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Kinetics of binding properties of 5′-GMP with cisplatin under simulated physiological conditions by capillary electrophoresis

Andrea Zenker[a], Markus Galanski[a], Thomas L. Bereuter[a], Bernhard K. Keppler[a,*], Wolfgang Lindner[b]

[a]*Institute of Inorganic Chemistry, University of Vienna, Währinger Strasse 42, A-1090 Vienna, Austria*
[b]*Institute of Analytical Chemistry, University of Vienna, Währinger Strasse 38, A-1090 Vienna, Austria*

Abstract

Kinetics of the adduct formation of cisplatin and its hydrolysis products with 5′-GMP was investigated using capillary electrophoresis (CE) under 'simulated physiological conditions'. Therefore, chloride ion and phosphate concentration as well as the pH were chosen comparable to intracellular conditions. Furthermore, the influence of the sulfur-containing α-amino acids L-methionine and L-cysteine on the kinetics of the adduct formation have been studied. Both amino acids increased the half-time of the reaction significantly but did not influence the formation of the major adduct *cis*-$[Pt(NH_3)_2(N7\text{-}GMP)_2]^{2-}$. The reaction products were separated using CE, and characterised using UV and NMR spectroscopy.

Keywords: Cisplatin; Kinetics; Nucleoside monophosphates; Capillary zone electrophoresis; Methionine; Cysteine

1. Introduction

Cisplatin, *cis*-$[Pt(NH_3)_2Cl_2]$, is a frequently used anticancer drug successfully applied for the treatment of various malignancies [1]. Best results are obtained for cases of testicular and ovarian cancer [2]. The antitumour activity of platinum anticancer drugs is due to the platination of the bases of DNA with a clear preference for guanine. Adduct formation, based on intrastrand chelation involving two adjacent guanines, triggers DNA degradation and apoptosis [3–6]. The reaction follows a two-step mechanism and rate-determining for the initial binding to DNA is the hydrolysis of the first chloride ion of cisplatin [7–11]. The activated complex *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ coordinates primarily to the N7 position of guanine as well as to a lower extent to adenine [3,11–13]. Hydrolysis of the second chloride results in coordinative binding to a second base enabling bifunctional adducts. The ring closure of monofunctional to bifunctional adducts has been investigated by different methods [14–18]. Values of the half-time determined for the ring closure strongly depend on the applied method as well as on the conditions. For the determination of rate constants pseudo-first-order kinetics have been assumed, mostly under acid conditions (pH about 4) [19–21]. These assumptions are of importance with regard to the evaluation of the complexity but at the same time these simplifications may reduce to some extent the relevance in biological systems.

*Corresponding author. Tel.: +43-1-4277-52600; fax: +43-1-4277-52680.

E-mail address: Bernhard.Keppler@ap.univie.ac.at (B.K. Keppler).

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However, interactions between platinum complexes and DNA were studied using general model systems (e.g. oligonucleotides, calf thymus DNA, nucleoside monophosphates) and different analytical techniques (high-performance liquid chromatography [HPLC], nuclear magnetic resonance [NMR], fast protein liquid chromatography [FPLC]) [7–11,22–24].

Recently, we have shown that capillary electrophoresis (CE) combined with a diode array detector (DAD) is a very suitable analytical technique to investigate interactions of cisplatin and nucleoside monophosphates (NMP) including small oligonucleotides [25]. Meanwhile, we make extensive use of this technique as the basis for a first screening of new synthesised metal complexes with regard to their potential binding towards DNA. In order to understand the course of the binding properties of these compounds, we investigated the kinetics of cisplatin–guanosine–5′-monophosphate (5′-GMP) interaction under 'simulated physiological conditions' implementing CE technology. This screening method represents an important step forward to determine the properties of new metal ion complexes, which are then selected for further investigations.

The CE technology offers several advantages. It requires low sample amounts (reducing the costs for synthesis of new compounds), is an easy-to-handle and rapid technique and allows adjustment of the buffer conditions, and, in contrast to HPLC, no organic solvents are required. These advantages are important parameters for simulation of physiological conditions. Moreover, samples can be analysed directly without prior sample preparation avoiding unintended changes.

Of special interest are also the possible interactions of cisplatin–GMP complexes with selected endogenous molecules. As known, platinum(II) compounds have a very high affinity for sulfur ligands [26–29], and therefore sulfur-containing molecules are frequently applied as rescue agents in cancer therapy [30,31] and in this context the bis-chelate [Pt(Met-H-S,N)$_2$] has been isolated from the urine of patients treated with cisplatin [32]. This chelate is, however, inactive towards DNA. Nevertheless, cisplatin reacts with 5′-GMP in the presence of L-methionine resulting in mixed-ligand adducts and reversible methionine binding [26]. Accordingly, we focused on the influence of the sulfur-containing α-amino acids L-methionine (L-Met) and L-cysteine (L-Cys) on the complex formation reactions.

We studied the kinetics of the adduct formation of 5′-GMP with cisplatin under 'simulated physiological conditions' in the absence and presence of L-methionine and L-cysteine as well as the influence of chloride ions employing CE technology. The determination of the half-time ($t_{1/2}$) of the complex formations allowed us to compare binding properties under different reaction conditions. Due to the lack of structural information about the coordinative binding site of the activated metal complex to nucleoside monophosphates, NMR investigations have been applied as a complementary analytical technique.

2. Materials and methods

2.1. Capillary electrophoresis

Analyses were performed on a Hewlett-Packard ^{3D}Capillary Electrophoresis system equipped with a diode-array detector (DAD). Fused-silica capillaries with extended lightpath (50 μm I.D.; effective length 56 cm) were purchased from Hewlett-Packard (Waldbronn, Germany). Once a day the capillary was flushed with sodium hydroxide solution (0.1 *M*, HPCE grade; Fluka, Buchs, Switzerland) for 2 min. Prior to each analysis the capillary was flushed with the incubation/separation buffer (sodium phosphate buffer, 1 m*M*, sodium chloride, 4 m*M*, pH 7.4) for 5 min. The temperature of the capillary as well as of the sample tray was kept constant at 37°C. All samples were dissolved in the separation buffer and injected by pressure (p=10 mbar, t=15 s). During the separation the voltage was kept constant at 30 kV. The wavelengths for data presentation were chosen due to the absorption maxima of the major analytes. Deviations from these conditions are described in the corresponding figure legends.

2.2. Standards

Cisplatin, *cis*-[PtCl$_2$(NH$_3$)$_2$], was prepared according to the method published by Dhara [33]. Syntheses as well as purity control of the standards

$[PtCl(NH_3)_2(H_2O)]^+$ (monoaqua) and $[Pt(NH_3)_2(H_2O)_2]^{2+}$ (diaqua) was reported previously [25]. These standards were required for identification of the mono- and diaqua species. For structures see also Fig. 1.

2.3. Kinetics studies

Stock solutions of cisplatin (5 m*M*) and GMP (10 m*M*) were dissolved in the incubation/separation buffer. GMP (0.2 m*M*) and cisplatin (0.1 m*M*) were mixed resulting in a ratio of 2:1 and filtered prior to injection. Aliquots were always taken from the same sample vial. During analyses the sample tray was kept at 37°C (Julabo, Seelbach, Germany). The kinetics study is monitored at 200 nm in the case of hydrolysis products of cisplatin and at 252 nm for GMP adducts.

Calibration was done externally by dissolving GMP in the incubation buffer (0.02 m*M*–0.4 m*M*) and by analysing under equal conditions (calibration curve: $r^2=0.9991–0.9929$, $n=3$, RSD$<0.5\%$).

2.4. Chemicals

Disodium guanosine 5′-monophosphate (5′-GMP), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), disodium carbonate (Na_2CO_3), sodium hydrogen carbonate ($NaHCO_3$), L-cysteine (L-Cys) and sodium chloride (NaCl) were purchased from Fluka (Buchs, Switzerland). L-methionine (L-Met) was purchased from Roth (Karlsruhe, Germany). All chemicals were at least of HPLC quality. Water and acetonitrile (both HPLC quality) were purchased from Merck (Darmstadt, Germany). Stock solutions of phosphate buffer (100 m*M*) were diluted with water to obtain the final concentration of the incubation/separation buffer (1 m*M*, pH 7.4). All solutions were filtered prior to analysis through a 0.2-μm filter (Millipore, Bedford, MA, USA) and degassed using ultrasonication.

2.5. NMR experiments

Samples for NMR investigations were prepared under similar conditions as described for the kinetics studies but of higher concentrations (10 m*M* for GMP and 5 m*M* for cisplatin) and lyophilised after a certain incubation period. Prior to analysis the samples were dissolved in 0.7–1 ml D_2O (Cortec, Paris, France). Spectra were recorded at 400.13 MHz using an Avance DPX 400 spectrometer (Bruker, Rheinstetten, Germany).

3. Results

In general the peak identities were elucidated by their migration behaviour and spectral properties as described previously [25]. Each kinetics of the reaction between the platinum complex and 5′-GMP

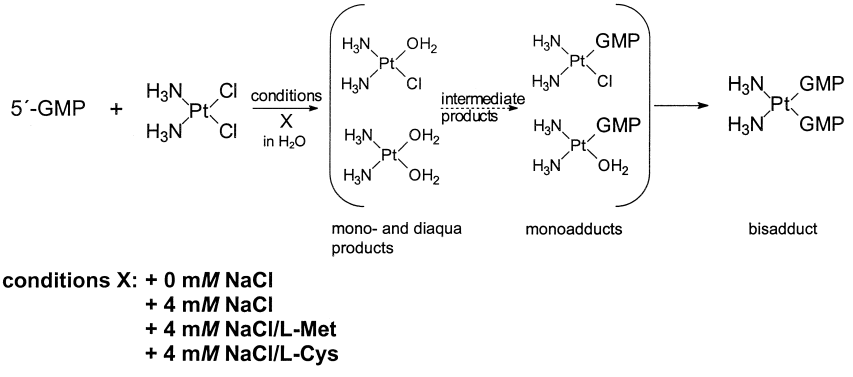

Fig. 1. Scheme of different reactions and adduct formations of cisplatin with 5′-GMP in the presence and absence of sodium chloride as well as L-methionine and L-cysteine.

was obtained by following the decrease of free 5′-GMP by means of CE. These measurements do not differentiate between mono- or bisadducts and monitor only the binding behaviour of the monophosphate towards the metal complex or its hydrolysis products. The described half-time, which was determined graphically, comprises the total reaction to the bisadduct with disregard of hydrolysis products of cisplatin and the formation of the monoadducts. Fig. 1 shows a scheme of the different reaction and adduct formation steps and indicates the various conditions in solution.

3.1. Influence of chloride ions on the coordinative binding of cisplatin to 5′-GMP

In order to study the hydrolysis and binding kinetics under physiological conditions we simulated inter- and intracellular conditions especially with respect to the chloride ion concentration. The formation of adducts depends on the presence or absence of chloride ions. After i.v. injection of cisplatin, the drug circulates in the blood, where the Cl^- concentration is about 100–150 m*M*, and therefore, hydrolysis is largely prevented. However, it is assumed that after its passing through the cell membrane the parent metal complex becomes activated in the intracellular area due to the lower Cl^- concentration of about 3–5 m*M*. The equilibrium of the hydrolysis reaction shifts towards the monoaqua product *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$.

In order to study the influence of chloride ions on the formation of adducts, especially on the formation of monoadducts, 100 m*M* chloride ions were added to the incubation buffer. This concentration corresponds to the extracellular area of most human cells. The 5′-GMP–cisplatin ratio was incrementally varied from 2:1 (as in the kinetics study) to 1:1 and incubated with, and, as a control, without, chloride ions, at 37°C. According to the literature the formation of the monoadduct should be preferred in the 1:1 experiment. Analysis was carried out at the beginning and after 40 h of incubation. Absolute concentrations could not be determined due to the lack of corresponding standards. Therefore, we set the largest detected area of one species (monoadduct *cis*-$[Pt(NH_3)_2Cl(N7\text{-}GMP)]^-$ or bisadduct *cis*-$[Pt(NH_3)_2(N7\text{-}GMP)_2]^{2-}$ or GMP) of all four experiments to 100%. All other corresponding areas of the same species were calculated relative to this value. Consequently, each species had once reached a value of 100%. Comparison of the values of all four experiments applies only among one species. Results are summarised in Fig. 2.

3.1.1. Comparison of free 5′-GMP

The comparison of uncoordinated GMP in the four experiments showed the following results. Maximum concentration of free GMP was found in the 2:1 experiment in the presence of 100 m*M* Cl^- and the obtained value of the peak area was therefore set to 100%. In contrast, only 12% (referred to the 100% mentioned above) were detected in the same experiment but without any addition of chloride. In the 1:1 experiment 58% of GMP were not coordinated to the platinum complex (+chloride), whereas all of the nucleotide was coordinated to platinum in the experiment without any chloride in the buffer.

3.1.2. Comparison of the formation of bisadducts

The largest area of bisadduct was formed in the 2:1 experiment without any chloride in the incubation buffer and was therefore set to 100%. In the presence of chloride only one third (33%) of the bisadduct was detected in comparison to the experiment mentioned before. Incubation of GMP with cisplatin in a molar ratio of 1:1 in the chloride-containing buffer resulted in a decrease of the formation of bisadduct to 46%, whereas the area slightly increased to 55% in the absence of chloride.

3.1.3. Comparison of the formation of monoadducts

Maximum formation of monoadducts was found in the 1:1 experiment in the presence of chloride (100%). In the absence of chloride only 60% of monoadducts were formed. Comparison of the monoadduct formation in the 2:1 experiments showed nearly the same value (59%) in the presence of chloride in the incubation buffer, whereas no monoadducts could be detected in the absence of chloride.

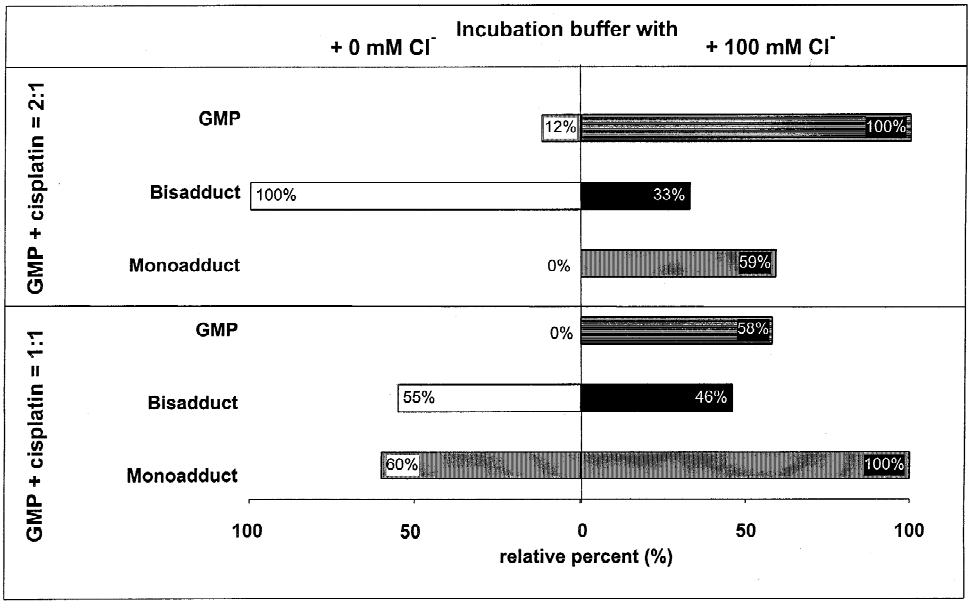

Fig. 2. Formation of GMP–platinum adducts displayed in relative percentages in the absence (left) and presence (right) of chloride ions at 37°C.

3.2. Kinetics of cisplatin and 5′-GMP complex formation in the presence or absence of chloride ions

3.2.1. Kinetics of cisplatin and 5′-GMP in the presence of chloride ions

Cisplatin was incubated with GMP in a ratio of 1:2 in order to obtain the final bisadduct *cis*-$[Pt(NH_3)_2(N7\text{-}GMP)_2]^{2-}$. Fig. 3 shows the time-dependent decrease of GMP (peak 1) as well as the formation and the increase of the major adduct (peak 2), which was detected for the first time after 5 h at 252 nm. Investigations of this major component using ^{1}H-NMR revealed the typical H8 proton shift of GMP from 8.1 ppm (H8 of free GMP [peak V, Fig. 3(b)]) to 8.6 ppm (H8 of bound GMP [peak IV, Fig. 3(b)]). This proves the coordinative binding of GMP to the metal complex. Due to this fact, the electrophoretic behaviour, the absorption maximum (λ_{max}=258 nm) of this adduct and the complete turnover of free GMP at the end of the observation period we assumed the formation of the major bisadduct *cis*-$[Pt(NH_3)_2(N7\text{-}GMP)_2]^{2-}$. After the same incubation time (5 h) the monoadduct *cis*-$[Pt(NH_3)_2Cl(N7\text{-}GMP)]^{1-}$ (peak 3) could also be detected and was identified on the basis of its migration time, which was determined using the experiment described above (Section 3.1) as well as by its spectral properties. Further evidence for this conclusion is the complete disappearance of this peak at the end of the reaction. The half-time of the complex formation reaction, which means that 50% of total GMP are bound, was reached after 8 h and about 75% of GMP were bound after 13 h (Fig. 4). After 80 h GMP was significantly reduced and the bisadduct became the predominant species. Hardly any changes were observed for the following 120 h, and, therefore, the observations were stopped after 200 h. At the end of the reaction the final bisadduct *cis*-$[Pt(NH_3)_2(N7\text{-}GMP)_2]^{2-}$ could be detected as the major constituent with a minor amount of GMP being still present. No additional adducts as well as no charged hydrolysis products of cisplatin could be detected during the reaction.

In an additional experiment investigating the hydrolysis of cisplatin [Fig. 3(a)], two hydrolysis products, *cis*-$[Pt(NH_3)_2(H_2O)Cl]^{+}$ (peak II, mono-aqua) and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (peak I, diaqua)

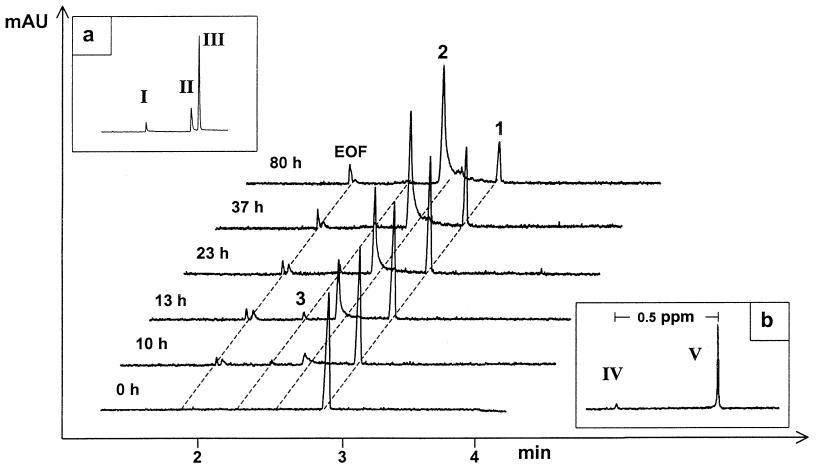

Fig. 3. Kinetics of the reaction of 5′-GMP (peak 2) with cisplatin incubated in a molar ratio of 2:1 in 1 m*M* phosphate buffer with 4 m*M* sodium chloride (37°C) recorded at 252 nm. (a) Cisplatin (III) and its hydrolysis products *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (peak II) and *cis*-$[Pt(NH_3)_2(H_2O)]^{2+}$ (I) detected at 200 nm (in the absence of 5′-GMP). (b) Downfield shift of H8 proton of 5′-GMP before (V) and after (IV) coordinative binding of the activated metal complex to 5′-GMP investigated using ^{1}H-NMR (for details see Section 2).

were separated and identified by adding standards to the sample solution (for the structure see also Fig. 1). Nevertheless, in the presence of GMP neither the monoaqua nor the diaqua species were found.

3.2.2. Kinetics of cisplatin and 5′-GMP in the absence of chloride ions

In order to study the influence of the chloride concentration on the kinetics behaviour of cisplatin–

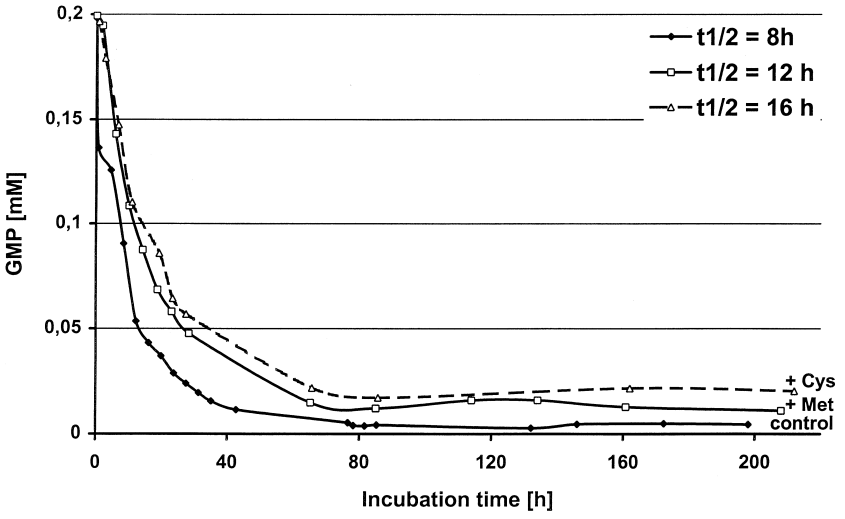

Fig. 4. Kinetics of 5′-GMP with cisplatin incubated in a molar ratio of 2:1 in 1 m*M* phosphate buffer with 4 m*M* sodium chloride, pH 7.4 (37°C) in the absence (—♦—) of any sulfur-containing compounds and in the presence of (—□—) methionine and (—▲—) cysteine, respectively.

GMP interactions the concentration of chloride ions in the phosphate buffer was varied. For comparison of the kinetics obtained the amount of free GMP was determined. In the absence of any chloride ion 50% of GMP was coordinated to the platinum complex within 7 h, whereas the half-time increased to 8 h in the presence of 4 m*M* Cl^-. As in the previous experiment, after 80 h the GMP concentration hardly decreased further. At the end of these studies (200 h) the major component of these reactions was identified as the bisadduct and neither cisplatin hydrolysis products nor significant amounts of free GMP could be detected.

3.3. Kinetics of cisplatin–5′-GMP complex formation in the presence of L*-methionine and* L*-cysteine*

Biological relevant molecules containing sulfur atoms like the α-amino acids methionine and cysteine or the tripeptide glutathione are competing ligands for GMP as they might react with the activated form of cisplatin [26–31]. By coordination of such molecules an inactivation of platinum-containing chemotherapeutica is observed. Investigations showed that L-methionine coordinates to cisplatin in the presence of 5′-GMP; nevertheless, interactions with the monophosphate are still feasible. Sadler et al. proposed a reaction mechanism explaining these unexpected results [27]. They concluded that $[Pt(L\text{-}Met\text{-}S,N)(GMP\text{-}N7)(NH_3)]^+$ is the major product (charges of 5′-GMP ignored) which arises via labilization of NH_3 from the intermediate complex $[Pt(L\text{-}Met\text{-}S,N)(NH_3)_2]^+$ due to the high *trans* effect of the coordinated sulfur.

Therefore, we were interested in the kinetics of the adduct formation of GMP with cisplatin in the presence of L-methionine or L-cysteine. The intracellular concentration of L-methionine is about 32 μ*M* and of L-cysteine about 33 μ*M* [34]. These concentrations correspond to a molar ratio of about 0.3:1 (amino acid:metal complex) as the concentration of the metal complex was kept comparable to the previous experiment. At pH 7.4 L-methionine as well as L-cysteine should have a nearly zero net charge (due to their zwitter ion properties). Consequently, the applied electrophoretic method was not suited to separate cisplatin from the amino acids.

In the presence of L-methionine the half-time of the chelation reaction was reached after 12 h, whereas in the presence of L-cysteine the half-time was about 16 h (Fig. 4). In both cases free GMP could still be detected at the end of the observation period (200 h). In the case of L-methionine about 5% (0.01 m*M*) of GMP still remained unreacted, whereas in the case of L-cysteine 10% of GMP (0.02 m*M*) could be detected. These results are in general accordance with those published by Barnham et al. [27], where the formation of a final monoadduct including a chelated L-methionine product $[Pt(L\text{-}Met\text{-}S,N)(GMP\text{-}N7)(NH_3)]^+$ has been suggested (charges on 5′-GMP ignored). Forming this adduct the remaining GMP should be found if it is added to the reaction solution in a ratio of 2:1 (according to the two available coordinative binding sites of cisplatin). Again the major component formed was the bisadduct with two GMP molecules coordinated to the platinum complex.

The increase of the molar ratio of cisplatin to L-methionine or to L-cysteine to 1:1 (instead of 1:0.3) in the kinetics studies showed quite different results. In the case of L-methionine more then 50% of GMP were coordinatively bound to cisplatin within 19 h. After this relatively fast, initial reaction there was consequently a minor decrease during the next 180 h as only 0.02 m*M* GMP reacted. After 180 h the reaction course seemed to get stimulated and finally stopped after 350 h of incubation so that no free GMP could be detected anymore. Incubation of GMP with the metal complex in the presence of L-cysteine clearly showed a two-step reaction. After 20 h about 40% of GMP were bound. This initially high reaction rate is followed by a plateau as hardly any further decrease of GMP was observed for the following 80 h. The reaction rate increased again after 100 h. GMP decreased continuously until the end of the observation period (350 h). The half-time of this reaction is therefore retarded to 114 h. In both kinetics studies the major reaction product was finally the bisadduct *cis*-$[Pt(NH_3)_2(N7\text{-}GMP)_2]^{2-}$ (confirmed by its migration time and spectral properties). No platinum–L-methionine or –L-cysteine complexes could be identified at the end of the study. In all four experiments at least one reaction component was eluting with the EOF (and therefore it has to be electrically neutral) but without any

Table 1
Overview of the different reaction conditions and the corresponding reaction half-time of the kinetics (pH of all buffer solutions: 7.4; incubation temperature: 37°C)

Reactions	Conditions	$t_{1/2}$ (h)	n[a]
5′-GMP+cisplatin (molar ratio=2:1)	1 m*M* phosphate buffer	7	3
5′-GMP+cisplatin (molar ratio=2:1)	1 m*M* phosphate buffer 4 m*M* NaCl	8	5
5′-GMP+cisplatin+L-Met (molar ratio=2:1:0.3)	1 m*M* phosphate buffer 4 m*M* NaCl	12	3
5′-GMP+cisplatin+L-Cys (molar ratio=2:1:0.3)	1 m*M* phosphate buffer 4 m*M* NaCl	16	3
5′-GMP+cisplatin+L-Met (molar ratio=2:1:1)	1 m*M* phosphate buffer 4 m*M* NaCl	19	2
5′-GMP+cisplatin+L-Cys (molar ratio=2:1:1)	1 m*M* phosphate buffer 4 m*M* NaCl	114	2
5′-GMP+cisplatin (molar ratio=2:1)	20 m*M* phosphate buffer	73	2

[a] *n*, number of repetitions.

characteristic absorption spectrum. As we were dealing with minor components identification failed.

An overview of the observed reaction half-time values of the described kinetics studies are summarised in Table 1.

4. Discussion

In this work the versatility of CE technology to study reaction kinetics of 5′-GMP with cisplatin under ‘simulated physiological conditions’ could be demonstrated.

Under physiological conditions injected cisplatin circulates in the blood stream followed by an active or passive transport through the cell membranes to finally reach the nucleus in the form of its hydrolysis products or adducts. Therefore, the interaction of the metal complexes with different biological targets has to be considered, which can partially be simulated by competitive studies and CE technology. Addition of chloride ions to the incubation buffer resulted in an increase of the reaction half-time of complex formations (as outlined in Table 1). The addition of L-methionine and L-cysteine at physiological concentrations had a significant influence as the reaction half-time increased from 8 h to 12 h and 16 h, respectively. The reaction half-time increased dramatically by adding the amino acids to the metal complex in equimolar concentrations. In the case of L-cysteine an increase from 8 h to 114 h was observed. In this special case a two-step reaction mechanism was monitored, which may be interpreted as the reversible binding of sulfur-containing amino acids to platinum compounds. It is known from the literature that complex formation of thioethers (L-methionine) with cisplatin is reversible by the addition of 5′-GMP [29], whereas platinum–thiolates (L-cysteine) complexes are known to be more stable. In this work we showed that 5′-GMP coordinates to the hydrolysis products of cisplatin even in the presence of L-cysteine although the kinetics observed were very slow compared to L-methionine (114 h compared to 19 h). Binding of platinum complexes to sulfur-containing molecules might play an important role in the design of a drug reservoir of novel platinum anticancer drugs. A direct comparison of the presented data to those published in the literature is difficult due to the use of different analytical methods and conditions but the tendency is in line. We observed quite different results studying the kinetics of 5′-GMP with cisplatin under the conditions described above but changing only the concentration of the incubation buffer. For example, 20 m*M* phosphate buffer instead of 1 m*M* resulted in a 9-fold increase of the half-time from 8 h to 73 h. Lowering the incubation temperature by 3°C also retarded the reaction half-time from 8 h to 17 h.

It has been shown that CE proved to be an attractive alternative to HPLC for the analysis of nucleotides with metal complexes, with much potential for extension. CE, especially in combination

with mass spectrometry, will play a leading role in the analysis of DNA adducts.

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