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ARTICLE TYPE

Stitching of Tyrosine and 10*H*-Acridin-9-one: *Turn-ON* Fluorescence in the Narrow pH range 7.4 – 8.5 and Intracellular Labelling of Cancer Cells†

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We tailored 10*H*-acridine-9-one and (*S*)-tyrosine into 3-(4-hydroxyphenyl)-2-[(9-oxo-9,10-dihydroacridine-4-carbonyl) amino]propionic acid (2). 2 underwent pH dependent protonation/deprotonation and the effect was harnessed in terms of change in the fluorescence. The characteristic fluorescence change of the molecule at pH 7.5 ±1 range and its cell permeability allowed us to label the cancer cells.

The plethora of fundamental information that can be gained by monitoring the cellular pH[1,2] led to the development of a number of pH responsive probes[3] for fluorescence microscopic imaging. It is probably reasoned to the difficulty in the working of the probe (ON-OFF of fluorescence) within the narrow range of physiological pH and under the biological conditions that the pH dependent fluorescence microscopy finds limited use as a diagnostic tool for the early detection of cancer. Towards this end, inspired by the photoinduced intramolecular charge transfer (ICT)[4] quenching of flavin fluorescence by a non-covalently bonded *Tyr* in flavin reductase (**1**, Figure 1),[5] we designed a new probe. Further, it was motivated by the conviction that the acridine part and amino acid residue undergo pH dependent protonation-deprotonation and consequently due to the altered intramolecular charge transfer make change in the fluorescence of the molecule; we stitched together 10*H*-acridin-9-one (analogue to flavin) and amino acids. Amongst the resulting compounds screened for their pH dependent fluorescent properties, **2** (Figure 1) was identified as exhibiting fluorescence *ON* in the pH 7.5±1 range and fluorescence *OFF* at pH <6.5 and >8.5.

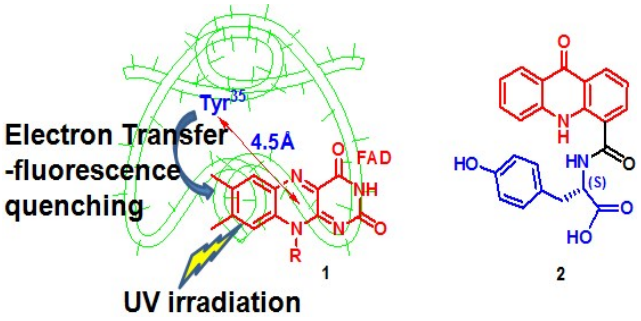

Figure 1. Schematic representation of flavin reductase bound to FAD placed 4.5 Å from Tyr[35] (**1**) and structure of compound **2**.

Absorption and emission spectra of **2** (10 μM) in HEPES buffer – DMSO (9:1; pH 7.0) exhibited λ_{abs} at 370 nm (Figure S27) and λ_{em} at 450 nm (λ_{ex} 250, 370 nm) (Figure 2), respectively. When the pH was changed to acidic (pH 3.0), a slight decrease in the fluorescence intensity was observed. On the other hand, considerable increase in the fluorescence emission was observed when the pH of the solution was varied between 7.0–8.0 (Figure 2). Remarkably, further increase in the pH of the solution (>8.5) resulted in quenching of fluorescence. The 6 – 8 fold increase in fluorescence at pH 7.4–8.0 can be attributed to protonation-deprotonation dependent variation in the ICT process of the molecule which was confirmed from the NMR spectral data.

COOH group at δ_H 12.9 was deprotonated at pH 3.5 (Figure 3, S28, S29). Acridine NH (δ_H 12.1) was protonated at pH 6.5 and shifted downfield but it was restored to its original δ_H at pH 7.5 and ultimately disappeared (deprotonated) at pH >11.0. The signal due to phenolic OH at δ_H 9.19 was disappeared at pH ≥8.5. Therefore, the molecule undergoes protonation – deprotonation within a narrow pH range. Theoretical calculations comply with the ^{1}H NMR spectral results (Figure S30). The combined experimental and theoretical results favour the possibility of ICT at pH 6.5 due to the positive charge on acridine and negative charge on the *Tyr* residue (**2a**, Scheme 1), and hence low fluorescence emission. Deprotonation of acridine NH at pH 7.5 – 8.0 decreased the ICT and probably species **2b** is responsible for sharp increase in the fluorescence in this pH range. At higher pH, the deprotonation of phenolic OH (**2c**, Scheme 1, Figure S31) and hence higher electron density with the *Tyr* residue (donor) enhanced ICT and consequently diminished the fluorescence. The fluorescence quantum yield (Φ) at pH 7.6 was 0.63 and the probe does not undergo photobleaching (Figure S32). Reversing the pH of the solution to 3.5-6.5, species **2a** was regenerated (^{1}H NMR spectrum). Molecular orbital calculations revealed difference in the energy of HOMO-LUMO of **2** at different pH values (Table S1). In **2a**, the difference in the energy of the HOMO of acceptor (**2a(A)**) and HOMO of donor (**2a(D)**) allows ICT whereas in **2b**, the ICT is considerably reduced due to the reduction in $HOMO_A$-$HOMO_D$ gap. It is pertinent to mention that the COOH and phenolicOH is not in the H-bond range with acridine $N_{10}H$ rather it showed nOe with amidicNH (Figure S14).

† The authors declare no competing interests.

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The role of COOH and phenolic OH in **2** was evident as the fluorescence behavior of compounds **3-5** (Figure 4, Figure S33) was entirely different from **2**. Moreover, the distance of COOH, OH from acridine moiety and their orientation w.r.t. acridine (C_α stereochemistry) are also critical parameters for getting the desired fluorescence as compounds **6**–**8** exhibit minor fluorescence change in the pH 3-10 range (Figure S33). Excitingly, similar to the PET quenching of flavin fluorescence by non-covalently bonded *Tyr* in flavin reductase,[5] the covalent combination of acridine and *Tyr* in compound **2** enabled it to display a marked PET (Figure S34) fluorescence behavior as a function of pH.

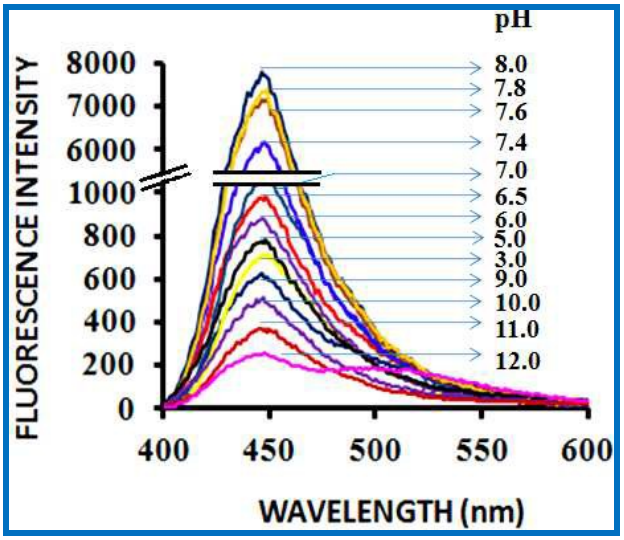


Figure 2. Fluorescence spectra of compound **2** (10 μM, DMSO:HEPES buffer, 1:9) as a function of pH.

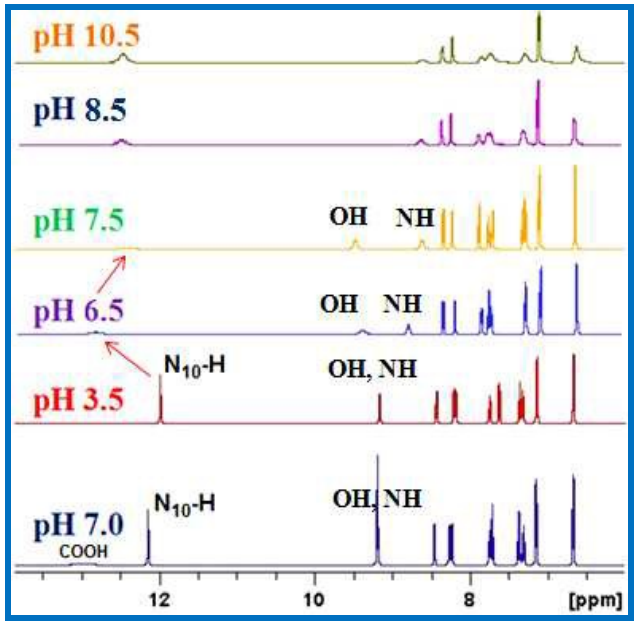
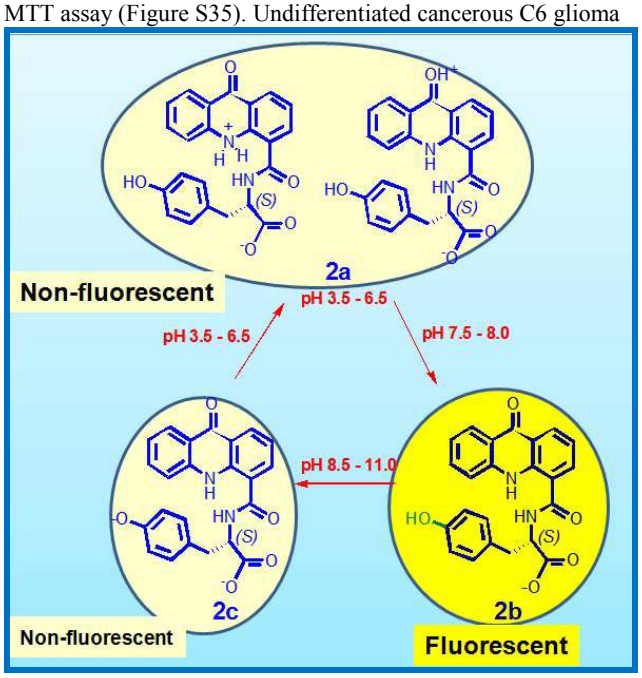


Figure 3. ^{1}H NMR spectrum of compound **2** as a function of pH.

Based on the characteristic fluorescence features of **2** and available reports of acidic extracellular microenvironment (pH_e 6.0–6.9) and basic pH in the intracellular region (pH_i 7.12-7.65)[6,7] of human and animal tumor cells, we sought to utilize probe **2** to label the cancer cells. Unequivocally, the change in pH of tumor cells (normal cell pH_e 7.3–7.4 and pH_i 7.0–7.2) enhances the metastasis, mutation rate and cell viability.[8-11] Negligible cytotoxicity of probe **2** for C6 glioma cells was measured by MTT assay (Figure S35). Undifferentiated cancerous C6 glioma



Scheme 1. Protonation-deprotonation of **2** at different pH. Charge development in the acceptor and donor part of the molecule significantly affects ICT.

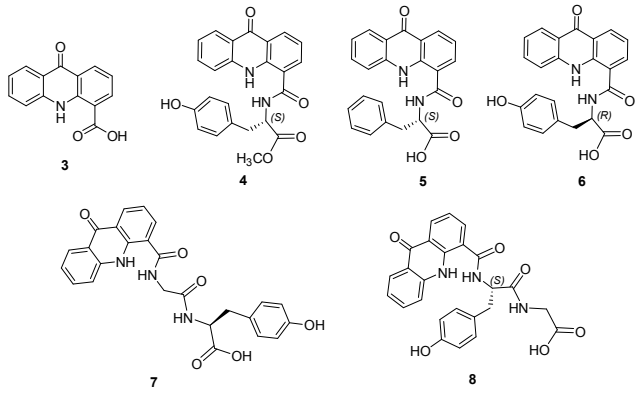


Figure 4. Compounds used for control experiments.

cells were incubated with 30 μM probe **2** for 24 h. After exposure to the probe, cells were washed with 1X PBS thrice for 5 min and fixed with acetone : methanol in 1:1 ratio for 10 min. This was followed by mounting of the cells on the slides using antifading medium and intriguingly, considerable fluorescence was observed in the confocal microscope images (Figure 5A-a). C6 glioma cells showed high intracellular fluorescence in the cytosol not in nucleus because probe signal was not colocalized with nuclear stain, DAPI staining as illustrated in the fluorescent images (overlay column of the Figure 5A). In contrast to the cancerous cells, confocal microscopic images of RA treated[12] differentiated (normal) cells showed decrease in the intracellular fluorescence intensity with compound **2** as compared to the undifferentiated cancerous cells which may reflect the change in cellular pH due to differentiation (Figure 5A-b). The relative

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expression of probe **2** in the cancerous cells was almost three times to that observed in the normal cells (Figure 5B). As control experiments, we did not observe difference of fluorescence in cancer and non-cancer cells with compound **4**, **6** and **7**.

Although we did not measure cellular pH directly but the combined results of biological and non-biological experiments along with the literature support (difference in pH of cancer and non-cancer cells) confirm that probe **2** is capable to label the cancerous cells and hence the notion of pH change of tumor cells can be translated to the paradigm of a diagnostic technique. The sensitivity of the technique may certainly be helpful in initial stage detection of cancer and consequently reduction in the cancer mortality rate which otherwise is responsible for 15% of the total deaths worldwide.[13,14]

In conclusion, inspired by a natural process, the working of a pH dependent, highly photostable (Figure S32, S36A) fluorescent probe for differentiating the cancer cells from non-cancer cells without using a tumor-targeting tag is demonstrated. The signal is potentially switchable and non-switchable depending on the local conditions, being non-fluorescent at pH 6.5 and fluorescent at pH 7.5 and hence providing the advantage of signal amplification. The effect gets reversed at pH >8.5. Both experimental results and theoretical calculations show that the protonating/ deprotonating features of the molecule contribute to the fluorescence changes within a narrow pH range. The non-toxicity of the probe to normal cells under the working concentration, permeability to the cell membrane and reversibility of its action further add to the biomedical applications of the probe.

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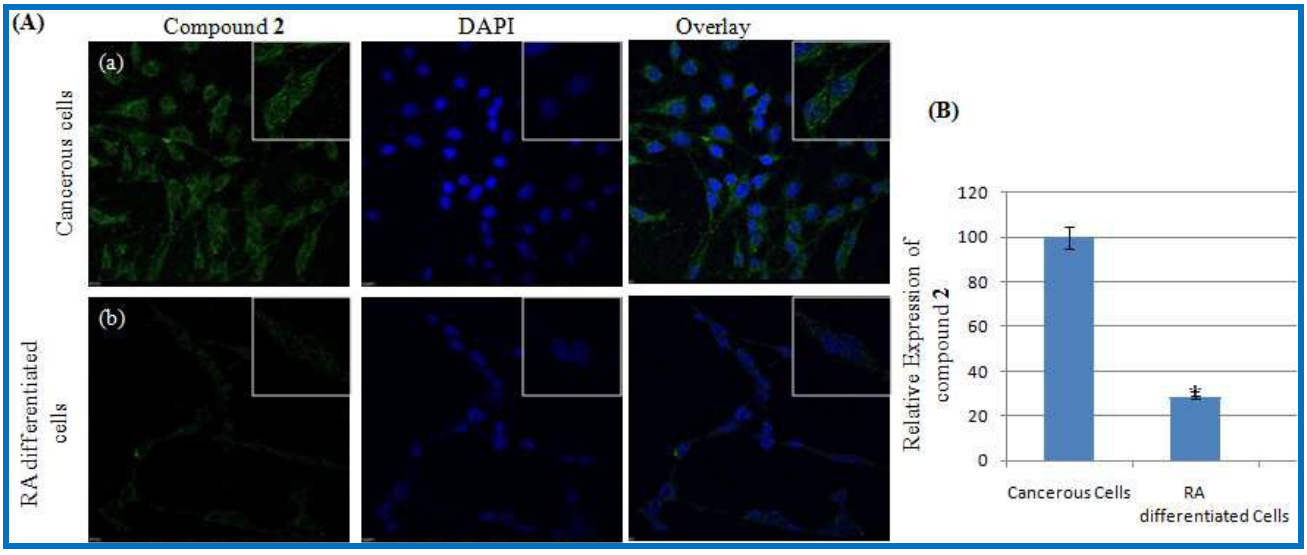

Figure 5: (A) Confocal images of the compound **2** expression in C6 glioma cells. (Scale Bar 50 μm) **(a)** Undifferentiated C6 glioma cells exposed to compound **2** for 24 h. **(b)** RA differentiated C6 glioma cells group**:** Undifferentiated C6 glioma cells first treated with 10 μM RA a potent differentiation inducer, after every 48 h for 5 days, then exposed to the compound **2** for 24 h. DAPI (4',6-diamidino-2-phenylindole) is the fluorescent stain that emits blue fluorescence on binding to the A-T rich region of the DNA. Both cancer and normal cells showed fluorescence intracellularly in the cytosol of the cell. Images were captured using A1R Nikon Confocal Laser Scanning Microscope at λ_{ex} = 488 nm and λ_{em} range 500–550 nm. To highlight the function of probe 2, a negative control sample treated by DMSO has been performed both in cancer cells and normal cells in DIC channel as DMSO itself is non-fluorescent and no fluorescence signal was detected (Figure S36B). **(B)** Histogram represents the relative change in the optical intensity of compound **2** in different treated groups as compared to control. *p<0.05 represents the statistically significance difference between the treated groups and control.

Notes and references

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†Electronic Supplementary Information (ESI) available: [General procedure, mass spectra, NMR spectra, Fluorescence spectra]. See DOI: 10.1039/b000000x/

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