Published in final edited form as:

J Biol Inorg Chem. 2013 February ; 18(2): 249–260. doi:10.1007/s00775-012-0970-4.

Influence of extracellular pH on the cytotoxicity, cellular accumulation, and DNA interaction of novel pH-sensitive 2-aminoalcoholatoplatinum(II) complexes

Seied Mojtaba Valiahdi,

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Alexander E. Egger,

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Walter Miklos,

Institute of Cancer Research, Department of Medicine I and Comprehensive Cancer Center, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria; Research Platform “Translational Cancer Therapy Research”, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria

Ute Jungwirth,

Institute of Cancer Research, Department of Medicine I and Comprehensive Cancer Center, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria; Research Platform “Translational Cancer Therapy Research”, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria

Kristof Meelich,

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Petra Nock,

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Walter Berger,

Institute of Cancer Research, Department of Medicine I and Comprehensive Cancer Center, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria; Research Platform “Translational Cancer Therapy Research”, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria

Christian G. Hartinger,

B. K. Keppler bernhard.keppler@univie.ac.at.

Electronic supplementary material The online version of this article (doi:10.1007/s00775-012-0970-4) contains supplementary material, which is available to authorized users.

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria; Research Platform "Translational Cancer Therapy Research", University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Mathea Sophia Galanski,

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria; Research Platform "Translational Cancer Therapy Research", University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Michael A. Jakupec,

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria; Research Platform "Translational Cancer Therapy Research", University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Bernhard K. Keppler

Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria; Research Platform "Translational Cancer Therapy Research", University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

Abstract

Extracellular acidity is a frequent pathophysiological condition of solid tumors offering possibilities for improving the tumor selectivity of molecular therapy. This might be accomplished by prodrugs with low systemic toxicity, attaining their full antitumor potency only under acidic conditions, such as bis(2-aminoalcoholato- $\kappa^2 N,O$)platinum(II) complexes that are activated by protonation of alcoholato oxygen, resulting in cleavage of platinum–oxygen bonds. In this work, we examined whether the pH dependency of such compounds is reflected in differential biological activity in vitro. In particular, the pH dependence of cytotoxicity, cellular accumulation, DNA platination, GMP binding, effects on DNA secondary structure, cell cycle alterations, and induction of apoptosis was investigated. Enhanced cytotoxicity of five of these complexes in non-small-cell lung cancer (A549) and colon carcinoma (HT-29) cells at pH 6.0 in comparison with pH 7.4 was confirmed: 50 % growth inhibition concentrations ranged from 42 to 214 μM in A549 cells and from 35 to 87 μM in HT-29 cells at pH 7.4 and decreased at pH 6.0 to 11–50 and 7.3–25 μM , respectively. The effects induced by all five pH-sensitive compounds involve increased 5'-GMP binding, cellular accumulation, and DNA platination as well as stronger effects on DNA secondary structure at pH 6.0 than at pH 7.4. As exemplified by treatment of A549 cells with a 2-amino-4-methyl-1-pentanolato complex, induction of apoptosis is enhanced at pH 6.5. These results confirm the increased reactivity and in vitro activity of these compounds under slightly acidic conditions, encouraging further evaluation of ring-closed aminoalcoholatoplatinum(II) derivatives in solid tumors in vivo.

Keywords

Anticancer drug; Apoptosis; Cellular accumulation; DNA binding; pH-sensitive prodrug

Introduction

Three platinum-based anticancer drugs have made their way into worldwide clinical use in the last 35 years. The great advances in the treatment of certain types of cancer brought about by cisplatin as well as the introduction of carboplatin and oxaliplatin in clinical therapy promoted the development of a plethora of other platinum compounds [1, 2]. The major drawbacks of platinum drugs (as with many other anticancer agents) are their severe side effects and various forms of resistance [3]. Therefore, successful strategies to enhance the tumor selectivity of these compounds would yield a benefit for a large number of patients.

Suitable strategies may be developed by consideration of tumor pathophysiology. Solid tumors are prone to hypoxia as a result of being insufficiently supplied with blood vessels. Upregulation of glycolysis and large amounts of lactate lead to tumor acidosis, forcing evolution to phenotypes resistant to acid-induced cell toxicity [4]. Overexpression of Na^+/H^+ exchanger, monocarboxylate transporters, and carbonic anhydrase leads to acidification of the extracellular space [5]. Reorganization of the extracellular matrix and local ion and water transport across the plasma membrane depend on both intracellular and extracellular pH [6]. The circumstance that the extracellular liquid of solid tumors is substantially more acidic than that of normal tissues [7, 8] can cause a decrease of cellular accumulation and activity of weakly basic chemotherapeutic drugs [9]. Drugs that are suitable for treatment of hypoxic cells, such as mitomycin C [10], or drugs that display increased cytotoxicity under acidic conditions [11–13] may be more appropriate to combat such tumors.

In the search for new platinum-based anticancer drugs, great efforts are being devoted to the design of complexes that are more efficient and less toxic than the drugs already in clinical use. For this purpose, the rational design and the study of relevant structure–activity relationships have been extended to families of new compounds having high structural diversity, such as aminoalcohol and aminoalcoholatoplatinum(II) complexes [14, 15]. The platinum(II) complexes with aminoalcoholato ligands show moderate but pH-dependent reactivity toward human serum albumin under physiological conditions [16]. A similar picture was observed for reaction with 5'-GMP, as determined by capillary zone electrophoresis and electrospray ionization mass spectrometry [17, 18].

The coordination sphere around the central metal in most of the antineoplastic square-planar platinum(II) complexes established so far features two “nonleaving” am(m)ine nitrogen donors and two leaving groups (chloride or oxygen donors). In the case of aminoalcohol complexes, e.g., (*SP-4-2*)-[bis(2-aminoethanol- κN)dichloridoplatinum(II)], the hydroxy groups of the ligands enhance the water solubility of the complexes, as well as their affinity for DNA, which is known to be the crucial target of most platinum drugs. This was the impetus for synthesizing these cisplatin derivatives in the first place [19]. Moreover, the bis(aminoalcohol)dichlorido complexes are capable of forming ring structures via intramolecular ring formation in aqueous solution upon deprotonation and release of one of the leaving ligands [20]. Doubly ring closed species can be isolated and are stable in the solid state as well as in aqueous solution. These doubly ring closed complexes show a remarkable pH-dependent behavior with respect to their chemistry and cytotoxicity. To yield

a more complete picture of the biological behavior of this class of compounds, we decided to investigate the pH dependence of their cytotoxic potency, cellular accumulation, DNA platination, cell cycle alterations, and induction of apoptosis in vitro in two human tumor cell lines (HT-29 and A549) as well as their 5'-GMP binding and effects on DNA secondary structure in cell-free experiments.

Materials and methods

Compounds

Cisplatin, oxaliplatin, and the aminoalcoholato complexes **1–5** (Fig. 1) were synthesized according to literature procedures [14, 21–23]. Of the four chiral complexes **2–5**, only **3** is enantiomerically pure (*S* enantiomer), whereas the other three compounds are racemates.

Cell lines and culture conditions

Human A549 (non-small-cell lung cancer) and HT-29 (colon carcinoma) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in 75-cm² culture flasks (Iwaki/Asahi Technoglass, Gyouda, Japan) as adherent monolayer cultures in complete culture medium, i.e., minimal essential medium supplemented with 10 % heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 4 mM L-glutamine (all purchased from Sigma-Aldrich, Vienna, Austria) without antibiotics. Cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO₂ and 95 % air.

Antiproliferative activity in cancer cell lines

Antiproliferative effects were determined by means of a colorimetric microculture assay—3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay—[24] with modifications as described previously [25]. Cells were harvested from culture flasks by trypsinization and seeded in 96-well microculture plates (Iwaki/Asahi Technoglass, Gyouda, Japan) at cell densities of 4×10^3 (A549) or 3×10^3 (HT-29) cells per well, in order to ensure exponential growth of untreated controls throughout the experiment, and were allowed to resume adherent growth for 24 h. The test compounds were dissolved in Dulbecco's modified Eagle's medium (DMEM) (without sodium bicarbonate) supplemented with 10 % heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine, 1 % nonessential amino acids (from 100× ready-to-use stock), and sodium bicarbonate in either 8.8 mM (pH 6.0) or 39.6 mM (pH 7.4) concentration (all purchased from Sigma-Aldrich, Vienna, Austria). Cells were exposed to the compounds for 24 h under an atmosphere containing 8 % CO₂ and 92 % air, followed by incubation in drug-free DMEM (pH 7.4, 200 µl per well) for a further 72 h (5 % CO₂). At the end of the incubation, all media were replaced with RPMI 1640 medium (150 µl per well; supplemented with 10 % heat-inactivated fetal bovine serum and 4 mM L-glutamine) plus MTT solution (20 µl per well) in phosphate-buffered saline (PBS; 5 mg/ml). After incubation for 4 h, the medium/MTT mixtures were removed, and the formazan crystals formed by viable cells were dissolved in 150 µl dimethyl sulfoxide per well. Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic), using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of viable cells

was expressed in terms of treated to control (T/C) values by comparison with untreated control microcultures. To correct for differences in growth kinetics, concentration–effect curves were transformed by taking the optical densities of separate MTT-stained control microcultures at the beginning of drug exposure as zero values, and 50 % growth inhibition (GI_{50}) concentrations were calculated by interpolation. Evaluation was based on means from at least three independent experiments, each comprising six replicates per concentration.

DNA platination

A549 and HT-29 cells grown to approximately 70 % confluence in 90-mm tissue culture plates (Iwaki/Asahi Technoglass, Gyouda, Japan) were exposed to the platinum complexes at pH 6.0 and pH 7.4 as described in the previous section under an atmosphere containing 8 % CO_2 and 92 % air for 24 h at 37 °C. Afterwards, cells were washed once with tris(hydroxymethyl)aminomethane (Tris)-buffered saline (0.0027 M KCl, 0.137 M NaCl, 0.025 M Tris, pH 7.4) and harvested by trypsinization. The number of viable cells was determined with a hemocytometer using trypan blue staining. Cells (3×10^6 – 6×10^6) were washed three times with Tris-buffered saline and centrifuged at 900g for 2 min. Cell pellets were resuspended in 500 μ l of lysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 20 μ g/ml RNase, and 0.2 % Triton X-100) and incubated for 30 min at 37 °C. The lysate was centrifuged at 10,000g for 8 min, and supernatants mixed with a 0.7-fold volume of 2-propanol were centrifuged for 30 min at 10,000g and 4 °C. The pellets were washed with 70 % ethanol, again centrifuged for 30 min at 10,000g and 4 °C, and dried in air, then dissolved in Milli-Q water (18.2 M Ω cm) and the mixture was shaken for 20 min at 65 °C. Inductively coupled plasma mass spectrometry (7500ce instrument, Agilent Technologies, Waldbronn, Germany, equipped with a CETAC ASX-520 autosampler and a MicroMist nebulizer at a sample accumulation rate of approximately 0.25 ml/min) was used for the measurement of r_b values (number of platinum atoms per nucleotide). To minimize the impact of $^{15}N^{16}O$ interference with ^{31}P , aliquots (100 μ l) were diluted to 5 ml with 2 % (w/w) hydrochloric acid. Beryllium and indium were added as internal standards for phosphorus and platinum, respectively, in concentrations of 1 ppb, and concentrations were determined via the isotopes ^9Be , ^{31}P , ^{115}In , and ^{195}Pt . All experiments were performed in triplicate.

Cellular accumulation

All experiments were performed according to the procedure described previously [26] and in triplicate (three wells per row of a six-well plate), and corresponding adsorption blanks were located on the same plate. HT-29 and A549 cells were seeded at densities of 3×10^5 and 3.5×10^5 cells per well, respectively. Plates were kept at 37 °C for 48 h prior to drug exposure. Consecutively, the medium was completely removed and replaced with 2.5 ml of a fresh solution of the test compound in complete medium of pH 6.0 or 7.4, prepared as described earlier, and drug exposure lasted for 2 h at 37 °C. After termination of exposure, the medium was completely removed and wells were washed twice with PBS, followed by addition of 0.5 ml of subboiled HNO_3 per well for more than 1 h. Subsequently, aliquots of 400 μ l were transferred into 15-ml polypropylene tubes, which were filled to a total volume of 8 ml with Milli-Q water, and internally standardized with indium (0.5 ppb). The quantity of platinum was determined by inductively coupled plasma mass spectrometry on the same day, and the ratio of cellular amounts at pH 6.0 and 7.4 was calculated. The precision is indicated by

standard deviations resulting from error propagation (most probable error) considering the uncertainties of the concentrations in blank and uptake experiments at pH 6.0 and 7.4.

Binding to 5'-GMP

The disodium salt of 5'-GMP, sodium chloride, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Fluka. All buffer solutions were filtered prior to analysis through 0.2- μ m membrane filters (Sartorius, Göttingen, Germany) and degassed in an ultrasonic bath. All analyses were performed with a Hewlett-Packard 3D capillary electrophoresis system equipped with a diode-array detector. Fused-silica capillaries (inner diameter 50 μ m, total length 50 cm, effective length 42 cm) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Before the first use, the capillary was flushed with 1 mM sodium hydroxide solution (high-performance capillary electrophoresis grade, Fluka, Buchs, Switzerland) for 20 min and water (high-performance liquid chromatography grade, Roth, Karlsruhe, Germany) for 10 min. Afterwards, the capillary was conditioned for 30 min with the background electrolyte ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, 50 mM, pH 6.0 or 7.4). Before each analysis, the capillary was purged for 2 min with 0.1 mM sodium hydroxide, 1 min with water, and 2 min with the background electrolyte. The background electrolyte was replenished after every third run. The temperature of the capillary as well as that of the sample tray was kept constant at 37 °C. All samples were dissolved in 20 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 6.0 or 7.4), and were injected by applying pressure (10 mbar) for 15 s. The voltage was maintained at 15 kV.

5'-GMP (1.0 mM) was incubated with the platinum complexes (0.5 mM) in the respective buffer to obtain a twofold molar excess of 5'-GMP. Immediately after incubation, the first aliquot was taken for analysis by capillary electrophoresis. The peak area of 5'-GMP was monitored continuously at 200 nm during time intervals covering up to 20 h, and the experiment was performed in triplicate. The results are expressed in terms of the half-life ($t_{1/2}$) evaluated graphically from the relative peak area–time curve or percentage. Half-lives greater than 20 h were estimated on the basis of the extrapolated graphs.

Effects on DNA secondary structure

The effects of the compounds on the secondary structure of double-stranded plasmid DNA were studied as described in a previous publication [27]. For this purpose, plasmid pTZ18u (2,860 base pairs) from Bio-Rad Laboratories (Munich, Germany) was transformed in competent TOP10F cells, isolated, and purified according to standard procedures (Promega, Pure Yield™ plasmid) and dissolved in doubly distilled water. Stock solutions of the compounds were prepared in water. All solutions were immediately used for incubation with 1 μ g plasmid in 10 mM phosphate buffer at pH 6.0 or 7.4 in a final volume of 40 μ l and a final concentration of the test compounds of 60 μ M for different incubation times (up to 3 h) at 37 °C. The reaction products were separated in a 1 % agarose gel at 80 V for 1 h, and the electrophoretic mobility of DNA was visualized under UV light after staining with ethidium bromide.

Cell cycle distribution

A549 cells (1.5×10^5) were exposed to **5** for 24 h. To analyze cell cycle progression, cells were collected, washed with PBS, resuspended in 0.9 % NaCl solution, and fixed in 70 % ethanol (-20°C). Then, cells were incubated with RNase (10 $\mu\text{g/ml}$; Sigma) for 30 min at 37°C and stained with propidium iodide (PI; 10 $\mu\text{g/ml}$, Sigma-Aldrich) for 30 min at 4°C . Cells were analyzed by flow cytometry using fluorescence-activated cell sorting (FACS; FACSCalibur, Becton, Dickinson, Palo Alto, CA, USA). CellQuest Pro (Becton, Dickinson, New York, NY, USA) was used to analyze the resulting DNA histograms. Experiments were repeated three times, and representative data are shown.

Western blot analysis

A549 cells (2×10^5) were exposed to 50 and 100 μM **5** for 24 h. Total protein lysates were prepared, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto a poly(vinylidene difluoride) membrane for Western blotting as described previously [28]. Primary antibodies against p53 (Thermo Scientific Neomarkers, Fremont, CA, USA; clone DO-1), cyclin A (sc751, Santa Cruz Biotechnology), cyclin B1 (sc245, Santa Cruz Biotechnology), cyclin D1 (sc753, Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PARP; 9542, Cell Signaling Technology), cleaved PARP (9541, Cell Signaling Technology), and β -actin (AC-15; A1978, Sigma-Aldrich) were used in dilutions of 1:1,000. Cyclin E antibody (sc247, Santa Cruz Biotechnology) was used in a dilution of 1:500. Secondary antibodies labeled with horseradish peroxidase from Santa Cruz Biotechnology were used in working dilutions of 1:10,000.

Annexin V/PI staining

A549 cells (1.5×10^5) were exposed to the platinum compounds for 24 h. Cells were stained with annexin V (annexin V–fluorescein isothiocyanate; BD Biosciences) and PI (200 ng/ml, Sigma-Aldrich) and analyzed according to the manufacturer's protocol by flow cytometry using FACS (FACSCalibur, Becton, Dickinson, Palo Alto, CA, USA). CellQuest Pro (Becton, Dickinson, New York, NY, USA) was used to analyze the data. The experiments were repeated three times, and representative examples are shown.

Mitochondrial membrane potential detection—5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide staining

Breakdown of the mitochondrial membrane potential was determined by FACS analysis using 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1). For this purpose, a mitochondrial membrane potential detection kit (Stratagene, La Jolla, CA, USA) was used following the manufacturer's instructions. In brief, 1.5×10^5 A549 cells were exposed to the test compounds for 24 h. After trypsinization and washing with PBS, cells were incubated for 10 min in freshly prepared JC-1 solution (10 mg/ml in medium) at 37°C . Spare dye was removed by washing in PBS, and cell-associated fluorescence was measured by FACS (FACSCalibur, Becton, Dickinson, Palo Alto, CA, USA). CellQuest Pro (Becton, Dickinson, New York, NY, USA) was used to analyze the data.

Results

Antiproliferative activity in cancer cell lines

A549 and HT-29 cells were chosen to study the antiproliferative effects of the pH-sensitive 2-aminoalcoholatoplatinum(II) complexes. For comparison, the clinically established drugs cisplatin and oxaliplatin were included as well. Cells were exposed to the compounds either at pH 6.0 or at pH 7.4 for 24 h, followed by a drug-free incubation with DMEM for 72 h at pH 7.4. Concentration–effect curves for the aminoethanolato complexes and clinically established platinum drugs are depicted in Fig. 2, S1, and S2. The activity of all aminoalcoholato complexes increased in both cell lines under slightly acidic conditions, as indicated by a shift of GI_{50} concentrations toward lower micromolar concentrations (Table 1) and a shift of concentration–effect curves toward lower T/C values (Fig. 2). At pH 7.4, GI_{50} concentrations of the bis(2-aminoalcoholato- κ^2N,O)platinum(II) complexes were in the range from 42 to 214 μM in A549 cells and from 35 to 87 μM in HT-29 cells. At pH 6.0, these concentrations decreased to 11–50 μM for A549 cells and 7–25 μM for HT-29 cells. Acidification of the medium during exposure resulted in 3.3-fold to 4.3-fold enhanced cytotoxicity in A549 cells and 2.8-fold to 11-fold enhanced cytotoxicity in HT-29 cells. The strongest increase in cytotoxicity was observed in HT-29 cells treated with **1** (11-fold increase). The GI_{50} concentrations of cisplatin are dependent on the pH to some extent as well (4.9-fold decrease at pH 6.0 in A549 cells and twofold decrease in HT-29 cells), whereas the antiproliferative activity of oxaliplatin is independent of the pH in both cell lines.

The structure–activity relationships with respect to the aminoalcoholato ligand are similar in both cell lines at pH 6.0, with cytotoxic potency decreasing depending on the ligand in the following order: 2-amino-4-methyl-1-pentanolato (**5**) > 2-aminoethanolato (**1**) > 2-amino-1-hexanolato (**4**) > (2*S*)-amino-3-methyl-1-butanolato (**3**) > 2-amino-1-butanolato (**2**).

DNA platination and cellular accumulation

HT-29 and SW480 human cancer cell lines were exposed to cisplatin, oxaliplatin, and five pH-sensitive platinum complexes at pH 7.4 and 6.0. The resulting η_b values are given in Fig. 3. When comparing the absolute amounts of DNA-bound platinum, one should bear in mind that cisplatin and oxaliplatin were used in much lower concentration (5 μM) owing to their higher cytotoxicity than the pH-sensitive complexes (50 μM).

DNA platination by each compound was similar in the two cell lines, with decreasing η_b values depending on the aminoalcoholato ligand in the following order: 2-amino-1-hexanolato (**4**) > 2-amino-4-methyl-1-pentanolato (**5**) > (2*S*)-amino-3-methyl-1-butanolato (**3**) > 2-amino-1-butanolato (**2**) > 2-aminoethanolato (**1**). However, it is remarkable that in A549 cells cisplatin led to DNA platination equal to or greater than that caused by most of the tested pH-sensitive platinum(II) complexes at pH 7.4, although cisplatin was 10 times less concentrated.

Exposing cells in acidic conditions (pH 6.0) strongly increased cellular accumulation and DNA platination by all substances. For comparison of the effect of different pH values on cellular accumulation and DNA platination, the respective ratios were calculated for all

complexes (see Table S1) and are depicted in Fig. 4. Although the impact of pH on cellular accumulation differs for the individual compounds (1.5-fold to 4.0-fold increase), DNA platination is roughly threefold enhanced for all 2-aminoalcoholatoplatinum(II) complexes in both cell lines. This is in accordance with the proposed mechanism of action, as the ring-opened form, which is mainly formed under acidic conditions, is believed to be the species forming DNA adducts. The strongest enhancement of DNA platination and cellular accumulation was observed in the case of **3**. Although a roughly fourfold increase was observed in HT-29 cells at pH 6.0, the enhanced accumulation/DNA platination is less pronounced in the case of A549 cells (2.5-fold and 3.0-fold, respectively), but is still among the highest ratios in this cell line. As expected, DNA platination and cellular accumulation of cisplatin and oxaliplatin are much less influenced by the pH (with the exception of a threefold increase in DNA binding of oxaliplatin in A549 cells) as they do not possess this pH-dependent ring opening and closing mechanism.

Binding to 5'-GMP

The binding of the platinum complexes to 5'-GMP was studied by capillary electrophoresis in phosphate buffer at pH 6.0 and 7.4 (37 °C) in order to characterize the pH-dependent reactivity of the compounds with nucleophiles. The peak area of unbound 5'-GMP was monitored for up to 20 h (Fig. 5). The results are expressed in terms of the half-life ($t_{1/2}$) evaluated graphically from the relative peak area–time curve or percentage. Whereas the half-lives of 5'-GMP binding exceeded 50 h at pH 7.4 in all cases, acidic conditions (pH 6.0) strongly accelerated 5'-GMP binding to half-lives of mostly less than 10 h (Table 2), indicating a strong influence of the pH on the reactivity of the compounds. Similarly to cisplatin, formation of mono-5'-GMP and bis-5'-GMP adducts was observed for the aminoalcoholato complexes (Fig. 5).

Effects on secondary structure of DNA

Alterations of DNA secondary structure can be easily detected by changes in the electrophoretic mobility of a circular double-stranded DNA plasmid (pTZ18u) that exists in a supercoiled and an open circular form. The faster migration of the more compact supercoiled form in a neutral agarose gel is diminished by local untwisting of DNA upon interaction with cross-linking (or intercalating) agents. This is exemplified by the effect of 60 μ M cisplatin and (2-aminoalcoholato)platinum(II) complexes at pH 6.0 which cause a marked retardation of the supercoiled plasmid already after incubation for 1 h (with the exception of **2** and **3**). In contrast, at pH 7.4, the mobility of neither the supercoiled form nor the open circular form of the plasmid is altered by interaction with the ring-closed complexes at the same concentration, arguing against the formation of DNA cross-links (Fig. 6).

Impact of pH on cell cycle distribution

The impact of **5** on A549 cell cycle progression at different pH values was determined by FACS analysis (Fig. 7). A 24-h drug exposure induced a significant dose-dependent decrease in the proportion of cells in G₀/G₁ phase, whereas the proportion of S-phase cells increased distinctly and that of cells in G₂/M phase increased slightly. Regarding the impact of pH

conditions, the differences were minor and concerned the absence of an increase in the G₂/M fraction under acidic conditions. These results were supported by protein expression changes of cyclin E and cyclin B1 (Fig. 8). These data indicate that the stronger anticancer potency at lower pH is not mainly based on cytostatic effects.

Induction of apoptosis and p53 induction

On the basis of the minor cytostatic effects, we investigated whether **5** induces its pH-dependent anticancer effects by more pronounced induction of apoptosis. Therefore, A549 cells were treated with **5** for 24 h, and cleavage of the caspase substrate PARP was determined by Western blotting. Indeed, only a minor cleavage of PARP was induced at 50 μ M and pH 7.4. In contrast, cleavage of PARP was almost complete at 50 μ M under acidic conditions (Fig. 9). Incubation with 100 μ M **5** resulted in complete PARP cleavage under both conditions. A549 cells harbor a wild-type p53 gene. Thus, p53 induction by **5** was determined as a measure of induced DNA damage. Indeed, dose-dependent p53 stabilization was observed. Under acidic conditions, only 50 μ M **5** was required to induce maximum p53 stabilization, which was almost undetectable at physiological pH.

As further proof of apoptosis induction, combined annexin V/PI staining of cells treated for 24 h with **5** was performed. At pH 7.4, primarily necrosis and late apoptosis were observed, but in a minor cell fraction (Fig. 10a). In contrast, especially the fraction of early apoptotic cells (annexin V-positive but PI-negative) was distinctly increased when drug exposure was performed under acidic conditions (Fig. 10b). Furthermore, the fraction of late apoptotic cells was increased.

To investigate whether pH-dependent induction of apoptosis by **5** involves the intrinsic mitochondrial pathway, JC-1 staining experiments were performed. This marker indicates the loss of mitochondrial membrane potential as an early event in apoptosis by the intrinsic pathway. A dose-dependent increase of the fraction of cells with depolarized mitochondrial membrane potentials in A549 cells was observed after exposure to **5**. However, this effect tended to be more pronounced only under acidic conditions (Fig. 11a, b).

Discussion

Targeting strategies are among the most intensively discussed approaches in drug design. Activation of pH-sensitive anticancer agents may selectively target and kill slightly acidic solid tumor tissue, whereas normal tissue should remain virtually unaffected. In recent years, (2-aminoalcoholato)platinum(II) complexes have been extensively studied because they are known to be activated under acidic conditions [14]. In acidic solution, the chelate ring can be opened (Fig. 12), yielding a complex with significantly higher affinity for DNA bases and enhanced cytotoxicity in vitro [14, 17]. This is a convincing example of how small differences in chemical properties can be exploited to design compounds with specific anticancer properties. Furthermore, derivatization can be used to control these properties within very narrow margins.

A study of platinum(II) complexes with 2-aminoethanol and 2-amino-1-butanol ligands under cell-free conditions has confirmed that these compounds form adducts resulting in

alterations of DNA secondary structure in general and DNA interstrand cross-links in particular to a higher extent at pH 6.0 than at pH 7.4 [27]. In the present study, we demonstrated that the biological activity of five different 2-aminoalcoholatoplatinum(II) complexes is dependent on the extracellular pH. In particular, cytotoxicity, cellular accumulation, and DNA platination in cancer cells as well as binding to GMP in addition to the capacity of altering DNA secondary structure in cell-free settings were shown to be higher at pH 6.0 than at the physiologically prevailing pH 7.4. Furthermore, one selected compound (**5**) was shown to induce DNA damage, as indicated by p53 stabilization, mitochondrial membrane depolarization, and consequently, apoptosis to a higher extent at slightly acidic pH than at normal pH. Although pathophysiological changes of extracellular pH in tumor tissue are conceived as the driving force for activation of these compounds, activation does not have to be exclusively extracellular. Even if speculative, a certain extent of activation in acidic cellular compartments cannot be excluded and might even explain why the complexes exert some activity also under normal (slightly basic) extracellular conditions.

The observed effects are consistent with the known chemical behavior of the compounds, since the alcoholato oxygen atoms are protonated under acidic conditions. As a consequence, the platinum–oxygen bonds are cleaved, rendering the platinum center accessible for nucleophilic attack and facilitating adduct formation with biological target molecules. Furthermore, acidification seems to facilitate the accumulation of these compounds in tumor cells by increasing passive diffusion or active transport. A previous comparison of one ring-opened (dichlorido) complex with the analogous chelate (**1**) at both pH 7.4/7.0 and pH 6.0 suggested that the pH-dependent equilibrium of the ring opening/closure reaction is established early within the exposure time, as the observed differences between cytotoxic potencies of the two species were marginal and within the range of statistical errors. Under the assumption that this behavior can be generalized to all representatives of this class of compounds, no further experiments were performed with ring-opened species, which are much less stable in solution than the ring-closed species and are therefore not being considered for drug development.

In this context, it should be mentioned that a certain pH dependence of activity can even be observed in platinum complexes lacking acid-labile N,O-chelating ligands. Cytotoxicity of cisplatin, for instance, was shown to be enhanced under slightly acidic conditions in cultures of EMT6 mammary tumor cells, which the investigators tried to explain by ion trapping of hydrolysis products [29]. Cellular accumulation of cisplatin was reported by other investigators to depend on extracellular pH and to be increased by acidification of the culture medium [30], and enhanced induction of apoptosis by activation of caspase 3 was observed in prostate tumor cell lines treated with cisplatin in slightly acidic medium [31]. Furthermore, formation of DNA adducts by cisplatin in cell-free experiments was shown to be enhanced at pH 6.8 compared with pH 7.5, and cellular pH was found to be increased in cisplatin-resistant cells compared with cisplatin-sensitive parental cells in three of four isogenic pairs of cell lines [32]. These observations, which are paralleled to some extent by those presented here, may at least be partially explained by the different reactivities of the hydrolysis products of cisplatin, which are readily formed in aqueous solution. Acidification

of the medium shifts the equilibrium between aqua and hydroxo species in favor of the more reactive aqua complexes [33]. Aquated cisplatin can then react with carbonate or phosphate in a monodentate or bidentate manner [34]. At lower pH, a larger fraction of both carbonate and phosphate ligands is protonated, weakening their coordination to the platinum center.

Introducing chelating ligands that are rather inert at pH 7.4 but open upon protonation in acidic solution was expected to enhance the pH dependence of reactivity compared with cisplatin, which is largely supported by the results presented here for aminoalcoholato complexes. In particular, the (2-aminoalcoholato)platinum(II) investigated complexes show a strong increase in DNA binding at pH 6.0 in comparison with pH 7.4. Furthermore, the time-dependent 5'-GMP binding at pH 6.0 roughly correlates with the compounds' antiproliferative activities at the same pH (see Table 1): Complex **5** reacts most rapidly with 5'-GMP and exhibits the lowest GI₅₀ concentrations, whereas **2** and **3** are the least reactive derivatives and show the highest GI₅₀ concentrations. Furthermore, a correlation between 5'-GMP binding (Table 2) and DNA interaction under cell-free conditions was observed. In both assays, binding at pH 6.0 decreases in the following order: **5** > **1** > **4** > **2** > **3**.

Although the cytotoxic potencies of the new cisplatin derivatives are lower than that of the parent drug cisplatin, their more pronounced activation by lowered pH argues for their suitability for targeting the acidic microenvironment of solid tumors. Compound **1** (with 2-aminoethanolato ligands) and compound **5** (with 2-amino-4-methyl-1-pentanolato ligands), the most potent compounds, with the most pronounced acidic activation in HT-29 cells, emerge as a reasonable choice for further studies (whereas intermediate structures such as **2** and **3** appear less attractive in this respect). Whether this behavior translates into reduced adverse effects and hence an improved therapeutic index and efficacy will be the subject of in vivo studies in solid tumor models. These protonation-activated agents may allow an effective therapy without severe toxicity, in which an inactive, nontoxic prodrug is administered, is activated only at site of acidic tumors, destroying tumor cells, and is excreted in the inactive form from the body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Austrian Research Promotion Agency (FFG, grant M811591; B.K.K.); and the Austrian Science Fund (FWF, grant L568; W.B.). Sara Daraei (St.-Marien-Hospital Lünen, Akademisches Lehrkrankenhaus der Westfälischen Wilhelms-Universität Münster) is gratefully acknowledged for assistance in writing parts of the manuscript.

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
FACS	Fluorescence-activated cell sorting
GI₅₀	Fifty percent growth inhibition

JC-1	5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 <i>H</i> -tetrazolium bromide
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PI	Propidium iodide
T/C	Treated to control
Tris	Tris(hydroxymethyl)aminomethane

References

- Galanski M, Jakupec MA, Keppler BK. *Curr Med Chem*. 2005; 12: 2075–2094. [PubMed: 16101495]
- Galanski M, Keppler BK. *Anticancer Agents Med Chem*. 2007; 7: 55–73. [PubMed: 17266505]
- Heffeter P, Jungwirth U, Jakupec M, Hartinger C, Galanski M, Elbling L, Micksche M, Keppler B, Berger W. *Drug Resist Updates*. 2008; 11: 1–16.
- Gatenby RA, Gillies RJ. *Nat Rev Cancer*. 2004; 4: 891–899. [PubMed: 15516961]
- Swietach P, Vaughan-Jones RD, Harris AL. *Cancer Metastasis Rev*. 2007; 26: 299–310. [PubMed: 17415526]
- Stock C, Schwab A. *Pflugers Arch Eur J Physiol*. 2009; 458: 981–992. [PubMed: 19437033]
- Tannock IF, Rotin D. *Cancer Res*. 1989; 49: 4373–4384. [PubMed: 2545340]
- Izumi H, Torigoe T, Ishiguchi H, Uramoto H, Yoshida Y, Tanabe M, Ise T, Murakami T, Yoshida T, Nomoto M, Kohno K. *Cancer Treat Rev*. 2003; 29: 541–549. [PubMed: 14585264]
- Raghuhanand N, Martinez-Zaguilan R, Wright SH, Gillies RJ. *Biochem Pharmacol*. 1999; 57: 1047–1058. [PubMed: 10796075]
- Kennedy KA, Rockwell S, Sartorelli AC. *Cancer Res*. 1980; 40: 2356–2360. [PubMed: 7388797]
- Mayer LD, Bally MB, Loughrey H, Masin D, Cullis PR. *Cancer Res*. 1990; 50: 575–579. [PubMed: 2297698]
- Siemann DW, Chapman M, Beikirch A. *Int J Radiat Oncol Biol Phys*. 1991; 20: 287–289. [PubMed: 1991690]
- Skarsgard LD, Chaplin DJ, Wilson DJ, Skwarchuk MW, Vinczan A, Kristl J. *Int J Radiat Oncol Biol Phys*. 1992; 22: 737–741. [PubMed: 1544846]
- Galanski M, Baumgartner C, Meelich K, Arion VB, Fremuth M, Jakupec MA, Schluga P, Hartinger CG, Keyserlingk N, Graf v. Keppler BK. *Inorg Chim Acta*. 2004; 357: 3237–3244.
- Galanski M, Baumgartner C, Arion V, Keppler BK. *Eur J Inorg Chem*. 2003; 14: 2619–2625.
- Aleksenko SS, Hartinger CG, Semenova O, Meelich K, Timerbaev AR, Keppler BK. *J Chromatogr A*. 2007; 1155: 218–221. [PubMed: 17240384]
- Hartinger CG, Schluga P, Galanski M, Baumgartner C, Timerbaev AR, Keppler BK. *Electrophoresis*. 2003; 24: 2038–2044. [PubMed: 12858373]
- Schluga P, Hartinger CG, Galanski M, Meelich K, Timerbaev AR, Keppler BK. *Analyst*. 2005; 130: 1383–1389. [PubMed: 16172663]
- Zimmermann W, Galanski M, Keppler BK, Giester G. *Inorg Chim Acta*. 1999; 292: 127–130.
- Galanski M, Zimmermann W, Baumgartner C, Keppler BK. *Eur J Inorg Chem*. 2001; 5: 1145–1149.
- Dhara SC. *Indian J Chem*. 1970; 8: 192–193.
- Kidani Y, Inagaki K, Iigo M, Hoshi A, Kureitani K. *J Med Chem*. 1978; 21: 1315–1318. [PubMed: 722741]

23. Meelich K, Galanski M, Arion VB, Keppler BK. *Eur J Inorg Chem.* 2006; 12: 2476–2483.
24. Mosmann T. *J Immunol Methods.* 1983; 65: 55–63. [PubMed: 6606682]
25. Grguric-Sipka S, Kowol CR, Valiahdi SM, Eichinger R, Jakupc MA, Roller A, Shova S, Arion VB, Keppler BK. *Eur J Inorg Chem.* 2007; 2007: 2870–2878.
26. Egger AE, Rappel C, Jakupc MA, Hartinger CG, Heffeter P, Keppler BK. *J Anal At Spectrom.* 2009; 24: 51–61. [PubMed: 22723721]
27. Zorbas-Seifried S, Hartinger C, Meelich K, Galanski M, Keppler BK, Zorbas H. *Biochemistry.* 2006; 45: 14817–14825. [PubMed: 17144675]
28. Heffeter P, Jakupc MA, Körner W, Chiba P, Pirker C, Dornetshuber R, Elbling L, Sutterlüty H, Micksche M, Keppler BK, Berger W. *Biochem Pharmacol.* 2007; 73: 1873–1886. [PubMed: 17445775]
29. Laurencot CM, Kennedy KA. *Oncol Res.* 1995; 7: 371–379. [PubMed: 8747600]
30. Stewart DJ. *Crit Rev Oncol Hematol.* 2007; 63: 12–31. [PubMed: 17336087]
31. Schwerdt G, Freudinger R, Schuster C, Weber F, Thews O, Gekle M. *Toxicol Sci.* 2005; 85: 735–742. [PubMed: 15716484]
32. Murakami T, Shibuya I, Ise T, Chen ZS, Akiyama SI, Nakagawa M, Izumi H, Nakamura T, Matsuo KI, Yamada Y, Kohno K. *Int J Cancer.* 2001; 93: 869–874. [PubMed: 11519050]
33. Berners-Price, SJ, Appleton, TG. *Platinum-based drugs in cancer therapy.* Kelland, LR, Farrell, NP, editors. Humana; Totowa: 2000. 3–35.
34. Todd RC, Lovejoy KS, Lippard SJ. *J Am Chem Soc.* 2007; 129: 6370–6371. [PubMed: 17465550]

Fig. 1. Novel pH-sensitive platinum compounds with 2-aminoethanolato (**1**), 2-amino-1-butanolato (**2**), (2*S*)-amino-3-methyl-1-butanolato (**3**), 2-amino-1-hexanolato (**4**), and 2-amino-4-methyl-1-pentanolato (**5**) ligands

Fig. 2.

Antiproliferative effects of selected pH-sensitive cisplatin derivatives (**1**, **5**), as determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide assay. Concentration–effect curves in A549 cells (*left panels*) and HT-29 cells (*right panels*) at pH 7.4 and 6.0 (24 h exposure) indicate enhanced effects in slightly acidic medium

Fig. 3.

Influence of the extracellular pH (7.4 vs. 6.0) on DNA platination by the platinum(II) complexes in the human cancer cell lines HT-29 (*left*) and A549 (*right*) during exposure for 24 h. The concentration of the test compounds during exposure (5 μ M or 50 μ M) is given in parentheses. Means and corresponding standard deviations of three experiments are given as the number of platinum atoms per nucleotide (r_b value)

Fig. 4.

The pH-dependent enhancement of cellular accumulation and DNA platination, expressed as the pH 6.0/pH 7.4 ratio in the human cancer cell lines HT-29 and A549

Fig. 5.

Electropherograms recorded at 200 nm during a kinetic study of the reaction of **3** with 5'-GMP (in a molar ratio of 1:2). The incubation was performed in 20 mM phosphate buffer at pH 6.0 and 37 °C. Phosphate buffer (50 mM, pH 6.0) was used as the background electrolyte. The traces are normalized to the most abundant peak in the electropherogram

Fig. 6.

Interaction of cisplatin (A), **1** (B), **2** (C), **3** (D), **4** (E), and **5** (F) with double-stranded DNA (plasmid pTZ18u). Electropherograms after incubation of the plasmid with 60 μ M test compound at pH 6.0 or 7.4 for the times indicated as compared with untreated controls (C). Complexes **1**, **4**, and **5** strongly affect the mobility of the supercoiled (sc) form of the plasmid at pH 6.0, whereas the effects at pH 7.4 are negligible. Cisplatin shows strong effects on mobility at pH 7.4 and signs of DNA degradation at pH 6.0. *oc* open circular

Fig. 7. Cell cycle distribution of A549 cells under normal (**a**) and acidic (**b**) conditions after treatment with 5. Upon 24 h exposure, the amount of cells in S phase was dose-dependently enhanced and that of G₁/G₀ phase cells was reduced without a major impact of the pH

Fig. 8.
Expression of cell-cycle-regulated cyclins in A549 cells under normal (pH 7.4) and acidic (pH 6.5) conditions after 24 h treatment with 5. β -Actin served as the loading control

Fig. 9.

Influence of pH on induction of apoptosis and p53 expression in response to **5**. Expression of cleaved poly(ADP-ribose) polymerase (*PARP*), *PARP*, and p53 in A549 cells after 24 h of treatment was determined by Western blotting. β-Actin served as the loading control. *cl.* cleaved

Fig. 10.

Effects of pH on apoptosis induction by **5** as measured by annexin V staining. A549 cells were treated with **5** for 24 h with the concentrations indicated. Under acidic conditions (**b**), induction of apoptosis was significantly stronger than under normal conditions (**a**)

Fig. 11.

Loss of mitochondrial membrane potential (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide staining, fluorescence-activated cell sorting) in A549 cells under normal (a) and acidic (b) conditions after 24 h exposure to **5**. No significant difference between the two pH groups was observed in the case of the drug-free controls or at the indicated concentrations of **5** (two-way analysis of variance with Bonferroni postcorrection using GraphPad Prism). Only under acidic conditions did 100 μM **5** induce a significant ($p < 0.01$) increase of the fraction of cells with depolarized mitochondria, whereas this effect was comparable but did not reach significance at pH 7.4 (one-way analysis of variance with Bonferroni postcorrection)

Fig. 12.
pH-dependent ring opening and closing reactions of (2-aminoalcoholato)platinum(II) complexes

Table 1

Antiproliferative effects of pH-sensitive platinum compounds at pH 6.0 and 7.4, and factors indicating the extent of acidic activation in two human cancer cell lines

Compound	HT-29			A549		
	GI ₅₀ (μM) ^a		Factor	GI ₅₀ (μM) ^a		Factor
	pH 6.0	pH 7.4		pH 6.0	pH 7.4	
1	7.4 ± 0.7	81 ± 8	11	19 ± 8	77 ± 36	4.1
2	25 ± 15	87 ± 15	3.5	50 ± 14	214 ± 88	4.3
3	16 ± 8	44 ± 4	2.8	46 ± 20	151 ± 34	3.3
4	8.7 ± 3.6	38 ± 17	4.4	29 ± 11	110 ± 63	3.8
5	7.4 ± 0.8	36 ± 5	4.9	11 ± 6	42 ± 9	3.8
Cisplatin	1.1 ± 0.1	2.2 ± 0.3	2.0	0.7 ± 0.1	3.4 ± 1.3	4.9
Oxaliplatin	2.2 ± 0.3	2.2 ± 0.1	1.0	1.1 ± 0.1	1.2 ± 0.1	1.1

See Fig. 1 for an explanation of **1–5**.

GI₅₀ 50 % growth inhibition after exposure for 24 h in the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay

^aMeans ± standard deviations from at least three independent experiments

Table 2 Half-lives ($t_{1/2}$) of 5'-GMP binding of the aminoalcoholatoplatinum(II) complexes

Compound	$t_{1/2}$ (h)	
	pH 6.0	pH 7.4
1	6.1 ± 0.7	62.2 ± 3.4
2	8.5 ± 2.0	>60
3	11.0 ± 0.5	>50
4	6.6 ± 0.1	>50
5	2.9 ± 0.1	>50

Incubation was performed in 20 mM phosphate buffer (pH 6.0 or 7.4) at 37 °C at a concentration ratio of 5'-GMP to platinum(II) complexes of 2:1.