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THE MECHANISM OF THE NEOCARZINOSTATIN-INDUCED CLEAVAGE OF DNA [1]

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The single strand scission of DNA by neocarzinostatin (NCS) requires oxygen and is inhibited by free radical and thiol group trapping agents. Photoreduction of the two disulfide links in NCS in the presence of sodium hypophosphite affords the sulfhydryl group, the oxidation of which is responsible for nicking the DNA. The latter process and consequently the nicking of DNA by NCS is catalyzed by Cu(II) ion. Cleavage of DNA by NCS is prevented by selective oxidation with performic acid of the two disulfide links, the removal of which is monitored by a fluorescein mercuric acetate assay. In contrast to bleomycin, NCS is not activated by traces of Fe(II) ion and there is no evidence for strong binding of NCS to DNA.

INTRODUCTION

Neocarzinostatin (NCS 157365) an acidic protein antibiotic produced by streptomyces carzinostaticus variant F-41 exhibits a high chemotherapeutic index against murine leukemia SN-36 (1,2), leukemia L-1210 (3,4) and human lymphoblastic leukemia cells (5) and is currently in Phase II clinical trials (6). The primary effect of NCS is arrest of mitosis (7) and the principal cell target is DNA. Studies on the mode of action (8) indicate NCS inhibits DNA synthesis and also causes degradation of existing DNA (8,9). DNA cleavage can be reproduced in vitro in the presence of 2-mE (10) although in view of the reported nicking of DNA by thiols this required reinvestigation (11). Hitherto no nicking of DNA by NCS has been reported in the absence of thiol even at a drug concentration of 100 μg/ml (10). The mechanism whereby NCS cleaves DNA and the role of the reductant remained to be clarified (12). NCS resembles bleomycin (13) and phleomycin (14)

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Abbreviations: CCC, covalently closed circular, OC, open circular. FMA, fluorescein mercuric acetate, NCS, neocarzinostatin, 2mE, 2-mercaptoethanol.

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in its ability to nick DNA and in its requirement for a reducing agent. While the nicking of DNA by bleomycin is greatly enhanced by traces of Fe(II) (15) there have been no reports of the effects of metal ions on the NCS induced scission of DNA. We recently reported the application of ethidium fluorescence assays in conjunction with PM2-CCC-DNA for sensitive detection of DNA strand scission by several antitumor antibiotics (15-18). The further application of these techniques to provide new evidence on the chemical mechanism of NCS-induced DNA scission and the role of certain reducing agents and metal ions is described.

METHODS AND MATERIALS

Highly purified NCS was a generous gift of Dr. T. S. A. Samy of the Sidney Farber Cancer Institute, Harvard Medical School. ℓ-Cysteine hydrochloride and 2-mE were purchased from Baker Chem. Co., $CuSO_4$, sodium benzoate and NaH_2PO_2 were from Fisher Scientific Co., N-methylmaleimide was purchased from Aldrich and glutathione was obtained from Matheson, Coleman and Bell Co. FMA was prepared by a literature procedure (19). Ethidium bromide and disodium EDTA were purchased from Sigma and PM2-CCC-DNA (80% CCC) was prepared as described before (20).

Ethidium Fluorescence Assay for Nicking of DNA

The fluorometric methods of measuring strand breakage of PM2-CCC-DNA and its inhibition by free radical scavengers have been described (16,20). The conversion of PM2-CCC-DNA to PM2-OC-DNA results in a 30% increase in fluorescence in the pH 11.8 ethidium assay solution (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA and 0.5 μg/ml of ethidium bromide) and 100% loss of fluorescence after a heating and cooling cycle (96°/4 min and then 23° for 5 min) since the strands are now separable. The reactions were performed at 37° in a volume of 250 μl containing potassium phosphate pH 7.2; 0.36 - 0.18 A_{260} units of PM2-CCC-DNA (80% CCC) and other components as indicated in the legends to the Figures.

Reaction of NCS with PM2-DNA After Removal of O_2

A solution containing 245 μl of a mixture of 40 mM phosphate buffer pH 7.2 and 0.18 A_{260} units/ml of PM2 was placed in a 10 ml two necked pear shaped flask. On the wall of the flask separated from the main bulk of solution was carefully placed 5 μl of 7 mg/ml NCS solution. The contents of the flask were then degassed by successive freezing, evacuation and thawing. The solutions were then mixed and heated in a constant temperature bath at 37° for 2 hr, the flask filled with nitrogen and aliquots of solution withdrawn for ethidium bromide assay.

Determination of Disulfide Groups

The method developed by Karush, Klinman and Marks using the fluorescence quenching of FMA by disulfide group was employed (19). The appropriate volume of sample and a 10^{-6} M solution of FMA in 0.01 N sodium hydroxide were mixed and brought to a final volume of 5 ml in 1 N sodium hydroxide. Under high pH conditions the disulfide bridges are hydrolyzed to thiol anion which quench the

fluorescence of FMA. After incubation for 15-60 min the fluorescence of the control and sample solution were recorded using a Turner 430 fluorometer with excitation wavelength 500 nm and emission wavelength 600 nm.

Photochemical Reduction of NCS and Cystine

A solution containing 0.025 N HCl, 0.025 N NaH_2PO_2 and 0.35 mg/ml NCS (or 10^{-4} M cystine) was placed in a 1 mm quartz cell and irradiated with a 140 watt Hanovia utility lamp at 10 cm distance for 3 hr at room temperature (21). The amount of -SH group present in the solution was estimated from the fluorescence quenching of 10^{-7} M FMA at pH 7.5 (19). Under pH 7.5 conditions disulfide bridges are not hydrolyzed and only free thiol groups quench the fluorescence of FMA.

Oxidative Cleavage of Disulfide Group in NCS with Performic Acid

To a solution of 0.4 mg of NCS in 100 µl of cold 98% formic acid was added 100 µl of cold 30% hydrogen peroxide and the mixture was kept in ice for 3 hrs (24). The reagent was removed under vacuum, the residue dissolved in 500 µl of water, and the solution dialyzed at 4° for 47 hrs to remove residual oxidant. Dialysis of a control solution of NCS (0.7 mg/ml) for the same period of time did not detectably change its activity.

RESULTS AND DISCUSSION

1.4×10^{-5} M NCS in deionized water cleaves 70% of PM2-CCC-DNA in 120 min at 37° and requires oxygen (Figure 1). The rate of scission decreases substantially when oxygen is removed and increases in the presence of oxidants like H_2O_2. The nicking is strongly inhibited by free radical scavengers (sodium benzoate) or -SH traps, e.g. N-methylmaleimide. All of these observations suggest the cleavage is due to free thiol groups in NCS and resemble closely the cysteine induced scission of DNA (Figure 2) (11). Other thiol reducing agents e.g. 2-mE and dithiothreitol which have been used in conjunction with NCS behave in a similar manner (21,22).

Addition of 1×10^{-4} M $CuSO_4$ considerably increases the rate of nicking of DNA by both NCS and cysteine (Figures 1 and 2) which is ascribed to the known catalytic effect of the Cu(II) on the oxidation of thiols (23). Unlike bleomycin, (15) low concentrations of Fe(II) ion (2×10^{-6} M) have no detectable effect on the rate and extent of NCS-induced DNA scission. Thus, although NCS resembles bleomycin in its ability to nick DNA alone (15) and in the enhancement by a reducing agent, it does not sequester traces of Fe(II) nor require this ion for its activity.

Single strand scission of SV-40 DNA by NCS in the presence of 2-mE has been reported by Beerman and Goldberg (12) who did not observe DNA nicking by NCS

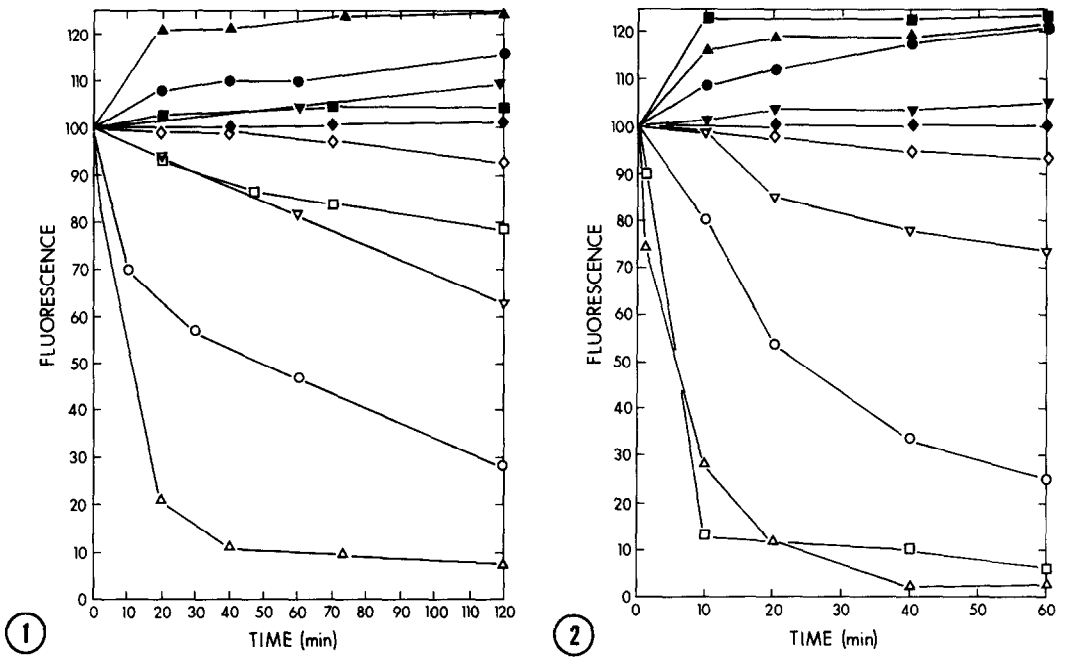
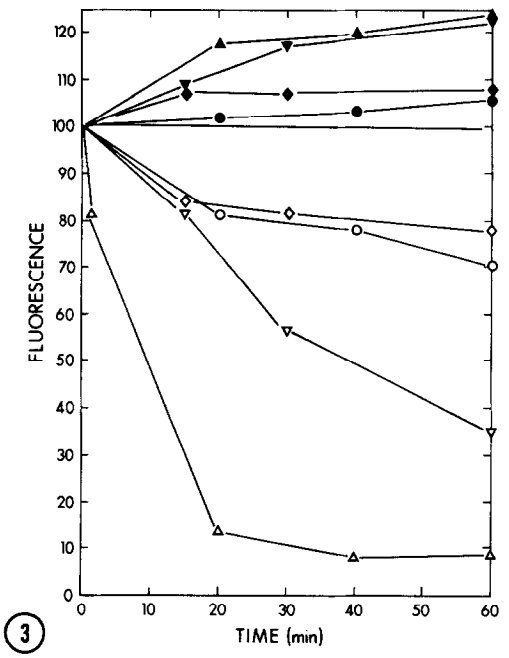


FIGURE 1. Single strand scission of PM2-CCC-DNA by NCS in the presence of oxidants. Reactions were performed in deionized water at 37° in 0.05 M potassium phosphate buffer, pH 7.2, and contained 0.36 A_{260} units/ml of PM2-CCC-DNA (80% CCC) and 1.4 x 10^{-5} M NCS. The before heat fluorescence reading are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96°, and rapid cooling. Additional components were (○) none (△) 2 x 10^{-4} M H_2O_2 (▽) NCS under oxygen free conditions, (◇) 40 mM sodium benzoate (□) 9 x 10^{-2} M N-methyl-maleimide.

FIGURE 2. Single strand scission of PM2-CCC-DNA by cysteine. Reactions were performed using conditions described in the legend for Figure 1 with the additional components (○) 4 mM ℓ-cysteine (△) 4 mM ℓ-cysteine and 10^{-4} M H_2O_2 (□) 4 mM ℓ-cysteine and 10^{-4} $CuSO_2$ (▽) 4 mM ℓ-cysteine, 10^{-4} M H_2O_2 and 0.02 M sodium benzoate (◇) 4 mM ℓ-cysteine preincubated with 0.02 M N-methylmaleimide at 37° for 3 hr.

alone up to a concentration of 100 µg/ml. We confirmed that 10^{-3} M 2mE as well as 5 x 10^{-4} M glutathione and 1 x 10^{-4} M $NaBH_4$ (24) (not shown) also enhance the rate of DNA cleavage by NCS (Figure 3).

Thiols are commonly used to maintain the -SH group in the reduced form in sulfhydryl enzymes and coenzyme A for example (25). Thus it is likely that the catalytic effect of 2-mE, dithiothreitol and glutathione is due to their gen-



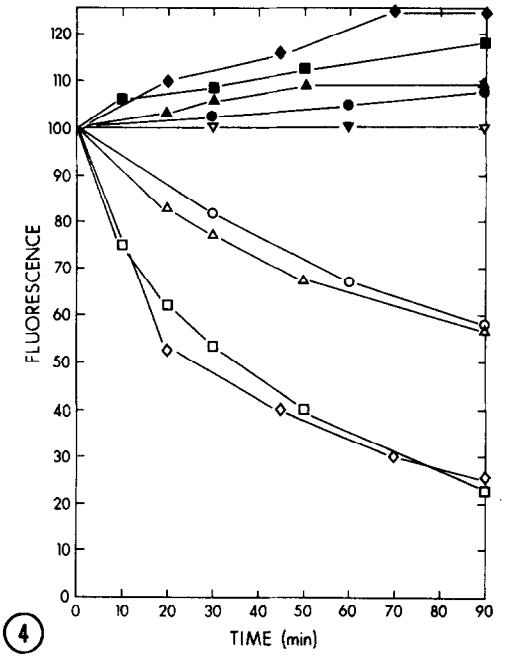


FIGURE 3. Single strand scission of PM2-CCC-DNA by reduced NCS. Reactions were performed using the conditions described in the legend for Figure 1 with 0.36 A_{260} units of PM2-CCC-DNA and 7 x 10^{-6} NCS. Additional components were (○) none (◇) 25 mM HCl, 25 mM NaH_2PO_2 and 10^{-4} M $CuSO_4$ (▽) 10^{-4} M $CuSO_4$ and a photolyzed solution of the NCS, 25 mM HCl and 25 mM NaH_2PO_2. (△) 5 x 10^{-4}M glutathione.

FIGURE 4. Single strand scission of PM2-CCC-DNA by performic acid treated NCS in the presence and absence of reducing agents. Reactions were performed using conditions described in the legend for Figure 1 with 0.36 A_{260} units/ml of PM2-CCC-DNA and 1.4 x 10^{-5}M NCS and the following additional components (○) none (□) 1 mM 2-mE and substituting 1.4 x 10^{-6} M NCS (▽) substituting 1.4 x 10^{-5} M performic acid treated NCS (△) 1.4 x 10^{-5} M performic acid treated NCS and 1 mM 2-mE (◇) 10^{-4} M $CuSO_4$.

eration of free -SH groups in NCS which now appear to be responsible for the DNA scission.

To substantiate this conclusion and to avoid competitive DNA cleavage by added thiols, a non-interfering photochemical reduction of the disulfide linkages in NCS was employed (21). Photolysing a 10^{-2} M solution of cystine in 0.05 M HCl with 0.05 M NaH_2PO_2 with a 140 watt Hanovia lamp for 3 hr gave cysteine quantitatively. Similarly irradiation of 7 x 10^{-6} M NCS under the same conditions gave a sample which cleaved PM2-CCC-DNA at a much faster rate than

control NCS (Figure 3). That this was due to the production of free -SH groups upon irradiation of NCS was confirmed by an assay for -SH which depends on the fluorescence quenching of FMA at pH 7.5 developed by Karush and co-workers (19).

In addition NCS was treated with performic acid (which oxidizes the disulfide links (24) to $-SO_3H$ groups) then dialyzed to remove excess oxidant and analyzed for residual disulfide by the FMA method (19). Less than 5% of the original S-S links remain and correspondingly the ability to nick DNA with or without reducing agent is greatly reduced (Figure 4). Although it is recognized that treatment of NCS by HCO_3H could also effect the oxidation of other groups besides the S-S links [e.g. tryptophan residues (24)] the strong correlation between the disulfide links, their selective reduction and consequent cleavage of DNA is evident. Activated NCS nicks comparable amounts of DNA at a much lower concentration (~1×10^{-5} M) than cysteine or similar thiols (~4 mM) (15). There are two possible explanations (a) the -SH group in reduced NCS is more readily oxidized and can generate $O_2^{\cdot-}$ and $OH^{\cdot}$ radicals more rapidly and (b) NCS may be bound to the DNA and as in the case of bleomycin (15) and daunorubicin (27) generate $OH^{\cdot}$ radicals close to the target. At present the first explanation seems more likely since we found no evidence that NCS binds to DNA. Since a correlation has been established between the extent of strand scission of DNA and the inhibition of DNA synthesis by DNA polymerase 1 by NCS (26), the present studies should contribute to the understanding of the mode of action of this valuable antineoplastic agent.

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REFERENCES

1. Ishida, N., Miyazaki, K., Kumagai, K. and Rikimaru, M., (1965), J. Antibiot. (Tokyo) Ser. A. 18, 68-76.

2. Kumagai, K. and Miyazaki, K. (1965), J. Antibiot. (Tokyo) Ser. A. 16, 55.
3. Bradner, W. T. and Hutchison, D. J. (1966), Cancer Chemotherapy Rept. 50, 79-84.
4. Goldin, A., Serpick, A. A. and Mantel, N. (1966), Cancer Chemotherapy Rept. 50, 173-218.
5. Lazarus H., Raso, V. and Samy, T. S. A. (1977), Cancer Res., 37, 3731-3736.
6. D.C.T. Newsletter (N.I.H.) (1977) 3, 2.
7. Kumagai, K., Ono, Y., Nishikawa, T. and Ishida, N. (1966), J. Antibiot., 19, 69-74.
8. Ono, Y., Watanabe, Y. and Ishida, N. (1966), Biochim. Biophys. Acta, 119, 46-58.
9. Ono, Y., Ito, Y., Maeda, H. and Ishida, N. (1968), Biochim. Biophys. Acta 155, 616-618.
10. Kappen, L. S. Goldberg, I. H. (1977), Biochemistry, 16, 479-485.
11. Rosenkranz, H. S., and Rosenkranz, S. (1971) Arch. Biochem. Bi ophys. 146, 483-487.
12. Beerman, T. A. and Goldberg I. H. (1974), Biochem. Biophys. Res. Commun., 59, 1254-1261.
13. Suzuki, H., Nagai, K., Akutsu, E., Yamaki, H., Tanaka, N. and Umezawa, H. (1970), J. Antibiot. 23, 473-480.
14. Stern, R., Rose, J. A., and Freidman, R. M. (1974), Biochemistry 13, 307-312.
15. Lown, J. W., and Sim, S. K. (1977), Biochem. Biophys. Res. Commun. 77, 1150-1157.
16. Cone, R., Hasan, S. K., Lown, J. W. and Morgan, A. R. (1976), Can. J. Biochem., 54, 110-119.
17. Lown, J. W., and Sim, S. K. (1976), Can. J. Biochem., 54, 441-452.
18. Lown, J. W., Sim, S. K., Majumdar, K. C. and Chang, R. Y. (1977), Biochem. Biophys. Res. Commun., 76, 705-710.
19. Karush, F., Klinman, N. R., and Marks, R. (1964), Anal. Biochem., 9, 100-114.
20. Morgan, A. R. and Pulleyblank, D. E., (1974), Biochem. Biophys. Res. Commun., 61, 396-403.
21. Swanepoel, C. A. (1963). J. South. Afri. Chem. Inst. 16, 48-50 (Chem. Abst. 60, 5635).
22. Bode, V. C. (1967), J. Mol. Biol., 26, 125-129.
23. Robert, B. (1954), Bull Soc. Chim. Biol. 36, 253-269.
24. Meienhofer, J., Maeda, H., Glaser, C. B. and Czombos, J. (1970) Progress in Peptide Res. S. Lande Ed. pp. 295-306.
25. Cleland, W. W. (1964), Biochemistry 3, 380-482.
26. Poon, R., Beerman, T. A., and Goldberg, I. H. (1977), Biochemistry 16, 486-493.
27. Lown, J. W., Sim, S. K. Majumdar, K. D. and Chang, R. Y. (1977), Biochem. Biophys. Res. Commun., 76, 705-710 (see Figure 2).