RSC Advances

ROYAL SOCIETY OF CHEMISTRY

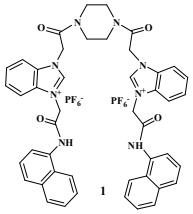
www.rsc.org/advances

GRAPHICAL ABSTRACT

Benzimidazolium-based new flexible cleft built on piperazine unit: A case of selective fluorometric sensing of ATP

Kumaresh Ghosh*, Debojyoti Tarafdar, Asmita Samadder and Anisur Rahman Khuda- Bukhsh

A benzimidazole- based compound **1** which is highly selective and sensitive towards ATP over ADP and AMP in CH_3CN/H_2O (1:1, *v/v*, using 10 mM HEPES buffer, pH 6.4) has been designed and synthesized. Compound **1** is cell permeable and can detect the presence of ATP in Human cervical cancer cells (HeLa).



1

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Benzimidazolium-based new flexible cleft built on piperazine unit: A case of selective fluorometric sensing of ATP

Kumaresh Ghosh*[a], Debojyoti Tarafdar[a], Asmita Samadder[b] and Anisur Rahman Khuda-Bukhsh[b]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

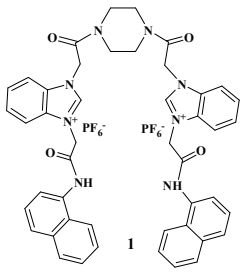
A benzimidazole- based compound **1** built on piperazine motif has been designed and synthesized. The chemosensor **1** is highly selective and sensitive towards ATP over ADP and AMP in CH_3CN/H_2O (1:1, *v/v*, using 10 mM HEPES buffer, pH 6.4) as evidenced from the significant change in emission. Furthermore, the sensor **1** is cell permeable and can detect the presence of ATP in Human cervical cancer cells (HeLa).

Introduction

Anion binding and recognition by design-based synthetic receptors is of current interest in supramolecular chemistry due to many implications that anions have in both abiotic and biotic systems.[1] Among anions of biological interest, ATP, ADP, AMP and related inorganic phosphates draw attention. ATP is a multifunctional nucleotide, important as a molecular currency of intracellular energy transfer and is involved in DNA duplication and transcription.[2] Hydrolysis of ATP under cellular condition gives ADP, AMP, and inorganic phosphates. Deficiency of ATP results in ischemia, Parkinson's disease and hypoglycaemia. Therefore, selective detection of ATP is considered to be important in anion recognition chemistry.[3] Detection of ATP can offer useful information about the production and consumption of ATP in real time and thus the metabolic rate of a cell can be realized. Owing to the benefits of fluorescence detection, there has been great interest in developing a selective fluorescent chemosensor for ATP. Careful scrutiny of the literature shows that among various approaches, metal ion complexes are usually considered to be ideal for phosphate and phosphate-based biomolecule recognition.[4] On contrary, the use of organic cation as the fluorescent sensor for nucleotides is less explored.[5]

Use of polyammonium cations by Lehn[6] in the binding of ATP stimulated the researchers to entire in this area to develop simple to complex molecular architectures for the selective recognition of ATP.[7] Though there are examples of fluorescent sensors that are efficient in selective recognition of ATP[7j] there is a need of developing new easy-to-make simple molecular receptors for the same.

Our longstanding work in molecular recognition has inspired us to report herein a simple and easy-to-make chemosensor **1** that selectively recognizes ATP over ADP and AMP fluorimetrically in aqueous CH_3CN at pH 6.4.



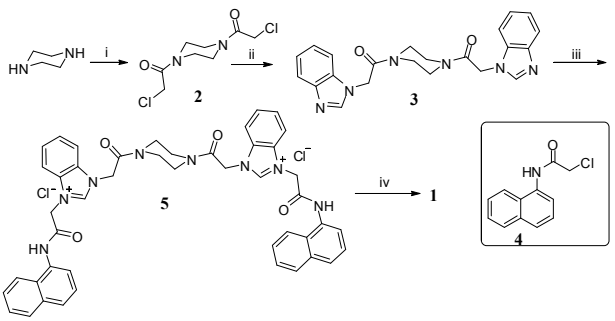
1

Results and discussion

Compound **1** was prepared according to the Scheme 1. Reaction of piperazine with chloroacetyl chloride yielded the dichloroamide **2** which was treated with benzimidazole in the presence of NaH in dry THF to afford the compound **3**. Compound **3** was next refluxed with the chloroamide **4** (obtained from 1-naphthylamine on reaction with chloroacetyl chloride in $CHCl_3$–H_2O (1:1, *v/v*)) in dry CH_3CN–DMF (5:1, *v/v*) mixture solvent for 2 days to give the dichloride salt **5**. Anion exchange reaction of **5** with NH_4PF_6 in MeOH–H_2O gave the desired compound **1** in appreciable yield. All the compounds were characterized by usual spectroscopic techniques.

[a]*Department of Chemistry, University of Kalyani, Kalyani-741235, India. Email: ghosh_k2003@yahoo.co.in, Fax: +913325828282; Tel: +913325828750;* [b]*Department of Zoology, University of Kalyani, Kalyani-741235, India.*

†Electronic Supplementary Information (ESI) available Figures showing the change in fluorescence and UV-vis titrations of receptor **1** with various anions, NOESY spectrum, MTT assay, spectral data. See http://dx.doi.org/10.1039/b000000x/



Scheme 1. (i) Chloroacetyl chloride, K_2CO_3, $CHCl_3$-H_2O, $TBAHSO_4$, stirring for 4h; (ii) NaH, benzimidazzole, THF, reflux, 8h; (iii) **4**, CH_3CN–DMF (5:1, *v*/*v*), reflux, **2** days; (iv) NH_4PF_6, MeOH/H_2O, stirring for 30 min.

In **1**, benzimidazolium-coupled naphthylamide part in each side of the conformationally flexible piperazine motif is considered to provide the polarized C-H and amide N-H bonds as hydrogen bond donors to anion. The positive charge on each benzimidazolium moiety under piperazine spacer will make the anion-complex stable involving charge-charge interaction. In view of this fact, compound **1** was explored in anion binding. For this, we initially investigated its interaction spectrophotometrically with the sodium salts of some biologically relevant phosphate anions such as ATP, ADP, AMP, $H_2PO_4^-$,

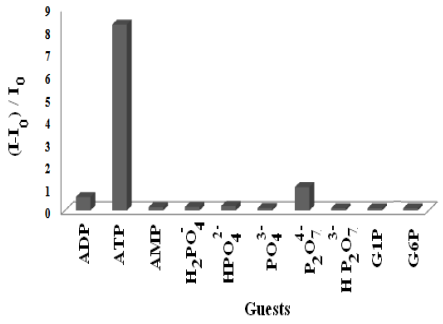


Fig 1. Change in fluorescence ratio of **1** (c = 2.5 x 10^{-5} M) at 415 nm upon addition of 10 equiv. of various sodium salts of anions (c = 1 x 10^{-3} M) [λ_{ex} = 290 nm] in CH_3CN–H_2O (1:1, *v*/*v*, using 10 mM HEPES, pH 6.4).

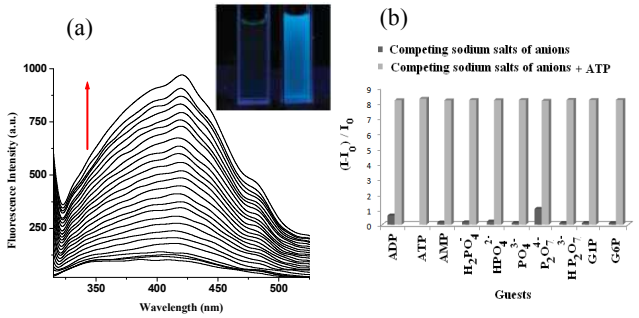


Fig 2. (a) Emission titration spectra of **1** (c = 2.5 x 10^{-5} M) in CH_3CN-H_2O (1:1,v/v, using 10 mM HEPES, pH 6.4) upon gradual addition of 25 equiv. of ATP (c = 1 x 10^{-3} M) [λ_{ex} = 290 nm], inset shows colour change under UV irradiation; (b) Change in fluorescence ratio of **1** (c = 2.5 x 10^{-5} M) in the presence of 6 equiv. amounts of ATP in presence of sodium salts of various anions in CH_3CN–H_2O (1:1, *v*/*v*, using 10 mM HEPES, pH 6.4).

HPO_4^{2-}, PO_4^{3-} and $P_2O_7^-$, glucose-1-phosphate and glucose-6-phosphate by adding standard solutions of these salts to the fixed concentration of **1** in CH_3CN-H_2O (1:1, *v*/*v*, 10 mmol HEPES, pH 6.4). In fluorescence, the change in emission of **1** upon incremental addition of the said anions in CH_3CN-H_2O (1:1, *v*/*v*, using 10 mM HEPES, pH 6.4) was monitored by exciting the probe at 290 nm. Figure 1, in this regard, represents the change in fluorescence ratio of **1** at 415 nm. As can be seen form Fig. 1, only ATP brought about significant change in emission of **1** upon interaction. Upon titration with ATP, the broad emission of naphthalene unit at 385 nm in **1** shifted to ~420 nm and became intense on progression of titration (Fig. 2a). The receptor solution under exposure of UV radiation also became bluish in color in the presence of ATP (inset, Fig. 2a). Figure 2b describes the selective fluorescence response $(I-I_0)/I_0$ of **1** towards ATP in the presence of individual sodium salt and corroborates the pronounced OFF–ON type of ATP selectivity. To our opinion, the intense emission centred at ~420 nm in Fig. 2a in the presence of ATP is attributed to the formation of exciplex (between naphthalene and adenine) that results in from the complexation either in the mode **A** or mode **B** as proposed in Fig. 3.

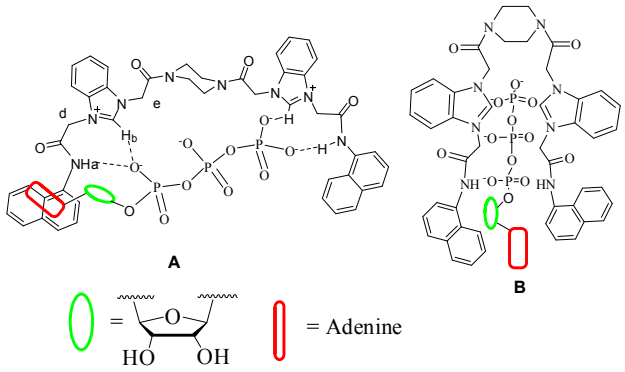


Fig 3. Probable modes of binding with ATP.

DFT optimization[8] of the structure **1** in the gas phase using B3LYP functional and 3-21G basis set reveals that the benzimidazolium moieties are away from each other and adopts an extended conformation (Fig. 4). We presume that this extended form interacts with ATP more favourably *via* the mode **A** rather than the mode **B** in Fig. 3.

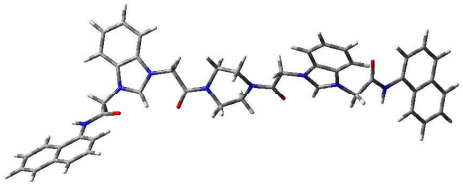


Fig 4. DFT optimized geometry of **1** in gas phase.

It is further to be mentioned that the receptor **1** under similar condition did not show any measurable interaction with other several anions such as F^-, Cl^-, Br^-, I^-, NO_3^-, AcO^-, ClO_4^-, HSO_4^- and $H_2PO_4^-$ (taken as tetrabutylammonium salts) in CH_3CN-H_2O (1:1, *v*/*v*, 10 mmol HEPES, pH 6.4) (ESI). Figure 5, in this regard, displays the change in fluorescence ratio of **1** in the presence of different anions.

In UV-vis titration, the change in absorbance of **1** was not significant and thus revealed weak interaction in the ground state. While in the presence of ATP, ADP and AMP the change in absorbance of **1** around 260 nm was increased markedly, the absorbance at ~ 290 nm decreased to the smaller extent giving a ratiometric change in the spectra. But these ratiometric spectral changes were almost same in each case and thus were not of much informative in distinction.

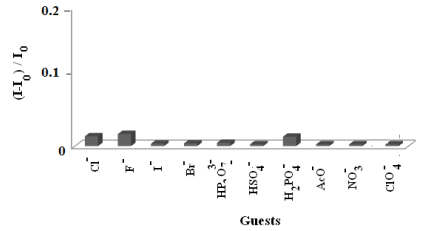


Fig 5. Change in fluorescence ratio of **1** (c = 2.5 x 10^{-5} M) at 410 nm upon addition of 5 equiv. amounts of tetrabutylammonium salts of various anions (c = 1 x 10^{-3} M) in CH_3CN–H_2O (1:1, *v*/*v*, 10 mM HEPES, pH 6.4).

We next recorded ^{1}H NMR of **1** in the presence of selective anion ATP to identify its interacting protons. For this, ^{1}H NMR of **1** (c = 1.5 x 10^{-3} M) was initially taken in d_6-DMSO. Then to this solution, ATP dissolved in minimum amount of D_2O was added in equivalent amount and the change in position of the signals of the different protons was noticed carefully (Fig. 6a). In presence of ATP, amide proton H_a and benzimidazole proton H_b of **1** exhibited downfield chemical shifts of 0.12 ppm and 0.06 ppm, respectively. The presence of minimum amount of D_2O was unable to exchange the amide proton immediately. However, the downfield chemical shifts of both H_a and H_b typically accounts for their involvement in hydrogen bonding with the phosphate chain of ATP. A small downfield chemical shift (0.07 ppm) of methylene protons of types d and e also suggested their involvement in hydrogen bonding with the phosphate group of ATP. In addition, complexation-induced small upfield chemical shift of the adenine ring protons by 0.03–0.07 ppm can be considered due to its closeness to the π- surface of naphthalene unit either *via* mode **A** or mode **B** in Fig. 3. In this context, small upfield chemical shift (0.03 ppm) of the naphthyl ring protons centered at 7.68 ppm substantiates the proposition.

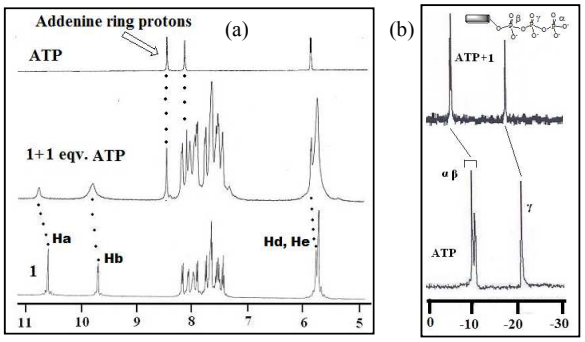


Fig 6. (a) Partial ^{1}H NMR (d_6-DMSO, 400 MHz) of **1** (c = 1.5 x 10^{-3} M) with equiv. amount of ATP (see labeled structure in Fig. 3); (b) ^{31}P NMR of ATP (c = 4 x 10^{-3} M) and with equiv. amount of **1** (c = 4 x 10^{-3} M) in d_6-DMSO-D_2O (5:1, *v*/*v*).

In ^{31}P NMR, α, β and γ P-atoms of ATP in the presence of equiv. amount of **1** showed marked downfield chemical shifts of 4.41 ppm, 4.61 ppm and 4.66 ppm, respectively and thereby confirmed the intimate involvement of phosphate chain in binding into the open cavity of the receptor (Fig. 6b).

In order to be acquainted with the binding structure in solution phase, we recorded the NOESY spectra of **1** both in the absence and presence of ATP. The correlation of the signals in NOESY spectrum of **1** in Fig. 7 suggests a conformation where the amide proton (type 'a') and the benzimidazolium proton of type 'b' are aligned in the same face. In the presence of equivalent amount of ATP, the correlations of the indicated signals were not observed. This is presumably due to conformational behavior of the piperazine motif[9] which controls the binding arms to complex the phosphate chain into cavity. Only a weak cross peak between adenine ring proton and the methylene proton of type 'd' was noticed (ESI). This indicated the closeness of the adenine ring to the naphthalene moiety although there was no direct correlation between the adenine and naphthalene ring protons. This observation is in accordance with the suggested binding mode **A** rather than the mode **B** in Fig. 3.

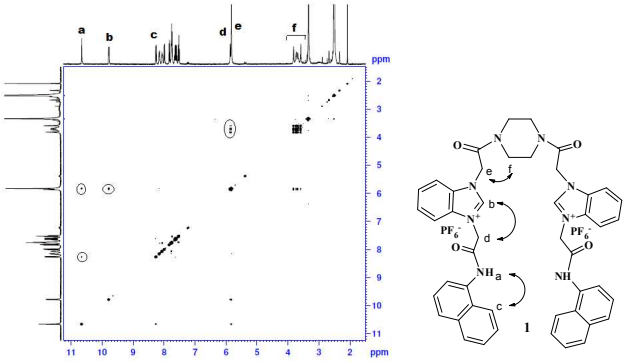


Fig 7. NOESY spectrum of **1** in d_6-DMSO.

The Benesi-Hilderband curve for interaction of ATP with receptor **1** is demonstrated in Fig. 8.[10] The linearity of the plot clearly suggests the 1:1 host-guest interaction with an association constant of 1.83 x 10^3 M^{-1}.

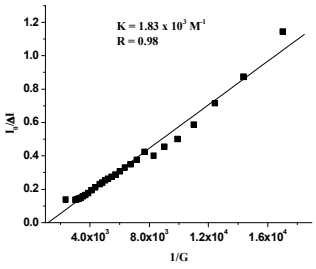


Fig 8. Benesi-Hilderband plot for **1** (c = 2.5 x 10^{-5} M) with ATP (c = 1 x 10^{-3} M) at 420 nm.

With this information in hand, we further explored the possibility of using **1** for the recognition of ATP in living cells (Human cervical cancer cells, HeLa) using confocal imaging experiments (Fig. 9). Images of confocal microscopy revealed that the control

(B) and host treated (C) cell sets did not show any fluorescence, whereas cells treated with host and further treated with ATP showed blue fluorescence (E). This suggests that the host is cell permeable and can sense the presence of ATP in the environment of cellular medium.

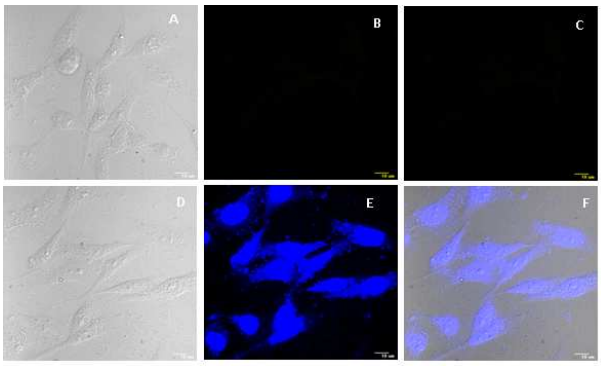


Fig 9. Confocal microscopy of different sets of normal untreated and experimental sets of HeLa cells: (A) Bright field image of normal cells, (B) Fluorescence image of normal cells, (C) Fluorescence image of cells treated with **1** (10 μM) for 1 h at 37 °C, (D) Bright field image of cells treated with **1** and ATP, (E) Blue fluorescence images of cells upon treatment with receptor **1** (10 μM) and then ATP (50 μM) for 30 min at 37 °C, (F) The overlay image of (D) and (E) is shown.

For quantitative estimation of percentage of fluorescent cells in control and different experimental set of cells, we performed 'Fluorescence Activated Cell Sorter' (FACS) study (Fig. 10). Results show that there was a rise in percentage of fluorescing cells set treated with both receptor **1** and ATP (C) (shown by the shifting in the quadrant) which were absent in both sets of untreated normal control set (A) and cells treated with receptor only (B). This result along with confocal microscopy image data corroborates that the receptor is potential in sensing of ATP under the cellular environment thereby suggesting its use in biological application. It is fact that the addition of receptor **1** to the cells did not show any cytotoxicity as evident from the morphology of the cells (Fig. 9) as well as MTT assay depicted in ESI. The viability

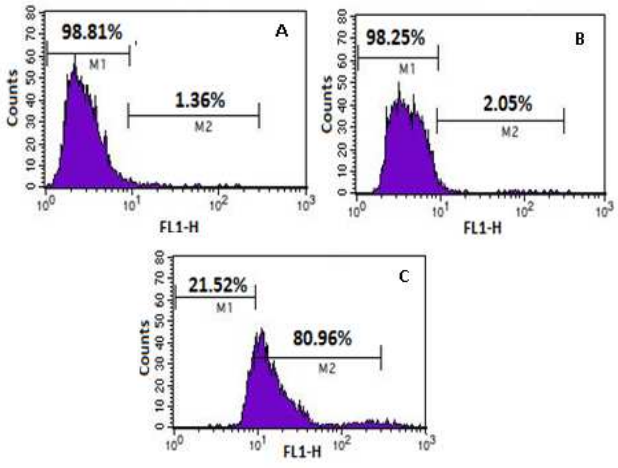


Fig 10. Quantitative assessment of fluorescence by Fluorescence Activated Cell Sorter (FACS)- (A) Control (untreated HeLa cells), (B) HeLa cells incubated with receptor **1**, (C) HeLa cells incubated with receptor **1** further incubated with ATP.

was more than 90%, for each normal, acetonitrile and receptor-treated cells. In each case of the experimental cell sets receptor was dissolved in minimum amount of solvent (acetonitrile) and then it was diluted in PBS (20 μl of receptor in 500 μl PBS) prior to treatment maintaining the optimum condition of the cells to sustain. Only in case of MTT assay receptor was dissolved in minimum of solvent (acetonitrile) and diluted in DMEM media before treatment. Since the percentage of viable cells of all the series was observed to be above 90% this would suggest that the receptor was not cytotoxic when exposed to cultured cells in vitro.

Conclusion

In conclusion, we have designed and synthesized benzimidazolium-based receptor **1** built on piperazine motif, which acts as fluorescent sensor for ATP over ADP, AMP and other anions in aqueous CH_3CN at pH 6.4. During interaction with ATP the non fluorescent solution of the chemosensor turns into bluish colour under UV light irradiation. However, under similar conditions, a series of other anions were non interacting and the solution was non emissive under UV exposure. The conformational mobility of piperazine motif, hydrogen bonding features of benzimidazolium and naphthylamide units of **1** are the possible factors that control the impressive selectivity in the recognition process. The chemosensor is cell permeable and is successfully applied in microscopic imaging for detection of ATP in Human cervical cancer cells (HeLa). Thus the present example in this account is simple, easy-to-make and a new addendum to the existing reported ATP sensors.[7]

Experimental

Syntheses:

1,1'-(Piperaine-1,4-diyl(2-chloroethanone) 2 (*8*): To a stirred solution of piperazine (1 g, 11.6 mmol) in 40 mL dry CH_2Cl_2, chloroacetylchloride (2.77 ml, 34.83 mmol) and dry Et_3N (4.85 ml, 34.82 mmol) were added dropwise. The reaction mixture was allowed to stir at room temperature for 8h. After completion of reaction, solvent was evaporated under vacuum. The residue was dissolved in water (40 mL) and the product was extracted with $CHCl_3$ (40 mL x 3). The combined organic layer was dried over anhydrous sodium sulfate and evaporated in *vacuo*. The crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:1) as eluent to give the desired product as pale brown solid (2 gm, yield 72.1%); mp.133ºC; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.10 (s, 4H), 3.72-3.56 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.4 (-CO-), 165.3 (-CO-), 46.1 (-NCH_2-), 45.8 (-NCH_2-), 42.0 (-NCH_2-), 41.6 (-NCH_2-), 40.6 (-CH_2Cl-) (more signals due to rotamers); FTIR (KBr, cm^{-1}): 2991, 2908, 2879, 1338; HRMS (TOF MS ES+) calcd for $C_8H_{12}Cl_2N_2O_2$: 239.0354 $(M+H)^+$; found: 239.0305 $(M+H)^+$.

2-Benzoimidazol-1-yl-1-[4-(2-benzoimidazol-1-yl-acetyl)-piperazin-1-yl]-ethanone (3): To a stirred solution of benzimidazole (1.28 g, 10.87 mmol) in 15 mL dry THF, NaH (0.300 g, 12.55 mmol) was added and the reaction mixture was refluxed for 30 mins. Reaction mixture was cooled to room temperature and dichloro amide **2** (1 g, 4.18 mmol) was added to the reaction mixture. The mixture was refluxed again for 6h. A

white precipitate was obtained and it was filtered off, washed with water and THF for several times to afford **3** (1.50 g, yield 89.1%) as white solid; m.p. 215˚C; ^{1}H NMR (400 MHz, d_6-DMSO): δ 8.12 (s, 2H), 7.66 (d, 2H, *J* = 8 Hz), 7.50 (brd, 2H), 7.26-7.18 (m, 4H), 5.37 (d, 4H, *J* = 12 Hz), 3.74-3.51 (m, 8H); ^{13}C NMR (100 MHz, d_6-DMSO) δ 166.0, 145.4, 143.3, 135.0, 122.7, 121.9, 119.6, 111.0, 45.8, 44.5, 42.0; FTIR (KBr, cm^{-1}): 3535, 3347, 1654, 1506, 1231; HRMS (TOF MS ES+) calcd for $C_{22}H_{22}N_6O_2$: 403.1882 $(M+H)^+$; found: 403.1800 $(M+H)^+$.

2-Chloro-N-naphthalen-1-yl-acetamide (4): To a stirred solution of 1-napthylamine (2.50 g, 17.46 mmol) in 30 mL $CHCl_3$, chloroacetylchloride (2.08 mL, 26.19 mmol) followed by 10 mL water was added. Anhydrous K_2CO_3 (3.61 g, 26.19 mmol) and tetrabutylammonium hydrogensulfate (0.006 g, 0.017 mmol) were then added to the reaction mixture. The reaction mixture was allowed to stir at room temperature for 8h. After completion of reaction, solvent was evaporated under vacuum. The residue was dissolved in water (40 mL) and the product was extracted with $CHCl_3$ (3×40 mL). The organic phase was dried over anhydrous sodium sulfate and evaporated in vacuo to afford the compound **4** as pure white solid (3 gm, yield 78.2%); mp.154 ^{o}C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (s, 1H), 7.99 (d, 1H, *J* = 8 Hz), 7.89 (t, 2H, *J* = 8 Hz), 7.76 (d, 1H, *J* = 8 Hz), 7.60 – 7.49 (m, 3H), 4.36 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.4, 134.0, 131.1, 128.8, 127.0, 126.6, 126.5, 126.2, 125.6, 120.7, 120.3, 43.3 ; FT-IR: ν in cm^{-1} (KBr): 3256, 3052, 1665, 1556, 1505, 1349; HRMS (TOF MS ES+) calcd for $C_{12}H_{10}ClNO$: 242.0349 $(M+Na)^+$; found: 242.0349 $(M+Na)^+$.

Receptor 1: To a stirred solution of **3** (0.300 g, 0.745 mmol) in DMF (2 mL) compound **4** (0.500 g, 2.28 mmol) in dry CH_3CN (15 mL) was added. The reaction mixture was refluxed with stirring for 3 days under nitrogen atmosphere. After completion, the reaction mixture was cooled to room temperature and filtered. The precipitate was washed with CH_3CN for several times to give pure dichloride salt **5** (0.436 g, yield 69%). The dichloride salt **5** (0.273 g, 0.324 mmol) was dissolved in 3 mL hot DMF-CH_3OH (1:3, *v*/*v*) mixture solvent and NH_4PF_6 (0.080 g, 0.486 mmol) was added to it in one portion. After stirring the reaction mixture for 20 min water was added. The precipitate was filtered and washed with water to give pure **1** (0.309 g, yield 89.9%) as white solid; m.P. 195˚C.

^{1}H NMR (400 MHz, d_6-DMSO): δ 10.64 (s, 2H), 9.78 (d, 2H, *J* = 4 Hz), 8.25 (d, 2H, *J* = 8 Hz), 8.14 (d, 2H, *J* = 8 Hz), 8.05 (brt, 2H), 7.98 (d, 2H, *J* = 8 Hz), 7.83 (d, 2H, *J* = 8 Hz), 7.75-7.73 (m, 6H), 7.65-7.38 (m, 4H), 7.52 (t, 2H, *J* = 8 Hz), 5.85 (s, 2H), 5.82 (s, 2H), 3.82 (s, 2H), 3.73 (d, 4H, *J* = 12 Hz), 3.59 (s, 2H); ^{13}C NMR (100 MHz, d_6-DMSO) δ 164.2, 163.5, 144.6, 133.7, 132.4, 131.5, 131.3, 128.2, 127.5, 126.6 (two carbons unresolved), 126.2, 126.1, 125.9, 125.5, 122.5, 121.6, 113.8, 113.7, 49.0, 47.8, 30.6; FT-IR: ν in cm^{-1} (KBr): 3337, 3166, 1690, 1657, 1432, 842; HRMS (TOF MS ES+) calcd for $C_{46}H_{42}F_6N_8O_4P$: 915.2965 $(M–PF_6)^+$; found: 915.2971 $(M –PF_6)^+$.

General procedure for fluorescence and UV-vis titrations

Stock solutions of the receptors were prepared in CH_3CN-H_2O (1:1, *v*/*v*, using 10 mM HEPES, pH 6.4) in the concentration range of ~ 10^{-5} M. Stock solutions of guests in the concentration range of ~ 10^{-3} M, were prepared in the same solvents and were individually added in different amounts to the receptor solution (2 mL) taken in the cuvette. The change in emission of the receptor was noted. Same stock solutions for receptor and guests were used to perform the UV-vis titration experiment. Solutions of guests were successively added in different amounts to the receptor solution (2 mL) taken in the cuvette and the absorption spectra were recorded. Both fluorescence and UV-vis titration experiments were carried out at 25 ^{o}C.

Cell culture

Human cervical cancer cells (HeLa) were procured from National Centre for Cell Science, Pune, India. 5×10^5 cells/mL were cultured in DMEM supplemented with 10% fetal bovine serum and 1% PSN antibiotic at 37 ^{o}C with a constant supply of 5% CO_2.

Confocal microscopy

The cells were prepared for the fluorescence analysis by the following method: In brief, the cells were seeded at 10^7 cells per plate and grown to 70% confluency. Three sets of cells were cultured. First set was devoid of any treatment which served as normal control HeLa cells. To the second and third set of cells, the cells were washed in phosphate buffered saline (PBS) and 40µl of host was added and incubated for 20 mins. To the third set of cells, after further washing in PBS, 20µl of guest was added and further incubated for 20 mins. Finally the cells were observed under confocal microscope (Carl Zeiss LSM 510 META Laser Scanning Microscope).

FACS study

The cells were prepared for the fluorescence analysis by the following method: In brief, the cells were seeded at 10^7 cells per plate and grown to 70% confluency. Three sets of cells were cultured. First set was devoid of any treatment which served as normal control HeLa cells. To the second and third set of cells, they were washed with PBS and 40µl of host was added and incubated for 20mins. To the third set of cells, after washing with PBS, 20µl of guest was added and further incubated for 20mins. The cells were then isolated in PBS and fixed in 70% ethanol. Finally the cells were analyzed FACS (BD Calibur). The cell samples were analyzed in Cyflogic v.1.2.1 software and each determination was based on mean fluorescence intensity of 10,000 events.

Method for MTT assay

Reagents

MTT [3-4, 5-Dimethyl-thiazol-2-yl)-2, S-diphenyltetrazolium bromide], and DMSO were purchased from Sigma-Aldrich Inc. (St-Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and antibiotics, namely, penicillin, streptomycin, and neomycin (PSN) were purchased from Gibco BRL (Grand Island, NY, USA). All organic solvents used were of high performance liquid chromatography grade.

Assessment of percentage of viable cells

The percentage viability of HeLa cells, after being exposed to both the receptors, was evaluated by MTT assay.[11] The cells were incubated in 96-well microplates for 24 hours along with the receptors at different concentrations. A set of HeLa cells not exposed to any of the receptors were kept as untreated control. Other sets of cells were incubated with receptor **1** through 5µl of stock solution. The cells were allowed to grow for the next 24 hrs. MTT was then added to each well and incubated for the next 4hr. The intracellular formazan crystals formed were solubilized with dimethyl sulfoxide (DMSO) and the absorbance of the

solution was measured at 595 nm by using a microplate reader (Thermo scientific, Multiskan ELISA, USA). The percentage of cell survival was calculated as: (mean experimental absorbance/mean control absorbance) × 100%.

Acknowledgments

We thank DST, New Delhi, India for providing facilities in the department. DT thanks CSIR, New Delhi, India for a fellowship.

References

1 (a) J. L. Sessler, P. A. Gale and W. S. Cho, *Anion Receptor Chemistry, Royal Society of Chemistry*, Cambridge, 2006; (b) *Supramolecular Chemistry of Anions,* ed. A. Bianchi, K. Bowman-James and E. Garcia-Espana, Wiley-VCH, New York, 1997; (c) J.-M. Lehn, *Supramolecular Chemistry, Concepts and Perspectives*, VCH, Weinheim, 1995; (c) H.-J. Schneider and A. K. Yatsimirsky, *Chem. Soc. Rev*., 2007, **37**, 263; (d) E. Garcia-Espana, P. Diaz, J. M. Llinares and A. Bianchi, *Coord.Chem. Rev*., 2006, **250**, 2952; (e) S. O. Kang, Md. A. Hossain and K. Bowman-James, *Coord.Chem. Rev*., 2006, **250**, 3038; (f) V. McKee, J. Nelson and R. M. Town, *Chem. Soc. Rev*., 2003, **32**, 309; (g) H. Dugas, *Bioorganic Chemistry: a Chemical Approach to Enzyme Action,* Springer, New York, 1996.

2. (a) P. Ritter, *Biochemistry, a foundation*, Creation Research Society Quarterly June 1999, Books/Cole, Pacific Grove, CA, vol. 36, 1996; (b) W. N. Lipscomb and N. Strater, *Chem. Rev*., 1996, **96**, 2375; (c) P. Nyron, *Anal. Biochem*., 1987, **167**, 235; (d) T. Tabary and L. Ju, J. *Immunol. Methods*, 1992, **156**, 55.
3. K. T. Bush, S. H. Keller and S. K. Nigam, *J. Clin. Invest.,* 2000,**106**, 621.
4. M. J. Kim, K. M. Swamy, K. M. Lee, A. R. Jagdale, Y. Kim, S. J. Kim, K. H. Yoo and J. Yoon, *Chem. Commun.,* 2009, 7215.
5. C. Schmuck and M. Heller, *Org. Biomol. Chem.,* 2007, **5**, 787 and references cited therein.
6. M. W. Hosseini, J.–M. Lehn and M. P. Mertes, *Helv. Chim. Acta,* 1983, **66**, 2454.
7. (a) Z. C. Xu, N. J. Singh, L. Lim, J. Pan, H. Kim, S. Park, K. S. Kim and J. J. Yoon, *J. Am. Chem. Soc.,* 2009, **131**, 15528; (b) J. Y. Kwon, N. J. Singh, H. Kim, S. K. Kim, K. S. Kim and J. Yoon, *J. Am. Chem. Soc.,* 2004, **126**, 8892; (c) C. Bazzicalupi, S. Biagini, A. Bencini, E. Faggi, C. Giorgi, I. Matera and B. Valtancoli, *Chem. Commun*., 2006, 4087; (d) J. Kaur and P. Singh, *Chem. Commun.,* 2011, **47**, 4472; (e) D. Amilan Jose, S. Mishra, A. Ghosh, A. Shrivastav, S. K. Mishra and A. Das, *Org. Lett*., 2007, **9**, 1979; (f) F. Sancenon, A. B. Descalzo, R. Martinez-Manez, M. A. Miranda and J. Sato, *Angew. Chem., Int. Ed*., 2001, **40**, 2640; (j) Y. Zhou, Z. Xu and J. Yoon, *Chem. Soc. Rev*., 2011, **40**, 2222; (k) K. Ghosh and I. Saha, *New J. Chem.,* 2011, **35**, 1397; (l) K. Ghosh and I. Saha, *Org. Biomol. Chem.,* 2012, **10**, 9383.
8. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr. T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez and J. A. Pople, *Gaussian 09* (*Revision A.02*): Gaussian, Inc., Wallingford CT, 2004.
9. Z. Xu, J. Yoon and D. R. Spring, *Chem. Commun*., 2010, **46**, 2563.
10. P. T. Chou, G. R. Wu, C. Y. Wei, C. C. Cheng, C. P. Chang and F. T. Hung, *J. Phys. Chem B.*, 2000, **104**, 7818.
11. T. Mossam, *J. Immunol. Methods*, 1983, **65**, 55.

