Reactions of cisplatin and *cis*-$[PtI_2(NH_3)_2]$ with Molecular Models of Relevant Protein Sidechains: a Comparative Analysis.

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Abstract

Quite surprisingly, cisplatin and *cis*-$[PtI_2(NH_3)_2]$ were found to manifest significant differences in their reactions with the model protein lysozyme. We decided to explore whether these differences recur when reacting these two Pt compounds with other proteins. Notably, ESI MS measurements carried out on cytochrome c nicely confirmed the reaction pattern observed for lysozyme. This prompted us to exploit a computational DFT approach to disclose the molecular basis of such behavior. We analyzed comparatively the reactions of *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$ with appropriate molecular

models (Ls) of the sidechains of relevant aminoacids. We found that when Pt(II) complexes are reacted with sulfur ligands both quickly lose their halide ligands and then the resulting *cis*-$[Pt(L)_2(NH_3)_2]$ species loses ammonia upon reaction with a ligand excess. In the case of imidazole, again *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$ quickly lose their halide ligands but the resulting *cis*-$[Pt(L)_2(NH_3)_2]$ species does not lose ammonia by reaction with excess imidazole. These results imply that the two platinum complexes manifest a significantly different behavior in their reaction with representative small molecules in agreement with what observed in the case of model proteins. It follows that the protein itself must play a crucial role in triggering the peculiar reactivity of cis-$[PtI_2(NH_3)_2]$ and in governing the nature of the formed protein adducts. The probable reasons for the observed behavior are critically commented and discussed.

Keywords: cisplatin, cis-diiodo-diamino-platinum, DFT, ESI-MS, protein sidechain, ligand substitution, trans effect.

Introduction

Cisplatin is a leading anticancer drug in wide clinical use.[1] Yet, its severe side-effects and the limitations due to the development of platinum-resistant tumors have given rise to an intensive search of innovative platinum(II) compounds that might exhibit improved biological profiles, produce a wider spectrum of anticancer activities and overcome platinum resistance. Relevant examples are polynuclear platinum compounds,[2] platinum(IV) complexes,[3,4] monofunctional platinum(II) complexes,[5] and – among them – platinum(II) complexes where chlorido ligands are replaced by bulkier halides, such as iodide.[6-11] The iodido analogue of cisplatin, i.e. *cis*-$[PtI_2(NH_3)_2]$, was earlier found to be inactive in sarcoma mice models,[12] such inactivity being explained by the lower reactivity of Pt−I bonds compared to Pt−Cl bonds in aqueous solution.[6] However, it was reported more recently that various iodido platinum(II) complexes may display pronounced antiproliferative properties *in vitro*, often superior to those of cisplatin.[11] At the same time, several studies on the mechanism of action of these complexes revealed a substantial and unanticipated reactivity for iodido Pt(II)

complexes toward proteins,[13] suggesting that functional proteins might be alternative important targets for these Pt compounds. In this context, although a quite general consensus now exists on DNA being the main biomolecular target for cisplatin, there has been a growing attention for the interactions of cisplatin and related compounds with proteins as these interactions may be at the basis of the relevant toxic effects of these drugs, contributing to their antiproliferative action and also affecting the intracellular fluxes and their distribution.[14]

The interactions of cisplatin and related Pt drugs, including iodido platinum(II) complexes, with model proteins were extensively investigated in recent years thanks to the use of advanced mass spectrometry methods and to the obtainment of several crystal structures of Pt-protein adducts.[15] On the whole, those studies highlighted that a few protein side chains such as Cys, His and Met represent the main, almost exclusive, anchoring sites for platinum fragments on the protein outer surface. Surprisingly, it was observed that, at variance with most Pt(II) compounds, the diiodido analogue of cisplatin may form adducts with small model proteins where the halide ligand is retained and the ammonia ligand is replaced, this being a truly unexpected feature.[13] This unconventional behavior is in contrast with the solution behavior of these two platinum drugs that undergo a similar aquation process in aqueous buffers, typically involving halide release.[6]

To go further in the investigation of these systems we have applied a combined experimental and theoretical strategy. First, we have explored the reaction of *cis*-$[PtI_2(NH_3)_2]$ with cytochrome c, a small model protein suitable for mass spectrometry experiments, bearing solvent accessible residues able to coordinate the platinum center.[16-18] Through application of an established ESI-MS protocol,[15] we could confirm the peculiar reactivity of *cis*-$[PtI_2(NH_3)_2]$ with cyt c compared to cisplatin leading to formation of distinct adducts. Then, to understand this aspect in more depth, we have taken advantage of a computational strategy aimed at evaluating the thermodynamic and kinetic basis of the proposed mechanistic hypotheses. Indeed, computational studies have often and successfully been applied to describe the reactivity of metals and metallodrugs with proteins.[19-24]

In particular, we addressed the reactivity of cisplatin and its iodido analogue with three specific molecular models of protein side chains to predict the consequences of halide replacement on Pt complexes reactivity. Although most experimental and theoretical studies on the mechanism of

interaction between antitumor Pt(II) complexes and proteins have been carried out with amino acids,[25-27] the simplest protein units, the reactivity of these latter species with Pt drugs can diverge significantly from that of protein side chains, due to the simultaneous presence of the reactive amino group and carboxylic group expected to exist in the zwitterionic form. Indeed, the latter functional groups, involved in the amide bonds of the backbone, are not available for the coordination in real proteins.

Accordingly, we have investigated comparatively the reactions of *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$ with three simple molecules – i.e. ethanethiol, dimethyl sulfide, and imidazole – that are in our opinion realistic molecular models (L) of cysteine, methionine and histidine sidechains. These reactions were investigated at DFT level of theory explicitly taking into account the first, second and third substitution step.

MATERIALS AND METHODS

Experimental details

Chemicals, reagents and the cytochrome c (horse heart, C7752) were supplied by Sigma-Aldrich and used without further purification.

cis-$[PtI_2(NH_3)_2]$ was synthesized as reported in literature.[6] Interactions between *cis*-$[PtI_2(NH_3)_2]$ and cytochrome c were assessed by high-resolution ESI-MS as described in our previous works:[15, 27-29] a 10^{-3} M stock solution of complex was prepared in water and an aliquot was added to a solution of cytochrome c in 20 mM ammonium acetate buffer at pH 6.8. Metal complex to protein ratio was 3:1, with final protein concentration of 10^{-4} M. The reaction mixture was incubated up to 72 h at 37 °C. Aliquots were sampled at increasing time intervals (6, 24, 48, 72 h) and injected after dilution with bidistilled water to a final concentration of 10^{-7} M. Before the analysis formic acid (0.1% v/v) was also added. The ESI mass spectra were acquired through direct infusion at 7 μL/min flow rate in a TripleTOF® 5600^+ mass spectrometer (Sciex, Framingham, MA, U.S.A.), equipped with a DuoSpray® interface operating with an ESI probe. The ESI source parameters were optimized and were as follows: positive polarity, Ionspray Voltage Floating 5500 V, Temperature 30 °C, Ion source

Gas 1 (GS1) 45 L/min; Ion source Gas 2 (GS2) 0 L/min; Curtain Gas (CUR) 15 L/min, Declustering Potential (DP) 100 V, Collision Energy (CE) 10 V. For acquisition, Analyst TF software 1.7.1 (Sciex) was used and deconvoluted spectra were obtained by using the Bio Tool Kit micro-application v.2.2 embedded in PeakView™ software v.2.2 (Sciex).

Computational details

All calculations were performed by using the Jaguar programs package.[30, 31] The local minimum geometry of each Pt(II) species was calculated in gas phase with the hybrid exchange-correlation functional B3LYP,[32, 33] which is known to give a good description of geometries and reaction profiles for transition-metal-containing compounds[34-37] including platinum(II)[38-40] and a few platinum(IV)[4, 39, 41] based anticancer compounds, and the LACVP** basis set,[42, 43] consisting, for the platinum atom, of the 1s−4d core electrons described with the Hay and Wadt core−valence relativistic (i.e., with an implicit treatment of scalar-relativistic effects) effective core-potential (ECP), whereas the outer 18 electrons are explicitly treated by a basis set of double-ζ quality; the all-electrons 6-31G** basis set was employed to treat the remaining atoms. Vibrational frequencies analyses were performed at the same level of theory to confirm the correct nature of the optimized stationary points and calculate zero-point energy and thermal corrections at 298.15 K and 1 atm (under the hypothesis of an ideal gas behavior) for enthalpy and free energy estimations. Intrinsic reaction coordinate (IRC) calculations were employed to correctly locate reagents and products minima connected with the transition states for each considered reaction step. Solvation energies were evaluated using the Poisson−Boltzmann continuum solvent method implemented in Jaguar.[44] The energies of all stationary points were evaluated performing single point calculations with a larger basis set: 6-311++G** for the main group elements and LACV3P++**,[42, 43] consisting of the Hay and Wadt core−valence ECP basis set of triple-ζ quality plus diffuse d function, for the metal atom.

The degree of trigonal bipyramidal / square pyramidal coordination of the five coordinate transition states was characterized using the shape index parameter τ_5 proposed by Allison et al.[45]- defined as $\tau_5 = (\beta - \alpha)/60°$, β and α being the angles between the two couples of trans basal ligands in the square pyramidal extreme, and assuming a value of 0 for an ideal square pyramidal and 1 for an ideal trigonal bipyramidal geometry.

RESULTS

ESI-MS studies

To confirm the unconventional reactivity of *cis*-[$PtI_2(NH_3)_2$] toward proteins, we have studied its interaction with the model protein cytochrome c. Similarly to lysozyme and other model proteins[15] cytochrome c can be easily investigated through ESI-MS measurements and is well prone to bind metal centers. Indeed, cyt c is characterized by a suitable molecular weight of ~12 kDa and possesses solvent exposed amino acid residues bearing S and N donors able to bind efficiently metal centers. The reactivity of this protein with cisplatin has been already investigated by different bio-physical methods including mass spectrometry and X-ray crystallography, revealing that, upon incubation, cisplatin reacts in a classical fashion i.e. by the release of the chloride ligand. The amino acid residues involved in the platinum coordination are Met65 or Met65 and Glu61 simultaneously, His18 and His33.[17, 18] These evidences suggest that also in the case of *cis*-[$PtI_2(NH_3)_2$] with cytochrome c, the binding of metal center should occur at the level of the same residues.

In Figure 1 the mass spectra recorded at different time intervals for *cis*-[$PtI_2(NH_3)_2$] incubated with cytochrome c are reported.

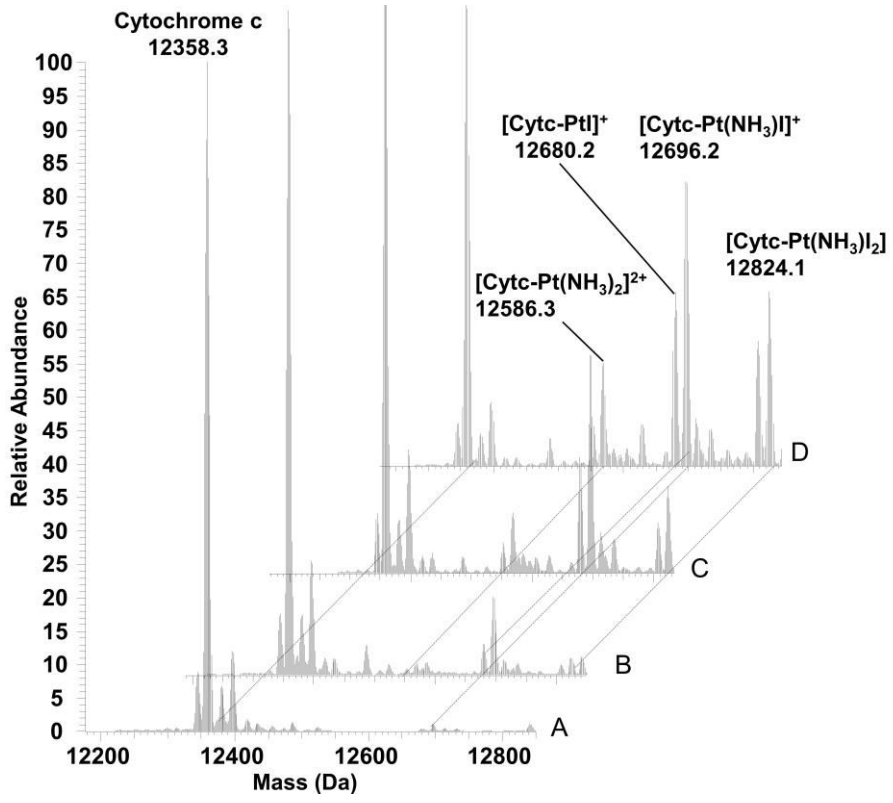
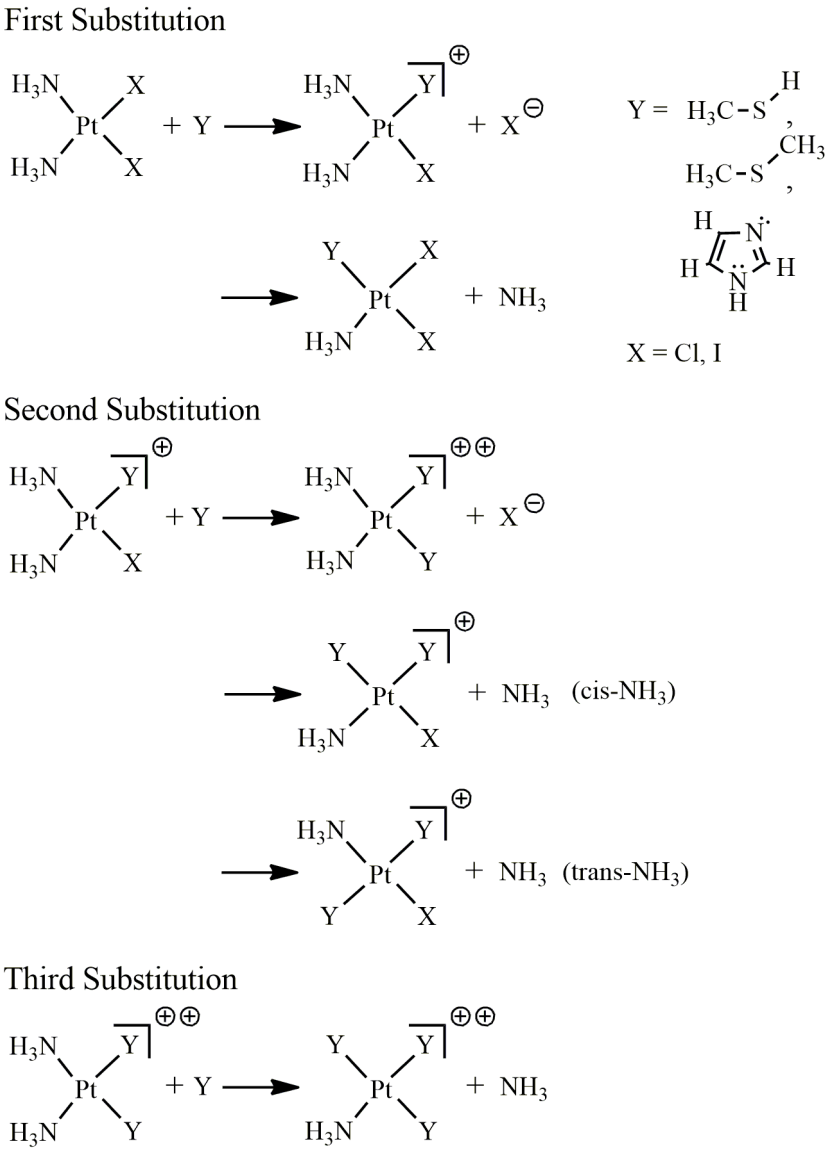


Figure 1. Deconvoluted ESI-MS of cytochrome c incubated with *cis*-$[PtI_2(NH_3)_2]$ in 20 mM ammonium acetate buffer at pH 6.8, 37° C. The metal complex to protein ratio was 3:1, with final protein concentration of 10^{-4} M. Spectra were recorded after 6 (A), 24 (B), 48 (C) 72 h (D) of incubation.

Four main peaks were detected at 12586, 12680, 12696 and 12824 Da assigned to cyt c respectively bearing $[Pt(NH_3)_2]^{2+}$, $[PtI]^+$, $[Pt(NH_3)I]^+$ and $[Pt(NH_3)I_2]$ bound fragments. Thus, these results clearly confirmed that the unexpected reactivity observed with lysozyme[13] is conserved also in the case of cytochrome c.

Computational studies

The reactions of either cisplatin or diiodido diamine platinum(II) with the appropriate molecular models, (Ls), of Cys, Met, or His side chains, i.e. ethanethiol, dimethyl sulfide and imidazole, were investigated at DFT level of theory. More specifically, we have initially studied the first and second substitution process according to which either the halido or the amino ligand are replaced by L (Scheme 1). Although cisplatin and its iodide analogue are known to undergo aquation of the halide ligands in water, this process is relatively slow, with experimental barriers of the order of 23-24 kcal mol^{-1} for both first and second halide substitution, significantly higher than the barriers expected for the much stronger nucleophilic species considered here (as confirmed by our calculations showing values in the order of 14-21 kcal mol^{-1}). We therefore assumed that cisplatin and its iodide analogue are quite stable in water on the timescale of their reaction with the considered model nucleophiles.



Scheme 1. First, second, and third substitution of either the halido or the amino ligand by L .

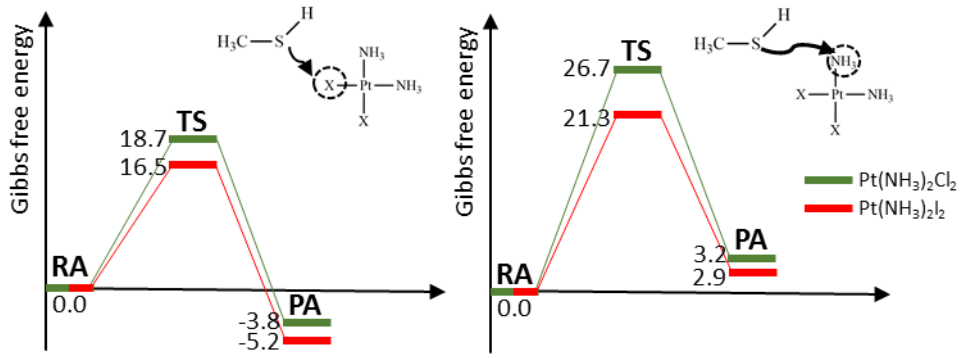
First Substitution. In order to study both the thermodynamics and the kinetics of the considered ligand substitution processes, we have used a pseudo-molecular model in which a reactant adduct (RA) is initially formed between the platinum complex and the attacking amino acid model and evolves into a product adduct (PA) in which the leaving group is still non-covalently bound to the product metal complex, passing through a penta-coordinated transition state. ESI-MS and IRMPD studies and related computational investigations indeed showed that such a mechanism takes place in gas phase and in solution for reaction of cisplatin with several aminoacids, including histidine and methionine.[46-48] The formation of RA was found to be thermodynamically favored, so we assumed it to

be the initial state for the ligand substitution process. For both cis-$[PtCl_2(NH_3)_2]$ and cis-$[PtI_2(NH_3)_2]$ and all aminoacid models, the first substitution of halide resulted to be moderately exergonic with reaction free energies in the range –3.8 to –9.9 kcal mol^{-1} for *cis*-$[PtCl_2(NH_3)_2]$ and –4.1 to –11.1 kcal mol^{-1} for cis-$[PtI_2(NH_3)_2]$ while amine substitutions are slightly endergonic with reaction free energies in the range 2.4 to 3.2 kcal mol^{-1} for cis-$[PtCl_2(NH_3)_2]$ and +1.7 to +2.9 kcal mol^{-1} for *cis*-$[PtI_2(NH_3)_2$ (Table S1, Figures 1-3). Calculations also evidenced the following trend of thermodynamic preference for both halide and ammine substitution imidazole > $S(CH_3)_2$ > CH_3SH, although the kinetics of ligand substitution shows a different trend (vide infra).

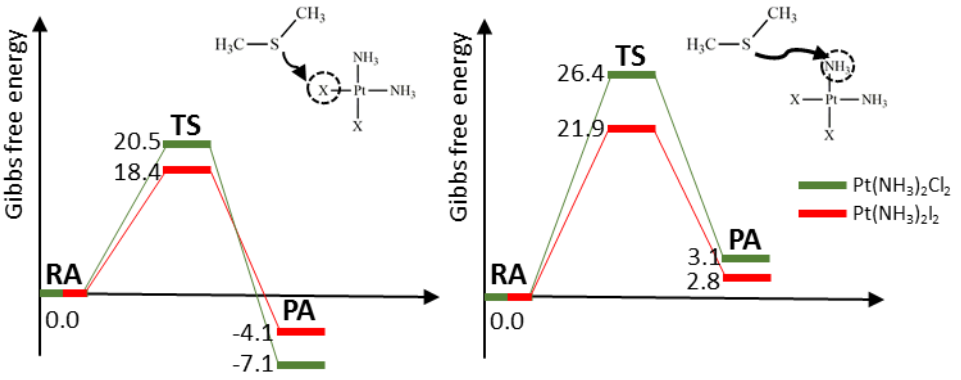
An intermediate trigonal bipyramidal / square pyramidal coordination of Pt was found for the transition state (TS) of ligand substitution (see Figure 4 for Cys model) as shown by values around 0.5 of the shape index parameter τ_5 for the five coordinate transition metal complexes (Table S2), assuming a value of 0 for an ideal square pyramidal and 1 for an ideal trigonal bipyramidal geometry, see Computational Details paragraph for the shape index definition. although some difference was appreciated in the orientation and non-coordinative interactions of the approaching nucleophile. In particular, we found that the S-H bond of CH_3SH was oriented towards the leaving halide thus providing for some energy stabilization of the corresponding TS. The activation barriers were estimated in terms of both enthalpy and free energy differences between TS and the reaction adduct (RA) (Table S1). The activation free energies for the halide substitution by ethanethiol, dimethyl sulfide, and imidazole in *cis*-$[PtCl_2(NH_3)_2]$ are 18.7, 20.5, and 22.8 kcal mol^{-1}, respectively, whereas the corresponding values for *cis*-$[PtI_2(NH_3)_2]$ are slightly lower: 16.5, 18.4, 20.3 kcal mol^{-1} (Figures 1-3), presumably due to the better leaving group ability of iodide compared to chloride; calculations indicate a trend of reactivity of CH_3SH > $S(CH_3)_2$ > imidazole opposite to that of thermodynamic stability. These activation free energy values are relatively low, allowing quite fast reaction rates for the considered ligand substitutions at physiological temperature – with half times calculated with the Eyring equation on the scale of seconds to minutes. The activation free energies for the ammine substitution by ethanethiol, dimethyl sulfide, and imidazole in *cis*-$[PtCl_2(NH_3)_2]$ are 26.7, 26.4, and 28.0 kcal mol^{-1}, respectively, whereas the corresponding values for *cis*-$[PtI_2(NH_3)_2]$ are much lower: 21.3, 21.9, 24.3 kcal mol^{-1} (Figures 1-3). The much higher reactivity of iodoplatin toward ammine

substitution can be attributed to the higher trans-effect of I^- causing a higher labilization of the Pt-N bond.

The activation free energies for ammine substitution in *cis*-$[PtCl_2(NH_3)_2]$ are quite high and indicate very slow reactions, especially for the substitution by histidine, thus suggesting that the reaction of *cis*-$[PtCl_2(NH_3)_2]$ with the considered amino acids molecular models invariably leads to the halide substituted product *cis*-$[PtCl(L)(NH_3)_2]$. On the other hand, the corresponding activation energies for ammine substitution in *cis*-$[PtI_2(NH_3)_2]$ are low enough, in particular for substitution by cysteine and methionine models, to allow relatively fast reactions at physiological temperature. However, due to the significantly lower barriers for the iodide substitution, by 2-4 kcal mol^{-1} and to their much higher thermodynamic stability, also the reactions of *cis*-$[PtI_2(NH_3)_2]$ with cysteine and methionine models are expected to lead almost exclusively to the halide substituted product *cis*-$[PtX(L)(NH_3)_2]$.



a)



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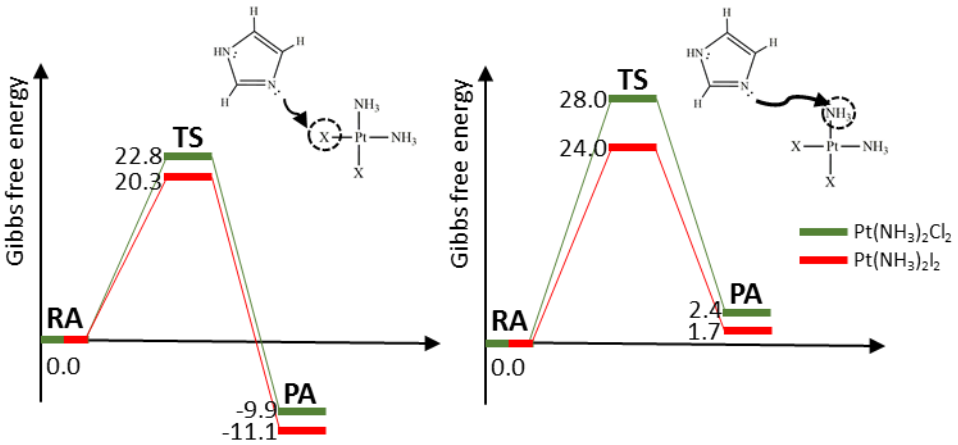

c)

Figure 1. Calculated reaction profiles for the first substitution of chloride/iodide (X), and ammonia with models of a) Cys, b) Met, and c) His side chains for *cis*-[$PtCl_2(NH_3)_2$] and *cis*-[$PtI_2(NH_3)_2$]. Reactant-adduct (RA), transition state (TS), and product-adduct (PA) are considered and all values are in kcal/mol.

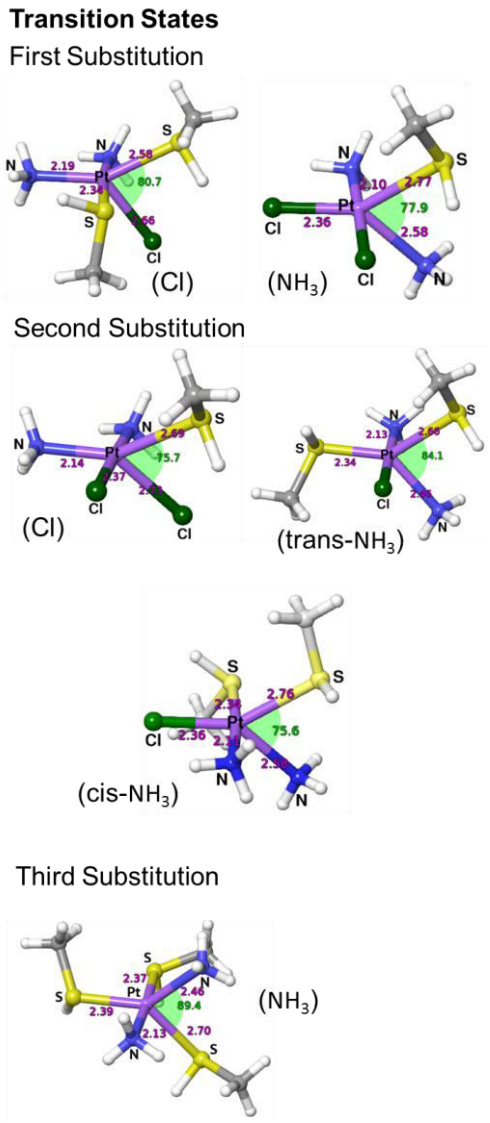

Figure 2. Transition states for the first, second, and third substitution of chloride, and *trans*- and *cis*-ammonia by model of Cys side chain for *cis*-$[PtCl_2(NH_3)_2]$. All distances in Å.

Second Substitution. Due to the much greater thermodynamic and kinetic preference for the halide substituted product *cis*-$[PtX(L)(NH_3)_2]$ in the first substitution of both *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$, the second substitution by a further model molecule was considered only for the *cis*-$[PtX(L)(NH_3)_2]$ complex and not for the other possible *cis*-$[PtX_2(NH_3)(L)]$ product of the first substitution step.

For both *cis*-$[PtCl(L)(NH_3)_2]$ and *cis*-$[PtI(L)(NH_3)_2]$, the second substitution of halide resulted to be moderately endergonic for cysteine and methionine and barely exergonic for histidine models with

reaction free energies, respectively, of +6.5 and +3.8 and –0.2 for the chloro and +7.7, +1.9 and –4.6 kcal mol^{-1} for the iodo complex. When ammine substitution is considered, two possibilities should be taken into account, corresponding to the substitution of the ammine either in trans or cis to the remaining halogen: while for cysteine model the substitution of the ammine trans to halogen is clearly thermodynamically favored over that trans to S for the iodo and unfavored for the chloro complex, probably due to well-known $I^- > RS^- > Cl^-$ trend of trans effect, for methionine and histidine models the trend is similar but less clear. In essentially all cases, amine substitution is slightly endergonic, with reaction free energies in the range –0.6 to 8.4 kcal mol^{-1}, without a clear trend for *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$ (Table S1, Figures 5-71-3). The second substitution of both the halide and – on average – of the ammine ligand shows the same imidazole > $S(CH_3)_2$ > CH_3SH thermodynamic preference trend observed for the first substitution – for both *cis*-$[PtCl(L)(NH_3)_2]$ and *cis*-$[PtI(L)(NH_3)_2]$.

For the second substitution, too, intermediate trigonal bipyramidal / square pyramidal geometries around Pt were found for the transition states of ligand substitution (see Figure 4 for Cys model), with higher square pyramidal character for halogen substitution (τ_5=0.32) but higher trigonal bypiramidal character for the substitution of the ammonia trans to S (τ_5=0.68), see Table S2. The activation free energies for the halide second substitution by Cys, Met, His models in *cis*-$[PtCl(L)(NH_3)_2]$ is 16.6, 17.8, and 21.1 kcal mol^{-1}, respectively, whereas the corresponding values for *cis*-$[PtI(L)(NH_3)_2]$ are on average similar: 21.2, 16.5, 18.8 kcal mol^{-1} (Figures 5-7). These activation free energy values are similar or slightly lower than those for the first substitution, suggesting that also halide second substitution is quite fast at physiological temperature. The activation free energies for the ammine substitution by Cys, Met, His models in *cis*-$[PtCl(L)(NH_3)_2]$ are, respectively, 22.4, 22.4, 30.7 kcal mol^{-1} for the ammine trans to the L ligand and 24.3, 24.5, 25.8 kcal mol^{-1} for the ammine trans to the halogen ligand, whereas the corresponding values for *cis*-$[PtI(L)(NH_3)_2]$ are: 23.0, 22.6, 28.9 kcal mol^{-1} for the ammine trans to the L ligand and 20.6, 20.3, 23.4 kcal mol^{-1} for the ammine trans to the halogen ligand (Figures 5-7). It is worth noting that the values of the activation barriers for the ammine substitution by cysteine or methionine models are around 22-23 kcal mol^{-1} for the chloro complex, while those for the iodo complex are ca. 2 kcal mol^{-1} lower, around 20-21 kcal mol^{-1}. These

results indicate that, although the rate of ammine substitution is rather fast at physiological temperature, with half times of the order of minutes, it is slower than that of halide substitution which remains favored.

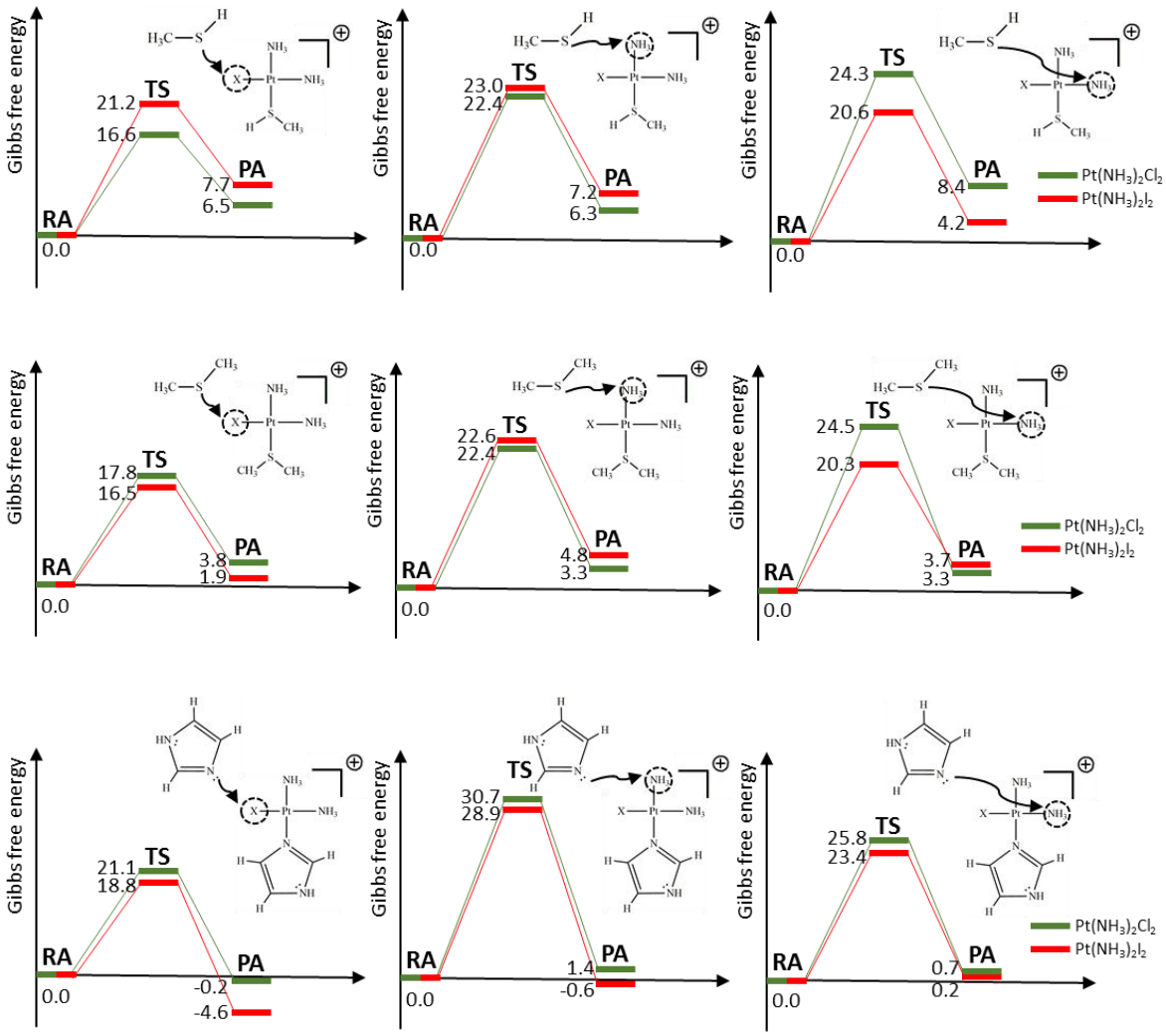

Figure 2. Calculated reaction profiles for the second substitution of chloride/iodide (X), *trans*- and *cis*-ammonia (with respect to the first substituent) with model of Cys, Met, and His side chains for *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$. Reactant-adduct (RA), transition state (TS), and product-adduct (PA) are considered and all values are in kcal/mol.

Third Substitution. Due to the much higher thermodynamic and kinetic preference for the halide substitution in both first and second steps of *cis*-[$PtCl_2(NH_3)_2$] and *cis*-[$PtI_2(NH_3)_2$], the third substitution of one of the remaining ammines by a further L model molecule was considered only for the *cis*-[$Pt(L)_2(NH_3)_2$] complex.

Thermodynamically, the third substitution is slightly endergonic for cysteine and methionine and barely exergonic for histidine (Figure 8) with the values for reaction free energies being, respectively, 6.7, 4.1 and –1.5 kcal/mol^{-1}. A distorted trigonal bipyramidal transition state was found (τ=0.32) for ammonia substitution with an activation free energy of 14.7 kcal mol^{-1} for the methyl sulfide, 21.8 kcal mol^{-1} for dimethyl sulfide and 27.3 kcal mol^{-1} for imidazole, indicating that at physiological temperature ammonia substitution is very fast for cysteine, relatively fast for methionine but very slow for histidine models.

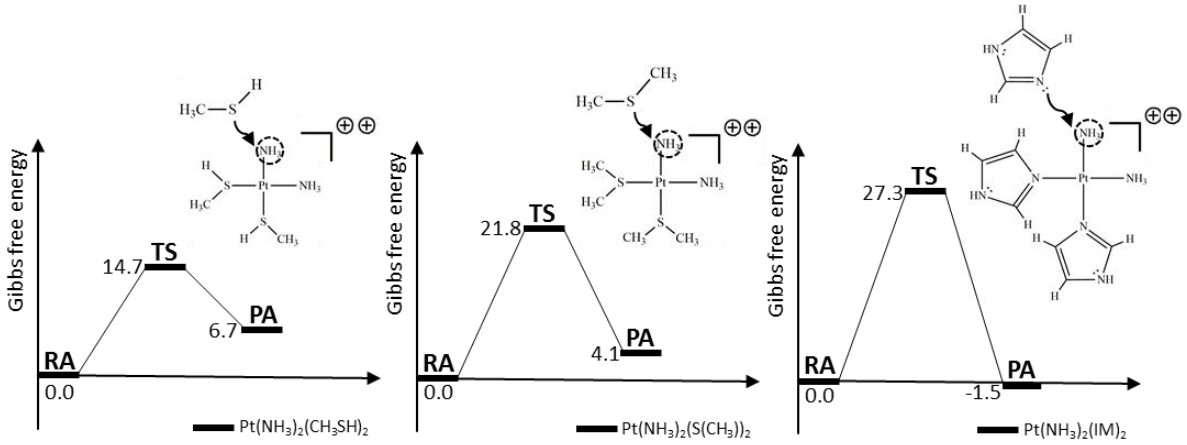

Figure 8. Calculated reaction profiles for the third substitution of ammonia with models of Cys (above), Met (middle), and His (below) side chains for *cis*-[$Pt(NH_3)_2$(substituent)$_2$].

DISCUSSION

The calculated paths for the reactions of Pt(II) drugs with aminoacid sidechains models

The above described theoretical calculations indicate the following sequence of reaction paths for the release of ammonia from *cis*-$[PtCl_2(NH_3)_2]$and *cis*-$[PtI_2(NH_3)_2]$ complexes when interacting with the molecular models of three relevant aminoacid sidechains.

The activation free energies for ammine substitution by cysteine and methionine models in *cis*-$[PtCl_2(NH_3)_2]$ are much higher than those for chloride substitution so that the reaction of *cis*-$[PtCl_2(NH_3)_2]$ with the considered amino acids invariably leads to the halide substituted products *cis*-$[PtCl(L)(NH_3)_2]$. Although the activation energies for ammine substitution in *cis*-$[PtI_2(NH_3)_2]$ are significantly lower, to allow relatively fast reactions at physiological temperature, they are still significantly higher than those for iodide substitution, by 2-4 kcal mol^{-1}, so that, also in this case, the reaction of *cis*-$[PtI_2(NH_3)_2]$ is expected to lead almost exclusively to the halide substituted products *cis*-$[PtI(L)(NH_3)_2]$.

These halide substituted *cis*-$[PtX(L)(NH_3)_2]$ complexes show low activation free energies for the second halide substitution by Cys and Met Ls– between 16 and 21 kcal mol^{-1} – so that they are expected to give easily rise to the dihalide substituted complex *cis*-$[Pt(L)_2(NH_3)_2]$. However, while for the chloro species, *cis*-$[PtCl(L)(NH_3)_2]$, the barriers for ammine substitution are significantly higher than those for chloride, for the iodo species, in *cis*-$[PtI(L)(NH_3)_2]$, the barriers for ammine substitution are similar or slightly higher than those for iodide so that *cis*-$[PtI(L)_2(NH_3)]$ could be formed, although with a lower rate, with simultaneous release of ammonia.

The final product of dihalide substitution by cysteine or methionine MMs, for both chloro and iodo complexes, *cis*-$[Pt(L)_2(NH_3)_2]$, shows a very low barrier for ammine substitution by cysteine model, 14.7 kcal mol^{-1}, so that a very fast ammonia release – with half times of the order of milliseconds at physiological temperature – is expected, while a slightly higher barrier is calculated for ammine substitution by methionine model, 21.8 kcal mol^{-1}, so that a lower rate of ammonia release – with half times of the order of minutes at physiological temperature – is expected.

When the reaction of the histidine model molecule, imidazole, with *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$ is considered, our calculations indicate relatively low barriers for the first and second halide substitution (19-20 kcal mol^{-1}) which are expected to be quite fast, with reaction rates of the order of seconds at physiological temperature. However, while the chloro compound or its halide substituted *cis*-$[PtCl(L)(NH_3)_2]$ species show high barriers for ammine substitution – 26-28 kcal mol^{-1} – and vanishingly slow reactions rates, the iodido analogue and its halide substituted *cis*-$[PtI(L)(NH_3)_2]$ species show accessible barriers for ammine substitution – 23-24 kcal mol^{-1} – and low but observable rates. On the other hand, once the dihalide substituted species, *cis*-$[Pt(L)_2(NH_3)_2]$, is formed, it shows a high barrier for ammine substitution by imidazole, 27.3 kcal mol^{-1}, so that no ammine substitution is expected.

Inferences for the reactions of Pt(II) complexes with proteins

Our studies were first inspired by the comparative analysis of the crystal structures and ESI-MS spectra of the adducts of a few small model proteins with *cis*-$[PtCl_2(NH_3)_2]$and *cis*-$[PtI_2(NH_3)_2]$. We were surprised in detecting retention of the halide ligand in the case of the iodide derivative, a behavior that patently contradicted the current opinion concerning the reactivity of platinum complexes with biomolecules. ESI-MS studies on the binding of *cis*-$[PtI_2(NH_3)_2]$ at cyt c unveiled the presence of four bound metal fragments, i.e. $[Pt(NH_3)_2]^{2+}$, $[PtI]^+$, $[Pt(NH_3)I]^+$ and $[Pt(NH_3)I_2]$, upon incubation time of 72 hours. By assuming these fragments involved in square planar coordination with suitable protein sites, we may draw some hypothesis on the ligand substitution sequence leading to their formation. Indeed, $[Pt(NH_3)I_2]$ is the only fragment coordinated by one protein site, thus apparently suggesting that the NH_3 release is the first step in the binding of iodoplatin. However, the presence of both $[Pt(NH_3)_2]^{2+}$ and $[Pt(NH_3)I]^+$ fragments yielded by two subsequent substitutions, testifies that substitution of either two iodides or one iodide and one ammonia (whatever order) has to be occurred. Our computational investigation on simple models to reproduce the reactions of interest, is fully consistent with these experimental evidences, indeed a generalized labilization of ammonia in the case of *cis*-$[PtI_2(NH_3)_2]$ reacting with the considered molecular models L was clearly indicated.

More specifically, calculations showed how the activation free energy values for the (first) substitution of iodide and ammonia by Ls are <24 kcal/mol, thus, both processes can occur with estimated half-times of less than 3 hours which is well below the duration of the ESI-MS experiments, so that the reaction is under thermodynamic rather than kinetic control.

The calculated reaction free energy values are also in agreement with the experimental evidences. The reaction free energy values for the iodide substitution were estimated in the range –5 - +1 kcal/mol, thus approximately only 5-6 kcal mol^{-1} lower than those calculated for the corresponding ammonia substitution (Table 2). This result indicates that, although a higher fraction of iodide substitution product is predicted, a small fraction of ammonia substitution must be expected under a thermodynamic control of the process. Moreover, the specific environment in the protein coordination pockets may strongly affect the reactivity of complexes. In particular, it is well known that inside the protein cavities as well as on the protein surface the dielectric constant is generally rather lower (respectively <10 and around 20-30) than that of the water (about 80),[49] and this effect should reduce the stabilization of the leaving iodide anion but not that of the leaving neutral ammonia thus making ammonia substitution thermodynamically more favorable and stabilizing the binding of $[Pt(NH_3)I_2]$ and $[Pt(NH_3)I]^+$ fragments.

We can thus speculate that, the different coordination of *cis*-$[PtI_2(NH_3)_2]$ compared with cisplatin, when incubated with model proteins in the same experimental conditions, is strongly related to the features of the coordination pockets on the proteins and different reactivity for *cis*-$[PtI_2(NH_3)_2]$ might be expected depending on the specific coordination sites. This is the consequence of the presence of multiple coordination sites in the cytochrome c able to bind the platinum center, but it is also an independent indication that different fragments may be coordinated depending on the specific features of the binding site.

On the other hand, when cisplatin is considered, the substitution of chlorides in the reaction with molecular models L was estimated to show activation free energies much lower than for ammine substitution, by at least 5 kcal mol^{-1}, and a much more favorable thermodynamics, by more than 7 kcal mol^{-1} for first substitution, thus indicating that only chloride substitution may occur.

Notably, the hypothetical regiochemistry for the reaction of either *cis*-[$PtCl_2(NH_3)_2$] or *cis*-[$PtI_2(NH_3)_2$] with a protein hosting available CH_3SH, $S(CH_3)_2$, and IM sites was also correctly predicted by our calculations: the fraction of targeted sites in the trend IM > $S(CH_3)_2$ > CH_3SH is in full agreement with the observation that either cisplatin and its iodide analogue were found to be coordinated by Met65 and/or His18/His33 of cyt c, and by exclusively His15 of lysozyme.[13, 50]

Table 2. Reaction free energies ($\Delta G_{R\rightarrow P}$) and calculated half-time ($t_{1/2}$) for the first substitution of either halide or ammonia by molecular models L in *cis*-[$PtCl_2(NH_3)_2$] and *cis*-[$PtI_2(NH_3)_2$]. Half-time values are calculated by applying the Eyring equation (unitary transmission coefficient and T = 310 K). Reported energy values in kcal/mol.

L	*cis*-[$PtCl_2(NH_3)_2$]				*cis*-[$PtI_2(NH_3)_2$]			
	NH_3		Cl^-		NH_3		I^-	
	$\Delta G_{R\rightarrow P}$	$t_{1/2}$	$\Delta G_{R\rightarrow P}$	$t_{1/2}$	$\Delta G_{R\rightarrow P}$	$t_{1/2}$	$\Delta G_{R\rightarrow P}$	$t_{1/2}$
CH_3SH	8.4	8 days	-7.0	1.64 s	6.6	1.86 m	1.0	46 ms
$S(CH_3)_2$	7.3	5 days	-8.5	30.4 s	3.5	4.92 m	-0.6	1.0 s
IM	3.5	68 days	-12.8	21.2 m	2.3	2.48 h	-5.2	22.0 s

CONCLUSIONS

ESI-MS experiments and DFT calculations were performed to compare the reactivity of cisplatin and its iodide analogues toward protein targets. The reaction of *cis*-[$PtI_2(NH_3)_2$] with cytochrome c was investigated through a well-established ESI-MS protocol. This iodido complex, analogously to cisplatin, was found to target cyt c on histidine sites, however it is characterized by a peculiar reactivity in which not only the halide but also the ammonia ligand was replaced by available protein sites. DFT calculations corroborated such finding by showing that a significant labilization of the Pt-N bond occurred on *cis*-[$PtI_2(NH_3)_2$] because of the strong trans influence exerted by the iodide ligands. Calculations also indicated that the substitution of both iodide and ammonia by protein side chains may take place within some hours or less, whereas ammonia release from cisplatin would occur in

much longer times (in the order of days), thus explaining why the products of chloride substitution are the only ones experimentally detectable at physiological conditions. The regiochemistry of the protein side chains involved in either cisplatin or *cis*-[$PtI_2(NH_3)_2$] binding was also elucidated by the DFT investigation. Indeed, by assuming long times (>>2 hours) of incubation between the target protein and a solution of either platinum complexes, and, thus, under a thermodynamic control of the ligand exchange processes, DFT calculations clearly indicated the imidazole of histidine residue as the preferred protein sites, in agreement with several experimental evidences.

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DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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