ROS dependent antitumour activity of photo-activated iron(III) complexes of amino acids

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Abstract. Several amino acid-based photo-active monomeric iron(III) complexes of the general formula, $[\text{Fe}(\text{L})_2]^-$, where L = Schiff base ligands (salisalidene arginine, salicylidenetryptophan, 3,5-di-tert-butyl benzalidine arginine and salicylidene tryptophan) were synthesized, characterized and explored for photo-activated anticancer activity to Chang Liver Cells, HeLa and MCF-7 cells. Complexes exhibited remarkable photo-cytotoxicity with IC_{50} value to the extent of $0.7 \mu\text{M}$ to Chang Liver Cells in visible light and there was a 40-fold enhancement in cytotoxicity in comparison to the cytotoxicity in dark. Complexes were non-toxic to MCF-10A (normal cells) in dark and visible light ($\text{IC}_{50} > 100 \mu\text{M}$ in dark; $\text{IC}_{50} > 80 \mu\text{M}$ in visible light) signifying target-specific nature of the anti-tumour activity of the complexes. Increased ROS concentration, as probed by DCFDA assay, in the cancer cells was responsible for apoptotic cell death. Decarboxylation or phenolate-Fe(III) charge transfer of photo-activated iron(III) complexes generating $\bullet\text{OH}$ radicals (ROS) were responsible for the apoptosis. Overall, the tumour-selective photo-activated anticancer activity of the amino acid-based iron(III) complexes have shown a promising aspect in developing iron-based photo-chemotherapeutics as the next generation PDT agents.

Keywords. Iron(III) complexes; amino acid; photocytotoxicity; photo-activation; ROS generation.

1. Introduction

Transition metal complexes with a wide range of coordination number and geometry, accessible redox chemistry, ‘tunability’ of the thermodynamics and kinetics of ligand substitution, electron transfer reaction have emerged as the potential tools for biological and medicinal applications as exemplified by cisdiamino dichloroplatinum(II), a popular anticancer agent that targets genomic DNA.^{1–5} However, systemic side effects including nephrotoxicity, drug resistance, thiol deactivation and lack of target-specificity have limited their clinical applications. Therefore, efforts are needed to find potent, cost-effective and safer cancer treatment

modalities. Photodynamic therapy (PDT) has emerged as a tumour-specific, non-invasive treatment.^{6–10} It involves simultaneous presence of tumour-localizing photosensitizer (PS), non-toxic red light for activating the PS and molecular oxygen to exhibit anti-cancer activity. The excited triplet state of the PS transfers its energy to triplet oxygen ($^3\text{O}_2$) to generate singlet oxygen ($^1\text{O}_2$) to oxidize key cellular macromolecules like proteins, DNA, cell-membrane, etc. leading to tumour ablation. Photofrin®, a first generation PDT drug, was clinically tested primarily against oesophageal cancer. Due to lack of ideal PS and difficult clinical execution, PDT has yet to gain acceleration in clinical use. Photo-activated coordination compounds exhibiting ligand exchange reaction, an isomerization reaction, intramolecular redox

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Scheme 1. Schematic drawing of the complexes, $\text{NHEt}_3[\text{Fe}(\text{L})_2]$, **1–4**, where L = Schiff base ligands of salicylidene arginine and salicylidenetriptophan (**1,2**), 3,5-di-tert-butyl benzalidine arginine and salicylidene tryptophan (**3,4**).

reaction or activating $^3\text{O}_2$ into $^1\text{O}_2$ by transferring the energy are suitable for photo-chemotherapeutic applications, as the alternatives for porphyrin-based photosensitizers.^{11–15} Bio-essential and kinetically labile first-row transition metal complexes absorbing light in PDT window (600–700 nm) are, however, preferable for photo-chemotherapeutic applications. Considering greater tissue penetration, longer wavelength absorbing and stable photo-active complexes of bio-compatible metal ions like vanadium, cobalt, copper or iron have been explored in PDT window to various cancer cell lines.^{16–21} The present work originated from our interest to develop iron(III)-based complexes for photo-activated chemotherapy by exploring the photo-labile nature of the iron(III)-carboxylates or iron(III)-phenolates on photo-activation at LMCT band generating cytotoxic hydroxyl radical.^{17,22,23} Recently, we explored the photochemistry of the oxo-bridged diiron(III) complexes for photochemotherapeutic applications to various cancer cell lines.²⁴ In the present work, we have synthesized several monomeric iron(III) complexes of amino acid-based Schiff base ligands (Scheme 1) to probe photo-activated cytotoxicity to various cancer cells with the mechanistic insight of the chemistry and cell death.

2. Experimental

2.1 Materials and methods

All reagents and chemicals were procured from HIMEDIA (India), MERCK chemicals (India), Sigma-Aldrich (USA)

and used as received without any further purification. The solvents used were purified by standard procedures.²⁵ Aqueous solutions were prepared by using double distilled water.

FT-IR spectra were recorded in solid phase using Perkin-Elmer UATR TWO FT-IR Spectrometer operating from 400 to 4000 cm^{-1} . UV-vis spectrum was recorded on Perkin-Elmer UV/VIS spectrometer. Molar conductivity measurements were done by using a EUTECH INSTRUMENT Control Dynamics (India) conductivity meter. Cyclic voltammetric measurements were made at 25 °C on a EG&G PAR model 253 VersaStat potentiostat/galvanostat using a three-electrode setup comprising of a glassy carbon as working, a platinum wire as an auxiliary, and saturated calomel as the reference (SCE) electrode. Tetrabutylammonium perchlorate (TBAP) (0.1 M) was used as supporting electrolyte in dimethylformamide (DMF).²² Electrospray ionization mass spectral measurements were done using Esquire 3000 plus ESI (Bruker Daltonics) Q-TOF Mass Spectrometers. Experimental and calculated ESI-MS values for each complex were identical to a significant last figure above the decimal point.

2.2 Hydroxyl radical generation study (ROS)

Photo-induced generation of hydroxyl radicals by the iron(III) complexes was studied by UV-visible spectroscopy. In this experiment, benzoic acid (50 μM) was initially incubated with 500 μM complex in aqueous DMF (10% v/v) and then photo-activated by using visible light. Hydroxyl radicals generated on photo-activation of Fe(III) complexes were responsible for the formation of salicylic acid which gave a reddish-brown solution of Fe(III)-salicylate complex when methanolic solution of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (100 μM) was added externally.^{26,27} UV-visible spectra of Fe(III)-salicylate (λ_{max} , 520 nm) was recorded in the presence of complexes (50 μM)

exposed to visible light (400–700 nm) for 200 s. Gradual increase in A_{520} of Fe(III)-salicylate indicated the generation of OH radical. We plotted the extent of increase of absorbance of Fe(III)-salicylate against time to correlate the relative photo-activated generation of hydroxyl radicals.

2.3 Synthesis

A reported synthetic procedure was used to synthesize amino acid-based Schiff base ligands (L^1 – L^2).^{28,29} Ligands L^3 and L^4 were synthesized following same protocol with minor modification and characterized by IR spectroscopy prior to the synthesis of the complexes.

2.3a General synthetic procedure for ligands L^3 and L^4 : Amino acid (L-arginine, 0.087 g, 1.0 mmol; L-tryptophan 0.204 g, 1.0 mmol) was dissolved in 1% KOH in absolute MeOH and stirred gently to warming for 30 min. A methanolic solution of 3,5-di-tert-butyl-2-hydroxybenzaldehyde (0.234 g, 1.0 mmol) was added and stirred at room temperature for 1 h. The mixture was left for overnight till complete precipitation. The yellow precipitate was collected by filtration, washed with cold ethanol and dried over anhydrous $CaCl_2$. Yield: 60–70%. $R_f = 0.32$ (L^3) and 0.45 (L^4) in $CH_2Cl_2/MeOH$ (5% MeOH). Typical C=N str at 1580 cm^{-1} was observed for the ligands L^3 and L^4 indicating Schiff-base ligands.

2.3b General procedure for synthesizing complexes (1–4): Complexes (1–4) were prepared by a general synthetic procedure in which a mixture of $Fe(NO_3)_3 \cdot 9H_2O$ (0.404 g, 1 mmol) and the Schiff base ligands (H_2L^1 , 0.558 g; H_2L^2 , 0.408 g; H_2L^3 , 0.70g; H_2L^4 , 0.841g) was refluxed for 3 h in 30 mL MeOH to deep purple solution in the presence of 1 mL of triethylamine. Complexes were precipitated from ethyl acetate. The reddish-purple precipitate was washed with cold ethanol and dried over vacuum. Yield: 50–70%.

Anal. Calc. for $C_{32}H_{48}N_9O_6Fe$ (1): Yield: 57%; C, 54.09; H, 6.81; N, 17.74. Found: C, 53.85; H, 6.75; N, 17.67. Molar conductance (Λ_M , $Sm^2\text{ mol}^{-1}$) in DMF- H_2O (10% v/v DMF: H_2O) at $30^\circ C$: 110; FT-IR (KBr phase), cm^{-1} : 3205 br (H_2O), 1620s($C=O_{str}$), 1377s, 1155w($C=N_{str}$), 566w. (br, broad; vs, very strong; s, strong; m, medium; w, weak). (br, broad; w, weak; m, medium; s, strong). Q-TOF ESI MS (MeOH): m/z 610.1951 $[MH_2]^+$. UV-visible in DMF- H_2O (10% v/v DMF: H_2O) [λ_{max} , nm ($\epsilon \times 10^{-4}$, $L\text{ mol}^{-1}\text{ cm}^{-1}$)]: 485 (648), 418 (725).

Anal. Calc. for $C_{42}H_{44}N_5O_6Fe$ (2): Yield: 65%; C, 65.46; H, 5.75; N, 9.09; Found C, 65.26; H, 5.59; N, 9.01. Molar conductance (Λ_M , $Sm^2\text{ mol}^{-1}$) in DMF- H_2O (10% v/v DMF: H_2O) at $30^\circ C$: 105. FT-IR (KBr phase), cm^{-1} : 3384s (H_2O), 3040br (NH), 1610s ($C=O_{str}$), 1367s, 1170s ($C=N_{str}$), 736s, 523s. Q-TOF ESI MS: m/z 670.0283 $[MH_2]^+$. UV-visible in DMF- H_2O (10% v/v DMF: H_2O) [λ_{max} , nm ($\epsilon \times 10^{-4}$, $L\text{ mol}^{-1}\text{ cm}^{-1}$)]: 493 (725), 414 (960).

Anal. Calc. for $C_{48}H_{80}N_9O_6Fe$ (3): Yield: 51%; C, 61.66; H, 8.62; N, 13.48; Found: C, 61.49; H, 8.54; N,

13.39. Molar conductance (Λ_M , $Sm^2\text{ mol}^{-1}$) in DMF- H_2O (10% v/v DMF: H_2O) at $30^\circ C$: 100. FT-IR (KBr phase), cm^{-1} : 3331br (H_2O), 2950br (NH), 1618s ($C=O_{str}$), 1528w, 1428w, 1360w, 1253w, 1170s ($C=N_{str}$). Q-TOF ESI MS: m/z 834.4455 $[MH_2]^+$. UV-visible in DMF- H_2O (10% v/v DMF: H_2O) [λ_{max} , nm ($\epsilon \times 10^{-4}$, $L\text{ mol}^{-1}\text{ cm}^{-1}$)]: 526 (379), 423 (1114).

Anal. Calc. for $C_{58}H_{76}N_5O_6Fe$ (4): Yield: 72%; C, 70.01; H, 7.70; N, 7.04; Found: C, 69.69; H, 7.65; N, 6.93. Molar conductance (Λ_M , $Sm^2\text{ mol}^{-1}$) in DMF- H_2O (10% v/v DMF: H_2O) at $30^\circ C$: 95. FT-IR (KBr phase), cm^{-1} : 3231 br (H_2O), 2988m, 2942m (C-H), 2608s (COO^-), 2502s, 1618s ($C=O_{str}$), 1443s, 1140s ($C=N_{str}$), 737m, 553m. Q-TOF ESI MS: m/z 894.4076 $[MH_2]^+$. UV-visible in DMF- H_2O (10% v/v DMF: H_2O) [λ_{max} , nm ($\epsilon \times 10^{-4}$, $L\text{ mol}^{-1}\text{ cm}^{-1}$)]: 520 (302), 440 (221).

2.4 Biological assay

Experimental details of Cell viability assay, ROS generation are provided in the Supplementary section.

3. Results and Discussion

3.1 Synthesis

All the four new amino acid-based binary complexes of the formulation $Et_3NH[Fe(L)_2]$ were prepared by general synthetic procedure in which methanolic solution of $Fe(NO_3)_3 \cdot 9H_2O$ was refluxed with the methanolic solution of amino acid-based Schiff base ligands (L^1 – L^4) in the presence of stoichiometric amount of triethylamine (Et_3N). Complexes were precipitated as a reddish-brown powder on the addition of ethyl acetate. Dried complexes were used for further characterization, analytical studies and photo-activated cytotoxic investigations. The purity of the complexes was determined by elemental analysis (C, H, N analysis) before characterization and other studies.

3.2 Solubility and stability

Complexes (1–4) were soluble in aqueous dimethylformamide (10% v/v, DMF), DMF and dimethyl sulfoxide (DMSO). Stability of the complexes was determined by UV-visible spectral measurements in which spectral traces were recorded in different time intervals till 72 h in DMF: H_2O (10% DMF v/v). We observed no change in UV-visible spectra of the complexes in aqueous DMF indicating the stability of the complexes for biological studies. However, UV-A (365 nm) light had a significant effect on the complexes in aqueous DMF.

Figure 1. The UV-visible spectrum of the complexes **1–4** in DMF-H₂O (10% v/v DMF:H₂O) with the inset showing their d–d bands.

3.3 Characterization and general aspects

Negatively charged complexes were 1:1 electrolyte as evident from the molar conductance data typically in the range 95–115 Sm² mol^{−1} determined in aqueous-DMF (10% v/v DMF).³⁰ Complexes were characterized by the molecular ion peak as [MH₂]⁺, where M was the molecular ion, in Q-TOF ESI mass spectra determined in MeOH (Figures S1–S4, Supplementary Information). Strong C=N_{str} was observed at ~1500 cm^{−1} in the IR spectra of the complexes (Figure S5, Supplementary Information).³¹ Complexes exhibited a broadband with λ_{max} in the range 480–550 nm, assignable to typical phenolate(p_π)–to–iron(III)(d_{π*}) or carboxylate(p_π)–to–iron(III)(d_{π*}) charge-transfer transition in DMF-H₂O (10% DMF v/v) (Figure 1).^{22–24} Other ligand-centred (LC) bands for the complexes were observed at <400 nm. The LMCT band is broad extending to the PDT window that enabled us to use visible light (400–800 nm) for photocytotoxicity of the complexes. Complexes (**2,4**) showed quasi-reversible cyclic-voltammetric response at 0.00 V and 0.04 V, respectively, for the Fe(III)/Fe(II) couple in DMF containing 0.1 M TBAP as supporting electrolyte. The negative reduction potential of the redox couple indicated facile redox chemistry of the complexes rendering them suited for photo-chemo-therapeutic applications (Figure S6, Supplementary Information).

3.4 DFT calculations and optimized geometry

Optimized geometry and the corresponding energetics of the complexes (**1–4**) was determined

by using unrestricted B3LYP density functional theory.³² The optimized energy of all complexes (**1–4**) are **1**(−2023 a.u.), **2**(−2184 a.u.), **3**(−2453 a.u.) and **4**(−2648 a.u.) (Figure 2). The optimized energy of the complexes is lower than their corresponding ligands, L¹(−950.97 a.u.), L²(−1030.36 a.u.), L³(−1263.87 a.u.) and L⁴(−1343.74 a.u.) (Figure S10, Supplementary Information) which indicated the formation stable complexes (**2–4**). We have determined the energies of HOMO and LUMO of the respective complexes. Larger energy gap in HOMO and LUMO indicated the thermodynamic stability of the complexes.

3.4a Photo-cytotoxicity assay: Ability of mitochondrial dehydrogenases in the viable cells to cleave the tetrazolium rings of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) forming dark violet membrane impermeable crystals of formazan was explored to probe cytotoxicity of the complexes *in vitro* to Chang Liver Cell, HeLa (human cervical carcinoma), MCF-7 (human breast carcinoma) and normal breast cells line (MCF-10A) in the dark or under photo-illuminated condition using visible light (400–700 nm, 10 J cm^{−2}) (Table 1, Figure 3, Figure S7).²² MCF-10a cell line was chosen as a control cell line to probe the tumour-specificity of the complexes.³³ Cells were treated with the complexes in dose-dependent manner and the IC₅₀ values were obtained by the non-regression analysis of the dose–response plot for the complexes and the same are presented in Table 1.

Complexes (**1–4**) were non-toxic to HeLa and MCF-7 cells in dark (IC₅₀ ~ 60–90 μM), while they are moderately toxic (IC₅₀ ~ 40 μM) to Chang Liver Cells although the difference in cytotoxicity behavior in different cell lines are not clearly known. We observed the remarkable effect of visible light on the cell viability of the cancer cells treated with the complexes (IC₅₀ ~ 6–20 μM). However, complexes (**1–4**) were enormously cytotoxic to Chang Liver Cells with an approximate IC₅₀ value of 1.0 μM. The photocytotoxicity index (PCI), which may be defined as the ratio of IC₅₀ values in dark and light, of the complexes (**1–4**) in Chang Liver Cells are in the range 24–56. Complex **4** with photo-active indole moiety exhibited highest photo-cytotoxicity index. However, the photocytotoxicity index of the complexes in HeLa and MCF-7 cells were <15, with the complex **4** exhibited highest among the complexes. Level of toxicity of the complexes in normal cells (MCF-10A) in dark and light was significantly low (IC₅₀ in dark, >100 μM; IC₅₀ in light ~90 μM) revealing state of the art of the iron-based compounds as targeted photo-chemotherapy. Lower toxicity of the complexes in MCF-10A cells may

Figure 2. Energy-minimized structures and HOMO/LUMO of all complexes (1–4).

be due to the natural efflux mechanism in the normal cells or could be due to the reduced concentration of ROS. Ligands (L^1 – L^4) and $Fe(NO_3)_3 \cdot 9H_2O$ were almost non-toxic to HeLa cells excluding any possibility of the artefact as the involvement of only ligands or the Fe^{3+} salt in photo-cytotoxicity. It was the ligands in $Fe(III)$ -bound form, has shown photo-cytotoxicity. Preferential exposure of light to tumours only in PDT has led the method to be tumour-specific. In the present case, we compared the cytotoxicity of the complexes to the cancer cells in light and to the normal cells in dark mimicking the condition in PDT. We observed remarkable differences in cytotoxicity for the complexes considering the protocol of clinical exercise of PDT and this could be of paramount importance in developing iron(III)-based next generation photo-chemotherapeutics.

3.5 Intracellular Reactive Oxygen Species (ROS) generation

Enhanced cytotoxicity of the photo-activated complexes (1–4) in cancer cells compared to the normal cells might

be due to the enhanced generation of ROS. The intracellular ROS generation in MCF-7 cells treated with complex 4 on photo-exposure was studied using non-polar cell permeable 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye (Figure 4). The dye which is cleaved by the intracellular esterase is converted into highly fluorescent 2',7'-dichlorofluorescein (DCF) on oxidation by intracellular ROS or ROS generated *in situ*, emitting green light. Intracellular ROS generation was probed in our present study by flow cytometric analysis (FACS) on MCF-7 cells using DCFH-DA dye.³⁴ Shift in fluorescence intensity of the MCF-7 cells treated with the complexes gave us the measure of the intensity of ROS generation. A greater shift signified a higher amount of oxidized DCF and thus greater ROS generation.

Nature of ROS was probed by the 0.8% agarose gel electrophoresis where we have confirmed the photo-oxidation of SC pUC18DNA in UV-A light (365 nm) (Figure S8, Supplementary Information). Complexes (1–4) were able to cleave SC DNA to the extent of 60% into the nicked circular (NC) form. However, we observed a significant reduction in the cleavage of

Table 1. Cytotoxicity data ($IC_{50}/\mu M$) of the complexes in dark and on visible light exposure.

Complexes	Chang liver cells			HeLa			MCF-7			MCF-10A		
	$IC_{50}/\mu M$			$IC_{50}/\mu M$			$IC_{50}/\mu M$			$IC_{50}/\mu M$		
	Dark ^a	Light ^b	PCI ^c	Dark ^a	Light ^b	PCI ^c	Dark ^a	Light ^b	PCI ^c	Dark ^a	Light ^b	PCI ^c
1	40.0 (± 5.0)	1.25 (± 0.5)	32	67.5 (± 7.0)	21.3 (± 4.0)	3	77.5 (± 5.0)	20.1 (± 3.1)	4	>100	92.8 (± 8.0)	—
2	44.0 (± 3.0)	1.89 (± 0.3)	23	61.6 (± 5.0)	14.5 (± 1.7)	4	67.5 (± 3.0)	12.2 (± 2.2)	5	>100	84.3 (± 4.0)	—
3	40.7 (± 5.0)	1.19 (± 0.2)	34	68.1 (± 5.0)	12.1 (± 1.2)	6	79.8 (± 3.0)	9.1 (± 1.8)	9	>100	89.1 (± 4.0)	—
4	38.2 (± 3.0)	0.72 (± 0.2)	53	64.2 (± 5.0)	7.2 (± 1.5)	9	87.1 (± 3.0)	6.1 (± 0.7)	14	>100	94.4 (± 4.0)	—
L¹	—	—	—	>100	91.3 (± 4.3)	—	—	—	—	—	—	—
L²	—	—	—	>100	94.7 (± 8.1)	—	—	—	—	—	—	—
L³	—	—	—	>100	88.4 (± 4.6)	—	—	—	—	—	—	—
L⁴	—	—	—	>100	97.9 (± 6.2)	—	—	—	—	—	—	—
Fe(NO₃)₃ · 9H₂O	—	—	—	>100	>100	—	—	—	—	—	—	—
[{Fe(μ-O)(L-his)(phen)}₂](ClO₄)₂	—	—	—	30.0 (± 4.0)	17.0 (± 2.0)	—	36.0 (± 4.0)	21.0 (± 3.0)	—	>100	88.0 (± 6.0)	—
[Fe₂(μ-O)(μ-OAc)(phen)₄](ClO₄)₃	—	—	—	58.0 (± 6.0)	14.0 (± 3.0)	—	60.0 (± 7.0)	16.0 (± 2.0)	—	>100	76.0 (± 5.0)	—
Cisplatin^d	—	—	—	24.0	—	—	—	—	—	—	—	—
Photofrin^{@d}	—	—	—	>41.0	4.3 (± 0.2)	—	—	—	—	—	—	—

^a IC_{50} values correspond to 24 h incubation in dark. ^b IC_{50} values correspond to 4 h incubation in the dark followed by exposure to visible light (400–700 nm, 10 J cm⁻²). ^c IC_{50} values of the complexes were adapted from the ref. ²⁴. ^dThe IC_{50} values of cisplatin and photofrin[@] were adapted from ref. ²². ^ePCI is defined as a photocytotoxicity index which is the ratio of IC_{50} value in dark to the IC_{50} value in visible light.

Figure 3. Cell viability (MTT assay) plots showing cytotoxicity of the iron complexes (1–4) to MCF-7 and MCF-10a cells in dark (black squares) and in the presence of visible light (red symbols, 400–700 nm, 10 J cm^{-2}). MCF-7 and MCF-10a cells: visible light exposure (400–700 nm, 10 J cm^{-2}). The black symbols denote cells treated with the complexes in dark whereas the red symbols denote cells treated with the complexes in presence of visible light.

Figure 4. The shift in fluorescence band of DCFH-DA of MCF-7 cells treated with the complex 4 in dark and in visible light (400–700 nm, 10 J cm^{-2}) indicating the extent of ROS generation by the complexes.

SC pUC19 DNA in the presence of hydroxyl radical ($\bullet\text{OH}$) radical scavengers like DMSO, KI and mannitol indicating generation of hydroxyl radical (Figure S10, Supplementary Information) as the oxidant responsible for DNA cleavage, when the complex 4 was photo-activated.³⁵

3.6 UV-visible spectral probe for hydroxyl radical ($\bullet\text{OH}$) generation

Later we probed the photo-activated generation of hydroxyl radical ($\bullet\text{OH}$) by photochemical hydroxylation of benzoic acid into salicylic acid.^{26,27} Photo-activated

Figure 5. Studies on the generation of hydroxyl radical when complex **4** was photo-activated in visible light (400–700 nm, 10 J cm^{-2}). The inset figure shows the changes in the spectral traces of the complex **4** with exposure to the UV-A light for different intervals.

complexes those were able to generate hydroxyl radical, efficiently transform benzoic acid into salicylic acid which form Fe(III)-salicylate complex on external addition of a methanolic solution of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$. Formation of Fe(III)-salicylate was probed by UV-visible spectroscopy with the intense band (LMCT) at 520 nm. The intensity of the band was observed to be increased with respect to the UV light irradiation time (Figure 5, Figure S9, Supplementary Information).

For complex **4**, although, we observed the appearance of new bands at 550 nm instead of typical Fe(III)-salicylate band at 520 nm. Complex **4** could generate an

excess amount of $\bullet\text{OH}$ radical leading to the formation of catechol and Fe(III)-catecholate has typical $\lambda_{\text{max}} > 550 \text{ nm}$.³⁶ Generation hydroxyl radical ($\bullet\text{OH}$) as ROS from the photo-activated complexes can be explained according to the Scheme 2.

Photo-activation at the phenolate to Fe(III) or carboxylate to Fe(III) LMCT band localized in 450–550 nm could lead to the charge transfer from phenolate to Fe(III) or photodecarboxylation. Either way *in situ* generated iron(II) is readily susceptible for Fenton type reaction with molecular oxygen to generate hydroxyl($\bullet\text{OH}$) radical.^{23,24} Level of hydroxyl radical generation accounted for the relative order of photocytotoxicity of the complexes (**1–4**). The presence of *t*-Bu group in the complex **4** might be responsible in facilitating rapid charge transfer from phenolate $\rightarrow$ Fe(III) on photo-activation and stabilizing the resulting phenolate radical, Fe(II) as well. This resulted in the generation of enhanced hydroxyl radical ($\bullet\text{OH}$) and accounted for higher photo-cytotoxicity of the complex **4**.

3.7 Apoptosis

To investigate the mode of photo-induced cell death, Annexin-V-FITC/PI assay was performed with MCF-7 cell line (Figure 6).³⁷ This assay was based on the dual staining by annexin V-FITC (fluorescein isothiocyanate) and the DNA binding dye propidium iodide (PI). Cells in the early apoptosis stage are identified as a population in Q1-LR, necrotic cells population will be identified in Q1-UL. The population in Q1-UR represents cells in the late apoptotic stage. The MCF-7 cells

Phenolate-*O*-Fe(III) LMCT

Carboxylate-*O*-Fe(III) LMCT

Scheme 2. The proposed mechanism of photo-activated generation of hydroxyl radical by the complexes (**1–4**).

Figure 6. Annexin V-FITC/PI coupled to flow cytometry analysis showing apoptosis induced by complex **4** (10 μ M) in the presence of visible light (400–700 nm, 10 J cm⁻²).

were treated with complex **4** (10 μ M) followed by 1 h photo-irradiation with 400–700 nm light and 4 h post photo-irradiation incubation. Around 42% populations of the cells were in apoptotic stage (Q1-LR+ Q1-UR) without any significant necrotic population. In the dark, however, the complex induced about 20% necrotic cell deaths. Overall, this assay suggests an apoptotic mechanism of photo-induced cell death by the complex **4**.

4. Conclusions

In summary, we have explored the photo-activated antitumour activity of selected monomeric amino-acid-based iron(III) complexes by modulating intracellular levels of ROS when exposed to the visible light. The cytotoxicity was purely ROS dependent. Complexes when photo-activated at their broad LMCT band (400–600 nm), hydroxyl ($\bullet$ OH) radical was generated *via* photo-redox mechanism (Type-I photoprocess) and enhanced concentration of ROS might have knocked down the mitochondrial redox cascades which led

to the apoptosis. Cytotoxicity of the complexes was remarkably tumour-specific leaving normal cells almost unaffected. Therefore, the present study has ended up with a considerable scope to explore iron(III) complexes further for *in vivo* applications and extensive testing towards the clinical applications of photodynamic therapy (PDT) *in lieu* of current drugs.

Supplementary Information (SI)

SI has details on biological assays like cell-viability assay, studies in intracellular ROS generation, Figures related to Q-TOF ESI mass spectra, solid-phase IR, Cyclic voltammetry, MTT plots of the ligands (L¹–L⁴), 0.8% agarose gel electrophoresis and studies on hydroxyl radical generation. Supplementary Information is available at www.ias.ac.in/chemsci.

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