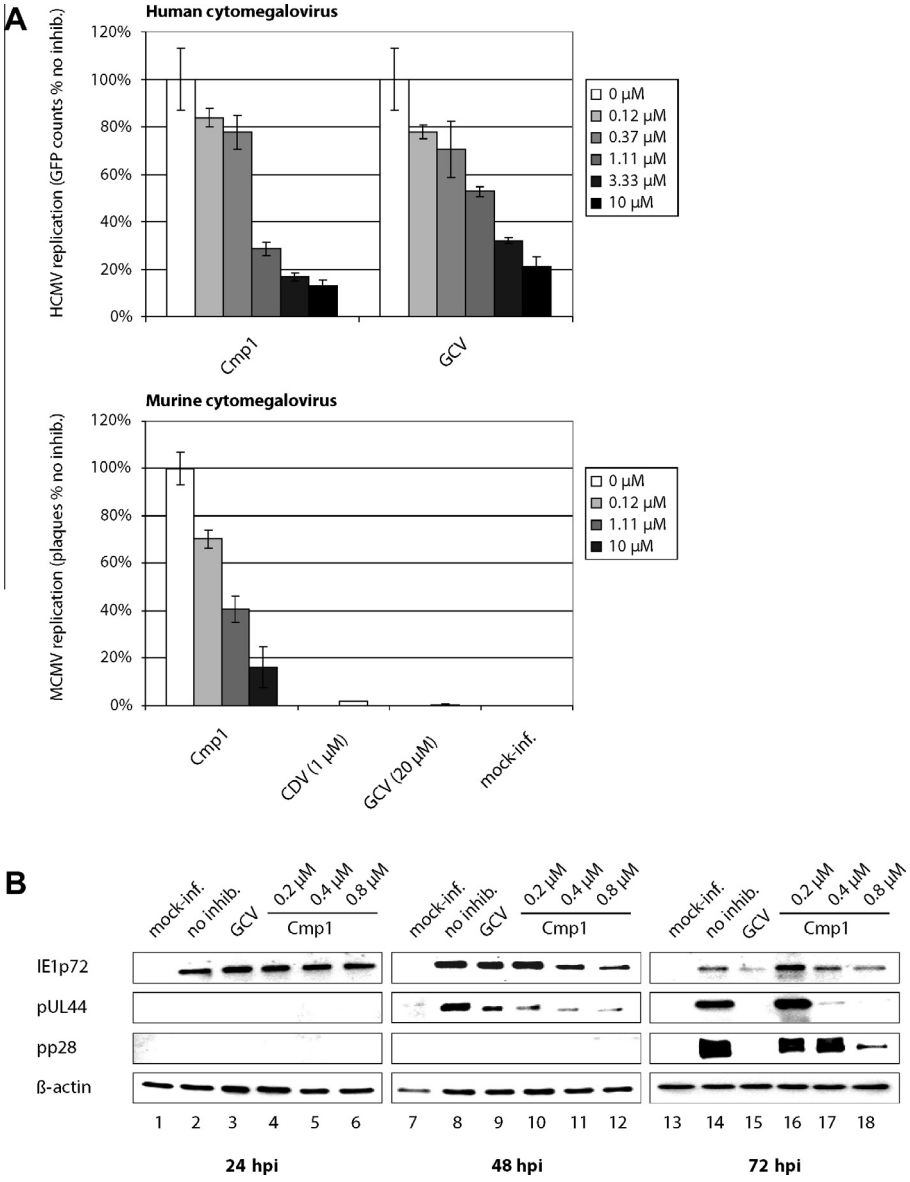

Fig. 2. Anti-cytomegaloviral activity of Cmp**1**. (A) The measurement of inhibitory effects on replication of human and murine cytomegaloviruses was determined by the use of established assay systems, i.e. an HCMV GFP-based replication assay (upper panel) (Marschall et al., 2000) and a MCMV plaque reduction assay (lower panel) (Schleiss et al., 2008). All determinations were performed in quadruplicate (infections in duplicate, GFP fluorometry/microscopic counting in duplicate) and mean values ± SD are given. (B) Western blot analysis was performed to verify the mode of antiviral activity of Cmp**1**. HFFs were infected with HCMV GFP-AD169 at MOI of 0.5 or remained uninfected as a control (mock-inf.). Cmp**1** was added in serial concentrations (0.2, 0.4, 0.8 µM) immediately after infection (reference drug GCV, 10 µM). Cells were harvested 24, 48 and 72 h post-infection and cell lysates were subjected to SDS-PAGE and standard Western blotting procedures. Detection of specific proteins was carried out with the following antibodies: MAb6327 (IE1p72), MAb-UL44 (BS510), MAb-pp28 (41-18) and MAb-β-actin (Ac-15).

Table 2
Comparative analysis of the antiviral activity of Cmp**1**.[*]

	HCMV	MCMV	HHV-6	VZV	HSV-1	HSV-2	EBV	Vaccinia	HAdV-2
IC_{50}[†] Cpm1	0.78 ± 0.14 µM (n = 4)	3.69 ± 0.49 µM (n = 4)	1.55 ± 0.31 µM (n = 8)	3.55 ± 0.26 µM (n = 4)	>10 µM (n = 4)	>10 µM (n = 4)	>10 µM (n = 4)	9.85 ± 6.61 µM (n = 4)	0.56 ± 0.00 µM (n = 8)
IC_{50}[†] GCV	1.49 ± 0.58 µM (n = 4)	4.26 ± 0.99 µM (n = 4)	n.d.	0.81 ± 0.05 µM (n = 4)	0.70 ± 0.01 µM (n = 4)	2.70 ± 0.70 µM (n = 4)	>10 µM (n = 4)	n.d.	n.d.
Cytotoxicity (1–10 µM)[‡]	None	None	None	none	None	None	None	None	None

[*] Viruses and cells used: HCMV, human cytomegalovirus, strain AD169-GFP, primary human fibroblasts; MCMV, murine cytomegalovirus, strain Smith, primary murine fibroblasts; HHV-6, human herpesvirus type 6A, strain U1102, J-JHAN; VZV, varicella zoster virus, strain Oka, primary human fibroblasts; HSV-1 and HSV-2, herpes simplex virus types 1 and 2, strain 166v VP22-GFP and isolate 01-6332, respectively, Vero; EBV, Epstein–Barr virus, strain B95-8, 293T (T81GFP); vaccinia virus, strain IHD-5, primary human fibroblasts; HAdV-2 human adenovirus type 2, A549.
[†] The 50% inhibitory concentrations of virus replication were determined by GFP-based reporter assay (HCMV, HSV-1) or plaque reduction assay, respectively. Mean values and standard deviations derived from n-fold measurements are given.
[‡] Drug-induced cytotoxicity was determined by microscopic evaluation performed at two separate time points during the course of the experiment.

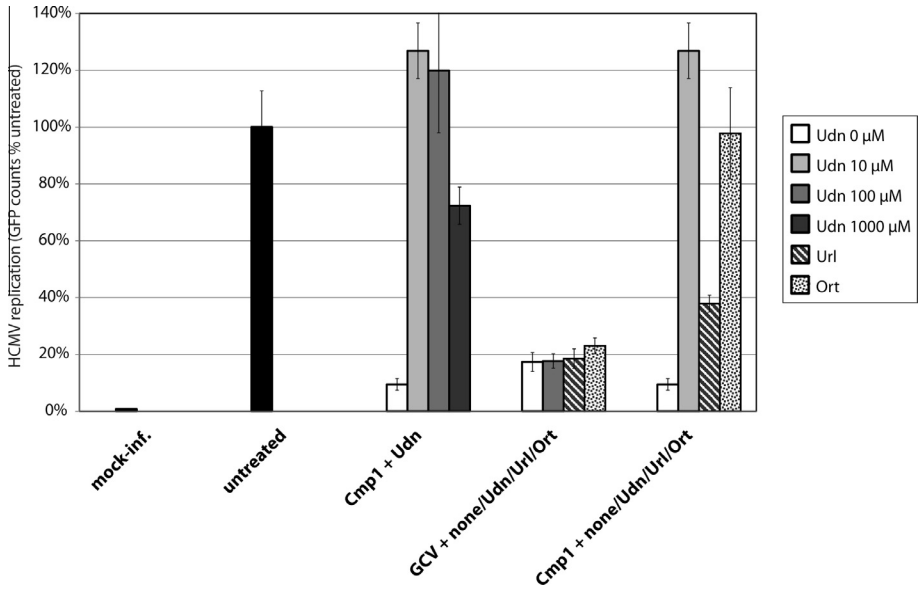

Fig. 3. Rescue of Cmp**1**-mediated antiviral effect by precursors of pyrimidine biosynthesis. HFFs were used for infection in an HCMV GFP-based replication assay as described for Fig. 2 (uninfected control, mock-inf.) and treated with 3.3 μM of Cmp**1** or GCV (or remained untreated, no inhib.). For experimental rescue of the antiviral effect of Cmp**1**, uridine (Udn, Sigma–Aldrich) was co-applied at concentrations 10, 100 or 1000 μM. As further comparative settings, GCV or Cmp**1** were co-applied together with Udn, uracil (Url) or orotate (Ort) at the concentration of 100 μM (combined with 3.3 μM GCV) or 10 μM (combined with 3.3 μM Cmp**1**). Cells were harvested 7 days post-infection and lysed for measurement in the automated GFP fluorometry (determinations in quadruplicate; mean values ± SD are given).

the inhibitory potential of Cmp**1**, a finding strongly encouraging further steps of drug development.

3.4. Early-stage inhibition of cytomegalovirus replication is consistent with a block in DHODH activity

The activity of DHODH mediates *de novo* biosynthesis of pyrimidine ribonucleotides and is thus thought to represent a rate-limiting step of viral replication at the levels of genome synthesis and early gene expression. Therefore DHODH inhibition suggests an inhibitory profile of HCMV replication similar to that known for the currently used drugs GCV, CDV and FOS (i.e. pharmacological inhibitors of viral genome synthesis and DNA polymerase activity). To address this point, we analyzed the expression of HCMV proteins during the course of replication in primary human fibroblasts. As a characteristic feature, Cmp**1** had low effects on viral immediate early protein production (Fig. 2B; IE1p72) and no effect on a cellular house-keeping protein (β-actin). In contrast, the expression of viral early (pUL44) and true late (pp28) proteins was substantially reduced by Cmp**1** treatment in a concentration-dependent manner (Fig. 2B, lanes 4–6, 10–12, 16–18). Expression levels at 72 h post-infection were quantitated by densitometric evaluation of signal intensities using AIDA Image Analyzer (pUL44: 100% no inhib., 116% 0.2 μM Cmp**1**, 23% 0.4 μM Cmp**1**, 12% 0.8 μM Cmp**1**; pp28: 100% no inhib., 75% 0.2 μM Cmp**1**, 77% 0.4 μM Cmp**1**, 51% 0.8 μM Cmp**1**). The pattern of inhibition was comparable to that observed for GCV (lanes 3, 9 and 15). In order to exclude a cytotoxic or anti-proliferative activity of Cmp**1** as the reason for blocking viral early protein production, an analysis of cell viability was performed. First, confluent layers of HFFs were treated with Cmp**1** in a range of concentrations between 0.1 and 10 μM and monitored by microscopy for 7 days. No sign of drug-induced changes in cell morphology or cytotoxicity was detected at any time. Next, a commercially available proliferation assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay; Promega) was performed using subconfluently seeded, proliferating HFFs as described previously (Hutterer et al., 2013). Also in this setting, no or only low effects of antiproliferative activity were detectable within the range of relevant concentrations (i.e. the measured signals representing cell viability refer to 100% at 0 μM Cmp**1**, 83% at 0.2 μM, 82% at 0.4 μM and 79% at 0.8 μM, respectively). Thus, the data shown in Fig. 2B are based on an early antiviral block in the absence of cytotoxicity and are consistent with a block of DHODH activity, stressing the postulated importance of DHODH for HCMV replication.

Table 3
Analysis of the anti-adenoviral activity of Cmp**1** against a selection of clinically relevant virus types and well-characterized laboratory strains.*

HAdV	Cmp1		
	0 μM	0.37 μM	1.11 μM
A31†	100	72	3
B03‡	100	127	29
B03k‡	100	70	11
C02$	100	100	9
C05$	100	82	6
D08†	100	6	0
D19†	100	81	4
D37‡	100	96	3
E04‡	100	128	23
E04k‡	100	115	9

* Antiviral activity was determined by adenovirus yield assay. A549 cells were infected at MOI 20 and continuously treated with the given concentrations of Cmp**1**. Virus-containing supernatants were transferred to uninfected cells at 4 d†, 6 d‡ or 2 d$ post-infection, respectively, and used for quantitation of infectious virus. Percentages of virus-positive cells per total cells were determined on the basis of indirect immunofluorescence staining (monoclonal anti-adenovirus hexon protein antibody; Abcam B025-AD51/ab7428) by microscopic countings: $n = 4$ for C02, C05, D19, D37; $n = 3$ for B03k, E04k; $n = 1$ for A31, B03, D08, E04.

3.5. An animal model demonstrates the anti-cytomegaloviral effect in vivo

An established animal model was applied to investigate the anti-cytomegaloviral efficacy of Cmp**1** *in vivo*. For this, a recombinant MCMV carrying a reporter module for luciferase expression and a deletion of the immunostimulatory genomic region M157 (MCMV157luc) (Klenovsek et al., 2007) was used for intraperitoneal infection (i.p., 5×10^5 PFU/mouse) of Rag1−/− Black6 mice. Mice were daily treated with Cmp**1** (30 mg/kg/d or 100 mg/kg/d,

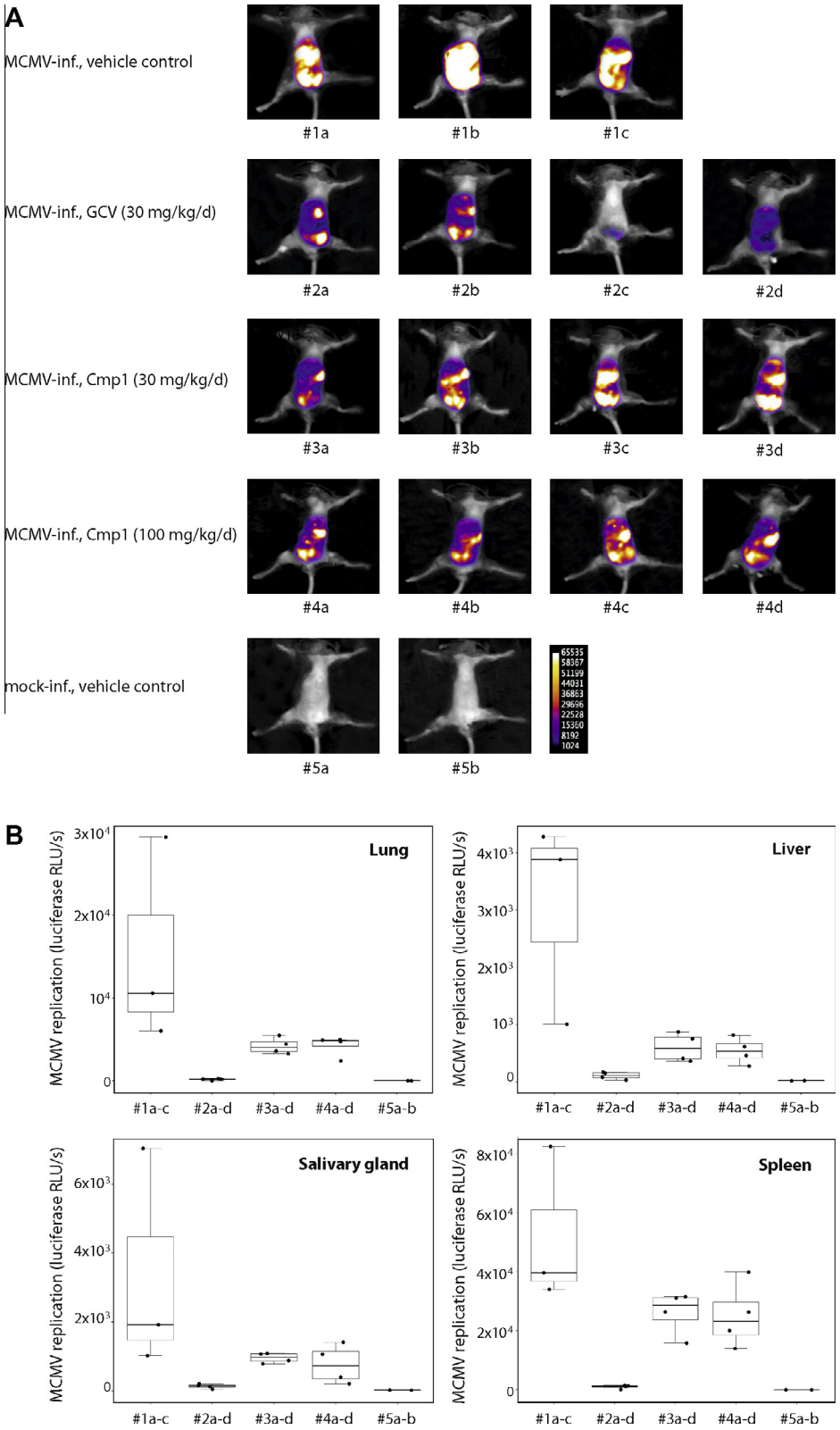

Fig. 4. *In vivo* model demonstrating antiviral efficacy of Cmp**1**. (A) A luciferase reporter system was used for *in vivo* imaging of MCMV-infected mice. Rag−/− mice were used for i.p. infection (5×10^5 PFU) with indicated doses of luciferase expressing MCMV-del157luc. Antiviral drug treatment was performed daily with 30 or 100 mg/kg as indicated. At 5 days post-infection, animals were anesthetized and analyzed for viral replication by *in vivo* luciferase imaging. (B) Quantitation of MCMV replication under drug treatment in four different organs. At 7 days post-infection, the animals were sacrificed for preparation of organs. Homogenates of organ samples were adjusted to equivalent protein concentrations and used for quantitative measurement of luciferase activity under standard conditions. Determinations were performed in triplicate and medians of the values are given (RLU/s, relative light units per second). Boxes cover the inner 50% of data ranges, i.e. spanning from 25 to 75 percentiles (interquartile range, IQR). Whiskers extend to the most extreme data points found within a 1.5× IQR area.

i.p.), GCV (30 mg/kg/d, i.p.) or a vehicle control (Fig. 4A). Caging was performed in groups, and individual animal parameters, such as body weight and putative signs of toxicity, were monitored on days 0, 5 and 7 post-infection (Table S1). No drug-related side effects or toxicity were observed. At 5 days post-infection, animals were anesthetized and analyzed for viral replication by *in vivo* luciferase imaging. Importantly, animals treated with Cmp**1** (Fig. 4A, animals #3a–d and #4a–d) or GCV (#2a–d) showed a reduction of viral infection and organ dissemination of the virus compared to the vehicle control group (#1a–c). Thus, i.p. treatment with Cmp**1** produced visually detectable antiviral efficacy. For quantitative evaluation of viral replication, animals were sacrificed 7 days post-infection and organs were prepared to homogenates. Equivalent protein concentrations were adjusted and used for measurements of luciferase activity under standard conditions (Klenovsek et al., 2007). As an important result, an inhibitory effect of Cmp**1** on MCMV replication was detected in all organs analyzed, i.e. lung, salivary gland, liver and spleen (Fig. 4B). It should be stated, however, that at least under these experimental conditions the antiviral efficacy of Cmp**1** remained quantitatively below that of GCV. Nevertheless, statistical evaluation by student's *t*-test indicated a significant effect ($P < 0.05$, for both drug concentrations in lung, salivary gland and spleen) or highly significant effect ($P < 0.001$, for both drug concentrations in liver) when compared to the vehicle control. Combined, the data provide first evidence for the antiviral activity of a compound directed to the target DHODH *in vivo*.

4. Discussion

In this study, we describe an experimental approach to identify cellular targets and drug candidates for putative use in future antiviral therapy. This study was based on the *in silico* technology 4SCan® drug design, successfully applied in combination with a medicinal chemistry campaign to screen and nominate experimental drugs. This was further exemplified by several levels of chemical, biological, cell culture-based and *in vivo* model testings. Here we report the following: (i) cellular dihydroorotate dehydrogenase (DHODH) represents an interesting antiviral target, as further validated in this study confirming earlier reports, (ii) *in silico* and *in vitro* analyses identified hit compounds as potent inhibitors of human DHODH revealing the optimized lead compound Cmp**1**, (iii) experimental data provided evidence for a pronounced and broad antiviral activity of Cmp**1** (directed against at least four herpesviruses and ten adenoviruses), (iv) IC_{50} values of antiviral activity were in the sub-micromolar range, (v) for HCMV, an inhibitory block was demonstrated for the early stage of viral replication, and (vi) an *in vivo* model confirmed the anti-cytomegaloviral efficacy of Cmp**1** in mice as expressed by significantly reduced viral load in various organs. The present findings show, on the one hand, that 4SCan® technology is useful in identifying hit compounds and supporting their improvement into optimized leads for potential application in pharmaceutical treatments including antiviral therapy; on the other hand, data suggest that DHODH is a prospective target for further antiviral research.

Interestingly, data provided in this study showed similarities in the profiles of antiviral activity between Cmp**1** and the anti-herpesviral reference drug GCV. Hereby, it is crucial to note that both compounds have different and distinct mechanisms of action. While GCV is an analogue of 2′-deoxy-guanosine and acts as a prodrug that is phosphorylated by viral and cellular kinases to effect chain termination of viral genome synthesis, Cmp**1** restricts the nucleotide pool by inhibition of DHODH. Our data strongly suggest that Cmp**1** binds into the ubiquinone cofactor binding site acting competitively towards ubiquinone and non-competitively towards the pyrimidine precursor orotate. This inhibition of pyrimidine *de novo* synthesis may result in a restriction of available nucleotides for DNA chain elongation, leading to a stall of the viral DNA polymerase at the growing nucleotide chain, finally followed by a premature stop of polymerization and detachment from the template. Thus, although Cmp**1** and GCV possess individual modes of antiviral action, both drugs produce very similar profiles of an early block of HCMV replication.

Recently, DHODH has been considered as a drugable target for antiviral and antimalarial therapy (Phillips and Rathod, 2010; Chacko and John, 2012), as derived from the use of leflunomide is an approved drug for the treatment of rheumatoid arthritis (Goldenberg, 1999; Herrmann et al., 2000; Olsen and Stein, 2004). The immunosuppressive activity of leflunomide is mediated by pyrimidine depletion, exerting critical shortage of pyrimidine biosynthesis mainly in activated lymphocytes but only poorly in resting lymphocytes (Rückemann et al., 1998). In addition, Waldman et al. (1999a) reported anti-cytomegaloviral properties of leflunomide, effected through a novel mechanism independent from the inhibition of HCMV DNA synthesis. Waldman et al. (1999b) demonstrated a block in the late stage of HCMV replication, as particularly exerted on cytoplasmic maturation of virions. However, the fact that leflunomide apparently attacks further targets in addition to DHODH, i.e. protein kinases and signaling (Chacko and John, 2012), suggests a more complex mode of action. The clinical evaluation of this drug for antiviral treatment has not been completed to date. Although first individual clinical trials were seen favourably (Henao-Martínez et al., 2012; Avery et al., 2010; John et al., 2004), the present situation does not provide a clear picture whether leflunomide will be broadly beneficial in antiviral therapies.

In contrast to leflunomide and other inhibitors of DHODH described so far, Cmp**1** appears to act in a more selective manner. No inhibitory activity on cellular protein kinases has been detected in this study (Table 1); a proof of true monoselective way of action, however, can only be provided by broader test panels in the future. Recently, a novel antiviral compound targeting DHODH, NITD-982, was described (Wang et al., 2011). The inhibitor was identified through screenings using a dengue virus (DENV) infection assay. The compound showed strong anti-DENV activity in the nanomolar range and broad antiviral spectrum in cell culture-based testings. However, in contrast to its potency *in vitro*, the compound did not show any *in vivo* efficacy in a DENV mouse model system (33). Thus, the present study provides for the first time experimental evidence that the novel DHODH inhibitor Cmp**1** shows efficacy as an antiviral drug in an *in vivo* model.

5. Conclusions

Combined, data from our study and from related studies underline that DHODH is a promising target for the development of antiviral drugs. Hereby, the application of the 4SCan® technology showed a clear practical benefit for antiviral research. To our knowledge, Cmp**1** represents the first DHODH-targeted experimental drug that inhibits DHODH at nanomolar concentrations in a selective way (secondary targets have not been revealed by ongoing selectivity testings). Thus, Cmp**1** and additional leads derived from this compound class are prospective for further medicinal development. In particular, the broad antiviral activity of Cmp**1** may gain new therapeutic options for the treatment of infections with human pathogenic herpesviruses and adenoviruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.10.003.

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