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Inhibition of herpes simplex virus replication by anthracycline compounds

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Summary

The replication of type 1 and type 2 strains of herpes simplex virus (HSV) was inhibited >99.9% by low concentrations (0.1–0.2 μM) of anthracycline compounds. The degree of viral inhibition was dependent upon the host cell. *N,N*-dimethyl daunomycin (NDMD), a non-mutagenic compound, was more potent as an inhibitor of HSV synthesis than either daunomycin (DM) or adriamycin (AD). The depression of viral yield by DM or AD was attributable, in part, to a temperature-dependent direct effect on infectious virions. Tritium-labeled DM bound tightly to HSV particles. NDMD did not directly inactivate virions in spite of superior potency in reducing viral yields. All three anthracyclines could be added late in the infectious cycle (6–8 h p.i.) and retain effectiveness. Cesium chloride density gradient analysis verified that viral DNA synthesis was blocked by addition of all three anthracyclines early in the infectious cycle. The inhibition of HSV replication was not a simple consequence of the suppression of host DNA synthesis since treatment of cells with compounds for 24 h before infection did not reduce virus yields even though host DNA synthesis was inhibited by 90%. Further, the kinetics of inhibition of cellular DNA synthesis by anthracyclines was similar in HFF or Vero cells but the degree of inhibition of virus replication was markedly different. The data suggest that anthracyclines with substitutions on the sugar moiety may be useful anti-herpes agents.

Herpes simplex virus; Anthracycline; Adriamycin; Daunomycin, *N,N*-Dimethyldaunomycin

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Introduction

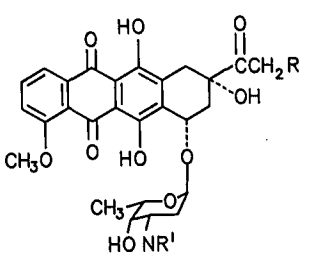
The anthracycline antibiotics daunomycin (DM) and adriamycin (AD) are clinically useful anticancer agents. These compounds were also shown to inhibit the replication of vaccinia, adeno, herpes simplex, and fowl plague viruses (Bossa et al., 1975; Cohen et al., 1969; Minor and Dimmock, 1977; Shortridge and Squires, 1977). Many of these experiments, however, were performed in HeLa cells which are sensitive to the cell killing effects of anthracyclines (Kim and Kim, 1972). This fact makes it difficult to separate specific effects on virus synthesis from nonspecific effects on the host cell. Minor and Dimmock (1977) showed that DM selectively inhibited viral but not cellular protein synthesis in fowl plague virus-infected BHK-21 cells. The direct effect of AD or DM on virions was not examined in previous studies.

AD and DM are mutagenic while the *N,N*-dimethyl derivative of daunomycin (NDMD, Fig. 1) is not (Umezawa et al., 1978; Westendorf et al., 1984). We have examined the antiviral activity of these compounds in cells which are not destroyed by treatment with high drug concentrations. We have also determined the direct effect of the agents on the infectivity of HSV. The most potent antiviral effect in cell cultures was obtained with NMDM, an anthracycline compound which does not directly inactivate virions. AD and DM decrease virus infectivity in a time- and temperature-dependent manner. This direct effect by AD and DM may account for much of the decrease in HSV yields observed previously (Bossa et al., 1975; Cohen et al., 1969).

Materials and Methods

Cells

Vero cells and a continuous line of human foreskin fibroblasts (HFF) were propagated in Eagle's basal medium (EBM) containing 5% fetal calf serum (FCS, GIBCO, Inc.), 100 U penicillin/ml and 100 μg streptomycin/ml.



AD, R=OH $R^1 = H_2$
DM, R=H, $R^1 = H_2$
NDMD, R=H, $R^1 = (CH_3)_2$

Fig. 1. Structure of anthracycline compounds.

Viruses

Three strains of HSV (obtained from Dr. H.C. Bubel, University of Cincinnati) were used: KOS, MP (both type 1), and EK (type 2). All virus stocks were prepared by infecting Vero cells at an input multiplicity of 0.1 PFU/cell. Cultures were sonically disrupted at 20–24 h p.i. and stored at −70°C after inclusion of DMSO to 5% (v/v) to prevent freeze damage (Wallis and Melnick, 1968). All viruses were assayed by a plaque method on Vero cells with a methylcellulose overlay (0.5% in EBM + 1% FCS). Plaques were counted at 72 h p.i.

Virus purification

HSV was partially purified by the following method: Infected cell extracts were centrifuged at 1000 × *g* for 10 min. The supernatant fluid was centrifuged at 121,000 × *g* for 90 min and the resulting virus pellet was treated with pancreatic DNAase I (500 μg/ml, 2000 u/mg) for 60 min at 4°C and 15 min at 37°C. Virus was concentrated by ultracentrifugation as above and banded on a 10–40% (w/w) potassium tartrate density gradient (121,000 × *g* for 3 h). Virus from the density gradient was used for the [^{3}H]DM binding experiments (see Results).

DNA, RNA, and protein synthesis

The ability of cells to synthesize macromolecules after anthracycline treatment was determined by exposing cultures in 35 mm plastic dishes to EBM containing 1 μCi/ml of [^{3}H]thymidine (72 Ci/mmol, ICN Radiochemicals), [^{3}H]uridine (45.9 Ci/mmol, New England Nuclear, Inc.), or [^{3}H]leucine (50 Ci/mmol, New England Nuclear, Inc.) for 30 min at 36°C. In all experiments reported the incorporation of radiolabeled compound into cellular macromolecules was linear for at least 60 min. Labeled cells were scraped into ice cold saline after brief (2 min) trypsin (0.25%) treatment and then added to an equal volume of cold 10% TCA. After standing at 4°C overnight, precipitates were collected on glass fiber filters (GF, Schleicher and Schuell, Inc.) and washed 8 times with cold 5% TCA. Each filter was counted in 15 ml Ready Solv HP (Beckman Instruments, Inc.).

CsCl analysis of DNA

Cultures were labeled with [^{3}H]thymidine (2 μCi/ml) before extraction of DNA. Cells were lysed with 1% SDS in 10 mM Tris-HCl (pH 7.4) containing 5 mM EDTA. Lysates were incubated with RNAase (200 μg/ml, 100 U/mg, preheated 90°C for 10 min) for 60 min at 37°C. Predigested pronase was added to 1 mg/ml and incubation continued for 18 h. DNA was precipitated with cold ethanol, collected, and solubilized in 1 × SSC containing 0.5% SDS. Samples were treated again with RNAase and pronase as described and DNA was precipitated with ethanol. DNA solubilized in 1 × SSC was added to CsCl solution (initial density 1.700 g ml^{-1}). Three ml volumes were covered with mineral oil and centrifuged at

121,000 × *g* for at least 50 h. Fractions were collected and the refractive indices determined prior to acid precipitation of labeled DNA which was subsequently collected on glass fiber filters and counted in Ready Solv HP. All enzymes were from Sigma Chemical Co.

Uptake of [^{3}H]daunomycin

Cells were exposed to EBM + 2% FCS + [^{3}H]DM (New England Nuclear, 3.8 Ci/mmol) at different concentrations for 15 min at 37°C. At the end of the labeling period cells were scraped into the medium, filtered on to Millipore HA filters, and washed 3 times with cold physiological saline (3 ml/wash). Filters were placed in vials, 0.5 ml ethyl acetate added followed 15 min later by 15 ml Ready Solv HP cocktail.

Anthracycline compounds

The sources were as follows: adriamycin, Aldrich Chemical Co.; daunomycin, Sigma Chemical Co.; *N,N*-dimethyl daunomycin, National Cancer Institute.

Results

Inhibition of HSV synthesis by adriamycin

The effect of AD on the synthesis of HSV was tested by incubating infected cells in the presence of various drug concentrations and determining virus titers 24 h p.i. The replication of both type 1 and type 2 HSV strains was inhibited to about the same extent by a given concentration of compound (Fig. 2). There was a marked difference in the degree of inhibition depending on which host cell was used. At 1 μM AD, for example, HFF cells showed > 3 $\log_{10}$ decrease in titer compared to Vero cells.

Inhibition of HSV by different anthracyclines

To test inhibition of HSV synthesis by anthracyclines, infected cells were treated with each compound after virus adsorption and viral yields determined 24 h later. The degree of inhibition by all 3 compounds was dependent upon the host cell (Fig. 3). NDMD proved to be more potent than AD or DM; e.g. at low concentrations (0.1 μM in HFF, 0.5 μM in Vero cells) there was > 400-fold difference in viral yield between NDMD-treated cells and either AD- or DM-treated cultures. Vesicular stomatitis, polio and rhinoviruses were not inhibited by any of the anthracyclines at concentrations up to 10 μM in either HFF or Vero cells (data not shown).

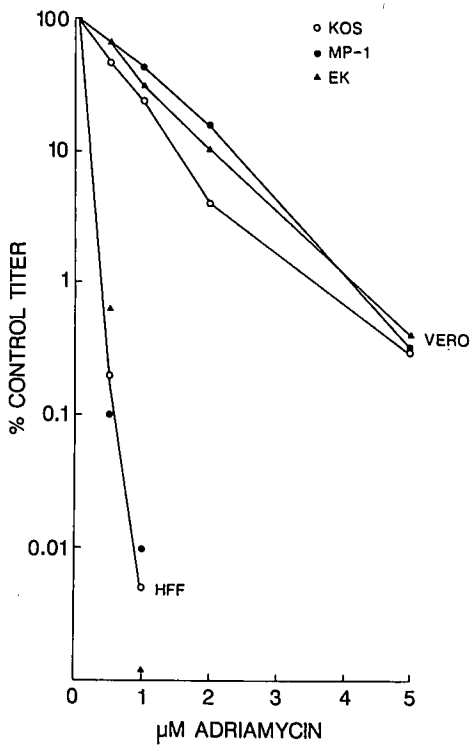


Fig. 2. Inhibition of HSV in Vero and HFF cells by adriamycin. Vero or HFF cells were infected with each HSV strain at 1 PFU/cell. At the end of the adsorption period (60 min, 36°C) unattached virus was removed and cell sheets were washed twice with EBM + 2% FCS. Fresh medium containing AD was added and viral yields determined at 24 h p.i.

Effects of anthracyclines on the host cells

The effects of anthracyclines on host cells were tested by incubating HFF or Vero cells in medium containing 1 μM AD, DM or NDMD. At time intervals indicated in Fig. 4, drug was removed and DNA synthesis measured by [^{3}H]thymidine incorporation. All drugs caused a rapid decrease in the ability of cells to synthesize DNA (Fig. 4). It is important to note, however, that these cells were resistant to cytotoxic effects of anthracyclines. HFF and Vero cells are not destroyed after 24 h incubation with anthracyclines and cellular protein synthesis continues, albeit at a decreased level (Table 1). Both HFF and Vero cells tolerated relatively high concentrations of AD (40 μM) for 24 h without obvious cytological damage as estimated by Trypan Blue exclusion and light microscopy. In these cells, therefore, anthracyclines exert only a cytostatic effect at concentrations much higher than those required to completely block cellular DNA synthesis. The inhibition of DNA synthesis by anthracyclines does not correlate with cytotoxicity in HFF or Vero cells as it does in HeLa cells used by other workers studying inhibition of virus replication by DM (Cohen et al., 1969; Shortridge and Squires, 1977).

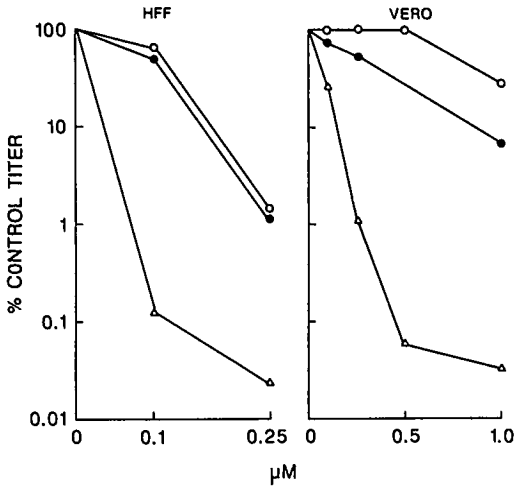


Fig. 3. Inhibition of HSV-MP in Vero and HFF cells by anthracyclines. Experimental conditions were as outlined in Fig. 2 except that Ad (○), DM (●), and NDMD (△) were tested. The concentrations of compounds are given on the abscissa.

The uptake of [^{3}H]DM by HFF and Vero cells is shown in Fig. 5. The amount of compound taken up in 15 min by the cells appeared to be equal when the data were expressed in terms of DPM per mg protein. There was a significant difference, however, when data were plotted as DPM/cell. This was obviously a reflec-

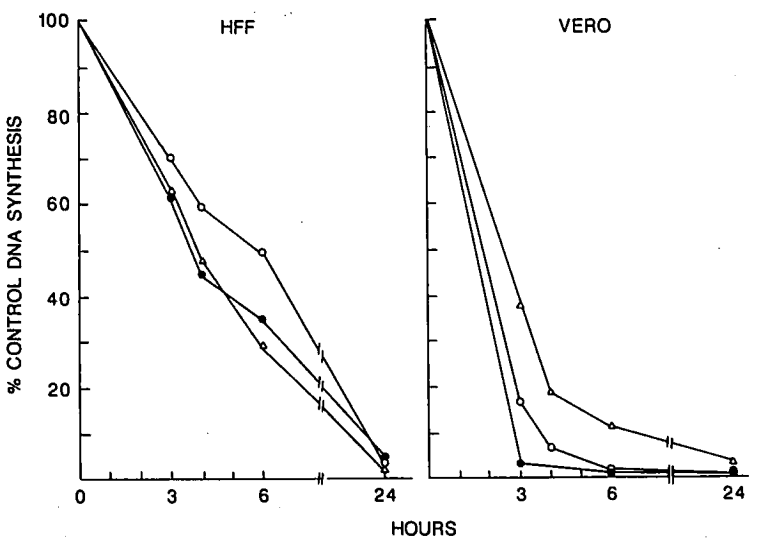


Fig. 4. Inhibition of DNA synthesis by anthracyclines. Vero or HFF cells were incubated with 1 μM AD (○), DM (●) or NDMD (△) for the time periods indicated. Drug was then removed and cultures were labeled for 30 min with [^{3}H]thymidine as outlined in Materials and Methods.

TABLE 1

Inhibition of macromolecule synthesis by anthracyclines.

Cell	Compound	% Inhibition DNA	RNA	Protein
Vero	Adriamycin	97 (±1)	96 (±2)	17 (±6)
	Daunomycin	98 (±1)	97 (±1)	32 (±6)
	NDMD	85 (±10)	38 (±6)	39 (±8)
HFF	Adriamycin	94 (±4)	94	61
	Daunomycin	96 (±1)	94	65
	NDMD	94 (±4)	94	61

Cells were incubated with 1 μM compound for 24 h before determining the incorporation of [^{3}H]thymidine, uridine, or leucine into acid insoluble material. Average values of 2–3 determinations (with standard deviations) are given. Values without SD represent single determinations.

tion of the larger size and protein content of HFF cells. Since the kinetics of inhibition of DNA synthesis in HFF and Vero cells were similar (Fig. 4) it would seem that if any differences in uptake occurred at longer incubation times they did not account for the increased sensitivity of HFF cells to anthracycline inhibition of HSV synthesis.

Effect of time of addition of anthracyclines on HSV inhibition

Incubation of cells with compounds for 24 h before infection failed to inhibit HSV

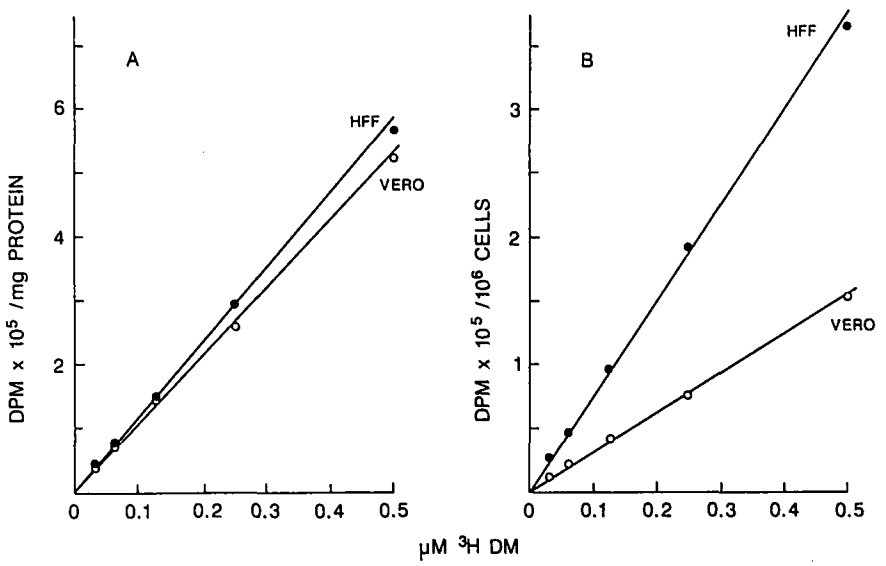


Fig. 5. Uptake of [^{3}H]DM by HFF and Vero cells. Conditions are as outlined in Materials and Methods. Average values of duplicate samples are presented.

TABLE 2

Effect of drug pretreatment on HSV synthesis in Vero cells.

Compound	μM	Virus yield (PFU/ml)	% Control
None	-	6.50×10^7	100
AD	0.5	6.20×10^7	95
	1.0	5.80×10^7	89
DM	0.5	8.35×10^7	128
	1.0	4.95×10^7	76
NDMD	0.5	1.12×10^8	172
	1.0	5.30×10^7	81

Vero cells were treated with the indicated concentrations of each compound for 24 h before infection with HSV-MP at an input moi of 1 PFU/cell. Fresh medium without compound was added after infection and cultures were harvested at 24 h p.i.

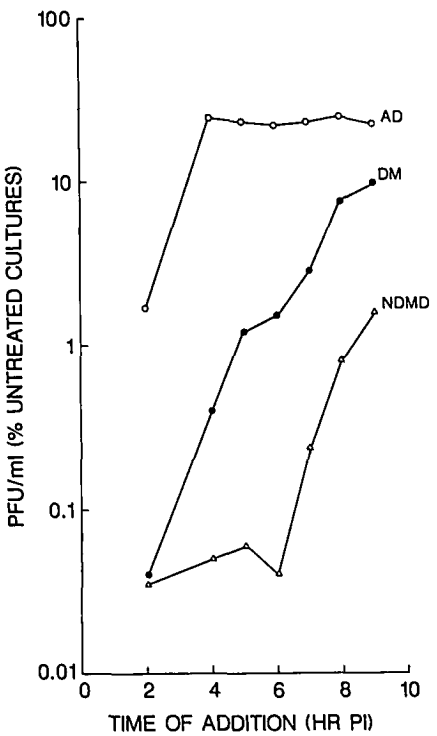


Fig. 6. Inhibition of HSV synthesis by anthracyclines added at intervals after infection. Vero cells were infected with HSV-MP at 1 PFU/cell. At the end of the adsorption period cells were washed twice with EBM and fresh EBM + 5% FCS was added to each well. At intervals thereafter, AD (○ = 2.5 μM), DM (● = 2.5 μM) or NDMD (△ = 1 μM) was added and incubation continued. At 24 h p.i. cultures were harvested and assayed for HSV.

synthesis significantly (Table 2) even though cells treated for 24 h with anthracyclines showed 90% inhibition of DNA synthesis (Fig. 4).

Anthracyclines could be added late in the replicative cycle and still exert virus inhibitory effects (Fig. 6). NDMD completely inhibited HSV synthesis when added as late as 6 h p.i. in Vero cells. AD and DM were less effective when added by 4 h p.i. although DM depressed the virus yield by 90% when added as late as 8 h p.i.

Direct effect of anthracyclines on HSV

The fact that the compounds could be added late in the infectious cycle and still be effective inhibitors suggested that they might be directly inactivating virions. To test this possibility, HSV was mixed with anthracyclines and incubated at 37°C. Infectious virus was assayed at the intervals shown in Fig. 7.AD (1 μM) decreased virus titers by more than 90% after 4 h incubation at 37°C. DM was somewhat less effective and NDMD was without effect. HSV-KOS displayed the same pattern of anthracycline inactivation as HSV-MP (data not shown). Long-term exposure (24 h) to NDMD did not inactivate HSV at concentrations sufficient to suppress virus synthesis by > 99.9%.

The direct inactivation of HSV by AD or DM was temperature-dependent with little or no loss occurring at 0°C (Table 3). Incubation of DNAase-treated, partially purified HSV with [^{3}H]DM resulted in binding of the compound to virions as determined by equilibrium sedimentation in potassium tartrate density gra-

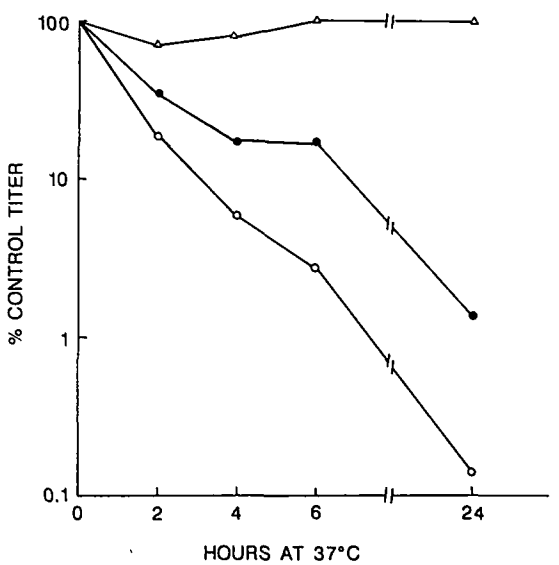


Fig. 7. Direct inactivation of HSV-MP by anthracycline compounds. HSV-MP (10^8 PFU) was mixed with an equal volume of AD (○), DM (●) or NDMD (△) to give a final drug concentration of 1 μM. Incubation was continued at 37°C and samples takes as indicated for virus assay.

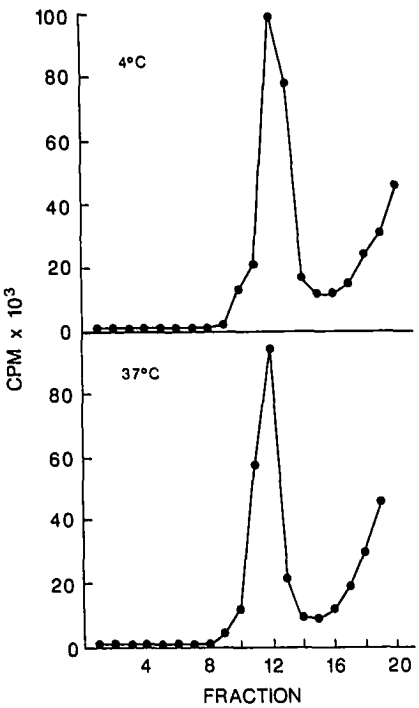


Fig. 8. Density gradient centrifugation of HSV-KOS after exposure to [^{3}H]DM. HSV was incubated with [^{3}H]DM (10 μCi) at 4°C or 37°C for 18 h. Samples were then layered over 10–40% (w/w) potassium tartrate gradients and centrifuged for 3.5 h at 115,000 × *g*. Fractions were collected and assayed for radioactivity and PFU. The radioactive peak in the top panel coincides with the peak of HSV PFU in that gradient.

dients (Fig. 8). [^{3}H]DM bound to virus particles equally well at 4°C or 37°C even though there was no decrease in titer after incubation of HSV with DM at 4°C (data not shown).

TABLE 3

Effect of temperature on the direct inactivation of HSV-MP by adriamycin.

Adriamycin	Temp. (°C)	Titer (PFU)	% Inactivation
–	0	3.90×10^5	–
+	0	3.40×10^5	13
–	22	3.55×10^5	–
+	22	1.08×10^5	70
–	37	3.95×10^5	–
+	37	3.75×10^3	99

Virus was incubated with 1 μM adriamycin at the indicated temperature for 6 h prior to dilution and assay on Vero cells.

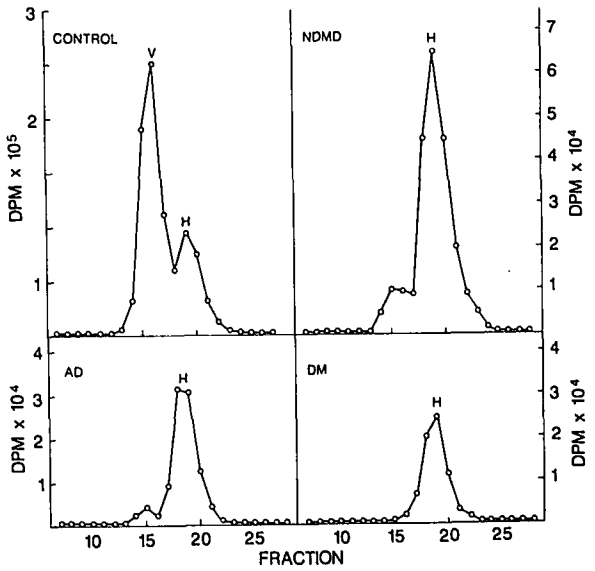


Fig. 9. CsCl density gradient analysis of DNA isolated from HFF cells infected with HSV in the presence or absence of anthracycline compounds. Compounds were added to medium after virus adsorption at the following final concentrations: AD = 250 nM; DM = 250 nM; NDMD = 50 nM. On the figures V denotes viral DNA (density, 1.726 g/ml) and H denotes host cell DNA (density, 1.700 g/ml).

Effect of anthracyclines on viral DNA synthesis

The effect of anthracyclines on HSV DNA synthesis was determined by labelling infected cells with [^{3}H]thymidine after infection. The amount of viral DNA synthesized was estimated by CsCl density gradient analysis. The results demonstrate the inhibition of viral DNA synthesis in HFF cells with 50 nM NDMD, and 250 nM AD or DM (Fig. 9). At these concentrations HSV DNA synthesis was inhibited to a greater extent than cellular DNA synthesis although the latter was severely inhibited in infected cells with any compound.

Discussion

The anthracycline compounds used in this study were effective inhibitors of HSV-1 or HSV-2 replication in either HFF or Vero cells. The extent of viral inhibition, however, was related to the host cell used for infection with HFF cells being the most sensitive cell line. Concentrations of compounds less than 1 μM inhibited HSV by over 3 $\log_{10}$ units in HFF cells. Differences in the inhibition of viruses by anthracycline compounds noted by some authors may be due to properties of the particular host cells used for their experiments. For example, Cohen et al. (1969)

found that daunomycin did not inhibit influenza virus synthesis in chick embryo cells while Minor and Dimmock (1977) observed complete inhibition of the related avian influenza virus in BHK 21 cells. Such differences may be due to uptake of anthracyclines although the present work suggests that other factors may also be involved.

The kinetics of inhibition of host cell DNA synthesis was about the same for AD, DM or NDMD in HFF cells even though the inhibition of HSV synthesis was markedly different when NDMD was compared to the other two compounds. The same was true for Vero cells. Even if uptake of these compounds is different in the short term, the effects on DNA synthesis suggest that substantial intracellular accumulation of AD, DM or NDMD occurs well before the end of the viral eclipse phase. The rate of uptake of the compounds by cells may not be a major factor in establishing the degree of viral inhibition.

A significant difference between the present work and previous studies on the inhibition of DNA-containing viruses also relates to the choice of host cells for virus infection. We have employed Vero and HFF cells, both of which are resistant to the cytotoxic effects of anthracyclines. The fact that three RNA-containing viruses replicated in these cells in the presence of high drug concentrations indicates a lack of general cellular toxicity by the compounds. By contrast, the HeLa cells used in other studies (Cohen et al., 1969; Shortridge and Squires, 1977) are extremely sensitive to cytotoxicity of anthracyclines. Concentrations of 1 μM AD or DM, for example, will destroy HeLa cells after overnight incubation. Such is not the case for Vero or HFF cells.

Previous reports have not considered direct inactivation of virions in relation to reduction in virus titers by anthracyclines. Both AD and DM were capable of directly inactivating HSV in a time- and temperature-dependent fashion. The amino group on the daunosamine moiety appears to be necessary for inactivation of HSV by AD or DM. Two pieces of evidence support this view; (1) NDMD does not directly inactivate HSV at concentrations comparable to AD or DM (Fig. 7), and (2) AD bound by its amino group to a solid agarose support with a 10 atom spacer (Affigel 10, Bio-Rad Laboratories, Inc.) does not inactivate HSV (unpublished data).

The inactivation of HSV by AD or DM was apparently not a photodynamic effect. Incubation of HSV with compounds followed by irradiation with bright light failed to reveal differences in titer from virus incubated in darkness during or after compound treatment (data not shown).

Modification of the amino group on daunosamine appears to result in two favorable properties; (1) loss of mutagenicity (Umezawa et al., 1978; Westendorf et al., 1984), and (2) increased antiviral potency. The influence of size and type of substitution at the sugar amino group on the antiviral activity of anthracycline compounds has not yet been determined.

The addition of AD, DM or NDMD immediately after the adsorption period resulted in an inhibition of HSV DNA synthesis. It was surprising, therefore, to find that addition of compounds relatively late in the infectious cycle could result in a decrease in virus yield. Much of the inhibition by AD and DM in this exper-

iment could be attributed to direct inactivation. This was not the case for NDMD which did not inactivate HSV and was fully active when added as late as 6 h p.i. Progeny virus was first detected at 7–8 h p.i. in our hands. This suggested that NDMD might be capable of interfering with some late event in the virus replicative cycle although an effect on the final stages of viral DNA synthesis cannot be ruled out. AD was reported to interact with membranes and to influence membrane activities (Bossa et al., 1977; Murphree et al., 1981; Siegfried et al., 1983). It is possible, therefore, that NDMD is capable of disrupting the HSV synthetic cycle by altering membrane functions necessary for viral protein synthesis or virion assembly.

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