Antiviral Research 161 (2019) 116–124

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Targeting the terminase: An important step forward in the treatment and prophylaxis of human cytomegalovirus infections

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ARTICLE INFO

Keywords:
Human cytomegalovirus
Terminase
Benzimidazole ribonucleosides
Deoxyribosylindole nucleoside analogs
Tomeglovir
Letermovir

ABSTRACT

A key step in the replication of human cytomegalovirus (HCMV) in the host cell is the generation and packaging of unit-length genomes into preformed capsids. Enzymes required for this process are so-called terminases, first described for double-stranded DNA bacteriophages. The HCMV terminase consists of the two subunits, the ATPase pUL56 and the nuclease pUL89, and a potential third component pUL51. The terminase subunits are essential for virus replication and are highly conserved throughout the *Herpesviridae* family. Together with the portal protein pUL104 they form a powerful biological nanomotor. It has been shown for tailed dsDNA bacteriophages that DNA translocation into preformed capsid needs an extraordinary amount of energy. The HCMV terminase subunit pUL56 provides the required ATP hydrolyzing activity. The necessary nuclease activity to cleave the concatemers into unit-length genomes is mediated by the terminase subunit pUL89. Whether this cleavage is mediated by site-specific duplex nicking has not been demonstrated, however, it is required for packaging. Binding to the portal is a prerequisite for DNA translocation. To date, it is a common view that during translocation the terminase moves along some domains of the DNA by a binding and release mechanism. These critical structures have proven to be outstanding targets for drugs to treat HCMV infections because corresponding structures do not exist in mammalian cells. Herein we examine the HCMV terminase as a target for drugs and review several inhibitors discovered by both lead-directed medicinal chemistry and by target-specific design. In addition to producing clinically active compounds the research also has furthered the understanding of the role and function of the terminase itself.

1. Introduction

The first-line pharmacotherapy option for the treatment of systemic human cytomegalovirus (HCMV) infections is ganciclovir (GCV) and its orally bioavailable form valganciclovir (Biron, 2006). GCV is a prodrug that undergoes initial phosphorylation via the virus-encoded pUL97 protein kinase (Littler et al., 1992; Sullivan et al., 1992). Following further phosphorylation to a triphosphate (Biron et al., 1985; Boehme, 1984), GCV-TP directly inhibits the virus-encoded pUL54 DNA polymerase and/or incorporates into replicating viral DNA resulting in early chain termination (Frank et al., 1984; Freitas et al., 1985; Matthews and Boehme, 1988; Reardon, 1989). Upon therapy failure due to the emergence of drug resistance and/or adverse effects, foscarnet (FOS) or cidofovir (CDV) are utilized. These therapy options also elicit their antiviral effect through inhibition of the virus-encoded DNA polymerase and/or incorporation into elongating viral DNA causing early chain termination, but lack the virus specific activation associated with GCV (Erice et al., 1989; Lea and Bryson, 1996; Wagstaff and Bryson, 1994). Due to the nature of infection (infection followed by a prolonged latent state), patients with compromised immune systems may have to endure extended periods of prophylactic therapy to prevent infection (Sinclair and Sissons, 2006) or undergo repeated regimens of pre-emptive therapy (Bodro et al., 2012). However, currently approved pharmacotherapies for the treatment of HCMV suffer from two major drawbacks: 1) GCV has a moderate to high incidence of neutropenia (Crumpacker, 1996) while CDV and FOS suffer from a high incidence of nephrotoxicity (Lea and Bryson, 1996; Wagstaff and Bryson, 1994). 2) Prolonged antiviral therapy can lead to the selection of virus with decreased susceptibility to drugs (Erice, 1999; Gilbert and Boivin, 2005; Jabs et al., 1998; Lurain and Chou, 2010). In addition and

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https://doi.org/10.1016/j.antiviral.2018.11.005
Received 25 September 2018; Received in revised form 7 November 2018; Accepted 13 November 2018
Available online 23 November 2018
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because most of the currently approved anti-HCMV drugs share a common target, the incidence of cross-resistance (resistance to one drug conferring decreased susceptibility to another) is also a growing concern (Drew, 2010; Drew et al., 2001). Considering the significant drawbacks of the currently available viral DNA polymerase inhibitors, there is a growing need for new pharmacotherapies with novel antiviral mechanisms of action to combat drug resistance and/or improve toxicity profiles.

Processing and packaging of the HCMV genome is facilitated, in part, by the terminase, a complex of proteins that assists with packaging the genome into preformed capsids and cleaving the HCMV DNA concatemer into individual genomes; these processes are conducted in the nucleus of the infected cell. This is an essential process in the lifecycle of HCMV and, therefore, is a prime target for antiviral therapy. In this article, we explore the role the HCMV terminase plays in the replication of the virus, discuss novel compounds that target this enzyme including letermovir (the first FDA approved HCMV terminase inhibitor), present possible mechanisms by which these compounds elicit an antiviral effect, and offer thoughts on the future of antiviral therapy utilizing these compounds.

2. The HCMV terminase – structure-function relationships

2.1. General outline

A key step in HCMV maturation is the packaging of unit-length genomes into capsids; this topic has become a major focus in viral research. Although HCMV has a linear genome, the DNA circularizes immediately after translocation into the nucleus of infected cells (Stinski, 1991). Therefore replication of the covalently closed circular DNA is thought to occur via rolling circle mechanism leading to head-to-tail linked genomes, the so called concatemer (McVoy and Adler, 1994). For formation of infectious virus these concatemers have to be cleaved into unit-length genomes and packaged into preformed capsids. Enzymes involved in the viral DNA packaging process, terminases, are responsible for site-specific duplex nicking and insertion of the DNA into the procapsids (Fig. 1). HCMV terminase is a heterotrimer consisting of the ATPase pUL56, the nuclease pUL89 and a third component, pUL51 (Bogner, 2010; Bogner et al., 1998; Borst et al., 2013; Scheffczik et al., 2002; Scholz et al., 2003). The nuclear egress of nucleocapsids occurs via budding through the inner and outer nuclear membrane including a temporary envelopment in the perinuclear space (Mettenleiter et al., 2009). For final envelopment the capsids are translocated through the cytoplasm to the assembly compartment where those, together with tegument proteins, bud into endosomal vesicles (Schauflinger et al., 2013; Tooze et al., 1993). The enveloped virions are transported to the cell surface and released by exocytosis.

2.2. The nanomotor

It has been shown that all dsDNA viruses employ a DNA-packaging motor, the so-called nanomotor, to pump the viral genome into the preformed nucleocapsids (Casjens and Huang, 1982; Rao and Feiss, 2015). The nanomotor is able to convert chemical energy into physical energy in order to manage encapsidation of dsDNA. The energy source is ATP-hydrolysis mediated by one component of the motor. This energy leads to conformational changes of the motor components, resulting in movement. Since the dsDNA is highly condensed to near crystalline structure in the capsids, hydrolysis of many nucleoside triphosphates are necessary to drive the process (Guo and Lee, 2007; Smith et al., 2001). Smith et al. (2001) demonstrated that in the case of the motor of bacteriophage phi29, 60 pN are required for packaging of one genome into the preformed capsid. More recently it has been shown that this motor rotates the DNA during packaging. While the rotation rate increases according to filling of the capsid, the ATP activity decreases (Liu et al., 2014).

The HCMV nanomotor consists of three units: two packaging proteins, the terminase subunits pUL56, pUL89, and pUL51, and the portal protein, pUL104.

2.3. Components of the nanomotor

2.3.1. Portal protein pUL104

A critical packaging component is the portal protein, a viral capsid channel for DNA translocation into preformed capsids. Previous studies have shown that *UL104* encodes the HCMV portal protein (Dittmer and Bogner, 2005). All portal proteins are located at a single vertex of the capsid and are thought to be dodecamers (Guasch et al., 2002; Lurz et al., 2001; Trus et al., 2004). Through oligomerization of twelve radially organized pUL104 monomers a channel is formed. Furthermore, monomers assemble immediately *in vitro* (Holzenburg et al., 2009) and interaction of all components are required for the nanomotor to function. In HCMV infected cells, the portal protein directly interacts with the terminase subunit pUL56 (Dittmer et al., 2005) (Fig. 1, *4*). Another feature of portal proteins in general is the short-term binding of DNA during the insertion process (Fig. 1, *5*); this prerequisite for a portal protein is fulfilled by HCMV pUL104 (Dittmer and Bogner, 2005). A structure-based model for DNA translocation through the portal involves cooperation between the ATPase activity of the terminase and a "valve" or stepping mechanism or revolution without rotation provided by the portal (Guo et al., 2016; Jing et al., 2010).

2.3.2. The terminase

Human cytomegalovirus terminase consists of the subunits pUL56 and pUL89 and a possible third component pUL51. HCMV is one of the most complex viruses and it seems to have evolved different functional arrangements of its terminase subunits compared to most herpesviruses and bacteriophages.

2.3.2.1. Terminase subunit pUL56. The HCMV terminase subunit pUL56 is highly conserved throughout the herpesviruses and, according to its function, was shown to be an essential protein. The protein is expressed with early-late kinetics (Giesen et al., 2000). The HCMV pUL56 is associated with sequence-specific binding of DNA containing packaging motifs (pac 1 and pac 2) leading to the hypothesis that pUL56 plays a key role in DNA packaging (Bogner, 2010; Bogner et al., 1998). In addition, pUL56 is translocated to viral replication centers via its own nuclear localization signal and further evidence was provided that nuclear translocation of pUL56 is mediated by the importin-dependent pathway (Giesen et al., 2000) (Fig. 1, *2*). DNA translocation has been shown to be an energy-dependent process associated with ATPase activity of one terminase subunit (Fig. 1, *4*). Even though pUL56 does not have a typical Walker box, it was demonstrated that amino acids YNETFGKQ (aa709-716) represent an ATP-binding site with glycine G714 as an invariant amino acid (Hwang and Bogner, 2002; Scholz et al., 2003).

Almost all DNA metabolizing enzymes analyzed thus far are ring-like molecules. Image analysis of recombinant-expressed, purified pUL56 revealed that the enzyme exists as a dimer formed by the association of two ring-like structures positioned on top of each other and connected by a pronounced density on one side (Savva et al., 2004). While each ring measures about 9 nm in diameter, the central protein deficit of approximately 3.5 by 2.5 nm is sufficient for DNA-binding (Fig. 1, *3*).

It has been demonstrated that the C-terminal region of pUL56 is able to interact with the pUL104 portal protein as a necessary step toward the packaging of the viral genome into the procapsid (Dittmer et al., 2005) (Fig. 1, *4*). Through examination of the interaction domain of pUL89 with pUL56, it is reasonable to hypothesize that the interaction domain of pUL56 with pUL104 is an alpha helical structure. However, the defined motif (i.e. the amino acid sequence and secondary protein structure) within pUL56 that interacts with pUL104 has yet to be

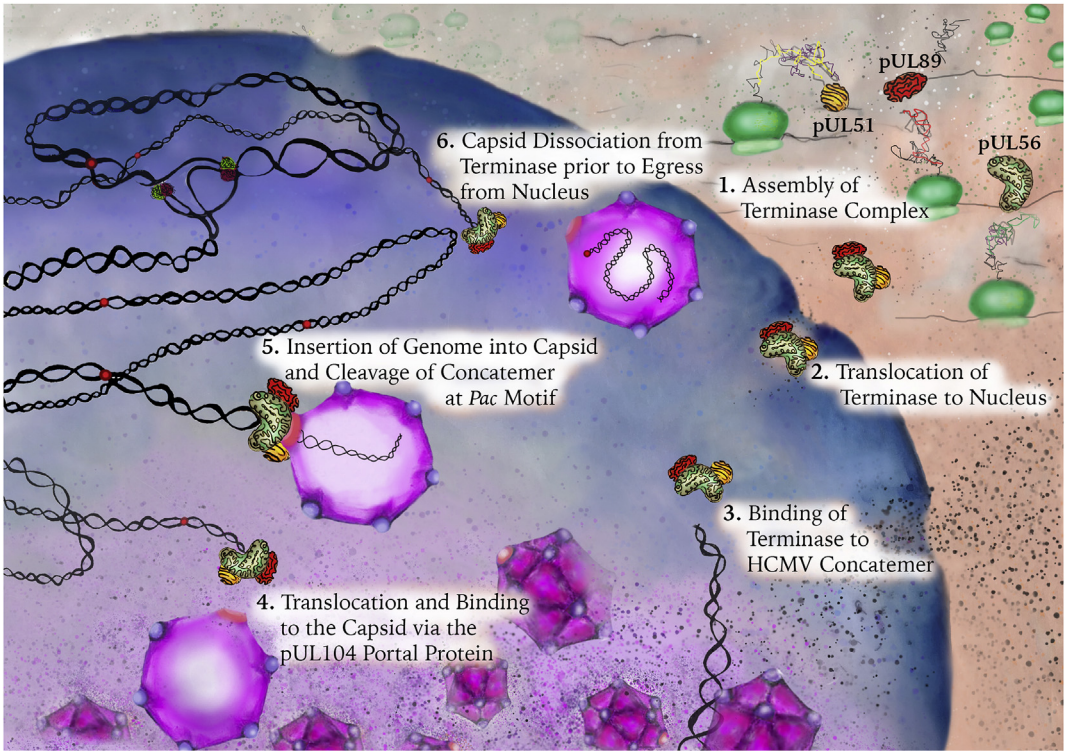


Fig. 1. The Role of the Terminase in the Replication of HCMV and Targets for Antiviral Pharmacotherapy. *1.* Upon biosynthesis of the terminase proteins in the cytoplasm, the three components of the terminase (pUL51, pUL56, pUL89) form a heterotrimer. *2.* The nuclear localization signal contained on pUL56 allows the terminase complex to migrate from the cytoplasm to the nucleus. *3.* Upon entry into the nucleus, the terminase binds with the multi-genome concatemer. *4.* The concatemer-terminase complex migrates toward the viral procapsid and binds to the portal protein (pUL104) which is contained within the viral procapsid wall. *5.* The HCMV genome is inserted into the capsid followed by cleavage of the double-stranded DNA at the *Pac* motif contained within the *a* sequence. *6.* The concatemer-terminase complex dissociates from the portal protein. Processes *4–6* are repeated allowing multiple packaging events from a single concatemer. Disruption of any of these events would result in the inhibition of packaging of the HCMV genome into the procapsid. For example, disruption of the terminase heterotrimer formation in the cytoplasm would result in the inability of pUL89 and/or pUL51 to translocate to the nucleus since pUL56 contains the nuclear localization signal. Lack of pUL89 in the nucleus would result in a marked reduction of genome cleavage since pUL89 contains the nuclease domain of the terminase complex. Data reviewed herein strongly indicate that letermovir inhibits the binding of the terminase to the concatemer (step *3*) whereas the benzimidazole D-ribonucleosides, deoxyribosylindole nucleoside analogs, and tomeglovir inhibit the binding of the complex to the portal protein (step *4*) and/or disrupt proper genome cleavage (step *5*).

reported.

In order to endow the cleavage process with specificity, both subunits of the terminase complex must come into close proximity. We identified the interaction domain of pUL89 and the corresponding region in pUL56 has very recently been confirmed (Ligat et al., 2017; Thoma et al., 2006) (Fig. 1, *1*). Ligat et al. (2017) demonstrated that the required amino acids (aa671-680) are located in the C-terminus of pUL56 and are essential for binding the two terminase subunits together and, thus, essential for HCMV replication.

2.3.2.2. Terminase subunit pUL89. Within HCMV, pUL89 is unique as it is the only protein that has been shown to be both spliced and has early-late kinetics of expression. In view of its putative function, the small terminase subunit pUL89 is translocated into the nucleus and, in particular, into viral replication centers of infected cells (Thoma et al., 2006) (Fig. 1, *2*). Further, the terminase subunit pUL89 interacts with the C-terminal half of pUL56 (Hwang and Bogner, 2002) (Fig. 1, *1*). It also was clearly shown that the 21 amino acid sequence GRDKALAVEQFISRFNSGYIK (aa580-600) is sufficient for the interaction with pUL56 and is required for DNA packaging (Thoma et al., 2006). Single particle analysis with DNA containing the *a* sequence (sequence of viral DNA that contains the *pac* motifs (motif where concatemer cleavage occurs)) revealed that pUL89 is required for cleavage of concatemers into unit-length genomes (Scheffczik et al., 2002). More importantly, nuclease activity assays demonstrated that there is a positive cooperativity between pUL56 and pUL89 cleavage of DNA (Scheffczik et al., 2002) (Fig. 1, *5*). Whether this cleavage is mediated by site-specific duplex nicking or not is unknown, although it is a prerequisite for packaging. Nadal et al. (2010) reported that the nuclease motif of pUL89 resides at the C-terminus. Further structural analysis of purified pUL89 revealed that pUL89 is arranged as a two-domain monomer incorporating sites involved in DNA binding and nuclease activity (Theiss et al., submitted, unpublished work).

2.3.2.3. Terminase subunit pUL51. Recently a third HCMV terminase subunit, pUL51, was identified. Since knock-down experiments lead to prevention of infectious particles and a block in cleavage of viral concatemers, it has been proven that this protein represents the third component of the terminase (Borst et al., 2013). This protein is expressed at the late phase of infection and, according to its function, is translocated to the nuclear replication centers (Borst et al., 2013). Further evidence demonstrated that nuclear translocation requires the interaction of all three terminase subunits (Neuber et al., 2017) (Fig. 1, *2*). pUL51 forms a stable complex with the other terminase subunits, pUL56 and pUL89, and is crucial for viral cleavage. The C-terminus of pUL51 plays a particularly important role for the interaction during complex formation (Neuber et al., 2018) (Fig. 1, *1*).

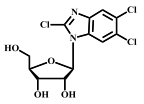
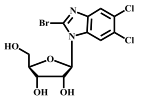
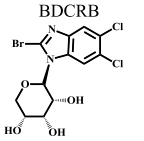
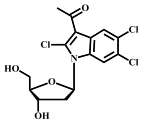
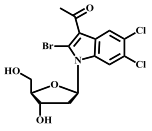
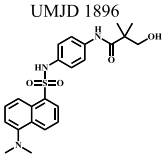
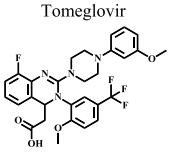
3. HCMV terminase inhibitors

3.1. Benzimidazole D-Ribonucleosides

3.1.1. TCRB and BDCRB

The first set of compounds confirmed to inhibit the HCMV terminase

Table 1
Summary of terminase inhibitors.

Compound	EC_{50}	Mutations[a]	Stage of Development
 TCRB	2.9 μM	pUL89 D344E, A355T pUL56 Q204R	Pre-Clinical
 BDCRB	0.7 μM	pUL89 D344E, A355T pUL56 Q204R	Pre-Clinical
 GW275175X	1.4 μM	pUL89 D344E, A355T pUL56 Q204R	Phase 1 Trials – Completed
 UMJD 1853	0.3 μM	pUL89 D344E, E256Q pUL56 Q204R	Pre-Clinical
 UMJD 1896	0.3 μM	pUL89 D344E, E256Q pUL56 Q204R	Pre-Clinical
 Tomeglovir	1.2 μM	pUL89 M360I, V362M, H389N pUL56 P202A[b], I208N[b]	Phase 1 Trials – Discontinued
 Letermovir	4–5 nM	pUL56 V236M, L241P, C325Y, R369 M/G/S	Phase 3 Trials – Completed; Approved for Prophylactic Use in HSCT recipients

[a] Only mutations that confer amino acid substitutions resulting in high level resistance are denoted here.
[b] Determined against MCMV.

were the benzimidazole D-ribonucleosides. Originally synthesized as a potential anti-cancer agent (Townsend and Revankar, 1970), 2,5,6-trichloro-1-(β-D-ribofuranosyl)benzimidazole (TCRB; Table 1) was highly selective against HCMV (EC_{50} = 2.9 μM) at non-cytotoxic concentrations (CC_{50} > 100 μM). It's 2-bromo homolog BDCRB (Table 1) was more active (EC_{50} = 0.7 μM) and no more cytotoxic. These activities are at least 2-fold more than that of GCV (Drach et al., 1992; Townsend et al., 1995). The spectrum of antiviral activity was limited to cytomegalovirus (both human and rhesus) with less activity observed in guinea pig and rodent cytomegalovirus (Drach et al., 1992; Nixon and McVoy, 2004; North et al., 2004).

The first evidence that TCRB and BDCRB did not act like the other nucleoside antiviral analogs was that unlike acyclovir and GCV, they did not inhibit viral DNA synthesis. Nor did they inhibit viral RNA or protein synthesis (Drach et al., 1992). Furthermore TCRB and BDCRB were active against strains of HCMV with clinically significant GCV-, CDV-, or FOS-resistant mutations in either *UL97* and/or *UL54* (Drach et al., 1992; Evers et al., 2004). These data clearly demonstrate that TCRB and BDCRB do not share a mechanism of action with the currently approved nucleoside analogs.

The metabolic profile of BDCRB provided additional evidence for a unique mechanism of action. Unlike GCV or CDV, both of which are prodrugs that must be phosphorylated to become active, BDCRB is not phosphorylated into a mono-, di-, or triphosphate nor is it incorporated into DNA or RNA (Krosky et al., 2002). In addition, the 5′-deoxy analogs, which are incapable of being phosphorylated, are more active than TCRB or BDCRB (Drach et al., 1995; Krosky et al., 2002). Thus these compounds elicit their antiviral effect without being phosphorylated.

Time-of-addition studies gave additional evidence that a unique mechanism was involved and suggested what it could be. Unlike GCV, which begins to lose potency around 48 h post-infection, BDCRB does not begin to lose potency until around 80 h post-infection (Evers et al., 2004). These results showed that BDCRB elicits an effect through inhibition of a process after viral DNA synthesis has occurred. Electron micrographic examination of HCMV inhibited by TCRB provided further evidence of a unique mechanism of action. Virus-infected cells incubated with TCRB contained viral particles lacking encapsulated DNA. Since HCMV replication proceeds with the synthesis of high molecular weight concatemers that must be cleaved into genome length units, this led to the hypothesis and proof that TCRB and BDCRB inhibit viral DNA processing (maturation) and not DNA synthesis. Further experimentation demonstrated that genome maturation is not completely inhibited by BDCRB but can occur at a low frequency by continuing beyond the cleavage point for a unit-length genome. This gave rise to a small amount of non-functional structures termed “monomer plus” DNA (McVoy and Nixon, 2005; Underwood et al., 1998).

The best and probably most significant evidence to define the mechanism of action of the benzimidazole D-ribonucleosides came as a result of selection of HCMV resistant isolates and marker transfer studies. Under increasing concentrations of either TCRB or BDCRB, virus able to replicate in the presence of these compounds was selected. Resistance to both drugs mapped to amino acid substitutions in pUL89 (specifically D344E and A355T) and/or pUL56 (specifically Q204R) (Krosky et al., 1998; Underwood et al., 1998). These results show that the target(s) of TCRB and BDCRB are pUL89 and pUL56 and not the virus encoded DNA polymerase pUL54. In addition to mutations in *UL56* and *UL89*, a mutation conferring an amino acid change within pUL104 (specifically L21F) was discovered in the two TCRB- or BDCRB-resistant HCMV strains (Komazin et al., 2004). However this mutation did not in and of itself confer resistance to BDCRB suggesting that this site is not involved with drug binding. Nonetheless, since pUL56 interacts with pUL104 as part of the normal process of concatemer cleavage and genome packaging into the capsid (Dittmer et al., 2005), this mutation in drug resistant isolates may compensate for the conformational changes in pUL56 and/or pUL89 that confer TCRB and BDCRB resistance.

The mechanism by which TCRB and BDCRB affect the terminase at a molecular level resulting in inhibition of terminase function is not entirely clear. The mutations in *UL89* that confer TCRB and BDCRB resistance map to a region of the terminase that is the ATPase-coupled helicase site – a site which unwinds the concatemer prior to cleavage (Champier et al., 2007). Therefore, it is possible that TCRB and BDCRB interfere with the ability of the terminase to unwind the DNA prior to cleavage (Fig. 1, *5*). Since pUL89 does not bind to packaging motifs, this site on pUL89 must be in close proximity to another of the terminase subunits (pUL56) which does bind the viral concatemer. In fact, the mutation in *UL56* that confers TCRB and BDCRB resistance maps to a zinc-finger, a metal binding motif that binds DNA (Champier et al., 2008; Krosky et al., 1998). Thus, TCRB and BDCRB may interfere with DNA binding since a mutation in *UL56* could result in a protein that has lower affinity for drug without affecting DNA binding (Fig. 1, *3*). However, it is also possible that these drugs inhibit the formation of the quaternary terminase structure, i.e. these drugs inhibit pUL89 and pUL56 from interacting with each other (Fig. 1, *1*). Such inhibition of interaction could manifest in pUL89 not translocating to the nucleus since it does not have a nuclear localization domain, likely, utilizes the interaction with pUL56 (which does have a nuclear localization

domain) to translocate to the nucleus following synthesis in the cytoplasm. Therefore, if TCRB and BDCRB inhibit the interaction between pUL89 and pUL56, pUL56 would translocate to the nucleus while pUL89 remains in the cytoplasm. Thus, without pUL89 to provide nuclease activity, terminase activity would be effectively inhibited. A final possibility is that the binding of TCRB and BDCRB results in a distortion of the three dimensional terminase structure which could have a profound impact upon binding and processing of concatemeric DNA and/or binding to other protein structures necessary to carry out replicative function (Fig. 1, *4–5*). In support of this hypothesis, previous studies have found an altered processing of concatemeric DNA by BDCRB (McVoy and Nixon, 2005). This is consistent with an inability of the large terminase subunit pUL56 to bind to the portal protein pUL104 in the presence of BDCRB thereby suggesting that BDCRB blocks the insertion of DNA into capsids by preventing a necessary interaction of pUL56 and pUL104 (Dittmer et al., 2005). Supporting this conclusion is the observation mentioned above – the mutation found in *UL104* in BDCRB-resistant HCMV that did not confer resistance (Komazin et al., 2004).

3.1.2. GW275175X

Despite the excellent activity of TCRB and BDCRB against HCMV *in vitro*, pharmacokinetic studies in rats and monkeys demonstrated that these compounds were metabolized via glycosidic bond cleavage too rapidly to be considered good clinical candidates (Good et al., 1994; Lorenzi et al., 2006). Thus, the structure-activity relationship of the series was expanded by the synthesis of many new analogs (Chan et al., 2000; Migawa et al., 1998; Townsend et al., 1997; Zou et al., 2000). From greatly expanded efforts, two clinical candidates were identified. The first, GW1263W94 (maribavir) is a benzimidazole L-ribonucleoside with a good pharmacokinetic profile (Trofe et al., 2008; Wang et al., 2003). Detailed investigation of this compound revealed a mechanism of action different from that of TCRB and BDCRB – that is, inhibition of viral DNA synthesis via inhibition of the virus-encoded pUL97 protein kinase (Biron et al., 2002). Because it is not a terminase inhibitor, we will not discuss it further; the reader is referred to Biron et al. (2011) and/or Prichard (2009) for additional information (Biron et al., 2011; Prichard, 2009).

The second compound, GW275175X (Table 1), is a D-ribopyranose derivative of BDCRB with an antiviral spectrum of activity similar to that of BDCRB (EC_{50} against HCMV = 1.4 μM) (Underwood et al., 2004). Animal studies using GW275175X against HCMV demonstrated a 30-fold enhancement of effect when compared to the vehicle control (Kern et al., 2004). Pharmacokinetic evaluation of GW275175X in mice and monkeys demonstrated a moderate volume of distribution, a low clearance rate, and a relatively long half-life (probably due to the lack of significant glycosidic bond cleavage). A Phase 1 trial was conducted and demonstrated that the drug was well tolerated without serious adverse effects or clinical abnormalities (De Clercq, 2011). However, further development of GW275175X was halted in favor of GW1263W94 (maribavir), which was in a more advanced stage of development.

Further examination of GW275175X revealed a time-of-addition profile similar to that of BDCRB. A BDCRB-resistant HCMV isolate (1038rB) demonstrated 20-fold resistance to GW275175X. Biochemical assays of HCMV DNA synthesis and maturation in the presence of GW275175X exhibited a lack of concatemer cleavage analogous to that shown by TCRB and BDCRB (Underwood et al., 2004). Incubation of HCMV with increasing concentrations of GW275175X consistently selected the pUL89 D344E amino acid substitution. The pUL56 Q204R substitution, observed with BDCRB resistance (Krosky et al., 1998), was also selected on a regular basis in a separate series of experiments (Chou, 2017a). Taken together, we hypothesize that GW275175X binds to the HCMV terminase in a manner similar to that of BDCRB and, as such, would share a similar molecular mechanism of action (Fig. 1, *4–5*).

3.2. Deoxyribosylindole nucleoside analogs

Another chemical approach to overcome the rapid metabolism problem posed by TCRB and BDCRB was to replace the benzimidazole heterocycle with a chemically similar indole. Chemical synthesis and subsequent testing demonstrated that the deoxyribosylindole derivatives of TCRB (UMJD 1853, Table 1) and BDCRB (UMJD 1896, Table 1) have submicromolar potency against HCMV (EC_{50} = 0.3 μM for both; approximately 2-fold greater activity than BDCRB). Due to the chemical nature of the glycosidic bond in indole ribonucleoside analogs, this bond is much more stable compared to that in the benzimidazole nucleosides. Thus it should be able to resist metabolic degradation that primarily contributed to the rapid half-life of TCRB and BDCRB (Williams et al., 2004). In support of this hypothesis, *in vivo* metabolic studies using 10 mg/kg of UMJD 1896 in rats resulted in little to no metabolic degradation (Gentry, data not shown).

Exploration of the mechanism of action for the deoxyribosylindole nucleoside analogs demonstrated a time-of-addition profile similar to that of BDCRB. In addition, the BDCRB-resistant isolate containing the pUL89 D344E and pUL56 Q204R amino acid substitutions was also resistant to the deoxyribosylindole nucleoside analogs (Williams et al., 2004). Further genetic studies examining the resistance profile of the deoxyribosylindole nucleoside analogs resulted in the identification of another amino acid substitution (pUL89 E256Q) that conferred resistance (Gentry et al., 2015). Surprisingly, this mutation did not confer resistance to BDCRB. Thus, the mutations that confer TCRB and BDCRB resistance also confer resistance to the deoxyribosylindole nucleoside analogs, but the converse is not true. The pUL89 E256Q substitution is situated between two motifs in pUL89 that have been implicated in ATP binding (Champier et al., 2007). If pUL89 provides the nuclease activity for the terminase (which requires energy) but is not the primary subunit that binds ATP (pUL56 is), then corresponding motifs on pUL89 that would assist with binding of ATP would be on the interface between the two subunits. Taken together, we conclude that the deoxyribosylindole nucleoside analogs and TCRB/BDCRB have similar, but not necessarily identical, binding sites. Given that they have similar binding sites, we hypothesize that the molecular mechanism of action for the deoxyribosylindole nucleoside analogs is identical to that of BDCRB and TCRB (Fig. 1, *4–5*). The pUL89 E256Q substitution, which also appears to be at the interface between the pUL89 and pUL56 interface, adds weight to our molecular mechanism hypothesis.

3.3. Tomeglovir

3-Hydroxy-2,2-dimethyl-N-[4({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)-phenyl]propanamide (tomeglovir, BAY 38–4766; Table 1) is a nonnucleoside inhibitor of HCMV replication. *In vitro* studies determined that tomeglovir is 5.5-fold more potent against HCMV (EC_{50} = 1.2 μM) compared to GCV (EC_{50} = 6.9 μM) (Buerger et al., 2001). In addition, tomeglovir also has activity against clinical isolates of HCMV including several that are GCV-resistant (McSharry et al., 2001). *In vivo* studies determined that the antiviral activity of tomeglovir against murine, guinea pigs, and HCMV as measured by survival, viremia, or lethal challenge was as good if not better than GCV in all cases (Reefschlaeger et al., 2001; Schleiss et al., 2005; Weber et al., 2001). In addition, tomeglovir has a favorable pharmacokinetic and low toxicity profile and, as such, was scheduled by Bayer to enter into Phase 1 clinical trials, but those trials were discontinued.

Genetic studies to determine the target for tomeglovir and provide a mechanism of action for the drug found mutations in multiple genes (*UL56, UL89*, and *UL104*), all of which are involved in the processing and packaging of the HCMV concatemer into the viral capsid (Buerger et al., 2001; Chou, 2017a; Reefschlaeger et al., 2001). Further experimentation revealed that several amino acid substitutions in HCMV (mainly in pUL89) that conferred high-level resistance to tomeglovir conferred low-level resistance to BDCRB or GW275175X and vice versa

suggesting that these drugs may bind to similar, but not identical locations on the HCMV terminase (Buerger et al., 2001; Chou, 2017a). In support of this, the conserved regions of pUL56 and pUL89 where the high-level tomeglovir resistance substitutions are located are the same conserved regions that confer TCRB and BDCRB resistance (Champier et al., 2007, 2008). This would also offer a reasonable explanation on why the combination of BDCRB and tomeglovir against HCMV confers a mixture of synergy and antagonism (Evers et al., 2002). In addition, several substitutions that confer resistance of HCMV to tomeglovir are in an adjacent conserved region of pUL89, one that has been implicated in binding to the portal protein (pUL104) (Champier et al., 2007; Chou, 2017a). While pUL56 is primarily responsible for binding of the terminase complex to the portal protein (Dittmer et al., 2005), pUL89 also has a region adjacent to the helicase and nuclease domains that have some properties consistent with binding of the portal protein. Therefore, the molecular mechanism of action of tomeglovir could be similar to the benzimidazole D-ribonucleosides (since they have similar binding sites on the target protein) (Fig. 1, *4–5*), but more likely differs since tomeglovir appears to bind in a conserved region of the pUL89 protein that the benzimidazole D-ribonucleosides do not. In addition to the molecular mechanisms of action proposed for TCRB/BDCRB, it is also possible that tomeglovir directly interferes with the binding of the terminase complex with the portal protein. In support of the different molecular mechanism hypothesis, tomeglovir produced only marginal or negligible amounts of monomer plus viral DNA (Buerger et al., 2001); this is in sharp contrast to the levels of monomer plus viral DNA produced by BDCRB (Krosky et al., 1998; McVoy and Nixon, 2005).

3.4. Letermovir

{(4S)-8-Fluoro-2-[4-(3-methoxyphenyl)-1-piperazinyl]-3-[2-methoxy-5-(trifluoromethyl)phenyl]-3,4-dihydro-4-quinazolinyl}acetic acid (letermovir; AIC246; MK 8228; Table 1) was discovered in an attempt to identify novel compounds that could inhibit HCMV replication by a mechanism different and distinct from that of the currently available viral DNA polymerase inhibitors (Lischka et al., 2010). Preclinical *in vitro* studies of letermovir demonstrated high potency against laboratory and clinical strains of HCMV (EC_{50} = 4–5 nM) with toxicities observed at concentrations well above the EC_{90}. In addition, letermovir retained activity against isolates of HCMV that are resistant to GCV and was highly selective for HCMV demonstrating little to no activity against other human pathogenic viruses (hepadnavirus, adenovirus, retroviruses, flaviviruses, orthomyxoviruses) (Lischka et al., 2010; Marschall et al., 2012). *In vivo* experiments found high levels of activity against HCMV in a mouse xenograft model with an ED_{90} dose of 8 mg of letermovir/kg/day (in comparison to the ED_{90} of GCV = > 100 mg/kg/day) (Lischka et al., 2010).

Phase 1 clinical trials for letermovir demonstrated good pharmacokinetic profiles for both oral and intravenous formulations with renal or hepatic impairment increasing the exposure of the drug to the patient when compared to healthy matched controls (Erb-Zohar et al., 2017; Kropeit et al., 2017a, 2017b). For all dosing regimens, letermovir was generally well tolerated indicating a good safety profile. In another phase 1 trial, co-administration of letermovir with either cyclosporine A or tacrolimus, immunosuppressive agents frequently administered to solid organ transplant patients (Holt, 2017), resulted in an increase in cyclosporine A or tacrolimus exposure presumably through inhibition of hepatic CYP450 3A4, an enzyme known to metabolize both cyclosporine A and tacrolimus (Kropeit et al., 2018). Although co-administration with cyclosporine A did alter the pharmacokinetic profile of letermovir, co-administration with tacrolimus did not. In phase 2 clinical trials, the optimal dose of 240 mg/day resulted in complete suppression of viremia in hematopoietic stem cell transplant patients when compared to placebo while maintaining an acceptable safety profile. However, suboptimal dosing of 60 mg/day resulted in the emergence of a drug-resistant isolate prior to a viremic episode (Chemaly et al., 2014; Lischka et al., 2016). In another phase 2 study with kidney transplant patients with active HCMV replication, a 14-day regimen of letermovir resulted in viral clearance for 50% of the patients when compared to 28.6% of the patients using the current standard of care (GCV) (Stoelben et al., 2014). In addition, letermovir was well tolerated and no patient developed HCMV disease during the course of treatment. Finally in a phase 3 clinical trial to assess letermovir prophylaxis for HCMV infection in hematopoietic stem cell transplant (HSCT) recipients, fewer patients had clinically significant HCMV infection or were attributed with having a primary end-point with letermovir (37.5%) when compared to placebo (60.6%) (Marty et al., 2017). In that same study, adverse events with letermovir were determined to be mainly low grade. Finally, a lung transplant patient that developed refractory multi-drug resistant HCMV disease was administered letermovir in addition to a reduction in the immunosuppressive pharmacotherapy (tacrolimus and corticosteroid). After 28 days of treatment, viral load was below the limit of detection and the patient remained HCMV disease free off treatment for at least 3 months (Kaul et al., 2011). While it is hard to distinguish if the reduction in immunosuppressive therapy or the administration of letermovir is responsible for the reduction in viral load, it is likely that both contributed. Taken together, clinical trials for the use of letermovir for prophylaxis and possible treatment of HCMV infection were successful. As such, letermovir under the brand name Prevymis was approved for prophylaxis against HCMV in HSCT recipients in late 2017 (Kim, 2018).

Initial characterization of the mechanism of action for letermovir in time-of-addition experiments gave a profile similar to that of tomeglovir indicating inhibition of viral DNA processing (Lischka et al., 2010). Further experimentation determined that letermovir blocks viral replication without inhibiting the synthesis of viral DNA or viral proteins (Goldner et al., 2011) similar to previous studies with other terminase inhibitors (McVoy and Nixon, 2005; Underwood et al., 1998). Finally, genetic studies involving the generation of letermovir-resistant isolates resulted in a myriad of mutations in all three of the primary terminase subunits (*UL51*, *UL56*, and *UL89*) (Chou, 2015, 2017a, b; Chou et al., 2018; Goldner et al., 2014; Goldner et al., 2011). Many of the mutations discovered in *UL56* and all of the mutations in *UL51* and *UL89* confer low-level resistance and are therefore unlikely to cause any clinical concern. However, several mutations that confer amino acid substitutions discovered in pUL56 (V236M, L241P, C325Y, R369 M/G/S) confer high levels of resistance and could result in manifestation of clinical resistance. In fact, the pUL56 V236M substitution that confers letermovir resistance was identified in a phase 2 clinical trial patient prior to a viremic episode (Lischka et al., 2016). This development occurred using a suboptimal dosing regimen and represents the only clinical manifestation of letermovir resistance reported to date. In addition, the mutations that confer high levels resistance to letermovir did not confer resistance to BDCRB and only the pUL56 R369M substitution conferred low level resistance to tomeglovir (Goldner et al., 2014). The converse is also true – the HCMV isolate 1035rB, which confers high level resistance to BDCRB (Underwood et al., 1998), does not confer resistance to letermovir (Gentry, unpublished data) and mutations that confer high levels of resistance to tomeglovir do not confer resistance to letermovir (Chou, 2017a). Although some have speculated that letermovir will have a low barrier to resistance because some mutations that confer high levels of resistance do not confer any disadvantage to the virus (Chou, 2015; Goldner et al., 2014), the manifestation of high incidences of letermovir resistance in a clinical setting has not been observed to date.

Although the molecular mechanism by which letermovir exerts its antiviral effect has not been determined, mutations that confer amino acid substitutions in pUL56 that confer the highest levels of letermovir resistance are the most likely location of drug-target binding and can therefore give us insight into the possible molecular mechanism of action for this drug. Amino acids 236 and 241 are hypothesized to be in a region of pUL56 that constitutes a leucine zipper, a basic DNA binding

motif (Champier et al., 2008). The other two amino acids (325, 369) are in or near another conserved region of the pUL56 protein with unknown function, but are likely involved with DNA binding since the ATP binding region, pUL89 binding domain, and pUL104 binding domain are located near the C-terminus of pUL56 (Dittmer et al., 2005; Ligat et al., 2017; Scholz et al., 2003). Taken together, we hypothesize that letermovir directly interferes with binding of viral concatemer DNA to the pUL56 subunit of the terminase complex (Fig. 1, *3*).

4. Concluding remarks

The multi-functional aspects of the HCMV terminase complex allow for a myriad of potential drug targets. In addition to conformational changes that affect complex orientation (Fig. 1, *4–5*), direct inhibition of binding of the complex to the portal protein (Fig. 1, *4*), and inhibition of DNA binding that were discussed above (Fig. 1, *3*), there remain other possibilities. These include but are not limited to i) preventing the three primary subunits of the terminase from forming a quaternary complex (Fig. 1, *1*), ii) directly inhibiting helicase and/or nuclease function (Fig. 1, *5*), iii) inhibiting the binding of ATP to pUL56, and iv) prohibiting terminase-portal protein dissociation upon insertion of genomic DNA (Fig. 1, *6*). In support of this, raltegravir, an antiretroviral drug that inhibits the HIV integrase, inhibits nuclease activity presumably through binding to the terminase at the active site and interfering with the Mg^{2+} or Mn^{2+} ions that are necessary for terminase function (Nadal et al., 2010).

With standard-of-care therapy regimens, especially for viral infections, moving more toward combination rather than monotherapy (Cihlar and Fordyce, 2016; Pletz et al., 2017; Tamma et al., 2012), terminase inhibitors have the potential to play a pivotal role in the treatment of HCMV. Current and previous studies with HCMV terminase inhibitors in combination with other antivirals for the treatment of HCMV have demonstrated an additive if not synergistic enhancement of effect *in vitro* (Evers et al., 2002; O'Brien et al., 2018; Wildum et al., 2015). In addition to positive pharmacodynamic interactions occurring with terminase inhibitors in combination with other antivirals with different mechanisms of action, combinations of terminase inhibitors with distinct binding sites and, thus, distinct molecular mechanisms of action also have the potential to result in a synergistic effect; this was observed with the combination of BDCRB and letermovir (O'Brien et al., 2018). Although many of these combinations have yet to be tested *in vivo*, the *in vitro* data suggest that at the very least, combinations involving HCMV terminase inhibitors will be additive in a clinical setting.

With approval of letermovir by the FDA for HCMV prophylaxis in HSCT recipients, the terminase was validated as a target for the treatment and/or prophylaxis of HCMV infections. The distinct viral target without a mammalian counterpart offers the potential for a large therapeutic index (i.e. lack of serious adverse effects at therapeutic doses). In addition, the favorable pharmacodynamic drug interaction profile with other antivirals gives the terminase inhibitors the potential to be a part of any combination regimen for the treatment of HCMV infections. The future of treatment for HCMV infections is changing and we believe that the terminase inhibitors will play a prominent role in that future.

Acknowledgments

The authors thank Ellie Ross, Pharm.D. for creating the figure for this review.

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