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Benzothiadiazole Derivatives as Fluorescence Imaging Probes: Beyond Classical Scaffolds

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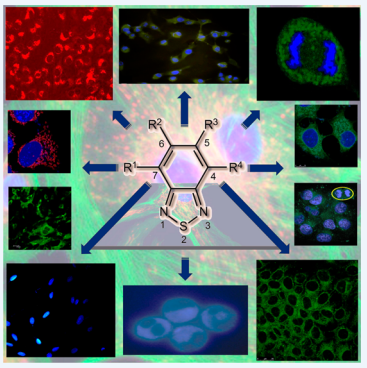
CONSPECTUS: This Account describes the origins, features, importance, and trends of the use of fluorescent small-molecule 2,1,3-benzothiadiazole (BTD) derivatives as a new class of bioprobes applied to bioimaging analyses of several (live and fixed) cell types. BTDs have been successfully used as probes for a plethora of biological analyses for only a few years, and the impressive responses obtained by using this important class of heterocycle are fostering the development of new fluorescent BTDs and expanding the biological applications of such derivatives. The first use of a fluorescent small-molecule BTD derivative as a selective cellular probe dates back to 2010, and since then impressive advances have been described by us and others. The well-known limitations of classical scaffolds urged the development of new classes of bioprobes. Although great developments have been achieved by using classical scaffolds such as coumarins, BODIPYs, fluoresceins, rhodamines, cyanines, and phenoxazines, there is still much to be done, and BTDs aim to succeed where these dyes have shown their limitations.



Important organelles and cell components such as nuclear DNA, mitochondria, lipid droplets, and others have already been successfully labeled by fluorescent small-molecule BTD derivatives. New technological systems that use BTDs as the fluorophores for bioimaging experiments have been described in recent scientific literature. The successful application of BTDs as selective bioprobes has led some groups to explore their potential for use in studying membrane pores or tumor cells under hypoxic conditions. Finally, BTDs have also been used as fluorescent tags to investigate the action mechanism of some antitumor compounds.

The attractive photophysical data typically observed for π-extended BTD derivatives is fostering interest in the use of this new class of bioprobes. Large Stokes shifts, large molar extinction coefficients, high quantum yields, high stability when stored in solution or as pure solids, no fading even after long periods of irradiation, bright emissions with no blinking, good signal-to-noise ratios, efficiency to transpose the cell membrane, and irradiation preferentially in the visible-light region are just some features noted by using BTDs.

As the pioneering group in the use of fluorescent small-molecule BTDs for bioimaging purposes, we feel pleased to share our experience, results, advances, and personal perspectives with the readers of this Account. The readers will clearly note the huge advantages of using fluorescent BTDs over classical scaffolds, and hopefully they will be inspired and motivated to further BTD technology in the fields of molecular and cellular biology.

1. INTRODUCTION

Fluorescent compounds that can provide useful information from biological responses through specific interactions are of paramount importance for medicine, molecular and cellular biology, chemistry, chemical biology, and other interdisciplinary sciences. The continuous effort to develop new fluorescent small-molecule compounds that can perform selective biological interactions inside different cell types with positive and specific responses is justified by the high sensitivity of fluorescent techniques and the possibility to visualize the process under analysis with very low concentrations of the fluorophore. These very attractive tools (fluorescent techniques) are known for their excellent results in the study of cellular processes mostly because they are the most sensitive of the available spectroscopic techniques.[1] The possibilities of biological applications of new fluorescent small-molecules are indeed virtually unlimited.[2] The reader is urged to peruse comprehensive surveys and reviews of the literature that describe the use and importance of fluorescent probes applied in the visualization of cellular processes.[3−5] For instance, it is possible to observe different biological processes in living cells and tissues,[6] to monitor cell secretion,[7] to visualize mitochondrial dynamics,[8] and many others.[9]

Both the challenge and the development of a better comprehension of those aforementioned cellular processes and mechanisms start with an equally challenging step, namely, the rational design and synthesis of stable fluorescent compounds that can selectively stain molecules of the amazing cellular machinery with their stunning processes. In this context, the biological application of fluorescent small-molecule 2,1,3-benzothiadiazole (BTD) derivatives (Figure 1) has begun to flourish in the past few years with impressive results and excellent perspectives. The large number of available fluorescent probes have indeed been built over a limited number of "core scaffolds",

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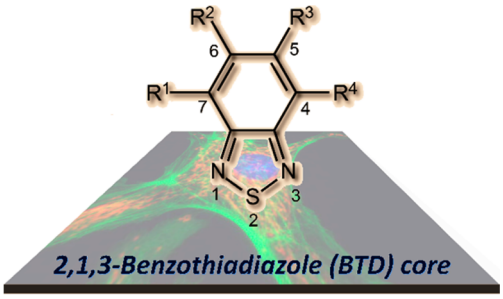

Figure 1. Illustrative view of the 2,1,3-benzothiadiazole basic structure found in some fluorescent derivatives applied for bioimaging purposes. Note that R^2 and R^3 are usually H atoms and that R^1 and R^4 are the groups in the positions commonly used for π-extensions.

and this issue has been recently reviewed elsewhere.[10] Coumarins, boron−dipyrromethenes (BODIPYs), fluoresceins, rhodamines, cyanines, and phenoxazines are among the most used fluorescent derivatives.[10] The use of these fluorescent small-molecules is undoubtedly of paramount importance, but the limitations associated within them are already well-known.[10]

Coumarin-based bioprobes are usually not suitable for use in chemical fixed samples (adhered cells), which reduces the range of potential biological and medical applications. Although their Stokes shifts are generally good, their emission spectra are too wide (typically from yellow to far red), therefore impairing their use in combination with other red-fluorescent probes. Commercially available BODIPYs are, for instance, frequently used to stain lipidic structures. Although the manufacturer claims that this class of compounds has great photostability, this is not always correct, but the photostability is typically better than that of fluorescein-based fluorophores. BODIPY dyes may quickly lose their emission potency through photobleaching. Because of their characteristic lipophilicity, sometimes they must be diluted in organic solvents that are normally toxic to mammalian cells, thus impairing long-term live-cell assays. Fluorescein dyes are in general water-soluble, but some structural features of these compounds limit their use. Their high rate of photobleaching, their broad emission spectra, and changes in their signal because of pH variation are harmful for multistaining procedures on live-cell systems. Fluorescein-based bioprobes are also widely used conjugated with antibodies, therefore requiring storage at −20 °C. Rhodamine dyes are among the most widely used. These dyes typically have high hydrophobicity, which produces an elevated aggregation state in water solution. Aggregation usually affords dye−dye interactions and a self-quenching effect. Their broad emission spectra (commonly noted from orange to far red) also reduce their use in multistaining assays. Cyanine probes have low polarity, and the cyanine ester must be diluted in organic solvents such as dimethylformamide or dimethyl sulfoxide that are toxic for mammalian cells at levels above 0.01%. The Stokes shifts are typically not large, and therefore, time-consuming efforts related to the detector setting are required in order to perform the cell imaging. The manufacturers suggest that storage conditions as low as −20 °C and repeated freeze−thaw cycles reduce their life span. Phenoxazine bioprobes are less frequently used but have typical small Stokes shifts, resulting in some difficulties related to the required adjustment of cellular image acquisition, in particular when laser scanning confocal microscopy is used.

BTDs are a new class of bioprobes that are just beginning to have their potential explored, aiming to succeed where those classical scaffolds have shown their limitations. We have already reviewed[11] the beneficial features of π-extended BTDs for photoluminescence applications as well as their potential for mitochondrial-selective staining,[12] but this issue will be better evaluated herein. We had already explored the use of fluorescent BTDs for different purposes[13−15] related to light technologies rather than bioimaging. Among BTDs' attractive chemical and photophysical properties, it is interesting to highlight the following:

(i) Fluorescent BTD derivatives are usually efficient and stable fluorophores, even in excited states.
(ii) The BTD ring has a strong electron-withdrawing capacity and facilitates intramolecular charge transfer (ICT) stabilizing processes (among others).
(iii) BTDs have highly polarized properties that lead to intermolecular interactions such as heteroatom contacts and $\pi-\pi$ interactions, therefore normally affording well-ordered crystal structures.
(iv) Fluorescent BTDs typically display large Stokes shifts.
(v) Fluorescence emission of BTD-based bioprobes usually does not fade off even after long periods of irradiation.
(vi) BTDs typically display bright emission with no blinking during cellular experiment time periods.
(vii) The signal-to-noise ratio, which is crucial for the success of the experiment, is typically very good for BTDs bioprobes.
(viii) Almost all of the described BTDs used in bioimaging applications have proved to be capable of efficiently transposing the cell membrane (with a few exceptions), which is one of the most desired features of fluorogenic dyes.[16]
(ix) BTDs may be stored (pure or in solution) for years at room temperature, as will be discussed. Many commercial staining kits, which are typically fluorescent small-molecule derivatives of the classical scaffolds, must be stored between −20 to −80 °C and are therefore prone to degradation by repeated freeze−thaw cycles.
(x) BTDs are typically excited in the visible-light region, facilitating laser intensity at minimum levels in order to avoid temperature elevation, preventing morphological alterations and oxidative stress, and also returning high-quality image acquisitions. Any signal of autofluorescence emission from the cells can therefore be easily avoided.
(xi) Theoretical calculations may be used as an important tool for predicting the photophysical properties of fluorescent BTDs, thus allowing the synthesis of new structures to be guided by theoretical predictions, as we have described elsewhere.[17]

Because of their attractive physicochemical and photophysical properties, it is surprising that biological applications of fluorescent small-molecule BTDs have been reported starting only a few years ago. In 2007, we demonstrated the first application of fluorescent small-molecule BTD derivatives as sensitive fluorophores for double-stranded DNA (dsDNA)-selective detection and quantification.[18] In the same year, another group reported that polymeric BTD-containing derivatives are also efficient for DNA concentration determination.[19] Later, we also demonstrated that some BTDs proved to be efficient fluorophores for real-time PCR analyses.[20] Indeed, different groups have also applied BTD-containing derivatives for biological investigations[21−23] but not yet as cellular selective markers in bioimaging readings.

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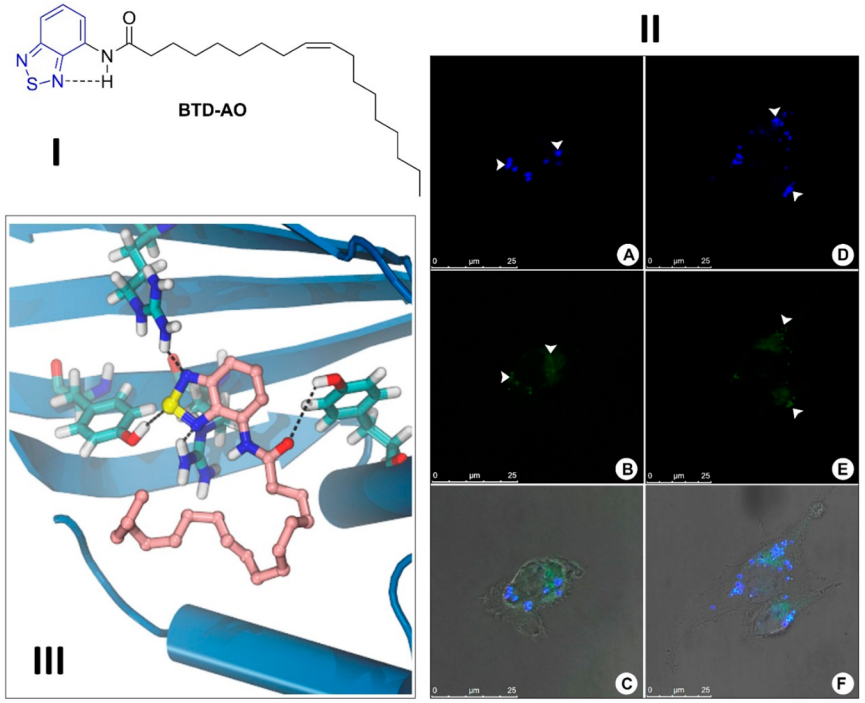

Figure 2. (I) Structure of the fluorescent oleic acid derivative **BTD-AO**. (II) Images of MDA-MB-231 breast cancer cells incubated with **BTD-AO** at (A–C) 4 °C and (D–F) 37 °C for 5 min. The bioprobe accumulated on vesicles (lipid droplets) near the peripheral region of the cells. The images show the same results at both temperatures tested, as indicated in (A), (B), (D), and (E) arrowheads. A blue fluorescence signal is seen in (A) and (D), whereas green emission is seen in (B) and (E). (C) and (F) show the normal morphology of the cells as measured by phase-contrast microscopy. Scale bars are 25 μm. (III) X-ray structure of FABP (PDB ID 2FLJ) and predicted conformation of **BTD-AO** upon binding to FABP. A total of 8.1×10^8 conformers were sampled per run. Black dashed lines depict potential H-bonding interactions between the ligand and the protein. Adapted from ref 29. Copyright 2014 Royal Society of Chemistry.

In view of all the positive effects of the photoluminescent properties of π-extended BTDs, it is surprising that bioimaging (cellular) applications of BTD dyes have been reported only in this decade. In 2010, we described for the first time the use of small-molecule BTDs with impressive subcellular localization, staining only nuclei of human stem cells.[24] In the same year, some groups independently demonstrated the high potential of micellar BTD-containing red emitters preferentially localized in the cytoplasm (mouse leukemic monocyte macrophage cell line, RAW 264.7).[25] Since then, several advances and new BTD bioprobes have been described by us and other groups, as will be presented. Herein we wish to disclose the advances, challenges, and trends in the use of fluorescent small-molecule BTDs for cell-imaging applications and how this new class of bioprobes may fill the gap not occupied by the use of the classical scaffolds and be "key players" in the future of molecular bioprobes. BTD-containing polymers (copolymers) applied for bioimaging of cellular models have been reviewed elsewhere[26,27] and are outside the scope of this Account.

2. CELLULAR CHALLENGES FOR THE DEVELOPMENT OF NEW BIOPROBES AND THE ROLE OF BTD BIOPROBES

2.1. Lipid-Based Structures and Cell Membrane Transposition

The first challenge for a molecular bioprobe is that it must be designed to reach the cell cytoplasm, which is enclosed by the cell membrane. For some, membrane permeability is among the most desired feature of a fluorogenic bioprobe.[16] The cell membrane consists primarily of a thin layer of amphipathic lipids and sterols that are spontaneously arranged with their hydrophobic regions (known as "tails") facing toward to each other, thereby isolating these regions from the surrounding polar fluid. The hydrophilic regions of these lipid molecules (known as "heads") maintain the molecular association with the intracellular and extracellular polar environments of the resulting bilayer. The cell membrane also has a large content of proteins, and both the lipids and proteins of the membrane can show different levels of glycosylation. All of these molecules are applied in various essential biological activities, and as a result of their compositions and vital roles in cell metabolism, these molecules symbolize a resistant barrier to the fluorescent probes and commonly prevent transposition of the cell membrane.[28]

The cell membrane itself may be labeled by some specific bioprobes. BODIPY dyes and nitrobenzoxadiazole (NBD) and dimethylaminonaphthalenesulfonyl (dansyl) fluorophores may be used for such purposes. Although the use of BODIPY dyes as lipid-based selective probes has been suggested, the selectivity typically noted may not be satisfactory, and their successful application is usually for staining of lipid droplets because of their affinity for neutral lipids. NBD has a lower lifetime when diluted in aqueous solvents. This feature reduces its application to imaging assays with live cellular models. Dansyl mainly associates in the polar headgroup region in the lipid bilayer of the cell membrane. Because of this characteristic, the bioprobe can also bind proteins, and although this feature can be exploited to analyze fatty-acid-binding proteins, the expected selectivity of the bioprobe is compromised. Because most live cellular models have an abundant quantity of lipid-binding proteins in the cytoplasm, which will be unspecifically targeted, the net result is a noise fluorescence signal. In this context, studies of the lipidic

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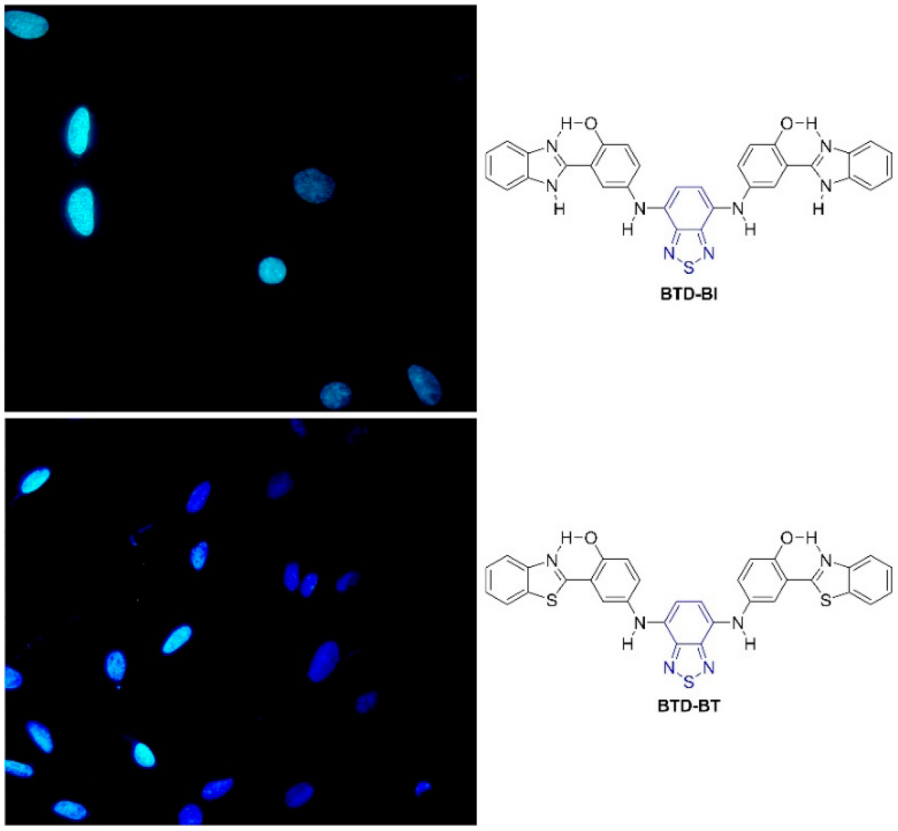

Figure 3. Selective nuclear staining of dsDNA with neutral fluorescent BTD derivatives (the blue emitters **BTD-BI** and **BTD-BT**) in live-cell-imaging assays using a confluent monolayer of human stem cells. Adapted from ref 24. Copyright 2010 Elsevier Ltd.

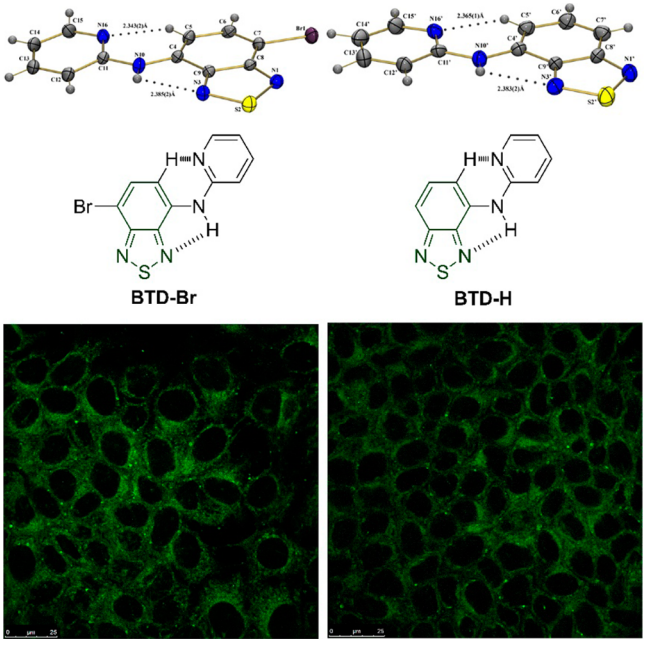

Figure 4. Selective mitochondrial staining using **BTD-H** and **BTD-Br** in MCF-7 cancer cells. As deduced from X-ray diffraction on single crystals, both molecules have flat structures, each with two intramolecular H-bonds. Adapted from ref 35. Copyright 2012 Royal Society of Chemistry.

incorporation and metabolism are compromised by the limitations of the available dyes.

To overcome these aforementioned drawbacks, a lipophilic fluorescent BTD derivative named **BTD-AO** has been developed by our group.[29] The lipophilic nature of **BTD-AO** and its structural similarity with oleic acid (the principle of the structural design of the new fluorophore) enabled a new approach for studying the uptake and dynamics of fatty acids (and derivatives) in live cells, especially at low temperatures (4 °C), at which all endocytic pathways (passive processes of membrane trafficking) are completely inhibited.[30] Figure 2 shows the images obtained using the BTD fluorophore at 4 and 37 °C after 5 min, which indicate the high selectivity of **BTD-AO** toward lipid droplets and the fast cellular membrane transposition. The internalization kinetics were monitored for 60 min, and no significant difference could be noted at these two temperatures. The study was able to point to a complete oleic acid cellular pathway and elucidate the role of active dynamic process of internalization once the endocytic pathway was inhibited. The probe proved to be more strongly associated for fatty acid binding protein (FABP) or albumin than with natural oleic acid (ca. 2−3 kcal mol^{-1}) because of additional H-bonding interactions. Five different cell lines (MDA-MB 237, MCF-7, Caco-2, HUVEC, and HeLa) showed similar results.

The use of the lipophilic BTD derivative proved to be better by far than any other commercially available or previously described fluorophore based on the classical scaffolds. **BTD-AO** also exhibited large Stokes shifts (100−140 nm) and large molar extinction coefficients (log ε = 3.02−3.48) and did not form micelles in solution.

2.2. Nuclear Selective Staining

Staining of the nuclei of live cells is one of the most important tasks for cellular biology studies. The majority of the available

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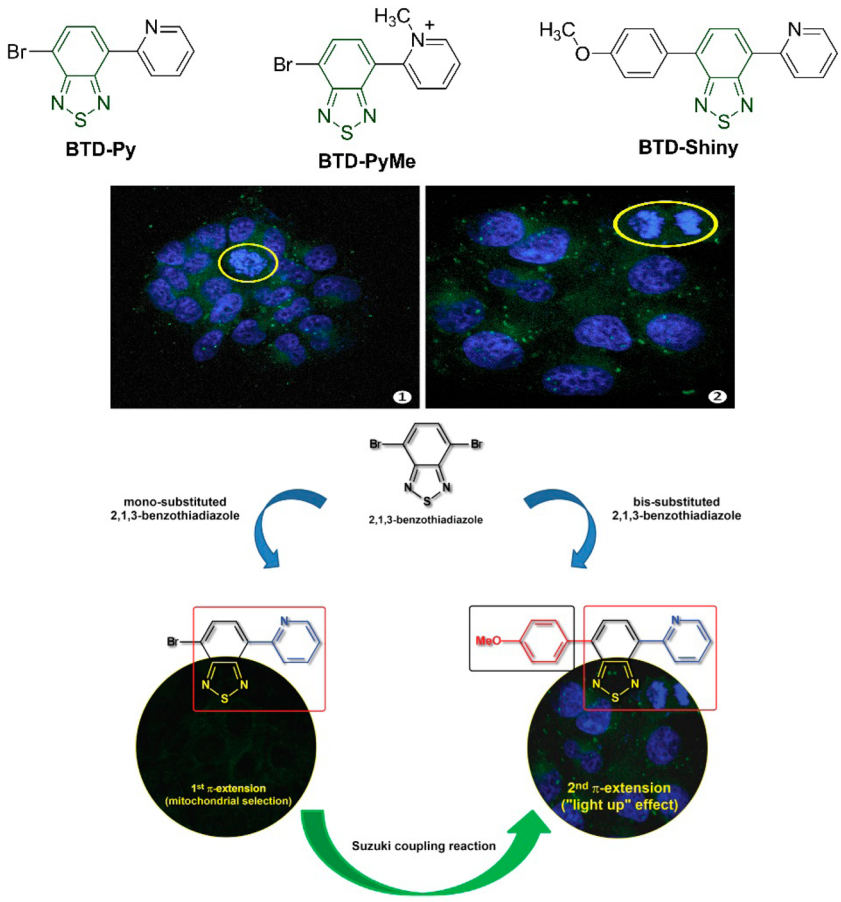

Figure 5. Selective mitochondrial staining and dynamics using **BTD-Shiny** in MCF-7 cancer cells and the molecular architecture of the small-molecule fluorophore. Nuclei were stained with DAPI (blue). The monosubstituted derivatives stained only mitochondria but exhibited weak fluorescence emission. Adapted from ref 36. Copyright 2012 Brazilian Chemical Society.

dyes produced to stain nuclei selectively have some kind of interaction mechanism with the DNA content, which may impair the normal cell function.[31] These dyes are typically cationic derivatives displaying common mechanisms of DNA interaction such as intercalation into the DNA strands, minor groove binding, major groove binding, or external strand binding, as we have reviewed elsewhere.[32] The representative net result of the interactions of these cationic dyes with DNA is the induction of DNA alterations and subsequent cell death. The molecular design of a selective nuclear probe must also take into account the fact that the molecule will have to cross at least two major natural barriers, namely, the plasmatic membrane and the nuclear pores. The nuclear membrane is much more difficult to cross because it is organized as a double membrane with an intermembrane space. This array is capable of retaining a large number of molecules that passively cross the external nuclear membrane. The nuclear envelope has several pores that act as selective spots to integrate both the nuclear and cytosol contents, allowing passive molecular traffic of structures with molecular weights down to 40 kDa.[33] The main success of DNA-staining molecules relies on the inclusion of a prior procedure of cell treatment with fixative agents or a direct DNA content analysis over polyacrylamide or agarose gels. The design of nuclear fluorescent markers must also consider specific spectroscopic and photophysical properties such as high molar absorptivity, large extinction coefficients at visible wavelengths, maximal light-up effect upon DNA binding, low affinity for RNA molecules, and no affinity to other biomolecules. Alternative techniques may also provide selective cell nucleus staining. Among these techniques, recombinant nuclear proteins with fluorescent tags or direct nuclear protein detection by antibodies are the most commonly used. The recombinant methodology is very time-consuming and requires a specialized staff and a medium-sized laboratory. Antibody detection has all of the problems associated with cell membrane permeabilization procedures, cross-reactions, and background emissions and is also time-consuming. Small fluorescent molecules are in general still preferred for nuclear DNA staining.

In this context, two new neutral (noncationic) fluorescent small-molecule BTD derivatives have been proposed as selective markers for nuclear dsDNA and returned high-quality images in live cell-imaging assays (Figure 3) using a confluent monolayer of human stem cells.[24] The new markers proved to be an excellent alternative to the commercially used blue emitters Hoechst 33342 and 4′,6-diamidino-2-phenylindole (DAPI) and are considered to be the first BTD small-molecules used as selective imaging probes for cellular experiments.

Both compounds proved to be capable of transposing both the cellular and nuclear membranes. Another advantage of these two BTD fluorophores was that they could be easily excited with visible light and could be used in either live or adhered cells. Their molecular architectures were planned to display two well-known sites capable of performing excited-state intramolecular proton transfers (ESIPTs) and two sites prone to ESIPT using

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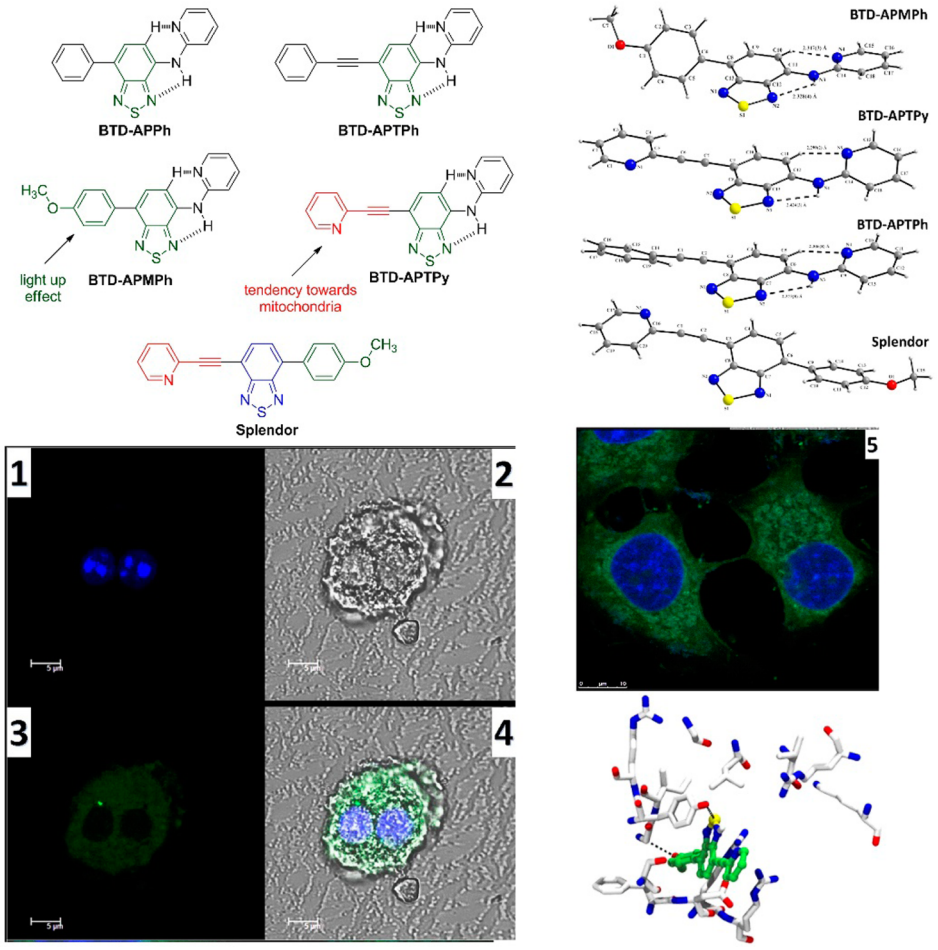

Figure 6. Staining experiments on live MCF-7 cancer cells during a cellular division cycle in the anaphase step (1−4) aimed at mitochondrial imaging and tracking and the late cytokinesis step (5). The nuclei were stained with commercially available DAPI (blue emission). The use of **Splendor** (green emission) enabled the clear observation of the mitochondrial distribution and dynamics between the two nuclei during the anaphase until the cytokinesis step. The mitochondrial distribution during the anaphase (3) is also very clear. **Splendor** binds to the cytosolic-open form of adenine nucleotide translocase (ANT), as shown at the bottom right. Adapted from ref 37. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

the nitrogens of the BTD core. The designed compounds displayed large Stokes shifts (103−127 nm), a typical ESIPT signature. We have recently shown, however, that ESIPT is not a preferred stabilizing process in BTD derivatives,[34] although it cannot be completely ruled out. The advantages of the synthesized BTDs included not only the high-quality images but also the fact that they could be stored at room temperature as pure solids for months without the need for freezing and that they selectively labeled only nuclear dsDNA, whereas some leakage may be noted for some commercially available dyes such as DAPI.

2.3. Staining of Mitochondria with BTD Fluorophores

Mitochondria are the most studied eukaryotic organelles because of their complexity and pivotal role in maintenance of cellular homeostasis. MitoTracker Red and rhodamine 123 are widely used for cellular experiments with selective mitochondrial staining. All of the commercially available and recently developed mitochondrial probes depend on the membrane potential of the organelle (ca. −180 mV). For instance, although rhodamine 123 is retained in active mitochondria, the dye is easily removed from the cell by washing procedures or after the mitochondria loses its membrane potential. This undesirable effect during standard staining protocols highly limits their use under experimental conditions in which the cellular models must be exposed to fixative agents. These probes are also not recommended for experimental conditions that affect the energetic state of the mitochondria because of variations in the membrane potential.

In contrast, fluorescent BTD derivatives could be used to selectively stain mitochondria in either live or fixed cells independent of the membrane potential of the mitochondria. Two previous works by our group had shown the potential of BTD dyes to stain mitochondria. The first was based on the use of monosubstituted derivatives[35] (Figure 4), whereas the other required π extensions on both sides of the BTD ring[36] (Figure 5).

A clear mitochondrial preference was noted for the bright green emitters **BTD-H** and **BTD-Br** in three distinctive cell lines (MCF-7, Caco-2, and normal fibroblasts). Both structures are almost completely flat and showed bright green emission (Figure 4). Their molecular architectures were planned to display two intramolecular H-bonds. The photophysical analyses of these derivatives indicated the possibility of ESIPT as the stabilizing process. Many features pointed toward ESIPT: (i) nearly perfectly planar ground-state and excited-state structures; (ii) large Stokes shifts; (iii) the charge transfer (CT) nature of the intramolecular electronic transitions; (iv) significant changes in the optical spectra with pH variations; and (v) support from the theoretical calculations.

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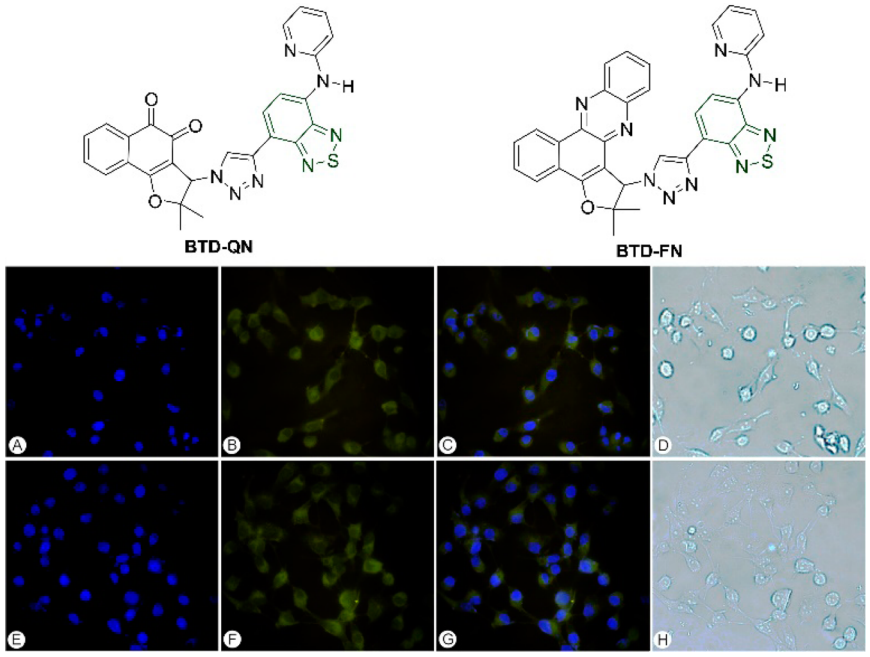

Figure 7. MDA-MB-231 staining with **BTD-FN** plus commercially available DAPI and phase-contrast images. (A) and (E) show nuclei stained with DAPI (blue). (B) and (F) show a fluorescence pattern associated with the perinuclear region (green) obtained with **BTD-FN**. (C) and (G) are the overlaps of the DAPI and **BTD-FN** staining patterns, and (D) and (H) show phase-contrast images of the MDA-MB-231 cells' normal morphology. Adapted from ref 40. Copyright 2014 Royal Society of Chemistry.

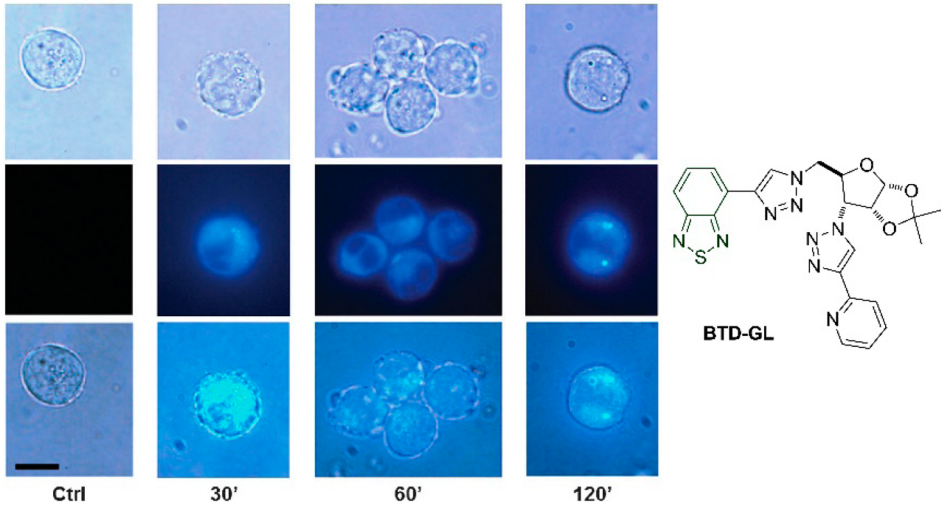

Figure 8. U937 cells after 30, 60, or 120 min of incubation in the absence (control) or presence of **BTD-GL** at 40 μM. The same cells were detected in optical phase-contrast (top row) and under fluorescence (middle row), and the images were merged (bottom row). The scale bar is 10 μm. Adapted from ref 42. Copyright 2013 Royal Society of Chemistry.

The monosubstituted structures shown in Figure 5 are not as planar as those with two intramolecular H-bonds (Figure 4) and showed no intense fluorescence emission inside the tested cell lines. Despite their weak fluorescence emissions, these monosubstituted BTDs stained only mitochondria. In this sense, it was expected that a second substitution would afford a light-up effect, whereas the first substitution would be responsible for the mitochondrial selection. **BTD-Shiny** indeed showed the predicted behavior, and only mitochondria were selectively stained (bright green emission) in the tested cell lines (Figure 5), allowing us to follow the mitochondrial dynamics during a cell division cycle. **BTD-Shiny** also displayed attractive photophysical data such as large Stokes shifts (102−159 nm), large molar extinction coefficients (log ε = 4.04−4.38), and a fluorescence quantum yield of 0.85.

A breakthrough in the use of fluorescent BTDs for mitochondrial staining has recently been described by us.[37] Five derivatives were synthesized and tested. Four of them did not show mitochondrial selection; of these four derivatives, one showed a tendency toward this organelle, while another displayed a significant light-up effect (see Figure 6). The knowledge gained by analyzing their molecular architectures allowed the rational design of the fifth derivative, named **Splendor**, which showed superior mitochondrial selection with outstanding results in bioimaging experiments in living cells (Figure 6).

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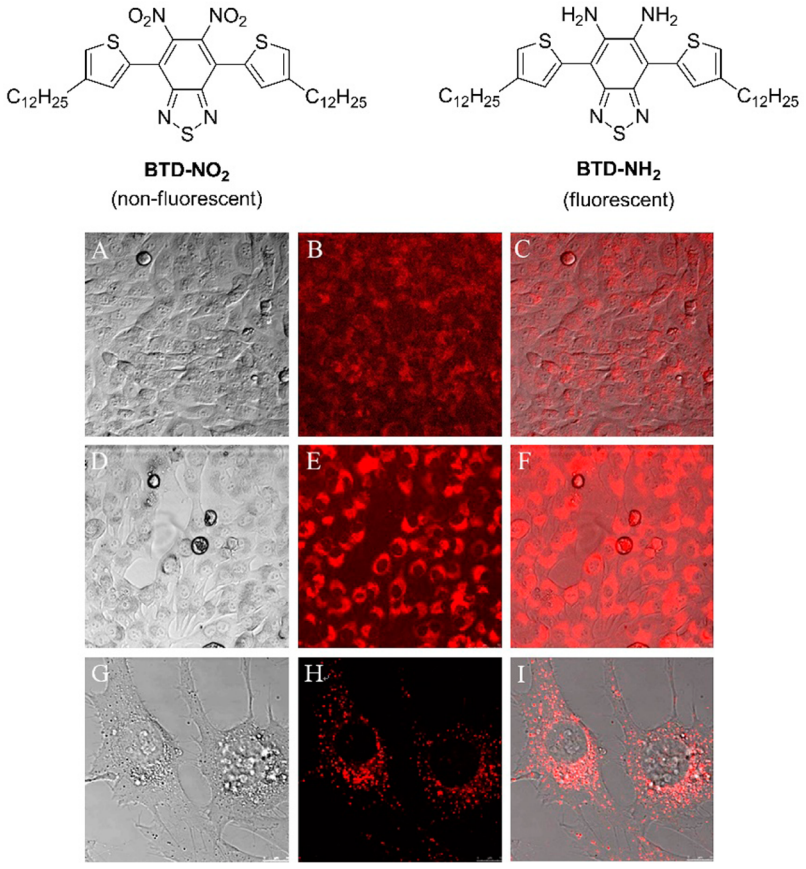

Figure 9. MG-63 cancer cells incubated with **BTD-NO_2** for 24 h under (A−-C) normal and (D−I) hypoxic conditions: (left column) normal morphology of the cells by phase-contrast microscopy; (middle column) fluorescence channel; (right column) merged images. Strong fluorescence was observed in the cytoplasm when the cells were cultured under hypoxic conditions (E and H) compared with those under normal conditions (B). Adapted from ref 43. Copyright 2013 Elsevier Ltd.

The most important derivative has been stored in solution at room temperature for 5 years with no loss in selectivity (subcellular localization) and no degradation. The results using a freshly synthesized derivative were similar to those for a sample that had been stored for a long time. In the study it was also determined that **Splendor** binds to the cytosolic-open form of adenine nucleotide translocase (ANT), analogous to the crystallographic inhibitor atractyloside,[38] whereas the other four derivatives did not show any expressive interaction with this important protein found in the mitochondrial machinery. These results helped to explain the high mitochondrial affinity of **Splendor** and the lack of selectivity of the other four derivatives.

All of the BTD-based mitochondrial markers generally followed the same principles expected for a mitochondrial directing group, that is, they encompassed good geometry, spacing, and rigidity. The lipophilic and electron-deficient character of the BTD core would also in principle help with transposition of the mitochondrial membrane and interaction.

2.4. Other Applications

BTDs may also be used as fluorescent tags to disclose the cellular dynamics and action mechanism of bioactive compounds. Although it is true that the incorporation of a fluorescent tag may change the biological response of a bioactive compound, this elegant strategy has been successfully used in several studies.[39] Until recently, BTDs had not been used as fluorescent tags for bioimaging studies of bioactive compounds. In this sense, we have incorporated a BTD core as the fluorescent tag in β-lapachone derivatives[40] to probe their cellular dynamics and migration inside live cells (Figure 7).

The bioimaging experiments revealed that the bioactive compounds were most distributed in the cytosol near in the cells' nuclei, therefore giving important insights into the subcellular location and the action mechanism of such substances. **BTD-FN** showed a fluorescence pattern associated with the perinuclear region plus a slight homogeneous stain through the cytosol. The results allowed us to understand the preferential site of action of the planned BTD, namely, in the perinuclear region, which is known to be mitochondria-rich.[41] These compounds proved to induce the formation of reactive oxygen species (ROS) and to affect the mitochondrial membrane potential, inducing apoptosis, as we demonstrated by complementary experiments.[40]

Artificial (giant unilamellar vesicles) and live-cell (U937 human cancer cell) systems for membrane interactions and trafficking of a glycoligand tagged with a BTD as the fluorophore have been described by Policar and co-workers (Figure 8).[42] Glicoligands are known to be sugar-based molecules that can complex metal cations. The studies revealed that the probe **BTD-GL** was most accumulated at the outer and inner membranes, therefore showing that it may be used in future experiments as a membrane probe. This designed probe was also able to passively cross the cell membrane, reaching the inner compartments.

An elegant study with a red BTD emitter generated inside the cells has been described by Jiang and co-workers.[43] In that study, the two nitro groups of a nonfluorescent BTD were reduced to

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amines by a nitroreductase under hypoxic conditions (Figure 9). The presence of the two nitro groups made the structure virtually nonfluorescent, and under hypoxic conditions, the presence of **BTD-NO_2** caused a 4-fold increase in the gene expression of nitroreductase in the tested cells after 24 h. In turn, the nitroreductase reduced the nitro groups inside the cells, affording the strong red emitter **BTD-NH_2**. The nonfluorescent **BTD-NO_2** could distinguish normal and cancer cells and was capable of selectively tracing the hypoxic tumor cells with strong red fluorescence.

3. SUMMARY AND OUTLOOK

Fluorescent BTDs have the potential to go beyond where classical scaffolds have gone already while pushing the limits of state-of-the-art molecular probes for bioimaging applications. The high chemical and photostability, intense fluorescence emission, ease of handling, excellent stability in solution for long periods of time without the need for freeze−thaw cycles, and bright images of high quality are also fostering the interest in BTD derivatives as bioprobes. To date, many important organelles and cell components have been successfully and selectively labeled with fluorescent BTDs, including nuclear DNA, mitochondria, lipid droplets, and membranes, among others. In general, most of the described fluorescent small-molecule BTDs proved to be capable of transposing the cell membrane with a few exceptions in micellar systems.[25]

A survey of the recent literature reveals that some new technological fluorescent systems, such as silica-based nanoparticles,[44] copolymers,[45] etc., are successfully utilizing BTDs as the fluorophore for bioimaging applications. To date, many achievements have been made by using fluorescent BTD derivatives as bioprobes, but much is yet to be done. Lysosomes, endosomes, the endoplasmic reticulum, and other components and organelles have not yet been selectively labeled using fluorescent small-molecule BTDs. The current status of BTD bioprobes is transitioning from the proof-of-principle stage to widespread adoption by the scientific community. We do hope that this Account will serve to inspire and motivate chemists and biologists around the world to develop new fluorescent BTD bioprobes, which no doubt will be part of the technology of the future for bioimaging experiments. The wide avenue for new findings using fluorescent BTDs is just waiting to be explored with the promising perspective that the works described herein have highlighted.

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Author Contributions

The manuscript was written through contributions from all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Pedro H. P. R. Carvalho received his degree in chemistry from UnB in 2012 and completed his M.Sc. at the same university in 2014. He has been working under the supervision of Prof. Brenno A. D. Neto since the beginning of his undergraduate program and is currently developing his Ph.D. project in the synthesis of new BTDs.

Jose R. Correa obtained his M.Sc. and Ph.D. degrees in cellular and molecular biology at Instituto Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil. After a period as a postdoctoral fellow, he became a professor of cellular biology at UnB (Biology Institute). His research interests are focused on cancer biology, photoluminescent selective cell markers, and antineoplastic agents.

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ABBREVIATIONS

BTD, 2,1,3-benzothiadiazole; ICT, intramolecular charge transfer; ESIPT, excited-state intramolecular proton transfer; CT, charge transfer; ROS, reactive oxygen species

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