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# Synthesis and anticancer activity of gold porphyrin linked to malonate diamine platinum complexes

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## Synopsis

Novel anticancer agents containing a gold porphyrin appended to a platinum complex were synthesized and characterized. Interestingly, the heterobimetallic conjugates showed enhanced cytotoxic activity due to cooperative effects from the two metals in addition to selectivity to cancer cells.

## Abstract

Recently, Gold(III) porphyrins have gained great interest as anticancer drugs not only for stability of gold(III), but also for the functionalization of porphyrin to allow bridging with another metal such as platinum(II). We report here, for the first time, the synthesis of three new bi-metal compounds containing gold(III) porphyrin conjugated to a platinum diamine moiety through malonate bridging to obtain enhanced cytotoxicity from both metals combined to the phototoxicity of the porphyrin. The three complexes differ by type of diamine ligands around platinum(II) which are ammonia (NH<sub>3</sub>), cyclohexane diamine (CyDA) and pyridine methyl amine (Py). The synthesis was carried out using complexation of activated malonic acid derivatives with aqua diamino-platinum(II) complexes and the products were characterized by IR, NMR, mass spectra and by elementary analysis. Cytotoxic activity of the conjugates was screened in both healthy and cancer cell lines, human fibroblast cells (FS-68) and human breast cancer cells (MCF-7), and was compared to the corresponding platinum(II) complexes. The cyclohexyldiamine (CyDA) derivative exhibited the most cytotoxic effect among the series. The results showed that gold(III)/Pt(II)conjugates are more potent by 2 to 5.6-fold than the corresponding platinum complexes. Moreover, the dyad AuP-PtCyDA is 18% more potent and also more selective towards cancer cells than the parent gold porphyrin substituted with malonic acid. On the other hand, the IC<sub>50</sub> of dyad AuP-PtCyDA is 43% lower than that of AuTPP, but more selective towards healthy cells. Singlet oxygen measurements indicated that gold(III) porphyrin derivatives are poor oxygen sensitizers and cell death occurred potentially due to generation of others reactive oxygen species (ROS) upon reductive quenching of the gold porphyrin excited state. In addition, the increase in cancer cell death obtained after light irradiation is totally absent in healthy cells, demonstrating the specificity of this PDT treatment on cancer cells. Our findings imply that the incorporation of two different cytotoxic metals in the same molecule represents a remarkable cytotoxic effect compared to traditional homometallic Pt(II) drugs.

## Introduction

Cancer still remains one the major cause of mortality in the world in spite of the many progresses made in medical treatments for several decades. Conventional cancer treatment includes surgery, chemotherapy and radiotherapy. Platinum based compounds, such as Cisplatin, are widely used as anticancer therapeutic agents, but they have dose-limiting side effects and some cancer lines have become resistant to these drugs.<sup>1</sup>

Photodynamic therapy (PDT) is an approved clinical treatment in oncology. It has many advantages such as low-cost, less invasiveness and minimal side-effects than conventional chemotherapy.<sup>2</sup> PDT requires a photosensitizer, such as a porphyrin derivatives, which are known to better concentrate within the tumor tissue.<sup>3</sup> Currently, many porphyrin based photosensitizers are developed used in the clinic.<sup>2c,34</sup> The Photofrin was the first photosensitiser approved by the Food and Drug Administration (FDA) in 1993 for bladder cancer therapy. Now, it is used for several cancers. Then, a second generation of photoactive compounds was produced and developed for clinical use such as Foscan, Metvix, Laserphyrin, Redaporphin. In addition, several compounds such as Tookad, Radachlorin are under clinical trials for cancer treatment. Light excitation of photosensitizers produces reactive oxygen species (ROS) in tumor and ultimately leads to cancer cell death, a method that confers higher selectivity to cancer cells than Cisplatin-based chemotherapy treatments.

The combination of a porphyrin or a phthalocyanine with an appended platinum,<sup>5</sup> gold,<sup>6</sup> or ruthenium,<sup>7</sup> complex has been attempted to design innovative anticancer drugs. Interesting synergetic anticancer properties are obtained thanks to the dark chemostatic effect of the metal complex amplified by the PDT effect generated upon light excitation of the porphyrin or the phthalocyanine.<sup>5a</sup> <sup>b</sup> Recently, Che and co-workers reported the great pverotential of cationic gold(III) porphyrins as cytotoxic agent towards various cancer cell lines.<sup>8</sup> This discovery has stimulated many studies with gold porphyrins to develop anticancer drugs.<sup>9</sup> Interestingly, while Cisplatin anticancer drugs bind to DNA and induce cell death by binding to purine nucleic bases such as guanine and adenine,<sup>1a</sup> gold porphyrins target mitochondria and particularly the heat shock protein.<sup>10</sup>As a consequence, the combination of Cisplatin derivative with gold porphyrin could lead to synergistic effect owing to different modes of action.

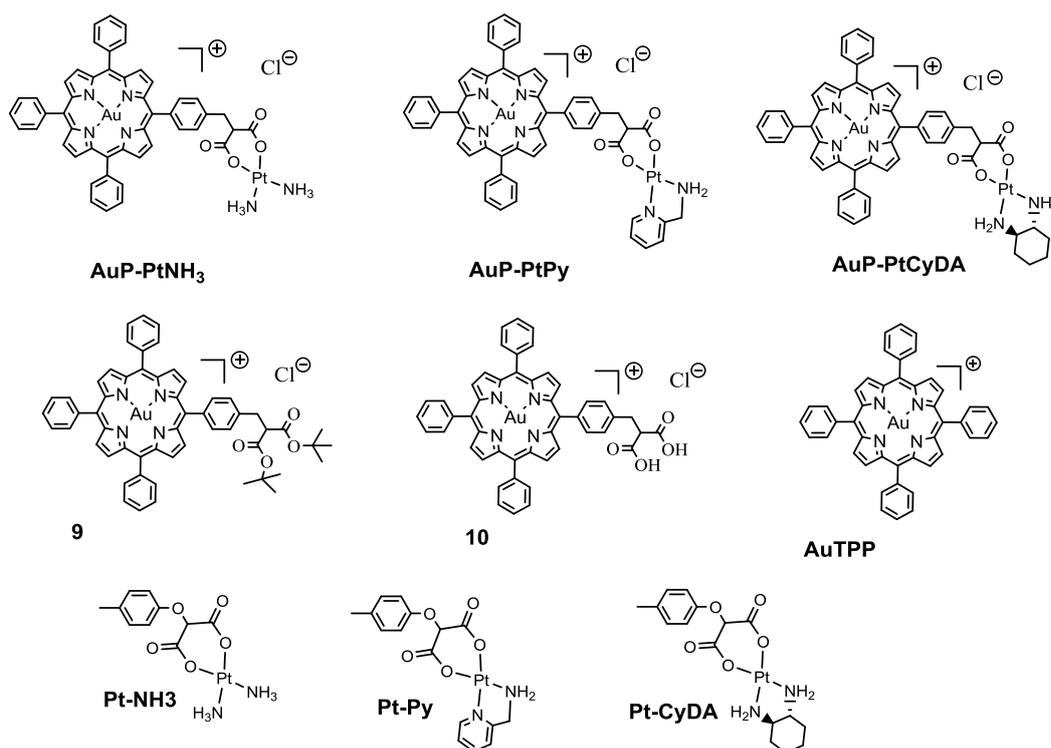
The most general mechanism of action of PDT is the generation of singlet oxygen by energy transfer from the photosensitizer excited triplet state to the triplet ground state of oxygen (classified as type II mechanism in PDT). It is well-accepted that photosensitizers with triplet excited states are more favorable than singlet ones to sensitize oxygen, because oxygen has a triplet ground state therefore energy transfer process from excited states of similar multiplicity are more efficient.<sup>11</sup>

Gold porphyrins are known to undergo intersystem crossing with unity quantum yield owing to the heavy atom effect induced by the presence of gold.<sup>12</sup> As a result, it is reasonable to anticipate that gold porphyrins could be promising candidates for PDT, such as it was shown for palladium porphyrins.<sup>11d,e</sup> However, to the best of our knowledge, there is so far no single study of the phototoxic activity of gold porphyrin derivatives.

In this work, we have investigated the combination of Cisplatin derivatives covalently connected to a gold porphyrin in order to take advantage, on one hand, of the intrinsic cytotoxicity of the platinum complex added to that of the gold porphyrin and, on the other hand, of the potentially

high phototoxicity of gold porphyrin upon light excitation (Chart 1). In addition, the affinity of porphyrin for tumor cells could also enhance selectivity and concentration of Cisplatin into tumor and thus increase the anticancer activity. However, this affinity is not sufficient legitimating that targeting strategies are currently explored to enhance the selectivity towards cancer cells.<sup>13</sup> Overall, the gold porphyrin platinum complex conjugates could have superior anticancer activity than individual compounds.

To test this idea, we report herein the preparation and the biological properties of three new dyads composed of gold porphyrin connected to a platinum diamine complex liganded *via* a malonate anchor (Chart 1). The diamine ligands around platinum(II) used in this work are ammoniac, cyclohexane diamine (CyDA) and pyridine methyl amine (Py). These three amine ligands were selected in this study, because related platinum complexes, such as Cisplatin, Picoplatin and oxaliplatin, have shown effective anticancer activities.<sup>1a, 5f, 14</sup> The three dyads were tested in human healthy and tumor cell lines to quantify their cytotoxicity without light excitation and their phototoxicity under light excitation.



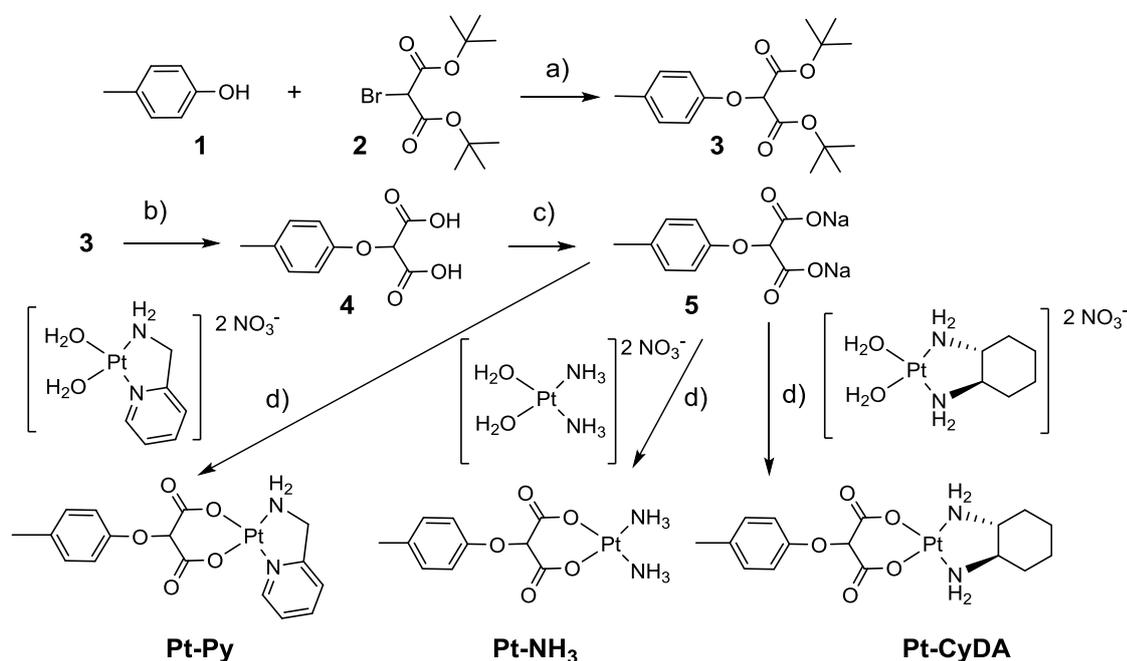
**Chart 1.** Structures of the compounds investigated in this study.

It was demonstrated that gold(III)/Pt(II)conjugates are more potent by 2 to 5.6-fold than the corresponding platinum complexes. The cyclohexyldiamine (CyDA) derivative exhibited the most cytotoxic effect among the series. Singlet oxygen measurements indicated that gold(III) porphyrin derivatives are poor oxygen sensitizers and cell death occurred potentially due to generation of others

reactive oxygen species (ROS) upon reductive quenching of the gold porphyrin excited state. Overall this study indicates that the incorporation of two different cytotoxic metals in the same molecule represents a remarkable cytotoxic effect compared to traditional homometallic Pt(II) drugs and that an increased cancer cell death was obtained after light irradiation only in cancer cells.

### Synthesis of the compounds

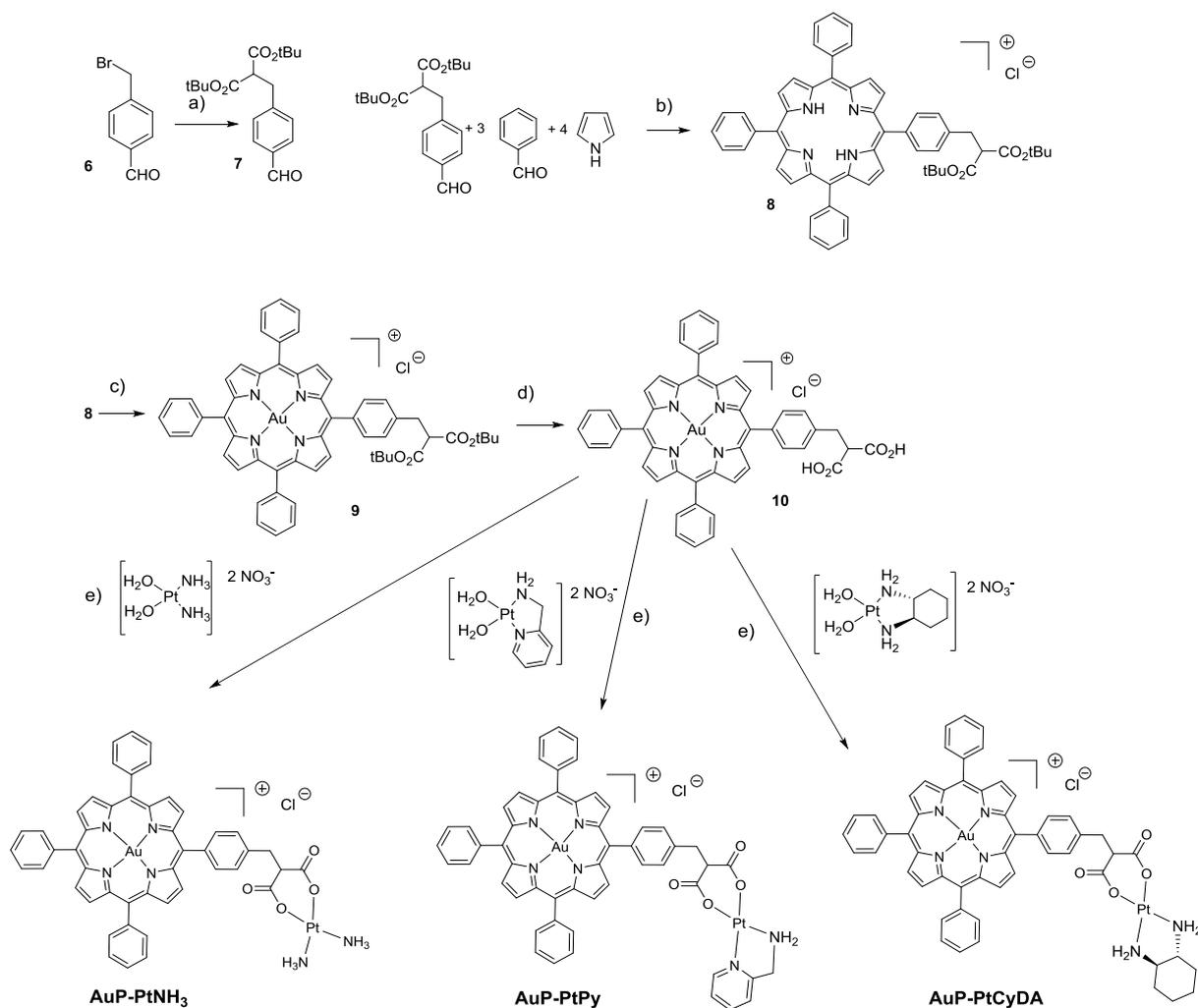
The synthesis of the reference platinum compounds (**Pt-NH<sub>3</sub>**, **Pt-Py** and **Pt-CyDA**) is illustrated in Scheme 1. First, p-cresol **1** was O-alkylated with bromoditertbutyl malonate **2** according to a Williamson substitution reaction affording **3** in 36% yield. We suspect that this lower yield stems from a second O-alkylation of compound **3**, concomitant with its bromination and further nucleophilic substitution by p-cresol as already reported on a similar compound.<sup>15</sup> The tertbutyl esters of the malonate were subsequently hydrolyzed with trifluoroacetic acid (TFA) to give compound **4**. Finally, the dicarboxylato platinum complexes were obtained in 92% yield according to Dhara's methodology, which consists in activating the carboxylic acid groups by deprotonation using sodium hydroxide before being reacted with the platinum amino precursor containing two weakly bound aqua ligands.<sup>14a, 16</sup>



**Scheme 1.** Synthetic route for the preparation of the reference platinum complexes. Reagents and conditions: a) THF, NaOH, RT, 24h, 36% ; b) TFA, 70°C, 1h, 100% ; c) EtOH, NaOH ; d) EtOH/Water (5/5), diamine(dinitro)platinum(II), RT, 48h, 92%.

The preparation of the dyads made of gold porphyrin/platinum complex required the porphyrin **8** as key intermediate (Scheme 2). Towards this goal, the ditertbutyl 4-(4-formylbenzyl)malonate **7** was first prepared by C-alkylation of the 4-(bromomethyl)- benzaldehyde **6** in 64% yield. The tertbutyl ester was preferred over methyl or ethyl malonate owing its fast and quantitative hydrolysis in acidic conditions. Indeed, gold porphyrins are electron deficient molecules and in basic conditions they can undergo nucleophilic addition of hydroxide in *meso* position.<sup>17</sup> The key porphyrin **8** was synthesized by cross-condensation of pyrrole with benzaldehyde and ditertbutyl 4-(4-formylbenzyl)malonate **7**. Using Adler conditions (reflux of the reagents in propionic acid),<sup>18</sup> the porphyrin was obtained in 12% yield, while Lindsey conditions (reaction conducted at RT in CH<sub>2</sub>Cl<sub>2</sub>)<sup>19</sup> with BF<sub>3</sub>-OEt<sub>2</sub> as catalyst afforded **8** in lower yield (5%). However, Lindsey conditions with a mixture of two catalysts BF<sub>3</sub>-OEt<sub>2</sub>/TFA: 0.019/1 at 0.01 M improved the yield of **8** to 22%.

The insertion of Au(III) into the porphyrin was performed according to Sauvage methodology<sup>20</sup> using a Au(I) based complex surrounded with weakly binding ligand (THT=tetrahydrothiophene) and can be conducted under mild reaction conditions (Scheme 2). Classical conditions, using the salt KAuCl<sub>4</sub> in refluxing acetic acid, were too harsh and caused hydrolysis of the ester groups and partial decarboxylation. Lastly, the introduction of the platinum complex was accomplished with Dhara's methodology similarly as for the reference complexes except that the solvent was adapted to these more hydrophilic compounds (mixture of ethanol and water). The complexes were characterized by proton NMR and IR spectroscopy, elemental analysis as well as by high resolution mass spectrometry. The dyads **AuP-PtNH<sub>3</sub>**, **AuP-PtPy** and **AuP-PtCyDA** are soluble in ethanol, DMSO and acetone.



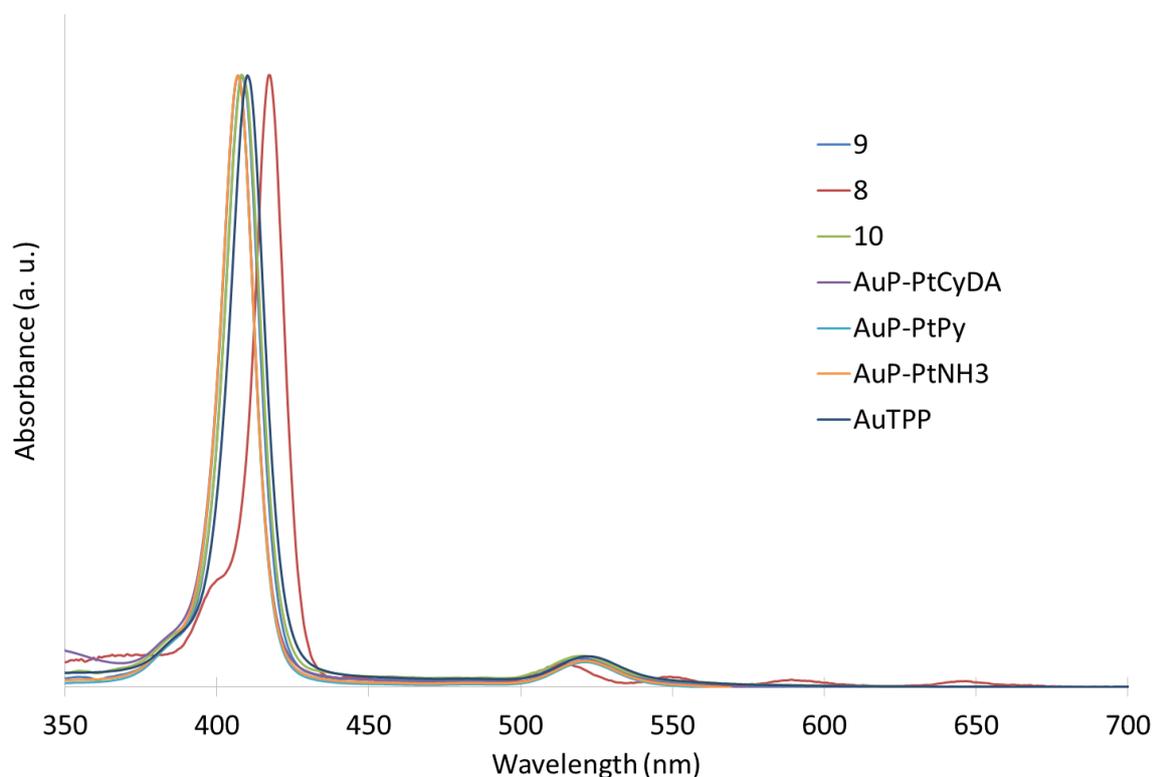
**Scheme 2.** Synthetic route for the preparation of the dyads gold porphyrin linked to platinum complex. Reagents and conditions: a) THF, NaH, di-tert-butyl malonate, 1h, 78% ; b) CH<sub>2</sub>Cl<sub>2</sub>, BF<sub>3</sub>·Et<sub>2</sub>O, TFA, 2h, DDQ, 2h, 22% ; c) CHCl<sub>3</sub>, Au(THT)<sub>2</sub>BF<sub>4</sub>, lutidine, reflux, 3h, 70% ; d) TFA, 70°C, 7h, 94% ; e) ethanol/water, NaOH, diamine(dinitro)platinum(II), RT, 48h, 90%.

### UV-vis electronic absorption spectra

The absorption spectra of the dyads gold porphyrin/platinum complex along with that of the reference **AuTPP** recorded in dichloromethane are shown in Figure 1 and the spectroscopic data are gathered in Table 1. The spectra of the dyads are essentially dominated by the transitions of the gold(III)–porphyrin, since the Pt complexes does not exhibit any absorption band in the visible region. The presence of the malonate on one phenyl substituent does not modify the transition on the porphyrin, since there is no  $\pi$ -conjugation with it. In addition, the *meso* aryl substituents of porphyrins are known to orient with *circa* 60° angle preventing electronic interaction between them.<sup>21</sup>

**Table 1.** Maximum absorption wavelengths and molar extinction coefficients of the porphyrins recorded in dichloromethane at room temperature.

Compounds	$\lambda_{\text{abs}} / \text{nm}(\log \epsilon / \text{M}^{-1}\text{cm}^{-1})$
<b>AuP-PtNH<sub>3</sub></b>	407 (5.49); 522 (4.18)
<b>AuP-PtPy</b>	407 (5.45); 526 (4.01)
<b>AuP-PtCyDA</b>	407 (5.52); 529 (4.03)
<b>8</b>	417 (5.69); 507 (4.12); 542 (3.76); 584 (3.64); 637 (3.39)
<b>9</b>	407 (5.60); 527 (4.23)
<b>10</b>	407 (5.49); 524 (4.16)
<b>AuTPP</b>	409 (5.44); 527 (4.11)



**Figure 1.** Normalized absorption spectra of the porphyrin derivatives recorded in dichloromethane.

Unsurprisingly, the presence of the appended platinum complex does not alter the spectrum of the gold porphyrin owing to the deconjugation induced by the methylene spacer between the porphyrin

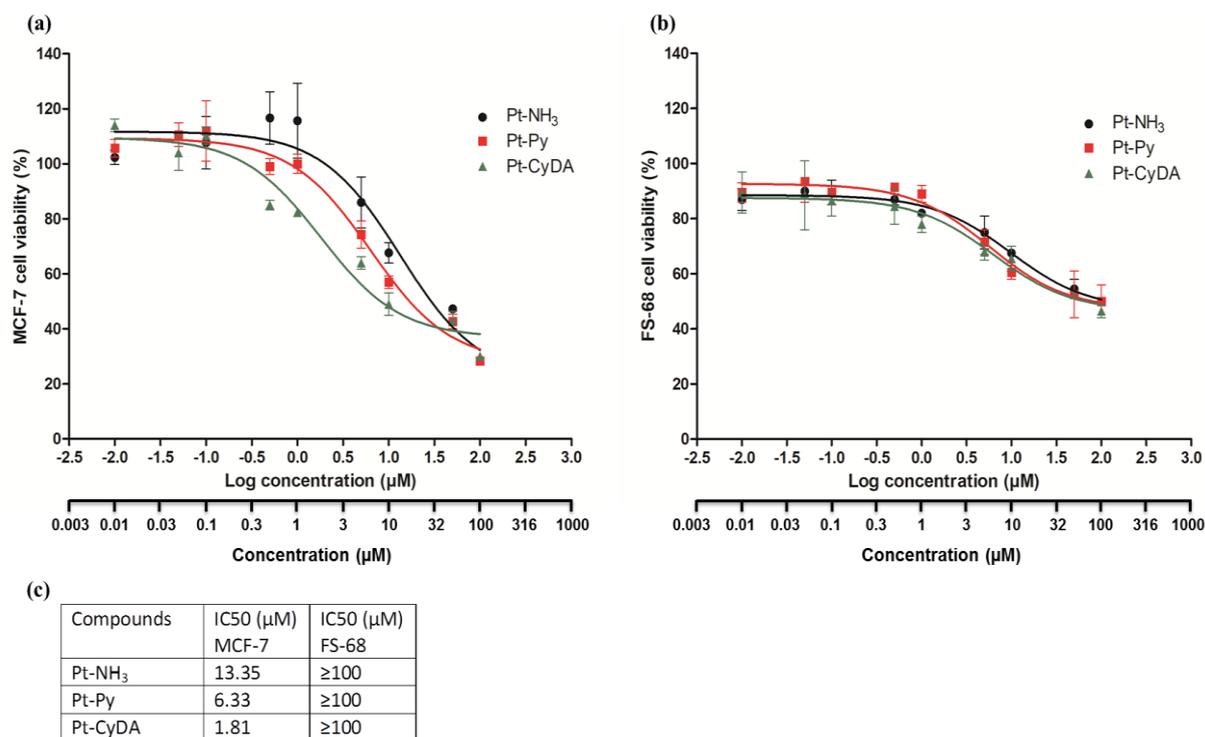
and the Pt complex. The absorption bands of the gold(III)–porphyrin are characterized by a hypsochromic shift of both the Soret and the Q-bands relative to those in the free base porphyrin (Figure 1). These spectral features are explained by Au<sup>III</sup>-N bonding interactions, consequently leading to stabilization of the HOMO levels, and an increase in the HOMO–LUMO gap with respect to that of the free base.<sup>22</sup>

### Singlet oxygen measurements

Most of the porphyrins used in PDT operate according to a type II mechanism, that is the production of singlet oxygen by energy transfer from the photosensitizer excited state to oxygen present in the biological tissues.<sup>2a, b</sup> The singlet oxygen quantum yield production was therefore measured to assess the potential of these gold porphyrin systems to act as sensitizers for PDT. The measurements were recorded in methanol and the production of singlet oxygen was assayed by recording the band at 1270 nm of singlet oxygen emission after gold porphyrin systems illumination. Unfortunately, none of the gold porphyrin and dyads prepared in this work produced detectable luminescence signal setting, thus a singlet oxygen quantum yield was below 1%. This unexpected result probably stems from the short lived triplet excited state of gold(III) porphyrins ( $\tau \approx 1.5$  ns), which decay to ground state before the energy transfer to surrounding oxygen could take place.<sup>12, 23</sup>

### Biological activity

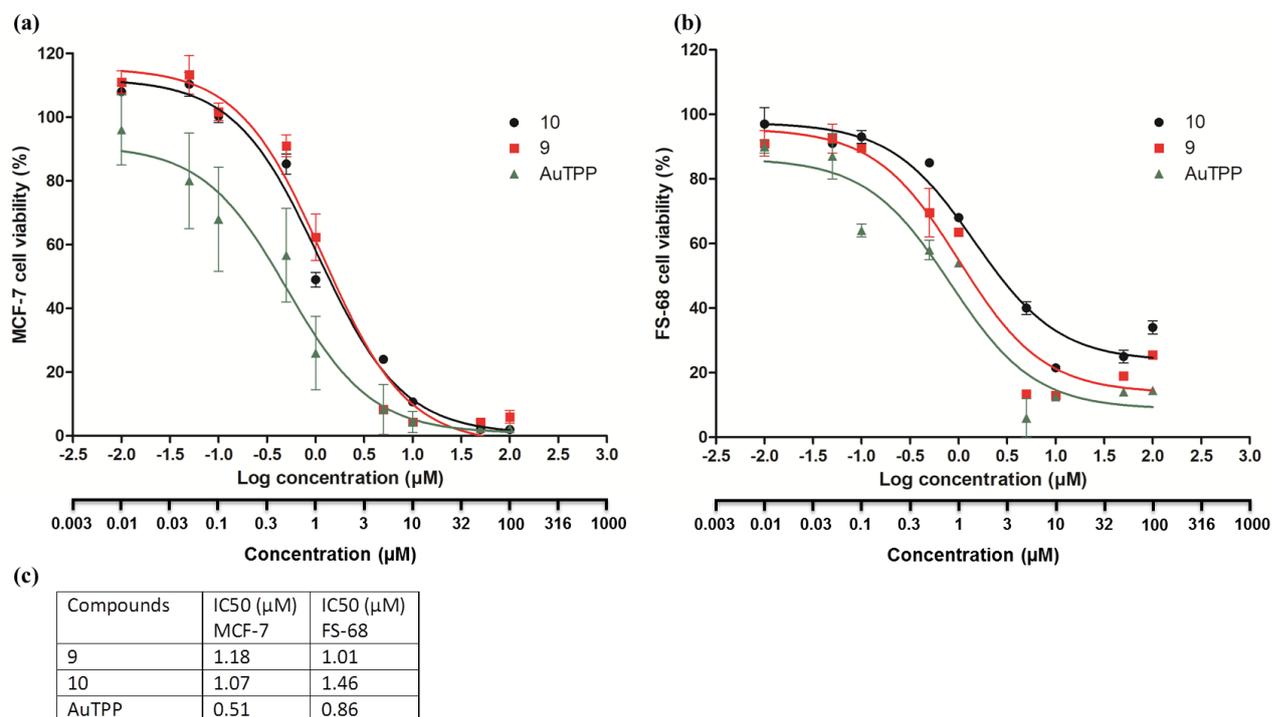
In order to determine the therapeutic potential of these new compounds, we have first studied the cytotoxicity induced in human breast cancer cells (MCF-7) and in healthy human fibroblasts (FS-68). The cells were incubated in darkness during 72 h with increasing concentrations of each compound (from 0.01 to 100  $\mu$ M). The concentrations of the drugs which led to 50% cell mortality ( $IC_{50}$ ) were determined. As shown in Figure 2, obtained cytotoxicity data in both cell lines showed the classical sigmoidal dose-response curves when plotted as a logarithmic function of concentration ( $\mu$ M). Figure 2a,b illustrates the dose dependent cytotoxic effect of the reference platinum complexes **Pt-NH<sub>3</sub>**, **Pt-Py** and **PtCyDA** which are in the range of those reported with similar compounds and the ranking is consistent with previous studies<sup>5f, 24</sup>: **Pt-NH<sub>3</sub><Pt-Py<PtCyDA**. Overall, the Pt complex with the ligand CyDA is the most cytotoxic of the series with a lower  $IC_{50}$  value than Cisplatin ( $IC_{50}(Cisplatin) \approx 5$   $\mu$ M).<sup>25</sup> Importantly, the cell death is very low in healthy fibroblasts,  $IC_{50}$  is too high to be calculate precisely ( $\geq 100$   $\mu$ M) (Figure 2c). This result highlights the targeting of cancer cells by these compounds.



**Figure 2.** Cytotoxic study of **Pt-NH<sub>3</sub>**, **Pt-Py** and **PtCyDA**. (a) Human breast cancer cells (MCF-7) and (b) healthy fibroblasts cells (FS-68) were incubated 72 h with increasing concentrations of **Pt-NH<sub>3</sub>**, **Pt-Py** and **PtCyDA** and maintained in darkness. Values are means  $\pm$  standard deviation of 3 experiments. (c) IC<sub>50</sub> values are reported for each cell line.

Then, the cytotoxic activity of gold porphyrins **AuTPP**, **9** and **10** was investigated in both human breast cancer cells (Figure 3a) and in healthy fibroblasts cells (Figure 3b). The results first show that overall, the gold porphyrins are more potent than the reference platinum complexes. Although the difference of cytotoxicity in cancer cells between **Pt-CyDA** and **10** is small, but on healthy fibroblasts this difference become very high; close to 100 fold (Table 3c). In addition, the introduction of the malonate substituent on the porphyrin decreases its cytotoxic activity, since the simple **AuTPP** has the lowest IC<sub>50</sub>. This result is consistent with the report of Che and co-workers, showing that any substituent pattern on **AuTPP** results in a weakening of its cytotoxicity.<sup>8a</sup> Important features for the anticancer activity of gold(III) porphyrins are the size of the macrocycle, which must fit in the cavity of the target receptor (certainly heat shock protein)<sup>10</sup> and the lipophilicity of the porphyrin because its biological properties might be related to the fact it is a planar aromatic organic cation.<sup>26</sup> Thus, complex **9** with bulky hydrophobic malonate substituent displayed the least activity, which might be attributed to traffic entrance into cells. A long floppy alkyl chain might have been more favorable to tether the platinum complex and to maintain the cytotoxic activity of gold(III) porphyrin. Finally, we note that for

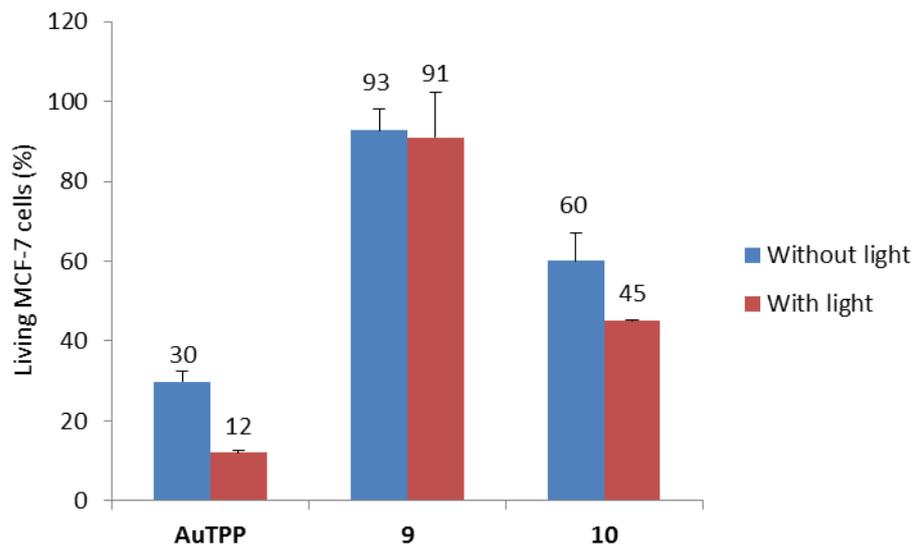
gold porphyrins **AuTPP** and **10**, the cytotoxicity is lower on healthy cells than cancerous ones, a significant advantage to decrease side effects during the treatment.



**Figure 3.** Cytotoxic study of **AuTPP**, **9** and **10**. (a) Human breast cancer cells (MCF-7) and (b) healthy fibroblasts cells (FS-68) were incubated 72 h with increasing concentrations of **AuTPP**, **9** and **10** and maintained in darkness. Values are means  $\pm$  standard deviation of 3 experiments. (c) IC<sub>50</sub> values are reported for each cell line.

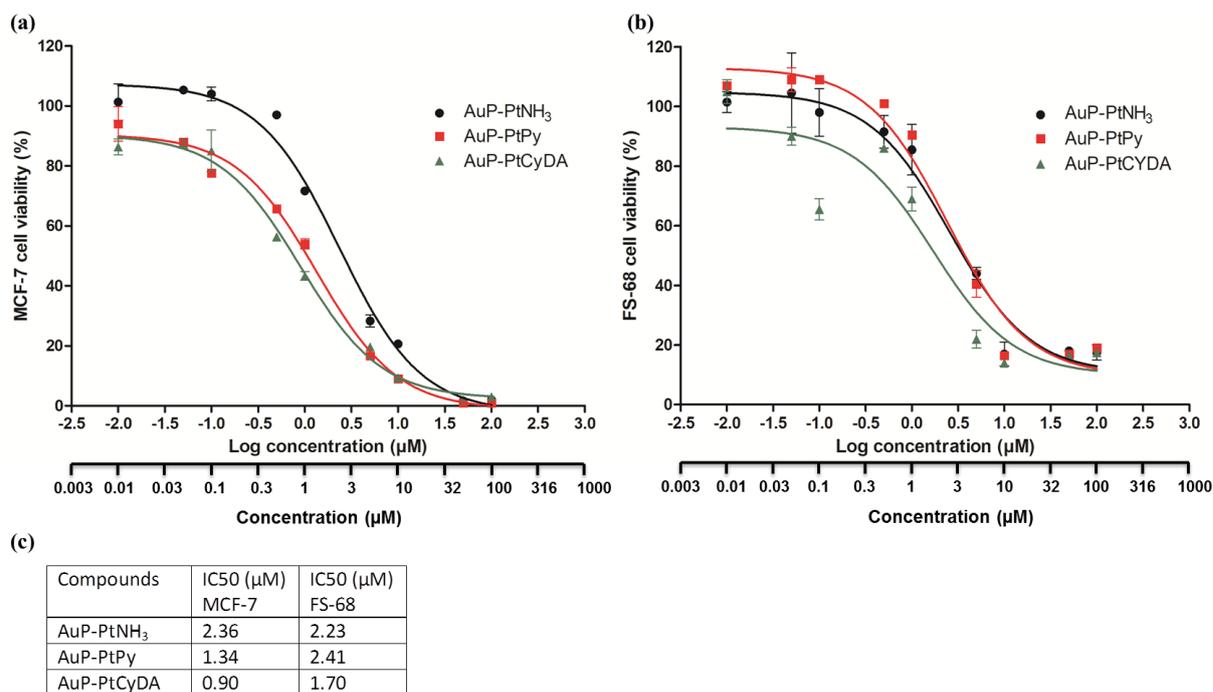
Then, the photodynamic therapy efficiency of the gold porphyrins was studied in order to determine the possibility of a dual therapy that could increase, after light excitation, the cell death already due to the cytotoxicity of the compound (Figure 4). Cancer cells were thus incubated for 24 h with **AuTPP**, **9** and **10** at a concentration of 0.5 μM and then irradiated or not with the mercury lamp of a fluorescent microscope (20 min,  $\lambda_{exc} = 390 - 420$  nm, 39 J.cm<sup>-2</sup>). The cell death quantification performed 2 days after illumination demonstrates a decrease in living cells treated and submitted to photoexcitation. In fact, light excitation induces a supplementary cell death in addition to their intrinsic cytotoxicity and the most significant effect was obtained by the photoexcitation of **AuTPP** for which the illumination induced 18% of cell death to add to the 70% of cell death obtained without light. However, the additional cell mortality generated upon light excitation remains modest, indicating that gold porphyrins have weak, but not null, cytotoxic as compared for other sensitizers, such as free base porphyrins.<sup>13a, 27</sup> As mentioned above, the low phototoxicity of gold porphyrins is certainly explained

by the very weak singlet oxygen generation quantum yield of these compounds, since porphyrins usually display higher ones.<sup>27a, 28</sup>



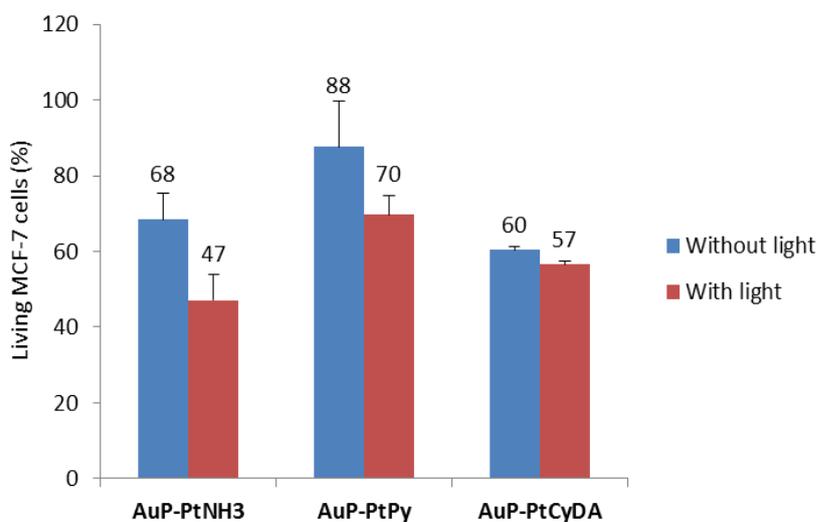
**Figure 4.** Phototoxicity of **AuTPP**, **9** and **10** incubated at 0.5  $\mu\text{M}$  with MCF-7 cells during 24 h and irradiated 20 min at  $\lambda_{\text{exc}} = 390 - 420 \text{ nm}$  ( $39 \text{ J}\cdot\text{cm}^{-2}$ ). Values are means  $\pm$  standard deviation of 3 experiments.

Finally, the toxicity (Figure 5) and phototoxicity (Figure 6) of the three dyads were tested. On cancer and healthy cells (Figure 5a,b,c), all of them display  $\text{IC}_{50}$  values at the micromolar level, which are lower than those of the reference platinum complexes, but remain in the same range, let alone a bit better, as that of the reference gold porphyrin **10**. Again, the most active dyad is **AuP-PtCyDA**, where the Pt is liganded with cyclohexane diamine. The cytotoxicity effect of the bimetal porphyrin conjugates **AuP-PtNH<sub>3</sub>**, **AuP-PtPy** and **AuP-PtCyDA** was increased by 5.6, 4.7 and 2 fold enhancement, respectively, compared to their corresponding Pt-complexes. These results would suggest that porphyrins granted the transport of the complex inside the cancer cell for more favorable cytotoxicity. As expected, the CyDA complex showed the highest effect.



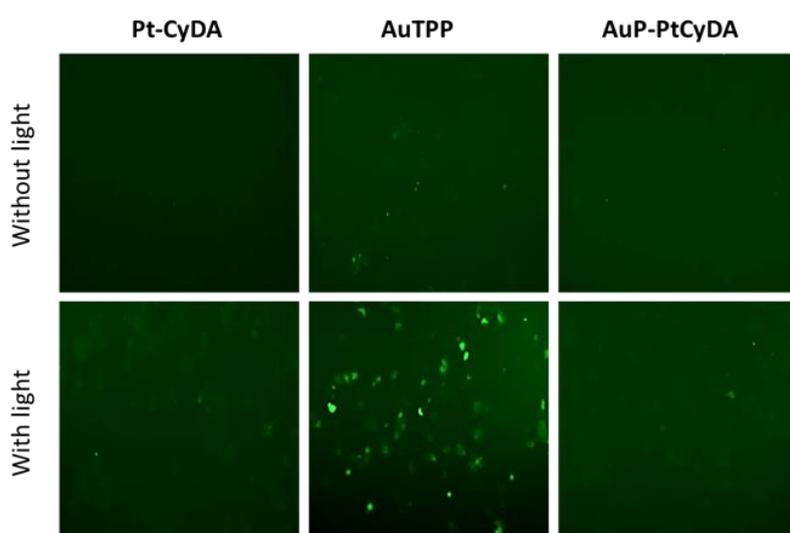
**Figure 5.** Cytotoxic study of dyads **AuP-PtNH<sub>3</sub>**, **AuP-PtPy** and **AuP-PtCyDA**. (a) Human breast cancer cells (MCF-7) and (b) healthy fibroblasts cells (FS-68) were incubated 72 h with increasing concentrations of dyads **AuP-PtNH<sub>3</sub>**, **AuP-PtPy** and **AuP-PtCyDA** and maintained in darkness. Values are means  $\pm$  standard deviation of 3 experiments. (c) IC<sub>50</sub> values are reported for each cell line.

Similarly as for the reference gold porphyrins (**AuTPP**, **9** and **10**), light excitation increases cell mortality, but to a low extent (Figure 6). Similarly as for the reference gold porphyrins (**AuTPP**, **9** and **10**), light excitation increases cell mortality, but to a low extent (Figure 6). In addition, **AuP-PtCyDA** does not exhibit PDT efficiency in these conditions.”



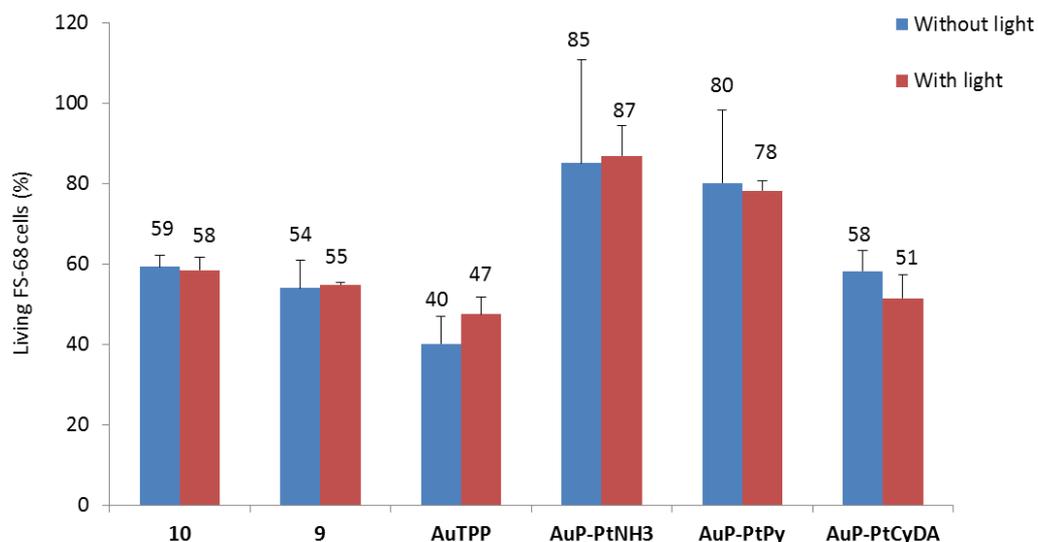
**Figure 6.** Phototoxicity of dyads **AuP-PtNH<sub>3</sub>**, **AuP-PtPy** and **AuP-PtCyDA** incubated at 0.5  $\mu\text{M}$  with MCF-7 cells during 24 h and irradiated 20 min at  $\lambda_{\text{exc}} = 390 - 420 \text{ nm}$  ( $39 \text{ J}\cdot\text{cm}^{-2}$ ). Values are means  $\pm$  standard deviation of 3 experiments.

To confirm that the phototoxicity induced by photoexcitation was due to PDT effect, we studied the reactive oxygen species (ROS) production in MCF-7 cells human cells treated with the compounds **Pt-CyDA** (as negative control), **AuTPP** and **AuP-PtPy** with or without laser irradiation. Cells were first incubated with DCFH<sub>2</sub>-DA (2',7'-dichlorodihydrofluorescein diacetate), which is a non-fluorescent molecule. In the presence of ROS produced within the cell, this molecule is oxidized into the fluorescent 2',7'-dichlorofluorescein (DCF) and its green luminescence can be detected at  $\lambda = 450 \text{ nm}$  using fluorescent microscope. The results, reported in Figure 7, showed that the light induced an increase in green fluorescence inside the cells incubated with **AuTPP** and **AuP-PtPy** traducing thus a ROS production and suggesting a cell death induced by PDT. The singlet oxygen measurements have shown that all these porphyrin derivatives are poor oxygen photosensitizers, therefore it is likely that the ROS production is induced by generating cytotoxic radicals *via* electron transfer from excited  $\text{AuP}^+$  to surrounding organic molecules such as glutathione, a ubiquitous antioxidant in cells, upon reductive quenching of  $\text{AuP}^+$  excited state, which is a strong oxidant.<sup>12</sup> Accordingly, PDT effect can arise by a type I mechanism, which passes *via* the formation of oxidized species, inducing thus an oxidative stress by oxidizing GSH to glutathione disulfide. Photosensitizers operating upon a type I mechanism is particularly interesting in hypoxic environments found in many solid tumours.



**Figure 7.** ROS production in MCF-7 cells incubated with **Pt-CyDA**, **AuP-PtCyDA** and **AuTPP** at 0.5  $\mu\text{M}$  during 24 h and irradiated 20 min at  $\lambda_{\text{exc}} = 390 - 420 \text{ nm}$  ( $39 \text{ J}\cdot\text{cm}^{-2}$ ). Green luminescence shows the generation of ROS detected at  $\lambda = 450 \text{ nm}$ .

Finally, we decided to study the PDT effect in healthy fibroblasts in the same conditions as those used for cancer cells (Figure 8). For this, FS-68 cells were incubated 24 h with 0.5  $\mu\text{M}$  of compounds and were submitted to light excitation at  $\lambda_{\text{exc}} = 390 - 420 \text{ nm}$ ,  $39 \text{ J}\cdot\text{cm}^{-2}$ . The quantification of living cells 2 days after the PDT experiment showed a total absence of phototoxic effect. This result highlights the specific effect of PDT on cancer cells and suggests that there could be no phototoxicity on healthy cells around the tumor.



**Figure 8.** Phototoxicity of gold porphyrins **10**, **9**, **AuTPP** and dyads **AuP-PtNH<sub>3</sub>**, **AuP-PtPy**, **AuP-PtCyDA** incubated at 0.5  $\mu\text{M}$  with healthy fibroblasts (FS-68) during 24 h and irradiated 20 min at  $\lambda_{\text{exc}} = 390 - 420 \text{ nm}$  ( $39 \text{ J}\cdot\text{cm}^{-2}$ ). Values are means  $\pm$  standard deviation of 3 experiments.

## Conclusion

For the first time, we report three new dyads consisting of two appended cytotoxic moieties such as a gold(III) porphyrin and a platinum complex and their biological properties towards cancer cells were investigated in the dark (chemostatic effect) and upon light excitation (PDT effect) and compared to their corresponding individual platinum(II) complexes. To evaluate the decrease in side effects that could occur with such compounds, the effect on healthy cells was also studied. The main results of this work are: i) the diamine ligand around the Pt centre has a high impact on the cytotoxicity of the resulting complex and cyclohexane diamine is the best ligand; ii) the gold porphyrins are poor singlet

oxygen photosensitizers and thereby exhibit low phototoxicity with the present systems, but improvement can be obtained with modification of the porphyrin core, such as fluorination. The presence of fluorine atoms on meso phenyl substituents could enhance both the oxidizing power<sup>29</sup> and the lifetime of the triplet excited state. Indeed, it is postulated that gold porphyrins display ligand to metal charge transfer transitions, which are responsible of the fast decay of the triplet excited state.<sup>23</sup> However, the phototoxicity is not null and most probably result by a type I mechanism; iii) the introduction of a malonate group of one phenyl substituent of AuTPPs lightly decreases cyto- and photoxicity the gold porphyrin; iv) the dyad **AuP-PtCyDA** displays interesting cytotoxic activity towards cancer cells, for which the mechanism of action would be interesting to be investigated. The enhanced cytotoxicity indicates that porphyrins allow the delivery of the complex into cancer cells. The complex might have dual biological functioning between Au(III) and Pt(II) arms. The former as reported would interact with mitochondria and the latter would target DNA; both mechanisms cause cancer cell death.

Importantly, all compounds present a close or clearly higher cytotoxic activity in cancer cells than healthy cells and the PDT effect, even slight, induced around 15 or 20% more cancer cell death for **AuTPP**, **AuP-PtNH<sub>3</sub>** and **AuP-PtPy** in conditions totally harmless for healthy cells (0% cell death).

Further biological analysis would clarify the anticancer activity if it is based on specific metal or both. Additional analysis of the intracellular metal contents such as ICP-analysis to monitor the uptake of the whole stable bimetal conjugate by cancer cells would clarify the delivery mechanism. On the other hand, the incorporate mode of action exhibited by the two metals may be even amplified using an alternative photosensitizer. These studies are in due course in our laboratory.

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## **Experimental part**

### **Synthesis of the compounds**

#### ***Generalities***

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on an *AVANCE 300 UltraShield BRUKER* and *AVANCE 400 BRUKER*. Chemical shifts for  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra are referenced relative to residual proton in the deuterated solvent ( $\text{CDCl}_3$   $\delta = 7.26$  ppm for  $^1\text{H}$ ,  $\text{DMSO-d}^8$   $\delta = 2.50$  ppm for  $^1\text{H}$ ) or to an internal reference (TMS,  $\delta = 0$  ppm for both  $^1\text{H}$ ). NMR spectra were recorded at room temperature, chemical shifts are written in ppm and coupling constants in Hz. High-resolution mass (HR-MS) spectra were obtained by electrospray ionization coupled with high resolution ion trap orbitrap (LTQ-Orbitrap, ThermoFisher Scientific), working in ion-positive or ion-negative mode.

Chemicals were purchased from Sigma-Aldrich or Alfa Aesar and used as received. Thin-layer chromatography (TLC) was performed on silica sheets precoated with Merck 5735 Kieselgel 60F<sub>254</sub>. Column chromatography was carried out with Merck 5735 Kieselgel 60F (0.040-0.063 mm mesh). Bromo ditertbutyl malonate **2**, 4-(bromomethyl)-benzaldehyde **6** and platinum precursors  $\text{Pt}(\text{OH}_2)_2(\text{NH}_3)_2(\text{NO}_3)_2$ ,  $\text{Pt}(\text{OH}_2)_2(\text{Py})(\text{NO}_3)_2$  and  $\text{Pt}(\text{OH}_2)_2(\text{CyDA})(\text{NO}_3)_2$  were synthesized according to literature.

**Ditertbutylmalonate p-cresol (3).** A total of 1 g (9.24 mmol) of p-cresol **1** and 2.7 g (9.24 mmol) of bromoditertbutyl malonate **2** were dissolved under argon in 20 ml of distilled THF. 0.36 g (9 mmol) of pulverized NaOH was added, and the mixture was stirred for 24 h at room temperature. The organic solution was diluted with water and extracted with dichloromethane. The combined organic phases were washed with water and dried over  $\text{Na}_2\text{SO}_4$ . The solvent was evaporated and the crude product was purified by column chromatography ( $\text{SiO}_2$ ;  $\text{CH}_2\text{Cl}_2$ /petroleum ether 5/5). Yield: (1.08 g, 36%). NMR ( $^1\text{H}$ ,  $\text{CDCl}_3$ , 300 MHz)  $\delta$  (ppm): 7.07-7.04 (d, 2H,  $J = 8.63$  Hz), 6.86-6.84 (d, 2H,  $J = 8.63$  Hz), 4.92 (s, 1H), 2.26 (s, 3H), 1.48 (s, 18H). HRMS (ES+) [M+]  $m/z$  345.1672 found, 345.1678 calc.

**Compound (4).** 206 mg (0.63 mmol) of **3** was dissolved in 1 ml TFA and heated at 70°C for one hour. The solvent was evaporated and the product was dried under vacuum. Yield: (134 mg, 100%). IR (ATR):  $1706\text{ cm}^{-1}$  (C=O). NMR ( $^1\text{H}$ ,  $\text{CDCl}_3$ , 300 MHz)  $\delta$  (ppm): 7.10-7.08 (d, 2H,  $J = 8.63$  Hz), 6.82-6.80 (d, 2H,  $J = 8.61$  Hz), 5.25 (s, 1H), 2.22 (s, 3H). HRMS (ES+) [M+]  $m/z$  209.0454 found, 209.0450 calc.

**General procedure 1 (GP 1).** Compound **4** was dissolved in water and the carboxylic acid functions were activated into sodium carboxylates, using 2 eq of NaOH. 1.2 eq of the respective diammine(dinitro)platinum(II) complex was dissolved in ethanol and added. The solution was stirred for 48 h at room temperature. The precipitated complexes **Pt-Py**, **Pt-NH<sub>3</sub>**, **Pt-CyDA** were filtered and dried.

**Pt-NH<sub>3</sub>**. According to GP1, 20 mg (0.095 mmol) of **4** was dissolved in 2 ml water, 7.6 mg (0.19 mmol) of NaOH was added, combined with 40 mg (0.11 mmol) of diamine(dinitro)platinum(II) dissolved in 2 ml EtOH, and stirred for 48 h. The precipitated complex **Pt-NH<sub>3</sub>** was filtered, washed with water and dried. Yield (38 mg, 92%). IR (ATR): 1669 cm<sup>-1</sup> (C=O). NMR (<sup>1</sup>H, DMSO, 300 MHz) δ (ppm): 7.03 (br, 2H), 6.66 (br, 2H), 5.70 (s, 1H), 4.29 (br, 6H), 2.20 (s, 3H). HRMS (ES+) [M<sup>+</sup>] m/z 459.0412 found, 459.0427 calc. Elem. Anal. Exp C, 27.68; H, 3.20; N, 6.19. calc C, 27.47; H, 3.23; N, 6.41.

**Pt-CyDA**. According to GP1, 40 mg (0.19 mmol) of **4** was dissolved in 3 ml water, 15 mg (0.38 mmol) of NaOH was added, combined with 90 mg (0.2 mmol) of diamine(dinitro)platinum(II) dissolved in 3 ml EtOH, and stirred for 48 h. The precipitated complex **Pt-CyDA** was filtered, washed with water and dried. Yield (90 mg, 92%). IR (ATR): cm<sup>-1</sup> (C=O). NMR (<sup>1</sup>H, DMSO, 300 MHz) δ (ppm): 7.06-7.03 (d, 2H, *J* = 8.53 Hz), 6.68-6.65 (d, 2H, *J* = 8.9 Hz), 6.16-6.03 (dd, 2H, *J* = 9.9, 30.91 Hz), 5.66 (s, 1H), 5.45-5.33 (dt, 2H, *J* = 37.28, 8.96 Hz), 2.21 (s, 3H), 2.09 (br, 2H), 1.84-1.80 (d, 2H, *J* = 12.95 Hz), 1.46-1.44 (d, 2H, *J* = 8.86 Hz), 1.22 (br, 2H), 1.04-0.97 (t, 2H, *J* = 10.22 Hz). HRMS (ES+) [M<sup>+</sup>] m/z 539.1061 found, 539.1053 calc.

**Pt-Py**. According to GP1, 40 mg (0.19 mmol) of **4** was dissolved in 3 ml water, 15 mg (0.38 mmol) of NaOH was added, combined with 89 mg (0.2 mmol) of diamine(dinitro)platinum(II) dissolved in 3 ml EtOH, and stirred for 48 h. The precipitated complex **Pt-Py** was filtered, washed with water and dried. Yield (89 mg, 92%). IR (ATR): 1681, 1649 cm<sup>-1</sup> (C=O). NMR (<sup>1</sup>H, DMSO, 300 MHz) δ (ppm): 8.44-8.42 (d, 1H, *J* = 5.97 Hz), 8.18-8.12 (td, 1H, *J* = 7.73, 1.58 Hz), 7.66-7.63 (d, 1H, *J* = 7.8 Hz), 7.54-7.50 (t, 1H, *J* = 6.96 Hz), 7.05-7.02 (d, 2H, *J* = 8.36 Hz), 6.71-6.68 (d, 2H, *J* = 8.9 Hz), 6.64-6.62 (d, 1H, *J* = 5.57 Hz), 5.70 (s, 1H), 4.15-4.14 (br, 2H), 2.21 (s, 3H). HRMS (ES+) [M<sup>+</sup>] m/z 511.0761 found, 511.0764 calc.

**Porphyrin (8)**. BF<sub>3</sub>.Et<sub>2</sub>O 16 μl (1.2 × 10<sup>-2</sup> mmol) and 0.5 ml (6.46 mmol) of trifluoroacetic acid (TFA) were added to a solution of 0.55 ml (5.38 mmol) of benzaldehyde, 600 mg (1.79 mmol) of **7**, and 0.35 ml (5 mmol) of freshly distilled pyrrole in 1 l of CH<sub>2</sub>Cl<sub>2</sub>. The mixture was kept at room temperature for 2 h. 1.2 g (5.2 mmol) of DDQ was added when the aldehydes were completely reacted, and the mixture was kept at room temperature for an additional 2 h. The solvent was evaporated and the crude product was purified by column chromatography (SiO<sub>2</sub>; CH<sub>2</sub>Cl<sub>2</sub>/hexane 6/4) to remove tetraphenylporphyrin. The monosubstituted porphyrin was then eluted with CH<sub>2</sub>Cl<sub>2</sub> in form of a thick, purple band. Yield: (331 mg, 22%). UV-Vis (CH<sub>2</sub>Cl<sub>2</sub>, nm) λ<sub>max</sub> (nm): 418 (ε 4.2 × 10<sup>5</sup> mol<sup>-1</sup>.L.cm<sup>-1</sup>), 522 (1.1 × 10<sup>4</sup>), 557 (4.1 × 10<sup>3</sup>), 655 (2.3 × 10<sup>3</sup>). NMR (<sup>1</sup>H, CDCl<sub>3</sub>, 300 MHz) δ (ppm): 8.41-8.80 (m, 8 H), 8.23-8.20 (dd, 2H, *J* = 7.08, 7.67 Hz), 8.14-8.11 (d, 2H, *J* = 8.02 Hz), 7.76-7.74 (m, 9H), 7.61-7.58 (d, 2H, *J* = 8.15 Hz), 3.83-3.77 (t, 1H, *J* =

8.41 Hz), 3.49-3.46 (d, 2H,  $J = 7.94$  Hz), 1.55 (s, 18H). HRMS (ES+) [M+]  $m/z$  843.3908 found, 842.3910 calc.

**Porphyrin (9).** A total of 700 mg (0.83 mmol) of porphyrin **8** and 1.9 g ( $4.1 \times 10^{-3}$  mmol)  $[\text{Au}(\text{THT})_2]\text{BF}_4$  were dissolved in 5 ml chloroform. 193  $\mu\text{l}$  ( $1.6 \times 10^{-3}$  mmol) of 2,6-lutidine was added and the reaction was heated to reflux. A gold mirror very rapidly deposited on the walls of the flask, and the reaction mixture turned reddish. After refluxing for three hours, the solution was evaporated to dryness. The residue was taken up in dichloromethane and purified through a silica column, eluting the free base porphyrin with dichloromethane and the desired red-orange band with  $\text{CH}_2\text{Cl}_2/\text{AcOEt}$  (9/1). Yield: (602 mg, 70%). UV-Vis ( $\text{CH}_2\text{Cl}_2$ , nm)  $\lambda_{\text{max}}$  (nm): 408 ( $\epsilon 3.1 \times 10^5 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$ ), 526 ( $1.2 \times 10^4$ ). NMR ( $^1\text{H}$ ,  $\text{CDCl}_3$ , 300 MHz)  $\delta$  (ppm): 9.26-9.23 (m, 8H), 8.24-8.22 (d, 6H,  $J = 6.53$  Hz), 8.16-8.14 (d, 2H,  $J = 8.09$  Hz), 7.90-7.83 (m, 9H), 7.71-7.69 (d, 2H,  $J = 7.78$  Hz), 3.83-3.78 (t, 1H,  $J = 7.76$  Hz), 3.51-3.49 (d, 2H,  $J = 8.09$  Hz), 1.55 (s, 18H). HRMS (ES+) [M+]  $m/z$  1037.3326 found, 1037.3341 calc.

**Porphyrin (10).** 630 mg (0.607 mmol) of gold porphyrin **9** was dissolved in 2 ml TFA and heated at 70°C for seven hours. After removal of the solvent under reduced pressure, the product was dissolved in methanol and purified using exclusion size chromatography. Yield: (530 mg, 94%). IR (ATR): 1634  $\text{cm}^{-1}$  (C=O). UV-Vis ( $\text{CH}_2\text{Cl}_2$ , nm)  $\lambda_{\text{max}}$  (nm): 408 ( $\epsilon 2.8 \times 10^5 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$ ), 526 ( $1.3 \times 10^4$ ). NMR ( $^1\text{H}$ , DMSO- $d_6$ , 300 MHz)  $\delta$  (ppm): 9.44-9.40 (m, 8H), 8.22-8.18 (m, 6H), 8.09-8.06 (d, 2H,  $J = 8.13$  Hz), 7.94-7.86 (m, 8H), 7.76-7.73 (d, 2H,  $J = 7.98$  Hz), 3.92-3.88 (t, 1H,  $J = 6.18$  Hz), 3.71-3.69 (d, 2H,  $J = 5.85$  Hz). HRMS (ES+) [M+]  $m/z$  925.2075 found, 925.2089 calc.

**General procedure 2 (GP 2).** Compound **10** was dissolved in EtOH and the carboxylic acid functions were activated into sodium carboxylates using 2 eq of NaOH. 1.2 eq of the respective diammine(dinitro)platinum(II) complex was dissolved in Ethanol/Water (1/1) and added. The solution was stirred for 48 h at room temperature. The solvents were evaporated. The complexes were dissolved in DMF and precipitated with a saturated solution of NaCl. The precipitated complexes **AuP-PtNH<sub>3</sub>**, **AuP-PtPy**, **AuP-PtCyDA** were filtered, washed with water and dried.

**AuP-PtNH<sub>3</sub>.** According to GP2, 60 mg ( $5.6 \times 10^{-2}$  mmol) of gold porphyrin **10** was dissolved in 16 ml EtOH, 4.5 mg (0.11 mmol) of NaOH was added, combined with 23.74 mg ( $67.2 \times 10^{-3}$  mmol) of diamine(dinitro)platinum(II) dissolved in 12 ml of a mixture EtOH/H<sub>2</sub>O(1/1). The mixture was stirred for 48h at room temperature. The solvents were evaporated. The complex was dissolved in DMF and precipitated with a saturated solution of NaCl. The precipitated complex **AuP-PtNH<sub>3</sub>** was filtered,

washed with water and dried. Yield (58 mg, 90%). IR (ATR): 1619  $\text{cm}^{-1}$  (C=O). UV-Vis ( $\text{CH}_2\text{Cl}_2$ , nm)  $\lambda_{\text{max}}$  (nm): 408 ( $\epsilon$   $2.8 \times 10^5 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$ ), 526 ( $1.3 \times 10^4$ ). NMR ( $^1\text{H}$ , DMSO- $d_6$ , 300MHz)  $\delta$  (ppm): 9.35-9.28 (m, 8H), 8.29-8.27 (d, 6H,  $J = 6.64$  Hz), 8.26-8.17 (d, 2H,  $J = 8.01$  Hz), 8.14-7.91 (m, 9H), 7.82-7.79 (d, 2H,  $J = 7.55$  Hz), 4.38-4.34 (t, 1H,  $J = 7.08$  Hz), 4.36-4.28(br, 6H), 3.55-3.54 (d, 2H,  $J = 5.31$  Hz). HRMS (ES+) [M+]  $m/z$  1151.2090 found, 1151.2090 calc. Elem. Anal. Exp C, 48.69; H, 3.48; N, 6.35. calc C, 48.56; H, 3.61; N, 6.5.

**AuP-PtCyDA.** According to GP2, 60 mg ( $5.6 \times 10^{-2}$  mmol) of gold porphyrin **10** was dissolved in 16 ml EtOH, 4.5 mg (0.11 mmol) of NaOH was added, combined with 27.66 mg ( $63.8 \times 10^{-3}$  mmol) of diamine(dinitro)platinum(II) dissolved in 12 ml of a mixture EtOH/H<sub>2</sub>O(1/1).The mixture was stirred for 48h at room temperature. The solvents were evaporated. The complex was dissolved in DMF and precipitated with a saturated solution of NaCl. The precipitated complex **AuP-PtCyDA** was filtered, washed with water and dried. Yield (61 mg, 90%). IR (ATR): 1626  $\text{cm}^{-1}$  (C=O).UV-Vis ( $\text{CH}_2\text{Cl}_2$ , nm)  $\lambda_{\text{max}}$  (nm): 408 ( $\epsilon$   $2.8 \times 10^5 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$ ), 526 ( $1.3 \times 10^4$ ). NMR ( $^1\text{H}$ , DMSO- $d_6$ , 300MHz)  $\delta$  (ppm): 9.34-9.30 (m, 8H), 8.29-8.26 (dd, 6H,  $J = 7.27, 7.63$  Hz), 8.18-8.15 (d, 2H,  $J = 8$  Hz), 7.98-7.91 (m, 9H), 7.81-7.78 (d, 2H,  $J = 8.36$  Hz), 6.13-5.99 (m, 2H), 5.45-5.32 (m, 2H), 4.35-4.30 (t, 1H,  $J = 6.56$  Hz), 3.44-3.42 (d, 2H,  $J = 6.82$  Hz), 2.15-2.12 (m, 2H), 1.86-1.82 (t, 2H,  $J = 8.76$  Hz), 1.48-1.46 (d, 2H,  $J = 9.28$  Hz), 1.23-1.21 (m, 2H), 1.06-1.01 (m, 2H). HRMS (ES+) [M+]  $m/z$  1231.2739 found, 1231.2716 calc. Elem. Anal. Exp C, 49.61; H, 3.79; N, 6.02. calc C, 49.3; H, 3.45; N, 6.34.

**AuP-PtPy.** According to GP2, 60 mg ( $5.6 \times 10^{-2}$  mmol) of gold porphyrin **10** was dissolved in 16 ml EtOH, 4.5 mg (0.11 mmol) of NaOH was added, combined with 23.88 mg ( $56 \times 10^{-3}$  mmol) of diamine(dinitro)platinum(II) dissolved in a mixture of 2.5 ml EtOH and 17.5 ml H<sub>2</sub>O. The solution was stirred for 48h at room temperature. The solvents were evaporated. The complex was dissolved in DMF and precipitated with a saturated solution of NaCl. The precipitated complex **AuP-PtPy** was filtered, washed with water and dried. Yield (61 mg, 90%). IR (ATR): 1631  $\text{cm}^{-1}$  (C=O). UV-Vis ( $\text{CH}_2\text{Cl}_2$ , nm)  $\lambda_{\text{max}}$  (nm): 408 ( $\epsilon$   $2.8 \times 10^5 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$ ), 526 ( $1.3 \times 10^4$ ). NMR ( $^1\text{H}$ , DMSO- $d_6$ , 300MHz)  $\delta$  (ppm): 9.33-9.30 (m, 8H), 8.52-8.50 (d, 1H,  $J = 5.78$  Hz), 8.27-8.25 (d, 6H,  $J = 6.39$  Hz), 8.16-8.13 (d, 2H,  $J = 7.92$  Hz), 8.13-7.90 (m, 9H), 7.83-7.80 (d, 2H,  $J = 7.92$  Hz), 7.67-7.64 (d, 1H,  $J = 7.94$  Hz), 7.55-7.50 (t, 1H,  $J = 7.12$  Hz), 6.61-6.59 (br, 2H), 4.39-4.35 (t, 1H,  $J = 6.75$  Hz), 4.17-4.13 (t, 2H,  $J = 5.44$  Hz), 3.46-3.44 (d, 2H,  $J = 5.44$  Hz). HRMS (ES+) [M+]  $m/z$  1225.2269 found, 1225.2247 calc. Elem. Anal. Exp C, 49.16; H, 3.55; N, 5.92. calc C, 48.81; H, 3.17; N, 6.28.

**Singlet oxygen detection.** Singlet oxygen emission was detected through a double ruled grating SPEX monochromator (600 grooves/mm blazed at 1  $\mu\text{m}$ ) and a long-wave pass (780 nm). All spectra were

measured in 4 faces quartz cuvettes. All the emission singlet oxygen luminescence have been displayed with the same absorbance (less than 0.2) with the lamp and photomultiplier correction.

### **Biological experiments**

**Cell culture.** Human breast adenocarcinoma cells (MCF-7) (purchased from ATCC) were cultured in Dulbecco Eagle's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Adult Human Dermal Fibroblast cells (FS-68) were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and 1% P/S. Both cell lines were allowed to grow in humidified atmosphere at 37 °C under 5 % CO<sub>2</sub>. For cytotoxic and phototoxic studies, the compounds (powder) are first diluted in DMSO at the concentration of 10 mM. Then, they are sonicated during 30 seconds and diluted at the required concentrations in culture medium of each cell line”.

**Cytotoxicity study.** MCF-7 and FS-68 cells were seeded into 96-well plates at a density of 10<sup>3</sup> cells/cm<sup>2</sup>. One day after cell growth, cells were incubated with or without different concentrations of compounds (from 0.01 to 100 μM) for 3 days. After the incubation time, cells were incubated with 0.5 mg mL<sup>-1</sup> MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) during 4 h to determine mitochondrial enzyme activity. Then, MTT precipitates were dissolved in ethanol/DMSO (1:1) solution and absorbance was measured at 540 nm.

**Phototoxicity assay.** Both cell lines, MCF-7 and FS-68 cells, were seeded into 384-well plates at a density of 10<sup>3</sup> cells/cm<sup>2</sup> and allowed to grow for 24 h. Cells were incubated or not (control) with 0.5 μM concentration of compounds solution for 24 h. After incubation, cells were exposed, or not, to light using mercury lamp of a fluorescence microscope at excitation ranges between 390-420 nm for 20 min (39 J.cm<sup>-2</sup>). Two days after, MTT assay was performed to evaluate the phototoxic effect of compounds.

**ROS production.** The detection of ROS was performed during the phototoxicity experiment. Forty five minutes before irradiation, cells were incubated with 20 μM concentration of DCFH<sub>2</sub>-DA (Cellular ROS Assay Kit, Abcam, USA). Cells were exposed to light using mercury lamp of a fluorescence microscope at excitation ranges between 390-420 nm for 20 min (39 J.cm<sup>-2</sup>). After irradiation, cells were washed and fluorescence emission of DCF was detected at 450 nm using fluorescent microscope.

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