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Treatment of DNA with ammonium bicarbonate or thiourea can lead to underestimation of platinum-DNA monoadducts*

Gregory R. Gibbons, Jimmy D. Page, and Stephen G. Chaney

Department of Biochemistry and Biophysics and Curriculum in Toxicology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA

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Summary. Thiourea and NH_4HCO_3 are widely used to block the conversion of Pt-DNA monoadducts to diadducts prior to the enzymatic digestion of DNA and subsequent analysis of the relative proportion of the different types of Pt-DNA adducts. Our data show that NH_4HCO_3 (100 mM, 18 h, 25° C) is much less effective than thiourea (10 mM, 10 min, 25° C) at blocking monoadducts, apparently because considerable monoadduct-to-diadduct conversion occurs during the incubation of platinated DNA with NH_4HCO_3. Under these incubation conditions, neither NH_4HCO_3 nor thiourea treatment causes significant diadduct-to-monoadduct conversion. At 25° C, thiourea causes no significant removal of either ethylenediamine(en)- or diaminocyclohexane(dach)-Pt monoadducts. However, at 37° C, both en-Pt and dach-Pt monoadducts are selectively removed. Pt-DNA diadducts are stable to 10 mM thiourea at either temperature. These data suggest that previous experiments using NH_4HCO_3-blocked DNA are likely to have underestimated Pt-DNA monoadducts and to have overestimated diadducts. As a consequence, such studies are likely to produce inaccurate estimates for the repair of individual adducts. The data also show that although thiourea treatment is suitable for blocking Pt-DNA monoadducts under the conditions generally used (10 mM, 10 min, 25° C), it can selectively remove Pt-DNA monoadducts at higher temperatures.

Introduction

Platinum anticancer compounds react preferentially with the N7 of guanine and adenine residues in DNA to form G monoadducts; GG, AG, and GNG intrastrand diadducts; and GG interstrand diadducts [20]. Each of these adducts may have different effects on the inhibition of replication [21, 23], on mutation rates [2, 3], and on lethality [17, 20, 22]. Moreover, each may also be repaired at different rates both in vitro [13, 18] and in vivo [14]. Thus, considerable effort has been directed toward determining the relative proportion of these adducts in DNA that has been treated with platinum drugs. Most of the techniques used to determine the relative proportion of these adducts in DNA implement the enzymatic digestion of DNA followed by high-performance liquid chromatography (HPLC) [5, 6], fast protein liquid chromatography (FPLC) [8, 10], or FPLC followed by competitive enzyme-linked immunosorbent assay (ELISA) [11, 12, 14, 15]. To prevent monoadduct-to-diadduct conversion during the digestion and chromatography steps of these procedures, the monoadducts are generally blocked with either thiourea [5, 6] or NH_4HCO_3 [8, 10–12, 14, 15]. We found that NH_4HCO_3 treatment can lead to a significant underestimation of monoadducts in the DNA. Thiourea treatment at 25° C appears to give an accurate estimate of monoadducts but leads to a selective loss of Pt-DNA monoadducts at 37° C.

Materials and methods

Materials. Both unlabeled and tritiated dichloroethylenediamineplatinum(II) [$PtCl_2$(en)] and dichloro(*trans*-d,l)1,2-diaminocyclohexaneplatinum(II) [$PtCl_2$(dach)] were synthesized by Dr. S. Wyrick as previously described [24]. Stock solutions were prepared in 150 mM NaCl just prior to their use.

Effect of thiourea and NH_4HCO_3 on the proportion of platinum adducts in DNA. Salmon-sperm DNA (0.5 mg/ml) was incubated with 100 μg/ml [^{3}H-dach]-$PtCl_2$(dach) in 25 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM TRIS-HCl (pH 7.4) for 15 or 30 min at 37° C. The reaction was stopped using one of two protocols. In the first, NH_4HCO_3 was added to a final concentration of 0.1 M and the sample was dialyzed for 18 h vs 0.1 M NH_4HCO_3 at 25° C, followed by two 2-h dialyses vs TEN 7.4 [10 mM TRIS-HCl (pH 7.4) 10 mM NaCl, 1 mM EDTA] at 4° C. This procedure has been reported to trap monoadducts in the $(NH_3)_3$Pt-DNA form [9]. The second protocol involved

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Offprint requests to: S. G. Chaney, Department of Biochemistry and Biophysics, FLOB CB# 7260, University of North Carolina, Chapel Hill, NC 27599-7260, USA

Table 1. Effect of treatment conditions on the type of Pt-DNA adducts formed

Treatment conditions[a]	% Monoadduct[b,c]	% GG[b]	%AG+GNG[b,d]
Experiment 1 – DNA treated with $PtCl_2$(dach) for 15 min, then with TU or NH_4HCO_3:			
(a) Dialysis only	68.4 ± 2.1	29.6 ± 1.8	2 ± 0.3
(b) 1 mM TU	69.2 ± 1.3	30.8 ± 2	ND
(c) 10 mM TU	71 ± 0.4	29 ± 0.3	ND
(d) 100 mM NH_4CHO_3	15.6 ± 1.8	69.3 ± 4.2	15.1 ± 1.1
Experiment 2 – DNA treated with $PtCl_2$(dach) for 15 min, then aged for 16 h, then treated with TU or NH_4HCO_3:			
(a) No further treatment	23.8 ± 1.9	61.3 ± 2.7	14.8 ± 0.3
(b) 1 mM TU	23.6 ± 2.9	58.4 ± 1.1	18 ± 1.4
(c) 10 mM TU	21.6 ± 0.4	60 ± 3.6	18.4 ± 1.6
(d) 100 mM NH_4HCO_3	13.4 ± 0.8	71.4 ± 3.1	15.2 ± 2.7
Experiment 3 – DNA treated with $PtCl_2$(dach) for 30 min, then with TU or NH_3HCO_3, then aged for 16 h:			
(a) 1 mM TU	61.2 ± 1.2	33.6 ± 1.2	5.2 ± 0.5
(b) 1 mM TU + aging	59.7 ± 0.8	34.4 ± 0.8	5.9 ± 0.2
(c) 100 mM NH_4HCO_3	33.1 ± 2.5	56 ± 3.4	10.9 ± 0.8
(d) NH_4HCO_3 + aging	34.1 ± 4.2	56.1 ± 1.3	59.8 ± 0.7

[a] In experiment 1, salmon-sperm DNA was treated with $PtCl_2$(dach) for 15 min at 37° C. The DNA was then either (a) dialyzed vs TEN 7.4 + 0.5 M NaCl under conditions designed to preserve monoadducts [19], (b) dialyzed vs TEN 7.4 + 0.5 M NaCl followed by treatment with 1 mM TU for 1 h at 25° C, (c) dialyzed vs TEN 7.4 + 0.5 M NaCl followed by treatment with 10 mM TU for 10 min at 25° C, or (d) dialyzed vs 0.1 M NH_4HCO_3 for 18 h at 25° C. In experiment 2, the DNA was treated with $PtCl_2$(dach) for 15 min at 37° C, dialyzed vs TEN 7.4 + 0.5 M NaCl, and then aged for 16 h at 37° C to enable the conversion of most monoadducts to diadducts [19]. The DNA then (a) received no further treatment, (b) was incubated with 1 mM TU for 1 h at 25° C, (c) was incubated with 10 mM TU for 10 min at 25° C, or (d) was dialyzed vs 0.1 M NH_4HCO_3 for 18 h at 25° C. In experiment 3, the DNA was treated with $PtCl_2$(dach) for 30 min at 37° C and either (a) dialyzed vs TEN 7.4 + 0.5 M NaCl followed by treatment with 1 mM TU for 1 h at 25° C or (c) dialyzed vs 0.1 M NH_4HCO_3 for 18 h at 25° C; aliquots of both the TU-treated (b) and the NH_4HCO_3-treated DNA (d) were then aged for 16 h at 37° C. In each case, the relative proportion of the different types of adducts was determined by enzymatic digestion and HPLC separation. Details of these procedures are described in Materials and methods

[b] These data are reported as mean values ± SEM ($n = 3$)

[c] This fraction includes monoadduct, blocked monoadduct, or protein-Pt-dG, depending on the incubation conditions [5, 6]. Most of the monoadduct occurred in the form of protein Pt-dG following the digestion of DNA that had not been treated with blocking reagents [5], as TU-Pt-dG in the thiourea-treated samples, and as NH_3-Pt-dG in the NH_4HCO_3-treated samples. All of these products were considered to have been derived from monoadducts; thus, they were quantitated together for the purposes of this table

[d] As the GNG and AG peaks overlapped, they were quantitated together

TU, Thiourea; ND, not detectable

adding NaCl to a final concentration of 0.5 M and dialyzing the sample for 1 h vs TEN 7.4 + 0.5 M NaCl at 4° C, followed by two 1-h dialyses vs TEN 7.4 at 4° C. This procedure has been shown to preserve monoadducts in the reactive form [18]. For experiments designed to measure the effects of thiourea on monoadducts, the DNA was processed by the second protocol, immediately treated with either 1 mM thiourea for 1 h at 25° C or 10 mM thiourea for 10 min at 25° C, and dialyzed vs TEN 7.4 for 4 h at 4° C (one change of buffer). For experiments designed to measure the effects of thiourea and NH_4HCO_3 on diadducts, the DNA was processed by the second protocol and then incubated in TEN 7.4 for 16 h at 37° C; this additional incubation in the absence of drug, which we refer to as aging, was carried out to enable the conversion of most of the monoadducts to diadducts. The aged DNA was treated with 1 mM thiourea for 1 h at 25° C, with 10 mM thiourea for 10 min at 25° C, or with 100 mM NH_4HCO_3 for 18 h at 25° C. The DNA was then dialyzed vs TEN 7.4 for 4 h at 4° C (one change of buffer). In each case, the DNA was digested essentially as described by Eastman [6].

HPLC chromatography was carried out on a Whatman ODS-3 Partasil column. Buffer A was 20 mM ammonium acetate (pH 5.5) and buffer B was methanol. The gradient was run as 0–40% buffer B over the first 40 min followed by 40%–100% buffer B over the next 10 min using a flow rate of 1 ml/min; 0.5-ml fractions were collected and aliquots were counted directly in ScintiVerse LC (Fisher Scientific, Raleigh, N. C.). The elution profiles were analyzed and plotted using the Spectrodata Software package (Spectrofuge Corp., Carrboro, N. C.). The columns were calibrated with Pt(dach)(Cl)dG (monoadduct), Pt(dach)(TU)dG (thiourea-blocked monoadduct), Pt(dach)(dGpG) (GG diadduct), Pt(dach)(dApG) (AG diadduct), and Pt(dach)(dG)(dG) (GNG diadduct) standards prepared essentially as described elsewhere [4–6]. The HPLC procedure used was optimal for separating monoadduct, thiourea-blocked monoadduct, and GG and AG diadducts (data not shown), but the AG and GNG peaks overlapped. Previous studies have shown that the digestion of platinated DNA results in the release of most of the monoadducts as protein-blocked monoadducts [6] and that the treatment of platinated DNA with NH_4HCO_3 causes the conversion of many of the monoadducts to NH_3-blocked monoadducts [10]. The protein-blocked monoadducts eluted at the same position as the thiourea-blocked monoadducts, whereas the NH_3-blocked monoadducts were resolved from the other digestion products by this HPLC procedure.

Effect of thiourea on the stability of Pt-DNA adducts. L1210 DNA (0.5 mg/ml) was incubated with either 100 μg/ml $PtCl_2$(dach) or 80 μg/ml $PtCl_2$(en) in 25 mM NaCl, 0.2 mM EDTA, and 2 mM TRIS-Cl (pH 7.4) for 30 min at 37° C. The reaction was stopped by adding NaCl to a final concentration of 0.5 M and dialyzing the sample for 1 h vs TEN 7.4 + 0.5 M NaCl at 4° C, followed by two 1-h dialyses vs TEN 7.4 at 4° C. For experiments designed to determine the effect of thiourea on monoadducts, this DNA was divided into two aliquots, one of which was incubated with 10 mM thiourea for 10 min at either 37° or 25° C. The DNA samples were then dialyzed vs TEN 7.4 for 4 h at 4° C (one change of buffer), followed by dialysis vs water for 48 h at 4° C (two changes of buffer). For experiments designed to determine the effect of thiourea on diadducts, the DNA was aged at 37° C for 48 h prior to the thiourea treatment. Levels of Pt-DNA adducts were determined by atomic absorption as described elsewhere [19].

Results

Fichtinger-Schepman et al. [9] have demonstrated that *cis*-$Pt(NH_3)_2(Cl)(dGMP)$ reacts with NH_4HCO_3 to form *cis*-$Pt(NH_3)_3(dGMP)$ and that *cis*-$Pt(NH_3)_3(dGMP)$ is nonreactive with added dGMP. Although these data have been interpreted to show that NH_4HCO_3 is effective at blocking monoadduct-to-diaduct conversion [8–10], it must be kept in mind that the reaction of monoadducts with NH_4HCO_3 is relatively slow. These data do not exclude the possibility of monoadduct-to-diadduct conversion during the 18-h incubation with NH_4HCO_3 that is required for quantitative conversion of $Pt(NH_3)_2(Cl)$-G monoadducts to blocked $Pt(NH_3)_3$-G monoadducts. In experiments designed to determine the inactivation of pBR322 plasmids by very brief incubations of $PtCl_2$(dach), we observed that a 15-min incubation with $PtCl_2$(dach) followed by a 10-min treatment with 10 mM thiourea at 37° C resulted in far less inactivation of the plasmid than that observed when the incubation was followed by an 18-h treatment with 100 mM NH_4HCO_3 at room temperature (data not shown). Since diadducts are thought to be more lethal than monoadducts [17, 19, 21], these data suggested either that the NH_4HCO_3 had failed to prevent monoadduct-to-diadduct conversion or that thiourea had reacted with many of the diadducts and converted them to monoadducts.

To distinguish between these possibilities, control experiments were carried out using salmon-sperm DNA (Table 1). In the first experiment, the DNA was incubated with $PtCl_2$(dach) for 15 min at 37° C and then treated with either 1 mM thiourea for 1 h at 25° C, 10 mM thiourea for 10 min at 25° C or 100 mM NH_4HCO_3 for 18 h at 25° C. The relative proportion of the different types of adducts was determined by a modification of the enzymatic digestion/HPLC separation procedure described by Eastman [6]. Obviously, the thiourea-treated DNA exhibited a much greater proportion of monoadducts than did the NH_4HCO_3-treated DNA. The percentage of monoadduct found in these experiments was somewhat higher than that previously reported by Eastman [6] but was fully consistent with the slower rate of monoadduct-to-diadduct conversion observed for dach-Pt adducts [19]. In the second experiment, the effects of thiourea and NH_4HCO_3 treatment were determined on DNA that had been aged for 16 h to increase the proportion of diadducts. Once again, thiourea completely blocked monoadduct-to-diadduct conversion, whereas the NH_4HCO_3 treatment did not. More importantly, the thiourea treatment did not result in any detectable diadduct-to-monoadduct conversion. The third experiment confirmed that both thiourea and NH_4HCO_3 treatment effectively prevented any monoadduct-to-diadduct conversion during subsequent incubations of the DNA. Taken together, these data strongly suggest that considerable monoadduct-to-diadduct conversion occurred during the initial incubation with NH_4HCO_3. Thus, in all subsequent experiments we used thiourea to block Pt-DNA monoadducts.

The other important consideration for any blocking reagent is that it not remove platinum adducts from the DNA. Most previous experiments carried out to determine the effect of thiourea or similar reagents on the stability of Pt-DNA adducts have used DNA that had been treated with platinum for at least 16 h at 37° C, conditions under which Pt-DNA diadducts predominate [5, 6, 19] (Table 1). Using DNA prepared in this manner, Eastman [6] has previously shown that thiourea (10 mM, 10 min, 25° C) does not remove significant amounts of platinum from DNA. Similar control experiments conducted in our laboratory have shown no loss of platinum from DNA that had been treated with thiourea at either 37° C or 25° C. However, in experiments designed to determine levels of Pt-DNA monoadducts and diadducts in L1210 cells that had been treated for very brief periods with $PtCl_2$(dach) or $PtCl_2$(en), we observed lower r_b values (Pt adducts per base pair) in cells that had been treated with 10 mM thiourea for 10 min at 37° C prior to the purification of the DNA (data not shown). These data suggested that the thiourea was removing Pt monoadducts from the DNA.

This possibility was assessed in a series of control experiments using purified L1210 DNA. In the first experiment, the DNA was treated with $PtCl_2$(dach) or $PtCl_2$(en) for 30 min at 37° C. From the data shown in Table 1 and

Table 2. Effect of thiourea on the stability of Pt-DNA adducts

Treatment conditions[a]		Pt-DNA adducts	% Loss of Pt
Drug used	Incubation with thiourea	r_b ($\times 10^2$)[b]	
Experiment 1 – DNA treated with 10 mM TU at 37° C for 10 min, then aged for 48 h:			
$PtCl_2$(dach)	No	9.68 ± 0.44	
$PtCl_2$(dach)	Yes	6.33 ± 0.64	35%
$PtCl_2$(en)	No	7.39 ± 0.56	
$PtCl_2$(en)	Yes	6.76 ± 0.36	8%
Experiment 2 – DNA aged for 48 h, then treated with 10 mM TU at 37° C for 10 min:			
$PtCl_2$(dach)	No	2.37 ± 0.16	
$PtCl_2$(dach)	Yes	2.39 ± 0.12	0
$PtCl_2$(en)	No	2.33 ± 0.12	
$PtCl_2$(en)	Yes	2.39 ± 0.07	0
Experiment 3 – DNA treated with 10 mM TU at 25° C for 10 min, then aged for 48 h:			
$PtCl_2$(dach)	No	1.54 ± 0.03	
$PtCl_2$(dach)	Yes	1.57 ± 0.03	0
$PtCl_2$(en)	No	0.87 ± 0.06	
$PtCl_2$(en)	Yes	0.91 ± 0.06	0

[a] L1210 DNA was treated with either $PtCl_2$(dach) or $PtCl_2$(en) for 30 min at 37° C and dialyzed vs TEN 7.4 + 0.5 M NaCl under conditions designed to preserve monoadducts [19]. The platinated DNA samples were then divided into two aliquots, one of which underwent treatment with 10 mM thiourea for 10 min and one of which did not. In experiment 1, the thiourea treatment was carried out at 37° C and was followed by aging of the DNA at 37° C for 48 h. In experiment 2, the DNA was aged for 48 h to convert monoadducts to diadducts and was then treated with thiourea at 37° C. In experiment 3, the thiourea treatment was carried out at 25° C and was followed by aging of the DNA for 48 h at 37° C. Levels of Pt-DNA adducts were determined by atomic absorption as previously described [19]. Details of these procedures are described in Materials and methods

[b] These data are reported as the average ± range ($n = 2$)

TU, Thiourea

those reported elsewhere [6, 19], it is clear that Pt-DNA monoadducts should predominate under these conditions. The DNA was then dialyzed at 4° C to remove excess drug and was split into two aliquots. One of the aliquots received no further treatment, whereas the other was incubated with 10 mM thiourea for 10 min at 37° C. Platinum adduct levels were determined by atomic absorption as described elsewhere [19]. Under these conditions, thiourea removed 8% of the en-Pt adducts and 35% of the dach-Pt adducts (experiment 1). However, once the DNA had been aged long enough to convert all of the monoadducts to diadducts (experiment 2), thiourea treatment at 37° C had no effect on either en-Pt or dach-Pt adducts. Under the conditions originally proposed by Eastman [5, 6], thiourea had no effect on either dach-Pt or en-Pt monoadducts (experiment 3).

Discussion

NH_4HCO_3 is widely used to block Pt-DNA monoadducts both in vitro and in vivo [8, 10–12, 14, 15]. Our data suggest that such experiments are likely to underestimate significantly the proportion of monoadducts in the DNA, with the extent of this error depending on the proportion of monoadducts initially present in the DNA. The half-life for monoadduct-to-diadduct conversion appears to be 1–2 h for $(NH_3)_2Pt$ and en-Pt monoadducts [6, 7, 19] and about 4 h for dach-Pt monoadducts [19]. Thus, one would expect significant levels of Pt-DNA monoadducts to persist through at least the first several hours of treatment with the corresponding platinum drugs [1–2 h for *cis*-$PtCl_2(NH_3)_2$ or $PtCl_2$(en) or 4–6 h for $PtCl_2$(dach)]. Likewise, since the initial values for monoadducts and the various diadducts would likely be in error, this would also affect experiments in which repair rates for individual adducts have been estimated based on the analysis of NH_4HCO_3-blocked DNA [14, 15]. Of course, our data (Table 1) directly demonstrate only that NH_4HCO_3 treatment leads to an underestimation of dach-Pt monoadducts. However, Eastman [6] has previously reported that thiourea treatment results in a much higher proportion of monoadducts than that seen following NH_4HCO_3 treatment in *cis*-$PtCl_2(NH_3)_2$- and $PtCl_2$(en)-treated DNA. Thus, we feel that our observations are likely to apply to other platinum drugs as well.

Our data confirm that thiourea is an effective blocking agent for dach-Pt, en-Pt, and possibly for $(NH_3)_2$-Pt monoadducts under the conditions first reported by Eastman [5, 6]. However, at higher temperatures, both en-Pt and dach-Pt monoadducts are preferentially removed, with the latter being removed to a greater extent than the former. There is precedent for the selective removal of Pt-DNA monoadducts by sulfur-containing nucleophiles. Bodenner et al. [1] have shown that diethyldithiocarbamate removes Pt monoadducts but not Pt diadducts from DNA. Our data show that thiourea can be used to block monoadducts, but that care must be taken to select the correct incubation conditions. Our findings also demonstrate that the dach carrier ligand influences the stability of Pt monoadducts. The reason for this effect is not known. However, the dach ligand does cause considerable distortion of the square-planar platinum(II) configuration [16, 18], and this might make the dach group a slightly better leaving ligand than the en group. In any case, since it is clear that the carrier ligand can influence the stability of Pt monoadducts, it is essential that the correct incubation conditions for thiourea treatment be reestablished when platinum complexes are tested with novel carrier ligands. Furthermore, such control experiments should be carried out using DNA that contains a preponderance of monoadducts so as to enable the selection of conditions that minimize the removal of Pt-DNA monoadducts.

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