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# Doubt on a dogma: aquation of equatorial ligands of Pt(IV) complexes under physiological conditions

Alexander Kastner, Isabella Poetsch, Josef Mayr, Jaroslav V. Burda, Alexander Roller, Petra Heffeter, Bernhard K. Keppler and Christian R. Kowol*

Dedicated to Prof. Dr. Wolfgang Weigand on the occasion of his 60th birthday.

**Abstract** Due to their higher kinetic inertness and consequently reduced side reactions with biomolecules, Pt(IV) complexes are considered as the future of anticancer platinum drugs. We investigated the aqueous stability of a series of biscarboxylato Pt(IV) complexes under physiologically relevant conditions. Unexpectedly and in contrast to the current chemical understanding, especially oxaliplatin and satraplatin complexes revealed fast hydrolysis in equatorial position (even in cell culture medium and serum). Notably, the formed hydrolysis products strongly differ in their reduction kinetics, a crucial parameter for activation of Pt(IV) drugs also changing the anticancer potential of the compounds in cell culture. The discovery that intact Pt(IV) complexes can hydrolyze at equatorial position, contradicts the dogma on the general kinetic inertness of Pt(IV) compounds and needs to be considered in the screening and design for novel anticancer drugs.

Pt(II) complexes still play a very important role in cancer treatment[1], being part of about 50% of all chemotherapies.[2] Cis-, carbo- and oxaliplatin, are widely used against various forms of cancer.[3] Furthermore, very recent clinical data show impressive synergistic effects of platinum drugs together with checkpoint inhibitor immunotherapy.[4] Their mode of action involves aquation, binding to DNA and thereby inducing apoptosis.[5] However, Pt(II) complexes do not only bind to DNA in the tumor tissue, but also in healthy cells, resulting in (severe) side-effects like nephro-, neuro- and gastrointestinal-tract toxicity.[6] To reduce these adverse effects, Pt(IV) complexes attract increasing interest.[7] Such low-spin $d^6$ octahedral complexes are considered to be kinetically more inert[8] and consequently far less reactive towards biomolecules.[9] Activation by reduction to the active Pt(II) complexes can occur via low-molecular weight compounds like ascorbate and glutathione[10], and/or high-molecular weight proteins.[11] Another advantage of Pt(IV) complexes are the two additional axial ligands, which can be used to optimize chemical and biological properties, like lipophilicity and reduction potential. Furthermore, bioactive substances or drug-targeting moieties can be used as axial ligands.[8b, 12] Despite these benefits, no Pt(IV) drug has been clinically approved so far.[2] Nevertheless, some of them entered clinical trials. However, Tetraplatin, ctc-$[Pt(DACH)Cl_4]$ (DACH = (1*R*,2*R*)-(−)-1,2-diaminocyclohexane), was discontinued because of its neurotoxicity[13], while Iproplatin, ctc-$[Pt(IPA)_2(OH)_2Cl_2]$ (IPA = isopropylamine) did not show superior anticancer activity.[14] The most prominent representative Satraplatin, ctc-$[Pt(NH_3)(CHA)(OAc)_2Cl_2]$ (CHA = cyclohexylamine) ultimately failed to show improved overall survival in a phase III study as well.[2] Nevertheless, current research strongly focuses on Pt(IV) complexes, as they are considered as the next generation of platinum-based anticancer drugs.[7] Consequently, the underlying chemical properties and reactivities are of high importance for the specific design of novel drugs with optimized biological properties. As already mentioned above, Pt(IV) complexes are considered as kinetically inert. However, there are a few reports that the axial ligands can be hydrolysed when electron-withdrawing ligands like dichloroacetate are present.[15] Concerning the equatorial ligands of Pt(IV) complexes, a study using plasma from patients treated with satraplatin showed not only the parent Pt(II) drug JM118, but unexpectedly also the mono- and di-hydrated Pt(IV)-species, where one or two equatorial chlorido ligands were exchanged with hydroxido ligands.[16] This is in strong contrast to the “dogma” of kinetic inertness of Pt(IV) drugs. As such a hydrolysis would strongly impact on the chemical characteristics (e.g. reduction potential) and consequently also the biological properties, in this study we investigated the hydrolysis of the equatorial ligands for a panel of different Pt(IV) complexes in comparison to satraplatin (Figure 1).

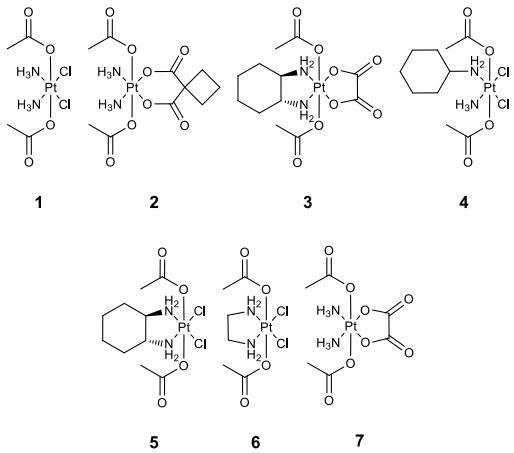


**Figure 1.** Investigated Pt(IV) compounds.

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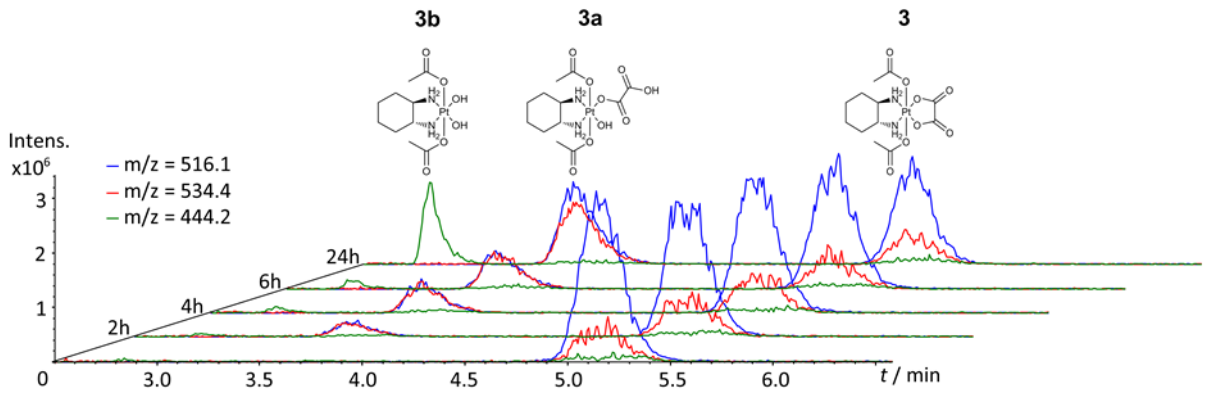


**Figure 2.** Time-dependent Hydrolysis of **3** (50 µM in PB) at 37°C and pH 7.4 with formation of the hydroxido (**3a**) and dihydroxido (**3b**) complex**.**

To get a first overview, we analyzed the hydrolytic stability of the model complexes **1-3** and satraplatin **4** (Figure 1) under physiologically relevant conditions. All complexes possess two acetato ligands in axial position and a cisplatin (**1**), oxaliplatin (**2**), carboplatin (**3**) or *cis*-amminedichlorido(cyclohexylamine) (**4**) equatorial core. The complexes were incubated in phosphate buffer (PB) at 37°C and pH 7.4 for 24 h and monitored using HPLC-MS. Indeed, after 24 h the majority of satraplatin (72% hydroxido and 3% dihydroxido species) and the oxaliplatin-based complex **3** (43% hydroxido and 14% dihydroxido; Figure 2) were already hydrolyzed. In contrast, the cisplatin analogue **1** was widely stable (5% hydroxido species) and the carboplatin derivative was completely unchanged (Figure S1). To exclude a significant impact of the column on the hydrolysis process the incubated solutions were also directly injected into the mass spectrometer. This revealed the same hydrolyzed products as observed with HPLC-MS (data not shown).

Notably, repetition of these experiments at pH values of 8.0 and 9.0 even resulted in distinctly accelerated hydrolysis (Table 1). At pH 9.0 the parental oxaliplatin derivative **3** completely disappeared with exclusive formation of the dihydroxido species. Also satraplatin was fully converted into its hydrolyzed species (25% hydroxido/75% dihydroxido). In contrast, in case of the cisplatin analogue **1** still ~70% of intact complex could be observed together with ~20% hydroxido and 5 % dihydroxido species. Carboplatin was again completely unaffected. These findings indicated a distinct pH-dependency of the reaction, which could be exemplarily proven for **3** revealing a perfect linear correlation between pH and the reaction rate (Figure S2).

To rule out the involvement of phosphate in the hydrolysis, other buffer systems i.e. HEPES and ammonium carbonate were investigated for **3** yielding comparable results (Figure S3). Next, **3** was investigated in cell culture medium (RPMI-1640), which showed similar ratios of hydroxido/dihydroxido complex formation as in pure PB solution (Figure S3). Notably, the pH value of fresh cell culture medium was about 7.4 but raised up to pH 8–9 within 24 h.

**Table 1.** Relative amount in % of the respective hydrolysis products of complexes **1**-**4** determined by HPLC-MS after incubation of 50 µM compound at 37°C for 24 h in PB at different pH values.

| | | Compound | | | |
|---|---|---|---|---|---|
| **pH** | **Species** | **1** | **2** | **3** | **4** |
| 7.4 | % Hydroxido | 5 | / | 43 | 72 |
| | % Dihydroxido | / | / | 14 | 3 |
| 8.0 | % Hydroxido | 10 | / | 32 | 76 |
| | % Dihydroxido | 2 | / | 53 | 19 |
| 9.0 | % Hydroxido | 22 | / | / | 25 |
| | % Dihydroxido | 5 | / | 100 | 75 |

Consequently, the cell culture medium was phosphate-buffered (150 mM), generating stable pH values over >24 h. Finally, the hydrolysis of **3** was also investigated in mouse serum (also buffered with 150 mM phosphate), again yielding similar results (Figure S3). This clearly indicates that all the components of cell culture medium or serum (amino acids, proteins, inorganic salts and vitamins) have no influence on the hydrolysis process of the Pt(IV) complex. Instead this reaction is solely dependent on the presence of water as a solvent and the appropriate pH value.

Notably, the hydrolysis of oxaliplatin with ~10%[17] is distinctly slower compared to **3** with ~50% after 24 h at pH 7.4. A likely explanation is, that the Pt(II) complex with a monodentate oxalate ligand possesses a $pK_a$ of 7.23[18] and therefore an aqua ligand is partially present. This enables a fast ring-closing reverse reaction to the bidentate-bound oxalate and reformation of oxaliplatin. Consequently, the release of oxalate is suppressed. In contrast, the $pK_a$ of the Pt(IV) complex **3a** is much lower at 3.5 (see below). Therefore a hydroxido ligand is still present at pH 7.4 and the reverse reaction back to **3** is hindered. This results in a shift of the equilibrium towards the dihydroxido species and as a consequence in faster hydrolysis than in case of Pt(II).

To investigate if the hydrolysis of **3** takes place via an attack on the electrophilic carboxylic group of the oxalate ligand or the platinum itself, we analyzed the hydrolysis process in $H_2{}^{18}O$ (buffered with 50 mM phosphate). This measurement resulted in an exact mass of *m/z* = 488 for ctc-$[Pt(DACH)(OAc)_2(^{18}OH)_2] + Na^+$, which proves that the platinum

core is directly attacked and not the oxalato ligand (Figure S4). This is in line with $H_2{}^{18}O$ studies of the hydrolysis of the axial dichloroacetate ligands of mitaplatin.[19]

To gain more insights on the exact impact of the different equatorial ligands, the additional Pt(IV) model complexes **5-7** (Figure 1) were synthesized. Table 2 presents an overview on the speed of hydrolysis of all complexes **1**-**7** after incubation in PB at pH 7.4 and 37°C for 24 h.

**Table 2.** Overview on the impact of equatorial ligands on the rate of hydrolysis in % of complexes **1**-**7** determined by HPLC-MS of 50 µM compound after 24 h incubation in PB at 37°C.

| Complex | % Hydroxido | % Dihydroxido | Stable ligand[b] | Labile ligand |
|---|---|---|---|---|
| **1** | 5 | 0 | $NH_3$ | Cl |
| **2** | 0 | 0 | $NH_3$ | CBDCA |
| **3** | 41 | 13 | DACH | oxalate |
| **4** | 72[a] | 3 | $NH_3$/CHA | Cl |
| **5** | 81 | 18 | DACH | Cl |
| **6** | 42 | 0 | en | Cl |
| **7** | 0 | 0 | $NH_3$ | oxalate |

[a] two different mono-hydroxido species can be observed with a ratio of ~ 1:6 [b] DACH = (1*R*,2*R*)-(−)-1,2-diaminocyclohexane; CHA = cyclohexylamine; en = ethylenediamine; CBDCA = 1,1-cyclobutanedicarboxylic acid.

The following trends could be observed: 1) when comparing the derivatives with a chlorido ligand as leaving group, the oxaliplatin-like complex (DACH; **5**) has the fastest hydrolysis with complete disappearance of the parental compound after 24 h, followed by satraplatin ($NH_3$/CHA; **4**), the ethylenediamine complex (en; **6**) and the cisplatin derivative ($NH_3$; **1**) with just 5% hydrolysis. 2) Comparison of the complexes with two $NH_3$ equatorial ligands shows generally very slow hydrolysis rates with the cisplatin derivative **1** as the fastest (5% hydrolysis), followed by the completely stable oxalate-bearing complex **7** and the carboplatin analogue **2**. 3) When comparing complexes with the same amine ligand but chlorido vs. oxalato leaving groups (e.g. **5** vs **3**), the complex with chlorido ligands hydrolyses faster (81% hydroxido for **5**) than the one with the bidentate oxalate ligand (41% for **3**). However, when exchanging the DACH ligand in complexes **5** and **3** for two $NH_3$ moieties, the hydrolytic stability dramatically increases with only 5% hydroxide species for **1** and no hydrolysis for **7**. This impressively shows that the influence of the leaving group (chlorido vs. oxalate) has much less impact on the hydrolysis compared to the stable amine ligand (DACH vs. $NH_3$). Notably, incubation of **1**-**7** in cell culture medium (RPMI-1640) resulted in similar amounts of hydrolysis products. As RPMI-1640 contains ~100 mM NaCl, this also confirms that the presence of chloride ions cannot significantly change the rate of hydrolysis (this could be also supported by comparison of **4**, **5** and **6** in 50 mM PB vs. 50 mM PBS with 150 mM NaCl).

In order to obtain deeper insight into the underlying binding energies (BE) and heights of activation barriers, a set of quantum-chemical data was calculated. The BE obtained from energy decomposition of the individual reactants (Table S1) are in good agreement with the trends mentioned above. For example: comparison of the BE of the leaving chlorido ligands resulted also in the order **5**~**4**>**6**>**1** (in case of **4** the weaker-bound chlorido ligand was considered). Furthermore, BE of the ammine ligands in complex **1**, **2** and **7** were in line with the experimental data, although the BE of the acetato ligands are quite different. These results were also verified by extended-transition state natural orbitals for chemical valence (ETS-NOCV)[20] analysis and average ionization local potential (ALIP) maps[21]. Additionally, the heights of activation barrier calculations of the hydrolysis (Scheme S1) of the equatorial ligands correlate very well with the HPLC-MS experiments (Table 1). As summarized in Table 3, the activation energies for the hydrolysis in case of **3** and **4** are distinctly lower compared to **1** and **2**. This confirms that the higher σ-donor properties of a secondary amine compared to simple $NH_3$ ligands are important for an accelerated reaction, which explains the differences observed in the HPLC-MS experiments.[22] Furthermore, also the second hydrolysis step ΔE(TS2) of **3**, where the monodentate-bound oxalate ligand is finally released, possesses a distinctly lower activation barrier compared to **4** with two chlorido ligands in exact agreement with the HPLC-MS studies (Table 1). The calculations in case of **4** further revealed a higher *trans*-effect of the CHA ligand compared to $NH_3$ explaining the two peaks observed with HPLC-MS. This is in accordance with a report on the respective Pt(II) complex JM118.[23]

**Table 3.** Energy of activation barriers (in kcal/mol) for replacement of the first (ΔE(TS1)) and the second (ΔE(TS2)) equatorial-leaving group.

| Complex | **1** | **2** | **3** | **4**[a] | **4**[b] |
|---|---|---|---|---|---|
| ΔE(TS1) | 31.1 | 31.6 | 27.0 | 25.7 | 26.1 |
| ΔE(TS2) | 27.3 | 30.4 | 22.6 | 24.8 | 25.0 |

[a] (trans to CHA); [b] (trans to $NH_3$)

To test how the hydrolysis alters the chemical and biological properties the two derivatives of **3** were synthesized. This was achieved through incubation of **3** at pH 8–9 and 37°C and subsequent purification via preparative HPLC. The hydroxido (**3a**) and the dihydroxido (**3b**) species was characterized by $^1$H and $^{13}$C-NMR, mass spectrometry and elemental analysis. Furthermore, the $pK_a$ values of **3a** and **3b** were determined via $^1$H-NMR and found to be 3.5 and 4.0, respectively (Figure 3).

This is in line with data of **3a** after ~3 h of incubation at pH of 2.5, which resulted in a complete transformation back to its parental complex **3**. According to the $pK_a$ value the hydroxido ligand gets protonated at such low pH values and the thereby generated aqua ligand can be released under re-formation of the bidentate oxalato complex **3**. In contrast, **3a** is stable at pH 5.5, which again fits to the $pK_a$ value of 3.5.

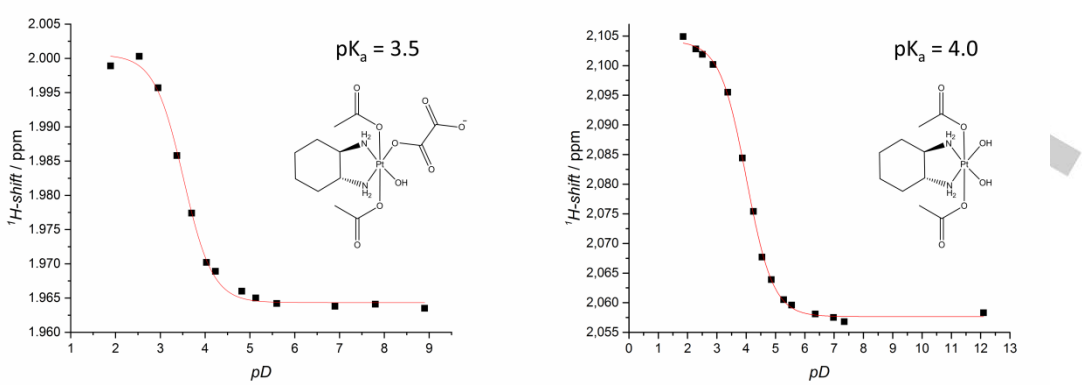


**Figure 3.** Determination of $pK_a$ values via correlation between ppm-shift of the acetato ligand and pD for **3a** (left) and **3b** (right).

After incubation of **3b** in MeOH/$Et_2O$ a few single crystals could be obtained and analyzed by X-ray diffraction. The structure reveals an octahedral geometry with two axial acetato ligands and the equatorial DACH moiety. However, the two hydroxido groups were exchanged by methoxido ligands (Figure 4; for bond lengths and angles see Table S5/S6) Notably, the crystals contain both the *R*,*R* and *S*,*S* isomers as a racemic mixture. This can be explained by the ~2% *S*,*S* isomer present in the commercially available DACH compound and the often observed preference of compounds to crystallize as a racemate and not as the pure isomers.[24]

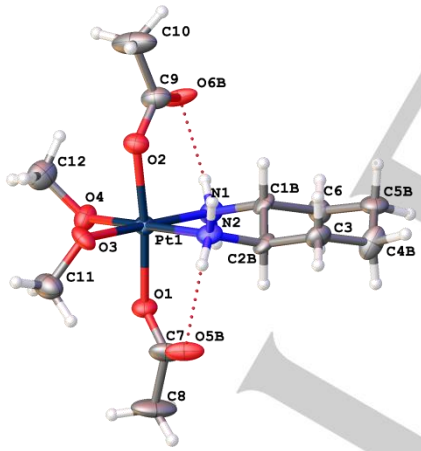


**Figure 4.** X-ray crystal structure of **3b** incubated in MeOH/$Et_2O$ (the disorder of the DACH ligand is omitted).

As a next step, the reactivity of **3b** with different organic solvents was investigated. In contrast to aqueous cell culture medium or serum, incubation of **3b** with e.g. DMSO, acetonitrile, MeOH or EtOH for 1 h resulted in the exchange of one hydroxido ligand, which could be proven by mass spectrometry and an altered HPLC retention time (Figure S5). This indicates that at very high excess, the hydroxido ligands indeed can be substituted, which could also be used as a new synthetic pathway of introducing equatorial ligands into already existing Pt(IV) complexes.

As a next step, the thermodynamic reduction properties of **3**, **3a** and **3b** were compared using cyclic voltammetry. All three complexes showed irreversible reduction peaks with decreasing potentials the more hydroxido ligands are present in the molecule (**3**: –630 mV vs. NHE; **3a**: –670 mV vs. NHE; **3b**: –920 mV vs. NHE). This trend is in line with data from similar Pt(IV) complexes, however with one or two axial hydroxido groups.[25] The kinetic reduction rates of **3**, **3a,** and **3b** were investigated by HPLC after incubation with 10 eq. of L-ascorbic acid at 20°C. While **3** was completely stable within 6 h, **3a** and **3b** were reduced much faster and fully converted to the respective Pt(II) species already after 3-4 h (Figure 5). Consequently, these hydroxide species are even faster reduced than the cisplatin complex **1** which is well-known to be much more sensitive than oxaliplatin or carboplatin derivatives.[26] Thus, although the thermodynamic reduction potential decreases with increasing number of OH groups, the reduction rate extremely accelerates. Although this seems to be unexpected, these data are in line with a study of Gibson *et al*.[25] using axial mono- and dihydroxido derivatives of complex **3** and support the importance of the Pt(IV) reduction kinetics.

**Figure 5.** Reduction rate of 1 mM compounds **1**-**3**, **3a** and **3b** at 20°C with 10 eq. L-ascorbic acid in 250 mM phosphate buffer at pH 7.4 monitored by HPLC.

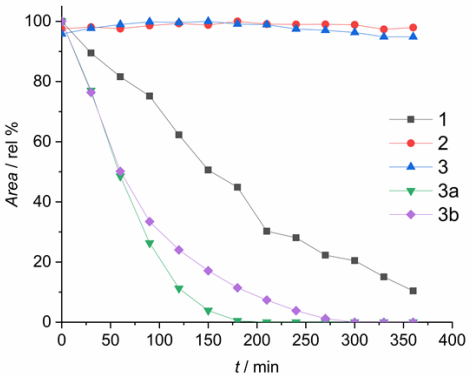


**Table 4.** $IC_{50}$ *values of **3**, **3a** and **3b** against cancer cells after 72 h exposure. Values represent mean ±SD from 3-4 biologically independent experiments performed in triplicates.*

| Cell line | 3 | | 3a | | 3b | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ | ±SD | $IC_{50}$ | ±SD | $IC_{50}$ | ±SD |
| **HCT116** | 16.2 | ±2.0 | 16.6 | ±2.4 | 11.8 | ±2.1 |
| **RKO** | 12.5 | ±2.8 | 15.6 | ±4.9 | 9.4 | ±2.9 |
| **CT-26** | 18.7 | ±2.2 | 13.7 | ±1.8 | 8.2 | ±2.1 |

To evaluate, whether the changed chemical properties of the hydrolysis products result in differences in biological activity, the anticancer activity of **3**, **3a** and **3b** against three cancer cell lines (HCT116, RKO and CT-26) was evaluated. These experiments revealed that **3b** had a significantly lower $IC_{50}$ value (up to 2-fold more active) compared to the parental species **3** or the monohydroxido species **3a** (Figure 6, Figure S6 and Table 4). An explanation for this could be that after reduction of **3a,** the hydroxido group in the respective Pt(II) complex is protonated ($pK_a$ = 7.23)[18]. This aqua ligand represents a good leaving group and facilitates ring-closure to the bidentate-bound oxalate and reformation of oxaliplatin. Consequently, a very similar cytotoxic activity **3** and **3a** can be expected. In contrast, in case of **3b**, oxaliplatin cannot be regenerated, and instead the Pt(II) complex $[Pt(DACH)(H_2O)OH]^+$ is formed, able to directly interact with biological targets. In addition, cellular uptake of **3**, **3a** and **3b** was studied on HCT-116 and CT-26 cells after 3 h incubation. Notably, no significant differences in the uptake could be observed, with the parental complex **3** showing the highest cellular platinum levels (Figure S7).

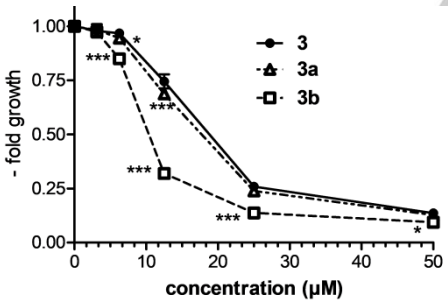


**Figure 6.** Anticancer activity of **3**, **3a** and **3b** after 72 h against HCT116 cells measured by MTT assay. The values given are means ± SD of one representative experiment performed in triplicates. * p<0.05, *** p<0.001.

Taken together, the here presented data indicates that we have to rethink the current knowledge on anticancer Pt(IV) complexes and doubt on the dogma of a generally very high hydrolytic stability of Pt(IV) complexes. Depending on the exact equatorial coordination sphere, there are massive differences in their stability at physiological pH and the resulting hydrolyzed Pt(IV) complexes possess vastly altered physicochemical properties. Especially their rate of reduction, the crucial factor in the activation of Pt(IV) prodrugs, is strongly accelerated upon prior hydrolysis. These observations are also important for e.g. simple physiologically buffered solutions of oxaliplatin(IV) complexes, where more than 50% hydrolysis can be expected within 24 h. Furthermore, not the leaving group itself, but the substituents at the equatorial amine ligands have the major impact on the rate of hydrolysis. In addition, it is important to mention that (cancer) cells possess different pH values in their organelles, e.g. up to pH 8 in mitochondria.[27] Consequently, even higher levels of hydrolysis can be expected, as this reaction is further accelerated at more alkaline pH values. Under such conditions also cisplatin derivatives start to hydrolyze to a significant degree.

Thus, it is essential to carefully select the equatorial core of Pt(IV) prodrugs not only according to the biological activity of the active Pt(II) analogue, but also to the hydrolytic stability of the Pt(IV) prodrugs (satraplatin<oxaliplatin<<cisplatin<carboplatin) and the rate of reduction (satraplatin>cisplatin>>oxaliplatin~carboplatin). All these parameters influence the biological properties and have to be considered in the future design of novel Pt(IV) anticancer prodrugs.

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## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** Platinum(IV) complexes • Anticancer • Hydrolysis • Prodrug • Reduction

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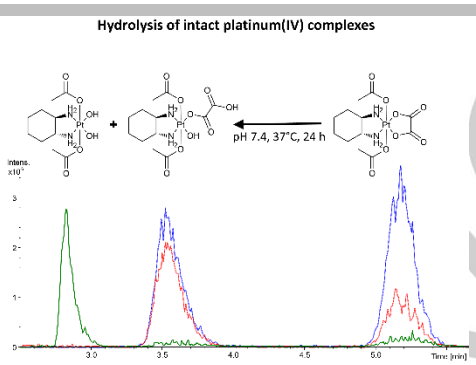
Layout 1:

## COMMUNICATION

**Less inert than assumed:** The hydrolytic stability of platinum(IV) complexes was investigated and substantial hydrolysis of equatorial ligands could be observed for various platinum(IV) compounds under physiological relevant conditions.

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***Page No. – Page No.***

**Doubt on a dogma: aquation of equatorial ligands of Pt(IV) complexes under physiological conditions**