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Neocarzinostatin chromophore-DNA adducts: evidence for a covalent linkage to the oxidized C-5' of deoxyribose

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ABSTRACT

The nonprotein chromophore of neocarzinostatin forms a variety of adducts with DNA. The predominant adduct recovered from nuclease digests of chromophore-treated poly(dA-dT)·poly(dA-dT) is a compound with structure chromophore-d(TpApT). Mild acid hydrolysis of this compound released free adenine, while snake venom exonuclease (pH 6.5) released 5'-dTMP leaving in both cases adducts of slightly altered chromatographic mobility. These results eliminate adenine and 5'-dTMP as possible sites of covalent chromophore attachment. Electrophoresis data suggest that the adduct is not a phosphotriester. At pH 8.6, chromophore-d(TpApT) spontaneously hydrolyzed, releasing chromophore and 3'-dTMP, leaving a modified d(ApT) which contained deoxyadenosine-5'-aldehyde. Deoxyadenosine-5'-aldehyde was released from the modified d(ApT) by snake venom exonuclease, and identified by a series of derivatizations including 1) mild oxidation to deoxyadenosine-5'-carboxylic acid, 2) $NaBH_4$ reduction to deoxyadenosine, and 3) formation of a hydrazone with phenylhydrazine. Since deoxyadenosine-5'-aldehyde cannot exist as such in the chromophore-d(TpApT) adduct, we suggest that the chromophore may be covalently attached to the C-5' of deoxyadenosine as a phosphorylacetal or similar structure. Hydrolysis of the chromophore-acetal bond at pH 8.6 would leave a phosphorylhemiacetal on C-5', which would be expected to spontaneously decompose to yield the observed 3'-phosphate and 5'-aldehyde groups.

INTRODUCTION

Neocarzinostatin (NCS) is a DNA-degrading antitumor antibiotic consisting of a protein plus a noncovalently bound nonprotein chromophore (1); a partial structure of the nonprotein chromophore has been reported (2). Recently we showed that the isolated nonprotein chromophore, in addition to causing DNA strand breaks with both 5'-aldehyde and 5'-phosphate termini, formed novel covalent adducts with DNA (1,3-5). In this report, we present a more detailed characterization of the predominant adduct formed by the chromophore with the synthetic polymer poly(dA-dT)·poly(dA-dT).

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MATERIALS AND METHODS

Adduct Preparation

Preparation and purification of chromophore-poly(dA-dT)·poly(dA-dT) adducts have been described (5). Briefly, [^{3}H]adenine- or [^{3}H]thymine-labeled poly(dA-dT)·poly(dA-dT) was incubated with the isolated nonprotein chromophore of neocarzinostatin in the presence of 2-mercaptoethanol. The poly(dA-dT)·poly(dA-dT) was precipitated with ethanol and digested at pH 5 to the level of single nucleosides with DNAse II, endonuclease S_1 and phosphatase. Reverse-phase high-pressure liquid chromatography (HPLC) separated various adduct species from the unaltered nucleosides.

High-Pressure Liquid Chromatography

Samples (0.5-1.5 ml) were adjusted to pH 5 with sodium acetate or acetic acid, loaded onto a Waters μBondapack C_{18} or an Altex Ultrasphere ODS column and eluted at 1 ml/min with a linear or convex gradient of increasing methanol in water containing 5 mM ammonium acetate and 5 mM acetic acid.

Borohydride Reduction

Compounds to be reduced were treated with $NaBH_4$ for 40 min at 22°C in 0.5 M sodium phosphate pH 6.5. $NaBH_4$ was added every 10 min in four equal portions totalling 150 mM.

Phenylhydrazine Treatment

Compounds were incubated at 37°C for 45 min in the presence of 10 mM phenylhydrazine and 0.05 M sodium acetate-0.1 M acetic acid, pH 4.5 (3).

Electrophoresis

Paper electrophoresis was performed on Whatman 3 MM strips at 20 V/cm and 0°C. Buffers were i) 0.1 M ammonium carbonate (pH 8.9) or ii) 0.05 M sodium acetate-0.05 M acetic acid (pH 4.9) in 7:3 water-ethanol.

Hypoiodite Oxidation

The reaction mixture (0.1 ml) contained 10 mM I_2, 10 mM KI and 50 mM sodium carbonate, pH 9 (6). After a 30 min incubation at 22°C, 4.8 μl of 1 M HCl and 10 μl of 0.5 M sodium thiosulphate were added to reduce residual I_2.

Preparation of Authentic Deoxyadenosine-5'-carboxylic acid

Platinum-catalyzed oxidation of several nucleosides to 5'-carboxylic acids has been described (7). Similar treatment of deoxyadenosine gave three ultraviolet-absorbing, HPLC-separable products in significant (>5%) yield, only one of which was negatively charged at pH 8.9 as judged by paper electrophoresis. This compound was repurified by HPLC in 10 mM acetic acid and subjected to high-resolution mass spectroscopy. The neat compound gave

a molecular ion at m/e = 265.0812 (calc. for $C_{10}H_{11}N_5O_4$ 265.0811) confirming its identity as deoxyadenosine-5'-carboxylic acid (dAdo-5'-COOH). It formed the expected tri(trimethylsilane) derivative (m/e = 481).

RESULTS

Identification of Deoxyadenosine-5'-Aldehyde

As we reported previously (5), the adduct recovered from combined DNAse II and endonuclease S_1 digests as chromophore-d(TpApT) spontaneously hydrolyzes at pH 7.0 or pH 8.6, releasing simultaneously chromophore and 3'-dTMP, leaving a modified d(ApT) which can be cleaved by snake venom exonuclease to yield 5'-dTMP plus a modified deoxyadenosine. Identification of thymidine-5'-aldehyde at the 5'-end of NCS-induced strand breaks (3) led us to suspect that the modified deoxyadenosine derived from the adduct might be deoxyadenosine-5'-aldehyde, and we have found that the modified deoxyadenosine exhibits chemical behavior expected for a 5'-aldehyde, and nearly identical to that of thymidine-5'-aldehyde.

The modified deoxyadenosine was completely hydrolyzed by mild alkali (6 hr at pH 12 and 37°C), releasing all the adenine label as the free base, presumably by β-elimination. Reduction of the modified deoxyadenosine with $NaBH_4$ produced an alkali-stable compound which was indistinguishable from authentic deoxyadenosine (but distinguishable from the parent compound) in three chromatography systems: i) HPLC, ii) paper chromatography in ethanol-aqueous 1 M sodium acetate (7:3) and iii) thin-layer silica gel chromatography in n-propanol-water (4:1) (data not shown). These results suggest an oxidized deoxyadenosine sugar.

The modified deoxyadenosine (but not its $NaBH_4$-reduced derivative) reacted with phenylhydrazine to form a much more hydrophobic derivative, presumably the phenylhydrazone addition product (Fig. 1). This result indicates that the $NaBH_4$-reducible group is either an aldehyde or a ketone.

Mild oxidation of the modified deoxyadenosine by potassium hypoiodite produced a negatively-charged compound indistinguishable from deoxyadenosine-5'-carboxylic acid by both HPLC and paper electrophoresis (Fig. 2). This result strongly suggests that the modified deoxyadenosine is deoxyadenosine-5'-aldehyde. Although it is possible that another carboxylic acid derivative of deoxyadenosine might cochromatograph with the 5'-carboxylic acid, only the C-5' position of deoxyadenosine can be oxidized to an aldehyde while retaining all carbons from the sugar, as is implied by the $NaBH_4$ reduction experiments.

Like the modified deoxyadenosine, the modified d(ApT) which was derived

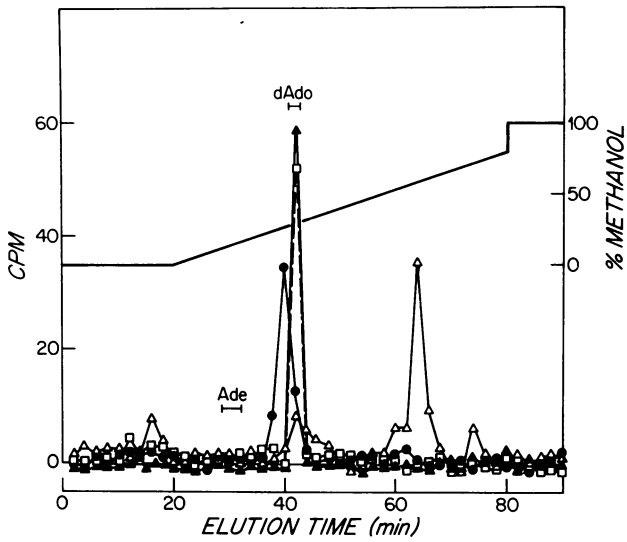

Figure 1. Phenylhydrazine derivatization. The modified deoxyadenosine released from chromophore-d(TpApT) by pH 8.6 hydrolysis plus snake venom exonuclease, was incubated in the presence (Δ) or absence (●) of phenylhydrazine and subjected to HPLC on the Waters column. Another aliquot of the modified adenosine was reduced with $NaBH_4$, reisolated by HPLC, and incubated in the presence (□) or absence (▲) of phenylhydrazine.

from hydrolysis of chromophore-d(TpApT) decomposed in alkali (releasing adenine plus 5'-dTMP), formed a hydrazone with phenylhydrazine, and was reduced by $NaBH_4$ to an alkali-stable compound indistinguishable from authentic d(ApT) (not shown). These results imply that the modified d(ApT) also contains a carbonyl group, presumably the 5'-aldehyde of the deoxyadenosine moiety. Since the intact chromophore-d(TpApT) adduct retains an intact phosphodiester at the deoxyadenosine C-5' position, it cannot also contain deoxyadenosine-5'-aldehyde; rather, the 5'-aldehyde must be formed by its decomposition. Consistent with this proposal, intact chromophore-d(TpApT) showed no detectable change in chromatographic properties after treatment with either phenylhydrazine or $NaBH_4$, and still decomposed in alkali (releasing 3'-dTMP, 5'-dTMP and adenine) following $NaBH_4$ treatment.

Removal of Adenine and 5'-dTMP from Chromophore-d(TpApT)

As discussed below, formation of an aldehyde at the deoxyadenosine C-5' position upon decomposition of chromophore-d(TpApT) implicates this C-5' as a likely site of covalent chromophore attachment. To test this hypothesis, we sought to eliminate other moieties as possible chromophore attachment

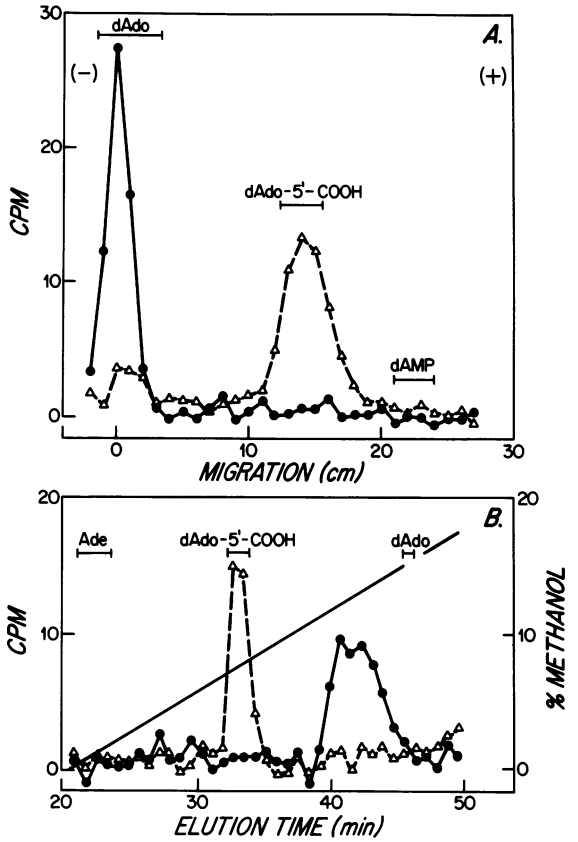

Figure 2. Hypoiodite oxidation. The modified deoxyadenosine was incubated in the presence (Δ) or absence (●) of potassium hypoiodite and subjected to paper electrophoresis (A) (8 hr, buffer i), or HPLC (B) on the Waters column.

sites.

Chromophore-d(TpApT) is hydrolyzed completely by a 16 hr incubation in 1 N HCl at 37°C releasing adenine, 3'-dTMP and 5'-dTMP (5). A variety of milder acid treatments induced partial hydrolysis, and adenine label was consistently lost from the adduct at a faster rate than thymine label, suggesting that the hydrolysis proceeded via a depurinated intermediate. This proposal was confirmed by subjecting such a partial acid hydrolyzate to HPLC on the Altex column with a convex methanol gradient. Under these conditions, a new adduct peak, presumably chromophore-d(Tp-ribose-pT), was resolved, which contained only thymine label and eluted just ahead of chromophore-d(TpApT) (Fig. 3A). As expected, pH 12 hydrolysis of this depurinated compound released thymine label as a one-to-one mixture of 3'-dTMP and 5'-

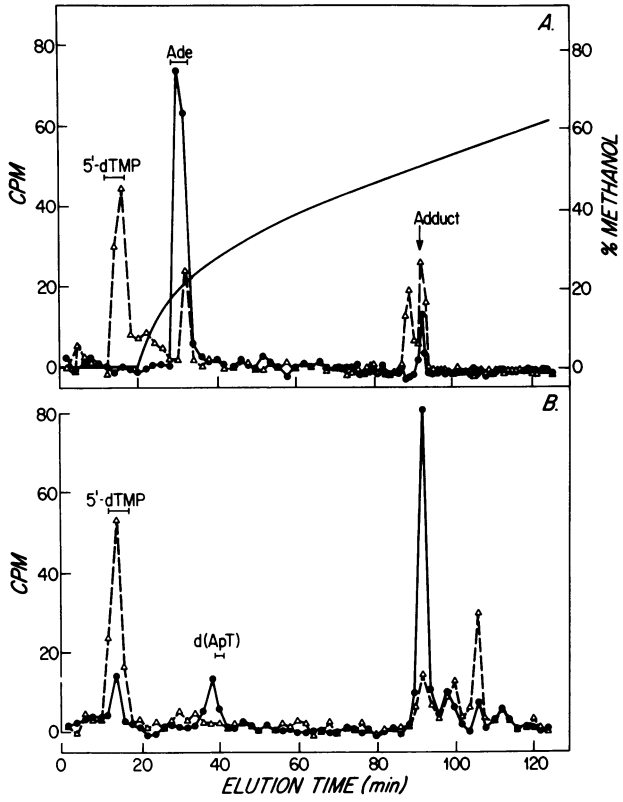

Figure 3. Partial degradation of the adduct. A. Thymine-labeled (Δ) or adenine-labeled (●) chromophore-d(TpApT) was incubated for 40 hr in 0.5 N HCl at 37°C and subjected to HPLC on the Altex column. Under these conditions untreated chromophore-d(TpApT) eluted as a single peak at 92 min (arrow). The thymine-labeled peak at 88 min is the new adduct peak, presumably chromophore-d(Tp-ribose-pT). The thymine-labeled peak at 32 min may be a depurinated form of the modified d(ApT). B. Thymine-labeled chromophore-d(TpApT) was incubated in 0.1 M Tris-malonate, pH 6.5 for 5 hr at 37°C in the presence (Δ) or absence (●) of snake venom exonuclease (25 μg/ml, Sigma type VII), and subjected to HPLC on the Altex column. In the control, (●) the peak at 14 min is 3'-dTMP, and the peak at 38 min is the modified d(ApT). The increase in dTMP in the enzyme-treated sample is attributable to 5'-dTMP released by the enzyme from both the modified d(ApT) and chromophore d(TpApT) (which elutes at 92 min). The peak at 106 min is the new adduct peak, presumably chromophore-d(TpA).

dTMP (separated as in ref. (5), not shown). The existence of this depurinated intermediate indicates that adenine cannot be the site of covalent chromophore attachment.

When thymine-labeled chromophore-d(TpApT) was incubated at pH 6.5, most

of the thymine label still eluted at the position characteristic of chromophore-d(TpApT). Only a small fraction was hydrolyzed to a one-to-one mixture of 3'-dTMP and modified d(ApT). However, when excess snake venom exonuclease was added to the incubation mixture, nearly all the chromophore-d(TpApT) was hydrolyzed, and, in addition to dTMP, a new thymine-labeled compound, presumably chromophore-d(TpA), was formed which eluted even later than chromophore-d(TpApT) (Fig. 3B). This result implies that the site of covalent chromophore attachement cannot be on the 5'-dTMP moiety, since 5'-dTMP can be removed while other moieties remain attached to the chromophore.

Electrophoresis of the Adduct

To test the possibility of a phosphotriester adduct, thymine-labeled chromophore-d(TpApT) and chromophore-d(TpA) were subjected to paper electrophoresis (Fig. 4). In a pH 5 buffer containing 30% ethanol, chromophore-d(TpApT) migrated slowly toward the anode, indicating a net negative charge. It is unlikely that this migration was due to solvent electroosmosis, since a neutral marker (thymidine) moved slightly toward the cathode under these conditions and chromophore-d(TpA) was nearly stationary. Since the chromophore is expected to bear a positive charge due to the aminosugar moiety (2), the net negative charge on chromophore-d(TpApT), and the apparent neutrality of chromophore-d(TpA) suggest that both phosphates remain as negatively-charged diesters, and that the adduct is not a phosphotriester. However, in the absence of a more complete understanding of the chromophore chemistry, we cannot exclude the possibility that oxidation-reduction reactions may have generated a negative charge on the chromophore itself (giving it a net zero charge) or that the chromophore may have lost its positively charged sugar moiety.

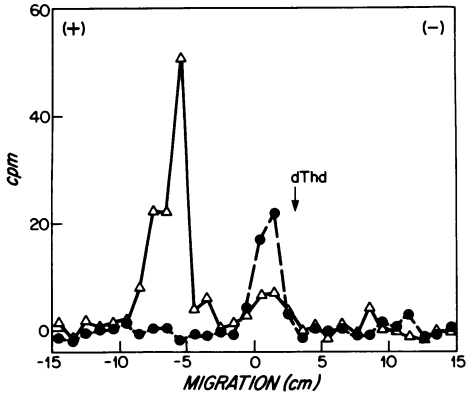

Figure 4. Electrophoresis of the adduct. [^{3}H]thymine-labeled chromophore-d(TpApT) (Δ) or chromophore-d(TpA) (●) (prepared as described in Fig. 3), were subjected to paper electrophoresis for 35 hr in buffer ii. Similar results were obtained with [^{3}H]adenine-labeled and [^{3}H]chromophore-labeled chromophore-d(TpApT).

DISCUSSION

When NCS chromophore reacts with poly(dA-dT)·poly(dA-dT), only 2% of the chromophore becomes covalently attached to DNA and no single adduct species accounts for more than 0.2% of the initial added chromophore or more than 0.04% of the total DNA nucleotides (5). These factors have prevented the preparation of a sufficient quantity of any single adduct for direct chemical analysis. However, by various hydrolysis experiments and derivatizations, we have identified an endonuclease S_1-resistant compound with structure chromophore-d(TpApT) which decomposes at neutral pH, releasing simultaneously three products: chromophore, 3'-dTMP and a modified d(ApT) which contains deoxyadenosine-5'-aldehyde. Based primarily on these decomposition products, we suggest that the most likely linkage of chromophore to DNA is at the C-5' of deoxyribose, as a 5'-phosphorylacetal or similar structure. Such an acetal could form the 5'-aldehyde by a simple decomposition. However, the stability of chromophore-d(TpApT) at pH 5 (5) suggests that the proposed acetal is not a simple hemiacetal, but is terminated by some blocking group. The covalently attached chromophore is the logical candidate for such a blocking group, especially since its release is kinetically linked to cleavage of the phosphodiester bond and generation of the 5'-aldehyde (5).

Experiments with [5'-^{3}H]thymidine-labeled DNA suggest that a fraction of neocarzinostatin-induced strand breaks involve release of the 5' carbon as formic acid (8). If the initial oxidation of C-5' were a hydrogen abstraction, the resulting free radical on C-5' could either undergo further oxidation resulting in the release of formic acid (9) or react by addition with any of several moieties on the chromophore, resulting in the proposed acetal adduct on C-5'. Since acetals are not intrinsically unstable at neutral pH, the lability of the chromophore-acetal bond at pH $\geq$ 7 must be attributed to instability of the chromophore itself. If the chromophore were linked to deoxyribose via its methylamino group (2), i.e., as an aminal rather than an acetal, this might explain the acid stability and alkaline lability of chromophore-d(TpApT). However, it is difficult to speculate on the exact chemistry of either the formation or the hydrolysis of the chromophore-deoxyribose bond, since the oxidation-reduction chemistry of the chromophore is so poorly understood.

Another candidate for the proposed acetal blocking group would be the 2-mercaptoethanol used for chromophore activation (as a thioacetal). While we have not rigorously eliminated this possibility, we have found that a similar chromophore-d(TpApT) adduct is formed when a non-thiol reducing agent, $NaBH_4$,

is used for activation (Zhang Chengbo, unpublished data).

Although dimethylbenzanthracene forms an adduct at the 2'-OH of ribose in RNA (10), formation of covalent adducts with deoxyribose in DNA, by any agent, has not been previously reported. Based on work with other adduct-forming compounds (11), more likely sites of adduct formation in d(TpApT) would be either the adenine base, or one of the phosphates (as a phosphotriester). Therefore, we have sought to verify the proposed chromophore-deoxyribose linkage by eliminating these moieties as possible chromophore attachment sites in chromophore-d(TpApT). This strategy has been successful in two cases: formation of chromophore-d(Tp-ribose-pT) excludes an adenine base adduct, and formation of chromophore-d(TpA) excludes the 5'-dTMP moiety (and therefore its phosphate) as an adduct site. However, to exclude a phosphotriester on the other phosphate, there is only indirect evidence, namely the net negative charge on chromophore-d(TpApT) and the absence of the expected diesters (chromophore-pdApdT, dTp-chromophore, or some form of d(TpApT)) as hydrolysis products (12,13). However, it should be noted that proposing an adduct on any moiety other than the deoxyadenosine sugar, requires that one also propose a second chromophore-DNA reaction to explain oxidation of this sugar. Thus, while the chromophore-5'-phosphorylacetal adduct is not a proven chemical structure, it is clearly a reasonable proposal consistent with all the known chemical properties of chromophore-d(TpApT).

The chromophore-DNA adduct is a minor product of the reaction between chromophore and DNA. The major product is a direct DNA strand break bearing 3'-phosphate and 5'-aldehyde termini (3). The fact that chromophore-d(TpApT) decomposes at neutral pH to form a "strand break" with these same termini, raises the possibility that the adduct might be a normal intermediate in formation of the strand break. However, when chromophore-treated poly(dA-dT)·poly(dA-dT) was precipitated, redissolved, and incubated at pH 8, no decrease was seen in the amount of chromophore-d(TpApT) recovered from the nuclease digest. Thus, while isolated single-stranded chromophore-d(TpApT) is labile at neutral pH, the same structure appears to be quite stable in native double helical DNA. Therefore, if an adduct is an intermediate in strand break formation, it must be a more labile form than the one we have characterized, possibly because of tertiary structure considerations. Attempts to "trap" a larger fraction of strand breaks as the putative adduct intermediate by lowering the pH and temperature of the reaction have failed; nearly the same distribution of adducts was always seen in the nuclease digests.

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