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Suppression of Herpes Simplex Virus Type 1 Reactivation from Latency by (±)-9-{[(Z)-2-(Hydroxymethyl)Cyclohexyl]Methyl} Guanine (L-653,180) In Vitro

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Latent herpes simplex virus type 1 (HSV-1) infection was induced in human embryonic lung cells in vitro by using a combination of viral replication inhibitors and elevated temperature. Under reactivating conditions (superinfection by human cytomegalovirus or temperature manipulation), a nonantiviral thymidine kinase inhibitor (L-653,180) was found to suppress or delay reactivation of HSV-1 from latently infected human embryonic lung cells. L-653,180 alone or in combination with interferon was ineffective as a primary or acute viral replication inhibitor and was unable to induce latent HSV-1 infection in cell culture. These data suggest that initial or acute virus replication and replication resulting from reactivation from latency are separate events.

The establishment and maintenance of herpes simplex virus (HSV) latency in infected hosts have been subject to intense investigation for many decades (4–8, 12, 13, 17, 18, 22, 23). However, the mechanisms involved in the induction and maintenance of latency are not fully understood. Virus latency is achieved in vivo when infectious viral particles cannot be isolated from the host, yet the HSV genome is shown to be present in HSV type 1 (HSV-1) ganglionic explants during cocultivation. In vitro induction of HSV-1 latency has been accomplished through the use of viral inhibitors and temperature manipulation (11, 15–18, 21, 24–27).

The pathogenesis of HSV-1 infection in humans usually involves virus replication at the point of entry. The virus proceeds to the nerve endings in the area, where it localizes in the nuclei of the nerve cells. It is in the neuronal nuclei that the establishment and maintenance of the latent state of the virus infection are probably initiated. At a later time, the occult latent infection may be reactivated as a result of nonspecific stimuli. The presence of virus-specific thymidine kinase (TK) in infected cells has been implicated in the reactivation of HSV from latency. Tenser et al. (22) and others (4, 14) have shown that TK has no role in the establishment and maintenance of HSV-1 latency; however, there is some indication that TK-deficient mutants, when latent, reactivate either poorly or not at all (4, 14, 22).

Since TK plays an unknown role in the reactivation of latent HSV-1, a virus-specific TK inhibitor might affect the reactivation of latent virus. Therefore, we studied the use of (±)-9-{[(Z)-2-(hydroxymethyl)cyclohexyl]methyl}guanine (L-653,180), a nonantiviral TK inhibitor, to suppress the reactivation of HSV-1 from latently infected cell cultures. L-653,180 is a potent selective inhibitor of virus-coded TK, although it is not a substrate for this particular enzyme (3). L-653,180 is ineffective against acute or primary virus infection in MRC cells (3). However, in mouse trigeminal ganglionic explants treated with the drug, reactivation was delayed significantly compared with reactivation in untreated controls under the same conditions, and the amount of reactivated infectious virus was reduced (3).

Our results show that the drug (L-653,180) is ineffective against virus replication in cell culture but suppresses HSV-1 reactivation from latently infected cells. The data suggest that the mechanisms involved in the replication of HSV-1 during acute infection differ from those controlling the reactivation of HSV-1 from the latent phase of infection.

MATERIALS AND METHODS

Effect of L-653,180 on virus growth. To determine the effect of L-653,180 and interferon (IFN) on the replication of HSV-1, confluent cell monolayers were treated with IFN and infected with virus in the presence or absence of the drug (15 μg/ml). Briefly, one group of cultures was pretreated with the inhibitor and/or IFN combination for 24 h. Another group was mock treated with 10% Dulbecco minimal Eagle medium (DMEM) for the same period. Then cells were infected with approximately 15 to 20 PFU of HSV-1 per cell for 60 min and then washed three times with tri-buffered saline to remove unadsorbed inoculum. The washing was used in place of anti-HSV-1 serum to prevent more than one cycle of virus multiplication. All cultures except the mock-treated infected controls were refed with medium containing the inhibitor and then incubated at 37°C for 12 h. Cultures were sampled and assayed for infectious virus after multiple cycles of freezing and thawing at 1, 2, 4, 6, 8, 10, and 12 h after infection.

Induction, maintenance, and reactivation of HSV-1 from latency. The induction of HSV-1 latency in cell culture by inhibitors, temperature manipulation, or both has been described previously (15). Briefly, human embryonic lung (HEL) cells (kindly provided by Brian Wigdahl, The Pennsylvania State University College of Medicine, Hershey) were grown to confluency in 10% DMEM at 2×10^6 to 3×10^6 cells per T-25 flask. To induce latency, confluent monolayers of cells were pretreated 24 h or overnight with 4.0 ml of medium containing 200 IU of IFN (Roferon alpha; Hoff-

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mann-La Roche Inc.) and one of the following: acyclovir (ACV) (34 μg/ml), bromovinyl deoxyuridine (BVDU) (15 μg/ml), 2'-nor-cyclic GMP (2'-nor-cGMP) (15 μg/ml) (1, 2, 9, 10, 12, 23), or L-653,180 (15 μg/ml) (3). (ACV and BVDU were generously supplied by Brian Wigdahl; 2'-nor-cGMP and L-653,180 were obtained from Merck Sharp & Dohme, Rahway, N.J.) After pretreatment of cells with the inhibitor, the medium was removed and the cells were infected with 0.5 ml of HSV-1 (strain Patton) at a multiplicity of infection (MOI) of 0.5 PFU per cell for 1 h with intermittent agitation of the culture flasks. After infection, duplicate cultures were refed 4.0 ml of medium containing 200 IU of IFN and DMEM, 2'-nor-cGMP, ACV, or L-653,180. Unless otherwise noted, all cultures, including infected and uninfected (treated and untreated) controls, were incubated at 37°C in 5% CO_2 at 90% humidity. Randomly selected duplicate cultures were scored for the appearance of virus-induced cytopathic effect (CPE) and then sampled for infectious virus every 24 h during medium changes.

The maintenance of latently infected cultures was accomplished by manipulation of the incubation temperature or maintenance of cultures in L-653,180 alone or in combination with IFN. Every other day, cultures were randomly sampled by plaque assay for infectious virus and virus-induced CPE. Reactivation of latent HSV-1 was carried out in one of two ways: (i) by superinfection of cells with human cytomegalovirus (HCMV) (strain AD169) or (ii) by reduction of the incubation temperature from 40.5 to 37°C. Cell-associated latent HSV-1 was reactivated from latency by superinfection with HCMV in the presence or absence of L-653,180 at permissive or elevated temperatures (5, 25).

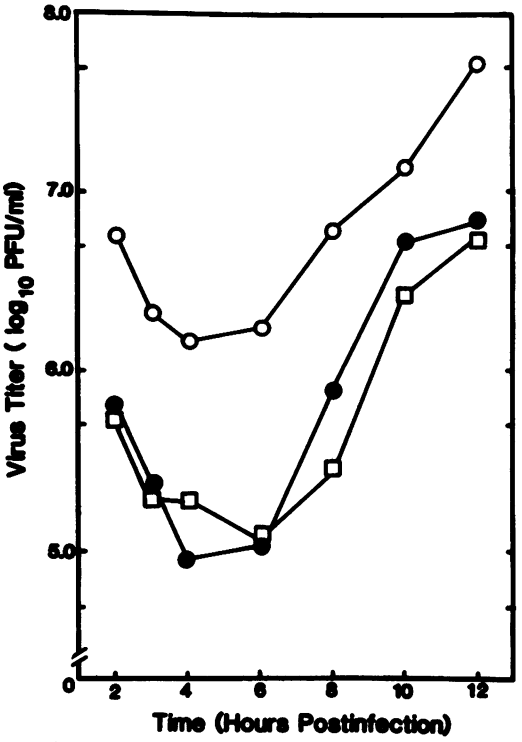

FIG. 1. Effect of L-653,180 (15 μg/ml) and IFN (200 IU) on growth of HSV-1. Confluent monolayers of cells were infected at an MOI of 15 PFU per cell as described in the text. The inhibitor used was L-653,180 plus IFN. Symbols: ○, control (10% DMEM); ●, pretreated cells; □, posttreated cells. Data shown are representative of three experiments. At 100 μg of L-653,180 per ml, there was no toxic effect on the viability of the cells.

RESULTS

Effect of L-653,180 on growth of HSV-1. L-653,180 alone or in conjunction with IFN was ineffective as an inhibitor of virus replication during a 12-h growth cycle (Fig. 1). We did not obtain an inhibition of virus replication by increasing L-653,180 to as high as 100 μg/ml in culture. There was no adverse toxic effect on the cells during this period as measured by cell death (data not shown). These data support the finding that TK has no role in virus replication (4, 14, 22).

Establishment of latent HSV-1 infection. The fact that L-653,180 could not induce latent HSV-1 infection is consistent with previous observations that TK plays no role in the establishment and maintenance of HSV-1 latency in trigeminal ganglia. In contrast, L-653,180 used alone or in combination with IFN did not inhibit acute primary virus infection, nor did it induce latent herpetic infection (Table 1). However, cultures pretreated with IFN and either ACV or 2'-nor-cGMP for 24 h before infection with HSV-1 could be maintained thereafter with L-653,180 and IFN for the first 7 days with no visible virus-induced CPE. Increasing the concentration of L653-180 to as much as 100 μg/ml did not result in any noticeable cytoxicity.

As shown previously, both BVDU and ACV inhibited acute primary virus replication and induced latency when used in combination with IFN. Induction of HSV-1 latency in vitro was accomplished by methods previously described (15, 16), with modifications. 2'-Nor-cGMP, a known potent antiviral nucleoside analog, also was found to be a potent inducer of latent HSV-1 infection in HEL cells (Table 1).

Maintenance and reactivation of latently infected cells. Having established latent HSV-1-infected cultures, maintenance of the cultures was carried out by elevation of the incubation temperature or by adding L-653,180 and IFN to the cultures. Reactivation of latent HSV-1 was suppressed or delayed during the period between 10 to 26 days postinfection in the presence of L-653,180 and IFN (Fig. 2). Subsequent removal of L-653,180 resulted in the reactivation of latent virus ($10^{2.5}$) on day 30 postinfection. The infectious virus titer rose to approximately $10^{6.0}$/ml (Fig. 2) 9 days after the removal of L-653,180 and IFN. Elevation of the incubation temperature to 40.5°C in similar experiments inhibited reactivation in the presence or absence of L-653,180. The suppression of reactivated latent virus was not dependent upon the initial HSV-1 input multiplicity (MOI, 0.5, 0.1, and 0.001 PFU per cell), nor was the inhibition a function of the yield of reactivated virus.

Treatment of latent cultures by superinfection with HCMV at 40.5°C resulted in the reactivation from latency of the infectious virus (5, 24–26). To ascertain whether latent HSV-1 could be reactivated in the presence of L-653,180 and IFN, cultures were superinfected with 0.2 PFU of HCMV per cell on day 26 postinfection. In the presence of L-653,180 and IFN, the reactivation of infected cultures by HCMV was suppressed. For instance, in L-653,180-free cultures, infectious virus reactivated to titers that were high ($10^{4.8}$ to $10^{5.3}$/ml) compared with the titers obtained from the cultures maintained in L-563,180 (Fig. 2). This observation suggests a possible role for TK in the reactivation process.

Duration of latency after superinfection with HCMV. We examined the duration of latency in the presence or absence

TABLE 1. Establishment of HSV-1 latency in HEL cells in vitro

Pretreatment[a]	Maintenance	Virus titer (PFU/ml)[b] and CPE at day postinfection				
		1	2	3	5	7
DMEM	DMEM	2.3×10^8 (++)[c]	2.4×10^8 (+++)	3.8×10^7 (+++)	1.4×10^5 (++++)	1.0×10^3 (++++)
IFN	IFN	5.5×10^8 (+)	2.4×10^8 (+++)	4.8×10^7 (++++)	5.8×10^3 (++++)	3.3×10^3 (++++)
ACV-IFN	ACV-IFN	$<10^1$ (−)	$<10^1$ (−)	$<10^1$ (−)	$<10^1$ (−)	$<10^1$ (−)
ACV-IFN	L-653,180–IFN	$<10^1$ (−)	$<10^1$ (−)	$<10^1$ (−)	$<10^1$ (−)	$<10^1$ (−)
BVDU-IFN	BVDU-IFN	2.3×10^2 (−)	2.8×10^3 (−)	4.4×10^4 (+)	1.8×10^5 (++)	1.3×10^5 (++)
BVDU-IFN	L-653,180–IFN	2.0×10^2 (−)	1.9×10^4 (−)	1.5×10^5 (++)	3.8×10^4 (+++)	9.5×10^2 (+++)
2′-Nor-cGMP	2′-Nor-cGMP	2.5×10^3 (+)	5.0×10^3 (++)	1.3×10^4 (++)	$<10^1$ (+++)	$<10^1$ (+++)
L-653,180	L-653,180	3.5×10^8 (++)	1.3×10^8 (+++)	3.3×10^8 (+++)	4.4×10^6 (++++)	5.0×10^2 (++++)
2′-Nor-cGMP–IFN	L-653,180–IFN	2.3×10^2 (−)	$<10^1$ (−)	$<10^1$ (+)	$<10^1$ (+)	$<10^1$ (+)
2′-Nor-cGMP–IFN	2′-Nor-cGMP–IFN	4.0×10^2 (−)	$<10^1$ (−)	$<10^1$ (+)	$<10^1$ (+)	$<10^1$ (+)
L-653,180–IFN	L-653,180–IFN	4.3×10^8 (++)	8.2×10^4 (++)	1.5×10^6 (+++)	1.9×10^4 (++++)	5.0×10^2 (++++)

[a] Inhibitors: BVDU, 15 μg/ml; ACV, 34 μg/ml; 2′-nor-cGMP (MSD-L-648,164-002L011), 10 to 15 μg/ml; L-653,180 (MSD-L-653,180-000Y002), 10 to 15 μg/ml.
[b] Representative of three separate experiments in duplicate at an MOI of 0.5 PFU per cell.
[c] Scoring of CPE: −, 0%; +, 25%; ++, 50%; +++, 75%; ++++, 100% CPE.

of L-653,180. In cultures maintained in L-653,180 and IFN, the reactivation of HSV-1 by HCMV occurred after 8 and 11 days, compared with 3 to 4 days for the inhibitor-free cultures (Table 2). Without superinfection of latent cultures, it took 11 to 13 days for infectious virus to be measured in the presence of L-653,180 and IFN and 2 to 4 days to be measured in L-653,180-free medium. For unknown reasons, the inhibitory effect of L-653,180 was not observed in BVDU-induced, latently infected cultures in similar experiments.

DISCUSSION

We have succeeded in suppressing or delaying the activation of infectious HSV-1 from latently infected HEL cells by using a nonantiviral virus-specific TK inhibitor (L-653,180). The inhibitory effect of L-653,180 was not dependent on the yield of reactivated virus or on the initial input multiplicity of virus (in this study, MOIs ranged from 0.5 to 0.001 PFU per cell) used to establish the latent phase of infection. Under reactivating conditions (incubation temperature manipula-

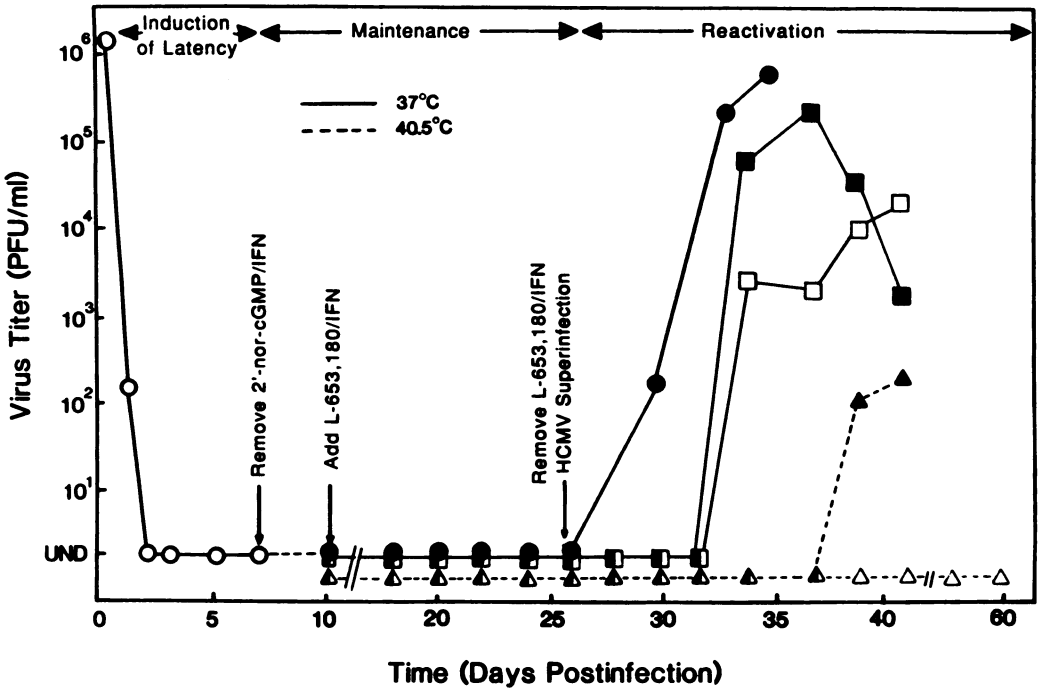

FIG. 2. Induction of HSV-1 latency by 2′-nor-cGMP and reactivation in the presence or absence of L-653,180 (15 μg/ml) and IFN. Latency was established as described in Materials and Methods. HEL cells were infected (HSV-1, 0.5 PFU per cell) and then maintained in 2′-nor-cGMP and IFN for 7 days. At day 7, the drugs were removed and the temperature was elevated to 40.5°C. At day 10, some of the cultures were returned to 37°C and the rest were continued at 40.5°C. From this point on, all cultures were maintained in the presence of L-653,180 and IFN, except for one maintained in 10% DMEM (●); this culture was not subsequently superinfected. The drugs were removed from all other cultures at day 26, and the cells were superinfected with HCMV. In similar experiments, cultures were kept at 37°C in the presence (□) or absence (■) of L-653,180 and IFN; others were kept at 40.5°C in the presence (△) or absence (▲) of L-653,180 and IFN. Virus titers were determined by plaque assays (in triplicate) in Vero cells. Results are representative of three experiments.

TABLE 2. Effect of HCMV superinfection on latent HSV-1 reactivation at 37°C

Pretreatment	Maintenance	Virus titer (PFU/ml)[a]			
		HCMV with:		No HCMV with:	
		No inhibitor[b]	Inhibitor[b]	No inhibitor	Inhibitor
BVDU-IFN	BVDU-IFN	3.0×10^2 (2)	3.0×10^2 (2)	ND[c]	1.0×10^4 (<2)
2'-Nor-cGMP–IFN	2'-Nor-cGMP–IFN	1.2×10^4 (11)	4.0×10^5 (15)	3.4×10^5 (7)	$<10^1$ (15)
ACV-IFN	L-653,180–IFN	4.7×10^4 (4)	1.1×10^4 (8)	1.7×10^5 (4)	2.0×10^4 (11)
2'-Nor-cGMP–IFN	L-653,180–IFN	4.1×10^3 (3)	4.9×10^4 (11)	2.6×10^3 (2)	1.3×10^2 (13)

[a] Representative of three separate experiments in duplicate at an MOI of 0.5 PFU per cell. Numbers in parentheses refer to days postinfection before the appearance of infectious virus.
[b] Presence or absence of inhibitor (L-653,180 and IFN) with HCMV (0.2 PFU per cell) postsuperinfection.
[c] ND, Not determined.

tion or superinfection of latent cultures with HCMV), L-653,180 alone or in conjunction with IFN suppressed the reactivation of HSV-1 from latency. Not only was L-653,180 ineffective as a replication inhibitor, but it did not induce latent HSV-1-infected cultures in vitro. This initial finding supports the fact that TK is not necessary for HSV replication and does not play any role in the establishment and maintenance of HSV latency (4, 14, 22).

Although L-653,180 is a potent and specific inhibitor of HSV TK, it is not a substrate for this enzyme (3). Its inhibitory effect on the viral TK may reduce the amount of TMP available for viral DNA replication, but there are other inherent biochemical de novo and salvage pathways of TMP in cell cultures. Nevertheless, TK-deficient viral mutants are infectious in culture. However, TK-deficient mutants are much less virulent than the wild type in animal studies and do not usually establish latency or at least establish recurrent infections. The inability of TK-deficient mutants to establish recurrent infections suggests that TK may play an important role in the process of reactivation of latent HSV, which would supply TMP in cells such as the neurons harboring the latent virus, where there is very little, if any, endogenous TMP. It is also possible that TK is involved in the mechanism that triggers the reactivation of latent virus (20). Therefore, a viral TK inhibitor like L-653,180 may effectively prevent or delay the reactivation of latent virus, as clearly demonstrated in this study.

We also reported that 2'-nor-cGMP, a known inhibitor of viral replication, can be used to induce and establish latent HSV-1 and that L-653,180 inhibits the virus-specific TK. Although this cyclic phosphate of ganciclovir is converted to the linear monophosphate of ganciclovir and further phosphorylated to the triphosphate, a potent inhibitor of the HSV DNA polymerase, there is evidence suggesting that its main mode of action is not in vivo. It also acts by another mechanism (12) that has not, as yet, been elucidated. Whatever the mechanism might be, the presence of a replication inhibitor is expected to prevent the reactivation of latent virus.

The end result of elevated temperature and L-653,180 on the reactivation of latent HSV-1 in infected cells is the suppression of reactivation. However, both L-653,180 and elevated temperature may act through a different mode of action. For instance, HCMV reactivates latent HSV-1 from infected cultures at elevated temperatures (17, 19, 20–27); on the other hand, L-653,180 suppressed reactivation at both elevated and permissive incubation temperatures (Fig. 2). The effect of L-653,180 was not dependent on the latency-inducing agent, since ACV-induced latently infected cultures exhibited the same effect as those induced by 2'-nor-cGMP (data not shown).

The idea that L-653,180 probably inhibits the replication of small amounts of virus that can be reactivated does not appear to be valid in this case, because the suppression of reactivated virus was independent of the input multiplicity or the amount of reactivated latent virus. Thus, the ability of L-653,180 TK inhibitor to "distinguish" between acute infection and reactivation events indicates that TK may be involved in reactivation. In summary, reactivation of HSV-1 by HCMV in HEL cells was suppressed in the presence of L-653,180 and IFN. It appears from this study that L-653,180 plus IFN, although ineffective as an antiviral agent against HSV-1 during acute or initial infection, effectively blocks the reactivation of latent HSV-1. Although the findings of this study with regard to HSV pathogenesis are not clear, the results suggest (i) that viral TK inhibitors may have utility in chemotherapeutic intervention of recurrent herpes infections to prevent reactivation of the latent virus and (ii) that virus replication and reactivation are separately controlled events, with a role for TK in the latter case.

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