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Alexandra Binter
Syracuse University

Jerry Goodisman
Syracuse University

James C. Dabrowiak
Syracuse University

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Formation of monofunctional cisplatin-DNA adducts in carbonate buffer

Alexandra Binter, Jerry Goodisman, James C. Dabrowiak *

Department of Chemistry, Syracuse University, 111 College Place, Rm 1-014 CST, Syracuse, NY 13244-4100, United States

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Abstract

Carbonate in its various forms is an important component in blood and the cytosol. Since, under conditions that simulate therapy, carbonate reacts with cisplatin to form carbonato complexes, one of which is taken up and/or modified by the cell [C.R. Centerwall, J. Goodisman, D.J. Kerwood, J. Am. Chem. Soc., 127 (2005) 12768–12769], cisplatin-carbonato complexes may be important in the mechanism of action of cisplatin. In this report we study the binding of cisplatin to pBR322 DNA in two different buffers, using gel electrophoresis. In 23.8 mM HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid, 5 mM NaCl, pH 7.4 buffer, cisplatin produces aquated species, which react with DNA to unwind supercoiled Form I DNA, increasing its mobility, and reducing the binding of ethidium to DNA. This behavior is consistent with the formation of the well-known intrastrand crosslink on DNA. In 23.8 mM carbonate buffer, 5 mM NaCl, pH 7.4, cisplatin forms carbonato species that produce DNA-adducts which do not significantly change supercoiling but enhance binding of ethidium to DNA. This behavior is consistent with the formation of a monofunctional cisplatin adduct on DNA. These results show that aquated cisplatin and carbonato complexes of cisplatin produce different types of lesions on DNA and they underscore the importance of carrying out binding studies with cisplatin and DNA using conditions that approximate those found in the cell.

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Keywords: Cisplatin; Monofunctional; Carbonate; Supercoiled DNA; Binding

1. Introduction

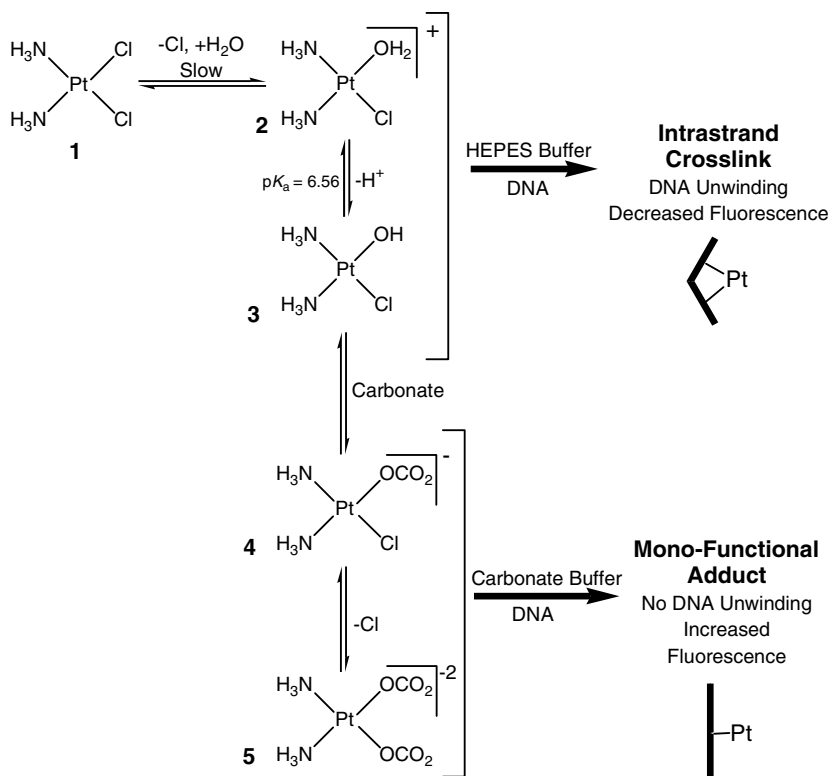
Cisplatin, *cis*-diamminedichloroplatinum(II), **1**, Scheme 1, is one of the most successful drugs currently in use for treating cancer [1]. In the clinical formulation of cisplatin called Platinol, the hydrolysis of **1** is suppressed by the addition of 154 mM NaCl but some hydrolysis (~15%) to *cis*-[Pt(NH₃)₂(H₂O)(Cl)]⁺, **2**, still occurs [2,3] (Scheme 1). As the drug enters the blood, where the chloride concentration is 105 mM, additional hydrolysis of **1** to **2** can take place. Since the p*K_a* for the deprotonation of the mono-aquo form, **2**, to produce the corresponding hydroxo species, *cis*-[Pt(NH₃)₂(OH)(Cl)], **3**, is 6.56 [4], both **2** and **3** are present at physiological pH (pH 7.4). Compounds **1**–

3 may react with components in the blood or they may enter the cytosol and react with components inside the cell to elicit their biological effects.

It is widely accepted that the target for platinum drugs is nuclear DNA [1]. However, DNA binding studies involving cisplatin and its analogs have usually been done using conditions, which do not simulate those found in blood or the cytosol. For example, earlier we studied the binding of cisplatin and *cis*-Pt((CH₃)₂CHNH₂)₂Cl₂ to PM2 DNA in the buffer tris(hydroxymethyl)aminomethane-nitrate (TRIS-NO₃) [5]. Nitrate ion is a poor ligand for binding to metal ions [6] and, because the primary amine of TRIS is largely protonated at physiological pH, it has limited availability for reaction with Pt⁺² [7]. In this buffer, the platinum drugs formed aquated species that bound to and unwound closed circular PM2 DNA [5].

In contrast, carbonate, which is a good nucleophile for metal ions [8,9], is a major component of blood, yet it is

* Corresponding author. Tel.: +1 315 443 4601; fax: +1 315 443 4070.
E-mail address: jcdabrow@syrr.edu (J.C. Dabrowiak).



Scheme 1. Reaction products of cisplatin in HEPES and carbonate buffers and type of adducts formed with pBR322 DNA.

rarely present in DNA binding studies involving cisplatin and its analogues. The total concentration of all carbonate forms in blood (carbonate ion, bicarbonate, carbonic acid, and dissolved carbon dioxide), which are in equilibrium with each other, is ~ 24 mM. Although the intra- and extra-cellular cellular concentrations of carbonate are similar, stress applied to the cell can cause the former to be much greater than 24 mM [10].

Extensive work by Harris, van Eldik, and others [8,11,12] has shown that carbonate in its various forms can react with metal ions to form carbonato complexes. This can occur by attack of carbonate ion with the subsequent displacement of a metal-bound ligand (usually water), or by the reaction of dissolved carbon dioxide gas with a hydroxo species which has a metal-bound hydroxide ligand. Since the latter reaction does not involve a metal-ligand bond-breaking step, it occurs on the millisecond time frame, allowing a hydroxo complex to rapidly convert to its corresponding carbonato species. Depending on the pH of the medium and the pK_b for the protonation of the bound carbonato ligand, the complex can remain as a carbonato species or it can protonate to its bicarbonato form. In most cases the bicarbonato complex rapidly decarboxylates to its hydroxo form (loses CO_2) but some stable bicarbonato complexes are also known [8,11,13]. These transformations have been implicated in the mechanism of action of human carbonic anhydrase, which catalyzes the reversible hydration of CO_2 [8].

Earlier we used ^1H , ^{15}N HSQC NMR and other techniques to show that cisplatin reacts with carbonate in car-

bonate buffer to produce the carbonato complexes **4** and **5**, Scheme 1 [14]. If Jurkat cells are present in the medium, **4** is readily taken up and/or modified by the cells [15], suggesting that it may be the platinum species responsible for the biological effects of cisplatin. In this report we study the binding of cisplatin to purified closed circular pBR322 DNA using agarose gel electrophoresis with two different buffers. One buffer contains 23.8 mM HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid, which interacts only weakly with platinum. This buffer allows cisplatin aquo species to form and interact with DNA. The second buffer, which contains 23.8 mM carbonate (the approximate concentration of carbonate in the cytosol), interacts with cisplatin to form carbonato species as shown in Scheme 1 [14]. Both buffers contained 5 mM NaCl, the approximate chloride ion concentration in the cytosol. We found that, as expected, cisplatin binding to DNA in HEPES buffer significantly changes the supercoiling (writhing) of Form I DNA and decreases ethidium binding to DNA. This behavior is consistent with the formation of the aquated species **2** (**3**), which reacts with DNA to form an intrastrand crosslink. However, in carbonate buffer which allows the formation of cisplatin-carbonato species, cisplatin binding to DNA causes little or no change in the amount of supercoiling but it significantly increases ethidium binding to DNA. This behavior is consistent with the formation of a monofunctional cisplatin adduct on DNA. Since cisplatin carbonato complexes can form under physiological conditions and since they may enter the cell, understanding the interaction of platinum carbonato

species with DNA should be important in understanding the molecular mechanism of action of cisplatin.

2. Materials and methods

The DNA binding studies were done in a total volume of 20 μl containing 5.0 mM NaCl and 19.2 μM (bp) pBR322 DNA (Sigma–Aldrich), and either 23.8 mM HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid, (pH 7.4) or 23.8 mM carbonate (NaHCO_3 , Sigma–Aldrich, St. Louis, MO) (pH 7.4). The stock solution of cisplatin (Sigma–Aldrich) (3.0 mM) in aqueous 150 mM NaCl was diluted immediately before use to give working solutions containing 10 mM NaCl and cisplatin concentrations in the range 10–200 μM . Appropriate volumes of the working solutions and 10 mM NaCl were added to solutions containing the buffer and DNA to give the final concentrations given above and values of r , $r = [\text{I}]/[\text{DNA-bp}]$, given in the captions to figures. The samples were incubated at 37 $^\circ\text{C}$ for 24 h in sealed Eppendorf tubes after which time 8 μl of solution were removed from the reaction tube and 1 μl of a solution containing 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added. A 8 μl volume of each solution containing the loading the glycerol and dyes was loaded in the

wells of a 1% agarose gel and electrophoresis was carried out for a period of ~ 4 h, at 75 V. After electrophoresis, the gel was immersed in ~ 1 l of deionized water containing 100 μl of a 10 mg/ml solution of ethidium bromide for 15 min to stain DNA and then soaked in water alone for 15 min to de-stain the background of the gel. A digital image of the stained gel was captured using a Kodak Gel Logic 100 equipped with Fisher Biotech IT-88A transilluminator and band intensities (in arbitrary units) were determined using Kodak software and SigmaScan (v. 4.0). All captured images were below saturation, in the linear response range of the image capture system, and independent of the orientation of the gel on the transilluminator.

3. Results and discussion

Figs. 1–3 are the results of cisplatin binding to pBR322 DNA in two different buffers, 23.8 mM carbonate pH 7.4 (left side of gels) and HEPES buffer, pH 7.4 (right side of gels). The fastest migrating form of the DNA in the gel is the closed circular form, Form I, while the slower moving band is the nicked circular form, Form II [16–18]. While Forms I and II DNA in carbonate buffer exhibit only small changes in mobility with changes in r , the staining intensity of the DNA in the gel clearly *increases* as r *increases*. Fig. 4

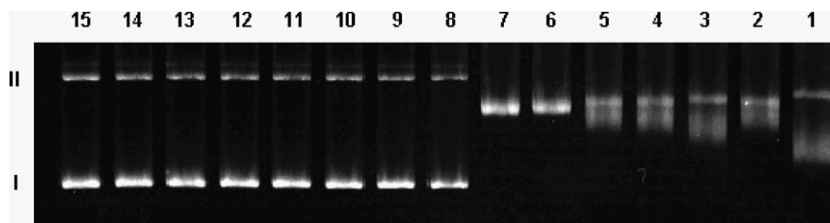


Fig. 1. Ethidium stained agarose gel of pBR322 DNA (19.2 μM bp) in the presence of cisplatin. 23.8 mM HEPES buffer, pH 7.4, lanes 1–7; 23.8 mM carbonate buffer, pH 7.4, lanes 8–15. The lane numbers and value of r : 1, 9, 0.078; 2, 10, 0.091; 3, 11, 0.104; 4, 12, 0.117; 5, 13, 0.130; 6, 14, 0.143; 7, 15, 0.156; 8, 0 (control). $r = [\text{cisplatin}]/[\text{DNA-bp}]$.

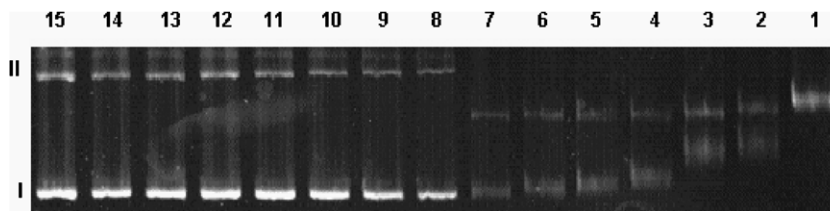


Fig. 2. Ethidium stained agarose gel. See Fig. 1 caption for more details. The lane numbers and value of r : 1, 9, 0.26; 2, 10, 0.52; 3, 11, 0.78; 4, 12, 1.04; 5, 13, 1.30; 6, 14, 1.56; 7, 15, 1.82; 8, 0 (control).

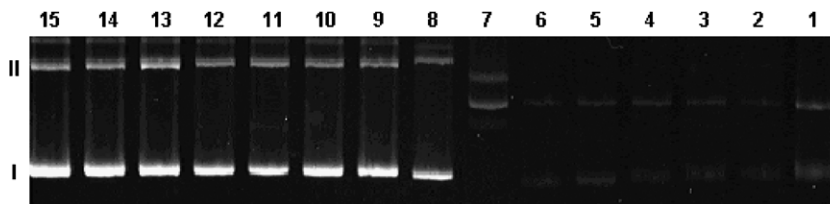


Fig. 3. Ethidium stained agarose gel. See Fig. 1 caption for more details. The lane numbers and value of r : 1, 9, 1.56; 2, 10, 1.82; 3, 11, 2.08; 4, 12, 2.34; 5, 13, 2.60; 6, 14, 2.86; 7, 15, 3.13; 8, 0 (control).

shows band intensities for Forms I and II (in arbitrary units), from the gels shown in Figs. 1–3, plotted as functions of r , with linear least-square fits to the points. Fig. 4a corresponds to $r \leq 0.156$ (gel shown in Fig. 1) and Fig. 4b, which combines results from the gels shown in Figs. 2 and 3, corresponds to a 20-fold greater range of r .

The slope of each linear fit is divided by the y-intercept to give the relative slope. For Form I pBR322 DNA, the relative slope is 2.1 in the upper panel and 0.37 in the lower. For Form II, the relative slope is 3.8 in the upper panel and 1.7 in the lower. Thus the increase of band intensity with drug concentration in carbonate buffer is faster for lower concentrations of cisplatin, and slower for higher drug concentrations. The increase in band intensity with increased cisplatin concentration implies that cisplatin binds to Forms I and II of pBR322 DNA in carbonate buffer. (However, the binding hardly affects DNA mobility, as shown below.) This conclusion, that cisplatin binds to DNA in carbonate solution, was also reached by Fichtinger-Schepman, et al. [19] who studied the binding of cis-

platin to salmon sperm DNA ($r \sim 0.06$) in the presence of 20 mM NaCl, NaHCO₃, and NH₄HCO₃. They used atomic absorption spectroscopy to show that, when compared to an incubation medium which has no salt or buffer, all three salts allowed about the same amount of platinum to bind to DNA (~30% of the amount in a salt-free medium). Assuming that the intensity is a linear function of the number of cisplatin adducts on DNA, the intensity data in Fig. 4 can be fitted reasonably well by a Langmuir isotherm, which is appropriate for ligand binding to a multiple-site DNA lattice [20].

As expected, cisplatin binds to and unwinds closed circular Form I DNA in HEPES buffer, Figs. 1–3, right side of gels. Since cisplatin binding is believed to produce an intrastrand crosslink on DNA which reduces the twist angle at the site [21], the lesion causes the DNA form to reduce its writhing which is manifested by a change in the mobility of the form (measured by the distance traveled in the gel). Since Form I pBR322 DNA is negatively supercoiled, low values of r make the writhing less negative, i.e., closer to zero, which should open up the structure. The effect of this can be seen in Fig. 1, for $r \leq 0.156$, which shows that the mobility of Form I *decreases* as r increases. At higher values of r , Figs. 2 and 3, platinum binding to Form I increases the writhing to zero and then changes its sign (positive supercoiling), so that increased binding should *increase* the mobility of this form in the gel, as observed. Also evident from Figs. 1–3 is that in HEPES buffer the mobility of the nicked circular Form II DNA, which has no supercoiling, increases at all values of r . Although this effect was previously observed [17], its structural origin is unknown. Cisplatin binding to DNA in HEPES causes a *decrease* in the staining intensity of the DNA forms which, at large r , makes DNA difficult to detect in the gel, Fig. 3. Formation of the intrastrand crosslink is known to reduce ethidium binding to DNA [16–18,22]

In Fig. 5 we show the mobilities as functions of r for Forms I and II pBR322 DNA in carbonate buffer. The lines are least-square linear fits. For $r \leq 0.156$, the mobilities are not changed much by cisplatin in carbonate buffer, indicating that binding does not greatly change the degree of supercoiling of Form I DNA and it does not change the structure of Form II. The slopes of both lines in Fig. 5a are small and negative, -17 ± 8 and -8 ± 7 for Forms I and II respectively (compare -764 ± 93 and 305 ± 34 for Forms I and II in HEPES). In Fig. 5b, however, where $r \leq 3.2$, the line for Form I DNA has a small negative slope (-2.4 ± 0.4) but the line for Form II DNA has a small positive slope ($+2.2 \pm 0.3$). The negative slopes for Form I indicate a decrease in supercoiling for small r ; since the supercoiling (writhing) is originally negative, it is becoming less negative.

As we earlier showed [14], cisplatin reacts with carbonate to form carbonato complexes, one of which, **4**, is taken up and/or modified by Jurkat cells in culture. In carbonate buffer, a second carbonato complex, *cis*-[Pt(NH₃)₂-

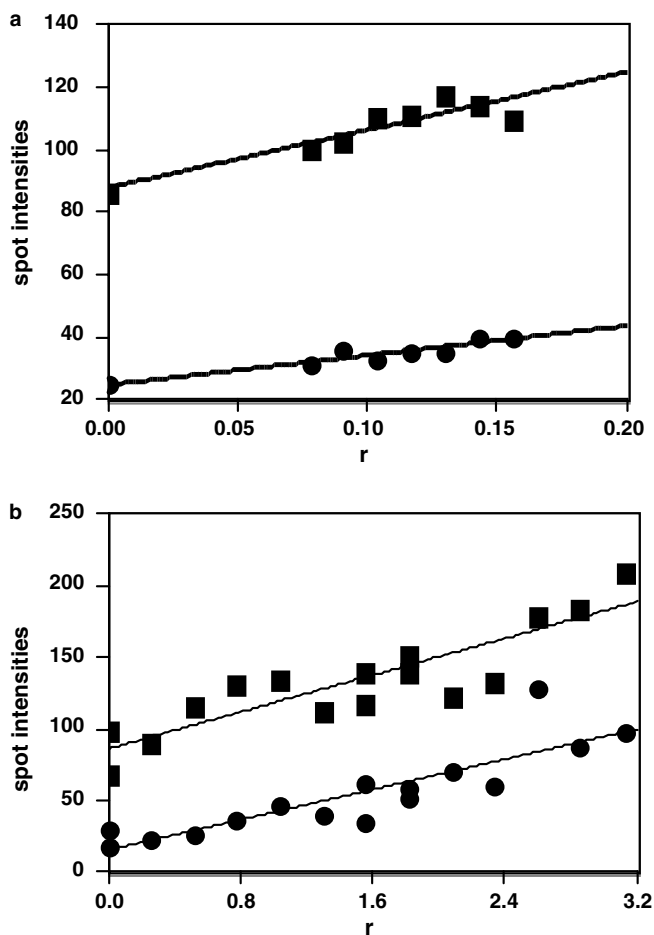


Fig. 4. Gel spot intensities (in arbitrary units) of Forms I and II in carbonate increase with r . (a) Top, squares, closed circular Form I pBR322 DNA; bottom, circles, nicked circular Form II DNA for gel shown in Fig. 1. (b) Top, squares, Form I DNA; bottom, circles, Form II DNA for gels shown in Figs. 2 and 3. Lines are linear least-square fits.

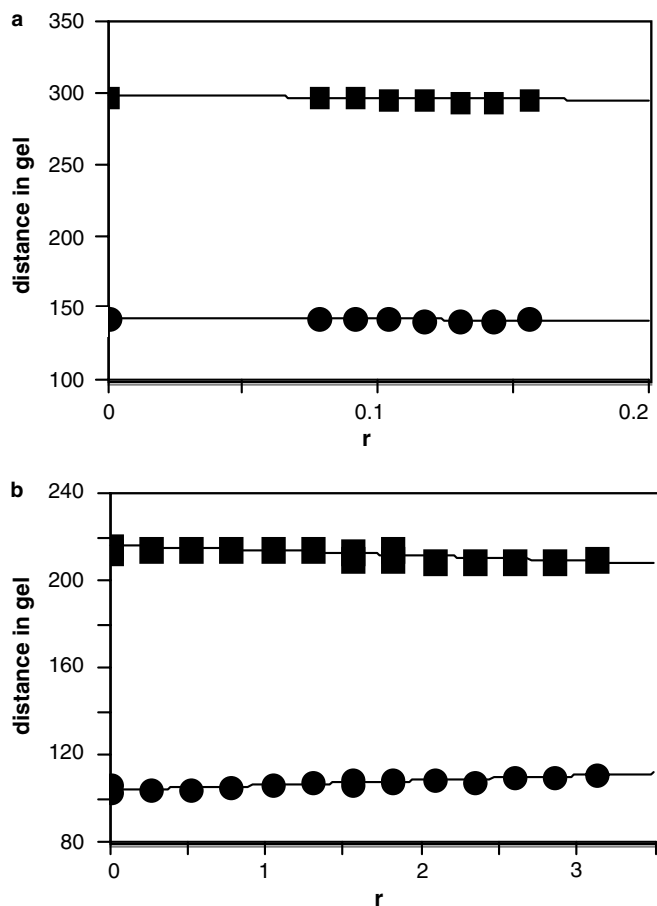


Fig. 5. Mobilities (in arbitrary units) of Forms I and II in carbonate vary little with r . (a) Top, squares, closed circular Form I pBR322 DNA; bottom, circles, nicked circular Form II DNA for gel shown in Fig. 1. (b) Top, squares, Form I DNA; bottom, circles, Form II DNA for gels shown in Figs. 2 and 3. Lines are linear least-square fits.

$(\text{CO}_3)_2]^{-2}$, **5**, forms and one or both of these species could be interacting with pBR322 DNA in the gel studies. In addition to carbonate, the buffer medium contains a small amount of chloride ion (5 mM) to simulate the concentration of this ion in the cytosol. Since the carbonate complexes could be partially protonated at pH 7.4, to give their bicarbonato and/or hydroxo forms [8], the equilibria defining the interaction of **4** and/or **5** with DNA are complex. However, it is possible that the DNA adduct formed in carbonate buffer retains a platinum-bound carbonato ligand, which is a poorer leaving ligand than, for example, chloride. The presence of an anionic carbonato ligand at the site of the platinum lesion could allow additional ethidium, which is a cation, to associate with DNA thus enhancing fluorescence. In HEPES buffer, and in other media having poor metal binding ligands, cisplatin aquates to form **2** (**3**) which subsequently attacks DNA with the loss of bound water [23,24]. This initially formed monofunctional adduct ultimately loses the chloro ligand to form a bifunctional intrastrand crosslink, which changes the degree of supercoiling. Reduced fluorescence in HEPES could be caused by the ability of cationic platinum adducts

to reduced binding of cationic ethidium near the lesion and/or to platinum-induced structural changes in DNA which hinder intercalation of ethidium [18,22].

The small changes observed in the mobility for DNA exposed to cisplatin in carbonate buffer are similar to those recently observed for a potentially new antitumor drug, *cis*-K[Ru(eddp)Cl₂], reported by González-Vílchez and coworkers [25]. The authors attributed the small changes in mobility at low values of r to monofunctional binding of the ruthenium complex to pBR322 DNA. Small changes in mobility of supercoiled DNA also occur for a platinum complex that forms a monofunctional adduct with guanine residues on DNA [26] as well as with sterically hindered Pt⁺² and Pd⁺² complexes containing mepirizole as the non-leaving ligand [27]. Thus the lack of DNA unwinding by cisplatin in carbonate buffer strongly suggests that the drug is binding to DNA in a monofunctional manner.

Clearly, the composition of the medium significantly influences the outcome of the binding experiment and additional work will be necessary to determine the structural relationship between the lesion formed in carbonate buffer to that produced when carbonate is absent from the medium. As was earlier pointed out, carbonate in its various forms is ubiquitous in biological systems and it readily reacts with metal ions to form carbonato complexes [8,11–13]. In particular, carbonate can react with cisplatin to form carbonato species and, as we have recently shown [28], it can open the bidentate dicarboxylate chelate ring of the related platinum drug carboplatin. We hope that this report will stimulate new interest on the potential role of carbonate in the molecular mechanism of action of platinum-based anticancer drugs.

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