REVIEW

Toward inhibition of human cytomegalovirus replication with compounds targeting cellular proteins

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ABSTRACT

Anti-viral therapy for human cytomegalovirus (HCMV) currently relies upon direct-acting antiviral drugs. However, it is now well-known that these drugs have shortcomings, which limit their use. Here I review the identification and investigation of compounds targeting cellular proteins that have anti-HCMV activity and could supersede those anti-HCMV drugs currently in use. This includes discussion of drug repurposing, for example the use of artemisinin compounds, and discussion of new directions to identify compounds that target cellular factors in HCMV infected cells, for example screening of kinase inhibitors. In addition, I highlight developing areas such as the use of machine learning and emphasize how interaction with fields outside virology will be critical to development of anti-HCMV compounds.

TEXT

Human cytomegalovirus. The herpesvirus human cytomegalovirus (HCMV) infects over 60% of the human population worldwide and is a significant cause of human morbidity and mortality (1). HCMV affects immunocompetent and immunosuppressed individuals, including neonates and pregnant women. Congenital HCMV infection can be found in up to 2% of live births worldwide (2) and most commonly causes numerous neurological abnormalities in newborns and infants, including hearing and/or vision loss and cerebral palsy (1, 2). In addition to productive replication, HCMV also exhibit latent infection and reactivation from latency resulting in productive replication. Therefore, HCMV infection is life-long and HCMV reactivation from latency can be a major cause of human disease. For example, HCMV reactivation from latency in women is a common cause of congenital infection (3) and HCMV reactivation from latency can be found in up to 50% of all solid organ transplant recipients and is a major factor in organ transplant rejection (4). HCMV replication and reactivation from latency is also a major factor in the progression of acquired immunodeficiency syndrome (AIDS) (1, 5). It is likely the full breadth of pathologies associated with HCMV infection has yet to be uncovered. Links between HCMV infection and cardiovascular disease have been suggested (6) and growing evidence indicates HCMV plays a role in susceptibility to tuberculosis infection (7) and exhaustion of the immune system in later life (8). Because HCMV infection is life-long and affects some of society's most vulnerable individuals, the care and treatment of HCMV-infected individuals has notable social and economic impact (1).

HCMV vaccine development is an area of research widely acknowledged to be important to human health (9-11). Thus far, there has been little success in producing highly efficacious HCMV vaccine candidates that can be widely used, although it must be noted that HCMV vaccines with even modest efficacy still have potential for clinical benefit in some patient populations (9, 10) and results of on-going vaccine trials of several vaccine candidates have yet to be reported (11). HCMV vaccines utilizing novel technologies such as mRNA-based expression of viral proteins, similar to that used recently to develop vaccines to prevent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, are now being developed (12). However, their efficacy has yet to be determined. Therefore, in the absence of HCMV vaccines current management of HCMV related disease relies upon supportive care and the use of anti-HCMV drugs, all of which are direct acting antiviral drugs that inhibit productive HCMV replication.

I will briefly review currently used direct acting anti-HCMV drugs and will propose that compounds inhibiting cellular proteins should be developed to replace these drugs. I then review identification and investigation of compounds that inhibit cellular proteins during productive HCMV replication. This starts with the discussion of a common approach to identification of compounds with anti-HCMV activity; investigation of compounds already used to treat human disease that can be repurposed as anti-HCMV compounds (for example, the anti-malarial artemisinin compounds). I then go on to review new directions in identification of anti-HCMV compounds, for example high throughput screening kinase inhibitors, including examples from my own laboratory's experiences in these areas.

This review is timely as there is not only an urgent need to develop novel anti-HCMV drugs, but there is also an increasing interest in using compounds targeting host cell proteins for treatment of viruses other than HCMV (13, 14). Excellent recent examples of this include the continued clinical use of the anti-human immunodeficiency virus (HIV) CCR5-receptor antagonist maraviroc (13), and the identification and clinical testing of compounds inhibiting replication of SARS-CoV-2, for example the inhibitor of translation elongation factor eEF1A plitidepsin (15, 16). Therefore, I also make general comments regarding compounds and methodologies that may be of interest to researchers working with viruses other than HCMV.

Currently used direct acting HCMV drugs and novel direct acting anti-HCMV compounds. The most widely used anti-HCMV drug is ganciclovir (GCV), or its orally bioavailable prodrug valganciclovir (VGCV) (Fig. 1). The use of these nucleoside analogues have been reviewed in detail elsewhere, including most recently by Griffiths and Reeves (11). Briefly, inhibition of long chain HCMV DNA synthesis by these drugs can effectively inhibit HCMV replication and treat HCMV disease, but their shortcomings, including the development of GCV/VGCV resistant viruses, limits their use (Fig. 1). Also, while GCV/VGAV are often given to infants and newborns, GCV/VGAV are not licensed for use in these important patient populations, in part because of theoretical concerns of potential carcinogenic and teratogenic effects of GCV/VGCV. However, evaluation of these potential effects is problematic as carcinogenic and teratogenic effects of GCV/VGCV have been observed only in murine models, plus data on these effects in mice has yet to be published and is only found in

105 information provided within the medical packaging for GCV/VGACV. In addition, there is
106 a lack of data on any long-term effects of GCV/VGCV after administration early in life.

107 Efforts to improve the characteristics of GCV have been largely unsuccessful.
108 Modifications of GCV structure to potentially improve both anti-HCMV effects and
109 selectivity for HCMV infected cells have been reported by a number of laboratories, but
110 none of these modifications have found success in development of compounds for
111 clinical testing. Compounds structurally related to GCV, such as 9-[2-
112 (phosphonomethoxy)ethyl] guanine (PMEG), have increased anti-HCMV effects
113 compared to GCV, but also can have increased toxicity to cells (17, 18). Efforts to
114 modify nucleotide metabolism in infected cells to increase nucleoside analogue
115 efficiency and potentially decrease development of drug resistance have also been
116 attempted (19, 20), but could have detrimental effects to cells.

117 Drugs to supersede GCV are required. Drugs such as the viral DNA synthesis
118 inhibitor brincidofovir, the HCMV kinase UL97 inhibitor maribavir and the HCMV
119 genome packaging inhibitor letermovir (Fig. 1) all have anti-HCMV activity and have
120 been tested for their ability to inhibit transplant rejection associated with HCMV
121 replication in randomized clinical trials (11). Presently, only letermovir has been licensed
122 for use based on clinical trial data (11). However, as expertly reviewed elsewhere (11),
123 there are shortcomings in how clinical trials for the use of anti-HCMV drugs during
124 transplantation have been designed. These include the possibility that clinical trial
125 design and end points were not useful to access drug efficacy (11). Therefore, the use
126 of maribavir and brincidofovir could be reevaluated. Although there is hope that use of
127 drugs such as letermovir and maribavir will become more common, it is important to

note that, like GCV, it is possible to find a range of letermovir and maribavir resistant HCMV viruses in laboratory and clinical studies of these drugs (21-27) (Fig. 1). This suggests that letermovir and maribavir resistant HCMV viruses may become a problem in the future if the use of these drugs become widespread.

To supersede the direct acting antiviral drugs discussed above, novel direct acting antiviral compounds that inhibit productive HCMV replication have been sought. In general, efforts to find such compounds have fallen into two broad areas. First, identifying compounds that target the function of the HCMV protein immediate-early 2 (IE2) using a cell-based assay to monitor transcriptional transactivation by IE2 (28-35). Second, identifying compounds that inhibit HCMV protein-protein interactions required for HCMV replication by employing either *in vitro* or *in silico* screens to understand what compounds interact with the HCMV DNA polymerase (36-38). However, none of these compounds have progressed toward clinical use for various reasons. In most of these studies, the compounds tested did not have anti-HCMV efficacy *in vitro* that was greater than that reported for GCV (50% effective dose of 1-10 micromolar). In other cases, the compounds are not amenable to medicinal chemistry efforts to improve anti-HCMV activity or create favorable characteristics for clinical use (31, 37).

Furthermore, the future development of new direct acting antivirals targeting HCMV is confounded by technical limitations found in developing assays for anti-HCMV compound discovery. For example, ectopic expression of many HCMV proteins in bacteria or eukaryotic cells is inefficient. Also, linking florescent reporter proteins to HCMV proteins (e.g. linking green florescent protein (GFP) expression to IE2 expression in recombinant HCMV viruses) can decrease HCMV titre (39, 40) and it has

not yet been possible to produce recombinant HCMV viruses which express GFP linked to several HCMV proteins required for productive HCMV replication (B.L.S., unpublished observations). It worth noting that there are many HCMV proteins of unknown function and a considerable amount of research is underway to understand which of those proteins are viable targets for future anti-HCMV drug development.

Compounds targeting cellular proteins. The difficulties in use and development of direct acting antiviral agents has led to increasing interest in investigating the potential of compounds inhibiting cellular proteins as anti-HCMV drugs. As with other viruses, HCMV is an obligate intracellular pathogen and the list of cellular proteins required for its productive replication is long and growing (41). Several factors support the use of compounds targeting cellular proteins to inhibit productive HCMV replication. These include a high barrier to anti-viral compound resistance, as inherited mutations or mutations occurring spontaneously in the human genome that could facilitate drug resistance are rare compared to the occurrence of drug resistance mutations that arise in the HCMV genome during replication. Also, mutation of a cellular protein to facilitate anti-viral drug resistance may lead to inhibition of that cellular proteins function, which could lead to cell death and prevent virus replication. Importantly, there is the opportunity to treat HCMV disease with existing compounds that are safely used in humans to inhibit cellular protein function, which has the potential to greatly accelerate the process from identification of a compounds anti-HCMV activity to its clinical use.

Repurposing of drugs: Artemisinin compounds. A well-known approach to finding novel anti-viral compounds is to select compounds that are well characterized in pre-clinical or clinical studies and test them for anti-viral activity, a process commonly referred to as drug repurposing. A wide range of clinically tested drugs targeting cellular proteins have been selected and tested for anti-HCMV activity. These include drugs as varied as cardiac glycosides (which inhibit ion channel function) (42-45), anti-fungal drugs (inhibitors of cytochrome p450 51) (46, 47) and drugs that inhibit DNA metabolism (48), all of which have a reported anti-HCMV efficacy *in vitro* equivalent to or greater than the reported *in vitro* anti-HCMV efficacy of GCV. Arguably, the most advanced drug repurposing efforts to inhibit productive HCMV replication involves the study of artemisinin compounds, which are widely used as drugs to treat malaria and known to inhibit *in vitro* replication of several human DNA viruses such as papillomaviruses, polyomaviruses, hepatitis viruses and human herpesviruses, including HCMV (49).

Artemisinin compounds (including artemisinin monomers, dimers, trimers and compounds structurally related to artemisinin) (Fig. 2A and 2B) have effective anti-HCMV activity *in vitro* at concentrations similar to or less than GCV (50-54) and can synergize with GCV to efficiently inhibit HCMV replication (43, 55). As yet, there have been no reports of HCMV viruses resistant to artemisinin compounds, suggesting that artemisinin compounds inhibit the function of cellular factors. Recent data indicates that artemisinin compounds target the cellular protein vimentin (56) (Fig. 2C), which could modulate several processes required for efficient HCMV replication, including cell cycle control and transcription of HCMV immediate-early genes (56). Another report has indicated that an artemisinin compound may influence intracellular signaling involving

196 the NF-κB pathway (57), which may be required for transcription of HCMV immediate-
197 early genes (57) (Fig. 2D). It will be interesting to see in future if inhibition of molecular
198 mechanisms reported to be associated with anti-HCMV activity of artemisinin
199 compounds act together or independently to inhibit HCMV replication. This will not only
200 further our understanding of the interaction between HCMV and artemisinin compounds,
201 but will also allow us to understand how artemisinin compounds interact with the viruses
202 mentioned above and will indicate what range of viruses might be susceptible to
203 inhibition by artemisinin compounds.

204 What sets artemisinin compounds apart from other drugs repurposed for anti-
205 HCMV use is that artemisinin compounds can be modified to increase anti-HCMV
206 activity (for examples, see many reports from the Marschall, Efferth and Tsogoeva
207 laboratories, including (58, 59)) and, crucially, a general trend in *in vivo* data has been
208 reported which suggests that treatment of HCMV infected individuals with an
209 artemisinin-based drug can inhibit shedding or replication of HCMV (60, 61), especially
210 in children with high viral loads (60). These data suggested that artemisinin compounds
211 could have activity against HCMV *in vivo*, but considerably more clinical data is required
212 to understand how artemisinin compounds should be administered to HCMV infected
213 individuals and to understand in which HCMV infected individuals artemisinin
214 compounds could have the most effect (60). Further exploration of artemisinin
215 compounds as anti-HCMV drugs is supported by observations that an artemisinin
216 compound could inhibit HCMV replication in an *ex vivo* models of congenital HCMV
217 infection (53, 58).

Research involving artemisinin stem from selecting a drug of interest and subsequently investigating that drug. In contrast, there has also been interest in high throughput screening of compounds that can be repurposed for anti-viral use. Notable examples include screening of commercially available drug libraries using diverse screening detection strategies such as cell-based reporter assays, recombinant HCMV viruses expressing reporter proteins fused to HCMV proteins and detection of HCMV antigens using antibodies (28, 62-64). These screens identify specific compounds, that have the potential to inhibit cellular functions required for productive HCMV replication, for example the anti-protozoal drug emetine (which inhibits ribosomal protein S14 function) (62), or groups of compounds that act on essential cellular processes required for HCMV replication. For example, compounds potentially inhibiting DNA metabolism (carboplatin and floxuridine) (64) and compounds potentially inhibiting microtubule transport of virus (colchicine, podophyllotoxia, podofilox, vinblastine, vincristine) (63). Most of the aforementioned compounds have an anti-HCMV efficacy equivalent to or greater than the reported *in vitro* anti-HCMV efficacy of GCV. However, drugs that inhibit essential cellular processes would likely have significant toxicity *in vivo* and it is unlikely that they would be administered to the patent populations affected by HCMV disease. It is interesting to note that the compound screening experiments mentioned above also identify compounds with anti-HCMV activity whose function in HCMV infected cells is unclear or unknown (28, 63-65). For example, the ion channel inhibitor convallatoxin (63) and the natural product moiety deguelin (28, 65). Further investigation of these and other compounds with potential anti-HCMV activity may

240 uncover compounds that have the potential to be developed for clinical use against
241 HCMV.
242
243 **Screening and development of novel compounds: Kinase inhibitors.** A more
244 recent area of interest is identifying compounds not yet developed for clinical use that
245 could be inhibitors of productive HCMV replication. Of particular interest are compounds
246 that inhibit cellular kinase proteins. This is supported by the long-standing observations
247 that many cellular kinase proteins are required for productive HCMV replication (66, 67)
248 and various kinase inhibitors targeting cellular kinases that are not routinely used in
249 clinical settings can inhibit productive HCMV replication (35, 66, 68-70).
250 To identify kinase inhibitors that have anti-HCMV activity it is possible to select
251 and test well characterized kinase inhibiters individually. Examples of this include those
252 compounds that inhibit cyclin dependent kinases, a kinase involved in histone
253 phosphorylation or the AMP-activated proteins kinase (35, 66, 68-70). While many of
254 these kinase inhibitors have a reported anti-HCMV efficacy *in vitro* at least equivalent to
255 that reported for GCV, it is unlikely they will be of clinical benefit as they target essential
256 cellular processes, which could lead to notable cellular cytotoxicity *in vivo*. However, the
257 availability of high throughput screening methodologies, for example screening based
258 on expression of viral antigens which is detected by high throughput automated
259 microscopy (71, 72), has allowed the identification of multiple kinase inhibitors from
260 various compound collections. This is aided by the availability of kinase inhibitor
261 libraries from both academic laboratories (73) and biopharma companies (74, 75). For
262 the most part these are collections of structurally diverse compounds. Crucially, it is

thought that these kinase inhibitors do not target proteins involved in essential cellular processes, but rather kinase proteins that are non-essential for cellular viability, that have functional analogues in the cell or that are involved in intracellular signaling pathways that have redundancy in the cell (73-75). Therefore, they may have few cytotoxic effects *in vivo*.

Screening of kinase inhibitor collections has identified several compounds with anti-HCMV activity that can be investigated further (73-75). Many of these compounds have anti-HCMV efficacy *in vitro* equivalent to or greater than the reported *in vitro* anti-HCMV efficacy of GCV (73-75). Plus, our laboratory has observed (BLS, unpublished data) that HCMV does not become resistant to any of kinase inhibitors identified in the aforementioned works (73-75). However, a drawback to using kinase inhibitors is that it can be unclear which kinase proteins are inhibited by a kinase inhibitor. Many kinase inhibitors display polypharmacology in which they can inhibit the function of multiple related kinase proteins (76, 77). Therefore, kinase selectivity assays must be carried out to confirm the kinase targets of inhibitors being investigated as novel anti-HCMV compounds. Lack of selectivity for a single kinase may be viewed as a disadvantage in the development of anti-viral compounds. However, it should also be argued that polypharmacology could be advantageous, as it could be possible to find compounds able to inhibit multiple proteins required for viral replication, but not essential cellular processes, which could increase anti-viral activity of a compound.

To provide a better understanding of which kinase inhibitors could be potential anti-HCMV compounds, collections of structurally related kinase inhibitors whose targets have been rigorously characterized have been screened (75-77). This revealed

that a range of kinase inhibitor families (chemotypes) had anti-HCMV activity and also revealed that a very broad range of kinases had the potential to be anti-HCMV targets (75). Indeed, it was notable that compounds with potential anti-HCMV activity were not obviously from the same chemotype (75) and in many cases structurally related compounds within a chemotype could have either potential anti-HCMV or pro-HCMV effects (75). Although polypharmacology of some compounds made analysis of kinase targets challenging, what was notable was that compounds from different chemotypes targeting the same kinase proteins had the same effects. For example, kinases inhibited by compounds from multiple chemotypes targeting MAP4K4, MNK, PRKD1, PRKD2, PRKD3, CLK2, HIPK1, HIPK4 DYRK1A, DYRK1B and DYRK2 had potential anti-HCMV effects (75). It has been demonstrated that inhibitors of MAP4K4, CLK2 and DYRK proteins have anti-HCMV activity (73, 78, 79) and development of inhibitors targeting these and other kinases mentioned above could be a profitable route to developing inhibitors of productive HCMV replication. Interestingly, the above-mentioned screen (75) also suggested that inhibition of certain kinases may have pro-viral effects, for example inhibition of PRKG1, PRKG2, PRKX, PKA, ROCK1 and ROCK2. Although the potential pro-HCMV activity of these kinases requires confirmation, future studies of HCMV inhibitors should consider potential pro-HCMV effects of inhibiting these and possibly other kinase proteins. Additionally, those kinases whose inhibition may promote cytotoxicity was also considered in the study (75). For example, inhibition of PLK1 was likely to cause cytotoxicity (75). Inhibition of this kinase should be avoided in future studies of novel HCMV inhibitors.

An advantage of the screening well characterized compounds (75) is that the breadth and depth of information available on the kinase inhibitors screened allowed re-examination of screening data by machine learning (75, 80). For example, machine learning could identify kinase targets that were overrepresented in the list of kinase proteins inhibited in the screen (78). This confirmed the potential role of DYRK and MAP4K4 kinases in HCMV replication and stimulated experiments that demonstrated a novel kinase inhibitor targeting MAP4K4 had anti-HCMV activity (78). Machine learning analysis also indicated that a range of MAPK proteins were likely required for HCMV replication (78), although selective inhibitors of those proteins have either yet to be tested for anti-HCMV activity or have yet to be described. It is notable that in the experiments discussed above it was found that many very poorly characterized kinase proteins were found to be associated with HCMV replication (75, 78). Therefore, investigating kinase inhibition in virus infected cells has the potential to uncover or stimulate research into new areas of virus-host interaction.

A point of interest is that many of the kinase inhibitors in the studies mentioned above target kinase proteins required for production of HCMV IE proteins (73-75, 78). This is, perhaps, not surprising as multiple intracellular signaling pathways that require kinase proteins are required for activation of the HCMV major IE promoter and phosphorylation of IE proteins may be linked to their essential functions in productive HCMV replication (67, 81-83).

This point also reveals a further advantage to development of kinase inhibitors as anti-HCMV drugs. Thus far in this review I have focused on inhibition of productive HCMV replication. However, it is equally important to consider how novel anti-HCMV

drugs can be used to inhibit reactivation of HCMV from latency. As both productive replication and reactivation from latency require activation of the HCMV IE promoter (67, 84) it is possible that use of kinase inhibitors will prove to be an approach to inhibiting both productive HCMV replication and reactivation from latency. For example, our laboratory and others have shown that inhibitors of the kinase MSK1 can inhibit productive HCMV replication in fibroblasts and reactivation of HCMV from latency in a myeloid cells (75, 85) (Fig. 3A and 3B). A selective inhibitor of MSK1 that does not have broad polypharmacology, SB-747651A, has been characterized (86) (Fig. 3A) and it has been reported that SB-747651A was well tolerated in an uninfected animal model (87). Therefore, SB-747651A is a candidate kinase inhibitor that should be further explored as an inhibitor of both productive HCMV replication and reactivation of HCMV from latency.

As yet, kinase inhibitors have not reached clinical use for HCMV disease. This is due to limitations surrounding anti-HCMV efficacy and toxicity (potentially from on-target effects of compound polypharmacology). However, a key feature of kinase inhibitors that greatly aids their development is they are small molecules amendable to structural changes (88). Advances made elsewhere will likely speed development of selective kinase inhibitors as anti-viral compounds. For example, there is great technological development in fields such as oncology where a combination of medicinal chemistry campaigns to link compound structure and function and atomic resolution analysis of compound binding to its targets (88) can lead to the identification of highly selective kinase inhibitors. It is likely that these compounds may be useful inhibitors of productive HCMV replication and latency.

Moreover, as intracellular signaling pathways are utilized by many human viruses, there is the prospect that development of kinase inhibitors as antiviral drugs for HCMV may lead to the identification of kinase inhibitors that can be used as broad spectrum anti-viral compounds against a range of viruses important to human health. This would most likely involve discovery of inhibitors that can prevent replication of several the human herpesviruses, who utilize the same or similar molecular and cellular pathways for replication.

Concluding remarks and future perspectives. As discussed above, current direct acting antiviral drugs for HCMV have shortcomings and their development can be difficult. In this review I have presented arguments for the development of compounds that target cellular proteins as anti-HCMV compounds, including artemisinin compounds and kinase inhibitors. For artemisinin compounds to move forward to clinical use a critical step will be clinical studies to understand drug dosing (pharmacokinetics and pharmacodynamics) in neonates, children and adults. For other repurposed drugs the first major challenge will be generating clinical data that suggests these compounds have efficiency against HCMV *in vivo*. Progression of kinase inhibitors toward clinical use may be more complex. I have suggested at least one kinase inhibitor, SB-747651A, that should be considered for development. As outlined above, other kinase inhibitors may require substantial pre-clinical development before clinical testing.

Additionally, a diverse range of cellular functions are required for productive HCMV replication (41). Therefore, it is likely that many more compounds targeting cellular proteins that have anti-HCMV activity have yet to be discovered. This may be

achieved by reevaluating how screening experiments are performed and analyzed. A drawback to the screening approaches mentioned above is that they utilize collections of compounds and are typically screened using a single screening methodology. Compound collections typically contain compounds of extremely diverse structure and function. Therefore, a single screening methodology may find some, but not all, compounds with anti-HCMV activity during the screening process of any given compound collection. Therefore, it may be wise in future experiments to screen compounds collections with multiple orthogonal screening methodologies. Moreover, such is the potential of this machine learning technology to uncover new drug targets, it would be useful to further characterize kinase targets within kinase inhibitor collections already screened for anti-HCMV compounds (73, 74) and apply the machine learning approach mentioned above (78) to them. Indeed, if possible, it would be useful to understand what data can be mined using machine learning from previous screens for anti-HCMV compounds (28, 36, 38, 42, 64) and understand how future compound screens can be designed to take advantage of developments in machine learning data analysis.

We must also be mindful that it is likely the full range of cellular proteins required for HCMV replication has yet to be found. For example, a recent single cell genomic analysis of cellular protein function in HCMV infected cells has discovered Golgi and ER proteins hitherto unrecognized as required for HCMV replication (41). This study also identifies other proteins required for productive HCMV replication whose functions have not been well characterized, such as proteins required for lysosome function and nutrient sensing (e.g. proteins of the LAMPTOR complex) (41). The challenge now will

400 be to characterize these proteins, understand their role in HCMV infected cells and
401 understand what compounds can be used to inhibit their function.

402 Regardless of how potential anti-HCMV compounds are found in future, to
403 accelerate the process of testing novel anti-HCMV compounds it may be advantageous
404 to develop a standard testing model for anti-HCMV compounds so that compounds can
405 be easily compared for anti-HCMV activity. This will include the use of multiple
406 standardized cell lines, a reference HCMV strain and standardized anti-HCMV assays
407 and cellular cytotoxicity assays.

408 While I have focused on inhibition of cellular proteins in this review, it cannot be
409 guaranteed that compounds inhibiting cellular proteins will be available for or successful
410 in clinical use in the near future. Therefore, I also advocate considering inhibition of
411 other cellular molecules required for HCMV replication. For example, we have explored
412 inhibition of productive HCMV replication using compounds that appear to interact with
413 cellular DNA in chromatin (64). Importantly, because interference with the function of
414 viral or cellular DNA may affect the replication of many viruses other than HCMV, the
415 compounds investigated in our aforementioned project (64) have the potential to be
416 used as broad spectrum anti-viral compounds. For example, they may be potent
417 inhibitors of poxviruses, whose replication can be inhibited with DNA binding
418 compounds (89).

419 Other than HCMV vaccine strategies, an area of interest that I have not focused
420 on in this review is modulation of the anti-viral immunity to inhibit HCMV replication or
421 disease during productive infection. When inhibiting viruses other than HCMV,
422 treatment of infected cells with interferon has, arguably, been the most successful

strategy thus far (13, 14). However, HCMV encodes multiple countermeasures to the type I interferon response (11) making it very unlikely that type I interferon will be a useful tool against HCMV disease. That said, expression of cellular anti-HCMV proteins in infected cells can be controlled by ubiquitin mediated proteasomal degradation (90), for example degradation of SLFN11 by the Cullin4-RING E3 Ubiquitin Ligase complex (91). As part of a collaborative project our laboratory has argued that inhibition of proteins controlling proteasomal degradation could lead to anti-HCMV cellular protein expression in HCMV infected cells, which could inhibit productive HCMV replication (91). This is supported by the observation that the Cullin4-RING E3 Ubiquitin Ligase complex inhibitor MLN4924 is an effective inhibitor of productive HCMV replication (92). Future studies are required to assess the suitability of treating HCMV infected individuals with MLN4942, although clinical oncology studies with MLN4942 are now underway (93). The use of MLN4942 or other compounds influencing the proteasomal degradation of proteins may be a useful strategy to inhibit replication of a wide range of viruses, as the requirement for proteasomal degradation of proteins in infected cells to promote virus replication is widely recognized.

Looking forward, it is likely that development of novel anti-HCMV compounds will require meaningful interaction between virologists and fields outside of virology. I have highlighted examples of this above. For instance, interaction with medicinal chemistry to develop safe and efficacious anti-viral compounds, interaction with oncology research to identify novel kinase inhibitors and interaction with bioinformatics research to mine data from existing and future screening experiments. Bioinformatics may be the area that requires the most investment, as there are other technologies that could be used to

identify and develop anti-HCMV compounds. For example, there are few examples where *in silico* screening of compounds have been used to identify inhibitors of HCMV protein function (36). This approach could be aided by recent advances made elsewhere, including utilizing the structures of viral and cellular proteins generated by the machine learning tool alphafold (94-97) in *in silico* screening.

Finally, many of the compounds I have mentioned in this review have been approved for use in humans by regulatory authorities. However, the utility of the compounds I discuss here as anti-HCMV drugs can and should only be assessed by rigorous pre-clinical development and correctly designed and executed randomized clinical trials. Only then should compounds be considered for clinical use against HCMV.

ABBREVIATIONS

CLK2: cyclin-dependent kinase-like kinase 2. CREB: cAMP response binding protein. DYRK1A, DYRK1B, DYRK2: dual-specificity tyrosine phosphorylation-regulated kinase 1A, 1B and 2. HIPK1 and HIPK4: homeodomain-interacting protein kinase 1 and 4. IE: immediate-early. MAPK: mitogen activated protein kinase. GCV: ganciclovir. MAP4K4: mitogen-activated protein kinase kinase kinase kinase 4. MIEP: major immediate-early promoter. MNK: MAP kinase-interacting serine/threonine-protein kinase. MSK1: mitogen and stress activated kinase 1. PKA: protein kinase A. PLK1: polo-like kinase 1. PRKD1, PRKD2 and PRKD3: protein kinases D1-D3. PRKG1, PRKG2: cGMP dependent kinase 1 and 2. PRKX: protein kinase, x-linked. SLFN11: Schlafen protein 11. ROCK1, ROCK2: rho-associated, coiled-coil-containing protein kinase 1 and 2. VGCV: Valganciclovir.

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CONFLICTS OF INTEREST

The author declares that there are no conflicts of interest.

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FIGURE LEGENDS

Figure 1 Current anti-HCMV drugs. At the top of the figure are the chemical structures of anti-HCMV drugs (highlighted in pink). Ganciclovir is shown above Valganciclovir.

503 Shown below the chemical structures are the viral targets (highlighted in blue),
504 mechanism of action (highlighted in green) and location of resistance mutations
505 (highlighted in yellow). Data on drug targets and mechanism of action have been
506 reviewed elsewhere (11, 35). Data on the location of resistance mutations found in *in*
507 *vitro* and *in vivo* studies can be found in references such as (11, 21-27, 35, 98).
508
509 **Figure 2 Artemisinin compounds and inhibition of HCMV replication.** (A) and (B)
510 Chemical structures of an Artemisinin monomer and the commonly used Artemisinin
511 related compound Artesunate, respectively. Dimers and Trimers of Artemisinin are
512 described in references such as (58, 59) and can be formed by connecting Artemisinin
513 monomers together using different chemical linkers attached to monomers at various
514 sites, depending on the chemical synthesis strategy used. For information on other
515 modifications to artemisinin that are possible see the references found in this review.
516 (C) Inhibition of productive HCMV replication using a mechanism proposed in reference
517 (56). It was proposed that in HCMV infected cells phosphorylation (superscript P, in
518 green) of vimentin lead to its degradation, which was associated with progression of the
519 cell cycle toward G1/S and productive HCMV replication. However, binding of
520 Artesunate to vimentin could reduce vimentin phosphorylation, which resulted in
521 stabilization of vitmentin and fewer infected cells moving toward G1/S and may have
522 reversed the cell cycle to G0/G1. The ability of Artesunate to prevent movement of the
523 cell cycle toward G1/S in HCMV infected cells would not favor transcription of HCMV
524 immediate-early genes and productive HCMV replication. (D) Inhibition of productive
525 HCMV replication using a mechanism proposed in reference (57). Activation of the NF-

κB intracellular signaling pathway was proposed to be required for productive HCMV replication, where phosphorylation (superscript P, in green) of IκBα and its subsequent proteasomal degradation allowed the dimer p65-p50 to translocate from the cytoplasm to the nucleus and activate transcription of HCMV immediate-early genes. However, it was proposed that binding of Artesunate to p65 prevented the aforementioned steps in the NF-κB intracellular signaling pathway to occur.

Figure 3 Compounds inhibiting MSK and inhibition of HCMV replication. (A) Chemical structures of MSK inhibitors previously shown to inhibit productive HCMV replication (SB-734117) and reactivation of HCMV from latency (H-89) (75, 85), plus the chemical structure of the MSK inhibitor we propose in this review should be tested for anti-HCMV activity, SB-747651A (86). (B) Inhibition of productive HCMV replication or reactivation from latency using a mechanism proposed in references (75, 85) and reviewed in detail in reference (99). Briefly, productive HCMV replication and reactivation of HCMV from latency requires transcription from the HCMV major immediate-early promoter (MIEP). It was proposed that transcription from the MIEP requires the transcription factor CREB. MSK is recruited to the MIEP by CREB and subsequent phosphorylation of CREB and histone H3 by MSK lead to transcriptional activation of the MIEP.

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Anti-HCMV drugs targeting HCMV proteins

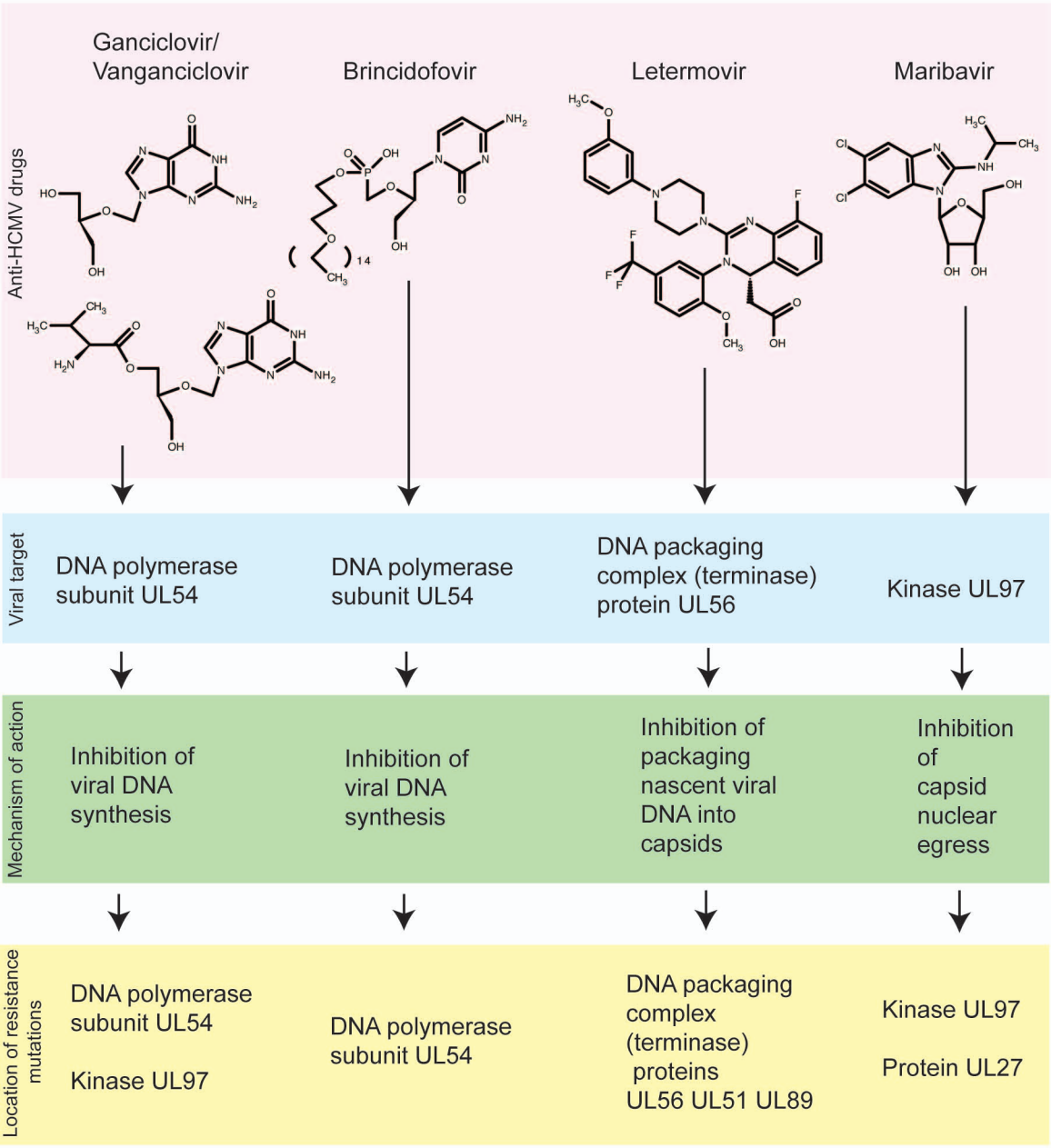
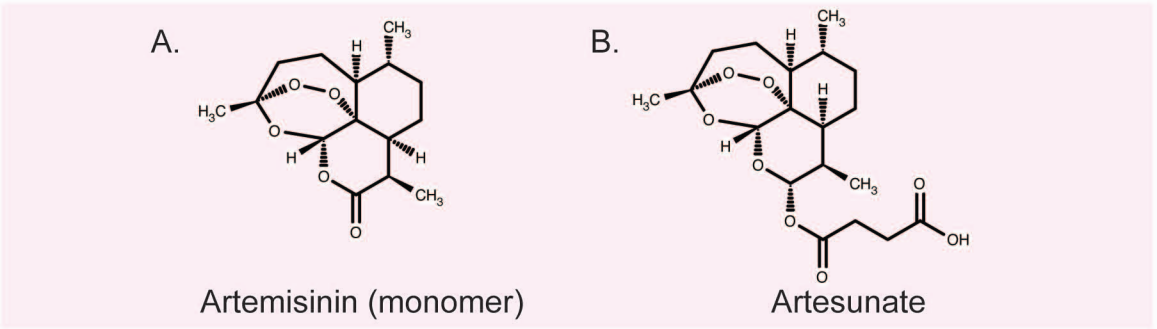
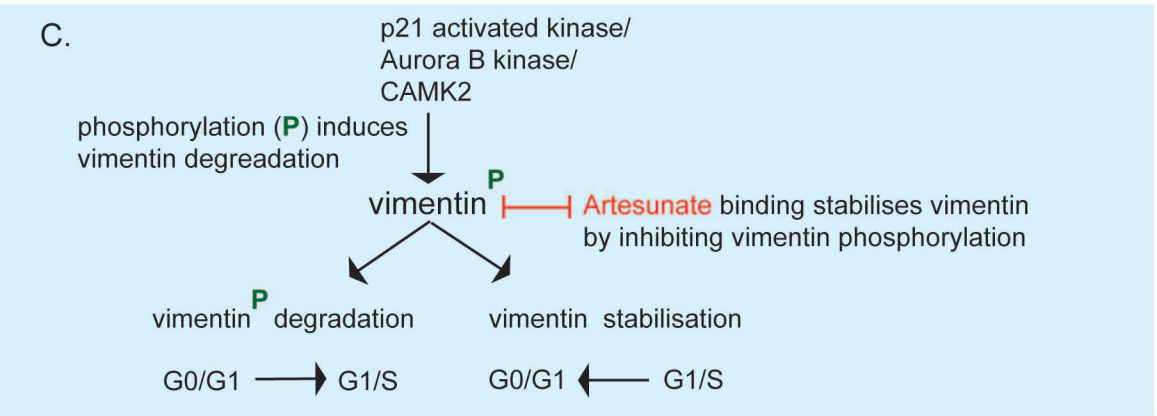


Figure 1


A.
B.
Artemisinin (monomer)
Artesunate


C.
p21 activated kinase/
Aurora B kinase/
CAMK2
phosphorylation (P) induces
vimentin degreadation
vimentin
P
Artesunate binding stabilises vimentin
by inhibiting vimentin phosphorylation
vimentin P degradation
vimentin stabilisation
G0/G1
G1/S
G0/G1
G1/S

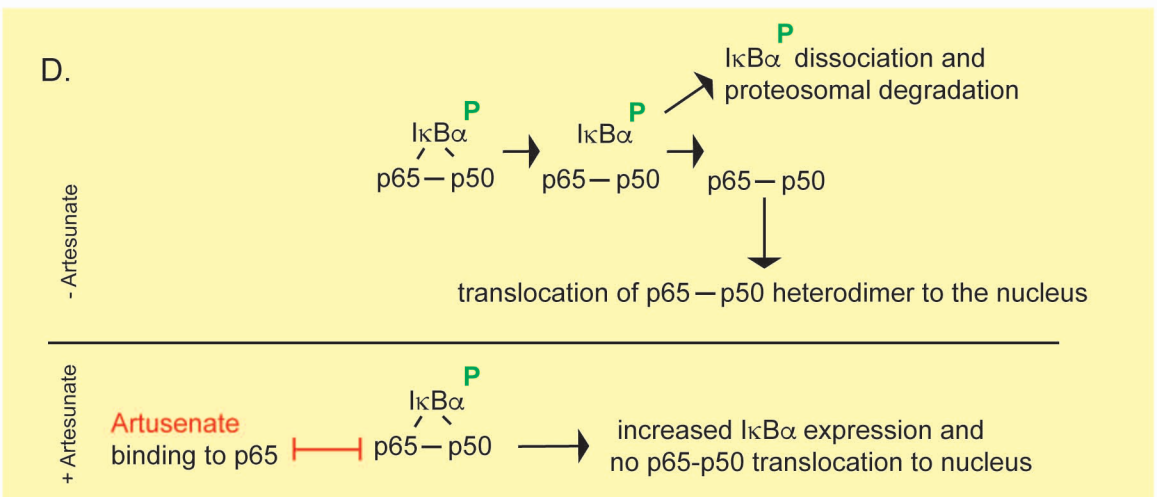
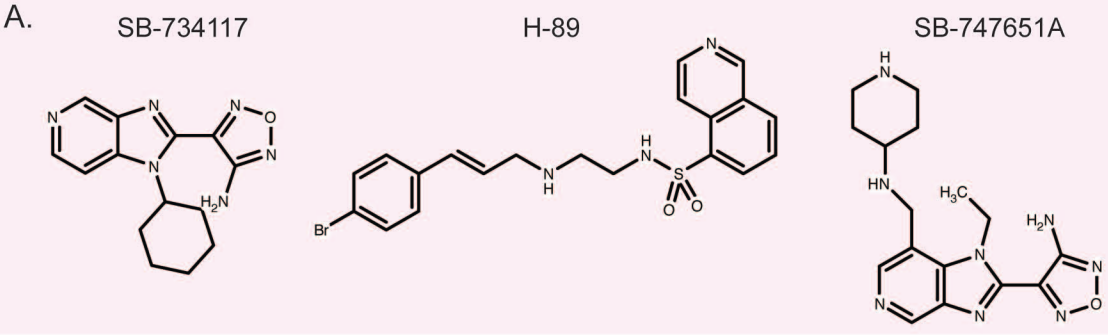

D.
- Artesunate
IκBα
P
p65—p50
translocation of p65—p50 heterodimer to the nucleus
+ Artesunate
Artusenate
binding to p65
increased IκBα expression and
no p65-p50 translocation to nucleus

Figure 2

Compounds inhibiting MSK kinase activity

A.



B.

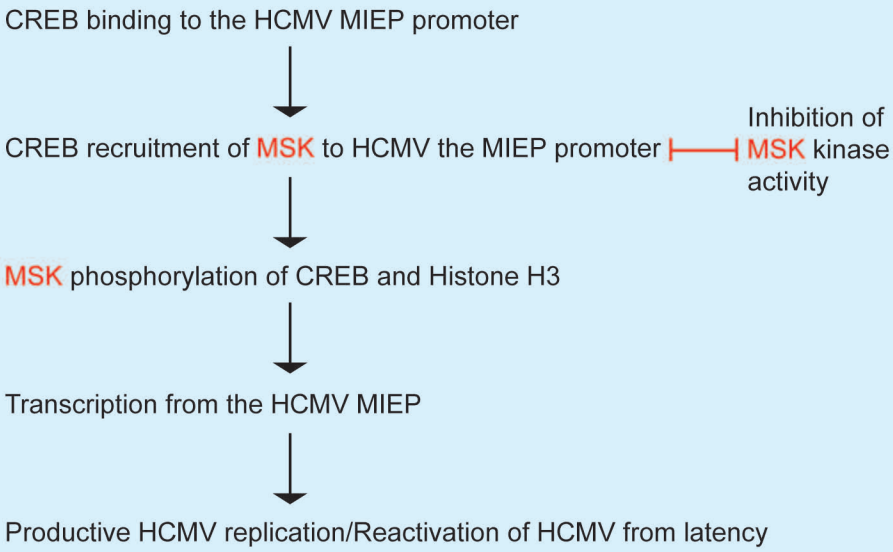


Figure 3