Communications to the Editor

Isotope Effects on the Sequence-Specific Cleavage of dC in d(AGC) Sequences by Neocarzinostatin: Elucidation of Chemistry of Minor Lesions

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We recently reported a new technique for analysis of the mechanism of DNA cleavers that uses specifically deuteriated ^{32}P end-labeled DNAs in combination with gel electrophoresis to detect and quantitate potentially rate-limiting carbon–hydrogen bond cleavages at individual sequence sites.[1] The approach was successful using Fe(II)·bleomycin and [4′-^{2}H]labeled DNAs. To establish the generality of the method, especially in the analysis of the chemistry at *minor* lesions, we herein report our initial study of the cleavage of deoxycytidine residues in d(AGC) sequences by neocarzinostatin.

Neocarzinostatin (NCS) binds to double-stranded DNA via intercalation of its naphthoate moiety and interaction of its unusual [7.3.0]dodecadien-diyne epoxide with the minor groove (Figure 1).[2a] A putative diradical species of NCS, generated by nucleophilic addition of a thiol at C-12 (Figure 1), abstracts hydrogen atoms from the deoxyribose residues of DNA to produce DNA strand breaks in the presence of O_2.[2] The predominant DNA damage is direct strand breaks at thymidylate and deoxyadenylate residues resulting in thymidine (deoxyadenosine) 5′-aldehyde at the 5′-terminus and phosphate at the 3′-terminus. Recent efforts have uncovered a less prevalent lesion thought to be involved in NCS-induced mutagenesis in *E. coli*: alkali-labile abasic sites at deoxycytidylate residues in d(AGC) sequences.[3,4]

Two approaches using oligomers have been previously undertaken to understand the mechanism of formation of this minor lesion. The first utilized d(AGCGAGC*G) containing commercially available [1′,2′,5-^{3}H]dC*. Incubation with NCS resulted in release of cytosine accompanied by transfer of 0.04 equivalents of [^{3}H] into the drug, suggesting a very large isotopic discrimination (k_H/k_T = 25!) on C-1′ chemistry.[4] However, the distribution of [^{3}H] label in dC* was not rigorously established. The second approach utilized the same labeled oligomer and demonstrated that cytosine release was accompanied by production of 0.6 equiv of 2-deoxyribonolactone.[5] We noted that our recently developed technique could permit a direct observation of the putative isotope effect on 1′-carbon–hydrogen bond cleavage solidifying earlier mechanistic proposals and at the same time test the premise that our new approach might provide a powerful

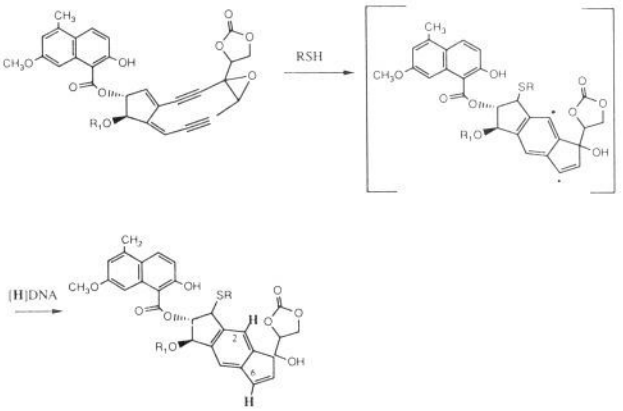

Figure 1. Proposed structure for NCS and its mechanism of activation.[2] One of the putative biradicals is postulated to be responsible for hydrogen atom abstraction of the C-1′ hydrogen of dC_4 and dC_8 from d-($GAGC_4GAGC_8$*G).

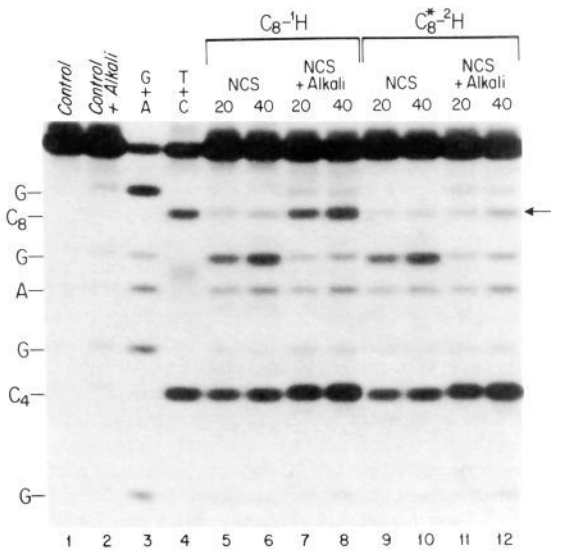

Figure 2. Isotope effects on alkali-dependent strand scissions by NCS at dC residues in d(AGC) sequences. 5′-^{32}P end-labeled d-($GAGC_4GAGC_8$*G)$_3$ annealed to d(CGCT)$_3$ was incubated with NCS (20 and 40 μM) and 5.0 mM glutathione in a standard reaction.[4] Lanes 5–8, all dCs are 1′-^{1}H, and lanes 9–12 dC_8* is 1′-^{2}H. Lanes 1 and 2 are (-drug) controls without and with alkali, respectively, for the nondeuteriated nanomer. Similar controls for the C_8*-deuteriated nanomer have been carried out (data not shown). Lanes 3 and 4 are Maxam–Gilbert markers and are shown as G + A and T + C. Lanes 5 and 9 and 6 and 10 are 20 and 40 μM NCS, respectively. Lanes 7 and 11 and 8 and 12 are 20 and 40 μM NCS + alkali.

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Table I. 5′-^{32}P-GAGC$_4$GAGC$_8$G(C_8, 1′-^{1}H) or 5′-^{32}P-GAGC$_4$GAGC*G(C_8*, 1′-^{2}H) and $(CGCT)_3$ with NCS Quantitation of Isotope Effects Using Densitometry

	C_8: 1′-^{1}H		C_8*: 1-^{2}H			
experiment	area C_4[a]	area C_8[a]	area C_4[a]	area C_8*[a]	$C_8[1'\text{-}^1H]$ / $C_8^*[1'\text{-}^1H]$	$C_4[1'\text{-}^1H]$ / $C_4[1'\text{-}^2H]$
NCS (20 μM + alkali)	2.8	1.4	2.7	0.4	3.3	1.0
NCS (40 μM + alkali)	4.1	2.0	4.0	0.5	4.2	1.0

[a] Areas under the peaks were established by using an LKB Ultroscan XL Laser densitometer. The areas reported were obtained after subtraction of a small amount of background observed in control experiments run in the absence of drug and in the presence of alkali (see for example, lane 2, Figure 2). Typical backgrounds are less than 10% of the reported areas. C_4 serves as the internal control as both oligomers contain C_4 with ^{1}H at C-1′.

method for the detection of the chemistry of minor lesions.

[1′-^{2}H]dC*TP and [1′-^{1}H]dCTP[6] were incorporated specifically into the penultimate residue of the nanomer d(GAGC$_4$GAGC$_8$ (or C_8*)G) as previously described.[4] The nanomer containing unlabeled (C_4) and (C_8) in the d(AGC) sequence was end labeled, annealed to $d(CGCT)_3$, and incubated with limiting concentrations of NCS, and the degradation products were analyzed by polyacrylamide gel electrophoresis. In Figure 2 it can be seen that there is alkali dependent cleavage at both C_8 and C_4 (compare lanes 5, 7, and 6, 8). Identical experiments were performed on the same oligomer containing deuteriated C_8 (C_8*). The direct observation of an isotope effect on NCS mediated oligomer damage is apparent on the alkali labile reaction by comparison of lanes 7 with 11 and 8 with 12. Quantitation of the isotope effect by densitometry is summarized in Table I. A k_H/k_D of 3.3 and 4.2 for 1′-C-H bond cleavage was calculated at 20 and 40 μM NCS, respectively. A similar analysis for cleavage at C_4, the internal control, gave the expected k_H/k_D of 1.0. The non-"alkali" dependent cleavage observed in lanes 5 and 6 with migration slightly faster than G_7 is presumably an intermediate d(GAG) moiety attached to a 5′-phosphorylated α,β-unsaturated lactone, that is, the precursor to C_4. Since the 1′-hydrogen of C_4 is unlabeled, there is no isotope effect on its formation (compare lanes 5, 6 with lanes 9, 10). These results provide the first direct evidence that NCS can effect removal of a 1′-hydrogen from a deoxycytidine residue in the d(AGC) sequence and demonstrate that a substantial isotope effect occurs on this reaction.

When the protio and deuterio nanomers were annealed to $d(CICT)_3$ instead of $d(CGCT)_3$, no significant isotope effect was observed (data not shown). Previous studies have shown that inosine (I), base-paired to C, enhances NCS-mediated cleavage 5-fold relative to G. If this enhancement is due to an increase in the relative rates of hydrogen abstraction by the activated drug versus dissociation from DNA of the activated drug, then the suppression of the isotope effect is explainable. A similar modulation of isotope effects was observed with BLM.[1]

These studies have recently been extended to an EcoRI-BamHI DNA fragment from pBR322 (375 bp) in which [1′-^{2}H]dC or [1′-^{1}H]dC has been incorporated.[1] Two d(AGC) sequences are present in this restriction fragment:

^{32}P–GACAGCTTATCATCGATAAGCT–

Results from densitometry scans (data not shown) indicated an alkaline labile isotope effect of 3.7 on the cleavage of dC closest to the 5′-end. No detectable damage was observed at the second d(AGC) sequence. The extent of damage at dC in the first d(AGC) sequence was less than 0.1% of the total damage. Such pathways, while difficult to investigate from a mechanistic point of view, may be the pathway of major biological significance. The direct observation of this C-1′ C–H bond cleavage thus demonstrates the power of this method in establishing the chemistry involved in minor pathways.

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Unusual Molecular Hydrogen Complex of Rhenium: A Long Hydrogen–Hydrogen Bond and Inertness to Substitution

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Since the discovery of $M(H_2)(CO)_3(PR_3)_2$ (M = Mo, W; R = *i*-Pr or Cy) by Kubas and co-workers,[1] transition-metal polyhydrido complexes have been the subject of intensive research and close scrutiny with respect to the nature of metal–hydrogen bonding,[2] e.g., M–$(H)_3$ (classical), H–M–(H_2) (nonclassical), or possibly M–(H_3). In this context, the question of the bonding in the rhenium pentahydrides ReH_5L_3 has been particularly intriguing, since initially ^{1}H NMR (T_1 values) and X-ray results pointed to different conclusions when L = PPh_3.[3] Here we report that protonation of an analogous rhenium pentahydride containing a tridentate phosphine ligand, viz., ReH_5(Cyttp) (**1**, Cyttp = $PhP[CH_2CH_2CH_2PCy_2]_2$), affords an unusual dihydrogen complex, $[Re(H_2)H_4(Cyttp)]SbF_6$ (**2**). This product features a surprisingly long H–H bond; it also exhibits a remarkable lack of reactivity in substitution reactions with D_2 and typical 2e donor ligands.

1 was prepared by the action of excess $NaBH_4$ on $ReCl_3$(Cyttp) in ethanol and characterized as a classical pentahydride by ^{1}H NMR spectroscopy (T_1) and X-ray crystallography.[4] It reacts with excess $HSbF_6$ (65% aqueous) in benzene solution to afford **2**[5-7] as an air-stable white solid in high (>88%) yield. **2** is remarkably resistant to loss of H_2; e.g., it remains intact after storage at 0.1 Torr for more than 2 days at ambient temperatures.

Variable-temperature ^{1}H NMR spectra of **2** at 250 MHz in CD_2Cl_2 solution are shown in Figure 1. At ambient temperature,

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(5) **2**: ^{31}P{^{1}H} NMR (C_6D_6): 12.49 (d), -8.06 ppm (t) (J_{PP} = 23.7 Hz). Anal. Calcd for $C_{36}H_{67}F_6P_3ReSb$: C, 42.61; H, 6.65. Found: C, 42.73; H, 6.68.

(6) Similar protonation reactions of ReH_5L_3 (L = PPh_3,[7a] PMe_2Ph[7b]) are reported to give $[ReH_6L_3]^+$; $[ReH_6(PPh_3)_3]BF_4$ was formulated as a nonclassical, η^2-H_2-containing complex from electrochemical evidence.[7c]

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