Platinum(IV)-chlorotoxin (CTX) conjugates for targeting cancer cells

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Abstract

Cisplatin is one of the most widely used anticancer drugs. Its side effects, however, have motivated researchers to search for equally effective analogs that are better tolerated. Selectively targeting cancer tissue is one promising strategy. For this purpose, a platinum(IV) complex was conjugated to the cancer-targeting peptide chlorotoxin (CTX, TM601) in order to deliver cisplatin selectively to cancer cells. The 1:1 Pt-CTX conjugate was characterized by mass spectrometry and gel electrophoresis. Like most platinum(IV) derivatives, the cytotoxicity of the conjugate was lower in cell culture than that of cisplatin, but greater than those of its Pt(IV) precursor and CTX in several cancer cell lines.

1. Introduction

Chlorotoxin (CTX, TM601) is a 36-amino-acid peptide with four disulfide bridges (Figure 1). It is a component of the venom of the giant yellow Israeli scorpion *Leiurus quinquestriatus hebraeus*, which can be synthesized in the lab using solid phase synthesis. CTX is thought to bind to functional proteins like matrix metalloproteinase-2 (MMP2) [1] and chloride ion channels [2]. Upon binding of CTX, these proteins are eliminated following internalization into the cell [3], leading to inhibition of cell invasion and migration [1, 2]. Chloride ion channels are among the many membrane proteins overexpressed in different types of cancers, and MMP-2 is specifically up-regulated in gliomas and related cancers [1, 2], properties that make these proteins attractive targets for selective drug targeting. Recently, the protein annexin A2 was also identified as a receptor for CTX on the surface of a number of human cancer cell lines [4].

The binding of CTX to proteins on the surface of tumor cells results in specificity for tumor tissue. For example, several studies reveal that CTX binds preferentially to glioma cells [5]. Thus, CTX has been investigated as a targeting agent for the specific delivery of radioisotopes (^{131}I-CTX) to brain cancer cells, and the radiolabeled peptide has entered clinical development. CTX also binds many non-glioma tumor cell lines including those derived from lung, prostate, and melanoma cancers, but not normal non-transformed cells [4, 5]. It has also been demonstrated that CTX has robust anti-angiogenic activity [6, 7].

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Appendix A: Supplementary Data Supporting information available: detailed small-scale synthetic procedure, calibration curve for determination of CTX concentration, mass spectral data, representative kill curves.

Because of these unique biological properties, there are many ongoing efforts to implement CTX as a targeting platform for anticancer applications. Included are the use of CTX to achieve intraoperative visualization of cancer foci with a CTX:Cy5.5 bioconjugate [8] and in vivo MRI detection of gliomas by CTX conjugated superparamagnetic iron oxide nanoparticles [9]. Multifunctional CTX nanoprobes comprising iron oxide nanoparticles and fluorescent dyes for magnetic resonance and optical visualization have been developed for brain tumor imaging [10-12]. Finally, CTX-targeted nanovectors composed of polyethyleneimine (PEI) nanoparticles and fluorophores [13] as well as iron oxide nanoparticles [14] have been used as gene delivery systems. Despite the utility of CTX as a broad tumor-targeting platform, no applications besides gene therapy appear to have been described.

Here we report the use of CTX as a carrier for the classical anticancer drug cisplatin. Cisplatin is one of the most successful anticancer drugs, especially for the treatment of ovarian and testicular cancer. Resistance phenomena and undesired side-effects have, however, limited its utility in the clinic [15]. Among other reasons, these deficiencies can be caused by reactions of cisplatin with proteins before reaching the therapeutically relevant target – nuclear DNA [16, 17]. These side-reactions may be attenuated when Pt is applied in a higher oxidation state, Pt(IV), compared to Pt(II) as in cisplatin. Pt(IV) compounds are more kinetically more inert than their Pt(II) analogs and therefore might reach the cancer cell without premature chemical transformation. Inside cells, however, reactions of Pt(IV) compounds with cellular reductants give rise to DNA-reactive Pt(II) species [18].

There have been prior reports of the use of conjugates of cancer-cell targeting peptides for the delivery of Pt drugs [19]. We previously described tri- and pentapeptide conjugates based on a Pt(IV) compound designed to target tumor vasculature [20]. Very recently, another Pt(IV) conjugate system was presented using a cell penetrating peptide as a carrier. [21] Here we use CTX to target cancer cells instead of angiogenic vessels and provide a modified synthetic procedure suitable for larger peptides like the 36-amino acid containing CTX. In contrast to the systems comprising short peptides (3, 5, and 11 amino acids respectively) neither standard liquid [20] nor solid phase conjugation [21] were applicable for generating the CTX conjugate. By combining the concepts of milder toxicity with Pt(IV) and targeting by the carrier-peptide CTX through the chemical fusion of cisplatin and chlorotoxin, we have the potential for delivering an anticancer drug selectively to the sites of the tumor with reduced side-effects in vivo. In the present article we describe the conjugation of a Pt(IV) complex to CTX by selective amide coupling, analysis of the conjugate, and in vitro studies determining its efficacy and potential as an anticancer drug candidate.

2. Experimental section

2.1 Materials and methods

The compound *cis, cis, trans*-[PtCl$_2$(NH$_3$)$_2$(succinato)$_2$] (**1**) was prepared according to literature methods [22]. CTX was provided by Transmolecular lnc. (King of Prussia, PA, USA). A stock solution of trypsin (Promega, Madison, WI, USA) was obtained by dissolution in 1 mM acetic acid (1 μg/μL, pH 3.0). HeLa, MCF7, and A549 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 4.5 g/L glucose and L-glutamine, no sodium pyruvate, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from cellgro, Manassas, VA, USA) at 37 °C, in a humidified atmosphere at 5% CO$_2$. Analytical and preparative HPLC were performed on an Agilent 1200 system.

2.2 Synthesis of 1-CTX – Small-scale optimization

Two sets of EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride)/NHS (*N*-hydroxy-succinimide) coupling reactions between CTX and **1** were carried out to determine optimal synthetic conditions similar to a literature reported method [23]. Reactions were carried out at 5:1 and 10:1 Pt:CTX molar ratios, respectively, with changes in pH (6.5, 7.5, 8.5), incubation time (1h, 24 h), and temperature (16 °C, RT) (for details see Supporting Information). The reaction products were analyzed by HPLC (solvent A: 0.1% TFA in H_2O, solvent B: 0.1% TFA in MeCN, Vydac C_{18} column, 4.6×250 mm, 25 °C, 1 mL/min, $\lambda =$ 220 nm, 273 nm, gradient 20% B for 15 min, 20-32% B in 25 min, 32% B for 5 min, 32-80% B in 5 min, 80-20% B in 1 min, 20% B for 9 min).

2.3 Synthesis of 1-CTX – Large scale

Large-scale liquid phase synthesis of **1**-CTX was carried out under optimized conditions. Briefly, the CTX peptide (2.98 μmol, 11.9 mg) was dissolved in 3.5 mL of PBS buffer (pH 6.5). The NHS ester of **1** was generated by dissolving **1** (29.8 μmol, 15.9 mg), NHS (37.3 μmol, 4.29 mg), and EDC (37.3 μmol, 7.15 mg) in 1 mL of PBS (pH 5.0) followed by incubation for 10 min at room temperature. The solution of **1**-NHS ester was added to the CTX solution and the reaction was incubated for 24 h at 16 °C. The reaction was quenched with 500 μL 1% TFA. The conjugate was purified by HPLC as described above, but the column was 9.4×250 mm and the flow rate, 4 mL/min.

2.4 Determination of the peptide concentration

A Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) was used applying the microplate procedure (2 - 40 μg/mL linear working range) with CTX as a standard. Briefly, 100 μL of each standard or unknown sample replicate was pipetted into a microtiterplate well, after which 100 μl of the working reagent (1.5 mL reagent A, 1.44 mL reagent B, 60 μL reagent C) was added to each well. The plate was mixed thoroughly and incubated at 37 °C for 2 h. After cooling the plate to room temperature, the absorbance at 562 nm was measured on a plate reader (BioTek, Winooski, VT, USA). The average absorbance of the blank (0 μg/mL peptide) was subtracted from all other individual standard and unknown sample replicates.

2.5 Peptide gel electrophoresis

The sample (2 μg **1**-CTX) was mixed with Tricine sample buffer (Bio-Rad, Hercules, CA, USA) including 2% β-mercaptoethanol followed by heating for 5 min at 95 °C. A 16.5% Ready Gel Tris Tricine (Bio-Rad) was used for electrophoresis. The running buffer consisted of 0.1 M Tris, 0.1 M Tricine, 0.1% SDS (cathode buffer), and 0.2 M Tris-HCl pH 8.9 (anode buffer), respectively. The gel was stained with 3 g/L Coomassie and destained in water.

2.6 Trypsination and mass analysis

Reduction of disulfides with DTT (dithiothreitol), alkylation of cysteines with iodoacetamide, and digestion of the peptide with trypsin were carried out using standard protocols [24]. Peptide characterization was performed by LC-MS analysis in the following manner. Chromatographic separation of proteolytic peptides, which had been loaded onto a reversed phase peptide microtrap for on-line desalting, was carried out on a C_{18} reversed phase capillary HPLC column with a H_2O/MeCN solvent gradient elution, using a Tempo nanoflow HPLC instrument (Eksigent, Dublin, CA, USA). Mass spectral data were acquired with a QSTAR Elite quadrupole time-of-flight mass spectrometer (AB Sciex, Foster City, CA, USA).

2.7 Cytotoxicity determination

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assays were carried out in three independent experiments in triplicate as previously described with slight modifications as follows [25]. HeLa, A549, and MCF7 cells were plated in flat-bottomed 96- well plates at 2,000 cells/well. A serial two-fold dilution (1-16 μM) of CTX, cisplatin, and **1**- CTX, respectively, in growth medium was prepared to give five concentrations (16, 8, 4, 2, and 1 μM), and the plates were incubated for 72 h. After incubation, administration of MTT and absorbance measurements were carried out as described previously [25].

3. Results and discussion

3.1 Synthesis

The Pt(IV) compound *cis, cis, trans*-[PtCl$_2$(NH$_3$)$_2$(succinato)$_2$] (**1**) [22] was coupled to CTX using EDC/NHS chemistry (Scheme 1). A similar strategy has been used previously by us to conjugate a small tripeptide with **1** [20]. For CTX, however, the coupling reaction is less straightforward owing to different possible binding sites (ɛ-amino groups of lysine and the *N*-terminus). Therefore, the reaction conditions for generating a well-defined 1:1 Pt:peptide conjugate had to be established.

The coupling conditions of CTX to **1** according to Scheme 1 were investigated based on a strategy for labeling *N*-terminal amino groups in peptides [23]. During the course of this study, a protocol for preferential conjugation of N-terminal amino groups was developed by carrying out the coupling reaction under low pH conditions. Using a "lower-than-normal" reaction pH (i.e. pH 7-8 [26]) ensures that the ɛ-amino group in lysine stays protonated, thus attenuating its nucleophilic reactivity. This strategy exploits the significant difference in pK_a values between the α-amino group (pK_a = 8.9) and the ɛ-amino group of lysine (pK_a = 10.5) [27].

To identify the reaction product **1**-CTX, preparative RP-HPLC purification was carried out first, and the peptide and Pt concentrations in the fractions eluted from the column were measured. The peptide concentration was determined by the Micro BCA™ assay within the linear range (2 - 40 μg/mL, Supporting Information, Figure S1). This method was modified by replacing the commonly used high molecular weight standard BSA with CTX. The Pt concentration was determined by flameless atomic absorption spectroscopy (AAS). In Figure 2 is depicted a typical HPLC profile (a), as well as the analytical HPLC trace (b) of the main product (1:1 Pt:CTX), which elutes at ~26.6 min. The starting materials had retention times of 3-5 min (**1**-NHS ester) and 18.6-19.7 min (CTX), as determined in separate HPLC runs.

The following coupling parameters were then varied in small-scale reaction setups to determine optimal conditions for obtaining the desired coupling product in the highest yield: pH (6.5, 7.5, 8.5), Pt:CTX ratio (10:1, 5:1), incubation time (1 h, 24 h), and temperature (4 °C, 16 °C, room temperature). Two rounds of 12 reactions each on a 12.5 nmol CTX scale were performed. In the first round, the pH, Pt:CTX ratio, and incubation time were varied with all reactions conducted at 4 °C. In the second round, pH, Pt:CTX ratio, and reaction temperature (16 °C and room temperature) were varied while all reactions were incubated for 24 h. HPLC analysis was used to determine whether and how much the desired **1**-CTX, as identified before by HPLC and Micro BCA™/AAS, was synthesized under the various reaction conditions tested (for details and tabulated reaction conditions see Supporting Information). HPLC analysis of round 1 showed the reactions incubated for 24 h gave considerably better yields than those incubated for only 1 h. In round 2, yields of **1**-CTX varied between 25 and 45% according to integrated HPLC peaks. Among round 2

conditions, the reaction carried out at a Pt:CTX ratio 10:1, pH 6.5, at 16 °C for 24 h gave a moderate yield of only 31%. This condition was chosen as the optimal, however, because it afforded a purer product upon preparative HPLC later on. Instead of having side-products with similar retention times like the product eluted, a large amount of starting material CTX with a much shorter retention time on the RP-HPLC column was observed (Figure 2 a). Thus, unreacted CTX can be recovered after the separation from the product **1**-CTX.

Large-scale synthesis of **1**-CTX (3 μmol) for use of the conjugate in cytotoxicity studies was carried out under the optimized condition described above. After purification by HPLC, analysis by Micro BCA™ assay and AAS revealed the isolated species to have a Pt:CTX ratio of 0.99:1, close to the expected ratio of 1:1. We were not able to attribute the other HPLC peaks to species with a defined Pt:CTX ratios except for the peptide that eluted at ~29.8 min. This species could be identified as a Pt complex bearing two CTX moieties, for the Pt:CTX ratio was 0.5 as determined by analysis of peptide and Pt concentration. A doubly Pt labeled peptide was not observed, probably because the pH conditions chosen allowed only the *N*-terminus to react.

3.2 Analysis of 1-CTX

The molecular weight of the desired product (HPLC t_R = 26.6 min), which exhibits a 1:1 Pt:CTX ratio, was determined by ESI-TOF-MS(+) (Figure S2). These data further confirmed the stoichiometry of the Pt-CTX conjugate as having one platinum atom per peptide molecule. In TOF-MS three peptide species **A**-**C** could be identified (Table 1). The molecular ion peaks **A**-**C** originate from peptides having a *C*-terminal amide and all cysteines in an oxidized state. Peak **C** corresponds to the species **1**-CTX, peak **B** to the platinum-free but succinylated species, and peak **A** to the peptide CTX as a small impurity. Owing to the results of the HPLC analysis of **1**-CTX carried out beforehand (Figure 2 b), we assume that the species giving rise to peak **B** can only be generated during sample preparation or measurement. The latter possibility is unlikely, however, because the ESI interface was at ambient temperature and the ESI needle voltage was relatively low (2600 V) such that fragmentation caused by cleavage of the Pt–O bond should not be induced.

To confirm that the 1:1 Pt-CTX conjugate does not exist as a dimer or oligomer, a peptide gel electrophoresis experiment was carried out (Figure 3). Interestingly, no dimers or higher order oligomers were noted by HPLC, MS, or gel electrophoretical analysis despite the presence of two functionalizable carboxylic acid groups on the Pt(IV) complex **1**. Although we observed a species with a Pt:CTX ratio of 1:2 (section 3.1), the pH conditions chosen presumably prevent reaction of CTX already bound to **1** to yet another Pt complex generating a 2:2 Pt-CTX conjugate. This kind of dimer would have given the same analytical results from AAS and the MicroBCA test.

Identification of the actual position(s) of attachment of the platinum complex required digestion of the peptide into smaller fragments, followed by tandem mass spectrometry to observe any amino acid modifications. Because the peptide contains eight cysteines, all of which were linked via disulfide bonds as confirmed by MS measurements, disulfide reduction with DTT and cysteine alkylation were performed in order to facilitate digestion of the peptide with trypsin. Trypsin cleaves at the carboxy side of K and R (except when followed by P); five such cleavage sites occur in CTX [28]. A representative fragment ion mass spectrum of the *N*-terminal peptide fragment MCMPCFTTDHQMAR is shown in Figure S3. Here the *N*-terminus is succinylated nearly quantitatively and the two cysteines are carbamidomethylated. Reduction with DTT most likely also results in loss of the platinum complex due to reduction of six-coordinate Pt(IV) to afford four-coordinate Pt(II), with one succinyl moiety remaining attached to the peptide. There was no evidence for succinylation at any other position in the sequence. We found the *C*- terminal peptides

(GK)CYGPQCLCR not to be modified, whereas the tryptic peptides in the middle of the sequence (K)CDDCCGGK(GR) could not be located in the mass spectrum. We presume that these peptides were lost during on-line sample desalting with the reversed phase peptide trap. This assumption is supported by detection of a very small amount of non-succinylated *N*-terminal peptide, which is consistent with the molecular weight measurements of intact **1**-CTX described above.

3.3 In vitro studies

Oxidation of cisplatin to the more kinetically inert Pt(IV) pro-drug form gives rise to a platinum complex with increased stability in the biological environment of the cytosol. The conjugate can then be reduced intracellularly to the more reactive cisplatin. In vitro, this increased stability can lead to decreased cytotoxicity when compared to the parental compound cisplatin [29].

The cytotoxicity of **1**-CTX, CTX, and cisplatin was measured in cervical (HeLa), breast (MCF7), and lung (A549) cancer cells, which express different levels of receptors targeted by CTX. A summary of data from this study and comparison to literature are presented Figure 4. The IC_{50} value of CTX in all cell lines is $\gg$ 16 μM (Figure S4). Because of the availability of only limited quantities of the CTX peptide, concentrations above 16 μM could not be investigated while at the same time guaranteeing an adequate number of replicates and repetitions of experiments (section 2.7). By comparison to results obtained with cisplatin and **1**- CTX, however, an $IC_{50} \gg$ 16 μM suggests that CTX is relatively non-toxic in the concentration range screened. The role of CTX in **1**-CTX, therefore, is most likely to serve as a carrier and not a toxin.

The average IC_{50} value of **1**-CTX determined from three independent experiments in HeLa cells is 10.7 $\pm$ 5.0 μM. Similarly, the IC_{50} values of **1**-CTX in MCF7 and A549 cells were determined as 14.0 $\pm$ 3.5 μM and 12.0 $\pm$ 4.6 μM, respectively. IC_{50} values in HeLa, MCF7, and A549 for cisplatin are 2.5 $\pm$ 1.3, 11.0 $\pm$ 4.6 and 6.2 $\pm$ 2.0 μM (bar chart in Figure S4).

These data indicate that, in all three tested cell lines, the IC_{50} values for the conjugate are lower than that for CTX, but greater than that for cisplatin. The decreased cytotoxicity of **1**-CTX relative to cisplatin was expected because of the relative kinetic inertness of the Pt(IV) moiety **1** in **1**-CTX. For example, the IC_{50} of the Pt(IV) compound **1** in HeLa cells is 82 $\pm$ 30 μM after 96 h incubation time, whereas the IC_{50} for cisplatin is 0.37 $\pm$ 0.06 μM under the same conditions (IC_{50} ratio ~ 200, Table 2) [22]. In MCF7 [30] and A549 cells [31], however, the Pt(IV) complex **1** exhibited cytotoxicity similar to that of cisplatin (IC_{50} ratios 1.0 and 1.2, respectively, Table 2, Figure 4). It is therefore valid to compare the cytotoxicities of **1** and **1**-CTX relative to that of cisplatin.

The cell line in which **1**-CTX appears to be closest in cytotoxicity to cisplatin is MCF7, for which the conjugate is approximately only 1.3 times less cytotoxic than cisplatin (as compared to two times less effective in A549 cells and four times less cytotoxic in HeLa cells, Table 2). The different expression levels of receptors for CTX in these cell lines may account for the observed trends in cytotoxicity. Whereas the chloride ion channel protein CLC-3 is expressed in virtually every cell line,[32] MMP2 and Annexin A2 expression levels differ among the cell lines investigated.

MCF7 cells show low levels of MMP2 and thus bind CTX poorly [8]. Annexin A2 is also not expressed in MCF7 cells [33]. Thus, the cytotoxicities of **1** and **1**-CTX are expected to be similar, as observed with IC_{50} ratios of 1.0 and 1.3 related to cisplatin (Table 2, Figure 4). In A549 cells, Annexin A2 shows a high affinity for CTX [4], and MMP2 is expressed in these cells [34]. Thus, targeting effects are expected to increase the cytotoxicity of the

Pt(IV) complex **1** by CTX conjugation. We did not observe a significant increase in cytotoxicity, however, when comparing **1** with an IC_{50} ratio of 1.2 related to cisplatin [31] to **1**-CTX with an IC_{50} ratio of 1.9. This result indicates that targeting does not play a significant role here (Table 2, Figure 4). Reported IC_{50} values in A549 cells of Pt(IV) compounds with other biologically active ligands like cell-penetrating peptides are also similarly close to the value for cisplatin [21],[31],[35]. Although receptors for CTX are present on A549 cells, the relatively high cytotoxicity of **1** by comparison to cisplatin seems to outweigh any targeting effect. This behavior is similar to that for the MCF7 cells, which by contrast have low affinity for CTX.

One might speculate that the missing targeting effect of CTX for A549 cells is due to lower affinity of CTX when coupled to Pt(IV) within the conjugate. In HeLa cells, however, where **1** has a very high IC_{50} [22], we did observe an obvious targeting effect by **1**-CTX. In these cells, besides CLC-3 expression [36], there is expression of CTX receptors Annexin A2 [37] and MMP2 [38]. The cytotoxicity of **1**-CTX was significantly increased due to the targeting of receptors in comparison to non-toxic **1** and CTX. Whereas the Pt(IV) complex **1** is 200 times less cytotoxic than cisplatin, as a conjugate it is only 4 times less cytotoxic (Table 2, Figure 4). This difference corresponds to an increase in cytotoxicity of factor 50 due to targeting.

For comparison, the targeting tripeptide conjugate of **1** that we presented previously [20] showed an increase by a factor of 6-7 compared to the cytotoxicity of **1** in HeLa cells.

These in vitro results suggest that our Pt-CTX conjugate might be a viable drug candidate for the treatment of cervical cancer, able to target cancer cells and selectively release the powerful anticancer drug cisplatin on-site. Because of this targeting, fewer side effects would be expected in vivo for the conjugate than for cisplatin itself.

In conclusion, we prepared a conjugate of chlorotoxin (CTX), a cancer-targeting peptide, with the Pt(IV) succinic acid complex **1** and identified the 1:1 conjugate **1**-CTX by determining its Pt and peptide content and by mass spectrometric analysis. The Pt complex is conjugated selectively via an amide bond with the *N*-terminus of the peptide. Cytotoxicity studies revealed increased efficacy in three different cell lines when compared to the peptide CTX or the Pt(IV) precursor. The cell killing effect of the conjugate approached that of cisplatin, indicating that a Pt-CTX construct might be valuable as a cancer-targeting drug candidate. Particularly in HeLa cells, the Pt-CTX conjugate was more cytotoxic than the Pt(IV) building block alone because of targeting. Further in vitro studies including cellular uptake measurements comparing cells expressing/not expressing CTX receptors, as well as in vivo studies will give more insight into the viability of introducing Pt-CTX as a cancer drug candidate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AAS	atomic absorption spectroscopy
CTX	chlorotoxin
DTT	dithiothreitol
EDC	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NHS	*N*-hydroxy-succinimide
PEI	polyethyleneimine
TFA	trifluoroacetic acid

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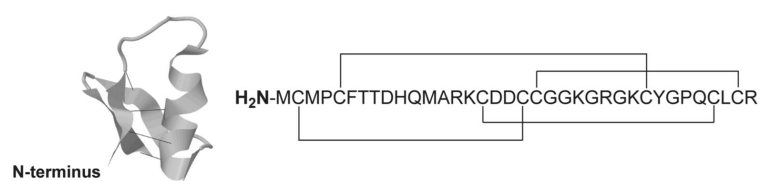
Figure 1.
The CTX structure as determined by NMR spectroscopy is shown to the left (PDB ID: 1CHL, view in Jmol), and the sequence is shown at the right. The four disulfide cross-links joining the eight cysteine residues are indicated.
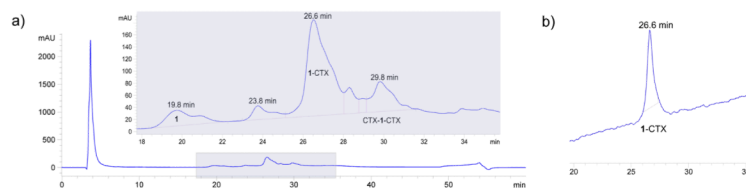
Figure 2.
a) Representative RP-HPLC profile of the large-scale reaction of **1** with CTX (λ=220 nm).
b) Analytical HPLC for the peak eluted at 26.6 min, the desired reaction product **1**-CTX as determined by Micro BCA™ and AAS.
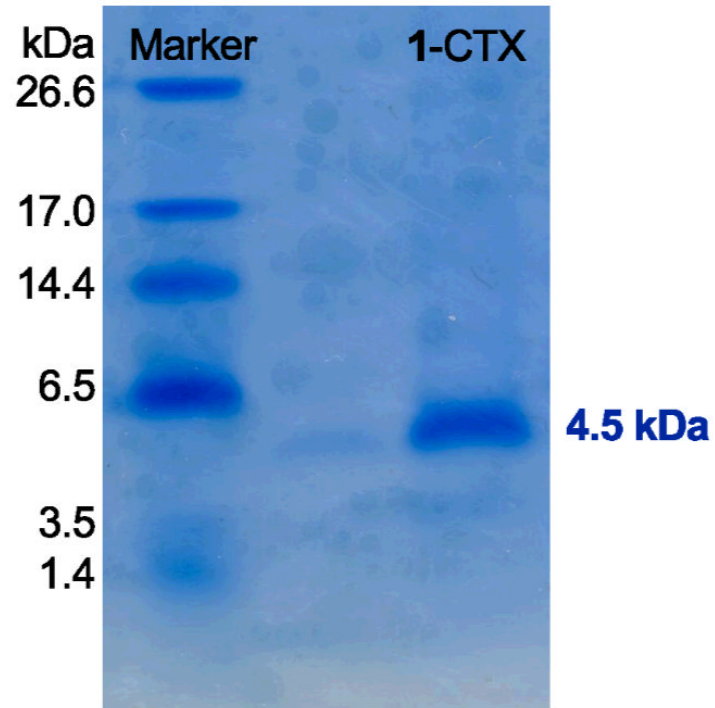
Figure 3.
16.5% Tris Tricine gel with 2 µg **1**-CTX and Polypeptide SDS-PAGE molecular weight standard, 100 min, 100 V, Coomassie staining.
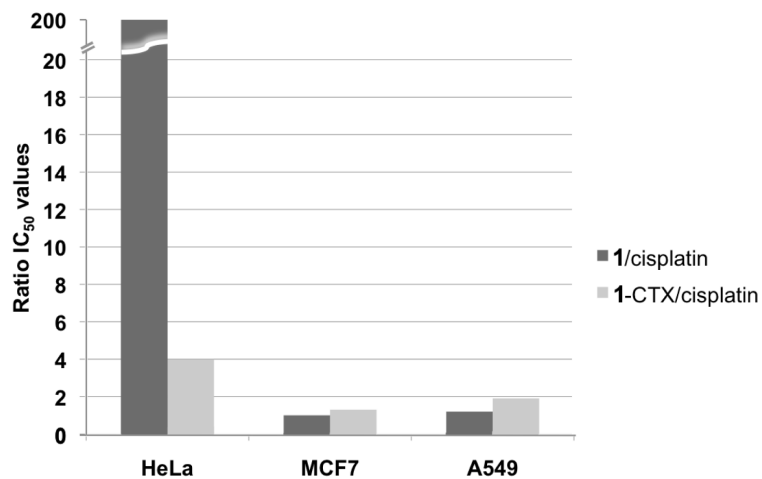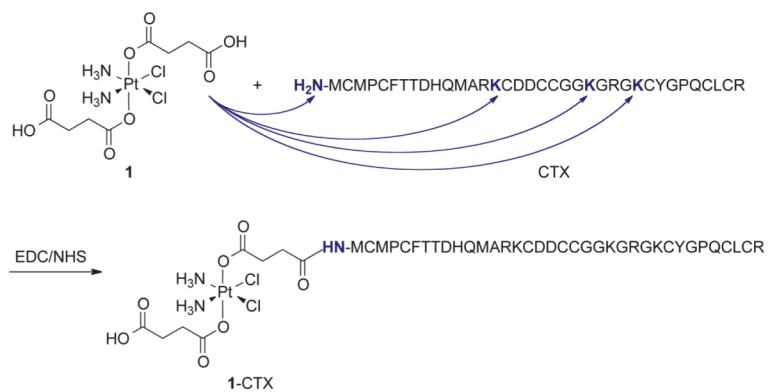
Figure 4.
Ratios of IC$_{50}$ values of **1**-CTX and cisplatin (light gray bars) in comparison to literature values of **1** and cisplatin [22], [30], [31] (dark gray bars) in HeLa, MCF7, and A549 cells. Representative kill curves and original bar-graphed IC$_{50}$ data are supplied in the Supporting Information (Figure S4).

Scheme 1.
Coupling of cis, cis, trans-[PtCl$_2$(NH$_3$)$_2$(succinato)$_2$] (**1**) to CTX. The blue arrows show possible binding sites for the platinum complex **1**. In **1**-CTX, the complex is bound to the N-terminus (Section 3.2).

Table 1

Overview of calculated masses and m/z for species **A-C** found in the TOF-MS (Figure S2).

	Formula	Average mass	Mono-isotopic mass	+3H	+4H	+5H	+6H
CTX (**A**)	$C_{158}H_{249}N_{53}O_{47}S_{11}$	3995.77	3992.57	1331.86	999.15	799.52	666.44
Succinylated CTX (**B**)	$C_{162}H_{253}N_{53}O_{50}S_{11}$	4095.84	4092.58	1365.20	1024.15	819.52	683.10
1-CTX (**C**)	$C_{166}H_{263}Cl_2N_{55}O_{54}PtS_{11}$	4511.96	4507.55	1503.52	1127.89	902.52	752.27

Ions of the +3, +4, +5 and +6 series of species **A-C** were identified. The isotope pattern of **C**+5H matches the calculated one (Figure S2).

Table 2

Ratios of IC_{50} values of **1** and cisplatin [22], [30], [31] and **1**-CTX and cisplatin, respectively. Standard deviations are given in the text (section 3.3) where available.

	HeLa IC$_{50}$ [μM]	ratio	MCF7 IC$_{50}$ [μM]	ratio	A549 IC$_{50}$ [μM]	ratio
1	82	~ 200	11.1	~ 1.0	14.0	~ 1.2
cisplatin	0.4		10.7		12.0	
1-CTX	10.7	~ 4.3	14.0	~ 1.3	12.0	~ 1.9
cisplatin	2.5		11.0		6.2	