Targeting Platinum Compounds: synthesis and biological activity

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Probing the potential of platinum(II) complexes for the inhibition of thiol-dependent enzymatic activity*

Abstract - The synthesis and biological evaluation of platinum(II) amine complexes designed to act as inhibitors of the human cysteine protease cathepsin B, a thiol-dependent enzyme, is described. The complexes, composed of a cathepsin targeting ligand and a platinum(II) moiety with varying degrees of reactivity towards nucleophiles were characterized by physical-analytical methods and the proof of the principle was illustrated in a model reaction. In biological tests for inhibitory activity against cathepsin B the presented compounds did not show significant inhibitory activity.

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6.1 INTRODUCTION

Disturbance of the natural equilibrium of enzymatic activity of the cysteine proteases, responsible for protein degradation, may lead to a variety of pathological conditions, including rheumatoid arthritis, cancer and neurological disorders [1]. Protein inhibitors can restore the normal balance through regulation of these enzymes and therefore present an opportunity for drug development [2,3]. At the active site of the thiol-dependent cathepsin enzymes a conserved cysteine residue catalyses the hydrolysis of amide bonds in peptides and proteins [4]. Synthetic cathepsin inhibitors generally contain an electrophilic functionality that can react with this cysteine. Examples of such electrophilic groups include aldehydes, disulfides, vinylsulfones and halomethyl ketones [5,6]. This chapter describes the results of an investigation on whether platinum(II), a well-established pharmacophore for the development of cytotoxic agents [7] with high thiophilicity [8], could be used as an electrophilic cysteine binding moiety, for the possible development of a novel class of cathepsin inhibitors.

In the treatment of various types of cancer the reaction of cisplatin and related drugs with cellular DNA is a key step in the triggering of apoptotic pathways in the cell. More specifically the binding of platinum to the N7-nitrogens of two adjacent guanosine bases is thought to disrupt DNA duplication leading to cell death [9]. However, platinum drugs are also known to react with many other cell components. The chemical preference of the 'soft' metal platinum for 'soft' ligands, such as sulfur-donating ligands accounts for much of the drug binding to glutathione and other S-containing biomolecules, present in relatively high concentrations inside the cell [10]. Ligand design for new platinum-based antitumour drugs is focused on minimizing these side reactions that may be involved in the many undesirable side effects associated with platinum-based chemotherapy, as well as drug resistance [11].

The aim of this study is to investigate whether the susceptibility of platinum(II) to react with thiols could be used as a starting point in the development of a novel class of cysteine protease inhibitors. Such compounds would contain, next to a platinum entity, a protease-specific recognition element, inspired by the morpholine-carbonyl-leucinyl-homophenyl-

alanyl moiety, as present in the broad-spectrum vinyl sulfone based cysteine protease inhibitor **1** and its disulfide analogue **2** (Figure 6.1). To test this hypothesis, peptide platinum complexes **3**-**5** (Figure 6.1) were synthesised and evaluated for their inhibitory efficacy in cell lysates, as well as on purified recombinant cathepsin B (CatB). The outcome of these studies is presented here.

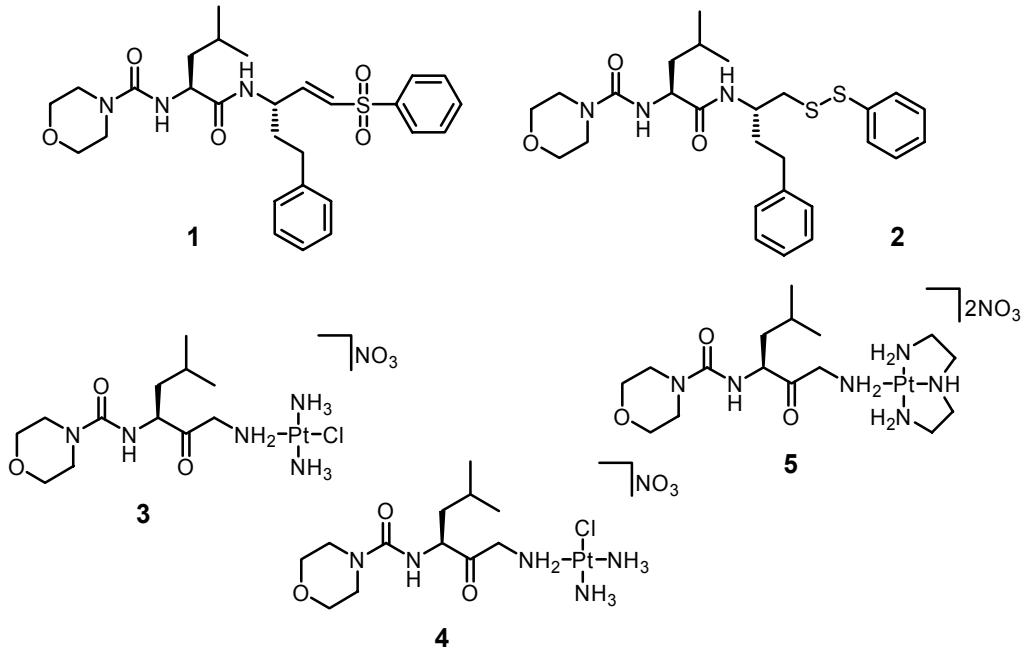
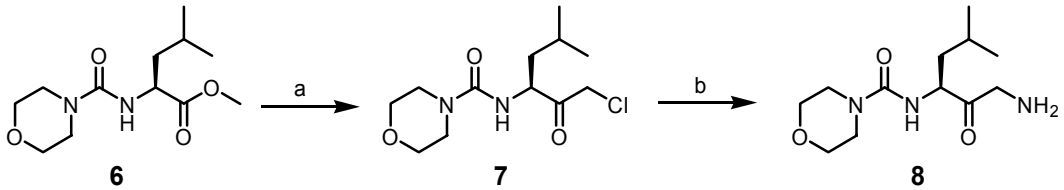


Figure 6.1: Cathepsin inhibitors containing vinylsulfone (**1**) [5], disulfide (**2**) [6] or platinum (**3**-**5**) as electrophilic groups.

6.2 SYNTHESIS OF THE COMPLEXES

The synthesis of the targeting ligand **8** was accomplished as illustrated in scheme 6.1. Addition of morpholine acetyl chloride to leucine methyl ester gave compound **6,** which was efficiently converted to the chloroketone **7** by the Kowalski method, utilizing excess of LDA and CH_2ICl [12]. This method is preferable to the classical method, involving conversion of an N-acyl-α-amino acid to an α-diazoketone and subsequent acidolysis with HX [13], as the use of diazomethane is avoided. The resulting chloroketone was converted to amino methyl ketone **8** using excess NH_3 in diethyl ether over 72 h [14].



Scheme 6.1: Synthesis of ligand **8**. Reagents and conditions: (a) 6 equiv. LDA, 5 equiv. CH_2ICl in dry THF; (b) NH_3/MeOH in Et_2O.

For the synthesis of complexes **3**-**5**, the platinum compounds *trans*-[Pt(NH$_3$)$_2$Cl$_2$], *cis*-[Pt(NH$_3$)$_2$Cl$_2$] and [Pt(dien)Cl]Cl were activated by overnight treatment with 0.9 equivalent of AgNO$_3$ in the dark. After removal of AgCl the activated platinum species were reacted overnight with an equimolar amount of ligand **8**, yielding the target compounds **3**-**5**. The final compounds precipitated from methanol by slow diffusion of diethyl ether, to give pale brown solids, in overall yields of 41% (**3**), 57% (**4**) and 51% (**5**), respectively. Their structural integrity was verified using ^{1}H NMR, ^{195}Pt NMR and ESI-MS.

6.3 MODEL REACTION MONITORED BY NMR

To obtain insight in the reactivity of the compounds with thiol-containing biomolecules their overnight reaction with one equivalent of cysteine in dilute solution (D$_2$O, pH 7.5, 310 K) was followed using ^{195}Pt NMR. In aqueous solution reactive thiols can displace the coordinated chloride of compounds **3** and **4**, either through direct substitution, or via a hydrated intermediate species [15]. Compound **5** is not endowed with an obvious labile ligand coordinated to platinum and is therefore expected to react at a much lower rate with nucleophiles.

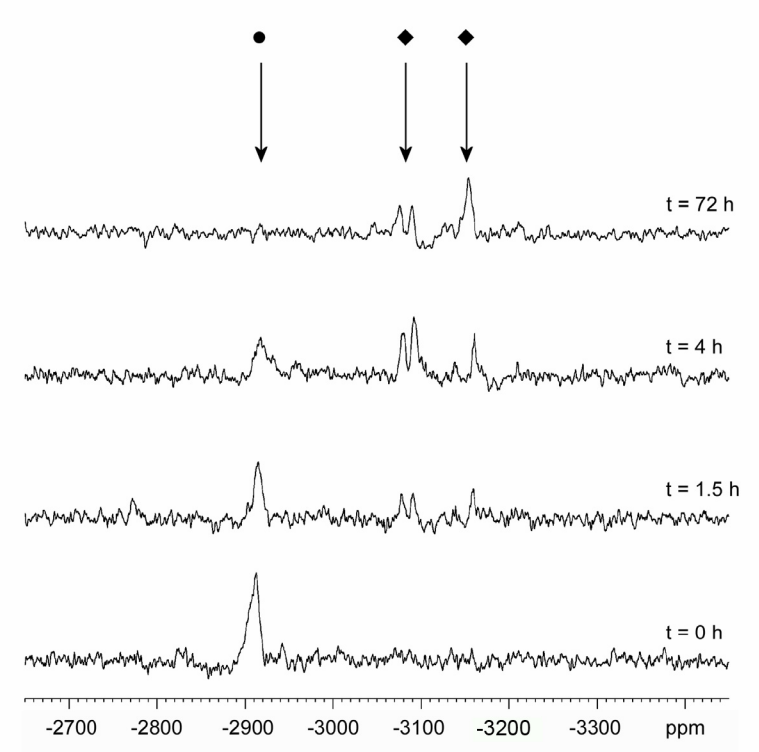


Figure 6.2: Time-dependent ^{195}Pt NMR for the reaction of compound **5** with 1 equiv of cysteine at 310 K in D$_2$O at pH 7.5 showing the disappearance of the starting material (●) with the gradual appearance of new signals around 200 ppm upfield (◆).

Indeed, for compound **3** the starting material disappeared entirely within the first 30 min of measurement, started directly after the addition of the cysteine, to give two new peaks at -3165 and -3203 ppm typical for the [PtN_3S] chromophore [16]. In the case of compound **5** the starting material disappeared gradually, while simultaneously three new species appeared at about 200 ppm upfield 1½ h from the start of the reaction (Figure 6.2). After 72 h the starting material had disappeared entirely and only the three new peaks were found present at -3077, -3091 and -3155 ppm in an approximate ratio of 1 : 1 : 2, respectively, which can be assigned to three different [PtN_3S] species formed in the reaction [16]. The ability of cysteine to replace all, or at least three of the four amine ligand groups surrounding the platinum atom, suggests that compound **5**, though not as reactive as compound **3** or **4**, might be sufficiently reactive to bind the active site of a thiol-dependent enzyme.

6.4 BIOLOGICAL ASSAYS

As the next research objective, the viability of compounds **3**-**5** as cysteine protease inhibitors was investigated in a selection of biological assays. Cisplatin was always included in the tests as a reference compound. Inhibition of CatB activity on a functional scale was tested in competition-type experiments, using the active site-restricted affinity label DCG-0N, a derivative of DCG-04 that shows identical labelling characteristics [17,18]. This probe selectively binds at the active site cysteine of several cathepsins in a covalent fashion. The amount of binding of the probe is inversely proportional to the amount of bound inhibitor. The activity of such an inhibitor can hence be detected in a semi-quantitative fashion via the biotin moiety of the probe. When total cell lysates from human monocytes containing active CatS, CatH, CatB and CatZ were incubated with the platinum compounds, subsequently probed with DCG-0N and visualised using streptavidin-horse radish peroxidase (HRP) and a chemiluminescent detection reagent (ECL) [19], no effect on the amount of active cathepsin polypeptides was observed (data not shown). To eliminate the possibility that the lack of enzyme inhibition in cell lysates was due to non-specific interactions with cellular proteins and/or DNA, the inhibitor activity of each compound was also measured using commercially available CatB from human liver that was purified by affinity chromatography. CatB was incubated with compounds **3-5** and cisplatin for 30 min at pH 5.0 *in vitro* prior to addition of DCG-0N, followed by resolution by SDS-PAGE under fully denaturing conditions and visualization of active polypeptide species by the streptavidin-HRP blot. None of the compounds were found to inhibit CatB at concentrations up to 500 μM (Figure 6.3). Traces of CatS and CatZ were also visible as minor contaminations of the CatB preparation. These enzymes were not inhibited consistently by the compounds either.

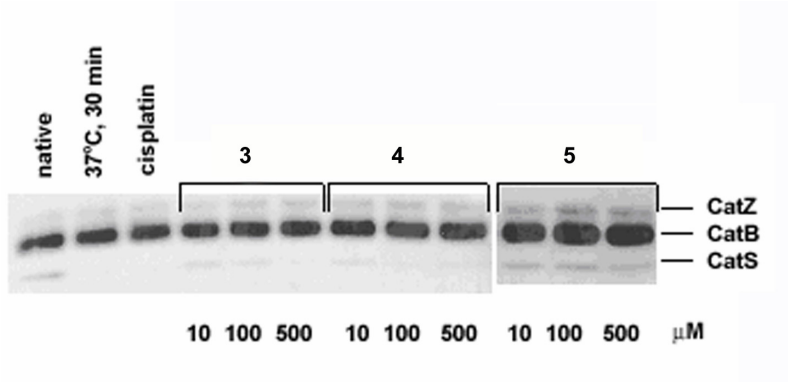


Figure 6.3: Streptavidin-HRP blot of purified CatB treated with cisplatin and compounds **3**-**5**.

To confirm this result by an independent method, the influence of the compounds **3**-**5** and cisplatin on the turnover of the fluorogenic substrate benzoxy-phe-arg-aminomethylcumarin (Z-FR-AMC), which detects the combined activity of CatB, CatL and CatS, was measured [20]. Again, none of the compounds functionally inhibited cathepsin activity (data not shown). At the higher level of sensitivity in this assay compared to the affinity-labelling method, the data even suggested a concentration-dependent slight increase in cathepsin activity with some of the compounds, most prominently observed with compound **4** (Figure 6.4). Given that this increase was not detected using the affinity labelling method, it is unlikely to be functionally meaningful. Clearly, the compounds lack significant activity on cathepsin activity *in vitro*.

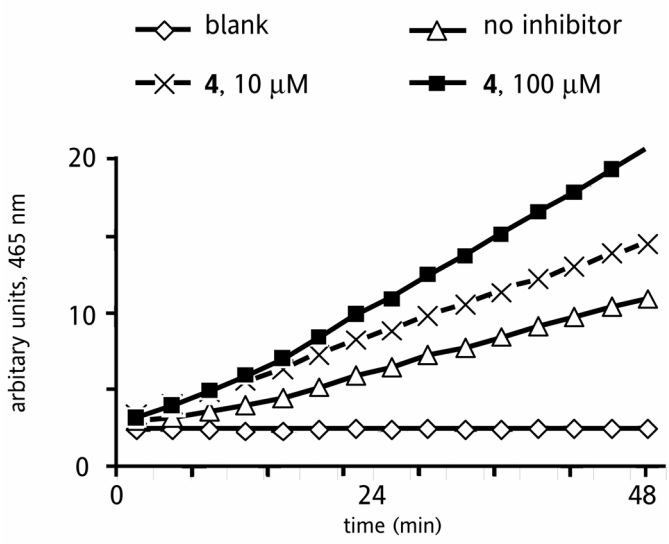


Figure 6.4: Cathepsin activity measured as a function of time on incubation of purified CatB with compound **4,** using the Z-FR-AMC assay.

6.5 CONCLUSION

In conclusion, the NMR-model study clearly shows the ability of the complexes described in this chapter to react with thiols under conditions mimicking those encountered in the cell, thereby underlining the proof of principle that platinum(II) complexes have the potential to bind the active site of thiol-dependent enzymes. Unfortunately, this reactivity is not translated to enzyme inhibition in the assays carried out with cell lysates containing a number of cathepsins, or with purified CatB. Although this observation does not rule out activity of these compounds in other cysteine proteases, or in thiol-dependant enzymatic activities of an altogether different nature, a better understanding of the activity of this class of compounds in cathepsin enzymes is a useful starting point for the evaluation of the potential of platinum-based enzyme inhibitors. The active site of CatB [21-23] should be large enough to accommodate a platinum(II) moiety as found in the presented compounds, which is typically around 4-5 Å in size [24]. It is therefore probable that the lack of activity observed is due to a lack of affinity for the active site. This can be due, either to insufficient targeting induced by the morpholine-leucine-amino-ketone ligand, or to insufficient reactivity of the platinum at the active site. Further variation of both the targeting and the non-targeting ligands surrounding the platinum moiety therefore needs to be undertaken to investigate whether platinum compounds can act as inhibitors of thiol-dependent enzymatic activities. Finally, the binding kinetics of platinum(II) in general may be too slow for efficient inhibition of this class of enzymes. In this case, other transition metals, such as palladium, may be more suitable electrophiles in novel inhibitors of thiol-dependent enzymatic activity.

6.6 EXPERIMENTAL SECTION

6.6.1 General

All NMR measurements were performed on a 300 MHz Bruker DPX300 spectrometer with a 5 mm multi-nucleus probe. Temperature was kept constant at 298 K using a variable temperature unit. The water signal for the spectra taken in D_2O was minimized using a WATERGATE pulse sequence. MS spectra were taken on a ThermoFinnegan AQA ESI-MS. Reagents were purchased from Aldrich, unless otherwise stated. Solvents were obtained from Applied Biosystems Inc. THF was distilled over sodium prior to use. The platinum compounds, *cis*-$[Pt(NH_3)_2Cl_2]$, *trans*-$[Pt(NH_3)_2Cl_2]$ and [Pt(dien)Cl]Cl were obtained using literature procedures, from K_2PtCl_4, provided by Johnson and Matthey on a generous loan scheme.

6.6.2 Preparation of the ligands

N-morpholine-leucine methyl ester **6***:* To L-leucine methyl ester hydrochloride (2 g, 13.4 mmol) and triethyl amine (3.64 ml, 26.8 mmol) dissolved in dichloromethane (50 ml) was added 4-morpholinecarbonyl chloride (1.98 ml, 14 mmol) under nitrogen. The solution was stirred for 4 h and concentrated in vacuo, washed with brine and extracted with DCM (3 × 20 ml). The combined organic layers were dried ($MgSO_4$) and evaporated in vacuo to yield the product as an off-white solid (3.46 g, 13.4 mmol) in quantitative yield. ^{1}H NMR ($CDCl_3$, TMS) δ (ppm): 0.92 (d, J = 6.3 Hz, 6H; $(CH_3)_2$), 1.55 (t, J = 7.2 Hz, 2H; CH_2CH, 1.71 (m, 1H; $CH(CH_3)_2$), 3.38 (m, 4H; 2 CH_2N morph.), 3.63 (m, 4H; 2 CH_2O morph), 3.69 (s, 3H; OCH_3), 4.41 (d, J = 4.3 Hz,1H; α-H leu), 5.80 (m, 1H; NH). ^{13}C ($CDCl_3$): δ (ppm): 20.9 CH(CH_3), 22.0 CH(CH_3), 24.0 ($CH(CH_3)_2$), 40.1 (CH_2CH), 43.3 (CH_2NH morph), 51.1(*CH*CO) 51.4 (CH_3O), 65.5 (CH_2O morph), 156.8 (*C*(O)OMe), 174.1 (*C*(O)NH). ESI-MS: *m/z*: 260 $[M+H]^+$, 281 $[M+Na]^+$, 297 $[M+K]^+$.

N-Morpholine-leucine chloroketone **7***:* LDA was prepared by addition of nBuLi (18.8 ml, 30 mmol) to diisopropylamine (4.62 ml, 33 mmol) in dry THF (40 ml) at -70 °C under N_2. The LDA was added dropwise to **6** (1.29 g, 5 mmol) and chloroiodomethane (1.45 ml, 20 mmol) in dry THF (28 ml) over 30 min keeping the temperature below -70 °C and the mixture was stirred for another 45 min at -70 °C. Glacial acetic acid (7.5 ml) in THF (40 ml) was added dropwise keeping the temperature below -60 °C. The reaction mixture was poured on brine (400 ml) and extracted with EtOAc (2 × 200 ml). The combined organic layers were washed with $NaHCO_3$ (2 × 500 ml), 5 % $NaHSO_3$ (2 × 400 ml) and brine (500 ml), dried ($MgSO_4$) and concentrated in vacuo to yield a dark yellow oil. Purification by column chromatography (EtOAc : Hex 1 : 2 – 2 : 1) gave the purified product as a light yellow solid (581 mg, 2.1 mmol) in 42 % yield. ^{1}H NMR ($CDCl_3$, TMS): δ (ppm): 0.96 (d, ^3J(H,H) = 6.1 Hz, 6H; $(CH_3)_2$), 1.53 (m, 2H; CH_2CH), 1.71 (m, 1H; $CH(CH_3)_2$), 3.38 (m, 4H; 2 CH_2N morph.), 3.68 (m, 4H; 2 CH_2O morph), 4.40, 4.34 (ab, J = 16.2 Hz, 2H; CH_2Cl), 4.66 (m, 1H; α-H leu), 5.32 (m, 1H; NH). ^{13}C ($CDCl_3$): δ (ppm): 21.5 CH(CH_3), 23.0 CH(CH_3), 24.9 ($CH(CH_3)_2$), 39.9 (CH_2CH), 43.9 (CH_2NH morph), 47.0 (CH_2Cl) 55.7 (*CH*CO), 66.2 (CH_2O morph), 157.2 (*C*(O)CH_2Cl), 203.5 (*C*(O)NH). ESI-MS: *m/z*: 299 $[M+Na]^+$, 315 $[M+K]^+$.

N-morpholine-leucine aminoketone **8***:* To **7** (350 mg, 1.26 mmol) in ether (10 ml) was added NH_3/MeOH (2.5 ml) under N_2. After 24 h and 48 h another portion of NH_3/MeOH (2.5 ml) was added. After 72 h the reaction was evaporated to dryness, redissolved in EtOAc (5 ml), washed with brine (10 ml) and extracted with EtOAc (3 × 5 ml). The combined organic layers were dried ($MgSO_4$) and evaporated to dryness to yield the product as a yellow solid (227 mg, 0.88 mmol) in 70 % yield. ^{1}H NMR ($CDCl_3$ with drops MeOD-d_3, TMS) δ (ppm): 0.94 (m; $(CH_3)_2$), 1.02 (q, ^3J(H,H) = 3.3 Hz, 2H; CH_2CH), 1.51 (m, 1H; $CH(CH_3)_2$), 3.34 (m, 2H; NH_2), 3.38 (t, J = 4.8 Hz, 4H; 2 CH_2N morph.), 3.67 (t, J = 4.8 Hz, 4H; 2 CH_2O morph), 3.78 (m, 1H; α-H leu), 3.95 (m, 2H; CH_2NH_2). ESI-MS: *m/z*: 281 $[M+Na]^+$.

6.6.3 Preparation of the platinum complexes

trans-Diamminechloro(N-morpholine-leucine aminoketone)platinum(II) nitrate **3***:* To *trans*-diamminedichloroplatinum (57 mg, 0.19 mmol) in DMF (3 ml) was added silver nitrate (31 mg, 0.18 mmol) in DMF (0.5 ml) in small portions over 3 h. The mixture was stirred overnight in the dark. The resulting suspension was centrifuged to remove the grey silver chloride and the solution was added to compound **8** (50 mg, 0.19 mmol) in DMF (1 ml). The resulting solution was stirred overnight at room temperature, after which it was evaporated to dryness. By dissolving the resulting solid in a minimum amount of methanol and careful layering with diethyl ether the product was obtained as a pale brown solid (45 mg, 0.08 mmol) in 41 % yield. ^{1}H NMR (MeOD-d_3): δ (ppm): 0.92 (m; $(CH_3)_2$), 1.10 (m, 2H; CH_2CH), 1.68 (m, 1H; $CH(CH_3)_2$), 3.39 (m, 4H; 2 CH_2N morph.), 3.65 (m, 4H; 2 CH_2O morph), 3.84 (m, 2H; CH_2NH_2). ^{195}Pt NMR (MeOD-d_3): δ=2322 [PtN_3Cl]. Calculated Mass: 583. ESI-MS: *m/z*: 543 $[M\text{-}NO_3\text{+}Na]^+$, 560 $[M\text{-}NO_3\text{+}K]^+$.

cis-Diamminechloro(N-morpholine-leucine aminoketone)platinum(II) nitrate **4***:* To *cis*-diamminedichloroplatinum (57 mg, 0.19 mmol) in DMF (3 ml) was added silver nitrate (31 mg, 0.18 mmol) in DMF (0.5 ml) in small portions over 3 h. The mixture was stirred overnight in the dark. The resulting suspension was centrifuged to remove the grey silver chloride and the solution was added to compound **8** (50 mg, 0.19 mmol) in DMF (1 ml). The resulting solution was stirred overnight at room temperature, after which it was evaporated to dryness. By dissolving the resulting solid in a minimum amount of methanol and careful layering with diethyl ether the product was obtained as a pale brown solid (63 mg, 0.11 mmol) in 57 % yield. ^{1}H NMR (MeOD-d_3): δ (ppm): 0.94 (m; $(CH_3)_2$), 1.02 (q, J = 3.3 Hz, 2H; CH_2CH), 1.51 (m, 1H; $CH(CH_3)_2$), 3.34 (m, 2H; NH_2), 3.38 (t, J = 4.8 Hz, 4H; 2 CH_2N morph.), 3.67 (t, J = 4.8 Hz, 4H; 2 CH_2O morph), 3.78 (m, 1H; α-H leu), 3.95 (m, 2H; CH_2NH_2). ^{195}Pt NMR (MeOD-d_3): δ=2291 [PtN_3Cl]. Calculated Mass: 583. ESI-MS: *m/z*: 522 $[M\text{-}NO_3]^+$, 584 $[M+H]^+$.

Diethylenetriamine(N-morpholine-leucine aminoketone)platinum(II) dinitrate **5***:* To diethylenetriaminechloroplatinum chloride (111 mg, 0.3 mmol) in DMF (3 ml) was added silver nitrate (46 mg, 0.27 mmol) in DMF (0.5 ml) in small portions over 3 h and the mixture was stirred overnight in the dark. The resulting suspension was centrifuged to remove the white silver chloride and the solution was added to compound **8** (76 mg, 0.3 mmol) in DMF (3 ml). The resulting solution was stirred overnight at room temperature, after which it was evaporated to dryness. By dissolving the resulting solid in a minimum amount of methanol and careful layering with diethyl ether the product was obtained as a pale brown solid (104 mg, 0.15 mmol) in 51 % yield. ^{1}H NMR (MeOD-d_3): δ (ppm): 0.98 (m; $(CH_3)_2$), 1.38 (m, 2H; CH_2CH), 1.60 (m, 1H; $CH(CH_3)_2$), 2.61 – 3.05 (m; CH_2 dien), 3.44 (t, J = 4.8 Hz, 4H; 2 CH_2N morph.), 3.75 (t, J = 4.7 Hz, 4H; 2 CH_2O morph), 3.98 (m, 2H; CH_2NH_2), 4.16 (m, 1H;

α-H leu). ^{195}Pt NMR (MeOD-d_3): δ=2904 [PtN_4]. Calculated Mass: 679. ESI-MS: *m/z*: 617 $[M\text{-}NO_3]^+$.

6.6.4 Biological evaluation

Affinity-labeling of active cysteine proteases

Compounds **3-5** were dissolved in reaction buffer (50 mM citrate/phosphate pH 5.0, 1 mM Na_2H_2EDTA, 50 mM DTT). Affinity-purified CatB (Sigma) or cell lysates (5 µg) were either directly labelled with DCG-ON (native) or labelled after incubation at 37 °C for 30 min without inhibitor (37 °C, 30 min) or in the presence of compounds **3-5** at the concentrations indicated. Cisplatin (500 µM) served as additional control. Reactions were terminated by addition of SDS reducing sample buffer and immediate boiling. Samples were resolved by 12.5 % SDS-PAGE gel, then blotted on a PVDF-membrane and visualized, using streptavidin-HRP and the ECL-detection kit.

Determination of cathepsin activity

In order to establish combined CatBLS activities using the fluorogenic substrate Z-FR-AMC (0.1 M citrate buffer pH 5.0, 4 mM DTT, 4 mM Na_2H_2EDTA, 6 µM Aprotinin), affinity-purified CatB was incubated with Z-FR-AMC for 45 min in the presence or absence of compound **3-5** at 10 µM and 100 µM, respectively. Liberated fluorescence was determined at 465 nm and expressed in arbitrary units.

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