Synthesis, Characterization of Gold (III) Complexes and an *in vitro* Evaluation of their Cytotoxic Properties

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***Abstract*— Three tetradentate Schiff base ligands and gold (III) complexes have been synthesized by a 2:1 molar condensation of salicylaldehyde with ethylenediamine and *o*-phenylenediamine. The ligands are N,N'-bis (salicylaldehyde) ethylenediamine (1), N,N'-bis (salicylaldehyde) *o*-phenylenediamine (2) and N,N'-bis (salicylaldehyde)-dimethylbenzene-1,2-diamine. The prepared ligands and their complexes have been characterized by elemental analyses, melting points, infrared spectroscopy and ^{1}H NMR. The spectral data of the ligands and their complexes are discussed in relation to structural changes due to complications. In vitro cytotoxic study results show that the gold complexes exhibit moderate activity and block the proliferation of WHCO1 cells with an IC_{50} range of 19.02 - 45.27µM, and IC_{50} range of 10.03 – 68.54µM for the WHCO6 lines.**

***Keywords*—** Schiff base ligands, Gold (III) complexes, characterization, anticancer

I. Introduction

OVER the last three decades several studies have been devoted to the evaluation of gold compounds as anti-tumor agents [1]-[3]. Gold (III) complexes, iso-structural and isoelectronic with platinum (II) complexes, are emerging as a new class of metal complexes with outstanding cytotoxic properties and are presently being evaluated as potential antitumor agents [4]. The strict relationship to platinum (II) compounds makes gold (III) complexes good candidates for development and testing as anticancer drugs. Since all the investigated compounds exhibited sufficient stability under physiological conditions, their cytotoxic properties were evaluated *in vitro*, via the MTT assay on the representative WHCO1 and WHCO6 oesophageal cancer cell lines. Remarkably, all these gold (III) complexes showed significant cytotoxic effects. The cytotoxic properties of the free ligands were also determined under the same solution conditions and the results are discussed here.

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Au (III) coordination chemistry is relatively scarce in the literature. Gold (III) is difficult to work with because it is unstable to reduction; it readily undergoes hydrolysis reactions, and Au (III) complexes often precipitate as $AuCl_4^-$, $AuCl_2^-$, or mixed $AuCl_4^-/AuCl_2^-$ salts that exhibit limited solubility and prove to be difficult to characterize. Reports of gold (III) complexes in the literature show them to be predominantly square planar in geometry, with a variety of ligands coordinated to the metal center [5]. This is because they possess the same electronic configuration (d^8). Ligand exchange kinetics is relatively faster in gold (III) complexes than in platinum (II) complexes. Thus, the strict relationship to platinum (II) compounds rendered gold (III) complexes attracting candidates for development and testing as potential anticancer drugs [6]. Schiff base ligands have been reported to form gold (III) complexes [7]-[11]. Metal complexes of the ligands that have been prepared here have only been reported for nickel, copper and zinc [12].

II. Methodology

All manipulations were carried out under an atmosphere of nitrogen or argon using standard Schlenk techniques. All other glassware was thoroughly dried at 210ºC for at least four hours prior to use. Anhydrous magnesium sulphate or sodium sulphate were used for drying reaction solutions. Melting points were determined on the Kofler Hotstage microscope (Reichart Thermovar) and are uncorrected. Microanalysis data were obtained using a Carlo Erba EA1108 elemental analyzer. Infrared Spectra were recorded on a Perkin-Elmer 1000 FT-IR spectrometer, as Nujol Mull for solids. All data are given in wavenumbers (cm^{-1}). ^{1}H NMR spectra were recorded on a Varian Unity-400 instrument. Mass spectra (MS) were recorded on a Waters atmosphere pressure ionization quadruple time-of-flight (API Q-TOF) Ultima (electronspray ionization (ESI), 70 eV) and/or SA VG70-SEQ (Fast atom bombardment (FAB), 7 kV) instrument.

*A. General procedure for the preparation of the Schiff base ligands (**1** – **3**)*

Salicylaldehyde (0.90 ml, 8.45 mmol), EtOH (30 ml), and the appropriate amine (0.30 ml, 4.49 mmol) were refluxed for 2 hours and cooled to room temperature. The precipitate was further cooled at -15°C for 2 hours. The precipitate was filtered on a Buchner funnel and washed with cold EtOH (3 x 10 ml). The residue was dried at 50°C *in vacuo*, to yield the Schiff base ligand.

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*1) Ligand **1** N,N'-bis(salicylaldehyde)-ethylenediamine*

Yellow shiny crystalline solid. Yield 85%. Anal. Calcd. for $C_{16}H_{16}O_2N_2$: C,71.64 %; H, 5.97 %; N 10.44 %. Found: C 71.15%; H 5.98%; N 10.26%. M.p.:120-122°C. ^{1}H NMR: (400 MHz, d_6-DMSO): 13.00 (2H, br s), 8.40 (2H, s), 7.30 (4H, dd), 6.95 (2H, d), 6.85 (2H, t), 3.95 (4H, s). IR (Nujol): 1634 (vs), 1581 (w), 1498 (s), 1461 (s), 1284 (s), 940 (m), 806 (w).

2) Ligand 2 N,N'-bis(salicylaldehyde)o-phenylenediamine

Orange powder. Yield 90%. Anal. Calcd. for $C_{20}H_{16}O_2N_2$: C,75.94%; H, 5.06%; N 8.86%. Found: C 75.68%; H 5.08%; N 8.73%. M.p.:180-183°C. ^{1}H NMR: (400 MHz, d_6-DMSO): 12.95 (2H, br s), 8.95 (2H, s), 7.60 (2H, d),7.40 (4H, dd), 6.85 (4H, dd). IR (Nujol):1613 (s), 1584 (w), 1561 (w), 1462 (vs), 1370 (s), 1276 (w).

3) Ligand 3 N,N'-bis(salicylaldehyde)-dimethylbenzene-1,2-diamine

Red crystalline powder. Yield 80%. Anal. Calcd. for $C_{22}H_{20}O_2N_2$: C,76.72%; H, 5.85%; N 8.13%. Found: C 76.62%; H 5.65%; N 8.25%. M.p.:190-193°C. ^{1}H NMR: (400 MHz, d_6-DMSO): 12.95 (2H, br s), 8.95 (2H, s), 7.60 (2H, d),7.40 (4H, dd), 6.85 (4H, dd). IR (Nujol):1625 (s), 1585 (w), 1555 (w), 1440 (vs), 1365 (s), 1270 (w), 1230 (w).

B. General procedure for the preparation of the Gold (III) complexes (4–6)

To a solution of the appropriate ligand (1–3) in dry CH_2Cl_2 (10 ml) was added an equimolar amount of [$NaAuCl_4.4H_2O$] dissolved in dry EtOH (10 ml). The reaction was allowed to stir in an ice bath overnight before reducing the solvent to *ca* 5 ml and precipitating the products using hexane. The crude solid was dissolved in hot MeOH and recrystallized from benzene, affording crystalline complexes which were washed with Et_2O and dried under vacuum.

*1) Complex **4***

Reddish brown solid. Yield 60% Anal. Calcd. for $C_{16}H_{14}O_2N_2AuCl$: C,38.53%; H, 2.83%; N 5.62%. Found: C 38.19%; H 2.91%; N 5.56%. M.p.:145-148°C. MS (EI, m/z): 463.26, $[M\text{-}Cl]^+$. ^{1}H NMR: (400 MHz, d_6-Acetone):

2) Complex 5

Dark brown solid. Yield 65% Anal. Calcd. for $C_{20}H_{14}O_2N_2AuCl$: C,43.95%; H, 2.56%; N 5.12%. Found: C 43.59%; H 2.81%; N 5.56%. M.p.:165-168°C. MS (EI, m/z): 511.38, $[M\text{-}Cl]^+$. ^{1}H NMR: (400 MHz, d_6-Acetone): 8.39 (2H, d), 7.94 (4H, q), 7.63 (4H, q), 7.35 (2H, d), 7.20 (2H, t).

3) Complex 6

Dark red solid. Yield 65% Anal. Calcd. for $C_{22}H_{18}O_2N_2AuCl$: C,45.97%; H, 3.16%; N 4.87%. Found: C 45.19%; H 3.81%; N 4.56%. M.p.:195-198°C. MS (EI, m/z): 539.38, $[M\text{-}Cl]^+$. ^{1}H NMR: (400 MHz, d_6-Acetone): 7.52 (2H, d), 7.45 (2H, d), 7.35 (4H, dd), 7.04 (2H, d), 6.92 (2H, d), 1.81(6H, t).

C. Cell culture

All cell lines were maintained at 37ºC, in a 5% CO_2 humidified incubator.

1) Sub-culturing

All cell lines were sub-cultured by removing media, rinsing once with 2 ml trypsin, then trypsinising with 3 ml trypsin. Once cells had rounded, 3 ml of media were added to neutralize the trypsin, and cells were centrifuged out of the trypsin/media solution. Cells were then re-suspended in fresh media and a portion added to a new dish.

2) Freezing and thawing cells

A confluent dish was trypsinised, neutralized with fresh media, then the cells were centrifuged out of the trypsin/media solution. Cells were re-suspended in 3-4 ml of freezing media (90%) complete culture medium, 10% DMSO), and aliquoted into cryotubes (1 ml per tube). Tubes were kept at -70°C overnight and then transferred to liquid nitrogen.

D. Screening

1) Crystal violet assay

Media was removed from the cells in the culture dish before rinsing with trypsin, and trypsinising (standard procedure). Cells were quenched with trypsin before spinning them down. The cells were then re-suspended in fresh media, squirting up and down to get a single cell suspension and a cell count was done, to enable us to determine the final volume of cell suspension to get 15,000 cells per 90 µl DMEM. A multi-channel pipette was used to transfer 90 µl of the cell suspension into the microplate wells. For each concentration of the test drug – high (50 µg/ml), medium (10 µg/ml) and low (1 µg/ml), cells were plated in duplicate. 2 wells each for a media blank and for the untreated control were also included. The presence of cells in each well was confirmed by viewing under the microscope and cells were incubated for 24 hours at 37°C to allow them to settle. The test drugs were prepared at 10X in the concentration range to be tested in 10 µl of DMEM with a final concentration of 0.2% DMSO. The 10 µl drugs in the correct dilutions were then added to the cells making a final volume of 100 µl in each well before incubating at 37°C for 48 hours. Plates were observed and cell morphology and survival recorded at 24 and 48 hours respectively. Media in wells was then discarded. 100 µl methanol was added to each well (fixes the cells), and left for 10 minutes before discarding the methanol. 50 µl crystal violet solution (DNA stain) was added to each well and left for 20 minutes. The crystal violet was discarded and the wells rinsed thoroughly but gently with H_2O, being careful not to lift the cells. The dish was drained out by placing face down on a paper towel for a few min. 50 µl of 50% acetic acid solution was added to each well, to solubilize the crystal violet, and left for 4 hours. The plates were then read on a micro-plate reader at 595 nm.

2) MTT assay

Media was removed from cells and cells rinsed with trypsin *ca* 1-2 ml and trypsinisinisation was for *ca* 1 minute before sucking off the trypsin. 2 ml trypsin was added and quenched with an equal volume of media before spinning down the cells for 5 minutes at 1000 rpm. The pellet was re-suspended in 1 ml of fresh media squirting up and down to get a single cell suspension. A cell count using a haemocytometer was done in order to determine the final

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volume of the cell suspension needed to get 1500 cells per 90 µl DMEM. A multi-channel pipette was used to transfer 90 µl of cells into a 96 well plate. 3 wells for the untreated cells and 3 wells for the media blank at 100 µl/ well were also included. The presence of the cells in each well was confirmed and cells were incubated at 37°C for 24 hours to allow them to settle. The drugs at 10X the concentration range to be tested in 10 µl DMEM with a final concentration of 0.2% DMSO were prepared. 10 µl samples in correct dilutions of the drugs were then added to the cells making the total volume to be 100 µl in each well. The cells were then incubated for 48 hours at 37°C. 10 µl MTT reagent was added per well and cells incubated for 4 hours at 37°C. 100 µl of solubilization solution was added into each well and cells incubated at 37°C for 24 hours before reading the plates at 595 nm on a micro-plate reader.

3) IC_{50} data analysis

The resulting dose response curves were analyzed by non-linear regression analysis using GraphPad Prism version 4.00 for Windows to yield IC_{50} values which are specific for the compound for the particular cell line.

III. Results and Discussion

The three ligands (1-3) were readily prepared in good yields by the condensation of salicylaldehyde and ethylenediamine (1), salicylaldehyde and *o*-phenylenediamine (2), salicylaldehyde and N,N-dimethylbenzene-1,2-diamine respectively, with a 2:1 molar ratio. 1 is bright yellow crystalline material and 2 an orange powder and 3 a red crystalline powder, Fig. 1. The prepared ligands are soluble in MeOH, DMSO, CH_2Cl_2 and EtOH, and insoluble in nonpolar solvents e.g. *n*-pentane. The ligands are tetradentate in nature having an N_2O_2 donor site capable of complexing transition metal ions.

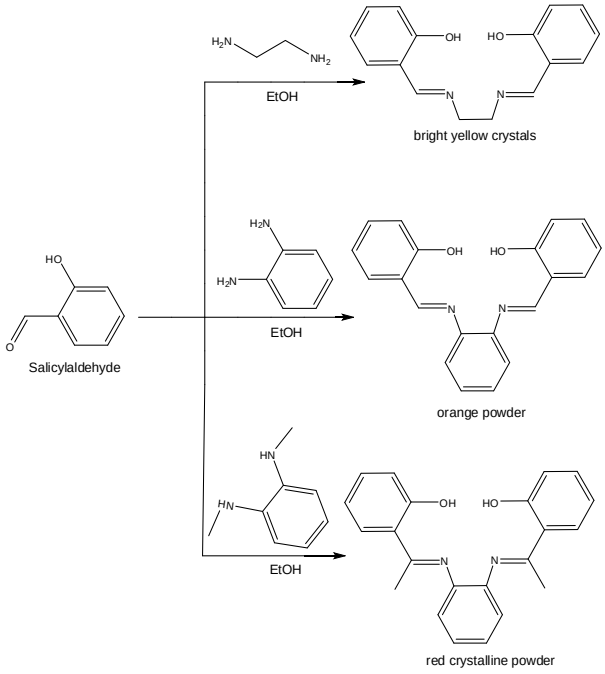

Fig. 1 - The synthetic preparation of Ligands 1-3

The complexes were prepared from equimolar amounts of the ligands and gold salt in ethanol in an ice bath, Fig. 2. The elemental analysis of the Schiff bases are consistent with calculated results from the empirical formula of each compound.

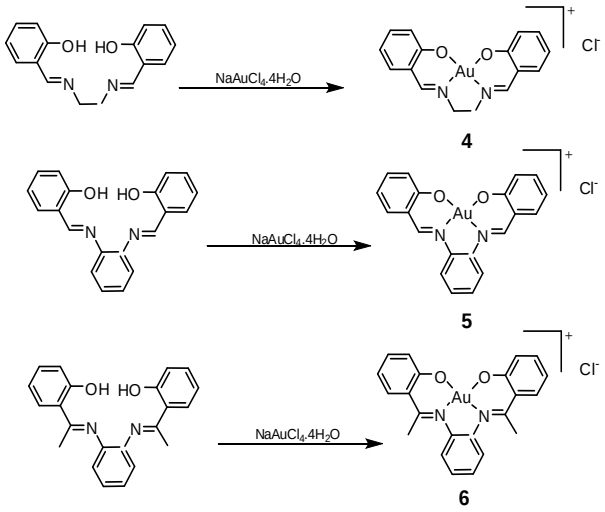

Fig. 2 - The synthetic preparation of analogous complexes

A. *IR-spectra*

The characteristic frequencies observed for the ligands at 1634 and 1613 cm^{-1} can be attributed to the C=N stretching vibration. In the gold (III) complex, these frequencies are slightly shifted to higher frequencies (15-40 cm^{-1}) suggesting that the ligands coordinate with the metal ion through the N_2O_2 donor atom set of the tetradentate Schiff bases. The ring skeletal vibrations (C=C) occur between 1584 and 1462 cm^{-1} and are unaffected by complexation. The phenolic stretching vibrations appeared at *ca*. 1275 cm^{-1} in the ligands and undergo a shift towards higher frequencies (40-60 cm^{-1}) in the complexes.

B. *^{1}H NMR-Spectra*

The ^{1}H NMR spectra of the compounds were carried out in d_6-DMSO or d_6-Acetone. The chemical shift observed for the OH protons in Fig. 3 (13.1 ppm) is not observed in the gold complex and this confirms the bonding of oxygen to the gold ion. The presence of a sharp singlet for the –C(CH)=N proton in Fig. 4 (8.9 ppm) indicates that the magnetic environment is equivalent for all protons, suggesting the presence of a planar ligand in the complexes. The multiplets of the aromatic protons appeared within the range 6.9 to 7.6 ppm and are not affected by chelation.

C. *Preliminary Screening*

A total of three gold (III) complexes have been screened together with three free ligands. Samples were tested against WHCO1 and WHCO6 oesophageal cancer cell lines at three different concentrations and the number of cells remaining after 48 hours of treatment were assessed using the crystal violet assay.

1) Crystal violet assay

This is a simple assay useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes. The dye, crystal violet stains DNA and protein. In essence there, there is more DNA to stain when the cells thrive and multiply than when they are killed by the drug. On final solubilization, the amount of dye taken up by the mono-layer i.e. the DNA of the cells on the dish can be quantified in a spectrophotometer or plate reader.

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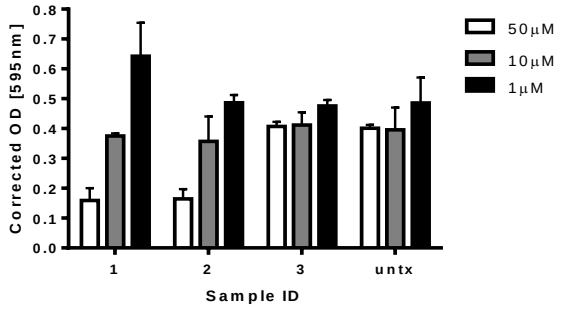

Fig. 3. Screening data against the oesophageal cell line WHCO1 by the free ligands

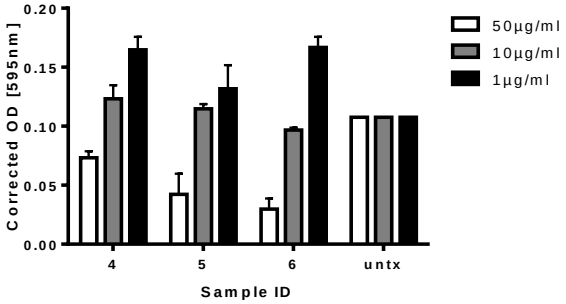

Fig. 4. Screening data against the oesophageal cell line WHCO1 by the Gold (III) complexes

Samples were tested against WHCO1 oesophageal cell line at three different concentrations and the number of cells remaining after 48 hours of treatment were assessed using the crystal violet assay. Plates were processed using crystal violet staining, which is a protein stain- the number of cells remaining after treatment stain blue, and when the stain is solubilized in acetic acid, the absorbance gives a reading proportional to the number of cells remaining.

From the crystal violet data that was obtained it was seen that all the gold (III) complexes together with the free ligands did exhibit killing properties and hence further investigation was indeed needed.

2) IC_{50} determination

The cells are cultured as usual, then subjected to a "treatment". At the end of the treatment period, the MTT assay is then used to check cell survival, viability or proliferation. MTT can be metabolized by all living cells, so the assay can be used with all cell types. MTT solution is added to cells cultured in microplates, then the cells were incubated for 4 hrs. During this period, MTT is converted into a coloured, water-insoluble formazan salt by the metabolic activity of viable cells. The insoluble formazan is solubilized by adding a solvent and the amount of formazan is quantified on a plate reader at 595 nm. Table 1 shows the IC_{50} values of the ligands and gold (III) complexes in WHCO1 and WHCO6 oesopesophageal cancer cell lines, after 72 hours of exposure to the drugs. All experiments were done three times and experimental points within an experiment were done in triplicate.

It emerges that the gold (III) complexes are reasonably toxic and the free ligands also show some remarkable activity. Both free ligands and gold (III) complexes exhibit important cell killing properties towards the reference WHCO1 and WHCO6 oesophageal cancer cell lines, with IC_{50} values lying in the low micromolar range.

TABLE 1
IC_{50} VALUES (µM) OF THE LIGANDS AND GOLD (III) CMPLEXES IN WHCO1 AND WHCO6 OESOPESOPHAGEAL CANCER CELL LINES

Compound	IC_{50} in WHCO1 (µM)	95% CI	IC_{50} in WHCO6 (µM)	95% CI
1	11.25	9.96 – 13.01	32.26	27.96 –35.35
2	15.65	13.99 –17.94	46.55	42.91 49.35
3	13.43	11.97 –15.60	43.11	38.8545.41
4	8.50	5.89 – 9.90	20.58	16.29 22.12
5	10.30	8.81 – 12.63	27.17	24.24 29.25
6	9.25	8.28 –11.58	25.23	22.73 29.75
cisplatin	3.75	2.25 -3.95	14.45	12.25 15.55

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