Articles

Platinum Complexes Can Inhibit Matrix Metalloproteinase Activity: Platinum−Diethyl[(methylsulfinyl)methyl]phosphonate Complexes as Inhibitors of Matrix Metalloproteinases 2, 3, 9, and 12

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Platinum complexes able to inhibit matrix metalloproteinases (MMPs) through a noncompetitive mechanism are reported for the first time in this study. [$PtCl_2$(SMP)] and [Pt(dimethylmalonato)(SMP)], characterized by the bisphosphonate-analogue ligand diethyl[(methylsulfinyl)methyl]phosphonate (SMP), are slight inhibitors of MMP-2 (IC_{50} = 258 ± 38 and 123 ± 14 μM, respectively) but markedly inhibit MMP-9 (IC_{50} = 35.5 ± 6 and 17 ± 4 μM), MMP-3 (IC_{50} = 5.3 ± 2.9 and 4.4 ± 2.2 μM), and MMP-12 (IC_{50} = 10.8 ± 3 and 6.2 ± 1.8 μM). In contrast, cisplatin, carboplatin, and the SMP ligand are inactive, and the bisphosphonate clodronate shows a broad-spectrum inhibitory activity in the high micromolar range (mean IC_{50} > 200 μM). These results, along with mechanistic investigations (DNA interaction and tumor cell growth inhibition), demonstrate that ligand modifications of platinum compounds can be exploited to target also biological substrates distinct from DNA.

Introduction

Platinum drugs represent a unique class of DNA-damaging antitumor agents. In combination with other chemotherapeutic agents, *cis*-diamminedichloridoplatinum(II) (cisplatin, *cis*-DDP), *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II) (carboplatin), and, more recently, *cis*-[(1*R*,2*R*)-1,2-diaminocyclohexane-*N*,*N*']][oxalato(2−)-*O*,*O*']platinum(II) (oxaliplatin) have enjoyed significant success in the treatment of a variety of solid tumors. Progress in understanding both the chemical properties and the mechanism of action of cisplatin has guided the development of new analogues having either different leaving ligands or different amines. In general, modifications in the chlorido leaving groups result in compounds with different pharmacokinetic properties, whereas modifications in the carrier ligands can alter the efficacy and/or the spectrum of activity of the resulting complex. Modifications of the carrier ligands and, to a lesser extent, of the leaving groups have also been exploited for achieving tumor tissue specificity. Homing moieties such as galactose and bile acid for liver,[1−3] and estrogen or tamoxifen derivatives for breast have been employed.[4,5] Platinum complexes containing aminobisphosphonate and diethyl[(methylsulfinyl)methyl]phosphonate (SMP[a]) ligands have also been proposed to achieve bone tissue specificity.[6−8] With respect to aminobisphosphonates, the SMP ligand is characterized by having a sulfoxide in place of a substituted amine, and phosphonic groups blocked as diethyl esters. The neutral Pt−SMP complexes [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] (dmm = dimethylmalonate) are shown in Figure 1, along with SMP and the bisphosphonate clodronate. The X-ray structures of [$PtCl_2$-(SMP)] and [Pt(dmm)(SMP)] are not available; however, the two complexes were spectroscopically characterized, and the SMP ligand was shown to form a five-membered chelate ring, contributing to the chemical stability of the complexes.[8] The SMP ligand is structurally analogous to the bisphosphonates, a class of drugs originally developed for disturbances of calcium homeostasis and, more recently, for palliation and prevention of bone metastases.[9,10] Because bisphosphonates are known for their ability to inhibit matrix metalloproteinases (MMPs),[11,12] we wondered whether [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] could be endowed with anti-MMP activity, thus exploring the possibility of targeting platinum complexes toward enzymes critically linked to the invasive and metastasizing phenotype.[13] The MMPs are a family of more than 21 zinc-dependent endopeptidases, divided into eight structural classes, three of which are membrane-bound. MMPs mediate the homeostasis of the extracellular matrix, are upregulated in almost every type of human cancer, and their expression is frequently associated with poor prognosis. Whereas some MMPs are expressed mainly by cancer cells, other are expressed by tumor microenvironment cells. This indicates that MMPs are altered in various aspects of malignant phenotype, i.e., cancerogenesis, invasion/metasta-

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[a] Abbreviations: SMP, diethyl[(methylsulfinyl)methyl]phosphonate; dmm, dimethyl malonate; MMP, matrix metalloproteinase; EtBr, ethidium bromide; dien, diethylenetriamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; RF, resistance factor.

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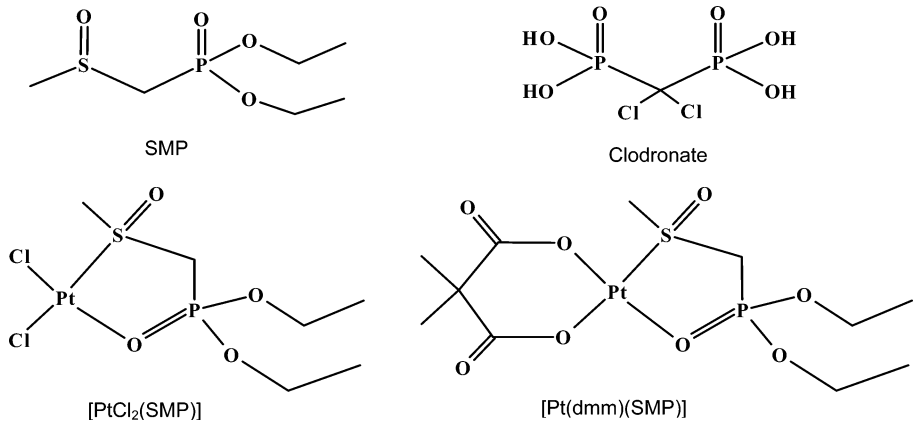


Figure 1. Structures of SMP, clodronate, [$PtCl_2$(SMP)], and [Pt(dmm)(SMP)] (dmm = dimethyl malonate).

Table 1. Inhibitory Effect of [$PtCl_2$(SMP)], [Pt(dmm)(SMP)], and Clodronate upon MMP-2, MMP-9, MMP-3, and MMP-12 Proteolytic Activity (IC_{50}, μM)[a]

compd	MMP-2	MMP-9	MMP-3	MMP-12
[$PtCl_2$(SMP)]	258 ± 38	35.5 ± 6	5.3 ± 2.9	10.8 ± 3
[Pt(dmm)(SMP)]	123 ± 15	17 ± 4	4.4 ± 2.2	6.2 ± 1.8
clodronate	145 ± 9	320 ± 27	300 ± 20	121 ± 11

[a] IC_{50} (mean value ± SD calculated over at least three experiments) represents the compound's concentration able to inhibit the enzymatic activity by 50% after 1 h of interaction between compound and enzyme.

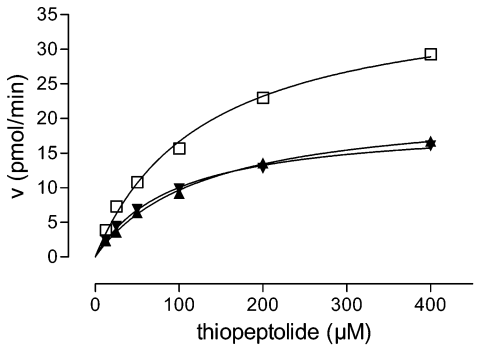
sis, and angiogenesis,[14] therefore representing promising therapeutic targets.[15]

This study demonstrates that ligand modification of platinum compounds can be exploited to target also biological processes distinct from platinum−DNA interaction.

Results

Inhibition of Matrix Metalloproteinase Activity. The inhibitory effects of [$PtCl_2$(SMP)], [Pt(dmm)(SMP)], cisplatin, carboplatin, clodronate, and free SMP ligand have been investigated upon MMP-2, MMP-9, MMP-3, and MMP-12, four metalloproteinases involved in cancer and characterized by distinct structural features.[13] Recombinant catalytic domains were used for inhibition studies, and the proteolytic activity was evaluated through spectrophotometric measurements of a thiopeptolide substrate. The concentrations of compounds able to inhibit the enzymatic activity by 50% (IC_{50}) after 1 h of interaction between compound and enzyme are reported in Table 1. As expected for a bisphosphonate, clodronate inhibited MMP-2, MMP-9, MMP-3, and MMP-12; however, it was effective only at rather high concentrations and showed a poor selectivity (IC_{50} of 145 ± 9, 320 ± 27, 300 ± 20, and 121 ± 11 μM, respectively). In contrast, [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] showed inhibitory activities similar to that of clodronate against MMP-2 (IC_{50} of 258 ± 38 and 123 ± 15 μM, respectively) but much greater toward MMP-9, MMP-3, and MMP-12 {IC_{50} of 35.5 ± 6, 5.3 ± 2.9, and 10.8 ± 3 μM for [$PtCl_2$(SMP)] and IC_{50} of 17 ± 4, 4.4 ± 2.2, and 6.2 ± 1.8 μM for [Pt(dmm)-(SMP)]}. Interestingly, neither cisplatin and carboplatin (up to 300 μM) nor SMP (up to 600 μM) displayed inhibitory activity (data not shown). In order to check if the inefficacy of cisplatin could be a consequence of a kinetic inertness that does not allow the complex to react with the enzyme in a 1-h reaction time, the inhibitory activity of cisplatin toward the four MMPs was evaluated also after longer reaction times (up to 8 h). Even under these conditions, cisplatin did not inhibit the activity of the metalloproteinases with respect to control, thus indicating that the lack of inhibition was not due to kinetic inertness.

To investigate the mechanism of inhibition of Pt−SMP complexes, the enzymatic activity was measured as a function of thiopeptolide concentration, in the presence of [$PtCl_2$(SMP)] or [Pt(dmm)(SMP)] at the IC_{50} concentration. The plot of the measured rate of proteolysis (v, pmol/min) against substrate concentration is shown in Figure 2 for the case of MMP-12. The rate of substrate conversion as a function of substrate concentration follows the Michaelis−Menten equation [$v = v_{max}$-[S]/([S] + K_m)], where [S] is the substrate concentration, v_{max} is the maximum rate of conversion, and K_m is the Michaelis constant (the substrate concentration at which the rate of conversion is half of v_{max}). The v_{max} and K_m values were determined by fitting the data directly to the Michaelis−Menten equation using nonlinear regression (GraphPad Prism Software). In the presence of IC_{50} concentrations of [$PtCl_2$(SMP)] and [Pt-(dmm)(SMP)], v_{max} values decreased with respect to control (22 ± 0.8, 19.4 ± 0.4, and 38.3 ± 1.3 pmol/min, respectively),



	v_{max} (pmol/min)	K_m (μM)
Control (□)	38.3±1.3	129.7±10.8
[$PtCl_2$(SMP)] (▲)	22 ±0.8	128.3±11.2
[Pt(dmm)(SMP)] (▼)	19.4 ±0.4	93.3±6.2

Figure 2. MMP-12 inhibition by [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] complexes. The graph shows the rates of proteolysis by MMP-12 enzyme, as a function of substrate concentration, in the absence (control, □) and in the presence of [$PtCl_2$(SMP)] (▲) or [Pt(dmm)(SMP)] (▼) IC_{50} concentrations (10.8 and 6.2 μM, respectively). The rates of proteolysis (v, pmol/min) were calculated from linear plots of (OD × reaction volume)/($\epsilon \times l$) against time (min), where ϵ is the extinction coefficient of 2-nitro-5-thiobenzoic acid (13 600 $M^{-1}cm^{-1}$) and l is the path length of light through the sample in cm. Points are mean values ± SE over three experiments; no SE bars are seen, since they are smaller than the size of the symbols. Kinetic parameters (v_{max} and K_m, ± SE) were determined by fitting the data to the Michaelis−Menten equation using nonlinear regression analysis (GraphPad Prism, San Diego, CA).

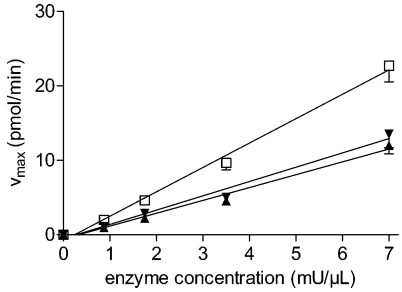


Figure 3. MMP-12 v_{max} variation as a function of enzyme concentration. The graph shows the v_{max} variation of MMP-12 reaction as a function of enzyme concentration in the absence (control, □) and in the presence of [$PtCl_2$(SMP)] (▲) or [Pt(dmm)(SMP)] (▼) IC_{50} concentrations (10.8 and 6.2 μM, respectively). Points are mean values ± SE over three experiments; no SE bars are seen when smaller than the size of the symbol. One U = 100 pmol/min at 37 °C, 100 μM thiopeptolide.

whereas K_m values were not significantly modified (128.3 ± 11.2, 93.3 ± 6.2, and 129.7 ± 10.8 μM, respectively). Effects similar to those observed for MMP-12 (i.e., a decrease of v_{max} and no change on K_m) were also observed in the case of MMP-2, MMP-3, and MMP-9 (data not shown), thus suggesting a similar mechanism of action of [$PtCl_2$(SMP)] and [Pt(dmm)-(SMP)] complexes upon all enzymes under investigation.

The above-described results suggest either a noncompetitive inhibition mechanism or an irreversible inhibition. To discriminate between these two possibilities, experiments were designed to study v_{max} variation as a function of enzyme concentration. As shown in Figure 3 for MMP-12, all straight lines have equal intercept but different slopes (lower in the presence of [$PtCl_2$-(SMP)] or [Pt(dmm)(SMP)] IC_{50}). Therefore, it can be concluded that Pt−SPM complexes act on MMP-12 through a noncompetitive inhibitory mechanism.

Comparative Analysis of MMP Sequences and Models of Their Interaction with Pt−SMP. Studies on the interaction of platinum complexes with proteins indicate that histidine and sulfur-containing amino acids (methionine and cysteine) represent preferential binding sites.[16−20] By inspection of the primary sequence of the catalytic domains of the MMPs (Supporting Information), it appears that cysteine residues are only present at conserved positions in the fibronectin repeats of MMP-2 and MMP-9 and are involved in disulfide bridges. The fibronectin repeats are inserted in a loop of the catalytic domains of these two MMPs and form an independent folding unit that extends relatively far from the enzyme active site.[21] Because of their involvement in disulfide bridges, it seems unlikely that cysteines of the fibronectin repeats may represent a potential binding site for the platinum substrates; moreover, their conserved positions in MMP-2 and MMP-9 would not justify the differential inhibition by Pt−SMP complexes. As far as histidine residues are concerned, they occur at various positions on the structure of the catalytic domains of all four MMPs. Six of them act as ligands for catalytic and structural zinc sites and can be excluded from the analysis of specificity determinants because of their highly conserved character and their low accessible surface area, which is similar for corresponding zinc-binding histidines of different MMPs. The same holds for the invariant methionine of the so-called metzincin motif ($HEX_2HX_5HX_7M$, where X means any amino acid) at the catalytic zinc site. In conclusion, only nonconserved histidines and methionines may represent platinum anchoring sites that can account for inhibition specificity. MMP-2 contains such residues, but quite far from the substrate pocket, the closest being a Met at the end of helix αB. In contrast, MMP-3 and MMP-9

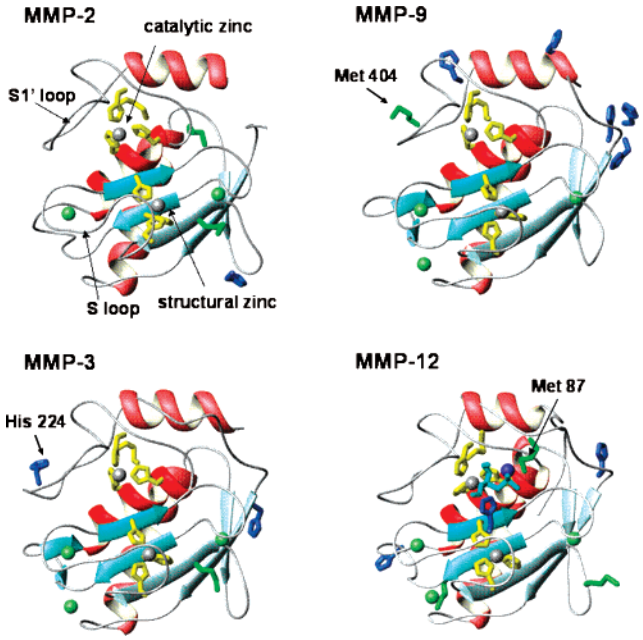


Figure 4. Structures of catalytic domains of MMPs and model of their interaction with Pt−SMP. Ribbon representation of the structures of catalytic domains of MMP-2 (PDB ID 1HOV), MMP-9 (1GKC), MMP-3 (1SLN), and MMP-12 (theoretical model with Pt−SMP). Helices are colored in red and strands are in cyan. The zinc atoms are shown as gray spheres and the calcium atoms as green spheres. The catalytic and structural zinc sites as well as the loops S and S1′ are indicated on the structure of MMP-2. Fully conserved His and Met residues are shown as yellow sticks, while nonconserved His and Met residues are shown as blue and green sticks, respectively. Nonconserved His and Met residues close to the catalytic zinc site are indicated. The SMP ligand of Pt−SMP bound to MMP-12 is shown in cyan and the platinum atom as a blue sphere.

contain, respectively, a His (His224) and a Met (Met404) residue at the edge of the pocket, in the S1′ loop (Figure 4). The S1′ loop represents the most variable region and a source of specificity within the MMP family, encompassing a segment of prime importance for binding of susbtrates and various inhibitors.[22−24] Finally, MMP-12 contains a Met residue (Met87), located three positions downstream from the N-terminus. The conformation of the N-terminus appears to be important in the activation mechanism of this enzyme.[25] It should be noted, however, that available structures of MMP-12 do not contain the correct N-terminal extension and are truncated a few residues after the physiological cleavage site (Phe84). Therefore, we modeled the conformation of the N-terminal segment of MMP-12 on the closely related structure of MMP-3 (PDB ID 1SLN). In this latter protein, the NH_3^+-terminal group of Phe83 (homologous to Phe84 in MMP-12) forms a surfacial salt bridge with the side chain carboxylate of Asp251, a residue conserved among all MMPs, which allows the N-terminal amino acids to pack against a hydrophobic groove over the active site.[26] The blocking of the N-terminus through this salt bridge appears to be quite a general feature of MMPs and results in a several-fold greater activity (a phenomenon called superactivation) of wild-type enzymes versus N-truncated MMPs.[25]

In summary, MMP-3, -9, and -12 contain candidate anchoring sites in regions critical for enzyme activity. These are, in particular, His224 of MMP-3 and Met404 of MMP-9, both located in the S1′ specificity loop, and Met87 of MMP-12, which ends up very close to the active site upon formation of a salt bridge between N-terminal Phe84 and Asp237 (Figure 4).

To model the interaction of the Pt−SMP moiety to candidate residues of MMP-3, -9, or -12, the available X-ray coordinates

of the K[$PtCl_3$(SMP-*S*)] complex were used. K[$PtCl_3$(SMP-*S*)] is characterized by having monodentate SMP with detached phosphonate group and can be easily formed in the cation-rich districts associated with bone tumors.[27] Structure calculations show that the platinum complex partially obstructs the substrate pocket, and the detached P═O group of the SMP ligand can be located at binding distance from the catalytic zinc site (Figure 4). It is also possible that one of the histidines ligating the catalytic zinc ion can bind to platinum using the second imidazole nitrogen as donor atom. It can be therefore hypothesized that, after interaction of Pt−SMP at primary anchoring sites on the MMPs surface, other binding events can take place, further stabilizing the inhibitor−enzyme interaction. MMP-2 does not have potential anchoring residues in such a favorable position to allow concerted binding events, and this can justify its lower inhibition by Pt−SMP complexes. We want to emphasize that, differently from Pt−SMP's, ciplatin and carboplatin are not able to efficiently inhibit any of the MMPs selected for this investigation. We believe that a key role is played by the SMP ligand, which, in the semidetached form, can have the P═O group reaching over the zinc ion while the platinum-bound sulfoxide moiety can confer to the metallic center an additional lability, promoting further reactions after the initial anchoring event.

DNA Interaction Properties. A primer extension footprinting assay was performed to determine, through a replication mapping experiment, the sequence-selective DNA binding of platinum compounds under investigation. The DNA-polymerase blocking sites resulting from the presence of DNA adducts formed after 24 h reaction time at 0.001 platinum/nucleotide molar ratio are shown in Figure 5a. It is possible to assume that in our experimental conditions (low platinum/nucleotide ratio, quite long reaction times, and temperature of 37 °C) the extent of platination is quantitative. Both [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] complexes (lanes 2 and 3) and cisplatin (lane 1) displayed quite similar patterns of stop bands, corresponding to purine sites on DNA template; similar results were obtained also after 48 h reaction time (not shown). Under the experimental conditions used, blocking adducts of cisplatin are of bifunctional nature;[28,29] however, it cannot be excluded that blocking adducts of Pt−SMP complexes could also be of monofunctional nature. In order to investigate this possibility, we have employed ethidium bromide (EtBr) as a fluorescent probe to distinguish between DNA perturbations induced by monofunctional or bifunctional adducts.[30,31] Intercalation of EtBr into DNA is blocked in a stoichiometric manner by bifunctional adducts of a series of platinum complexes, including cisplatin, and results in a loss of fluorescence intensity.[30] In contrast, modification of DNA by platinum complexes having only one leaving ligand, e.g., [Pt(dien)Cl]Cl, results only in a slight decrease of EtBr fluorescence intensity as compared to that of control DNA. Modification of DNA by [$PtCl_2$(SMP)], [Pt(dmm)(SMP)], or cisplatin at 0−0.08 platinum/nucleotide molar ratios resulted in a decrease of EtBr fluorescence, as shown in Figure 5b. The interaction with [$PtCl_2$(SMP)] determined a decrease of fluorescence comparable to that of cisplatin;[30−32] in contrast, the interaction with [Pt(dmm)(SMP)] led to a much smaller decrease of fluorescence intensity, comparable to that induced by monodentate [Pt(dien)Cl]Cl. Similar results were obtained also after a longer incubation time (48 h, results not shown). Therefore, the fluorescence analysis suggests that [$PtCl_2$-(SMP)] forms mainly bifunctional adducts similar to those of cisplatin, whereas the DNA adducts of [Pt(dmm)(SMP)] behave as monofunctional adducts. A monofunctional binding of the latter complex to DNA could be fostered by detachment of the

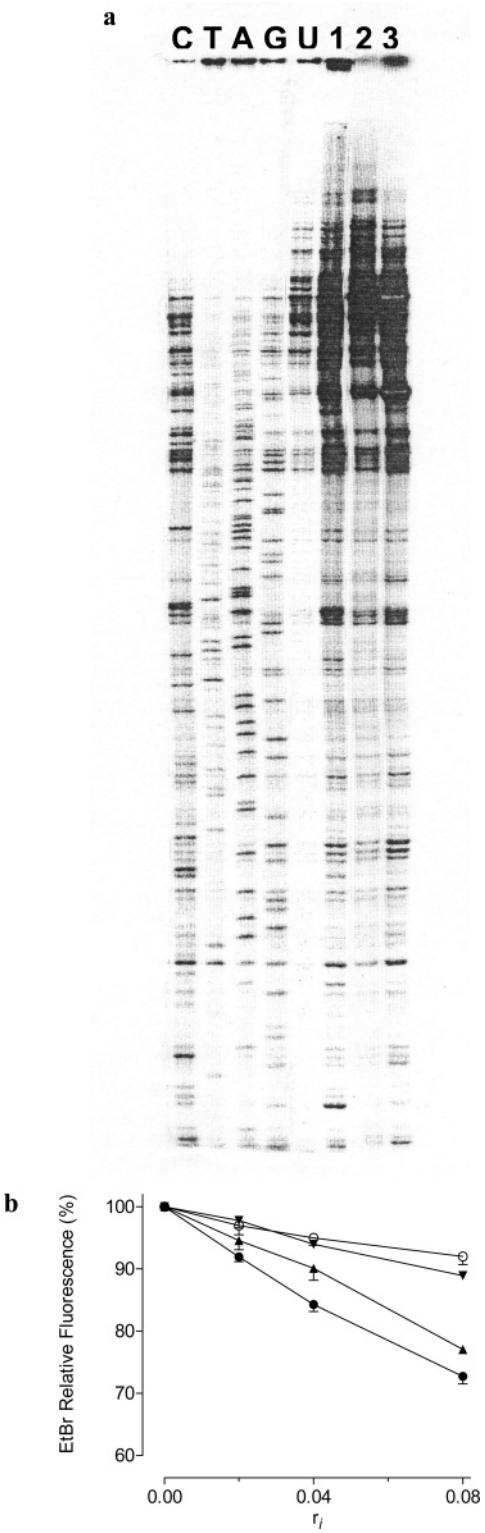


Figure 5. DNA interaction properties of [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] complexes. (a) Autoradiogram of 6% polyacrylamide/7 M urea sequencing gel, showing the stop sites of Sequenase 2 polymerase on pSP73KB DNA by cisplatin (lane 1), [Pt(dmm)(SMP)] (lane 2), and [$PtCl_2$(SMP)] (lane 3). pSP73KB plasmid DNA (2455 bp) was modified with platinum compounds at 0.001 r_i value (r_i is the platinum/nucleotide molar ratio at the onset of incubation) in $NaClO_4$ (10 mM) for 24 h at 37 °C. Lanes C, T, A, and G refer to the base positions on the template strand, U = no reagent. (b) Dependence of the ethidium bromide fluorescence upon r_i for DNA modified by platinum complexes in $NaClO_4$ (10 mM) at 37 °C for 24 h: [$PtCl_2$(SMP)] (▲), [Pt(dmm)(SMP)] (▼), [Pt(dien)Cl]Cl (○), cisplatin (●). Points are mean values ± SD over three experiments; when no SD bars are seen, they are smaller than the size of the symbols.

Table 2. In Vitro Growth Inhibitory Activity of [$PtCl_2$(SMP)], [Pt(dmm)(SMP)], SMP, Cisplatin, and Carboplatin toward A2780 and A2780cisR Ovarian Cancer Cells[a]

	[$PtCl_2$(SMP)]	[Pt(dmm)(SMP)]	SMP	cisplatin	carboplatin
A2780	100 ± 13	25 ± 6	300 ± 40	0.2 ± 0.01	1.3 ± 0.2
A2780cisR	70 ± 10	35 ± 5	260 ± 35	3.2 ± 0.5	23 ± 2.1
RF	0.7	1.4	0.8	16	17.6

[a] IC_{50} (mean value ± SD calculated over at least three experiments) in μM (96 h compound exposure) or RF (resistance factor, IC_{50} A2780cisR/IC_{50} A2780).

loosely bound phosphonate group of SMP ligand while dmm remains still bound to platinum.

In Vitro Growth Inhibition Assay. [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] complexes, along with the free ligand SMP and the reference compounds cisplatin and carboplatin, were tested for their growth inhibitory activity toward cisplatin-sensitive A2780 and cisplatin-resistant A2780cisR ovarian cancer cells (Table 2). [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] complexes displayed growth inhibitory effects markedly lower than those of cisplatin and carboplatin toward A2780 cells ($IC_{50} = 100 \pm 13$, 25 ± 6, 0.2 ± 0.01, and 1.3 ± 0.2, respectively), and the SMP ligand itself showed a negligible effect ($IC_{50} = 300 \pm 40$ μM). Interestingly, [Pt(dmm)(SMP)] was about 4 times more active than [$PtCl_2$(SMP)], and both complexes maintained their inhibitory activity toward cisplatin-resistant A2780cisR cells, the resistance factors (RF) (IC_{50} A2780cisR/IC_{50} A2780) being 1.4 and 0.7, respectively (RF for cisplatin and carboplatin = 16 and 17.6, respectively). This result suggests that Pt−SMP complexes are able to elude one or more molecular mechanisms responsible for resistance of A2780cisR cells, i.e., reduced uptake, increased levels of glutathione, and increased DNA repair.[33,34]

Discussion

With the aim of developing more selective platinum anticancer agents, compounds with biologically active carrier ligands have been proposed, an important objective being the achievement of tissue specificity.[1−7] The diethyl[(methylsulfinyl)-methyl]phosphonate ligand was included in our design strategy on the grounds that platinum complexes with a bisphosphonate-analogue ligand could be characterized by bone specificity,[8,27] as well as by the possibility of targeting biological substrates distinct from DNA,[35] in this particular case the matrix metalloproteinase enzymes. Matrix metalloproteinases represent an interesting target for anticancer drugs, and the most extensively studied inhibitors include collagen peptidomimetics and non-peptidomimetics, tetracycline derivatives, and bisphosphonates.[13] Here, the proof-of-principle that MMP activity can be inhibited also by platinum complexes is reported for the first time. Unlike bisphosphonates, which are broad-spectrum MMP inhibitors acting mainly through a Zn^{2+} cation chelation mechanism,[12] [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] selectively inhibit MMP-9, -3, and -12 and act through a noncompetitive inhibition. Importantly, ongoing experiments indicate that MMP inhibition by [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] is observed also in cellular systems, thus confirming the approach appropriateness.

At the present stage of the study, we cannot provide a clear mechanism for the inhibition selectivity of the Pt−SMP complexes. However, in a parallel investigation we showed how the Pt−SMP complex can easily detach the phosphonate group and offer three donor atoms to a cation: the detached P═O oxygen atom, the oxygen bound to the sulfur atom, and a platinum coordinated chloride ligand.[27] Moreover, detachment of the phosphonate group opens a free coordination site on platinum, which could favor anchoring of the complex moiety to a suitable nucleophile sited on the MMP surface. These properties of the Pt−SMP complexes (to be susceptible of attack by a nucleophile and to act as a chelating ligand toward a cation) could be involved in their inhibitory activity toward MMPs. This hypothesis appears to be supported by modeling results. Platinum coordination either to the S1′ loop or to the blocked N-terminal end of the active enzymes appears to be rather feasible. Moreover, such a coordination would not directly affect the residues responsible for substrate binding but still might alter the shape and accessibility of the active site, in accordance with a noncompetitive mechanism of inhibition. On the basis of the structure of MMP catalytic domains, it is presumable that simple platinum coordination to residues far away from the substrate pocket should exert little or no influence on the enzyme active site, as in the case of MMP-2.

As far as the DNA interaction properties of [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] are concerned, the primer extension footprinting assay indicates that DNA regioselectivities of both complexes are similar to that of cisplatin. Interestingly, the ethidium bromide fluorescence assay shows that DNA adducts formed by [Pt(dmm)(SMP)], unlike those of [$PtCl_2$(SMP)] and cisplatin, behave as monofunctional adducts, even after prolonged incubation times. It can be hypothesized that the latter complex can bind monofunctionally to DNA by displacing the phosphonato group of SMP ligand while dmm is still bound to platinum.

In a preliminary assay of in vitro antitumor activity toward A2780 tumor cells, [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] resulted to be markedly less active than cisplatin or carboplatin. It can be hypothesized that the low inhibitory ability of Pt−SMP complexes depends upon a rapid inactivation in the case of [$PtCl_2$(SMP)] and upon a slow formation of bifunctional adduct with DNA in the case of [Pt(dmm)(SMP)]. A recent study has indeed shown that the complex [$PtCl_2$(SMP)] undergoes fast solvation in aqueous solution, because of the *trans* effect exerted by the sulfur atom, whereas the [Pt(dmm)(SMP)] complex does not form solvato species in measurable concentration.[8] Therefore, inside the cell, where the chloride concentration is rather low, solvato species such as $[PtCl(H_2O)(SMP)]^+$ could be easily inactivated by platinophiles in their way to the target. In contrast, the greater inertness of the [Pt(dmm)(SMP)] complex could retard formation of bifunctional adducts with DNA, thereby reducing the growth inhibitory activity.

Interestingly, [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] are characterized by a different inhibitory potency. This property, along with the ability of overcoming cisplatin resistance of A2780cisR cells,[33,34] is worthy of further development aimed at improving the pharmacological actions of Pt−SMP complexes at the cellular level.

Conclusions

This contribution shows that novel platinum anticancer drug candidates can be rationally designed in such a way that the DNA-damaging property is associated with an inhibitory effect toward matrix metalloproteinase enzymes. To the best of our knowledge, no platinum complex able of inhibiting MMP activity has been reported so far, even though platinum complexes with aminobisphosphonate and hydroxamate have already been synthesized and investigated for anticancer activity.[6,7,36]

This result has been achieved by using the bisphosphonate-analogue diethyl[(methylsulfinyl)methyl]phosphonate (SMP) ligand, selected with the aim of obtaining bone specificity and

of modifying pharmacodynamic properties of platinum complexes. Modification of carrier ligands has already been explored to modify pharmacodynamic properties of cisplatin. Since DNA is the key pharmacological target of platinum compounds, a widely exploited strategy for the development of novel platinum drug candidates has been that of changing the profile of platinum−DNA adducts, as in the case of *trans*-platinum complexes[37] or multinuclear platinum complexes.[38] Moreover, DNA-targeting groups such as intercalators have also been conjugated to the metal, with the anticipation that the compounds would localize in the vicinity of DNA and exert superior growth inhibitory activity.[39,40] This study shows for the first time that the pharmacodynamic properties of platinum compounds can be modified even more markedly by selecting appropriate ligands, so that novel platinum compounds can be characterized by a dual mechanism of antitumor action: DNA targeting on one hand and inhibition of matrix metalloproteinase activity on the other hand.

Further investigations can lead to optimization of the inhibitory activity of this new class of antitumor platinum compounds toward matrix metalloproteinase and/or tumor cell growth.

Experimental Section

Starting Materials. The SMP ligand and the [$PtCl_2$(SMP)] and [Pt(dmm)(SMP)] complexes were synthesized as previously reported.[7] Cisplatin, carboplatin, clodronate, ethidium bromide, and calf thymus DNA were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Culture media and reagents were from Euroclone (Paignton, U.K.).

Matrix Metalloproteinase Activity Inhibition and Kinetic Assays. MMP activity inhibition and kinetic assays were performed by using the colorimetric method Biomol QuantiZyme (Biomol International L.P.), following the manifacturer's protocol with minor modifications. All experiments were done by using recombinant human MMP-2, MMP-3, MMP-9, and MMP-12 catalytic domains (amino acids 81−423, 83−255, 88−438, and 84−255, respectively) purchased from Biomol. Briefly, proteolytic activity was measured using a thiopeptolide substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5) in which the peptidic bond that is cleaved by the MMP is replaced by a thioester bond.[41,42] MMP-mediated thioester hydrolysis produces a sulfhydryl group that reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB) to form 2-nitro-5-thiobenzoic acid, the concentration of which can be evaluated by measuring the absorbance at 412 nm. In 96-well microplates, recombinant human MMP catalytic domains were incubated (1 h, 37 °C, if not otherwise indicated) with different concentrations of compounds under investigation in HEPES (MES in the case of MMP-3) (50 mM), $CaCl_2$ (10 mM), Brij-35 (0.05%), and DTNB (1 mM) at pH 7.5 (pH 6.0, in the case of MMP3). After addition of the thiopeptolide substrate to the incubation mixture, the increase of absorbance was recorded at 1 min time intervals for 60 min. Data were plotted as OD versus time for each sample to obtain the reaction rate in OD/min. The percentage of residual activity for each compound concentration was calculated by the formula % remaining activity = (rate in the presence of inhibition/control rate) × 100, and compound concentration able to inhibit enzymatic activity by 50% (IC_{50}) was calculated from semilogarithmic dose−response plots. The obtained results exclude the possibility that the reduction of enzymatic activity stems from interaction of the platinum complexes with the thiopeptolide and not with the enzyme. Generally, the thiopeptolide concentration was much greater than those of the platinum complexes; therefore, a mere reaction of the platinum complexes with the thiopeptolide would have resulted in a reduction of the proteolytic activities much smaller than those observed.

The kinetic parameters v_{max} and K_m were investigated by using thiopeptolide concentrations ranging from 12.5 to 400 μM, either in the absence or in the presence of IC_{50} concentrations of platinum complexes. Thiopeptolide conversion rates (v, pmol/min) were calculated from reaction rates (OD/min) by using the equation v = (reaction rate × reaction volume)/($\epsilon \times l$), where ϵ is the extinction coefficient of 2-nitro-5-thiobenzoic acid (13 600 $M^{-1}cm^{-1}$), and l is the path length of light through the sample in cm. K_m and v_{max} were calculated by using the software GraphPad Prism for nonlinear-regression analysis (GraphPad Software, Inc.). In order to discriminate between noncompetitive inhibition or irreversible inhibition mechanisms, the variation of v_{max} as a function of MMP-12 concentration was investigated.

Modeling of MMPs and of Their Adducts with Pt−SMP. Multiple sequence alignment was obtained with the program Clustalw (http://www.ebi.ac.uk/clustalw/). The structural model of the catalytic domain of MMP-12 (residues 84−255) was generated with the program Modeller-8v2,[43] using the structures of MMP-12 (PDB ID 1OS9) and MMP-3 (1SLN) as templates, and adding restraints for all the metal ions (2 Zn^{2+} and 3 Ca^{2+}). Models of the adducts between MMPs and Pt−SMP complex were obtained with the minimization protocol of the program PSEUDODYANA,[44] starting from the experimental structures of MMP-3 (1SLN) and MMP-9 (1GKC) and from the theoretical model of MMP-12. The Pt−SMP complex was introduced in the PSEUDODYANA library as a nonstandard residue using the available X-ray coordinates of the K[$PtCl_3$(SMP-*S*)] complex having monodentate SMP and detached phosphonate group[27] and defining nine rotatable dihedral angles for the SMP ligand. Structure calculations were performed by linking the platinum ion to the MMP and constraining the metal−ligand bond length in the range 1.9−2.1 Å for N donor histidine and 2.2−2.4 Å for S donor methionine. The platinum geometry was imposed to be square planar. Model analysis and display were performed with the program MOLMOL.[45]

Primer Extension Footprinting Assay. The primer extension footprinting assay was performed to evaluate the DNA interaction regioselectivity of the new platinum compounds.[28,29] Briefly, pSP73KB (2455 bp) plasmid DNA was modified with platinum compounds at 0.001 r_i value (r_i is the platinum/nucleotide molar ratio at the onset of incubation) in $NaClO_4$ (10 mM) for 24 h (if not otherwise indicated) at 37 °C. After removal of drug excess by ethanol precipitation and alkaline denaturation, the DNA was primed with 18-mer T7(+) primer (Promega); DNA synthesis was then performed by Sequenase 2 enzyme (Amersham Pharmacia) in the presence of [α-^{32}P]dATP (370 kBq, 111 TBq/mmol, Amersham Pharmacia) and unlabeled dNTPs, following the manufacturer's protocol. The products of synthesis were separated by electrophoresis on a denaturing gel (6% polyacrylamide/7 M urea) in parallel to a sequence ladder performed on untreated control DNA.

Characterization of DNA Adducts by Ethidium Bromide Fluorescence. Fluorescence measurements of DNA modified by platinum complexes in the presence of ethidium bromide (EtBr) were performed to investigate the nature of platinum−DNA adducts.[30−32] Briefly, calf thymus DNA (0.25 mg/mL) in $NaClO_4$ (10 mM) was incubated with platinum complexes at 0−0.08 r_i values at 37 °C in the dark. After 24 or 48 h incubation, aliquots of the DNA solution (0.12 mL), corresponding to 0.03 mg of DNA, were withdrawn and added to an EtBr solution (0.04 mg/mL in 0.4 M NaCl) to a final volume of 3 mL. Fluorescence measurements of DNA modified by platinum in the presence of EtBr were performed at 25 °C using a Perkin-Elmer LS 5B spectrofluorimeter. The excitation wavelength was 525 nm and the emitted fluorescence was measured at 590 nm. The percentage of relative fluorescence was determined by the formula % relative fluorescence = (f_t/f_n) × 100, where f_t = fluorescence of treated samples and f_n = fluorescence of control untreated samples.

Tumor Cell Lines and Cell Growth Inhibition Assay. A pair of ovarian cancer cell lines, A2780 (parent line from untreated patient) and A2780cisR (derived cisplatin-resistant subline), kindly supplied by Dr. L. Kelland (The Institute of Cancer Research, Surrey, UK), was used.[33,34] A2780 and A2780cisR were maintained at 37 °C in a 10% CO_2 humidified air in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum, glutamine (2 mM), insulin (10 μg/mL), hydrocortisone (0.5

μg/mL), amphoterycin B (2.5 μg/mL), and gentamicin (50 μg/mL). The growth inhibitory effect of platinum complexes was evaluated by using the Sulforhodamine B (SRB) assay.[46] Briefly, cells were seeded into 96-well microtiter plates (1000 cells/well, in 100 μL culture medium). After seeding, plates were incubated at 37 °C for 24 h prior to addition of the compounds. After 24 h, several samples were fixed in situ with cold trichloroacetic acid (TCA), to represent a measurement of the cell population at the time of compound addition. The compounds under investigation were freshly dissolved in culture medium and stepwise diluted to the desired final concentrations. After the addition of different compound concentrations to quadruplicate wells, the plates were further incubated at 37 °C for 96 h. Then, cells were fixed in situ by the gentle addition of cold 50% (w/v) TCA (50 μL, final concentration 10%) and incubated for 1 h at 4 °C. The supernatant was discarded, and the plates were washed four times with tap water and air-dried. Sulforhodamine B solution (100 μL, 0.4% w/v) in 1% acetic acid was added to each well, and plates were incubated for 30 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was then solubilized with Trizma base (10 mM) and the absorbance was read on an automatic plate reader at 515 nm. The compound concentration able to inhibit cell growth by 50% ($IC_{50} \pm SD$) was then calculated from semilogarithmic dose−response plots.

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Supporting Information Available: Figure S1 showing multiple alignment of catalytic domains of MMP-2, -3, -9, and -12 sequences, corresponding to residues 81−423, 83−255, 88−438, and 84−255, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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