The design and synthesis of novel heterodinuclear complexes combining a DNA-cleaving agent and a DNA-targeting moiety

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Chapter 3

A New Approach for the Preparation of Efficient DNA Cleaving Agents: Ditopic Copper-Platinum Complexes Based on 3-Clip-Phen and Cisplatin.*

The design and synthesis of new heterodinuclear DNA-targeting agents are described. The abilities of cisplatin and Cu(3-Clip-Phen) – an artificial DNA-cleaving agent – have been combined through their "covalent coupling". This strategy has led to bifunctional complexes, that are able to cleave the DNA in a double-stranded fashion in contrast to Cu(3-Clip-Phen) alone, and have promising cytotoxicities compared to cisplatin in several cell lines.

* This chapter is based on Paul de Hoog, Christophe Boldron, Patrick Gamez, Karen Sliedregt-Bol, Isabelle Roland, Marguerite Pitié, Robert Kiss, Bernard Meunier, and Jan Reedijk, *J. Med. Chem.* **2007**, *50*, 3148

3.1 Introduction

Among the strategies which find application in clinical anticancer chemotherapy, the cancer cells eradication induced by DNA-interacting molecules has proven its efficiency.[1, 2] First of all, cisplatin is one of the most widely used anticancer agents. It is generally accepted that the distortion of DNA generated upon binding of cisplatin is largely responsible for its antitumor properties.[3] Secondly, the therapeutic anticancer activity of the natural antibiotic bleomycin has been attributed to the ability of its metal complexes to perform oxidative DNA cleavage via oxidative degradation of the deoxyribose units.[4] This important discovery has led to the design and preparation of synthetic models of bleomycin such as 3-Clip-Phen.[5] Copper complexes of 3-Clip-Phen mediate single-strand cleavage of DNA from the minor groove, through the oxidation of the sugar moiety, albeit without sequence specificity.[5, 6]

Intrinsic or acquired drug resistance is a common problem in cisplatin chemotherapy.[7-9] Therefore, combination therapy was developed.[1] For example bleomycin, etopside and cisplatin are simultaneously used for testicular cancer treatment resulting in a curing rate of >99% if applied at the early stage of the syndrome.[10] Proceeding from this success, multifunctional drugs were developed.[11] For instance, cisplatin was combined with intercalating agents, known as Topoisomerase blockers,[12-17] and also with a photoactivated cleaving agent.[18]

The combined interest in (i) improving the DNA cleavage specificity of Cu(3-Clip-Phen) complexes together with their ability to perform double strand breaks (DSB) and (ii) circumventing drug resistance owing to the use of cisplatin,[7-9, 19, 20] has inspired the design of bifunctional molecules (Figure 3.1) containing both active entities.

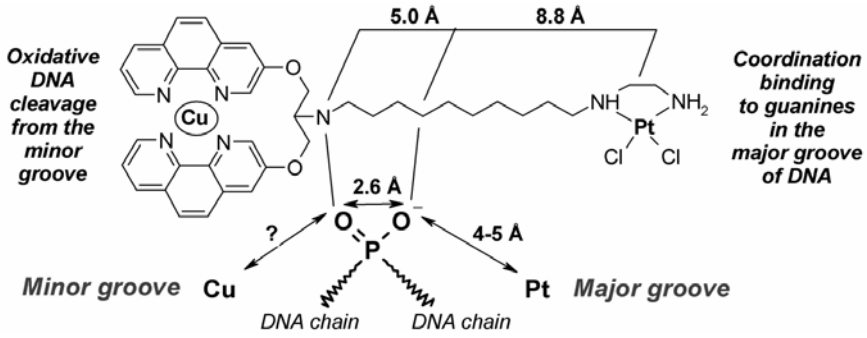


Figure 3.1 Strategy adopted for the synthesis of heterodinuclear minor/major groove interacting complexes.

Thus, compounds **Cu3CP-6-Pt** and **Cu3CP-10-Pt** have been prepared (Scheme 3.1) considering the intrinsic DNA-interaction characteristics of the two separate active metallic centers. Depending on their mutual degree of freedom, both moieties will or will not reach their preferential site of interaction simultaneously, namely the nitrogen atom N7 of guanine in the

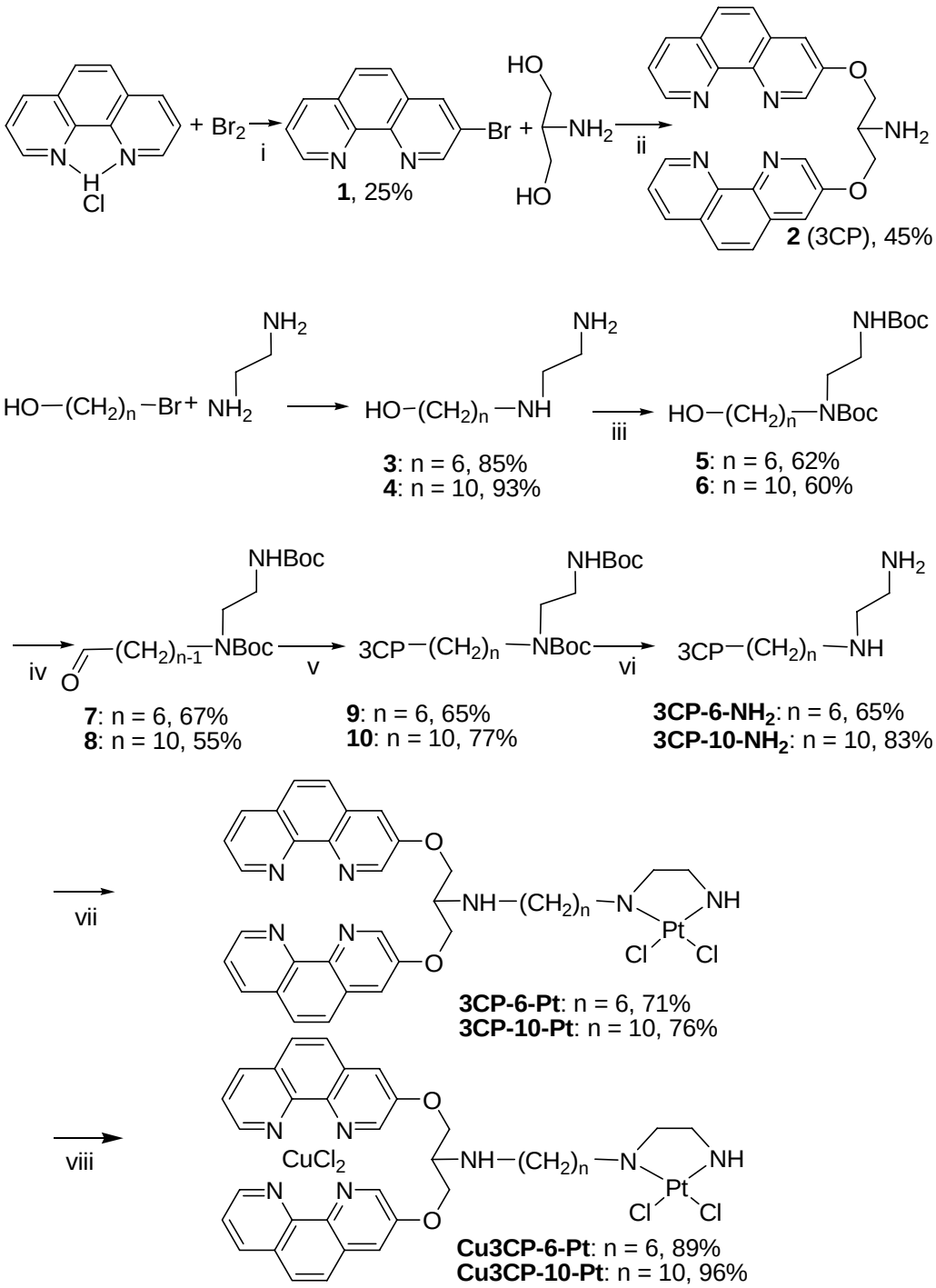
major groove for the platinum component, and the minor groove for the copper unit.[6] The minimum separation distance required to achieve such concomitant minor-major groove interactions has been determined from the crystal structure of a DNA-cisplatin adduct.[21] The phosphate oxygen atom pointing toward the major groove and the platinum ion of cisplatin are 4-5 Å apart from each other (Figure 3.1). The distance between both oxygen atoms of the same phosphate group is about 2.6 Å.

It has been proposed that the amino group of Cu(3-Clip-Phen) could be protonated, favoring its interaction with the polyanionic structure of DNA.[22] Thus, it could interact via hydrogen bonding with the oxygen atom of the phosphate, thereby pointing toward the minor groove.[23, 24]

Compound **Cu3CP-10-Pt** has therefore been designed with a bridge long enough to allow the interaction of both metal centers with their respective preferential target site. In the case of a shorter bridge, i.e. compound **Cu3CP-6-Pt**, both moieties will be forced to sit in the same DNA groove. Nevertheless, the complex retains its flexibility during the interaction with DNA.

3.2 Results and discussion

The general synthetic pathway to prepare ligands **3CP-6-NH_2** and **3CP-10-NH_2** is depicted in Scheme 3.1. The selective and complete platination of the ethylenediamine unit using 1 equivalent of K_2PtCl_4 was monitored by ^{195}Pt NMR and UV spectroscopy (Figure 3.2). The in situ reactions of the so-obtained platinum derivatives **3CP-6-Pt** and **3CP-10-Pt** with 2 equivalents of $CuCl_2$ yielded the heterobimetallic complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt,** respectively. The UV-Vis spectrum recorded for the solution of **3CP-6-NH_2** or **3CP-10-NH_2** exhibits the typical features for the free 3-Clip-Phen ligand. The spectrum recorded for the solution of **3CP-6-Pt** or **3CP-10-Pt** is comparable to the one of the solution of **3CP-6-NH_2** or **3CP-10-NH_2**. As expected, platinum(II) does not coordinate to the Clip-Phen part of **3CP-6-NH_2**, but to the ethylenediamine part as clearly indicated by the ^{1}H NMR and ^{195}Pt NMR studies. No significant shift of the 3-Clip-Phen peaks were observed by ^{1}H-NMR, and the ^{195}Pt-NMR peak around –2310 ppm corresponds to the coordination of platinum to the ethylenediamine moiety.[25-27] The spectrum recorded for a solution of **Cu3CP-6-Pt** or **Cu3CP-10-Pt** is typical for a Cu(3-Clip-Phen) complex.[5] As anticipated, Copper(II) coordinates to the Clip-Phen moiety of the platinum complex.



Scheme 3.1 Reagents and conditions: (i) 140 °C, nitrobenzene; (ii) 50 °C, NaH, 2,6-di-*tert*-butyl-4-methylphenol, DMF; (iii) (1) NaH, Boc_2O, THF, overnight, (2) K_2CO_3, MeOH, reflux, 1 h; (iv) pyridinium chloridotrioxidochromate (PCC), CH_2Cl_2, 2 h; (v) (1) 3-Clip-Phen (**2**), MeOH, reflux, 2 h, (2) $NaBH_4$, reflux, 2 h; (vi) TFA, 0 °C, 1 h; (vii) K_2PtCl_4, $MeOH/H_2O$, 5 h; (viii) $CuCl_2$, DMF, 50 °C.

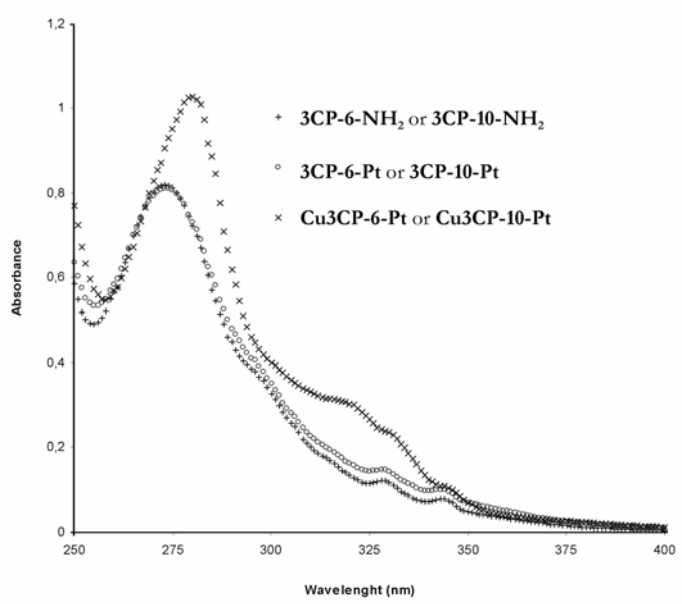


Figure 3.2 UV-Vis spectroscopic studies of a solution of ligand **3CP-6-NH$_2$** or **3CP-10-NH$_2$,** a solution of **3CP-6-Pt** or **3CP-10-Pt**, and a solution of **Cu3CP-6-Pt** or **Cu3CP-10-Pt**. The UV-vis spectra were recorded after dilution of the solutions with H_2O to a concentration of 20.8 μM.

The relaxation of supercoiled circular ΦX174 DNA (form I) into the relaxed (form II) and the linear (form III) conformations is monitored in order to compare the aerobic cleavage abilities of complexes **Cu3CP-6-Pt**, **Cu3CP-10-Pt** and Cu(3-Clip-Phen) in the presence of a reducing agent (Figure 3.3). The bifunctional complexes are incubated for 20 h in order to allow the formation of platinum-DNA adducts. The nuclease activity is subsequently initiated by the addition of 5 mM mercaptopropionic acid (MPA) in air.

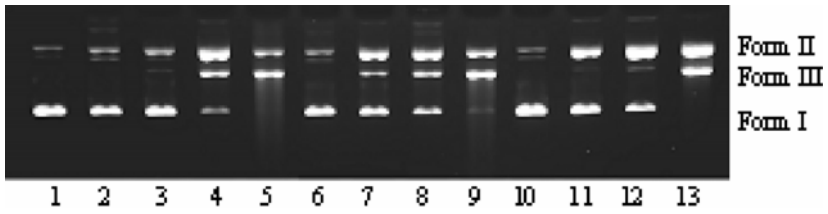


Figure 3.3 Comparison of the oxidative cleavage of ΦX174 plasmid DNA performed by **Cu3CP-6-Pt**, **Cu3CP-10-Pt** and Cu(3-Clip-Phen) in the presence of 5 mM MPA. Lane 1: control DNA. Lane 2: 250 nM **Cu3CP-6-Pt** without MPA. Lane 3: 100 nM **Cu3CP-6-Pt**. Lane 4: 150 nM **Cu3CP-6-Pt**. Lane 5: 250 nM **Cu3CP-6-Pt**. Lane 6: 250 nM **Cu3CP-10-Pt** without MPA. Lane 7: 100 nM **Cu3CP-10-Pt**. Lane 8: 150 nM **Cu3CP-10-Pt**. Lane 9: 250 nM **Cu3CP-10-Pt**. Lane 10: 250 nM Cu(3-Clip-Phen) without MPA. Lane 11: 100 nM Cu(3-Clip-Phen). Lane 12: 150 nM Cu(3-Clip-Phen). Lane 13: 250 nM Cu(3-Clip-Phen).

Lanes 4, 8 and 12 clearly show that the nuclease activities of **Cu3CP-6-Pt** and **Cu3CP-10-Pt** are higher than the one achieved with Cu(3-Clip-Phen), and more linear DNA is formed. Indeed, quantifications show that **Cu3CP-6-Pt** and **Cu3CP-10-Pt** cleaved the largest quantities of form I (starting material). Using the same experimental conditions (lanes 4, 8 and

12), Form III is generated by the platinum/copper complexes and not by Cu(3-Clip-Phen). Moreover, smears (resulting from multi-fragmented DNA) are observed when using complexes **Cu3CP-6-Pt** or **Cu3CP-10-Pt** at 250 nM concentration, which is not the case with Cu(3-Clip-Phen) (lanes 5, 9 and 13). The most striking feature observed for these hybrid platinum/copper complexes is that form III already appears while form I is still present. This result obviously indicates that the heterobimetallic complexes are able to perform direct double strand cuts, whereas Cu(3-Clip-Phen) is only capable of carrying out successive single strand cuts.[5] Time-course studies of the cleavage of the complexes **Cu3CP-6-Pt**, **Cu3CP-10-Pt** and Cu(3-Clip-Phen) have been performed to further investigate the direct double strand cleavage (Figure 3.4). From these studies, it appears that linear DNA is formed for both complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt** directly from the start of the reaction in contrast to Cu(3-Clip-Phen). Even after most of the supercoiled DNA has disappeared in the reaction with Cu(3-Clip-Phen), only less then 8% of linear DNA has formed. Also, these studies confirm the higher cleaving activities of complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt** compared to Cu(3-Clip-Phen) alone, since a fast disappearance of form I, associated with the appearance of form III is observed with these bifunctional molecules. If the concentration of Cu(3-Clip-Phen) is increased to 250 nM, the full disappearance of the supercoiled DNA is observed (Figure 3.4d). Although, small quantities of linear DNA are still observed before the complete disappearance of supercoiled DNA, its amount is inferior to the one noticed with complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt**. In addition, more than 80 % of circular DNA is formed, while the reaction with complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt** only generate a maximum of 60 % of form II.

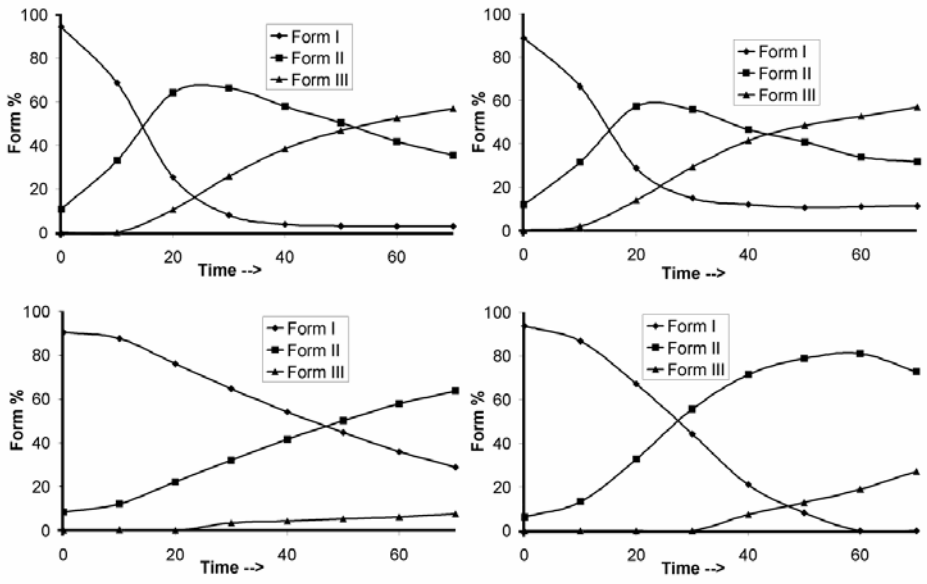


Figure 3.4 Time-course experiments of DNA cleavage (20 μM base pairs) over a period of 70 minutes in the presence of 5 mM MPA and air. Before addition of the reductant, the complexes were incubated for 24 h. (a) 200 nM **Cu3CP-6-Pt**, (b) 200 nM **Cu3CP-10-Pt**, (c) 200 nM Cu(3-Clip-Phen), (d) 250 nM Cu(3-Clip-Phen).

The DSB formation was further explored by a statistical test developed by Povirk *et al.*, which has been used to assay other complexes such as bleomycin.[28, 29] This test assumes a Poisson distribution of strand cuts, and allows calculating the average number of DSB per molecule, n_2. n_2 is obtained from the fraction of linear DNA after cleavage, and the total average number of single- plus double-strand breaks ($n_1 + n_2$), from the fraction of remaining supercoiled DNA after reaction. In order to determine n_1 and n_2 both supercoiled and linear DNA should be present in the experiments. For complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt**, the n_1/n_2 values determined at 20 minutes (Figure 3.4), amounts to respectively 10.5 and 6.8 and respectively 5.8 and 3.1 at 30 minutes. The n_1/n_2 values for Cu(3-Clip-Phen) at 40 and 50 minutes are respectively 18.5 and 16.4. The DSB values of **Cu3CP-6-Pt** and **Cu3CP-10-Pt** are in the range of bleomycin,[29] while the cleavage occurs in a more random manner for Cu(3-Clip-Phen). These results emphasize the ability of complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt** to induce direct double stranded cuts.

Therefore, it appears that the platinum moiety of complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt** acts as an anchor to DNA through a kinetically inert coordination bond. Subsequently, the Cu(3-Clip-Phen) component can function as a cleaving agent only in the close proximity of the platinum moiety, thereby favoring double stranded scissions.

To further investigate the platinum coordination of the complexes to DNA, high resolution analyses with a 36 bp DNA fragment were performed (Figure 3.5a). This fragment contains the two major platination sites on the 5'-end-labelled ODN I strand (highlighted in bold in Figure 3.5a). Complexes able to coordinate to DNA retard the migration rate of the complexed DNA during denaturing polyacrylamide gel electrophoresis (PAGE), as a result of the increase in molecular weight and the change of the overall charge. Therefore, complexes **3CP-6-Pt**, **3CP-10-Pt**, **Cu3CP-6-Pt** and **Cu3CP-10-Pt** were incubated for 24 h with the 36 bp DNA fragment, followed by PAGE in order to detect the platinum-DNA adducts (Figure 3.5b). The complexes are all able to coordinate to ODN I, because free ODN I has partially disappeared and clear bands have emerged above the free ODN fragment (Figure 3.5b). Cisplatin has almost fully reacted, and a clear band corresponding to the adducts is observed (Figure 3.5b, lane 2). Complexes **3CP-6-Pt**, **3CP-10-Pt**, **Cu3CP-6-Pt** and **Cu3CP-10-Pt** have partially reacted with the ODN fragment with the following order of efficiency: **3CP-6-Pt** ≈ **3CP-10-Pt** > **Cu3CP-6-Pt** = **Cu3CP-10-Pt**. In contrast to cisplatin, the products engendered by complexes **3CP-6-Pt**, **3CP-10-Pt**, **Cu3CP-6-Pt** and **Cu3CP-10-Pt** give rise to a smear (Figure 3.5b). This is especially true for complexes **Cu3CP-6-Pt** and **Cu3CP-10-Pt** whose copper moieties are coordinated. No clear bands can be observed (lanes 4 and 6), possibly as the result of unselective DNA binding of the platinum part caused by the Cu(3-Clip-Phen) moiety, which preferentially coordinates in the minor groove of DNA.

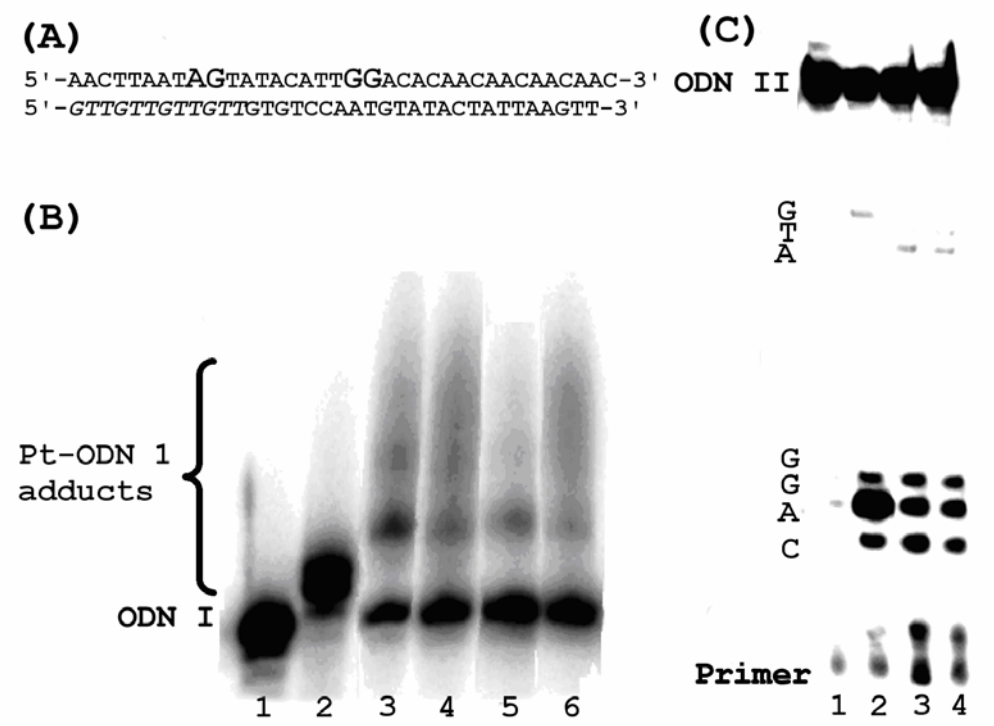


Figure 3.5. (a) 36 bp DNA fragment used for these studies. The two major platination sites are shown in bold and the primer is visualized in italic on ODN II. (b) PAGE analysis of the platinum-ODN I adducts. ODN I-ODN II duplex was ^{32}P-labeled on the 5'-end of ODN I. The complexes were incubated for 24 h with the ODN I-ODN II duplex before analyses. Lane 1: ODN I. Lane 2: 3 μM cisplatin. Lane 3: 10 μM **3CP-6-Pt**. Lane 4: 10 μM **Cu3CP-6-Pt**. Lane 5: 10 μM **3CP-10-Pt**. Lane 6: 10 μM **Cu3CP-10-Pt**. (c) Phosphor image of a DNA sequencing gel comparing the sequence specificity of cisplatin, **3CP-6-Pt** and **3CP-10-Pt** with primer extension. ODN I-ODN II duplex was incubated with platinum-complexes then precipitated to remove unlinked complexes. 5'-end labeled primer was added and all the samples were extended using TAQ polymerase, starting from the 5'-end-labelled primer. Lane 1: blank experiment. Lane 2: 3 μM cisplatin Lane 3: 10 μM **3CP-6-Pt**. Lane 4: 10 μM **3CP-10-Pt**. Note that the AG and GG sites give the sequence of the opposite strand.

Primer extension experiments are performed to investigate the sequence selective binding of platinum complexes to DNA.[30-36] TAQ polymerase has proven to effectively stop at platination sites, and is therefore used for these studies.[37-39] The cisplatin-specific damage sites are the GG and AG sites, but the majority of cisplatin is detected at the GG site. Complexes **3CP-6-Pt** and **3CP-10-Pt** lead to similar results, suggesting that the platinum part is interacting with its preferred site (Figure 4c). However, the bulkiness of the complexes also affects the bases in the close proximity of the adduct. Therefore, the stops of the TAQ polymerase are also influenced by this steric issue. In the experiment with cisplatin, the polymerization is halted at the GG site, primarily on the A before the GG spot, but also at the cytosine before the adenine, and at the first guanine. Near the AG site, the TAQ polymerase mainly stops at the G base. For complexes **3CP-6-Pt** and **3CP-10-Pt**, the enzyme halts at the GG site are equally distributed

between the GAC nucleobases. At the AG site, the damage is mainly observed at the A base. However, no stops are detected at the G base.

The cytotoxic activities of complexes **3CP-6-Pt**, **3CP-10-Pt**, **Cu3CP-6-Pt**, **Cu3CP-10-Pt**, Cu(3-Clip-Phen) and cisplatin have been determined for breast (MCF7 and EVSA-T), colon (WIDR), ovarian (IGROV), melanoma (M19), renal (A498), non-small lung (H226), two glioblastomas (Hs683 and U373), two colorectal (HCT-15 and LoVo) and lung (A549) cancer cell lines. The results of the most relevant activities are summarized in Table 3.1. The cytotoxicities of all complexes are insignificant for the cell lines HCT-15, Hs683, LoVo and A498. Therefore, the corresponding values have been excluded from Table 3.1. Indeed, a complex with an IC_{50} value higher than 10 μM (>10) is considered to be inactive.[40]

Table 3.1 In vitro cytotoxicity assays for **3CP-6-Pt**, **3CP-10-Pt**, **Cu3CP-6-Pt**, **Cu3CP-10-Pt**, Cu(3-Clip-Phen), and cisplatin, against a selection of cancer cell lines.

	IC_{50}[a] values (μM)					
Cell lines	**3CP-6-Pt**	**3CP-10-Pt**	**Cu3CP-6-Pt**	**Cu3CP-10-Pt**	Cu(3-Clip-Phen)	Cisplatin
WIDR	3.7	3.6	7.0	>10	0.58	3.2
EVSA-T	5.6	4.0	1.6	2.7	0.53	1.4
H226	3.0	4.1	5.1	>10	1.6	>10
A549	>10	7.4	>10	>10	>10	1.5
MCF-7	0.9	5.4	3	>10	>10	9
M19	6.0	5.2	0.86	0.37	0.30	1.9
IGROV	>10	>10	7.7	>10	5.4	0.56
U373	>10	6.5	7.1	>10	>10	5

[a]IC_{50} = concentration of drug required to eradicate 50% of the cancer cells.

The IC_{50} values observed for Cu(3-Clip-Phen) are in some cases superior to those achieved with cisplatin (Table 3.1, WIDR, EVSA-T, H226 and M19). The significant antiproliferative activity of 3-Clip-Phen and the corresponding copper complex has been previously measured on the L1210 murine leukemia cell line.[28] These good activities confirm the high potential of Cu(3-Clip-Phen) as an antitumor agent, and strengthen the potential of our strategy to prepare hybrid Cu(3-Clip-Phen)/cis-Pt derivatives. The IC_{50} values obtained with the copper-free complexes (**3CP-6-Pt** and **3CP-10-Pt**) are similar or higher compared to the ones achieved with cisplatin, for the cell lines WIDR, H226, and MCF-7. Complex **3CP-6-Pt** is even ten times more active than cisplatin for the cell line MCF-7, where Cu(3-Clip-Phen) has been

found to be inactive. Interestingly, in some cell lines, the cytotoxicities of **3CP-6-Pt** or **3CP-10-Pt** are even higher than those reached with **Cu3CP-6-Pt** or **Cu3CP-10-Pt**. However, for the EVSA-T and M19 cell lines, the hybrid platinum/copper complexes are more active, which is in agreement with the known activity of Cu(3-Clip-Phen). However, Cu(3-Clip-Phen) is equally good or better in both cell lines. In most cases, compound **3CP-10-Pt** is more active than **3CP-6-Pt,** and surprisingly, **Cu3CP-6-Pt** is more efficient than **Cu3CP-10-Pt**.

3.3 Conclusions

The new synthetic approach to produce highly efficient DNA-cleaving agents herein reported now also offers the prospect to improve the nuclease activity of well-known effective drugs through their synergistic combination within a single molecule. The resulting ditopic (or even multitopic) bio-active molecules thus expand the mechanism of action of its well established antitumor-active components. The fine-tuning of the cleaving ability of such Cu-Pt complexes by further varying the nature and the length of the bridge between the platinum and the Cu(3-Clip-Phen) moieties is reported in chapters 4 and 5. Investigations on the cleavage mechanism with these potential anticancer drugs are reported in chapter 4.

3.4 Experimental section

Synthesis of 3-bromo-1,10-phenanthroline (1) The synthesis of **1** was previously reported,[41] but the purification has been optimized. 45 mL of nitrobenzene were added to 1,10-phenantroline hydrochloride (25 g, 107.5 mmol) into a two necked flask and heated at 140 °C. Then a solution of bromine (8.25 mL, 160 mmol) in 23 mL of nitrobenzene was added drop wise to the flask using a dropping funnel. The total amount of the bromine was added over a period of one hour. The mixture was left for three hours at 140 °C and the mixture was cooled to room temperature. The precipitate was filtered and 100 mL of 25% aqueous NH_4OH were added to the filtrate. The solution was extracted three times with 50 mL of CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The raw product was purified by column chromatography (SiO_2, DCM:MeOH, 99:1) to eliminate 1,10-phenanthroline and obtain a mixture of multi-bromo-phenanthroline products. This solid mixture (3-bromo-phenanthroline (43.3 mmol)) was dissolved in 280 mL of DMF and heated to 100 °C to dissolve it. After dissolution, the mixture was cooled to room temperature and 150 mL H_2O were added. The resulting solution was allowed to stand in the refrigerator for two days. The precipitate was filtered and the solvent was evaporated under reduced pressure yielding the pure product. Yield 25%.

Synthesis of 1,3-di(1,10-phenanthrolin-3-yloxy)propan-2-amine (2) The synthesis of **2** was previously reported,[5] but the synthetic procedure has been optimized. Serinol (0.352 g, 3.86 mmol) was dissolved in 64 mL of DMF under argon. Then 2,6-di-*tert*-butyl-4-methylphenol (0.851 g, 3.86 mmol) and NaH (60% dispersion in mineral oil, 1.85 g, 46.4 mmol) were added. The mixture was allowed to react for 20 minutes at RT and at 4 °C for further 20 minutes with an ice bath. **1** (2 g, 7.7 mmol) was added as a solid at once to the mixture and the wall of the flask were rinsed with 32 mL of DMF. The mixture was

stirred for 20 minutes at 4 °C and for 30 minutes at room temperature. Then the reaction mixture was stirred for 40 hours at 50 °C. 100 mL of water were added to quench the excess of NaH and the mixture was extracted with 3 × 100 mL of CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure leading to a dark brownish oil. This oil was purified by column chromatography (SiO_2, DCM:MeOH:NH_4OH, 95:5:0.5) giving the desired compound. Yield 45%.

Synthesis of 6-(2-Amino-ethylamino)-hexan-1-ol (3) and 10-(2-Amino-ethylamino)-decan-1-ol (4) 6-Bromohexan-1-ol or 10-bromohexan-1-ol (3.74 mmol) was added to 2 mL of ethylenediamine (30.00 mmol) at 4 °C over a period of 2 h. The reaction mixture was then stirred for 1 h at RT, and 10 mL of ethyl acetate were added. The resulting solution was further stirred for 15 min. After overnight decantation, the ethyl acetate phase was separated, evaporated under reduced pressure, and stirred under vacuum during 2 h at 50 °C, in order to remove the residual ethylenediamine starting material. Data for **3**: Color less oil (yield = 85%). ^{1}H NMR ($DCCl_3$, 300 MHz) δ 3.59 (t, 2H, *J* = 6.52 Hz), 2.79 (m, 2H), 2.52-2.70 (m, 4H), 1.51 (m, 4H), 1.35 (m, 4H) ppm. Low resolution MS (ESI >0) *m*/*z* 161.2 [(M+H)$^+$; calcd for $C_8H_{21}N_2O^+$: 161.3]. Data for **4**: Color less oil (yield = 93%). ^{1}H NMR ($DCCl_3$, 300 MHz) δ 3.56 (t, 2H, *J* = 6.61 Hz), 2.76 (t, 2H, *J* = 5.97 Hz), 2.63 (t, 2H, *J* = 6.02 Hz), 2.56 (t, 2H, *J* = 7.15 Hz), 1.48 (m, 4H), 1.25 (m, 12H) ppm.

Synthesis of (2-*tert*-Butoxycarbonylamino-ethyl)-(6-hydroxy-hexyl)-carbamic acid *tert*-butyl ester (5) and (2-*tert*-Butoxycarbonylamino-ethyl)-(10-hydroxy-decyl)-carbamic acid *tert*-butyl ester (6). NaH (0.830 g, 20.8 mmol) was added in portions to a solution of **3** or **4** (4.6 mmol) in 6 mL of THF. The solution was then heated to 50 °C during 1 h under argon. The heterogeneous reaction mixture was cooled down to 4 °C using an ice bath, and di-*tert*-butylcarbonate (Boc_2O) (4.5 g, 20.8 mmol) in 20 mL of THF was added dropwise over a period of 30 min. The ice bath was removed and the mixture was allowed to react overnight at RT. The excess of NaH was neutralized carefully with 50 mL of water, and the resulting solution was extracted with diethyl ether (3 × 50 mL). The pooled organic phases were dried over sodium sulfate, and the solvent was evaporated under reduced pressure. The crude residue was refluxed with 5 g of K_2CO_3 in 50 mL of MeOH during 1 h. The reaction mixture was cooled down to RT and filtered. The filtrate was extracted with heptane (2 × 20 mL). After evaporation of the solvent, the residue was dissolved in 50 mL of DCM, washed with distilled water (3 × 50 mL), dried over sodium sulfate, and the solvent was evaporated under reduced pressure. Data for **5**: Color less oil (yield = 62%).^{1}H NMR ($DCCl_3$, 300 MHz) δ 5.10-4.60 (br, 1H), 3.62 (m, 2H), 3.35-3.10 (m, 6H), 1.60-1.25 (m, 26H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 63.45, 47.08, 40.38, 33.37, 29.19, 27.30, 26.21 ppm. Low resolution MS (ESI >0) *m*/*z* 383.2 [(M+Na)$^+$; calcd for $C_{18}H_{37}N_2NaO_5^+$: 383.5]. Data for **6**: Color less oil (yield = 60%). ^{1}H NMR ($DCCl_3$, 300 MHz) δ 3.63 (t, 2H, *J* = 6.59 Hz), 3.35-3.10 (m, 6H), 1.60-1.15 (m, 34H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 63.1, 47.7, 46.4, 39.7, 32.8, 29.5, 29.4, 29.3, 28.4, 26.8, 25.7 ppm. Low resolution MS (ESI >0) *m*/*z* 417.2 [(M+H)$^+$; calcd for $C_{22}H_{45}N_2O_5^+$: 417.6].

Synthesis of 2-*tert*-Butoxycarbonylamino-ethyl)-(6-oxo-hexyl)-carbamic acid *tert*-butyl ester (7) and (2-*tert*-Butoxycarbonylamino-ethyl)-(6-oxo-decyl)-carbamic acid *tert*-butyl ester (8). **5** or **6** (0.96 mmol) and pyridinium chloridotrioxidochromate (PCC, 0.310 g, 1.44 mmol) were stirred separately during 15 min in 4.5 mL of DCM containing 0.5 g of activated powdered molecular sieves. The solution of alcohol **5** or **6** was added to the solution of PCC using a cannula. The resulting reaction mixture was stirred during 1.5 h. Diethyl ether (10 mL) was then added and the solid materials were

separated by filtration over celite. The filtrate was evaporated under reduced pressure to afford **7** or **8** as color less oils. Data for **7**: Color less oil (yield = 67 %). ^{1}H NMR ($DCCl_3$, 300 MHz) δ 9.76 (s, 1H), 3.35-3.10 (m, 6H), 2.44 (t, 2H, *J* = 6.60 Hz), 1.70-1.20 (m, 24H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 202.2, 156.1, 79.7, 47.4, 46.4, 43.5, 39.5, 28.3, 26.2, 21.7 ppm. Low resolution MS (ESI >0) *m/z* 381.2 [$(M+Na)^+$; calcd for $C_{18}H_{34}N_2O_5Na^+$: 381.5]. Data for **8**: Color less oil (yield = 55 %). ^{1}H NMR ($DCCl_3$, 300 MHz) δ 9.76 (s, 1H), 3.35-3.10 (m, 6H), 2.41 (t, 2H, *J* = 7.33 Hz), 1.75-1.15 (m, 32H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 202.7, 79.5, 47.6, 46.3, 43.8, 39.6, 29.2, 29.0, 28.3, 26.7, 22.0 ppm. Low resolution MS (ESI >0) *m/z* 437.3 [$(M+Na)^+$; calcd for $C_{22}H_{42}N_2O_5Na^+$: 437.6].

Synthesis of (2-*tert*-Butoxycarbonylamino-ethyl)-{6-[3-Clip-Phen]-hexyl}-carbamic acid *tert*-butyl ester (9) and (2-*tert*-Butoxycarbonylamino-ethyl)-{10-(3-Clip-Phen)-decyl}-carbamic acid *tert*-butyl ester (10). **7** or **8** (0.86 mmol), 3-Clip-Phen (**2**) (0.38 g, 0.86 mmol) and 1 g of molecular sieves were stirred under argon in 20 mL of MeOH under reflux during 3 hours. After cooling down to 4 °C, $NaBH_4$ (0.063 g, 1.72 mmol) was added and the reaction mixture was further stirred for 2 h. 50 mL of DCM were added and the molecular sieves were removed by filtration. The filtrate was transferred in a separating funnel, washed with water (2 × 50 mL), dried over sodium sulfate, and evaporated under reduced pressure. The crude product was purified by column chromatography (SiO_2, DCM:MeOH:NH_4OH, 95:5:0.5). Data for **9**: Light brown powder (yield = 65 %). ^{1}H NMR ($DCCl_3$, 300 MHz) δ 9.13 (dd, 2H, *J* = 4.30, 1.49 Hz), 8.94 (d, 2H, *J* = 2.70 Hz), 8.19 (dd, 2H, *J* = 8.05, 1.49 Hz), 7.76 and 7.70 (AB, 4H, *J* = 8.86 Hz), 7.61 (d, 2H, *J* = 2.70 Hz), 7.56 (dd, 2H, *J* = 8.05, 4.30 Hz), 4.99 (br, 0.5H), 4.78 (br, 0.5H), 4.40 (m, 4H), 3.60 (m, 1H), 3.00-3.40 (m, 7H), 2.86 (t, 2H, *J* = 7.15 Hz), 1.10-1.70 (m, 26H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 153.9, 150.2, 146.1, 142.5, 140.5, 135.8, 129.4, 127.2, 126.0, 122.0, 115.1, 67.5, 56.5, 47.8, 46.3, 39.5, 30.3, 28.3, 26.9, 26,6 ppm. Low resolution MS (ESI >0) *m/z* 812.57 [$(M+Na)^+$; calcd for $C_{45}H_{55}N_7NaO_6^+$: 812.95], 828.55 [$(M+K)^+$; calcd for $C_{45}H_{55}N_7KO_6^+$: 829.06], 790.57 [$(M+H)^+$; calcd for $C_{45}H_{56}N_7O_6^+$: 790.97]. Anal. Calcd for $C_{45}H_{55}N_7O_6 \cdot 0.7$ CH2Cl2: C, 64.62; H, 6.69; N, 11.54. Found: C, 44.62; H, 6.93; N, 11.81. Data for **10**: Light brown powder (yield = 77%). ^{1}H NMR ($DCCl_3$, 300 MHz) δ 9.02 (dd, 2H, *J* = 4.34, 1.65 Hz), 8.85 (d, 2H, *J* = 2.83 Hz), 8.06 (dd, 2H, *J* = 8.05, 1.65 Hz), 7.62 and 7.57 (AB, 4H, *J* = 8.88 Hz), 7.49 (d, 2H, *J* = 2.83 Hz), 7.42 (dd, 2H, *J* = 8.05, 4.34 Hz), 5.03 (br, 0.5H), 4.79 (br, 1H), 4.30 (m, 4H), 3.47 (m, 1H), 2.95-3.30 (m, 7H), 2.75 (t, 2H, *J* = 7.06 Hz), 1.00-1.60 (m, 34H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 154.7, 151.0, 146.9, 143.3, 141.3, 136.6, 130.2, 128.0, 126.8, 122.8, 115.9, 68.4, 57.2, 48.7, 48.5, 47.1, 40.4, 31.1, 30.2, 30.0, 29.1, 28.0, 27,5 ppm. Low resolution MS (ESI >0) *m/z* 868.4 [$(M+Na)^+$; calcd for $C_{49}H_{63}N_7NaO_6^+$: 869.1], 846.4 [$(M+H)^+$; calcd for $C_{49}H_{64}N_7O_6^+$: 847.1]. Anal. Calcd for $C_{49}H_{63}N_7O_6 \cdot 1.5$ CH_2Cl_2: C, 62.31; H, 6.83; N, 10.07. Found: C, 62.03; H, 7.33; N, 10.44.

Synthesis of [N*1*-{6-[3-Clip-Phen]-hexyl}-ethane-1,2-diamine] (3CP-6-NH_2) and N*1*-{10-[3-Clip-Phen]-decyl}-ethane-1,2-diamine (3CP-10-NH_2). **9** or **10** (0.1 mmol) was dissolved in 2 mL of trifluoroacetic acid (TFA) at 4 °C, and stirred during 1 h. The TFA was then evaporated under reduced pressure and the crude product was purified by column chromatography (SiO_2, DCM:MeOH:NH_4OH, 90:10:1). Data for **3CP-6-NH_2**: Light brown powder (yield = 65 %). NB: the NMR spectra chemical shifts are dependent on the sample concentration. ^{1}H NMR (MeOD-d_3, 300 MHz) δ 9.03 (d, 2H, *J* = 2.83 Hz), 8.91 (d, 2H, *J* = 2.84 Hz), 8.40 (d, 2H, *J* = 6.56 Hz), 7.92 (d, 2H, *J* = 2.83 Hz), 7.89 (d, 2H, *J* = 8.95 Hz), 7.83 (d, 2H, *J* = 8.92 Hz), 7.69 (dd, 2H, *J* = 8.09, 4.48 Hz), 4.78 (m, 4H), 4.31 (m, 1H), 3.44 (t, 2H, *J* = 7.66), 3.35 (m, 4H), 3.04 (t, 2H, *J* = 7.49), 1.90 (m, 2H), 1.71 (m, 2H), 1.48 (br,

4H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 153.1, 149.1, 144.6, 141.6, 139.4, 136.8, 129.5, 127.5, 127.2, 126.1, 122.5, 116.0, 64.7, 56.4, 44.5, 35.6, 25.3, 25.1 ppm. Low resolution MS (ESI >0) *m/z* 590.37 [$(M+H)^+$; calcd for $C_{35}H_{40}N_7O_2^+$: 590.74], 295.98 [$(M+2H)^{2+}$; calcd for $C_{35}H_{41}N_7O_2^{2+}$: 295.86]. Data for **3CP-10-NH$_2$**: Light brown powder (yield = 83 %). NB: the NMR spectra chemical shifts are dependent on the sample concentration. ^{1}H NMR (MeOD-d_3, 300 MHz) δ 8.98 (dd, 2H, *J* = 4.41, 1.61 Hz), 8.84 (d, 2H, *J* = 2.82 Hz), 8.34 (dd, 2H, *J* = 8.12, 1.62 Hz), 7.90 (d, 2H, *J* = 2.83), 7.85 (d, 2H, *J* = 8.98 Hz), 7.80 (d, 2H, *J* = 8.93 Hz), 7.65 (dd, 2H, *J* = 8.09, 4.45 Hz), 4.60 (d, 4H, *J* = 4.75), 3.87 (br, 1H), 3.10 (m, 6H), 2.84 (m, 2H), 1.70 (m, 2H), 1.54 (m, 2H), 1.41-1.16 (m, 12H) ppm. ^{13}C NMR ($DCCl_3$, 75 MHz) δ 154.7, 151.0, 146.9, 143.3, 141.3, 136.6, 130.2, 128.0, 126.8, 122.8, 115.9, 68.4, 57.2, 48.7, 48.5, 47.1, 40.4, 31.1, 30.2, 30.0, 29.1, 28.0, 27.5 ppm. Low resolution MS (ESI >0) *m/z* 646.3 [$(M+H)^+$; calcd for $C_{39}H_{48}N_7O_2^+$: 646.8], 323.8 [$(M+2H)^{2+}$; calcd for $C_{39}H_{49}N_7O_2^{2+}$: 323.9]. Anal. Calcd for $C_{39}H_{47}N_7O_2 \cdot 2CH_2Cl_2$: C, 60.37; H, 6.30; N, 12.02. Found: C, 60.44; H, 6.16; N, 11.87.

Synthesis of Pt[N*1*-{6-[3-Clip-Phen]-hexyl}-ethane-1,2-diamine]Cl$_2$ (3CP-6-Pt) and Pt[N*1*-{10-[3-Clip-Phen]-decyl}-ethane-1,2-diamine]Cl$_2$ (3CP-10-Pt). A solution of K_2PtCl_4 (0.1 mmol) in 3 mL of H_2O was added dropwise to a solution of **3CP-6-NH$_2$** or **3CP-10-NH$_2$** (0.1 mmol) in 6 mL of MeOH over a period of 2 min. The resulting off-white precipitate was filtered after a reaction time of 5 h. The product was washed consecutively with 3 × 10 mL of H_2O, 3 × 10 mL of MeOH, and 2 × 20 mL of diethyl ether. The coordination compounds were dried overnight at 50 °C under reduced pressure. The integrals of the ^{1}H-NMR peaks around 4.60 ppm may be inaccurate due to the suppression of the water signal by the program used during the scanning of the sample. Also, the signal owing to DMSO, used to dissolve the samples, is partially overlapping the peaks in the 2.70 ppm region. Data for **3CP-6-Pt**: Off-white powder (yield = 71 %). ^{1}H NMR (DMSO-d_6 and D_2O, 300 MHz) δ 8.82 (br, 2H), 8.64 (br, 2H), 8.42 (br, 2H), 7.78 (br, 2H), 7.64-7.54 (br, 6 H), 4.84 (br, 1H), 4.57-4.53 (br, 2H) 4.15 (br, 1H), 3.07-2.69 (m, 6H), 1.80 (br, 2H), 1.67 (br, 2H), 1.41 (br, 4H) ppm. ^{13}C NMR (DMSO-d_6, 75 MHz) δ 153.7, 148.2, 142.4, 139.3, 137.4, 129.9, 127.5, 127.1, 123.0, 116.4, 65.2, 55.3, 46.7, 46.0, 44.2, 35.4, 25.3 ppm. ^{195}Pt NMR (DMSO-d_6, 300 MHz) δ –2308 (complex) and –2949 (complex with coordinated DMSO) ppm. MS (MALDI-TOF) *m/z* 898.6, 897.6, 899.6, 900.6, 901.6, 902.6 [$(M+DMSO(solvent)–Cl^-)^+$; calcd for $C_{37}H_{45}ClN_7O_3PtS^+$:898.3, 897.3, 899.3, 900.3, 901.3,902.3], 784.4, 785.4, 783.4, 786.4, 787.5, 788.4 [$(M –HCl –Cl^-)^+$; calcd for $C_{35}H_{38}N_7O_2Pt^+$:784.3, 785.3, 783.3, 786.3, 787.3, 788.3]. Data for **3CP-10-Pt**: Off-white powder (yield = 76 %). ^{1}H NMR (DMSO-d_6 and D_2O, 300 MHz) δ 8.78 (br, 2H), 8.68 (br, 2H), 8.23 (br, 2H), 7.67-7.49 (m, 4H), 4.81 (br, 1H), 4.58 (br, 3H), 4.11 (br, 2H), 2.82-2.69 (br, 7H), 1.76 (br, 16H) ppm. ^{13}C NMR (DMSO-d_6, 75 MHz) δ 154.5, 150.7, 145.5, 143.3, 138.5, 130.8, 128.5, 127.8, 123.9, 117.4, 66.4, 56.7, 53.2, 48.0, 47.4, 36.6, 29.9, 27.4-26.8 ppm. ^{195}Pt NMR (DMF-d_7, 300 MHz) δ -2329 ppm. MS (MALDI-TOF) *m/z* 839.1, 840.1, 838.1, 841.1, 842.1, 843.1 [$(M –HCl –Cl^-)^+$; calcd for $C_{39}H_{46}N_7O_2Pt^+$:839.3, 840.3, 838.3, 841.3, 842.3, 843.3]. ICP-AES Pt to K ratio = 1:0.37. Anal. Calcd for $C_{39}H_{47}Cl_2N_7O_2Pt \cdot 0.37\ KCl \cdot 6H_2O$: C, 44.72; H, 5.68; N, 9.36. Found: C, 44.25; H, 5.04; N, 9.19.

Synthesis of CuPt[N*1*-{6-[3-Clip-Phen]-hexyl}-ethane-1,2-diamine]Cl$_4$ (Cu3CP-6-Pt) and CuPt[N*1*-{10-[3-Clip-Phen]-decyl}-ethane-1,2-diamine]Cl$_4$ (Cu3CP-10-Pt). $CuCl_2$ (0.1 mmol) was added as a solid to a suspension of **3CP-6-Pt** or **3CP-10-Pt** (0.05 mmol) in DMF (25 mL). The reaction was stirred overnight at 50 °C. The DMF was partially evaporated under reduced pressure, and the crude was precipitated in 100 mL of diethyl ether. The solid material was filtered and washed with 3 × 20 mL of diethyl ether and dried overnight at 50 °C under reduced pressure. The resolution of the mass

measurements was not sufficient enough, because only broadened peaks were observed. Data for **Cu3CP-6-Pt**: MS (MALDI-TOF) m/z 954.1 [$(M–Cl^–)^+$; calcd for $C_{35}H_{39}Cl_3N_7O_2PtCu^+$: 954.7], UV-Vis (DMSO/$H_2O$ 10/90) λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}): 281 (41500), 318 (14400), 346 (5700), Data for **Cu3CP-10-Pt**: MS (MALDI-TOF) m/z 1055.1 [$(M + DMSO –2Cl^–)^+$; calcd for $C_{41}H_{53}Cl_2N_7O_3SPtCu^+$: 1053.5], 1077.1 [$(M + DMSO –2Cl^–)^+$; calcd for $C_{41}H_{53}Cl_2N_7O_3SNaPtCu^+$: 1076.5], UV-Vis (DMSO/$H_2O$ 10/90) λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}): 282 (43600), 319 (17500), 347 (8000)

Cleavage studies. Complexes were prepared as 1 mM solutions in DMSO, and diluted to respectively 400, 600 and 1000 nM with MilliQ water. 5 µL of complex solution were added to 10 µL of supercoiled ΦX174 DNA ((Invitrogen)7 nM, 40 µM base pairs) in 6 mM NaCl, 20 mM sodium phosphate buffer (pH 7.2), and incubated for 20 h at 37 °C. To initiate the cleavage, 5 µL of a 20 mM mercaptopropionic acid solution in water were added, and the resulting reaction mixture was incubated at 37 °C for 1 h. The reaction was quenched at 4 °C, followed by the addition of 4 µL of loading buffer (bromophenol blue) prior to its loading on a 0.8 % agarose gel containing 1 µg mL^{-1} of ethidium bromide. The gels were run at a constant voltage of 70 V for 90 minutes in TBE buffer containing 1 µg mL^{-1} of ethidium bromide. The gels were visualized under a UV transilluminator, and the bands were quantified using a BioRad Gel Doc 1000 apparatus interfaced with a computer. A correction factor of 1.47 has been applied to quantify the amount of supercoiled DNA (form I) present in all samples.

Time-course experiments of DNA cleavage: the solutions of the different complexes were prepared as 1 mM solutions in DMSO, which were subsequently diluted to respectively 800 and 1000 nM (only Cu(3-Clip-Phen) with MilliQ water. 50 µL of complex solution were added to 100 µL of supercoiled ΦX174 DNA ((Invitrogen) 7 nM, 40 µM base pair) in 6 mM NaCl, 20 mM sodium phosphate buffer (pH 7.2), and the resulting reaction mixture was incubated for 20 h at 37 °C. To initiate the cleavage, 50 µL of a 20 mM mercaptopropionic acid were added, and a sample was taken out every 10 minutes. 4 µL of loading buffer (bromophenol blue) were added, and the sample was directly frozen in liquid nitrogen. When all samples were collected they were loaded on a 0.8% agarose gel containing 1 µg mL^{-1} of ethidium bromide.

The DSB formation was calculated from a test developed by Povirk *et al.*[28, 29] which assumes a Poisson distribution of strand cuts, and allows to calculate the average number of DSB per molecule, n_2. n_2 is obtained from the fraction of linear DNA after cleavage, and the total average number of single- plus double-strand breaks ($n_1 + n_2$), from the fraction of remaining supercoiled DNA after reaction. In order to determine n_1 and n_2 both supercoiled and linear DNA should be present in the experiments. The fraction of linear DNA after scission chemistry, form III = $n_2\exp(-n_2)$. The supercoiled fraction remaining after treatment is form I = $\exp[-(n_1 + n_2)]$.[29]

Analysis of platinum adducts by high resolution polyacrylamide gel electrophoresis. (Experiments have been performed at the CNRS in Toulouse) The ODNs I, II and the primer were purchased from Eurogentec, and purified on a 15% polyacrylamide gel. Concentrations of single-stranded ODNs were determined by UV titration at 260 nm.[42] The ODNs were end-labeled with ^{32}P using standard procedures with T_4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP for the 5'-end, before being purified on a MicroSpin G25 column (Pharmacia).[43]

Analysis of the platinum-DNA adducts: The 36mer ODN I-ODN II duplex target (2 μM, 5'-end labeled on ODN I) was annealed in 1100 μL of Tris-HCl (20 mM, pH 7.2) by heating to 90 °C for 5 minutes, followed by slow cooling to room temperature. Samples were divided in shares of 60 μL before addition of 60 μL of complex solution (6 μM cisplatin or 20 μM of complexes **3CP-6-Pt**, **3CP-10-Pt**, **Cu3CP-6-Pt** and **Cu3CP-10-Pt**). Samples were incubated for 20 hours at 37 °C, and subsequently precipitated with 100 μL of sodium acetate buffer (3M, pH 5.2), and 1300 μL of cold ethanol. The resulting pellets were rinsed twice with 200 μL of cold ethanol, and then lyophilized. The samples dissolved in 5 μL of a bromophenol blue/xylene cylenol/formamide solution were separated by denaturing 20 % polyacrylamide gel electrophoresis.

Primer extension experiments: The 36mer ODN I-ODN II duplex target (2 μM) was annealed in 1100 μL of Tris-HCl (20 mM, pH 7.2) by heating to 90 °C for 5 minutes, followed by slow cooling to room temperature. Samples were divided in shares of 60 μL before the addition of 60 μL of complex solution (6 μM cisplatin or 20 μM complex **3CP-6-Pt** and **3CP-10-Pt**). Samples were incubated for 20 hours at 37 °C, and then precipitated with 100 μL of sodium acetate buffer (3M, pH 5.2), and 1300 μL of cold ethanol. The resulting pellets were rinsed twice with 200 μL of cold ethanol, and then were lyophilized. The samples were dissolved in MilliQ H_2O to a 1.25 μM concentration of ODN I/ODN II. To 2 μL of this solution, was added 1 μL of enzyme 10 × buffer, 2 μL of primer/ODN I solution (1 equivalent of 5'-end labeled primer + 1 equivalent of ODN I) and 2 μL of MilliQ H_2O. The samples were hybridized by heating to 90 °C for 5 minutes, followed by slow cooling to RT. To this solution, 2 μL of a 250 μM dGTP, dCTP, dATP and dTTP solution were added and 1 μL of enzyme (2.5 units TAQ polymerase). The reaction time with the TAQ polymerase was 30 minutes at 37 °C. TAQ polymerase is a thermally stable enzyme; therefore, the samples were directly frozen in liquid nitrogen. 5 μL of a bromophenol blue/xylene cylenol/formamide solution were added to a 5 μL sample, followed by separation by denaturing 20 % polyacrylamide gel electrophoresis. Maxam and Gilbert sequencing scale, where the 3'-phosphate ends of the resulting fragments were removed with polynucleotide kinase, was used to analyze DNA fragments.[44]

Cytotoxicity tests. (Experiments have been performed in the group of R. Kiss in Brussels) The following human tumor cell lines were used to determine the cytotoxic abilities of the compounds prepared:

MCF7	breast cancer
EVSA-T	breast cancer
WIDR	colon cancer
IGROV	ovarian cancer
M19 MEL	melanoma
A498	renal cancer
H226	non-small cell lung cancer
Hs683	glioblastomas
U-373MG	glioblastomas
HCT-15	colorectal cancer
LoVo	colorectal cancer
A549	lung cancer

The experimental procedure reported below has been followed for the cell lines: MCF7, EVSA-T, WIDR, IGROV, M19 MEL, A498 and H226. The test and reference compounds were dissolved to reach a concentration of 250000 ng mL^{-1} in the full medium, by a 20 fold dilution of a stock solution containing 1 mg compound/200 μL. The complexes were dissolved in DMSO. The cytotoxicity of the different compounds was estimated via the microculture sulforhodamine B (SRB) test. The experiment was started on day 0 when 150 μL of trypsinized tumor cells (1500 – 2000 cells/well) were plated in 96-wells flat bottom microtiter plates (falcon 3072, BD). The plates were preincubated for 48 h at 37 °C, with 8.5 % CO_2 to allow the cells to adhere.

On day 2, a three-fold dilution sequence of ten steps was performed in the full medium, starting with the 250000 ng mL^{-1} stock solution. Every dilution was used in quadruplicate by adding 50 μL to a column of four wells. This procedure resulted in the highest concentration of 62500 ng mL^{-1} for column 12. Column 2 was used for the blank. PBS was added to column 1 in order to diminish interfering evaporation.

On day 7, the incubation was terminated by washing the plate twice with PBS. Subsequently, the cells were fixed with 10% trichloroacetic acid in PBS and placed at 4 °C for one hour. After five washing steps with tap water, the cells were stained for at least 15 minutes with 0.4% SRB dissolved in 1% acetic acid. After staining, the cells were washed with 1% acetic acid to remove the free stain. The plates were air-dried and the bound stain was dissolved in 150 μL of 10 mM Tris-base. The absorbance was read at 540 nm using an automated microplate reader (Labsystems Multiskan MS). The data were processed for the construction of concentration-response curves and the determination of the ID_{50} value, using the Deltasoft 3 software.

The experimental procedure described below has been used for the following cell lines: Hs683, U373MG, HCT-15, LoVo, and A549. The cells were cultured at 37 °C in sealed (airtight) Falcon plastic dishes (Nunc, Gibco, Belgium) containing Eagle's minimal essential medium (MEM, Gibco) supplemented with 5 % fetal calf serum (FCS). All the media were supplemented with a mixture of 0.6 mg mL^{-1} glutamine (Gibco), 200 IU mL^{-1} penicillin (Gibco), 200 IU mL^{-1} streptomycin (Gibco), and 0.1 mg mL^{-1} gentamycin (Gibco). The FCS was heat-inactivated for 1 h at 56 °C.

The MTT test is an indirect technique, which allows the rapid measurement (5 days) of the effect of a given product on the global growth of a cell line.[40] This test is based on the measurement of the number of metabolically active living cells able to transform the yellowish MTT product (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) into the blue product, formazan, via mitochondrial reduction performed by living cells.[40] The amount of formazan obtained at the end of the experiment is measured with a spectrophotometer, and is therefore directly proportional to the number of living cells at that moment. The measurement of the OD (Optical density) provides a quantitative measurement of the effect of the product investigated as compared to control (untreated cells), and enables it to be compared to other reference products.[40]

The cells are put to grow in flat-bottomed 96-well micro-wells with 100 μL of cell suspension per well and between 1,000 and 5,000 cells/well depending on cell type. Each cell line is seeded in its own cell culture medium. After a 24-hour period of incubation at 37 °C, the culture medium is replaced by 100 μL

of fresh medium in which the substance to be tested has been dissolved at the different concentrations required. In our experiments, we tested the 6 compounds (**3CP-6-Pt**, **3CP-10-Pt**, **Cu3CP-6-Pt**, **Cu3CP-10-Pt**, Cu-3-Clip-Phen and cisplatin) at 10^{-5} M to 10^{-9} M by ½ log step. Each experimental condition is carried out in six different wells. After 72 hours of incubation at 37 °C with the drug (experimental conditions) or without the drug (control), the medium is replaced by 100 µL MTT at the concentration of 1 mg mL^{-1} dissolved in RPMI. The micro-wells are then incubated for 3 h at 37 °C and centrifuged at 400 G for 10 minutes. The MTT is removed and the formazan crystals formed are dissolved in 100 µL of DMSO. The micro-wells are then shaken for 5 minutes and read on a spectrophotometer at 2 wavelengths (570 nm: the maximum formazan absorbance wavelength; 630 nm: the background noise wavelength).[40]

The IC_{50} value of the 3-Clip-Phen ligand is for: Hs683; 8.4 µM, U373MG; 1 µM, HCT-15 > 10 µM, LoVo; 8.6 µM, A549; 5 µM and MCF-7; 4.5 µM.

3.5 References

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