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Letermovir for the Prevention of CMV Infection in Transplant Recipients

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1.1 Background

1.1.1 Cytomegalovirus

Human cytomegalovirus (CMV; also known as HHV5) is the prototype member of the β-herpesviridae and the largest member of the virus family Herpesviridae. CMV is a DNA virus with an enveloped capsid including a linear, double-stranded DNA genome of ca. 230 kb, encoding approximately 200 viral proteins [1].

CMV is a very common infection in the human population worldwide, with prevalence rates in the United States ranging from 40% to nearly 100%, depending on the region and socioeconomic status [2]. Primary infection results from close interpersonal contact (bodily fluids) [3, 4] and is generally symptomless, often passing unnoticed [5]. Once established, the infection is lifelong as the virus enters latency in different cell types, including leukocytes (lymphocytes, monocytes, dendritic cells) and CD34+ hematopoietic progenitor cells [6]. For healthy, immune-competent individuals, the infection remains asymptomatic. However, primary infection or reactivation from latency poses a significant threat to those with weakened or compromised immune systems, particularly transplant recipients [7]. While other clinical scenarios do occur, e.g. congenital CMV infections or HIV/CMV coinfections, and while CMV infection may exhibit other complex interactions with its host, e.g. in intensive care patients or certain cancer patients, these are not the focus of the development of letermovir (LET) described here and are therefore not discussed further.

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1.1.2 Immunocompromised Patients

The situation is very different in the immunocompromised versus the immune-competent populations, particularly for transplant recipients, including human stem cell transplant (HSCT) and solid organ transplant (SOT) patients [1]. In transplant recipients, the use of immune suppressants, crucial to the prevention of graft organ rejection (or graft vs. host disease [GvHD] in the case of HSCT recipients), also weakens containment of the latent/persistent virus, enabling (i) the reactivation of viral replication, (ii) virus spread, and ultimately (iii) the development of CMV end-organ disease. CMV continues to be the most common opportunistic viral infection in transplant recipients [8]. If not rapidly contained, the infection can be serious or even life-threatening, being associated with increased morbidity and mortality from CMV directly and indirectly through the increased risk of GvHD (HSCT recipients), graft organ failure (SOT patients), or an increased risk of other opportunistic infections [9, 10]. Indeed, it is now understood that any level of active CMV replication may negatively impact the overall all-cause mortality of HSCT patients, even independently of successfully controlling virus replication with preemptive therapy (PET) (see below) [11, 12].

1.1.3 Patient Groups with a High Risk for CMV Complications

Although the risk of a primary CMV infection or a reactivation of latent virus is increased in all patients on immune suppression, that risk is not uniformly distributed. In the case of HSCT, the risk of CMV reactivation and disease is known to be markedly higher in D−/R+ recipients (see Figure 1.1, upper panel). These are highly immunosuppressed, seropositive HSCT recipients with an established/latent CMV infection (R+) given blood-forming stem cells from a CMV-naïve, seronegative donor (D−), meaning that these patients can no longer control the persistent virus and are almost defenseless once the virus fully reactivates from latency [13]. Among HSCT patients, approximately 60% are CMV-seropositive at the time of transplant and thus are particularly vulnerable to CMV reactivation [14]. Due to its cytopathogenic potential, CMV may cause end-organ disease in these patients, most commonly involving the gastrointestinal (GI) tract or the lungs, as a result of uncontrolled viral replication in tissues facilitated by impaired functional virus-specific T-cell responses and a lack of neutralizing antibodies [15, 16].

Conversely, CMV-seronegative SOT recipients (R−) given an organ from a CMV-seropositive donor (D+) become infected via latent virus, reactivating in the transplanted donor organ (see Figure 1.1; lower panel). These patients are initially defenseless against the primary infection due to their own CMV-naïve immune system, which is additionally under medical suppression. As described above for HSCT recipients, CMV infection in SOT recipients can also have both direct and indirect effects, with a significant impact on transplant outcomes. Direct effects in SOT include CMV syndrome and tissue-invasive organ disease, whereas CMV-associated indirect effects include, among others, acute and chronic organ rejection and opportunistic secondary infections [17].

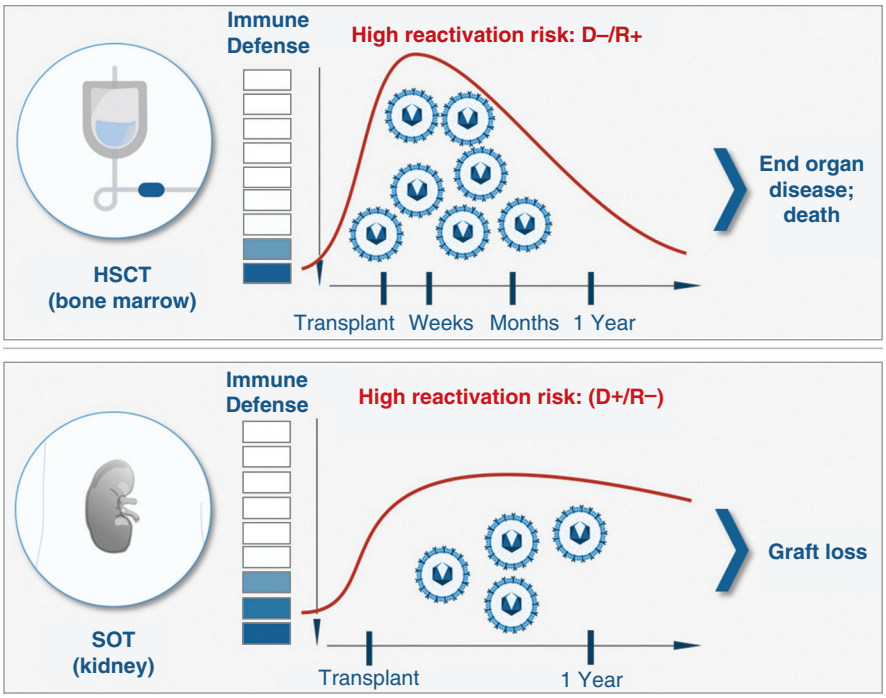


Figure 1.1 High-risk organ donor/organ recipient configurations for CMV reactivation and disease in immunocompromised HSCT and SOT patients.

D, donor; R, recipient; HSCT, hematopoietic stem cell transplantation; SOT, solid organ transplantation

HSCT, D−/R+ describes the situation where a CMV-naïve immune system from a seronegative donor is transplanted into a latently infected, highly immunosuppressed seropositive recipient, leaving the recipient unprotected in the absence of some intervention to prevent/control virus reactivation.

SOT, D+/R− describes the situation where a latently CMV-infected organ from a seropositive donor is transplanted into a seronegative recipient with a pharmacologically suppressed immune system, leaving the recipient unprotected against the virus which has effectively hitch-hiked on the transplanted organ.

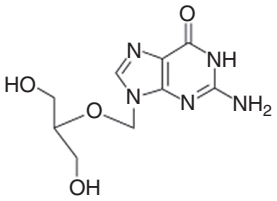
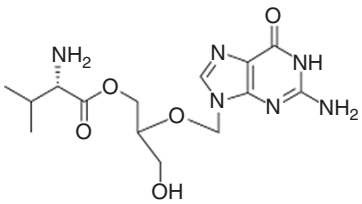
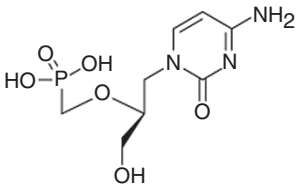
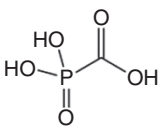
Source: Peter Lischka and Holger Zimmermann (2024).

1.1.4 Available CMV Treatments Before Letermovir

1.1.4.1 Antiviral Drugs

When the LET project began, only a few drugs were available for the treatment or prevention of CMV infections, namely ganciclovir (GCV), cidofovir (CDV), and foscarnet (FOS) (see Table 1.1). All drugs were approved in the 1990s (the GCV prodrug valganciclovir [VGCV] was approved in 2001) and all are limited in their use by toxic side effects, pharmacokinetic drawbacks, and resistance development. Moreover, since all of these drugs ultimately share the same primary molecular target, the viral DNA polymerase pUL54, cross-resistance between drugs has been observed in the clinic, in some cases extending to all available polymerase inhibitors, thus eliminating all effective treatment options. In addition to this pharmaceutical armamentarium,

Table 1.1 Overview of approved anti-CMV drugs prior to LET.

Drug name	Structure	Intracellular activation	Location of resistance mutations
Ganciclovir		Viral kinase + cellular kinases	UL97 UL54
Valganciclovir		Viral kinase + cellular kinases	UL97 UL54
Cidofovir		Cellular kinases	UL54
Foscarnet		No	UL54

UL54, CMV DNA polymerase; UL97, CMV protein kinase.
All approved anti-CMV drugs prior to LET ultimately target the viral DNA polymerase pUL54 and thus are prone to cross-resistance. Some drugs initially require intracellular activation (phosphorylation) via the viral kinase UL97 and/or cellular kinases (see text for details).

only a handful of new small molecules or monoclonal antibodies were in clinical development at that time. Moreover, there was no effective vaccine on the horizon. This situation has not really changed to date [1, 18].

Ganciclovir (GCV, Cymeven®, Roche) or its oral prodrug valganciclovir (VGCV, Valcyte®, Roche) is an analog of deoxyguanosine [19, 20]. (V)GCV is efficacious against CMV as well as other Herpesviridae and represented the CMV therapy of choice for HSCT and SOT patients at the time [19]. GCV/VGCV requires specific intracellular phosphorylation by the viral protein kinase pUL97 (to the monophosphate), as well as intracellular kinases to form the active triphosphate. Once activated, it inhibits the viral DNA polymerase and is also incorporated into progeny viral DNA, which drastically suppresses the rate of chain extension [21]. For this reason, (V)GCV resistance may be mediated by mutations in either the viral protein kinase pUL97 or the viral polymerase pUL54.

Despite its efficacy, (V)GCV's use is associated with restrictive toxicities, including neutropenia, leucopenia, anemia, and thrombocytopenia, as well as renal and longer-term genetic/reproductive toxicity. The former is particularly relevant in the HSCT recipient population, as the myelotoxic side effects of the drug are not compatible with a prolonged prophylactic treatment regimen, limiting treatment options in HSCT with this drug to so-called PET (see below).

Cidofovir (CDV, Vistide®, Gilead), a phosphonomethoxy analog of cytosine, is a competitive inhibitor of the viral DNA polymerase, acting as a chain terminator. CDV is activated solely by intracellular kinases, making it independent of the viral kinase pUL97. CDV is efficacious and has a long intracellular half-life. However, it also exhibits profound renal toxicity as well as carcino- and teratogenicity.

Foscarnet (FOS, Foscavir®, AstraZeneca), a pyrophosphate analog, does not require intracellular activation for activity. It binds directly to the pyrophosphate binding site of the viral DNA polymerase and inhibits viral DNA replication by blocking the cleavage of the pyrophosphate group from the terminal nucleoside triphosphate and preventing DNA chain extension. While efficacious, the use of FOS is associated with substantial and treatment-limiting toxicities, especially nephrotoxicity and electrolyte disruption, which also restrict or even prohibit its use in combination with other commonly used nephrotoxic drugs in this population such as the immunosuppressive agents tacrolimus or cyclosporine [22, 23]. In addition, FOS requires frequent intravenous (IV) infusions (every 8–12 h). For these reasons, its use was (and is) largely restricted to rescue therapy.

1.1.4.2 Dominant Treatment Strategies

As outlined above, CMV treatment or prevention in the immunocompromised population before LET was entirely restricted to inhibitors of the viral pUL54 DNA polymerase, with substantial and often treatment-limiting toxicities and significant risk of (cross-)resistance. An obvious approach to the problems presented by therapeutic toxicity is simply to wait for clinical signs related to a CMV infection, and only treat once pathology is evident. In the case of CMV, this approach is accompanied by considerable additional risk: (i) a local reactivation of the virus rapidly becomes systemic, resulting in end-organ disease, GvHD in the case of HSCT recipients or organ rejection in the case of SOT recipients, and substantially increases the risk of morbidity and mortality, (ii) CMV is itself immune-modifying, meaning that essentially any degree of active replication may facilitate so-called indirect CMV effects including an increased risk of secondary infections. Therefore, ideally, active CMV replication must be prevented or minimized before it becomes uncontrollable [9, 10, 17, 24].

Considering the toxicity profiles of the available treatments at the time, two preferred strategies were available to combat/prevent CMV infection and disease:

1. Prophylaxis: Antiviral prophylaxis, ideally starting immediately following organ transplantation and continuing during the period of increased risk for CMV reactivation. Although a prophylactic approach is typically easy to coordinate and there is ample evidence demonstrating the efficacy of this strategy for preventing CMV reactivation and disease and positively affecting

CMV-associated indirect effects, prophylaxis could be associated with increased drug costs and, more importantly, the prolonged drug exposure increases the risks of treatment-limiting toxicities. Prophylaxis also carries an elevated risk of late-onset reactivation and disease following cessation of treatment compared with PET (see below). While prophylaxis with (V)GCV for 3–6 months post-transplantation is the dominant strategy for CMV prevention in SOT recipients, in the case of HSCT patients, (V)GCV prophylaxis, even after engraftment, is usually contraindicated due to (i) the myelosuppressive side effects (neutropenia/leucopenia) of the drug and the fact that (ii) reduction of immunosuppression is excluded due to the risk of GvHD [25]. This situation led to the development of the following second option.

2. PET: Antiviral treatment is initiated when viral replication is detected in blood and when viremia/DNAemia reaches a specific threshold. The intention here is to (i) efficiently control virus replication, (ii) prevent symptomatic infection, and (iii) permit a more rapid/effective immune reconstitution by limiting exposure to potentially toxic (myelosuppressive) drugs. However, the risk of direct and indirect CMV effects, such as end-organ disease, rejection, graft loss, and opportunistic infections, is increased. Indeed, due to its immune modulatory activity, the mere presence of CMV replication (including low-level viremia without CMV disease) is associated with increased mortality in the HSCT population [11, 12, 26]. It is also of note that preemptive treatment requires regular time- and resource-intensive monitoring for CMV reactivation in blood.

1.1.4.3 Unmet Medical Need

Clearly, none of the available treatment options at that time could be considered ideal for the prevention or control of CMV in transplant patients, and thus there was a clear and unmet need for an effective CMV therapy that (i) is sufficiently safe to allow prophylaxis and (ii) acts via an alternative mechanism of action (i.e. not mediated by inhibition of pUL54).

1.2 Discovery Phase

The journey of anti-CMV drug development at AiCuris began at the Bayer Anti infective Research Laboratories even before the discovery of LET. Ongoing library screening for anti-CMV substances led to the identification of several new hits, which were selected for lead development, and evaluation of their antiviral and safety properties. It is noteworthy that the applied screening assay set-up (cell culture-based replication assay) appeared to be sensitive to targets late in the virus replication cycle as especially components targeting the viral capsid or the viral terminase complex (see below) were identified, though the reasons for this are unclear. Among these was a member of a new chemical class: the 3,4-dihydroquinazolines.

Structure–activity relationship (SAR)-based studies to improve the candidate's drug characteristics (see Figure 1.2), including efficacy and physical–chemical properties, was based on thousands of derivates and ultimately concluded with BAY 73-6327 (also known as AIC246, LET, and Prevymis®).

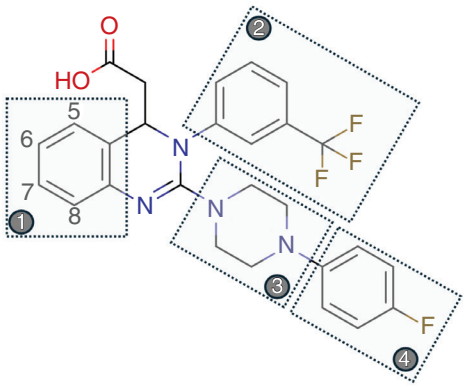


Figure 1.2 SAR for LET.

(1) Lipophilic and polar substituents well tolerated in 6- and 8-position; no improvement by pridyls, bis-substitution favorable.
(2) Lipophilic substitution most active; 3-position better than 4-position better than 2-position; no activity improvement through bis-substitution; benzyl substituents not tolerated.
(3) Loss of activity by replacement with ethylenediamines, piperidines, cyclohexyl, and phenyl.
(4) Substitutions in 3- and 4-positions well tolerated.

Source: Peter Lischka and Holger Zimmermann (2024).

At this early stage, as the initial mechanism of action (MoA) studies and Phase 1 trial planning for LET were commencing, the Anti-infectives Research Division was spun out from Bayer AG and became what is now AiCuris Anti-infective Cures AG, taking a comprehensive early pipeline of anti-infective projects including the anti-CMV drugs program with it.

AiCuris quickly grew from a spin-out of 22 people to an integrated R&D biotech company, including in-house expertise in preclinical, clinical, and chemistry, manufacturing, and controls (CMC) development. Further development of LET up to and including Phase 2b was thus performed and steered by AiCuris.

1.3 Preclinical Characterization

1.3.1 Antiviral Potency/Selectivity/Inhibitory Profile

Initial virology studies revolved around determining the new candidate's potency, first against very well-characterized CMV laboratory strains, then moving on through clinical strains, drug-resistant CMV strains, other herpesviruses, and ultimately to other viral pathogens.

1.3.1.1 *In Vitro* Potency Versus Laboratory CMV Strains

Early *in vitro* potency studies either examined LET's ability to inhibit the viral cytopathic effect on cell cultures [27] or used a recombinant virus expressing the green fluorescent protein and examined LET's effect on the fluorescence of infected cell cultures [28]. CMV laboratory strains exhibit a very narrow cell tropism mainly restricted to fibroblasts; therefore, initial *in vitro* studies used primary fibroblast cells from different tissues (e.g. skin, lung) for efficacy analyses [28, 29]. These studies delivered remarkable results; LET consistently demonstrated both EC_{50} and EC_{90} values in the low nanomolar range and was >400 (based on EC_{50}) to >2000 times (based on EC_{90}) more potent than the contemporary gold standard CMV treatment (i.e. GCV, see Figure 1.3, Part A and Table 1.2).

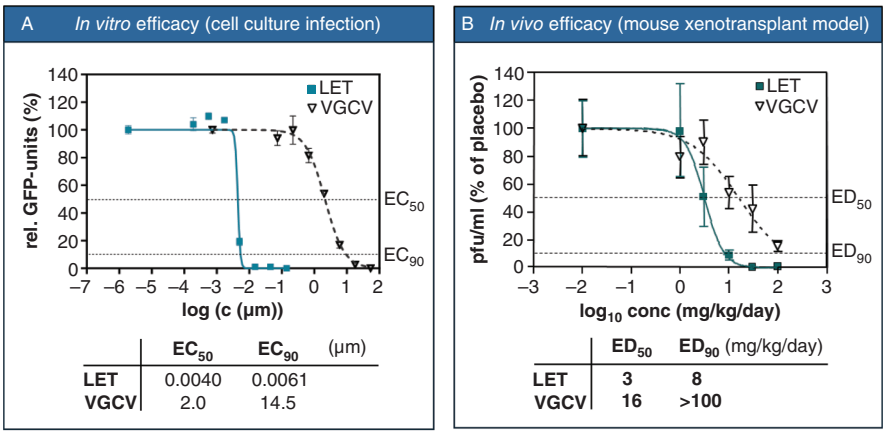


Figure 1.3 *In vitro* and *in vivo* dose-response curves for LET and VGCV.

(A) Comparison of the *in vitro* dose-response curves showing >400- to >2000-fold greater potency of LET compared to VGCV as well as the remarkably steep dose response of LET. Data were obtained from a cell culture infection model using human fibroblast cells and a GFP-expressing CMV laboratory strain. EC_{50} and EC_{90} values for both drugs are indicated.

(B) Comparison of the *in vivo* inhibition curves obtained from a murine xenotransplant model. Data confirm the relevance of the *in vitro* observation and demonstrate a substantially greater efficacy and a steeper dose response for LET vs. VGCV. ED_{50} and ED_{90} values for both drugs are indicated.

Source: Adapted from [28] / American Society for Microbiology.

Perhaps more important than the absolute potency is the selectivity index (SI), i.e. the ratio of cytotoxicity expressed as the 50% cytotoxic concentration (CC_{50}) to EC_{50}, since this underlies the subsequent therapeutic potential *in vivo*. The results showed a consistently high CC_{50} >33 µM (highest concentration tested) in various fibroblast lines, resulting in a selectivity index of at least 12 000 (median 18 000) and indicating good tolerability [28].

Interestingly, at this early stage, it was already recognized that mutant laboratory strains encoding various GCV resistance mutations in UL97 and/or UL54 retained sensitivity to LET (see Table 1.2 and Figure 1.4). This was the first indication that the target site or inhibitory mechanism of AIC246 is different from that of classical polymerase inhibitors like GCV [28, 30].

1.3.1.2 *In Vitro* Potency Versus Clinical CMV Isolates and Resistance-breaking Profile

Potency studies were then extended to clinical CMV isolates including both GCV-susceptible (GCV-S) and GCV-resistant (GCV-R) strains. Ultimately, >70 CMV field isolates were tested for LET sensitivity, out of which 35% demonstrated a GCV-R phenotype due to known GCV resistance mutations that were mapped either to the viral protein kinase UL97 or the viral polymerase UL54. Overall, there was no change in the potency profile between laboratory strains and clinical isolates or between GCV-S and GCV-R strains [28, 30, 31] (Figure 1.4). This demonstrated the broad anti-CMV

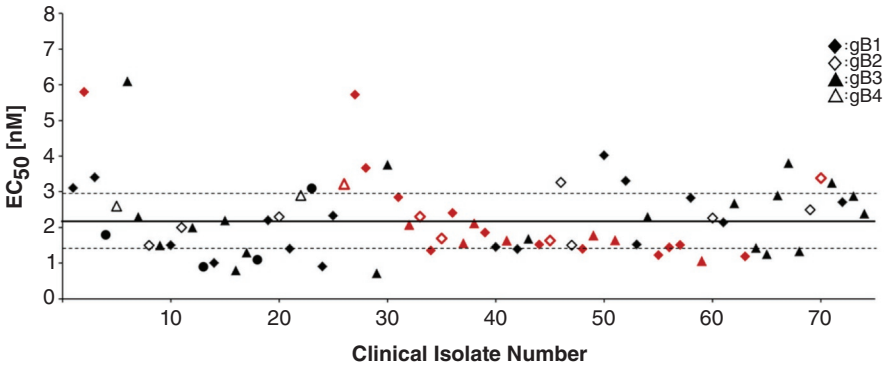


Figure 1.4 LET EC_{50} values from 74 different clinical isolates, including GCV-R strains, by gB subtype.

Black symbols show GCV-S isolates.

Red symbols show GCV-R isolates.

Filled/open diamonds or triangles indicate the individual gB subtype of each virus isolate.

Comparison of individual EC_{50} values from 74 clinical CMV isolates determined by plaque reduction assays. The overall mean EC_{50} value (thick line) ± standard deviation (dashed lines) of the tested viruses is shown. The individual EC_{50} values were shown to be in roughly the same low nanomolar range. The susceptibility of the clinical virus isolates to LET was unaffected by both gB subtype and phenotypic/genotypic GCV resistance.

Source: Adapted from [31] / with permission of Elsevier.

spectrum of this drug and confirmed that LET had an alternative MoA to the currently approved drugs, giving it a potentially resistance-breaking profile.

In addition, the studies with CMV field isolates also revealed that LET susceptibility is not associated with one of the four described CMV glycoprotein B (gB) subtypes (Figure 1.4). This is of importance since due to its involvement in CMV entry and cell-to-cell spread, some studies suggest an impact of different gB genotypes on pathogenesis or disease outcomes in immunocompromised CMV-infected patients [31, 32].

1.3.1.3 *In Vivo* Efficacy (Xenotransplant Model)

As outlined below, LET was found to be inactive against rodent CMVs like mouse- or rat CMV (MCMV; RCMV). Therefore an engineered xenograft mouse model, originally described by Chong et al. [33], was employed for the first *in vivo* evaluation of LET's efficacy. Essentially, gelfoam sponges carrying CMV-infected human fibroblasts were transplanted into mice. After vascularization of the pseudo-organ, mice were treated once daily per os for 9 days, the sponge was explanted, and the CMV titer in the human cells within the sponge was quantified.

Consistent with the prior *in vitro* data, the results showed a significant and dose-dependent reduction of the viral titer in the gelfoam after treatment. Potency was notably greater for LET than GCV, as was the gradient of the dose-dependency curve (Figure 1.3B). While 30 and 100 mg/kg/day LET dose groups achieved a 2-log reduction in viral titer by the end of treatment, 100 mg/day GCV achieved only a 1-log reduction. As seen in the previous *in vitro* studies, LET also appeared to be notably more effective than GCV *in vivo*.

1.3.1.4 *In Vitro* Antiviral Specificity

Having established LET's potency and resistance-breaking potential against CMV, LET was screened against a broad panel of other herpesviruses and other human-pathogenic viruses, as shown in Table 1.2. Interestingly, the data indicated that

Table 1.2 Summary of *in vitro* potency data for LET against CMV laboratory strains and clinical isolates, other herpesviruses, and representative human-pathogenic viruses.

Virus strain	Range of activity EC50 (μM)	
	LET	GCV
Potency vs. human CMV		
Laboratory strains	0.0020–0.0051	1.5–3.5
GCV-resistant laboratory strains	0.0016–0.0039	4.5–32
Clinical isolates	0.0001–0.0058	0.6–5
GCV-resistant clinical isolates	0.0014–0.0061	5.9–55
Potency vs. nonhuman CMV (strain)		
MCMV (Smith)	4.5	4.3
RCMV (Maastricht)	>10	0.9
RhCMV (68-1)[a]	>10	0.6
GpCMV (22122)[a]	>10	ND
Potency vs. other herpesviruses (strain)		
Alphaherpesviruses		
VZV (Oka)	>10	0.8
HSV-1 (166v VP22-GFP)	>10	2.2
HSV-2 (01-6332)	>10	2.5
Betaherpesviruses		
HHV-6 (typeA-GS)	>10	ND
Gammaherpesviruses		
EBV (B95-8)	>10	ND
Potency vs. other human-pathogenic viruses		
Adenovirus (HAdV-2)	>10	ND
Hepadnavirus (HBV HepG2.2.15)	>30	ND
Retrovirus (HIV-1 LAI)	>11	ND
Orthomyxovirus (influenza A A/WSN/33)	>10	ND
Flavivirus (HCV replicon)	>32	ND

EBV, Epstein–Barr virus; EC50, concentration at 50% maximum efficacy; GCV, ganciclovir; GpCMV, guinea pig cytomegalovirus; HAdV, human adenovirus; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV-6, human herpes virus 6; HIV, human immunodeficiency virus; HSV, herpes simplex virus; MCMV, murine cytomegalovirus; ND, not determined; RCMV, rat cytomegalovirus; RhCMV, rhesus monkey cytomegalovirus; VZV, varicella-zoster virus.

a. Lischka et al., unpublished data.

Source: Adapted from [28, 30].

LET is remarkably specific for human CMVs since no significant activity was noted against other alpha-, beta-, and gamma-herpesviruses (including CMVs from other species), or unrelated pathogenic viruses (including adeno-, hepadna-, retro-, orthomyxo-, and flaviviruses) [30].

1.3.1.5 Other Characteristics of Letermovir's Inhibitory Profile

In Vitro Inhibition of Both Focal and High Titer Infections Assays examining the cell-to-cell spread of the virus *in vitro* showed that LET could contain an active infection, almost completely preventing the spread to neighboring cells.

In addition, replication assays using varying multiplicities of infection (MOI) showed that LET's potency was more robust against high MOI infections than GCV, exhibiting only a 3-fold increase in EC_{50} across a 300-fold increase in MOI while the EC_{50} of GCV increased by a factor of 5. This interesting drug property was already suspected given the very steep dose-response curve of LET when compared to that of GCV (Figure 1.3) [28].

In Vitro Drug Combination Profile In anticipation of possible clinical comedication scenarios, *in vitro* drug combination assays were performed and showed that LET exhibited additive, i.e. nonantagonistic, effects with all approved anti-CMV drugs, indicating the theoretical potential for combination therapy if needed. Moreover, the same studies showed there were neither agonistic nor antagonistic effects apparent between LET and a large panel of anti-HIV drugs supporting the potential use of LET in CMV-HIV-coinfected individuals undergoing highly active antiretroviral therapy (HAART) [34].

1.3.1.6 Summary

The early *in vitro* and *in vivo* studies of LET's potency, selectivity, and antiviral profile demonstrated a highly potent drug with low toxicity and remarkable specificity for CMV.

While the results of these studies showed that LET may have considerable therapeutic potential and likely had an alternative MoA versus GCV and the other approved anti-CMV drugs, neither these results nor the screening process used to identify LET as a potential anti-CMV drug candidate suggested a likely MoA.

1.4 Mechanism of Action Studies

1.4.1 Target Identification

The replication cycle of CMV is divided into three sequential phases: immediate early (IE), early (E), and late (L). The IE stage begins as soon as viral DNA enters the nucleus through the expression of the IE regulatory genes. During the E phase, viral DNA replication occurs, while viral DNA packing, virus assembly, and egress of progeny virus take place during the L phase of the replication cycle (Figure 1.5, panel C). Accordingly, early MoA studies attempted to identify the point in the replicative cycle affected by LET, in order to narrow the field of likely molecular targets.

In vitro assays were used to examine the effects of drug treatment on viral protein and DNA expression profiles. LET-mediated suppression of viral replication was shown

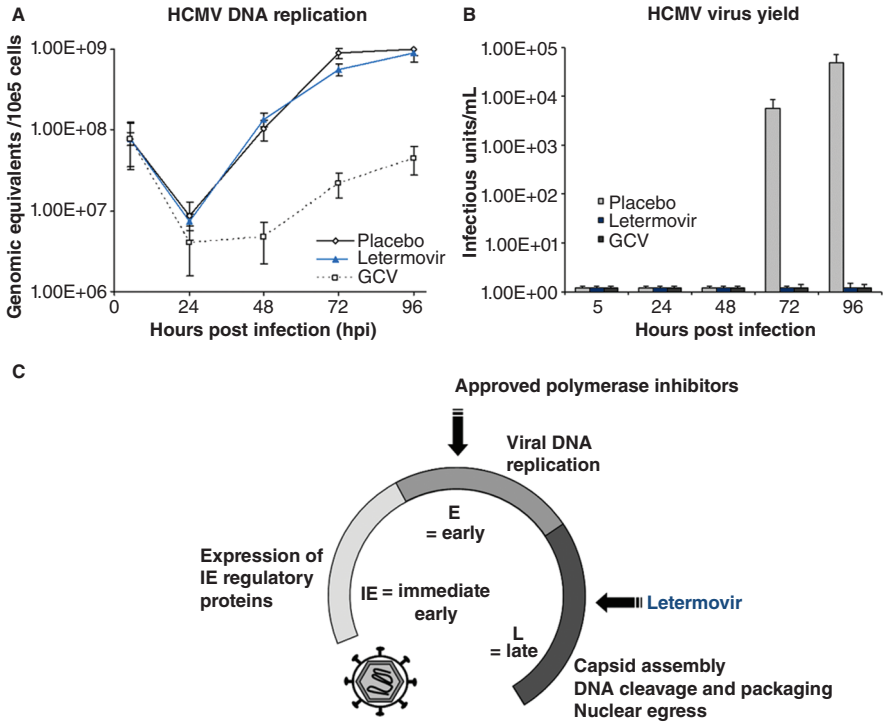


Figure 1.5 *In vitro* inhibition of viral replication without suppression of viral DNA synthesis.

CMV-infected human fibroblasts were treated with LET, GCV, or placebo at 10-fold EC_{50}. (A) Like the placebo treatment, LET had no effect on viral DNA replication; nonetheless, (B) LET treatment effectively inhibited the generation of infectious virus particles. This suggests that LET acts during the L phase of viral replication, i.e. after viral DNA replication and via a mechanism distinct from that of the DNA polymerase inhibitors. (C) CMV replication cycle.

Source: Adapted from [29] / American Society for Microbiology.

to have no apparent effect on either viral protein expression over the three phases or on viral DNA replication [29]. Thus, LET's MoA appeared to be different from that of GCV or other viral DNA polymerase inhibitors in that LET appeared to act later in the viral replication cycle, targeting a process after DNA replication that is not associated with viral protein expression (Figure 1.5). This assumption was confirmed by time-of-addition studies and is consistent with LET being active against CMV strains that are resistant to the CMV polymerase inhibitors GCV, CDV, or FOS (see above) [28, 29].

The first indication that LET might affect the cleavage and packaging of viral DNA also came from time-of-addition and recovery kinetic studies in which LET showed antiviral kinetics similar to a previously identified viral terminase inhibitor (BAY 38-4766), which was used as a "late-acting" control compound in these experiments [28, 29].

The CMV terminase is an essential multiprotein complex responsible for viral genome maturation and packaging by cleaving the concatenated viral genomic DNA formed in

the nucleus from rolling circle viral DNA replication into single genomic units and packaging these genome units into preformed viral capsids (Figure 1.6). The core viral terminase complex is composed of at least three subunits: the proteins pUL56, pUL89, and pUL51, which work together with other packaging proteins and the portal protein pUL104 to complete this process. Recent cryoEM studies suggest that the functional viral terminase complex is a hexameric structure, with each monomer itself comprising the three subunits where pUL51 and pUL56 function as regulator/fixer proteins and pUL89 harbors the ATPase/nuclease function. The terminase complex is highly conserved among different CMV strains and within herpesviruses [35–37].

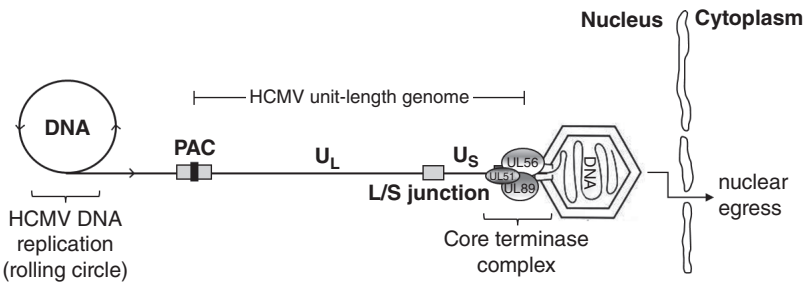


Figure 1.6 Function of the viral terminase complex.

Schematic model of CMV DNA synthesis and packaging. CMV DNA replication, the terminase complex, and the CMV genome structure are illustrated. The CMV genome is comprised of unique long (U_L) and unique short (U_S) regions separated by a region designated the L/S junction. The CMV viral terminase complex cleaves the concatemeric viral DNA at the terminase cleavage site, located within the PAC region at the genome terminus. The resulting individual genomic units are then packaged into preformed viral capsids followed by the nuclear export of mature virions into the cytoplasm (nuclear egress).

Source: Adapted from [29].

The initial idea that LET targets the CMV terminase was confirmed through molecular characterization of LET-resistant strains obtained from *in vitro* drug resistance selection studies. CMV genotyping identified individual mutations in the UL56 terminase gene capable of causing LET resistance as shown by marker transfer experiments (see also Section 1.8) [28, 29, 38]. Since all these data supported the hypothesis that LET exerts its effects via the viral terminase complex, these functions were then directly investigated using a terminase cleavage assay to investigate the processing of viral genome concatemers and electron microscopy to evaluate packaging of viral genomic DNA into nascent pre-capsids. The results showed that LET inhibits the formation of properly processed unit-length CMV genomes (Figure 1.7) and thus prevents packaging of viral genomic DNA into premature viral capsids. As a consequence, empty viral capsids (B-capsids) accumulate in the nucleus of infected cells and are not exported to the cytoplasm for further particle maturation.

Overall, the data confirmed that LET targets the terminase subunit pUL56 and thereby interferes with cleavage and packaging of CMV progeny DNA, i.e. it has a similar MoA to that proposed for the discontinued anti-CMV terminase inhibitors of the benzimidazole-ribonucleoside of sulfonamide class described below.

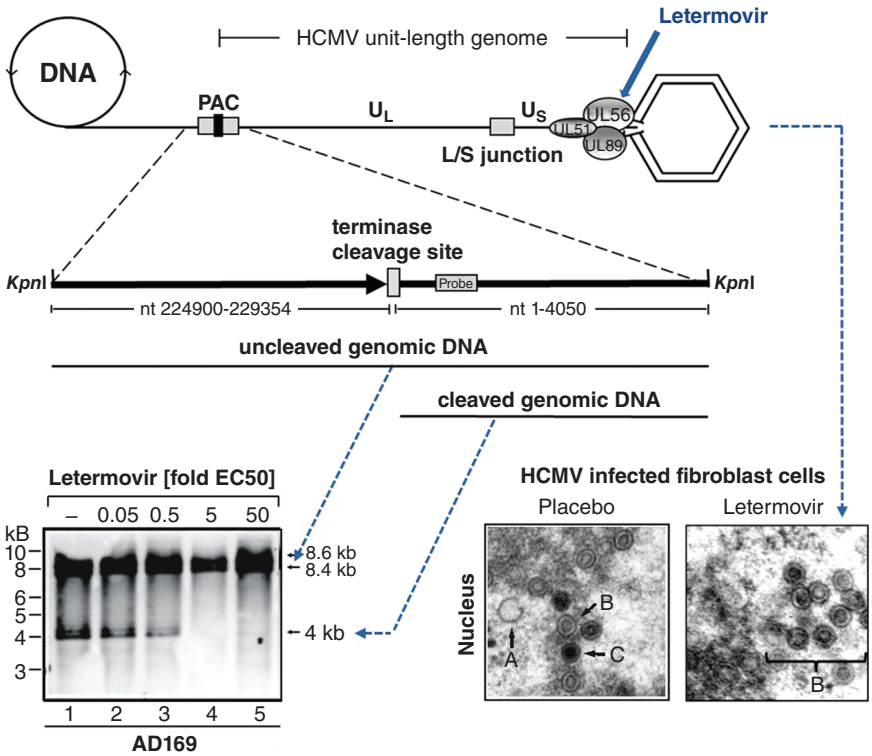


Figure 1.7 Effect of LET on viral DNA processing and packaging.

In concatemeric viral DNA, the PAC region, containing the terminase cleavage site, is flanked by two KpnI restriction sites approximately 8.6 kb apart. Inhibition of the terminase complex by increasing concentrations of LET prevents cleavage of the viral DNA concatemer at the terminase cleavage site, resulting in 8.6 kb fragments after digestion of the isolated viral DNA with KpnI. In the absence of LET, viral DNA is naturally cleaved by the terminase complex at the terminase cleavage site, resulting in ca. 4 kb fragments after digestion with KpnI.

In addition, the failure of genomic viral DNA cleavage due to the presence of LET prevents viral DNA packaging into preformed capsids and the subsequent nuclear egress of mature C-capsids into the cytoplasm. This results in the accumulation of empty B-capsids in the nucleus of infected, LET-treated cells, as shown in the electron micrographs.

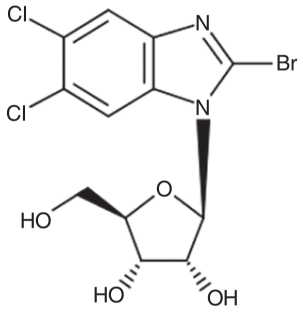
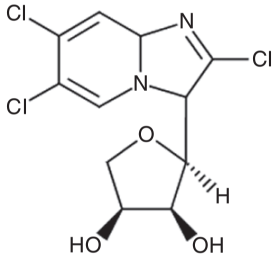
Source: Adapted from [29] / American Society for Microbiology.

1.5 Terminase Inhibitors

1.5.1 Previous and Contemporary Drug Candidates Targeting the Terminase Complex

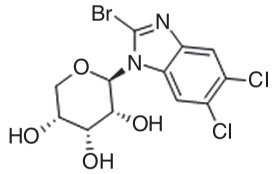
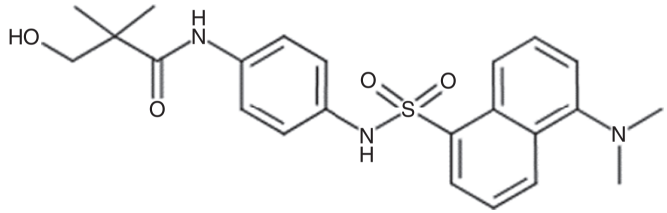
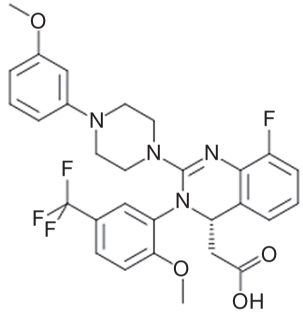
Prior to LET, there were two published chemical classes of terminase inhibitors: the benzimidazole ribonucleosides (e.g. BDCRB/TCRB and GW275175X) and the sulfonamides (e.g. BAY 38-4766) (see Table 1.3).

Table 1.3 Overview of published CMV terminase inhibitors.

Chemical class	Substance	Structure	Range of activity EC_{50} (μM)[a]	Viral genes harboring resistance mutations[b]	*In vitro* drug combination with GCV (antiviral effect)	Activity against nonhuman CMVs
Benzimidazole ribonucleoside	BDCRB		0.3–1.4	UL89 + UL56	Additive-synergistic	Yes
	TCRB		2.9	UL89 + UL56	n.d.	Yes

(Continued)

Table 1.3 (Continued)

Chemical class	Substance	Structure	Range of activity EC_{50} (μM)[a]	Viral genes harboring resistance mutations[b]	*In vitro* drug combination with GCV (antiviral effect)	Activity against nonhuman CMVs
	GW275175X		0.3–1.4	UL89 + UL56	n.d.	Yes
Sulfonamide	Tomeglovir (BAY 38-4766)		0.3–1.2	UL89 + UL56	Antagonistic	Yes
Quinazoline	LET		0.002–0.005	UL56	Additive	No

a. Antiviral activity determined against CMV laboratory strains adapted partly from [36].
b. Only genes harboring mutations conferring high-level drug resistance are listed (see text body for references).

The benzimidazole ribonucleosides were originally synthesized by Townsend et al. [39] and later found to exert their anti-CMV activity by interfering with the viral terminase complex [40, 41]. Although clinical development of the first set of these terminase inhibitors (BDCRB and TCRB) was not pursued due to unfavorable pharmacokinetics (PK) properties, medicinal chemistry efforts led to the identification of GW275175X, a D-ribopyranose derivative of BDCRB with improved *in vivo* stability. *In vitro* studies confirmed the MoA of this molecule as a terminase inhibitor, and the drug was selected by GlaxoSmithKline (now GSK plc) as a clinical candidate for the treatment of CMV diseases [42]. The drug progressed to Phase 1, where it demonstrated good safety and tolerability, but further development as an anti-CMV drug was discontinued for strategic decisions [36].

An early terminase inhibitor, identified at Bayer using the same discovery approach as LET, was the sulfonamide BAY 38-4766. It was an effective anti-CMV drug *in vitro*, and it did not inhibit viral DNA synthesis; rather, it interfered with viral genomic processing and packaging [27, 43]. BAY 38-4766 had an EC_{50} in the 0.5–2 μM range and was active against GCV-R strains of CMV [27]. Drug resistance selection studies demonstrated that BAY 38-4766 acted via inhibition of the viral terminase complex, mediated mainly by interaction with the UL89 gene product [43]. Despite its very promising antiviral properties, the development was stopped in Phase 1 because the drug induced CYP enzymes, which led to very low exposure levels in humans.

1.5.2 Letermovir: Same Target, Different Interaction

Although LET shares the overall mode of action with other terminase inhibitor molecules, studies have shown that the molecular interaction between LET and the terminase complex appears to be quite distinct. This is supported by a comparison of the resistance and activity profiles of LET with those of other terminase inhibitors of the structurally distinct sulfonamide or benzimidazole-ribonucleoside classes (see Table 1.3). There is no relevant cross-resistance between LET and other terminase inhibitors [29, 38]. Indeed, at the molecular level, and in contrast to other cleavage-packaging inhibitors [29, 38–41, 43, 44], LET resistance mutations are essentially confined to UL56 and almost entirely to a single and distinct region (see Section 1.8) while the UL89 terminase subunit gene is essentially irrelevant to LET resistance. In addition, the activity spectrum of LET is also highly selective for human CMV and inactive against CMVs of other species, in marked contrast to the broader anti-CMV activity of the other terminase inhibitors [27, 30, 45–47]. Finally, a distinct LET drug combination profile in terms of agonism/antagonism has also been described for LET versus other approved anti-CMV drugs (i.e. polymerase inhibitors) [34].

1.5.3 Advantages of Terminase Inhibitors

To date, LET is the only terminase inhibitor to be granted marketing approval. LET's paradigm-shifting potential is largely a result of its molecular target (pUL56 of the viral terminase complex), or rather, it results from (i) the fact that it does not target the same molecule (viral DNA polymerase) as *all* available anti-CMV drugs at the

time and (ii) the fact that LET's target has no direct equivalent in the human host cells. This makes the development of LET resistance independent of resistance to other anti-CMV treatments and thus also enables a genuine rescue therapy option.

In addition, the lack of an equivalent mammalian target also reduces the likelihood of toxicities by minimizing potential interaction with host pathways.

Interestingly, a further potential advantage of LET over polymerase inhibitors has been proposed in the context of prophylaxis: the potential low-level exposure to viral proteins without the release of viable capsids. Depending on the duration of treatment, this low-level exposure may facilitate immune training and reduce the risk of late-onset disease, though the impact of this remains to be confirmed.

1.6 Preclinical Safety Evaluation

To support a long-term prophylactic treatment in patients, a comprehensive evaluation of potential safety pharmacological and toxicological risks was conducted before testing of LET in humans was initiated.

For safety pharmacological investigations, LET was administered to rats and dogs at single doses of up to 45 mg/kg to determine the effect on electrocardiogram (ECG), the cardiovascular system, the respiratory system, the central nervous system, renal function, lipid metabolism, or hematology, blood glucose concentration, and the GI tract. Taking all the data from these safety pharmacological studies together, there were no findings that hindered the clinical development of LET.

Of note, discouraging bioavailability data and emesis noted in the dogs after oral administration of LET disqualified the dog as a model species in this drug development program, and, therefore, only very limited toxicity and toxicokinetic data were generated in dogs. Instead, the monkey was selected as the appropriate nonrodent species for toxicity profiling of LET. The monkey showed high systemic exposure to the compound after oral administration, and it is phylogenetically close to humans.

As a prerequisite for first human studies, subacute (4-week) oral toxicity studies with LET were also performed in a rodent (rat) and a nonrodent (monkey) species. Acute toxicity studies were conducted in rats and mice (oral and IV).

To evaluate the genotoxic potential of LET, *in vitro* and *in vivo* mutagenicity studies were performed.

Subsequently, subchronic to chronic oral toxicity studies in mice (13-week), rats (26-week), and monkeys (39-week), fertility studies in rats and monkeys (sexually mature), carcinogenicity studies in rats and mice, and developmental toxicity studies in rats and rabbits were conducted. In addition, data from a pre- and postnatal toxicity study in rats were evaluated together with appropriate toxicokinetic evaluations.

Moreover, IV dose toxicity studies in rats and monkeys and a local tolerability study in rabbits were carried out to support IV dosing in humans as well.

In summary, LET demonstrated an overall favorable toxicological profile leading to a very positive risk/benefit ratio for the prophylactic treatment in the indicated patients.

1.7 Clinical Development and MAA/NDA Submission

1.7.1 Regulatory Support for Clinical Development

In both the United States and European Union, supportive programs are offered by the regulatory agencies to encourage the development of new drugs where there is a serious unmet medical need (Fast Track) and/or the disease is rare (orphan drug; <200 000 patients in United States, and 5 patients in 10 000 residents in European Union).

These programs facilitate frequent communication with regulatory agencies during drug development for serious or life-threatening diseases. They offer accelerated review and approval times, extended market exclusivity, and cost/tax benefits, which are crucial incentives for drug development in complex niche indications.

The US Food and Drug Administration (FDA) granted Fast Track designation for LET in May 2011. European Medicines Agency (EMA) granted Orphan Drug Designation in April 2011 and the FDA in December 2011.

1.7.2 Phase 1

Phase 1 data confirmed that LET was well suited to IV and oral dosing; oral tablet dosing is particularly advantageous in such a patient population, facilitating simple dosing without the need for an outpatient visit as would be required for IV administrations. However, the anticipated poor condition of the target patient population and the broad range of expected concomitant medications necessitated an extensive Phase 1 program in addition to the standard safety, dose finding, and absorption, distribution, metabolism, and excretion (ADME) characterization studies before LET development could be moved into patients.

1.7.2.1 Drug–Drug Interaction Studies

Early *in vitro* enzyme inhibition studies had suggested that LET may interact with other drugs, including commonly used concomitant medications for immune suppression, such as tacrolimus or cyclosporine [48]. Considering especially the key immune suppressants essential for clinical management of organ or stem cell recipients, appropriate Phase 1 studies were performed.

Drug–drug interaction data show LET to be a substrate and moderate inhibitor of CYP3A and a substrate of 2D6 and is predicted to induce CYP2C9 and 2C19. LET is also a substrate and inhibitor of OATP1B1, 1B3, P-gp, and an inhibitor of OAT3, BCRP, BSEP, and MRP-2.

PK analyses based on the Phase 2b trial (see below) in HSCT patients resulted in LET dose adjustment (50% dose reduction) for patients receiving concomitant cyclosporine [49, 50].

1.7.2.2 Special Populations

In addition, based on the predictable comorbidities in the target population, studies were performed examining the effect of renal and hepatic impairment on LET PK. As may be expected based on LET's predominantly biliary excretion [51, 52],

moderate and severe hepatic impairment led to increased exposure [51]. Perhaps less predictably, considering the negligible renal excretion, renal impairment also led to slight but not clinically relevant increases in exposure, but no dose adjustment based on renal function was required [52].

Taken together, this meant that LET could not be recommended for patients with severe hepatic impairment or for those with moderate hepatic impairment with at least moderate renal impairment.

1.7.2.3 IV Formulation

In HSCT recipients, a period of high risk for CMV reactivation is immediately following transplantation and prior to engraftment. LET's safety profile enables administration during this time. However, in the first days post-transplant, patients may be unable to swallow oral formulations. Therefore, an effective CMV prophylaxis in the HSCT population must also be available in a parenteral formulation. Following successful proof-of-concept (PoC), AiCuris developed the hydroxypropyl beta-cyclodextrin formulation of LET to permit parenteral administration [53].

Please note: Phase 2 studies in HSCT subjects proceeded with the oral formulation only, partly because established clinical practices at the time had developed to specifically avoid using anti-CMV drugs pre-engraftment due to their myelosuppressive toxicities. The IV formulation was introduced in Phase 3 when inclusion was allowed directly after transplantation.

1.7.3 Clinical Proof-of-concept

Based on the population of greatest need, the development program was clearly focused on CMV prophylaxis in the HSCT recipient population. Especially HSCT patients (and particularly the R+/D− patients) were effectively unprotected during the window of maximum risk, i.e. before immune reconstitution, and therefore at profound risk from CMV viremia and disease (see Figure 1.1). The well-known toxicities of available drugs prohibited their use in prophylaxis, forcing the use of preemptive strategies. However, although this may lessen the risk of disease, any degree of virus replication, including subclinical, can negatively impact patient outcomes [11, 12]. This population, therefore, showed the greatest probability of achieving a superior risk/benefit for LET versus existing CMV treatments.

1.7.3.1 Phase 2a Clinical Trial (AIC001-2-001)

An initial PoC trial was designed for the preemptive treatment of CMV in HSCT recipients as the intended primary target population, reflecting the need for (i) a like-for-like comparison of LET versus standard of care treatment regimens, (ii) a maximal permitted LET treatment period of just 14 days, and (iii) a maximum dose of 80 mg daily due to limited preclinical and clinical data at the time. Based on these considerations, the AIC001-2-001 trial was initially designed using a preemptive treatment approach in HSCT recipients, i.e. treatment would be initiated upon detection of viremia [54], although the overall goal of the development program remained CMV prophylaxis. In practice, the risk associated with the use of a novel and, therefore, clinically unproven preemptive treatment in a highly vulnerable

viremic HSCT patient was perceived as too high, and recruitment failed with only one patient recruited.

The trial population was therefore switched by protocol amendment to the kidney or kidney/pancreas transplant population, again using preemptive treatment with 40 mg LET twice daily (b.i.d.), 80 mg LET once daily (q.d.), or local standard of care determined using a 1 : 1 : 1 randomization scheme. Patients were CMV-positive with no signs of severe systemic infection, end-organ CMV disease, GvHD, liver/renal dysfunction, positive hepatitis B virus (HBV)/HIV infection, diarrhea, severe gastrointestinal (GI) disease, recent treatment with GCV, CDV, or FOS, or treatment with CYP3A4 inducers/inhibitors. This time recruitment progressed well, and 27 subjects were selected and treated.

The primary endpoint for the trial was the decline in CMV DNA versus baseline over a 14-day treatment period. Observations of changes from baseline in viral load at day 15 showed statistically significant reductions in CMV copy numbers in all three treatment groups (LET 40 mg b.i.d.: $P = 0.031$, 80 mg q.d.: $P = 0.018$, standard of care [SoC]: $P = 0.001$); differences between groups were not significant and could reasonably be attributed to baseline differences and the small trial population. Interestingly, the proportion of subjects achieving viral clearance appeared higher for the LET groups (6/12, 50%) compared with the SoC (2/7, 29%). Presumably due to the measure used (viral DNA copy number in blood), the apparent kinetic of viral inhibition was also notably different between LET and SoC (GCV/VGCV), with reported viremia values dropping from day 4 under GCV treatment versus day 11 under LET. This likely reflects the different MoAs of the two drugs (LET does not inhibit DNA replication but does prevent the maturation of infectious virus particles) and provides a caveat in the interpretation of such data.

PoC could, therefore, be considered established. Overall, LET appeared to be at least similarly effective to SoC within the analyzed time frame, and the drug was safe and well tolerated [54].

1.7.3.2 Emergency IND Treatment of a Lung Transplant Patient with Multiresistant CMV Disease

Shortly after the completion of the PoC trial, further successful demonstration of LET's potential was provided via the Emergency Investigational New Drug (eIND) treatment of a lung transplant recipient with severe refractory and multidrug-resistant CMV disease [55]. The patient had been viremic for 5 months and exhibited severe, disseminated, refractory, multidrug-resistant CMV disease of the lungs, GI tract, and retina. By the time of the LET eIND treatment, the patient had already received numerous approved and off-label drugs: GCV, FOS, CDV, leflunomide, CMV hyperimmune globulin, CMX-001 (an experimental, lipid-conjugated derivative of CDV), as well as an artemisinin derivative, all without success.

LET was obtained for emergency treatment since all other treatment options had been exhausted. The subject received 120 mg/day LET for 16 days. Because plasma concentrations were then shown to be toward the lower end of the established safety range based on early Phase 1 data in healthy volunteers, the dose was increased to 240 mg/day.

By the end of the 49-day total treatment, the CMV viral load was below the LLOQ (<600 copies/mL), and clinical evidence indicated ongoing resolution of the lung,

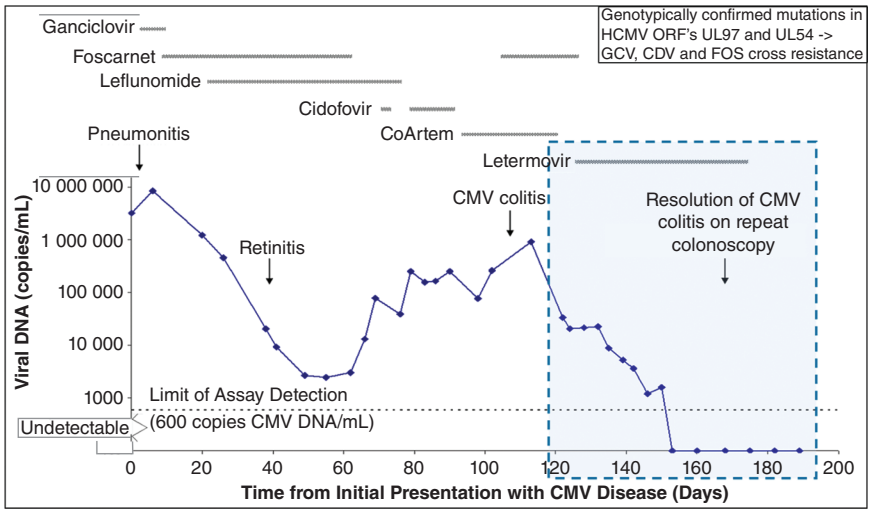


Figure 1.8 Clinical demonstration of LET's efficacy in treatment of an eIND patient with multidrug-resistant CMV.

Viral load in a lung transplant recipient with disseminated, refractory, multidrug-resistant CMV infection and CMV disease in lungs, GI tract, and retina. After exhausting all available approved and off-label anti-CMV treatments, as well as some experimental drugs (top panel), LET treatment was initiated under an emergency IND obtained from the FDA.

By the end of a 49-day LET treatment period, the virus was undetectable and the disease was resolving.

Source: Adapted from [55] / with permission of Elsevier.

GI, and retinal disease (see Figure 1.8). Three months after completion of treatment, the subject still had no detectable CMV viremia, and there was no sign of rejection in the lungs.

These results not only supported the efficacy and safety conclusions of the Phase 2a trial, but they also demonstrated the tolerability of the 240 mg/day dose and the enormous potential of LET for life- and organ-saving interventions in such multidrug-resistant cases.

1.7.3.3 Credentials Established

Based on the results from the PoC trial and the additional eIND treatment of a severely ill lung transplant patient with severe, disseminated, drug-resistant CMV disease, the case for LET's potential had been well made. On the clinical side, this appeared to be sufficient to allay remaining concerns relating to LET's use in a highly vulnerable patient population. On the business side however, current offers from out-licensing partners still contained substantial price reductions because they assessed the remaining development risks as high. In contrast, AiCuris shareholders were rather convinced by the existing data and saw further investment into Phase 2b clinical trials as justified taking into account the future potential of the novel drug. Development up to end-of-phase 2 was therefore pursued at AiCuris rather than taking the less advantageous out-licensing deals already available.

1.7.4 Letermovir for CMV Prophylaxis in HSCT Patients

1.7.4.1 Phase 2b: First Prophylaxis Trial in HSCT Patients

Having established PoC, the first clinical trial in the ultimate target HSCT population was a dose-ranging trial (AIC246-01-II-02) designed to compare the efficacy and safety of LET with placebo for CMV prophylaxis [50]. Because the potential for hematotoxicity through LET could not yet be excluded at that time, prophylaxis was started post-engraftment.

A total of 131 R+ HSCT recipients were recruited and randomized 1 : 1 : 1 : 1 to 12 weeks CMV prophylaxis with either 60, 120, or 240 mg/day LET or placebo. Placebo was a justified comparator since there was no alternative prophylaxis available, and patients who developed CMV viremia or disease discontinued and received local SoC preemptive treatment as a rescue therapy.

The primary endpoint in this trial was the rate of all-cause prophylaxis failure. Secondary analyses included specific analyses of virological failure rates and time-to-onset analyses. After 12 weeks of treatment, LET showed a powerful, dose-dependent, and statistically significant reduction versus placebo in all-cause prophylaxis failure (LET: 48% at 60 mg/day, 32% at 120 mg/day, 29% at 240 mg/day compared with placebo: 64%) (Figure 1.9; Panel A). In line with this, the time to onset of prophylaxis failure was also significantly reduced by LET. However, the clearest demonstration of LET's efficacy in this setting was the complete prevention of virological failure at the maximum 240 mg/day LET dose after the exclusion of patients who were already CMV-positive at baseline (Figure 1.9b). Again, safety data showed no findings of concern associated with LET prophylaxis. Especially regarding potential future treatment regimens, comparisons versus placebo showed no indication of either (i) hematotoxicity, i.e. leukopenia or neutropenia, or (ii) renal toxicity. LET's safety profile was, therefore, sufficiently clean to open the way to early initiation of prophylaxis, even before engraftment, meaning LET may even fulfill the clinical ideal of immediate prophylaxis following transplantation, covering the period of maximum risk.

The importance of these results was not lost on the wider anti-infectives industry either. LET's emerging product profile and the lack of a potential alternative CMV prophylactic drug enabled AiCuris to secure the largest German pharmaceutical deal of 2012, by out-licensing LET to Merck & Co., Inc., Rahway, NJ, USA (hereafter: MSD) [56]. AiCuris received a €110 million upfront payment and was eligible for milestone payments of up to €332.5 million based on the successful achievement of development, regulatory, and commercialization milestones. This was a remarkable result for such a young pharmaceutical company and one which earned the company's leadership the German Future Prize of 2018.

Subsequent pivotal trials, marketing approval applications, and indication extensions proceeded through MSD.

1.7.4.2 Phase 3 CMV Prophylaxis Trial in HSCT Patients

The pivotal trial MK-8228-001 compared the safety and efficacy of LET or placebo for CMV prophylaxis in HSCT recipients [49]. Over a period of nearly 2 years, a total of 565 patients from 67 centers in 20 countries were randomized (2 : 1) and

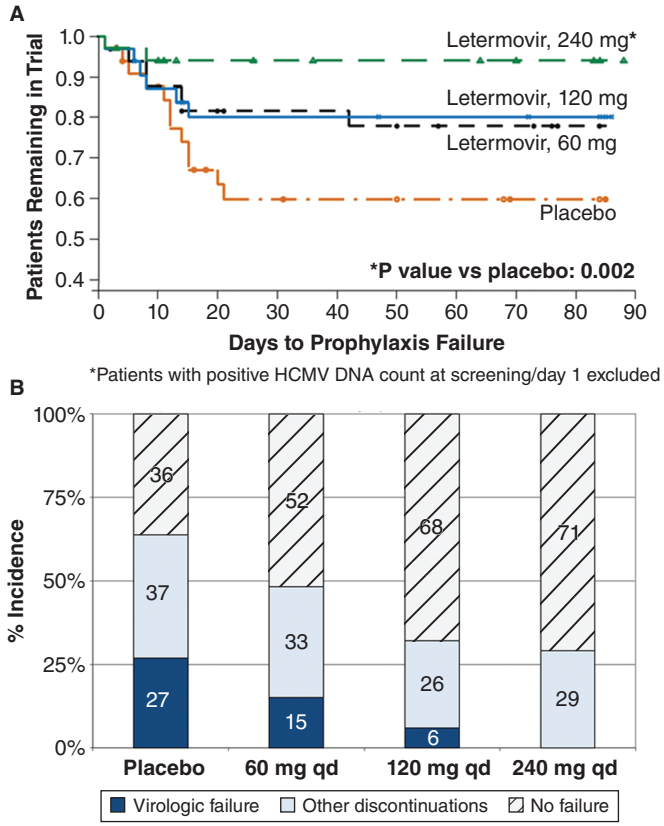


Figure 1.9 Dose-dependent protection of CMV reactivation in HSCT patients (Phase 2b trial).

(A) Kaplan–Meier plot of time-to-failure in the first (Phase 2b) trial of LET prophylaxis in HSCT patients. Subjects who discontinued treatment for reasons other than treatment failure or who were lost to follow-up were censored at the time of discontinuation or at the time of last contact with the center. All subjects who completed treatment were censored at the time of last dose.
LET significantly reduced time-to-failure (all-cause failure including CMV viremia or disease). The effect was dose-dependent and statistically significant (P = 0.007) for the highest dose group of 240 mg/day).

(B) Treatment failure rates are shown as virological failure (dark blue; viremia or CMV disease) or any other cause for discontinuation (light blue). LET treatment completely prevented CMV viremia and disease at the highest test dose of 240 mg, once daily (P = 0.002). The effect was profound and dose-dependent.

Source: Adapted from [50].

treated with either 480 mg/day LET (240 mg/day if on cyclosporine) or placebo. Treatment was started within 28 days post-transplantation and lasted up to week 14 (ca. 100 days) post-transplantation. Importantly, engraftment was not a prerequisite for treatment initiation in this trial.

The primary endpoint agreed with regulatory agencies and clinicians was clinically significant CMV (csCMV, defined as either CMV disease or CMV viremia

warranting preemptive treatment) up to week 24 post-transplantation in patients without viremia at baseline (per central laboratory), thereby including a further 10-week observational period after cessation of prophylaxis. Early discontinuations or missed week 24 data were imputed as meeting the csCMV endpoint (i.e. as prophylaxis failures). Patients were followed up to week 48.

The results again showed LET to be highly effective, this time at reducing the clinically defined CMV endpoint. Of the 495 patients treated in the trial and confirmed as viremia negative at baseline, 37.5% (122/325) of LET-treated patients versus 60.6% (103/170) placebo-treated patients met the primary endpoint; this result was highly statistically significant ($P < 0.001$) and did not result from differences in discontinuations/missing data that were comparable between groups. Looking only at the csCMV rates, 17.5% (57/325) of LET-treated patients had csCMV compared with 41.8% (71/170) of placebo subjects (Figure 1.10; Panel A). Importantly, mortality rates were also significantly reduced in the LET group at 24 weeks post-transplantation (10.2% vs. 15.9%; $P = 0.03$), and numerically at week 48 ($P = 0.12$) (Figure 1.10; Panel B) compared to the placebo group. Subgroup analyses showed that these benefits (differences between treatment groups) were greater for high-risk patients.

Particularly important, nearly 2/3 of patients treated did not have engraftment at the start of treatment. LET treatment had no discernable effect on engraftment times and did not affect the observed rates of GvHD, infections, or relapse (in underlying hematologic disease).

Although minor side effects were noted in this larger Phase 3 population, LET continued to demonstrate a very positive safety profile, with no major side effects.

1.7.4.3 Marketing Approval in HSCT Recipients

The results of the HSCT development program demonstrated effective CMV prophylaxis, with very few side effects and no discernable myelo- or nephrotoxicity, and are suitable for use immediately after transplantation. This was clearly a huge advancement on the only previous option of preemptive intervention with a toxic drug to limit an already active and, therefore, harmful infection.

In November 2017, marketing approval was granted by the US FDA [57], and in January 2018 [58], by the EMA for prophylaxis of CMV infection and disease in adult R+ allogeneic HSCT recipients.

1.7.4.4 Further Clinical Development and Real-world Data

After approval, the number of HSCT patients to benefit from CMV prophylaxis rapidly increased. With the growing number of patients, the amount of real-world data available for meta-analyses also grew, and confirmed the picture already suggested by the Phase 3 controlled clinical trial, that LET primary prophylaxis was a highly effective and safe new drug associated with a significant reduction in (i) CMV reactivation, (ii) csCMV infection, and (iii) CMV disease after allogeneic HSCT. Building on these new data, a recent meta-analysis combining multiple real-world studies, including a large number of outcome events, has now also demonstrated what was already suggested by the Phase 3 study, that primary prophylaxis with LET is also associated with a significant reduction in all-cause mortality and nonrelapse mortality beyond

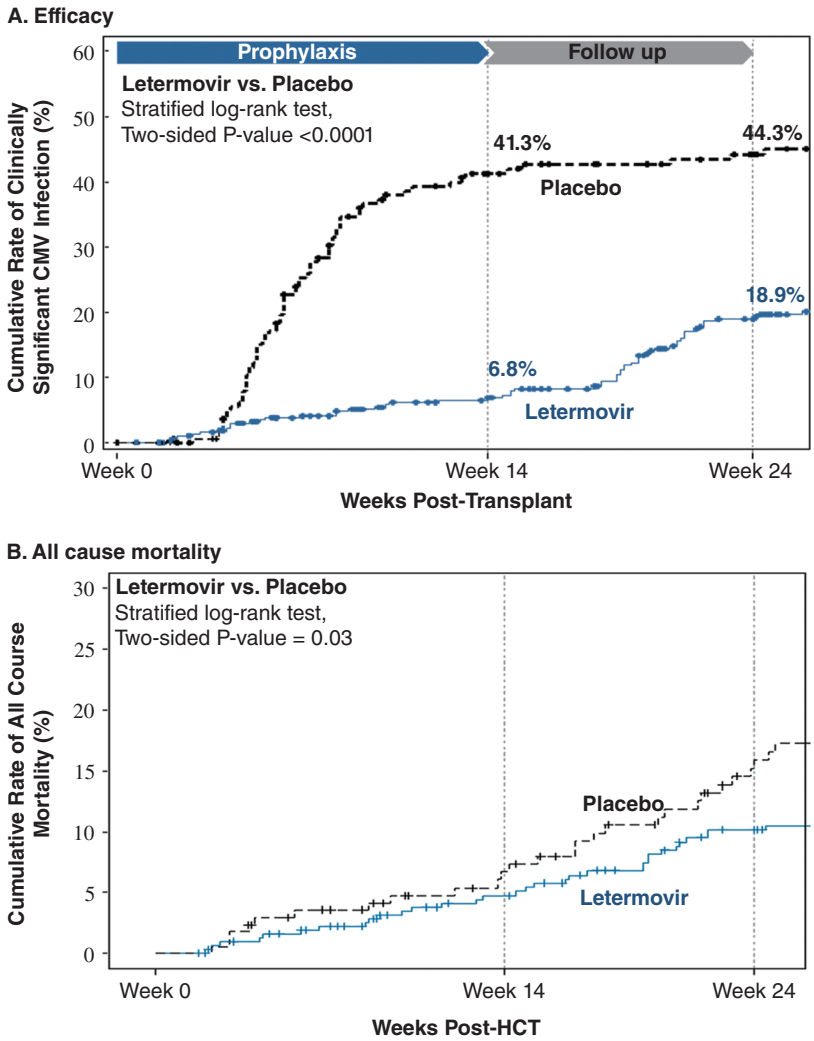


Figure 1.10 Efficacy and all-cause mortality for LET prophylaxis vs. placebo-treated HSCT patients (Phase 3 trial).

(A) The incidence of csCMV was significantly reduced by LET prophylaxis vs. placebo in HSCT recipients, both during prophylaxis and during follow-up after cessation of therapy.

(B) Mortality was significantly reduced in the LET prophylaxis group vs. placebo at week 24.

Source: Adapted from [49].

week 24 post-HSCT [26]. Reasons for the observed mortality benefit of LET prophylaxis compared to the (V)GCV PET control group are (i) the potent antiviral activity of the drug preventing, reducing, or delaying CMV-related complications and (ii) the reduced need to treat patients preemptively with antiviral agents that are associated with serious toxicities.

Though LET is very efficient in suppressing virus reactivation while patients are on therapy, the incidence of csCMV increased again after discontinuation of the drug on day 100 post-HSCT, particularly in patients who remained at high risk of

CMV reactivation (see Figure 1.10). This finding indicates that some patients experience late CMV infections after cessation of the drug [26, 49].

Considering LET's safety profile, along with the aforementioned observations and taking into account results of some observational studies showing that high-risk patients benefited from extended LET prophylaxis [59–61], it was felt likely that extension of the prophylaxis period would be safe and may help reduce the incidence of these so-called late-onset infections [62]. This would also be consistent with the approach taken in other transplant populations (e.g. KT, below), and a new Phase 3 trial was started to compare the safety and efficacy of 100 vs. 200-day prophylaxis periods for safety and efficacy.

1.7.4.4.1 Phase 3 Trial for Extension of the Prophylaxis Period

In this randomized, placebo-controlled trial, 255 HSCT patients who had completed 100 days of LET prophylaxis with no incidence of csCMV and who remained at high risk for CMV reactivation were randomized 2 : 1 to 100 additional days of prophylaxis with either LET or placebo [63]. The primary endpoint was the occurrence of csCMV from randomization (on day 100 after HSCT) up to day 200 (week 28 after HSCT). Secondary comparisons included csCMV up to week 48 as a follow-up.

The results showed that the incidence of csCMV at 28 weeks post-transplantation was significantly reduced by 200 vs. 100 days LET prophylaxis (3% vs. 19% incidence). At the same time, tolerability and safety (including all-cause mortality) were comparable between 100- and 200-day treatment periods.

Interestingly, there was no additional difference in all-cause mortality from randomization to week 28 or week 48 when comparing the 100- and 200-day prophylaxis groups. This suggests that the survival benefit demonstrated for LET prophylaxis (see above) is most likely related to the fact that LET prevents CMV reactivation in the first 100 days post-transplant, when the patients are most vulnerable. Whether there is still a "small" survival benefit beyond 100 days will become apparent when a more extensive database of patients treated for 200 days is available.

In summary, the results of this study suggest that extended-duration LET may be a good option for patients who have multiple risk factors for CMV reactivation. Prolonging LET prophylaxis in HSCT patients further reduces the incidence rates of (i) csCMV infections, (ii) indirect CMV effects like secondary infections (e.g. invasive fungal), (iii) exposure to toxic drugs like GCV, FOS, and CDV, (iv) drug resistance, and (v) is safe and well tolerated.

Later occurrence of CMV infection might mean that reactivation occurs in the setting of a more mature immune system, which could be beneficial, potentially reflected in the early survival benefit of LET compared with the absence of survival benefit seen in this study.

The extension of HSCT treatment to 200 days was granted by the FDA in August 2023 [64] and by the EMA in October 2023 [65].

1.7.4.4.2 Follow-up Trials in Specific Populations

Pediatric HSCT Recipients The pediatric population remained a group in urgent need with no approved treatment. Although publications demonstrated off-label use of LET, no formal approved guidance was available [66, 67].

A further Phase 2b trial was undertaken to investigate the PK, safety, tolerability, and efficacy of 100 days of LET prophylaxis in the pediatric HSCT population [68]. Starting with 12–18-year-olds, the trial progressed stepwise through <12 to 2-year-olds, and then 2-year-olds to newborns. Dosage was determined (and reduced for patients on concomitant cyclosporin A [CsA]) to achieve comparable exposure to the approved adult dose. Safety data and PK models were reviewed and modified as necessary between age cohorts.

The results confirmed the efficacy and safety for all age groups. Incidence rates of csCMV after 100 days of prophylaxis were broadly comparable to previous trials in adult HSCT patients (19.6% at week 14, 25.0% at week 24).

1.7.5 Letermovir for CMV Prophylaxis in KT Patients

As described in the background section, the high-risk population among SOT recipients is different from that of HSCT recipients – but for the same reasons: the elevated risk arises from a CMV infection/reactivation in the setting of a CMV-naïve immune system. In this case, D+/R− [69, 70], representing ca. 20% of all KT recipients [71]. Interestingly, and perhaps somewhat unexpectedly, the proportion of R− recipients and, therefore also, the total number of D+/R− high-risk patients appears to be increasing in the kidney recipient population and is projected to continue to grow for at least the next 20 years [71]. This is of particular importance because a primary CMV infection in these high-risk KT patients is associated with more severe illness and worsening outcomes [72]. Given this, effective measures to prevent or control CMV infections are warranted.

Prevention of CMV disease is not restricted solely to preemptive treatment in this population. According to the current consensus guidelines, VGCV and GCV are the recommended agents of choice for CMV prophylaxis and preemptive treatment in kidney transplantation, whereby high-risk D+/R− patients generally receive universal prophylaxis with VGCV [70, 73]. SOT patients generally have intact hematopoietic systems and are relatively resilient to GCV toxicity compared to HSCT recipients. In this setting, prophylaxis may be extended for up to 200 days, as has been standard for over a decade [74]. This extends protection beyond the period of maximum risk and reduces the likelihood of late-onset CMV thereafter [74, 75]. Despite this, CMV disease still occurs in up to 50% of high-risk D+/R− SOT patients despite prevention and in 17% of R+ patients [17].

Although 200-day prophylaxis with VGCV is quite effective in preventing CMV reactivation, it is nonetheless associated with toxicities, including increased rates of leukopenia/neutropenia (especially when given with toxic concomitant medications) and these can necessitate interruptions in GCV, immunosuppression (IS), or antimicrobial treatments, leading to increased rates of infection, rejection, and organ loss. Indeed, neutropenia is reported by 30–40% of SOT patients in the first year post-transplantation, largely drug-related [17].

Long-term use of GCV also increases the likelihood of resistance to polymerase inhibitors (discussed below). Beyond the risk to the patient, these issues also bring substantial complications to case management, consume more resources, and increase costs.

In the KT population, especially the D+/R− high-risk patients, there was a clear medical need for an effective anti-CMV drug without the issues of myelotoxicity and the need for dose adjustments related to renal function [49, 75]. Based on its safety and efficacy profile in the PoC trial and in the Phase 2b and Phase 3 HSCT trials, LET appeared well placed to offer equivalent efficacy to (V)GCV while avoiding the risks to patients' outcomes from renal- and myelo-toxicity.

1.7.5.1 Phase 3 Noninferiority Trial in KT Recipients

In 2018, following FDA approval of LET for prophylaxis of CMV infection and disease in adult R+ allogeneic HSCT recipients in 2017, MSD began a Phase 3 noninferiority comparison of LET versus VGCV in high-risk (D+/R−) kidney transplant recipients.

Over a 2-year period, the trial recruited a total of 601 patients, randomized 1:1 to 480 mg/day LET (240 mg/day if taking cyclosporine) or 900 mg/day VGCV (adjusted for renal function) beginning not later than day 7 post-transplantation [75]. Consistent with the SoC in this population, the treatment period in this trial was up to 200 days post-transplant, with follow-up at 1 year post-transplantation. The primary comparison was the incidence of CMV disease up to week 52 based on blinded adjudicator assessments, and secondary analyses included CMV disease by 28 weeks post-transplantation.

The results confirmed the noninferiority of 200 days prophylaxis with LET versus VGCV in the prevention of CMV disease with failure rates of 10.4% (LET) versus 11.8% (VGCV) up to 52 weeks post-transplantation. The majority of failures resulted from late-onset disease from a viral rebound in the post-treatment period. Indeed, no LET-treated patients developed CMV disease during the 200-day treatment period, compared to 1.9% of VGCV patients. Incidence rates of viral DNAemia during and post-treatment were consistent with adjudicator-based disease rates. Clearly, LET was at least as effective as VGCV in this population. Nonetheless, the data show some patients had reactivation even after 200 days of prophylaxis, suggesting further extension of prophylaxis may benefit some patients. Ultimately, monitoring of CMV immunity may help in determining the optimal time point for cessation of prophylaxis [72].

As in previous clinical trials, the safety data showed the clear advantage of LET over VGCV: rates of leukopenia or neutropenia were significantly reduced in LET-treated patients versus VGCV (26% vs. 64%, $P < 0.001$) (Figure 1.11) and led to treatment discontinuation in just 1.4% (LET) versus 5.4% (VGCV). Overall drug-discontinuation rates due to adverse events were also notably reduced: 4.1% (LET) versus 13.5% (VGCV).

Overall, the results showed a clear safety advantage for patients receiving LET rather than VGCV. The lack of need for dose adjustment is a further advantage for the clinical management team, particularly in the KT population, resulting in reduced burden and costs.

1.7.5.2 Marketing Approval in KT Recipients

Marketing approval of LET for the prevention of CMV disease in high-risk adult KT recipients was received from the US FDA in June 2023 [64] and EMA in October 2023 [65].

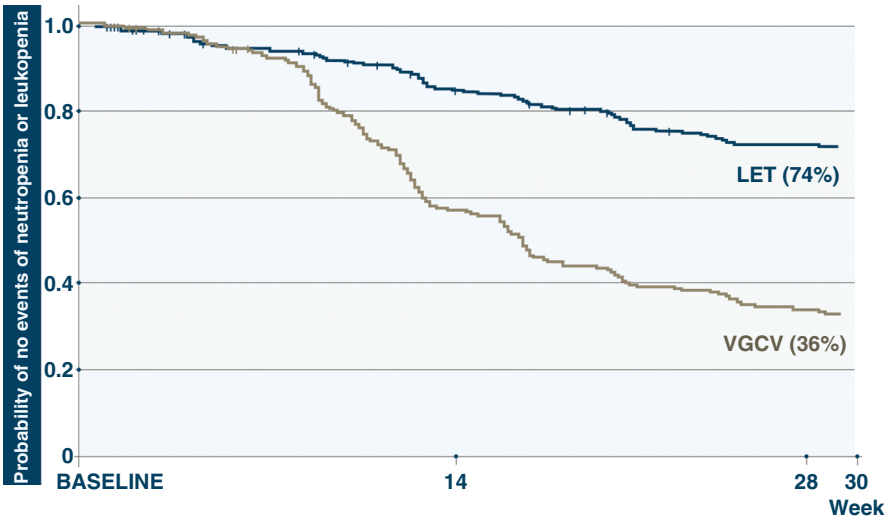


Figure 1.11 CMV prophylaxis with LET greatly reduces the rates of neutropenia and leukopenia compared with VGCV in KT patients (Phase 3 trial).

Event free (neutropenia/leukopenia) probability.

In addition to the comparable efficacy of LET vs. VGCV for prevention of csCMV in KT patients, the rates of neutropenia/leukopenia were significantly reduced in the LET group (26%) vs. GCV (64%), $P < 0.001$.

Source: Adapted from [75] / American Medical Association.

1.7.5.3 Further Clinical Development and Real-world Data

Real-world data examining the risks and benefits of LET versus VGCV in kidney transplantation have already begun to emerge. In addition to at least comparable prophylactic efficacy versus VGCV, LET's safety profile allows maintenance or even increase of mycophenolate dosing without the need for supplemental granulocyte colony-stimulating factor (G-CSF), making the conversion from VGCV particularly useful in cases of VGCV-induced leukopenia [76].

1.8 Drug Resistance

1.8.1 Genetic Characterization of Letermovir Resistance

Knowledge on potential drug resistance is important for any antiviral project. In the context of CMV in transplant recipients, clinical resistance historically meant, e.g. VGCV dose escalation or a transition to an alternative DNA polymerase inhibitor like foscarnet or cidofovir with greater toxicity. Cross-resistance due to the common molecular target, the viral polymerase, was a further complicating issue leading to worsening treatment outcomes. Rapid identification and characterization of amino acid mutations conferring resistance to a novel drug are therefore important to guide and optimize treatment options [77].

As a direct-acting antiviral drug (DAAD), the development of LET resistance is essentially inevitable. Therefore, identification and characterization of potential resistance mutations began early in the development program. Partly, this was essential for understanding LET's MoA, especially considering the target complex comprises three distinct subunits (UL56, UL89, and UL51), and partly because these data are crucial for effective clinical management. Similarly, cataloging natural polymorphisms unrelated to resistance is also important to permit rapid interpretation of sequencing data from patients with CMV viremia [77–79].

First, a number of *in vitro* drug resistance studies were conducted using cell culture selection of LET-resistant virus strains, followed by genotyping of potential target genes involved in cleavage/packing of viral progeny DNA. Amino acid (aa) mutations were identified against a standard genomic reference sequence (CMV AD169) and then transferred to a cloned LET-susceptible wild-type strain for *in vitro* drug susceptibility testing (marker transfer) [38, 79]. This approach, referred to as recombinant phenotyping, has become the standard for attributing a resistant or susceptible phenotype to a given CMV genotype [77, 80]. The work confirmed the principle molecular target as the terminase subunit pUL56 and showed that single-site aa mutations in the UL56 gene could confer LET resistance. Interestingly, all these single-site resistance mutations were seen to cluster in the UL56 region aa 231–369, which was therefore considered the LET-resistance region, a designation that still applies. The different aa resistance mutations in this region were shown to range in their impact from low-level (<20-fold) to high-level (>100-fold) increases in EC_{50}, with only minimal loss of viral growth fitness. The most significant mutations so far are aa exchanges at codon C325, which confer >3000-fold increase in EC_{50}, and thus essentially absolute drug resistance [29, 38, 79, 80].

Following on from this foundational work, a series of subsequent *in vitro* studies using variations of the same approach identified additional unknown resistance mediating mutations or additional aa exchanges at codons already known to confer drug resistance. Interestingly, the results showed that almost all LET-resistance mutations were still located in the UL56 LET resistance region, with the minor exceptions of one low-level resistance mutation at codon 25 in UL56, four low-level resistance mutations in the UL89 gene, and one in the UL51 gene [81–83]. A summary of the known LET resistance mutations is given in Figure 1.12.

Knowledge on LET resistance mutations also enabled screening of wild-type CMV genotype data, either from publicly available databases or from collections of LET-naïve clinical isolates, for the potential presence of preexisting LET-resistant genotypes. In summary, the data showed no evidence of preexisting LET-resistant genotypes circulating in the population [79, 84].

It is of note that in some cases, engineered mutant virus strains lacking the viral polymerase proofreading activity have been used to accelerate the selection of resistant virus mutants and the subsequent identification of novel resistance mutations in cell culture. *In vitro* resistance selection studies using such engineered strains also showed that LET resistance mutations emerged at a lower selection passage compared to foscarnet and GCV, suggesting that LET may have a lower genetic barrier to resistance than polymerase inhibitors [81–83]. However, a caveat to this comparison

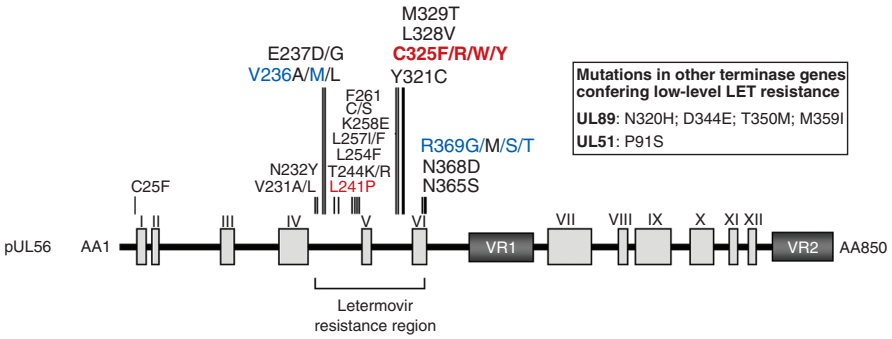


Figure 1.12 Known LET-resistance mutations.

Schematic representation of the UL56 domain organization according to Champier et al. (2008). Conserved regions are indicated as gray boxes (I–XII); variable regions (VR1 and VR2) as black boxes. LET resistance mutations in UL56 are indicated above, resistance mutations in UL89 and UL51 are listed in the box. Resistance mutations are color-coded by the degree of resistance conferred:

Black: EC_{50} ratios <20-fold (most probably not of clinical relevance).

Blue: EC_{50} ratios 20- to 100-fold.

Red: EC_{50} ratios >100-fold.

Note: Mutations at position UL56 C325 confer >3000-fold resistance (i.e. absolute resistance).

Source: References [28, 29, 38, 31, 79, 81–84, 93, 99].

is the inherent bias against drugs that act downstream from DNA replication and thus are less effective than polymerase inhibitors in preventing the formation of DNA mutations generated by a proofreading-deficient viral DNA polymerase. Therefore, the clinical relevance of these data can only be meaningfully interpreted in light of clinical data (see below).

1.9 Letermovir Resistance in Clinical Trials

Resistance genotyping was performed retrospectively for all subjects with csCMV in the Phase 2b, and the Phase 3 HSCT and KT trials. In addition, unknown polymorphisms were characterized by recombinant phenotyping, an approach now considered standard [77].

The first reported clinical case of LET resistance (V236M) was reported in the dose range finding Phase 2b trial in an HSCT patient [78]. It occurred after ~7 weeks of treatment in a subject receiving low-dose LET (60 mg/day), indicating the dose to be at least partially permissive to viral replication and so suboptimal. No cases of resistance were reported in the higher dose groups (120 and 240 mg/day LET).

In the Phase 3 trials, the LET doses used were higher (480 or 240 mg/day with concomitant cyclosporine), and the overall number of resistance events was very low in both HSCT and KT subjects [75, 85].

In total, three subjects in the Phase 3 HSCT trial were shown to have four LET-resistance mutations in UL56. Two of these were already known from preclinical

in vitro work (V236M and C325W), and two were newly identified mutations, however, at codons already associated with LET resistance (UL56 aa237 and aa369) [85].

In the Phase 3 KT trial, no subjects (of 292 LET-treated) had detectable LET resistance. This is compared to 12 of 297 VGCV-treated subjects with VGCV resistance mutations. However, it is of note that subsequent genetic analyses of VGCV failures, including UL56 sequencing, showed that a single VGCV-treated subject had two low-level LET-resistance mutations that were present with low frequency (<10%). However, re-sequencing of the pUL56 from the same sample failed to confirm the presence of any LET-resistance mutation, which suggests that the detection of such mutations in a LET-naïve patient may have been an artifact of the sequencing process supporting the assumption that no LET-resistant viral strains are circulating in the population [86, 87].

1.10 Real-world Resistance

In the first period after marketing approval, multiple reports of drug resistance were published, which, however, have been largely restricted to the context of secondary prophylaxis or CMV treatment rather than the approved primary prophylaxis indication [88–92].

In the meantime, however, with increasing experience with the clinical use of LET, the resistance rates reported in the clinical trials (see above) appear to be representative of the real world. As concluded by Limaye et al. [75], though resistance to LET is rare in prophylaxis, it appears to be rather more common in cases of therapeutic/rescue off-label use. At an intuitive level, this may reflect the considerable differences between the prophylaxis and treatment settings regarding ongoing virus replication rates (little-to-none vs. potentially high viral loads) and the resulting opportunities for resistance mutations to arise; however, this is not known.

A recent frequency analysis of >1100 clinical samples from suspected LET treatment failures suggested that when LET resistance is detected, single-step mutations conferring high LET resistance (RI ≥3000), especially at the codon 325 position, appear to be more common than low-level resistance mutations (RI <20), confirming the intuitive expectation [93]. This difference can also be explained because in vitro resistance selection experiments often used LET concentrations escalating from a low nanomolar level, whereas therapeutic concentrations of LET at the standard dose are expected to be in the micromolar range, far exceeding the EC_{50} values of many resistant mutants [85]. The occasional appearance of low-grade LET resistance mutations in clinical specimens suggests insufficient drug exposure in those cases [93].

1.11 Outlook for Letermovir

In the high-risk HSCT and kidney recipient populations, LET is now well-established as a safe and effective prophylactic drug to prevent CMV infection/reactivation. Early data reporting the use of LET prophylaxis in other SOT indications

likewise suggest good anti-CMV efficacy, safety, and low rates of resistance and thus support the general use of LET as CMV prophylaxis in all transplant patients [76, 94, 95].

LET treatment regimens are specifically optimized for prophylaxis. Nevertheless, an increasing number of literature case reports show that the drug is also being used off-label as a PET or therapy in both HSCT and SOT settings, including cases of resistant/refractory CMV [25, 88, 92, 96–98]. While these are still case reports and small studies, it appears that LET may be efficacious in the treatment setting, though drug resistance rates seem to be higher than in prophylaxis use [75]. The latter observation suggests that dosing regimens may need optimizing for LET to be used as PET or rescue therapy. This cannot be easily addressed without targeted clinical trials but may open new avenues for LET while preventing widespread resistance.

Generally, available data show promise for LET as a first-line agent for universal prophylaxis in high-risk transplant patients. Although this review revolves around the development program that specifically targeted CMV in HSCT/KT transplant recipients, it is also worth remembering the ongoing need for efficacious CMV treatments in the neonatal and HIV populations. Specific investigations should be undertaken to evaluate the potential of this safe and potent drug in these indications.

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