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Two Specific Topoisomerase II Inhibitors Prevent Replication of Human Cytomegalovirus DNA: An Implied Role in Replication of the Viral Genome

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In this study, we show that human cytomegalovirus DNA synthesis is inhibited in infected confluent human embryonic lung cells treated with the DNA-intercalative topoisomerase II inhibitor 4-9′-(acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA). Similar inhibitory effects were observed with VM-26, a nonintercalative topoisomerase II inhibitor. This antiviral effect is not attributable to cytotoxic effects per se. Furthermore, *m*-AMSA appears to have a notably irreversible inhibitory effect on human cytomegalovirus DNA replication. No inhibition of viral DNA synthesis was observed with *o*-AMSA, a DNA-intercalative isomer of *m*-AMSA that does not inhibit topoisomerase II.

Human cytomegalovirus (HCMV) infections are serious, often life-threatening complications in immunocompromised patients, including individuals with acquired immunodeficiency syndrome and recipients of organ and bone marrow transplants (1, 11, 14). Historically, many of the HCMV antiviral drug strategies have targeted the virus-encoded DNA polymerase. Nucleoside analogs such as the guanine analog 9-(1,3-dihydroxy-2-propoxymethyl)guanine (12, 20) and pyrophosphate analogs including phosphonoformate and phosphonoacetate (10, 30) selectively inhibit the viral DNA polymerase. However, in vitro viral DNA synthesis and virus production resume upon removal of these compounds. We have, therefore, pursued other possible targets for anti-HCMV drug action which do not exhibit such reversible antiviral effects.

DNA topoisomerases are enzymes that control and modify the topological state of DNA. Type II topoisomerases do this by a strand-crossing reaction involving a double-strand break, which gives them the unique ability to catalytically relax, unknot, and decatenate covalently closed DNA molecules (31). Studies of yeast temperature-sensitive topoisomerase II mutants (6, 9, 28) and recent in vitro studies of simian virus 40 replication (33) have shown that topoisomerase II has an essential role in the terminal replicative phases of both systems.

4′-9′-(Acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) is a DNA-intercalative acridine derivative that has been shown to induce reversible double- and single-stranded-DNA breaks, the ends of which are covalently bound to topoisomerase II (2, 15, 26, 34). Thus, topoisomerase II is believed to be the primary, if not the sole, intracellular target of *m*-AMSA. Another class of compounds, which include such compounds as VP-16 and VM-26, also appear to be specific inhibitors of topoisomerase II but do not have DNA-intercalative properties (3, 18).

Effects of *m*-AMSA on HCMV DNA replication. Human embryonic lung (HEL) cells were maintained and infected as previously described (10). Confluent cells were infected with HCMV (Towne strain, passage 40) at a multiplicity of 1 to 2 PFU per cell. Following viral adherence for 1 h, medium containing various concentrations of *m*-AMSA was added (Drug Synthesis Branch, National Cancer Institute, Bethesda, Md.). Intracellular viral DNA levels were quantitated by lysing cells with 0.5 ml of ice-cold alkaline lysis buffer (0.5 M NaOH, 1.0 M NaCl) per 2×10^5 cells. Lysates were heated to 70°C for 15 min, neutralized with an equal volume of neutralization buffer (1 M Tris [pH 7.4], 1.0 M NaCl), and blotted onto nitrocellulose filters by using a dot blot apparatus as described by the manufacturer (Schleicher & Schuell, Inc., Keene, N.H.). Filters were baked, prehybridized, and hybridized with ^{32}P-labeled nick-translated HCMV DNA as previously described (4). All data were normalized by subtracting the amount of hybridized signal from an equal number of mock-infected cells.

Figure 1 shows HCMV DNA levels 48 and 72 h postinfection in cultures treated with *m*-AMSA concentrations ranging from 0 to 1.2 μM. The 50% effective dose of *m*-AMSA, determined from two independent experiments, appears to be 0.6 μM. No viral DNA synthesis was detected even 9 days after infection when *m*-AMSA at concentrations greater than 1.2 μM was used.

HCMV DNA synthesis is not affected by *o*-AMSA. *o*-AMSA is an isomer of *m*-AMSA. Although *o*-AMSA does possess DNA-intercalative and membrane-interactive effects similar to those of *m*-AMSA, it does not inhibit topoisomerase II (15). In experiments in which HCMV-infected HEL cells were treated with concentrations of *o*-AMSA of up to 2.5 μM, no HCMV DNA-inhibitory effects were observed.

Cytotoxic effects of *m*-AMSA. The cytotoxic effects of *m*-AMSA on dividing cells are well documented (2, 5, 27, 32); *m*-AMSA at concentrations far below those which inhibit HCMV DNA replication shows severe cytotoxic effects on subconfluent HEL cells. Thus, one possible trivial explanation for the antiviral effects of *m*-AMSA is a cytotoxic effect on the host cell. To test this possibility, mock-infected confluent HEL cells were treated with various concentrations of *m*-AMSA for 4 days. Cell toxicity was assessed by trypsinization and by counting in a hemacytometer the percentage of suspended cells able to exclude a 0.05% trypan blue dye solution (Table 1). *m*-AMSA at concentrations well above those required to inhibit viral

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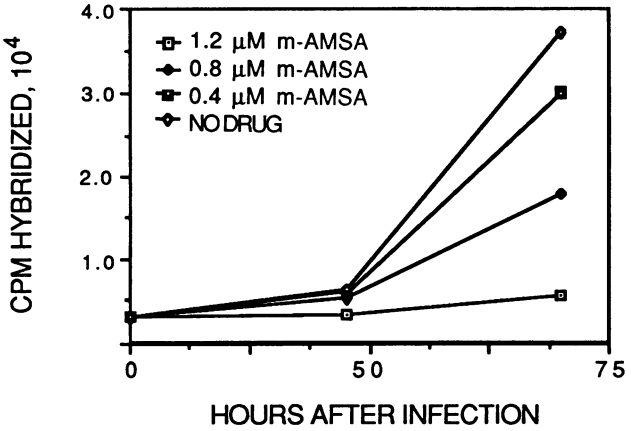

FIG. 1. HCMV DNA synthesis inhibition by *m*-AMSA. Infected confluent HEL cells were treated with the indicated concentrations of *m*-AMSA. Cells were lysed at indicated times. Dot blots and hybridizations were performed as described in the text.

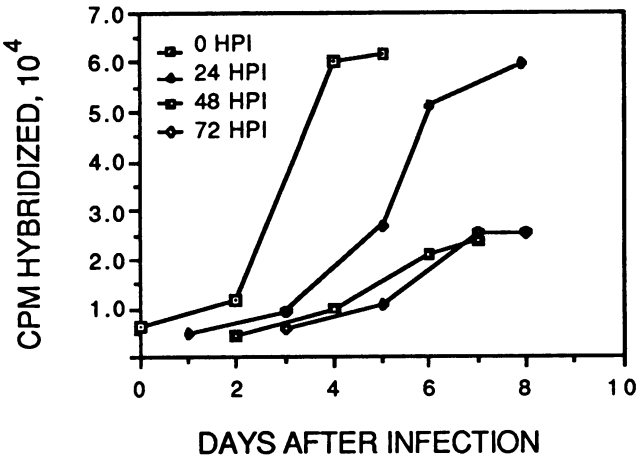

FIG. 2. Reversibility of *m*-AMSA antiviral effects. Infected confluent HEL cells were treated with 1.2 μM *m*-AMSA, which was removed at the times indicated by three 30-min washes with drug-free medium at 37°C. Two sets of data (with identical values) were collected for mock-treated cells and cells which received drug treatment upon infection followed by immediate removal after virus attachment (⊡). HPI, Hours postinfection.

DNA synthesis exhibited no significant effects on cell confluent survival.

Reversibility of *m*-AMSA treatment. To determine the reversibility of the inhibitory effects of *m*-AMSA on HCMV viral DNA synthesis, confluent HEL cells were simultaneously infected and treated with 1.2 μM *m*-AMSA. Drug was then removed at indicated times by three 30-min washes with drug-free medium at 37°C (Fig. 2). The presence of *m*-AMSA for up to the first 24 h of infection appeared to inhibit the onset but not the outcome of viral DNA synthesis. However, if *m*-AMSA was removed at 48 or 72 h after infection, HCMV DNA synthesis did not recover completely. HCMV DNA synthesis begins around 24 h after infection, reaching maximal rates at 72 h postinfection. Thus, it appears that *m*-AMSA exhibits an irreversible antiviral effect only if present during significant viral DNA synthesis. The complete reversibility of 24-h drug treatment demonstrates that *m*-AMSA, which is known to be a membrane-permeable compound (5, 17, 27), was removed in these experiments.

In separate experiments, confluent HEL cells were pretreated with 1.2 μM *m*-AMSA for 3 days. At this time, drug was removed and these cells were infected with HCMV at a multiplicity of 1 to 2 PFU per cell. Dot blot analysis of viral DNA levels in pretreated cells 4 days after infection showed that *m*-AMSA pretreatment had no effect on HCMV DNA replication, compared with that in mock-pretreated infected cells (Fig. 3). These results indicate that *m*-AMSA does not reduce the intrinsic ability of HEL cells to support HCMV DNA replication.

TABLE 1. Effects of *m*-AMSA on HEL cell viability[a]

m-AMSA concn (μM)	No. of cells		
	Live	Dead	% Live
No drug	400	107	78.9
0.4	455	87	83.9
0.8	333	71	82.4
1.2	415	75	84.7
1.6	411	52	88.8
2.0	482	80	85.8

[a] HEL cells were grown to 95% confluence in six-well Corning dishes. *m*-AMSA was then added directly to the media to the final concentrations indicated.

***m*-AMSA effects are not due to intercalation.** Although *m*-AMSA is thought to be a specific topoisomerase II inhibitor, this enzyme has not previously been directly implicated in herpesvirus replication. Therefore, we sought to rule out other explanations for its antiviral effects. *m*-AMSA is known to be a DNA-intercalative compound. To eliminate the possibility that intercalation per se explains the replication-inhibitory effects of *m*-AMSA, we tested the anti-HCMV effects of a specific nonintercalative topoisomerase II inhibitor, VM-26. (VM-26 was a generous gift of T.-S. Hseih, Duke University, Durham, N.C.) This topoisomerase II inhibitor appeared to be quite effective in inhibiting

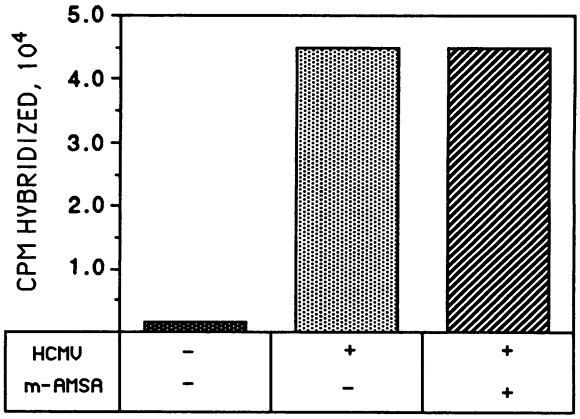

FIG. 3. Effects of *m*-AMSA pretreatment on HCMV DNA replication. Confluent HEL cells were pretreated with 1.2 μM *m*-AMSA for 3 days prior to drug removal and infection by HCMV (1 to 2 PFU per cell). Mock-infected and mock-pretreated controls were included. Cultures were lysed 4 days after infection (8 days after pretreatment), and HCMV DNA was quantitated as described in the text. Presence (+) and absence (−) of HCMV and *m*-AMSA pretreatment in each experiment are indicated.

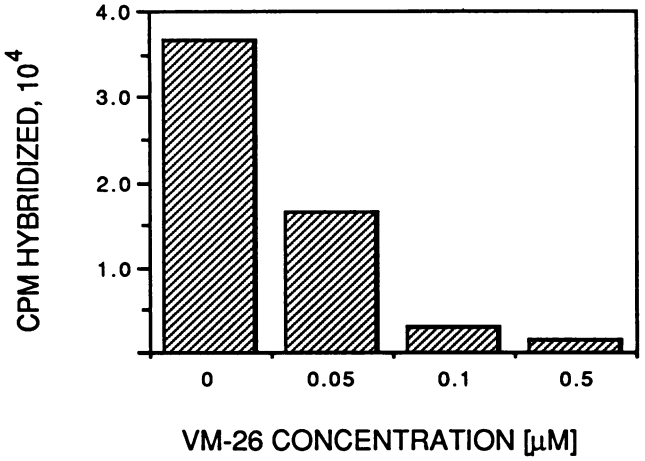

FIG. 4. Effects of a nonintercalative topoisomerase II inhibitor, VM-26, on HCMV DNA replication. Infected confluent HEL cells were treated with the indicated concentrations of VM-26. Cells were lysed 4 days after infection, and DNA was blotted, probed, and quantitated as described in the legend to Fig. 1 and in the text.

HCMV DNA replication, with a 50% effective dose of 0.05 μg/ml (Fig. 4).

We further considered the possibility that *m*-AMSA DNA intercalation could adversely affect the activity of the HCMV-encoded DNA polymerase. HCMV DNA polymerase was isolated and assayed as previously described (13). All *m*-AMSA concentrations tested (up to 250 μM) had no effect on the rate of nucleotide incorporation by the HCMV DNA polymerase (data not shown). VM-26, a nonintercalative topoisomerase II inhibitor, also appeared not to have nonspecific effects on the viral DNA polymerase. Additionally, neither drug inhibited DNA polymerase α or the herpes simplex virus-encoded DNA polymerase.

Topoisomerases have long been thought to play essential roles in DNA replication (24, 25, 29). Recently, several workers have used the simian virus 40 as a model for studying the exact relationship of topoisomerases with DNA replication both in vivo and in vitro (21, 22, 33). Current data from that system suggest that topoisomerase I is required for the swivelase reaction, which would act to dissipate accumulated supercoiling brought about by the progressive movement of the replication fork. Topoisomerase II appears to be essential only at the terminal steps of replication by acting to decatenate the daughter products (21, 22). Such a scheme seems to be applicable to other DNA replication systems. Similar relationships have been demonstrated in yeast cells by using temperature-sensitive topoisomerase I and II conditional mutants (6, 9, 28).

From this previous work, it was reasonable to expect that topoisomerase II would also be essential for DNA replication of herpesviruses. Recent results with herpes simplex virus (23) have demonstrated inhibition of reactivation in a trigeminal-ganglion model system by novobiocin, an antibiotic with noted nonspecific effects on DNA polymerase α (7) and chromatin aggregation (8, 18, 19), as well as on topoisomerase II.

The drug effects we observed strongly support a role for topoisomerase II in replication of HCMV DNA in three ways. First, our results were obtained by using *m*-AMSA and VM-26, two compounds thought to specifically inhibit topoisomerase II which act by completely independent mechanisms. Second, *o*-AMSA, a very closely related isomer of *m*-AMSA that does not inhibit mammalian type II topoisomerases, had no effect on viral DNA synthesis. Third, these topoisomerase II inhibitors had no other measured effect that would explain their antiviral properties. No topoisomerase II inhibitor tested had any effect on the rate of nucleotide incorporation by host cell- or virus-encoded DNA polymerase. In addition, the antiviral effects of these compounds did not appear to be the result of effects on transcription and ordered expression of viral genes, since the major immediate-early and late (pp65 tegument protein) products were detected equally in treated and mock-treated infected cells by immunological visualization (data not shown).

Some investigators have postulated that topoisomerase II inhibitors elicit broad nonspecific metabolic changes in treated cells, including an SOS-type response. Thus, the irreversible antiviral response to *m*-AMSA could have been secondary to a nonspecific change in the metabolic state of the treated infected cells. This appears not to be the case, however, since extended pretreatment of confluent cells with *m*-AMSA had no effect on the ability of these cells to replicate HCMV viral DNA upon infection after drug removal. This result also reinforces the contention that *m*-AMSA was removed by our washing methods.

Perhaps most notable is the fact that *m*-AMSA treatment is not reversible if the drug is present well into the onset of viral DNA replication. It is interesting that upon removal of *m*-AMSA at 48 or 72 h postinfection, a small amount of viral DNA synthesis did resume but did not approach the levels of synthesis in untreated cells. These results, in conjunction with the complete inhibition of viral DNA synthesis observed with continuous exposure of infected cells to *m*-AMSA, suggest the possibility of a role for topoisomerase II subsequent to initiation and throughout the elongation and termination phases of the replicative process. Such a role would be consistent with recent results with prostatic adenocarcinoma cells, in which topoisomerase II was found to interact with newly replicated daughter DNA molecules near replication forks (16).

In contrast, when *m*-AMSA was present for only the first 24 h of the HCMV infectious cycle, the transient presence of the drug delayed the onset of DNA replication but did not affect the ultimate outcome. If an *m*-AMSA block does occur at initiation, this effect must be reversible.

The slow infectious cycle of HCMV and an almost complete lack of information about the molecular events of its replication make it presently impossible to infer the exact nature of topoisomerase II involvement in replication of HCMV DNA. However, our data are consistent with the indications from other experimental systems that topoisomerase II plays an important role in DNA replication.

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