MOLECULAR PHARMACOLOGY, 17, 388–394

DNA Damage in Neocarzinostatin-, Macromomycin-, and Bleomycin-Treated SV40 Virions

TERRY A. BEERMAN* AND MARY W. GUTAI†

*Department of Experimental Therapeutics, Grace Cancer Drug Center, and †Department of Viral Oncology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, New York 14263

Received October 8, 1979; Accepted November 12, 1979

SUMMARY

BEERMAN, T. A., AND M. W. GUTAI. DNA damage in neocarzinostatin-, macromomycin-, and bleomycin-treated SV40 virions. *Mol. Pharmacol.* 17: 388–394 (1980).

The DNA strand scission antitumor drugs neocarzinostatin, macromomycin, and bleomycin have been found to introduce single-strand nicks in encapsidated SV40 DNA. The nicking of encapsidated DNA by neocarzinostatin was demonstrated to have occurred within purified SV40 virions rather than as a result of drug-induced leakage of DNA from the virus. The relationship between sulfhydryl agent and reactivity of these drugs with encapsidated SV40 DNA is similar to that reported for naked SV40 DNA. The reactivity of neocarzinostatin at 0.5 μg/ml with naked and encapsidated SV40 form I DNA is quite similar as measured by the conversion of superhelical DNA to the nicked circular duplex form. However, at higher drug levels (1.0–100.0 μg/ml) the encapsidated DNA is less susceptible to further DNA damage.

INTRODUCTION

Neocarzinostatin is an antibiotic with a molecular weight of 10,700 that has antitumor activity (1–4). The drug has been studied extensively and found to be an inhibitor of DNA synthesis in both mammalian cells and a cell-free assay (5–7). Incubation of mammalian cells with neocarzinostatin results in damage to the cellular DNA (8, 9). The breakdown of cellular DNA is related to the drug's ability to inhibit both DNA synthesis and cell growth (6).

In a reaction stimulated by 2-mercaptoethanol, neocarzinostatin possesses endonucleolytic activity with DNA in a cell-free assay (8, 10, 11). The drug's cytotoxic activity *in vivo* can be correlated with the drug's ability to nick DNA *in vitro* (10). The nature of the DNA strand scissions has been determined to be predominantly random single-strand breaks that generate both 3′-phosphate and 5′-phosphate ends, and the release of a base possibly followed by a β-elimination reaction (12, 13).

Macromomycin is another protein antibiotic of similar size to neocarzinostatin that also shows potential as an antitumor agent (14–16). Though this drug has not been as well studied as neocarzinostatin, the two drugs appear to have many similarities. Macromomycin inhibits cellular DNA synthesis and nicks cellular DNA (17–19). The drug's ability to inhibit cell growth appears to be correlated with the ability of the drug to nick cellular DNA (18). In addition, macromomycin cleaves DNA in a cell-free assay; but, unlike neocarzinostatin, sulfhydryl agent is not necessary for maximal nicking activity (20, 21).

Bleomycin is a glycopeptide antitumor drug that is a similar to both neocarzinostatin and macromomycin in that this drug causes the breakdown of cellular and cell-free DNA and inhibits cellular DNA synthesis (1).

The ability of all three of these drugs to cut DNA in cell-free assays has been studied with a variety of DNA types including both linear and superhelical DNA (20, 21). These drugs possess endonucleolytic activity that can place one or more breaks in DNA, although the exact mechanism of action has yet to be determined.

SV40[1] is a small DNA tumor virus whose genomic DNA of approximately 3×10^6 daltons is a covalently closed double-stranded molecule which is packaged in a viral coat. The virus produces a lytic infection in monkey cells, transforms mouse cells, and causes tumors in hamsters (22).

In this paper we will examine the effects of neocarzinostatin, macromomycin, and bleomycin on the supercoiled DNA of intact SV40 virions. Since these drugs have the ability to penetrate mammalian cells and to cause extensive damage to the cellular DNA, it was of interest to determine whether they could penetrate a

This work was supported in part by the United States National Cancer Institutes Grants CA-24538, CA-24906, CA-14801, and CP-71062.

[1] Abbreviations used: SV40-I, covalently closed duplex DNA; SV40-II, nicked circular duplex DNA; PFU, plaque-forming units; SV40, Simian virus 40.

0026-895X/80/030388-07$02.00/0
Copyright © 1980 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

Downloaded from molpharm.aspetjournals.org at University at Buffalo on December 9, 2012

virus and cleave the viral DNA. A detailed comparison is made between neocarzinostatin's ability to nick encapsidated viral DNA as opposed to naked SV40 DNA.

MATERIALS AND METHODS

Cells and virus. SV40 virus (strain 776) was grown on the BSC-1 line of African green monkey kidney cells in minimum Eagle's medium (MEM) supplemented with fetal calf serum (2%), glutamine, penicillin, and streptomycin.

Purification of ^{3}H-labeled SV40-DNA. Viral DNA was labeled at 24 h after infection with 20 μCi of [^{3}H]thymidine/10-cm dish and harvested at approximately 60 h after infection. Viral DNA was extracted according to the Hirt procedure (23), treated with RNase A (10 μg/ml), extracted with an equal volume of phenol (containing 0.1 vol 10 mM Tris–HCl, pH 8.0), and precipitated in 2.5 vol of cold ethanol. The form I DNA was purified by equilibrium centrifugation in CsCl/ethidium bromide gradients followed by sucrose density gradient centrifugation through a 5 to 20% neutral sucrose gradient as previously described (24).

Purification of ^{14}C- or ^{3}H-labeled SV40 virions. BSC-1 cells were infected with SV40 virus at a multiplicity of 0.001 PFU/cell. The virion DNA was labeled at 48 h after infection with 30 μCi [^{3}H]thymidine or 12 μCi [^{14}C]-thymidine/75-cm^{2} flask, and harvested when greater than 90% of the cells had become detached from the surface (12–14 days after infection). Clarified cell lysates were centrifuged to equilibrium twice in CsCl gradients (P = 1.34 g/ml). The purified virions were dialyzed against MEM–30 mM Hepes, pH 7.6, and stored at −20°C.

Drugs and reagents. Neocarzinostatin (NSC-157365) was provided by the Investigational Drug Branch of the National Cancer Institute, and crude macromomycin (NSC-170105) was obtained from the Developmental Therapeutics Program of the National Cancer Institute. Macromomycin was purified by a modification of the procedure of Sawyer *et al.* (20). Bleomycin was kindly donated by Bristol Laboratories of Syracuse, New York. [*Me*-^{3}H]Thymidine was obtained from New England Nuclear. [*Me*-^{14}C]Thymidine was from Amersham Corp., as was the ACS scintillation fluid. DNase I and RNase were obtained from Worthington. Endo R *Bam* and *Hpa*II were purchased from Biolabs. Agarose was purchased from Sigma.

Analysis of SV40 DNA damage by sedimentation on alkaline sucrose gradients. SV40-I DNA was analyzed for DNA damage on a 5–20% alkaline sucrose gradient containing 0.7 M NaCl, 0.3 M NaOH, and 0.01 M EDTA. The entire reaction mixture of 100 μl was centrifuged in a Spinco SW50.1 rotor for 85 min at 49,000 rpm and 20°C. Placement of the reaction mixture on the alkaline sucrose gradient terminated all drug activity. The gradients were fractionated from the top and the fractions counted in ACS scintillation fluid. The percentage conversion of SV40-I to type II DNA was determined from the proportion of radioactivity appearing in the band positions for supercoiled and open circular duplex DNA, although the resulting SV40-II consists of DNA containing one or more breaks. The percentage form I DNA in the untreated control was taken as 100% and all calculations of the percentage form I remaining were normalized to this 100% value.

Analysis of encapsidated SV40 DNA damage by sedimentation on alkaline sucrose gradients. The procedure used is essentially that described previously except the gradient was overlaid with 0.5 ml of lysis solution consisting of 0.6% sodium dodecyl sulfate and 0.01 M EDTA, pH 7.4. After a 20-min lysis period, the gradients were processed as described previously.

Analysis of encapsidated SV40 DNA damage by sedimentation on neutral sucrose gradients. Encapsidated SV40 DNA was analyzed for strand scissions by neutral sucrose gradient analysis as described previously except the gradient consisted of 5–20% sucrose in 0.1 M Tris–HCl, pH 7.0, 0.7 M NaCl, and 0.01 M EDTA and was run for 180 min.

Analysis of encapsidated SV40 DNA damage by agarose gels. Encapsidated SV40 DNA was incubated in an equal volume of lysis solution (see the preceding) for 20 min at 20°C and then placed upon a 0.7% agarose gel (7 × 125-mm tubes) prepared in TAE buffer consisting of 0.05 M Tris–HCl, 0.02 M sodium acetate, and 0.002 M EDTA, pH 8.0. The gels were electrophoresed at 100 V for 2 h using TAE buffer in the upper and lower reservoirs. After electrophoresis the gels were removed from the tubes, stained for 30 min in the presence of 0.5 μg/ml of ethidium bromide, visualized under ultraviolet light, and then photographed using a Polaroid MP3 camera and Kodak Tri-X pan film.

RESULTS

Sedimentation analysis of neocarzinostatin-treated purified viral DNA and neocarzinostatin-treated virus lysed directly on an alkaline sucrose gradient. The introduction of a single-strand scission in superhelical SV40 DNA (form I) results in conversion of the DNA to an open circular duplex DNA (form II). Centrifugation analysis on an alkaline sucrose gradient results in a 53 S DNA for the collapsed duplex superhelical DNA, while the form II molecule strand separates into 21 S and 17 S circular and linear DNA, respectively, although the centrifugation conditions used to resolve form I and form II DNA on gradients are not sufficient to resolve form II DNA into its component forms. Using purified SV40 virus containing radiolabeled DNA, it was possible to duplicate the centrifugation patterns seen with purified DNA by placing the virus on alkaline sucrose gradients overlaid with lysis solution. A mixture of [^{14}C]thymidine-labeled SV40 virus (90% form I) and purified [^{3}H]thymidine-labeled SV40 DNA (60% form I) was run on such a gradient and resulted in denatured form I DNA from either the virus or the naked DNA sedimenting to identical positions on the gradient (Fig. 1A). Also denatured form II DNA sedimented to the same gradient position whether it was from naked or encapsidated DNA. In addition, there appeared to be complete lysis of the virus as all the radioactivity applied to the gradients was recovered in the forms I and II DNA peaks.

In a similar experiment neocarzinostatin at 2.0 μg/ml

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

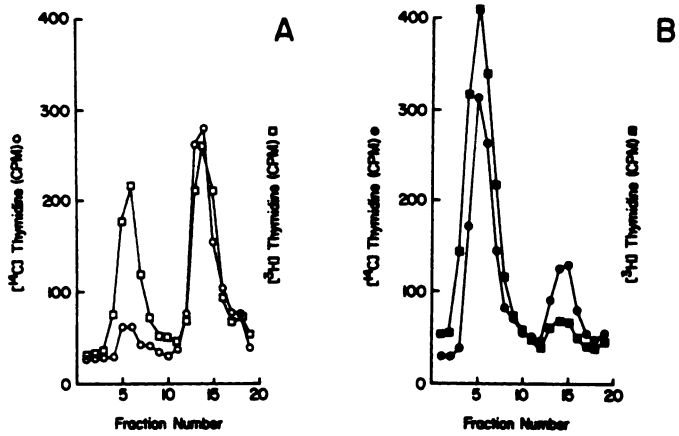

FIG. 1. *A comparison of neocarzinostatin-induced strand scission of naked and encapsidated SV40 DNA*

[^{3}H]Thymidine-labeled SV40 DNA (0.25 μg of specific activity, 4.1 × 10^4 cpm/μg) and [^{14}C]thymidine-labeled encapsidated SV40 DNA (0.12 μg of specific activity, 1.3 × 10^4 cpm/μg) were incubated together in a 100-μl reaction mixture consisting of 0.1 M Tris–HCl, 1.0 mM 2-mercaptoethanol, pH 7.0, and the appropriate amount of drug. After 30 min at 24°C, the samples were placed on top of an alkaline sucrose gradient overlaid with lysis solution and centrifuged as described in Materials and Methods. (A) A comparison of the sedimentation profiles of naked SV40 DNA (□——□) and encapsidated SV40 DNA (○——○) in the absence of drug. (B) A comparison of the sedimentation profiles of naked SV40 DNA (■——■) and encapsidated SV40 DNA (●——●) after treatment with neocarzinostatin (2.0 μg/ml). Sedimentation was from left to right.

was incubated with a mixture of ^{3}H-labeled naked DNA and ^{14}C-labeled virus, and the resulting DNA sedimentation analysis is shown in Fig. 1B. The purified SV40 DNA was nicked by the drug as expected (8), but there was also cleavage of the ^{14}C-labeled encapsidated DNA which then sedimented to the denatured form II DNA position. When the percentage conversion of form I to form II DNA was compared, normalizing the control value to 100% form I, 14% of the purified DNA remained as form I compared to 24.7% of the virion DNA.

Concentration dependence of neocarzinostatin and macromomycin cleavage of virion DNA. Both neocarzinostatin and macromomycin were found to cleave superhelical DNA upon incubation of the virus with drug (Table 1). The conversion of form I to form II DNA was dependent upon the drug concentration, though it must be remembered that in this assay only the first strand break would be measured.

Time course dependence of neocarzinostatin and macromomycin cleavage of virion DNA. Neocarzinostatin and macromomycin, when incubated with SV40 virus, were both found to cleave SV40 DNA in a manner that was dependent upon the time of incubation of drug with virus prior to lysis (Table 2). Since the extent of DNA damage is proportional to the time of drug treatment, it would appear that the drug action is taking place prior to lysis of the virus. To further ensure that damage to the DNA is not occurring after lysis, a high level of neocarzinostatin (100 μg/ml) was placed in the lysis solution, followed immediately by the virus maintained in a drug reaction mixture (0.1 M Tris, pH 7.0, and 2-mercaptoethanol at 10.0 mM). Under these conditions, there was no breakdown of SV40 form I DNA as measured on an alkaline sucrose density gradient (data not shown).

TABLE 1

Drug concentration dependence of strand scission of encapsidated SV40 DNA

The experimental conditions and method of analysis were as described in Fig. 1. The data from the gradient analysis were converted to percentage form I DNA by dividing the radioactivity found in the form I DNA position by that in the forms I and II DNA positions. All the data were based upon a control form I value (85%) normalized to a value of 100%. The neocarzinostatin-treated samples were incubated for 30 min at 24°C in 0.1 M Tris–HCl and 1.0 mM 2-mercaptoethanol, pH 7.0, while macromomycin-treated samples were incubated in the dark for 60 min at 24°C in 0.1 M Tris–HCl, pH 7.0.

Drug concentration	Percentage form I DNA
μg/ml	
Neocarzinostatin	
0	100
0.02	98
0.1	87
0.25	70
0.5	55
1.0	42
5.0	20
Macromomycin	
0	100
1.2	93
2.5	47
5.0	19
10.0	9
20.0	7
40.0	0

Effects of 2-mercaptoethanol on drug cutting of encapsidated DNA. Neocarzinostatin is known to damage DNA in a cell-free assay in a reaction that is almost completely dependent upon the presence of sulfhydryl agent (8, 10). Cleavage of DNA during neocarzinostatin incubation with virus was also found to be nearly totally

TABLE 2

Time course dependence of drug-induced strand scission of SV40 encapsidated DNA

The experiment was performed as described in Table 1 except the drug concentration for neocarzinostatin was fixed at 1.0 μg/ml and that for macromomycin at 10.0 μg/ml. The samples were reacted at 24°C for the indicated times.

Incubation time	Percentage form I DNA
min	
Neocarzinostatin	
Control	100
0	91
5	62
15	52
30	64
60	38
Macromomycin	
0	100
15	42
30	37
60	22
120	9

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

MOL PHARM

dependent upon the presence of 2-mercaptoethanol (Table 3). In control experiments without drug, 2-mercaptoethanol under the assay conditions used did not cause DNA damage. Cutting activity was stimulated by 10 mM 2-mercaptoethanol when compared with levels of activity at 1.0 mM. At the lower 2-mercaptoethanol level (1.0 mM), it took a 10-fold higher drug level (1.0 μg/ml) to cleave DNA to nearly the same extent as at 0.1 μg/ml of drug with 10 mM 2-mercaptoethanol. In the absence of 2-mercaptoethanol, even extreme levels of drug (100 μg/ml) were not able to nick the viral DNA.

Macromomycin is known to cleave cell-free DNA, but the reaction is independent of sulfhydryl agent (20, 21). In the drug-treated viral system, it was also found that macromomycin damage to DNA was not influenced by the absence or presence of 2-mercaptoethanol (Table 3).

Bleomycin is a glycopeptide drug that is also known to damage DNA in a cell-free assay (1). When encapsidated SV40 DNA was treated with bleomycin, there was a conversion of DNA from form I to form II DNA (Table 3). Previously it had been determined that 2-mercaptoethanol stimulates the drug's nicking activity nearly 20-fold (1). In our studies there was also a dramatic stimulation of drug activity by 2-mercaptoethanol (Table 3).

Determination of the effect of nucleases on SV40. One possible explanation for the cleavage of DNA in drug-treated virus is that the drug induces the DNA to "leak" from the virus and subsequent DNA damage occurs outside the virus. To exclude this possibility, a comparison was made between DNA damage in neocarzinostatin-treated virus in the presence and absence of DNase I. As seen in Table 4, DNase I is unable to cleave SV40 DNA when incubated with virus as compared to the extensive digestion of naked DNA. After neocarzinostatin treatment of virus or DNA, 42 and 25% of the DNA remained as form I, respectively. The presence of DNase I in addition to neocarzinostatin did not significantly alter the percentage DNA damage seen in the viral system, while naked DNA was extensively cleaved (Table 4). Any DNA leaking from the drug-treated virus would have received multiple breaks, as was found in the naked DNA system.

Other nucleases were examined for their effects on encapsidated DNA. Neither micrococcal nuclease, venom phosphodiesterase, nor Endo R *Bam*HI enzymes were found to induce damage to DNA when incubated with virus under conditions which caused degradation to naked DNA (data not shown).

Neutral gradient analysis of neocarzinostatin-treated SV40. Superhelical SV40 DNA sediments at 21 S on neutral gradients, while the nicked form II DNA sediments at 17 S. Neocarzinostatin has been shown to nick superhelical DNA by converting the 21 S form I DNA to the 17 S form (8). This cleavage of superhelical DNA in the absence of alkaline conditions indicated that the DNA damage was a true strand scission as opposed to alkali-labile bonds. In a similar analysis with drug-treated virus, neocarzinostatin at 0.5 μg/ml was found to nick form I DNA as analyzed by neutral sucrose gradients (Fig. 2). The percentage form I DNA remaining was 40%, a value close to that seen with a similar drug level using alkaline gradient analysis (40–59%).

Analysis of drug-treated virion DNA by electrophoresis in agarose gels. It is possible to interpret the neutral sucrose data as drug-induced double-strand breaks in form I DNA, as the sedimentation conditions would not resolve form II DNA from a linear duplex

TABLE 3

Effect of 2-mercaptoethanol on drug-induced strand scission of encapsidated SV40 DNA

The experiment was performed as described in Table 1 except the concentration of 2-mercaptoethanol was varied. All reactions were incubated for 30 min at 24°C except those of macromomycin, for which the reaction time was 60 min.

Drug	2-Mercaptoethanol	Percentage form I DNA
μg/ml	mM	
Neocarzinostatin		
0	0	100[a]
0	10.0	98–100
0.1	10.0	35
0.5	1.0	59
0.5	10.0	0
1.0	1.0	49
1.0	10.0	0
5.0	0	98
5.0	1.0	20
5.0	10.0	0
100.0	0	97
Macromomycin		
0	1.0	100
2.5	0	50
2.5	1.0	66
Bleomycin		
0	1.0	100
1.0	1.0	63
2.0	0	98
2.0	1.0	0
10.0	0	81

[a] The percentage form I in untreated controls was 82–84% and was normalized to 100%.

TABLE 4

A comparison of neocarzinostatin and DNase I strand scission activity on naked and encapsidated SV40 DNA

The experiment was performed as described in Table 1. Two sets of reactions were run, one for naked SV40 DNA and a second for encapsidated SV40 DNA. Reactions were prepared as described in Fig. 1 except the samples contained 0.1 M Tris–HCl, 1.0 mM 2-mercaptoethanol, and 5 mM $MgCl_2$, pH 7.0. The reaction was run for 30 min at 24°C. Under these conditions there was no DNA nicking in untreated samples. When drug and DNase I were used in the reaction system, they were added simultaneously.

Treatment	Percentage form I DNA	
	SV40 virus	SV40 DNA
Control (−neocarzinostatin, −DNAse I)	100[a]	100[a]
Neocarzinostatin (0.5 μg/ml)	42	25
DNAse I (10.0 ng/ml)	100	0[b]
Neocarzinostatin (0.5 μg/ml) and DNAse I (10.0 ng/ml)	37	0[b]

[a] These values represent the normalization of the percentages of form I DNA, which were 85% for the SV40 encapsidated DNA and 61% for SV40 DNA.

[b] Peak position was at the top of the gradient, indicating that multiple breaks were placed in the DNA.

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

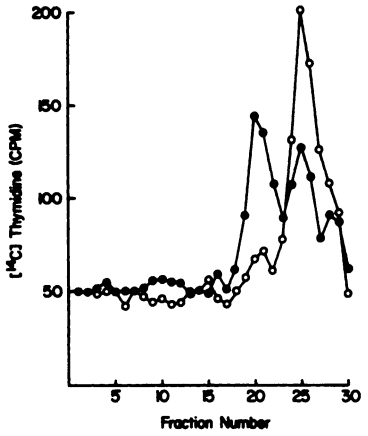

FIG. 2. *Analysis of neocarzinostatin-induced strand scission of encapsidated SV40 DNA on neutral sucrose gradients*

The incubation conditions were as described in Fig. 1 except the drug concentration was 0.5 μg/ml. Sedimentation of the drug-treated DNA was done on neutral sucrose gradients as described in Materials and Methods and was from left to right. Control (O——O) and neocarzinostatin-treated (●——●).

molecule. Forms I and II DNAs, as well as the linear duplex DNA, are easily distinguishable by electrophoresis on agarose gels.

An initial experiment determined that lysed virus could be analyzed for its DNA composition on agarose gels (Fig. 3e). If virus was not lysed prior to analysis in gels, no bands were visible (Fig. 3d). A comparison of DNA from lysed SV40 virus and from purified SV40 DNA showed that DNA from both sources run at equidistant positions on the gel (Figs. 3a and e). Analysis of drug-treated virus by this technique resulted in similar conversion of form I to form II DNA at a neocarzinostatin level of 0.5 μg/ml whether the analysis was done on gels or sedimentation gradients. At this level of drug, there was no increase in the amount of linear duplex DNA, indicating that the first strand scission in the DNA is a single-strand break (Fig. 3f). A high drug level of 50 μg/ml resulted in some small amounts of linear duplex DNA, but under the same reaction conditions there was found to be even more linear duplex DNA when using naked SV40 DNA (Figs. 3g and h).

Neocarzinostatin induced multiple breaks in encapsidated DNA and purified SV40 DNA. The assays presented thus far have been concerned with the determination of the percentage conversion of supercoiled DNA to a nicked form by the introduction of one strand break. Under the centrifugation conditions used, it would be difficult to determine if more than one break is occurring per SV40 molecule. By centrifuging the DNA under alkaline conditions and an extended time period, it is possible to resolve multiple breaks in the DNA.

Treatment of SV40 DNA with high concentrations of neocarzinostatin and a more optimal level of 2-mercaptoethanol (10.0 mM) resulted in further fragmentation of the DNA (Fig. 4A). These data are in agreement with similar results obtained by Beerman and Goldberg (8). Even at the lowest drug concentration (1.0 μg/ml), nearly all the DNA was reduced to linear pieces. A similar analysis of neocarzinostatin-treated SV40 virus is shown in Fig. 4B. Clearly there is much less damage to the viral DNA than that seen at comparable drug levels with purified DNA.

DISCUSSION

Drugs such as neocarzinostatin, macromomycin, and bleomycin have seen studied for their endonucleolytic effect on naked DNA and also cellular DNA (1). This paper demonstrates for the first time the ability of these DNA-reactive antitumor drugs to cleave encapsidated SV40 DNA. The fact that virus in general is extremely resistant to nucleolytic activity makes this finding unique.

A comparison of neocarzinostatin's endonucleolytic activity on SV40 DNA and on encapsidated SV40 DNA demonstrated that the rate of conversion of superhelical

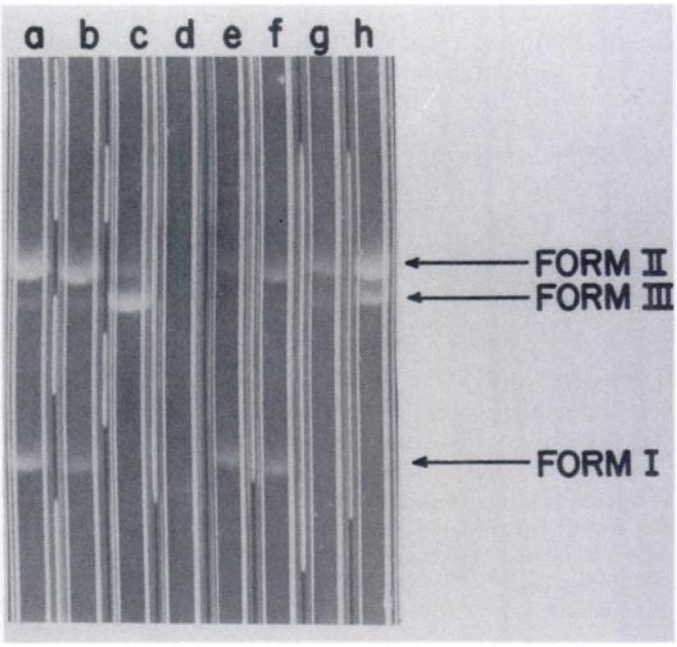

FIG. 3. *Agarose gel analysis of neocarzinostatin-treated naked and encapsidated SV40 DNA*

All reaction systems were 50 μl and contained 0.1 M Tris-HCl, 1.0 mM 2-mercaptoethanol, pH 7.0, and also DNA and drug as indicated. The reactions with drug were run for 30 min at 20°C and stopped by the addition of a lysis solution (50 μl of 0.6% sodium dodecyl sulfate and 0.01 M EDTA, pH 7.4 for 20 min at 20°C). Electrophoresis was done on 0.7% agarose for 2 h at 100 V and 24°C. (a) Naked SV40 DNA (0.35 μg) containing forms I, II, and linear duplex (form III) DNA—no lysis solution was added. (b) Naked SV40 DNA (0.30 μg) containing forms I and II DNA—treated with lysis solution. (c) Naked SV40 DNA (0.25 μg) digested with *Hpa*II enzyme to produce linear duplex DNA (Digestion with *Hpa*II enzyme was included to serve as a marker for linear (form III) DNA.) (3 units of enzyme was incubated with 20 μg/ml DNA in a 60-μl reaction for 1 h at 37°C using the buffer specified by New England Biolabs)—treated with lysis solution. (d) Encapsidated SV40 DNA (0.12 μg)—no lysis solution. (e) Encapsidated SV40 DNA (0.12 μg)—treated with lysis solution. (f) Encapsidated SV40 DNA (0.12 μg) incubated with neocarzinostatin at 0.25 μg/ml—treated with lysis solution (The lysis solution was found to completely inactivate the drug.). (g) Encapsidated SV40 (0.12 μg) incubated with neocarzinostatin at 50.0 μg/ml—treated with lysis solution. (h) Naked SV40 DNA (0.35 μg; see sample a) incubated with neocarzinostatin at 50.0 μg/ml—treated with lysis solution.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

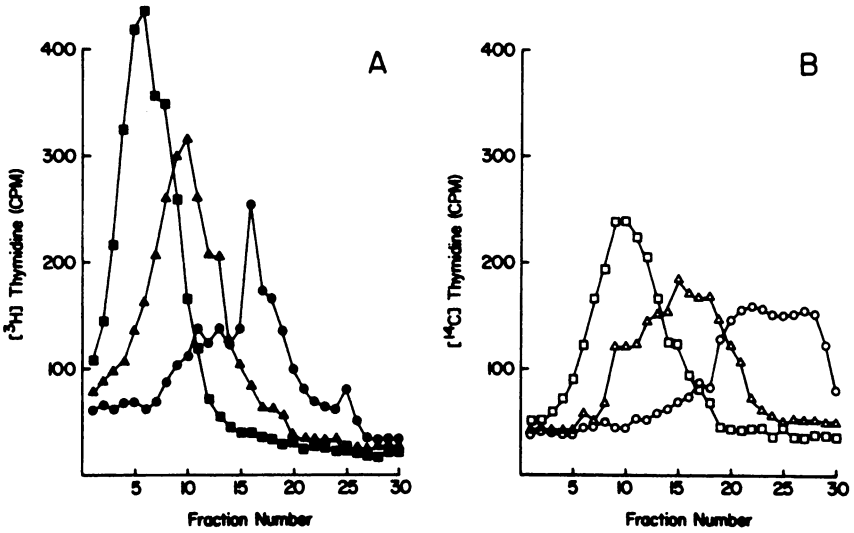

FIG. 4. *Analysis of multiple strand scissions induced by neocarzinostatin in naked and encapsidated SV40 DNA*

The incubation conditions were as described in Fig. 1 except that the 2-mercaptoethanol concentration was increased to 10 mM (under the experimental conditions this concentration of 2-mercaptoethanol did not by itself cause nicking of the DNA). (A) Naked SV40 DNA was treated with neocarzinostatin at 1.0 μg/ml (●——●), 10 μg/ml (▲——▲), and 100.0 μg/ml (■——■). Encapsidated SV40 DNA was treated with neocarzinostatin at 1.0 μg/ml (○- - - -○), 10.0 μg/ml (△- - - -△), and 100 μg/ml (□- - - -□). (B) The samples were then centrifuged for 330 min at 49K and 20°C. The sedimentation was from left to right. No attempt was made to recover superhelical DNA which, when present at the lowest drug concentration, was sedimented to the bottom. Denatured form II DNA appeared in fractions 22 and 26.

DNA to form II DNA is similar for both systems (Figs. 1A and B). Both neocarzinostatin and macromomycin nicked superhelical encapsidated SV40 DNA in a concentration-dependent manner. That the nicking of DNA occurred prior to lysis of the virus is suggested by the dependence of DNA cleavage upon the time of drug treatment of the virus (Table 2). Also, the addition of a high level of neocarzinostatin (a concentration 1000-fold higher than is necessary to see cleavage of the viral DNA) to the lysis solution did not result in breakdown of viral DNA upon the addition of SV40 virus to the lysis solution.

The possibility that drug treatment of the virus caused DNA to "leak" from the virus so that the drug effect on DNA occurred outside the virus was eliminated by analysis of neocarzinostatin and DNase I effects on SV40 virus. DNase I levels sufficient to cause multiple breaks in naked DNA (25 breaks per strand) were unable to cause any nicking of encapsidated SV40 DNA. Treatment of virus with neocarzinostatin at a concentration sufficient to convert over half the DNA to form II and treatment of virus with a similar drug level in combination with enough DNase I to make 25 breaks per strand of DNA resulted in both systems showing nearly identical levels of DNA damage (Table 4). A similar system of neocarzinostatin and DNase I with naked SV40 DNA demonstrated that DNase I was fully active in the presence of drug (Table 4) and that any leakage of DNA in the virus system would have resulted in extensive nicking of the DNA by DNase I.

A comparison of the endonucleolytic activity of these DNA-reactive drugs as a function of 2-mercaptoethanol concentration demonstrated that all three drugs showed a dependence or lack of dependence on sulfhydryl agent that is similar to that seen with naked DNA (1). Likewise, neocarzinostatin at 0.1 μg/ml in the presence of 10.0 mM 2-mercaptoethanol showed significant nicking of superhelical encapsidated SV40 DNA, while a 1000-fold increase in drug in the absence of 2-mercaptoethanol resulted in no detectable breakdown of DNA. Neocarzinostatin-nicking of naked viral DNA is known to be dependent upon 2-mercaptoethanol concentration up to 10 mM levels (7, 8, 10). Also drug levels of 0.1 μg/ml in the presence of 10.0 mM 2-mercaptoethanol resulted in greater breakdown of encapsidated DNA than neocarzinostatin at 1.0 μg/ml in the presence of 1.0 mM 2-mercaptoethanol (Table 3). In contrast, neocarzinostatin damages cellular DNA in the absence of 2-mercaptoethanol.

Sausville and Horwitz (25) recently reported that the addition of neocarzinostatin (10 μg/ml) to infected cells does not cause detectable breakage of preexisting SV40 DNA molecules, although new viral synthesis is inhibited greater than 90% and the cellular DNA is substantially damaged. Thus, the failure of drug to nick viral DNA in their system could be explained by the absence of 2-mercaptoethanol.

Bleomycin nicking of naked SV40 is known to be stimulated 20-fold by 2-mercaptoethanol (1). It was found that bleomycin cutting of encapsidated SV40 DNA was also dependent upon 2-mercaptoethanol. Macromomycin endonucleolytic activity on naked DNA has been shown to be unaffected by the presence of 2-mercaptoethanol (20, 21). This appears also to hold true in the virion, where the presence of 1.0 mM 2-mercaptoethanol did not alter the drug's endonucleolytic activity.

Another similarity between neocarzinostatin activity on naked and encapsidated SV40 DNA is the production of true single-strand scissions as opposed to alkali-labile bonds in both systems. The results of a neutral sucrose gradient analysis of encapsidated SV40 DNA treated with drug were similar to previously published data for

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

naked DNA (8) showing that nearly equal amounts of DNA breakdown occur on alkali or neutral gradients.

Superhelical DNA, upon receiving one single strand break, is converted to an open duplex DNA. It has been shown that neocarzinostatin is able to cleave this DNA to smaller pieces (8). A comparison of neocarzinostatin endonucleolytic activity, as measured by multiple breaks per strand on SV40 DNA and on encapsidated SV40 DNA, showed that the naked DNA was more rapidly degraded by five fold than the encapsidated DNA (26) (Figs. 4A and B). There also appeared to be less linear DNA on agarose gels in the drug-treated (50 μg/ml) encapsidated SV40 DNA system compared to naked SV40 DNA. The initial break in superhelical DNA occurs at the same rate for drug-treated naked and encapsidated DNA (Fig. 1). After introduction of the first nick, the availability of additional cleavage sites appear to be decreased in the virus. During the conversion of superhelical DNA to form II DNA, it is known that the altered secondary structure of form I DNA that renders this DNA more susceptible to single strand-specific chemicals and nucleases is lost (27). If an analogous mechanism occurs in virions, it is possible that the initial attack by these drugs cleaves the encapsidated DNA at a unique exposed site such as regions of altered secondary structure which disappear after the first strand scission or at the nuclease sensitive regions reported by Varshavsky *et al.* and Scott *et al.* (28, 29). A recent study by Das *et al.* (30) suggests that susceptibility to restriction endonuclease cleavage depends on the compactness of the SV40 chromatin structure. It could thus be argued that because of special features of the chromatin structure, there are fewer available sites for drug attack than in naked DNA.

To study the question of availability of DNA nicking sites in encapsidated SV40 DNA compared to naked SV40 DNA, we are proceeding to map the sites of drug damage in both DNA systems by restriction enzyme digestion and nucleotide sequence analysis. By comparison of the DNA site specificity of drug-treated naked and encapsidated SV40 DNA, we hope to understand the susceptibility of chromatin structure to DNA-reactive agents and get a step closer to knowing the mechanism of antitumor drug activity which to date remains obscure. In addition, the ability of these drugs to penetrate virions and cause DNA damage warrants the examination of these agents as antiviral drugs.

ACKNOWLEDGMENTS

The authors would like to thank Charlene Van Dusen and Janet Arnone for their excellent technical assistance and Nina Ruth Wright for her aid in editing the manuscript.

REFERENCES

1. Goldberg, I. H., T. A. Beerman and R. Poon. *Cancer: A Comprehensive Treatise, Vol. 5. Chemotherapy* (F. F. Becker, ed.). Plenum Press, New York, 427–456 (1977).
2. Meienhofer, J., H. Maeda, C. B. Glaser, J. Czombos and K. Kuromizu. Primary structure of neocarzinostatin, an antitumor protein. *Science* **178**: 875–876 (1972).
3. Ishida, N., K. Miyazaki, K. Kumagai and M. Rickimaru. Neocarzinostatin, an antitumor antibiotic of high molecular weight. *J. Antibiot. (Tokyo) Ser. A* **18**: 68–76 (1965).
4. Bradner, W. T. and D. J. Hutchison. Neocarzinostatin (NCS-69856): An antitumor antibiotic effective against ascitic leukemia L-1210 in mice. *Cancer Chemother. Rep.* **50**: 79–84 (1966).
5. Homma, M., T. Koida, T. Saito-Koide, I. Kamo, M. Seto, K. Kumagai and N. Ishida. Specific inhibition of the initiation of DNA synthesis in HeLa cells by neocarzinostatin. *Prog. Antimicrob. Anticancer Chemother.* **2**: 410–415 (1970).
6. Beerman, T. A. and I. H. Goldberg. Relationship between DNA strand-scission and DNA synthesis inhibition in HeLa cells treated with neocarzinostatin. *Biochim. Biophys. Acta* **475**: 281–293 (1977).
7. Kappen, L. S. and I. H. Goldberg. Effect of neocarzinostatin-induced strand scission on the template activity of DNA for DNA polymerase I. *Biochemistry* **16**: 479–485 (1977).
8. Beerman, T. A. and I. H. Goldberg. DNA strand scission by the antitumor protein, neocarzinostatin. *Biochem. Biophys. Res. Commun.* **59**: 1254–1261 (1974).
9. Tatsumi, K., T. Nakamura and G. Wakisaka. Damage of mammalian cell DNA *in vitro* and *in vivo* induced by neocarzinostatin. *Gann* **65**: 459–461 (1974).
10. Beerman, T. A., R. Poon and I. H. Goldberg. Single-strand nicking of DNA *in vitro* by neocarzinostatin and its possible relationship to the mechanism of drug action. *Biochim. Biophys. Acta* **475**: 294–306 (1977).
11. Tatsumi, K., T. Sakane, H. Sawada, S. Shirakawa, T. Nakamura and G. Wakisaka. Unscheduled DNA synthesis in human lymphocytes treated with neocarzinostatin. *Gann* **66**: 441–444 (1975).
12. Poon, R., T. A. Beerman and I. H. Goldberg. Characterization of the DNA strand-breakage *in vitro* by the antitumor protein neocarzinostatin. *Biochemistry* **16**: 486–493 (1977).
13. Kappen, L. S. and I. H. Goldberg. Gaps in DNA induced by neocarzinostatin bear 3′- and 5′-phosphoryl termini. *Biochemistry* **16**: 729–734 (1978).
14. Yamashita, T., N. Naoi, K. Watanabe, T. Takeuchi and H. Umezawa. Further purification and characterization of macromomycin. *J. Antibiot.* **29**: 415–423 (1976).
15. Chimura, H., M. Ishizuka, M. Hamada, S. Hori, K. Kumura, J. Iwanage, T. Takeuchi and H. Umezawa. A new antibiotic, macromomycin, exhibiting antitumor and antimicrobial activity. *J. Antibiot.* **21**: 44–49 (1968).
16. Lippman, M. M., W. R. Laster, B. J. Abbott, J. Venditti and M. Baratta. Antitumor activity of macromomycin B (NSC 179105) against murine leukemias, melanoma, and lung carcinoma. *Cancer Res.* **35**: 939–945 (1975).
17. Kunimoto, T., M. Hori and H. Umezawa. Macromomycin, an inhibitor of the membrane function of tumor cells. *Cancer Res.* **32**: 1251–1256 (1972).
18. Beerman, T. A. Cellular DNA damage by the antitumor protein macromomycin and its relationship to cell growth inhibition. *Biochem. Biophys. Res. Commun.* **83**: 906–914 (1978).
19. Zuzuki, H., T. Nishimura, K. Muto and N. Tanaka. Mechanism of action of macromomycin: DNA strand scission, inhibition of DNA synthesis and mitoses. *J. Antibiot.* **31**: 875–883 (1978).
20. Sawyer, T. H., A. W. Prestayko and S. T. Crooke. Purification and mechanism of action of macromomycin. *Cancer Res.* **39**: 1180–1184 (1979).
21. Beerman, T. A. Strand scission of superhelical and linear duplex DNA by the antitumor protein macromomycin. Relationship of *in vitro* DNA damage to cell growth inhibition. *Biochim. Biophys. Acta* (in press).
22. Kelly, J. L. and D. Nathans. The genome of simian virus 40. *Adv. Virus Res.* **21**: 85–173 (1977).
23. Hirt, B. Selective extraction of polyoma DNA from infected mouse cultured cells. *J. Mol. Biol.* **26**: 365–369 (1967).
24. Danna, K. and D. Nathans. Specific cleavage of simian virus 40 DNA by restriction endonuclease of hemophilus influenzae. *Proc. Natl. Acad. Sci. U.S.A.* **68**: 2913–2917 (1971).
25. Sausville, F. A. and S. B. Horwitz. Inhibition of SV40 DNA synthesis by camptothecin and neocarzinostatin. *Mol. Pharmacol.* **14**: 1156–1166 (1978).
26. Abelson, J. N. and C. A. Thomas. The anatomy of the T5 bacteriophage DNA molecule. *J. Mol. Biol.* **18**: 262–291 (1966).
27. Woodworth-Gutai, M. and J. Lebowitz. Introduction of interrupted secondary structure in super-coiled DNA as a function of superhelix density: Consideration of hairpin structures in superhelical DNA. *J. Virol.* **18**: 195–204 (1975).
28. Varshavsky, A. J., O. H. Sundin and M. J. Bohn. SV40 viral minichromosome: Preferential exposure of the origin of replication as probed by restriction endonucleases. *Nucleic Acids Res.* **5**: 3469–3478 (1978).
29. Scott, W. A. and D. S. Wigmore. Sites in simian virus 40 chromatin which are preferentially cleaved by endonuclease. *Cell* **15**: 1511–1518 (1978).
30. Das, G. C., D. P. Allison and S. K. Niyogi. Sites including those of origin and termination of replication are not freely available to single-cut restriction endonucleases in the supercompact form of simian virus 40 minichromosome. *Biochem. Biophys. Res. Commun.* **89**: 17–25 (1979).

Send reprint requests to: Terry A. Beerman, Department of Experimental Therapeutics, Grace Cancer Drug Center, 666 Elm Street, Buffalo, New York 14263.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012