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Ligand Substitution Reaction On Platinum(II) Complex With Bio-relevant Thiols: Their Kinetics, Mechanism And Bioactivity In Aqueous medium

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Abstract: The kinetics of the interaction between $[Pt(pic)(H_2O)_2](ClO_4)_2$, *cis*-diaqua(2-Aminomethylpyridine)platinum(II) perchlorate **1** and selected thiols (L-cysteine and N-acetyl-L-cysteine) has been studied spectrophotometrically in aqueous medium as a function of complex concentration as well as thiol, pH, and temperature at constant ionic strength. The observed pseudo-first-order rate constants k_{obs} (s^{-1}) obeyed the equation $k_{obs} = k_1[thiol]$. At pH = 4.0, complex **1** interacts with the thiols via two distinct consecutive steps. The first step is dependent while the second step is independent of ligand concentration in each case. The rate constants for the process are of the order: $k_1 \approx 10^{-3}$ $M^{-1}s^{-1}$ and $k_2 \approx 10^{-5}$ s^{-1}. The association equilibrium constant (K_E) for the outer sphere complex formation has been evaluated together with the rate constants for the two subsequent steps. Both the steps are ligand-assisted anation and the final step is the ring closure process. The activation parameters for both the steps were evaluated using Eyring's equation. The low $\Delta H^{\ddagger}_1$ = (34.91 ± 0.97 kJmole^{-1}) and $\Delta H^{\ddagger}_2$ = (29.10 ± 0.72 kJmole^{-1}) values and large negative values of $\Delta S^{\ddagger}_1$ = (-174.68 ± 2.18 $JK^{-1}mole^{-1}$) and $\Delta S^{\ddagger}_2$ = (-233.74 ± 2.4 $JK^{-1}mole^{-1}$) for both the anation steps were evaluated. On the basis of the kinetic observations, evaluated activation parameters and spectroscopic data, a plausible associative mechanism is proposed for both the processes. Antibacterial property on both gram positive and gram negative bacteria and anticancer property of complex **1** and its substituted complexes **2** and **3** on HeLa cells have been investigated. Complexes **1** to **3** show growth inhibition remarkably on bacteria. The same of them show anticancer activity near about 70% when compared to *cis*platin. The complexes bind to DNA and change its electrophoretic mobilization pattern in Agarose gel.

Key words: Kinetics and mechanism. 2-Aminomethylpyridine. L-cysteine. N-acetyl-L-cysteine. Associative mechanism, Anti-bacterial and anticancer property

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Introduction:

Since the last forty years *cis*platin has been routinely used as an anti-cancer drug[1-5] throughout the world with limited knowledge of its mechanism of action in biological system. Recently, few platinum based anticancer agents are clinically successful for the treatment of cancer. Majority of the metal based commercially available therapeutic agents for cancer treatment are Pt(II) complexes[6,7]. In order to understand the specific and selective role of the metal ions in biological system, thermodynamic and kinetic investigations are of interest [5, 8].

Owing to the high intracellular concentration (about 10 mM) of thiols, like L-cysteine and glutathione (GSH), it is assumed that most of the platinum complexes form adducts with sulfur-containing biomolecules before it reaches the DNA. Due to the soft nature of Pt(II), it forms stable adduct with soft basic nature of sulfur. Binding of platinum complexes with sulfur-donor ligands are responsible for the occurrence of toxic effects[8,9] such as nephrotoxicity, neurotoxicity, and gastrointestinal disorder, and the thiols are also supported to involved in resistance mechanism[10]. Binding of platinum complexes[9-12] with the thiols is kinetically favoured. The resulting Pt-S (thio-ether) bond may be terminated in the presence of DNA, i.e. N^7 atom of 5′-GMP can substitute the molecule of thio-ether. For this reason, it is believed that these Pt-S(thio-ethers)[13] adduct can act as drug reservoir in the body before interaction with DNA, i.e. they are suitable intermediates of the reaction of Pt(II) complexes and DNA.

The carrier ligand, 2-Aminomethylpyridine (pic) in [*cis*-$Pt(pic)(H_2O)_2](ClO_4)_2$ behaves as a σ donor and π-acceptor with Pt(II) centre, which facilitates substitution of the labile water molecules with biologically important ligands like thiols or thio-ethers. Simultaneously, the additional influences like steric and electronic effects are also considered for the mechanism and reaction rate. The influence of Pt(II) complexes on the growth of both bacteria and cancerous cells (HeLa) and the direct interaction between Pt(II) complexes with DNA have been investigated.

Experimental Section

Instrumentation

Conductivity measurements were made with a Systronics conductivity meter (Model 308), where the cell constant was calibrated with standard buffer solution. The pH measurements were done with the help of a Systronics digital pH meter (Model-335) with accuracy of ±0.01 U, calibrated with a Standard phosphate buffer solution (KH_2PO_4/Na_2HPO_4). The IR spectra were recorded on a Nicolet-IS-10 spectrometer (KBr disc, 4,000–400 cm^{-1}) and 400-100cm^{-1} was measured using CsI. The kinetic measurements were conducted on a Shimadzu UV 1601 spectrophotometer attached to a thermoelectric cell temperature controller (Model TCC-240A, accuracy ± 0.1°C). The development of a characteristic peak in the product complexes **2** and **3** at 240 nm was monitored as a function of time at different fixed temperatures. NMR was carried out in Bruker Ascend-400 using D_2O as solvent.

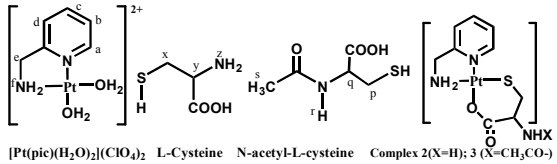
Chemicals, Complex Synthesis and Solutions

All the chemicals including L-cysteine and N-acetyl-L-cysteine were purchased from Sigma-Aldrich Pvt. Ltd. and used without further purification (purity 99.9%). For the biological assay, all reagents were purchased form Hi-media. The buffer solution (KH_2PO_4/Na_2HPO_4) was purchase from Thermo Fisher Scientific (USA) for calibration of pH-meter.

The complex $[Pt(pic)Cl_2]$ was prepared according to the literature method[14] with slight modification and elemental analysis gave; C, 18.78 %(19.23); H, 1.97 %(2.13); N, 7.41 %(7.48); and Cl, 9.47 % (9.67), where the numbers in parentheses were calculated from the chemical formula. The diaqua complex, $[Pt(pic)(OH_2)_2](ClO_4)_2$, **1** (where pic = 2-Aminomethylpyridine) was prepared in solution by the method of Hay and Basak[15]. The chloro complex $[Pt(pic)Cl_2]$ (100.0 mg; 0.267 mM) was converted into the diaqua analogue in solution by adding two equivalents of $AgClO_4$. Great care was taken to remove Ag^+ ions and the chloro complex to convert into the corresponding diaqua species. To ensure the complete removal of Ag^+ ion from the solution a slightly less amount of $AgClO_4$ (109.0 mg; 0.525 mM) was taken than the calculated amount (110.9 mg; 0.534 mM) for complete utilization of Ag^+ to remove the chlorides from complex **1**. It was kept overnight, and the AgCl precipitate was removed by filtration through a special pressure drive membrane (pore size 0.05 μm, GFC) filter (ultra filtration). Though the slight amount of chloro complex remains unchanged and that will be removed along with the AgCl precipitate. The strength of complex **1** solution is calculated on the basis of consumed amount of $AgClO_4$. To check the presence of eventual remaining of Ag^+ is done by adding dil HCl and no precipitate was found. The diaqua complex was characterized by elemental analysis[16], C=20.71 % (21.21), H= 2.81% (2.94), and N=8.08% (8.25), where the numbers in parentheses were calculated from the chemical formula. Electronic absorption spectrum of **1** in H_2O, (λ_{max}/nm($\varepsilon/M^{-1}cm^{-1}$): 272(4032). Selected IR frequencies (KBr disk, cm^{-1}): 3541-3456(br), 3400(s), 3231(s), 2955(s), 1650-1620(br), 1611(s), 1589(br), 1390(s), 1360-1080(br), 1089(s), 626(s) and 555(s).

^1H NMR (400 MHz, D_2O) of complex **1** (δ in ppm and J in Hz): δ 8.4 (d, J = 5.6, $1H^a$), δ 8.1 (t, J=4.8, 2.8, $1H^d$) δ 7.74 (t, J=7.6, 8.0, $1H^c$), δ 7.56^b (d, J= 6.4, 1H), δ 4.44 (s, $2H^e$), δ 2.26 (s, $2H^f$).

The ionic strength of the solutions was adjusted to 0.1 M with $NaClO_4$. Double distilled water was used in the preparation of all solutions. The pH of the solutions was adjusted at 4.0 by adding $NaOH/HClO_4$. The reaction products of both the ligands with complex **1** were prepared by mixing them in different ratios, viz. 1:1, 1:2, 1:3, 1:5 and 1:10 and then thermosetting them at 50 °C for 24 h. The absorption spectra of the resulting solutions were almost same, recorded at 240 nm for both L-cysteine and N-acetyl-L-cysteine ligands. The spectral differences between the product complexes **2** and **3** [*cis*-Pt(pic)(thiol)], with the substrate complex **1** are shown separately in (see in SI Fig. S1 and S2).



$[Pt(pic)(H_2O)_2](ClO_4)_2$ L-Cysteine N-acetyl-L-cysteine Complex 2(X=H); 3 (X=CH_3CO-)

Structures of Ligands and Complexes

The composition of substituted products in solution was determined by Job's method of continuous variation and the metal: ligand ratio was found to be 1:1 (see in SI Fig. S3 and S4) in both the cases. The solid products **2** and **3** were obtained by slow evaporation of respective reaction mixtures of L-cysteine and N-acetyl-L-cysteine with complex **1** separately. UV/Vis, FT-IR and NMR spectroscopic data of **2** are as follows : Electronic absorption spectrum in H_2O,(λ_{max}/nm($\varepsilon/M^{-1}cm^{-1}$): 284(4732): Selected IR frequencies (KBr disk, cm^{-1}): 3384(br), 2991(w), 1616(s), 1606(s), 1388(s), 1089(s), 630(s), 559(s), 420(br) and 450(br) and ^1H NMR (400 MHz, D_2O) (δ in ppm and J in Hz) : δ 8.51 (d, J = 5.2, $1H^a$), δ 7.94 (t, J = 8.0, 7.6, $1H^d$), δ 7.51 (t, J = 8.0, $1H^c$), δ 7.45 (t, J = 5.6, J=2.4, $1H^b$), δ 4.7 (br, $2H^z$), δ 4.43 (t, J=5.2, J=4.4, $2H^f$), δ 4.28 (s, $2H^e$), δ 3.24 (d, J= 4.45,$1H^y$), δ 3.24 (d, J= 3.35,$1H^x$), δ 2.88 (d, J = 4.0, $1H^x$):

Complex **3**: Electronic absorption spectrum in H_2O,(λ_{max}/nm ($\varepsilon/M^{-1}cm^{-1}$): 266(3932): Selected IR frequencies (KBr disk, cm^{-1}): 3398(br), 2995(br) 1622(s), 1587(br), 1425(br), 1370(s), 1330(s), 1093(s), 624(s), 553(s), 428(br) and 414(br).

^1H NMR (400 MHz, D_2O (δ in ppm and J in Hz) δ 8.51 (d, J = 5.2, $1H^a$), δ 8.03 (m, , $1H^r$), δ 7.45 (d, J = 6.4, $1H^d$), δ 7.59 (t, J = 6.8, $1H^c$), δ 7.39 (t, J=5.4, J=8,4, 4.5, $1H^b$), δ 4.69 (m, $1H^q$), 4.4 (s, $2H^e$), δ 3.24 (s, $1H^x$), δ 2.88 (t, J = 7.2, J=6.8, $1H^x$), δ 2.08 (s, $2H^f$), δ 1.96 (s, $3H^s$).

Kinetic Investigation

The conventional mixing technique was followed and pseudo-first-order reaction conditions were employed for all the kinetic runs. The progress of the reactions was followed by measuring the increase in absorbance at 240 nm, where the spectral difference between the complex **1** and the product complexes **2** and **3** is at a maximum. Plots of ln (A_α - A_t) versus time, where A_α and A_t are the absorbances at infinite time (after the completion of the reaction) and at time, for the interaction of the ligands (L-cysteine or N-acetyl-L-cysteine) with complex **1** were found to be nonlinear. The plot being nonlinear curved (Fig. 1) at

the initial stage and subsequently of constant slope, indicating that the reaction proceeds via two consecutive steps. From the limiting linear portion of the curve, values of $k_{2(obs)}$ were obtained. The $k_{1(obs)}$ values were obtained from plots of lnΔ versus time, where time is small (Fig. 2). Origin software was used for computational analysis. The method of Weyh and Hamm [17] was adopted to calculate the rate constants for the two consecutive steps. The rate data, represented as an average of duplicate runs, are reproducible to within ± 4 %.

In vitro antibacterial assay

Test microorganisms

Antibacterial activity of complex **1** and L-cysteine and N-acetyl-L-cysteine substituted complexes **2** and **3** were studied on both the gram positive (Bacillus subtilis) and gram negative (E.coli Dh5α) model organisms.

Determination of antibacterial activity by tube dilution method

Antimicrobial activity of complex **1** was tested by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by using tube dilution method. The set of 10 sterile tubes were prepared by dispensing the LB broth each tube to attain desired volume. The blank and control were maintained for each setup. These tubes were distributed with appropriate volume Pt(pic) complexes from the 1μM stock solution of tested compound. The final concentration of compounds ranges from 0.05 μM to 0.50 μM. 10 μl of diluted bacterial suspension was inoculated to each tube to give a final concentration of 5×10^5 CFU/ml (Jackie Reynolds et. al. 2005). The same steps were carried for three sets with appropriate blank and incubated at 37 ^oC at 120 rpm for 12 hours. Optical density at 600 nm was determined which represents the number of cells in each tube. Tetracycline was used as a positive control (Data not shown). All tests were performed in triplicate and minimal bactericidal growth was determined as inhibition by observing the absorption pattern in LB broth. The average values of the absorbance by Escherichia coli DH5α and Bacillus subtilis at 600 nm were considered and % of growth inhibition was calculated for plotting the graph by Graph Pad Prism software**.**

Antitumor Property

Cells and culture conditions

Human cervical carcinoma cell lines (HeLa) were used in this study. The cells were grown in DMEM (Hi-Media) containing 10% FBS (Hi-Media) and 1% of penicillin/streptomycin (50 IU/ml and 500 μg/ml), cells were cultured for two days at 37 ^oC in 5% CO_2 incubator.

In vitro cytotoxicity assay

Cell viability was investigated by using the MTT colorimetric assay [18,19]. Approximately 10,000 cells were added to each well of a 96-well plate. After 24 h of incubation, the cells were treated with desired concentration of Pt(pic) complexes. *Cis*platin [*Cis*gland from Gland Pharma Limited] used as positive control in the same [20]. The plates were incubated for 48 and 72 hrs. After that 20 μl MTT (5 mg/ml in PBS) was added to each well and incubated for another 3 hrs. Then the media was carefully removed and 150 μl of DMSO was added to each well to dissolve the blue formazan product. The absorbance of this product was measured at 540 nm, using ELISA plate reader (Stat Fax™® 2100 Microplate Reader, USA)[21].

Direct DNA interaction studies of Pt(II) Complexes by Agarose Gel Electrophoresis

Aliquots of 1–3 μg of plasmid PcDNA3 containing complex **1** and the other L-cysteine and N-acetyl-L-cysteine substituted complexes **2** and **3** were used for this study. Pt(pic) complexes and the other L-cysteine and N-acetyl-L-cysteine substituted Pt(pic) complexes were directly incubated with plasmid DNA in a final volume of 20 μl for 10 min at 37 ^oC. The reaction was terminated by the addition of 5 μl loading buffer consisting 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water. The plasmids were analyzed in 1% agarose gel.

Results and discussion

Spectroscopic Properties

UV/Vis and FT-IR spectra: UV/Vis and FT-IR have been used to determine the bonding nature and structures of the metal complexes. λ_{max} (272 nm) of (**1**) shifted to (284 nm) in **2** and λ_{max} (266 nm) in case of **3**. The structures of the complexes **2** and **3** were elucidated by comparing the IR spectra of the ligands and their corresponding complexes (see SI in Fig. S5-S9) [22-24]. νS-H str. frequency of L-cysteine and N-acetyl-L-cysteine at 2540 & 2538 cm^{-1} respectively disappeared in their corresponding Pt(II) complexes[25], proving that thiol-sulfur of L-cysteine and N-acetyl-L-cysteine coordinates with Pt(II) center. Moreover, (S,O) chelation has been suggested for Pd(II) and Pt(II) complexes with such ligands[26]. The OH stretching bands of the carboxylic group in the free ligands have low intensity, and appear at quite low wave numbers (1622(w) cm^{-1}) and are split into several components. A couple of strong bands, assigned to ν_{as} (COO) and ν_s (COO) appear in the spectra of **2** and **3** at around 1612 – 1587 cm^{-1} and 1390 – 1384 cm^{-1} respectively, as should be expected for a coordinated carboxylate group [22,23,27]. In the substituted products **2** and **3**, νPt-O band appears at 420 & 428 and νPt-S band at 450 and 414 cm^{-1} respectively. No change in the position of the strong band $\delta(NH_2)$ around 1606 cm^{-1} in **2** and **3** clearly indicates that nitrogen of the amino acids are not ligated site to the Pt(II) metal[28-30]. It is very difficult to assign all the vibrational frequencies but attempts have been made to specify characteristic frequencies which supports the (O, S) coordination of the ligands in their respective Pt(II) complexes.

Kinetic study: The acid dissociation constants pKa_1 (–COOH) and pKa_2 (–SH) of L-cysteine [31] are 1.71 and 8.35 at 25 ^oC respectively. Similarly, first pKa_1 and pKa_2 (-SH) value of N-acetyl-L-cysteine [32] are 3.24 and 9.52 at 25 ^oC respectively. Both L-cysteine and N-acetyl-L-cysteine exist mainly as a neutral species (LH) at pH = 4.0. The equilibria are:

$$LH^+_2 \overset{K'_1}{\rightleftharpoons} LH + H^+$$

$$LH \overset{K'_2}{\rightleftharpoons} L^- + H^+$$

Since the pKa_1 and pKa_2[33] of **1** are 5.82 and 6.83 respectively, we can assume that at pH 4.0, the reactant complex (**1**) exists as the diaqua complex ion. At constant temperature, pH 4.0 and fixed concentration of complex **1**, the $\ln(A_\alpha - A_t)$ versus time plots (Fig. 1) for different thiol concentrations were curved at the

initial stage and subsequently of constant slope. This indicates that the reaction proceeds through a two-step consecutive process. We suggest that in the first step, one water ligand from **1** is replaced by the thiol (-SH) group of both L-cysteine and N-acetyl-L-cysteine. The second step is a slow process, where another water ligand is substituted by carboxylate ($-COO^-$) group leading to ring closure. The rate constant for such a process can be evaluated by assuming the following scheme:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where A is the diaqua species **1**, B is the single substituted intermediate, and C is the final product **2** or **3**. Formation of C from B is predominant after some time has elapsed. To further characterize the product, complex **1** and the thiols were mixed in 1:1 molar ratio at pH 4.0 and a pale yellow solution was obtained in each case. At pH 4.0, the complex **1** exists as purely diaqua species. Since the complex **1** does not exist as di-μ-hydroxo dimer[34-36] at this pH, so di-μ-S- bridge Pt(II) dimer substituted products are not possible. Di-μ-sulfur bridge dimer[37-39] is possible only when the reaction is followed at higher pH> 6.5. Bridge dimer formation of Pt(II) complex depends on the carrier ligand (NH_3, Pic) and along with pH of the reaction medium. The carrier ligand, pic have the properties of σ donor and π acceptor, whereas in case of NH_3[37] have no such π accepting property. Moreover, nitrogen donor center of thiols L-cysteine and N-acetyl-L-cysteine is not considered in complex **2** and **3**, because the complex **1** does not react at pH 4.0 with dipeptides like Gly.Gly or only nitrogen donor biologically important molecules.

Evaluation of rate constant k_1

The A→ B step is dependent on ligand concentration. At a particular temperature, the rate constant $k_{1(obs)}$ for both the ligands(L-cysteine and N-acetyl-L-cysteine) with the complex (**1**) were obtained from plots of ln Δ (the variation of Δ is shown in the Fig. 1) versus time t. Typical plots are shown in Fig. 2 (See SI Fig. S10 and S11

for N-acetyl-L-cysteine) and are collected in Table 1. Sulfur atom of the thiols is good nucleophile towards the Pt(II) center and substitutes one water molecule, this associative path is fast and dependent on ligand concentration.

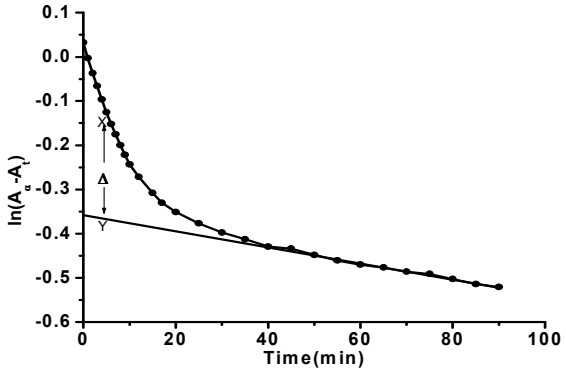


Fig. 1. A typical kinetic plot of ln (A_α - A_t) versus time(min): [complex **1**] = 2.43×10^{-4} M; [L-cysteine] = 2.43×10^{-3} M; Temperature = 25 °C

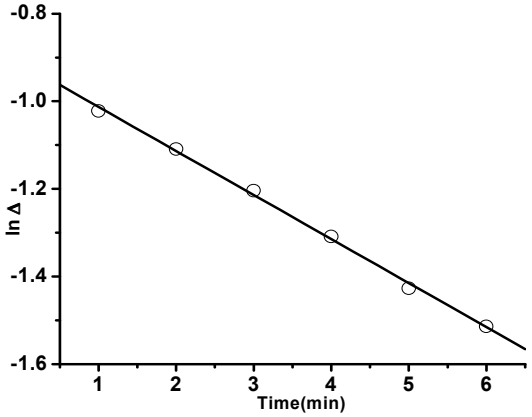


Fig. 2. A typical kinetic plot of lnΔ versus time(min), [complex **1**] = 2.43×10^{-4} M; [L-cysteine] = 2.43×10^{-3} M;Temperature = 25 °C

Table 1: 10^3 $k_{1(obs)}$ (s^{-1}) values at different [L-cysteine] and [N-acetyl-L-cysteine] at different temperatures. [Complex **1**] = 2.43×10^{-4} M, pH= 4.0, ionic strength= 0.1 M $NaClO_4$

10^3 M^{-1}[Thio	L-cysteine					N-ac-L-cysteine				
	Temp(°C)					Temp (°C)				
	25	30	35	40	45	25	30	35	40	45
2.43	3.41±0.03	3.84±0.08	4.41±0.05	5.30±0.06	6.70±0.05	2.32±0.07	3.00±0.03	3.86±0.07	4.16±0.04	4.40±0.06
3.64	3.22±0.07	3.64±0.04	4.31±0.04	5.58±0.04	7.07±0.08	2.50±0.09	3.43±0.07	3.790.05 ±	4.05±0.03	4.30±0.05
4.86	3.33±0.06	3.92±0.07	4.16±0.08	5.36±0.07	7.00±0.05	2.42±0.05	3.81±0.05	3.92±0.07	4.17±0.08	4.54±0.07
6.07	3.05±0.02	3.43±0.05	4.76±0.03	5.56±0.05	8.00±0.04	3.07±0.08	3.89±0.07	4.07±0.05	4.30±0.07	4.41±0.05
7.29	3.12±0.03	3.39±0.06	3.92±0.06	6.11±0.04	6.66±0.04	2.19±0.08	3.05±0.07	3.33±0.03	3.57±0.05	3.86±0.04
R^2	0.98	0.99	0.975	0.98	0.99	0.97	0.98	0.97	0.99	0.97

The rate constant $k_{1(obs)}$ for the A → B step was evaluated by the method of Weyh and Hamm [14] using the usual consecutive rate law (graphing software was avoided for calculation of k_1, as we

know that where the plots shows initial curvature, Weyh and Hamm method gives good results. But in other cases, we have used Origin 6.0 software for calculations). The rate constant $k_{1(obs)}$ for the A→ B step can be calculated by the method of Weyh and Hamm using the usual consecutive rate law;

$(A_\infty - A_t) = a_1 \exp(-k_{1(obs)}t) + a_2\exp(-k_{2(obs)}t)$ (6)

Or, $(A_\infty - A_t) - a_1 \exp(-k_{1(obs)}t) = a_2 \exp(-k_{2(obs)}t)$ (7)

Where, a_1 & a_2 are constants dependent on the rate constant and extinction coefficient. The value of $[(A_\infty - A_t) - a_1 \exp(-k_{1(obs)}t)]$ are obtained from X- Y at different time (Fig. 1) when time is small. So, $\Delta = a_1 \exp(-k_{1(obs)}t)$

Or, $\ln\Delta = \text{constant} - k_{1(obs)}t$

The $k_{1(obs)}$ values are derived from the slope of the lnΔ versus time (Fig. 2). Where, Δ is the difference of $\ln(A_\alpha - A_t)$ values between the observed and extrapolated part of the linear portion of $\ln(A_\alpha - A_t)$ versus time curve at any time 't'. A similar method of calculation was followed for each ligand concentration from 2.43×10^{-3} to 12.15×10^{-3} M range at constant [complex(**1**)] (2.43×10^{-4} M) at pH 4.0, μ = 0.1 M $NaClO_4$ and at different temperatures 25, 30, 35, 40 and 45 °C respectively. Reaction rate increases with the increase of [thiol] up to a limiting rate (Fig. 3), which is probably due to the completion of outer sphere association complex formation [40,41]. In the next step of the reaction, interchange of the ligands from the outer sphere to inner sphere occurs, i.e. ligand attacks at the metal centre through sulfur to give the intermediate complex (B). In second step, carboxylate oxygen ($-COO^-$) substitutes another water molecule and complete the ring closed final product (C). The ligand concentration dependence $k_{1(obs)}$ values can be explained in terms of rapid formation of inner sphere association complex between the reactant complex **1** and the thiol in A → B step. The following scheme can be proposed.

$$\text{Complex 1} + \text{thiol} \underset{}{\overset{K_E}{\rightarrow}} \underset{\text{(outer sphere complex)}}{\text{Complex 1.thiol}} \overset{k_1}{\rightarrow} \text{B} \overset{k_2}{\rightarrow} \text{C}$$

Scheme 1, where thiol = L-cysteine or N-acetyl-L-cysteine; B = sulphur chelated inner sphere intermediate, and C = final product(S, O chelated)

Again, k_1 is the rate constant for the formation of (B) and K_E is the outer sphere association equilibrium constant.

$d[B]/dt = k_{1(obs)}K_E[A][\text{thiol}]/(1+K_E[\text{thiol}])$,

$d[B]/dt = k_{1(obs)}[A]_T$

Where, subscript T stands for total concentration of complex (**1**). Thus it can be written as

$k_{1(obs)} = k_1K_E[\text{thiol}]/(1+K_E[\text{thiol}])$ (8)

The equation can be written as:

$1/k_{1(obs)} = 1/k_1 + 1/k_1K_E[\text{thiol}]$ (9)

The $k_{1(obs)}$ values thus obtained are also dependent on the studied ligand concentration range. However, studies at further higher concentration up to 12.15×10^{-3} M not followed the linearity (Fig. 3) (see in SI Fig. S12 for N-acetyl-L-cysteine). The $k_{1(obs)}$ values at different ligand concentration at different temperatures are presented in (Table 1) and $k_{2(obs)}$ are independent of concentration but increase with increase of temperature.

The plot of $1/k_{1(obs)}$ versus 1/[thiol] is found linear(Fig. 4) (see in SI Fig. S13 for N-acetyl-L-cysteine) with an intercept of $1/k_1$ and a slope of $1/k_1K_E$. This was found to be so at all temperatures studied. The k_1 and K_E values (Table 4) were obtained from the intercept and from the slope-to-intercept ratios. The first step forward rate constants k_1 are obtained from the intercept of the plot $1/k_{1(obs)}$ versus 1/[thiol] and simultaneously outer sphere association equilibrium constants (K_E) are also obtained from the slope of same plot.

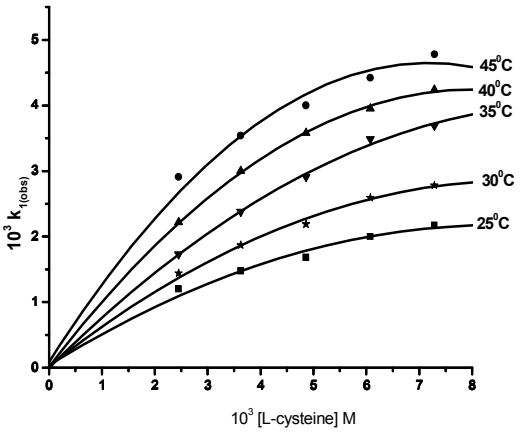


Fig. 3. Plot of 10^3[L-cysteine] versus $10^3k_{1(obs)}$ at different temperatures

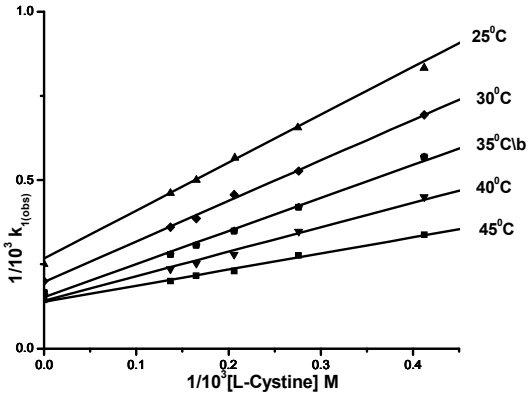


Fig. 4. Plot of $1/10^3k_{1(obs)}$ versus $1/10^3$[L-cysteine] at 25 °C, 30 °C, 35 °C, 40 °C and 45 °C

Evaluation of rate constant k_2

The B → C step is assigned to ring closure in which oxygen of carboxylate ($-COO^-$) group binds the metal centre. According to SHAB principle, Pt(II)-oxygen binding is more favourable than Pt(II)-nitrogen of $-NH_2$ group, this process is slower and independent of ligand concentration. At each temperature, the k_2 values were calculated from the limiting linear portion (when t is small) of the $\ln(A_\alpha - A_t)$ versus time curves and are collected in Table 2. Unlike k_1, k_2 was found to be independent of ligand concentration at each of the temperature studied. At particular temperature, the slope of $\ln(A_\infty - A_t)$ versus time plot for different ligand concentration was found to be constant in the region, the rate constant $k_{2(obs)}$ for B → C step can be evaluated from the method of Weyh and Hamm[14] using the consecutive rate law.

Table 2. $10^5 k_{2(obs)}$ (s^{-1}) values at different [L-cysteine] and N-acetyl-L-cysteine at different temperatures; [Complex **1**] = 2.43×10^{-4} M, pH= 4.0, ionic strength= 0.1 M $NaClO_4$

$10^3 M^{-1}$ [Thiol]	L-cysteine					N-acetyl-L-cysteine				
	Temp(^oC)					Temp (^oC)				
	25	30	35	40	45	25	30	35	40	45
2.43	3.41±0.03	3.84±0.08	4.41±0.05	5.30±0.06	6.70±0.05	2.32±0.07	3.00±0.03	3.86±0.07	4.16±0.04	4.40±0.06
3.64	3.22±0.07	3.64±0.04	4.31±0.04	5.58±0.04	7.07±0.08	2.50±0.09	3.43±0.07	3.79±0.05	4.05±0.03	4.30±0.05
4.86	3.33±0.06	3.92±0.07	4.16±0.08	5.36±0.07	7.00±0.05	2.42±0.05	3.81±0.05	3.92±0.07	4.17±0.08	4.54±0.07
6.07	3.05±0.02	3.43±0.05	4.76±0.03	5.56±0.05	8.00±0.04	3.07±0.08	3.89±0.07	4.07±0.05	4.30±0.07	4.41±0.05
7.29	3.12±0.03	3.39±0.06	3.92±0.06	6.11±0.04	6.66±0.04	2.19±0.08	3.05±0.07	3.33±0.03	3.57±0.05	3.86±0.04
R^2	0.98	0.99	0.975	0.98	0.99	0.97	0.98	0.97	0.99	0.97

The ligand concentration dependence $k_{1(obs)}$ can be explained by considering the scheme 3, involving the formation of an intermediate with increased coordination number.

Table 3: Rate constants for the $[Pt(pic)(H_2O)_2]^{2+}$ with different ligands at pH 4.0 and 0.1M $NaClO_4$

Temp. (^oC)	pH	L-cysteine				N-acetyl-L-cysteine			
25	4.0	$k_1\times10^3$ M	3.75±0.02	M K_E	156.86±5.50	$k_1\times10^3$ ($mol^{-1}.s^{-1}$)	2.22±0.01	M K_E	317.90±10.50
30			5.08±0.01		162.75±7.23		2.56±0.01		328.58±12.00
35			6.13±0.01		178.79±4.25		2.70±0.02		342.93±09.25
40			7.04±0.01		197.28±7.20		2.85±0.01		358.04±12.00
45			7.52±0.02		222.56±7.00		3.33±0.02		361.80±08.75
25		$k_2\times10^5$ (s^{-1})	3.22±0.14			$k_2\times10^5$ (s^{-1})	2.50±0.34		
30			3.64±0.24				3.43±0.42		
35			4.31±0.37				3.79±0.28		
40			5.58±0.32				4.05±0.28		
45			7.07±0.55				4.30±0.26		

Effect of Ionic Strength on the Reaction Rate

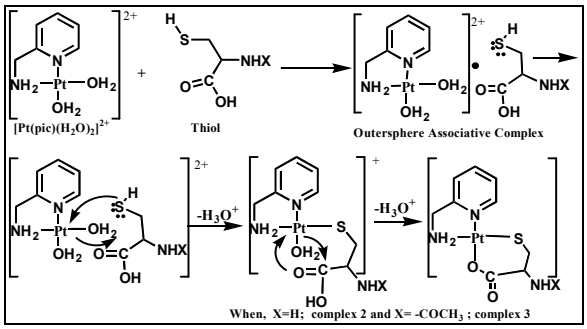
The individual complex formation reactions between complex **1** with the ligands L-cysteine and N-acetyl-L-cys are a function of the ionic strength. As expected, the rate of complex formation increases with increase of ionic strength (0.1 M $NaClO_4$) in aqueous medium for both the ligands. It was found that, at pH = 4.0, the rate of the reaction decreases with increase of ionic strength. It is assumed that a probable competition between the reactant ligands (each ligand individually with the Pt(II) complex) and ClO_4^- from inert salt ($NaClO_4$), for the metal center. An increase of concentration of the inert salt (0.1 M) affects the surrounding concentration in the vicinity of the Pt(II) center and consequently decreases the rate constant. No influence of pH variation was observed in the variable ionic strength experiments in our studied pH range.

Effects of pH and temperatures

The reactions were studied at four different pH values. The $k_{(obs)}$ values increased with increase in pH; at fixed concentrations of 2.43×10^{-4} M of [complex **1**], 2.43×10^{-4} M of [thiol] and 0.1 M of ionic strength the $10^3 k_{1(obs)}$ values at 30 ^oC were 0.55, 0.82, 1.23, 1.44 and 1.61 s^{-1} and $10^5 k_{2(obs)}$ values were 2.17, 2.83, 3.34, 3.84 and 4.14 s^{-1} at pH 2.5, 3.0, 3.5, 4.0 and 4.5, for L-cysteine and N-acetyl-L-cysteine respectively. The increase in rate may be explained based on the acid dissociation equilibria of the thiols and the complex. In the studied pH range, the complex exists as the diaqua species. The enhancement in rate is therefore explained by the deprotonation and increased donor ability of the thiols. The characteristic pH dependence for the substitution reaction can be theoretically explained by considering the following derived rate expression:

$$k_{obs} = k_a K_a [L]_{total}/(K_a + [H^+])$$

If we consider COOH is a donor center where, k_a, K_a and $[L]_{total}$ are the rate constant, acid dissociation constant of the L-cysteine and N-acetyl-L-cysteine's carboxylate groups and total thiol concentration, respectively. Consequent in kinetic study, the substitution reactions were followed at a constant pH of 4.0 to avoid complication from an additional parameter of $[H^+]$ in the rate equation. To study the effects of temperature, the reactions were studied at five different temperatures of different thiols concentrations and the anation rate constants for both the steps (k_1 and k_2) steps are given in Table 3. The activation parameters calculated from Eyring plots **(Fig. 5)** (see in SI Fig. S14-S16 for N-acetyl-L-cysteine) (R^2 for k_1 is 0.9865 and R^2 for k_2 is 0.9885) are given in Table 4 and compared with those for analogous systems involving the substitution in square planar Pt(II) complexes. On the basis of kinetic data (k_1, k_2 and K_E values) and activation parameters($\Delta H_1^{\ddagger}$ and $\Delta S_1^{\ddagger}$), the associative mechanism is proposed (**Scheme 3**) for both the ligand substitutions, which is also supported by Job's method(1:1 metal ligand ratio) and low $\Delta H_1^{\ddagger}$ and high negative $\Delta S_1^{\ddagger}$ values were found from the Eyring plot.



Scheme 3: plausible mechanism of the reaction between complex **1** with the thiols (L-cysteine and N-acetyl-L-cysteine)

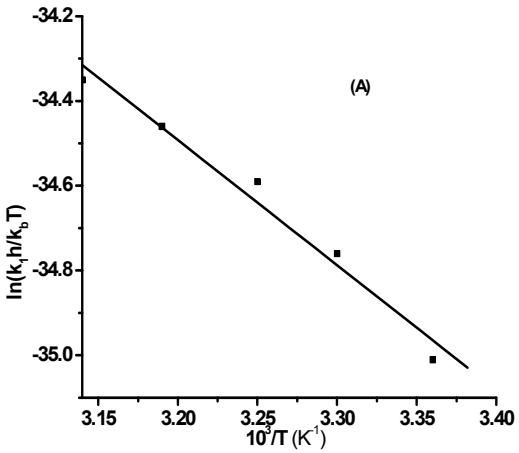


Fig. 5. Eyring plot for ($\ln k_1 h/k_B T$ vs. $10^3 1/T$) of L-cysteine

Table 4: Activation parameters for analogous systems

Systems	$\Delta H_1^{\ddagger}$ (kJ mol^{-1})	$\Delta S_1^{\ddagger}$ (JK mol^{-1})	$\Delta H_2^{\ddagger}$ (kJ mol^{-1})	$\Delta S_2^{\ddagger}$(JK mol^{-1})	Ref.
$[Pt(pic)(H_2O)_2]^{2+}$/ L-cysteine	34.91±0.97	-174.68±2.18	29.11±0.72	-233.74±2.4	This work
$[Pt(pic)(H_2O)_2]^{2+}$/ N-acetyl-L- cysteine	21.12±0.35	-294.25±1.05	19.45±0.47	-267.68±1.6	
$[Pt(phen)(H_2O)_2]^{2+}$/ Thiourea	41.0 ± 2.0	-41.0 ± 6.0	37.3 ± 0.7	-61.0 ± 2.0	[42]
$[Pt(en)(H_2O)_2]^{2+}$/ Thiourea	57.0 ± 3.0	-24.0 ± 1.1	36.0 ± 2.0	-69.0 ± 6.0	[43]
$[Pt(terpy)(H_2O)]^{2+}$/ Glutathione	23 ± 1	-116 ± 3	----	----	[44]
cis-$[Pt(pic)(H_2O)_2]^{2+}$ /Glutathione	52.37±2.10	-112.35±2.98	37.29±1.84	-130.12±3.16	[45]
[*cis*-$Pt(en)(H_2O)_2]^{2+}$/ L-aspargine	45.39±0.96	-116.98±2.9	33.78±0.51	-221.43±1.57	[46]
[*cis*-$Pt(en)(H_2O)_2]^{2+}$/ thiourea	61.90±1.6	-71±6	26.70±0.8	-186.80±2.7	[47]
[*cis*-$Pt(en)(H_2O)_2]^{2+}$/ L-thiosamecarbazide	35.60±0.8	-166±3	44.50±1.3	-182±4.0	[48]
[*cis*-$Pt(dach)(H_2O)_2]^{2+}$ /Glutathione	32.9±1.3	-187.20±4.2	30.50±0.1	-223.1±4.3	[49]
[*cis*-$Pt(dach)(H_2O)_2]^{2+}$ /DL-penicillamine	36.10±4.1	-175±12	44.4±1.1	-189±3.0	[41]

In vitro antibacterial activity

The results of in vitro antibacterial activities of Pt(pic) complex and its ligands L-cysteine and N-acetyl-L-cysteine substituted complex **2** and **3** are shown in graphs. The rate of growth inhibition of both kinds of bacteria is proportional to the concentration of the complexes in the media. L-cysteine and N-acetyl-L-cysteine substituted complex **2** and **3** do not show better inhibition compare to **1** alone. Gram negative Escherichia coli shows comparably less inhibition (SI Fig. S-17) than Gram positive Bacillus subtilis bacteria (SI Fig. S-18).

In vitro cytotoxicity activity

The cells were incubated for 48 h with continuous exposure of complex **1** and the other L-cysteine and N-ac- L-cysteine substituted complex **2** and **3** at 37 ^{o}C. The viability of Hela cells were then analyzed by MTT assay. As shown in graph, Pt(II) complexes are less toxic compare to *cis*platin. Like bacterial growth inhibition, L-cysteine and N-acetyl-L-cysteine substituted complex **2**, **3** and Complex **1** alone shows similar cytotoxic effect to HeLa cells (Fig. 6).

Direct DNA interaction studies of Pt(II) Complexes by Agarose Gel Electrophoresis

DNA interaction studies with the complexes were done by Agarose Gel Electrophoresis Lane 2-4 mobility of plasmid DNA is slightly retarded compare to Lane-1(only DNA). This result suggests that all the compounds including cisplatin have binding affinity to plasmid DNA (Fig. 7).

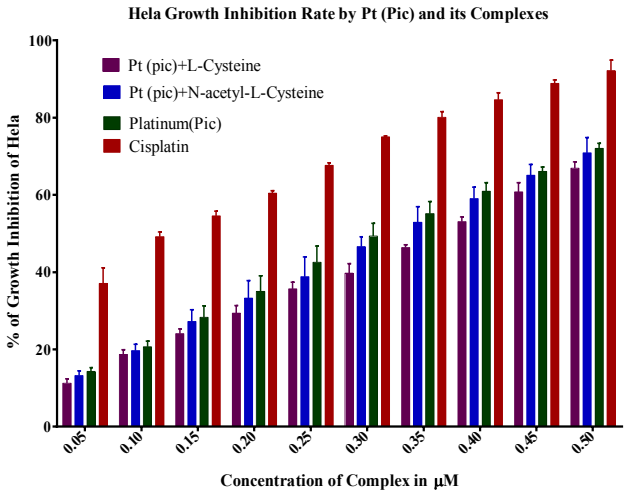


Fig 6. % of growth Inhibition of Hela cell in presence of $[Pt(pic)(H_2O)_2](ClO_4)_2$ and its L-cysteine & N-acetyl-L-cysteine substituted complexes from 0.05 µM to 0.5 µM concentration compared with cisplatin

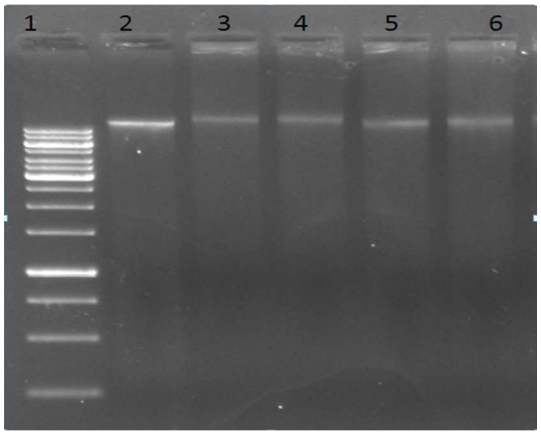


Fig 7. Agarose (1%) gel electrophoresis mobility pattern of pcDNA3, incubated at 37 °C for 5 minutes Lane-1;1kb DNA ladder; Lane-2;Plamid Control; lane-3; Complex (1); Lane-4; Complex (1) & N-Acetyl-L-Cysteine; Lane-5; Complex (1) & L-Cysteine; Lane-6; Cisplatin

Conclusion

The thiols, L-cysteine and its acetyl derivatives N-ac-L-cysteine both exist as a zwitter ion at pH 4.0 and have three potential donor centers (S, N and O) in their functional groups. In the substitution process, these ligands react through two consecutive steps, the first step is faster and bonded through sulfur and substitute one water molecule of the complex again the second step is the ring closure through carboxylic (COO^-) oxygen chelation process, which is slower than the first step and independent of thiols concentration. Substitution reaction rate of L-cysteine is faster than the N-acetyl-L-cysteine, most probably due to electromeric effect of acetyl substituent in substituted thiol, which slow down donor properties of sulfur and oxygen centers towards the metal. From the calculated activation parameters ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$), it is observed that low $\Delta H_1^{\ddagger}$ & $\Delta H^{\ddagger}_2$ and negative $\Delta S^{\ddagger}_1$ & $\Delta S^{\ddagger}_2$ values for the first and second steps (Table 4) suggest an associative mode of activation for the substitution process. The activation parameters imply a good degree of ligand participation in the transition state.

Complex **1** and other substituted complexes **2** and **3** are showing notable broad spectral bacterial inhibition property more or less 65 to 70 % in comparison with broad spectrum commercial antibiotic tetracycline. All these three Pt(II) complexes **1**, **2** and **3** also possess enough potentially anticancer property on Hela cells, which is 70 % in comparison with cisplatin. It is confirmed that these Pt(pic) complexes **1**, **2** and **3** interact directly with the naked DNA as similar as cisplatin. Hence there might be chances to sue these Pt(pic) complexes to treat tumour proliferations after clinical trials.

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