Strand Scission of Deoxyribonucleic Acid by Neocarzinostatin, Auromomycin, and Bleomycin: Studies on Base Release and Nucleotide Sequence Specificity†

Masaru Takeshita, Lizzy S. Kappen, Arthur P. Grollman,* Moiseś Eisenberg, and Irving H. Goldberg

ABSTRACT: The nucleotide sequence specificity of neocarzinostatin (NCS), auromomycin (AUR), bleomycin (Blm), phleomycin (Phlm), and tallysomycin (Tlm) has been determined by using these antibiotics and their associated chromophores to create strand scissions in end-labeled restriction fragments of DNA and then determining the base sequence of the oligonucleotides formed. NCS and the NCS chromophore induce similar patterns of cleavage in DNA fragments labeled at the 5′ terminus. The pattern produced by the AUR chromophore also resembles that of its holoantibiotic. Dithiothreitol enhances the rate of cleavage of DNA by the AUR chromophore but does not alter the sequence specificity. The results suggest that the polypeptide component of AUR and NCS serves primarily as a carrier for the chromophore. When tested with a fragment labeled at the 3′ terminus, the products of NCS and AUR cleavage do not display the patterns of chemically produced oligonucleotides cleaved at phosphodiester bonds, suggesting that the 5′ terminus is modified by a sugar fragment. NCS primarily attacks thymine (75% of the total bases attacked) and, to a lesser extent, adenine (19%) and cytosine (6%). AUR preferentially attacks guanine (67% of total bases), while attacking less often thymine (24%) and adenine (9%). Bleomycin and its analogues preferentially cleave purine–pyrimidine (5′ → 3′) and pyrimidine–pyrimidine (3′ → 5′) sequences. All (5′ → 3′) GT and GC sequences were cleaved. Phlm G and Phlm-Pep are less active than bleomycin toward purines while Tlm was more active. The patterns of cleavage produced by Blm A2 and Blm B6 are similar, while those produced by Phlm-Pep, Phlm G, Blm-B1′, and Blm-Pep resemble one another. The cleavage pattern of Tlm shows quantitative differences from the other analogues tested. Differences between bleomycin and its analogues may be related to structural differences in these molecules.

Neocarzinostatin (NCS)[1] and auromomycin (AUR)[1] are antibiotics with antitumor properties. They contain two components, an acidic polypeptide and a tightly bound chromophore moiety (Napier et al., 1979; Yamashita et al., 1979; Kappen et al., 1980a,b; Woynarowski & Beerman, 1980; Suzuki et al., 1980; Ohtsuki & Ishida, 1980; Kappen & Goldberg, 1980; Albers-Schönberg et al., 1980). Both drugs interact with DNA causing strand scissions and release of free bases (cf. Goldberg et al., 1981). The chromophores appear to be responsible for DNA strand scission and for the cytotoxic properties of these compounds (Napier et al., 1979; Kappen et al., 1980a,b; Suzuki et al., 1980; Ohtsuki & Ishida, 1980; Albers-Schönberg et al., 1980; Napier et al., 1981).

The bleomycins comprise a family of metalloglycopeptide antibiotics differing in their terminal amine side chain (cf. Haidle & Lloyd, 1979). Phleomycins are structurally related to bleomycins, differing in that one of the two thiazole ring moieties is partially saturated (Earhart, 1979). Tallysomycins also resemble the bleomycins but contain an additional amino-sugar moiety and a methyl group linked to the backbone of the molecule (Strong & Crooke, 1978). The therapeutic properties and toxicities of the active bleomycins, phleomycins, and tallysomycins differ, quantitatively; however, all of these antibiotics are capable of causing strand scission of DNA in vitro and in vivo (Haidle & Lloyd, 1979; Earhart, 1979; Strong & Crooke, 1978; Asakura et al., 1975).

NCS (Hatayama et al., 1978; D'Andrea & Haseltine, 1978) and the bleomycins (Takeshita et al., 1978; D'Andrea & Haseltine, 1978) display different sequence specificities with respect to strand scission and the release of free bases from DNA. The experiments reported here were designed to determine, in more detail, the cleavage patterns of DNA induced by these drugs. These observations allow us to establish the relative roles of chromophore and protein (NCS and AUR) as well as the effects of reducing agents (AUR) and molecular modifications (bleomycins, phleomycins, and tallysomycins) on the mechanism of action of these antibiotics.

Materials and Methods

Materials. Restriction enzymes *Hae*III, *Taq*I, *Hha*I, and terminal deoxynucleotide transferase were purchased from Bethesda Research Laboratories, bacterial alkaline phosphatase was from Worthington Biochemical, polynucleotide kinase was from P-L Biochemicals, and [γ-^{32}P]ATP (specific activity ~3000 Ci/mmol) and [α-^{32}P]ATP (specific activity ~2000 Ci/mmol) were from ICN. Copper-free samples of bleomycin A2 (Blm A2) (lot 71L 489) and tallysomycin B (Tlm), (lot 35F-1-21) were provided by Bristol Laboratories, Syracuse, NY; bleomycin B6 (Blm B6), phleomycin G (Phlm G) and phleomycin Pep (Phlm-Pep) were gifts from G. W. Grigg, Sydney, Australia; pepleomycin (Blm-Pep), NSC-276382, was obtained from the National Cancer Institute, Bethesda, Md. Bleomycin B1′ (Blm B1′) was a gift from Professor H. Umezawa; NCS was provided by Dr. W. T. Bradner of Bristol Laboratories, and AUR was obtained from Dr. T. S. A. Samy through the courtesy of Professor H. Umezawa. The non-

† From the Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794 (M.T., A.P.G., and M.E.), and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 (L.S.K. and I.H.G.). *Received April 21, 1981.* Supported by U.S. Public Health Service Research Grants GM 12573 (I.H.G.) and CA 17395 (A.P.G.) from the National Institutes of Health. A preliminary report of these findings was presented at the 182nd National Meeting of the American Chemical Society, Aug 1981 (Takeshita et al., 1981).

[1] Abbreviations used: NCS, neocarzinostatin; AUR, auromomycin; Blm, bleomycin; Phlm, phleomycin; Tlm, tallysomycin; DTT, dithiothreitol; MSH, β-mercaptoethanol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/81/0420-7599$01.25/0

protein chromophores of NCS and AUR were prepared as described earlier (Kappen et al., 1980a,b).

Preparation of DNA Fragments. Bacteriophage ϕX174 DNA was purified and digested with restriction enzymes as previously described (Takeshita et al., 1978). The restriction fragment designated Z7 in Sanger's map (Sanger et al., 1977) was phosphorylated at the 5′ terminus with [γ-^{32}P]ATP and polynucleotide kinase and then cleaved with *Taq*I into fragments containing 180 and 55 base pairs. Fragment Z5 in Sanger's map was phosphorylated at the 3′ terminus with [α-^{32}P]ATP and terminal deoxynucleotide transferase and then cleaved with *Hha*I. One of the products was a double-stranded fragment containing 50 base pairs which was isolated and purified by electrophoresis on a 8% polyacrylamide gel.

Measurement of Base Release. Reaction mixtures (100 μL) containing 100 mM Tris, pH 8.0, 1 mM dithiothreitol, 5.1 μg of [^{3}H]guanine-labeled DNA (3×10^3 cpm/μg) or 4.8 μg of [*methyl*-^{3}H]thymidine-labeled DNA (12.5×10^3 cpm/μg), and varying concentrations of chromophores prepared in methanol were incubated for 20 min at 0 °C. Reactions were started by the addition of chromophore. Concentrations of chromophore are expressed in terms of equivalent amounts of holoantibiotic. Control reactions contained an equivalent amount of methanol (final concentration 20%).

Reactions were terminated by addition of α-tocopherol (final concentration 0.5 mM). Aliquots (45 μL) of each reaction were distributed in duplicate. Five microliters of 3 M NaOH were added to one set of tubes; the remaining tubes received 5 μL of H_2O. After incubation for 30 min at 37 °C, the alkaline samples were neutralized with HCl, and the total volume of all samples was adjusted to the same level with H_2O.

Unlabeled guanine and thymine (15 μg) were added to all reactions, and the bases were separated on Whatman No. 1 paper by descending chromatography with the solvent system ethanol–1 M sodium acetate (7:3 v/v) for thymine (R_f 0.78) and 1-butanol–1-propanol–ethanol–58% NH_4OH–H_2O (4:4:1:2:4 v/v) for guanine (R_f 0.36). Positions of the bases were detected by ultraviolet absorption, and the radioactivity corresponding to the thymine and guanine regions was quantitated. The percent of base released was calculated from the amount of DNA applied on the chromatogram and the known specific activity of each base in the DNA. Amounts of thymine (0.028%) and guanine (0.15%) released in control reactions following treatment with 0.3 M NaOH were subtracted from the respective drug-treated samples. Bases were not released in other control reactions.

Quantitative Sequence Analysis. After the restriction fragments of DNA were incubated with various drugs, under conditions described in the legends to the figures, EDTA was added to a final concentration of 10 mM, and the solution was rapidly frozen and lyophilized. Samples were dissolved in 20 μL of 0.1 M NaOH containing 1 mM EDTA to which was added 20 μL of 10 M urea containing 0.05% bromophenol blue/xylene cyanole. The solution was heated at 90 °C for 15 s, and 5–10 μL aliquots were transferred to 20% polyacrylamide slab gels for sequence analysis. Gels were approximately 28 × 38 cm and 1.5-mm thick. Each track was 10 mm in width. Electrophoresis was conducted for 4–16 h at 850 V.

Nucleotide sequences of the restriction fragments were determined as described by Maxam & Gilbert (1977). Sequences determined by this method (Figure 1) were identical with those reported by Sanger et al. (1977).

Autoradiographs were prepared by exposing the polyacrylamide gels to Kodak SB-5 film at −20 °C. The time of exposure varied between 5 and 40 h, depending upon the amount of radioactivity in the sample. Autoradiographs were photographically reduced in size by a factor of 3. The optical density of these reductions was measured with an automated Optronics International Photoscan P1000 system by scanning at 50-μm resolution using a 50 × 50 μm^2 window. Occasionally, it was necessary to vary the contrast of the reduced autoradiograph to accommodate the range of observed optical densities to the resolution of the scanner.

FIGURE 1: Primary sequence of restriction fragments. Double-stranded fragments of ϕX174 DNA were prepared as described under Materials and Methods. Only the strand labeled with ^{32}P is shown. The 5′ termini of fragments A and B correspond to positions 669 and 436 in Sanger's map (Sanger et al., 1977), respectively. The 3′ terminus corresponds to position 490. Fragment A contains 179 bases. Only those shown in the figure were included in the sequence analysis. Fragment C was labeled at the 3′ terminus (position 979). The 5′ terminus of fragment C is located at position 929.

Data from 10 adjacent scans within each track were averaged over a total length of 8 cm. A total of 1600 data points was obtained for each track. This data was further smoothed by a standard five-point routine which eliminated spurious noise.

For standardization of these data, known amounts (12 to 18 000 cpm) of [^{32}P]ATP were subjected to polyacrylamide gel electrophoresis and autoradiography as described above. After photographic reduction and densitometer scanning, optical densities were integrated over the peaks and used, after base-line subtraction, to convert optical density to radioactivity. These parameters were not linearly related over the entire range; however, the nonlinear, empirically determined functions used were highly reproducible. The limit of sensitivity for this procedure is approximately 40 cpm.

Oligonucleotide lengths were assigned by comparing positions of the peaks in the drug-tested samples to the positions of oligonucleotides obtained from chemically cleaved DNA. The amount of each oligonucleotide was determined by integrating the area under each peak and, after base-line subtraction, converting optical density to radioactivity with the empirically obtained conversion functions described above.

Statistical Analysis of Base Sequence Specificity. The base sequence of each DNA fragment is known. Since the 5′ terminus of one of the strands is radioactively labeled, the length of each radioactive oligonucleotide found in the drug-treated digest corresponds to a known position of cleavage. The drugs studied are known to eliminate free bases. Thus, data were analyzed with respect to seven possible modes of recognition specificity: (a) base eliminated; (b) dinucleotide sequence reading from the 5′ end and terminating with the eliminated base; (c) trinucleotide sequences reading from the 5′ end and terminating with the eliminated base; (d) tri-

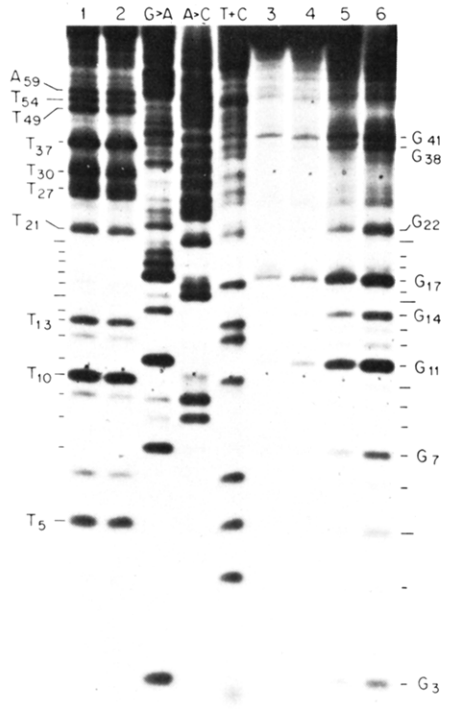

FIGURE 2: Cleavage of a 5′-labeled restriction fragment by NCS, AUR, and their chromophores. Reaction mixtures (40 μL) containing 100 mM Tris-HCl, pH 8.0, restriction fragment A (16 000 cpm), 5 μg/mL calf thymus DNA, and the drugs were incubated in the dark at 37 °C under the following conditions: (1) NCS (10 μg/mL) + 10 mM MSH, 30 min; (2) NCS chromophore (10 μg/mL) replaced NCS in reaction 1; (3) AUR (1 mg/mL), 7 h; (4) AUR chromophore (1 mg/mL), 2 h; (5) AUR chromophore (0.38 mg/mL) + 0.5 mM DTT, 2 h; (6) AUR chromophore (1 mg/mL) + 0.5 mM DTT, 10 min. The chromophore was dissolved in methanol, resulting in a final methanol concentration of 10% in the reaction mixture. Chromophore concentrations are expressed in terms of holoantibiotic. Samples were processed as described under Materials and Methods. G > A, A > C, and T + C represent the pattern obtained for fragment A after cleavage by chemical methods (Maxam & Gilbert, 1977).

nucleotide sequences reading from the 5′ end and centered on the eliminated base; (e), (f), and (g), respectively, as in (b), (c), and (d) but reading from the 3′ terminus.

From the known base sequence of the DNA fragment, we calculated the number of occurrences of each of the 4 bases, 16 dinucleotides, and 64 trinucleotides, reading from the 5′ and 3′ termini. A "relative probability" was assigned for each of the seven modes of recognition outlined above by dividing the total radioactivity for all oligonucleotides found in each specific drug-digested fragment by their number of occurrences in the original DNA fragment. Subgroupings were also analyzed in terms of purines, pyrimidines, and their combinations. The actual number of different oligonucleotides found for a given specificity was recorded and tabulated as "hits".

Statistical analyses, quantitative integrations, averaging, and smoothing were carried out by appropriate programs written in Fortran and Basic using a Perkin-Elmer/Interdata Model 7/16 16-bit minicomputer.

Results

Effect of NCS and AUR on a 5′-Labeled Restriction Fragment. NCS and the NCS chromophore induced identical patterns of cleavage in DNA fragments labeled at the 5′ terminus (Figure 2, tracks 1 and 2). Both preparations contained comparable amounts of chromophore and were tested in the presence of 2′mercaptoethanol. The AUR chromophore (track 4) produced a cleavage pattern similar to that of the

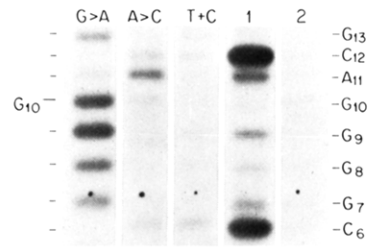

FIGURE 3: Cleavage of 3′-labeled restriction fragment by bleomycin. Blm A2, 25 μg/mL, was incubated with fragment C for 5 min at 37 °C in a reaction mixture (40 μL) containing 50 mM Tris–HCl, pH 8.5, 5 μ/mL calf thymus DNA, and 0.15 mM $Fe(NH_4)_2(SO_4)_2$. The mixture was subjected to polyacrylamide gel electrophoresis for 4 h followed by autoradiography as described under Materials and Methods (track 1). In the reaction shown in track 2, Blm was omitted and the concentration of $Fe(NH_4)_2(SO_4)_2$ was increased to 1.5 mM. G > A, A > C, and T + C represent the patterns obtained when the same restriction fragment was cleaved by chemical procedures (Maxam & Gilbert, 1977).

Table I: Base Release by AUR Chromophore and NCS Chromophore[a]

	[^{3}H]guanine released (%)		[^{3}H]thymine released (%)	
	−alkali	+0.3 M NaOH	−alkali	+0.3 M NaOH
AUR chromophore (2 mg/mL)	0.7	3.7	0.36	0.76
NCS chromophore (0.24 mg/mL)	0.26	0.4	3.9	10.3

[a] See Materials and Methods.

protein–associated drug (track 3). Dithiothreitol enhanced the rate of DNA cleavage by the AUR chromophore (Kappen et al., 1980b) but did not alter sequence specificity either at 37 °C (track 5 and 6) or at 0 °C (data not shown). Dithiothreitol alone does not produce detectable cleavage.

Effects of Blm A2, AUR, and NCS on a 3′-Labeled Restriction Fragment. The effect of bleomycin A2 on the cleavage pattern of a 3′-labeled restriction fragment was compared to that of Fe(II) (Figure 3). Sequence specificity is identical with that reported previously for a comparable restriction fragment labeled at the 5′ terminus (Takeshita et al., 1978; D'Andrea & Haseltine, 1978). In the latter case, however, labeled oligonucleotides produced by the action of bleomycin migrated slightly more rapidly than standard markers produced by chemical cleavage, reflecting the presence of a negatively charged fragment of deoxyribose bound to the 3′ terminus (Giloni et al., 1981). In contrast, the electrophoretic mobility of oligonucleotides produced by the action of bleomycin on 3′-labeled restriction fragments are precisely the same as those produced by standard markers or by Fe(II).

Effects of the NCS and AUR chromophores were also tested on the 3′-labeled fragment. In both cases, oligonucleotides migrated more slowly than standard markers of comparable length (data not shown). For example, the position of G_6 and G_{18} in the fragment cleaved with AUR corresponded, in electrophoretic mobility, to G_{12} and G_{23}, respectively, of the standard marker. Results obtained with 5′-labeled fragments correlate with measurements of base release from DNA, as reported previously for NCS (Poon et al., 1977; Ishida & Takahashi, 1976) and in Table I for AUR, although guanine release by AUR is considerably more dependent on alkaline treatment than is thymine release by NCS.

Base and Sequence Specificity of NCS. The base and dinucleotide specificities of the NCS chromophore on the cleavage of a 5′-labeled restriction fragment (containing 179 base pairs) is summarized in Table II. The first 72 bases in

Table II: Cleavage Patterns Produced by NCS and AUR Chromophores[a]

base	no. in fragment	hits NCS	hits AUR	rel. probability (%) NCS	rel. probability (%) AUR
A. Bases Attacked					
A	28	10	4	19	9
G	19	1	12	1	67
C	13	2	0	6	0
T	12	12	7	75	24
Pu	47	11	16	23	74
Py	25	14	7	77	26
B. Dinucleotide Sequences (5′ → 3′) Cleaved					
dinuc seq					
AC	5	2	0	4	0
AT	6	6	2	26	6
GC	5	0	0	0	0
GT	2	2	2	6	5
AA	11	3	1	3	1
AG	6	0	3	0	17
GA	7	1	1	0	2
GG	5	0	1	0	1
CC	1	0	0	0	0
CT	1	1	0	21	0
TC	1	0	0	0	0
TT	3	3	3	18	8
CA	7	5	2	12	5
CG	3	0	3	0	31
TA	3	1	0	8	0
TG	5	1	5	1	24
Pu-Py	18	10	4	34	11
Pu-Pu	29	4	6	4	18
Py-Py	6	4	3	42	16
Py-Pu	18	7	10	21	55
C. Dinucleotide Sequences (3′ → 5′) Cleaved					
AC	7	0	0	0	0
AT	3	3	1	20	8
GC	3	2	0	6	0
GT	5	5	3	22	6
AA	11	2	2	3	3
AG	7	0	4	0	12
GA	6	4	0	10	0
GG	5	0	5	0	29
CC	1	0	0	0	0
CT	1	1	0	20	0
TC	1	0	0	0	0
TT	3	3	3	11	6
CA	5	2	0	2	0
CG	5	0	1	0	7
TA	6	2	2	5	5
TG	2	1	2	1	24
Pu-Py	18	10	4	42	13
Pu-Pu	29	6	11	13	41
Py-Py	6	4	3	36	15
Py-Pu	18	5	5	9	30

[a] Components of reaction mixtures 2 (NCS chromophore) and 4 (AUR chromophore) are described in the legend to Figure 2. Values expressed as relative probability represent the total amount of oligonucleotides found corresponding to the base (or dinucleotide sequence) divided by the number of such oligonucleotides expected to be found if all positions were hit (this latter value is tabulated under number in fragment). The number of different oligonucleotides found for each base (or dinucleotide sequence) is tabulated under hits. Total radioactivities represented in this table are 19 671 and 17 524 cpm for NCS and AUR, respectively.

this sequence (A in Figure 1) were analyzed. This sequence was rich in adenine and contained twice as many purines as pyrimidines. As previously reported (Hatayama et al., 1978; D'Andrea & Haseltine, 1978), NCS primarily attacked thymine (75% of total bases) and, to a lesser extent, adenine (19%) and cytosine (6%). Virtually no guanine was attacked.

Bases most frequently found on the 5′ side of the base attacked followed the order A > C > T > G for thymine and C > T > A ≫ G for adenine. Reading from the 3′ side, G > A = C > T for thymine and G > T > A > C for adenine. Trinucleotide sequences (centered on the bases attacked) cleaved with high probability were ATG, TTT, CTC, ATA, and CAT (data not shown).

Further analysis of NCS-induced strand scission revealed that all 5′ → 3′ and 3′ → 5′ AT, GT, CT, and TT dinucleotide sequences were cleaved. In addition, five of seven CA (5′ → 3′), two of three GC (3′ → 5′), four of six GA (3′ → 5′), and one of two TG (3′ → 5′) sequences were cleaved. The following 5′ → 3′ dinucleotide sequences were *not* cleaved: GC, AG, GG, CC, TC, and CG. GA (5′ → 3′) and TG (5′ → 3′) sequences were cleaved only rarely. The following 3′ → 5′ sequences were *not* cleaved: AC, AG, GG, CC, TC, and CG. AA (3′ → 5′) sequences were rarely cleaved.

Base and Sequence Specificity of AUR. The cleavage pattern of AUR-treated DNA (Table II) reveals that guanine is preferentially attacked (67% of total bases). Lesser amounts of thymine (24%) and adenine (9%) and no cytosine were attacked under the conditions employed.

Bases most frequently found on the 5′ side of the base attacked followed the order C > T > A ≫ G for guanine and T > A > G ≫ C for thymine. The base found most frequently on the 3′ side followed the order G > T > A > C for guanine and A > G = T > C for thymine. Trinucleotide sequences (centered on the base released) preferentially attacked were CGG, TGT, and AGG (data not shown).

AUR cleaved all 5′ → 3′ GT, TT, CG, TG, and three of six AG dinucleotide sequences. AUR also cleaved all 3′ → 5′ GG, TT, TG, three of five GT, and four of seven AG sequences. The following 5′ → 3′ sequences were seldom if ever cleaved: AC, GC, AA, GA, GG, CC, CT, TC, and TA. The 3′ → 5′ sequences AC, GC, AA, GA, CC, CT, TC, CG, and CA were likewise spared.

Base Release and Sequence Specificity of the Bleomycins and Their Analogues. As previously reported, pyrimidines in DNA are preferentially attacked by bleomycin and released as free bases (Kuo & Haidle, 1974; Sausville et al., 1978; Povirk, 1979) or as base–propenals (Giloni et al., 1981). Specificities of some bleomycins, phleomycins, and their analogues tested with 5′-labeled restriction fragments for bases and dinucleotide sequences are summarized in Table III.

Cleavage by Blm A2 occurred preferentially at Pu–Py (5′ → 3′) and Py–Py (3′ → 5′) sequences. Other sequences were cleaved but less frequently. Py–Pu (5′ → 3′) sequences appear relatively protected from the effects of the drug.

All 5′ → 3′ GT and GC sequences in restriction fragment B were cleaved by Blm A2 and its analogues. The 5′ → 3′ sequences TA, TG, CA, CC, AG, and AC were spared. The 3′ → 5′ sequences most frequently cleaved by Blm A2, listed in order of relative probability, were TC, TT, GA, TG, and AT. The 3′ → 5′ sequences GC, GT, GG, AG, and CA were spared.

Compared to the four bleomycin analogues tested with respect to base release, Phlm G and Phlm-Pep showed significantly less activity while Tlm was more active toward purines with respect to the base attacked. In addition to the dinucleotide sequences which were not cleaved by Blm A2, Phlm G and Phlm-Pep did not significantly attack AA (5′ → 3′), GG (5′ → 3′), and CG (3′ → 5′) sequences. A general comparison of analogues (excluding sequences that occur only once) indicated that the patterns of cleavage produced by Blm A2 and Blm-B6 resemble one another and the patterns produced by Phlm-Pep, Phlm G, Blm-B1′, and Blm-Pep are similar. Tlm shows significant differences from the other analogues tested.

Table III: Cleavage Patterns Produced by Bleomycin Analogues[a]

base	no. in fragment	hits							rel. probability (%)						
		B6	PG	BP	Tlm	PP	B1′	A2	B6	PG	BP	Tlm	PP	B1′	A2
						A. Bases Attacked									
A	12	4	3	4	5	1	4	3	17	10	17	33	4	17	18
G	10	2	1	2	1	0	3	2	9	5	10	4	0	12	8
C	12	4	4	4	5	4	4	4	41	46	36	22	41	42	35
T	21	11	10	10	11	10	7	10	32	39	37	41	55	29	38
Pu	22	6	4	6	6	1	7	5	28	16	27	37	4	30	27
Py	33	15	14	14	16	14	11	14	72	84	73	63	96	70	73
						B. Dinucleotide Sequences (5′ → 3′) Cleaved									
dinuc seq															
AC	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT	5	3	2	3	3	2	2	2	10	9	13	10	19	11	15
GC	3	3	3	3	3	3	3	3	32	30	25	12	28	29	26
GT	1	1	1	1	1	1	1	1	23	37	27	40	38	29	22
AA	3	1	1	1	1	0	1	1	6	2	5	3	0	5	11
AG	3	0	0	0	0	0	1	0	0	0	0	0	0	1	0
GA	4	3	2	3	4	1	3	2	7	4	7	17	2	6	4
GG	2	1	0	1	1	0	1	1	4	0	4	4	0	4	4
CC	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CT	5	3	3	2	3	1	1	3	6	5	4	6	2	1	6
TC	4	1	1	1	2	1	1	1	3	3	4	4	3	3	4
TT	10	4	4	4	4	6	3	4	5	5	5	5	7	3	5
CA	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG	2	1	1	1	0	0	1	1	6	5	6	0	0	5	5
TA	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TG	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pu-Py	10	7	6	7	7	6	6	6	64	72	65	54	81	69	64
Pu-Pu	12	5	3	5	6	1	6	4	17	8	16	29	3	18	17
Py-Py	22	8	8	7	9	8	5	8	15	16	15	17	16	9	15
Py-Pu	10	1	1	1	0	0	1	1	5	4	5	0	0	4	3
						C. Dinucleotide Sequences (3′ → 5′) Cleaved									
AC	2	1	1	1	1	1	1	1	7	10	9	2	9	8	9
AT	3	2	2	1	2	2	2	2	10	8	5	7	15	7	11
GC	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GT	3	1	0	1	0	0	0	0	3	0	2	0	0	0	0
AA	3	1	1	1	1	0	1	1	4	4	4	10	0	5	3
AG	4	0	0	0	1	0	0	0	0	0	0	3	0	0	0
GA	3	1	1	1	1	0	1	1	7	3	6	6	0	7	14
GG	2	0	0	0	0	0	1	0	0	0	0	0	0	3	0
CC	3	1	1	1	1	1	1	1	11	14	10	6	13	12	7
CT	4	3	3	3	3	2	2	3	10	11	10	16	12	8	10
TC	5	2	2	2	3	2	2	2	17	18	14	13	21	17	15
TT	10	5	5	5	6	6	3	5	10	15	14	20	27	12	15
CA	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG	3	1	0	1	0	0	1	1	3	0	3	0	0	4	4
TA	5	2	1	2	3	1	2	1	4	2	4	17	3	4	2
TG	1	1	1	1	0	0	1	1	15	14	17	0	0	14	12
Pu-Py	10	4	3	3	3	3	3	3	21	19	17	8	22	15	20
Pu-Pu	12	2	2	2	3	0	3	2	12	7	11	16	0	15	17
Py-Py	22	11	11	11	13	11	8	11	48	63	53	50	73	51	51
Py-Pu	10	4	2	4	3	1	4	3	19	11	19	26	6	19	12

[a] Restriction fragment B was incubated for 15 min at 37 °C in a reaction mixture (40 μL) containing 50 mM Tris-HCl, pH 8.5, 5 μg/mL calf thymus DNA, and 0.15 mM $Fe(NH_4)_2(SO_4)_2$. Bleomycin analogues were present at the following concentrations: Blm A2, 12.5 μg/mL; Blm B1′, 12 μg/mL; Blm B6, 37.5 μg/mL; Blm-Pep, 12.5 μg/mL; Phlm G, 25 μg/mL; Tlm, 25 μg/mL; Phlm-Pep, 12 μg/mL. Reaction mixtures were subjected to polyacrylamide gel electrophoresis for 4–16 h. Values expressed as described in Table II. Phlm G, Phlm-Pep, and Blm-Pep are further abbreviated as PG, PP, and BP, respectively. Total radioactivities represented in this Table are 5281, 7830, 6059, 9263, 6762, 7622, and 5345 cpm for Blm B6, Phlm G, Blm-Pep, Tlm, Phlm-Pep, Blm B1′, and Blm A2, respectively.

Discussion

Quantitative Analysis of Sequence Specificity. The analyses we report here make use of programs and logic similar to those utilized for sequencing DNA (Gingeras & Roberts, 1980). However, in addition to identifying radioactive bands by position and assessing the length of each oligonucleotide represented, we measure the relative amount of radioactivity found in each band.

Assumptions made in these analyses, and possible sources of errors, are described under Materials and Methods and discussed further below. We have refrained from making comparisons either when the calculated relative probabilities are within close values or when the number of "hits" is too low to be statistically significant.

In determining the sequence specificity of drugs tested in our experiments, certain assumptions were made. Identical sequences were considered to have similar affinities for the drug in question. We recognize, however, that cooperativity phenomena may occur with initial binding of drugs affecting subsequent binding at other sites (Krugh et al., 1979). It is also possible that increased flexibility of the helix near the ends of the restriction fragment alters the pattern of drug binding.

We assume that a "single hit" occurs (each restriction fragment being cleaved only once); therefore, the calculated

"relative probability" is an accurate indication of the specificity. If this assumption is correct, small and large oligonucleotides should accumulate at similar rates during the reaction. This generally appeared to be the case, although in a few instances, a slight excess of smaller oligonucleotides accumulated at the time the strand scission reaction was terminated, indicating that more than one hit per fragment occurred in those instances.

A possible source of experimental error involves the procedure for measurement of optical density from the reduced autoradiographs. Segments representing one-sixth of the width of a given band were scanned. Great care was taken to align the scanner, and each of the 10 individual scans was visually observed before averaging. Variations among them were less than 5%. In addition, the empirically determined conversion function for optical density to radioactivity may introduce some error. At very high levels of radioactivity, the optical density reaches a plateau value. In these instances, we found that decreasing the exposure time in a standardized and reproducible way during the photographic reduction step allowed us to circumvent this problem. The overlap of well-resolved bands served as internal controls for the accuracy of the method.

Base Specificity of Auromomycin. The base specificity of AUR-induced strand scission of DNA has not previously been reported. The effect of AUR differs from that of NCS in that guanine, especially after alkaline treatment, is primarily attacked along with lesser amounts of thymine. Adenine is minimally affected while cytosine is not attacked. Further studies will be required to determine whether both guanine and thymine are attacked through the same mechanism. Furthermore, the marked (5-fold) stimulation of guanine released by alkali, even though about half of the breaks are alkali induced (L.S. Kappen and I.H. Goldberg, unpublished data), indicates that this base may attach to a sugar fragment on the 5′-phosphoryl end of the break, accounting for the abnormal electrophoretic mobility.

The neighboring base found most frequently on the 3′ side of the base cleaved by AUR was guanine. The effect of the nearest neighbor on the 5′ side was less prominent, with C and T being favored when G was attacked and T and A (with a relatively lower probability) when T was attacked.

Sequence analysis of the AUR-treated 5′-labeled DNA fragment correlates with the observed release of free bases. A similar correlation was not observed when a 3′-labeled fragment was used and compared with the chemically cleaved fragment described by Maxam & Gilbert (1977). A possible explanation of these results is that a residual deoxyribose fragment and/or modified base has been produced at the 5′ terminus which, by virtue of its size and/or charge, significantly alters the electrophoretic mobility of oligonucleotides formed by the cleavage reaction.

The AUR chromophore requires the presence of oxygen and a reducing agent for maximum biological activity, and the cleavage reaction occurs at 0 °C (Kappen et al., 1980b), suggesting that a radical-mediated redox mechanism is involved. The presence of reducing agents, such as DTT, does not alter the base specificity of the AUR chromophore toward DNA.

Role of the Protein in the Reaction of AUR and NCS with DNA. NCS and AUR were originally described as proteins with antimicrobial and antitumor activities (Yamashita et al., 1979; Goldberg et al., 1981). Recently, it was discovered that both proteins contain tightly bound chromophores (Napier et al., 1979; Yamashita et al., 1979; Kappen et al., 1980a,b). The structure of the NCS chromophore, which is biologically active, has been partially elucidated (Albers-Schönberg, 1980; Napier et al., 1981).

Since DNA functions are modified by certain proteins, it was of interest to determine the role of the proteins to which AUR and NCS are bound, in determining base release and sequence specificity. Our results indicate that the effects of the two chromophores are the same as those of the parent molecules, suggesting that the protein primarily serves as a carrier, possibly protecting the chromophore from degradation in vivo (Povirk & Goldberg, 1980; Kappen & Goldberg, 1980) and releasing it for interaction with DNA by an intercalative mechanism (Povirk & Goldberg, 1980, 1981).

Base Specificity of Bleomycins, Phleomycins, and Related Compounds. Bleomycin and the other analogues tested preferentially attack GC (5′ → 3′) and GT (5′ → 3′) sequences, releasing the pyrimidine base. The nearest neighbor effect was ascertained by comparing the eight possible dinucleotide sequences containing pyrimidines on the 3′ or 5′ side of the base released. Among the bases, GC (5′ → 3′) is preferred over GC (3′ → 5′) and GT (5′ → 3′) over GT (3′ → 5′), indicating a preference for 5′ → 3′ sequences. Among sequences of 3′ → 5′ polarity, thymine was the preferred nearest neighbor on the 3′ side of the pyrimidine released.

Although all of the bleomycin analogues display generally similar sequence specificities, certain differences were consistently observed. These can be related to structural differences between these antibiotics (Figure 4). Phlm-G/Blm B6 and Phlm-Pep/Blm-Pep represent pairs of molecules with identical side chains. Thus, differences in base specificity may be attributed to the presence of a saturated bithiazole ring in the phleomycins. Both phleomycin analogues tested show considerably less preference for purines than do any of the bleomycins. Furthermore, the four purine–purine dinucleotide sequences were cleaved with low frequency. We interpret these results as reflecting the increased difficulty encountered by the partly nonplanar phleomycin ring in forming an intercalation complex. This problem would be essentially apparent where purine–purine stacking forces are involved.

It has been reported that bleomycin (Povirk et al., 1979), but not phleomycin (Povirk et al., 1981), intercalates with DNA. This raises certain questions with regard to the observed patterns of base specificity. It seems rather unlikely that intercalative and nonintercalative binding could cause precisely the same specific cleavage of GC and GT sequences. In addition, several of us (M. Takeshita, R. Klett, and A.P. Grollman, unpublished studies) have observed that phleomycin induces supercoiling of relaxed closed circular DNA, suggesting that intercalation may occur under appropriate experimental conditions.

The binding specificity of the four bleomycin analogues was compared to determine the contribution of the side chain. Blm B1′ contains an uncharged amide, Blm A2, a singly-charged dimethylsulfonium group, Blm B6, three guanidino residues, and Blm-Pep, secondary amines and a terminal phenyl group (Figure 4). Marked differences in base specificity between these analogues do not occur, indicating that the terminal amine [the major structural difference between these analogues (Umezawa, 1979)] does not contribute significantly to the specificity of DNA binding.

Tallysomycin differs from bleomycin in that a second amino sugar and its group are attached to the core of the molecule. The side chain of tallysomycin consists of D-lysine linked to spermidine. Base specificity of tallysomycin differs quantitatively from that of the bleomycin analogues in that it shows the most marked preference for the GT sequence and a general

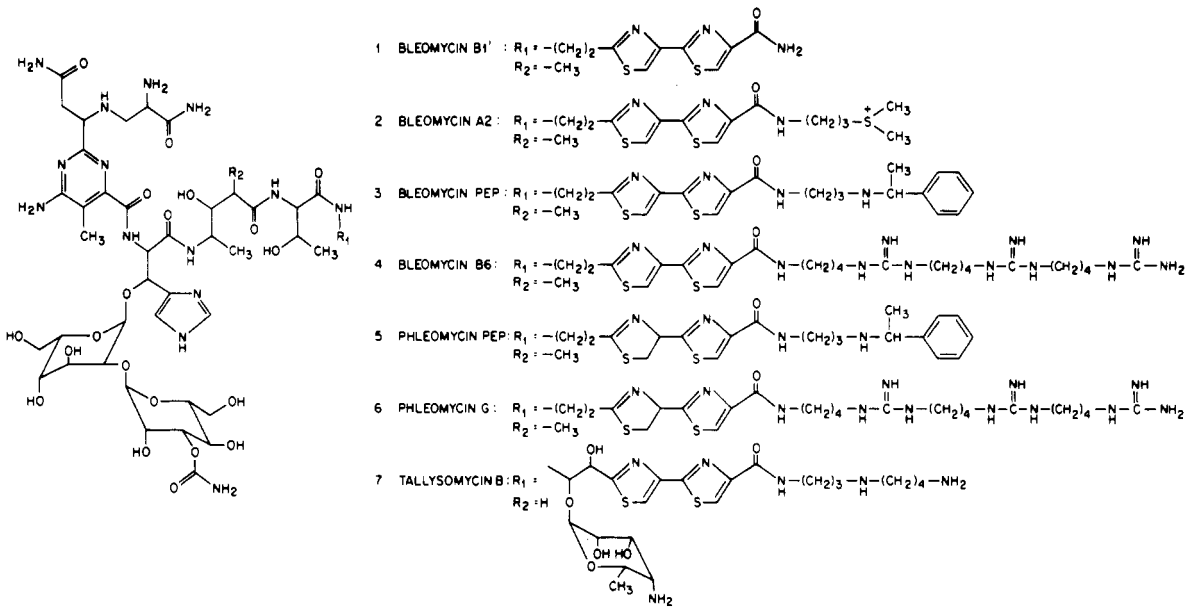

FIGURE 4: Structure of the bleomycins, phleomycins, and tallysomycin B.

preference for adenine over cytosine. As discussed above, the terminal amine does not contribute significantly to base specificity. Therefore, the altered specificity of tallysomycin must be attributed to the presence of the additional amino sugar and/or methyl moieties.

Considerable efforts have been made to modify the structures of bleomycin, phleomycin, and tallysomycin so as to enhance their therapeutic activity and to minimize the pulmonary and renal toxicities caused by these drugs (Umezawa, 1979). It appears from our in vitro studies that the base-specific cleavage of DNA (presumed to be responsible for the chemotherapeutic properties) is not markedly altered by these structural changes. Thus, modifications of the side chains may be expected to alter transport, organ distribution, and perhaps toxicities of the drug.

Site of Bleomycin Cleavage. Bleomycin attacks the deoxyribose moiety of DNA, leaving a stable fragment attached to the 3′ terminus of the resulting oligonucleotide. This residue, recently shown to be glycolic acid (Giloni et al., 1981), slightly alters the electrophoretic mobility of the cleavage products. Since the bases attacked are released as base–propenals (Giloni et al., 1981), it was predicted that the 5′ terminus of bleomycin-cleaved DNA should be free. Direct evidence on this point was obtained in experiments which compared the electrophoretic mobilities of bleomycin-cleaved and chemically cleaved DNA labeled at the 3′ terminus. In this case, the oligonucleotides formed have mobilities identical with those of the standard markers, indicating that a single base is removed leaving a free 5′-phosphate terminus.

This same approach was used to demonstrate that cleavage of DNA by NCS and AUR yielded oligonucleotides that migrate more slowly than oligonucleotides of comparable length with normal 3′ and 5′ termini. The chemical nature of the terminus produced by these antibiotics is unknown but is likely to be a deoxyribose fragment to which the base remains attached (Hatayama & Goldberg, 1980).

In the case of NCS, nearest neighbor effects are not evident. For bleomycin, there is a clear preference for 5′ → 3′ GC and GT sequences. It is also possible that the bases located to the 5′ side of the neighboring guanine or to the 3′ side of the neighboring base oof the pyrimidine released could contribute to the base specificity observed. A DNA fragment containing 16 specific trinucleotide sequences is required to examine this problem systematically. In the 72-base fragment used for our experiments, only 7 of these sequences exist; thus, it is not possible to accurately ascertain the effect of neighboring bases.

When only double-strand cleavage is considered (assuming that such breaks may be the result of two single-strand scissions on opposite strands, separated by 1–3 base pairs), the guanine–pyrimidine (5′ → 3′) specificity of bleomycin would be represented by several tetra-, penta-, and hexanucleotide sequences; for example

$$\begin{matrix}GCGC & GCAC & GCXAC \\ CGCG' & CGTG' & CGYTG'\end{matrix} \text{ or } \begin{matrix}GCXX'GC \\ CGYY'CG\end{matrix}$$

Our results appear to be consistent with observations of Lloyd et al. (1978), who showed that very low concentrations of bleomycin produce double-strand breaks in PM2 at a limited number of discrete sites.

Acknowledgments

We gratefully acknowledge the assistance of Dr. Robert Klett, who helped develop the procedure used for gel scanning and data analysis.

References

Albers-Schönberg, C., Dewey, P. C., Hensens, O. D., Leisch, J. M., Napier, M. A., & Goldberg, I. H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1351.

Asakura, H., Hori, M., & Umezawa, H. (1975) *J. Antibiot. 25*, 537.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608.

Earhart, C. F. (1979) in *Antibiotics: Mechanism of Action of Antieukaryotic and Antiviral Compounds* (Hahn, F. E., Ed.) Vol. V-2, p 298, Springer-Verlag, New York.

Giloni, L., Takeshita, M., Johnson, F., Iden, C., & Grollman, A. P. (1981) *J. Biol. Chem. 256*, 8608.

Gingeras, T. R., & Roberts, R. J. (1980) *Science (Washington, D.C.) 209*, 1322.

Goldberg, I. H., Hatayama, T., Kappen, L. S., Napier, M. A., & Povirk, L. F. (1981) in *Molecular Action on Targets for Cancer Chemotherapeutic Agents* (Sartorelli, A. C., Bertino, J. R., & Lajo, S. S., Eds.) pp 163–191, Academic Press, New York.

Haidle, C. W., & Lloyd, R. S. (1979) in *Antibiotics: Mechanism of Action of Antieukaryotic and Antiviral*

Compounds (Hahn, F. E., Ed.) V-2, p 124, Springer-Verlag, New York.
Hatayama, T., & Goldberg, I. H. (1980) *Biochemistry 19*, 5890.
Hatayama, T., Goldberg, I. H., Takeshita, M., & Grollman, A. P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3603.
Ishida, R., & Takahashi, T. (1976) *Biochem. Biophys. Res. Commun. 68*, 256.
Kappen, L. S., & Goldberg, I. H. (1980) *Biochemistry 19*, 4786.
Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1970.
Kappen, L. S., Napier, M. A., Goldberg, I. H., & Samy, T. S. A. (1980b) *Biochemistry 19*, 4780.
Krugh, T. R., Hook, J. W., III, Lin, S., & Chen, F.-M. (1979) in *Stereodynamics of Molecular Systems* (Sarma, R. H., Ed.) p 423, Pergamon Press, New York.
Kuo, M. T., & Haidle, C. W. (1974) *Biochim. Biophys. Acta 335*, 109.
Lloyd, R. S., Haidle, C. W., & Robberson, D. L. (1978) *Biochemistry 17*, 1890.
Maxam, A., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560.
Napier, M. A., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1979) *Biochem. Biophys. Res. Commun. 89*, 635.
Napier, M. A., Goldberg, I. H., Hensens, O. D., Dewey, R. S., Liesch, J. M., & Albers-Schönberg, G. (1981) *Biochem. Biophys. Res. Commun. 100*, 1703.
Ohtsuki, K., & Ishida, N. (1980) *J. Antibiot. 33*, 744.
Poon, R., Beerman, T. A., & Goldberg, I. H. (1977) *Biochemistry 16*, 486.
Povirk, L. F. (1979) *Biochemistry 18*, 3989.
Povirk, L. F., & Goldberg, I. H. (1980) *Biochemistry 19*, 4773.
Povirk, L. F., & Goldberg, I. H. (1981) *Biochemistry 20*, 4007.
Povirk, L. F., Hogan, M., & Dattagupta, N. (1979) *Biochemistry 18*, 96.
Povirk, L. F., Hogan, M., Dattagupta, N., & Buechner, M. (1981) *Biochemistry 20*, 665.
Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchinson, C. A., III, Slocombe, P. M., & Smith, M. (1977) *Nature (London) 265*, 687.
Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978) *Biochemistry 17*, 2746.
Strong, J. E., & Crooke, S. T. (1978) in *Bleomycin: Current Status and New Developments* (Carter, S. K., Crooke, S. T., & Umezawa, H., Eds.) p 343, Academic Press, New York.
Suzuki, H., Miura, K., Kumada, Y., Takeuchi, T., & Tanaka, N. (1980) *Biochem. Biophys. Res. Commun. 94*, 255.
Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983.
Takeshita, M., Grollman, A. P., Eisenberg, M., Kappen, L. S., & Goldberg, I. H. (1981) 182nd National Meeting of the American Chemical Society, New York, NY, Abstract 92.
Umezawa, H. (1979) in *Bleomycin: Chemical, Biochemical and Biological Aspects* (Hecht, S., Ed.) p 24, Springer-Verlag, New York.
Woynarowski, J. M., & Beerman, T. A. (1980) *Biochem. Biophys. Res. Commun. 94*, 769.
Yamashita, T., Naoi, N., Hidaka, T., Watanabe, K., Kumada, Y., Takeuchi, T., & Umezawa, H. (1979) *J. Antibiot. 32*, 330.