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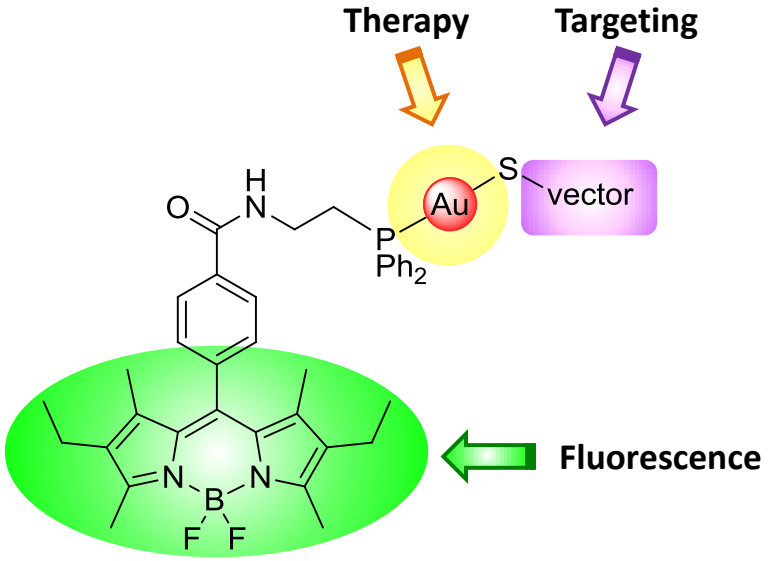

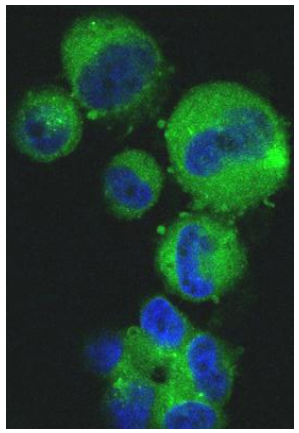

vector: thioglucose and bombesin derivatives

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Dalton Discussions

PREPRINT

Towards the elaboration of new gold-based optical theranostics

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Four new red BODIPY-gold(I) theranostic compounds were synthesized. Some of them were vectorized by tethering a biovector (glucose or bombesin derivatives) to the metallic center. Their photophysical properties were studied. Additionally, their cytotoxicity was examined on different cancer cell lines and on a normal cell line, they were tracked *in vitro* by fluorescence detection, and their uptake was evaluated by ICP-MS measurements.

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Introduction

The field of metallodrugs in medicinal inorganic chemistry, and especially in cancer chemotherapy is constantly being developed since the pioneer discovery of biological properties of cisplatin. Indeed, additionally to the cisplatin analogues, biological activities have been discovered and explored for numerous metals, in particular ruthenium and gold displayed promising results (NAMI-A, KP1019, auranofin…).[1]

However, recent advances in analytical techniques and more precisely in proteomic screening and gene expression revealed that the mechanism of action of these new metallodrugs can be completely different from that of cisplatin derivatives, and can undergo a wide range of biomolecular interactions beyond DNA.[1b] Most of the time their behaviour is difficult to elucidate, and this means that today, some of many metallodrugs available on the market are being used in patients without fully understanding their biological behaviour and their exact mechanism of action. Therefore, one challenge is to understand their mechanism of action in order to improve the efficiency of such drugs while limiting the side effects. One elegant way, which could circumvent these crucial issues is the attachment of a label to the metallodrug, which could be followed by molecular imaging, for example by radioisotopic imaging,[2] or optical imaging.[3] The resulting compounds that can be tracked *in vitro* and/or *in vivo* are considered as a kind of theranostic agents. Despite the incontestable advantages of this approach, the development of such theranostic agents is still underdeveloped, maybe because of the challenge in the synthesis of such systems.

Within the large variety of fluorescent dyes, BODIPY (borodipyrromethene) derivatives are highly suitable candidates for biological and medicinal applications. They exhibit rich photophysical properties, *i.e.* high fluorescence quantum yields, high thermal and photochemical stability, and a high degree of tuneable emission into the red-near infrared region from a simple variation of the molecular structure.[4] Recently, we investigated the potential of BODIPYs as optical imaging probes, for bimodal SPECT-PET/optical imaging.[5]
We also reported on the advantage of these BODIPYs in the field of medical imaging, by coupling them to transition metals (*i.e.* Ru^{II}, Os^{II}, and Au^{I} cations) *via* a phosphine ligand, which presents interesting anticancer properties.[3c] Among the different systems, which were studied, the BODIPY-phosphine-Au complex exhibits the most promising properties (Figure 1). We could observe that the Au atom had no influence on the absorption and emission of the dye. Moreover, the complexes showed a good stability in PBS (phosphate buffer saline, pH 7.4), as well as interesting cytotoxicities against A2780S and A2780cisR cell lines. However, this system was not optimized for its application *in vivo*, in particular it was not vectorized for tumor targeting (no significant active transport was observed).

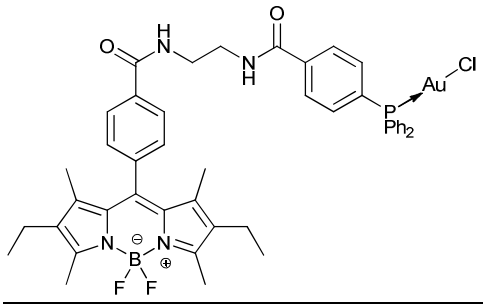

Figure 1: Structure of the BODI-Au-Cl theranostic complex previously studied.

In the present study, we decided to improve the gold(I) complex in order to obtain more suitable systems for biological applications. First, we changed the linker between the BODIPY fluorophore and the Au(I) complex. The phenyl moiety was removed, in order to increase the water solubility and simplify the synthesis. Secondly, we worked on the introduction of different biovectors: two sugar derivatives and one peptide (modified bombesin) in order to improve the water solubility of the compounds and for targeting them selectively on tumors, which overexpress sugar or/and bombesin receptors. The synthesis of the new compounds, their photophysical properties, and some biological studies will be presented.

Results and discussion

Synthesis

Compared to our previous study on metal-based BODIPY-phosphine derivatives,[3c,6] the synthesis of target **BODI-Au-1** was greatly improved: three steps less, synthetic intermediates easier to handle, and simpler purifications. Starting from BODIPY-acid

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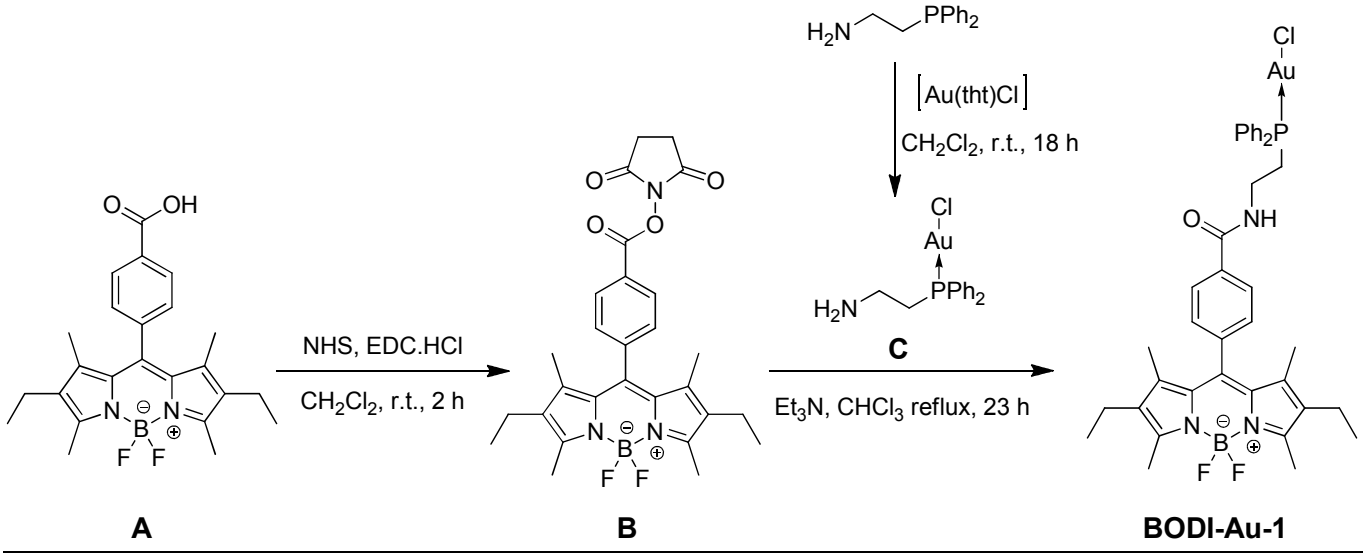
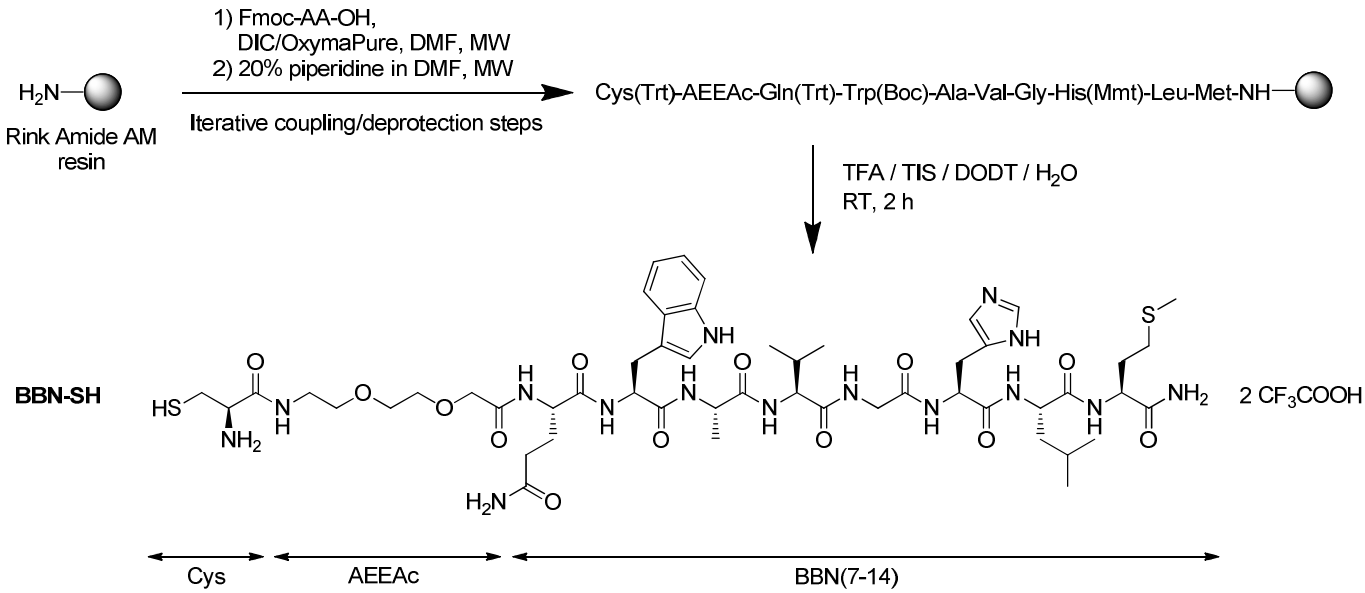
derivative **A**, the **BODI-Au-1** was obtained in two steps: activation of the acid moiety with *N*-hydroxysuccinimide (NHS), which yielded intermediate **B**, followed by the coupling reaction of **B** with the phosphine-gold complex **C** (Scheme 1).



Scheme 1: Synthesis of theranostic **BODI-Au-1**.

The introduction of the vectors (thioglucose analogues or modified bombesin) was then investigated. **BBN-SH** is a bombesin-analog agonist, which contains the minimal sequence required for high affinity binding to bombesin receptors (Scheme 2).[16] We decided to attach an hydrophilic spacer to this sequence to prevent the BODIPY-gold(I) moiety from decreasing the affinity of the octapeptide. A cysteine residue was finally added to enable the coupling reaction to the gold atom *via* the thiol group of the lateral chain. The **BBN-SH** peptide (Cys-AEEAc-bombesin$_{(7-14)}$) was obtained by automated solid phase peptide synthesis on a microwave synthesizer. The synthesis was carried out on a Rink Amide AM resin. All amino acids and the spacer 2-(2-(2-aminoethoxy)ethoxy))acetic acid (AEEAc) were N-α-terminal protected by a Fmoc group. The final peptide was cleaved from the resin with simultaneous removal of the side-chain protecting groups by treatment with TFA/TIS/DODT/water (92.5/2.5/2.5/2.5 v/v; 10 mL) at room temperature for 2 h. After treatment the crude peptide was purified by semi-preparative RP-HPLC and lyophilized to afford **BBN-SH** in 18% overall yield.



Scheme 2: Synthesis of the BBN-SH vector.

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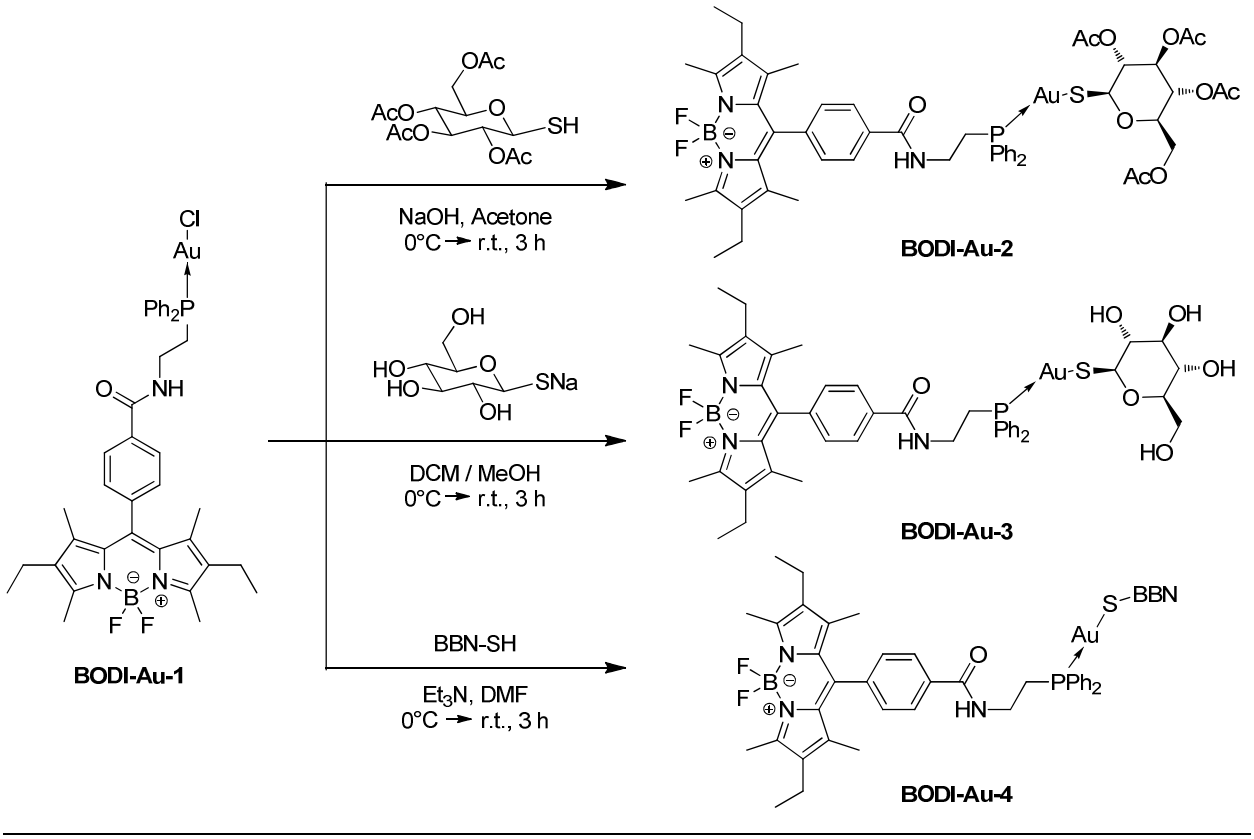
coupling reactions were easily performed by reacting **BODI-Au-1** with the corresponding thiolate derivatives (either commercially available or prepared *in situ*) (Scheme 3). It is worth noting that these reactions were performed in total conversion even for the modified bombesin.[7]



Scheme 3: Syntheses of theranostics **BODI-Au-2**, **BODI-Au-3**, and **BODI-Au-4**.

These reactions could be monitored by ^{31}P NMR. A significant shift of the resonance was observed in the thiolate complexes (singlets at 31.0 ppm for **BODI-Au-2**, at 30.2 ppm for **BODI-Au-3** one, and at 29.4 ppm for **BODI-Au-4** complexes) when compared with the starting **BODI-Au-1** complex (singlet at 25.7 ppm) (Figure 2). The shape of the signals - broad singlet - is characteristic of P-Au-S compounds.[8]

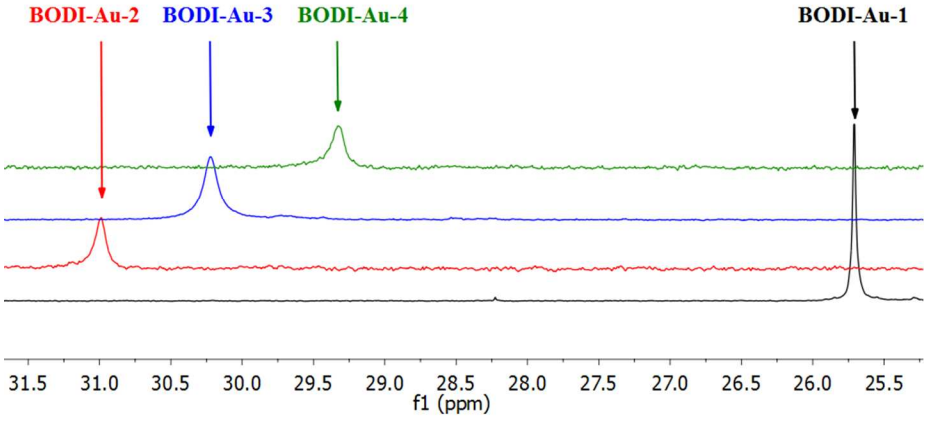


Figure 2: ^{31}P NMR spectra of theranostics **BODI-Au-1**, **BODI-Au-2**, **BODI-Au-3**, and **BODI-Au-4**.

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Photophysical studies

The photophysical studies of the different compounds were examined in DMSO, at room temperature. All data are collected in Table 1.

Table 1: Photophysical properties of selected BODIPY derivatives in DMSO at 298 K.

Compound	λ_{abs} (nm)	λ_{em} (nm)	ε ($M^{-1}.cm^{-1}$)	Φ_F[a] (%)	Brightness[b] ($M^{-1}.cm^{-1}$)
BODI-Au-1	525	543	33530	98	32850
BODI-Au-2	525	542	59650	97	57860
BODI-Au-3	525	542	46900	86	40330
BODI-Au-4	525	542	39300	21	8250

[a] Rhodamine 6G (Φ_F = 0.94 in ethanol, λ_{exc} = 488 nm) was used as a reference.[9] All Φ_f are corrected from refractive index. [b] Brightness = $\varepsilon^*\Phi_F$

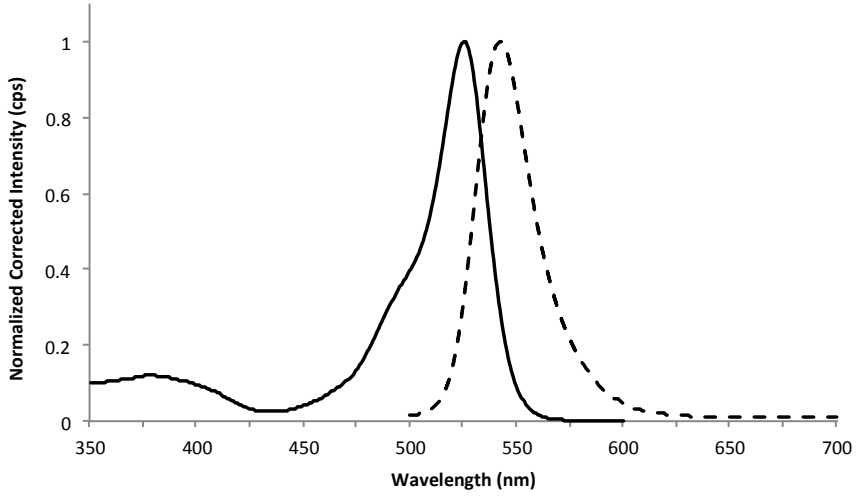

Figure 3: Absorption (solid line) and emission (dashed line) spectra of compound **BODI-Au-4**.

The three BODIPY based complexes **BODI-Au-1-3** present very high fluorescence quantum yields, as already described for similar compounds.[3c] Finally, **BODI-Au-4**, displays a fluorescence quantum yield of 21%, showing that the introduction of the bombesin derivative affects the intense luminescence properties of BODIPYs.[10] This quenching is most likely due to an Photoinduced Eletron Transfer between the Tryptophan and the BODIPY.[11] Nonetheless, the brightness remains sufficient for tracking the complex *in vitro*. Preliminary stability studies of the complexes have been performed by ^{31}P-NMR in a DMSO/ DMEM culture medium mixture. The spectra display no change in the phosphorus signal meaning that P-Au, Au-Cl, and Au-S bonds are stable under these conditions. Hence, a significant degradation of the complexes *in vitro* appears unlikely (see supporting information).

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Biological experiments

Bombesin affinity evaluation

The affinity of **BODI-Au-4** for bombesin receptors (BB1, BB2 and BB3 receptors) was evaluated on a competitive binding assay on rat cerebral cortex membranes.[12] Under these assay conditions, **BODI-Au-4** was able to displace the radioligand [^{125}I]-[Tyr^4]bombesin(1-14) (K_D = 0.71 nM) with an IC_{50} of displacement of 1.53 nM (Figure 4). A 5-fold loss of inhibitory activity was observed compared to the reference compound bombesin(1-14) (0.28 nM). This value remains in the low nanomolar range and compares favourably with the ones observed for several bombesin derivatives used successfully in imaging studies.[13]

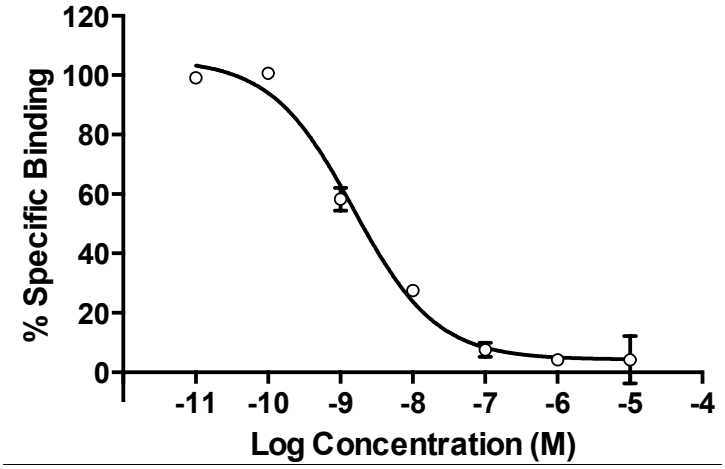

Figure 4. Displacement of radioligand [^{125}I]-[Tyr^4]bombesin(1-14) binding on rat cerebral cortex membranes by **BODI-Au-4**. Non-specific binding was defined by 1 µM cold bombesin (1-14).

IC_{50} determination on cancer cells

The antiproliferative activity of these novel BODIPY-phosphine-Au complexes was tested on two different cell lines known to overexpress the Glucose Transporter 1 (Glut1) and the bombesin receptor (GRPR).[14,15] Because the sensitivity of cancer cells to a treatment often depends on the type of cancer, we chose to use PC3 and MDA-MB-231 (MDA) cell lines as a model for human prostatic and breast cancers, respectively. We used auranofin as a benchmark gold complex.

Table 2: Determination of the IC_{50} values (µM) of BODIPY complexes on PC3 cells, MDA-MB-231 cells (MDA), and hMEC cells by MTS assays at 48h.

Compound	PC3	MDA	HMEC	$IC_{50(MDA)}/IC_{50(HMEC)}$
BODI-Au-1	74.9 ± 25.4	84.6 ± 11.3	25.1 ± 0.4	3.4
BODI-Au-2	39.7 ± 15.9	23.1 ± 13.4	9.8 ± 0.2	2.4
BODI-Au-3	29.4 ± 10.2	29.5 ± 6.3	24.5 ± 0.2	1.2
BODI-Au-4	115.9 ± 30.8	58.0 ± 20.5	10.9 ± 0.3	5.3
C	27.3 ± 5.7	25.5 ± 11.0	n.d.	n.d.
auranofin	11.2 ± 5.3	4.8 ± 1.2	< 4	> 1.2

The two thioglucose analogues-containing BODIPYs (**BODI-Au-2** and **BODI-Au-3**) and **C** displayed substantial toxicicity on both cell lines with similar IC_{50} values (around 30 µM), but were less efficient than auranofin (between 11 and 5 µM in PC3 and MDA cells, respectively) (Table 2).

BODI-Au-1, the BODIPY complex of reference that is not bound to a vector, turned out to be less potent, *i.e.* with IC_{50} values around 80 µM with both cell lines. The significant difference of cytotoxicity between **BODI-Au-1** and **C** highlights the non-innocence of the BODIPY moiety. This result proves the significance to initiate the study with an already labelled molecule rather than optimizing a drug and later tethering a label moiety. It also shows that a vector is needed to get higher cytotoxicities.

BODI-Au-4 also displayed different toxic effects between the two models, with IC_{50} values being 116 µM and 58 µM in PC3 and MDA cells, respectively.

IC_{50} have also been determined on HMEC (primary Human Mammary Epithelial Cells), which are normal cells and that can be compared to MDA (human mammary epithelial adenocarcinoma cells). It is worth noting that HMEC are really sensitive cells, *e.g.*

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a solution containing 1% of DMSO killed 50 % of HMEC cells, whereas a solution of containing up to 2% of DMSO induced less than 10% toxicity of MDA cells (see supporting information for details). So it is not surprising to get lower IC_{50} values for this cell line. The interesting point is the comparison of the IC_{50}(MDA)/IC_{50}(HMEC) ratio: it varies from 1 to 5 depending on the tested compounds. Such a comparison enables to address the point of using some vectors rather than others. Here the non-vectorized complex **BODI-Au-1** displays a ratio of more than **BODI-Au-3**. Clearly, the glucose derivatives improved this ratio (the complex **BODI-Au-3** is as efficient on cancer cells as on normal ones), whereas the bombesin derivative gives a bad ratio. Note that the ratio seems to be really bad with auranofin too, but the IC50 were too low to be reliable under these experimental conditions. Although these comparative cytotoxicity studies on cells are informative, only *in vivo* studies will enable us to evaluate the selectivity of the compounds.

In vitro confocal fluorescence microscopy

Fluorescence microscopy experiments were performed on both cell lines in order to determine the localization of the different compounds inside the cells. Figure 5 shows a series of pictures obtained with PC3 cells (results concerning MDA cells are presented in the supporting information). The fluorescence images prove that all compounds entered the cells. The colocalization with DAPI indicates that the BODI-Au systems are present in the cytoplasm of the cells, and not in the nucleus.
It is worth noting that all the compounds can be tracked *in vitro* by fluorescence microscopy at a concentration range used for the IC50 determination. Interestingly, **BODI-Au-3** and **4** were found brighter than **BODI-Au-1** and **BODI-Au-2** in both cell lines.

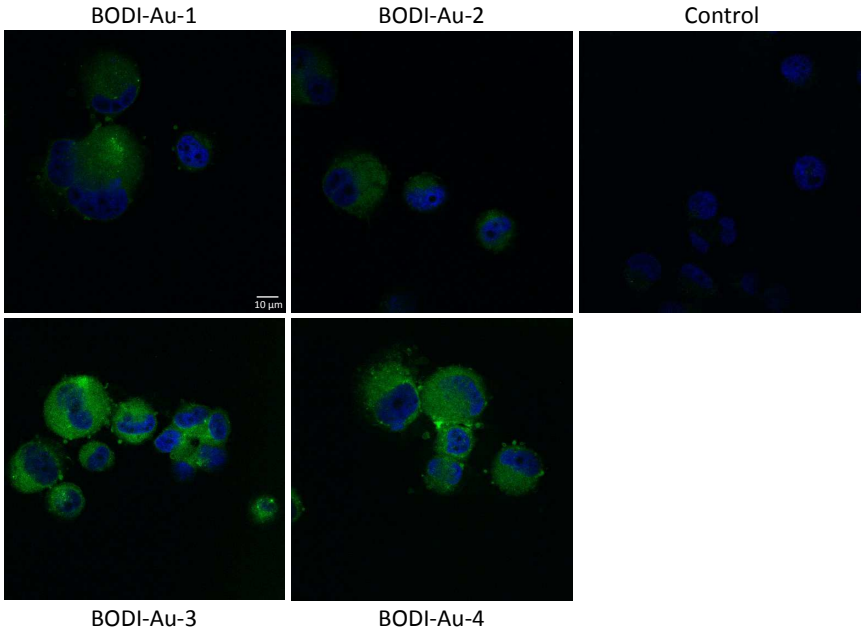

Figure 5. Confocal microscopy experiments of **BODI-Au-1**, **BODI-Au-2**, **BODI-Au-3**, and **BODI-Au-4** in PC3 cell line treated with 50 µM of metal complexes for 1 h at 37 °C (exc. 405 nm, detection 503-568 nm range for BODIPY complexes and 408-488 nm for DAPI).

ICP-MS uptake measurements of the BODIPY compounds in living cells

In order to evaluate whether the cell uptake of the compounds might affect their cytotoxic properties, cell extracts from MDA and PC3 cells (treated with 50 µM of the four BODIPY-complexes and auranofin for 1 h at 37 °C) were analyzed by ICP-MS. The results, expressed as pmol metal/mg protein, are reported in Table 3.

Table 3: Cell uptake of **BODI-Au-1**, **BODI-Au-2**, **BODI-Au-3**, **BODI-Au-4**, and auranofin in MDA and PC3 cells treated with 50 µM of metal complexes for 1 h at 37 °C analyzed by ICP-MS (in pmole/mg of protein).

Compound	MDA	PC3
BODI-Au-1	1277 ± 150	1251 ± 95
BODI-Au-2	5828 ± 170	5467 ± 240
BODI-Au-3	1702 ± 30	2139 ± 160
BODI-Au-4	1529 ± 120	1086 ± 45
auranofin	24752 ± 3850	30565 ± 3750

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First, whichever compound used, the amount of gold measured in either cell line was quite comparable . Additionally, there seems to be a global correlation between the IC50 values and the gold uptake in the cells. However, such an observation was taken with caution because of the different incubation times used for ICP-MS (1h) and IC50 measurements (48h) Indeed, the pharmacokinetics of the different compounds must be very dependent upon the internalization mechanism. This consideration could explain why **BODI-Au-2** and **BODI-Au-3** display comparable IC50 values, despite a very different gold uptake measured after 1h incubation.
Finally, the comparison of the internalization of auranofin and **BODIPY-Au-3** (two thioglucose tetraacetate containing species) highlights the non-innocence of the phosphine ligand with respect to their cell uptake.

In vitro fluorescence experiment

The detection of BODIPY compounds in living cells was performed by fluorescence measurements. MDA-MB-231 cells (MDA) and PC3 cells were incubated for one hour with these fluorophores at 4°C or at 37°C and rinsed twice with culture medium before fluorescence measurement. The intensity of fluorescence of the BODIPY complexes in the cells is a function of the type of cell line, incubation temperature, and type of BODIPY used (Figure 6). Furthermore, the fluorescence was measured at various times (up to 48 h) (data not shown). The intensity of fluorescence remained the same, which strongly suggests that the fluorophores reamined stable in the cell.

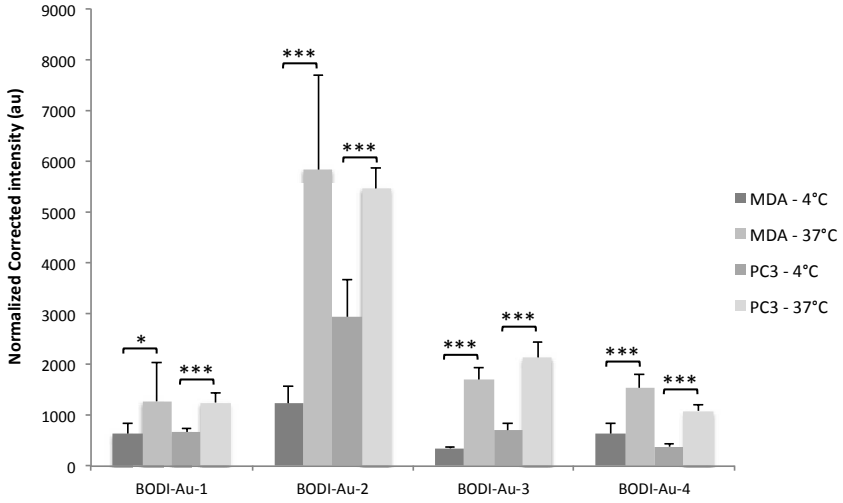

Figure 6: Uptake of BODIPY complexes in living cells evaluated by intensity of fluorescence. Fluorescence levels in MDA-MB-231 (MDA) and PC3 cells after 1 h incubation at 4°C or 37°C with **BODI-Au-1**, **-2**, **-3** and **-4** was detected on a fluorimeter Victor3 (excitation 485nm, emission 535 nm) and results were given after substraction for each compounds of basal fluorescence in the medium and correction by a response factor given by ICP-MS measurements. Significant difference in Mann-Whitney U test, *p<0.05, ***p<0.005.

Interestingly, the intensity of fluorescence measured at 4 and 37°C for **BODI-Au-1** complex is comparable with that measured upon incubation with MDA and PC3 cell lines. However, the fluorescence with all other BODIPY complexes, was found to be much lower upon incubation at 4°C than that upon incubation at 37°C. It may suggest that the cellular uptake of these molecules involved an active transport, at least partially. This is consistent with or without the presence of a vector on the compounds.

Conclusions

In summary, a series of promising new BODIPY-Au theranostic complexes has been developed: they wereconjugated with two types of biovectors (i.e. glucose/bombesin derivative). These complexes were found to have satisfactory photophysical properties, and good solubilities in biological media, which enabled us to track their *in vitro* fluorescence. Biological studies have highlighted the fact that the biovector plays an important role regarding the internalization of the resulting systems into cancer and normal cells. . Additionally, the affinity of the bombesin-theranostic conjugates for the bombesin receptors appeared to be in the same range than that of several bombesin derivatives successfully used in previous imaging studies. This may be an important point for future *in vivo* investigations. Moreover, all compounds were found to be cytotoxic. The cell uptakes determined by ICP-MS are in good agreement with IC_{50} values suggesting that one of the key points is to ensure that gold enters the cells. It was shown that fluorescence experiments are a good complement to ICP-MS measurements. They may be used to evaluate the extent of active transport involved in the internalization of a specific therapeutic agent. The biological study presented here, validates the concept

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of tethering a vector to a fluorophore *via* its metallic centre and its potential advantages: efficiency and easiness of the synthesis of the desired complexes, conservation of a significant cytotoxicity, satisfactory fluorescence intensity *in vitro*, and conservation of the primary function of the vector as to achieve the uptake of the complexes inside the cells. The former point implies a possible selective targeting between cancer *vs* healthy tissues, which do not express the same level of bombesin receptors.
With such promising, gold-based bioconjugated theranostic, future *in vivo* studies may be envisioned especially to evaluate the selectivity of the complexes *in vivo*. However, the emission wavelength of these fluorophores necessarily needs to be bathochromically shifted towards the therapeutic window, which will be achieved upon proper structural modification of the BODIPY core (i.e. upon extending the conjugation).

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Experimental

General information

Instrumentation. The analyses were performed at the "Plateforme d'Analyses Chimiques et de Synthèse Moléculaire de l'Université de Bourgogne". The identity and purity (≥95%) of the complexes were unambiguously established using high-resolution mass spectrometry and multinuclear NMR spectroscopy. The exact mass of the complexes was obtained on a Thermo LTQ Orbitrap XL ESI-MS. ^{1}H (300.13, 500.13, or 600.23 MHz), ^{13}C (125.77 MHz), ^{19}F (470.55 MHz) and ^{31}P (121.5, 202.45, or 242.9 MHz) NMR spectra were recorded on Bruker 300 Avance III, Bruker 500 Avance III, or Bruker 600 Avance II spectrometers. Chemical shifts are quoted in parts per million (δ) relative to tetramethylsilane, TMS (^{1}H and ^{13}C), using the residual protonated solvent (^{1}H) or the deuterated solvent (^{13}C) as an internal standard. Alternatively, 85% H_3PO_4 (^{31}P) and $CFCl_3$ were used as external standards. The coupling constants are reported in Hertz. Infrared spectra were recorded on a Bruker Vertex 70v FT-IR spectrophotometer (transmission mode). The elemental analyses were performed using a Thermo Electron Flash EA 1112 Series CHNS/O elemental analyser instrument.

Materials. All solvents used were analytical grade and when specified taken from a Mbraun solvent purification system or dried and distilled under Ar atmosphere. BODIPY–acid **A** derivative was synthesized as reported by Tomasulo *et al.*[17] The gold complex **C** was synthesized as reported by Lacôte *et al.*[18] All other reagents were commercially available and used as received.

Synthesis

Compound B. Bodipy acid **A** (500 mg, 1.18 mmol, 1.0 eq.), EDC.HCl (452 mg, 2.36 mmol, 2.0 eq.), *N*-hydroxysuccinimide (271 mg, 2.36 mmol, 2.0 eq.) were dissolved in dichloromethane (75 mL). The mixture was stirred at room temperature for 2 h. The reaction was monitored by thin layer chromatography (silica gel, eluent: ethyl acetate - heptane (1:1)). The solution was washed three times with distilled water (3 × 100 mL), dried over magnesium sulfate and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (eluent: ethyl acetate–heptane (gradient 3:7 to 1:1)) to afford the Bodipy-ester activated **B** as a red powder (70%).

Compound C. The reaction was carried out in a glove box. [Au(tht)Cl] (tht = tetrahydrothiophene) (279.7 mg, 0.872 mmol, 1.0 eq.) was dissoveld in dry dichloromethane. 2-(diphenylphosphino)ethanamine (200.0 mg, 0.872 mmol, 1.0 eq.) was added and the mixture was stirred overnight at room temperature in the glove box. The reaction was monitored by ^{31}P NMR. The solvent was removed under reduced pressure. The crude product was washed three times with pentane and dried under reduced pressure. The complex **C** was isolated as a white powder (87%).

BODI-Au-1. The reaction was carried out under an Ar atmosphere. Bodipy-ester activated **B** (250.0 mg, 0.480 mmol, 1.0 eq.), complex **C** (265.6 mg, 0.575 mmol, 1.2 eq.) and distilled triethylamine (135 µL, 0.969 mmol, 2.0 eq.) were dissolved in degassed chloroform (24 mL). The mixture was refluxed for 23 h. The reaction was monitored by ^{31}P NMR. The solvent was removed under reduced pressure. The crude product was dissolved in degassed dichloromethane and washed three times with degassed HCl 0.1 M

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and distilled water, dried over magnesium sulfate and the solvent was removed under reduced pressure. The product was washed three times with degassed pentane and dried under reduced pressure. **BODI-Au-1** was isolated as a red powder (77%).

BODI-Au-2. The reaction was carried out under an Ar atmosphere. In a schlenk tube, 1-thio-ß-D-glucose tetraacetate (42.0 mg, 0.115 mmol, 1.0 eq.) was dissolved in distilled acetone (3 mL) and degassed NaOH 1.0 M (115 µL, 0.115 mmol, 1.0 eq.) was added. The mixture was stirred 15 min at room temperature in the dark. In another schlenk tube, **BODI-Au-1** (100.0 mg, 0.115, 1.0 eq.) was dissolved in distilled acetone (3 mL) and stirred 5 min at 0°C. The first solution was slowly added to the other one at 0°C, and the resulting reaction mixture was stirred at room temperature for 3 h in the dark. The reaction was monitored by ^{31}P NMR. The solvent was removed under reduced pressure. The crude product was dissolved in degassed dichloromethane and filtrated to remove salts, washed three times with degassed pentane and dried under reduced pressure. **BODI-Au-2** was obtained as a red powder (82%).

BODI-Au-3. The reaction was carried out under an Ar atmosphere. In a schlenk tube, **BODI-Au-1** (40.0 mg, 0.046 mmol, 1.0 eq.) was dissolved in degassed dichloromethane (2 mL) and stirred 10 min at 0°C. In another schlenk tube, 1-thio-*β*-D-glucose sodium salt (10.1 mg, 0.046 mmol, 1.0 eq.) was dissolved in degassed methanol (2 mL) and stirred 5 min at room temperature in the dark. This solution was slowly added to the first one at 0°C, and the resulting reaction mixture was stirred at room temperature for 3 h in the dark. The reaction was monitored by ^{31}P NMR. The solvent was removed under reduced pressure. The crude product was dissolved in degassed dichloromethane and filtrated, washed three times with degassed pentane and dried under reduced pressure. **BODI-Au-3** was isolated as a red powder (74%).

BBN-SH. The peptide **BBN-SH** (Cys-AEEAc-bombesin$_{(7\text{-}14)}$) was obtained by automated solid phase peptide synthesis on a Liberty Blue microwave synthesizer (CEM). The synthesis was carried out on a 0.25 mmol scale on a Rink Amide AM resin (Iris Biotech, 0.48 mmol/g) using predefined CEM High Swelling cycles. All amino acids and the spacer 2-(2-(2-aminoethoxy)ethoxy))acetic acid (AEEAc), from Iris Biotech, were N-α-terminal protected by Fmoc. The final peptide was cleaved from the resin with simultaneous removal of the side-chain protecting groups by treatment with TFA/TIS/DODT/water (92.5/2.5/2.5/2.5 v/v; 10 mL) at room temperature for 2 h. The filtrate from the cleavage mixture was concentrated, precipitated in cold Et_2O, collected by centrifugation (twice), and lyophilized to afford crude peptide.
The crude peptide was purified by semi-preparative RP-HPLC on a Dionex Ultimate 3000 system (Thermo Scientific) equipped with a BetaBasic-18 column (ThermoFisher, 150 × 30 mm, 5 µm) with a gradient program from 20% to 35% of solvent B over 30 min (solvent A is water with 0.1% TFA and solvent B is acetonitrile with 0.1% TFA) at a flow rate of 15 mL/min with UV detection at 214 and 280 nm. Fractions were analysed by RP-HPLC on a Dionex Ultimate 3000 system, with a photodiode array detector, on a Kinetex C18 column (Phenomenex, 50 × 2.1 mm, 2.6 µm), at a flow rate of 0.5 mL/min with a gradient program from 5% to 100% of solvent B over 5 min. The pure fractions were collected and lyophilized to give **BBN-SH** as a white solid in 18% yield (TFA salt, 60 mg, purity at 214 nm > 98.5%, t_R = 3.07 min).

BODI-Au-4. The reaction was carried out under an Ar atmosphere. In a schlenk tube, **BBN-SH** (13.3 mg, 9.4 µmol, 1.0 eq.) was dissolved in distilled dimethylformamide (1 mL) and distilled triethylamine (7 µL, 50.2 µmol, 5.3 eq.) was added. The mixture was stirred for 20 min at room temperature in the dark. In another schlenk tube, **BODI-Au-1** (8.2 mg, 9.4 µmol, 1.0 eq.) was dissolved in distilled dimethylformamide (1 mL) and stirred 5 min at 0°C. The first solution was slowly added to the next one at 0°C, and the resulting reaction mixture was stirred at room temperature for 3 h in the dark. The solvent was removed under reduced pressure. The crude product was washed four times with dry tetrahydrofuran to remove traces of dimethylformamide and dried under reduced pressure. **BODI-Au-4** was obtained as a red powder (95%).

Photophysical studies. UV-Visible absorption spectra were recorded on a JASCO V630BIO spectrometer. Solutions of compound were prepared in DMSO to a final concentration of 10^{-5} M. The steady-state fluorescence emission and excitation spectra were obtained by using a JASCO FP8560 spectrofluorometer instrument. All fluorescence spectra were corrected for instrument response. The fluorescence quantum yield (Φ_F) was calculated from equation:

$$\frac{\Phi_F}{\Phi_{FR}} = \frac{n^2}{n_R^2} \times \frac{\int_0^\infty I_F(\lambda_E, \lambda_F) d\lambda_F}{\int_0^\infty I_{FR}(\lambda_E, \lambda_F) d\lambda_F} \times \frac{1-10^{-A_R(\lambda_E)}}{1-10^{-A(\lambda_E)}}$$

Φ_F and Φ_{FR} are fluorescence quantum yields of the compound and the reference respectively. $A(\lambda_E)$ and $A_R(\lambda_E)$ are the absorbance at the excitation wavelength, and n is the refractive index of the medium. I_F and I_{FR} are fluorescent intensities of the compound and the reference respectively. The reference system used was rhodamine 6G (Φ_F = 0.94 in ethanol, λ_{ex} = 488 nm)[10] for **BODI-Au-1** to **BODI-Au-4** and cyanine 5.29 (Φ_F = 0.30 in ethanol, λ_{ex} = 610 nm)[11]. The data are shown in Table 1.

Biological experiments

Bombesin receptors binding assay. The affinity of **BODI-Au-1** for bombesin receptors was evaluated by Cerep (Poitiers, France) on a radioactive binding assay. Briefly, rat cerebral cortex membranes, expressing GPR-R, NMB-R and BRS-3 receptor subtypes, were incubated in the presence of 0.01 nM [^{125}I]-[Tyr^4]bombesin ligand (K_D = 0.71 nM) and a range of concentrations of tested compounds for 60 min at room temperature. Non-specific binding was determined in the presence of an excess (1 µM) of

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"cold" bombesin(1-14). The results are expressed as a percent of control specific binding. The IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) were determined by non-linear regression analysis of the competition curves generated with duplicate values using GraphPad Prism version 6.00 for Mac. The corresponding inhibition constants (K_i) were calculated using the Cheng-Prusoff equation. Bombesin(1-14) was used as a reference compound (K_i = 0.28 nM). Further details of the methodology for the assay can be found at http://www.cerep.fr.

IC_{50} determinations on cancer cells. Human MDA-MB-231 breast cancer cells and PC3 protate cancer cells were obtained from the ATCC. These cells were maintained at 37°C in DMEM with 4.5 g/L glucose supplemented with 10% FBS and antibiotics (Dutscher, Brumath, France). DMSO at comparable concentrations did not show any effects on MDA and PC3 cell cytotoxicity. Human Mammary Epithelial Cells (HMEC) were purchased from Invitrogen (Cergy Pontoise, France) and grown into HUMEC ready medium (Invitrogen). IC_{50} on HMEC were corrected from the toxicity of DMSO.
Cells were grown at a density of $1x10^4$ cells per well in 96-well cell culture plates (Dutscher) the day before treatment with different concentrations of BODIPY complexes, compound C and Auranofin, during 48 h. Thereafter, 10 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega, Charbonnieres, France) was added in 200 µL of medium and absorbance at 490 nm was measured after 3h incubation at 37°C.

***In vitro* confocal fluorescence microscopy.** MDA and PC3 cells ($2x10^5$ cells per well) were incubated at 37°C one hour with 50 µM **BODI-Au-1**, **-2**, **-3** or **-4**. Thereafter, cells were rinsed two times with Phosphate buffer saline (PBS) before paraformaldehyde fixation and visualization by confocal microscopy (Nikon Eclipse TE 2000). The transmitted light images and confocal epifluorescence images are acquired simultaneously using the same illumination beam. Excitation was provided by a 405 nm laser diode. Fluorescence emission was collected in the 503-568 nm range (BODIPY fluorescence) or 408-488 nm range (DAPI fluorescence) using the Nikon spectral imaging system. Image analysis was performed with Image J software.

Uptake of gold in cells by ICP-MS. PC3 cells ($5x10^5$ cells per well) were incubated at 37°C one hour with 50 µM **BODI-Au-1**, **-2**, **-3**, **-4** or auranofin. Thereafter, cells were rinsed two times with Phosphate buffer saline (PBS) and lysed in water before protein concentration determination (Bio-Rad DC protein assay kit, BioRad, Marnes la Coquette, France). All samples were digested in ICP-MS grade concentrated hydrochloric acid (OPTIMA Grade, Fisher Chemical) for 3 h at room temperature and filled to a total volume of 10 mL with ultrapure water. Indium was added as an internal standard at a concentration of 1 ppb. Determinations of total metal contents were achieved using a Thermo Element 2 ICP-MS. The instrument was tuned daily using a solution provided by the manufacturer containing 1 ppb each of Ba, B, Co, Fe, Ga, In, K, Li, Lu, Na, Rh, Sc, Tl, U, Y (Tune-Up Solution ELEMENT, Thermo Fisher Scientific, Bremen, Germany). External standard was prepared gravimetrically in an identical matrix to the samples (with regard to internal standard and hydrochloric acid) with gold solution single element standard at 1000 ppm (ASSURANCE, SPEX CertiPrep).

Fluorescence experiment to track the BODIPY-complexes in living cells. The detection of the BODIPY compounds in living cells was performed by fluorescence measurements. MDA and PC3 cells ($1x10^5$ cells per well) were incubated, at 4°C or 37°C for one hour with either 25 µM **BODI-Au-2** or 50 µM **BODI-Au-1**, **-3** or **-4** in 96-well black plates (VWR, Strasbourg, France). Thereafter, cells were rinsed two times with medium and left in a final volume of 100 µL. Fluorescence levels were measured on a Victor3 (Perkin Elmer, Waltham, MA, USA) at 485 nm excitation wavelength and 535 nm emission wavelength. The normalized corrected intensity (N.C. Intensity) was obtained by subtraction for each compound of basal fluorescence in the medium and division by a response factor given by ICP-MS measurements. This factor correspond to the ratio of the intensity of fluorescence of the studied compound on the cell line considered at 37°C on the value of the uptake of this compound measured by ICP-MS (equation 1).

$$N.C.\ intensity_{(BODI-Au-x)} = \frac{Corr.intensity_{(BODI-Au-x)}}{Corr.intensity_{(BODI-Au-x\ in\ PC3\ at\ 37°C)} / Uptake_{(BODI-Au-x\ in\ PC3\ at\ 37°C)}}$$

Equation 1: Determination of normalized corrected intensity (x = 1, 2, 3, or 4 and Corr. intensity = the intensity of fluorescence measured – basal fluorescence).

Notes and references

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