New 1,2-Dihydropyridine-Based Fluorophores and Their Applications as Fluorescent Probes

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Supporting Information

ABSTRACT: New 1,2-dihydropyridine (1,2-DHP)-based fluorophores 1a-1h were designed and synthesized by a one-pot four-component condensation reaction using dienaminodioate, aldehydes, and an in situ-generated hydrazone mediated by trifluoroacetic acid. The photophysical properties of 1,2-DHPs were studied in detail, and a few of them exhibited selective mitochondrial staining ability in HeLa cell lines (cervical cancer cells). A detailed photophysical investigation led to the design of 1,2-DHP 1h as an optimal fluorophore suitable for its potential application as a small molecule probe in the aqueous medium. Also, 1,2-DHP 1h exhibited sixfold enhanced emission intensity than its phosphorylated analogue 1h′ in the long wavelength region (λem ≈ 600 nm), which makes 1,2-DHP 1h′ meet the requirement as a bioprobe for protein tyrosine phosphatases, shown in L6 muscle cell lysate.

INTRODUCTION

Small molecule-based organic fluorophores are essential for sensing and imaging of biological specimens with high sensitivity and fast response. Even though a large variety of fluorophores are known, only a few have optimal performance because a majority of them often suffer from photobleaching, autofluorescence, and cytotoxic behavior that limit their further applications in biology. A number of heterocyclic fluorophores were reported for fluorescent labeling of biomolecules, sensing, and bioimaging applications; however, for most of these molecules, the emission maxima were observed in the green window of less than 500 nm. Consequently, the discovery of new heterocyclic fluorophore scaffolds with improved photophysical properties is highly warranted. Fluorescent properties exhibited by 1,4-dihydropyridines (1,4-DHPs) and our recent interest in 1,2-dihydropyridines (1,2-DHPs) have inspired us to develop new 1,2-DHP-based fluorophores with improved photophysical features.

1,4-DHPs are known to exhibit blue fluorescence with appropriately substituted electron-donating groups at the 1-position and electron-withdrawing groups at 3- and 5-positions (Figure 1). Furthermore, a higher Stokes shift was observed by the presence of an electron-donating aryl system in the 4-position of 1,4-DHP, which is attributed to an internal charge transfer in the excited state between the two π-systems. The 4-aryl-substituted 1,4-DHP comprising two different chromophores separated by an sp3 carbon served as a tunable photoactivated dyad involving energy and electron transfer processes between them (Figure 1). The fluorophore ability of 1,4-DHP was further extended as a chemosensor where a water-soluble glucopyranosyl 1,4-DHP is used in the detection of 2,4,6-trinitrophenol. 1,2-DHPs, however, were not explored in detail for their photophysical properties to an extent as that of 1,4-DHPs, but 2-pyridones, which are structural analogues of 1,2-DHPs, were recently reported as fluorescent probes.

Recently, ylidenemalonitrile enamines were reported as fluorescent “turn-on” indicators for their ability to undergo cyclization with 1° amines to produce fluorescent 1,2-DHP products.

In the quest for developing new fluorophores with improved photophysical properties, herein, we have explored 1,2-DHPs with an extended π-conjugation as novel fluorophores. As N-phenyl-1,2-DHPs absorb in the near UV region (Table S1), the corresponding derivatives with absorption in the visible region would be preferred for biological applications. Hence, the present 1,2-DHP design (Figure 1) involves a push–pull...
system with different electron-rich N-benzylideneamine substitutions that offer tuning of their photophysical behavior.\textsuperscript{12} This new N-benzylideneamine-appended 1,2-DHP offered a remarkable bathochromic shift in the absorption and emission profiles with large Stokes shifts (Table 1). The application of these fluorophores was demonstrated as selective mitochondrial fluorophore tags. To assess the role of the phenyl group at the sixth position, we have designed and synthesized a water-soluble fluorophore 1,2-DHP \textit{h} by utilizing an aldehyde generated from triethylene glycol monomethyl ether and \( \text{N,N-diethyl salicylaldehyde} \), which further offers an appropriate hydroxyl group substituent for appending any cleavable targeting group such as phosphate for in vitro phosphatase-sensing applications (Scheme 1).

**Photophysical Properties.** The photophysical properties of 1,2-DHPs \textit{a}–\textit{h}, viz., absorption, emission, quantum yields, and emission lifetime measurements, are provided in Table 1 and Supporting Information (Figures S2 and S3). The present design involves a D–π–A or push–pull type system; thus, the nature and position of the substituents on the 1,2-DHP moiety are crucial to tune their intramolecular charge transfer (ICT) properties, which leads to different photophysical properties. 1,2-DHPs \textit{a}–\textit{h} exhibit maximum absorption wavelengths (\( \lambda_{\text{max}} \)) between 396 and 448 nm in methanol with strong molar extinction coefficients (\( \epsilon \)). As expected, 1,2-DHP \textit{a}–\textit{h} were synthesized with acetaldehyde to evaluate the effect of the phenyl group as a contributing factor behind 1,2-DHP’s fluorophore ability. This methodology offers a choice of appending any aliphatic or aromatic group at the sixth position, thus, a suitable place for conjugation with bioactives or biomolecules. In addition, these 1,2-DHPs can undergo regioselective hydrolysis of 5-CO\textsubscript{2}Me, which was supported by its single crystal X-ray structure (Figure S1). This selectivity can be realized by difference in nitrogen lone pair delocalization with 3- and 5-CO\textsubscript{2}Me, thus offering another site for conjugation via an amide linkage. Furthermore, we have designed and synthesized a water-soluble fluorophore 1,2-DHP \textit{h}, as a standard and was found to be in the range of...
0.032–0.125 with 1,2-DHP 1g being the highest. These compounds exhibited remarkable Stokes shift values, which can help in obtaining better fluorescence imaging with minimum self-absorption of the fluorophore. It is already established that for better cellular imaging, compounds should have absorption in the visible region and high fluorescence quantum yield. In this regard, on the basis of the observed photophysical properties, the present design of 1,2-DHPs possesses the potential for their application as bioprobes.

**Applications.** The mitochondrial membrane has a negative potential of $-180 \text{ mV}$; therefore, it is typical to use cationic dyes for imaging these organelles. The push–pull system in 1,2-DHPs makes the ring nitrogen of 1,2-DHP to attain a sufficient positive charge; thus, 1,2-DHPs may have an ability to serve as mitochondrial staining agents. Further, to assess the potential of 1,2-DHPs for specific mitochondrial staining, 1,2-DHPs 1a–1g were studied in HeLa cells. Initially, cytotoxicity of 1,2-DHPs was evaluated using MTT assay, and it was found that 1,2-DHPs exhibit greater than 80% cell viability at 30 $\mu\text{M}$ (Figure S4). HeLa cells were incubated with 30 $\mu\text{M}$ of 1,2-DHPs for 10 min, and the excess compound was washed with Hanks’ balanced salt solution (HBSS) buffer solution. As shown in Figures 2 and S5, 1,2-DHPs were localized mostly in the cytoplasm, specifically stained mitochondria in HeLa cells, and no nuclear uptake was observed. Additionally, the co-staining experiment with MitoTracker red chloromethyl-X-rosamine (CMXRos), a commercially available mitochondria-imaging dye, confirmed the localization of 1,2-DHPs in the mitochondria supported by Pearson’s correlation coefficient in the range of 0.75–0.89. Among all the 1,2-DHPs under study, 1,2-DHP 1b, 1d, and 1g were found to exhibit high fluorescence intensity compared to others.

As a proof of concept, to justify the importance of the new 1,2-DHP as a fluorescent probe, we have synthesized a...
phosphorylated analogue 1h′ from 1,2-DHP 1h (Scheme 1). It is well-known that direct and rapid analysis of the crude lysate for endogenous phosphatase enzymes such as PTPs are of prime interest owing to their significant role in insulin-signaling pathways and a variety of disease states including hepatocellular carcinoma as well as metabolic disorders. PTPs are significant targets in many diseases, and there is a growing need for direct determination of endogenous protein phosphatase activity. The UV−vis absorption spectrum of the 1,2-DHP 1h′ in methanol exhibited absorption maximum at 448 nm, and the corresponding emission spectrum shows a peak at 594 nm, whereas in aqueous buffer medium (25 mM Hepes buffer, pH 7.4), a small bathochromic shift was observed both in absorption and emission spectra (Figure 3). The quantum yield of 1,2-DHP 1h′ in Hepes buffer medium is reduced to 0.007, which can be rationalized by differences in the electron density involved in conjugation with phosphate and phenoxide groups. This difference of electronic distribution also reflected in the fluorescence lifetime profile. 1,2-DHP 1h in Hepes buffer exhibited a fluorescence lifetime of 1.94 ns, which is good enough for imaging experiments, whereas its phosphorylated analogue 1,2-DHP 1h′ did not show any decay profile because of its weak fluorescence property (Table 1). To get the structural details of 1,2-DHPs 1h and 1h′, both the structures in their ground state were optimized using density functional theory (DFT) with the B3LYP exchange correlation functional and the 6-31G** basis set with a Gaussian G09 package, and the corresponding structures have been given in Figure 4a,b. The highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of 1,2-DHP 1h have been given in Figure 4c,d, respectively, which shows that the HOMO of 1,2-DHP 1h is largely localized on the diethylaniline group, whereas the...
LUMO is predominantly confined on the 1,2-DHP core, thus supporting our concept of the push–pull system. In aqueous medium, at physiological pH (Hepes buffer, pH 7.4), the fluorescence emission properties of 1,2-DHPs 1h and 1h′ showed a distinct change. 1,2-DHP 1h with a free hydroxyl group exhibited a sixfold higher orange fluorescence than that of 1,2-DHP 1h′ appended with a phosphate group (Figure 4e). The corresponding fluorescence changes were also reflected in visual appearance of both the solutions (Figure 4e, inset).

This significant difference in emission intensity inspired us to explore 1,2-DHP 1h′ as a phosphatase sensor. As it is well-known that blinking and photobleaching of the fluorophores may cause problems for the imaging experiments, the photostability of 1,2-DHP 1h′ was first tested by monitoring the fluorescence intensity as a function of time upon continuous irradiation (λ = 445 nm) in Hepes buffer solution (25 mM, pH 7.4, 0.3% DMSO) over a period of 20 min under aerobic conditions and was found to be quite stable (Figure 25 mM, pH 7.4, 0.3% DMSO) for 20 min under photostability of 1,2-DHP 1h′. The corresponding fluorescence emission properties of 1,2-DHPs 1h and 1h′ were determined with the relative method, employing an Shimadzu HPLC instrument with C18-phenomenex reversed-phase column (250 × 2.1 mm, 5 μm) for the purification of 1,2-DHP 1h′ using methanol and water. 1H, 13C, and 31P NMR spectra were recorded on a Bruker AVANCE II spectrometer at 500, 125, and 202 MHz, respectively. Chemical shifts are given in ppm using solvent residual peaks of chloroform (δ 7.26) and methanol (δ 3.31 ppm) as reference, and coupling constants in hertz. High-resolution electrospray ionization mass spectrometry analysis was recorded on a Thermo Scientific Exactive-LCMS instrument with ions given in the m/z ratio. Absorption spectra were recorded using a Shimadzu UV-2450, UV–visible spectrophotometer using a quartz cuvette with a 1 cm path length. The fluorescence spectrum of the 1,2-DHPs were recorded on a FluoroLog-322 (Horiba) instrument, which was equipped with a 450 W Xe arc lamp as the excitation source. The fluorescence quantum yields were determined with the relative method, employing an optically matched solution of coumarin 153 in MeOH as the reference (ΦR = 0.46). The following equation was used for calculating the quantum yield

\[
\Phi_s = \frac{Abs_s}{Abs_r} \times \frac{area_s}{area_r} \times \frac{n_s^2}{n_R^2} \times \Phi_R
\]

where the subscripts R and S refer to the reference and samples, respectively. Abs, area, and n are the absorbance at the excitation wavelength, area under the fluorescence spectrum, and refractive index of the solvent, respectively. Fluorescence lifetimes were measured using an IBH (FluoroCube) TCSPC system. L6 myoblast and HeLa cells were obtained from the National Centre for Cell Sciences, Pune, India. Tris buffer (25 mM, pH 7.4, 0.3% DMSO), Hepes buffer (25 mM, pH 7.4, 0.3% DMSO) and HBSS (pH 7.4) buffers were used for the cell culture studies. The cells were visualized using a fluorescent microscope (Pathway 855, BD Bioscience, USA). Pearson’s correlation coefficients were calculated using ImageJ software with a JACoP plugin.

**General Experimental Methods.** All the reactions were conducted using undistilled solvents, whereas CH$_3$Cl$_2$ was distilled over CaH$_2$, which was used for the demethylation of the phosphate ester of 1,2-DHP 1h. Silica gel 60 F$_{254}$ aluminum thin-layer chromatography (TLC) plates were used to monitor the reactions with short and long wavelength UV and visible lights to visualize the spots. Column chromatography was performed on the silica gel 100–200 and 230–400 mesh. The Shimadzu HPLC instrument with C18-phenomenex reversed-phase column (250 × 2.1 mm, 5 μm) was used for the purification of 1,2-DHP 1h′ using methanol and water. 1H, 13C, and 31P NMR spectra were recorded on a Bruker AVANCE II spectrometer at 500, 125, and 202 MHz, respectively. Chemical shifts are given in ppm using solvent residual peaks of chloroform (δ 7.26) and methanol (δ 3.31 ppm) as reference, and coupling constants in hertz. High-resolution electrospray ionization mass spectrometry analysis was recorded on a Thermo Scientific Exactive-LCMS instrument with ions given in the m/z ratio. Absorption spectra were recorded using a Shimadzu UV-2450, UV–visible spectrophotometer using a quartz cuvette with a 1 cm path length. The fluorescence spectrum of the 1,2-DHPs were recorded on a FluoroLog-322 (Horiba) instrument, which was equipped with a 450 W Xe arc lamp as the excitation source. The fluorescence quantum yields were determined with the relative method, employing an optically matched solution of coumarin 153 in MeOH as the reference (ΦR = 0.46). The following equation was used for calculating the quantum yield

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**CONCLUSIONS**

In summary, we have designed and synthesized a new class of 1,2-DHP-based fluorophores by a facile one-step multi-component protocol, and their photophysical properties were studied in detail. The results indicate that 1,2-DHPs with an extended N-benzylidenamine appendage have an absorption and emission maxima around 420 and 600 nm, respectively, having prominent Stokes shift. In particular, 1,2-DHPs 1g and 1h showed remarkable photophysical properties with high fluorescence. Furthermore, 1,2-DHPs 1b, 1d, and 1g are recognized as well-suited mitochondrial staining agents in HeLa cells. The potential of fluorophore 1,2-DHP 1h′ as a fluorescent probe in tyrosine phosphatase activity on the cell lysate was also explored. Synthetic accessibility and scope for conjugation warrants the utility of 1,2-DHP as a potential fluorescent probe for biological applications.

**EXPERIMENTAL SECTION**

**General Experimental Methods.** All the reactions were conducted using undistilled solvents, whereas CH$_3$Cl$_2$ was distilled over CaH$_2$, which was used for the demethylation of the phosphate ester of 1,2-DHP 1h. Silica gel 60 F$_{254}$ aluminum thin-layer chromatography (TLC) plates were used to monitor the reactions with short and long wavelength UV and visible lights to visualize the spots. Column chromatography was performed on the silica gel 100–200 and 230–400 mesh. The Shimadzu HPLC instrument with C18-phenomenex reversed-phase column (250 × 2.1 mm, 5 μm) was used for the purification of 1,2-DHP 1h′ using methanol and water. 1H, 13C, and 31P NMR spectra were recorded on a Bruker AVANCE II spectrometer at 500, 125, and 202 MHz, respectively. Chemical shifts are given in ppm using solvent residual peaks of chloroform (δ 7.26) and methanol (δ 3.31 ppm) as reference, and coupling constants in hertz. High-resolution electrospray ionization mass spectrometry analysis was recorded on a Thermo Scientific Exactive-LCMS instrument with ions given in the m/z ratio. Absorption spectra were recorded using a Shimadzu UV-2450, UV–visible spectrophotometer using a quartz cuvette with a 1 cm path length. The fluorescence spectrum of the 1,2-DHPs were recorded on a FluoroLog-322 (Horiba) instrument, which was equipped with a 450 W Xe arc lamp as the excitation source. The fluorescence quantum yields were determined with the relative method, employing an optically matched solution of coumarin 153 in MeOH as the reference (ΦR = 0.46). The following equation was used for calculating the quantum yield

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**General Procedure for the Synthesis of Hydrazone.** To a solution of hydrazine hydrate (10 equiv) in ethanol (10 mL) was added pertinent aldehyde (1 equiv), and the resulting mixture was stirred under reflux overnight. After complete consumption of the aldehyde, as indicated by 1H NMR, the reaction mixture was diluted with water and extracted with CH$_2$Cl$_2$. The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated, and the resulting residue was used directly for the next step without further purification.

**General Procedure for the Synthesis of 1,2-DHPs 1a–1g.** To a solution of diaminodioate (0.77 mmol, 1 equiv) in CH$_3$CN (3 mL) were added aldehyde (1.15 mmol, 1.5 equiv), hydrazine (1.25 mmol, 1.5 equiv), and trifluoroacetic acid (0.77 mmol, 1 equiv) at room temperature. The reaction mixture usually develops a yellow to dark red coloration immediately, which is an indication of the formation of 1,2-DHP. After complete consumption of diaminodioate, as
observed on TLC, the reaction mixture was quenched with saturated aqueous NaHCO₃ and extracted with EtOAc. The organic layer was dried over anhydrous Na₂SO₄ and concentrated, and the resulting residue was purified by column chromatography to afford the desired 1,2-DHP.

**Cellular Studies. Cell Culture and Treatment.** Rat skeletal muscle cell lines (L6 myoblasts) and cervical cancer cell lines (HeLa) were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic mix at 37 °C under 5% CO₂ atmosphere.

**Cell Viability Study of 1,2-DHPs** 1h and 1h’ on L6 Myoblast. MTT assay was performed to check the cytotoxicity of the compounds. The viability of L6 myoblast was measured by means of MTT assay, as explained before for the cell pellet using 0.15 M KCl (4 °C, C) followed by the addition of the corresponding products was measured after 45 min using a microplate reader (BioTek-USA). Results were expressed as percentage of cytotoxicity.

\[
\text{Percentage of toxicity} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100
\]

**Preparation of Cell Lysate.** Cells were grown in T25 flasks and after attaining 60% confluency, cells were differentiated in DMEM containing 2% horse serum for 5 days. Differentiated cells were then washed three times with Heps buffer (25 mM, pH 7.4). Cells were scraped off from the plates using a cell scraper and centrifuged, and the proteins were extracted from the cell pellet using 0.15 M KCl (4 °C for 30 min). The protein content of the lysate was then measured using a BCA protein assay kit.

**Cell Viability on the HeLa Cell.** Viability of the HeLa cell was measured by means of MTT assay, as explained before for the L6 myoblast. Cytotoxicities of 1,2-DHPs 1h and 1h’ (1, 5, 10, 20, and 30 μM) were standardized based on the concentration. Briefly, cells after incubation with the compound were washed, and MTT (0.5 g/L), dissolved in DMEM, was added to each well for the estimation of mitochondrial dehydrogenase activity, as described previously by Mosmann.²⁵ After an additional 2 h of incubation at 37 °C in a CO₂ incubator, 10% of SDS in DMSO was added to each well, and the absorbance at 570 nm of solubilized MTT formazan was measured using a microplate reader (NanoDrop-2000, Thermo Fisher Scientific). Results were expressed as percentage of cytotoxicity.

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