An Artemisinin-Derived Dimer Has Highly Potent Anti-Cytomegalovirus (CMV) and Anti-Cancer Activities

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Abstract

We recently reported that two artemisinin-derived dimers (dimer primary alcohol 606 and dimer sulfone 4-carbamate 832-4) are significantly more potent in inhibiting human cytomegalovirus (CMV) replication than artemisinin-derived monomers. In our continued evaluation of the activities of artemisinins in CMV inhibition, twelve artemisinin-derived dimers and five artemisinin-derived monomers were used. Dimers as a group were found to be potent inhibitors of CMV replication. Comparison of CMV inhibition and the slope parameter of dimers and monomers suggest that dimers are distinct in their anti-CMV activities. A deoxy dimer (574), lacking the endoperoxide bridge, did not have any effect on CMV replication, suggesting a role for the endoperoxide bridge in CMV inhibition. Differences in anti-CMV activity were observed among three structural analogs of dimer sulfone 4-carbamate 832-4 indicating that the exact placement and oxidation state of the sulfur atom may contribute to its anti-CMV activity. Of all tested dimers, artemisinin-derived diphenyl phosphate dimer 838 proved to be the most potent inhibitor of CMV replication, with a selectivity index of approximately 1500, compared to our previously reported dimer sulfone 4-carbamate 832-4 with a selectivity index of about 900. Diphenyl phosphate dimer 838 was highly active against a Ganciclovir-resistant CMV strain and was also the most active dimer in inhibition of cancer cell growth. Thus, diphenyl phosphate dimer 838 may represent a lead for development of a highly potent and safe anti-CMV compound.

Introduction

Infection with CMV, a member of the herpesvirus family, is common in humans. Seroprevalence rates increase with age, reaching 90% in individuals older than 80 years [1]. The virus establishes lifelong persistent infection, which usually remains asymptomatic. In immunocompromised hosts such as transplant recipients and patients with AIDS, CMV infection is associated with significant morbidity and mortality [2,3]. CMV is also the most common congenitally-acquired infection causing mental retardation and deafness in congenitally-infected children [4]. Recently, the detection of CMV in immunocompetent individuals has been linked with outcomes of several syndromes including sepsis, pulmonary complications in patients in intensive care-units, and in a brain tumor, glioblastoma multiforme [5–7]. Although the direct role of CMV in these syndromes is unclear, virus replication may contribute to their natural history, and the role of anti-CMV therapy in these conditions is currently being investigated.

The available systemic anti-CMV drugs act by targeting the viral DNA polymerase. These compounds effectively suppress CMV replication, but their use is associated with considerable toxicities to the bone marrow (Ganciclovir-GCV) and kidneys (Foscarnet and Cidofovir) [8,9] and the emergence of drug-resistant mutants during prolonged courses of therapy [9,10]. Thus, new compounds with low toxicity and ideally with a distinct mechanism of CMV inhibition are needed for CMV therapy.

The artemisinin-derived monomer arteunate was originally reported to inhibit CMV replication in vitro and in vivo [11,12]. Recently, we reported on two artemisinin-derived dimers with significantly more potent activity against CMV replication in vitro as compared to artemisinin-derived monomers [13]. Artemisinin monomers are currently the drugs of choice for malaria therapy [14]. In addition, both artemisinin monomers and dimers were shown to possess anti-cancer activities [15–17]. The potent anti-CMV activity of two artemisinin-derived dimers [13] prompted us to evaluate a series of newly-synthesized artemisinin-derived dimers. We report on the anti-CMV and anti-cancer activities of the most potent compounds in this investigation.

Results

A comparison of anti-CMV activity of 17 artemisinin derivatives

We previously reported on the anti-CMV activity of four artemisinin monomers (artesunate, artether, and arteannulide) and two artemisinin-derived dimers (dimer primary alcohol 606 and dimer sulfone 4-carbamate 832-4) [13]. We now tested one new artemisinin-derived monomer and 10 additional new artemisinin-derived dimers and compared their anti-CMV
activities to those of previously tested compounds. The abbreviated names of the 17 compounds and their molecular weights are listed in Table 1. In this report, each compound is referred to by its molecular weight. For example, compound 606 refers to the dimer primary alcohol, and 832-4 refers to dimer sulfone 4-carbamate (Table 1). Sulfone carbamate 551 is the monomeric version of dimer sulfone carbamate 832-4. The chemical structure of dimer sulfone 4-carbamate 832-4 prevents it from being catabolized into monomer sulfone 4-carbamate 551. Compound 574, which is the deoxy version of 606, was chosen for testing because the anti-malarial and anti-cancer activities of artemisins are at least partially endoperoxide bridge-dependent [18,19].

The two previously tested artemisinin-derived dimers had potent anti-CMV activity at concentrations of 1 μM or lower, while the artemisinin-derived monomers achieved a similar degree of CMV inhibition only at concentrations higher than 10 μM [13]. Based on these data, all new compounds were initially screened for anti-CMV activities. The most active compounds were then tested in detail for their anti-CMV activities. All dimers were initially evaluated at concentrations of 1 μM, 0.3 μM, and 0.1 μM. The deoxy dimer 574 and monomer sulfone carbamate 551 were screened at 1 μM and 10 μM. At 1 μM, the dimers displayed potent inhibition of late pp28 gene expression (which highly correlates with plaque reduction) [20] measured by luciferase activity, but the deoxy dimer 574 and monomer sulfone carbamate 551 did not (Fig. 1A). Several dimers were also effective at 0.3 μM, but only two dimers, sulfone 4-carbamate 832-4 and diphenyl phosphate 838, were highly inhibitory at 0.1 μM. Three structural analogs of sulfone 832-4 were synthesized (832-3, 800-3 and 800-551) did not (Fig. 1C). Monomers overall displayed similar cytotoxicity pattern, but were significantly more toxic than dimers at 100 μM (P = 0.03 by two-tailed t-test). Dimers 832-4 and 838 had the best selectivity index (SI), which is the ratio of CC50 to EC50, and were therefore selected for further detailed analysis of anti-CMV activity.

**Table 1. List of artemisinin-derived monomers (first 5 compounds) and dimers evaluated for anti-CMV activity, and their molecular weight (MW).**

| Compound                   | MW  |
|----------------------------|-----|
| Artemisin                  | 282 |
| Artemether                 | 298 |
| Artesunate                 | 384 |
| Artefanilide (ref 33)      | 433 |
| Art-PhSO2Ph4-CH2OC(O)NMe2 | 551 |
| Deoxy-Dimer-isobu-OH      | 574 |
| Dimer isobu-OH             | 606 |
| Dimer isobu-COOH           | 620 |
| SC Dimer OH                | 644 |
| Dimer isobuSO2Ph4-CH2OH    | 760 |
| Dimer isobu-Ph3-CH2OC(O)NMe2 | 800-3 |
| Dimer isobu-Ph4-CH2OC(O)NMe2 | 800-4 |
| SC Dimer OC(O)PH4-5SO2Me  | 826 |
| Dimer isobuSO2Ph4-CH2OC(O)NMe2 | 832-4 |
| Dimer isobuSO2Ph3-CH2OC(O)NMe2 | 832-3 |
| Dimer isobu-OP(O)(OPh)2   | 838 |
| SC Dimer OC(O)CH3(NBoc)Bn | 891 |

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Inhibition of a GCV-resistant CMV strain by dimers 832-4 and 838

We evaluated the inhibition of a GCV-resistant strain (EC50 of GCV = 7.6 μM) by dimers 832-4 and 838 using a plaque reduction assay. Dimers 832-4 and 838 were applied to infected cells at a concentration of 0.1 μM; GCV was applied at 5 μM and 30 μM. Twenty one days after infection, cells were stained with crystal violet and plaques were counted in each condition (Fig. 3). Dimers 832-4 and 838 fully inhibited plaque formation at 0.1 μM, while this strain showed an obvious resistance to GCV at 5 μM.

Slopes of the anti-CMV dose response curves of the tested anti-CMV compounds

A classic description of dose-response relationships is the median effect model based on mass action indicating that [21]:

\[ f_a = \frac{1}{1 + \left( \frac{EC_{50}}{D} \right)^n} \]

In this function, \( f_a \) is the proportion of the inhibited fraction of viruses, or the inhibition percentage, \( D \) is drug concentration, \( EC_{50} \) is the drug concentration that achieves 50% of the maximum inhibitory effect and \( n \) is a slope parameter of the curve mathematically. The effect of the slope parameter on anti-HIV activity was recently evaluated, and proved to be a characteristic of drug class and a crucial parameter dimension in the analysis of antiviral activity [21].

We applied this model to our derivatives and calculated the slope parameter of the most potent dimers (832-4 and 838), GCV, and artemisinin-derived monomer artesunate (Table 2). Compared to
GCV and artesunate, which had an anti-CMV m value of approximately 1, the m values of 832-4, 838, 760 and 606 were around 4, an indication that these compounds are a highly potent class of anti-CMV compounds.

Inhibition of cancer cell growth by dimers 832-4 and 838

Artemisinin-derived monomers and several dimers have been reported to inhibit the growth of cancer cell-lines [16,17]. Four dimers (832-4, 838, 760 and 606), two monomers (artesunate and artefenilide) and GCV were applied to three cancer cell lines: HeLa (cervical adenocarcinoma), HCT116 (colorectal carcinoma) and 1205Lu (melanoma) at concentrations ranging from 0.01 μM to 1 μM. MTT assay was performed 72 hr after treatment and CC50 of each compound in each cancer cell line was calculated (table 3). The CC50 of each compound in cancer cells was compared to the CC50 in non-cancer primary HFF cells. All dimers were significantly more active than monomers in inhibiting the growth of cancer cell lines. For example, the dimer 838 was at least 200-fold more potent in inhibiting growth of HCT116 and 1205Lu cells and 37-fold more potent in inhibiting growth of HeLa cells, as compared to artesunate, the most potent monomer in this assay. A qualitative correlation was observed between the anti-CMV activity and the anti-cancer activity among the four dimers (838, 832-4, 606 and 760); dimer 838, the most potent of all tested dimers against CMV replication, also had the strongest effect on growth inhibition of cancer cells.

Discussion

We report here on the anti-CMV activity of ten new artemisinin-derived dimers, one deoxy dimer and one new artemisinin monomer, and the identification of the most potent anti-CMV dimer. The enhanced anti-CMV activity of dimers as compared to monomers was not a result of increased cytotoxicity but rather a specific anti-CMV activity. The two most highly potent dimers were effective in inhibition of the laboratory-adapted Towne strain as well as a GCV-resistant strain. They were also shown to have the strongest inhibitory effect on the growth of cancer cell lines.

All dimers exhibited potent CMV inhibition at 1 μM. However, only two compounds (832-4 and 838) could be selected as highly potent anti-CMV agents based on their high CMV inhibition at 0.1 μM and their low cytotoxicity. At this concentration, the other dimers had either no inhibition or at most 40% inhibition of luciferase expression, while GCV had no inhibition at all.

Artemisinin derivatives were initially developed as anti-malarial drugs, but later were also found to have additional pharmacological activities such as growth inhibition of cancer cells and inhibition of CMV replication [12,13,22–25]. Whether or not
These new activities are related to each other and indicate a shared mechanism, the endoperoxide bridge appears to be critical in all pharmacological activities of artemisinin derivatives.

As a generalized observation, the dimers were significantly more potent than the monomers in CMV inhibition. Using the EC_{50} values dimer 838 was 163-fold more potent than artemisin in anti-CMV activity and its SI was 134-fold higher compared to artemisin. In support of this finding, the model of dose response slope parameter which was recently used to characterize the activities of anti-HIV compounds [21] revealed that dimers had a much higher slope than monomers. Generally, a higher slope correlates with a more vigorous anti-viral activity. This could result either from very high affinity of drug binding to a single ligand or from cooperative effect of multiple ligands with similar affinity.

Several anti-cancer agents (such as kinase inhibitors) have been shown to inhibit CMV replication, but in general, the concentrations required to achieve CMV inhibition were toxic to cells and prohibited their use as anti-viral candidates [26]. Interestingly, Sorafenib, a multi-kinase inhibitor, appears to inhibit CMV replication without associated cellular toxicity [27]. The dimers reported here prevented growth of cancer cell-lines and were potent inhibitors of CMV replication without apparent cellular toxicities. Therefore, they may provide a new class of anti-CMV agents, with a distinct mechanism of action. Similar to the anti-CMV activity, dimers were also more effective than monomers in inhibition of cancer cells. Future studies may reveal whether the dual anti-CMV and anti-cancer activities of dimers result from a shared mechanism of action. We and others have shown that inhibition of CMV replication with artemisin derivatives appears early during the replication cycle and involves a mechanism which is different from the DNA polymerase inhibitors [28,29]. The anti-cancer activities of artemisinins have been postulated to involve cell cycle arrest, apoptosis, and/or angiogenesis [30], processes that are also affected by specific CMV genes, mostly immediate early genes. Future studies will address the mechanism of CMV inhibition by artemisinin-derived dimers and their potential overlap with oncogenic signaling events.

In conclusion, we have shown that: 1) Dimers are highly active against CMV replication, and dimer diphenyl phosphate 838 is the most potent in CMV inhibition. 2) The dose response slope parameter of dimers is significantly higher than that of artemisinin monomers and GCV, a possible indication that dimers are a distinct class of anti-CMV compounds. 3) Dimers are more potent inhibitors than monomers in both cancer cell lines and in CMV.

### Materials and Methods

**Compounds**

Ganciclovir (GCV) was obtained from Roche, USA. All artemisinin derivatives used in this study were synthesized at...
Johns Hopkins University [17,31–33]. Newly synthesized dimer sulfide 3-carbamate (800-3) and dimer sulfide 4-carbamate (800-4) as well as dimer sulfone 3-carbamate (832-3) were synthesized as described in Materials S1. Artemisinin-derived monomers and dimers were dissolved in dimethyl sulfoxide (DMSO) and stocks of 10 mM were stored in −20°C. Synthetic compounds were at least 98% pure based on proton NMR spectroscopy. The DMSO itself was tested in CMV-infected cells, and it did not have any anti-viral activity [13].

Viruses
The pp28-luciferase Towne strain was constructed as previously described [20]. Briefly, the recombinant virus, which expresses luciferase under the control of UL99 (pp28) late promoter was generated by insertion of the reporter gene between the US9 and US10 open reading frames (ORFs) in the Towne genome. The expression of pp28-luciferase is strongly activated 48–72 hours post infection (hpi) and is almost completely inhibited in the presence of DNA polymerase inhibitors such as GCV and foscarnet [20]. We have recently reported that the pp28-luciferase reporter system is sensitive, reproducible and highly correlates with plaque reduction [20]. The ganciclovir (GCV)-resistant strain was obtained from a patient with CMV disease. It has a UL97 mutation (H520Q) and an EC50 of 7.6 μM for GCV. This clinical isolate was provided by the clinical virology laboratory with no identifiers that can link to a specific subject. The Johns Hopkins Office of Human Subject Research Institutional Review Board determined that this research qualified for an exemption.

Cell Culture, Virus Infection and Anti-viral assays
Human Foreskin Fibroblasts (HFF) passage 12–16 (ATCC, CRL-2088™) were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Gibco, Carlsbad, CA) in a 5% CO2 incubator at 37°C and used for infections with pp28-luciferase Towne CMV or the GCV-resistant strain. One day prior to infection with the pp28-luciferase, 4 × 10⁴ HFF cells were seeded on each well of 24-well tissue culture plates. Infection was carried out at multiplicity of infection of 1 PFU/cell (MOI = 1). Following 90 minute adsorption, media containing virus was removed and replaced by DMEM with 4% fetal bovine serum (Gibco, Carlsbad, CA) containing anti-viral compounds. The concentration of each compound was calculated and adjusted by volume such that it was constant throughout the experiment. Infected and treated HFF cells were collected 72 hpi and washed once with PBS. The lysates were assayed for luciferase and cell viability using a Luciferase Assay Kit (Promega, Madison, WI) and CellTiter-Glo Luminescent Cell Viability Assay Kit, respectively, on GloMax®-Multi+ Detection System (Promega) according to manufacturer’s instructions.

### Table 3. CC50 in three types of cancer cell lines: HeLa, 1205Lu, HCT116, and in non-cancer primary HFF cells.

| Compound | HeLa | 1205Lu | HCT116 | HFF |
|----------|------|--------|--------|-----|
| 832-4    | 0.27±0.03 | 0.13±0.007 | 0.07±0.006 | 57.5±2.9 |
| 838      | 0.19±0.06 | 0.06±0.002 | 0.04±0.005 | 55.8±2.8 |
| 760      | 0.56±0.18 | 0.1±0.006 | 0.07±0.005 | 49±0.2 |
| 606      | 1.06±0.2 | 4.9±0.04 | 0.1±0.001 | 48.1±2.6 |
| Artesunate | 7.1±0.4 | 42±2.4 | 11.3±0.6 | 71.7±4 |
| Artefanilde | >50 | >50 | 40±7.9 | 44.9±3.4 |
| GCV      | >50 | >50 | >50 | 247±33.4 |

Figure 3. Activity of 832-4, 838 and GCV against a GCV-resistant CMV strain. A plaque reduction assay was used to quantify the inhibition of the GCV-resistant CMV. CMV-infected HFF were treated with 832-4, 838 and GCV at the indicated concentrations. Plaques were counted 21 days post infection.
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Human lung fibroblasts (HEL) passage 8–12 (ATCC, CCL-137T) were grown in DMEM containing 10% fetal bovine serum. One day prior to infection, 3×10^6 cells were seeded on each well of 12-well tissue culture plates. GCV-resistant CMV was diluted in DMEM to a desired concentration which gave around 1000 plaque forming units per well and added to each well in duplicates. Plates were incubated for 90 minutes with shaking every 10 min; thereafter drugs were added and a methyleneblue overlay applied to each well. After incubation for 21 days, cells were stained with crystal violet and plaques were counted under microscope at 40× magnification.

Anti-Cancer Cells Assay

HeLa (Human Cervical Adenocarcinoma), HCT116 (Human Colorectal Carcinoma) and 1205Lu (Human Melanoma) cells (all from ATCC) were maintained in DMEM containing 10% fetal bovine serum. 12–16 hours prior to drug treatment, 3×10^4 to 5×10^5 cells were seeded on each well of 96-well tissue culture plates. Drugs were diluted in DMEM containing 10% fetal bovine serum and applied to the cells. 72 hours after drug treatment, 20 μl of MTT solution (5 mg/ml in PBS) was added into each well, followed by shaking the plates at 150 rpm for 5 minutes and an incubation of 4 hours (37°C, 5% CO₂). Media was removed, plates dried and each well was resuspended in 100 μl MTT formazan/DMSO. The absorbance of each well was recorded at 560 nm on a GloMax®-Multi+ Detection System (Promega).

Supporting Information

Materials
1. Synthesis of Dimer Sulfide 3-Carbamate 800-3. 2. Synthesis of Dimer Sulfone 3-Carbamate 832-3. 3. Synthesis of Dimer Sulfide 4-Carbamate 800-4. 4. Synthesis of deoxy-artemisinin alcohol 574. 5. Synthesis of Monomer Sulfone Benzylic Dimethyl Carbamate 551

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Author Contributions

Conceived and designed the experiments: RH RA-B. Performed the experiments: RH BTM ASR DTG. Analyzed the data: RH RA-B. Contributed reagents/materials/analysis tools: BTM ASR DTG. Wrote the paper: RH RA-B. Reviewed and revised the manuscript: BTM ASR DTG GHP.

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