In Vitro and *In Cellulo* Anti-Diabetic Activity of Au^{I}- and Au^{III}-Isothiourea Complexes

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Abstract:

Gold complexes are known for different biological activities. Considering this precedent, and growing interest in developing metal-based enzyme inhibitors, we report here the dipeptidyl peptidase-IV (DPP-IV) inhibitory potential of cationic, and neutral chiral gold (I), and gold (III) isothiourea complexes. Colorimetric assay with recombinant DPP-IV enzyme was employed for initial screening. Kinetic based mechanistic studies were also performed for most active complexes. Efficiency of identified inhibitors in biological environment was assessed in *in cellulo* assay, using Caco-2 cell line. These complexes showed a good to moderate inhibition of DPP-IV with IC_{50} values in the range of 22.0 – 99.0 μM, as compared to standard inhibitor sitagliptin (IC_{50} = 0.033 ± 0.04 μM). It was observed that steric, and electronic properties of the isothiourea ligands have profound effect on the DPP-IV inhibitory activity of these complexes. To the best of our knowledge this study reports for the first time on isothiourea based gold complexes for the inhibition of DPP-IV enzyme. These results thus provide an approach for exploring new insights into the development of effective agents against diabetes using incretin-based therapy.

Key words: Gold complexes; Dipeptidyl peptdase-IV; Diabetes type 2; Caco-2 cells; Isothiourea; Bioinorganic chemistry

1. Introduction

Type II diabetes mellitus (T2DM) is termed as “disease of millenia”. It is the most prevailing disease worldwide. According to an estimation of the World Health Organization (WHO), 300 million people will suffer from diabetes in 2025 [1]. This alarming increase in the prevalence of diabetes deserves improved and effective strategies for the prevention, and cure of the disease. Management of diabetes requires a comprehensive and integrated knowledge, and understanding of various factors involved in the on-set, and progression of the disease. Involvement of multiple pathophysiological factors in the etiology of disease demands the use of combination therapy in most of the cases. Therefore, there is a perpetually increasing need of improved anti-diabetic agents, targeting novel aspects of diabetes [2].

Among the hypoglycemic agents, DPP-IV inhibitors are the leading class of oral anti-diabetic agents. Dipeptidyl peptidase-IV (DPP-IV) is an enzyme responsible for the instant cleavage and inactivation of incretin (glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide 1 (GLP-1) hormones. Specific DPP-IV inhibition can lead to an increased half-life of circulating gut hormones which results in increased insulinotropic effect, decrease plasma glucose, and improves impaired glucose tolerance [3,4]. DPP-IV inhibitors have better safety and tolerability over other conventional oral anti-diabetic agents. However, adverse effects, such as kidney failure and pancreatitis, associated with these inhibitors necessitates the development of better treatment options [5].

During the last few years, there have been substantial developments in the field of metallo-drug discovery. Metal ions and their complexes have been known to play a significant role in various molecular and cellular processes. Incorporation of metal ions has many advantages over conventional therapeutic agents [6]. Gold complexes are well known pharmaceuticals [7].The unique catalytic properties of gold complexes has attracted the attention of many scientists during current years, with significant established interest in gold catalysis, and its organometallic chemistry [8]. Auranofin, a gold (I)-thiolate drug, is used for the treatment of rheumatoid arthritis [9]. This drug has also been studied as a chemotherapeutic agent against triple negative breast cancer, and brain tumors [10, 11]. Gold complexes are well known for their role in biological processes, and are known to possess anti-arthritic [12], anti-cancer [13], anti-bacterial, anti-fungal [14], anti-leukemia [15], and anti-malarial properties [16]. Isothioureas are widely recognised as useful organocatalysts, while levamisole (Ergamisol) is a recognised medication used to treat

parasitic worm infections [17]. In the present study, we evaluated the DPP-IV inhibitory potential of isothiourea based gold (I), and gold (III) complexes.

2. Experimental

2.1. Material and Methods

All solvents i.e. DMSO (CAS 67-68-5; Scharlau, Spain), and ethanol (CAS 64-17-5; Merck Millipore, Germany) were of analytical grades, and used without further purification. De-ionized water was used for buffer preparation. Recombinant human DPP-IV (EC 3.4.14.5) was purchased from Prof. Dr. Mark D. Gorrell, Sydney, Australia. Tris-(hydroxymethyl)-aminomethane (reagent grade) was obtained from Merck, Germany. Colorimetric substrate Gly-pro-*p*NA was obtained from LeapChem Ltd (Hangzhou, China). Dulbecco's modified eagle medium (DMEM) was purchased from Caisson Labs, UT, USA.

2.2. DPP-IV Enzyme Inhibition Assay

Inhibition of DPP-IV enzyme will be monitored using colorimetric *in vitro* biochemical assay. This assay involves the cleavage of gly-pro-pNA by enzyme to release *p*-nitroaniline, which gives absorbance at 400 nm. Initially, 15 µL of DPP-IV enzyme (in assay buffer) at the final concentration of 25 mU/mL was pipetted into a 96-well plate containing test compound to make a final volume of 50 µL. Plate was left for an incubation of 20 min at 37 °C. Reaction was initiated by the addition of 50 µL of substrate dissolved in reaction buffer (Tris-HCl, pH 8.0). Cleavage of substrate by the DPP-IV enzyme will be monitored at 400 nm using Spectramax M5 (Molecular Devices, CA, USA). Relative absorbance units per unit of time was converted to the amount of the cleaved substrate per unit of time (µM/s) by fitting to the calibration curve of free substrate [18].

2.3. Assay Protocol for Kinetic Studies

To study different kinetic parameters, activity of enzyme was monitored at various concentrations of test substances in the presence of different substrate (gly-pro-*p*NA) concentrations (0.05, 0.1, 0.2, and 0.4 mM) at 400 nm using the same assay protocol as described above. All the experiments were performed in triplicates. The inhibition pattern was evaluated by Lineweaver–Burk plot, Dixon plot was constructed between inhibitor concentration [I] and 1/V*max* to determine the type of inhibition. K_i was determined using the curve fitting program, GraFit (Erithacus Software, UK).

2.4. *In Cellulo* DPP-IV Activity Assay

2.4.1. Cell Culture

Caco-2 cells (ATCC-HTB37), obtained from ATCC were routinely sub-cultured at 70-80% confluence, and maintained at 37 °C in a 5% CO_2 atmosphere in high glucose DMEM media containing 1% non-essential amino acids (GIBCO, Thermo Fisher Scientific, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Capricorn-Scientific, Germany).

2.4.2. *In Situ* Evaluation of the DPP-IV Inhibitory Effect of Gold Complexes

For the experiments, cells were seeded on 96-well plates with clear bottoms at a density of 1 x 105 cells/well. At confluence, spent media was discarded, cells were washed with sterile PBS, and treated with 50 µL of inhibitor or vehicle in HEPES buffer (20 mM, pH 7.4) for 1 h at 37 °C. Afterwards 50 µL of the substrate Gly-Pro-pNA (0.2 mM) was added and DPP-IV activity was recorded using a microplate reader (Molecular Devices, CA, USA) every 1 min for 60 min [19, 20].

2.5 Molecular Docking Studies:

The crystal structure of human DPPIV complexed with sitagliptin was downloaded from PDB. The protein was prepared prior to docking to assign partial charges, adding missing hydrogens and optimizing the heavy atoms and hydrogen *via* OPLS3e force field [21]. The gold complexes were sketched and optimized *via* Jaguar module of Maestro, Schrödinger [22]. The grid box on enzyme was defined by selecting one of the site points on the predicted allosteric binding site. The docking was carried out in standard precision mode keeping the enzyme rigid and ligands flexible *via* OPLS3e force-field [23, 24].

The docked complexes were further evaluated *via* rescoring with MMGBSA [25] to predict the binding energies i.e. ΔG_{bind} (Kcal/mol) by using the following equation:

$$\Delta G_{bind} = E\_{complex(minimized)} - E\_{ligand(minimized)} - E\_{receptor(minimized)}$$

where,

$E\_{complex(minimized)}$ = energy of protein–ligand complexes

$E\_{ligand(minimized)}$ = energy free ligands

$E\_{receptor(minimized)}$ = energy the free protein

2.6 Statistical Analysis

All the experiments were carried out in triplicate, and processed using SoftMax Pro 4.8 software (Molecular Devices, CA, USA). Results are expressed as mean ± standard error of mean. IC_{50} values were calculated using EZ-FIT enzyme kinetics software (Perrella Scientific Inc., NH, USA).

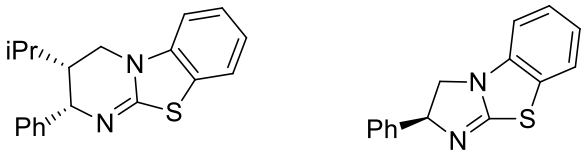
3. Results and Discussion

Isothioureas have been reported as biologically active class of compounds [26]. Heteroatoms in isothiourea structure provide chelating effect, and act as good ligands to facilitate complex formation [27]. There are examples in literature which showed enhanced biological activity of metal complexes as compared to their ligands [28]. Xie *et al* have reported the DPP-IV inhibitory activity of oxidovanadium complexes [29], but there are no reports on anti-diabetic effect of gold complexes targeting DPP-IV. Gasperini *et al*. have recently synthesized a range of novel isothiourea-gold complexes, and reported their biological activities against clinically important enzymes, such as β-glucuronidase, and phosphodiesterase-I. These compounds also showed cytotoxicity against MCF-7, and Hela cancer cell lines [30]. We, therefore, hypothesized that a fundamental study of these gold complexes against dipeptidyl peptidase-IV (DPP-IV) enzyme would be of significant interest for the management of type 2 diabetes mellitus.

3.1 In Vitro DPP-IV Inhibitory Activity of Gold-complexes

A series of cationic and neutral gold (I), and gold (III) complexes **1**–**18** were evaluated against human DPP-IV enzyme. These complexes exhibited a good to moderate inhibition of DPP-IV enzyme, with IC_{50} values in lower micromolar range (IC_{50} = 22.0 - 99.0 μM).

Detailed analysis of the DPP-IV inhibitory activity of these ligands indicated that the activity of these complexes is governed by the type of ligand and its absolute configuration, as well as the nature of the counterion. Both the ligand, and counterions play vital roles in the enzyme inhibitory activity of these complexes. Results of the present study are in good accord with the work of Xu et al., which showed that catalytic activity of gold (I) is affected largely by the nature of counterion, and ligands used [31]. During the current study the complexes derived from both enantiomers of two isothiourea Lewis bases, BTM and HyperBTM, were used (Figure-1).



(2*S*,3*R*)-HyperBTM (*R*)-BTM

Figure-1: Chiral isothiourea ligands used in the study.

The gold complexes evaluated in this study can be divided into the following three classes; cationic gold (I)-isothiourea complexes, neutral gold (I)–isothiourea complexes and gold (III)-isothiourea complexes:

3.1.1 Cationic gold (I)-isothiourea complexes

Compounds **1–12** (Figure-2) are cationic gold (I)-isothiourea complexes containing triflimide or tetrafluoroborate counterions. It is widely recognised that changes in structural or electronic properties around the active site of ligands can alter the enzyme inhibitory activities of the precursor metal compounds [32]. We have also observed that complexes coordinated with triphenyl phosphine (PPh_3) showed good enzyme inhibitory activity, as compared to their non-phosphine substituted analogues, such as compounds (*2R,3S*)-**1**, and (*2R,3S*)-**9** (IC_{50} = 99.0 ± 0.3 *vs* 59.13 ± 0.2 μM);and (*2S,3R*)-**3**, and (*2S,3R*)-**10** (IC_{50} = 66.53 ± 0.2 *vs* 37.46 ± 0.1 μM). This effect could be related to π-stacking and cation-π interactions between the phenyl phosphane ligand with the enzyme aromatic residues. It is stated in literature that the non-covalent to π-stacking, and cation-π interactions enhances activity and selectivity [33].

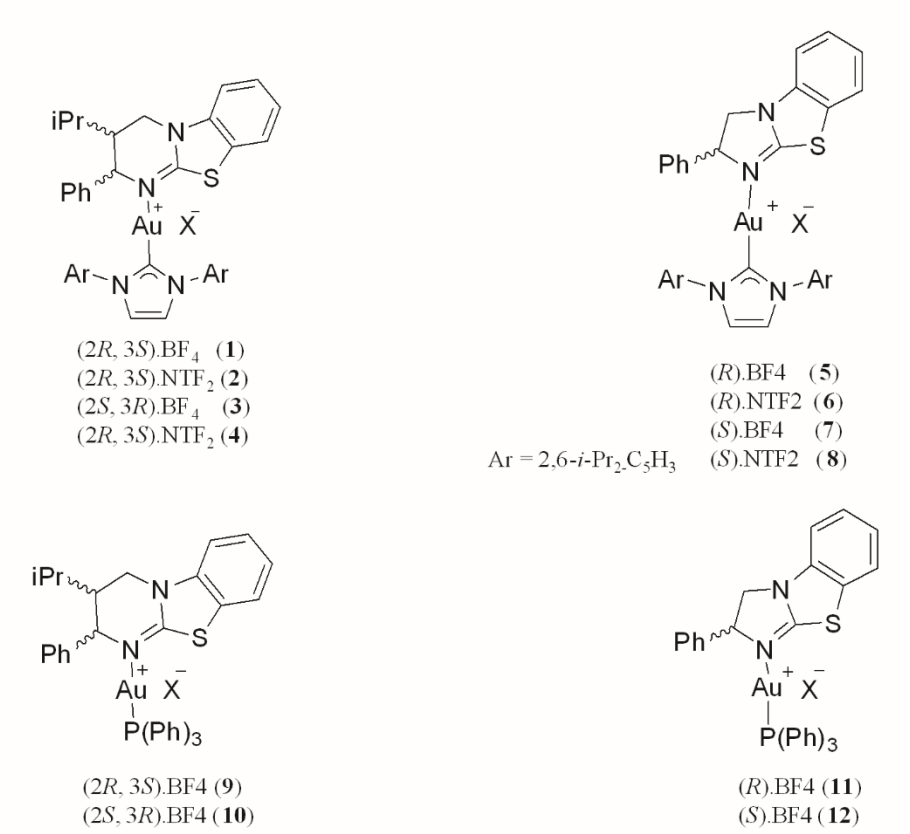
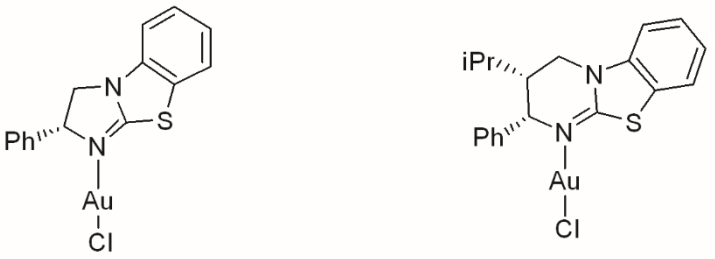


Figure-2: Cationic gold (I)-isothiourea complexes used in the study.

Interestingly, complexes (*R*)-**5** and (*R*)-**11**; and (*S*)-**7** and (*S*)-**12**, showed comparable enzyme inhibitory activity, indicating that the effect of the ligand (PPh_3 or NHC) is less pronounced when (R)/(S)-BTM was used as Lewis base (IC_{50} = 29.88 ± 2.0 and 22.6 ± 0.5; 23.47 ± 0.89 and 35.6 ± 1.0 µM, respectively). It is worthy to note the different activity of the complexes bearing BTM ((***R***)-**5**, (*S*)-**7**, (*R*)-**11** and (*S*)-**12** *vs* HyperBTM ((*2R,3S*)-**1**, (*2S,3R*)-**3**, (*2R,3S*)-**9** and (*2S,3R*)-**10**) isothiourea ligands, with the former showing IC50 lower than 35 µM while the latter being less active with IC_{50} values higher than 37µM (Table 1). This effect is presumably related to subtle differences in the steric environment associated with HyperBTM compared to the BTM isothiourea. It was also observed that the nature of the counterion significantly regulates the DPP-IV inhibitory activity of these complexes. Complexes (*2R,3S*)-**2**, (*2S,3R*)-**4**, (*R*)-**6**, and (*S*)-**8** with the triflimide counterion were inactive against DPP-IV enzyme, irrespective of the nature of the Lewis basic isothiourea ligand used.

3.1.2 Neutral gold (I)–isothiourea complexes

Neutral gold (I) complexes **13–16** (Figure-3) were evaluated against DPP-IV enzyme *in vitro*. The enantiomeric complexes (*R*)-**13** and (*S*)-**14** bearing BTM ligand showed a good DPP-IV inhibitory activity (IC_{50} = 45.88 ± 0.39, and 35.2 ± 0.9 μM, respectively) as compared to HyperBTM complexes (2*R*,3*S*)-**15**, and (2*S*,3*R*)-**16** with (IC_{50} = 81.8 ± 4.7 and 76.9 ± 3.7 μM, respectively). This trend is consistent with that observed for the cationic complexes **1 – 12**. Our results are in good accord with Inci *et al*., which suggested that the use of planar aromatic ligands would be of interest for the development of efficient DPP-IV inhibitors as anti-diabetic agents [34].



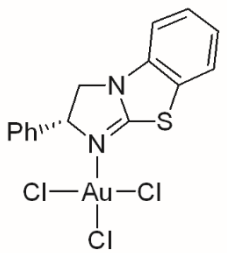
(R)- (13)
(S)- (14)

(2R, 3S)- (15)
(2S, 3R)- (16)

Figure-3: Structures of neutral gold (I) complexes **13** – **16**.

3.1.3 Gold (III)-isothiourea complexes

After the evaluation of series of gold (I) cationic, and neutral chiral gold complexes, DPP-IV inhibitory potential of the enantiomeric gold (III) complexes **(*R*)-**17 and (***S***)-18 was investigated (Figure-4). Only compound 18 showed a weak enzyme inhibitory activity (IC_{50} = 80.5 ± 1.5 μM). The increased Lewis acidity of the d^8 gold (III) center may have resulted in a weak inhibitory effect of the complex (Table 1).



(R)- (17)
(S)- (18)

Figure-4: Structures of neutral gold (III) complexes.

Table 1: DPP-IV Inhibitory Potential of gold complexes **1-18**.

Compounds	IC_{50}± SEM* (µM)
Cationic gold (I) complexes	
(2*R*,3*S*)-**1**	99.0 ± 0.3
(2*R*,3*S*)-**2**	NA**
(2*S*,3*R*)-**3**	66.53 ± 0.2
(2*S*,3*R*)-**4**	NA
(*R*)-**5**	29.88 ± 2.0
(*R*)-**6**	NA
(*S*)-**7**	23.47 ± 0.89
(*R*)-**8**	NA
(2*R*,3*S*)-**9**	59.13 ± 0.2
(2*S*,3*R*)-**10**	37.46 ± 0.1
(*R*)-**11**	22.6 ± 0.5
(*S*)-**12**	35.6 ± 1.0
Neutral gold (I) complexes	
(*R*)-**13**	45.88 ± 0.39
(*S*)-**14**	35.2 ± 0.9
(2*R*,3*S*)-**15**	81.8 ± 4.7
(2*S*,3*R*)-**16**	76.9 ± 3.7
Gold (III) complexes	
(*R*)-**17**	NA
(*S*)-**18**	80.5 ± 1.5

*IC_{50} Values are expressed as mean ± S. E. M. (Standard Error of Mean) where n=3; ** NA = not active

3.2 Mechanistic studies of gold (I) complexes

The most active cationic, and neutral gold (I) complexes (*R*)-**5**, (*S*)-**7**, (2*S*,3*R*)-**10**, (*R*)-**11**, (*S*)-**12**, and (*S*)-**14** were subjected to kinetic-based mechanistic studies to identify the type of inhibition. Mode of inhibition indicated a possible interaction of inhibitors in the active site of enzyme. Kinetic parameters indicated that complexes (2*S*,3*R*)-**10**, (*R*)-**5**, (*S*)-**7**, and (*S*)-**14** showed non-competitive type of inhibition, since K*m* remains unaffected whereas V*max* decreased. Mixed inhibition by complex (*R*)-**11** was indicated through altered values of both Km and V*max* demonstrate a binding of this complex with the enzyme at a site other than the substrate binding site with the catalytic residues of DPP-IV. An increase in the apparent affinity of the enzyme for the substrate (K*m*app> K*m*) in the presence of complex (*S*)-**12** was observed, whereas V*max* remains unaffected. This suggests that the inhibitor favours binding with the free enzyme, and inhibits DPP-IV activity in competitive manner (Table 2). Figure-5, and -6 represents the kinetic graphs for compounds (*S*)-**12**, and (*S*)-**14** respectively.

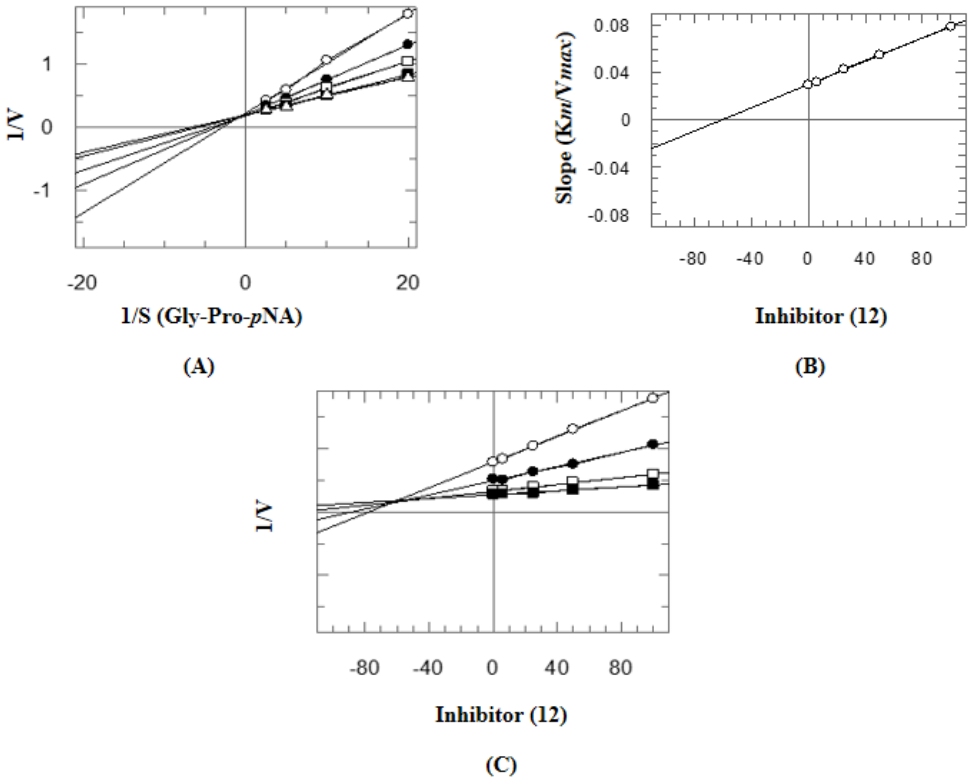


Figure-5: Steady state competitive inhibition of DPP-IV enzyme by inhibitor (*S*)-**12**. (A) Lineweaver–Burk plot between the reciprocal of 1/V*max vs* 1/substrate (gly-pro-*p*NA) in the presence of different concentrations of inhibitor, I = 100 µM (○), I = 50 µM (●), I= 25 µM (□), I= 6 µM (■), and in the absence of inhibitor, I = 0.00 µM (Δ). (B) Secondary re-plot from reciprocal plot for K*i* between slope *vs* [I], (C) Dixon plot, which further confirms the K*i,* and type of inhibition, which is competitive for compound (*S*)-12 against the DPP-IV.

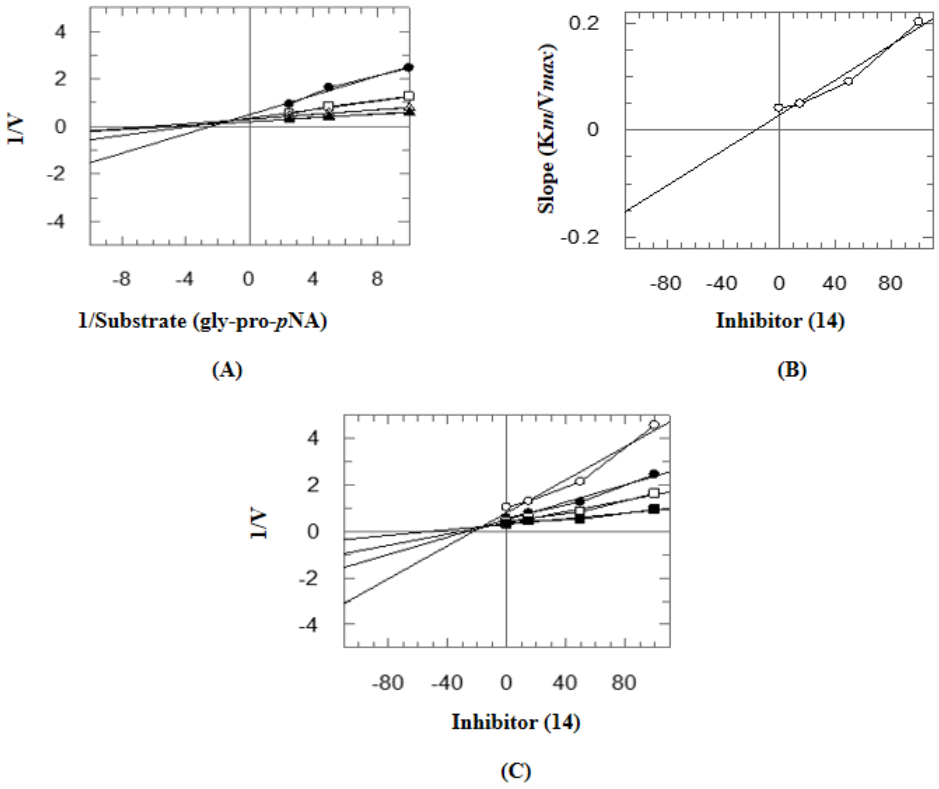


Figure-6: Steady state non-competitive inhibition of enzyme DPP-IV by (*S*)-**14**. (A) Lineweaver–Burk plot between the reciprocal of 1/V*max vs* 1/substrate (gly-pro-*p*NA) in the presence of different concentrations of inhibitor, I = 100 µM (●), I= 50 µM (□), I=15 µM (Δ), and in the absence of inhibitor I= 0.00 µM (▲). (B) Secondary re-plot from reciprocal plot for K*i* between slope *vs* [I], (C) Dixon plot, which further confirms the K*i,* and type of inhibition, which is non-competitive for compound (***S***)-**14** against the DPP-IV enzyme.

Table 2: Kinetics parameters for gold complexes.

Compounds	V*max* (µM/L/min)$^{-1}$	K*m* (mM)	V*max* app (µM/L/min)$^{-1}$	K*m* app (mM)	K*i* ± S.E.M. (µM)	Type of Inhibition
(***R***)-**5**	5.88	0.18	4.5	0.19	42.3 ± 0.001	Non-competitive
(***S***)-**7**	13.42	0.19	8.92	0.21	7.18 ± 0.004	Non-competitive
(***2S, 3R***)-**10**	9.35	0.23	16.35	0.19	35.89 ± 0.3	Non-competitive
(***R***)-**11**	11.75	0.24	7.45	0.48	7.4 ± 0.002	Mixed

(*S*)-12	5.03	0.25	4.99	9.8	59.2 ± 0.0002	Competitive
(*S*)-**14**	4.9	0.2	2.1	0.28	17.7 ± 0.01	Non-competitive

K*m* = Michaelis-Menten constant in the absence of inhibitor K*i* = dissociation constant of inhibitor, K*m* app = Michaelis-Menten constant in the presence of inhibitor, V*max* = the maximal velocity at which enzyme show catalysis per unit time, V*max* app = the apparent maximal velocity in the presence of inhibitor.

*Ki Values are expressed as mean ± S. E. M. (Standard Error of Mean) where n=3

3.3. *In Cellulo* DPP-IV Inhibition by Gold Complexes

Drug development using *in vitro* biochemical assays is often hampered by the lack of *in vivo* activity of identified leads due to factors, such as lower intracellular binding selectivity or membrane permeability [35]. We, therefore, have evaluated inhibitory potential of cationic, and neutral gold (I) complexes against DPP-IV enzyme in Caco-2 cell-based assay. Lower inhibitory potential of complexes in *in cellulo* assay, as compared to recombinant DPP-IV enzyme, might be attributed to complex biological environment in cellular assay. With the exception of complex (*S*)-**12** which showed the maximum inhibitory activity (IC_{50} = 20.5 ± 0.49 μM), all other complexes (*R*)-**11,** (*R*)-**13**, and (*S*)-**14** showed comparable inhibition of enzyme DPP-IV *in situ* cellular model, with IC_{50} values in the range of 45.8 – 53.6 μM (Table 3)

Table 3: Inhibitory activities of gold complexes in *in situ* Caco-2 DPP-IV inhibition assay.

Compound	IC_{50} ± S. E. M*(μM)
(*R*)·**11**	45.8 ± 1.83
(*S*)·**12**	20.5 ± 0.49
(*R*)·**13**	48.7 ± 5.4
(*S*)·**14**	53.6 ± 1.07

*IC_{50} Values are expressed as mean ± S. E. M. (Standard Error of Mean) where n=3

3.4. Molecular Docking Studies

Docking analysis was carried out for selected gold complexes. The compounds in DPP-IV inhibitory assay from subclass, cationic gold complexes were chosen *i.e.*, compounds **7**, and **11**.

The crystal structure of DPP-IV complexed with sitagliptin deposited in PDB (PDB ID: 1X70) was used for docking analysis. The compounds were docked into the predicted binding site of DPPIV

enzyme obtained *via* site map analysis. The docking score and predictive binding energies are presented in table 4.

Table 4: Molecular docking studies of selected gold complexes

Compounds	Docking Score kcal/mol	Predictive binding energy ΔG_{bind} kcal/mol
7	-5.3	-56.8
11	-2.9	-16.2

Compound **7** showed hydrogen bonding with Arg 358. While it made π-π interaction with Phe 357 (Figure-7). Compound **11** made π-cation interaction with Lys 554 of predicted allosteric site. (Figure-8).

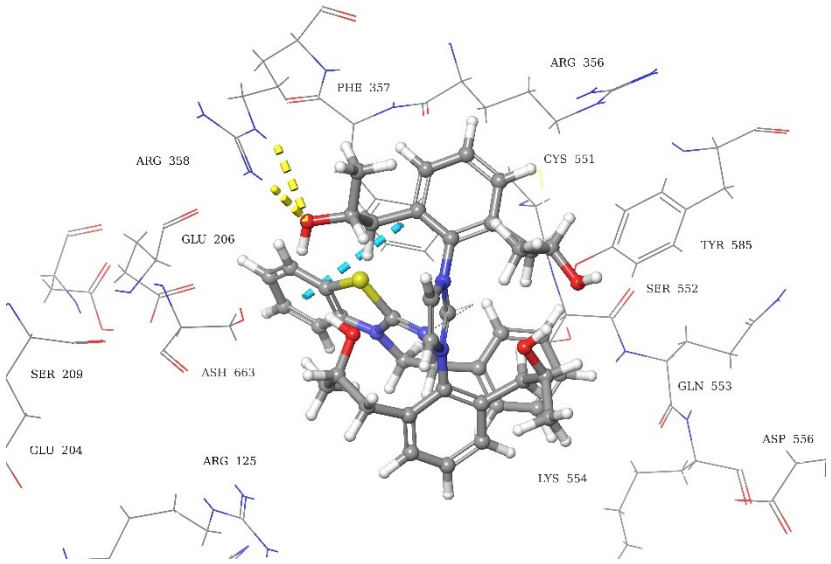


Figure-7: 3D ligand interaction diagram of compound **7** in the predicted allosteric site of DPPIV (PDB ID: 1X70). Hydrogen bonding is represented as yellow dashed line. π-π Interaction is represented as cyan dotted lines.

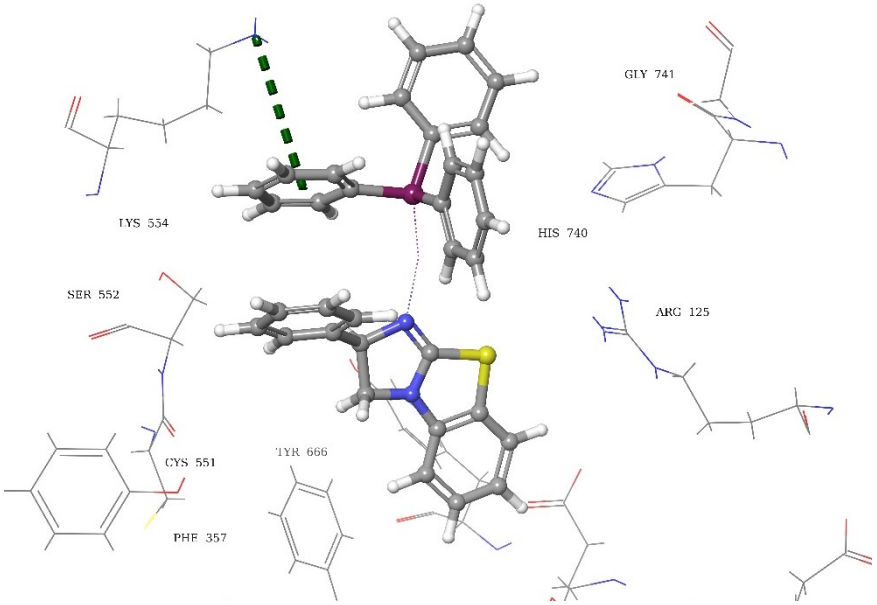


Figure-8: 3D ligand interaction diagram of compound **11** in the predicted allosteric site of DPPIV (PDB ID: 1X70). Dotted green line presents the π-cationic interactions.

These preliminary studies have demonstrated the anti-diabetic potential of gold (I), and gold (III)-isothiourea complexes through the inhibition of DPP-IV enzyme *in vitro*. These complexes will serve as leads for the development of DPP-IV inhibitors with improved activity. Our group further continues to investigate the biological interactions of different metal complexes inside the cell to better understand their biochemistry, and mechanism of action.

Conclusion

During the current study, it was found that both the ligand, and counterion play vital roles in the enzyme inhibitory activity. Triflimide isothiourea gold complexes were found to be inactive, irrespectively of the ligand used, whereas the presence of triphenyl phosphine in isothiourea gold complexes improved the activity. We have identified compound (*S*)-**12** as most promising complex of the series which showed good inhibition in *in cellulo* model. Inhibitory activity of these complexes is modulated by the electronic, and steric effects of the ligands. Moreover, the absolute configuration within the isothiourea might affect the activity of the complexes, with (*S*)-**12** being more active than (*R*)-**11**. Further studies are needed to fully disclose and understand the interactions of the different enantiomers with the enzyme. These results constitute the first report of gold

complexes capable of inhibiting DPP-IV in *in vitro*, and *in cellulo* models. Further research is currently underway to identify the mechanism of action, and to provide complexes with improved activity for incretin-based therapy.

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