

Research & Reviews: Journal of Pharmaceutics and Nanotechnology

PHARMACOLOGICAL ACTIVITIES OF PYRIMIDINE AND AMINO ACIDS COMPLEXES: A COMPARATIVE STUDY

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Research Article

Received date: 07/09/2015

Accepted date: 26/10/2015

Published date: 02/11/2015

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ABSTRACT

Some novel sequence of mixed ligand complexes (1–9) have been synthesised from 5–fluorouracil (5–FU) and amino acids via glycine (gly), L–alanine (ala) and L–valine (val) with Ni(II), Cu(II) and Zn(II) ions. These synthesized mixed ligand complexes were characterized by various physico–chemical and spectral studies. 5–Fluorouracil and its complexes were tested for their pharmacological activities via *in vitro* biological activities by the agar well diffusion method and *in vitro* antioxidant activities using the DPPH assay method. On comparison, Cu(II) complexes exhibit potent biological as well as antioxidant activities than 5–fluorouracil.

INTRODUCTION

The formation of mixed ligand complexes from bioactive ligands with transition metal(II) ions show biological, clinical, medicinal, agricultural, industrial, analytical and therapeutic applications ^[1,2]. Pyrimidines and its N–base analogues of uracil, thymine and cytosine play a significant role in human cellular life, coenzymes, antibiotics, drugs, several vitamins and their derivatives are the basic building units for nucleic acids of RNA and DNA ^[3]. They are also reported to a wide range of activities in antibacterial, fungicidal, antitumor, antiviral, anti–inflammatory, antipyretic, antimetabolites, anti–thyroid, agrochemicals and surface anaesthesia activities ^[4]. A mono fluorinated pyrimidine derivatives of 5–fluorouracil (5–FU; Aducril) perform as chemical mutagens in human as well as animals, nucleic acid synthesis and they are broadly examined as potent antibacterial and anticancer drugs ^[5]. They also act as an improved topical delivery and diminish the side effects ^[6].

Transition metal (II) ions are the primary elements for healthy life to human, plants and higher animals ^[7]. Amino acids were the fundamental components in living organisms ^[8] and its constituents are the basic building blocks of proteins in plants and human. Mixed ligand complexes of L–amino acids play a crucial role in biology, pharmacy, industry and laboratory reagents ^[9]. Currently, there is a great attention paid to the study the natural and safer antioxidant compounds because they play a vital role in food, cosmetics and pharmaceutical products ^[10] and also the prevention of disease caused by oxidative stress ^[11]. In the present paper, we investigate the biological and antioxidant activities of mixed ligand complexes derived from 5–fluorouracil and biologically active amino acids of glycine, alanine and valine with Ni(II), Cu(II) and Zn(II) ions were synthesised and characterized by means of analytical and spectral techniques. *In vitro* biological and antioxidant activities of 5–fluorouracil and their complexes were also reported and compared.

MATERIALS AND METHODS

All the ligands, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid and metal salts were extra pure AR grade (Sigma Aldrich, USA and Fluka, Switzerland) and used without further purification. Solvents for the physical measurements were of AR grade and purified by standard methods^[12]. Melting points of all the mixed ligand complexes were determined on Gallenkamp apparatus in open glass capillaries and are uncorrected. The elemental C, H and N analysis was performed on Elementar Vario EL III CHNS analyzer at STIC, CUSAT, India. Metal content of the complexes was estimated gravimetrically by the standard procedure. Molar conductance of the complexes was measured in DMSO solution using Elico CM 180 conductivity bridge by using 0.01 mol KCl solutions as calibrant. Magnetic susceptibility measurement on powder samples were carried out by the Gouy method using Hg[Co(SCN)₄] as calibrant and the diamagnetic corrections were applied in compliance with Pascal's constant. Electronic absorption spectra were recorded in DMSO medium using Hitachi U-2000 double beam spectrophotometer (cell length, 1 cm) in the 1100–200 nm range. Infrared spectra were recorded using KBr pellets on a JASCO FT/IR-410 spectrophotometer, in the 4000–400 cm⁻¹ range. ¹H NMR and ¹³C NMR spectra of the diamagnetic zinc complexes were carried out in DMSO-d₆ at room temperature using TMS as internal standard on Perkin Elmer R-32 spectrometer at IIT, Chennai, India.

In vitro biological activity

In vitro biological activities of free ligand 5-fluorouracil and their corresponding mixed ligand complexes (1–9) were tested against three gram-positive bacteria: *Bacillus cereus* (Bc), *Staphylococcus saprophyticus* (Ss) and *Staphylococcus aureus* (Sa), two gram-negative bacteria: *Escherichia coli* (Ec) and *Pseudomonas aeruginosa* (Pa) using Muller Hinton agar nutrient and three fungi namely *Aspergillus niger* (An), *Enterobacter species* (Es) and *Candida albicans* (Ca) using potato dextrose agar as the medium by well diffusion technique^[13]. About 2-8 h, older microorganism species inoculums containing approximately 10⁴-10⁶ colony forming units (CFU) per ml were used in these analyses. Sample solutions were prepared by dissolving the complexes in DMSO. Blank petri-dish for bacterial and fungal analysis were moistened with same solvent, cultured for 24 h and then incubated at room temperature. Twenty milliliters of molten nutrient agar solution was then poured into each petri-dish and allowed to solidify. Well with 5 mm diameter was dug in the media carefully with the help of sterile metallic cork borer with centers and this well was filled with the recommended concentration (40 µl) of the samples. These plates were transferred immediately to an incubator and incubated 24 h for bacteria and 72 h for fungi maintained at 35 °C. During the time, the sample solutions diffused and the growth of the inoculated microorganisms were affected. Inhibition in the growth was determined as a difference in growing between control and those treated with the sample solutions. The samples were subjected to further assay with a series of concentration (25, 50 and 75 µg) under the same conditions. The results were recorded as zone of inhibition in mm and compared with the standard drugs of tetracycline and nystatin, used as antibacterial and antifungal control respectively. Zone of inhibition is given as the average of three independent determinations.

In vitro antioxidant activity

5-Fluorouracil and their complexes have been tested for *in vitro* antioxidant activities by DPPH free radical scavenging assay method at 37 °C. DPPH radical scavenging effect was carried out according to the Blois method^[14]. Different concentration solutions (10, 20, 30, 40 and 50 µmol) of 5-fluorouracil and their complexes in DMSO medium were prepared. 1 ml of each compound solution have different concentrations was taken in different test tubes and 4 ml of a 0.1 mmol DPPH solution was added to each tube and the reaction mixture was shook vigorously for about 2-3 min. Then each tube was incubated in dark room for 20-30 min at room temperature. A blank DPPH solution was prepared without compound used for the baseline correction. After incubation, the absorbance values (λ_{max}) for each sample were measured at 517 nm using a UV-visible spectrophotometer. There was a change (decrease) in the absorbance values (λ_{max}) which indicated that the mixed ligand complexes show moderate scavenging activities. Ascorbic acid was used as the reference or positive control. Free radical scavenging effects in percentage was calculated using this formula as:

where A control=absorbance of the control (blank) and A sample=absorbance of the ligand or the complexes. All the analyses were made in three replicate for each and the results were compared with control.

General procedure for synthesis of mixed ligand complexes (1–9)

5-Fluorouracil (0.013 g/10 mM) was dissolved in water (10 ml) containing few drops of concentrated ammonia and stirred to get a clear solution. 10 ml of 10 mM of an appropriate metal salt solution in water (0.025 g of Ni(CH₃COO)₂•4H₂O, 0.020 g of Cu(CH₃COO)₂•H₂O and 0.022 g of Zn(CH₃COO)₂•2H₂O) was added dropwise and stirred at room temperature. To this solution 10 ml of 10 mM of amino acid (0.008 g of glycine, 0.009 g of L-alanine and 0.012 g of L-valine) in water was added and the reaction mixture was refluxed for 4-6 h (**Figure 1**). pH of the reaction medium was adjusted to 6.8 by adding few drops of aqueous Na₂CO₃ solution (0.011 g/10 mM). The resulting solution was reduced to half of its original volume and kept aside. The colored complexes (except 7-9 and 12) formed on standing were collected by vacuum filtration and washed several times with cold water, ethanol and diethyl ether. The resulting complexes were dried in air and stored in vacuum desiccator over anhydrous CaCl₂.

Various physico-chemical properties and IR and electronic absorption data of the synthesised complexes are given in **Tables 1 and 2** respectively. From the IR spectra, it has been concluded that the ligand 5-FU(A) bind to the M(II) ions via, N3 and C4=O

atoms and amino acids(B) bind through –N atom of amino group and carboxylato oxygen (COO-) during the formation mixed ligand complexes. The electronic absorption data confirms their distorted octahedral geometry. ¹H NMR spectra of Zn(II)–5-FU(A)–val(B) complex (9) reveals that the nonappearance of proton peak of –COOH for amino acids confirm that gly/ala/val(B) ligands are binded throughout bidentate coordination with the Zn(II) ion via amino –N and deprotonated carboxylato –O atoms. The spectrum of Zn(II) complexes show a characteristic new peak centered between 4.72–4.77 ppm which corroborate the presence of coordinated water molecule in the complexes and is shown in **Figure 2** and structure of all the complexes are given in **Figure 3**.

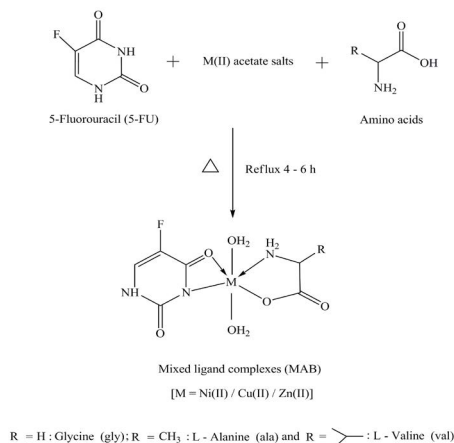


Figure 1. Synthetic route of mixed ligand complexes.

Table 1. Physico-chemical properties of mixed ligand complexes.

Complex	Color	Empirical formula ^a	Mol. wt	(m/z) value	Yield (%)	Elemental analysis, found (calculated) %				λ_m (S cm ² mol ⁻¹)	M.pt. (°C)
						M	C	H	N		
1	Green	NiC ₆ H ₁₁ FN ₃ O ₆	297.99	298.95	72	19.64(20.04)	24.11(24.49)	3.71(4.11)	14.06(14.41)	19.22	245
2	Green	NiC ₇ H ₁₃ FN ₃ O ₆	312.01	312.88	68	18.76(19.08)	26.87(27.11)	4.19(4.52)	13.43(13.76)	21.4	262
3	Pale green	NiC ₉ H ₁₇ FN ₃ O ₆	340.03	340.93	59	17.22(17.38)	31.71(32.01)	5.03(5.44)	12.36(13.68)	24.95	270
4	Dark blue	CuC ₆ H ₁₁ FN ₃ O ₆	302.98	303.7	75	20.92(21.21)	23.73(24.09)	3.65(4.02)	13.84(14.21)	22.25	252
5	Lustrous blue	CuC ₇ H ₁₃ FN ₃ O ₆	317	317.73	65	20.00(20.31)	26.46(26.60)	4.12(4.41)	13.22(13.45)	24.7	269
6	Pale blue	CuC ₉ H ₁₇ FN ₃ O ₆	345.03	345.78	60	18.38(18.61)	31.26(31.53)	4.96(5.17)	12.15(12.49)	25.65	>280
7	White	ZnC ₆ H ₁₁ FN ₃ O ₆	303.99	305.57	68	21.41(21.74)	23.58(23.91)	3.63(3.91)	13.75(14.11)	23.36	263
8	White	ZnC ₇ H ₁₃ FN ₃ O ₆	318	319.6	60	20.47(20.72)	26.31(26.55)	4.10(4.32)	13.15(13.44)	24.48	274
9	White	ZnC ₉ H ₁₇ FN ₃ O ₆	346.03	347.65	72	18.81(19.12)	31.09(31.35)	4.93(5.21)	12.09(13.37)	25.8	>280

^a C, H and N analysis values are within the limit of ± 0.3-0.4%.

Table 2. IR and electronic absorption (λ_{max}) spectral data of 5-FU(A) and its mixed ligand complexes.

System	IR spectral data (cm ⁻¹)									Electronic absorption value (cm ⁻¹)	
	$\nu(\text{OH}_2)$	$\nu(\text{C}=\text{O})$ of C ₂ & C ₄ pyrimidine ring	$\nu(\text{N-H})$ of N ₁ & N ₃ pyrimidine ring	$\nu(\text{C}_5\text{-F})$ pyrimidine ring	$\nu(\text{NH}_2)$	$\nu(\text{COO-})$ as	$\nu(\text{COO-})$ s	$\nu(\text{M-O})$	$\nu(\text{M-N})$	λ_{max}	Band assignments
5-FU(A)	-	1704, 1685 (s)	1513, 1430 (s)	1470 (s)	-	-	-	-	-	-	-
1	3250, 845(s)	1698, 1656 (s)	1510, 1409 (s)	1474 (s)	1563 (s)	1665 (s)	1442 (s)	436 (w)	552 (s)	27778, 16807, 10111	³ A _{2g} (F)→ ³ T _{1g} (P) ³ A _{2g} (F)→ ³ T _{1g} (F) ³ A _{2g} (F)→ ³ T _{2g} (F)
2	3255, 837(s)	1703, 1662 (s)	1515, 1412 (s)	1473 (s)	1560 (s)	1659 (s)	1438 (s)	418 (w)	534 (s)	27473, 16949, 10205	³ A _{2g} (F)→ ³ T _{1g} (P) ³ A _{2g} (F)→ ³ T _{1g} (F) ³ A _{2g} (F)→ ³ T _{2g} (F)
3	3240, 840 (s)	1701, 1658 (s)	1512, 1414 (s)	1474 (s)	1565 (s)	1660 (s)	1442 (s)	424 (w)	546 (s)	27028, 16949, 10152	³ A _{2g} (F)→ ³ T _{1g} (P) ³ A _{2g} (F)→ ³ T _{1g} (F) ³ A _{2g} (F)→ ³ T _{2g} (F)
4	3240, 824 (s)	1702, 1663 (s)	1516, 1405 (s)	1472 (s)	1558 (s)	1655 (s)	1440 (s)	430 (w)	578 (s)	26667, 15625	² E _g (D)→ ² T _{2g} (D) LMCT (n→π*)

5	3265, 820 (s)	1699, 1653 (s)	1510, 1414 (s)	1468 (s)	1562 (s)	1638 (s)	1412 (s)	432 (w)	568 (s)	26316, 15504	${}^2E_g(D) \rightarrow {}^2T_{2g}(D)$ LMCT ($n \rightarrow \pi^*$)
6	3255, 835 (s)	1703, 1657 (s)	1514, 1410 (s)	1472 (s)	1556 (s)	1642 (s)	1420 (s)	421 (w)	544 (s)	26178, 15335	${}^2E_g(D) \rightarrow {}^2T_{2g}(D)$ LMCT ($n \rightarrow \pi^*$)
7	3275, 838 (s)	1706, 1658 (s)	1511, 1403 (s)	1469 (s)	1556 (s)	1648 (s)	1436 (s)	405(w)	538 (s)	25974	LMCT (L \rightarrow M)
8	3258, 844 (s)	1699, 1655 (s)	1515, 1408 (s)	1472 (s)	1551 (s)	1625 (s)	1410 (s)	413 (w)	526 (s)	26110	LMCT (L \rightarrow M)
9	3264, 829 (s)	1703, 1660 (s)	1512, 1414 (s)	1468 (s)	1554 (s)	1632 (s)	1422 (s)	429 (w)	545 (s)	26385	LMCT (L \rightarrow M)

(s: sharp; w: weak; 5-FU(A): 5-Fluorouracil ligand)

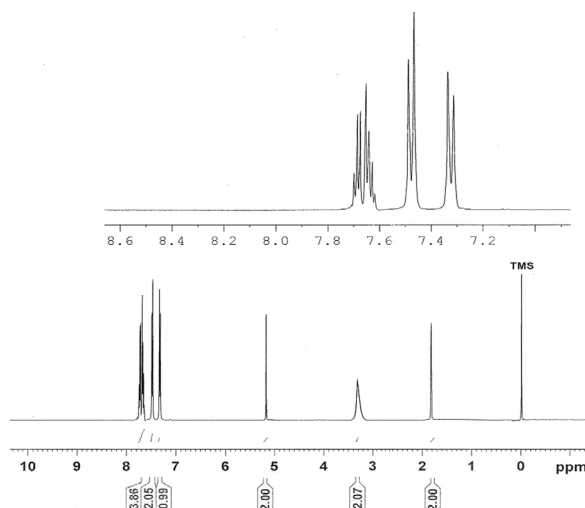


Figure 2. ${}^1\text{H}$ NMR spectra of Zn(II)-5-FU(A)-val(B) complex (9).

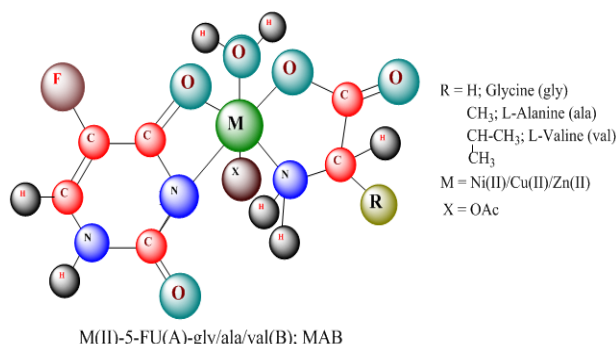


Figure 3. Proposed structure of complexes (1-9).

RESULTS AND DISCUSSION

In vitro biological activities of 5-fluorouracil and their complexes (1-9) were tested against five pathogenic bacterial and three fungal microorganism species by well diffusion method using agar as nutrient. Measured zone of inhibition (in mm) against the growth of bacterial and fungal species were summarized in **Tables 3 and 4**, respectively. On comparing the obtained results with commercially available tetracycline (antibacterial control) and nystatin (antifungal control) standard drugs which are used as control. All the complexes show remarkable biological activities at higher concentration (75 μg) against microorganisms (**Figure 4**). Biological activities of the complexes are normally higher than 5-fluorouracil and the activities depend upon the metal(II) ions i.e., size, charge distribution, shape and redox potential of the metal chelates^[15]. Also, this enhancement in the activity may also be rationalized^[16] on the basis of their structures by mainly possessing additional electron donor or withdrawing groups. Furthermore, the coordination reduces the polarity^[17] of the metal(II) ion mainly because of the partial sharing of its positive charge with the donor groups within the chelate ring system, which is mainly formed by chelation. This increased inhibition activities of the complexes can also be explained on the basis of Overtone's concept and Tweedy's chelation hypothesis^[18,19]. This consequence present in these complexes could be applied reasonably to heal some common diseases caused by *Bacillus cereus* and *Escherichia coli*^[20]. All the complexes are more bacteriostatic than fungistatic (**Tables 3 and 4**) and the zone of inhibition against the bacterial and fungal species for the mixed ligand complexes follows the order as: Control >> Cu(II) > Ni(II) > Zn(II) > 5-fluorouracil which follows the Irving-Williams stability. Furthermore, the mixed ligand valine complexes show higher activities which may be due to the presence of electron donating and electron withdrawing (C5-F) moieties in them.

Table 3. Antibacterial activities of mixed ligand complexes.

Compound	Diameter of inhibition zone in mm for different microorganism species														
	Bacterial species														
	Bc			Ss			Sa			Ec			Pa		
	Concentration in μg														
	25	50	75	25	50	75	25	50	75	25	50	75	25	50	75
Control	16	18	21	15	18	22	19	21	22	20	22	25	15	17	19
5-Flurouracil	9	10	12	-	-	-	11	13	15	9	11	14	-	-	-
1	11	13	15	10	12	16	-	-	-	13	14	16	11	13	14
2	-	-	-	11	15	18	14	15	17	15	17	20	-	-	-
3	13	16	18	13	15	19	-	-	-	18	19	22	13	14	16
4	13	14	18	-	-	-	15	17	19	-	-	-	12	15	17
5	14	16	19	-	-	-	17	18	21	16	19	22	-	-	-
6	15	17	20	14	17	20	-	-	-	18	21	23	14	15	18
7	-	-	-	9	11	14	12	13	17	13	15	15	-	-	-
8	10	12	15	-	-	-	14	16	18	-	-	-	10	12	13
9	12	15	17	10	13	16	-	-	-	15	17	19	13	15	16

Table 4. Antifungal activities of mixed ligand complexes.

Compound	Diameter of inhibition zone in mm for different microorganism species								
	Fungal species								
	An			Es			Ca		
	Concentration in μg								
	25	50	75	25	50	75	25	50	75
Control	19	22	25	16	18	20	14	16	17
5-Flurouracil	8	10	12	6	7	9	-	-	-
1	13	15	19	-	-	-	10	10	12
2	15	18	21	12	13	15	-	-	-
3	-	-	-	13	14	17	11	12	13
4	14	17	20	-	-	-	12	13	15
5	16	19	23	13	15	17	-	-	-
6	-	-	-	14	15	18	12	14	15
7	11	13	17	10	12	14	-	-	-
8	-	-	-	12	15	16	10	11	11
9	17	19	22	-	-	-	11	13	14

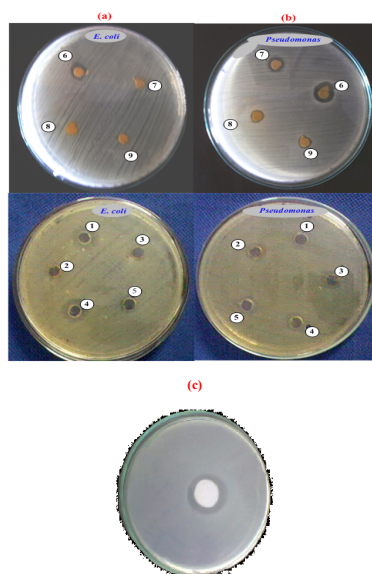


Figure 4. Antibacterial Activity Shown by complexes (1–9) (a): against *Escherichia coli*; (b): against *Pseudomonas aeruginosa*; (c): Antibacterial activity shown by 5-FU against *Escherichia coli*.

Antioxidant activities of 5-fluorouracil and their mixed ligand complexes were studied *in vitro* by DPPH free radical scavenging methods at 37 °C and their representative graph is shown in **Figure 5**. Ascorbic acid was used as the reference or positive control. All the analyses were done in three replicates. DPPH is a stable free radical that is often used for finding the radical scavenging activity in chemical analysis [21]. Reduction capability of DPPH radical is determined by the decrease in its absorbance at 517 nm

(blank) which can be induced by the antioxidant. From the results, the mixed ligand complexes have higher activities than 5-fluorouracil which is due to the presence of the metal(II) ions moiety in the complexes and also 5-fluorouracil alone shows some sensible activity^[21].

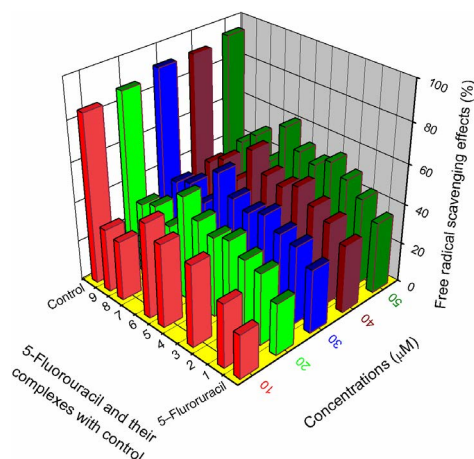


Figure 5. Antioxidant activities of mixed ligand complexes.

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