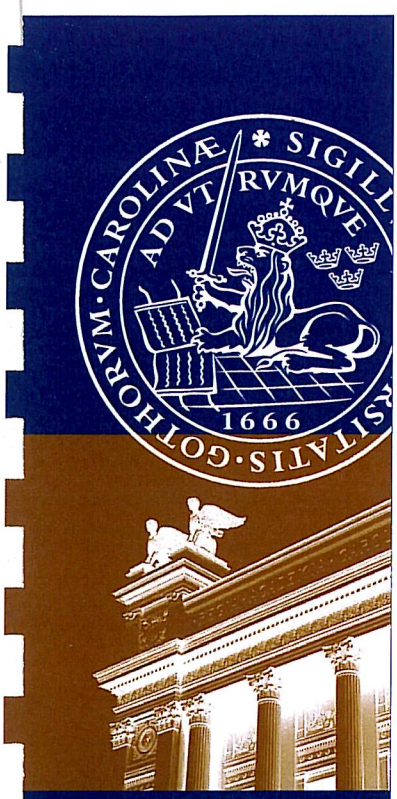

Influence of cisplatin on DNA and RNA stability

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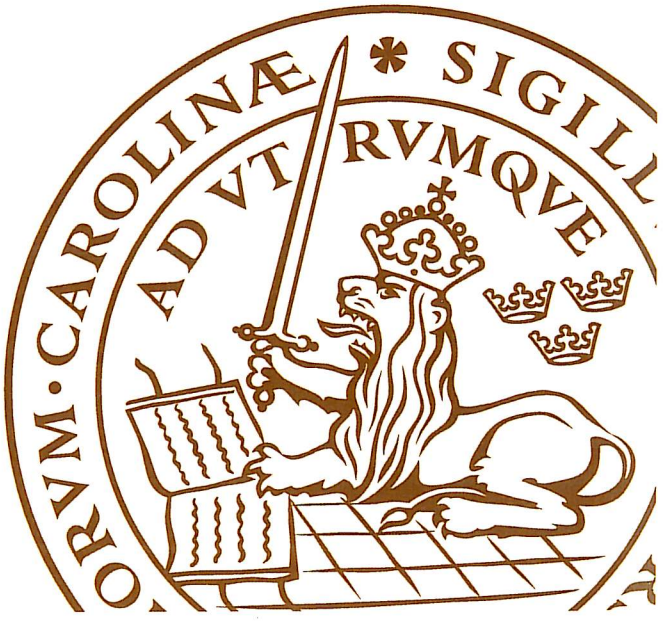
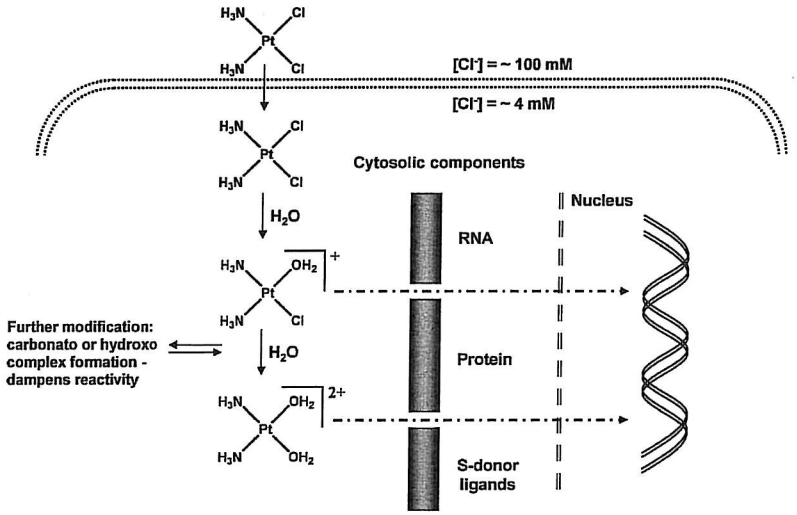


Populärvetenskaplig svensk sammanfattning.

Cisplatins inverkan på strukturen på DNA/RNA undersöktes med smältpunktsbestämning. Separation av nukleosider undersöktes med HPLC reverse column.

Cancer har idag, blivit allt vanligare i samhället. Det finns olika typer av cancer en del går att behandla med hjälp av cytostatika. Den vanligaste cancerformen bland män i åldrarna 20-35 är testikelcancer. Testikelcancer botas bland annat med cisplatin eller oxaliplatin. Cisplatin upptäcktes för 40 år sedan av Barnett Rosenberg. Cisplatins huvudsakliga uppgift är att binda in till DNA och att hindra cellerna från att dela sig. För att cisplatin ska fungera som cytostatika och binda till DNA måste cisplatin aktiveras. När cisplatin har kommit in i blodet är det stabilt, på grund av den höga salthalten i blodet. Då cisplatin kommer in i cellen byts kloridjonerna ut mot två vatten ligander och cisplatin kan binda in till DNA (Se figur 1). När cisplatin binder till DNA böjs DNAt.



Figur 1. Bilden visar hur cisplatin aktiveras och binder in till DNA.

Vid en temperaturökning separeras strängarna i DNA från dubbelsträngat till enkeltsträngat. Vid en smältpunktsbestämning ökar man temperaturen till dess att DNA separerats till enkelsträngat DNA. Då temperaturen har nått den punkt där hälften av DNA är in sin dubbelsträngad form och hälften är i enkelsträngad form har DNA nått sitt smältpunktsvärde (T_m).

När cisplatin binder in till DNA minskar T_m värdet, det beror på den ändrade strukturen på DNA:at. I denna studie uppmättes en skillnad i temperatur på ca 10 °C.

Smältpunktsbestämningen visade att RNA har högre T_m värde än DNA. Men det blev ingen skillnad i T_m värdet mellan platinerat RNA och oplatinerat RNA, vilket var oväntat och kräver fortsatt analys.

DNA och RNA sönderdelades även enzymatiskt. Beståndsdelarna separerades med kromatografi, för att studera var cisplatin bundit. De baser som är mer opolära fastnar i kolumnen och eluerar senare än de som är polära. DNA/RNA strängarna som har platinerats separerades addukterna som har bildats från de omodifierade nukleotiderna.

Abstract

Cancer is today a common disease in our society. There are several forms of cancer and some can be treated with cytostatic drugs. The most common form of cancer among men in the age of 20-35 is testicular cancer. Testicular cancer can be treated with cisplatin or oxaliplatin. The therapeutic use of cisplatin was discovered in the late 60's by Barnett Rosenberg. The main target of cisplatin is to bind DNA and prevent the cell from dividing. In order for cisplatin to work, it has to be activated. Cisplatin in the bloodstream is stable due to the high concentration of chloride ions, but when it enters the cell, the chloride ion concentration is lower the chloride ligands are exchanged for two water ligands. This makes it possible for cisplatin to bind to DNA.

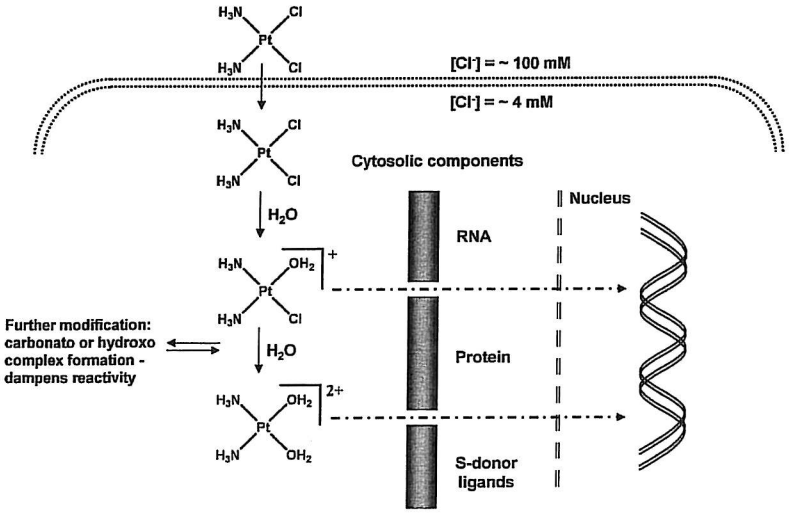


Figure 1 illustrates how cisplatin is activated and binds to DNA.

Upon temperature increase the two strands in double stranded DNA will separate into single strands. The definition of the thermal melting point is when half of the DNA is double stranded and half of the DNA is single stranded. The thermal melting point of the DNA decreases when cisplatin binds to it. This is due to the altered structure of the DNA. In this study, the difference was measured to be 10 °C. The thermal melting point measurement showed that RNA had a higher value than DNA. Though there was no difference between the platinated and unplatinated RNA.

The oligonucleotide was enzymatically digested, to be able to study were cisplatin was bound to the DNA. The components were separated by chromatography. A reverse phase C18 column was used. The polar bases will elute earlier than the more non-polar bases.

CONTENTS

1 Introduction

The aim with this project was to investigate how cisplatin influences the stability of DNA and RNA. To investigate this, thermal melting studies and HPLC measurements were used as methods.

1.1 The structure of DNA and RNA.

The DNA in all living cells is built up of four bases; Adenine, Guanine, Cytosine and Thymine. Adenine pairs with Thymine (A=T) and Cytosine with Guanine (C=G). Between A and T there are two hydrogen bonds and between C and G there are three hydrogen bonds (Biochemistry, Stryer 3rd eddition).

A sugar molecule, deoxyribose in DNA and ribose in RNA, is bound to the base forming a nucleoside and a phosphate group is bound to the sugar, whereby a nucleotide is obtained. In RNA the thymine is replaced with uracil.

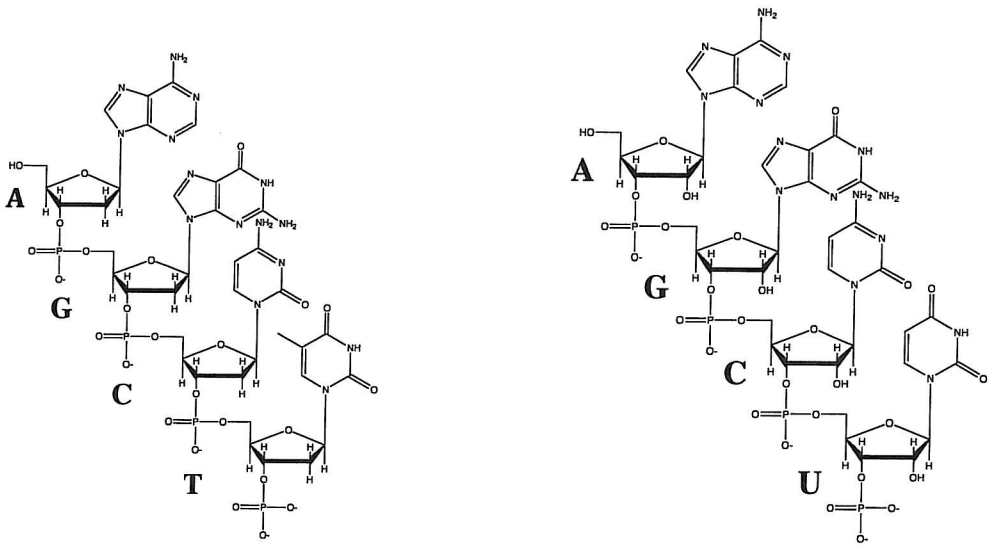


Figure 1 Shows the structure of DNA (left) and the structure of RNA (right).

The secondary structure of DNA is double helical with the phosphate groups on the outside. In the secondary structure there are grooves formed called the minor and major groove.

1.2 Cisplatin

Cisplatin is used to treat cancer

Cancer occurs when the cells in the body divide without any control. Eventually this uncontrolled cell division leads to a tumour. Sometimes the tumours will spread over the body and this is called metastasis. The criteria for cancer is that the cell growth is uncontrolled and that the cancer does not respond to body signals to arrest growth.

To understand the development of uncontrolled cell growth one has to look on the DNA level. The DNA in our body is exposed to different substances everyday that can lead to DNA damage. The body has mechanisms that can repair this damage. But sometime there will be a mutation in our body that cannot be fixed by our cell. The normal function of the cell is inhibited and the uncontrolled cell growth can start. (www.socialstyrelsen.se 2011-06-16)

The chemotheraputic aim is to stop the cell from growing. There are several different drugs that can be used to treat cancer, among these platinum based drugs. One of these is cisplatin (*cis*-dichloridodiamminplatinum(II)) (Figure 2). This drug is routinely used for the treatment of testicular cancer. Other platinum based drugs such as oxaliplatin and carboplatin are used for the treatment of colorectal cancer (Jung *et al* 2009).

The therapeutic use of cisplatin was discovered by Barnett Rosenberg approximetly 40 years ago. Cisplatins main intracellular target is to bind DNA.

Figure 2 shows the structure of cisplatin and oxaliplatin

Activation of Cisplatin,

In order for cisplatin to work as a therapeutic drug it has to be activated. (Kelland *et al* 2007). The mechanism behind the activation of cisplatin is aquation of one or two chlorides ions in the molecule. When cisplatin enters the body it keeps a stable configuration because of the high chloride concentration in the blood (100 mM).

Inside the cell the concentration of chlorides is lower (5 mM). The two chloride ions leave, and are replaced by two water ligands (Kelland *et al* 2007). Cisplatin binds covalently to the seventh position of guanine (G-N7) and forms an intrastrand crosslink with two adjacent guanines (Poklar *et al* 1996). Intrastrand means that the cisplatin binds to the same strand. The most common intrastrand crosslink is between guanine and guanine (60 - 65%) but it could also occur between adenine and guanine (20 - 25%) (Kelland *et al* 2007).

When adducts have been formed on the DNA they cause distortion of the DNA. The two adjacent guanine bases unwind the duplex and bend it towards the major groove (Jung *et al* 2009).

The bent DNA is recognized by a group of proteins called high mobility group proteins (HMGP). The proteins bind to the bent DNA and prevent the excision repair machinery (Kanika *et al* 2002).

RNA and proteins

Once the cisplatin enters the cell it will react not only with DNA, which is the main target, but it will also react with protein, RNA and other cellular components (Jung *al* 2009).

In this work I have studied the effect of cisplatin binding to two oligonucleotides sequence with either a central 1,2 GG binding site or a 1,3-GxG (x= U or T) binding site. See Table 1 and 2 for complete sequences

Table 1 shows the DNA that was used in this experiment. The DNA was obtained from IBA nucleic acid synthesis.

Name	**Sequnce 5'-3'**	**E (cm^2/µmol)**	**MW**
dGG22a	TCT CCT TCT TGG TTC TCT TCT C	167,94	6560.3
dGG22b	GAA GAG AAC CAA GAA GGA GAG A	167,94	6908.6
dGTG22a	TCT CCT TCT TGT GTC TCT TCT C	262,44	6560.3
dGTG22b	GAA GAG ACA CAA GAA GGA GAG A	262,44	6908.6

Table 2 shows the RNA that was used in this experiment. The RNA was obtained from IBA nucleic acid synthesis.

Name	Sequnce 5'-3'	E ($cm^2/\mu mol$)	MW
rGG22a	UCU CCU UCU UGG UUC UCU UCU C	190.7	6743.94
rGG22b	GAA GAG AAC CAA GAA GGA GAG A	190.7	7260.6
rGUG22a	UCU CCU UCU UGU GUC UCU UCU C	249.5	6743.94
rGUG22b	GAA GAG ACA CAA GAA GGA GAG A	249.5	7260.6

1.3 Melting studies

The structure of DNA and RNA are relatively stable under physiological conditions. However the structure cannot be too stable because that would inhibit the biochemical processes that occur in the cell. For example during transcription and replication the two strands in DNA have to separate and that is called unwinding. As mentioned before the residues on the DNA carry a negatively charge on the phosphate group. High ionic strength tends to stabilize the double helix (Biochemistry, 3rd edition Mathews)

The random coil structure has higher entropy than the double-helix form of the DNA. This is because the number of different configurations increases when the DNA has a coil structure. When the entropy changes, the free energy (ΔG) also changes (eq 1) (Biochemistry, 3rd edition Mathews).

$$\Delta G=\Delta H-T\Delta S \qquad \text{(eq 1)}$$

When the temperature increases above a certain degree the ΔG will be negative. That is denaturation and unwinding is favoured.

The bases absorb light at 260 nm. When the nucleotides in the DNA are in the helical form, the absorbance of light is reduced. When the temperature increases the absorbance will also increase due to configurational change of the DNA (Biochemistry, 3rd edition Mathews).

The melting temperature (T_m) denotes the temperature when half of the DNA population is found as random coild. The melting temperature depends on (G-C)/(A-

T) ratio. This is because there are three hydrogen bonds between G –C and only two between A-T. Hence the melting temperature is higher the higher the ratio G-C/A-T is.

When platinum based compounds like cisplatin are added to DNA the thermal stability of the DNA will change (Poklar *et al* 1996). The intrastrand crosslink that is formed when cisplatin is bound to DNA lowers the enthalpy and destabilizes the DNA (Poklar *et al* 1996).

1.4 HPLC

HPLC stands for high performance liquid chromatography. In this study the DNA/RNA oligonucleotide were first enzymatic digested into nucleosides and then dephosphorylated. A reverse phase C18 column was used to separate the resulting nucleosides.

A reverse column comprises a stationary non-polar phase, and a polar mobile phase.

Nuclease P_1 was used to digest the DNA/RNA This is a nuclease, which cleaves every nucleotide yielding 5´ monopohosphate.

Dephosphorylation is a process that eliminates phosphate groups, for this purpose intestinal alkaline phosphate (Sigma) was used, in the present study.

2 Methods

Buffer and solutions that was used in this study were the following,

For melting studies and gel preparation

3.0 M NaCl applied from Duchefa Biochemi

3.0 M $MgCl_2$ applied from Sigma Aldrich

500 mM MOPS applied from Sigma Aldrich

1.5 M NaOAc applied from Merck

10% APS applied from Sigma Aldrich

TEMED applied from Scharlon

250 mM MOPS and 250 mM NaOAc

2.1 Denaturating polyacrylamide gel electrophoresis

The gel preparation was made by mixing 110 ml 20% PAA gel/8 M Urea, 500 µl TEMED and 50 µl APS. The buffer used for the gel preparation was 1×TBE buffer.

Table 3 Solutions that used for denaturation polyacrylamide gel electrophoresis.

Urea	48 g
50%PAA/BizAA (48:2)	40 ml
1× TBE	20 ml
dH_2O 100 ml	
APS	500 µl
Temed.	50 µl

The voltage was set to 15 mA and the gel was run for 5 hours. When the bromophenol blue had migrated out of the gel, the glass was removed and the gel was covered by plastic film. The DNA was visualized by UV-shadowing and excised. The gel pieces were put in an Eppendorf tube and 400 µl NaOAc was added to each tube to allow the DNA be eluted over night at +4 °C.

The DNA was precipitated by mixing the 400 µl DNA+ NaOAc with 0.8 µl glycogen and 1 ml cold 100% ethanol. The tubes were centrifuged for 40 minutes at 13000 rpm. The supernatant was removed and 70 µl 70% cold ethanol was added to the pellet and centrifuged for another 15 minutes. The supernatant was removed and the DNA was air-dried. The pellet was resuspended in 50 µl water and the absorbance was measured by a NanoDrop spectrophotometer.

For all melting studies the concentration of the DNA was calculated using Lambeert Beers law (A= I×E×C). The absorbance measured by the NanoDrop spectrophotometer and the extinction coefficient was supplied by the manufactory (IBA, Biotechnology, Göthingen Germeny). The precipitated platinated DNA was used in thermal melting studies.

The four samples, 0.5 μM of each DNA strand, were mixed with 50 mM MOPS/NaOAc at pH 5.2 and 400 mM NaCl in a total volume of 800 μl.

The experiment was repeated for RNA oligonucleotides.

2.2 UV-spectrophotometer measurements

To evaluate the effect of salt concentration on thermal stability of the DNA duplex, three different salt concentrations were tested. In all samples the DNA concentration was 0.5 μM of each strand (GG22a with the complementary strand GG22b or GTG22a and GTG22b)

To sample 1: the buffer 50 mM MOPS/NaOAc was used.

To sample 2: the buffer was supplemented with in 400 mM NaCl

To sample 3: the buffer was supplemented with in 40 mM $MgCl_2$

The result is presented in (Figure 3). For further measurements 400 mM NaCl was used.

To be able to study the effect of cisplatin binding on the DNA duplex, GG22a or GTG22a was preplatinated and purified by denaturation gel electrophoresis. A two times excess of cisplatin to oligonucleotides was mixed in a 40 mM MOPS/NaOAc at pH 5.2.

For the RNA studies, the same methods were used. The RNA oligonucleotides are presented in Table 2.

2.3 HPLC studies

HPLC measurements where preformed to determine platinum adducts on RNA and DNA. As a first experiment plasmid DNA (pUC18) and tRNA, was incubated with cisplatin over night, ethanol precipitated, followed by digestion by P_1 nucelase. Finally the samples were treated with CiAP (Calf intestinal phosphate,).

For the measurements, six samples were made for both DNA and RNA. The first step was to platinate the DNA/RNA. The six samples had different ratio between the DNA and cisplatin (see Table 4). The concentration of the DNA was 3.0 mM, RNA 30 mM and cisplatin 1.5 mM. The table shows the ratio between DNA/RNA and Pt.

Table 4 shows the ratio between cis-platin and DNA/RNA

Experimnet #	DNA/RNA:Pt
1	10 : 1
2	5 : 1
3	1 : 1
4	1 : 5
5	1 : 10

Table 5 The following solution was made to platinate the DNA.

Sample	DNA (3 mM)	cisplatin(1,5 mM)	NaOAc (0.1 mM)	H_2O (µl)
1	12	2.4	38	327.2
2	12	4.8	38	325.2
3	12	24	38	306
4	12	120	38	210
5	12	240	38	90

The five samples were incubated at room temperature; whereafter 20 µl 3 M NaCl was added. The samples were precipitated by ethanol and the pellet dissolved in 30 µl water. The absorbance at 260 nm was measured by using Nanodrop spectrophotometer.

Table 6 shows the absorbance for each sample before the addition of the nuclease

Sample	Absorbance (260 nm)
1	11.527
2	6.375
3	5.416
4	5.102
5	4.258

The absorbance was used to calculate the concentration of the DNA in the sample.

The following concentrations of DNA were used for the P_1 nuclease addition. Sample six was used as control and no cisplatin was added to this sample. See Table 7.

Table 7 shows the calculated concentration,

Sample	Concentration
1	1.8 mM
2	1 mM
3	0.9 mM
4	0.8 mM
5	0.7 mM
6	3 mM

For the P_1 nuclease experiment the following solutions were made: The DNA/RNA was mixed with $ZnCl_2$ in 100 mM NaOAc, 100 mM NaOAc and water to an final volume of 100 µl. The solution was left shaking at (37°C) over night. For the dephosporylation assay, 15 µl 10x buffer CiAP(100 mM triss HCl pH 7.5, 100 mM $MgCl_2$) 5 µl enzyme and dH_2O were added to a final volume of 150 µl in an Eppendorph tube. The solutions were incubate at 37 °C for 2 h. The same methods were used for the RNA measurements. However a ratio of 1:10 for DNA: cisplatin and 1:8 for RNA: cisplatin was used. Table 8 shows the scheme for HPCL-gradient that was used to separate the nucleosides in the solutions.

The DNA/RNA oligonucleotides were first preplatinated and purified by denaturation polyacrylamide gel electrophoresis. Then the samples were treated as previously described.

Table 8 Scheme for the HPLC-gradient used to separate the nucleotides obtained after dephosphorylation.

Time(min)	40mM NH_4OAc	40%MeCn H_2O	MeOH	H_2O	Flow(ml/min)
0	95	5	0	0	1
5	95	5	0	0	1
35	60	40	0	0	1
40	0	100	0	0	1
45	0	0	0	100	1
50	0	0	0	100	1
55	95	5	0	0	1
60	95	55	0	0	1

3 Results

3.1 UV – spectrophotometer measurements

Several melting studies were preformed, first the effect of cations were evaluated. Figure 3 shows the result of this experiment.

The sample with no salt (control) showed a melting temperature (T_m) of 53 °C, the sample supplemented with 400 mM NaCl had a melting temperature (T_m) of 67 °C and the sample supplented with 40 mM $MgCl_2$ a melting temperature (T_m) of 65 °C.

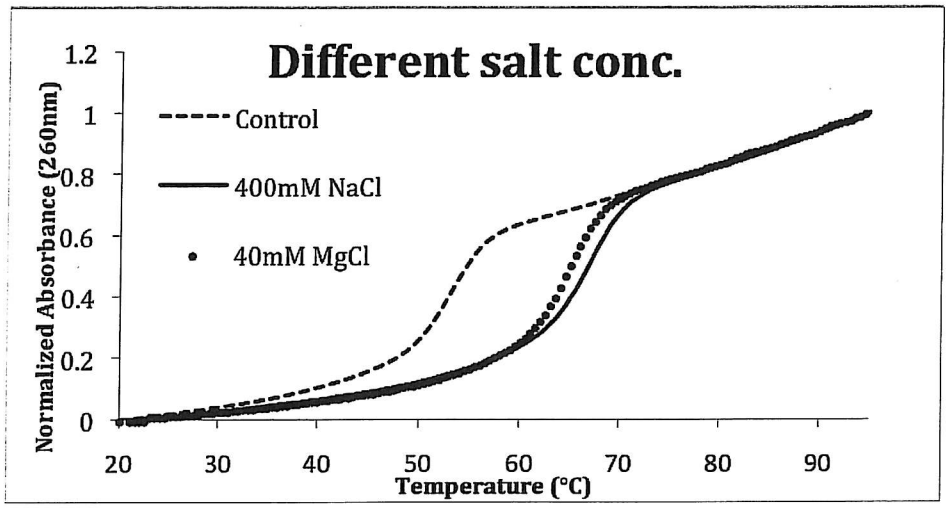


Figure 3 shows how salt concentration influences the melting temperature for DNA. The T_m value for sample 1(control) was found to be 53 °C, the T_m value with 400 mM NaCl was 67 °C and the T_m value with 40 mM $MgCl_2$ was 65 °C. The data was obtained on 0.5 µM DNA in 50 mM NaOAc/MOPS pH at 5.2.

Effect of Pt- adducts on thermal stability

Figure 4 shows the results obtained from the thermal stability of GG22-control (GG-c) and GG22-cisplatin(GG-Pt) DNA. The two curves have been normalized to range of 0 - 1. The platinated DNA (GG-Pt) has a lower melting temperature than the unplatinated DNA (GG-c). The difference is about 10 °C.

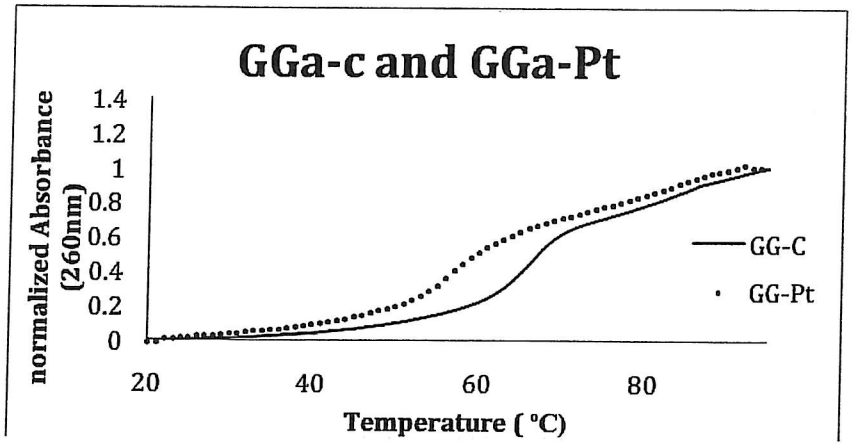


Figure 4 shows the melting curves obtained from the UV-absorbance measurements. The solid curve corresponds to the unplatinated DNA (GG-c) the dotted curve corresponds to the platinated DNA (GG-Pt). The T_m value for GG-c is 67 °C and the T_m value for GG-Pt is 56 °C. The data was obtained using 0.5 μM DNA in 50 mM NaOAc/MOPS pH at 5.2 the buffer supplemented with 400 mM NaCl and a temperature increase of 0.5 °C /min.

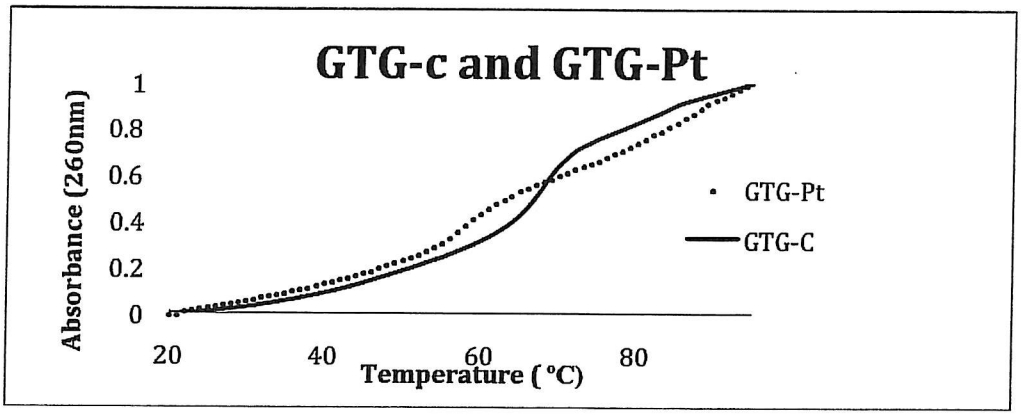


Figure 5 shows the melting curves obtained from the UV-absorbance measurements. The solid curve corresponds to the unplatinated DNA (GTG-C) and the dotted curve corresponds to the platinated DNA (GTG-Pt). The T_m value for GTG-c is 68 °C and the T_m value for GTG-Pt is 58 °C. The data was obtained using 0.5 μM DNA in 50 mM NaOAc/MOPS pH at 5.2 the buffer supplemented with 400 mM NaCl and a temperature increase of 0.5 °C /min.

The experiment with platinated GG22a was repeated and the results are presented in figure 6. In this graph, two peaks are seen from the first derivate. One peak is at approximately 56 °C, and the other peak at 66 °C. This observation suggests the presence of several species in the samples.

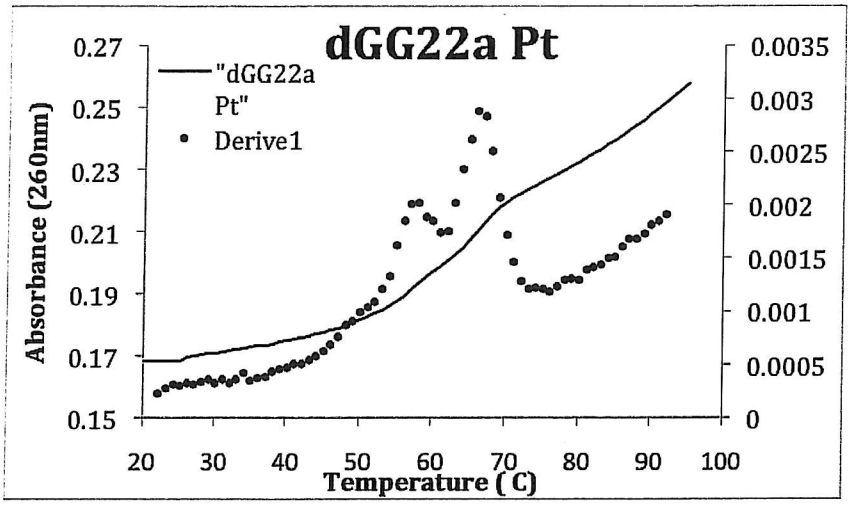


Figure 6 shows the graph obtained from melting studies of platinated dGG22. The dotted curve corresponds to the first derivate of the solid curve (Absorbance vs. temperature). The data was obtained using 0.5 μM DNA in 50 mM NaOAc/MOPS pH at 5.2 the buffer supplemeted with 400 mM NaCl and a temperature increase of 0.5 ° C /min.

The results from melting measurements of rGG22 and rGG22-Pt showed that the melting temperature T_m was the same for both rGG22 and rGG22-Pt. Figure 7 shows the graph that was obtained from melting studies with rGG22. The solid curve corresponds to the melting curve and the dotted curve corresponds to the first derivate from the same melting curve.

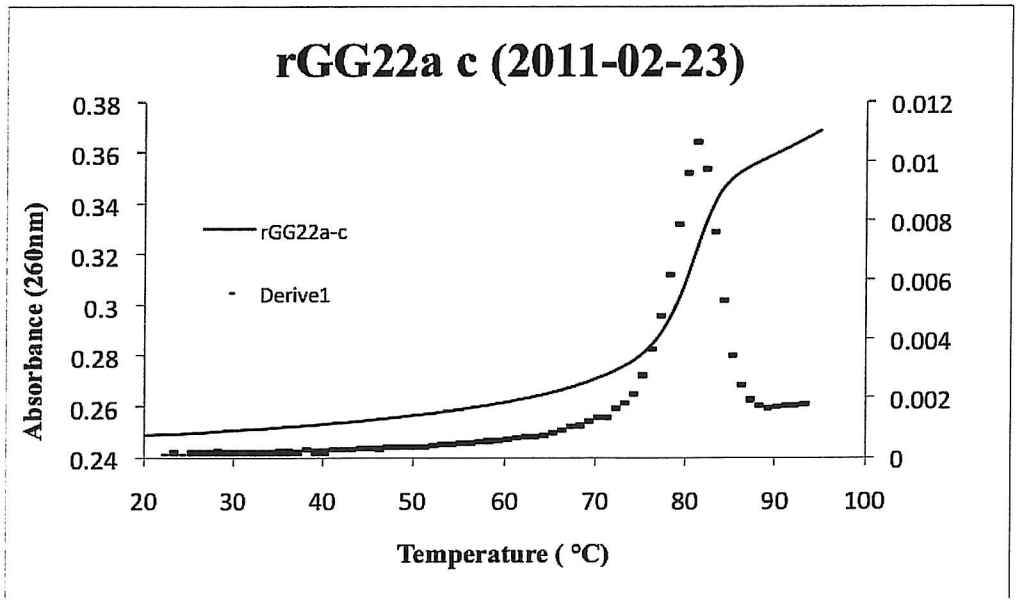


Figure 7 shows the melting curve for rGG22a. The solid line is the melting curve, and the dotted corresponds to the first derivate. The data was obtained using 0.5 μM DNA in 50 mM MOPS/NaOAc pH at 5.2 the buffer supplemented with 400 mM NaCl and a temperature increase of 0.5 °C /min.

The melting temperature for all thermal melting studies of DNA strands and RNA strands is presented in appendix B.

3.2 HPLC studies

The calculated concentration for the DNA samples is presented in Table 9. The concentration was used to calculate the amount of DNA to be used for the P1 nuclease assays

Table 10 Calculated concentration of the DNA used

Sample	Absorbance (260 nm)	Concentration (mM)
1	11.527	1.8 mM
2	6.375	1 mM
3	5.416	0.9 mM
4	5.102	0.8 mM
5	4.258	0.7 mM
6		3 mM

The retention time for each eluted peak is presented in table 10. To correlate the different peaks in the chromatogram, nucleoside standards were used as a reference. The first peak that was eluted corresponds to the base cytosine, the second peak corresponds to guanine, the third peak thymine and the forth peak adenine. In the RNA samples the base uracil eluted after cytosine but before guanine

The chromatograms were obtained from HPLC measurements with a reverse column. Figure 8, shows an example of a chromatogram. This chromatogram corresponds to the DNA control.

The other chromatograms obtained from HPLC is presented in Appendix B. No clear effect of the added cisplatin could be seen, except for tRNA reduction. Where the relative peak hight/area of G was reduced compare to the other bases.

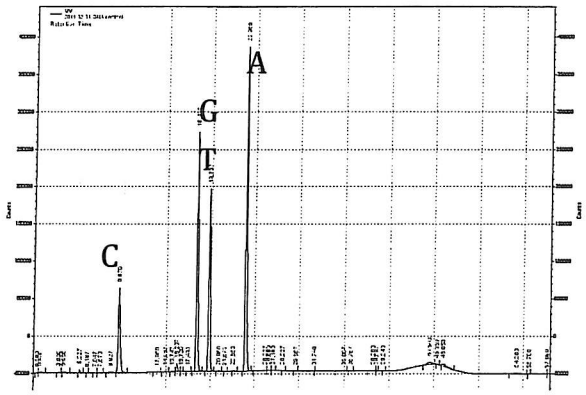


Figure 8: shows the control DNA (PUC18) without any cis-platin added after digestion with P1 nuclease. The retention time of the eluted peaks are from left to right; Cytosine 9.67; Guanine 18.44; Thymine 19.80 and Adenine 23.96

Table 11 corresponds to the eluted peaks for the different bases DNA and RNA

DNA: Pt	C	U	G	T	A
10:1	9.86		18.44	19.80	23.96
5:1	9.68		18.42	18.80	23.96
1:1	9.69		18.44	19.89	23.98
1:5	9.74		18.46	19.89	24
1:10	9.69		18.44	19.89	23.98
Control	9.67		18.42	19.79	23.96
RNA:Pt					
10:1	7.10	9.70	16.73		22.65
5:1	6.99	9.66	16.70		22.64
1:1	6.99	9.68	16.69		22.62
1:5	7.01	9.68	16.69		22.63
1:10	6.98	9.64	16.67		22.61
Control	6.98	9.647	16.69		22.623

The result from the HPLC measurements with the oligonucleotides dGG, dGG cisplatin, dGTG control, dGTG cisplatin and corresponded RNA nucleotides is present in table 11. The dGG control and dGG cisplatin, dGTG control and dGTG cisplatin, no clear different could be seen in the chromatogram.

Figure 9 shows the chromatograph with rGG22a and rGG22a cisplatin.

There is a new peak that is shown in the chromatogram rGG-cisplatin that has a retention time 14.42 and a peak area 57190 a.u. This peak corresponds to the GpG adduct.

From the chromatogram with the rGG control the peak at 19.92 has a peak area 208133 a.u this peak corresponds to guanine. The corresponded peak in the chromatogram with rGG cisplatin is smaller and it also eluted later. The retention time for this peak is 20.023 a.u and the peak area is 73291 a.u.

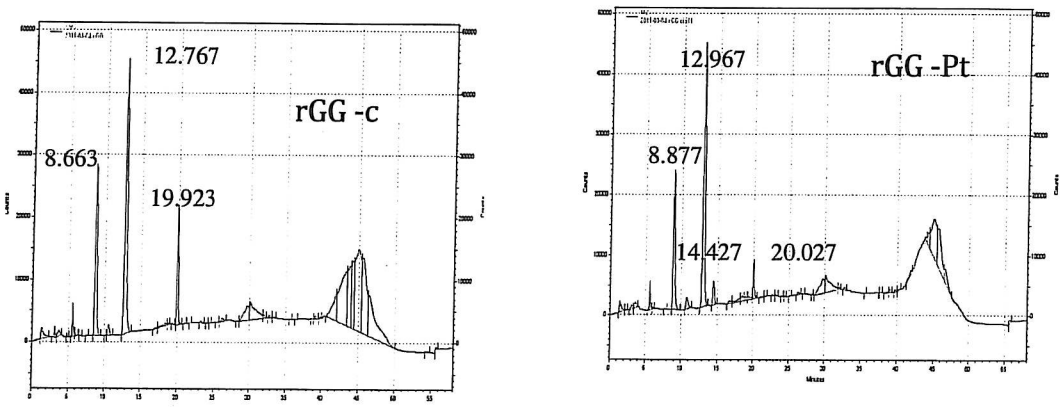


Figure 9 chromatogram that is obtained from HPLC measurements with a reverse phase column C18. The chromatogram to the left is the rGG control and the right is the rGG cisplatin. The retention time for each peak for the rGG control (left to right) is 8.66 (cytosine), 12.77 (uracil), and 19.92 (Guanine). The retention time for each peak for rGG cisplatin (left to right) is 8.89 (cytosine), 12.97 (uracil), 14.43 (GpG-Pt) and 20.03 (guanine).

There is a new peak that is shown in the chromatogram rGUG-cisplatin that has a retention time 21.96 and a peak area 85185 a.u. This peak corresponds to the GpUpG adduct.

From the chromatogram with the rGUG control the peak at 20.13 which has a peak area 2079827 a.u this peak corresponds to guanine. The corresponded peak in the chromatogram with rGUG cisplatin is smaller and it also eluted later. The retention time for this peak is 20.15 and the peak area is 197230 a.u.

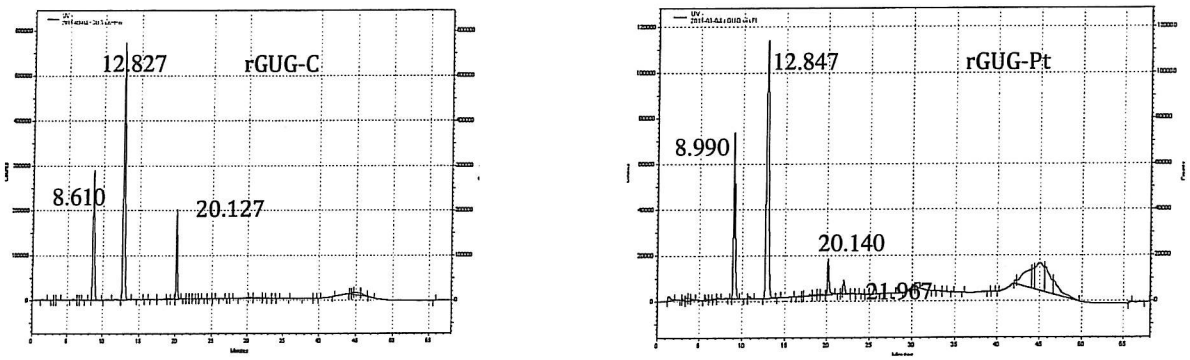


Figure 10 Chromatogram that is obtained from HPLC measurements with a reverse phase column C18. The chromatogram to the left is the rGUG control and the right is the rGUG cisplatin. The retention time for each peak for the rGUG control (left to right) is 8.61 (cytosine), 12.83 (uracil) and 20.13 (guanine). The retention time for each peak for rGUG cisplatin (left to right) is 8.99 (cytosine), 12.85 (uracil) and 20.14 (guanine) and 21.97 (GpUpG-Pt).

4 Discussion

Thermal melting studies.

The results from the thermal studies is presented below, the expected result is that the platinated DNA should have a lower melting temperature than the control due to the configurational change of the DNA.

In this study the melting temperature of DNA and platinated DNA has been investigated. The results from the thermal melting studies with different salt and concentration showed that salt increases the melting temperature of the DNA. The divalent Mg^{2+} ion was found to be more effective than the monovalent Na^{+} ion since ten times higher concentration of NaCl (aq) is needed to reach about the same melting point as $MgCl_2$ (aq). A higher ionic strength moves the melting temperature to a higher temperature. The reason why we chose NaCl (aq) in further studies is that $MgCl_2$ (aq) catalyzes intermolecular cleavage especially of RNA. The concentration also plays a major role in thermal studies; the higher concentration of the DNA gives higher melting point. See appendix B. The other experiment with platinated DNA showed that cisplatin lowered the melting temperature with almost 10 °C for both dGG22 and GTG22. The reason for a lower melting point is the configurational change of the DNA. However when the experiment was repeated, the platinated DNA showed the same melting temperature as the control. This could be due the fact that DNA had not been platinated. Figure 4 shows that the samples are not purified, since two peaks are shown. The

peak at 56° C indicate that the samples has been platinated. The second peak at 66 ° C indicates that maybe the DNA has been platinated. On the gel the GG22a-control and GG22a-Pt migrated differentely on the gel, the platianted migrated about 0.5 cm shorter than the control. This observation indicates that the samples have been platinated. The thermal melting studies with platinated RNA demonstrated a higher melting temperature than the corresponding platinated DNA. However there was no difference between T_m values for the control and the platinated RNA, respectively.

A large excess of Pt a new peak appears and the relative intensity of the G peak is reduced, this effect is most pronounced in the case of RNA.

For the HPLC measurement the retention time was the same for the platianted and unplatinated in both tRNA and PUC18 that indicates that the adducts have not been separated.

For the HPLC measurements with the DNA oligonucleotides no clear differences could been seen for the platinated and unplatinated samples. In contrast, the RNA oligonucleotides displays a visible different between rGG22a-C and rGG22a-Pt. From the rGG22a-C and rGG22a-Pt chromatogram (Figure 9) new peak can be seen in the rGG22a-Pt, which corresponds to the GpG-Pt adduct. Hence, the peak that corresponds to guanine in rGG22a-Pt is smaller compared to the same peak in rGG22a chromatogram. A similar observation was also made for the rGUG and rGUG-Pt oligonucleotides.

Abbreviations

APS	Adenosine 5′-phosphosulfate sodium salt
C1AP	Calf intestinal phosphatase, sigma
DNA	Deoxyribonucleic acid
$MgCl_2$	Magnesium chloride
MOPS	3-N Morpholino propanesulfonic acid
NaCl	Sodium chloride
NaOAc	Sodium acetate
PAA	Polyacrylamide
Pt	Platinum
RNA	Ribonucleic acid
TBE	Tris borate EDTA buffer
TEMED	Tetramethylethylenediamine

Appendix A

Table A.1 shows the solutions that were used during investigation of the melting temperature of the DNA.

	1	2	3
GG22a	0.5 μM	0.5 μM	0.5 μM
GG22b	0.5 μM	0.5 μM	0.5 μM
Buffer	50 mM	50 mM	50 mM
NaCl		400 mM	
$MgCl_2$			40 mM

The table below shows the experimental conditions used during platination of the DNA samples before nucelase P1 digestion. The experiments were performed in buffered solutions (0.10 mM NaOAc)

Table A.2 following solutions was made to paltinate the DNA samples before nucelase P1 digestion.

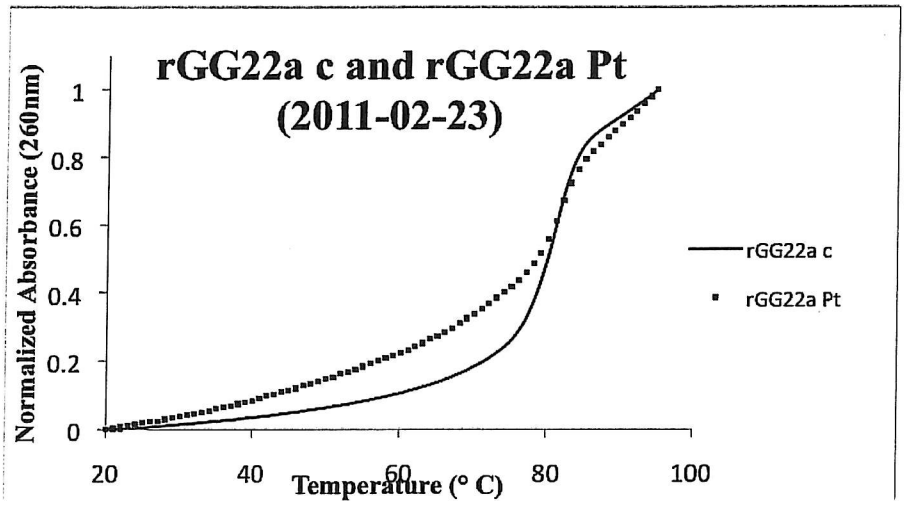
Sample	**DNA(3 mM)**	**Cisplatin (μl)**	**Buffer (μl)**	**dH_2O (μl)**
1	12	2.4	38	327.2
2	12	4.8	38	325.2
3	12	24	38	306
4	12	120	38	210
5	12	240	38	90

Table A.2 shows the solutions for P_1 nuclease. Water was added to a final volume of 100 μl.

Sampe	**DNA (mM)**	**NaOAc**	**$ZnCl_2$**	**P_1enzyme (μl)**
1	1.8	33 mM	3 mM	3
2	1	33 mM	3 mM	3
3	0.9	33 mM	3 mM	3
4	0.8	33 mM	3 mM	3
5	0.7	33 mM	3 mM	3
6	3	33 mM	3 mM	3

APPENDIX B

The graph shows the melting temperature for the other thermal melting temperatures that was made during this experiment. The table B.1 shows the melting temperature for all oligonucleotides that are obtained from thermal melting studies.



Figur B.1 shows the melting curves obtained from the UV-absorbance measurements. The black curve corresponds to the unplatinated RNA (GG-c) and the dotted curve corresponds to the platinated RNA (GG-Pt). The data was obtained using 0.5 μM DNA in 50 mM NaOAc/MOPS pH at 5.2 the buffer supplemeted with 400 mM NaCl and a temperature increase of 0.5 ° C /min.

Tabell B.1 shows the *Tm* value for the oligostrands that was obtained from the thermal melting stdudies.

Oligonucleotides		T_m (°C)	**Date**	A_{260}
dGG22a	control	53	2011-01-21	0.48
dGG22a	NaCl	65		0.49
dGG22a	$MgCl_2$	67		0.47
dGG22a	control	70	2011-01-24	3.38
dGG22a	cisplatin	61		0.66
dGTG22a	control	73		4.54
dGTG22a	cisplatin	88		3.72

dGG22a	control	66	2011-01-25	0.28
dGG22a Pt	cisplatin	58		0.25
dGTG22a	control	57		0.34
dGTG22apt	cisplatin	87		0.31
dGG22a	control	66	2011-01-31	0.38
dGG22a Pt	cisplatin	56		0.30
dGTG22a	control	68		0.45
dGTG22apt	cisplatin	58		0.31
dGG22a	control	65	2011-02-21	0.20
dGG22a Pt	cisplatin	66		0.20
dGTG22a	control	66		0.19
dGTG22a-Pt	cisplatin	67		0.16
dGG22a	control	65	2011-02-22	0.13
dGG22a Pt	cisplatin	66		0.20
dGG22a pt	cisplatin	66		0.16
rGG22a	control	80	2011-01-23	0.24
rGG22a Pt	cisplatin	81		0.21
rGUG	control	82		0.35
rGUG Pt	cisplatin	81		0.16
rGG22a oxPt	oxali platin	75		0.25
dGG22a	control	65	2011-02-24	0.24
dGTG22a	cisplatin	68		0.36
rGG22a	control	82		0.37
rGUG22a	cisplatin	81		0.25

The following figure was obtained from the HPLC measurements with tRNA and PUC18

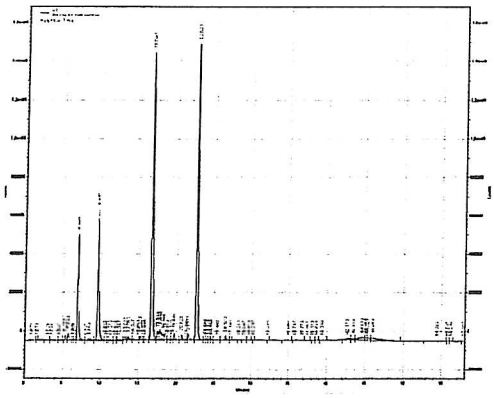
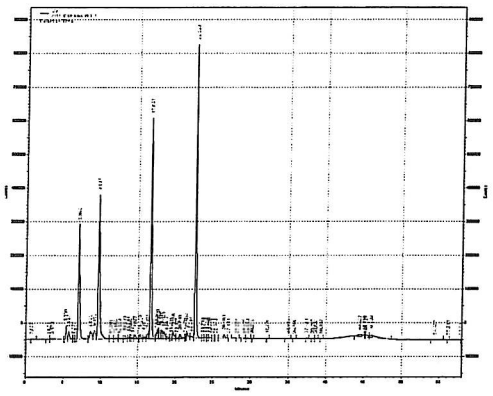
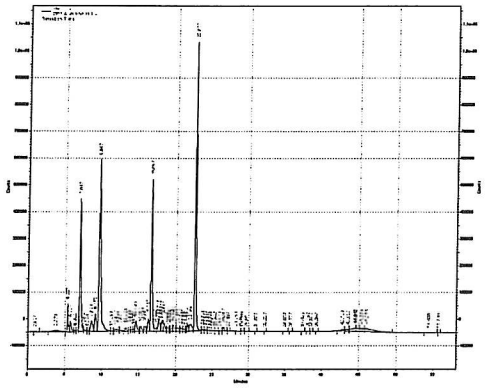


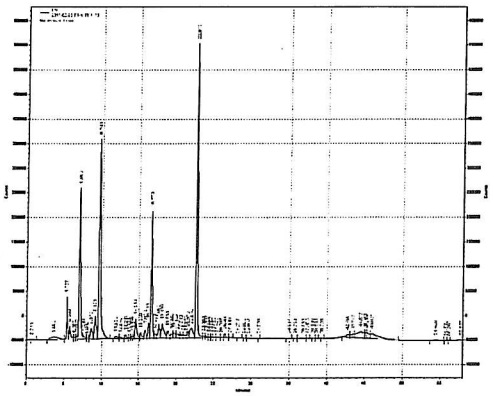
FIG1: RNA Control



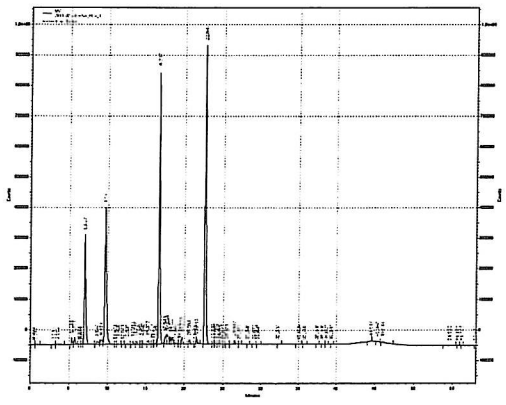
RNA:Pt 1_1



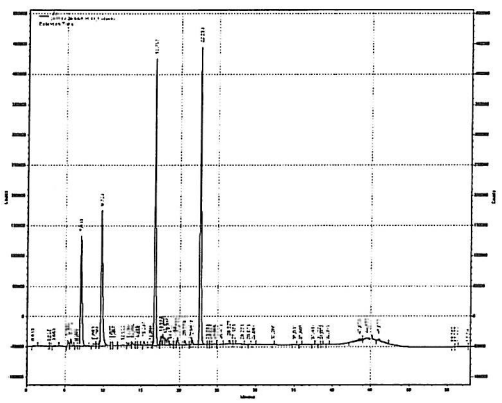
RNA :pt 1_5



RNA:pt 1_10

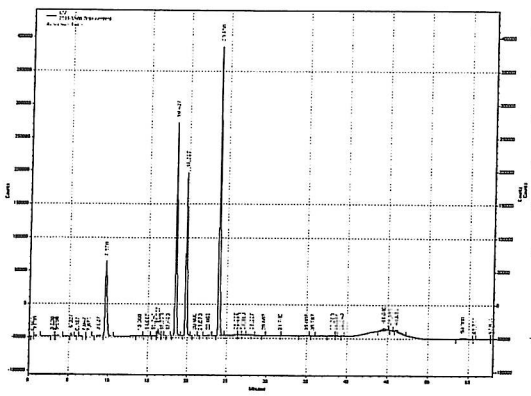


RNA :PT 5_1

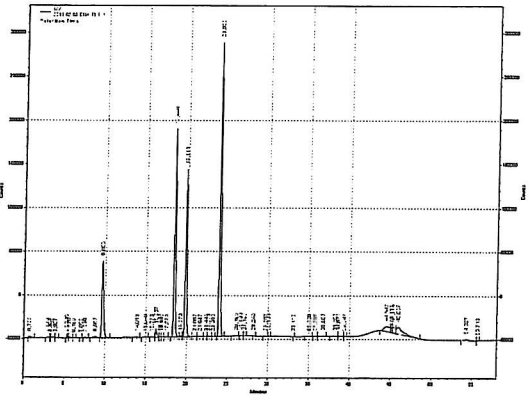


RNA Pt:10_1

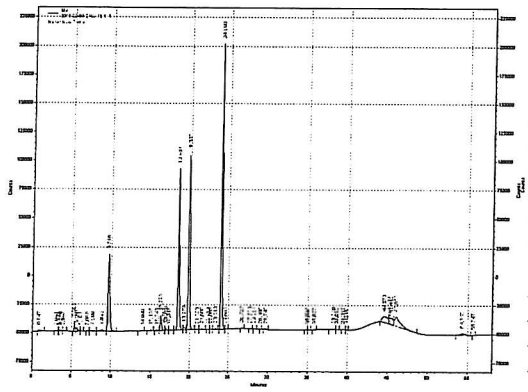
The following figure is obtained from HPLC measurements with the DNA/RNA oligo nucelosides.



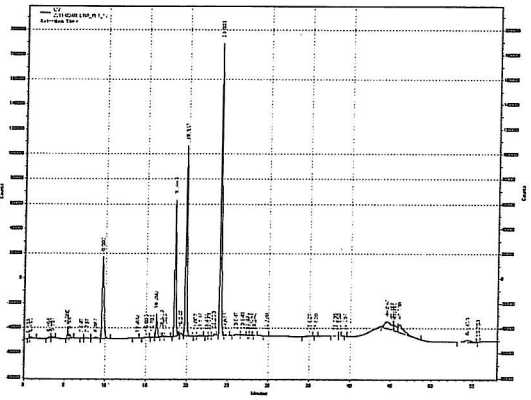
DNA:Control



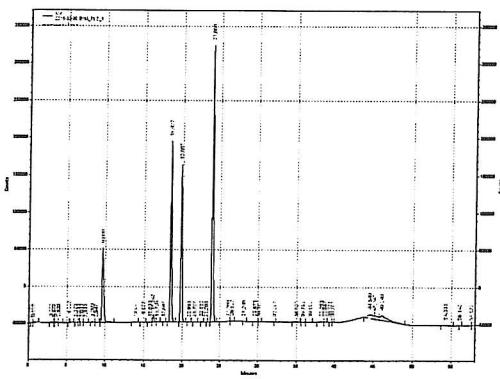
DNA:pt 1_1



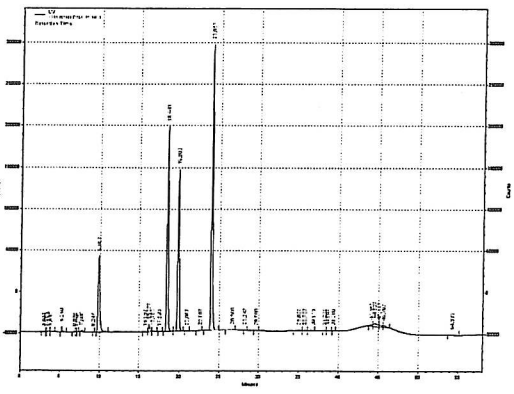
DNA:Pt 1_5



DNA:Pt 1_10

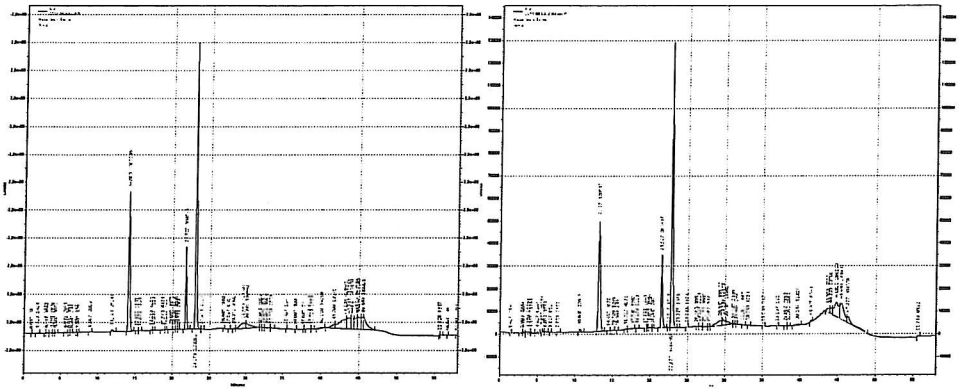


DNA:Pt 5_1



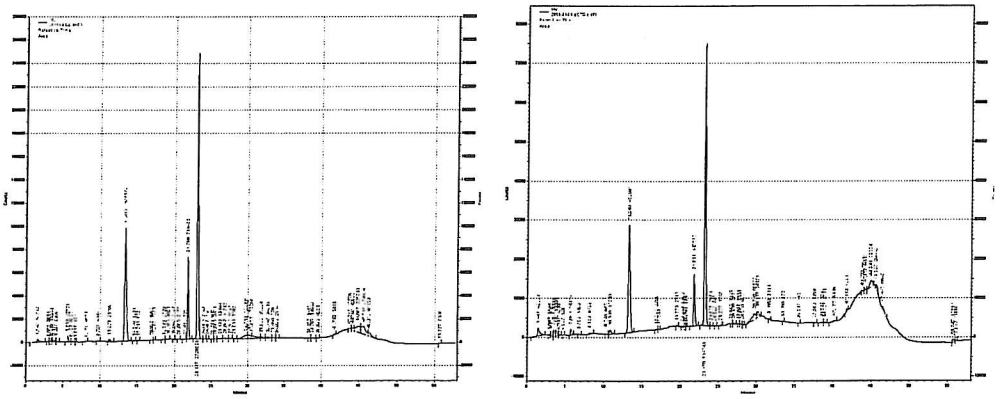
DNA:Pt 10_1

The following figure is obtained from HPLC measurements with the DNA/RNA oligo nucelosides.



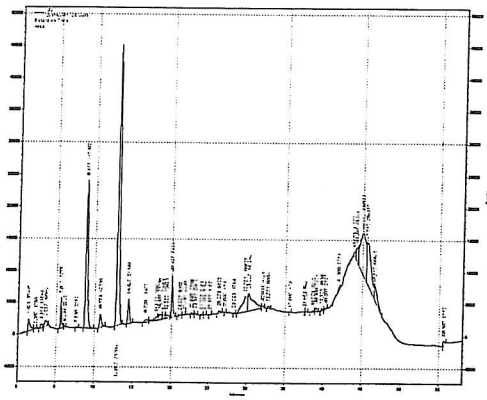
dGG22a control

dGG22a cisplatin

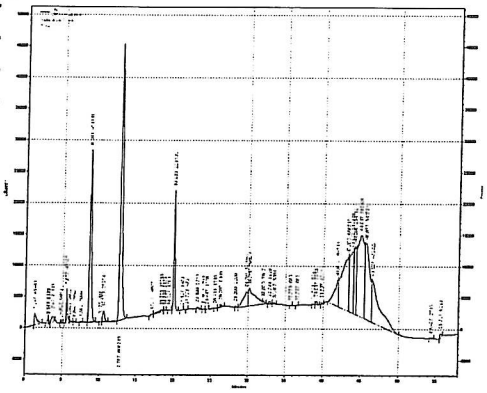


dGTG22a control

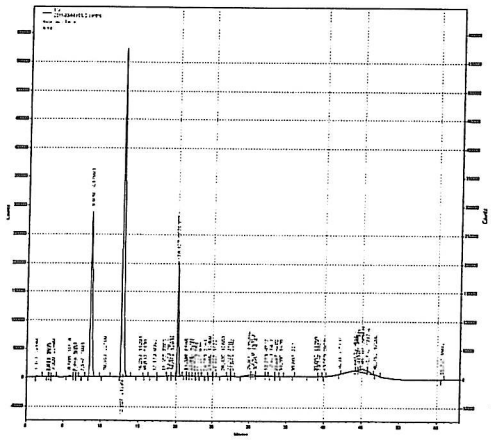
dGTG22a cisplatin



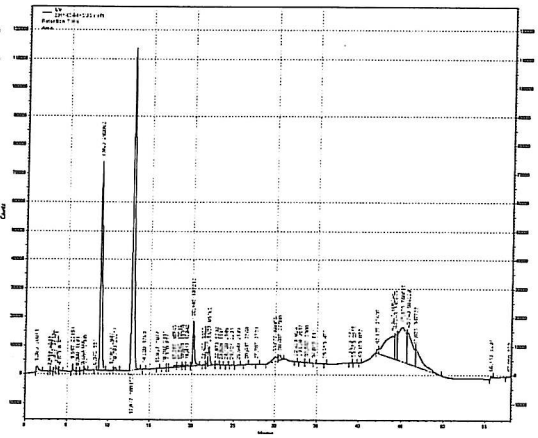
rGG22a control



rGG22a cisplatin



rGUG22a control



rGUG22a cisplatin

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