Effects of *S*-Adenosylhomocysteine Analogues on Vaccinia Viral Messenger Ribonucleic Acid Synthesis and Methylation†

Charles S. G. Pugh and Ronald T. Borchardt*

ABSTRACT: Structural analogues of *S*-adenosyl-L-homocysteine (AdoHcy), with modifications in the amino acid, base, or sugar portion of this molecule, were evaluated in vitro for their abilities to inhibit the *S*-adenosyl-L-methionine (AdoMet)-dependent transmethylations catalyzed by vaccinia virion mRNA (guanine-7)methyltransferase and mRNA (nucleoside-2′)methyltransferase. In addition, these analogues were evaluated for their effects on the vaccinia virion guanylyltransferase and on viral mRNA synthesis. These enzymatic activities are involved in the synthesis of the methylated capped structure at the 5′ terminus of viral mRNA. Kinetic inhibition constants were determined for those AdoHcy analogues that were found in preliminary studies to be good inhibitors of the virion mRNA methyltransferases. *S*-Adenosyl-L-homocysteine sulfoxide, *S*-adenosyl-L-homocysteine sulfone, Sinefungin, A9145c, *S*-(N^6-methyladenosyl)-L-homocysteine, *S*-(3-deazaadenosyl)-L-homocysteine, and *S*-aristeromycinyl-L-homocysteine were found to be the most potent inhibitors of the mRNA (guanine-7)methyltransferase. *S*-Tubercidinyl-L-homocysteine was found to be a poor inhibitor of the (guanine-7)methyltransferase but a very potent inhibitor of the (nucleoside-2′)methyltransferase. The synthetic analogues of AdoHcy were found not to affect guanylyltransferase activity or virion RNA synthesis. In contrast, Sinefungin and A9145c were found to stimulate guanylyltransferase activity and inhibit RNA synthesis. The inhibitory effects of RNA synthesis appear to result in a decrease in the amount of RNA released from the viral core. Similar effects were not observed with AdoHcy or its synthetic analogues. The core-bound RNA synthesized in the presence of A9145c contained very large RNA not produced in the absence of this AdoHcy analogue. These studies suggest that the natural AdoHcy analogues, A9145c and Sinefungin, have other biochemical effects in addition to their inhibitory effects in vaccinia virion methyltransferases. These inhibitory effects on RNA synthesis appear unique to these natural AdoHcy analogues and are not exhibited by the synthetic analogues studied. On the basis of these results, these effects must be considered as possible mechanisms for the reported antiviral effects of these compounds.

Most eukaryotic viral and cellular mRNAs contain the blocked and methylated structure m^7GpppN^m at the 5′ terminus (Shatkin, 1976; Shatkin et al., 1979). These blocked structures are present in newly formed RNA molecules and apparently are conserved during processing to cytoplasmic messengers (Salditt-Georgieff et al., 1976; Perry & Kelley, 1977). These methylated, blocked structures have been shown to be necessary for the efficient binding of the cytoplasmic messenger to the ribosome, subsequent polysome formation, and the translation of the mRNA to proteins (Both et al., 1975a,b; Breindl & Holland, 1976; Weber et al., 1977). Considering the importance of this form of posttranscriptional modification to the multiplication of the virus, several laboratories have attempted to identify specific inhibitors of the viral enzymes which are involved in these transmethylations (Pugh et al., 1977, 1978b; Borchardt & Pugh, 1979; Robert-Gero et al., 1975, 1979; Legraverend et al., 1977; Raies et al., 1976; Jacquemont & Huppert, 1977; Rottman et al., 1979; Kaehler et al., 1977, 1979; Vedel et al., 1978). Specific inhibitors of the viral enzymes could have potential as antiviral agents (Borchardt, 1980).

Both the sugar and base methylations at the 5′ terminus of mRNA are catalyzed by specific methyltransferases which require *S*-adenosyl-L-methionine (AdoMet)[1] as the methyl donor and are inhibited by the demethylated product *S*-adenosyl-L-homocysteine (AdoHcy) (Shatkin, 1976). The inhibitory activity of AdoHcy on AdoMet-dependent methyltransferases is a general characteristic of this class of enzymes (Borchardt, 1977). Analogues of AdoHcy, which have been found to inhibit mRNA methyltransferases in vitro, have also been shown to inhibit viral multiplication in vivo (Borchardt & Pugh, 1979; Pugh et al., 1978b; Jacquemont & Huppert, 1977; Robert-Gero et al., 1979; Nagarajan, 1979).

In an attempt to identify those structural features of AdoHcy which are essential for the binding of this ligand to viral mRNA methyltransferases, our laboratory studied the inhibitory properties of a series of synthetic analogues of AdoHcy toward Newcastle disease virion (NDV) mRNA (guanine-7)methyltransferase (Pugh et al., 1977). In addition, we (Pugh et al., 1978b; Borchardt & Pugh, 1979) have reported that two naturally occurring amino acid modified analogues of AdoHcy, e.g., Sinefungin and A9145c, are potent

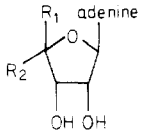
† From the Department of Biochemistry, Smissman Laboratories, University of Kansas, Lawrence, Kansas 66045. *Received July 22, 1981.* This work was supported by Research Grant GM-16364 from the National Institute of General Medical Sciences.

[1] Abbreviations: Ado-*N*-AcHcy, *S*-adenosyl-L-*N*-acetylhomocysteine; Ado-*N*-AcTpa, 3-(adenosylthio)-*N*-acetylpropylamine; AdoCys, *S*-adenosyl-L-cysteine; AdoDab, 5′-N^6-adenosyl-α,γ-diaminobutyric acid; L-AdoHcy, *S*-adenosyl-L-homocysteine; D-AdoHcy, *S*-adenosyl-D-homocysteine; AdoHcy sulfone, *S*-adenosyl-L-homocysteine sulfone; AdoHcy sulfoxide, *S*-adenosyl-L-homocysteine sulfoxide; AriHcy, *S*-aristeromycinyl-L-homocysteine; 2-aza-AdoHcy, *S*-(2-azaadenosyl)-L-homocysteine; 8-aza-AdoHcy, *S*-(8-azaadenosyl)-L-homocysteine; CytHcy, *S*-cytidyl-L-homocysteine; 3-deaza-AdoHcy, *S*-(3-deazaadenosyl)-L-homocysteine; AdoTba, 4-(adenosylthio)butyric acid; AdoTbMe ester, 4-(adenosylthio)butyrate methyl ester; 2′-deoxy-AdoHcy, *S*-(2′-deoxyadenosyl)-L-homocysteine; 3′-deoxy-AdoHcy, *S*-(3′-deoxyadenosyl)-L-homocysteine; N^6-dimethyl-3-deaza-AdoHcy, *S*-(N^6-dimethyl-3-deazaadenosyl)-L-homocysteine; InoHcy, *S*-inosyl-L-homocysteine; N^6-methyl-AdoHcy, *S*-(N^6-methyladenosyl)-L-homocysteine; N^6-methyl-3-deaza-AdoHcy, *S*-(N^6-methyl-3-deazaadenosyl)-L-homocysteine; TubHcy, *S*-tubercidinyl-L-homocysteine; UriHcy, *S*-uridyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; m^7G, 7-methylguanosine; N^m, 2′-*O*-methylnucleoside; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NDV, Newcastle disease virus; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol.

0006-2960/82/0421-1535$01.25/0

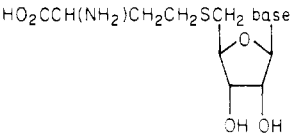
Table I: Structural Analogues of AdoHcy

Amino Acid Modifications:



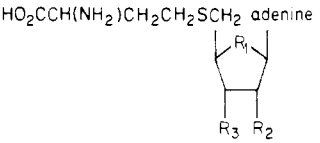
compd	R_1	R_2
L-AdoHcy	$-CH_2SCH_2CH_2CH(NH_2)CO_2H$ (L)	H
D-AdoHcy	$-CH_2SCH_2CH_2CH(NH_2)CO_2H$ (D)	H
AdoHcy sulfoxide	$-CH_2S(O)CH_2CH_2CH(NH_2)CO_2H$ (L)	H
AdoHcy sulfone	$-CH_2S(O)_2CH_2CH_2CH(NH_2)CO_2H$ (L)	H
Ado-*N*-AcHcy	$-CH_2SCH_2CH_2CH(NHAc)CO_2H$ (L)	H
AdoTba	$-CH_2SCH_2CH_2CH_2CO_2H$	H
AdoTbMe ester	$-CH_2SCH_2CH_2CH_2CO_2CH_3$	H
Ado-*N*-AcTpa	$-CH_2SCH_2CH_2CH_2NHAc$	H
AdoCys	$-CH_2SCH_2CH(NH_2)CO_2H$ (L)	H
Sinefungin	$-CH_2CH(NH_2)CH_2CH_2CH(NH_2)CO_2H$	H
A9145c	$=CHCH(NH_2)CH_2CH_2CH(NH_2)CO_2H$	
AdoDab	$-CH_2NHCH_2CH_2CH(NH_2)CO_2H$	H

Base Modifications:



compd	base
TubHcy	7-deazaadenine
N^6-methyl-AdoHcy	*N*^{6}-methyladenine
8-aza-AdoHcy	8-azaadenine
2-aza-AdoHcy	2-azaadenine
3-deaza-AdoHcy	3-deazaadenine
N^6-methyl-3-deaza-AdoHcy	*N*^{6}-methyl-3-deazaadenine
N^6-dimethyl-3-deaza-AdoHcy	*N*^{6}-dimethyl-3-deazaadenine
InoHcy	hypoxanthine
UriHcy	uracil
CytHcy	cytosine

Sugar Modifications:



compd	R_1	R_2	R_3
AriHcy	CH_2	OH	OH
2′-deoxy-AdoHcy	O	H	OH
3′-deoxy-AdoHcy	O	OH	H

inhibitors of NDV mRNA (guanine-7)methyltransferase and vaccinia virion mRNA (guanine-7)- and (nucleoside-2′-*O*)-methyltransferases. In this study, we have further defined the structural requirements for binding of AdoHcy to the vaccinia virion methyltransferases by using a series of synthetic and naturally occurring analogues of AdoHcy. In addition, we report here the effects of these analogues on other vaccinia virion enzymes including RNA polymerase and guanylyltransferase.

Materials and Methods

Materials. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]AdoMet, 10.7 Ci/mmol) was obtained from New England Nuclear, [5-^{3}H]uridine 5′-triphosphate ([5-^{3}H]UTP, 18 Ci/mmol) was from Schwarz/Mann, [8-^{3}H]guanosine 5′-triphosphate ([8-^{3}H]GTP, 16.7 Ci/mmol) was from ICN, AdoHcy, AdoMet, ribonucleoside triphosphates, *Crotalis atrox* venom nucleoside pyrophosphatase, and poly(adenylic acid) were from Sigma, vaccinia virus (WR) seed inoculum (VR119) was from the American type culture collection, Waymouth 752/1 medium was from Kansas City Biologicals, and DE-81 disks (DEAE-cellulose) were from Whatman. The AdoHcy analogues (Table I) used in this study were synthesized according to previously published procedures as cited below: D-AdoHcy, AdoHcy sulfoxide, AdoHcy sulfone, Ado-*N*-AcTpa, AdoTba, AdoTbMe ester, and Ado-*N*-AcHcy, Borchardt & Wu (1974); InoHcy, *N*^{6}-methyl-AdoHcy, 3-deaza-AdoHcy, *N*^{6}-methyl-3-deaza-AdoHcy, *N*^{6}-dimethyl-3-deaza-AdoHcy, UriHcy, and CytHcy, Borchardt et al. (1974); 2′-deoxy-AdoHcy and 3′-deoxy-AdoHcy, Borchardt & Wu (1975); AriHcy, Borchardt & Wu (1976); AdoCy, TubHcy, and 8-aza-AdoHcy, Borchardt et al. (1976); 2-aza-AdoHcy, R. T. Borchardt, D. Kuonen, J. A. Huber, and A. Moorman (unpublished experiments). Sinefungin and A9145c were generous gifts from Dr. R. Nagarajan, Lilly Research Laboratories. AdoDab was a gift from Dr. J. Coward, Yale University.

Viral Purification. Vaccinia (WR) virus was grown in monolayers of NCTC clone 929 mouse cells, strain L (Earle), which were cultured at 37 °C in serumless Waymouth's MD 752/1 (Kitos et al., 1962). Vaccinia virions were purified by rate zonal banding in sucrose gradients (Joklik, 1962).

Vaccinia Virion mRNA Methyltransferase Assay. The in vitro assays for mRNA (guanine-7)methyltransferase and mRNA (nucleoside-2′)methyltransferase were similar to those described earlier by Pugh et al. (1978a,b). The assay mixtures contained (final volume 100 μL) 50 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 0.05% NP-40, 10 mM dithiothreitol, 2.5 mM ATP, 2.5 mM CTP, 2.5 mM GTP, 1.0 mM UTP, variable amounts of [*methyl*-^{3}H]AdoMet (10.7 Ci/mmol), variable amounts of the AdoHcy analogues, and 0.05 A_{260} unit of purified vaccinia (WR) virus. The reaction mixtures were incubated for 40 min at 37 °C, and the reaction was terminated by the addition of 0.5 mL of buffer (50 mM Tris-HCl, 5 mM EDTA, and 0.5% $NaDodSO_4$, pH 7.8). Carrier RNA (40 A_{260} units) was added, and the RNA was ethanol precipitated. The RNA was dissolved and divided into two aliquots. To one aliquot was added 0.1 unit of *Crotalis atrox* venom nucleotide pyrophosphatase and to the other aliquot an equal volume of H_2O. Both aliquots were incubated at 37 °C for 1.5 h and then applied to DEAE disks as described below.

Vaccinia Virus Solubilized Guanylyltransferase Assay. A preparation of solubilized vaccinia virus guanylyltransferase was a generous gift from Dr. Bernard Moss (National Institutes of Health). The guanylyltransferase activity was assayed by using the procedure described earlier by Ensinger et al. (1975). The assay mixtures (100 μL) contained 50 mM Tris-HCl (pH 7.8), 2.5 mM $MgCl_2$, 2.5 mM [8-^{3}H]GTP (16.7 Ci/mmol), 10 μg of 5′-diphosphopoly(adenylic acid) [prepared as described earlier by Martin & Moss (1975)], 1.0 mM dithiothreitol, 2.5 μL of the solubilized vaccinia virus preparation, and the AdoHcy analogues. The reaction mixtures were incubated for 25 min at 37 °C, and the reaction was terminated by the addition of 0.5 mL of buffer (50 mM Tris-HCl, 5 mM EDTA, and 0.5% sodium dodecyl sulfate, pH 7.8). Carrier RNA was added, and the RNA was ethanol precipitated, resuspended, and applied to DEAE disks as described below.

Vaccinia Virion RNA Polymerase Assay. The assay for RNA polymerase activity was identical with the methyltransferase assays described above, except that [*methyl*-^{3}H]AdoMet and unlabeled UTP were replaced with unlabeled AdoMet and [5-^{3}H]UTP (1.0 mM, 0.05 Ci/mmol). After ethanol precipitation, the RNA was applied to DEAE disks as described below.

DEAE-cellulose Binding of RNA. The RNA-containing samples (90 μL) were pipetted directly onto 2.5-cm DEAE-

cellulose disks (DE-81). The disks were washed 6 times for 5 min each in 0.5 M Na_2HPO_4 buffer, twice in H_2O, and twice in ethanol as described by Roeder (1974). After the disks were dried, 10-mL aliquots of toluene-based scintillation fluid were added, and the samples were assayed for radioactivity.

Separation of Core-Bound and Released Vaccinia RNA. Vaccinia RNA was synthesized as described above in the presence of either [*methyl*-^{3}H]AdoMet or [5-^{3}H]UTP. The incubations (37 °C) were stopped with 0.9 mL of ice-cold buffer (50 mM Tris, 10 mM DTT, and 0.05% NP-40, pH 7.8), and the vaccinia cores were collected by centrifugation (60 s) in an Eppendorf 5412 microcentrifuge (Gershowitz et al., 1978). The supernatant which contained the released RNA was decanted. The pellet containing the core-bound RNA was resuspended in 1.0 mL of buffer (50 mM Tris, 10 mM DTT, and 0.05% NP-40, pH 7.8). To both the core-bound RNA and released RNA samples was added a solution (100 μL) containing 5.5% $NaDodSO_4$, 120 mM EDTA, and 10 A_{260} units of carrier RNA. The RNA from the two samples was then EtOH precipitated. After centrifugation, the RNA pellets were dissolved in 100 μL of buffer (50 mM Tris, 5.0 mM EDTA, and 0.5% $NaDodSO_4$, pH 7.8) and applied to DEAE-cellulose disks as described above or sedimented on sucrose gradients as described below.

Sedimentation of Vaccinia Virion RNA on Sucrose Gradients. RNA samples were dissolved in or diluted to 1.0 mL with 50 mM Tris, 5.0 mM EDTA, and 0.5% $NaDodSO_4$, pH 7.8 (buffer A). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The samples were heated to 60 °C, quenched in ice/H_2O, and centrifuged at 3400*g* for 10 min. The RNA in the aqueous phase minus the interphase material was then EtOH precipitated by adding 10 A_{260} units of carrier RNA if not already present. The RNA pellets were dissolved in 0.4 mL of buffer A, heated to 100 °C for 2 min, and quenched in ice/H_2O. The RNA was layered on linear gradients of 10–25% sucrose in buffer A plus 0.1 M NaCl. At the bottom of the gradient was a 0.5 mL of 60% sucrose (w/w) cushion. The RNA was sedimented in the Beckman SW41 rotor (25 °C, 5.0 h, 41 000 rpm). Fractions were collected from the bottom of the tube, 90 μL of each fraction was bound to DE-81 disks, and radioactivity was determined as described above.

Results and Discussion

Effect of AdoHcy Analogues on Virion mRNA (Guanine-7)methyltransferase and mRNA (Nucleoside-2′)methyltransferase. Vaccinia virus mRNA synthesized in vitro (Wei & Moss, 1975) and in infected cells (Boone & Moss, 1977) contains a methylated capped structure at the 5′ terminus. This methylated capped structure consists of a 7-methylguanosine residue connected from the 5′ position through a triphosphate bridge to the 5′ position of 2′-*O*-methyladenosine or 2′-*O*-methylguanosine. The 5′ terminus is biosynthesized by vaccinia virus core enzymes including mRNA guanylyltransferase (Monroy et al., 1978a,b), mRNA (guanine-7)-methyltransferase (Martin & Moss, 1975, 1976), and mRNA nucleoside 2′-methyltransferase (Barbosa & Moss, 1978a,b). Recently (Venkatesan et al., 1980) a RNA triphosphatase activity has been shown to be part of this viral complement of biosynthetic enzymes.

The effects of synthetic and natural analogues of AdoHcy on the (guanine-7)methyltransferase and (nucleoside-2′)-methyltransferase (Table II) were determined by using the in vitro assay procedures previously described by Pugh et al. (1978a,b). The AdoHcy analogues that contained modifications at the sulfur atom, e.g., AdoHcy sulfoxide, AdoHcy sulfone, and AdoDab, showed appreciable inhibitory activity toward the (guanine-7)methyltransferase. These results are consistent with our earlier observation (Pugh et al., 1978b) that the natural amino acid modified AdoHcy analogues, A9145c and Sinefungin, were very potent inhibitors of this same vaccinia mRNA methyltransferase. The other amino acid analogues tested were essentially devoid of inhibitory activity toward this particular methyltransferase. On the basis of these results, the structural features of importance in the binding of the amino acid portion of AdoHcy to the vaccinia (guanine-7)methyltransferase appear to include the following: (1) the chirality of the amino acid asymmetric carbon; (2) the terminal amino group; (3) the terminal carboxyl group; and (4) the three-carbon distance between the sulfur atom and the terminal amino and terminal carboxyl groups. The (guanine-7)methyltransferase appears capable, however, of accommodating changes in and around the sulfur atom of AdoHcy. The structure–activity relationships for the binding of amino acid modified analogues of AdoHcy to vaccinia (guanine-7)methyltransferase are qualitatively similar to that observed for the binding to Newcastle disease virus (NDV) (guanine-7)methyltransferase (Pugh et al., 1977).

The data for the base-modified AdoHcy analogues, shown in Table II, indicate that only N^6-methyl-AdoHcy and 3-deaza-AdoHcy show significant inhibitory activity toward the vaccinia (guanine-7)methyltransferase. The other base-modified analogues showed little or no inhibitory activity toward this enzyme. These results suggest that all of the general features of the adenine portion of AdoHcy are necessary for maximal binding to this vaccinia enzyme. It is of particular interest to compare these results with the data obtained for the NDV (guanine-7)methyltransferase. The NDV enzyme (Pugh et al., 1977) was shown to be very sensitive to inhibition by TubHcy and 8-aza-AdoHcy, suggesting some very basic qualitative differences between the site which binds the adenine portion of AdoHcy on the vaccinia enzyme vs. the NDV enzyme.

Of the sugar-modified analogues, only AriHcy showed significant inhibition of the vaccinia (guanine-7)methyltransferase. This substitution of the oxygen bridge in AdoHcy by a methylene in AriHcy appears to be in part tolerated by the enzyme binding site. The other sugar-modified AdoHcy analogues were substantially less active, suggesting crucial roles for the 2′- and 3′-hydroxy groups in the binding of AdoHcy. In the case of the NDV (guanine-7)methyltransferase (Pugh et al., 1977), both 2′-deoxy-AdoHcy and 3′-deoxy-AdoHcy exhibited some inhibitory activity (K_i = 26 ± 8 μM and K_i = 78 ± 3 μM, respectively), indicating perhaps less stringent roles for these functional groups on AdoHcy in enzymatic binding to the NDV enzyme.

Also shown in Table II are the results for inhibition of the vaccinia (nucleoside-2′)methyltransferase by the various synthetic and natural AdoHcy analogues. An analysis of these inhibition data is complicated by the fact that the (nucleoside-2′)methyltransferase requires a guanine-7-methylated and -capped RNA, e.g., m^7GpppN, as a methyl acceptor substrate (Barbosa & Moss, 1978a,b). Therefore, if the (guanine-7)-methyltransferase is inhibited by an AdoHcy analogue, the (nucleoside-2′)methyltransferase activity would be diminished due to a reduction in available substrate. Thus, an attempt to describe in detail the AdoHcy binding site on the (nucleoside-2′)methyltransferase based on the data shown in Table II might be misleading. However, any AdoHcy analogues that do not significantly inhibit the (guanine-7)methyltransferase but do inhibit the (nucleoside-2′)methyltransferase would be

Table II: Effects of AdoHcy Analogues on Vaccinia Virion mRNA (Guanine-7)methyltransferase, mRNA (Nucleoside-2′)methyltransferase, Guanylyltransferase, and RNA Polymerase[a]

compd	% control, [I] = 100 μM				inhibition constants, K_i ± SE (μM)[d]	
	guanylyltransferase[b]	RNA polymerase[c]	methyltransferases: guanine-7	methyltransferases: nucleoside-2′	mRNA (guanine-7)methyltransferase	mRNA (nucleoside-2′)methyltransferase
L-AdoHcy	102	100	6	2	1.04 ± 0.30	0.53 ± 0.04
amino acid modifications						
D-AdoHcy	105	96	78	23	56 ± 10	14 ± 4
AdoHcy sulfone	109	103	53	3	6.8 ± 2	1.2 ± 0.5
AdoHcy sulfoxide	ND	ND	60	2	9.3 ± 2	1.2 ± 0.2
Ado-*N*-AcHcy	ND	ND	91	90	ND[e]	ND[e]
AdoTba	ND	ND	100	82	ND	ND
AdoTbMe ester	ND	ND	100	80	ND	ND
Ado-*N*-AcTpa	ND	ND	100	60	ND	ND
AdoCys	99	106	91	51	120 ± 20	58 ± 10
Sinefungin	260	85	0	0	0.019 ± 0.002[f]	0.075 ± 0.016[f]
A9145c	236	83	0	0	0.0013 ± 0.0003[f]	0.0018 ± 0.0003[f]
AdoDab	ND	ND	52	48	ND	ND
base modifications						
TubHcy	102	96	80	0	140 ± 40	1.2 ± 0.3
N^6-methyl-AdoHcy	100	100	15	27	8.9 ± 3	9.0 ± 3
8-aza-AdoHcy	ND	ND	95	65	ND	ND
2-aza-AdoHcy	95	103	56	37	ND	ND
3-deaza-AdoHcy	106	100	16	0	6.1 ± 1	2.1 ± 0.4
N^6-methyl-3-deaza-AdoHcy	ND	ND	77	67	ND	ND
N^6-dimethyl-3-deaza-AdoHcy	ND	ND	88	75	ND	ND
InoHcy	ND	ND	71	52	ND	ND
UriHcy	ND	ND	100	69	ND	ND
CytHcy	ND	ND	100	70	ND	ND
sugar modifications						
AriHcy	96	101	34	19	6.3 ± 2	2.6 ± 0.5
2′-deoxy-AdoHcy	ND	ND	80	80	ND	ND
3′-deoxy-AdoHcy	ND	ND	83	81	ND	ND
3′-amino-AdoHcy	ND	ND	77	22	210 ± 50	4.6 ± 1.2

[a] Purified vaccinia virus was assayed in vitro for the methyltransferase, guanylyltransferase, and RNA polymerase activities as described under Materials and Methods. [b] Solubilized vaccinia virus guanylyltransferase was assayed by using 5′-diphosphopoly(adenylic acid) and [8-^{3}H]GTP, and data are expressed as percent inhibition of control (no inhibitor added). [c] RNA polymerase activity was assayed by using AdoMet (0.54 μM) and [5-^{3}H]UTP (1.0 mM, 0.05 Ci/mmol), and data are expressed as percent inhibition of control (no inhibitor added). [d] AdoMet was the variable substrate (0.12–1.0 μM). Each inhibitor showed linear competitive kinetics when plots of reciprocal velocity vs. reciprocal AdoMet concentration were made. Assays were done in duplicate, and the inhibition constants were calculated as described earlier (Pugh et al., 1977). [e] ND, not determined. When less than 50% inhibition of (guanine-7)methyltransferase was observed at an inhibitor concentration of 100 μM, the kinetic inhibition constants were not determined. [f] Data taken from Pugh et al. (1978b).

of particular interest. One analogue which apparently exhibits this property is TubHcy, which did not inhibit the (guanine-7)methyltransferase but produced significant inhibition of the (nucleoside-2′)methyltransferase.

For those analogues which exhibited substantial inhibition of the (guanine-7)methyltransferase or selective inhibition of the (nucleoside-2′)methyltransferase, we determined the kinetic inhibition constants (K_i) under conditions where AdoMet was the variable substrate. All of the AdoHcy analogues tested in this way exhibited competitive kinetic patterns. As reported earlier (Pugh et al., 1978b), the natural AdoHcy analogues, A9145c and Sinefungin, were extremely potent inhibitors of the vaccinia (guanine-7)methyltransferase, exhibiting inhibition constants 800- and 50-fold lower, respectively, than that of the parent compound, AdoHcy. AdoHcy sulfoxide, AdoHcy sulfone, N^6-methyl-AdoHcy, 3-deaza-AdoHcy, and AriHcy were only fair inhibitors of the (guanine-7)methyltransferase, exhibiting inhibition constants 6–9-fold higher than that of AdoHcy. The base-modified AdoHcy analogue TubHcy was particularly interesting since it exhibited a K_i comparable to that of AdoHcy against the (nucleoside-2′)methyltransferase, but the K_i toward the (guanine-7)methyltransferase was 140-fold higher for TubHcy than AdoHcy (Table II). AdoHcy sulfoxide, AdoHcy sulfone, 3-deaza-AdoHcy, and AriHcy were also comparable to AdoHcy in inhibiting the (nucleoside-2′)methyltransferase.

Effects of AdoHcy Analogues on Virion Guanylyltransferase and RNA Polymerase. The effects of the AdoHcy analogues on the vaccinia virion guanylyltransferase are shown in Table II. AdoHcy and the synthetic AdoHcy analogues did not significantly inhibit guanylyltransferase activity when tested at a concentration of 100 μM. However, as described above, the same concentration of several of the analogues produced significant inhibition of the virion methyltransferases. The natural AdoHcy analogues, A9145c and Sinefungin, both produced significant stimulation of the vaccinia guanylyltransferase. The mechanism of this stimulation is unknown at this point.

In addition to determining the effects of AdoHcy and AdoHcy analogues on the vaccinia methyltransferases and guanylyltransferase, we were also interested in their potential effects on virion RNA synthesis. The results shown in Table II indicate that only A9145c and Sinefungin produced an inhibitory effect on RNA synthesis as determined by incorporation of label from [5-^{3}H]UTP.

The time dependence of the effect on RNA synthesis of A9145c was investigated. The amount of RNA synthesized in the presence or absence (control) of A9145c is shown in Figure 1. With shorter incubation times, the rates of RNA synthesis are similar in the control and A9145c-treated sam-

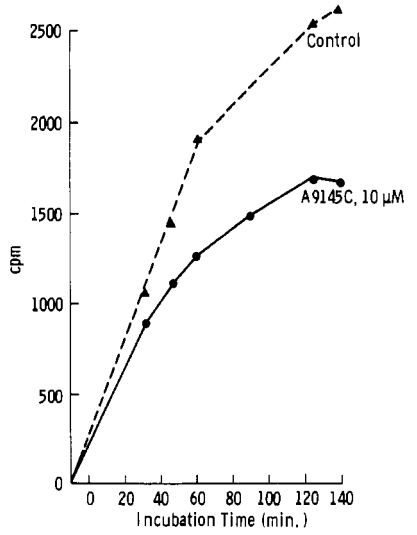


FIGURE 1: Effect of A9145c on vaccinia viral RNA synthesis. Vaccinia RNA was synthesized in vitro for various times with 0.5 mM [^{3}H]UTP (0.1 Ci/mmol) and 1.0 μM AdoMet in the absence (control) or presence of 10 μM A9145c and assayed as described under Materials and Methods. The data points represent the average of duplicate assays.

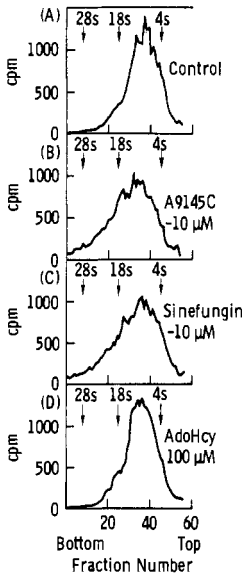


FIGURE 2: Effect of A9145c, Sinefungin, and AdoHcy on the size of vaccinia viral RNA. Vaccinia RNA was synthesized in vitro for 20 min with [^{3}H]UTP (0.05 mM, 1.0 mCi/mmol) and 1.0 μM AdoMet, including (A) no additions, (B) 10 μM A9145c, (C) 10 μM Sinefungin, or (D) 100 μM AdoHcy. After phenol extraction and heat denaturation, the total RNA was sedimented on sucrose gradients as described under Materials and Methods.

ples. However, with prolonged incubation, the rate of RNA synthesis in the presence of A9145c is diminished compared to that of the control.

Since the natural AdoHcy analogues, Sinefungin and A9145c, apparently inhibit vaccinia RNA synthesis in a time-dependent manner, we determined the effect these drugs had on the size of the total in vitro RNA synthesized. The sedimentation profiles of [^{3}H]UTP-labeled vaccinia RNA that were synthesized in the presence of A9145c, Sinefungin, or AdoHcy are shown in Figure 2. The control and AdoHcy-treated RNA sedimentation profiles were similar and were characterized by a lack of RNA greater than 28 S and had less than 15% of the total RNA greater than 18 S (Figure 2A,D). In contrast, the RNA that was synthesized in vitro in the presence of A9145c or Sinefungin contained small

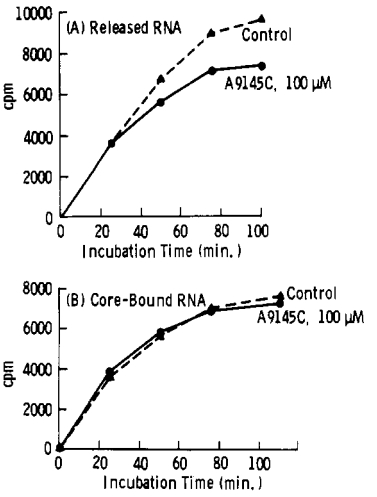


FIGURE 3: Time course of the effects of A9145c on core-bound and released RNA synthesis. Vaccinia RNA was synthesized in assay mixtures containing [^{3}H]UTP (0.05 mM, 0.1 Ci/mmol) and no AdoMet in the absence (control) or presence of 100 μM A9145c. The RNA was then separated into released (A) and core-bound RNA (B) as described under Materials and Methods.

amounts of RNA that sedimented faster than the 28S marker. About 30% of the total RNA in the Sinefungin and A9145c samples was greater than 18 S. Thus, Sinefungin and A9145c cause a small amount of very large vaccinia RNA to be produced in vitro.

Recent studies (Gershowitz et al., 1978) indicate that vaccinia RNA is synthesized, methylated, and blocked while closely associated with the core or nucleocapsid in vitro. The core-bound RNA seems to be a precursor to mRNA, since it is not polyadenylated as is the mature mRNA and is larger than the mature RNA (Gershowitz et al., 1978). The effect of A9145c on the time dependence of the synthesis of vaccinia core-bound and released RNA was therefore determined, and the results are shown in Figure 3. The amounts of released RNA produced in the control and A9145c samples were identical after 25 min (Figure 3A). However, the rate of RNA released from the A9145c sample decreased with increasing time, compared to the control. However, there was no effect of A9145c on the amount of RNA that was core bound, compared to the control. Thus, A9145c seems to inhibit RNA synthesis by decreasing the amount of RNA released from the core. A9145c is probably not affecting transcriptional initiation and elongation since the initial rates of RNA synthesis are identical and the RNA is synthesized on the core and A9145c does not result in a decrease in core-bound RNA. Thus, any attempt to construct a working model of the effect of A9145c on RNA synthesis must take into account its apparent lack of effect on initial RNA synthesis and release, as well as its effect to decrease the amount of released RNA with time.

In separate experiments, it was shown that neither AdoMet (100 μM) nor AdoHcy (1.0 mM) had any effect on the amount of newly synthesized core-bound or released vaccinia RNA (data not shown). In addition, AdoMet (100 μM) did not antagonize the effects of A9145c on released RNA (data not shown).

The influence of A9145c on the size of the in vitro released and core RNA was examined (Figure 4). The sedimentation profiles of the control and A9145c-released RNAs were qualitatively similar (Figure 4A). However, the released RNA synthesized in the presence of A9145c was less abundant than the control RNA, consistent with the experiments described above. In contrast, the sedimentation profiles of the control

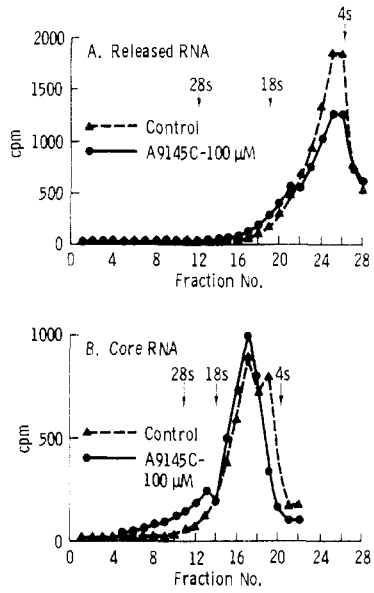


FIGURE 4: Effect of A9145c on the size of released and core-bound vaccinia viral RNA. Vaccinia RNA was synthesized in vitro for 60 min with [^{3}H]UTP (0.5 mM, 1.0 Ci/mmol) and 10 μM AdoMet in the absence (control) or presence of A9145c. The RNA was separated into core-bound and released RNA. The RNA was heat denatured and sedimented on sucrose gradients as described under Materials and Methods.

and A9145c-treated core-bound RNAs were quite different (Figure 4B). The core-bound RNA synthesized in the presence of A9145c contained very large RNA not present in the control core RNA. Thus, A9145c results in the synthesis of very large core-bound RNA, and the release of the core RNA is decreased.

In summary, the results of these studies suggest that the natural AdoHcy analogues, A9145c and Sinefungin, have biochemical effects in addition to their inhibitory effects on vaccinia (guanine-7)- and (nucleoside-2')methyltransferases (Pugh et al., 1978b). The effects of A9145c and Sinefungin on vaccinia guanylyltransferase and RNA synthesis appear unique to these natural AdoHcy analogues and are not exhibited by the synthetic analogues prepared to date. Based on the data described in this study, one must consider the possibility that the antiviral effects reported previously for A9145c and Sinefungin (Borchardt & Pugh, 1979; Pugh et al., 1978b) could result from a mechanism other than inhibition of mRNA methyltransferases. The answer to this question will have to await further investigation.

Acknowledgments

The authors gratefully acknowledge the support of the Center for Biomedical Research—University of Kansas.

References

Barbosa, E., & Moss, B. (1978a) *J. Biol. Chem. 253*, 7692.
Barbosa, E., & Moss, B. (1978b) *J. Biol. Chem. 253*, 7692.
Boone, R. F., & Moss, B. (1977) *Virology 79*, 67.
Borchardt, R. T. (1977) in *The Biochemistry of S-Adenosylmethionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. G., & Schlenk, F., Eds.) p 151, Columbia University Press, New York.
Borchardt, R. T. (1980) *J. Med. Chem. 23*, 347.
Borchardt, R. T., & Wu, Y. S. (1974) *J. Med. Chem. 17*, 862.
Borchardt, R. T., & Wu, Y. S. (1975) *J. Med. Chem. 18*, 300.
Borchardt, R. T., & Wu, Y. S. (1976) *J. Med. Chem. 19*, 197.
Borchardt, R. T., & Pugh, C. S. G. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) p 197, Elsevier/North-Holland, New York.
Borchardt, R. T., Huber, J. A., & Wu, Y. S. (1974) *J. Med. Chem. 17*, 868.
Borchardt, R. T., Huber, J. A., & Wu, Y. S. (1976) *J. Med. Chem. 19*, 1094.
Both, G. W., Furuichi, Y., Muthukrishnan, S., & Shatkin, A. J. (1975a) *Cell (Cambridge, Mass.) 6*, 185.
Both, G. W., Banerjee, A. K., & Shatkin, A. J. (1975b) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1189.
Breindl, M., & Holland, J. J. (1976) *Virology 73*, 106.
Chang, C.-D., & Coward, J. K. (1976) *J. Med. Chem. 19*, 684.
Ensinger, M. J., Martin, S. A., Paloetti, E., & Moss, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2525.
Gershowitz, A., Boone, R. F., & Moss, B. (1978) *J. Virol. 27*, 399.
Jacquemont, B., & Huppert, J. (1977) *J. Virol. 22*, 160.
Joklik, W. K. (1962) *Biochim. Biophys. Acta 61*, 290.
Kaehler, M., Coward, J., & Rottman, F. (1977) *Biochemistry 16*, 5770.
Kaehler, M., Coward, J., & Rottman, F. (1979) *Nucleic Acids Res. 6*, 1161.
Kitos, P. A., Sinclair, R., & Waymouth, C. (1962) *Exp. Cell Res. 27*, 307.
Legraverend, M., Ibanez, S., Blanchard, P., Enouf, J., Lawrence, F., Robert-Gero, M., & Lederer, E. (1977) *Eur. J. Med. Chem.—Chim. Ther. 12*, 105.
Martin, S. A., & Moss, B. (1975) *J. Biol. Chem. 250*, 9330.
Martin, S. A., & Moss, B. (1976) *J. Biol. Chem. 251*, 7313.
Monroy, G., Spencer, E., & Hurwitz, J. (1978a) *J. Biol. Chem. 253*, 4481.
Monroy, G., Spencer, E., & Hurwitz, J. (1978b) *J. Biol. Chem. 253*, 4490.
Nagarajan, R. (1979) U.S. Patent 4 158 056.
Perry, R. P., & Kelley, D. E. (1977) *Cell (Cambridge, Mass.) 8*, 433.
Pugh, C. S. G., Borchardt, R. T., & Stone, H. O. (1977) *Biochemistry 16*, 3928.
Pugh, C. S. G., Borchardt, R. T., & Stone, H. O. (1978a) *Anal. Biochem. 88*, 504.
Pugh, C. S. G., Borchardt, R. T., & Stone, H. O. (1978b) *J. Biol. Chem. 253*, 4075.
Raies, A., Lawrence, F., Robert-Gero, M., Loche, M., & Cramer, R. (1976) *FEBS Lett. 72*, 48.
Robert-Gero, M., Lawrence, F., Farrugia, G., Berneman, A., Blanchard, P., Vigier, P., & Lederer, E. (1975) *Biochem. Biophys. Res. Commun. 65*, 1242.
Robert-Gero, M., Blanchard, P., Lawrence, F., Pierre, A., Vedel, M., Vuilhorgne, M., & Lederer, E. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) p 207, Elsevier/North-Holland, New York.
Roeder, R. G. (1974) *J. Biol. Chem. 249*, 241.
Rottman, F. M., Kaehler, M., & Coward, J. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) p 361, Elsevier/North-Holland, New York.
Salditt-Georgieff, M., Jelinek, W., Darnell, J. E., Furuichi, Y., Morgan, M., & Shatkin, A. J. (1976) *Cell (Cambridge, Mass.) 7*, 227.
Shatkin, A. J. (1976) *Cell (Cambridge, Mass.) 9*, 645.
Shatkin, A. J., Furuichi, Y., & Sonenberg, N. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) p 341, Elsevier/North-Holland, New York.

Vedel, M., Lawrence, F., Robert-Gero, M., & Lederer, E. (1978) *Biochem. Biophys. Res. Commun. 85*, 371.
Venkatesan, S., Gershowitz, A., & Moss, B. (1980) *J. Biol. Chem. 255*, 903.
Weber, L. A., Hickey, E. D., Nuss, D. L., & Baglioni, C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3254.
Wei, C.-M., & Moss, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 318.

Viscometric and Fluorometric Studies of Deoxyribonucleic Acid Interactions of Several New Anthracyclines†

Jonathan A. Pachter,*,‡ Cheng-Hsiung Huang, Virgil H. DuVernay, Jr., Archie W. Prestayko,§ and Stanley T. Crooke§

ABSTRACT: Interactions involved in the binding of the anthracycline analogues adriamycin, carminomycin, pyrromycin, musettamycin, marcellomycin, and aclacinomycin to calf thymus DNA and covalently closed circular PM-2 DNA have been studied. Fluorescence quenching experiments revealed that denaturation of calf thymus DNA and increasing ionic strength each resulted in a marked decrease in the DNA binding affinities of all of the anthracyclines studied. These results suggest that intercalative and electrostatic interactions are both important in the DNA binding of these analogues. Viscometric studies indicated that under high ionic strength conditions which negated electrostatic effects, all of the anthracyclines induced an unwinding–rewinding process of the closed superhelical PM-2 DNA typically observed for DNA intercalators. Relative to the 26° unwinding angle of ethidium bromide, anthracyclines with a daunomycinone-like aglycon induced an unwinding angle of approximately 13°. This differed slightly from the unwinding angles of 10.3°–11.1° which were induced by anthracyclines with a pyrromycinone-like aglycon. Increases in the length of the glycosidic side chain did not elicit significant differences in PM-2 DNA unwinding ability, implying a lack of effect of glycosidic side chain length on the anthracycline intercalation process. The unwinding angles also showed little sensitivity to decreases in ionic strength, suggesting that the fraction of bound anthracycline molecules which are in a nonintercalated state is similar to the fraction of bound ethidium bromide molecules which are in a nonintercalated state.

The interaction of anthracycline antitumor antibiotics with DNA has been the subject of many studies using a wide variety of techniques including thermal denaturation (Kersten & Kersten, 1965; Zunino et al., 1972), X-ray diffraction (Pigram et al., 1972), spectrophotometric titration (Gabbay et al., 1976), fluorometric titration (Tsou & Yip, 1976; Zunino et al., 1977; DuVernay et al., 1979a), equilibrium dialysis (Zunino et al., 1972; Gabbay et al., 1976), and viscometry (Calendi et al., 1965; Kersten et al., 1966; DiMarco et al., 1971). This interaction is believed to inhibit DNA-dependent nucleic acid synthesis (Calendi et al., 1965; Zunino et al., 1972; Neidle, 1978; Crooke et al., 1978). It is now generally accepted that the biological activity of many anthracyclines may result, to a large degree, from their DNA interactions (Zunino et al., 1972).

At least two modes of interaction between anthracyclines and DNA have been distinguished (Zunino et al., 1972). Intercalation (Lerman, 1961), which consists of insertion of the hydrophobic chromophore between adjacent DNA base pairs, is thought to be the predominant mode of anthracycline–DNA interaction (Pigram et al., 1972) and has been most rigorously demonstrated (Kersten et al., 1966; Waring, 1970; Pigram et al., 1972; Gabbay et al., 1976). Electrostatic binding, which is thought to be a weaker mode of binding than intercalation, consists of an ionic interaction between the amino group on the anthracycline amino sugar and a phosphate group on the DNA backbone (Pigram et al., 1972; Zunino et al., 1972; Gabbay et al., 1976; Patel & Canuel, 1978).

Recently, carminomycin (CMM),[1] pyrromycin (PYM), musettamycin (MSM), marcellomycin (MCM), and aclacinomycin (ACM) have been isolated (Gause et al., 1974; Oki et al., 1975; Nettleton et al., 1977) and their molecular pharmacology has been investigated (Crooke et al., 1978; DuVernay et al., 1979a,b, 1980) in an attempt to develop less toxic anthracyclines for clinical use. Their structures, along with those of the prototypic anthracyclines adriamycin (ADM) and daunomycin (DNM), are shown in Figure 1. Previous studies in this laboratory have attempted to characterize the nature of anthracycline–DNA interactions. Structure–activity relationships regarding DNA-binding affinities and base composition preferences for DNA binding have been demonstrated (DuVernay et al., 1979a, 1980). The lack of DNA breakage activity of several of these analogues under moderate experimental conditions has also been reported (Mong et al., 1980). Using spectrofluorometric and viscometric techniques with linear DNA and covalently closed circular DNA, the present study investigates relationships between the structures of ADM, CMM, PYM, MSM, MCM, and ACM and the

† From the Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030 (J.A.P., C.H.H., V.H.D., A.W.P., and S.T.C.), and Bristol Laboratories, Syracuse, New York 13201 (A.W.P.). *Received April 6, 1981.* This work was supported in part by a grant from Bristol Laboratories and by a grant (CA-10893-P12) from the National Cancer Institute.

‡ Predoctoral trainee supported by Baylor College of Medicine Institutional Funds. Present address: Program of Neuroscience, Cullen Eye Institute, Baylor College of Medicine, Houston, TX 77030.

§ Present address: Smith Kline & French Laboratories, Philadelphia, PA 19101, and Department of Pharmacology, Baylor College of Medicine, Houston, TX 77030.

[1] Abbreviations: ADM, adriamycin; DNM, daunomycin; CMM, carminomycin; PYM, pyrromycin; MSM, musettamycin; MCM, marcellomycin; ACM, aclacinomycin; EtBr, ethidium bromide; NaP_i, sodium phosphate buffer; ccc DNA, covalently closed circular DNA.

0006-2960/82/0421-1541$01.25/0