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Enhancement in Anticancer Activity of Chitosan Tailored Imidazole-2-Thiosemicarbazones against MCF-7 Cancer Cell Line by Coordination with Copper(II) Ions

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Abstract

The coordination behavior of new batch synthesized chitosan imidazole-2-thiosemicarbazones tailored from both the low molecular weight commercial chitosan and high molecular weight crab shell chitosan was confirmed by Fourier Transform Infrared (FT-IR) and magnetic susceptibility measurements. Chlorine content in the complexes was estimated by potentiometric titration technique. The study showed the NNS tridentate coordination with copper(II) ion in a square planar orientation with the remaining valence satisfied by coordination with chloride ion. The MTT assay studies showed an enhancement in the anticancer activity of these chitosan thiosemicarbazone ligands upon their coordination with copper(II) ions against the human breast cancer (MCF-7) cell line *in vitro*. The minimal cytotoxicity of both the ligands and complexes against the normal mouse embryonic fibroblast (NIH3T3) cells revealed the selective attraction of these biomaterial chitosan derivatives towards the cancer cells with non-toxicity to healthy cells. There was only a marginal effect of molecular weight (M_w) and degree of deacetylation (DDA) of chitosan upon the anticancer activity of these chitosan derivatives.

Keywords: Anticancer activity; Chitosan imidazole-2-thiosemicarbazones; Coordination behavior; Copper(II) chitosan imidazole-2-thiosemicarbazones; MCF-7 cell line; NIH3T3 cells.

1. Introduction

Chitosan, a biopolymer that consists of β-1,4-linked 2-amino-2-deoxy-β-D-glucose and *N*-acetyl-D-glucosamine units [1] , is synthesized by deacetylation of chitin in an alkaline medium. [2] Chitosan is a bioactive material with wide pharmaceutical applications [3] aided by its biocompatible, biodegradable and non-toxic behavior in favor of its uses for clinical purposes. [4] It has been approved as 'Generally Recognized as Safe (GRAS)' material by United States Food and Drug Administration (USFDA).[3] The involvement of amino, acetamido and hydroxyl functional groups in the pyranose ring of chitosan can bring about the chemical modifications of enhanced solubility and biological activities. [4] Chitosan shows anticancer activity with low toxicity to healthy cells. [5] The previous works have shown that the amino group in C2 position of chitosan ring undergoes Schiff's addition with carbonyl moieties of different thiosemicarbazones such as salicylaldehyde thiosemicarbazones [6], pyridine-2-thiosemicarbazones [7], isatin thiosemicarbazones [8], thiophene-2-thiosemicarbazones [9] and imidazole-2-thiosemicarbazones [10], and the antitumorigenic and anticancer activity of these chitosan derivatives have been found enhanced upon coordination with copper(II) ions. [6-10] The current study comprised a continuum of *in vitro* anticancer activity assessment of chitosan functionalized imidazole-2-thiosemicarbazones and their copper(II) complexes against the human breast cancer (MCF-7) cell line. The thione sulphur and azomethine nitrogen of thiosemicarbazone moiety, and a heterocyclic nitrogen species in imidazole -2-carboxaldehyde ring have been reported to be involved in coordination with copper(II) ion [10-11] , and the current work accorded with the coordination of both the low and high molecular weight chitosan functionalized imidazole-2-thiosemicarbazones with copper(II) ion and chloride ion in addition from copper(II) chloride. The coordination of chitosan biomaterials with copper(II) ion increases positive charge intensity in amino group of ring chitosan and the copper(II) chitosan complexes show higher attraction to

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negatively charged cancer cell surfaces. [12] The cancer causing free radical species may abstract a proton from amino group at C2 and hydroxyl group at C3 and C6 position of the ring chitosan, and this phenomenon is associated with antioxidant [13-14] and anticancer activity [14] of chitosan and its derivatives. In addition, the anticancer activity of imidazole derivatives has been reported to be attributed to the interference with deoxyribonucleic acid (DNA) synthesis, and the subsequent discontinuation of cell division and cell growth. [15] The prognosis and treatment of human breast cancer as a frequently observed malignancy appear crucial for the researchers. [16] The MCF-7 as a cell line with an adequately high negative charge on the cell surfaces that could be maintained under a suitable ionic strength and pH of the medium [17] may lend itself to be selectively targeted by the cationic polysaccharide chitosan [14] and its derivatives. The dose dependent anticancer activity of chitosan against different cell lines [18] and the conspicuous MCF-7 cell growth inhibition by chitosan [19] could show the aptness of selecting MCF-7 cell line for the current work. The mouse embryonic normal fibroblast (NIH3T3) cells were used to determine the comparative *in vitro* anticancer activity against the normal cells.

2. Materials and Methods

2.1. Materials

Low molecular weight commercial chitosan (Mw < 3000 Da) with 87 % DDA was obtained from Sisco Research Laboratories Pvt. Ltd., Maharashtra, India. High molecular weight crab shell chitosan (M_w found = 350 kDa) with 67% DDA was synthesized from chitin isolation of crab shells collected from fishery wastages of city wet market, Kathmandu, Nepal. The chemicals and reagents used were imidazole-2-carboxaldehyde, ethanol 99.8% (Sigma-Aldrich), caustic soda, hydrochloric acid, copper(II) chloride and sodium acetate (Merck), hydrazine, acetone and sodium chloroacetate (Thermo Fisher Scientific), carbon disulphide (s d fine-chem limited), and ammonium hydroxide, methanol, and other reagents of analytical grade were used without further refining. The MCF-7 cancer cell line and NIH3T3 normal cells were obtained from Shikhar Biotech, Nepal. The tetrazolium 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide (MTT) dye, RPMI media (a mixture of glutamine and incomplete RPMI media, 1.2 % streptomycin and penicillin antibiotics, 25 mM 4-(2-hydroxyethyl)− 1-piperazineethanesulfonic acid (HEPES), 10 % fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) solution, phosphate buffer solution (PBS) were prepared in Central Department of Biotechnology, Kathmandu, Nepal.

2.2. Measurements

The spectroscopic studies were carried out with ATR-GeXPm experimentation using BRUKER 1 003 3610 FT-IR spectrophotometer in the frequency range 4000–400 cm^{-1}. Magnetic susceptibility data of the complexes were taken as effective magnetic moment (μ_{eff}) using Sherwood Scientific susceptibility balance, Cambridge, UK; X1 range, 1.5 cm (L) long weighing tube to be filled in with powdered sample, weight of the empty tube = 0.8233 g. The FT-IR spectroscopic and magnetic susceptibility data were taken at Indian Institute of science (IISc), Bangalore, India. The MTT assays with the cells culturing were carried out at Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal.

2.3. Experimental

2.3.1. Synthesis of copper(II) chitosan imidazole-2-thiosemicarbazone complexes

Low molecular weight commercial chitosan imidazole-2-thiosemicarbazone (CSITSC) and high molecular weight crab shell chitosan imidazole-2-thiosemicarbazone (CCSITSC) were prepared with minor modifications in the method of Schiff's base addition *via* the condensation of carbonyl moiety of imidazole-2-carboxaldehyde with chitosan thiosemicarbazide. [6-10, 20] This process involved partial incorporation of thiosemicarbazone moiety onto amino group at C2 position of ring chitosan as established by previous studies. [6-10, 20] Then, 1% acetic acid solution was added drop-wise to a mixture of 0.313 g of CSITSC or CCSITSC and 0.134 g of copper(II) chloride in the presence of 10 mL of methanol to maintain the pH at 6, and the mixture was stirred at 60 °C for four hours. The mixture was filtered with 2 µm pore size G4 sintered glass crucible, and the complex Cu-CSITSC or Cu-CCSITSC isolated as residue was dried for 24 hours at 40 °C. This process involved minor modifications in the reported method of preparation of chitosan-copper(II) complexes. [21]

2.3.2. Chlorine estimation in complexes

The chlorine percentages in the complexes were estimated with the help of potentiometric titration technique. The base of properly dried nickel crucible was covered with sodium carbonate and about 0.025 g of Cu-CSITSC or Cu-CCSITSC complex was made to get spread over the base of crucible. It was again covered with some sodium carbonate and kept in a furnace at 900 °C for one hour. The complex was cooled down, dissolved in 60 mL of 2 N HNO_3, and the solution was potentiometrically titrated against 0.02 N silver nitrate solution (Hg/Hg^+ , sat. KCl ‖ Ag^+ /Ag) using a salt bridge filled with a gel of a mixture of saturated solution of potassium nitrate and agar-agar powder in the ratio of 5:1 by weight. [11]

2.3.3. Characterization

***2.3.3.1. Cu-CSITSC*: ** $C_{11}H_{15}N_5O_4SCuCl$, unit formula wt. 412.35, chitosan unit weight 160.16, yield: 75%, color: yellowish green; m.p. > 300 °C, Calculated Cl %: 8.56; Cl % estimated (for 87% DDA of chitosan): 14.20, IR (cm^{-1} , w: weak, s: strong, m: medium): ν (O–H) and ν(N–H) stretches merged with a broad translocation at 3117s, ν(C=N) 1605s, ν(N-H) bend 1547s, ν(C=S) 1058s, ν(C–O-C) 1109w and ν (C=N ring) 1418 m, μ_{eff} (B. M.): 1.88

2.3.4. **2.3.3.2. *Cu-CCSITSC*:** $C_{11}H_{15}N_5O_4SCuCl$, unit formula wt. 412.35, chitosan unit weight 160.16, yield: 73%, color: yellowish green; m.p. > 300 °C, Calculated Cl %: 8.56; Cl % estimated (for 67% DDA of chitosan): 12.78, IR (cm^{-1} , w: weak, s: strong, m: medium): ν (O–H) and ν(N–H) stretches merged with a broad translocation at 3103s, ν(C=N) 1602s, ν(N-H) bend 1555s, ν(C=S) 1150s, ν(C–O-C) 1111s and ν (C=N ring) 1417s, μ_{eff} (B. M.): 1.87

2.3.5. Magnetic susceptibility measurement

From the equation,

$$\&g = \frac{ebal.\ L.\ (R - R0)}{m\ X\ 10^9}$$

where m =mass of weighing tube filled with sample (m_2) – weight of empty weighing tube (m_1), ebal is proportionality constant of unity, gram susceptibility ($\&g$) was calculated. Molar susceptibility ($\&m$) was obtained from the equation $\&m = \&g.\ molecular\ weight$. The Curie equation,

$$\mu eff = \mu B = \frac{2.84\sqrt{\&m.T}}{1}$$

BM was employed to find magnetic moment (μB) (T= 298 K, R0 = -34, L= 1.5 cm) [22-23]

2.3.6. Cells culturing and colorimetric MTT assays

The media used for cells culturing was the complete RPMI media prepared as a mixture of 1.2% solution of antibiotics (penicillin and streptomycin), 25 mM 4-(2-hydroxyethyl)− 1-piperazineethanesulfonic acid (HEPES), 5% fetal bovine serum (FBS) and glutamine in an incomplete RPMI media. The MCF-7 cancer cell line and NIH3T3 normal cells were separately cultured in the complete RPMI media with 5 % carbon dioxide for 24 hours. The cultured cells were washed with phosphate buffer solution (PBS) to remove the debris and the cells in a fresh medium were counted and distributed in the 96 well plate with about 105 cells in each well. The test sample solutions in the concentration range of 50–600 μgmL^{-1} DMSO with the cultured cells were taken at incubation for 48 hours. The MTT solution in 5 mg/mL PBS solution was added in each well of the 96 well plate, incubated at 37 °C for 4 hours in CO_2 until the purple crystals of formazan were visible under microscope in the intracellular spaces. The unreacted yellow tetrazole was removed. The purple formazan crystals were then dissolved in DMSO, the solutions were triturated and incubated for half an hour in CO_2 at 37 °C until the cells were lysed and formazan was dissolved to form the purple solutions. The purple color intensity of the solutions was quantified at an absorbance of 551 nm using the ELISA plate reader. The cells untreated with the sample were taken as positive control and the corresponding absorbance was controlled absorbance. The absorbance due to only media was taken as blank absorbance. The absorbance due to cells treated with the sample was sample absorbance. The cell viability % was determined by the relation [24]:

$$\%\ \text{cell viability} = \frac{(\text{sample absorbance} - \text{blank absorbance})}{(\text{control absorbance} - \text{blank absorbance})}\ X\ 100\ \%$$

3. **Results and discussion**

3.1. Characterization

3.1.1. Fourier transform infra-red (FT-IR) spectroscopy

The negative shifts of ν(C=N) reported at 1627-1619 cm^{-1} in free chitosan functionalized imidazole-2-thiosemicarbazone ligands [10] to 1605-1602 cm^{-1} in their copper(II) complexes showed the coordination of azomethine

nitrogen. [25] The negative shifts of ν(C=S) bands at 1160-1072 cm^{-1} in free chitosan functionalized imidazole-2-thiosemicarbazone ligands [10] to 1150-1058 cm^{-1} in complexes was indicative of coordination of thione sulphur with the metal ion. [26] The broad band translocations at a range 3500-3200 cm^{-1} were due to merging of ν(O-H) and secondary amide ν(N-H) stretches. [27] The imidazole ring ν(C-N), ν(C=C) and ν(C=N) stretches at the lower range of 1417-1418 cm^{-1} in these complexes [27] were the indications of coordination behavior of the ring with an increase in carbon-nitrogen double bond character due to resonance inside the ring.[28] This tridentate coordination behavior of the ligands showed deprotonation of H-N-C=S group and the ligand-metal ion charge balance due to coordination from the thiolate form of the ligand. [29]

3.1.2. Magnetic susceptibility measurement

The effective magnetic moment (μ_{eff}) values calculated with the magnetic susceptibility data (T= 298 K, R_0 = -34, L = 1.5 cm) were 1.87 BM for Cu-CSITSC and 1.88 B.M. for Cu-CCSITSC. These values in close proximity to spin only moment of 1.73 BM for a free electron indicated low spin-spin coupling of the electrons [30] and square planar geometry of complexes can be predicted on the basis of magnetic moment of < 1.90 B.M. [31]

The results of above spectroscopic and magnetic susceptibility measurement studies and the estimated chlorine percentages of 14.20% in Cu-CSITSC and 12.78% in Cu-CCSITSC can be utilized to propose the mononuclear square planar geometry of the complexes. The coordination behavior of chitosan imidazole-2-thiosemicarbazones to give the copper(II) complex of square planar structure is as shown in figure 3. The azomethine nitrogen and thione sulphur of the thiosemicarbazone moiety, heterocyclic ring nitrogen in imidazole -2- carboxaldehyde and one chlorine species, in addition, are involved in coordination with copper(II) ion.

3.2. Anticancer activity

The cytotoxicity profiles of CSITSC and CCSITSC ligands as shown by the MTT assays cell viability tests against the MCF-7 cancer cell line and the mouse normal embryonic fibroblast (NIH3T3) cells are presented in Table 1, and the cytotoxicity profiles of Cu-CSITSC and Cu-CCSITSC complexes as shown by the MTT assays cell viability tests against the MCF-7 cancer cell line and the mouse normal embryonic fibroblast (NIH3T3) cells are presented in Table 2.

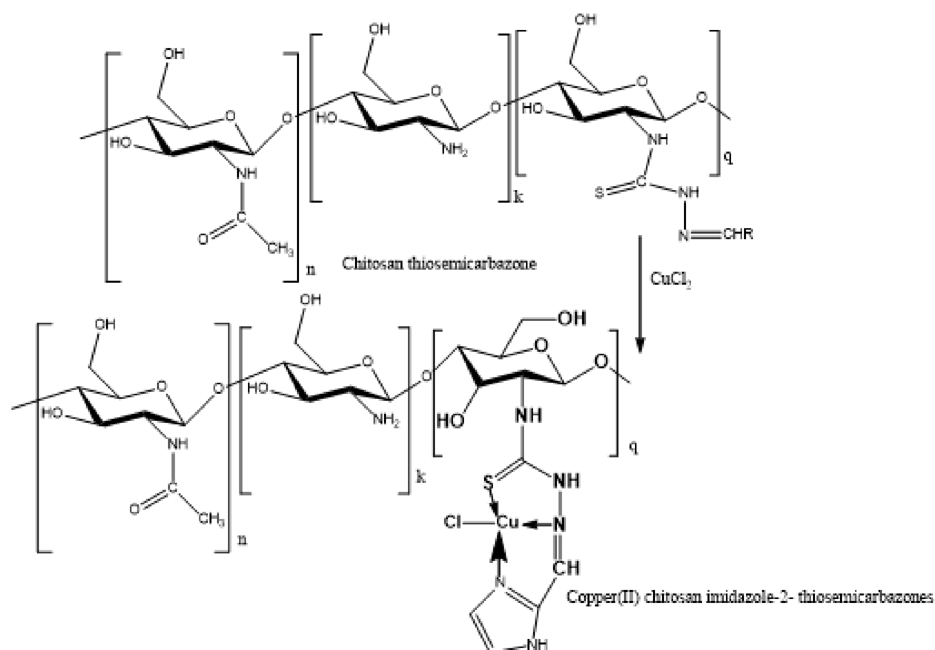

Fig. 1: Coordination behavior of chitosan imidazole-2-thiosemicarbazones to give the copper(II) complex of square planar structure

Table 1: Cytotoxicity profiles of chitosan thiosemicarbazone ligands against MCF-7 cancer cell line and NIH3T3 normal cells

Ligands	Activity against MCF-7 cancer cell line		Activity against NIH3T3 normal cells	
	Viability% (at 50–400 μgmL^{-1})	IC_{50} (μgmL^{-1}) ± S. D.	Viability% (at 50–600 μgmL^{-1})	IC_{50} (μgmL^{-1}) ± S. D.
CSITSC	75-32	368 ± 8.1	93-70	>600
CCSITSC	83-58	>400	94-74	>600

S.D. = Standard Deviation from the mean at n = 3.

Table 2: Cytotoxicity profiles of copper(II) chitosan thiosemicarbazone complexes against MCF-7 cancer cell line and NIH3T3 normal cells

Complexes	Activity against MCF-7 cancer cell line		Activity against NIH3T3 normal cells	
	Viability% (at 50–400 μgmL^{-1})	IC_{50} (μgmL^{-1}) ± S. D.	Viability% (at 50–600 μgmL^{-1})	IC_{50} (μgmL^{-1}) ± S. D.
Cu-CSITSC	72-19	277 ± 7.6	92-66	>600
Cu-CCSITSC	82- 53	>400	90-62	>600

S.D. = Standard Deviation from the mean at n = 3.

The enhancement in the *in vitro* anticancer activity against MCF-7 cancer cell line upon the coordination with copper(II) ions has been shown in figure 2, and the relative nontoxicity of the ligands and the corresponding copper(II) complexes towards NIH3T3 normal cells have been shown in figure 3. The colorimetric MTT assay data showed a concentration dependent rise in inhibition of MCF-7 cancer cells by these chitosan derivatives. The low molecular weight commercial chitosan-based imidazole-2-thiosemicarbazone was found to exhibit more cytotoxic activity than the high molecular weight crab shell chitosan-based imidazole-2-thiosemicarbazone. An enhancement in anticancer activity against the MCF-7 cell line was observable upon the coordination of these ligands to give the corresponding copper(II) complexes. Further, the anticancer activity enhancement was found more in the complex of low molecular weight chitosan imidazole-2-thiosemicarbazone. The study showed that these chitosan-based materials are selectively attracted towards the MCF-7 cancer cell surfaces, and show minimal cytotoxicity to healthy cells. The results accorded with the reports that copper(II) thiosemicarbazone complexes bring about the higher cytotoxic effects than thiosemicarbazones probably due to their inherent redox properties. [32-33] Chitosan nanomaterials have been reported to show more permeability towards more negatively charged MCF-7 cancer cell surfaces and exhibit anticancer activity by the cellular pathways. [34-36] So the MCF-7 cancer cell line was found appropriately selected for the study that may leave useful data for further mechanistic investigation of anticancer activity of this copper(II) chitosan complexes.

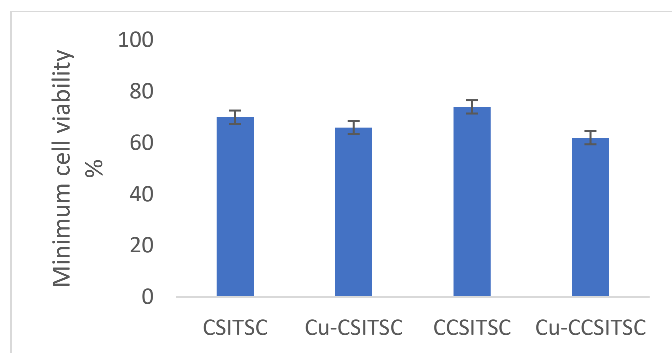

Fig. 2: Anticancer profile of ligand and the corresponding copper(II) complexes against MCF-7 cell line

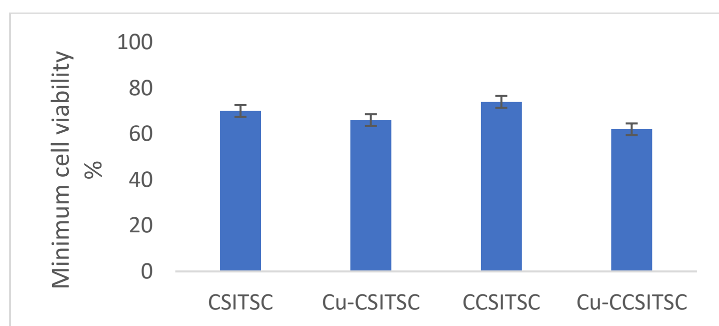

Fig. 3: Anticancer profile of ligand and the corresponding copper(II) complexes against NIH 3T3 normal cells

4. Conclusions

The *in vitro* anticancer activity of chitosan-tailored imidazole-2-thiosemicarbazones against the MCF-7 cancer cell line could be attributed to the synergistic anticancer effects of chitosan, thiosemicarbazone and imidazole moieties in the molecule. The lower cytotoxicity of these materials to NIH3T3 normal cells appears to be indicative of their minimal side effects. The lower M_w and higher DDA of the chitosan component in these compounds were observed to further increase their anticancer activity. The chitosan functionalized imidazole-2-thiosemicarbazones were found to show NNS tridentate coordination behaviour with copper(II) ion to give the square planar complexes, and this coordination with copper(II) ion brought about an enhancement in anticancer activity against the MCF-7 cancer cell line, but there was lower cytotoxicity against the normal NIH3T3 cells.

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6. Conflict of interest

The author declares no conflict of interest with anybody regarding the publication of this article.

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