JOURNAL OF
MEDICINAL
CHEMISTRY

Volume 39, Number 21 October 11, 1996

Communications to the Editor

Alkylation of DNA by the Anthracycline, Antitumor Drugs Adriamycin and Daunomycin

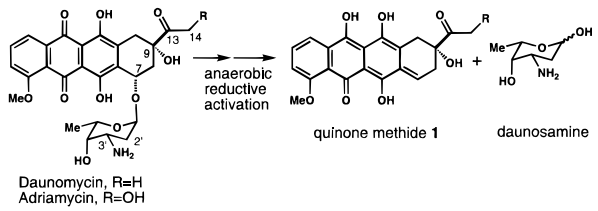
Dylan J. Taatjes,[†] Giorgio Gaudiano,[†,‡] Katheryn Resing,[†] and Tad H. Koch*[,†]

Department of Chemistry and Biochemistry University of Colorado, Boulder, Colorado 80309-0215, and Istituto di Medicina Sperimentale, CNR, Viale Marx, 15-43, 00137 Rome, Italy

Received July 18, 1996

Adriamycin is a broad spectrum anthracycline, antitumor drug which has been used extensively for the treatment of a number of cancers for a period of more than two decades. Daunomycin, which differs in structure only at the 14-position, has been used primarily for the treatment of acute leukemia. In spite of extensive investigation, the mechanism for selective tumor cell cytotoxicity is not precisely understood. An early proposal was the alkylation of DNA through bioreductive activation in an anaerobic medium.[1,2] Catalytic production of peroxides, including hydrogen peroxide, via reductive activation in the presence of molecular oxygen has also been proposed as a contributing factor.[3] For the past decade, topoisomerase-induced DNA strand breaks caused by anthracycline intercalation has been the most accepted explanation.[4] A recent observation by Phillips and co-workers of transcription blockages in DNA from reductively activated adriamycin and daunomycin has renewed interest in DNA alkylation as a source of tumor cell cytotoxicity.[5] They reported that reductive activation with dithiothreitol (DTT) in the presence of various DNAs led to adducts at isolated dGs and cross-links involving the 2-amino groups of dGs at the site 5′-GpC-3′. Similar reductive activation was achieved with xanthine oxidase.[5] The proposed intermediate was the quinone methide **1** (Scheme 1),[5] a well-established transient from reductive activation.[6] Cell culture experiments[7,8] and small animal experiments[9] also indicate a role for reductive activation in drug cytotoxicity. Drug−DNA covalent bond formation is proposed to trigger apoptosis.[8]

Scheme 1



We have investigated reactivity between the self-complementary synthetic deoxyoligonucleotide 5′-GCGCGCGC-3′, abbreviated $(GC)_4$, and adriamycin and daunomycin in the presence of three different reagents: the reducing agent DTT, the oxidizing agent hydrogen peroxide, and the alkylating agent formaldehyde. All three modes of activation yield the same DNA−anthracycline adduct mixture as established by HPLC, UV−vis spectroscopy, and mass spectrometry. The adduct (which we believe is actually the "cross-link" described in the literature[5,8]) is proposed to have the aglycon portion of the anthracycline intercalated in the DNA with a covalent linkage from the anthracycline 3′-amino to the 2-amino group of a dG via a methylene group originating from formaldehyde. Formaldehyde results from hydrogen peroxide oxidation of constituents in the reaction media including adriamycin.

Results and Discussion. $(GC)_4$ was selected for these studies because it is a short sequence bearing multiple copies of the 5′-GpC-3′ "cross-linking site" and has a relatively high melting temperature in the solutions employed: 61 °C in the Tris reaction buffer and 45 °C in the HPLC eluent. Reaction of 19 μM $(GC)_4$ with 37 μM adriamycin in pH 8 buffer (40 mM Tris, 100 mM KCl, 0.10 mM EDTA, 3 mM $MgCl_2$) containing 40 μM $FeCl_3$ and 7 mM DTT (the protocol of Phillips and co-workers[5]) in the dark at ambient temperature for 4 days resulted in complete reaction of the DNA. Three new peaks, one bearing a shoulder, appeared in the reverse phase HPLC chromatogram for compounds which showed UV−vis spectra consistent with DNA−adriamycin adduct structures. The same adducts were formed upon reaction of $(GC)_4$ with adriamycin in pH

[†] USA.
[‡] Italy.

S0022-2623(96)00519-5 CCC: $12.00

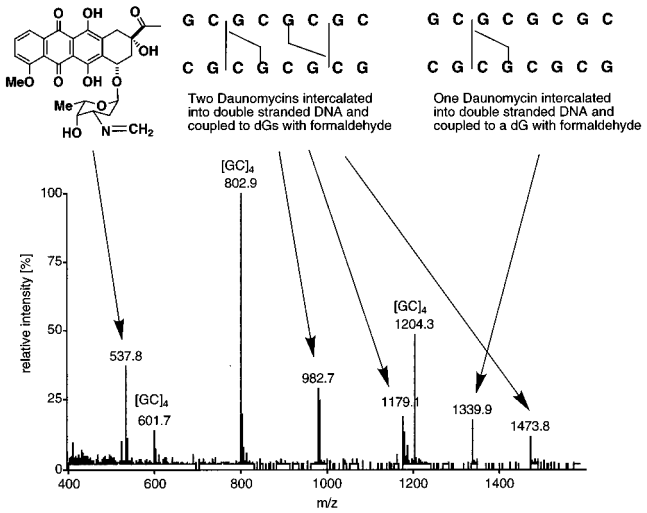
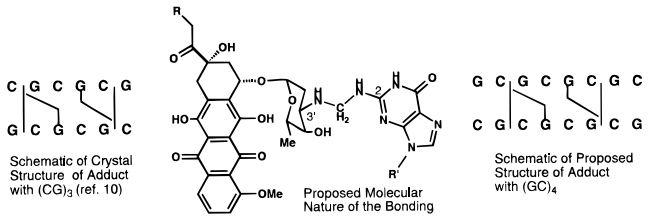


Figure 1. Negative ion, electrospray spray mass spectrum of the major adduct formed by reaction of daunomycin with the self-complementary DNA oligonucleotide $(GC)_4$ in pH 7.4 Tris/KCl/$MgCl_2$/EDTA buffer in the presence of hydrogen peroxide. The adduct is proposed to have two molecules of daunomycin, each intercalated at a CpG site in the double-stranded DNA and each linked from its 3′-amino to the 2-amino of a neighboring G base as shown in Scheme 2, consistent with the appearance of the ions at *m/z* 982.7 (calcd for $(M - 6)^{6-}$, 982.5), 1179.1 (calcd for $(M - 5)^{5-}$, 1179.2), and 1473.8 (calcd for $(M - 4)^{4-}$, 1474.3). Significant intensity at *m/z* 982.7 and 1473.8 also results from single stranded DNA covalently bound to one daunomycin as $(M - 3)^{3-}$ and $(M - 2)^{2-}$ ions, respectively. The adduct decomposes in the mass spectrometer to yield double-stranded DNA linked to a single daunomycin, *m/z* 1339.9 (calcd for $(M - 4)^{4-}$, 1339.5); single-stranded DNA, *m/z* 601.7, 802.9, and 1204.3; and daunomycin linked to a methylene (most likely the Schiff base as shown), *m/z* 537.8 (calcd for $(M - 1)^{1-}$, 538.2).

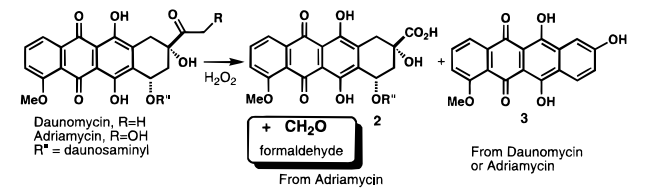
7.4 or pH 8 Tris/KCl/$MgCl_2$/EDTA buffer with 30−50 equiv of hydrogen peroxide or with 5 equiv of formaldehyde, both relative to adriamycin. The reaction with hydrogen peroxide required several days at ambient temperature; while with formaldehyde, all of the DNA formed products in 3 h. The UV−vis spectra associated with the adduct peaks showed ratios of absorbance at 260 to 480 nm of approximately 20:1 or 10:1, consistent with one or two molecules of adriamycin per double-stranded DNA, respectively. In this regard, the major product (50%) showed two molecules of adriamycin. Further, the maximum in the visible spectrum was actually 510 nm as opposed to 480 nm for free adriamycin, consistent with the adriamycin chromophore intercalated in the DNA. Isolation of the major DNA product was achieved by C18 reverse phase HPLC, eluting with a gradient created with 20 mM triethylammonium acetate and acetonitrile. All of the HPLC-isolated DNA−drug products were unstable with respect to conversion back to DNA and adriamycin, characteristic of the Phillips' "cross-links" and adducts.[5]

The negative ion, electrospray mass spectrum of the major product (Supporting Information) showed peaks for double-stranded DNA bound to two molecules of adriamycin, each via a methylene group, at *m/z* 1185.5 (30%, calcd for $(M - 5)^{5-}$ 1185.6); for single-stranded DNA bound to one molecule of adriamycin, via a methylene group, at *m/z* = 740.6 (62%, calcd for $(M - 4)^{4-}$, 740.6), at 987.8 (82%, calcd for $(M - 3)^{3-}$, 987.9), and at 1482.6 (18%, calcd for $(M - 2)^{2-}$, 1482.3); and for single-stranded DNA at *m/z* = 802.5 (65% $(M - 3)^{3-}$).

Scheme 2



Scheme 3



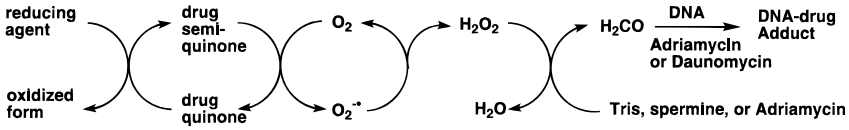
Double-stranded DNA bound to two molecules of adriamycin likely contributes some intensity at *m/z* 740.6, 987.8, and 1482.3 as $(M - 8)^{8-}$, $(M - 6)^{6-}$, and $(M - 4)^{4-}$, respectively; however, the amount could not be established unambiguously from isotope peaks because of insufficient signal to noise. In an MS/MS experiment, argon-induced decomposition of the ion at *m/z* 740.6 $(M - 4)^{4-}$ caused elimination of the adriamycin bearing the attached methylene group to produce an ion with *m/z* = 553.8 (calcd for $(M - 1)^{1-}$, 554.2) and restored the intact DNA (*m/z* = 802.8 ($(M - 3)^{3-}$). In another MS/MS experiment at lower accelerating potential, peaks were observed for both adriamycin and DNA bearing the methylene group. Further, an MS/MS experiment on *m/z* 554 established that the methylene group was linked to the sugar. Precise determination of the drug−DNA site of attachment by MS/MS sequencing of the oligonucleotide was not possible because of the facile elimination of the adriamycin bearing the attached methylene. However, the MS data indicate that each adriamycin is covalently bound to a separate DNA strand. The same mass spectrum was obtained for the major product (with the same HPLC retention time) from reaction of $(GC)_4$ with adriamycin and formaldehyde and from the reaction of $(GC)_4$ with adriamycin and DTT.

Daunomycin reacted with $(GC)_4$ in pH 7.4 or pH 8.0 Tris/KCl/$MgCl_2$/EDTA buffer in the presence of hydrogen peroxide or formaldehyde to yield the same mixture of DNA−daunomycin adducts. The negative ion, electrospray mass spectrum of the major product (50%) showed peaks for double-stranded DNA with each strand bound to one molecule of Daunomycin via a methylene group (Figure 1).

The actual structures for the major adriamycin and daunomycin adducts are suggested by the crystal structure of a product from reaction of $(CG)_3$ with daunomycin and formaldehyde by Wang and co-workers.[10] The structure showed two molecules of daunomycin intercalated in the duplex DNA at 5′-CpG with methylene groups linking the 3′-amino groups of the daunosamine to the 2-amino groups of dGs as shown in Scheme 2. The analogous structure proposed with $(GC)_4$ is similarly displayed. The formaldehyde coupling reaction has recently been demonstrated with natural DNA sequences in solution.[11]

What is the origin of the formaldehyde when the anthracyclines are activated with H_2O_2 or with DTT?

Scheme 4



With adriamycin a possibility is from a Baeyer−Villiger oxidation at the 13-position. Reaction of adriamycin with DTT in the presence of molecular oxygen (a source of H_2O_2[3]) or with H_2O_2 directly in pH 8 phosphate buffer at 25 °C yields two products indicative of Baeyer−Villiger oxidation, **2** and **3**, as shown in Scheme 3. With H_2O_2 the yield of **2** was 65% after 6 days. The products were characterized from spectroscopic and chromatographic data and comparison with independently prepared materials.[12] The byproduct from formation of **2** is formaldehyde as demonstrated by reaction with the Hantzsch reagent[13] and HPLC analysis; the byproducts from formation of **3** are presumed to be hydroxyacetic acid and daunosamine. Similar oxidation of daunomycin gives **3** but not **2**, indicating a different source of formaldehyde in the reactions with DNA. Control experiments showed that H_2O_2 oxidation of Tris, especially in the presence of Fe^{3+}/EDTA, produces a large excess of formaldehyde relative to that required for adduct formation; again quantitative analysis of formaldehyde was performed with the Hantzsch reagent. Further, formation of formaldehyde via a Fenton reaction with Tris and structurally related compounds has been reported.[14] Consequently, some of the formaldehyde in experiments with Tris buffer is artifactual relative to *in vivo* conditions. However, with adriamycin, but not daunomycin, DNA adducts are also observed in phosphate buffer, albeit in lower yield; in this case some of the adriamycin is sacrificed to produce formaldehyde as indicated by the formation of **2** in the reaction mixture.

Phillips and co-workers reported that degassing their reaction mixture with nitrogen for 10 min had no effect on the formation of adducts and "cross-links". This level of degassing was inadequate. A freeze−pump (7×10^{-6} Torr)−thaw degassed reaction mixture of adriamycin, DNA, DTT, and Fe^{3+} in Tris/KCl/$MgCl_2$/EDTA buffer showed virtually no reaction of the DNA after 4 days at ambient temperature, whereas the DNA in an identical reaction mixture equilibrated with atmospheric oxygen was completely transformed into the mixture of DNA−drug adducts described above. Further, the degassed reaction mixture produced the same DNA−drug adducts when subsequently exposed to air.

We propose that our DNA−drug adducts are equivalent to Phillips' DNA−drug "cross-links". Phillips' "cross-links" and adducts are distinguished only by differences in stability with respect to drug release.[5] In the GpC cross-link site, we propose that the dG of one strand is covalently bound to the drug via a methylene from formaldehyde and the dG of the second strand is hydrogen bonded to the 9-OH of the drug. Two hydrogen bonds from the 9-OH to the nitrogens at the 1- and 2-positions of the dG of the second strand are evident in the Wang crystal structure.[10] The H-bonding interaction then distinguishes a "cross-link" at a GpC site from an adduct at an isolated dG.

In concluding we summarize the proposed pathway from drug reduction to DNA−drug adduct formation in Scheme 4. Further, we ask why might tumor cells be more sensitive to the anthracyclines than normal cells? Some years ago lymphocytic leukemia cells were reported to bear higher levels of formaldehyde than normal lymphocytes.[15] More recently, breast cancer patients and tumor-bearing transgenic mice were shown to have higher levels of formaldehyde in their breath.[16] Although the discovery of these anthracycline−DNA adducts under reducing or oxidizing conditions resulted, at least in part, through artifactual production of formaldehyde, their facile formation, even with very low levels of formaldehyde, suggests a biological equivalent. As an example, we have observed that formaldehyde results from reaction of spermine, a polyamine associated with DNA *in vivo*, with H_2O_2 in the presence of iron/EDTA in phosphate buffer. A significant increase in urinary formaldehyde among other carbonyl compounds has been reported with adriamycin administration and linked to adriamycin-induced lipid peroxidation.[17] Further, if adduct formation via formaldehyde is an important component to cytotoxicity, then daunomycin, which would require cellular formaldehyde, might be more selective than adriamycin, which releases formaldehyde upon Baeyer−Villiger oxidation. Our results now suggest new strategies for anthracycline drug development.

Acknowledgment. We are grateful to the National Cancer Institute of the NIH (Grant CA24665) for financial assistance. We thank Pharmacia-Upjohn, Milan, Italy, and Nexstar Pharmaceuticals, San Dimas, CA, for generous samples of adriamycin and daunomycin.

Supporting Information Available: Negative ion, electrospray mass spectrum of the major DNA adduct from reaction of adriamycin with $(GC)_4$ in the presence of H_2O_2 and the MS/MS spectrum of the ion at *m/z* 740.6 (2 pages). Ordering information is given on any current masthead page.

References

(1) Lin, A. J.; Cosby, L. A.; Shansky, C. W.; Sartorelli, A. C. Potential Bioreductive Alkylating Agents. 1 Benzoquinone Derivatives. *J. Med. Chem.* **1972**, *15*, 1247−1252. Lin, A. J.; Sartorelli, A. C. Potential bioreductive alkylating agents. 7. Antitumor effects of phenyl-substituted 2-chloromethyl-3-phenyl-1,4-naphthoquinones. *J. Med. Chem.* **1976**, *19*, 1336−1338.

(2) Moore, H. W.; Czerniak, R. Naturally Occurring Quinones as Potential Bioreductive Alkylating Agents. *Med. Res. Rev.* **1981**, *1*, 249. Moore, H. W.; Czerniak, R.; Hamdan, A. Natural Quinones as Quinone Methide Precursors−Ideas in Rational Drug Design. *Drugs Exp. Clin. Res.* **1986**, *12*, 475−494.

(3) Bachur, N. R.; Gordon, S. L.; Gee, M. V. Anthracycline Antibiotic Augmentation of Microsomal Electron Transport and Free Radical Formation. *Mol. Pharmacol.* **1977**, *13*, 901−910. Lown, J. W.; Chen, H.-H.; Plambeck, J. A.; Acton, E. M. Further Studies on the Generation of Reactive Oxygen Species from Activated Anthracyclines and the Relationship to Cytotoxic Action and Cardiotoxic Effects. *Biochem. Pharmacol.* **1982**, *31*, 575−581. Dorshow, J. H. Role of Hydrogen Peroxide and Hydroxyl Radical formation in the Killing of Ehrlich Tumor Cells by Anti Cancer Quinones. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4514−4518.

(4) Liu, L. F. DNA Topoisomerase Poisons as Antitumor Drugs. *Annu. Rev. Biochem.* **1989**, *58*, 351−375. Pommier, Y. DNA Topoisomerases and Their Inhibition by Anthracyclines. In *Anthracycline Antibiotics*; Priebe, W., Ed.; ACS Symposium Series 574; American Chemical Society: Washington, DC, 1995; pp 183−203.

(5) Cullinane, C.; van Rosmalen, A.; Phillips, D. R. Does Adriamycin Induce Interstrand Cross-Links in DNA? *Biochemistry* **1994**, *33*, 4632−4638. Cullinane, C.; Cutts, S. M.; van Rosmalen, A.; Phillips, D. R. Formation of Adriamycin-DNA Adducts In Vitro. *Nucleic Acids Res.* **1994**, *22*, 2296−2303. Van Rosmalen, A.; Cullinane, C.; Cutts, S. M.; Phillips, D. R. Stability of Adriamycin-Induced DNA Adducts and Interstrand Crosslinks. *Nucleic Acids Res.* **1995**, *23*, 42−50. Cutts, S. M.; Phillips, D. R. Use of Oligonucleotides to Define the Site of Interstrand Cross-Links Induced by Adriamycin. *Nucleic Acids Res.* **1995**, *23*, 2450−2456.

(6) Abdella, B. R. J.; Fisher, J. A Chemical Perspective on the Anthracycline Antitumor Antibiotics. *Environ. Health Perspect.* **1985**, *64*, 3−18. Gaudiano, G.; Koch, T. H. Redox Chemistry of Anthracycline Antitumor Drugs and Use of Captodative Radicals as Tools for Its Elucidation and Control. *Chem. Res. Toxicol.* **1991**, *4*, 2−16.

(7) Bartoszek, A.; Wolf, C. R. Enhancement of Doxorubicin Toxicity Following Activation by NADPH Cytochrome P450 Reductase. *Biochem. Pharmacol.* **1992**, *43*, 1449−1457.

(8) Skladanowski, A.; Konopa, J. Adriamycin and Daunomycin Induce Programmed Cell Death (Apoptosis) in Tumour Cells. *Biochem. Pharmacol.* **1993**, *46*, 375−382. Skladanowski, A.; Konopa, J. Relevance of Interstrand DNA Crosslinking Induced by Anthracyclines for Their Biological Activity. *Biochem. Pharmacol.* **1994**, *47*, 2279−2287.

(9) Averbuch, S. D.; Gaudiano, G.; Koch, T. H.; Bachur, N. R. Radical Dimer Rescue of Toxicity and Improved Therapeutic Index of Adriamycin in Tumor-bearing Mice. *Cancer Res.* **1985**, *45*, 6200−6204.

(10) Wang, A. H.-J.; Gao, Y.-G.; Liaw, Y.-C.; Li, Y.-k. Formaldehyde Cross-Links Daunorubicin and DNA Efficiently: HPLC and X-ray Diffraction Studies. *Biochemistry* **1991**, *30*, 3812−3815. Wang, J. Y.-T.; Chao, M.; Wang, A. H.-J. Adducts of DNA and Anthracycline Antibiotics: Structures, Interactions, and Activities. In *Anthracycline Antibiotics*; Priebe, W., Ed.; ACS Symposium Series 574; American Chemical Society: Washington, DC, 1995; pp 168−182.

(11) Leng, F.; Savkur, R.; Fokt, I.; Przewloka, T.; Priebe, W.; Chaires, J. B. Base Specific and Regioselective Chemical Cross-Linking of Daunorubicin to DNA. *J. Am. Chem. Soc.* **1996**, *118*, 4731−4738.

(12) Fiallo, M.; Laigle, A.; Borrel, M. N.; Garnier-Sullerot, A. Accumulation of Degradation Products of Doxorubicin and Pirarubicin Formed in Cell Culture Medium Within Sensitive and Resistant Cells. *Biochem. Pharmacol.* **1993**, *45*, 659−665. Tong, G.; Lee, W. W.; Black, D. R.; Garnier-Sullerot, A.; Amirand, C.; Ballini, J.-C.; Chinsky, L.; Duquesne, M.; Jolles, B.; Sureau, F.; Turpin, P. Y.; Vigny, P. Interaction of Iron-Anthracycline Complexes with Living Cells: a Microspectrofluorometric Study. *Biochem. Biophys. Acta* **1993**, *1177*, 236−244. Henry, D. W. Adriamycin Analogs. Periodate Oxidation of Adriamycin. *J. Med. Chem.* **1976**, *19*, 395−398.

(13) Nash, T. The Colorimetric Estimation of Formaldehyde by Means of the Hantzsch Reaction. *Biochem. J.* **1953**, *55*, 416−421.

(14) Shirashi, H.; Kataoka, M.; Morta, Y.; Umemoto, J. Interaction of Hydroxyl Radicals with Tris (Hydroxymethyl) Aminomethane and Good's Buffers Containing Hydroxymethyl of Hydroxyethyl Residues Produce Formaldehyde. *Free Rad. Res. Commun.* **1993**, *19*, 315−321.

(15) Thorndike, J.; Beck, W. S. Production of Formaldehyde from N5-methyltetrahydrofolate by Normal and Leukemic Leukocytes. *Cancer Res.* **1977**, *37*, 1125−1132.

(16) Ebeler, S. E.; Zulim, R. A.; Hinrichs, S. H.; Clifford, A. J.; Shibamoto, T. Tumor-bearing Transgenic Mice and Cancer Patients Have Elevated Levels of Expired Formaldehyde. *FASEB J.* **1993**, *7*, A716. Ebeler, S. E.; Hinrichs, S. H.; Clifford, A.; Shibamoto, T. Analysis of Reactive Carbonyls in the Expired Air of Transgenic Mice. *Anal. Biochem.* **1992**, *205*, 183−186.

(17) Bagchi, D.; Bagchi, M.; Hassoun, E. A.; Kelly, J.; Stohs, S. J. Adriamycin-induced Hepatic and Myocardial Lipid Peroxidation and DNA Damage and Enhanced Excretion of Urinary Lipid Metabolites in Rats. *Toxicology* **1995**, *95*, 1−9.

JM960519Z