DNA Interactions of pH-Sensitive, Antitumor Bis(aminoalcohol)dichloroplatinum(II) Complexes[†,‡]

Stefanie Zorbas-Seifried,*[,||] Christian G. Hartinger,[§] Kristof Meelich,[§] Markus Galanski,[§] Bernhard K. Keppler,[§] and Haralabos Zorbas*[,||]

Max-Planck Institute of Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany and Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, A-1019 Vienna, Austria

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ABSTRACT: (*SP*-4-2)-Bis(2-aminoethanol)dichloroplatinum(II) (KP1356) and (*SP*-4-2)-bis[(*R*)-(−)-2-aminobutanol)]dichloroplatinum(II) (KP1433) are promising cytotoxic agents capable of changing their chemical structure depending on the pH value. On the basis of this, they are supposed to be active only in or preferentially in hypoxic tumors with low pH. In this study, we investigated the kinetics of changes of the DNA secondary structure, of the DNA modification degree, and of the formation of interstrand cross-links caused by these complexes in comparison to the parental compound *cis*-diamminedichloroplatinum-(II) (cisplatin). All examinations were performed at physiological pH 7.4 and at pH 6.0 mimicking the acidified environment of many tumor tissues. In general, cisplatin displayed a higher reactivity accompanied by more pronounced DNA compaction, untwisting, and formation of interstrand cross-links at both pH values. Additionally, it was shown for the first time that cisplatin generates interstrand cross-links faster at pH 6.0 than at 7.4. However, the difference between pH 7.4 and 6.0 was much larger for KP1356 and KP1433 than for cisplatin, since they were essentially nonreactive and induced almost no secondary structures at pH 7.4, as contrasted to cisplatin. Our data suggest that formed adducts, i.e., intra- and/or interstrand cross-links, may be the sole cause of the cytotoxicity of KP1356 and KP1433 at pH 6.0. The results of this study may stimulate and contribute to further improvement of these novel, specific cytotoxic drugs that are anticipated to exert their full power in the tumor while being reasonably inactive in normal tissue.

The anticancer drug cisplatin is one of the most successful anticancer drugs, mainly used for the treatment of testicular and ovarian cancer. However, therapy with cisplatin is often accompanied by severe side effects (e.g., nephrotoxicity and ototoxicity), innate or acquired resistance, and a comparatively narrow spectrum of application. To overcome these problems, great efforts were undertaken in the last decades to synthesize new complexes based on platinum. However, among thousands synthesized and a few analogues in clinical trials, besides cisplatin, only two are in worldwide routine clinical use today: carboplatin and oxaliplatin. Therefore, there is still great interest to generate improved, i.e., more efficient, more specific, and thus less toxic, cisplatin derivatives. An approach to gain control of the issues of specificity and toxicity is the "prodrug concept", i.e., creation of platinum complexes that should exert their activity mainly or exclusively in the tumor tissue exploiting the distinguishing features of the latter. Besides distinctive genetic differences compared to healthy cells, solid tumors are, for instance, often hypoxic due to insufficient blood supply (*1*, *2*). Thus, with increasing tumor size, the intra- and extracellular pH values decrease due to the production of large amounts of lactate during anaerobic glycolysis. Acidic conditions in tumor tissues as low as pH 5.5 are known. Consequently, such a prodrug is supposed to be activated in the resultant acidic environment of the tumor, in contrast to having lower or no reactivity in normal tissue.

In addition to the desired higher activity and thus specificity of the said kind of drugs against cancer cells, additional benefits may result from compounds directed specifically against hypoxic cells. First, very hypoxic tumors often follow an aggressive clinical course (*3*), facilitated by the expression or upregulation of several hypoxia-regulated proteins. These include growth factors governing the formation of new blood vessels and hypoxia-responsive transcription factors modulating the expression of genes, which promote tumor cell survival and metastasis formation (*2*). Second, it is known that hypoxic cells have numerous alterations in metabolic activity, which may lead to increased damage (*4*, *5*) and/or inefficient repair (*6*) of DNA. Decreased DNA repair is even more pronounced when hypoxia is accompanied by low pH (*4*, *7*). This may be due to the alteration of the structure and

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[‡]Dedicated to Prof. Bernhard Lippert on the occasion of his 60th birthday.

* Corresponding authors. (S.Z-S.): Telephone: +49-8985782367; fax, +49-8985783557; e-mail: seifried@biochem.mpg.de; (H.Z.) Telephone: +49-8989967926; fax, +49-8989967979; e-mail, zorbas@bio-m.de.

[||] Max-Planck Institute of Biochemistry.

[§] Institute of Inorganic Chemistry.

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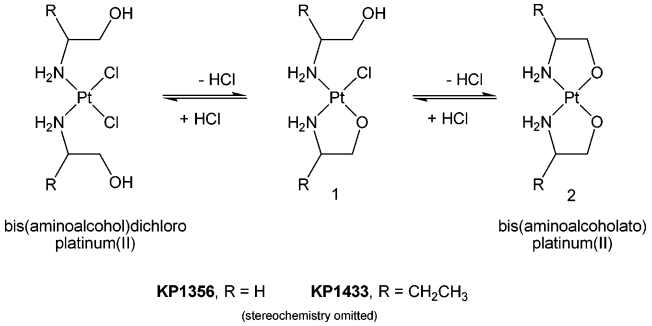

FIGURE 1: pH-dependent equilibrium of KP1356 and KP1433 resulting in single (1) and double (2) ring-closed species.

the function of a variety of cellular proteins (*8*, *9*) likely including repair enzymes. Consequently, exposure to hypoxia and its associated low pH have been shown to increase mutation frequencies (*10*) and, thus, to augment emergence of resistance against cytotoxic drugs such as cisplatin (*2*, *11*, *12*). For these reasons, development of prodrugs predominantly effective against hypoxic cancer cells may turn out not only to serve high specificity but also to be a quite an intelligent method—maybe in combination therapies with modern antiangiogenetic approaches—for preventing emergence of particularly harmful, i.e., metastatic and/or resistant, cancer cells.

DNA is the commonly accepted ultimate target of platinum complexes. Hence, to this end, compounds that may be expected to be rather inert in aqueous solution at pH 7.4 but reactive against DNA in acidic milieu have been synthesized. This concept, originated by Sadler and co-workers, led to synthesis of three classes of platinum-based complexes of this kind so far, i.e., platinum(II) complexes with two chelating aminophosphine (*13*−*15*), *O*-alkyldithiocarbonato (*16*, *17*), or aminoalcoholato ligands (*18*, *19*). These compounds were designed in a way that, at neutral or basic pH, the valences of the central platinum atom are blocked by intramolecular chelation. However, a shift of the pH to the acidic area should liberate the platinum, rendering it reactive against external ligands. In fact, all three types of complexes were shown to be activated in acidic media via ring-opening (for the aminoalcoholato complexes, cf. Figure 1; for a recent review see ref *20*). Most importantly, all investigated complexes of the bis(*O*-alkyldithiocarbonato)platinum(II) type were more cytotoxic at the acidic pH than at pH 7.4 (*16*). The bis(2-aminoalcoholato)platinum(II) type compounds were shown to become about 3-fold more cytotoxic against the human non-small cell lung cancer cell line A549 upon acidification of the growth medium (*19*). Consequently, these complexes indeed may become more reactive toward the cells' DNA at the lower pH.

However, before reaching and reacting with the cells' DNA, the complexes have to overcome several barriers such as cellular uptake; inactivation by blocking reagents in the cytoplasm; and efficient reaction with the DNA under nuclear conditions. In principle, lowering the pH may influence each one of these processes resulting in superior cytotoxicity. Moreover, mere enhanced reactivity with cellular DNA might not hold for increased cytotoxicity, if the adducts are readily removed by repair systems. Instead, formation of repair-resistant adducts that actively lead to programmed cell death is critical for the activity of the compounds (*21*). To help

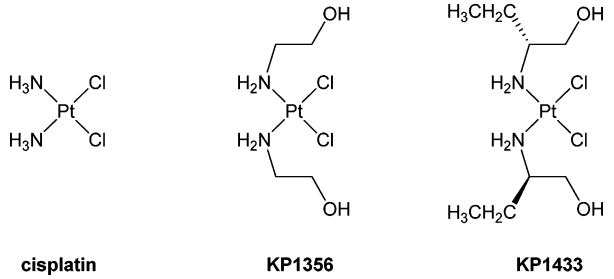

FIGURE 2: Structural formulas of platinum complexes investigated in this study and the parental compound cisplatin.

discern these possibilities, we analyzed the interaction of two compounds of the bis(2-aminoalcohol)platinum(II) type, KP1356 and KP1433,[1] as well as of the parental compound cisplatin (Figure 2) with dsDNA at pH 7.4 and 6.0. Our data support the notion that the sole or the main reason for the *whole* effect, i.e., enhanced cytotoxicity at pH 6.0, may be formation of potentially cytotoxic DNA adducts at this pH. These results contribute to the understanding of the molecular mode of action of these compounds and may aid the development of even more active complexes of this class.

EXPERIMENTAL PROCEDURES

Starting Material. Synthesis and characterization of KP1433 and KP1356 have been described (*18*, *22*). Cisplatin was purchased from Degussa. For all examinations, a stock solution of these drugs was prepared in doubly distilled water and stored immediately at −20 °C. Plasmid pTZ18u (2860 bp) was from Biorad. Plasmid P5 (3016 bp) was a gift from Dr. M. Ried. The plasmids were transformed in XL1 blue cells, isolated, and purified according to standard procedures and dissolved in TE buffer. Restriction endonuclease PvuII and the Klenow fragment of DNA polymerase I were purchased from New England Biolabs. Restricition endonuclease EcoRI and molecular weight marker GeneRuler 50 bp were from Fermentas. All radioactive products were purchased from Amersham Biosciences.

Changes in DNA Secondary Structure and DNA Modification Degree. Plasmid P5 was cleaved with EcoRI and PvuII to generate a linear double-stranded 177 bp fragment. The fragment was eluted from an agarose gel after electrophoretic separation, and 3′-end-labeled by the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP.

For each time point of the kinetics analysis, 1 μg of plasmid pTZ18u and 1.6 fmol of radioactively end-labeled 177 bp fragment and either one of the compounds at a final concentration of 60 μM were incubated separately in 10 mM phosphate buffer at pH 6.0 or pH 7.4 in a final volume of 40 μL at 37 °C. For detection of changes in DNA secondary structure, 5 μL of 5× "blue juice" sample buffer (final: 2.5% glycerol, 0.5% SDS, 10 mM EDTA, 0.025% bromphenol

[1] Abbreviations: KP1356, (*SP*-4-2)-bis(2-aminoethanol)dichloroplatinum(II); KP1433, (*SP*-4-2)-bis[(*R*)-(−)-2-aminobutanol)]dichloroplatinum(II); cisplatin, *cis*-diamminedichloroplatinum(II); carboplatin, diammine(cyclobutane-1,1-dicarboxylato(2-)-*O*,*O*′)platinum(II); oxaliplatin, [(*1R*,*2R*)-cyclohexanediamine-*N*,*N*′][oxalato(2-)-*O*,*O*′]platinum-(II); ICLs, interstrand cross-links; bp, base pair; ds, double stranded; ss, single stranded; ICP-MS, inductively coupled plasma mass spectrometry; sc, supercoiled; oc, open circular; CZE, capillary zone electrophoresis; ESI, electrospray ionization; EtBr, ethidium bromide; IC_{50}, 50% inhibitory concentration.

blue, 0.025% xylene cyanol) were added to a 20 μL aliquot of a specific time point. The reaction products were separated immediately in a 1% agarose gel in TBE buffer at 3 V/cm. The gel was stained with 0.2 μg/mL EtBr in 1× TBE, illuminated by UV light and photographed using a gel documentation system from Vilber Lourmat (Torcy Z.I. Sud, France). To visualize the DNA modification degree, 10 μL aliquots of each time point were mixed with 2.5 μL 5× "blue-juice" sample buffer. The samples were analyzed in a 4% polyacrylamide gel in 1× TBE buffer, 0.1% SDS at 15 V/cm. After electrophoresis, the gel was fixed in 7% acetic acid, 4% glycerol for 20 min and dried for 2 h at 65 °C under vacuum. The gel was exposed to an X-ray film overnight at −70 °C. Analyses of DNA secondary structure and of DNA modification degree were performed at least 3 times with virtually identical results.

To quantify the DNA platination extent, 4 μg of plasmid pTZ18u were incubated separately for each time point with KP1356, KP1433, or cisplatin at a final concentration of 60 μM in 160 μL of 10 mM phosphate buffer pH 7.4 or pH 6.0 at 37 °C. After addition of 5 μL of "blue-juice" sample buffer to a 20 μL aliquot of each reaction, the products were analyzed in a 1% agarose gel to reproduce the DNA secondary structures. To the remaining fraction, NaCl to a final concentration of 250 mM was added immediately after withdrawal of the 20 μL aliquots. DNA precipitation of this fraction with 2.5× vol ethanol was performed in presence of 75 μg/mL linear polyacrylamide. The amount of bound platinum of the dissolved pellet was determined by ICP-MS (Agilent 7500ce, Waldbronn, Germany; equipped with a CETAC ASX-520 autosampler, and a MicroMist nebulizer; software, Agilent ICP-MS Chemstation B.03.02, Microsoft Excel 2003 SP2). The r_B values were calculated by assuming 3.5 μg of DNA in each sample, which was dissolved in 1 mL of 2% HCl (HCl p.a. from Sigma Aldrich, further purified with a quartz sub-boiling system from Milestone-MLS GmbH, Leutkirch, Germany, 18.2 MΩ H_2O was obtained from a Synergy 185, Millipore, Bedford, MA) and 200 μL of each sample were filled up to 9 mL with 2% HCl. For internal standardization 1 mL of a 10 ppb In solution (CPI International, Santa Rosa, CA) was added. According to DIN 32645, the limits of detection and quantification for platinum were determined at 34 ng/L (r_B 0.0008) and 86 ng/L (r_B 0.002), respectively. For r_B determination, the reactions for each time point were performed twice, and each time point was determined 10 times to calculate the average r_B value.

Interstrand Cross-Link Assay. To analyze the ability of KP1356, KP1433, and cisplatin to form interstrand cross-links (ICLs) at pH 7.4 and pH 6.0, 0.8 fmol of radioactively end-labeled 177 bp fragment (see above) were incubated in 10 mM phosphate buffer pH 7.4 or pH 6.0 as described above at a final concentration of 60 μM and a final volume of 20 μL per sample. After incubation, all samples were instantly evaporated to complete dryness in a speed vac and resuspended in 10 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol blue), heated for 3 min at 95 °C and chilled in ice. The reaction products were separated in a denaturing 4% polyacrylamide gel, 7 M urea, 1× TBE, at 10 V/cm for 1 h. After fixing the gel in 7% acetic acid and 4% glycerol for 20 min and drying for 2 h at 65 °C under vacuum, it was exposed to an X-ray film at −70 °C for an appropriate time. ICL assays were repeated at least 2 times.

RESULTS

Induced Secondary Structures of DNA and Reactivity. It is largely documented that platinum-based complexes can untwist, locally melt, and/or bend dsDNA, depending on the kind of the specific adducts formed on DNA (*23*); for example, monofunctional or intercalating adducts may untwist dsDNA, whereas bifunctional adducts (intra- as well as interstrand cross-links), in addition, bend DNA. Conversely, analyzing the DNA secondary structure may provide valuable clues about the kind of the DNA adducts. Changes of DNA secondary structures can be easily monitored by evaluating the electrophoretic migration pattern in neutral agarose gels of a circular dsDNA plasmid prepared conventionally. Adducts that untwist dsDNA effect a slower migration of the negatively "supercoiled form" (sc) of the plasmid due to partial relief of the torsional stress and consequent relaxation of the compact sc form; a faster migration of the nicked, "open circular" (oc) form of a plasmid, on the other hand, is consistent with adducts that compact or apparently "condense" dsDNA (*24*).

Hence, to examine whether and how fast adducts of platinum complexes that alter the structure of dsDNA can be formed, we recorded the kinetics of the induced mobility alterations of the forms of a plasmid after reaction with these complexes at different pH values. However, since different DNA-interacting drugs may induce secondary structure changes of different magnitude, monitoring the kinetics of secondary structures does not necessarily reflect the degree of DNA modification or reactivity of the drug. Therefore, an additional direct control of reactivity was performed by including a minute amount of a linear, radioactively end-labeled 177 bp dsDNA fragment in each reaction. Increased molecular weight and accumulation of positive charges effect a mobility shift in a neutral polyacrylamide gel; tracking the kinetics of this mobility shift reflects the modification degree of all DNA in the reaction. Thus, this experimental setup allows for simultaneous analysis of a drugs' reactivity as well as of the possible kind of adducts. Typical results are shown in Figures 3−5.

Figure 3A shows the electrophoretic pattern of plasmid DNA incubated at 1 h time intervals up to 7 h with 60 μM KP1356 at pH 7.4. Alterations of DNA secondary structure could be barely detected apart from a very weak relaxation visible only after incubation time of 7 h. The slow formation of secondary structures of KP1356 was paralleled by a poor shifting of the radioactive fragment. In contrast, already after only 1 h of incubation at pH 6.0, pronounced relaxation of the sc form and mobilization of the oc form of the plasmid were evident (Figure 3C). This marked increase of formation of adducts that alter the DNA structure was mirrored in an immediate, prominent shifting of the radioactive fragment. By comparing the extent of shifting, we may conclude a higher reactivity of KP1356 of about 1 order of magnitude (∼10-fold) at pH 6.0 vs 7.4.

KP1433 apparently displayed no noticeable reactivity with DNA at pH 7.4 even after 7 h incubation and consequently no induction of DNA secondary structure whatsoever (data not shown). These results, however, were contrasted with

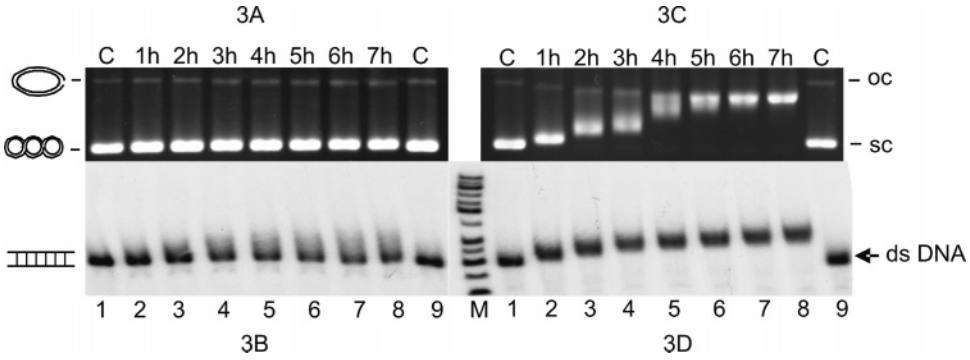

FIGURE 3: Interaction of KP1356 with dsDNA. Products of plasmid pTZ18u (A & C) and of a linear, radioactively labeled 177 bp DNA fragment (B & D) after incubation with 60 μM KP1356 at pH 7.4 (A & B) or pH 6.0 (C & D) for the indicated time points (lanes 2−8) and electrophoretic separation; A & C, 1% agarose gel stained with EtBr; B & D, autoradiograph of a dried, neutral 4% polyacrylamide gel. C (lanes 1), control DNA, not incubated; C (lanes 9), control DNA, mock incubated for 7 h; M: DNA molecular weight marker GeneRuler 50 bp.

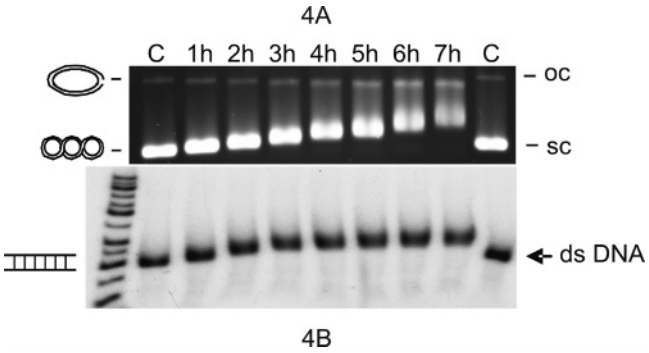

FIGURE 4: Interaction of KP1433 with dsDNA. Products of plasmid pTZ18u (A) and of a linear, radioactively labeled 177 bp DNA fragment (B) after incubation with 60 μM KP1433 at pH 6.0 for the indicated time points (lanes 2−8) and electrophoretic separation; A, 1% agarose gel stained with EtBr; B, autoradiograph of a dried, neutral 4% polyacrylamide gel. C (lane 1), control DNA, not incubated; C (lane 9), control DNA, mock incubated for 7 h; M: DNA molecular weight marker GeneRuler 50 bp.

data obtained at pH 6.0 (Figure 4). Gradual increase in reactivity accompanied by enhanced induction of DNA secondary structures (relaxation of the sc form and condensation of the oc form) was clearly visible already after 1 h reaction time. Comparison of Figure 4B with Figure 3D allows the conclusion that, at pH 6.0, KP1433 reacted about 2-fold slower with DNA than KP1356 possibly due to steric hindrance caused by the additional ethyl groups. Secondary structures were also formed with a 2-fold delay, as compared to KP1356.

We also determined the pH dependence of the interaction of DNA with the parental drug cisplatin. As shown in Figure 5, cisplatin experienced a boost in reactivity at pH 6.0 vs pH 7.4 too, documented by the increased shift of the radioactive fragment, accompanied by accelerated relaxation and condensation of the circular DNA. We estimated this difference to be only slightly greater than 2-fold. After 2 h of incubation at pH 6.0, cisplatin generated positive supercoils of the plasmid. Compared to the aminoalcohol platinum complexes, cisplatin appeared several orders of magnitude more reactive with DNA at pH 7.4. However, as evident from the shifting of the fragment at pH 6.0, cisplatin was only about 2 times more reactive than KP1356, and approximately 4 times more reactive than KP1433.

Overall, the increase with time of secondary structure induction of all investigated complexes corresponded quite precisely to the rise of the radioactive fragment. Hence, the faster formation of DNA secondary structures was likely a result of an increase in reactivity (equivalent to the degree of platination), rather than an altered mode of reaction with DNA, for example, an inherent increase of the capability of formation of bifunctional adducts at a lower pH. However, in spite of the higher reactivity of the aminoalcohol complexes at pH 6.0, careful inspection of the mobility of the oc form of the plasmid after reaction with the aminoalcohol compounds (Figures 3C and 4A) revealed that condensation was in general less pronounced compared to that of cisplatin (Figure 5C) at corresponding relaxation degrees. A possible reason for this is suggested in the Discussion.

Determination of r_B Values. To examine, whether our deductions regarding reactivity would also stand up to a method, which quantifies the modification degree of the DNA more accurately, the amount of the platinum load on the plasmid was determined by ICP-MS and the r_B values (Pt atoms per nucleotide) were calculated as well. Binding of aminoalcohol complexes to DNA at pH 7.4 was at a rather lowest limit of detection and not well above background. This hampered quantitative comparison with cisplatin. Hence, we waived evaluation of r_B values at pH 7.4. Results of a series of representative determinations of r_B values at pH 6.0 are shown in Figure 6. Comparison of the modification of DNA by KP1356 and KP1433 at this pH revealed a difference of ∼2−3-fold in favor of KP1356, in accordance with the results of the gel migration analysis (see previous section). Moreover, the determined r_B values at pH 6.0 verified the previous observation by gel analysis of a ∼2-fold higher reactivity of cisplatin vs KP1356 as well as of a ∼3−4 times higher reactivity of cisplatin vs KP1433. In conclusion, determinations of trends in r_B values essentially mirrored the results obtained by analyses in the gel electrophoresis system.

Formation of ICLs. In general, DNA bending leading to apparent condensation may be effected by several kinds of adducts, e.g., intra- (*25*, *26*) and interstrand cross-links (*27*−*31*). The experimental setup with neutral agarose gels does not allow discerning these possibilities. Therefore, the question whether KP1356 and KP1433 are able to form cross-links between the two DNA strands and how fast was approached by separate, independent experiments. For this purpose, the linear, radioactively end-labeled dsDNA frag-

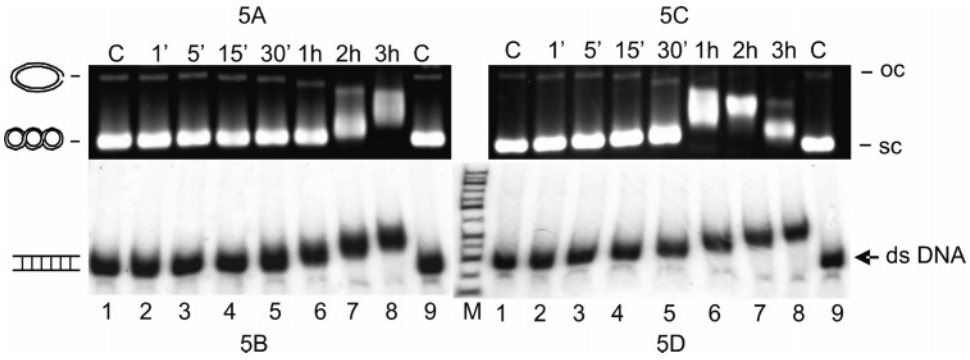

FIGURE 5: Interaction of cisplatin with dsDNA. Products of plasmid pTZ18u (A & C) and of a linear, radioactively labeled 177 bp DNA fragment (B & D) after incubation with 60 μM cisplatin at pH 7.4 (A & B) or pH 6.0 (C & D) for the indicated time points (lanes 2−8) and electrophoretic separation; A & C, 1% agarose gel stained with EtBr; B & D, autoradiograph of a dried, neutral 4% polyacrylamide gel. C (lanes 1), control DNA, not incubated; C (lanes 9), control DNA, mock incubated for 3 h; M: DNA molecular weight marker GeneRuler 50 bp.

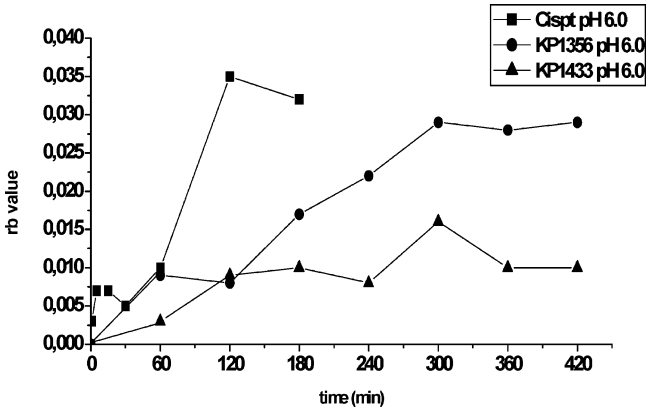

FIGURE 6: Graphic representation of the determined r_B values at pH 6.0 of cisplatin (■), KP1356 (●), and KP1433 (▲) at the indicated time points of incubation.

ment was analyzed in denaturing urea−polyacrylamide gels after reaction with the compounds for different times. In this system, the DNA single strands run separately to the lower regions of the gel; however, cohesion of the two complementary strands caused by at least one ICL retards their migration giving rise to discrete upward shifts representing unstructured, bulky drug−DNA complexes.

Results of the reaction of the fragment with KP1356 are shown in Figure 7A,B. Lowering the pH from 7.4 to 6.0 effected a drastic acceleration of ICL formation by a factor of more than 7. Apparently, the efficacy of ICL formation quite precisely reflected that of KP1356's general reactivity. Beyond 2 h of incubation at pH 6.0, all DNA bore at least one ICL.

KP1433, on the other hand, produced hardly any ICLs at pH 7.4 (Figure 7C), also paralleling its extremely weak reactivity at this pH. Upon acidification of the reaction conditions, however, ICL formation went faster, such that no DNA void of an ICL was left after 7 h of incubation. With regard to kinetics, KP1433 appeared to be about 2−4 times slower in ICL formation than did KP1356 at pH 6.0.

Investigation of the pH-dependence of cisplatin to form ICLs yielded the results shown in Figure 8. At pH 6.0, formation of ICLs by cisplatin was accelerated by more than 1 order of magnitude as compared to pH 7.4. This is slightly faster than that of KP1356, and about 1−2 orders of magnitude faster than that of KP1433. Increasing incubation led to gradually *faster* migration of the ICL-connected strands, best evident with cisplatin beyond 30 min (Figure 8B, lanes 5−8). We interpret this by assuming an increasing compactness of the DNA strands bearing more ICLs, thus preserving the form and migration properties of the linear dsDNA.

Since we did not relate increasing ICL formation at pH 6.0 to the accurate amount of adducts at each time point, we cannot distinguish unequivocally whether enhanced ICL formation was due to a higher *level of platination* or a *higher proficiency* of a compound to form ICLs at a certain level of platination. However, since the faster ICL formation paralleled the increase of the general reactivity of the compounds at pH 6.0 (see previous sections), we rather suggest a higher level of platination to be responsible. In conclusion, we have shown that both KP1356 and KP1433 can form ICLs. While ICL formation speed is rather low at pH 7.4, like that of cisplatin, it is greatly enhanced at pH 6.0, regardless of the actual reason.

DISCUSSION

In this report, we showed that, at pH 6.0, KP1356 and KP1433 readily formed adducts that effected visible DNA relaxation, DNA condensation, and DNA interstrand cross-linking. In contrast, such structures were much less or almost non-apparent at pH 7.4. Relaxation of the sc DNA was obviously effected by local untwisting at the sites of adducts. Condensed circular DNA modified with platinum complexes, first described for cisplatin and transplatin by Cohen et al. (*24*), was recognized by Bellon et al. as caused by *multiple bends* not in phase with the DNA periodicity leading to apparent diminished diameter of circular DNA (*32*). It must be emphasized that, in this context, a "bend" might be a *rigid*, directed DNA curve as well as a *flexible* hinge joint caused, for example, by the 1,3(dGpTpG) intrastrand adduct of transplatin, which can bend in at least two directions (*33*), or by "small loops", i.e., locally melted DNA of restricted extent, that may be formed at adducts sites. We cannot distinguish between the two variants of bending, stable or flexible, in this study.

In accordance with numerous investigations of platinum compounds, the detected untwisting is consistent with formation of monofunctional adducts at purine nucleobases. Bending, as defined above, may be caused by a variety of adducts. In general, platinum compounds with the cis geometry effect bending by bifunctional adducts, either intra-

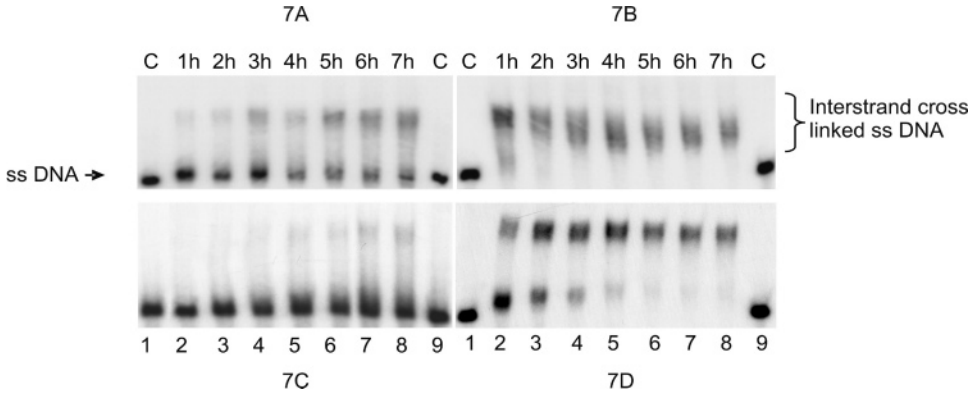

FIGURE 7: Formation of ICLs by KP1356 and KP1433. Products of a linear, radioactively labeled 177 bp DNA fragment after incubation with 60 μM KP1356 or KP1433 at pH 7.4 (A & C, respectively) or pH 6.0 (B & D, respectively) for the indicated time points (lanes 2−8) and electrophoretic separation in a denaturing 4% polyacrylamide gel; the panels depict the autoradiograph of the dried gels. C (lanes 1), control DNA, not incubated; C (lanes 9), control DNA, mock incubated for 7 h.

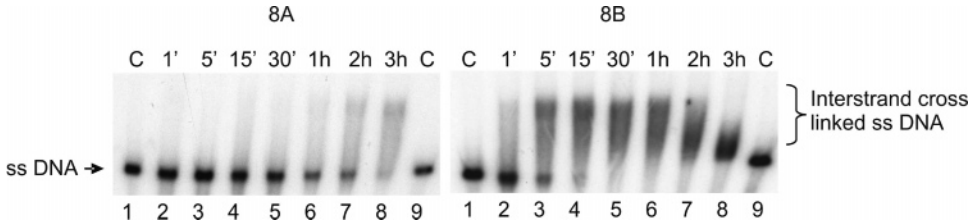

FIGURE 8: Formation of ICLs by cisplatin. Products of a linear, radioactively labeled 177 bp DNA fragment after incubation with 60 μM cisplatin at pH 7.4 (A) or pH 6.0 (B) for the indicated time points (lanes 2−8) and electrophoretic separation in a denaturing 4% polyacrylamide gel; the panels depict the autoradiograph of the dried gels. C (lanes 1), control DNA, not incubated; C (lanes 9), control DNA, mock incubated for 3 h.

(*25*, *26*) or interstrand cross-links (*27*−*31*). In addition, some cytotoxic compounds with the trans geometry have been reported to effect bending by monofunctional coordination to the central platinum atom (*34*−*37*). However, at least for the trans compounds with heterocyclic ligands, (stacking) interactions with DNA of these ligand(s) were proposed and/or modeled, which might give rise to *pseudobifunctional* adducts (*34*). Be that as it may, to our knowledge, there is no report claiming that monofunctional adducts of platinum complexes with the cis geometry may effect bending. Therefore, we conclude that the detected bending is a likely indication that KP1356 and KP1433 are able to form bifunctional adducts. Moreover, all mononuclear platinum complexes capable of bifunctional binding to DNA may react with close nucleobases on the *same* DNA strand producing intrastrand cross-links. Formation of interstrand cross-links, which may also bend dsDNA, was shown directly (Figures 7 and 8). In conclusion, KP1356 and KP1433 likely generate monofunctional adducts as well as bifunctional, intra- and interstand cross-links on dsDNA.

Accumulating evidence in the literature suggests that, to act cytotoxically, a platinum compound has, first, to form adducts that *persist* on DNA, i.e., which efficiently escape the DNA repair system(s); and second, to activate sensors that convey the DNA damage to a cell death signal (*38*, *39*). In spite of intensive research, the ultimate cytotoxic lesion, or lesions, of cells treated with the parental drug cisplatin remains elusive. Both intrastrand cross-links, like the 1,2-d(GpG) adduct of cisplatin, because of its unique structural features of which the main characteristic is a rigid, directed bend of 30−35 degrees into the major groove of dsDNA (*40*, *41*), as well as interstrand cross-links, which are dealt with by a different repair system than the latter, have been supposed to be cytotoxic lesions (*42*, *43*). Here, we showed that the aminoalcohol−platinum complexes, particularly KP1356, readily generated abundant ICLs at pH 6.0. On the basis of these considerations and by analogy, we hypothesize that each one or both kinds of bifunctional adducts (intra- and/or interstrand cross-links) may be the reason for the cytotoxic properties of KP1356 and KP1433.

Formation of lesions of KP1356 and KP1433 was found to be at least 10 times faster at pH 6.0 than at pH 7.4, yet cytotoxicity in the human non-small cell lung cancer cell line A549 was reported to be only 2.4−3.3-fold higher at pH 6.0 than at standard pH conditions (*19*). Hence, although we cannot conclusively eliminate further enhancing factors, the determined faster formation of potentially cytotoxic adducts on DNA is more than sufficient to serve as the sole explanation for the higher cytotoxicity at the acidic pH. Moreover, KP1356 and KP1433 seemed to be even partially prevented from exerting their whole potential at the more acidic pH. Faster repair of the adducts of these compounds, as a possible reason for this apparently paradoxical effect, would contradict work of other authors reporting that acidic intracellular milieu rather compromised efficient DNA repair (*4*, *6*, *7*) (see introduction). Another reason may be simply a reduced reactivity of the compounds inside the cells, the pH of which has been reported to be in general less acidic than that of an artificially acidified medium (*44*). Interestingly though, artificial acidification of cultured cells may also reduce the intracellular pH to a value *lower* than extracellular pH (*45*). Still an alternative suggestion may be that neutral or basic pH may favor the generation of neutral hydroxo metabolites, whereas at the more acidic pH, the compounds may generate charged aqua species (by replacing chloro ligands of mono or doubly ring-opened complexes). The

positively charged complexes should be hindered from penetrating the cell membrane (*46*). Analogous events were proposed to explain uptake and reactivity of cisplatin at the different pH values (*47*). Future experiments measuring uptake of compounds at different pH values, intracellular pH and accumulation as well as in vivo DNA modification should shed more light onto this issue.

KP1433, displaying two additional ethyl groups in comparison to KP1356, was synthesized by Keppler and coworkers (*18*) with the purpose to increase the lipophilicity and likely the cell membrane passage of the latter. However, cell culture experiments showed that this chemical modification of KP1356 resulted in a slight *decrease* at pH 6.0 (IC_{50} 55.0 vs 45.6 μM) and a greater *decrease* at pH 7.0 (IC_{50} 182.0 vs 110 μM) of cytotoxicity (*19*). Our results with KP1433 of both decreased DNA reactivity and accompanying alterations of DNA secondary structures (particularly bending) may indicate that, in spite of a possibly improved cell penetration of KP1433 vs KP1356, efficient formation of cytotoxic DNA adducts was nevertheless impaired.

To our knowledge, only the reaction of KP1356 with DNA and exclusively at neutral pH has been investigated so far (*48*). In this study, KP1356 reacted very slowly with calf thymus DNA at pH 7.2. More extensive investigations by different methods concerning reactivity of KP1356 and KP1433 have previously been assessed by reaction with the model nucleotide 5′-GMP. These studies confirmed slow reaction of KP1356 or KP1433 with 5′-GMP at neutral pH but fast reaction at pH 5.5−6.0 by different factors up to roughly 1 order of magnitude (*19*, *48*−*50*). In the cited studies, KP1356 was in general more reactive than KP1433 at either pH. Interestingly, published data showed that, upon reaction with 5′-GMP at pH 6.0, the first and dominating adduct of the complexes is a monoadduct of the mono ring-closed species resulting from the substitution of one chloro ligand by 5′-GMP. The Pt-GMP bisadduct was also gradually formed but, within the analysis time, never reached the magnitude of the monoadduct (*49*, *50*). These findings may serve an explanation for the observed slower mobilization of the oc form of the plasmid after reaction with the aminoalcohol compounds vs cisplatin (cf. Results). Presupposing that the detected DNA condensation is due to formation of bifunctional DNA adducts, the inefficient formation of the Pt-GMP bisadduct might indicate that reactivity of the second platinum valence of KP1356 and KP1433 and ensuing closure to bifunctional adducts on DNA is hampered.

In conclusion, previous work with 5′-GMP greatly agrees with the trends regarding reactivity of KP1356 and KP1433 determined in our report. However, investigations of reactivity with 5′-GMP and determination of the r_B value may not mirror faithfully the rate of formation of DNA adducts and, most significantly, they do not give consideration to KP1356 and KP1433 as cytotoxic and possibly antitumor drugs. Therefore, the results obtained by the present methodology not only substantiate previous findings concerning reactivity but also may contribute to the elucidation of the molecular mechanism of these compounds regarding their biological and pharmacological action.

What might be the reason for the detected general lower reactivity of the compounds at pH 7.4 vs 6.0? As shown by NMR spectroscopy, at pH 7.4 KP1356 and KP1433 undergo intramolecular ligand exchange reactions resulting in metabolites with one or two chelating aminoalcoholato ligands (ring-closed species 1 and 2; Figure 1) (*18*, *20*, *46*, *50*). Masking the central platinum atom may lead to the observed drastic reduction of reactivity. In contrast, cisplatin which lacks such ligands fails to block the platinum center with the consequence that it stays greatly reactive at pH 7.4. On the other hand, pH 6.0 favors the original, ring-opened structure of KP1356 and KP1433 yielding the detected higher reactivity. A further cause for the enhanced reactivity of all three complexes, KP1356, KP1433, and particularly cisplatin at pH 6.0 may derive from the fact that the above postulated positively charged, aquated metabolites at this pH, although disadvantageous for cell entry, may react more readily with nucleophilic sites on DNA than neutral, hydroxo species generated at more basic pH.

The positive impact of low pH on cytotoxicity, accumulation in the cytoplasm and in vitro reactivity of cisplatin traced in our report as a control is already known (*44*, *47*, *51*, *52*). Regarding cisplatin adducts, to our knowledge, only formation of interstrand and protein−DNA cross-links in vivo has been compared at different pH values (*44*). Although total DNA cross-linking increased almost 2-fold in cells cultured in pH 6.0 medium compared to that in cells cultured in pH 7.2 medium, ICLs were not found to be significantly more frequent at the more acidic pH. However, the ICLs determined in that work were the result of reaction of cisplatin with *and removal* from the cellular DNA, and, therefore, they do not reflect directly the efficiency of their formation. With our method instead, formation of ICLs was determined as such, and enhancement at pH 6.0 was clearly visible. Hence, to our knowledge, this is the first unambiguous demonstration that cisplatin forms considerably more ICLs at a slightly acidic than at basic/neutral pH. This novel finding may prove useful in attempts to understand cellular processing of cisplatin.

What is the distinctive advantage of developing pH-sensitive platinum complexes? The higher reactivity and ensuing faster formation of potentially cytotoxic adducts with DNA of KP1356 and KP1433 at pH 6.0 is excelled by parental cisplatin. Therefore, we deem their poor reactivity at pH 7.2−7.4 even more relevant in the effort to combat cancer with as few adverse side effects as possible. Cisplatin, in sharp contrast, still displays an appreciable reactivity at neutral pH. Moreover, the paradigm of KP1433 differing from KP1356 by two additional ethyl rests demonstrates that the gap between pH 6.0 and 7.4 in reactivity and cytotoxicity may be further enlarged by appropriate chemical modifications (*19*). An additional beneficial level of selectivity may derive from the enhanced formation of ICLs by KP1356 and KP1433 at low pH, which may confer on these compounds a desired organotropism toward cells that depend on homologous recombination for their proper function (*42*). Because of all these features, bis(aminoalcohol)dichloroplatinum(II) complexes may develop to highly selective antitumor compounds with particular potency against aggressive and resistant cancer cells but with low adverse side effects toward healthy cells and/or nontargeted organs.

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