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Invitro Cell viability and Antibacterial activity of novel Bismuth (V) complex

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Abstract : Bismuth (V) was incorporated in chloramphenicol and streptomycin keeping in view of finding new antibiotics. Formed complex was evaluated for their antibacterial and cell viability activity in mice. Murine peritoneal macrophage cell lines RAW 264.7 were used for *in vitro* antibacterial and cytotoxicty studies of various synthesized compounds. Bactericidal activity was assessed by Minimum inhibitory concentrations (MIC) of various compounds against different bacterial strains. Antibacterial activity of T-IV was found highest among all treatments given followed by T-III. Metal-ligand administered group showed comparatively higher *in vitro* cell viability than standard drug streptomycin. It was concluded that metal drug complex may have better antibiotic activity than parent antibiotics alone.
Key words: Chloramphenicol, Streptomycin, Bismuth (V)-ligand complex, antibiotics.

Introduction

Streptomycin, tetracycline and chloramphenicol are well known antibiotics. They have potential donor atoms to coordinate with metal ions and to cause their impact with a larger extent. Streptomycin is an aminoglycosidic antibiotic with three components: streptidine, streptose and N-methyl-L-glucosamine (Figure1). It is used to treat infections caused by Gram-negative bacteria and in the therapy of tuberculosis [1-4]. Serious toxicity is a major limitation of its usefulness, most notable is its ototoxicity, and causing deafness is severe cases. It has been proposed that the mechanism of action of this antibiotic could be related to an enhancement of the biological availability of metal ligand attached to it [5-6]. Some preliminary studies about the interaction in solution of some metal ions with streptomycin have been reported [7-9] but detailed characterization of the complexes is unavailable except for a neutral Cu (II) complex where Cu-O bonds with streptomycin are suggested [10-11]. Furthermore, literature on streptomycin as a ligand reveals that there is clear disagreement about the metal – ligand binding sites in complexes of this ligand [12].Therefore, in order to ascertain metal binding sites in streptomycin metal chelates, the synthesis and spectroscopic characterization of complexes of streptomycin with these transition/non-transition metal ions is attempted in this investigation.

Chloramphenicol (Chloromycetin, D-(7)-threo-di-choloacetamide-1-p-nitrophenyl propane-1-1, 3-diol, CAP) is an antibiotic that finds applications in combating a wide range of infections caused by Gram-negative and some Gram-positive organisms. It is currently the first choice antibiotic in developing countries and is still widely used in industrialized countries for the treatment of serious bacterial infections. The adverse effect to the eye of chloramphenicol appears to result from the interaction of the drug with metals ions. Chloramphenicol is still widely used in topical preparations (ointment and eye drops) for the treatment of bacterial conjunctivitis.

In view of the rapid development and also challenging demands, it has become necessary to synthesize newer compounds, which may show potential antimicrobial activity. In naturally occurring chloramphenicol, the amino nitrogen and deprotonated alcoholic oxygen may be involved in the binding with streptomycin in presence of aqueous alcoholic medium. This paper describes the synthesis of novel ligands and their complexing ability with bismuth metal ions. These synthesized compounds were assed for their potential antioxidant activities and hepatoprotective effects in rat (Communicated data). They were found to be a potent antioxidant agent and their activity was hepatoprotective. In further assessment of their biological activity synthesized compounds were evaluated for their anti-inflammatory and cell viability studies.

2. Experimental

2. 1. Material and methodology

Streptomycin and Chloramphenicol were purchased from Sigma Chemicals, St. Louis, MO. All the chemicals used in this study were of analytical grade and used as procured from Merck, India. Solvents used were of analytical grade and were purified by standard procedures. The stoichiometric analyses (C, H and N) of the complexes were performed using Elementar vario EL III (Germany) model. Metal contents were estimated on an AA-640-13 Shimadzu flame atomic absorption spectrophotometer in solution prepared by decomposing the respective complex in hot concentrated HNO3. Their IR spectra were recorded on Perkins–Elmer FTIR spectrophotometer in KBr and polyethylene pellets. The electronic spectra were recorded in water on Beckman DU-64 spectrophotometer with quartz cells of 1 cm path length. 1H NMR spectra were recorded in CDCl3 solvent on a Bruker Advance 400 instrument. Rigaku model 8150 thermoanalyser (Thermaflex) was used for simultaneous recording of TG-DTA curves at a heating rate of 10omin-1. For TG, the instrument was calibrated using calcium oxalate while for DTA, calibration was done using indium metal, both of which were supplied along with the instrument. A flat bed type aluminium crucible was used with α- alumina (99% pure) as the reference material for DTA. The activation energy and Arrhenius constant of the degradation process was obtained by Coats and Redfern method. The XRD powder pattern were recorded on a vertical type Philips 1130/00 x- ray diffractometer, operated at 40kVand 50Ma generator using the Cukα line at 1.54056 A° as the radiation sources. Sample was scanned between 5° to 70 °(2θ) at 25°C. The crystallographic data was analyzed by using the CRYSFIRE –2000 powder indexing software package and the space group was found by the GSAS program. Debye – Scherer relation with the help of 100% peak width determined the particle size. The density was determined by Archimedes method.

2.2 Ligand synthesis

Novel mixed ligands 12-((1R)-2, 2-dichloro-1-(1,3-dihydroxy-1-(4-nitrophenyl) propan-2-ylamino)-1-hydroxyethoxy)-3,6,10,12a-tetrahydroxy-4-isopropyl-6-methyl-1,11-dioxo-1,2,5a,6, 11, 11a, 12, 12a-octahydrotetracene-2-carboxamide and 1-(4-(2,2-dichloro-1-(1, 3-dihydroxy-1-(4-nitrophenyl)propan-2-ylamino)-1-hydroxyethoxy)-2-(4-(3,5-dihydroxy-6-(hydroxymethyl)-4 (methylamino)-tetrahydro-2H-pyran-2-yloxy)-5-formyl-5-hydroxy methyltetrahydrofuran-3-yloxy) -3,-dihydroxy-5-(methylamino) cyclohexyl) guanidine were prepared by mixing of chloramphenicol **(Fig.1a)** and streptomycin **(Fig.1b)** (0.5mmol of both, Sigma Chemicals, St. Louis, MO) in an aqueous solution of methanol in a round bottom flask. The reaction mixture was refluxed with stirring for 5 h under reduced pressure followed by cooling to room temperature. The product obtained was washed with a small amount of methanol and air dried. The above product was redissolved in excess warm methanol and clear solution was left undisturbed for weeks to give beautiful crystals of the ligands separately. The ligand was characterized by different physical techniques. Pertinent analytical and physico-chemical data for the ligand and its complexes are listed in **Table 1.** Ligands and their complexes have been characterized by elemental analysis, spectral UV, IR 1HNMR and XRPD studies. The optimized structure of the both complexes has trigonal bipyramdal.

2.3 Preparation of Bi (V) Complexes

To a methanolic solution of bismuth (V) (0.5 mmol) in a flask was added a methanolic solution of the novel ligand (0.5 mmol). The solution was stirred for 6h, after which the volume was reduced on a warm water bath. The product obtained was washed with a small amount of methanol and air dried. The above product was redissolved in excess warm methanol, and clear solution was left undisturbed for weeks to give beautiful crystals of the complexes. Proposed structure of bismuth complex is given in **figure 1(c).**

Table1: Color, reaction yield and elemental analysis of ligand and bismuth complex

Complex	Empirical formula	Color	Yield (%)	Analysis: found (calculated) (%) C	H	N	M	M.P.^{0}C
Ligand	$C_{32}H_{51}C_{l2}N_9O_{17}$	White	80	32.01 (32.11)	2.75 (2.77)	3.36 (3.30)	---	31
Complex	$C_{32}H_{49}BiC_{l5}N_7O_{29}$	White	75	32.01 (30.65)	2.75 (2.77)	3.36 (3.30)	16.45 (16.43)	56.5

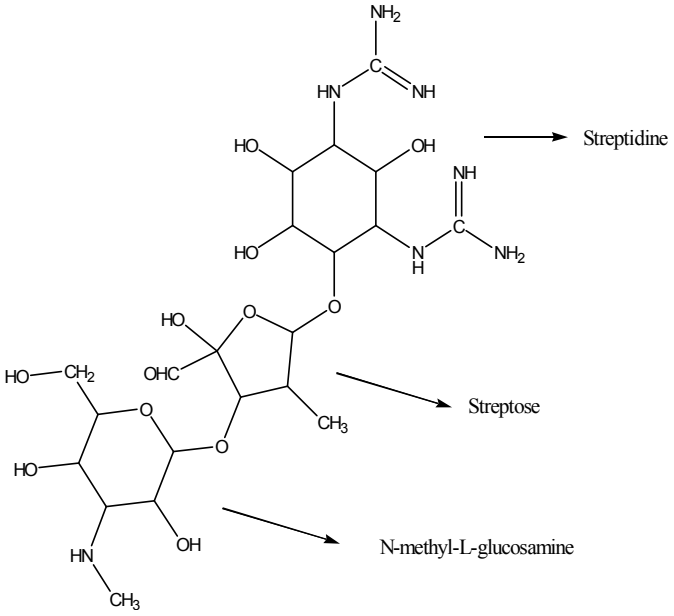

Figure.1(a): Streptomycin

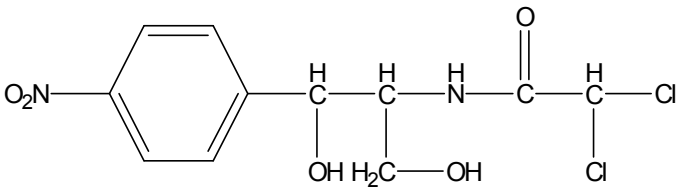

Figure.1(b): Chloramphenicol

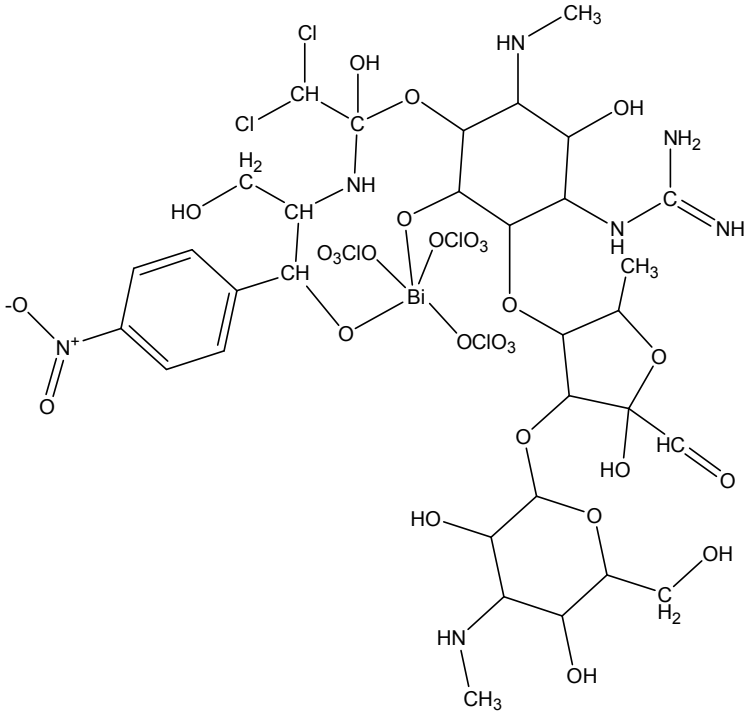

Figure.1 (c): Graphical structure of bismuth ligand complex

2.4 Experimental protocol

Five treatments were taken for all biological activities. T-I: Chloramphenicol, T-II: Streptomycin, T-III: STR+ CAP and T-IV: CAP+STR+Bi.

2.4 Isolation of macrophages

Peritoneal macrophages were isolated from Swiss albino mice by intraperitoneal injection of 5 ml of ice-cold PBS (10 mM, pH-7.4) [14]. The cells were harvested by peritoneal lavage after 5 min, and were subsequently cultured in RPMI- 1640 complete medium. The purity of the macrophages was checked by adherence and viability of macrophages (tested by crystal violet assay) was over 90%.

2.5 In vitro Cell viability assay

The toxicity of bismuth complex was studied by using MTT colorimetric assay [13]. MCF-7 cells were maintained in RPMI- 1640 medium supplemented with 10% heat inactivated FCS (Faecal Calf Serum) and100 U/ml of streptomycin. The cells were cultured in a humidified (5%) CO_2 incubator at 37 °C. Then MCF-7 cells were seeded onto 96-well plates at a density of 1×10^4 cells / well and incubated for 16 h for adherence. Afterwards, the media was aspirated from the wells and the cells were washed once with RPMI- 1640 without FBS. The various compounds (T-I, T-II, T-III and T-IV) in a concentration from 10 to 30 mg of each were diluted with RPMI- 1640 without FCS to a final volume of 75 µl and this media was added to separate wells, followed by incubation at 37 °C in humidified 5% CO_2 incubator for 4 h. Then the media containing the compounds was replaced with 200 µl of normal growth medium and cells were further incubated for 48 h under same conditions. After 48 h media was replaced by 200 µl of MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg dissolved in 1.0 ml of RPMI-1640) and further incubated for 2 h. Afterwards, the supernatant was aspirated, and the formazan crystals so formed were suspended in 100 µl iso-propanol containing 0.06 M HCl and 0.5% SDS. Aliquots were drawn from each well and the intensity of color was measured in a Fluorometer (Labtech, Australia) taking absorbance at 540 nm. Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank. The relative cell viability (%) compared to control cells was calculated by [abs] sample/ [abs] control × 100.

2.7 Antibacterial activity

For bactericidal activity, 2.5×10^6 macrophages in 0.1 ml balanced salt solution (BSS) were mixed with 0.3 ml vortexed diluted bacterial culture (overnight *Escherichia coli* culture) to prepare a 1:10 ratio of macrophage and bacteria [14]. Fifty microliters of normal serum was added to it and volume was made up to 1 ml with BSS. The tubes containing the above mixture were incubated for 15–20 min at 37 °C with shaking with different concentrations of synthesized compounds (1 µg ml^{-1} to 100 µg ml^{-1}). The extracellular bacteria were removed by washing and the cells were resuspended in 1 ml BSS. The macrophages were lysed by serial dilution in sterile water. The tubes were vortexed and 0.1 ml sample was plated in prewarmed LB plates. Colonies were counted after 24h. Three sets of experiments were set up for antibacterial activities of different concentrations of different compounds.

3. Result and Discussion

Satisfactory results of elemental analysis (Table .1) and spectral studies revealed that the complexes were of good purity. Various attempts to obtain the single crystals have so far been unsuccessful. X-ray diffraction studies indicate crystalline nature of the metal complexes. The complexes were soluble in polar solvents and water.

3.1. IR spectra

The IR spectra of ligand and their complexes have been assigned in **Table 2**.In both complexes ClO_4 ion appeared at 721,1064,962 cm-1 which coordinate with Bi(V) ion and in complex 2 922,1035and 724 cm-1 and band at 1521 and 1624 cm-1 appeared respectively suggesting that ligands occurs through the oxygen atoms from hydroxyl groups coordinated. The M-O bonding appeared at 419 and 1340cm-1 and in complex 2 M—O at 919 cm-1

3.2. ^{1}H N M R spectra

The ^{1}H NMR data of the ligand and complex is summarized in **Table.3.** The peak positions as well as the integrated intensities of these compounds are consistent with the proposed structures for them.

3.3. X-ray powder diffraction study

X-ray powder diffraction patterns of all ligands and their complexes were recorded between 9 and 80(2θ). The value of (2 θ), interplanar spacing d (_A) and the relative intensities (I/I0) of the compounds under study were recorded in **Table 4.**

Table 2: IR spectral data (cm^{-1}) of the metal complexes

Frequency	ν_{N-H}	OH	OH	NH_2	NH_2	M - O	M-ClO_4
$C_{32}H_{51}C_{12}N_9O_{17}$ Ligand	3365(s,b)	1686(s)	1529(m)	1317(w)	613(m)	472(m)	
$C_{32}H_{49}BiC_{15}N_7O_{29}$ Complex	3395(s,b)	1624(m)	1475(s)	1216(w)	627(s)	419(s)	922,1035,724

Table3: ^{1}H NMR data of ligand and complex

Compounds	δ (ppm)
$C_{34}H_{37}C_{l2}N_3O_{13}$ Ligand	[3.35(s)1H,OH],16.77(s)1H,OH,16.77(s),1H,OH,3.65(m),5H,OH,2.0(s) 1H, NH, 7.16(s), H,$NH_2$3.83(m)1H,CH,8.19(d)1H,CH(Ar),7.00(s)1H,CH(Ar),7.62(m),1H,CH(Ar),6.78(s)1H,CH(Ar),2.52-3.98 (m) 4H, (m) CH,3.98-4.73(d),2H,CH,1.35(s) 3H,Ch,1.06(s) 6H,CH_3].
$C_{32}H_{51}C_{l2}N_9O_{17}$ Complex	[3.65(m),5H,OH],[3.58(s),4H,OH,][2.0(s)4H,NH],8.56(s)1H,NH_2,]3.51-4.24,(m),4H, CH],[5.03(d) 1H,CH], 3.76-3.81(m),6H CH],8.19(s)1H ,CH] 8.89(m) 1H CH (Ar)], [7.62(s)1H,CH],[9.72(s)1H,CHO],4.73(m)1HCH,3.79,2H,CH2,3.17(m)6H,CH_3,1.18(m) 3H,CH_3].

Table 4: Crystallographic data for complexes

Compounds	ligand	Complex
Formula	$C_{34}H_{35}BiCl_5N_3O_{25}$	$C_{32}H_{49}BiC_{15}N_7O_{29}$
FW	1271.89	1382.01
Temp (K)	293	293
Wavelength	1.54056	1.54056
Crystal System	Orthorhombic	Orthorhombic
Space group	P N M A	I M M A
Unit cell dimension		
a(Å)	13.554540	22.094890
b(Å)	9.496137	8.983012
c(Å)	4.471746	8.483077
α	90.000	90.0000
β	90.000	90.000
γ	90.000	90.000
Volume (A^3)	575.58	1683.71
θ range (0)	19.0-70.0	7.0-30.0
Limiting indices	$0 \le h \le 8$	$0 \le h \le 7$
	$0 \le k \le 5$	$0 \le k \le 2$
	$0 \le l \le 2$	$0 \le l \le 2$
Particle size(nm)	49.123	50.341
Intensity (%)	7.2–100	5.9–100
R indices	0.0000106	0.000131
Density	1.405	1.343
Z	1	2

3.4 In vitro cytotoxicty assay

In vitro cytotoxicty of synthesized bismuth complexes and mixed ligand of streptomycin and Chloramphenicol, CIP (control) was measured by MTT colorimetric assay. (Fig.2.) The MCF-7 cells were incubated in increasing concentrations of T-I, T-II, T-III, T-IV and streptomycin STP as standard drug. All treatments showed ~70 % cell viability at 30 µg, significantly higher than standard drug streptomycin. At concentration of 30 µg of compound, T-IV showed more than 90 % of cell viability. T-I showed minimum (62%) cell viability among all compounds.

3.5 Antibacterial activity

The antibacterial activity was evaluated for all synthesized bismuth complexes and mixed ligand of streptomycin and Chloramphenicol, CIP was taken as control. Antibacterial activity was done by disc diffusion method using pathogenic strains of *S. aureus* (ATCC 29213) and *S. epidermidis* (MTCCB 1824) as gram positive, *P.aeruginosa* (MTCCB 741), and *E. coli* (ATCC 25922) as gram negative. Results for antibacterial activity are summarized in Table 5. Minimum inhibitory concentrations (MIC) of T-IV, against all bacterial strains were ranged in 8-12 µg/ml and zone of inhibition was optimum which proved it to be a strong antibiotic agent. This antibacterial activity of T-IV might be attributed to their heavy metal constituent and presence of nitrile group which enhances the activity [13]. T-III showed mild antibacterial activity in comparison to T-IV although it has long alkyl chain. Its MIC was ranged in 17-21 µg/ml and zone of inhibition was 212-325 mm.

Among T-I and T-II treated groups no significant difference was observed in antibacterial activity. In conclusion, all synthesized bismuth complexes and mixed ligand of streptomycin and Chloramphenicol were posed as a more potent class of antibacterial agents in comparison to already established antibacterial agents like Ciprofloxin. Further study is needed in order to deduce the effect of α, β-unsaturated compounds on antibacterial activity. Theses prepared compounds have great possibility of being used in all bacterial infection. Moreover they are economical to synthesize and which is of great significance for increasing population across the world.

Table 5: Antibacterial activity of synthesized Bismuth complex and ligand. Various treatments were taken against a number of pathogens. T-I is CAP, T-II is STR, T-III is STR+CAP and T-IV is CAP+STR+Bi. Ciprofloxin (CIP) is taken as control. Values are showing zone of inhibition (mm) and MIC values (µg/ml) against selected bacterial strains.

	E. coli	*S. epidermidis*	*S. aureus*	*P.aeruginosa*
Control	24/218.25	28/127.26	29/234.88	24/287.33
T-I	20/124.24	21/145.24	20/201.01	19/222.39
T-II	18/158.64	19/151.21	17/232.18	19/187.64
T-III	16/156.51	17/192.55	14/211.68	12/241.82
T-IV	11/36.54	12/44.98	11/124.61	11/129.54

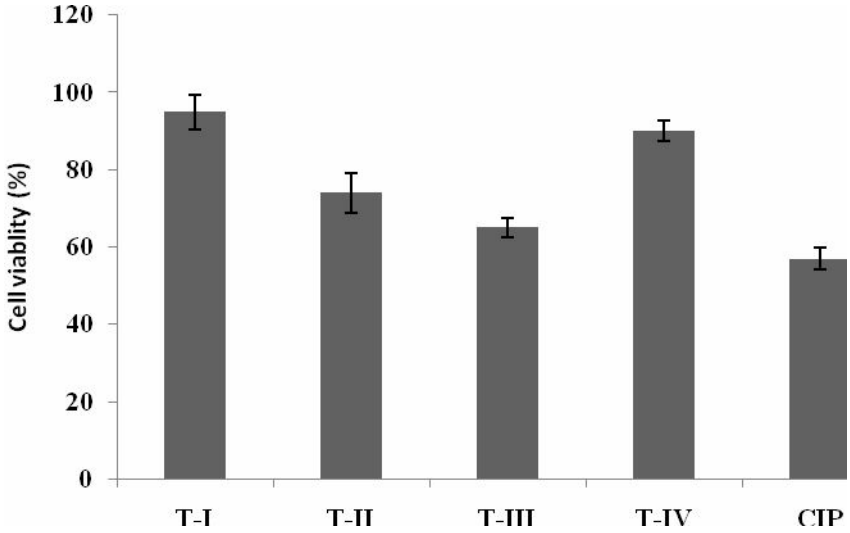

Figure.2.: *In vitro* cytotoxicty of synthesized Bismuth complex and ligands. Various treatments were taken against a number of pathogens. T-I is CAP, T-II is STR, T-III is STR+CAP and T-IV is CAP+STR+Bi. Ciprofloxin (CIP) is taken as control. Values are represented as ±SE.

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