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# Platinum Interference with siRNA Non-seed Regions Fine-Tunes Silencing Capacity

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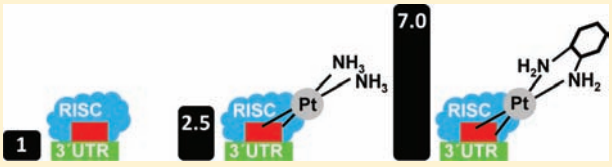
Supporting Information

**ABSTRACT:** Knowledge concerning the molecular mechanisms governing the influence of non-coding RNAs on protein production has emerged rapidly during the past decade. Today, two main research areas can be identified, one oriented toward the use of artificially introduced siRNAs for manipulation of gene expression, and the other one focused on the function of endogenous miRNAs. In both cases, the active molecule consists of a ∼20-nucleotide-long RNA duplex. In the siRNA case, improved systemic stability is of central interest for its further development toward clinical applications. With respect to miRNA processing and function, understanding its influence on mRNA targeting and the silencing ability of individual miRNAs, e.g., under pathological conditions, remains a scientific challenge. In the present study, a model system is presented where the influence of the two clinically used anticancer drugs, cisplatin and oxaliplatin, on siRNA's silencing capacity has been evaluated. More specifically, siRNAs targeting the 3′ UTR region of Wnt-5a mRNA (NM_003352) were constructed, and the biologically active antisense RNA strand was pre-platinated. Platinum adducts were detected and characterized by a combination of gel electrophoresis and MALDI-MS techniques, and the silencing capacity was evaluated in cellular luciferase-expressing systems using HB2 cells. Data show that platination of the antisense strand of the siRNAs results in adducts with protection against hydrolytic cleavage in the proximity of the platination sites, i.e., with altered degradation patterns compared to native RNAs. The MALDI-MS method was successfully used to further identify and characterize platinated RNA, with the naturally occurring platinum isotopic patterns serving as sensitive fingerprints for metalated sites. Expression assays all confirm biological activity of antisense-platinated siRNAs, here with platination sites located outside of the seed region. A significant reduction of silencing capacity was observed as a general trend, however. Of the two complexes studied, oxaliplatin exhibits the larger influence, thus indicating subtle differences between the abilities of cis- and oxaliplatin to interfere with si- and miRNA processing.




## INTRODUCTION

Non-coding RNAs are today recognized as important regulators of translation.[1,2] One of the better understood systems involves the processing and action of small RNAs by use of the RNA-induced silencing complex (RISC) and downstream interference with protein expression.[3−5] The regulatory function of RISC relies on the initial presence of a double-stranded non-coding RNA that, after incorporation of the antisense strand into the protein complex, allows recognition of the corresponding mRNA target site(s). The specificity of the target recognition process is primarily determined by the bases located at the 5′-end of the antisense strand with the bases 2−7 (or 8), i.e., the seed region, crucial for mRNA target selection.[6] Both endogenous and exogenous molecules take advantage of the processes initiated by the RISC complex formation.[7] In the case of endogenous molecules in mammals, e.g., non-coding micro-RNAs (miRNAs), the miRNA−mRNA interaction typically exhibits only partial base complementarity outside of the seed region. These non-stringent recognition requirements allow one miRNA to control protein production from several genes by repressing translation and promoting mRNA decay.[8−10] The tendency toward degradation of the mRNA can be modulated and often improved by increasing the complementarity between the antisense RNA and its target. The use of small interfering RNAs (siRNAs) for transient knock-down of protein production is an example where full-length complementarity is commonly used to optimize binding conditions and selectivity toward a single gene product.

During the past decade, substantial progress has been made toward an improved picture of how the RISC machinery can be used for both transient and long-term down-regulation of protein production.[11] Today, siRNAs are routinely used as biomolecular tools, and the technology holds good promise for therapeutic intervention, including both oral and particle vehicle formulations.[12−15] Currently, substantial research efforts are aimed at improved design of siRNA molecules with good systemic stability in body fluids. In the literature, a wide range of both phosphate backbone and sugar modifications are described as

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suitable.[16] The locked nucleic acids (LNAs)[17] and 2′-*O*-methyl-modified (O-Me) ribose units[18] are typical examples of well-functioning synthetic analogues that can be introduced to enhance siRNA efficacy. Ideally, the modified siRNAs should contain modifications preventing degradation yet allow for correct RISC loading and subsequent mRNA degradation.

In contrast to the well-established design criteria for use of, e.g., LNA and O-Me sugar modifications, knowledge related to effects arising after manipulation of the nucleobases is scarce.[16] An improved understanding of how base modifications may change RISC processing has an obvious relevance for development of novel types of siRNAs but—perhaps as importantly—should also contribute to clarify whether the RISC-induced cellular machinery is affected by exposure to drugs with nucleic acid affinity, e.g., platinum-based anticancer drugs.[19−21] Work in the Elmroth laboratory aimed at evaluating the latter types of effects was therefore initiated some years ago. In the initial studies conducted, our goal was to evaluate the effect of cisplatin and siRNAs on translational efficacy in a model system encompassing targeting of the 3′ UTR of Wnt-5a (NM_003392).[22−24] The Wnt-5a protein levels have a documented correlation with metastasizing ability in, e.g., malignant melanoma, breast cancer, and gastric cancers.[25] Thus, small-molecule intervention with protein expression here provides a tentative tool for manipulation of a disease-related phenotype.[26] In the initial study, we were able to observe that, first, both cisplatin and siRNAs were independently able to silence translation.[23] Second, the measured silencing ability after initial transfection of siRNA followed by subsequent cisplatin exposure was found to be additive. It was therefore concluded that cisplatin had a non-significant effect on direct siRNA-induced gene silencing under the experimental conditions used, with short-term evaluation on translational levels (48 h). In subsequent studies, the influence of sense-strand platinated siRNAs on silencing ability was investigated using cisplatin and oxaliplatin as metalation reagents.[22,24] The platinated siRNAs were found to be well tolerated by the RISC machinery and highly efficient, with silencing levels of 90% or better. Further, the silencing was found to be independent of the siRNA concentration, in the nanomolar range studied, with similar activity for native and platinated siRNAs. [22,24] Thus, neither the decrease in melting temperature of ∼10 °C caused by the platinum adduct nor the positioning of the platinum complex within the siRNA was found to affect silencing ability in these systems. These data are indicative of RISC being able to correctly identify platinated siRNA duplexes and provide efficient silencing by guidance of the non-platinated antisense strand with maintained efficacy. Together, these studies clearly show that metalation *per se* does not adversely affect biological processing of the siRNA prior to RISC loading of the active, non-metalated antisense strand.

In the present study we now, for the first time, turn our attention to the biolocially active antisense strand and its sensitivity to base modifications, with a focus on positions outside of the seed region. The assay described in earlier work was used to facilitate comparison of data between experiments. In the present study, we document the silencing capacity of three different cisplatin- or oxaliplatin-modified siRNAs (Chart 1).

In addition, the platinum binding sites were identified using PAGE methodology combined with a detailed MALDI-MS analysis of enzymatically digested platinated siRNAs. In a previous study, DeRose et al. highlighted the usefulness of MALDI-MS for documentation of platinum binding sites of partially digested RNA by use of average mass comparison of data output and expected cleavage fragments.[27] In our analysis, we now show direct evidence of platinum binding by identification of Pt-isotope patterns. The siRNAs studied contain at least one tentative soft-metal binding site (N7 of guanine, G-N7) located outside of the seed region and an unmodified phosphate backbone. The results obtained support our hypothesis that platinum-modified RNAs are functional molecules. However, a significant drug-induced reduction of silencing ability is observed, resulting from interactions outside of the seed region, an effect that here is most pronounced after interaction with oxaliplatin.

**Chart 1. Sequences of Studied siRNAs**[a]

**W-siRNA33**

sense: 5′-r(GGA CCC GCU UAU UUA UAG A)dTdT-3′
antisense: 3′-d(TT)r(CCU GGG CGA AUA AAU AUC U)-5′

**W-siRNA101**

sense: 5′-r(CCA AGA AUU GCA ACC GGA A)d(TT)-3′
antisense: 3′-d(TT)r(GGU UCU UAA CGU UGG CCU U)-5′

**W-siRNA147**

sense: 5′-r(GAA CUC UGU GGU UUA UUA U)d(TT)-3′
antisense: 3′-d(TT)r(CUU GAG ACA CCA AAU AAU A)-5′

[a]Observed platination sites are underlined, and the seed region is italic. The siRNAs were designed with full complementarity to their mRNA target sites, all located within the 3′ UTR of Wnt-5a mRNA (NM_003392).

## RESULTS

**Platinum Binding Site Determination by Gel Electrophoresis.** To determine the platinum binding sites, the single-stranded RNA antisense oligomers were radiolabeled and platinated with cisplatin or oxaliplatin ($C_{Pt}$:$C_{oligomer}$ = 5:1; see Materials and Methods for details). Platination of the RNA under these conditions typically resulted in formation of multiple adducts that could be separated using denaturing polyacrylamide gel electrophoresis (PAGE) (Figure S1). The content of the individual bands was analyzed further after excision, elution, and ethanol precipitation. Individual platination sites were determined after isolation of radio-labeled RNA products, followed by partial hydrolysis and analysis by PAGE. As can be seen in Figure 1, single-nucleotide resolution was obtained for fragments in the range from 3- to 19-mers, with good cleavage intensity at all guanine residues by RNase T1 (Figure 1, lane 2 of panels A−C), all in agreement with cleavage patterns expected for 21-mer siRNAs containing a 3′-d(TT) end. After platination, a pattern without cleavage after guanines was expected as a result of decreased flexibility around the phosphodiester linkage(s) surrounding the metal binding site(s).[28] Our experimental data are in agreement with this expectation, however with a clear difference between the platination products. For example, the fastest migrating band (Figure S1, P1) analyzed after exposure of W-siRNA33a to cisplatin results in cleavage protection of one single site, thus indicating G15 as the predominant platination site in this RNA (Figure 1A, lane 4). A similar cleavage pattern was obtained after exposure to oxaliplatin (Figure 1A, lane 5) and gives further support for G15 as the kinetically preferred interaction site. Analysis of the more slowly migrating bands (Figure S1, P2) shows that G12 serves as an additional metalation site for both metal complexes (Figure S2A, lanes 6 and 7).

The migration patterns caused by the presence of platinum adducts in fragments exceeding 13 nucleotides prevent detailed

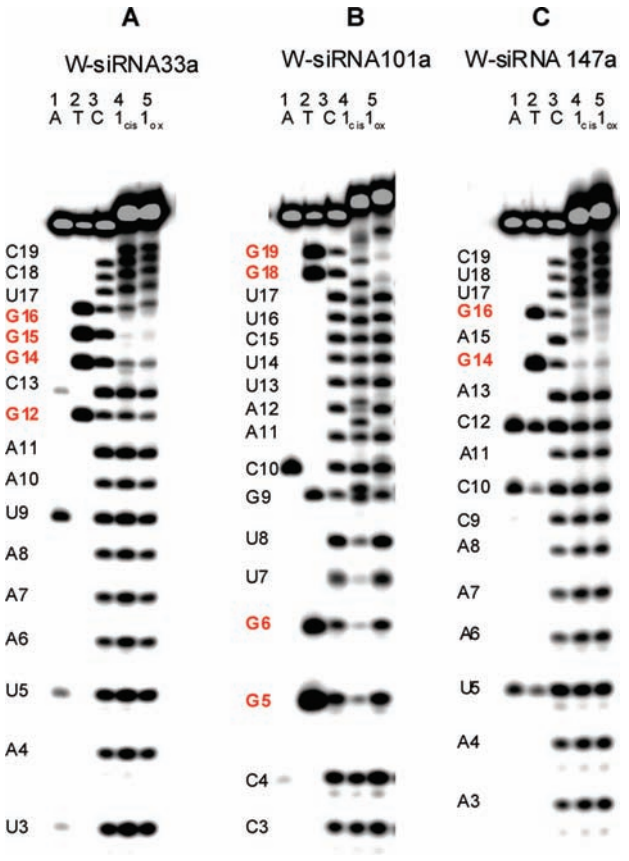


**Figure 1.** Representative cleavage products obtained after partial hydrolysis of native and platinated 5′-end-labeled RNA after separation on 20% denaturing PAGE: (A) W-siRNA33a, (B) W-siRNA101a, and (C) W-siRNA147a. From left to right in each panel, A−C: 1, partial RNase A digestion of native RNA; 2, partial RNase T1 digestion of native RNA; 3, alkaline hydrolysis of native RNA; 4, alkaline hydrolysis of cisplatin-platinated RNA with one Pt adduct; and 5, alkaline hydrolysis of oxaliplatin-platinated RNA with one Pt adduct.

interpretation of cleavage patterns for the longer fragments. In contrast, the good agreement between the cleavage patterns obtained for shorter fragments of both native and platinum-modified RNA, i.e., nucleotides U3−C13, indicates an unmodified W-siRNA33a 5′-end.

In the case of W-siRNA101a, tentative guanine binding sites are present at both the 5′- and 3′-ends, G5−G6 and G18−G19, respectively. A comparison of the results obtained for the fastest migrating bands reveals that cis- and oxaliplatin have different preferences with respect to initial binding sites. After exposure to oxaliplatin, platination primarily affects cleavage at G18 and G19 (Figure 1B, lane 5). In contrast, exposure to cisplatin (Figure 1B, lane 4) results in predominant loss of cleavage intensity at both G5 and G6, however also combined with reduction at G18 and G19. The apparent intensity loss at U7−U8 after cisplatin treatment is here likely due to the gel shift caused by the platinum adduct located at the 5′-end. It should be noted that, for siRNAs with more than one platinum adduct, both of these sites are subjected to loss of cleavage intensity indicating that both these sites are prone to platination provided the platinum compound is present in molar excess compared to the siRNA (Figure S2B, lanes 6 and 7). Our data thus imply that cis- and oxaliplatin have slightly different kinetic and/or thermodynamic preferences for the two GG-containing regions in W-siRNA101a, an effect that seems to dominate over contributions from electrostatic potential in the case of oxaliplatin.[29−31]

In a final experiment, the discriminating ability of cis- and oxaliplatin was investigated by analysis of the interaction pattern with W-siRNA147a. This RNA presents a single G-rich region with a GAG sequence, for which the cleavage intensity is significantly reduced after platinum exposure (Figure 1C, lanes 4 and 5). The cleavage pattern here suggests a GAG adduct with the two guanines as binding sites. Thus, we find a sequence preference with RNA in agreement with that reported for cisplatin on DNA;[32] this conclusion is also supported here by thermal melting studies, *vide infra*. In summary, the cleavage data show that the studied siRNAs form stable and specific adducts with both cis- and oxaliplatin. A preference for interaction with the guanine base is observed for both compounds, in agreement with previous studies by us and others.[27,33−36] Depending on the more extended sequence context, different binding preferences are observed for the two studied metal complexes, however.

**Platinum Binding Site Determination by MALDI-MS.** Mass spectrometry analyses were conducted on enzymatically digested platinated RNA oligomers to further characterize the platinum binding sites. Prior to analysis, the platinated and gel-purified oligonucleotides (Figure S1; products P1) were subjected to enzymatic digestion with RNase A, yielding selective digestion at the 3′-side of pyrimidines and leaving a 3′-phosphate end. A typical example of a MALDI-MS spectrum is shown in Figure 2A, where the spectrum of unplatinated, digested W-siRNA147a is presented. Four dominating peaks are identified at $m/z$ = 653.0, 983.1, 1311.2, and 1673.2 Da, corresponding to detection of the sequences AC, AAU, AAAC, and AGAGU, all in line with expected digestion patterns (compare Figure S3C). After exposure to cisplatin (Figure 2B), non-G-containing digestion products are detected at $m/z$ = 653.1, 983.0, and 1311.2 Da. However, the peak originating from AGAGU with $m/z$ = 1673.2 Da has disappeared. Instead, a new peak is detected at $m/z$ = 1900.3 Da, i.e., with mass increment of 227.0 Da. This mass shift fits well with the presence of one covalently bound $Pt(NH_3)_2$ moiety on the AGAGU fragment, which in the absence of other contributions should add a mass increment of 229.0 Da. However, due to the two positive charges associated with the adduct itself, detection of a singly protonated modified RNA oligonucleotide requires displacement of two protons,[27,37] thus explaining the observed mass shift of only 227.0 Da. A similar scenario is observed for the oxaliplatin−RNA adduct, where the added mass of a covalently bound $Pt(C_6H_{10}(NH_2)_2)$ moiety is 309.1 Da, but the observed mass increment is 2 mass units less, i.e., 307.1 Da (Figure S3). Thus, the mass spectra give further support for the assumption made from the gel cleavage studies (Figure 1C), indicating the GAG region of W-siRNA147a as the preferred binding site for both metal complexes. Close inspection of the peak patterns belonging to the corresponding fragments further confirms the presence of platinated RNA fragments with a distinct isotopic pattern caused by the presence of the four stable Pt isotopes ($^{194}$Pt, 32.9%; $^{195}$Pt, 33.8%; $^{196}$Pt, 25.3%; and $^{198}$Pt, 7.2%;[38] compare Figure 2B). W-siRNA33a and W-siRNA101a were also subjected to MALDI-MS analysis as described above. By combining information from expected digestion patterns with isotopic Pt fingerprints, the presence of two bidentate-modified platinated sites could be identified for both W-siRNA33a (Figures S4 and S5) and W-siRNA101a (Figures S6 and S7). In the case of W-siRNA33a, the identified fragments correspond

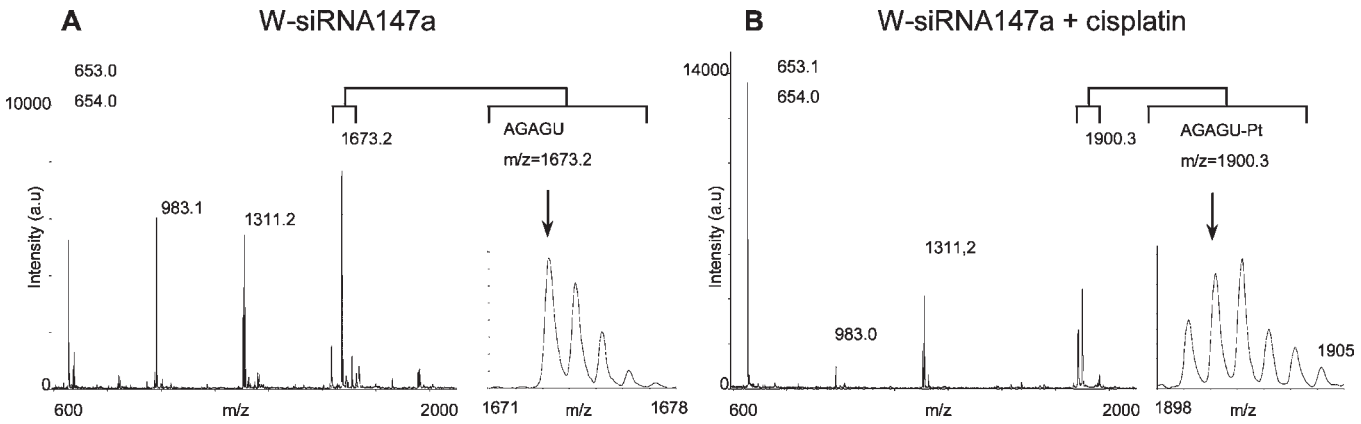


**Figure 2.** MALDI mass spectra of (A) native and (B) platinated W-siRNA147a after RNase A digestion. For native RNA (A), the inset shows an enlargement of the guanine-rich fragment with $m/z$ = 1673.2 Da. For cisplatin-platinated RNA (B), the inset shows an enlargement of the guanine-rich fragment, now with an additional mass corresponding to addition of one $Pt(NH_3)_2$ moiety, resulting in an observed $m/z$ = 1900.3 Da. The monoisotopic peak is indicated with an arrow in each panel.

to a mixture of bidentate-modified AAGC and GGGU oligomers, i.e., platinated regions A10−C13 and G14−C17 in the full-length RNA, and thus in good agreement with the PAGE analyses for the bis-modified RNA (Figure S2A, lanes 6 and 7). Finally, for W-siRNA101a, a mixture of bidentate-platinated GGU and GGdTdT oligomers was successfully detected (Figures S7 and S8; summary in Table S1).

**Thermal Melting Studies.** Thermal melting experiments were performed to study the effect of platinum binding on duplex stability. In all cases, purified mono-platinated antisense RNA (Figure S1; P1) was mixed with the sense strand prior to analysis. For all duplexes, platination resulted in a decrease of the melting temperature by 4−16 °C, depending primarily on sequence (see Table 1; experimental data in Figure S8). For the duplexes W-siRNA33 and W-siRNA101, the change in melting temperature is between 4 and 7 °C, whereas the reduction in melting temperature for W-siRNA147 is 16 °C. The larger decrease in the latter case suggests that platination is accompanied by more pronounced disruption of stacking and hydrogen-bonding patterns compared to the other two sequences. With the single GAG sequence as the only detected binding site, *vide supra*, such structural distortion can be envisioned by assuming formation of a bulge-type adduct with the platinum center bridging the two guanines rather than forming a sequential GA or AG adduct.

**W-siRNA Influence on Protein Expression.** The ability of the W-siRNAs to silence protein expression was studied in a luciferase expression assay as described before.[22−24] All three siRNAs studied were designed to interfere with full complementarity to three different target sequences located within the initial sequence of the 3′ UTR of Wnt-5a mRNA. The target sequence was inserted into a firefly luciferase-expressing plasmid. The W-siRNA silencing ability was studied in HB2 cells after co-transfection of siRNA, the firefly luciferase target plasmid, and a transfection control plasmid (see Figure 3 for a summary of results). As can be seen here, all native W-siRNAs exhibit good silencing capacity, with >90% suppression of firefly luciferase activity at a final siRNA concentration of 10 nM. The efficiency of the platinated siRNA is altered though, and the antisense-platinated siRNAs exhibit a reduced activity that is significant compared to that of the native siRNAs. The effect is here most pronounced for W-siRNA33 and W-siRNA147 ($P$ < 0.01, ***).

**Table 1. Summary of Experimentally Observed Melting Temperatures of Native siRNAs ($T_m$) and Melting Temperature Shifts Obtained after Platination ($\Delta T_m$)**

| | | $\Delta T_m$[b] | |
|---|---|---|---|
| name | $T_m$[a] (°C) | cisplatin-modified | oxaliplatin-modified |
| W-siRNA33 | 73.5(±1.0) | −4.7(±0.6) | −5.9(±0.6) |
| W-siRNA101 | 76.1(±1.9) | −6.7(±0.8) | −3.8(±0.4) |
| W-siRNA147 | 68.5(±0.6) | −16.2(±1.0) | −16.3(±0.2) |

[a] Data obtained in 1× MOPS buffer, pH 6.3; $C_{Na^+}$ = 140 mM, $C_{Mg^{2+}}$ = 2 mM with 0.6 $\mu$M of each strand. [b] Data obtained on purified platinated antisense strand hybridized with an unmodified sense strand.

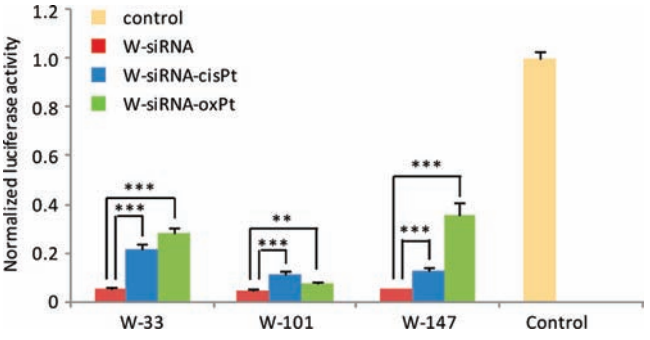


**Figure 3.** Illustration of the silencing ability of native (red) and antisense-platinated siRNAs (10 nM) modified with cis- and oxaliplatin (blue and green, respectively) after co-transfection with luciferase-expressing plasmids (pMiR Luc(1−260) and pRL-CMV) in HB2 cells. Data were normalized to experiments performed after transfection of pMiRLuc-(1−260) in buffer (yellow) and are presented with standard errors of the mean (±SEM) indicated. *** $P$ < 0.01, ** $P$ < 0.05.

It is noteworthy that, for those two siRNAs, the tentative platination site is located *outside* the 5′-seed region (cf. Chart 1).

In other words, platination interferes with protein expression when located in a region that is not directly involved in the seed-induced silencing mechanism. This observation suggests that interference might influence the biological activity of low-affinity miRNAs, for which subtle changes of affinity outside of the seed

region have been reported to affect silencing ability.[39,40] Off-target effects were also evaluated by use of firefly luciferase-expressing plasmids lacking the target site. In comparison to the data obtained in the presence of the target, the variations in protein expression were—with one exception (native W-siRNA101)—not significant ($P < 0.01$) and never deviated from the control by more than 25% (data in Table S2). This modest but significant down-regulation for W-siRNA101 can be explained by an antisense seed region match in the coding region of the firefly luciferase gene.

## DISCUSSION

Nucleic acids are well-characterized targets for many metal ions and complexes, both *in vitro* and *in vivo*. The nucleophilicity of guanine- and adenine-N7, in particular, makes these bases prone to interactions with metal ions of soft character in the intracellular environment.[41] However, metal adducts with other nucleobases have also been documented, although their relevance for biological function remains to be investigated in detail.

Among metal complexes, the interaction of cisplatin with DNA is currently one of the most well-documented types of metal−nucleic acid interactions.[19,21,42] Experimental data give a clear picture of cisplatin as an anticancer agent operating via a mechanism involving DNA interaction, disruption of repair, and induction of apoptosis. DNA interaction is also a likely mode of action for the second generation of metal-based complexes, e.g., oxaliplatin and JM216 and other promising anticancer-active metal complexes based on, for example, ruthenium and osmium.[43] Despite early documentation of RNA as an *in vivo* target for these types of drugs,[44,45] little attention has so far been paid to the biological consequences of metal complexes interfering with RNA function. However, studies by us and others during the past decade have pointed to RNA as an alternative or complementary target to DNA for platinum-based anticancer drugs. In addition to documentation of RNA as a bulk end-point pool for cisplatin,[44] there is evidence for RNA as both a kinetically competitive target[33,46] and a well-defined target site on the ribosome.[34] Recent work in our laboratory has also indicated tRNA as a plausible metalation target[36] and impediment of mi-/siRNA processing as an interesting route for further investigations.[22−24]

Processing of si- and miRNAs by the RISC machinery imposes delicate thermodynamic and steric constraints on siRNA candidates for efficient gene silencing. First, optimal loading of the antisense strand requires the use of a low-melting 5′-end.[47,48] Thus, provided that the duplex state predominates in solution, thermal melting asymmetry is the key feature required for function. Second, matching bases in the seed region, i.e., bases 2−8, have proven to be particularly important for recognition of the correct target.[6] From a synthesis point of view, knowledge concerning these requirements is essential, since they introduce restrictions onto which sites along the siRNA can be modified while maintaining or improving activity. So far, chemical modifications of siRNAs have primarily aimed at improving RISC loading and systemic stability by modification of the phosphodiester backbone with 2′-*O*-methyl and LNA as prime examples.[17,18] Recently, base-modified siRNAs have also been reported.[49−51] However, to our knowledge, this study presents the first examples of metal-modified antisense siRNAs with documented activity in cellular systems.

In previous studies, we have shown that combined use of cisplatin and siRNAs result in additive, but not synergistic, gene silencing.[23] In subsequent studies, aimed at elucidating the influence on silencing ability by preplatinated sense strands, it was revealed that sense-platinated siRNAs are well tolerated by the RISC machinery.[22,24] Further, we also showed that positioning of the platination site in close proximity to the 5′-end of the sense strand, in an attempt to change the loading preference, was without measurable change of silencing ability. The only effect of the platination was reduction of the off-target effect that was observed in a cell-free rabbit reticulocyte expession system for one of the siRNAs that showed partial complementarity to the coding region of the firefly luciferase gene.[22] In the present work, we now take advantage of these previous findings: our focus is on elucidating effects arising from platination of bases outside of the seed region, i.e., direct interaction with the biologically active part of the siRNA, but in regions usually not considered to be crucial for target selection. In case of endogenous miRNA silencing, the non-seed region has been shown to be able to compensate for poor complementarity within the seed region.[52] For siRNA targeting, which typically uses full complementarity between the antisense strand and the mRNA target, influence of suboptimal base-pairing outside the seed region has also been shown to have an effect on silencing ability.[50,53−55] In the system investigated here, we are now able to show that platinum adducts have an influence on silencing capacity when located outside the seed region. More specifically, metalation can be accompanied by a reduction in silencing capacity, e.g., for W-siRNA33 and W-siRNA147. For these two siRNAs, the measured relative changes of protein production are in the range of 2- to 7-fold, with the largest reduction in silencing ability obtained in the presence of oxaliplatin (cf. Figure 3). Common denominators for the siRNAs where the effect is most prominent are (i) the presence of at least two kinetically preferred G-N7 binding sites downstream of the seed region and (ii) location of the tentative initial binding sites in an area that also allows for formation of bulge-inducing bidentate 1,3-intrastrand adducts of GXG type with the guanines as interaction sites (G = G-N7 and X ≠ G; compare also Chart 1). In the case of W-siRNA101, where only small relative changes are observed (<2.4-fold change), these differences may be the result of the 3′-binding site being located too far away from the cleavage site to influence the silencing capacity compared to W-siRNA33 and W-siRNA147. Taken together, these results show that drug−siRNA interactions downstream of the seed region are able to modulate silencing capacity in a system where the seed−mRNA complementarity is unchanged. It is tempting to speculate that, for endogenous miRNA silencing, such effects could be even more pronounced, and that the differences between cis- and oxaliplatin with respect to activity spectrum and toxicity[56] might be due in part to their different abilities to interfere with miRNA activity. It should be noted that, for many cancers, up-regulation of Ago2 and increased miRNA activity has now been associated with a pathological state.[57] Our present observations thus indicate that in particular oxaliplatin could have the ability to modulate miRNA activity and allow for restoration of protein production with an impact on expression levels similar to those ∼2-fold changes typically found in biological systems.[40,52]

## CONCLUSIONS

The present study confirmed the tendency of RNA to form stable adducts with platinum(II)-based anticancer drugs. After platination of single-stranded RNA oligomers, well-defined binding sites were detected by both gel cleavage assays and MALDI-MS. Our data suggested the formation of adducts with

guanine (G) of three different types: bidentate GG adducts, bidentate GA or AG adducts, and bidentate GXG adducts (X ≠ G). By introduction of these binding sites into the antisense strand of three siRNAs, the effect of platination by both cis- and oxaliplatin on protein expression was investigated in a cell-based luciferase expression system. Our data showed that platination is compatible with siRNA activity when sites outside of the seed region were targeted. Typically, platination resulted in a decrease of siRNA activity, with the most pronounced influence on protein expression exerted by oxaliplatin. We speculate that such reduction of silencing activity might have biological consequences also *in vivo*, e.g., as a tool for restoration of miRNA silencing levels in cancers, where malignant conditions often are associated with abnormally high miRNA activity.

## MATERIALS AND METHODS

**Chemicals and Buffers.** All buffers and reagents were prepared in autoclaved water of Millipore quality. For gel electrophoresis, 1× TBE buffer pH 8.3 [89 mM Tris (Fluka), 89 mM boric acid (Sigma), and 2 mM EDTA (Sigma)] was used. Denaturing loading dye 6× was prepared [2.3 M urea (Duchefa biochemie), 1× TBE, 66% (v/v) formamide (Fluka), 0.05% (w/v) bromophenol blue (Sigma), and 0.05% (w/v) xylene cyanol FF (Sigma)]. Melting studies were performed in 1× MOPS buffer pH 6.3 [50 mM 4-morpholinopropanesulfonic acid (Sigma), 140 mM NaOAc (Sigma), and 2 mM $Mg(OAc)_2$ (Sigma)]. For annealing of siRNAs, a 20 mM sodium phosphate buffer pH 6.5 supplemented with 100 mM NaCl (Duchefa Biochemie) was used. Plasmids for the luciferase assays were Renilla luciferase control plasmids (pRL-CMV, Promega), firefly luciferase control plasmid (pMiR-Report, Ambion), and firefly luciferase plasmid with target insert pMiR-Luc(1−260), thoroughly described elsewhere.[23] In brief, pMiR-Luc(1−260) consists of pMiR-Report (Ambion) with the first 260 base pairs of the 3′ UTR of Wnt-5a ligated between the *Xho*I and *Hin*dIII sites in the 3′ UTR of the firefly luciferase gene.

**Oligonucleotides.** All oligonucleotides used were purchased in lyophilized form (IBA GmbH, Göttingen, Germany, of PAGE purification grade) and were dissolved in water to give a stock solution of 200 μM. Concentrations were determined on a Nanodrop spectrophotometer using the extinction coefficients supplied by the manufacturer (IBA). The short interfering RNAs used in this study target the 3′ UTR of the Wnt-5a mRNA (GenBank accession NM_003392). The names are derived from the first base targeted in the complementary 3′ UTR downstream the stop codon. W-siRNA33 consequently targets bases 33−51 of the 3′ UTR (bases 1494−1512 in NM 03392), W-siRNA101 targets bases 101−119 in the Wnt-5a 3′ UTR (bases 1562−1580 in NM_003392), and W-siRNA147 targets bases 147−165 in the Wnt-5a 3′ UTR (bases 1608−1626 in NM_003392). For co-transfection studies, siRNAs were rehybridized to a final concentration of 5.0 μM in sodium phosphate buffer by heating to 90 °C for 2 min and slowly cooling to room temperature.

**Platination of Antisense Strands.** Five nanomoles of RNA was mixed with 5× excess of cisplatin or oxaliplatin in 0.5× MOPS buffer and incubated at room temperature in the dark overnight. The platinated products were purified by 20% denaturing PAGE [acrylamide:*N*,*N*′-methylenebisacrylamide 24:1 (Gerbu), 8 M urea (Duchefa Biochemie), and 1× TBE]. The products were visualized by UV-shadowing, and the bands with the lower gel mobility, containing the platinated RNA, were excised (Figure S1). The RNA was eluted overnight at 4 °C in 2.5 M $NH_4OAc$ pH 4.9 or 1.0 M NaOAc pH 4.9, recovered by ethanol precipitation, and dissolved in water.

**Platinum Binding Site Determination with Radio-Labeled Single-Stranded RNA and Gel Electrophoresis.** Single-stranded RNA was radio-labeled at the 5′-end by T4 polynucleotide kinase (PNK, Fermentas). First, 200 nmol of RNA was mixed with 1.11 MBq [$\gamma$-$^{32}$P]-ATP (PerkinElmer) in PNK buffer [50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA (Fermentas)], incubated at 37 °C for 2 h, and finally purified by 20% denaturing PAGE. The labeled RNA was visualized by luminoradiography, excised, and eluted in 1.0 M NaOAc pH 4.9. The RNA was then recovered by ethanol precipitation in the presence of 20 μg of glycogen. In the following step, the labeled RNA was mixed with unlabeled RNA and 5× excess of cisplatin or oxaliplatin ($C_{Pt}$:$C_{oligomer}$ 5:1) in 0.5 × MOPS buffer and incubated at room temperature in the dark overnight. The platinated products were purified by 20% denaturing PAGE and recovered as described above. Alkaline hydrolysis was performed in a 10 μL reaction volume with 50 mM $NaHCO_3/Na_2CO_3$ buffer pH 9.2 and 1.0 mM EDTA for 15 min at 90 °C; 200 counts/s of labeled RNA was used. The samples were frozen in liquid nitrogen after addition of denaturing loading dye until gel loading. Enzymatic cleavage was performed in 10 μL reaction volume with 100 counts/s labeled RNA, 0.5 U RNase T1 (Fermentas), or 0.001 ng of RNase A (Fermentas). The samples were incubated at room temperature for 15 min, mixed with denaturing loading dye, and frozen in liquid nitrogen until further analysis. The cleaved products were analyzed by 20% denaturing PAGE.

**Platinum Binding Site Determination with MALDI Mass Spectrometry.** Platinated RNA, which had been eluted from gels in 2.5 M $NH_4OAc$, was cleaved by RNase A. Typically, 1 pmol of RNA was mixed with 10 ng of RNase A (Sigma) in 50 mM 3-hydroxypicolinic acid and incubated for 3 h at 37 °C. Samples for MALDI-MS were prepared by mixing the RNA digestion products (1 μL) on the target with 0.7 μL of 0.5 M 3-hydroxypicolinic acid and a small volume (∼0.1 μL) of ammonium-loaded cation exchange material.[58,59] Mass spectra were recorded on a PerSeptive Voyager-DE STR mass spectrometer (Applied Biosystems) with a reflector time-of-flight mass analyzer in positive ion mode. Mass spectrometric data were processed in the *m*/*z* free software (ProteoMetrics Inc.). RNA digestion spectra were smoothened and externally or internally calibrated from spectra of synthetic oligodeoxynucleotides. Peaks were initially assigned by the software's 'Auto label peaks' function, and the assignments were subsequently evaluated manually.

**Co-transfection Studies.** HB2 cells were cultured in Dulbecco's Modified Eagles medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma), 10 μg/mL bovine insulin (Sigma), 5 μg/mL hydrocortisone (Sigma), 5 units/mL penicillin, and 0.5 unit/mL streptomycin (Sigma) in 5% carbon dioxide at 37 °C. Approximately $10^5$ cells per well were seeded in a 24-well plate in media lacking antibiotics and were transfected the following day. Lipofectamine 2000 (Invitrogen) was diluted in DMEM and mixed with siRNA and plasmid pRL-CMV (Promega), pMiR-Report (Ambion), or pMiR-Luc(1−260) according to the manufacturer's instructions. The final concentration per well was 1 μL of Lipofectamine 2000, 0.2 μg of plasmid, and 10 nM gel-purified siRNA, *vide supra*. The cells were washed once in PBS and lysed in 50 μL of passive lysis buffer (Promega) 42 h post transfection. The luciferase activity was measured by using a Dual-Luciferase Reporter Assay system kit (Promega) on a BioOrbit 1250 luminometer, according to the manufacturer's instructions. All transfections were performed on three or more independent occasions. The firefly luciferase activity was internally normalized to the Renilla luciferase activity. The data were normalized to the buffer control, in each independent experiment, before the data were pooled. Data are presented as mean ± SEM (standard error of the mean). The difference in luciferase activity between the unmodified and the platinum-modified siRNA was analyzed by a two-tailed unequal variance *t*-test and found to be significant in all cases ($P < 0.01$).

**Thermal Melting Studies.** Thermal melting studies were performed in 1× MOPS buffer at a constant duplex concentration of 0.60 μM. A Cary 4000 UV−vis spectrophotometer (Varian) equipped with a thermal control unit was used. Prior to the melting analysis, the

samples were annealed by heating to 95 °C for 2 min and then cooled to 20 °C at a rate of 2 °C/min. Data were collected at 260 nm from 20 to 95 °C at a rate of 0.2 °C/min, with data collection intervals of 0.5 °C. The results were analyzed by the first-derivative method.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Summary of platinated fragments detected by MALDI-MS (Table S1), gels showing products produced after platination and results after alkaline hydrolysis (Figures S1 and S2), MALDI-MS spectra of platinated W-siRNA 33a and W-siRNA101a and theoretical cleavage products (Figures S3−S7), thermal denaturation curves (Figure S8), and complete ref 55. This material is available free of charge via the Internet at http://pubs.acs.org.

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