Ratiometric Cu$^{2+}$ Binding, Cell Imaging, Mitochondrial Targeting, and Anticancer Activity with Nanomolar IC$_{50}$ by Spiro-Indoline-Conjugated Calix[4]arene

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Supporting Information

ABSTRACT: A triazole-derivatized, spiro-indoline-linked, 1,3-di-derivative of calix[4]arene (L) has been synthesized to take advantage of its ion-binding capability in the ring-open form. Indeed, the spiro-indoline moiety is well known for its photochromic, acidochromic, and metallochromic properties. Therefore, the L has been explored for Cu$^{2+}$ binding, cell imaging, and anticancer activity of the corresponding complex since Cu$^{2+}$ complexes are known for such activity. The conversion from the closed to open form of L is expedited by light or proton, while the metal ion can open as well as stabilize it. The open form of L showed binding of Cu$^{2+}$ ratiometrically as demonstrated by absorption and fluorescence spectroscopy. This leads to the formation of 1:1 complex with a binding constant of (6.9 ± 2.3) × 10$^8$ M$^{-1}$, with the lowest detection limit being 1.9 nM. In the complex, the Cu$^{2+}$ is bound by two triazole-N and two phenolic-O groups resulting in a distorted tetrahedral coordination core of CuN$_2$O$_2$ as demonstrated based on density functional theory studies. To form such coordination core, the arms underwent considerable changes in some of the dihedral angles. The binding of Cu$^{2+}$ to L induces self-assembly of L by varying from simple particles to rodlike structures when bound to Cu$^{2+}$. The on–off fluorescence intensity of L and its Cu$^{2+}$-bound species are responsible for imaging cancer cells. The L shows red fluorescence in MDA-MB-231 cancer cells by targeting mitochondria as proved based on the colocalization study carried out using MitoTracker Green. While the L alone is nontoxic to cancer cells, the presence of Cu$^{2+}$ brings cell death to an extent of 90% with an IC$_{50}$ value of 165 nM by bringing a substantial quench in the fluorescence of L. A shift of population from G0/G1 and G2M phases to the Sub-G1 phase was observed as the concentration of the complex was increased, indicating cell death as studied by fluorescence-activated cell sorting. Thus, the present work clearly proved that a calix[4]arene functionalized at the lower rim with spiro-indoline moieties when complexed with Cu$^{2+}$ acts as an efficient anticancer agent and is capable of imaging cancer cells.

INTRODUCTION

Calix[4]arene platform is well known in the literature for its ease of functionalization at its upper and lower rims. The resulting derivatives of calix[4]arene have been developed for various applications, such as host–guest interactions, metal-ion, and anion sensing, and drug delivery. Spiro-indoline-based systems are known for their acido-, solvato-, and photochromic behaviors. Derivatives possessing spiro-indoline moieties were reported to have anticancer properties. Although the pyridinium moieties are known for their mitochondrial targeting ability, the quaternary nitrogen-containing spiro-indoline derivatives have not been explored in this direction. It is interesting to understand the anticancer activity of such groups when anchored onto the calix[4]arene platform, in particular when the same is bound to Cu$^{2+}$ since copper complexes are well proven to be anticancer agents as reported in the literature. Therefore, a lower-rim di-spiro-indoline-derivatized calix[4]arene has been synthesized (L), characterized, and explored for its Cu$^{2+}$-ion binding, followed by isolation, characterization, cell imaging, and anticancer activities of the Cu$^{2+}$ complex. In L, the spiro-indoline ring acts as the fluorescent probe and also binds to Cu$^{2+}$ in its open form and the resultant complex further exhibits anticancer activity.

RESULTS AND DISCUSSION

The L was synthesized by the condensation reaction of P$_1$ with P$_3$, while P$_3$ is obtained from the click reaction carried out between the di-alkyne derivative, P$_1$, with the azide derivative, P$_2$, according to Scheme 1. All of the products, including the precursors, were characterized by $^1$H and $^{13}$C NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS) (Figures S01–S05).

Closed and Open Forms of L in Solution. The L exhibits three major absorption bands centered at 202, 292, and 379 nm in acetonitrile and a major emission band at 475 nm with a strong shoulder at 570 nm when excited at 379 nm (excitation spectrum of L given in Figure S09). The spiro-indoline system is known to undergo ring opening, which is

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further expected to be stabilized by a transition-metal ion. Addition of trifluoroacetic acid (TFA) to \( L \) leads to the transformation of the closed ring form to that of the ring-open form, which was evident from the observed increase in the absorbance at 420 nm. The open form also exhibits increase in the emission intensity at 570 nm band in fluorescence spectra at the cost of the band at 475 nm, and the changes follow ratiometrically (Figure 1a,b). Similar results were seen in the literature for coumarin- and quinoline-conjugated spiro-pyran derivatives, wherein the ring opening was induced by TFA and metal ions, respectively.\(^{15−17}\)

\( \text{Cu}^{2+} \) Binding to \( L \) in Solution. The binding of \( \text{Cu}^{2+} \) to \( L \) in acetonitrile was explored by measuring the absorption spectra during the titration of the open form of \( L \) using \( \text{Cu}^{2+} \), which resulted in increasing the absorbance of 420 nm band at the expense of the band at 290 nm, and all of this results in anisosbestic point at 333 nm (Figure 2a,b) supporting the formation of the complex between \( L \) and \( \text{Cu}^{2+} \) as expected. The Job plot executed using these data yielded 1:1 complex between \( \text{Cu}^{2+} \) and \( L \) (Figure S10). The Job plot yielded an association constant, \( K_a = (6.9 ± 2.3) \times 10^5 \text{ M}^{-1} \), as deduced using the Benesi–Hildebrand equation (Figure S11), and the limit of detection (LOD) for \( \text{Cu}^{2+} \) by \( L \) was evaluated to be 2.5 nM (0.925 ppb) (Figure S12). The 1:1 complex formed was further confirmed by observing a peak at \( m/z = 1564.75 \) corresponding to the \( \{L + \text{Cu}^{2+} - \text{H}^+\} \) in ESI-MS (Figure 2c). The assignment of this peak to the copper complex was further confirmed by comparing the experimentally observed isotopic peak pattern to that of the calculated one.

The changes observed in the fluorescence spectra of \( L \) during the titration of \( \text{Cu}^{2+} \) were ratiometric (Figure 3b), wherein the intensity of the 570 nm band decreases and that of the 475 nm band increases, resulting in an isoemissive point at 507 nm (Figure 3a). The plot of the intensity ratio of these two bands w.r.t. the \( [\text{Cu}^{2+}] / [L] \) mole ratio is sigmoidal. Thus, both the absorption and fluorescence titrations exhibited evidence for the formation of the complex between \( L \) and \( \text{Cu}^{2+} \). The limit of detection (LOD) was derived based on the emission intensity, and this has been 1.9 nM (0.7 ppb) (Figure S13). The thermodynamics of the binding between \( L \) and \( \text{Cu}^{2+} \) has been studied by isothermal titration calorimetry (ITC) titration (Figure 3c). The binding was observed as exothermic with a change in the enthalpy being \( -4.17 \times 10^4 \text{ cal/mol} \). The titration data fit well with one site binding with a \( K_a \) value of \( 1.61 \times 10^4 \text{ M}^{-1} \) in the case of \( \text{Cu}^{2+} \). The negative \( \Delta S \) value (−131.6 cal/mol/deg) observed is suggestive of the complex formation between \( L \) and \( \text{Cu}^{2+} \) (Figure 3c). In effect, the ITC

**Scheme 1. Synthesis of Precursors and the Final Molecule, \( L^{a} \)**

\( (a) \) CuSO_4·5H_2O, sodium ascorbate, t-butanol/CH_2Cl_2/H_2O (1:1:2), room temperature (RT), 1.5 days; (b) ethanol, piperidine, reflux, 1 day; (c) methyl iodide, acetonitrile, inert atmosphere, reflux, 0.5 days.

![Scheme 1. Synthesis of Precursors and the Final Molecule, L^a](image)

**Figure 1.** (a) Absorption spectrum of \( L \) before (black) and after (red) addition of 0.02 M TFA. The inset is a photograph of the cuvettes containing acetonitrile solution of \( L \) (left one) and \( L + \text{TFA} \) (one drop). (b) Emission spectrum of \( L \) before (black) and after (red) addition of 0.02 M TFA.

**Figure 2.** (a) Absorption spectral traces obtained during the titration of \( L \) with 0–5 equiv of \( \text{Cu}^{2+} \) salt. The inset shows expanded region of the absorption spectra to identify the isosbestic point. (b) Plot of absorbance ratio of 420 nm band over 290 nm band as a function of mole ratio of \([\text{Cu}^{2+}]/[L]\). (c) ESI-MS image of the \( \text{Cu}^{2+} \) complex of \( L \). The inset shows the isotopic peak pattern for the molecular ion peak obtained from experiment (black) and that from the simulated (red).
observed in the presence of Cu²⁺ clearly reveals both incremental addition of Cu²⁺, the peak for the closed form opens up, whereas in the presence of TFA, the spiro-indoline moiety of 6.45, 6.80, 7.05, and 7.15 ppm and further broaden. Thus, the aromatic peaks from the disappearance, while the peaks for the open form gain in intensity and further broaden. The inset shows expanded region of the spectrum for the 7.5−8.0 ppm region. At higher mole ratios of Cu²⁺ addition, two of the singlets observed in the aromatic region of 7.0−7.2 ppm increase in the peak area besides being marginally shifted by 0.05 ppm (Figure 4ii−vi).

Characterization of the Isolated Cu²⁺ Complex of L. The broad nature of the peaks in the ¹H NMR spectrum and the comparison of the spectrum to that of L (Figure 4) confirm that the L is complexed by Cu²⁺ (Figure S16). The spectrum obtained for the isolated Cu²⁺ complex of L (Figure S16 and Figure 4vi) agrees well with that obtained when Cu²⁺ was added in situ to L. The presence of vibrational bands corresponding to L, such as those from the carbonyl group, triazole moiety, and the hydroxy group in the Fourier transform infrared (FTIR) spectra of the complex in comparison to the spectrum of L alone, supports the formation of the complex (Figure 5a). The diffuse reflectance spectroscopy (DRS) data obtained for the solids of L and its Cu²⁺ complex are similar to those observed for their solutions, supporting that the complex is retained even in the solution (Figure 5b). For the isolated complex, one would expect the fluorescence of L to be quenched due to the presence of paramagnetic Cu²⁺ ion, and the same has been observed (Figure 5c) as well. The presence of paramagnetic copper center in the complex was confirmed by electron paramagnetic resonance (EPR) spectrum, which shows the presence of characteristic peaks for Cu(II). The EPR spectrum showed 2.44 and 2.12 for the g∥ and g⊥ values, respectively, wherein g∥ > g⊥ (Figure 5d). In the transmission electron microscopy (TEM) image, the L alone (Figure 5e) shows particles of irregular shape, while the isolated complex exhibits nanorods with size ranging from 150 to 250 nm (Figure 5f). Although the L alone shows discrete particles, an extensive aggregation of these particles was observed in the presence of Cu²⁺. From the high-resolution TEM (HR-TEM) images and selected area electron diffraction (SAED) pattern, the crystalline nature of the complex has been established with an interplanar distance of 0.27 nm (Figure 5g,h).

Induced Self-Assembly in L by Cu²⁺ by Scanning Electron Microscopy (SEM). In the literature, the calix[4]-arene derivatives are known to exhibit self-assembly, and such aggregational features are generally altered when an ion or a molecule interacts with and/or binds to them. ⁴³⁻²⁴ SEM studies of L showed particles with sizes in the range of 19−32 nm (Figure 6a), which are mostly spherical in nature. In the presence of Cu²⁺, such particles are joined together to form rods with length ranging from 150 to 300 nm, supporting that the aggregation of the particles is induced by Cu²⁺ (Figures 6b and S17). A similar rod-shaped morphology of ~300 nm was also observed in the case of the isolated complex of Cu²⁺ with L (Figure 6c).

Complexation of L with Cu²⁺ by Density Functional Theory (DFT) Computations. The structure of L was optimized according to the details given in Experimental Section, and the corresponding optimized structure is shown in Figure 7a. The optimized structure for L possesses two O−H⋯O-type intramolecular hydrogen bonds at lower rim and stabilizes the cone conformation for the calix[4]arene platform. The time-dependent density functional theory (TDDFT) studies are able to predict the two absorption bands (429 and 507 nm), which were observed in the visible region (420 and 540 nm) of the spectrum for L correctly (Figure S18). The spiro-indoline functionality present at the lower rim of L is well suited for metal-ion binding. As the spectral titration data reported in this paper clearly showed the formation of 1:1

**Figure 3.** (a) Fluorescence spectral traces obtained during the titration of L with 0−5 equiv of Cu²⁺ salt. (b) Plot of intensity ratio of 475 nm band over that of 570 nm band as a function of mole ratio of [Cu²⁺]/[L]. (c) Titration of L with Cu²⁺ by isothermal titration calorimetry. (Top) Raw data obtained for each injection. (Bottom) Fitted graph for the ΔΗ data plotted upon background correction.

**Figure 4.** ¹H NMR spectral traces obtained during the titration in CD₃CN: (i) L, (ii) L + 0.02 equiv of TFA (after 60 min of incubation). Spectra obtained in the titration of (L + x equiv of Cu²⁺), (iii) x = 0.2, (iv) x = 0.7, (v) x = 1.0, (vi) x = 2.0. The peaks corresponding to the protons are labeled using a schematic structure. The inset shows expanded region of the spectrum for L (blue) and (L + 0.2 equiv Cu²⁺) (red).
complex between L and Cu\textsuperscript{2+}, the structural features of such complex were addressed by DFT computations. Several conjecture structures for the complex were generated by carefully placing the metal ion at all of the possible binding regions available at L (Figure S08). The optimized structure for the complex is shown in Figure 7b along with its geometrical features. The two O–H⋯O types of hydrogen bonds observed in L at the lower rim continue to be present even in the complex, suggesting that the conformation of the calix[4]arene platform does not change upon complexation (Table S1). The structure of the complex exhibits tetracoordinated Cu\textsuperscript{2+} center. Each arm of the lower rim of L contributes one phenolic-O and one triazole-N for coordination. Such coordination features were also noted for Mg\textsuperscript{2+} complex of triazole-linked

Figure 5. (a) FTIR spectra for L (black) and its complex (red) in KBr. (b) DRS and (c) emission spectra for L and for the complex in solid state (in 10\textsuperscript{4} scale). (d) EPR spectrum of the complex in solid state at RT. TEM images for (e) L and (f) its complex. (g) HR-TEM image of the complex and (h) its selected area electron diffraction (SAED) pattern.

Figure 6. SEM images of (a) L, (b) in situ generated Cu\textsuperscript{2+} complex, and (c) the isolated complex.

Figure 7. Computational calculations were performed at the M062X/6-31G(dp), SDD// B3LYP/6-31G(dp), solid-state drives (SDD) level of theory. (a) Optimized structure for the open form of L. (b) Optimized structure for the Cu\textsuperscript{2+} complex of L. The primary coordination core is shown enlarged. Bond angles (°): N\textsubscript{1}–Cu–N\textsubscript{2} = 105; N\textsubscript{1}–Cu–O\textsubscript{1} = 101; N\textsubscript{1}–Cu–O\textsubscript{2} = 99; N\textsubscript{2}–Cu–O\textsubscript{1} = 101; N\textsubscript{2}–Cu–O\textsubscript{2} = 104; O\textsubscript{1}–Cu–O\textsubscript{2} = 142. Bond distances (Å): N\textsubscript{1}⋯Cu\textsuperscript{2+} = 2.124 Å, N\textsubscript{2}⋯Cu\textsuperscript{2+} = 2.087 Å, O\textsubscript{1}⋯Cu\textsuperscript{2+} = 1.897 Å, O\textsubscript{2}⋯Cu\textsuperscript{2+} = 1.887 Å. (c) Atom numbering scheme of L is shown for one of the arms to follow the dihedral angles. (d) Frontier MOs with the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) gap for L and for its Cu\textsuperscript{2+} complex.
nitro-functionalized spiro-pyran-derivatized calix[4]arene in the literature. The complexation energy for the formation of the complex of Cu²⁺ of L is -518 kcal/mol. In the case of this complex, the Cu²⁺−O₁/−O₂ bond distances are 1.897 and 1.887 Å and the Cu²⁺−N₁/−N₂ bond distances are 2.124 and 2.087 Å, respectively. The bond angles in the primary coordination sphere range from 99 to 142° supporting that the geometry about Cu²⁺ is highly distorted tetrahedral. The coordinates of the optimized structures of L and its Cu²⁺ complex are given in Table S3. On going from L to its complex, substantial arm conformational changes occur to orient the ligating centers in a manner to accommodate Cu²⁺ coordinatively in the core. The dihedral angle of 5-6-7-8 shifts from 152 to 83° in one arm and 98–306° in the other arm. The dihedral angle of 6-7-8-9 (Figure 7c) shifts from 200 to 84° in one arm, while the other shows only minimal shift (Figure S19). Thus, the Cu²⁺-induced conformational changes are evident from these dihedral angles in both the arms to form a binding core.

To understand the binding strength, the corresponding molecular orbitals and their energies (Figure 7) were computed using DFT since the HOMO and LUMO gap (∆E<sub>gap</sub>) accounts for the stability of the complex. In L, ∆E<sub>gap</sub> is 3.90 eV and the HOMO is exclusively localized on the spiro-indoline group, and a similar observation is seen in the case of LUMO (Figure 7d). However, upon the complexation of L with Cu²⁺ ion, ∆E<sub>gap</sub> is reduced to 1.63 eV, indicating that the complexation stabilizes L. The HOMO is localized on the atoms present in the proximity to Cu²⁺ ion, while the LUMO is localized on the spiro-indoline groups of L. Based on the TDDFT computational studies, the complex exhibits vertical excitation energy at 435 nm. This is in agreement with the experimentally observed absorption spectrum showing a band at 420 nm. The optimized structure of the complex have been demonstrated for imaging cancer cells and also for their cancer cell-killing abilities using MDA-MB-231 cells. The MTT assay was carried out with these cells by both the untreated cells were used as control. From Figure 10a, it is observed that the population of G₀/G₁ phase was high in the untreated cells. Upon addition of the complex from 0 to 255 nM concentrations to these cells, a shift of population from G₀/G₁ and G₂M phases toward Sub-G₁ phase was observed (Figures 10b and S22). All of these suggest cell death as demonstrated using MDA-MB-231 cells by the FACS studies. These data support MTT assay results by suggesting that the treatment with higher concentration of the complex increases the cell death potency.

**Complex of L with Cu²⁺ by X-ray Photoelectron Spectroscopy (XPS) Data.** The complex formed between L and Cu²⁺ was also supported by XPS studies (Figures 8 and S20), and the results obtained for the isolated complex are similar to those of in situ generated 1:1 complex. The computational study supported the formation of an N₂O₂ primary coordination core with respect to Cu²⁺. This is expected to shift the XPS peaks of copper, nitrogen, and oxygen, and the same is observed supporting that the Cu²⁺ complex of L is formed. The 1s XPS spectra of C, N, and O showed shifts in the peaks of the complex compared to the L alone (Figure 8a–c). The C 1s spectrum of L showed a shift in the peak observed at 282.4–283.8 eV upon complexation with Cu²⁺ (Figure 8a). The N 1s spectra exhibited a shift in the peak by 1.08 eV on going from L to its Cu²⁺ complex that is observed at 399.18 eV (Figure 8b). In O 1s spectra, the peak observed at 530.4 eV for L was shifted to 531.25 eV in its complex (Figure 8c). The presence of copper was validated through Cu 2p XPS image of the complex, whereas such peak was absent in the uncomplexed L (Figure 8d). All of this reveals the binding of L by Cu²⁺ and thus supports the conclusions obtained from other experimental and computational studies.

**Cell Viability Study by Methyl Thiazolyl Tetrazolium (MTT) Assay.** The spiro-indoline-based molecular systems are known to exhibit anticancer properties, but similar moieties anchored on to the calix[4]arene platform have not been reported for such activity. In the present study, L and its Cu²⁺ complex have been demonstrated for imaging cancer cells and also for their cancer cell-killing abilities using MDA-MB-231 cells. The MTT assay was carried out with these cells by both L and its Cu²⁺ complex at different concentrations. The cell viability data given in Figure 9a showed that L alone does not exhibit much cell death (cell viability being ~95%) even at higher concentrations. However, the complex exhibited effective cancer cell-killing ability with an IC<sub>50</sub> value of 165 nM as derived from the plot of concentration vs percent live cells shown in Figure 9b. The complex exhibited higher cell death potency compared to simple Cu²⁺ salt at an equivalent concentration (Figure S21), and the comparative data clearly show that the presence of L augments the cell death supporting that the complex is essential.

**Mechanism of Cell Death by Fluorescence-Activated Cell Sorting (FACS) Analysis.** To study the mechanism of cell death, cell cycle analysis was performed by FACS with 230 nM L and its complex treated with MDA-MB-231 cells, while the untreated cells were used as control. From Figure 10a, it is observed that the population of G₀/G₁ phase was high in the control studies (only cells and L-treated cells). Upon addition of the complex from 0 to 255 nM concentrations to these cells, a shift of population from G₀/G₁ and G₂M phases toward Sub-G₁ phase was observed (Figures 10b and S22). All of these studies suggest cell death as demonstrated using MDA-MB-231 cells by the FACS studies. These data support MTT assay results by suggesting that the treatment with higher concentration of the complex increases the cell death potency.
Cell Imaging Studies. The spiro-indoline derivatives are reported for their red fluorescence due to the extended conjugation.\(^{15}\) Cancer cell imaging studies carried out with L and its complex showed red fluorescence in MDA-MB-231 cells (Figure 11). Colocalization studies carried out with MitoTracker Green show merging of red and green to form yellow color, which showed the accumulation of L as well as the complex in the mitochondrial region of the cells. So, L can be utilized as cancer cell imaging agent, whereas its Cu\(^{2+}\) complex can be used for cancer cell theranostics. Colocalization studies carried out in comparison to MitoTracker Green showed an overlap integral of 0.9 (Figure S23 and Table S2), which confirms that the L goes to the mitochondria. Other than this, the quenching of the red fluorescence intensity for the complex confirms the quenching of fluorescence of L by Cu\(^{2+}\) ions.

Cell Internalization Study. The MDA-MB-231 cells treated with different concentrations of the complex were analyzed for the cell-incorporated copper by inductively coupled plasma mass spectrometry (ICP-MS), and the corresponding data are given in Figure 12. These data supported the presence of increasing amount of copper in the cells with increase in the concentration of the complex used for treating the cells. The copper present inside the cell is substantially higher than the intrinsic copper of the cells, and hence all of this supports the internalization. The presence of L inside the cells is viewed through its fluorescence emission as studied by fluorescence imaging. Thus, the internalization of the complex by these cells was established by a combination of ICP-MS and fluorescence microscopy.

CONCLUSIONS AND COMPARISONS
A triazole-linked lower-rim bis-spiro-indoline-functionalized calix[4]arene conjugate (L) has been synthesized and characterized. The L can exist either in the ring-closed (spiropyran) or in the open (merocyanine) form due to the presence of spiro-indoline ring at its lower rim. It has been experimentally verified by us as well as others in the literature\(^{15−17}\) that the transformation from closed to open form can be achieved by (i) UV irradiation and (ii) adding acids such as trifluoroacetic acid (Figures 1 and S24). This transformation is noted by observing an increase in the absorbance at 420 nm band and emission intensity at 570 nm in the fluorescence spectra. The titration of L with Cu\(^{2+}\) showed ratiometric changes in the absorption as well as fluorescence spectra by exhibiting isosbestic and isosensitive points at 333 and 507 nm, respectively, supporting the formation of the complex of L. The binding constant of L by Cu\(^{2+}\) is \((6.9 \pm 2.3) \times 10^5 \text{M}^{-1}\) as obtained from the absorption spectral data, and this supports rather strong binding interaction between the ligand and Cu\(^{2+}\). While the Job plot yielded 1:1 complex, the ITC data confirmed the same. The 1:1 complex has been further established by observing a peak in ESI MS at \(m/z = 1564.757\), and the isotopic peak pattern supported the presence of the copper ion in that. The binding of Cu\(^{2+}\) to L was further supported by carrying out \(^1^H\) NMR titration, wherein the binding of this ion by the open form of

Figure 10. (a) Bar diagram of cell cycle phases of MDA-MB231 cells untreated (red) and treated for 24 h with L (blue) and its Cu\(^{2+}\) complex (green) based on the FACS data. (b) Cell cycle analysis with increasing concentrations of the complex for 6 h (0−255 nM). In both the cases: (i) SubG1, (ii) G0/G1, (iii) S, and (iv) G2M.

Figure 11. Confocal micrographs of MDA-MB231 cells untreated (control) and treated with L and the Cu\(^{2+}\) complex according to the labels given.

Figure 12. ICP-MS analysis of MDA-MB231 cells incubated with different concentrations of the Cu\(^{2+}\) complex of L (0−198 nM).
the spiro-indoline moiety as well as the paramagnetic broadening were evident from the spectral changes.

The copper complex of L was isolated, and the \(^1\)H NMR spectrum of this complex has been noted to have features similar to that obtained when the L was titrated in situ with Cu\(^{2+}\) salt (Figures 4iii–vi and Figure S16). The presence of 4.1 and 3.2 ppm peaks in \(^1\)H NMR spectrum supported that the cone conformation is maintained for the calix[4]arene platform even in the complex. The paramagnetic nature of the complex was also deduced from the EPR spectrum. The fluorescence spectrum obtained for the solid of the isolated complex also showed quenching of the fluorescence compared to simple L as expected. The quenching of fluorescence intensity of L was observed even during the in situ titration by Cu\(^{2+}\) salt. Even DRS spectra supported the presence of L in the complex. Irregular shaped particles of \(\sim 25\) nm size observed for L were joined together to form rodlike particles in the presence of Cu\(^{2+}\). The length of these rods ranges from 300 to 400 nm, suggesting that the formation of these rods is due to the induced aggregation of L and Cu\(^{2+}\). Similar rods were observed even when the microscopy study was performed using the isolated complex. All of this supports that the microscopic features observed for the isolated complex exists even in the solution. From the TEM studies it was observed that L alone exhibits nonuniform particles and these are further aggregated to rodlike shape in the presence of Cu\(^{2+}\). Thus, both SEM and TEM support Cu\(^{2+}\)-induced aggregation in L.

The HOMO–LUMO gap decreases from 3.9 eV in L to 1.63 eV in its Cu\(^{2+}\) complex, which supports the stabilization of L upon binding of Cu\(^{2+}\) as already demonstrated by the experimental studies. The DFT computational studies showed N\(_2\)O\(_2\) binding core of L for Cu\(^{2+}\) with distorted tetrahedral coordination geometry about the metal ion. These studies also showed that the binding of Cu\(^{2+}\) induces conformational changes in the lower rim of L, as evident from the dihedral angles. The complexation of Cu\(^{2+}\) did not alter the cone conformation of the calix[4]arene platform, as evident from the observed hydrogen-bonding interaction at lower rim, similar to that observed for L alone. The binding of L by Cu\(^{2+}\) is supported by the changes observed in the binding energies of N 1s and O 1s by 1.08 and 0.85 eV, respectively, from the XPS spectra.

The L alone does not kill cancer cells (MDA-MB-231 cells) (Scheme 2), but it shows red fluorescence when incubated with MDA-MB-231 cells due to the targeting ability of L toward mitochondria, which is strongly supported by the observed overlap coefficient of 0.9 from the colocalization study. The presence of Cu\(^{2+}\) in the incubated cells has been verified quantitatively by ICP-MS studies. The copper complex of L exhibited an I\(_{50}\) value of 165 nM, suggesting that the complex is an efficient anticancer agent. The cell cycle analysis (photomicroscopy) images on a maXis Impact high-resolution mass spectrometer (Bruker). Absorption and emission spectra (solid and solution) were measured on a Varian Cary UV 100 Bio UV–vis spectrophotometer and a Horiba Fluoromax-4 fluorescence spectrometer, respectively. DRS spectra were measured on Shimadzu (Japan) UV-NIR-3600. Transmission electron microscopy (TEM) images were recorded on a JEOL 2100F FEG-TEM microscope, and scanning electron microscopy (SEM) images were recorded on an FEI Quanta 200F FEG-SEM microscope. XPS spectra were recorded on an Rikagiken (Japan) UV-NIR-3600. Transmission electron microscopy (TEM) images were recorded on a JEOL 2100F FEG-TEM microscope, and scanning electron microscopy (SEM) images were recorded on an FEG-SEM-JSM-7600F microscope. XPS spectra were recorded on an AXIS Supra X-ray photoelectron spectrometer, Kratos Analytical, UK (Al K\(_{α}\) source, 225 W; pass energy, 160 eV; take-off angle, 90°), and confocal microscopy images on a Zeiss Axioplan-Observer Z1 microscope (inverted). ICP-MS spectra were measured on Element XR (Model: Thermo Fisher Scientific).
Germany), and the fluorescence-activated cell sorting (FACS) was carried out on FACS Aria Special Order System.

**Synthesis of the Precursors.** The precursor molecules, viz., P1, P2, P3, and P4 have been synthesized by following the literature reported procedures, and their characterization data are given here and the corresponding spectra in Figures S01–S04.

**Synthesis and Characterization of P1.** A known procedure was used to synthesize P1.1H NMR (400 MHz, CDCl3, 25 °C): δ = 7.06 (s, 4H, Ar−H), 6.72 (s, 4H, Ar−H), 6.49 (s, 2H, Ar−CH2), 4.74 (d, 4H, J = 3.1 Hz, OCH2−C), 3.43 (d, 4H, J = 13 Hz, ArCH2Ar), 3.32 (d, 4H, J = 13 Hz, Ar−CH2−Ar), 2.54 (t, 2H, CCH), 1.54 (s, 18H, C(CH3)3), 0.9 (s, 18H, C(CH3)3) ppm. 13C NMR (CDCl3, 25 °C): δ = 150.6, 149.7, 147.4, 141.8, 132.8, 128.2, 125.7, 125.2, 78.9, 63.4, 34.1, 34.0, 32.2, 31.9, 31.8, 31.2, 31.1 ppm. ESI-MS peak observed for P1 (C98H116N8O6) as [M + Na]+: 747.45 (observed) and 747.4389 (calculated).

**Synthesis and Characterization of P2.** The precursor P2 has been synthesized following the literature reported procedure.1H NMR (400 MHz, CDCl3): δ = 11.2 (br−s, 1H, Ar−OH), 9.91 (s, 1H, CHO), 7.57 (s, 1H, Ar−H), 7.52 (dd, 1H, Ar−H), 4.45 (s, 2H, Ar−CH2), 1.34 (s, 9H, Ar−(CH3)3) ppm. 13C NMR (CDCl3): δ = 196.9, 157.7, 142.9, 134.8, 130.2, 123.9, 120.2, 49.2, 34.3, 31.4 ppm.

**Synthesis and Characterization of P3.** The precursor P3 has been synthesized following the literature reported procedure.1H NMR (400 MHz, CDCl3): δ = 11.2 (br−s, 1H, Ar−OH), 9.91 (s, 1H, CHO), 7.57 (s, 1H, Ar−H), 7.52 (dd, 1H, Ar−H), 4.45 (s, 2H, Ar−CH2), 1.34 (s, 9H, Ar−(CH3)3) ppm. 13C NMR (CDCl3): δ = 196.9, 157.7, 142.9, 134.8, 130.2, 123.9, 120.2, 49.2, 34.3, 31.4 ppm.

**Synthesis and Characterization of P4.** The precursor P4 has been synthesized following the literature reported procedure.1H NMR (400 MHz, DMSO-d6): δ = 7.91 (m, 1H), 7.82 (m, 1H), 7.62 (m, 2H), 3.97 (s, 3H), 2.77 (s, 3H), 1.52 (s, 6H); 13C NMR (100 MHz, DMSO-d6): δ = 196.0, 142.0, 141.6, 129.3, 129.8, 123.3, 115.1, 53.9, 34.7, 21.6, 14.1 ppm.

**Synthesis and Characterization of L.** The precursor P4 was dissolved in 5–10 mL of ethanol, and the solution was heated to reflux. After 30 min, pipedine (0.1 mL) was added to this. A solution of P4 (0.2 g) in ethanol (20 mL) was added to the reaction mixture and refluxed for 24 h. Completion of the reaction was monitored by thin-layer chromatography. After completion of the reaction, the solvent was evaporated to dryness and the crude product was purified by silica gel chromatography using petroleum ether/ethyl acetate (2:3) as elution medium to get a light pink solid L (Figure S05). Yield: 0.1 g (64%). 1H NMR (400 MHz, CDCl3): δ = 6.97−7.16 (m, 15H), 6.84 (d, 1H), 6.83 (d, 1H), 6.80 (d, 1H), 6.78 (d, 1H), 6.76 (d, 1H), 6.71 (s, 2H, Ar−OH), 6.61−6.86 (m, 6H, Ar−H), 6.64−6.52 (q, 2H, Ar−CH2), 5.70 (d, 2H, spiroalkane), 4.50−5.14 (m, 9H), 4.12−4.29 (m, 5H), 3.16−3.25 (m, 5H), 2.5 (d, 6H), 1.30 (m, 6H), 1.26 (s, 18H), 1.16−1.23 (m, 6H), 0.88 (s, 18H); 13C NMR (CDCl3): δ = 150.6, 149.7, 147.4, 141.8, 132.8, 128.2, 125.7, 125.2, 78.9, 63.4, 34.1, 34.0, 32.2, 31.9, 31.8, 31.2, 31.1 ppm. ESI-MS peak observed for L (C39H37N9O9) as [M]+: 1502.78 (observed) and 1502.57 (calculated). Elemental analysis for L: Calculcd. 78.36; H, 7.78; N, 7.46; O, 6.39. Found., 78.21; H, 7.82; N, 7.48.

**Synthesis of [CuL] Complex.** A mixture of L (0.05 g) and Cu(ClO4)2·6H2O (0.012 g) was taken in a flask, and 10 mL of acetonitrile was added and the resulting mixture was stirred at room temperature. An excess amount of Et3N was added and stirring was continued for 1 day. A brownish yellow precipitate was found, which was filtered, washed with acetonitrile, and dried. Yield: 0.025 g. FTIR (KBr, cm−1): 3622, 2988, 2245, 1624, 1393 and 1028. XPS (Cu 2p, eV): 931 and 950.

**Spectral Titration Studies.** Stock solutions of L (0.6 mM) were incubated with 0.02 M trifluoroacetic acid (TFA) for 1 day and used for the studies. The 0.6 mM copper perchlorate solution was prepared in acetonitrile. The L was titrated with 0−5 equiv of Cu2+, and accordingly, the absorption and fluorescence spectra were measured. Fluorescence spectra were measured at λex = 379 nm in the range of 400−700 nm in a 10 mm cuvette containing 3 mL of analyte solution. 1H NMR titrations of L and TFA (0.02 equiv for 0−60 min) and Cu2+ (0−2 equiv) were carried out in CD3CN.

**Isothermal Titration Calorimetry (ITC) Experiments.** Calorimetric titration was carried out at 25 °C using a MicroCal ITC20 isothermal titration calorimeter (Northampton, MA). The 15 mM Cu2+ solution (40 μL) was taken in a syringe and titrated into 1 mM (270 μL) solution of ligand L in the cell through 20 injections. The one site binding model was used to fit the obtained data after appropriate subtraction of the solvent interaction with metal ion as control study.

**SEM, TEM, and XPS Analyses.** For SEM studies, 50 μM solution of L and the solution containing 1:1 mole ratio of L:Cu2+ and [CuL] in acetonitrile were drop-cast onto Al foil after ultrasonication for 15 min. For TEM and XPS analyses, the solutions of L and [CuL] were drop-cast onto carbon-coated copper grid and Al foil, respectively. The samples were dried in a vacuum desiccator before performing the measurements.

**DRS Study.** The diffuse reflectance spectroscopy (DRS) data of the isolated complex and L were measured in barium sulfate matrix using Shimadzu-UV-3600.

**IR and EPR Measurements.** IR spectra of L and its Cu2+ complex were measured using KBr pellet in the 400−4000 cm−1 region. The X-band EPR spectrum of [CuL] was recorded on a JES-FA200 ESR spectrometer in solid state at room temperature.

**DFT Studies.** All of the calculations were carried out by using Gaussian 16, Revision B01 software packages. The open structural form of L was generated, optimized, and subjected to TDDFT to generate the possible electronic transitions. The initial structure has been prepared from the reported crystal structure followed by certain modifications as given in Figures S06−S08. The optimized structure for the open form of L has been used for the complexation by Cu2+ ions by DFT and TDDFT in the gas phase as well as in acetonitrile solvent. The DFT computations adopted here are in unrestricted formalism since the metal ion is paramagnetic. During the geometry optimization, the B3LYP functional with SDD was used for Cu2+ ion and the 6-31G(d,p) basis set was used for the rest of the atoms. The vertical excitation energies were studied in acetonitrile solvent using the polarized continuum method using the self-consistent reaction field tool available in the Gaussian16 software package.

**Cell Viability Assay.** The anticancer property of the samples of L and its Cu2+ complex was tested on MDA-MB231 breast cancer cells using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells were
seeded in 96-well plate with ~15,000 cells per well in a Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 1% penstrep (antibiotic and antimycotic). After incubation for 24 h at 37 °C in 5% CO2 atmosphere, the cells were incubated for 24 h with various concentrations of L and its complex (0, 66, 132, 330, 528, and 660 nM). After 24 h treatment, each well was treated with 0.5 mg/mL methyl thiazolyl tetrazolium (MTT) reagent and incubated for 4 h. The medium was removed and 200 μL of dimethyl sulfoxide (DMSO) was added into each well to dissolve the crystals formed. The absorbance at λmax = 570 nm was measured using a plate reader.

Confocal Microscopy. MDA-MB-231 cells were seeded in 60 mm culture dishes containing glass coverslip with a seeding density of 0.5 × 10^5 cells in DMEM and incubated for 24 h at 37 °C. The untreated cells were used as control.

The cells were washed with phosphate-buffered saline (PBS) three times to remove excess of the compound and stained with 100 nM MitoTracker Green (M7514) in a fresh complete medium and incubated for 0.5 h. After that, the cells were washed again with PBS three times and the coverslips were placed on a glass slide with 4′,6-diamidino-2-phenylindole and observed under a confocal laser scanning microscope. The untreated cells were used as control.

Cell Cycle and Fluorescence-Activated Cell Sorting (FACS) Analyses. MDA-MB-231 cells (0.5 × 10^6) were incubated for 24 h in 60 mm dishes, treated with varying concentrations of L and its Cu2+ complex, and incubated again. After removing the medium and fixing the cells with ethanol, the cells were stained with 10 μL of propidium iodide (from a stock solution of 1 mg/mL) in 1 mL of complete medium cells observed using red filter in FACS.

Inductively Coupled Plasma Mass Spectrometry Analysis (ICP-MS). MDA-MB-231 cells were cultured in 60 mm dishes with complete DMEM for 24 h. The cells were treated with varying concentrations of L and its Cu2+ complex separately for 24 h at 37 °C followed by harvesting through centrifugation at 1000 rpm for 3 min and vortexed to rupture the cell membrane. These samples were further used for ICP-MS analysis. The untreated cells were used as control.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01402.

Characterization of P1, P2, P3, P4, and L; initial guess structure of L for complexation of Cu2+ by DFT; Job plot of L with Cu2+; binding constant derivation from the Benesi–Hildebrand equation; limit of detection for Cu2+ by L using absorption and fluorescence spectroscopy; 1H NMR titration data of L with TFA and Cu2+; SEM images of L in the presence of Cu2+; TDDFT data and atomic numbering for dihedral angle measurement; cell viability data, cell cycle data, intensity profile diagram from cell imaging, and overlap coefficient determination using colocalization; coordinates of optimized structures of L and its Cu2+ complex; irradiation study of L; and comparison with literature reports (PDF).

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Notes

The authors declare no competing financial interest.

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DEDICATION

The authors dedicate this paper to Professor C. N. R. Rao, FRS, on his 85th birthday.

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