Artemisinins target the intermediate filament protein vimentin for human cytomegalovirus inhibition

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The antimalarial agents artemisinins inhibit cytomegalovirus (CMV) in vitro and in vivo, but their target(s) has been elusive. Using a biotin-labeled artemisinin, we identified the intermediate filament protein vimentin as an artemisinin target, validated by detailed biochemical and biological assays. We provide insights into the dynamic and unique modulation of vimentin, depending on the stage of human CMV (HCMV) replication. In vitro, HCMV entry and viral progeny are reduced in vimentin-deficient fibroblasts, compared with control cells. Similarly, mouse CMV (MCMV) replication in vimentin knockout mice is significantly reduced compared with controls in vivo, confirming the requirement of vimentin for establishment of infection. Early after HCMV infection of human foreskin fibroblasts, vimentin level is stable, but as infection proceeds, vimentin is destabilized, concurrent with its phosphorylation and virus-induced calpain activity. Intriguingly, in vimentin-overexpressing cells, HCMV infection is reduced compared with control cells. Binding of artesunate, an artemisinin monomer, to vimentin prevents virus-induced vimentin degradation, decreasing vimentin phosphorylation at Ser-55 and Ser-83 and resisting calpain digestion. In vimentin-deficient fibroblasts, the anti-HCMV activity of artesunate is reduced compared with controls. In summary, an intact and stable vimentin network is important for the initiation of HCMV replication but hinders its completion. Artesunate binding to vimentin early during infection stabilizes it and antagonizes subsequent HCMV-mediated vimentin destabilization, thus suppressing HCMV replication. Our target discovery should enable the identification of vimentin-binding sites and compound moieties for binding.

Repurposing of the antimalarial agents artemisinins for treatment of human cytomegalovirus (HCMV) attracted interest, fueled by clinical experience and safety data from malaria therapy (1–5). The in vitro activity of artemisinins against HCMV, but not against herpes simplex virus 1 (HSV1), indicates unique and selective antiviral activities (6). We and others have reported that artemisinin-derived monomers (artesunate, artemether, artemisone) inhibit HCMV at micromolar concentrations, whereas the dimeric versions are inhibitory at nanomolar concentrations (1, 2, 7, 8). The endoperoxide bridge within the artemisinin trioxane pharmacophore is critical for compound activity (9, 10), and its chemical disruption (“deoxyartemisinin”) abolishes the anti-HCMV activity (2, 11). Until now, an artemisinin-resistant HCMV has not been selected, suggesting that virus inhibition primarily involves host-directed functions critical for virus replication (12, 13). The mechanisms of HCMV inhibition by artemisinins are different from the DNA polymerase inhibitor, ganciclovir (GCV), as they inhibit GCV-resistant HCMV. The combination of artemisinins and GCV is synergistic against HCMV (7, 14). The in vitro anti-HCMV activity of artesunate correlated with cell cycle stage (12), efficacious in contact-inhibited human foreskin fibroblasts (HFFs) but reduced in subconfluent HFFs. In contact-inhibited cells, HCMV induced cell cycle progression to G1/S at 24 h postinfection (hpi), but artesunate reverted it to early G0/G1, and decreased virus-induced expression of cyclin-dependent kinases (CDK1, -2, and -4).

The cellular/microbial targets of artemisinins have been of major interest to several disciplines, including infectious diseases and cancer. Studies have highlighted the complexity and promiscuity of these drugs toward multiple proteins in Plasmodium falciparum, but specific targets underlying the anti-infective or anti-cancer properties remain inconclusive (15, 16). To better define the anti-HCMV activities of artemisinins and identify cellular functions targeted by these drugs, we synthesized a biotin-labeled trioxane semisynthetically derived from artemisinin at the C10 position. Immunoprecipitation (IP)-MS followed by molecular, biological, and biochemical studies confirmed the type III-intermediate filament protein vimentin as an artemisinin target, resulting in post-translational modification and stabilization of vimentin. Because artemisinins inhibit CMV in vitro and in vivo, we determined the requirement of vimentin for virus replication. Reportedly, vimentin enables HCMV trafficking into the nucleus early after infection (17). However, its functions during later stages of infection have not been studied. Our data show distinct roles of vimentin at different stages of HCMV replication. In the early stage of infection, vimentin level is stable, likely providing support for virus transport into the nucleus, but subsequently,
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HCMV strategy is to destabilize and degrade vimentin. This latter process requires several mechanisms, including vimentin phosphorylation and induction of calpain activity. Binding of artemesunate to vimentin counteracts and reverses virus-induced vimentin degradation, primarily through decreasing its phosphorylation and inducing calpain activity, effects culminating in virus inhibition.

**Results**

**Identification of vimentin as artemisinin target**

Trioxane C10 primary alcohol (1), derived from dihydroartemisinin (DHA), the active metabolite of all monomeric artemisinins, was coupled with carboxylic acid (2) to produce biotin-labeled trioxane (552 kDa) (Fig. 1A). The biotin-labeled product will be referred to as 552. Lysates from noninfected HFFs were treated overnight at 4°C with DMSO, 552 (20 μM), or 552 with DHA as a competitor (200 μM), which was added 1 h prior to 552. 552-protein complexes were captured with streptavidin agarose beads followed by protein separation on SDS-PAGE and silver staining (Fig. 1B). Specific bands enriched in the 552 lane were cut and analyzed by MS. Vimentin was identified as one of the main 552-binding proteins (Table 1). To confirm the MS findings, IP with streptavidin agarose beads was performed in noninfected HFF cell lysates treated with DMSO, 552 (20 μM), or 552 with DHA as a competitor (200 μM). In the drug competition condition, DHA was added 1 h before 552. Lysates were incubated overnight, followed by Western blotting with anti-vimentin antibody. Vimentin was detected in the 552-treated lysates, but not in the DMSO or DHA (competitor)-treated samples (Fig. 1C). Next, equal quantities of purified His-vimentin protein (100 ng) were incubated for 1 h with increasing concentrations of DHA, followed by incubation with 552 for 1 h. Proteins were blotted and probed with streptavidin-HRP. Competition with increasing concentrations of DHA resulted in reduced biotinylated adducts (Fig. 1D), whereas vimentin level was similar in all conditions. A surface plasmon resonance (SPR) assay was performed after immobilizing purified His-vimentin on the CM5 sensor surface (Biacore T200, GE Healthcare). Artesunate (monomeric artemisinin) and an inactive metabolite, deoxyartemisinin, that lacks anti-HCMV activity were tested at concentrations ranging from 0.93 to 33.3 μM along with at least two zero concentrations. Artesunate showed fast association (kₐ) and dissociation (k₈) rates at all tested concentrations based on each sensogram (Fig. 1F). The binding affinity (Kₐ) of artemesunate binding to vimentin was calculated to be 12.3 ± 1.7 μM by steady-state affinity fitting with data at equilibrium (Fig. 1F). Deoxyartemisinin did not show any interaction with vimentin (Fig. 1, G and H), indicating that the endoperoxide bridge in the artemisinin pharmacophore is required for binding to vimentin.

**Vimentin modulation during HCMV infection**

To begin understanding whether binding of artemesinins to vimentin results in HCMV inhibition, we tested the effects of infection with two genotypically distinct strains of HCMV (TB40 and Towne) on vimentin expression and modulation. Vimentin level was gradually and reproducibly reduced in infected HFFs. At 4 hpi, vimentin level was stable, but starting at 24 hpi with HCMV-TB40 (Fig. 2A), its level decreased. Vimentin is a highly phosphorylated protein, and its phosphorylation strongly correlates with its disassembly (18). We selected two sites to measure vimentin phosphorylation during infection, Ser-55 and Ser-83 (19). Ser-55 is phosphorylated by CDK1, which also recruits Polo-like kinase (PLK1) to phosphorylated vimentin Ser-55, resulting in PLK1 activation and further vimentin phosphorylation at Ser-83 (20). The decrease in vimentin level during HCMV infection correlated with increased phosphorylation at the respective time points. Ser-55 phosphorylation was increased from 24 hpi onward, and Ser-83 was phosphorylated at 48 and 72 hpi (Fig. 2A).

**Artesunate treatment during infection stabilizes vimentin and reduces its phosphorylation**

Artesunate treatment resulted in reduced expression of HCMV Towne-encoded IE1/2 and pp65 as well as vimentin Ser-83 phosphorylation and recovered vimentin level (Fig. 2B). The changes in vimentin level and phosphorylation were similar to those observed with HCMV-TB40 (Fig. 2A), indicating that the two viral strains did not differ in their effects on vimentin. Deoxyartemisinin did not change vimentin level or Ser-83 phosphorylation and did not inhibit pp65 expression (Fig. 2C). The disappearance of the Ser-83 band at 50 kDa and the Ser-55 band at 57 kDa by phosphatase assay confirmed that these were phosphorylated proteins (Fig. 2D). In addition, MS analysis of the 50 kDa band identified vimentin.

Artesunate-mediated changes in vimentin were HCMV-specific, because in HSV1-infected HFFs artemesunate did not modify vimentin level and HSV1-encoded ICP8 was not reduced (Fig. 2E). We investigated the effects of artemesunate on vimentin level at different times during infection. When added from the time of infection up until 72 hpi, artemesunate treatment resulted in remarkable vimentin stabilization (Fig. 2F). When added from 24 to 42 hpi, or from 42 to 72 hpi, artemesunate had a minor effect on vimentin level. Vimentin stabilization required artemesunate to be present in the cell before the onset of viral DNA replication up until 72 hpi.

**Vimentin disassembly during infection**

Vimentin is a cytoskeleton protein, and its filaments are seen in attached and flattened cells (21, 22). We tested the localization of vimentin during HCMV infection and artemesunate treatment. An indirect immunofluorescence (IFA) was performed at 24 and 72 hpi with HCMV TB40 (Fig. 3 and Fig. S1), revealing an altered staining pattern and reduced vimentin signal during infection and recovery of vimentin signal with artemesunate. The inactive artemisinin metabolite could not rescue vimentin signal at 24 or 72 hpi. Altogether, the data indicate that vimentin is disassembled during infection and artemesunate maintains its assembly condition.

**Artesunate stabilizes vimentin**

To confirm the role of artemesunate in vimentin stabilization, we performed a cellular thermal shift assay (23, 24). HFFs were treated with artemesunate (30 μM) or DMSO, washed with PBS,
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A

B

C

D

E

F

G

H

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Table 1
MS analysis of proteins bound to 552 in HFFs

| Protein                                    | Molecular mass | No. of peptides | Accession no. | Coverage |
|--------------------------------------------|----------------|-----------------|---------------|----------|
| Cytoskeleton-associated protein 4          | kDa            | 11              | gi 19920317   | 21.0     |
| Vimentin                                   | 66             | 31              | gi 62414289   | 62.0     |
| Annexin A2 isoform 2                       | 39             | 2               | gi 209862831  | 5.9      |
| Annexin A1                                 | 39             | 6               | gi 4502101    | 23.0     |
| Actin, cytoplasmic 1                       | 42             | 6               | gi 4501805    | 19.0     |
| Cathepsin D preproprotein                  | 45             | 4               | gi 4503143    | 4.1      |
| Serpin B3                                  | 45             | 3               | gi 5902072    | 11.0     |
| Elongation factor 1-α1                     | 50             | 3               | gi 4503471    | 6.5      |
| Pyruvate kinase isozymes M1/M2             | 66             | 2               | gi 332164775  | 4.8      |
| Heat shock-70-kDa protein 1–like           | 70             | 3               | gi 12056496   | 6.2      |
| Junction plakoglobin                       | 82             | 2               | gi 12056486   | 2.8      |

Note: The table lists proteins identified by MS analysis of proteins bound to 552 in HFFs. The values in the Coverage column represent the percentage of the protein sequence covered by identified peptides.

Figure 1. Synthesis of 552 and identification of vimentin as a cellular target of artemisinin. A, synthesis of biotin-labeled artemisinin derivative (denoted 552) by EDC-mediated coupling reaction. The trioxane functionality is a six-membered ring that includes the peroxide bridge and other oxygen. B, cell lysates from noninfected HFFs were treated overnight at 4 °C with DMSO, 552 (20 μM), or 552 with DHA (200 μM) added 1 h before 552, and lysates were incubated overnight. Targeted protein was immunoprecipitated with streptavidin agarose beads, and samples were immuno blotted for vimentin. Vimentin level prior to pulldown (input, bottom) was similar in all conditions. C, purified His-vimentin was incubated with the indicated concentrations of DHA for 1 h followed by incubation with 552 (50 μM) for 1 h at 37 °C. DMSO bound to purified vimentin was detected after SDS-PAGE with streptavidin-HRP. DMSO samples were either untreated (lane 1) or treated with 552 (lane 2). E and F, sensograms of AS binding to immobilized vimentin and the steady-state affinity fitting curves at a series of increasing concentrations of AS. G and H, sensorgrams of deoxyartemisinin binding to vimentin at a series of increasing concentrations. The binding affinity (KD) of AS at equilibrium was determined to be 12.3 ± 1.7 μM. Data were fit to a single rectangular hyperbolic curve to calculate the KD value (see “Experimental procedures” for details). The S.D. (error bars) was calculated from three independent measurements. The chemical structure of AS and deoxyartemisinin that lacks the endoperoxide bridge is provided. The endoperoxide bridge is circled in red. Vim, vimentin; IB, immunoblotting.

Figure 2. Vimentin level is reduced during HCMV infection but maintained with artesunate. A, HFFs were infected with HCMV TB40. Expression of vimentin and its phosphorylation at serine residues 55 and 83 were measured by Western blotting at 4, 24, 48, and 72 hpi. B, HFFs were infected with HCMV Towne and treated with AS (30 μM). Expression of vimentin and its phosphorylation at Ser-83 were measured by Western blotting at 4, 48, and 72 hpi. C, left, HFFs were infected with HCMV Towne and treated with AS, deoxyartemisinin (Deoxy; 30 μM), or GCV (5 μM). The expression of HCMV-pp65, vimentin (Vim) and phosphorylation at Ser-55 and Ser-83 were measured at 72 hpi. Right, phosphatase assay showed disappearance of the Ser-83 band at 50 kDa and the Ser-55 band at 150 kDa. D, a phosphatase assay was performed on samples from C, and the relevant vimentin and phosphovimentin proteins were detected by Western blotting. E, HFFs were infected with HSV1 (MOI = 0.2) and treated with AS (30 μM) or GCV (5 μM). The expression level of vimentin and viral ICP8 was measured by Western blotting in cell lysates at 24 hpi. F, HFFs were infected with HCMV Towne, and AS was added/removed at the indicated times. Levels of pp65 and vimentin were measured by Western blotting at 96 hpi.

Figure 3. Vimentin level is not reduced during HCMV infection when treated with artesunate. A, HFFs were infected with HCMV Towne and treated with AS, deoxyartemisinin (Deoxy; 30 μM), or GCV (5 μM). The expression level of vimentin and viral ICP8 was measured by Western blotting in cell lysates at 24 hpi. B, HFFs were infected with HCMV Towne and treated with AS (30 μM). In the drug competition condition, DHA was added 1 h before 552, and lysates were incubated overnight. Targeted protein was immunoprecipitated with streptavidin agarose beads, and samples were immuno blotted for vimentin. Vimentin level prior to pulldown (input, bottom) was similar in all conditions. DMSO bound to purified vimentin was detected after SDS-PAGE with streptavidin-HRP. DMSO samples were either untreated (lane 1) or treated with 552 (lane 2). E and F, sensograms of AS binding to immobilized vimentin and the steady-state affinity fitting curves at a series of increasing concentrations of AS. G and H, sensorgrams of deoxyartemisinin binding to vimentin at a series of increasing concentrations. The binding affinity (KD) of AS at equilibrium was determined to be 12.3 ± 1.7 μM. Data were fit to a single rectangular hyperbolic curve to calculate the KD value (see “Experimental procedures” for details). The S.D. (error bars) was calculated from three independent measurements. The chemical structure of AS and deoxyartemisinin that lacks the endoperoxide bridge is provided. The endoperoxide bridge is circled in red. Vim, vimentin; IB, immunoblotting.

trypsinized and resuspended in PBS containing protease inhibitors, and incubated in a thermal cycler at the indicated temperatures. Vimentin level was measured by Western blotting. In the DMSO-treated samples, vimentin level decreased with increasing temperatures, whereas artesunate stabilized vimentin at all tested temperatures (Fig. 4A). Comparison of the cellular thermal shift by artesunate and deoxyartemisinin (30 μM) at 56 and 60 °C showed that only artesunate stabilized vimentin (Fig. 4B). Neither artesunate nor deoxyartemisinin had an effect on p53 stabilization (Fig. 4B).
Vimentin undergoes homodimerization, followed by anti-parallel association between homodimers to form soluble tetramers. Further assembly of vimentin filaments involves lateral association between eight tetramers to form a unit length filament, which is followed by longitudinal annealing of these into short filaments and radial compaction of the annealed filaments to produce mature filaments. These structures are dynamic and reorganize during mitosis or cell stress (25). Using glutaraldehyde cross-linking, we found that high-molecular weight forms of vimentin, representing soluble tetramers and possibly unit length filaments, were increased with artesunate treatment in noninfected and HCMV-infected HFFs (Fig. 4C). In sum, artesunate treatment results in vimentin stabilization.

**Proteasome-independent vimentin degradation during HCMV infection**

We investigated whether vimentin was degraded during HCMV infection through the proteasome. Using the proteasome inhibitor MG132, vimentin level did not recover at 72 hpi (Fig. 4D, top). Another experiment performed at 24 and 48 hpi revealed again that the vimentin level did not recover with MG132 treatment, whereas p53 level was recovered as expected (Fig. 4D, bottom), indicating that MG132 activity was intact. The data suggest proteasome-independent degradation of vimentin in HCMV-infected cells.

**Artesunate resists HCMV-mediated vimentin degradation via calpain**

Vimentin is subject to proteolytic cleavage by calpains (Ca$^{2+}$-activated neutral proteases) (26, 27), and HCMV reportedly activates calpains 1 and 2, which regulate p21cip (28). We measured calpain activity using a calpain-induced substrate cleavage assay. At 72 hpi, HCMV induced calpain activity (Fig. 4E). In the presence of the calpain inhibitor E64D (100 μM, 6 h), protease activity was reduced. HCMV-infected artesunate-treated cells showed similar relative fluorescence signal as infected-only cells, indicating that artesunate does not inhibit calpain. Next, we performed an in vitro calpain digestion assay using purified vimentin protein to determine whether artesunate-bound vimentin was rendered calpain-resistant. Recombinant human vimentin (1 μM) was digested with 2.5 units of calpain 1 for 10 min, and resultant vimentin fragments were visualized after Coomassie Blue staining. Without calpain, vimentin was uncleaved (Fig. 4F, lane 1). Calpain 1 was in a high-glycerol buffer, which altered the migration pattern of vimentin (lane 2). When incubated with calpain 1 under appropriate reaction conditions, vimentin cleavage generated a series of bands ranging from 15 to 37 kDa (lane 3). The calpain inhibitor E64D prevented vimentin cleavage (lane 6). Importantly, preincubation of vimentin with artesunate protected it from cleavage by calpain 1 (Lane 4 vs Lane 3). GCV did not confer protection against vimentin degradation (lane 5). Altogether, we confirm that vimentin cleavage results, at least in part, from...
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Figure 4. Artemisane targets vimentin for stabilization. HCMV degrades Vim by inducing calpain. Artemisane stabilizes vimentin against calpain-induced cleavage. A thermal shift assay was performed. HFFs were treated with AS (30 μM) or DMSO (A) or with AS (30 μM), Deoxy (30 μM), or DMSO (B) for 24 h, washed, and harvested in PBS containing protease inhibitors. Cell suspension was distributed into PCR tubes and incubated at the indicated temperatures for 3 min in a gradient thermal cycler. Heat-treated cells were lysed using a freeze-thaw cycle twice, followed by brief vortex, and centrifuged at 13,000 rpm for 10 min. Supernatants were transferred into fresh tubes and mixed with 2× SDS sample buffer, and Western blotting analysis was performed using anti-vimentin and anti-p53 antibody. β-Actin was used as a loading control. The experiments were performed three times, and data are from a representative experiment. Quantification of protein bands was performed by ImageJ and is shown below the blots. C, cell lysates from noninfected, infected, and infected AS-treated samples were cross-linked with glutaraldehyde 0.005%, and vimentin forms were detected by Western blotting. D, top, HCMV-infected or noninfected HFFs were treated with AS in the presence or absence of proteasomal inhibitor, MG132 (10 μM), added 8 h before harvesting. Expression of vimentin was measured by Western blotting at 72 hpi. Bottom, HFFs were infected with HCMV (MOI = 1), and MG132 was added 8 h before harvesting the cell lysates. Expression level of vimentin and p53 was measured at 24 and 48 hpi. E, HCMV-infected HFFs nontreated or AS-treated were used for a calpain activity assay using the fluorogenic calpain substrate Boc-Leu-Met-CMAC. At 72 hpi, cells were loaded with Boc-Leu-Met-CMAC (50 μM) for 30 min before the assay. E64D (labeled E) was used at 100 μM for 6 h prior to harvest in some of the conditions. Cells were harvested, and the relative fluorescence from the protease-cleaved product (AMC) of the fluorogenic substate was measured and summarized. Data show mean and S.D. (error bars) of triplicate values from one representative experiment, p < 0.001. F, purified recombinant vimentin (1 μg) and calpain were used in an in vitro vimentin cleavage assay, and shown is a Coomassie stain of vimentin cleavage. Lane 1, vimentin migration with activating reaction buffer (without calpain) in a 12% gel. Lane 2, in the absence of buffer, the enzyme does not digest vimentin; the glycerol in the calpain enzyme changes the migration pattern of vimentin. Lane 3, digestion pattern of vimentin in the presence of activated calpain in the buffer. Lane 4, pattern of vimentin with AS treatment. Artemisane is dissolved in DMSO, and the DMSO itself (lane 7) did not alter the migration pattern of vimentin. GCV (lane 5) was used as a negative control, and E64D (lane 6), a direct inhibitor of calpain, was used as a positive control.

HCMV-induced calpain activity. Artemisane binding to vimentin may prevent calpains from accessing several sites and digesting vimentin during infection, providing protection of vimentin structure/activity.

Vimentin plays distinct roles depending on the stage of HCMV replication

We reported that artemisins inhibit HCMV after virus entry, and their inhibitory effects persist during most stages of HCMV replication (6). We therefore investigated the role of vimentin during HCMV replication. The onset of infection and HCMV migration to the nucleus were reportedly delayed in the absence of an intact vimentin network (17), but later effects of vimentin on virus replication were not studied. Using lentivirus infection, we knocked down vimentin in HFFs to a degree that reduced virus progeny from vimentin knockdown cells starting from 48 hpi (Fig. 5E). Compared with control cells, inhibition of HCMV with artemisane was less effective in vimentin-deficient cells (5.2-fold versus 1.7-fold, respectively). Thus, vimentin level directly correlates with the anti-HCMV activity of artemisane.

Given the reported timing of HCMV inhibition by artemisins (post-entry), reduced levels of vimentin during the progression of infection, and the correlation between artemisins'
activity and vimentin stabilization, we attempted to dissociate the early role of vimentin from its later effects during HCMV replication. Vimentin was overexpressed in U373 glioma cells, followed by infection with the pp28-luciferase Towne. Luciferase activity measured at 96 hpi was significantly reduced in vimentin-overexpressing cells compared with controls (Fig. 5F). Vimentin overexpression was confirmed by Western blotting (Fig. 5G, top). Viral IE1/2 and pp65 expression was reduced at 72 hpi in vimentin-overexpressing cells (Fig. 5G, bottom), despite similar virus entry among all conditions (Fig. 5F). The data corroborate an earlier report that intermediate filament (IF) bundling in cells from a patient with giant axonal neuropathy results in HCMV suppression (17). Taken together, whereas the onset of HCMV replication requires an intact vimentin, its completion is hindered by it, indicating a dynamic role of vimentin depending on the stage of HCMV replication. These results further our understanding of the vimentin-arte- misinin interaction during HCMV replication. An intact vimentin network facilitates artesunate binding and HCMV suppression following virus entry. Indeed, at 4 hpi, both HCMV and artesunate maintained the level of vimentin, but starting from 24 hpi when virus strategy was to degrade vimentin, artesunate-bound vimentin counteracted these activities and stabilized it, mimicking an overexpression status.

Withaferin A, a vimentin-binding agent, inhibits HCMV replication and antagonizes the anti-HCMV activities of artesunate

Additional support for vimentin being a target of artesunate was obtained using withaferin A (WFA), a vimentin-binding compound. The anti-HCMV activity of WFA was first tested. The effective concentration resulting in 50% inhibition of pp28-luciferase activity and plaque formation (EC50) was 0.1 ±
0.01 μM, and the 50% cytotoxicity in noninfected HFFs (CC\textsubscript{50}) was 2.0 ± 0.0 μM (Fig. S2). The combination of WFA and arte- sumate (AS) was tested against HCMV Towne using a plaque reduction assay, and antagonism was found (Fig. 6 A). The com- bination of artesunate and GCV was synergistic, as reported (14) (Fig. 6 B). Western blots revealed that the combination of artesunate and WFA was less effective in reducing the expres- sion of viral IE1/2 compared with each drug alone (Fig. 6 C).

The cell cycle stage reflects vimentin dynamics during HCMV replication

Cell cycle regulation is critical for productive HCMV replication. Moving the cell cycle toward G\textsubscript{1}/S is a hallmark of HCMV infection (29, 30). Artemisinins were reported to reverse HCMV-mediated cell cycle changes and reduce the levels of CDKs. In addition, in confluent HFFs (at the time of infection), artesunate inhibited HCMV, but in subconfluent cells, its anti- HCMV activity was lost (12). Cell synchronization at G\textsubscript{0} with serum starvation, late G\textsubscript{1} with mimosine, or early G\textsubscript{1} with lova- statin resulted in effective HCMV inhibition with artesunate (12). Vimentin structure is highly dynamic and reorganizes in certain physiological situations, such as during cell cycle (25).

We investigated the correlation between cell cycle and vimen- tin changes in infected HFFs. HCMV inhibition by artesunate was associated with reduced Ser-83 phosphorylation and vimentin stabilization in contact-inhibited cells. However, in subconfluent cells, artesunate did not reduce HCMV pp65 expression, and although infection reduced vimentin expres- sion, artesunate could not rescue it or reduce Ser-83 phospho- rylation (Fig. 7 A). A bivariate flow cytometry was performed to further support the association between vimentin and cell cycle stage during infection (Table 2 and Fig. 7 B). The transition of infected cells into G\textsubscript{1}/S phase was accompanied by an overall reduction in vimentin signal (mean vimentin intensity 880 versus 485). Artesunate reverted the cells back to G\textsubscript{0}/G\textsubscript{1} along with an increased vimentin signal at all cell cycle stages. Next, the expression level of CDK1, -2, and -4 and PLK1 was measured in HCMV-infected cells. Infection induced CDK1, -2, and -4, and this effect was reversed by artesunate at 24 and 72 hpi (Fig. 7 C). PLK1, a kinase responsible for phosphorylating the Ser-55 and Ser-83 residues, was induced by HCMV and reduced by artesu- nate. GCV did not modify the level of CDKs or PLK1 during infection. Artesunate-mediated changes in CDKs and PLK1 were observed only in infected cells, and not in noninfected cells (Fig. 7 D), suggesting that the cell cycle in noninfected cells overcomes the effect of artesunate or that viral proteins
participate in vimentin reorganization. Taken together, the anti-HCMV activity of artesunate is mediated through vimentin, and the cell cycle drives changes in vimentin. HCMV harnesses cell cycle and vimentin; when vimentin is already degraded, artesunate loses its effectiveness. However, at the best cell cycle stage (early G1), artesunate will bind to vimentin and compete with HCMV to prevent its degradation, winning the battle on HCMV replication.

Discussion

Drug repurposing may expand the antiviral armamentarium. Repurposed agents may use host-directed antiviral mechanisms (31). Therefore, the identification of molecular targets critical for HCMV replication may accelerate drug development strategies. Here we identified vimentin as a major cellular

Table 2

Bivariate cell cycle analysis of vimentin distribution during the stages of the cell cycle in HCMV-infected HFFs and artesunate treatment

HFFs were infected with HCMV Towne and treated with artesunate (30 μM). At 72 hpi, cells were harvested for bivariate cell cycle analysis using propidium iodide and Alexa Fluor 488–conjugated mouse anti-human vimentin antibody. Infection increased the percentage of cells at G1/S from 5.8 to 33.76%. In noninfected cells, vimentin intensity increased from G0/G1 to G2/M and was overall reduced in HCMV-infected cells. Artesunate treatment recovered vimentin intensity during infection.

| Treatment          | G0/G1 | G1/S | G2/M |
|--------------------|-------|------|------|
| Noninfected        | 77 (710) | 6 (880) | 15 (1018) |
| HCMV               | 52 (484) | 34 (485) | 13 (641) |
| HCMV/AS            | 61 (667) | 23 (707) | 13 (888) |
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target of artemisinin monomers. We defined the dynamic changes of vimentin during HCMV replication and the role of artemisinins in HCMV inhibition through vimentin stabilization and modulation of its phosphorylation. A critical advantage of our assays is the availability of an artemisinin that lacks the endoperoxide bridge, providing a direct negative control for artemisinins’ bioactivity.

Vimentin was identified by MS using a biotin-labeled trioxane semisynthetically derived from artemisinin, and target confirmation studies included competition assays using cell lysates and purified His-vimentin protein, SPR, and stability assays. WFA, an agent reported to bind to vimentin (32), inhibited HCMV replication, but when used in combination with artesunate, an antagonism was found.

Vimentin is one of the most widely expressed and highly conserved proteins of the type III IF family. It is a marker of epithelial-mesenchymal transition, a process in which epithelial cells acquire a mesenchymal phenotype that causes them to alter their shape and exhibit increased motility (33, 34).

An intact vimentin network was reportedly required for the onset of HCMV replication, facilitating the trafficking of HCMV strains AD169 and TB40 into the nucleus (17). Disruption of vimentin with acrylamide, IF bundling in cells from a patient with giant axonal neuropathy, and absence of vimentin in fibroblasts from vimentin−/− mice reduced virus entry (17). During these initial steps of infection, vimentin was not disassembled, suggesting that maintenance of its network is necessary for the establishment of infection. Our studies corroborate this report and further characterize changes in vimentin throughout the early and late stages of HCMV replication. At 4 hpi, vimentin expression and phosphorylation were unmodified. However, as infection proceeded, vimentin structure was altered, its phosphorylation (Ser-55 and Ser-83) steadily increased, and its level was eventually reduced. Phosphorylation is a major post-translation modification that triggers vimentin disassembly (18). Artesunate binding to vimentin resulted in its stabilization, likely through interference with its phosphorylation. Multiple phosphorylation sites on vimentin have been identified (19, 20, 35–41). Among the kinases that phosphorylate vimentin are p21-activated kinase, Aurora B kinase, and CAMK2 (38). Vimentin interaction with phospho-ERK protects it from dephosphorylation (42). Phosphorylated vimentin interacts with 14-3-3 proteins, preventing the assembly of Raf-14-3-3 and similar complexes (43). The recognition of vimentin as a substrate for multiple kinases led to the hypothesis that it acts as a scaffolding protein involved in signal transduction (18). Significant in vivo phosphorylation sites on vimentin have been identified, favoring a depolymerized state, consisting of tetrameric subunits (18).

Additional mechanisms have been suggested to degrade and disassemble vimentin. Proteasomal inhibition did not result in recovery of vimentin level, suggesting that other mechanisms may contribute to its degradation (44). We confirmed that HCMV induces calpain. Based on our in vitro assay, artemesunate binding to vimentin likely blocks access from calpain.

Vimentin post-translational modification and its state of depolymerization may be virus-specific. Increased phosphorylation of vimentin by vaccinia virus resulted in its reorganization (45). Vimentin interacted with heterogeneous nuclear ribonucleoproteins and dengue virus NS1 and played a crucial role in the release of dengue virus (46). Epstein–Barr virus–encoded LMP1 colocalizes with vimentin in B cells, and chemical disruption of vimentin induces relocation of LMP1 to sites of vimentin redistribution (47). In addition, vimentin activates the PI3K-ERK-MAPK pathways and cellular transformation by LMP1 (48). Our results show that vimentin plays dual, stage-specific roles during HCMV infection. At early stages, its level and phosphorylation are maintained, a time when HCMV traffics into the nucleus and prepares it for viral DNA replication. However, at later time points, HCMV aims to disassemble vimentin. In contrast to changes induced by HCMV, vimentin organization during HSV1 infection is maintained (49, 50), and we show that vimentin expression is stable even at 24 hpi in HSV-1–infected fibroblasts (Fig. 2E), suggesting that it may play a distinct role during HSV-1 infection, mirrored by differences in cell cycle modulation between HSV-1 and HCMV. Whereas HCMV moves the cell cycle toward G1/S (29, 30), HSV-1 blocks it in early to mid-G1 (51), does not induce cyclin D or E, and reduces CDK2 activity (52). These differences in the modulation of CDKs and cyclins are reflected by changes in vimentin phosphorylation. Entry into the S phase would be accompanied by induction of calpain activity and reduced vimentin levels, whereas the exit from S phase is expected to restore vimentin levels (53–55). A correlation between vimentin levels and the cell cycle stage has been shown for mouse plasmacytoma cells (56). Our data reveal cooperation between vimentin and cell cycle stage (Fig. 8, model). The transition of HCMV-infected cells into the G1/S phase was marked by reduction in vimentin signal (Table 2). Artesunate binding to vimentin increased vimentin signal and reversed the cell cycle into G0/G1. Thus, vimentin levels are closely linked with cell cycle, likely in a bidirectional way, and by targeting vimentin, artesunate can not only stabilize it but also control the cell cycle in a way that is deleterious for HCMV. Whereas HCMV exploits vimentin strategically at the early and late stages of infection, the reversal of cell cycle cannot be tolerated by the virus. In fact, in subconfluent cells, artemesunate was inefficient in inhibiting HCMV, and although vimentin was reduced by infection, artemesunate could not restore its level or reduce its phosphorylation. In confluent cells, artemesunate reduced the levels of CDKs, PLK1, and vimentin phosphorylation, and HCMV was inhibited.

As the cellular target of artemisinins is now known, future studies should specify the binding sites of artemisinins, the required moieties for binding, and the role of vimentin as a cofactor for HCMV proteins. Our findings may also shed light on the anti-cancer activities of artemisinins as well as other pathogens. A more direct approach for drug development can now be taken.

Experimental procedures
Compounds and reagents

The artemisinin monomers (artesunate and DHA), GCV, and WFA were from Sigma. Deoxyartemisinin was provided by Dr. Jonathan Vennerstrom (University of Nebraska Medical
Center). Stock solutions of all compounds (10 mM) were stored at −80 °C. Recombinant human vimentin (catalog no. 10028-H08B-50) was purchased from Sino Biological Inc. (Beijing, China).

**Cell lines and viruses**

HFFs (passage 12–16) (ATCC, CRL-2088™), U373 glioma cells (from Dr. Gary Hayward, Johns Hopkins University), and mouse embryonic fibroblasts (MEFs) (passage 9–14) (ATCC CRL-1658) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) in a 5% CO₂ incubator at 37 °C. HCMV strains Towne (VR-977) and UL32-EGFP-TB40 (57) (VR-1578) were from ATCC. Unless otherwise specified, infections were performed at an MOI of 1 pfu/cell.

**Synthesis of artemisinin-biotin conjugate (552 kDa)**

A novel biotin-labeled trioxane, derived from DHA, was synthesized. To a round-bottom flask, biotin (2, 10 mg, 0.043 mmol, 1.0 eq), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; 8.1 mg, 0.052 mmol, 1.2 eq), 1-hydroxybenzotriazole (HOBt; 7.1 mg, 0.052 mmol, 1.2 eq), and dimethylformamide (DMF; 2 ml) were added. Artemisinin-derived monomer alcohol 1 (21.3 mg, 0.065 mmol, 1.5 eq) was added, and the reaction mixture was stirred at RT for 2 days. The reaction mixture was quenched with water and diluted with EtOAc. The organic layer was washed progressively with water, 3N LiCl, and brine and then dried over MgSO₄ and filtered. The solvent was removed under reduced pressure, and the residue was purified directly on silica. Gradient elution (0–8% MeOH (containing 10% NH₄OH) in CH₂Cl₂) afforded the desired compound as a colorless, amorphous solid: 10 mg, 44% yield; ¹H NMR (400 MHz, CDCl₃) δ 5.56 (brs, 1 H), 5.29 (s, 1 H), 5.25 (brs, 1 H), 4.50 (dd, J = 7.29, 5.21 Hz, 1 H), 4.31 (dd, J = 7.23, 4.89 Hz, 1 H), 4.22–4.15 (m, 1 H), 4.15–4.07 (m, 2 H), 3.21–3.11 (m, 1 H), 2.91 (dd, J = 12.79, 4.96 Hz, 1 H), 2.73 (d, J = 12.82 Hz, 1 H), 2.70–2.58 (m, J = 7.17, 6.92 Hz, 1 H), 2.37–2.29 (m, 3 H), 2.07–2.02 (m, 1 H), 2.02–1.98 (m, 1 H), 1.98–1.87 (m, 2 H), 1.86–1.75 (m, 3 H), 1.74–1.57 (m, 7 H), 1.52–1.42 (m, 4 H), 1.42–1.37 (m, 3 H), 1.35–1.20 (m, 2 H), 1.02–0.91 (m, 3 H), 0.86 (d, J = 7.52 Hz, 3 H); ¹³C NMR (100 MHz, CDCl₃) δ 173.7, 163.4, 103.1, 89.2.

**Figure 8. Model depicting vimentin modification during HCMV replication (left) and the effect of artemusate binding to vimentin on HCMV replication (right).** Following HCMV entry, vimentin maintains its stability to allow efficient virus trafficking to the nucleus. As infection proceeds, HCMV induces calpain as well as vimentin phosphorylation, leading to vimentin cleavage. Concurrently, HCMV induces CDKs and cell cycle progression to G₁/S. Overall, these linked events provide an environment conducive to lytic HCMV replication. Artemusate binding to vimentin early after virus entry prevents calpain access to vimentin as well as its phosphorylation, resulting in vimentin stability and maintenance of the cell cycle at G₁.

*Artemisinins target vimentin for HCMV inhibition*
Identification of 552-binding proteins by LC–MS/MS

Cell lysates from noninfected HFFs were incubated overnight at 4 °C with DMSO, 552 (20 μM) or 552 with DHA (200 μM) added 1 h before 552. Streptavidin agarose beads were used to capture 552-protein complexes, and bound proteins were eluted using 2× Laemmli sample buffer. Proteins were run on Tris-glycine gel and visualized using silver stain. Cut destained bands enriched in the 552 lane were submitted for MS. Silver staining was performed using the Silver Quest™ staining kit (Invitrogen, catalog no. LC6070), according to the manufacturer’s protocol.

Proteins in gel bands were proteolyzed with trypsin to cleave at lysines and arginines (Promega), as described previously (58). Protein identification by LC–MS/MS analysis of peptides was performed using a Velos Orbitrap MS (Thermo Fisher Scientific) interfaced with a two-dimensional nano-LC system (Eksigent). Peptides were fractionated by reverse-phase HPLC on a 75 μm × 100-mm C18 column with a 0–10% acetonitrile, 0.1% formic acid gradient over 60 min at 300 nl/min. Survey scans (full MS) were acquired from 350 to 1800 m/z with data-dependent monitoring of up to eight peptide masses (precursor ions), each individually isolated in a 1.9-Da window and fragmented using HCD activation collision energy 35 and 25-s dynamic exclusion. Precursor and fragment ions were analyzed at resolutions of 30,000 and 15,000, respectively, with AGC target values at 1e6 with 100-ms maximum injection time and 5e4 with 300-ms maximum injection time, respectively. A lock mass of siloxane (371.1012 Da) was used for on-the-fly recalibration.

Database searching was conducted as follows. Tandem mass spectra were extracted by Proteome Discoverer (version 1.4; Thermo Fisher Scientific). All MS/MS raw spectra were analyzed using Mascot (version 1.4.0.288; Matrix Science, London, UK). Mascot was set up to search RefSeqComplete2012_Homo sapiens (13,039 entries), assuming the digestion enzyme trypsin as described previously (58). Protein identification by LC–MS/MS analysis of peptides was performed using a Velos Orbitrap MS (Thermo Fisher Scientific) interfaced with a two-dimensional nano-LC system (Eksigent). Peptides were fractionated by reverse-phase HPLC on a 75 μm × 100-mm C18 column with a 0–10% acetonitrile, 0.1% formic acid gradient over 60 min at 300 nl/min. Survey scans (full MS) were acquired from 350 to 1800 m/z with data-dependent monitoring of up to eight peptide masses (precursor ions), each individually isolated in a 1.9-Da window and fragmented using HCD activation collision energy 35 and 25-s dynamic exclusion. Precursor and fragment ions were analyzed at resolutions of 30,000 and 15,000, respectively, with AGC target values at 1e6 with 100-ms maximum injection time and 5e4 with 300-ms maximum injection time, respectively. A lock mass of siloxane (371.1012 Da) was used for on-the-fly recalibration.
4× SDS sample buffer. Thereafter, samples were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

**Measurement of intracellular calpain activity (28)**

HFFs were infected with HCMV and loaded with the fluorogenic calpain substrate Boc-Leu-Met-CMAC (50 μM) for 30 min. Cells were harvested, and the relative fluorescence from the protease-cleaved product (AMC) of the fluorogenic substrate was measured.

**Collection of vimentin subunits**

Cell extracts were centrifuged at 200,000 × g, and supernatants were collected. Released subunits were cross-linked with glutaraldehyde (0.005%).

**Transfection and generation of vimentin knockdown cells**

The following vimentin plasmids were used for transient transfection in U373 (cells that achieve both good transfection and HCMV replication): pCDNA4-vimentin and pCDNA4 control. Knockdown of vimentin in HFFs was performed using lentivirus transduction with VIM shRNA: RHS3979-201759426-TRCN0000029119 and pLKO.1 control designed by the RNAi Consortium (GE Healthcare). Individual shRNA constructs were packaged using lentivirus as reported previously (61).

**SDS-PAGE and immunoblot analysis**

Cell lysates containing an equivalent amount of proteins were mixed with an equal volume of sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 5% β-mercaptoethanol) and boiled at 100 °C for 10 min. Denatured proteins were resolved in Tris-glycine polyacrylamide gels (10–12%) and transferred to polyvinylidine difluoride membranes (Bio-Rad) by electroblotting. Membranes were incubated in blocking solution (5% (w/v) nonfat dry milk and 0.1% Tween 20 in PBS (PBST)) for 1 h, washed with PBST, and incubated with antibody at 4 °C overnight. Membranes were washed with PBST and incubated with horseradish peroxidase–conjugated secondary antibodies in PBST for 1 h at RT. Protein bands were visualized by chemiluminescence using SuperSignal West Dura and Pico reagents (Pierce). Antibodies for HCMV proteins were used at 1:2000 and included the following: mouse monoclonal anti-HCMV IE1 and IE2 (MAB810, Millipore, Billerica, MA, USA), mouse monoclonal anti-HCMV UL83 (pp65, VP-C422, Vector Laboratories Inc., Burlingame, CA, USA), and mouse anti-HCMV UL44, 10E8 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Mouse anti-vimentin (V9) and rabbit anti phosphovimentin (Ser-83) were from Santa Cruz Biotechnology. Anti-HSV ICP8 NB100-2770 was from Novus (Novus, Centennial, CO, USA). Mouse anti-phosphovimentin (Ser-55) was from Enzo Life Sciences (Farmingdale, NY, USA). Rabbit polyclonal antibody HSP90β was from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-p53, anti-β-actin, and β-tubulin were obtained from Santa Cruz Biotechnology. Quantification of Western blots was performed with ImageJ software (National Institutes of Health).

**Artemisinins target vimentin for HCMV inhibition**

A phosphatase assay was performed to confirm the phosphovimentin bands. After transfer, the polyvinylidene difluoride membrane was washed twice with deionized H2O, followed by washing with buffer (5 min). The membrane was placed in FAST AP thermosensitive alkaline phosphatase buffer, and 100 units of FAST AP (Thermo) were added for 30 min in a 37°C shaker, followed by two washes with washing buffer. The membrane was blocked with blocking buffer for 1 h, and Ser-55 antibody (1:2000) was added overnight.

**IFA staining and confocal microscopy**

HFFs were plated on chamber slides, followed by infection with HCMV TB40 and treatment with artesunate (30 μM), deoxyartemisinin (30 μM), and GCV (5 μM) for 24 or 72 h. Cells were fixed with 3.7% paraformaldehyde for 20 min at RT, permeabilized with ice-cold methanol for 10 min at −20°C, and blocked with 5% BSA in 0.5% Tween 20 for 20 min at RT. Cells were incubated with primary antibodies at 4°C overnight, washed, and incubated with fluorescently labeled secondary antibodies (2 h, 37°C). Fluorescence microscopy was performed using a confocal laser-scanning microscope (Nikon EZ C1). Images were captured at ×60 magnification and processed under identical conditions with constant parameters (including scan speed and excitation and emission wavelengths) using Nikon EZ C1 software. Quantification of cell fluorescence was performed using ImageJ software; fluorescence intensity represents corrected total cell fluorescence/cell. Data shown are mean ± S.D. (n = 15).

**Cytotoxicity assay**

The MTT assay was performed per manufacturer’s instructions (Sigma–Aldrich). Noninfected HFFs were treated with WFA for 72 h, and 20 μl/well of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, 5 mg/ml in PBS) was added to each well. After shaking at 150 rpm for 5 min, the plates were incubated at 37°C for 2–3 h. Conversion of yellow solution to dark blue formazan by mitochondrial dehydrogenases was quantified by measuring absorbance at 560 nm.

**Drug combination studies**

HFFs were seeded in well-plates and infected with HCMV Towne at 75 plaques/well. First, a dose-response curve was generated for each drug individually to determine its EC50. Then drugs were combined at twice their EC50; diluted in DMEM with 4% FBS, followed by serial dilution; and added together after infection. Plaques were counted 10 days after infection. The Bliss model was used to calculate the effect of each drug combination on virus replication. In this model, drug combination represents the product of two probabilistically independent events as described in the following equation,

\[
F_{U1+2} = F_{U1} \cdot F_{U2} = \frac{1}{1 + \left( \frac{D_1}{EC_{50(1)}} \right)^m} \cdot \frac{1}{1 + \left( \frac{D_2}{EC_{50(2)}} \right)^m}
\]

(Eq. 2)

where \(D\) is the drug concentration, \(m\) is the slope, and \(EC_{50}\) is the effective concentration resulting in 50% virus inhibition.
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The combined effect of two inhibitors ($F_{1D}$ fractional unaffected) is computed as the product of individual effects of the two inhibitors, $F_{1I1} \text{ and } F_{1I2}$. If the ratio of observed -fold inhibition divided by the expected -fold inhibition is $> 1$, the compounds are synergistic. If the ratio is $< 1$, the combination is considered antagonistic, and if it equals 1, the combination is additive.

MCMV infection of vimentin knockout mice

Infection with MCMV Smith strain (ATCC VR-1399) was carried out in 3–4-week-old vimentin knockout (129S-Vimtm1Cba/MesDmarkJ) and WT 129S1/SvImJ (Jackson Laboratories, Bar Harbor, ME, USA). The Animal Care and Use Committee of Johns Hopkins University approved the experimental procedures. After 2–3 days of adaptation to the housing environment, mice were infected intraperitoneally with $10^4$ pfu/mice (0.1 ml in 0.8% saline). Mice were sacrificed at day 13 after infection. Salivary glands, spleen, and liver were harvested and stored at $-80^\circ$C until further use. Organs were homogenized in DMEM with 4% FBS at a final concentration of 100 mg/ml. Two million MEFs were seeded into 24-well plates. From each sample, 5% of the salivary gland homogenate or 10% of the spleen or liver homogenate was used for infection of MEFs in triplicates. Plaques were counted after 3 days.

Flow cytometry

HFFs were infected with HCMV Towne and treated with AS (30 μM). At 72 hpi, cells were harvested for bivariate cell cycle analysis on a FACSCalibur Flow Cytometer (BD Biosciences) using propidium iodide and Alexa Fluor 488-conjugated mouse anti-human vimentin antibody. Data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Statistical analysis

Statistical significance was assessed with GraphPad Prism 5.0 software. Data are presented as mean ± S.D. ($n \geq 3$). Statistical significance between two groups was analyzed by two-tailed Student’s $t$ test, and asterisks indicate the statistical significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. All experiments were performed at least twice.

Data availability

The raw MS data were deposited in 10.6084/m9.figshare.12407720 and 10.6084/m9.figshare.12814136.

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Abbreviations—The abbreviations used are: CMV, cytomegalovirus; HCMV, human cytomegalovirus; MCMV, mouse cytomegalovirus; HFF, human foreskin fibroblast; MEF, mouse embryonic fibroblast; HSV1, human herpesvirus 1; AS, artemesate; DHA, dihydroartemisinin; Deoxy, deoxyartemisinin; IF, intermediate filament; IP, immunoprecipitation; SPR, surface plasmon resonance; WFA, withaferin A; Vim, vimentin; hpi, hours postinfection; GCV, ganciclovir; pfu, plaque-forming units; CDK, cyclin-dependent kinase; PLK Polo-like kinase; HRP, horseradish peroxidase; IFA, indirect immunofluorescence assay; ERK, extracellular signal–regulated kinase; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; HCD, higher-energy collisional dissociation; AGC, automatic gain control; AMC, 7-aminomethyl coumarin; MOI, multiplicity of infection; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; RU, response units; RT, room temperature; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; HRP, horseradish peroxidase.

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