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Influence of Carbonate on the Binding of Carboplatin to DNA

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May 2008

APPROVED

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ABSTRACT

The reaction of aged carboplatin (reaction of carboplatin in 24 mM NaHCO₃ for 45 h, 37° C, pH 8.6) with pBR322 DNA for $0 \le r \le 2.8$, where r = [drug]/[DNA-bp], in 24 mM HEPES buffer, pH 7.4, for 24 h followed by agarose gel electrophoresis showed DNA mobility changes consistent with unwinding closed circular DNA. However, identical experiments conducted in a two buffer system, 24mM HEPES plus 24mM carbonate, showed no DNA mobility changes, indicating that carbonate blocks formation of the 1,2-intrastrand crosslink on DNA. Studies with aged carboplatin and with cisplatin carried out with $2.0 \le r \le 10.0$ in the two buffer system show that some DNA binding and unwinding occurs for both drugs. Since carbonate inhibits the binding of aged carboplatin and cisplatin to DNA, carbonate present in the body likely modulates the reactivity of these drugs with a variety of biological targets including DNA.

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INTRODUCTION

Platinum drugs have long been used in clinical cancer therapy. Since the serendipitous discovery of the antiproliferative properties of cisplatin, [*cis*-Pt(NH₃)₂Cl₂], by Barnett Rosenberg over 30 years ago (*1*,*2*), this compound has been successfully used in the treatment of ovarian, testicular, and many other cancers (*3*). However, the drug's severe side effects, often including kidney and nervous system toxicity, severe nausea, and high-frequency hearing loss (*4*), led the search for improved Pt²⁺ anticancer compounds.

One of the second-generation drugs developed to ameliorate these shortcomings was carboplatin, [*cis*-Pt(NH₃)₂(CBDCA-O,O')], where CBDCA is cyclobutane-1,1-dicarboxylate. Clinically, carboplatin is less oto- and nephrotoxic than cisplatin (*3*,*5*), and is useful against cisplatin-resistant malignancies (*6*). Carboplatin's bidentate dicarboxylate chelate makes the drug much less reactive than cisplatin, which may partly explain the reduction in side effects seen in chemotherapy (*7*).

Electrophilic platinum compounds have been shown to bind DNA in the cell, forming mono- and bifunctional adducts (7-9). It is generally thought that such DNA lesions induce apoptosis, destroying cancerous cells, though other mechanisms of action have been proposed (10). Research has shown that cisplatin and carboplatin both interact with DNA in roughly the same manner. Knox et. al. found in 1986 that, although carboplatin binds DNA at a much slower rate than cisplatin, both drugs form the same type of adduct on DNA (5). Their results showed that roughly 100 times as much carboplatin as cisplatin is needed

to achieve an equivalent amount of DNA binding, though smaller ratios have been reported (*11*); however, equal binding resulted in equal DNA lesions and cytotoxicity for the two drugs. Further research by Hongo et. al. supported the conclusion that the two drugs differ mainly in kinetics, and showed that both drugs preferentially target G,G sequences on DNA (*6*).

Reports of carboplatin's extremely slow reaction with DNA belied the drug's effectiveness in clinical use, leading researchers to look for a possible mechanism of activation *in vivo*. Carboplatin had been shown to first bind DNA monofunctionally as a monoaquo species, with loss of the CBDCA ligand leading to formation of a bifunctional adduct (5). However, the reaction of carboplatin with water proceeds extremely slowly ($k_1 = 7.2 \times 10^{-7} \text{ s}^{-1}$), indicating that aquation might not produce enough of the necessary ring-opened species *in vivo* to allow significant DNA binding (5). Further, DNA-binding carboplatin species were most prevalent under highly basic conditions not seen in the human system (*6*). Though direct interaction between unmodified carboplatin and DNA had been suggested (*7*,*11*), investigation into activation mechanisms which could produce ring-opened carboplatin species *in vivo* began.

One proposed activating agent was carbonate, found in equilibrium with bicarbonate, carbonic acid, and aqueous carbon dioxide and present at high concentration (23.8mM) in the blood (*12,13*). Carbonate has been shown to play an important role in the cellular uptake and toxicity of Ti^{4+} , Pb^{2+} and Cd^{2+} (*14-16*), and carbonato complexes of Pd^{2+} were shown to form via substitution by HCO_3^{-1} or CO_3^{2-} (*17*). Early studies showing carbonate's ability to displace

ligands from Pt^{2+} compounds indicated a possible role in platinum anticancer treatment (18).

The Dabrowiak Lab has investigated carbonate interactions with platinum drugs extensively. In 2005, Centerwall et. al. used 13 C NMR to show that a carbonate-modified cisplatin species forms when the drug is incubated in carbonate-containing tissue culture medium (*19*). Further study has shown that Jurkat cells are able to defend against cisplatin attack via production of a drug-modifying extracellular substance (*20*).

A study of the effect of carbonate on the DNA binding of cisplatin was also undertaken. Binter et. al. showed that a physiologically relevant amount of carbonate drastically alters the binding of cisplatin to pBR322 plasmid DNA, and it was theorized that carbonate leads to monofunctional adduct formation (21). While this theory was challenged by Todd et. al. (22), who suggested that carbonate mainly affects the rate of formation of the conventional 1,2-intrastrand bifunctional adduct, Binter's results nonetheless indicate that carbonate may play a large role in cisplatin activity *in vivo*.

The interaction between carboplatin and carbonate has also been studied by Dabrowiak and coworkers. In 2006, Di Pasqua et. al. used HSQC NMR to show that carboplatin is attacked by carbonate at physiological pH to produce a mixture of ring-opened anionic carbonato species (23). It was theorized that these ring-opened species could be the active agents responsible for carboplatin's anticancer activity in the body, and that carbonate ion is the attacking nucleophile responsible for activating the drug *in vivo*. This was supported by Di Pasqua's 2007 finding that carbonate-aged carboplatin is more cytotoxic to human neuroblastoma, proximal renal tube, and Namalwa-luc Burkitt's lymphoma cells than the native drug (24). It was also seen that Jurkat cells produce an unidentified extracellular substance which modifies carboplatin (25), similar to the defense mechanism against cisplatin reported by Dabrowiak and coworkers (20).

In this study, we sought to determine the effect of carbonate on the DNA binding of carbonate-activated carboplatin. We followed the carbonate aging procedure outlined by Di Pasqua (23,24), providing the full variety of carbonato-carboplatin species thought to develop under physiological conditions. By carrying out DNA binding in a carbonate-rich solution, we modeled the environment of cellular DNA *in vivo*. We used closed-circular plasmid DNA as our binding target, and measured the extent of binding through agarose gel electrophoresis, a benchmark technique in the study of platinum-DNA binding (5,6,9). We found that physiologically-relevant concentrations of carbonate in the DNA environment prevented binding of carbonate-aged carboplatin.

We then broadened the conditions of the study, using higher molar ratios of both carbonate-aged carboplatin and cisplatin to determine whether carbonate changed the mode of DNA binding, the rate, or both. We found that carbonate's limiting effect on DNA binding could be overcome by high molar ratios of both drugs, indicating that the 1,2-intrastrand crosslink could still form in the presence of carbonate. These findings were published in 2008 (*26*). We then attempted to quantify the carbonate effect. We sought to determine, using mass spectrometry, the amount of platinum bound to DNA in the presence and absence of carbonate, in order to obtain a rate for platinum-DNA binding in a carbonate-rich environment. Several tries at measuring the binding using this technique proved inconclusive. Additionally, we did a preliminary study using ethanol precipitation to isolate platinated DNA for binding measurement, but this was also unsuccessful.

Despite these difficulties in gathering quantitative data, our findings indicate that carbonate greatly reduces the rate of DNA binding of both cisplatin and carbonate-activated carboplatin. We show how platinum drugs, specifically the modified species which may form in the blood, interact with DNA in an environment similar to that inside the cell, and how this differs from previous models. Since it is critically important to observe how these drugs react under conditions of human chemotherapy, our findings may help clarify carbonate's role in the activity of these platinum drugs.

MATERIALS & METHODS

Materials: HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; sodium bicarbonate, >99% pure; and cisplatin (solid) were purchased from Sigma (St. Louis, MO). Carboplatin, 26.9 mM, was from Bedford Laboratories (Bedford, OH). Plasmid DNA (pBR322), 25μg/μL, was purchased from Invitrogen (Carlsbad, CA), and sodium chloride (A.C.S.) was purchased from Fisher Scientific (Pittsburgh, PA). Ethyl alcohol (190 proof, 95%, A.C.S.) was purchased from Pharmco-AAPER (Brookfield, CT), and sodium acetate (A.C.S.) was purchased from Fisher Scientific (Fair Lawn, NJ).

Preparation of drug solutions: A 0.5 M carbonate solution (pH 8.6) was made by dissolving solid NaHCO₃ in deionized water. Carboplatin infusion solution was added to a final concentration of 26.9 mM, and the resulting solution was incubated in a push-top Eppendorf tube for 45 hours at 37°C. Stock cisplatin solution was made by dissolving solid cisplatin in 154 mM NaCl to produce a 3 mM drug solution which was equilibrated in the dark for 24 hours at room temperature.

Reaction of drug with DNA: Plasmid pBR322 DNA was added to a concentration of 9.6 μ M in a pH 7.4 buffer solution consisting of either 24 mM HEPES plus 24 mM carbonate, or 24 mM HEPES alone. Appropriate concentrations of the drug solutions (carbonate-aged carboplatin or equilibrated

cisplatin) were added to give the indicated *r*-values, where r = [Pt]/[DNA-bp], and adjusted with dH₂O to a final volume of 20 µL. The drug-DNA solutions were incubated in push-top Eppendorf tubes at 37°C for 24 hours. In atmospherecontrolled experiments, screw-top Eppendorf tubes with a rubber o-ring seal were used; CO₂, argon, or nitrogen gas was blown at low pressure over the drug-DNA solution for ~30 seconds and the tubes quickly capped prior to 24 hour incubation.

Ethanol DNA precipitation: Double-volume binding samples were made (total volume = 40 μ L, same concentrations as above). After removal of an 8 μ L aliquot for gel electrophoresis, sodium acetate was added to each sample to a final concentration of 5.1 M, followed by an equal volume of ice-cold ethanol. Tubes were stored on ice 3 hours, then cold centrifuged at 12,000 *g* for 1 hour.

Gel electrophoresis: After the drug-DNA incubation, an 8 μ L sample of each binding experiment was mixed with 1 μ L of a 50% glycerol, 0.25% bromphenol blue and 0.25% xylene cyanol solution, and 8 μ L of the resulting solutions were loaded onto a 1% agarose gel. Electrophoresis was carried out at 75V for ~5 hours in TRIS buffer. Following electrophoresis, the gels were stained for 30 minutes in a 10⁻⁴ % ethidium bromide solution and de-stained in water for 15 minutes. A digital image of each gel was captured using a Kodak Gel Logic 100 imaging system with a Fisher Biotech IT-88A transilluminator. Band intensity data and migration distances were obtained using Kodak 1D software, version 3.6.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS): After gel electrophoresis and staining, individual bands of ethidium-stained DNA were located and excised from the gel using a razor blade. The bands were placed in push-top Eppendorf tubes by gel lane and digested in 1 mL 70% nitric acid. Samples were diluted to a volume of ~5 mL with water and analyzed on a Perkin Elmer Elan 6100 ICP-MS instrument. Platinum content was measured in nanograms, from which the bound platinum for each lane was extrapolated.

RESULTS

Carboplatin binding in HEPES: Carboplatin aged for 45 hours in 0.5 M carbonate was reacted with pBR322 DNA for 24 hours in a 24 mM HEPES buffer, pH = 7.4. After 24 hours, agarose gel electrophoresis showed significant changes in the mobility of both Form I and Form II DNA, Figures 1 and 2. Form I, closed circular DNA, shows minimum migration at r = 0.0 and maximum migration at r = 1.4, with migration steadily increasing between these two r-values. From r = 1.4 to r = 2.8 the Form I migration distance again increases, though the maximum migration distance at r = 2.8 is still less than that seen with no drug present. Form II nicked DNA migration distances increase with increasing r from r = 0.0 to r = 2.8, though the greatest changes in Form II migration are seen at $0.0 \le r \le 1.4$. At r = 1.4, Forms I and II have equal mobility, and the bands corresponding to the individual forms are indistinguishable.



Figure 1: Carboplatin bound to pBR322 plasmid DNA in HEPES buffer, stained with ethidium bromide. Lane and corresponding r-value ([carboplatin]/[DNA-bp]): 1, 2.8; 2, 2.6; 3, 2.4; 4, 2.2; 5, 2.0; 6, 1.8; 7, 1.6; 8, 1.4; 9, 1.2; 10, 1.0; 11, 0.8; 12, 0.6; 13, 0.4; 14, 0.2; 15, 0.0 (control).



Figure 2: Mobility of DNA with bound carboplatin in HEPES buffer measured in arbitrary units (from Fig. 1). Form I DNA indicated by circles and solid trendline ($R^2 = 0.9184$); Form II DNA indicated by squares and dotted trendline ($R^2 = 0.9788$).

Carboplatin binding in HEPES plus carbonate: Carbonate-aged carboplatin was reacted with pBR322 DNA under the same conditions as before, but with 24 mM carbonate present in addition to the 24 mM HEPES buffer, pH 7.4. After 24 hours, gel electrophoresis showed no change in migration of Form I DNA at any *r*-value, Figures 3 and 4. Form II DNA showed a slight increase in mobility, nearly indistinguishable to the naked eye, as *r* increased from 0.0 to 2.8.



Figure 3: Carboplatin bound to pBR322 plasmid DNA in combined HEPES and carbonate buffer, stained with ethidium bromide. Lane and corresponding *r*-value ([carboplatin]/[DNA-bp]): 1, 2.8; 2, 2.6; 3, 2.4; 4, 2.2; 5, 2.0; 6, 1.8; 7, 1.6; 8, 1.4; 9, 1.2; 10, 1.0; 11, 0.8; 12, 0.6; 13, 0.4; 14, 0.2; 15, 0.0 (control).



Figure 4: Mobility of DNA with bound carboplatin in combined HEPES and carbonate buffer, measured in arbitrary units (from Fig. 3). Form I DNA indicated by circles and solid trendline ($R^2 = 0.041$); Form II DNA indicated by squares and dotted trendline ($R^2 = 0.8362$).

Carboplating binding in HEPES plus carbonate, high *r***-values:** Carbonateaged carboplatin was again reacted with pBR322 DNA in 24 mM HEPES plus 24 mM carbonate buffer, pH 7.4, this time with $2.0 \le r \le 10.0$, Figures 5 and 6. Form I DNA showed a gradual decrease in migration distances with increasing *r*, similar to the trend seen at low *r*-values in HEPES alone but much less pronounced. Form II migration seemed to increase slightly where $2.0 \le r \le 6.0$, decreasing again for $6.5 \le r \le 10.0$.



Figure 5: Carboplatin bound to pBR322 plasmid DNA in combined HEPES and carbonate buffer, stained with ethidium bromide. Lane and corresponding *r*-value ([carboplatin]/[DNA-bp]): 1, 10.0; 2, 9.5; 3, 9.0; 4, 8.5; 5, 8.0; 6, 7.5; 7, 7.0; 8, 6.5; 9, 6.0; 10, 5.5; 11, 5.0; 12, 4.5; 13, 4.0; 14, 3.5; 15, 3.0; 16, 2.5; 17, 2.0; 18, 0.0 (control).



Figure 6: Mobility of DNA with bound carboplatin in combined HEPES and carbonate buffer, measured in arbitrary units (from Fig. 5). Form I DNA indicated by circles and solid trendline ($R^2 = 0.8937$); Form II DNA indicated by squares and dotted trendline ($R^2 = 0.0035$).

Cisplatin binding in HEPES plus carbonate, high *r***-values:** Cisplatin solution (3 mM, in 154 mM NaCl), equilibrated for 24 hours in the dark, was reacted with pBR322 DNA in combined HEPES/carbonate buffer at *r*-values of $2.0 \le r \le 10.0$ under the same conditions as the high *r*-value carboplatin experiment. Gel electrophoresis showed a notable decrease in mobility of Form I DNA for increasing *r* over the complete range of *r*-values, with Form II mobility increasing steadily. The mobility trends for cisplatin were more pronounced than with carboplatin for both DNA forms, and the change in migration of both DNA forms between *r* = 0.0 and *r* = 10.0 was much greater than for carboplatin.



Figure 7: Cisplatin bound to pBR322 plasmid DNA in combined HEPES and carbonate buffer, stained with ethidium bromide. Lane and corresponding *r*-value ([cisplatin]/[DNA-bp]): 1, 10.0; 2, 9.5; 3, 9.0; 4, 8.5; 5, 8.0; 6, 7.5; 7, 7.0; 8, 6.5; 9, 6.0; 10, 5.5; 11, 5.0; 12, 4.5; 13, 4.0; 14, 3.5; 15, 3.0; 16, 2.5; 17, 2.0; 18, 0.0 (control).



Figure 8: Mobility of DNA with bound cisplatin in combined HEPES and carbonate buffer, measured in arbitrary units (from Fig. 7). Form I DNA indicated by circles and solid trendline ($R^2 = 0.8912$); Form II DNA indicated by squares and dotted trendline ($R^2 = 0.9105$).

ICP-MS measurements of Pt bound to DNA: For each of the binding experiments described above, individual bands were excised from the agarose gel using a razor blade, digested in nitric acid, and analyzed using ICP-MS to determine platinum content. The amount of platinum (in nanograms) present in each lane was measured, and a general increase in platinum was shown for increasing *r*-values of both carboplatin and cisplatin. However, platinum was detected in control samples which received no drug; this indicated errors in the technique, making all ICP-MS reading suspect and leading us to discard data thus collected.

Binding under a CO₂ atmosphere: It was suggested that dissolved carbon dioxide in the carbonate buffer could be lost as CO_2 gas during the drug-DNA incubation step. To combat this, and ensure a consistent carbonate concentration throughout the 24 hour incubation, the carboplatin and cisplatin extended *r*-value experiments were repeated with a CO_2 atmosphere over the drug-DNA solution. Control experiments were performed under nitrogen, argon, and ambient air atmospheres. Various migration trends were seen, but the results could not be repeated and data from these experiments was discarded.

Ethanol DNA precipitation: A standard ethanol precipitation protocol (27) was performed to isolate platinated DNA after the 24 hour drug-DNA incubation step. After multiple attempts, no DNA was isolated, and this technique was abandoned.

DISCUSSION & CONCLUSIONS

In this study we use agarose gel electrophoresis to examine the effects of carbonate-aged carboplatin and cisplatin on the electrophoretic mobility of pBR322 plasmid DNA when binding is carried out in carbonate media. Since this gel technique does not require any work-up of the metalated DNA prior to gel analysis, it is especially well suited for Pt-DNA binding studies involving carbonate buffer.

Our preliminary study, testing the DNA binding of carbonate-aged carboplatin at low *r*-values, showed distinct migration changes when reaction took place in HEPES buffer only. The initial decrease of Form I mobility, followed by co-migration with Form II, then increased mobility, corresponds with trends seen for both cisplatin (9,21,22) and carboplatin (5,6) in previous gel electrophoresis studies. Using carbonate-activated carboplatin at low concentration, we were able to see migration patterns which were only observed using lengthy incubation times (6) or much higher drug concentrations (5) in prior studies on native carboplatin. Since platinum binding does not significantly affect the total charge of the DNA molecule, the observed changes in mobility are due to changes in the degree of supercoiling of DNA as would occur through formation of a 1,2-intrastrand crosslink. This result indicates that carbonate-aged carboplatin contains a greater number of DNA-reactive compounds than unmodified carboplatin, as was suggested by Di Pasqua et. al. (23). Our observations with carboplatin showed some key differences compared with earlier cisplatin studies. First, while the "coalescence point" (where Form I and Form II migrate to the same point on the gel) occurs at r = 1.4, this point does not seem to correspond to the migration for DNA with zero supercoiling (Form II when r = 0.0) as it did for Keck and Lippard (9). Second, the staining intensity trends seen here differ from those found in cisplatin studies (21). In our study, Form I intensity decreased with increasing drug concentration, opposite the published cisplatin trend. These observations may highlight differences between carboplatin and cisplatin DNA interactions.

When carbonate-activated carboplatin was reacted with DNA at low *r*-values in carbonate media, there were no readily-apparent changes in migration indicative of drug binding. Form I DNA showed the same migration for all *r*-values, while Form II migration increased a slight amount as *r* increased. This trend corresponds well with prior observations on the binding of cisplatin at similar *r*-values in carbonate media (*21,22*). From our observations, the 24 mM carbonate present in the reaction buffer was enough to prevent DNA binding of the drug. Here again, intensity decreased for Form I, counter to trends published for cisplatin studies.

Carbonate-aged carboplatin is known to contain carbonato/bicarbonato complexes (23); when the activated drug is added to a carbonate-free medium, these species lose CO_2 through mass action effects (28). This equilibrium shift results in the formation of aquo/hydroxo species, which are able to bind DNA via the formation of 1,2-intrastrand crosslinks, unwinding Form I supercoiled DNA and leading to the migration patterns here seen in HEPES alone. The addition of carbonate to the system blocks this process. At low drug concentration, the dissolved CO₂ in our 24 mM carbonate buffer is enough to prevent the loss of CO₂ from the carbonato/bicarbonato species of carbonate-aged carboplatin. The monoaquo species required for DNA binding cannot form, and thus DNA binding is blocked by the presence of carbonate. This leads to the lack of mobility change in HEPES/carbonate buffer when $r \leq 2.8$.

In order to determine whether the inhibitory effect of carbonate could be overcome by high concentrations of activated carboplatin, we extended the *r*values to a maximum of r = 10.0. At very high carboplatin doses, mobility changes were seen which indicated that the 1,2-intrastrand crosslink was forming despite the presence of 24 mM carbonate. This finding indicated that carbonate blocks the formation of the standard bifunctional adduct at low drug concentration; however, if the carboplatin concentration is increased with constant carbonate concentration, the equilibrium is shifted toward formation of aquo/hydroxo DNA binding species.

Following the publication of Dabrowiak and coworkers' initial report describing cisplatin binding to pBR322 DNA in carbonate buffer (21), Todd et. al. (22) further investigated binding in this system by increasing drug concentrations to r > 3.13. Using different techniques, they found that cisplatin can bind to closed circular DNA and unwind the superhelical structure even in the presence of carbonate. Their conclusion was that, while carbonate inhibits cisplatin binding, it does not change the type of platinum adduct which forms, and the 1,2intrastrand crosslink is capable of forming in both carbonate and carbonate-free systems.

Our results indicate that 1,2-intrastrand crosslinking occurs only at low ratios of cisplatin concentration to carbonate concentration. Since these conditions shift the equilibrium in favor of cisplatin aquo/hydroxo species, the observed binding is likely due to the interaction of the aquo/hydroxo species with DNA. As seen on our cisplatin gel, at high *r*-values the migration of Form I decreases while Form II migration increases, in a manner identical to that seen with no carbonate in the system (21). As with carboplatin, cisplatin in carbonate media forms carbonato complexes (19); these neutral or anionic species do not bind to DNA. The dissolved CO₂ present in carbonate buffer prevents aquated cisplatin species from forming and binding to DNA, and thus cisplatin binding can only occur when drug concentration is high enough to overcome carbonate's blocking effect. As with our carboplatin studies, the cisplatin staining intensity of Form I DNA decreased with increasing *r*-value, counter to prior published findings (21).

In an effort to prevent off-gassing of CO_2 from the carbonate buffer during the 24 hour drug/DNA incubation, several experiments were attempted which involved keeping an atmosphere of CO_2 gas over the reaction solution. This, it was theorized, would prevent the loss of dissolved carbon dioxide from the buffer, maintaining a consistent carbonate concentration throughout the experiment. Migration trends could not be replicated in these experiments, and studies using inert gases proved equally inconclusive (data not shown). Attempts at quantitatively describing the carbonate effect were similarly challenging. Excising DNA bands from stained gels and analyzing them for platinum content showed logical trends, where measured platinum content increased with increasing *r*-values. However, platinum was detected in samples which had not received any amount of carboplatin or cisplatin. This indicated that our control samples were being contaminated with platinum from some source, making the data untrustworthy (data not shown). Attempts at performing a standard ethanol DNA precipitation method were unsuccessful, and no DNA was isolated from the reaction samples.

Despite these difficulties, our gel electrophoresis findings give a reliable portrait of the effect of carbonate buffer on the DNA binding of these two platinum drugs. As mentioned, a strength of this electrophoresis technique is that it requires no isolation or treatment of the platinated DNA, eliminating the possibility of changing or removing platinum adducts through intermediate workup steps. Further, the carbonate aging process used to activate carboplatin provides modified drug species in the varieties and relative amounts which may form *in vivo*, while removing guesswork by not requiring us to identify individual species and concentrations. The system models the proposed *in vivo* activation mechanism from start to finish, operating on biochemical principles rather than operator input.

The system we have constructed carries out DNA binding under conditions identical to those of the human body in terms of carbonate concentration, H⁺ ion concentration, and temperature. The carboplatin activation step preceding DNA binding strays from strict physiological conditions with significantly increased reaction times and carbonate concentrations; these conditions are employed to provide a surplus of the carbonate-modified species expected to form in the blood stream. Overall, our system establishes a model which is similar to the DNA binding environment of both cisplatin and carboplatin, providing a relevant system to carry out studies on these two drugs.

In conclusion, our findings show that DNA binding of cisplatin and carbonate-activated carboplatin is affected by the presence of carbonate buffer at physiologically relevant concentrations. In particular, our high *r*-value studies show that the formation of the conventional 1,2-intrastrand crosslink is negatively influenced by the presence of carbonate for both drugs. Since carbonato/bicarbonate complexes can form by attack from carbonate, the presence of this material in the biological system may control the concentration of biologically reactive metal aquo species of cisplatin and carboplatin and their reactivity with targets including DNA. Clearly, elucidating the complex equilibria associated with these important reactions requires further investigation. This work demonstrating the role of carbonate in the mechanism of action of platinum anticancer drugs shows the importance of studying drug activity under biologically relevant conditions, and it is our hope that such investigations will continue in the field of anticancer drug research.

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