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## Anti-cancer and radio-sensitizing properties of new bimetallic (*N*-Heterocyclic Carbene)-amine-Pt(II) complexes

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### Abstract

Bioactive NHC-transition metal complexes have shown promise as anti-cancer agents, but their potential use as radiosensitizers has been neglected so far. We disclose here a new series of bimetallic platinum(II) complexes displaying NHC-type bridging ligands, (bis-NHC)[*trans*-Pt(RNH<sub>2</sub>)I<sub>2</sub>]<sub>2</sub> that have been synthesized *via* a simple, two-step procedure. They display cytotoxicity in the micromolar range on cancerous cell lines, accumulate in cells and bind to genomic DNA, by inducing DNA damages. Notably, these bimetallic complexes demonstrate significant radiosensitizing effects on both ovarian cells A2780 and non-small lung carcinoma cells H1299. Further investigations revealed that bimetallic species make irradiation-induced DNA damages more persistent by inhibiting repair mechanisms. Indeed, a higher and persistent accumulation of both  $\gamma$ -H2AX and 53BP1 foci post-irradiation was detected, in the presence of the NHC-Pt complexes. Overall, we provide the first *in vitro* evidence for the radiosensitizing properties of NHC-platinum complexes, that suggests their potential use in combined chemo-radio therapy protocols.

### Introduction

Over the last two decades, NHC-transition metal complexes (NHC = N-Heterocyclic Carbene) have attracted significant attention not only for uses in organometallic chemistry and catalysis,<sup>1-9</sup> but also as bio-organometallics, notably due to the high stability of the NHC-metal bonds under physiological conditions.<sup>10-18</sup>

Our group provided a seminal contribution to the field of bioactive NHC metal complexes, when we brought NHCs into the area of cytotoxic platinum derivatives,<sup>19</sup> at a time when only gold, silver, palladium, copper, rhodium and ruthenium NHC complexes had been reported as antitumor or antimicrobial species.<sup>20-21</sup> We have disclosed *trans*-(NHC)Pt(amine)X<sub>2</sub> complexes (Figure 1A) as a readily accessible and easily tunable platform for cisplatin-like compounds that demonstrate

antiproliferative activity against both cisplatin-sensitive (CEM and H460) and cisplatin-resistant cell lines (A2780/DDP, CH1/DDP and SK-OV-3) at a micromolar level. The *trans*-coordination mode of the labile ligands X, that crucially differentiates these species with respect to cisplatin, induces peculiar biological properties including specific DNA bonding modes.<sup>22-23</sup>

Since then, several groups have been actively committed to the development of Pt-NHC complexes as potential anticancer drugs, as shown in recent review articles.<sup>16, 18</sup> Many efforts have been devoted to the chemistry of NHC-platinum complexes, in order to make available a large array of structural motifs and substitution patterns. Structural tuning enabled notably conjugation of the Pt drug with targeting agents. Parallel studies were intended to elucidate their mechanisms of action. Driven by the cisplatin analogy,<sup>24</sup> special focus was given initially on their interaction with DNA targets, since the first known series of Pt-NHC complexes display labile ligands and affinity to DNA bases. Later on, alternative mechanisms of action and new pharmacological applications have been highlighted that mostly result from special structural features of the metal complexes themselves. For instance, a series of platinumacyclic NHC complexes has been demonstrated to target Asparagine Synthetase,<sup>25-26</sup> while other series induce Immunogenic Cell Death (Figures 1B-C).<sup>27-28</sup> It must be emphasized that, unlike complexes A, these last complexes do not display labile ligands in the coordination sphere of platinum.

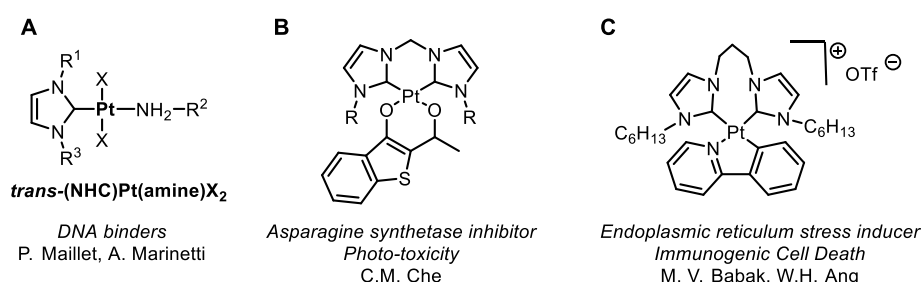


Figure 1. Antitumor NHC-platinum<sup>II</sup> complexes with different biological profiles

Despite the extensive research in the field of NHC-platinum complexes for various therapeutic applications, to the best of our knowledge, their usage as radiosensitizers in anti-cancer therapy has been overlooked so far. On the other hand, combination of cisplatin and derivatives with radiotherapy belongs to clinical standards of care for many types of cancers, including non-small cell lung cancer, ovarian cancer and cervical cancer, etc.).<sup>29</sup>

In this paper we report preliminary investigations on the radiosensitizing behavior of (NHC)PtI<sub>2</sub>(amine) complexes in cellular assays. We provide the first evidence that NHC-platinum complexes, including new bimetallic species, can have radiosensitizing effects on ovarian and non-small cell lung cancer cells.

## Results and Discussions

Since radiotherapy is based on the induction of DNA damage that leads to cell death if not correctly repaired, combination therapy relies notably on agents that impair DNA-damage repair and/or increase DNA damage.<sup>30-31</sup> Among them, metal-based DNA binding and damaging agents have emerged, including cisplatin<sup>®</sup> itself.<sup>32</sup> Indeed, patients treated with combined cisplatin-based chemotherapy and radiotherapy have significantly longer survival times than those treated with monotherapy protocols.<sup>29, 33</sup>

Thus, having in hand series of NHC-platinum complexes of the general formula (NHC)PtI<sub>2</sub>(amine) that operate as cytotoxic drugs through DNA-binding, we decided to explore their potential radiosensitizing properties, with the final aim to either potentiate radiotherapies or to increase their therapeutic windows. For these preliminary studies we have investigated radiosensitizing effects on the model ovarian A2780 and NSCLC H1299 cell lines, since the (NHC)PtI<sub>2</sub>(amine) complexes have demonstrated high levels of cytotoxicity and DNA binding on these cell lines.<sup>22-23</sup>

### Synthesis and characterization of the Platinum-NHC complexes

We focused our studies on the monometallic complexes **M1** and **M2**, as well as on the bimetallic (*bis*-NHC)[Pt(amine)I<sub>2</sub>]<sub>2</sub> complexes **D1-D4** in which two platinum<sup>(II)</sup> units are linked together by means of bis-NHC ligands (Figure 2).

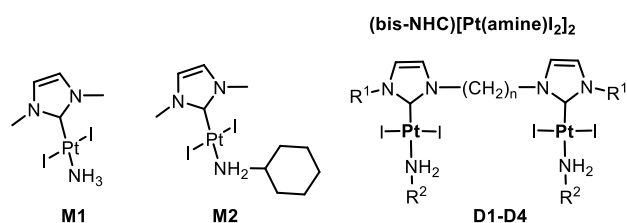


Figure 2. Mono- and bimetallic complexes displaying a *trans*-(NHC)Pt(amine)I<sub>2</sub> core

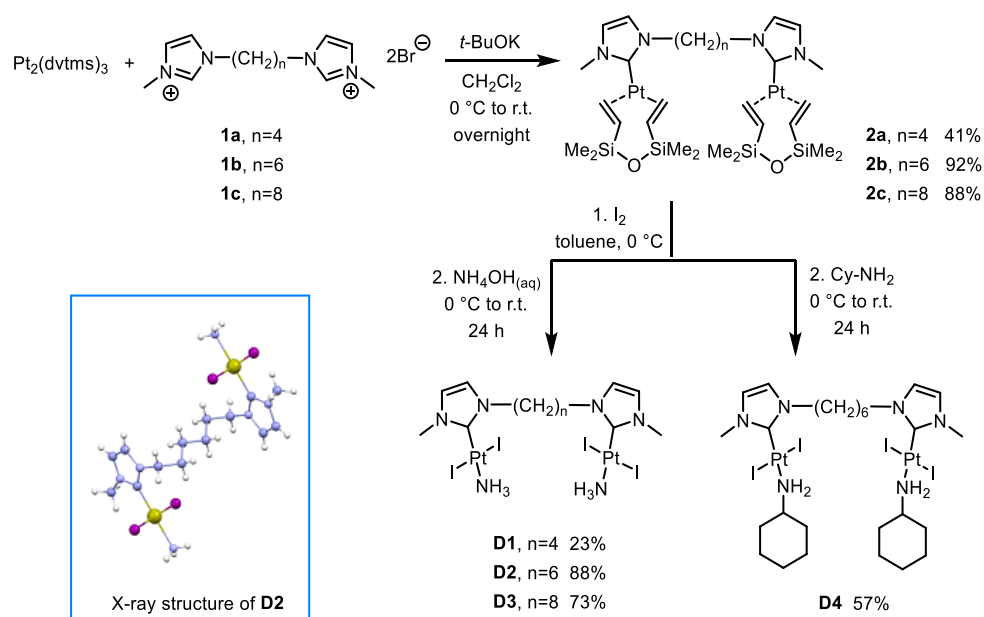
Complexes **M1** and **M2** belong to a known series.<sup>19</sup> Their synthesis and characterization, including the X-ray crystal structure of **M1**, are reported as Supporting Information (Tables S1, S2, Figures S1-S3). As far as we know, complexes of the general formula **D** haven't been reported before. In previous studies, analogous bis-NHC ligands have been used to build dinuclear cyclometalated platinum<sup>(II)</sup> complexes that induce apoptosis in cancer cells by inhibiting survivin expression, but do not interact with DNA.<sup>34</sup> Also, one binuclear Pt(II) and two Pt(IV) complexes displaying bis-NHC and pyridine ligands (instead of RNH<sub>2</sub> ligands) have been described previously which demonstrated antiproliferative activity on HCT116, MCF7 and PC3 cells.<sup>35</sup>

For the synthesis of the bimetallic complexes **D1-D4** we have adapted our general synthetic protocol<sup>19, 36</sup> that involves two main steps: coordination of an NHC ligand to a Pt<sup>(0)</sup>(dvtms) unit<sup>37</sup> and subsequent oxidation of Pt<sup>(0)</sup> to Pt<sup>(II)</sup> by addition of iodine, in the presence of the desired amine. The method is depicted in Scheme 1 hereafter.

The bis-imidazolium dibromides **1a-c** were prepared from 1-methylimidazole and the corresponding 1,n-dibromoalkanes.<sup>38-40</sup> They were treated then with potassium *tert*-butoxide to generate *in situ* the corresponding *N*-heterocyclic carbenes,<sup>41</sup> in the presence of a solution of platinum<sup>(0)</sup>-1,3-divinyl-1,1,3,3-tetramethyldisiloxane complex (Karstedt catalyst). The desired (bis-NHC)-Pt<sup>(0)</sup> complexes **2a-c** were obtained in 41%, 92% and 88% yields respectively. The second step involves oxidative addition of iodine to the Pt<sup>(0)</sup> complexes **2** and subsequent ligand exchange between the diene and ammonia (from aqueous ammonia solutions). The (bis-NHC)[Pt(NH<sub>3</sub>)I<sub>2</sub>]<sub>2</sub> complexes **D1**, **D2** and **D3** were obtained in good yields as yellow solids, after purification by column chromatography.

The bimetallic complex **D4** bearing a cyclohexylamine ligand, instead of an NH<sub>3</sub> ligand, has been prepared through the same procedure. It has been obtained in 57% yield.

Crystals suitable for X-ray diffraction studies could be obtained for complex **D2** (n=6). A view of the solid-state structure of this complex is provided in Scheme 1.



Scheme 1. Synthesis of the (bis-NHC)[Pt(RNH<sub>2</sub>)I<sub>2</sub>]<sub>2</sub> complexes and X-ray crystal structure of **D2**

The solubility of complexes **M** and **D** in water was evaluated by monitoring the UV-visible spectra of their solutions at various concentrations. All complexes are soluble in water in the concentration range 5-100 μM (Figure S4), except for **D1** that presents a linear relationship between absorbance and concentration only up to 20 μM. The stability of **D2** has been assessed by monitoring an aqueous 200 μM solution by UPLC-Mass during 24h (Figures S5 and S6). The aqueous solution of **D2** was prepared

under the conditions used for cell cultures, i.e. by dilution with water of a freshly prepared 5 mM DMSO solution. UPLC-MS data show that the complex undergoes iodide displacement, leading to four distinct compounds, but the resulting complexes retain the bimetallic core backbone. After initial equilibration, the mixture remains roughly unchanged over 24h. A DMSO adduct may be formed but it does not persist in water.

### ***Antiproliferative properties of the NHC-Pt complexes***

Prior to studies on radiosensitizing effects, we firstly established the anti-proliferative activities of the mono- and bimetallic NHC-Pt complexes on ovarian cancerous cells A2780 and non-small lung carcinoma (NSLC) cells H1299 since both cell lines are representative of cancers treated with a first-line platinum-based chemotherapy<sup>42</sup> or combined chemo-radiotherapy,<sup>29,33</sup> and are widely used as models for cell culture experiments. A third cell line, A2780/DDP, was chosen as a model for cisplatin resistant cells. All cell lines have been treated for 4 days (or 7 days) with increasing doses of the mono- and bimetallic complexes **M1**, **M2** and **D1-4**.

On the A2780 cell line, the bimetallic complexes **D1-4** showed  $IC_{50}$  values in the range 0.8-1.8  $\mu$ M. They give therefore the same levels of antiproliferative efficiency as previously found for the bimetallic complexes diamine $[(NHC)PtX_2]_2$  ( $IC_{50}$  around 1  $\mu$ M),<sup>22</sup> showing that the attachment site of the bridging chain (amine vs NHC nitrogen atoms) has moderate influence on the antiproliferative properties. However, the length of the linker chains in complexes **D** impacts their respective anti-proliferative activities, since **D1** (4 carbon atoms in the linker chain) is twice more potent than the bimetallic complexes **D2**, **D3** and **D4** that display 6 or 8 carbons in the linker chain (Table 1). The bimetallic complexes exhibit higher anti-proliferative activities than the corresponding monometallic complexes **M1** and **M2**, which might result from the twofold amount of possibly active platinum units in complexes **D**.

Most of the NHC-Pt complexes showed slightly higher anti-proliferative activities against H1299 cells than against A2780 cells, while cisplatin shows the opposite trend ( $IC_{50}$  = 0.3  $\mu$ M and 0.82  $\mu$ M for A2780 and H1299 cells respectively). Notably, the four bimetallic complexes showed no cross-resistance to cisplatin since they are all more potent than cisplatin against A2780/DDP cells. Especially complexes **D1** and **D2** demonstrate high cytotoxicity, with  $IC_{50}$  values lower than 1 $\mu$ M.

Interestingly, when the proliferation assays were run for seven days, the  $IC_{50}$  values did not change significantly with respect to the four days assays, except for complex **D1** in A2780 assays and complex **M1** in H1299 assays (Table 1). These seven days experiments prefigure the conditions of cell culture in the radiosensibilization assays that will be described hereafter.

It must be noted here that preincubation of the complexes for 6h in water or in culture media give the same IC<sub>50</sub> values as the fresh solutions prepared in water, indicating that the likely formation of protein-platinum adducts in the culture medium does not affect the cytotoxic activity.

Platinum complexes	A2780		A2780/DDP	H1299	
	4 days	7 days <sup>a</sup>	4 days	4 days	7 days <sup>a</sup>
<b>M1</b>	4.2± 0.9	3.5±0.2	5.9±0.9	2.5±0.8	5.6±1.2
<b>M2</b>	2.9±0.2	2.5±0.3	4.9±0.8	1.8±0.3	1.6±0.3
<b>D1</b>	0.8± 0.1	1.5±0.2	0.7±0.02	0.98±0.1	ND
<b>D2</b>	1.6± 0.2	1.6±0.2	0.95±0.1	1.3±0.3	1.45±0.4
<b>D3</b>	1.7± 0.3	1.9±0.2	2.7±0.2	1.1±0.3	2.5±0.1
<b>D4</b>	1.8± 0.1	1.9±0.2	1.7±0.15	1.7±0.5	ND
<b>Cisplatin</b>	0.3±0.01	ND	3.2±0.2	0.82±0.07	ND

Table 1. IC<sub>50</sub> (μM) values of the Pt-NHC complexes vs cisplatin on A2780, A2780/DDP and H1299 cells after 4 days or 7 days treatment. <sup>a</sup> Conditions used in the radiosensitization experiments hereafter. ND: not determined.

We also conducted anti-proliferation assays on the MCF10A cell, a spontaneously immortalized, non-malignant breast cell line, and hTERT-RPE1 cells, an immortalized human retinal epithelial cell line. All the complexes showed approximately two to five-fold lower cytotoxicity against these cell lines, than against the cancerous cell lines, indicating some selectivity for cancerous cell lines, mainly with the bimetallic complexes (see Table S3).

#### ***Cellular Platinum Accumulation and genomic DNA Platination***

Since the antiproliferative activity of the bimetallic complexes is almost twice that of the monometallic complexes in A2780 and H1299 cells, we wondered if it could be due to higher cellular uptake or DNA binding ability, or simply related to the twofold amount of platinum in their molecular structure. Thus, in order to clarify this point, we have compared the DNA binding properties of four representative complexes **M1**, **M2**, **D2** and **D4** at 1.5 μM concentration, which is around the IC<sub>50</sub> doses for the bimetallic species. We have determined both their cellular uptake and the amount of platinum bound to genomic DNA using the ICP-MS method.<sup>43</sup> The results in Table 2 show that the rate of intracellular accumulation of the bimetallic complexes **D2** and **D4** is almost 4 to 5 times higher than that of the monometallic complexes **M1**, **M2**. The same tendency has been found for their DNA binding efficiency, since the bimetallic complexes binds to genomic DNA up to 3 times more than monometallic complexes. Moreover, we also noticed that the cyclohexylamine ligand in **M2** and **D4** favors both the cellular uptake and DNA binding, compared to **M1** and **D2** respectively, that contain NH<sub>3</sub> ligands. While

the complexes behave similarly in both cell lines, their accumulation in H1299 cells is about 1.5 times higher than in A2780 cells.

Platinum complexes	A2780		H1299	
	Cellular uptake (ng Pt for 5x10 <sup>6</sup> cells)	Bound to DNA (pg Pt per mg of genomic DNA)	Cellular uptake (ng Pt for 5x10 <sup>6</sup> cells)	Bound to DNA (pg Pt per mg of genomic DNA)
<b>M1</b>	1.4±0.1	87±15	2.8±0.3	179±75
<b>M2</b>	3.2±0.1	112.1±20	6±0.2	91±35
<b>D2</b>	11.4±0.4 [5.7±0.2] <sup>a</sup>	388±70 [194±35] <sup>a</sup>	18±1 [8.8±0.1] <sup>a</sup>	327±90 [164±45] <sup>a</sup>
<b>D4</b>	30±4 [15±2] <sup>a</sup>	672.8±84 [336±42] <sup>a</sup>	48±4 [24±2] <sup>a</sup>	977±104 [488±52] <sup>a</sup>

Table 2. Cellular accumulation of platinum and platinum bound to DNA in A2780 and H1299 cells after 4 days treatment with **M1**, **M2**, **D2** and **D4** at 1.5µM. The amount of platinum is quantified on cell pellets or extracted genomic DNA digested with HNO<sub>3</sub> at 95°C and related to the total amount of cells (5 × 10<sup>6</sup> cells) or genomic DNA (mg). <sup>a</sup> Corrected value, representative of the amount of bimetallic complexes **D** entering the cells or binding DNA (1/2 of the measured amount of Pt).

Overall, these data indicate that the antiproliferative activities of the Pt-NHC complexes in A2780 and H1299 cells roughly correlate with their rate of cellular uptake and ability to bind DNA. Therefore, the increased bioactivity of bimetallic species might result not from a simple additive effect of the two platinum units, but rather from an increased uptake and DNA binding rate.

### **Induction of DNA damages**

In order to get more insights in the behavior of the NHC-Pt complexes above, we have explored next their capacity to induce DNA breaks. Most platinum complexes, including cisplatin, are known indeed to behave as genotoxic drugs<sup>24</sup> by inducing DNA cross-linking and subsequent DNA breaks, which stimulates H2AX phosphorylation and recruits γ-H2AX at the breaks.<sup>44</sup>

With this in mind, we have performed γ-H2AX foci immunofluorescence (IF) analysis assays on A2780 cells treated with the mono- and bimetallic NHC-Pt complexes, as well as with cisplatin, at their respective IC<sub>80</sub> concentrations for 4 days. Consistently with previous findings,<sup>44-45</sup> cisplatin induced significant accumulation of γ-H2AX foci. γ-H2AX foci were also detected in cells treated with the bimetallic NHC-Pt complexes **D1-D4** and the mono-metallic complex **M2**, but not with **M1** (Figure 3). This is consistent with the fact that **M1** binds less efficiently to DNA (Table 2) and suggests that **M1** might induce genomic toxicity through a different pathway, compared to the other complexes. Quantitative data show that the bimetallic complexes are more prone to induce γ-H2AX foci than the mono-metallic species **M2**. It is worth noting also that, in bimetallic NHC-Pt complexes, the length of



the linker strongly modulates their ability to induce DNA breaks, complex **D2** (n = 6) giving much higher induction than its analogue **D1** (n = 4), at their respective IC<sub>80</sub> concentrations.

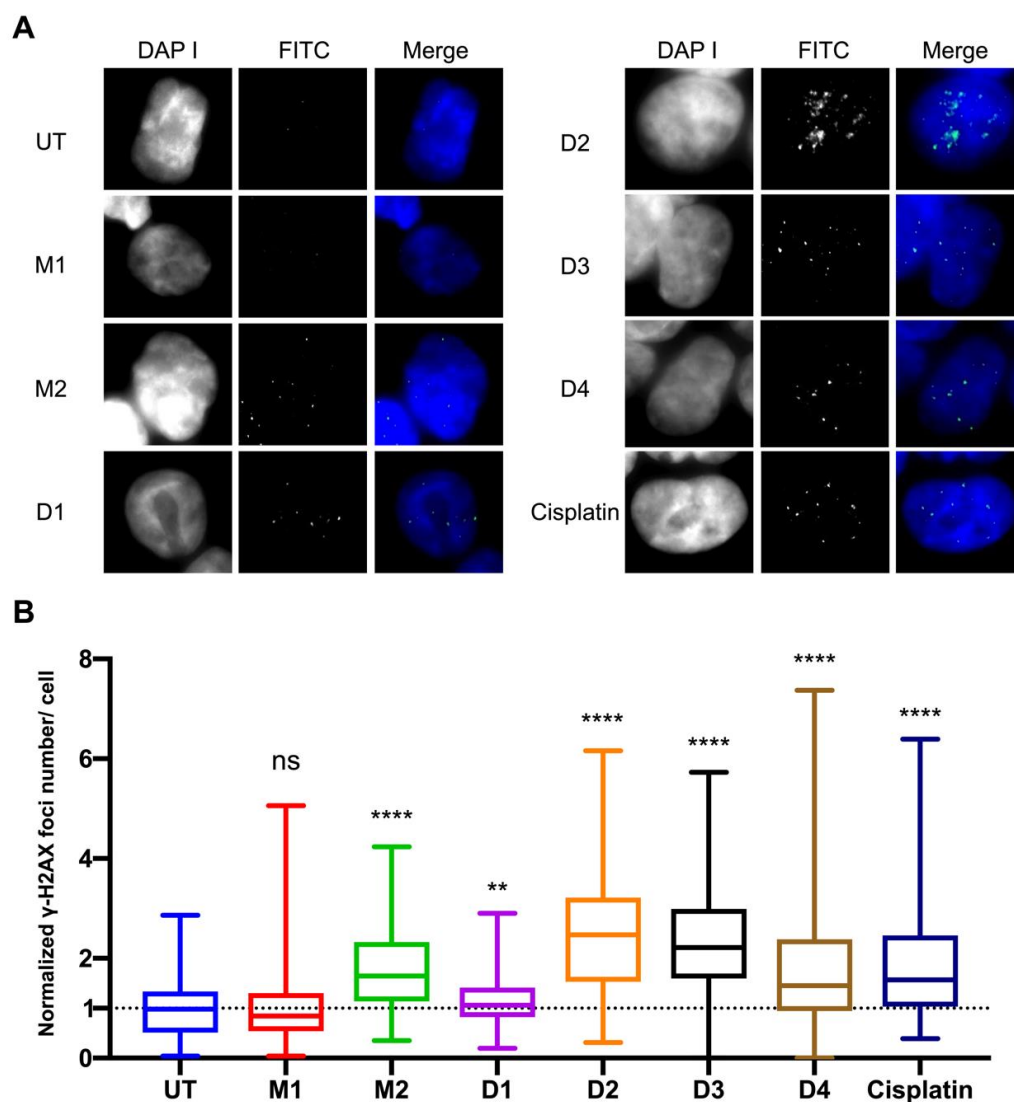


Figure 3. Activation of the DNA damages response. A2780 cells were treated for 96 h with **M1**, **M2**, **D1**, **D2**, **D3**, **D4** and cisplatin at doses that induce 80% growth inhibition (6, 3.5, 1, 2, 2, 2 and 0.5  $\mu$ M respectively). Cells were processed for IF using antibodies against  $\gamma$ -H2AX. (A) Representative Z projection of microscopy acquisitions of untreated and treated cells. (B) Number of  $\gamma$ -H2AX foci/cell, normalized to untreated sample (UT). The upper and lower ends of the boxes represent the interquartile range. The lines in the boxes represent the median values. Data result from two independent assays, each group having at least 200 cells counted for quantification. P values were calculated toward the UT: \*P < 0.05, \*\*P < 0.01, \*\*\*\*P < 0.0001, 2 paired t-Student test.

Taken together all these data indicate that complexes **D** and **M2** interact with DNA (Tables 1 and 2) and lead to DNA damages (Figure 3) that might account for their intrinsic cytotoxicity. This also open the way to possible uses of these complexes as radiosensitizing agents.

### ***Radiosensitizing properties***

Following the preliminary studies above on the antiproliferative and DNA binding properties of complexes **M** and **D**, we have considered the possibility of combining them with ionizing radiation in a synergistic manner. To the best of our knowledge, such radiosensitizing effects have never been reported before in the whole series of NHC-Pt complexes.

Complexes **M** and **D** have been evaluated for their radiosensitizing properties on A2780 and H1299 cell lines. We first showed that the two cell lines do not display the same radiosensitivity, based on their D10 values (D10 = doses of ionizing radiation allowing 10 % survival, six days post-irradiation). Indeed, D10 values determined by treating the cells with increasing doses of gamma-rays, were measured at  $3.5 \pm 0.2$  Gy for A2780 cells and  $9.7 \pm 0.3$  Gy for H1299 cells respectively (Figures 4A and 4B). The cells were treated then by increasing doses of gamma-rays in combination experiments, using the platinum complexes at 1  $\mu$ M concentration, that is at a dose that induces less than 20% proliferation inhibition in the absence of irradiation (see Table 1 for IC<sub>50</sub> values after 7 days treatment). Cells were treated with the platinum complexes one day before irradiation, then irradiated using a GSR D1 irradiator (gamma-ray, 662 keV) at the appropriate doses (1, 2, 3 and 4 Gys for A2780 and 2, 4, 6 and 8 Gys for H1299) and the D10 values were evaluated. Representative results obtained with complexes **M2** and **D2** are presented in Figures 4A and 4B for A2780 and H1299 cell lines respectively. We were pleased to see that both NHC-platinum complexes have radiosensitizing properties toward these cell lines. The radiosensitizing effects were reflected by the drop of the D10 values, with respect to those of untreated cells. The D10 values normalized to untreated cells (D10 ratios) are given in Figures 4C and 4D for all the mono- and bimetallic complexes, for the two cell lines. As shown in Figure 4, **M2** and **D2** display the most significant radiosensitizing effects at 1  $\mu$ M concentration in both cell lines.

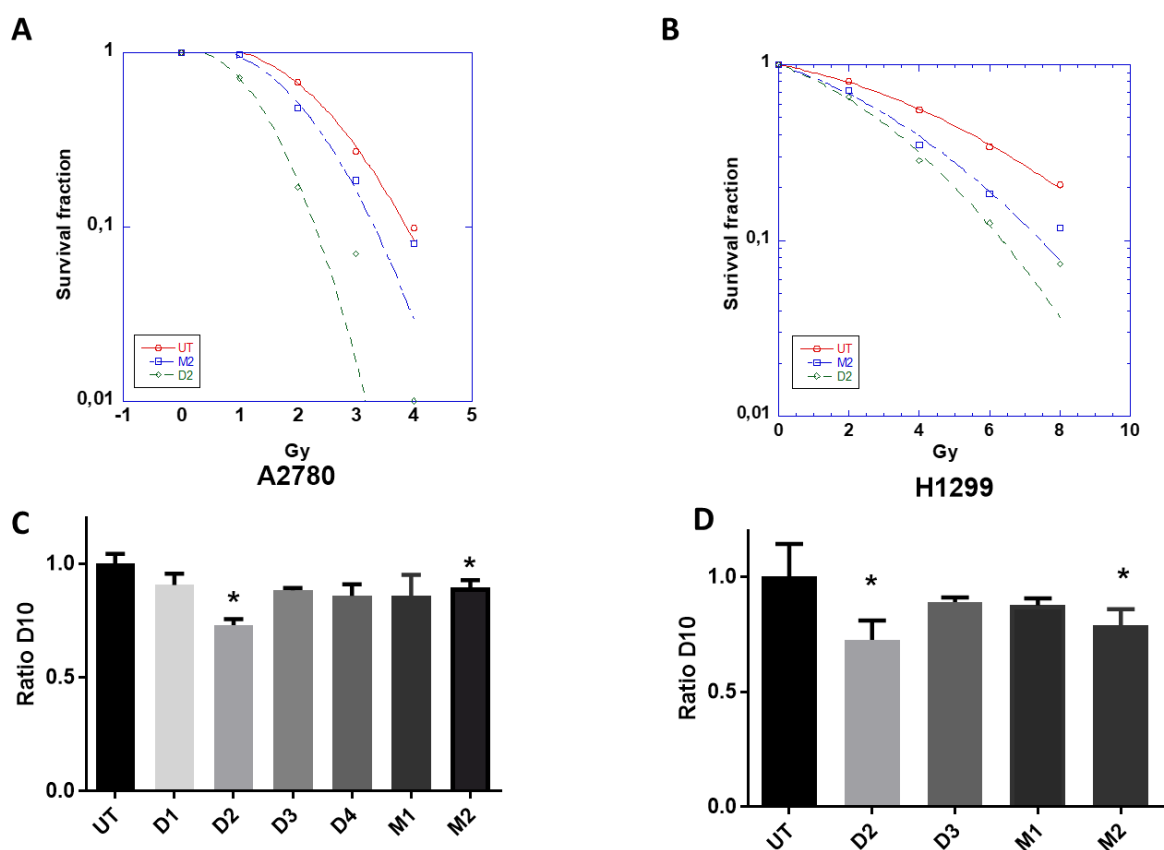


Figure 4. Survival curves of A2780 (A) and H1299 cells lines (B) following irradiation in the absence (UT) or in the presence of **M2** or **D2**. Ratios of D10 values for cells treated with  $1\mu\text{M}$  complexes vs untreated cells, in A2780 (C) and HT1299 (D) cell lines.  $\pm$  standard error from at least three replicates. P values were calculated toward the untreated sample (UT): \*P < 0.05, t-Student test.

Radiosensitization assays were then performed with increasing concentrations of complexes, at doses that induce less than 50% proliferation inhibition in the absence of irradiation (see Table 1 for  $\text{IC}_{50}$  values), that is in the range 1-5  $\mu\text{M}$  for monometallic complexes and 0.5-2  $\mu\text{M}$  for bimetallic complexes. Interestingly, under these conditions, radiosensitization is effective for both cell lines, for all complexes, while different trends can be noticed depending on the cell lines and the complexes used (Figures 5 and 6).

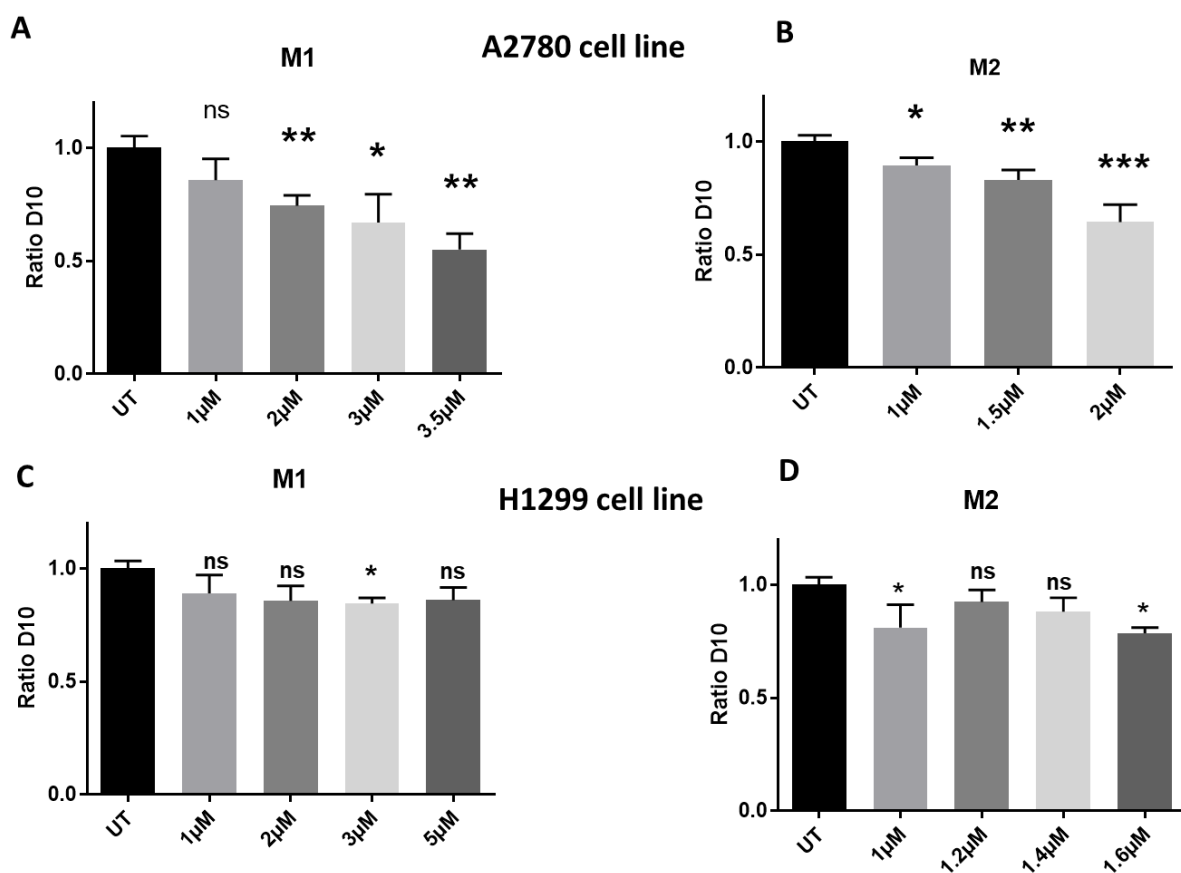


Figure 5. Radiosensitizing effects of monometallic NHC-Pt complexes at various concentrations. Ratios of D10 values for cells treated with complexes **M1** (A and C) and **M2** (B and D) vs D10 values for untreated cells (UT). A and B: A2780 cell lines; C and D: H1299 cell lines. Standard errors were calculated from at least three replicates. P values were calculated toward untreated samples: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, t-Student test.

The monometallic complexes proved to be more effective on the radiosensitive A2780 cells than on the radioresistant H1299 cell lines (Figure 5). For A2780 cells, the D10 ratio decreases up to 0.55 and 0.64 using **M1** and **M2** respectively, while for the H1299 cell line the D10 ratio reaches 0.87 and 0.78 for **M1** and **M2**, respectively. On both cell lines, **M2** is effective at lower doses than **M1**, which correlates with their respective cytotoxicity. The decrease of D10 is clearly dose dependent in A2780, whereas the trend is less pronounced in H1299 cells.

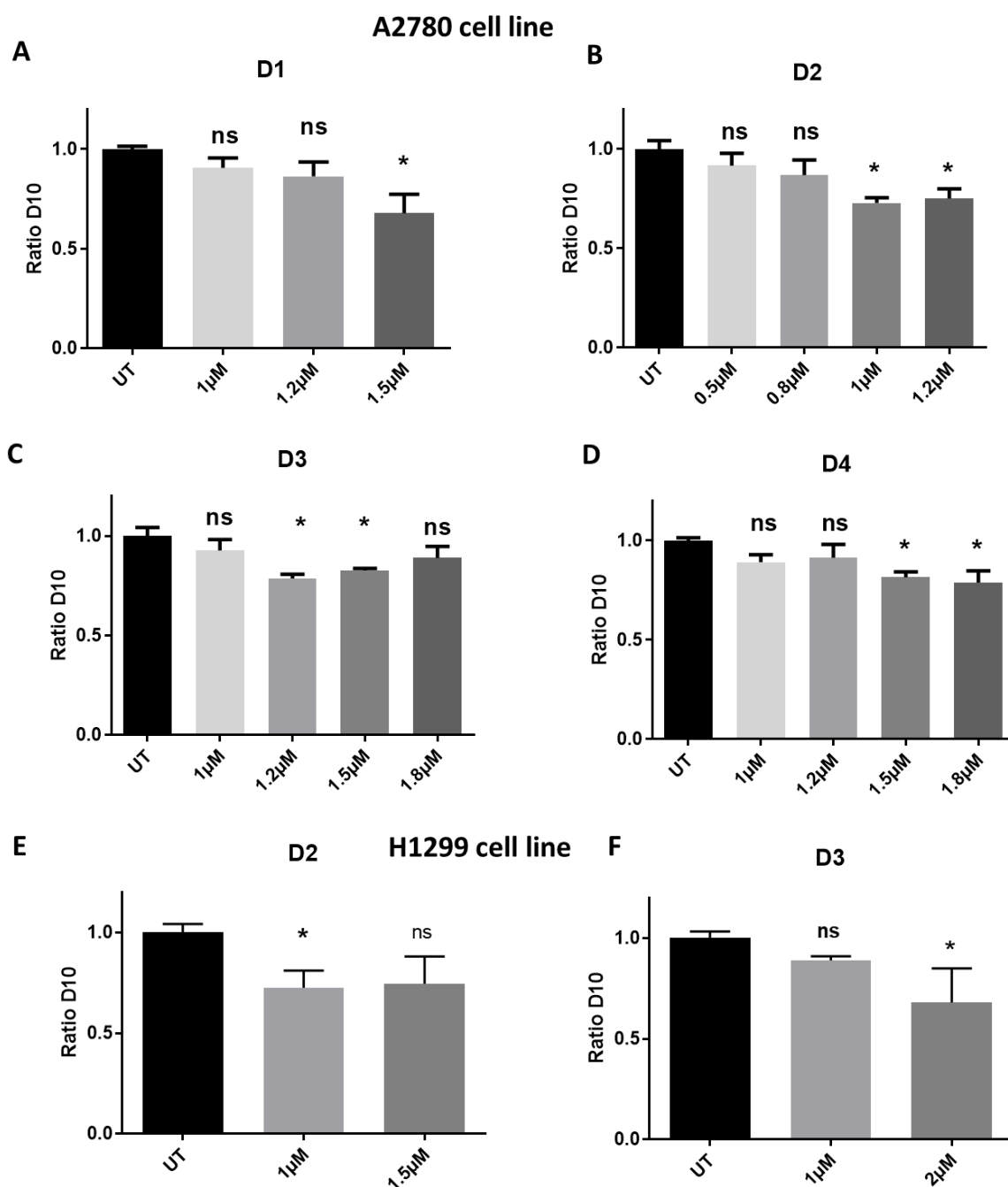


Figure 6. Radiosensitizing effects of the bimetallic complexes **D** at various concentrations. **Fig. 6A-D**: Ratios of D10 values for A2780 cells treated with complexes **D1-D4**, vs untreated cells (UT). **Fig. 6E-F**: Ratios of D10 values for H1299 cells treated with complexes **D2** and **D3**, to D10 values of untreated cells. Standard error from at least three replicates. P values were calculated toward the untreated sample (UT): \*P < 0.05, t-Student test.

On A2780 cells, the bimetallic complexes induce dose dependent decrease of the D10 ratios up to 0.67, 0.72, 0.78 and 0.81 for **D1**, **D2**, **D3** and **D4**, respectively (Figure 6). Complex **D2** is the most effective one, in a given dose range. It induces radiosensitization from 1 $\mu$ M concentration whereas radiosensitization is observed for the other complexes from 1.2 or 1.5 $\mu$ M. On H1299 cells, the two

representative complexes **D2** and **D3** display D10 ratios reaching 0.74 for **D2** and 0.68 for **D3**. Of note, except for **D1**, effective radiosensitization was obtained at concentration ranges that induce 20-50% inhibition in the absence of ionizing radiations.

### **Insights into the origin of the radiosensitization effect**

Overall, the results above demonstrate the radiosensitizing properties of the NHC-platinum complexes on the two cell lines. To go one step further, we have investigated then various radiosensitization protocols to get more insights into the mode of action of these complexes.

The treatment conditions, i.e. the time of incubation before irradiation as well as the removal or addition of the drug post-irradiation, have been varied in order to highlight how these parameters enable or hamper radiosensitizing effects. Representative results are given in Figure 7 for complex **D2** and additional examples are given in Figure S7. It appears that the NHC-platinum complexes must be preincubated for at least 1 to 4 days before irradiation to exert their radiosensitizing effects, whereas the presence of the complex post irradiation is not required. Figure 7 shows indeed that radiosensitization did not occur when the complexes were preincubated for only 10 min before irradiation, or added 10 min after irradiation. Thus, we can conclude that cellular uptake of NHC-Pt complexes and consequently their binding to DNA before IR is crucial in the radiosensitization process (Table 2).

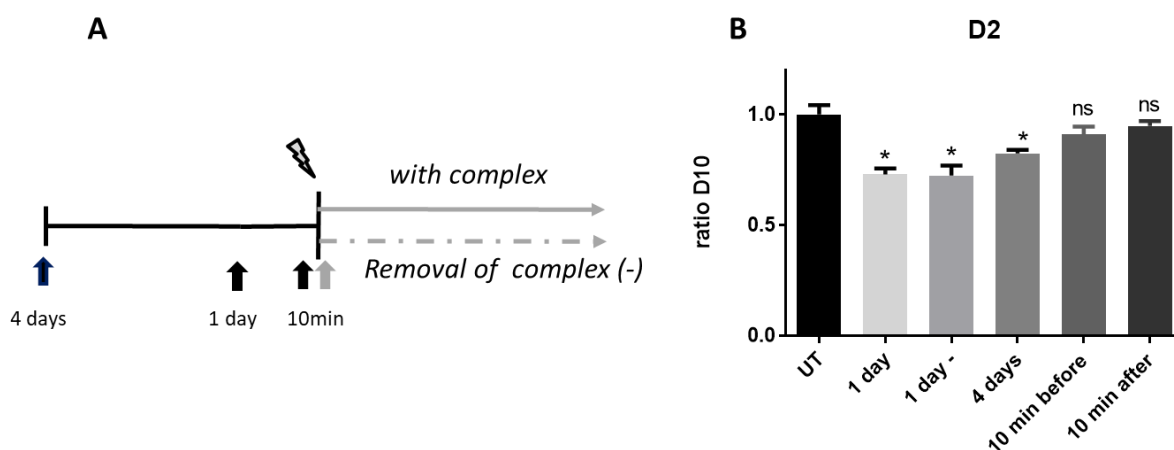


Figure 7. A) Chart of the treatment protocols used to measure radiosensitizing effects. B) Radiosensitizing effect of the bimetallic complex **D2** on A2780 cell lines, as a function of treatment conditions. Ratios of the D10 values for **D2** treated cells (1  $\mu$ M) vs untreated cells (UT). Conditions: 1 day incubation time before irradiation; 1 day incubation with removal of the residual complex from the culture medium after irradiation (1 day (-)); 4 days and 10 min incubation times before irradiation; addition of **D2** 10 min after irradiation. Standard error from three replicates. P values were calculated toward the untreated sample (UT): \*P < 0.05, t-Student test.

The radiosensitizing effects of DNA-interacting drugs can result from various biological responses, but are mainly due to increased IR-induced DNA damages and/or increased repair delays.<sup>46 47-48</sup> Thus, to

explore if NHC-Pt complexes' operate through one of these radiosensitization mechanisms, we have performed kinetic studies of IR-induced DNA damages, by comparing untreated A2780 cells and cells treated with representative NHC-Pt complexes. We have monitored the presence of both  $\gamma$ -H2AX (a DNA damage sensor) and 53BP1 foci (DNA damage repair sensor),<sup>49</sup> as a function of post-irradiation time. This study has been focused primarily on complexes **D2**, **M1** and **M2** since these three complexes have different abilities to induce DNA damages by their own (Figure 3), as well as different behaviors as radiosensitizing agents, complex **D2** being the most potent radiosensitizing agent among the NHC-Pt complexes tested so far (Figures 4-6).

In term of  $\gamma$ -H2AX foci induction post IR, 0.5 hours is the time span where the maximum of double strand breaks is induced.<sup>50</sup> After this time span, we detected a slight, but not statistically significant increase of the number of  $\gamma$ -H2AX foci in the presence of the NHC-Pt complexes **M2** and **D2** (Figures 8A and 8C). Remarkably, at 2 and 6 hours post IR, the combined **D2** treatment induced substantial and persistent  $\gamma$ -H2AX foci. At 24 hours post-IR, all the platinum complexes, **D2**, **M1** and **M2**, showed a significant induction of  $\gamma$ -H2AX foci.

In term of 53BP1 foci induction, at the short 0.5h time span post irradiation we did not detect a significantly higher induction in the presence of the platinum complexes as compared with untreated cells (UT) (Figures 8B and 8D). However, all the platinum complexes, and especially the bimetallic complex **D2**, showed a significantly higher number and larger size of 53BP1 foci at 24 hours post IR (Figures 8B, 8D and S8). These data correlate well with the  $\gamma$ -H2AX foci induction measurements above.

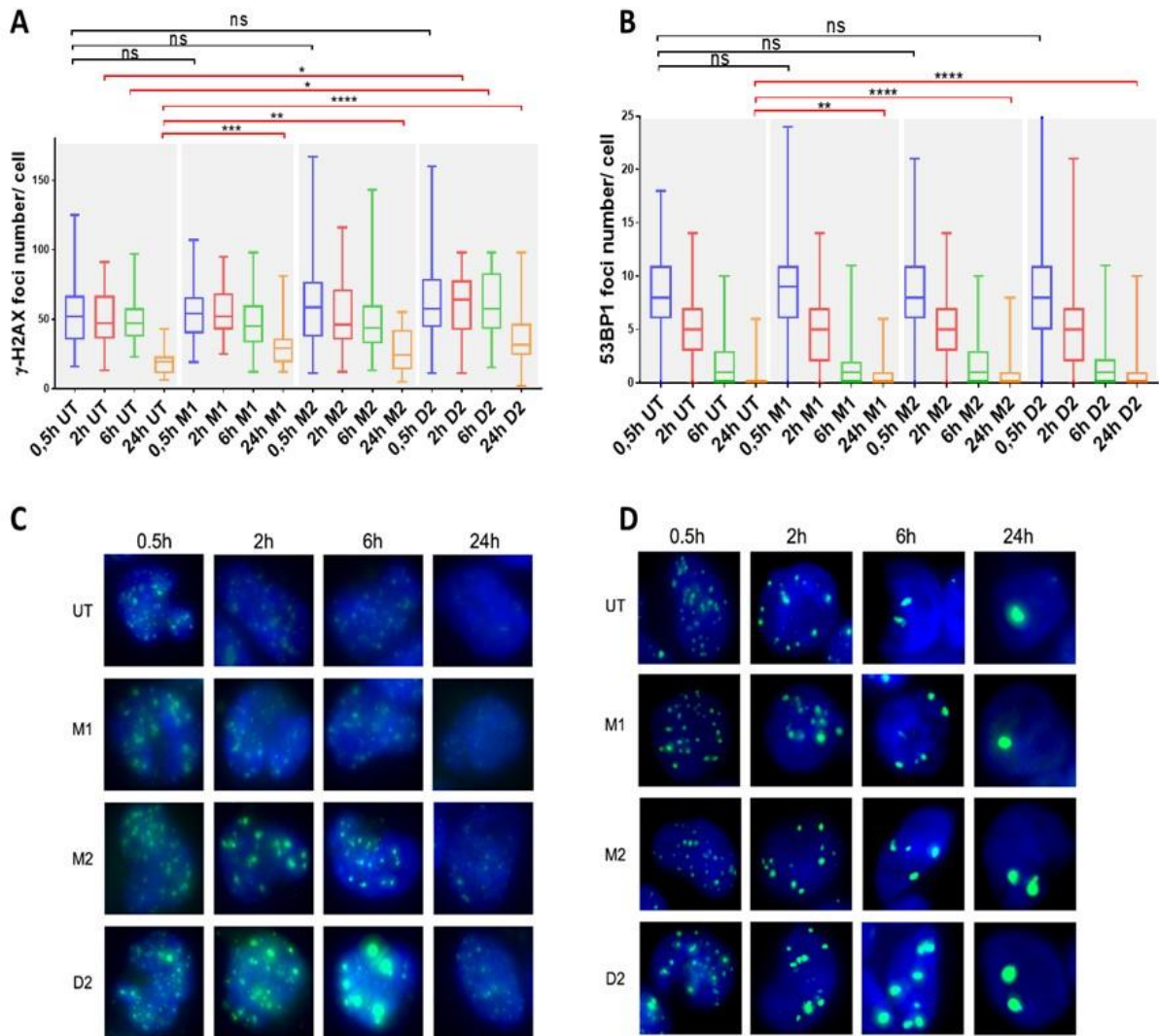


Figure 8: Kinetics of DNA damage repair post-irradiation. The A2780 cells were treated with **M1** (2  $\mu$ M), **M2** (1  $\mu$ M) and **D2** (1  $\mu$ M); after 1 day preincubation they were irradiated at 2 Gys. (A) Number of  $\gamma$ -H2AX foci formed at 0.5 h, 2 h, 6 h and 24 h post-irradiation. (B) Number of 53BP1 foci formed at 0.5 h, 2 h, 6 h and 24 h post-irradiation. The upper and lower ends of the boxes represent the interquartile range of foci number. The horizontal lines in the boxes represent the median foci number. Data from three independent assays, at least 200 cells have been used for quantification in each group. P values were calculated toward the untreated sample (UT): \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , t-Student test. (C) Representative Z projection of microscopy acquisitions of untreated and treated A2780 cells showing the  $\gamma$ H2AX foci at each time point post-irradiation. (D) Representative Z projection of microscopy acquisitions of untreated and treated A2780 cells showing the 53BP1 foci at each time point post-irradiation.

On the whole, these data suggest that the observed radiosensitizing effects of the complexes mainly correlate with their ability to inhibit DNA damage repair, while these complexes do not induce increased DNA damages during and shortly after irradiation. The most potent effects are produced by the bimetallic complex **D2** which slows down more efficiently the  $\gamma$ -H2AX and 53BP1 foci disappearance post-IR (Figures 8 and S8). The persistence of  $\gamma$ -H2AX and 53BP1 foci 24 hours post-



irradiation should result from DNA lesions that are hardly repaired in NHC-Pt treated cells and consequently continue to activate the 53BP1-mediated repair machine. The proposed radiosensitizing mechanism is sketched in Figure 9.

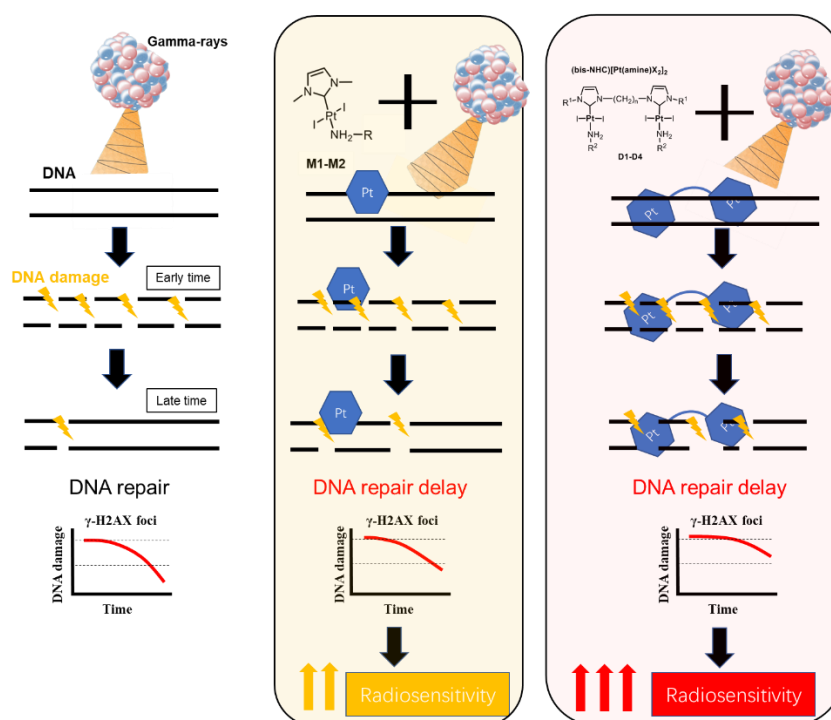


Figure 9: Incubation of NHC-Pt complexes before irradiation induces increased radiosensitivity, through inhibition of IR-induced DNA damage repair. Especially, the most potent radiosensitizing complex **D2** leads to a strongly delayed DNA repair.

## Conclusion

We have synthesized the unprecedented series of bimetallic  $[trans\text{-Pt}_2(\text{amine})]_2(\text{bis-NHC})$  complexes that have revealed high anti-proliferative activities in three cell lines. Notably these complexes are able to overcome cisplatin resistance in the ovarian A2780/DDP cells, with the most potent complexes **D1** and **D2** having  $IC_{50}$  values  $< 1\mu\text{M}$ . This complex also showed a two to five-fold higher selectivity for cancerous cell lines, with respect to non-cancerous cell lines. In most cases, the bimetallic NHC-Pt complexes are better antiproliferative agents and inducers of DNA damages than the corresponding monometallic complexes. This could be related to their increased cellular uptake and genomic DNA binding capacity as compared to monometallic complexes.

Although radiation/cisplatin combination therapy is well defined to be a standard care for many types of solid tumors,<sup>29</sup> the intrinsic high and broad toxicities of cisplatin, including gastrointestinal, myelosuppression, ototoxicity, neurotoxicity and nephrotoxicity, are always hindering its benefits for cancer patients and new radiosensitizing agents are needed to improve therapeutic windows.<sup>51</sup>

Therefore, in this study, the potential usage of the NHC-Pt complexes in combined radio-chemotherapy has been investigated.

Most interestingly, we have demonstrated that both bimetallic and monometallic complexes show dose-dependent radiosensitizing effects on two cell lines with different levels of intrinsic radiosensitivity, i.e. A2780 and H1299 cell lines. The concentration windows where they induce radiosensibilization, while having on their own a moderate antiproliferative effect (<50% inhibition), are in the range 1-2  $\mu$ M for complexes **D1**, **D2**, **D3**, **D4** and **M2**, and 2-3.5  $\mu$ M for **M1**. The monometallic complex **M2** and the bimetallic complex **D2** proved to be the most potent, displaying a radiosensitizing activity starting from 1  $\mu$ M doses.

In radiosensitization experiments, we have noticed that one day pre-incubation is sufficient for all the complexes, and their presence post-irradiation is not mandatory. This behavior suggests that only the NHC-Pt complexes bound to DNA before irradiation have a role in the radiosensitization process, and therefore the continuous treatment post-irradiation to form new DNA adducts is not required. The short duration of the treatment may represent an interesting feature from a pharmacological point of view, as it might reduce the side effects of the intrinsic toxicity of the drugs.

In line with the results above, it has been clearly noticed that the bimetallic complex **D2** and the monometallic complexes **M1** and **M2** induce delays in the repair of the IR-induced DNA damages. The repair slowdown has been highlighted by the increased number and size of 53BP1 foci, given that 53BP1 recruits proteins for repair. The persistence of 53BP1 foci up to two hours post-irradiation has been detected when using the bimetallic complex **D2**, while the effects are less marked with the two monometallic complexes. This divergent behavior can be assigned to different DNA coordination modes for the mono-metallic and bimetallic series of platinum complexes. Indeed, mono-metallic complexes are known to form predominantly short range intrastrand cross-links,<sup>22</sup> while bimetallic complexes are known to form long range intrastrand or interstrand cross-links.<sup>22, 52</sup> Moreover, our previous studies<sup>22</sup> have shown that bimetallic NHC-Pt complexes of the general formula  $[(\text{NHC})\text{PtI}_2]_2(\text{diamine})$  also form long range inter-strand DNA cross-links. Thus, we can postulate that the analogous bimetallic complexes (bis-NHC)[Pt(amine)I<sub>2</sub>]<sub>2</sub>, **D1-D4**, also bind to DNA via both platinum centers and form long range inter-strand DNA cross-links. These bulkier DNA adducts are expected to be more difficult to resolve by cell machinery than mono-metallic complexes, as they prevent the access of repair proteins to the DNA lesion sites.

Since the repair is crucial to genomic integrity and cellular survival after exposure to ionizing irradiation, this delay can explain, at least in part, the radiosensitizing properties of the NHC-Pt complexes. As proposed for other DNA binding radiosensitizers, such as platinum complexes<sup>46, 53</sup> and G-quadruplex ligands (RHPS4, Pt-ctpy),<sup>47-48</sup> the presence of platinum-DNA adducts near a double

strand break induced by IR, might impair its repair. Moreover, in addition to bind to DNA, NHC-Pt complexes were shown to induce DNA compaction.<sup>54</sup> If this is the case here, we can speculate that the access of the repair machinery to DNA damages will be compromised, which could explain the inhibition of IR-induced DNA damages.

Generally speaking, we cannot exclude that radiosensitization may also result from other biological effects.

In summary, we have synthesized a new series of NHC-Pt complexes by a rapid and efficient method. An *in vitro* radiotherapy testing protocol has been applied to different radiosensitive cells suggesting the possible use of the NHC-Pt complexes, and especially of bimetallic species such as **D2**, to modulate and increase the cells response to ionizing radiation via inhibition of DNA damage repair. The efficiency of these Pt(II) complexes in combined radio-chemotherapy must be confirmed by *in vivo* studies. These studies are ongoing in our group.

## EXPERIMENTAL SECTION

### General Methods for synthetic procedures.

All reagent-grade chemicals and solvents were used as received from the suppliers. Purifications were carried out either by flash chromatography using Macherey-Nagel Silica 60 M (40-63  $\mu\text{m}$ ) or on puriFlash XS 520 apparatus using Interchim columns loaded with 50  $\mu\text{m}$  silica.  $^1\text{H}$  NMR spectra (300.1 or 500.1 MHz) and  $^{13}\text{C}$  NMR spectra (75.5 and 125.8 MHz) were recorded on Bruker Avance spectrometers in deuterated solvent and the chemical shifts ( $\delta$ ) were calibrated using residual undeuterated solvent as an internal reference. Coupling constants to platinum refer to  $^{195}\text{Pt}$  couplings. IR were recorded on a Perkin-Elmer Spectrum 100 instrument. High-resolution mass spectra (HRMS) were recorded with a Micromass LCT Premier XE instrument (Waters), under electrospray ionization (ESI), coupled with a time of flight analyser (TOF).

The purity grade of the NHC-Pt complexes (>98%) was determined by reverse-phase liquid chromatography analysis, using an Aquity UPLC system (HSS T3 column) coupled with an ELS detector. Conditions: flow rate 0.6 mL/min, T = 40 °C, eluents water with 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B), gradients from 70% A to 100% B were used.

### General Procedures for the Synthesis of the (bis-NHC)[Pt(dvtmds)]<sub>2</sub> complexes 2a-c.

To a suspension of bis-imidazolium dibromide **1** in  $\text{CH}_2\text{Cl}_2$  (0.2 M) under argon was added a solution of the Karstedt's catalyst (platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane complex in xylene, ~2 % platinum). The mixture was cooled to 0 °C and solid *t*-BuOK was added in one portion. After 15 min., the mixture was allowed to warm up slowly to r.t. and stirred overnight. Then silica gel was added and

the solvents were removed under reduced pressure to provide a solid sample loading for purification by column chromatography.

**Complex 2a.** Obtained from 1,4-bis(3-methylimidazolium-1-yl)butane dibromide **1a**<sup>38</sup> (380 mg, 1.0 mmol), Karstedt's catalyst (23.0 mL, 2.0 mmol) and *t*-BuOK (315 mg, 2.8 mmol). Purification by chromatography (eluent: *n*-Heptane/EtOAc 7:3) afforded the title compound as a colorless oil (400 mg, 41% yield). **<sup>1</sup>H NMR (CDCl<sub>3</sub>, 500.1 MHz)** δ 6.98 (s, 2H, CH), 6.91 (s, 2H, CH), 3.83 (bs, 4H, NCH<sub>2</sub>), 3.49 (s, 6H, NCH<sub>3</sub>), 2.35-2.04 (m, 4H, C=CH<sub>2</sub>), 1.89-1.78 (m, 8H, C=CH<sub>2</sub> and SiCH), 1.60 (bs, 4H, CH<sub>2</sub>), 0.33 (s, 12H, SiCH<sub>3</sub>), -0.29 (s, 12H, SiCH<sub>3</sub>) ppm. **<sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.5 MHz)** δ 183.7 (Pt-C), 122.2 (CH), 120.4 (CH), 49.4 (NCH<sub>2</sub>), 40.2 (SiCH=C<sub>2</sub>H<sub>2</sub>), 37.0 (NCH<sub>3</sub>), 35.7 (SiCH=CH<sub>2</sub>), 27.6 (CH<sub>2</sub>), 1.6 (SiCH<sub>3</sub>), -1.6 (SiCH<sub>3</sub>) ppm.

**Complex 2b.** Obtained from 1,6-bis(3-methylimidazolium-1-yl)hexane dibromide **1b**<sup>40</sup> (391 mg, 0.96 mmol), Karstedt's catalyst (22.0 mL, 1.92 mmol) and *t*-BuOK (301 mg, 2.68 mmol). Purification by chromatography (eluent: *n*-Heptane/EtOAc 7:3) afforded the title compound as a colorless oil (889 mg, 92% yield). **<sup>1</sup>H NMR (CDCl<sub>3</sub>, 500.1 MHz)** δ 6.98 (s, 2H, CH), 6.96 (s, 2H, CH), 3.82 (t, *J* = 7.2 Hz, 4H, NCH<sub>2</sub>), 3.50 (s, 6H, NCH<sub>3</sub>), 2.26-2.12 (m, 4H, C=CH<sub>2</sub>), 1.93-1.75 (m, 8H, C=CH<sub>2</sub>, SiCH), 1.61 (t, *J* = 7.2 Hz, 4H, CH<sub>2</sub>), 1.19 (t, *J* = 7.2 Hz, 4H, CH<sub>2</sub>), 0.33 (s, 12H, SiCH<sub>3</sub>), -0.29 (s, 12H, SiCH<sub>3</sub>) ppm. **<sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.5 MHz)** δ 183.5 (Pt-C), 122.0 (*J*<sub>C-Pt</sub> = 37.5 Hz, CH), 120.4 (*J*<sub>C-Pt</sub> = 36.0 Hz, CH), 49.8 (*J*<sub>C-Pt</sub> = 40.5 Hz, NCH<sub>2</sub>), 40.1 (*J*<sub>C-Pt</sub> = 158.9 Hz, SiCH=C<sub>2</sub>H<sub>2</sub>), 37.0 (*J*<sub>C-Pt</sub> = 45.0 Hz, NCH<sub>3</sub>), 34.1 (*J*<sub>C-Pt</sub> = 118.4 Hz, SiCH=CH<sub>2</sub>), 30.5 (CH<sub>2</sub>), 26.3 (CH<sub>2</sub>), 1.6 (SiCH<sub>3</sub>), -1.7 (SiCH<sub>3</sub>) ppm. **HRMS (ESI+)** calcd. for C<sub>30</sub>H<sub>58</sub>N<sub>4</sub>O<sub>2</sub>Si<sub>4</sub>Pt<sub>2</sub>Na [M+Na]<sup>+</sup>: 1031.2830, found: 1031.2823. **IR (ν/cm<sup>-1</sup>)** 2952, 1456, 1401, 1296, 1244, 1170, 986, 859, 835, 780, 678.

**Complex 2c.** Obtained from 1,8-bis(3-methylimidazolium-1-yl)octane dibromide **1c**<sup>39</sup> (417 mg, 0.96 mmol), Karstedt's catalyst (22.0 mL, 1.92 mmol) and *t*-BuOK (300 mg, 2.68 mmol). Purification by chromatography (eluent: *n*-Heptane/EtOAc 7:3) afforded the title compound as a colorless oil (871 mg, 88% yield). **<sup>1</sup>H NMR (CDCl<sub>3</sub>, 500.1 MHz)** δ 6.99 (bs, 4H, CH), 3.84 (t, *J* = 7.7 Hz, 4H, NCH<sub>2</sub>), 3.50 (s, 6H, NCH<sub>3</sub>), 2.26-2.13 (m, 4H, C=CH<sub>2</sub>), 1.94-1.76 (m, 8H, C=CH<sub>2</sub>, SiCH), 1.63 (bs, 4H, CH<sub>2</sub>), 1.19 (bs, 8H, CH<sub>2</sub>), 0.32 (s, 12H, SiCH<sub>3</sub>), -0.29 (s, 12H, SiCH<sub>3</sub>) ppm. **<sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.5 MHz)** δ 183.4 (Pt-C), 122.0 (*J*<sub>C-Pt</sub> = 35.6 Hz, C<sub>imi</sub>), 120.4 (*J*<sub>C-Pt</sub> = 38.4 Hz, C<sub>imi</sub>), 50.0 (*J*<sub>C-Pt</sub> = 39.9 Hz, NCH<sub>2</sub>), 40.1 (*J*<sub>C-Pt</sub> = 158.1 Hz, SiCH=C<sub>2</sub>H<sub>2</sub>), 36.9 (*J*<sub>C-Pt</sub> = 45.6 Hz, NCH<sub>3</sub>), 34.1 (*J*<sub>C-Pt</sub> = 118.2 Hz, SiCH=CH<sub>2</sub>), 30.6 (CH<sub>2</sub>), 29.1 (CH<sub>2</sub>), 26.6 (CH<sub>2</sub>), 1.6 (SiCH<sub>3</sub>), -1.7 (SiCH<sub>3</sub>) ppm. **HRMS (ESI+)** calcd. for C<sub>32</sub>H<sub>62</sub>N<sub>4</sub>O<sub>2</sub>Si<sub>4</sub>Pt<sub>2</sub>Na [M+Na]<sup>+</sup>: 1059.3143, found: 1059.3125. **IR (ν/cm<sup>-1</sup>)** 2930, 2857, 1456, 1403, 1296, 1245, 1170, 1002, 989, 859, 835, 780, 678.

#### General Procedures for the Synthesis of the (bis-NHC)[Pt(amine)I<sub>2</sub>]<sub>2</sub> complexes D1-D4.

To a solution of (bis-NHC)[Pt(dvtmds)]<sub>2</sub>, **2**, in toluene (0.1 M) under argon at 0 °C was added dropwise a solution of iodine in toluene (0.5 M). Then, the desired amine was added and the mixture was allowed

to warm up slowly to r.t. and stirred for 24 hours. The crude product was purified either by recrystallization or by chromatography. After chromatography, the solvents were removed and the pasty solid residue was taken up with diethyl oxide and triturated to provide a solid suspension. After decantation, the supernatant liquid was carefully removed and the residue was dried under vacuum to afford the desired compounds as pale yellow crystalline powders.

**Complex D1.** Obtained from **2a** (360 mg, 0.37 mmol), iodine (205 mg, 0.81 mmol) and 28% aqueous ammonia (255  $\mu$ L, 1.83 mmol). The solvents were removed under reduced pressure and the crude product was purified by recrystallization from  $\text{CH}_2\text{Cl}_2/n$ -heptane to afford **D1** as a pale yellow powder (96 mg, 23% yield).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 500.1 MHz)  $\delta$  6.85 (s, 2H, CH), 6.83 (s, 2H, CH), 4.73 (bs, 4H,  $\text{NCH}_2$ ), 3.87 (s, 6H,  $\text{NCH}_3$ ), 2.84 (bs, 6H,  $\text{NH}_3$ ), 2.09 (bs, 4H,  $\text{CH}_2$ ) ppm.  $^{13}\text{C NMR}$  ( $\text{CDCl}_3$ , 125.8 MHz)  $\delta$  138.9 (Pt-C), 122.3 (CH), 120.4 ( $\text{CH}_i$ ), 49.4 ( $\text{NCH}_2$ ), 38.4 ( $\text{NCH}_3$ ), 25.8 ( $\text{CH}_2$ ) ppm. **Anal. calcd.** for  $\text{C}_{12}\text{H}_{24}\text{I}_4\text{N}_6\text{Pt}_2$ , C: 12.53, H: 2.10, N: 7.31; Found, C: 12.67, H: 2.11, N: 7.11.

**Complex D2.** Obtained from **2b** (112 mg, 0.11 mmol), iodine (62 mg, 0.24 mmol) and 28% aqueous ammonia (75  $\mu$ L, 0.55 mmol). The crude product was purified by chromatography (eluent:  $\text{CH}_2\text{Cl}_2/\text{acetone}$  100:0 to 98:2) to afford **D2** as a pale-yellow powder (115 mg, 88% yield).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 698.7 MHz)  $\delta$  6.85 (d,  $J = 2.0$  Hz, 2H, CH), 6.81 (d,  $J = 2.0$  Hz, 2H, CH), 4.35 (t,  $J = 7.9$  Hz, 4H,  $\text{NCH}_2$ ), 3.86 (s, 6H,  $\text{NCH}_3$ ), 2.63 (bs, 6H,  $\text{NH}_3$ ), 2.04-2.00 (m, 4H,  $\text{CH}_2$ ), 1.52-1.50 (m, 4H,  $\text{CH}_2$ ).  $^{13}\text{C NMR}$  ( $\text{CDCl}_3$ , 175.7 MHz)  $\delta$  137.7 (Pt-C), 122.1 (CH), 120.5 (CH), 50.7 ( $\text{NCH}_2$ ), 38.4 ( $\text{NCH}_3$ ), 29.3 ( $\text{CH}_2$ ), 26.0 ( $\text{CH}_2$ ) ppm. **HRMS (ESI+)** calcd. for  $\text{C}_{14}\text{H}_{28}\text{I}_3\text{N}_6\text{Pt}_2$   $[\text{M-I}]^+$ : 1050.8805, found: 1050.8848. **IR ( $\nu/\text{cm}^{-1}$ )** 3309, 3239, 3165, 2925, 2899, 1698, 1606, 1467, 1420, 1253, 1083, 728, 690.

**Complex D3.** Obtained from **2c** (144 mg, 0.14 mmol), iodine (78 mg, 0.31 mmol) and 28% aqueous ammonia (100  $\mu$ L, 0.70 mmol). The crude product was purified by chromatography (eluent:  $\text{CH}_2\text{Cl}_2/\text{acetone}$  100:0 to 99:1) to afford **D3** as a pale-yellow powder (123 mg, 73% yield).  $^1\text{H NMR}$  ( $\text{Acetone-}d_6$ , 300.1 MHz)  $\delta$  7.16 (d,  $J = 2.1$  Hz, 2H, CH), 7.13 (d,  $J = 2.1$  Hz, 2H, CH), 4.34 (t,  $J = 7.6$  Hz, 4H,  $\text{NCH}_2$ ), 3.83 (s, 6H,  $\text{NCH}_3$ ), 3.13 (br, 6H,  $\text{NH}_3$ ), 2.07-2.00 (m, 4H,  $\text{CH}_2$ ), 1.42 (bs, 8H,  $\text{CH}_2$ ) ppm.  $^{13}\text{C NMR}$  ( $\text{Acetone-}d_6$ , 75.5 MHz)  $\delta$  140.4 (Pt-C), 122.5 (CH), 121.2 (CH), 50.8 ( $\text{NCH}_2$ ), 37.9 ( $\text{NCH}_3$ ), 30.0 ( $\text{CH}_2$ ), 29.4 ( $\text{CH}_2$ ), 26.9 ( $\text{CH}_2$ ) ppm. **HRMS (ESI+)** calcd. for  $\text{C}_{16}\text{H}_{32}\text{I}_3\text{N}_6\text{Pt}_2$   $[\text{M-I}]^+$ : 1078.9118, found: 1078.9139. **IR ( $\nu/\text{cm}^{-1}$ )** 3306, 3237, 3164, 3125, 2926, 2854, 1696, 1605, 1466, 1420, 1250, 1047, 690.

**Complex D4.** Obtained from **2b** (126 mg, 0.12 mmol), iodine (70 mg, 0.26 mmol) and cyclohexylamine (30  $\mu$ L, 0.25 mmol). The crude product was purified by chromatography (eluent:  $\text{CH}_2\text{Cl}_2/\text{toluene}$  8:2) to afford **D4** as a pale-yellow powder (96 mg, 57% yield).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 300.1 MHz)  $\delta$  6.84 (d,  $J = 2.0$  Hz, 2H, CH), 6.79 (d,  $J = 2.0$  Hz, 2H, CH), 4.33 (t,  $J = 7.5$  Hz, 4H,  $\text{NCH}_2$ ), 3.85 (s, 6H,  $\text{NCH}_3$ ), 3.31-3.19 (m, 2H, Cy), 2.99-2.83 (m, 4H,  $\text{NH}_2\text{Cy}$ ), 2.30-2.27 (m, 4H, Cy), 2.06-1.96 (m, 4H,  $\text{CH}_2$ ), 1.81-1.76 (m, 4H, Cy),

1.66-1.45 (m, 8H, Cy, CH<sub>2</sub>), 1.36-1.27 (m, 4H, CH<sub>2</sub>), 1.20-1.11 (m, 4H, Cy) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.5 MHz) δ 138.8 (Pt-C), 121.9 (CH), 120.5 (CH), 54.9 (NCH), 50.6 (NCH<sub>2</sub>), 38.2 (NCH<sub>3</sub>), 36.0 (Cy), 29.4 (CH<sub>2</sub>), 26.2 (CH<sub>2</sub>), 25.5 (Cy), 25.0 (Cy) ppm. HRMS (ESI+) calcd. for C<sub>26</sub>H<sub>48</sub>I<sub>3</sub>N<sub>6</sub>Pt<sub>2</sub> [M-I]<sup>+</sup>: 1215.0370, found: 1215.0388. IR (ν/cm<sup>-1</sup>) 3218, 3125, 2925, 2853, 1713, 1571, 1466, 1447, 1376, 1224, 1139, 911, 730, 700.

*trans*-Diiodo(amine)(1,3-dimethylimidazol-2-ylidene)platinum (**M1**). Obtained from (1,3-dimethylimidazol-2-ylidene)(dvtmds)]platinum<sup>37</sup> (52 mg, 0.11 mmol), iodine (30 mg, 0.12 mmol) and 28% aqueous solution of ammonia (35 μL, 0.26 mmol). The crude product was purified by chromatography (eluent: *n*-heptane/EtOAc 7:3) to afford **M1** as a pale-yellow powder (44 mg, 72% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300.1 MHz) δ 6.80 (s, 2H, CH), 3.87 (s, 6H, NCH<sub>3</sub>), 2.60 (bs, 6H, NH<sub>3</sub>) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>, 175.7 MHz) δ 138.5 (Pt-C), 121.9 (CH), 38.2 (NCH<sub>3</sub>) ppm. HRMS (ESI+) calcd. for C<sub>7</sub>H<sub>14</sub>IN<sub>4</sub>Pt [M-I+CH<sub>3</sub>CN]<sup>+</sup>: 475.9911, found: 475.9918.

*trans*-Diiodo(*N*-cyclohexylamine)(1,3-dimethylimidazol-2-ylidene)platinum (**M2**). Obtained from (1,3-dimethylimidazol-2-ylidene)(dvtmds)]platinum<sup>37</sup> (310 mg, 0.65 mmol), iodine (181 mg, 0.71 mmol) and cyclohexylamine (75 μL, 0.65 mmol). The crude product was purified by chromatography (eluent: *n*-heptane/EtOAc 7:3) to afford **M2** as a pale-yellow powder (270 mg, 65% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300.1 MHz) δ 6.79 (s, 2H, CH), 3.85 (s, 6H, NCH<sub>3</sub>), 3.27-3.23 (m, 1H, NCH), 2.95-2.89 (m, 2H, NH<sub>2</sub>), 2.31-2.27 (m, 2H, Cy), 1.80-1.61 (m, 4H, Cy), 1.36-1.11 (m, 4H, Cy) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.5 MHz) δ 139.6 (Pt-C), 121.8 (CH), 55.0 (NCH), 38.1 (NCH<sub>3</sub>), 36.1 (CH<sub>2</sub>), 25.5 (CH<sub>2</sub>), 25.0 (CH<sub>2</sub>) ppm. HRMS (ESI+) calcd. for C<sub>13</sub>H<sub>24</sub>IN<sub>4</sub>Pt [M-I+CH<sub>3</sub>CN]<sup>+</sup>: 558,0694, found: 558,0719.

### Cell culture.

The A2780 ovarian carcinoma cell lines were purchased from ECACC (Salisbury, UK) and were grown in complete RPMI medium supplemented with 10% fetal calf serum, in the presence of penicillin-streptomycin. The resistance of A2780/DDP cells to cisplatin was maintained by monthly treatment with 1 μM cisplatin for 4 days. H1299 Non-Small-Lung Carcinoma cell lines (ATCC® CRL-5803™) were grown in RPMI medium supplemented with 10% fetal calf serum, 1% HEPES, 1% Sodium Pyruvate in the presence of penicillin and streptomycin. MCF10A (ATCC® CRL-10317™) were grown in DMEM/F12 supplemented with 5% Horse serum + 20ng/ml EGF, 100 ng/ml cholera toxin E, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone. hTERT-RPE1 (ATCC®-CRL-4000™) cells were grown in DMEM medium supplemented with 10% fetal calf serum, in the presence of penicillin-streptomycin.

### Cell proliferation assays.

Cells were treated with various concentrations of **NHC-Pt** complexes, at 37°C, under humidity and 5% CO<sub>2</sub> concentration, for 96h. Cellular growth was quantified using the Moxi Z Mini automated cell

counter (Orflox technology). CellTiter Glo® Luminescent Cell Viability Assay following the kit instructions (Promega) was also used, however we privileged cell counting since IC<sub>50</sub> values not always correlated to the number of cells, as previously observed.<sup>55</sup> **NHC-Pt** complexes were dissolved in DMSO, at a 5 mM concentration and conserved at -20°C. Diluted solutions in water were freshly prepared.

### **Platinum uptake measurement**

The platinum cellular uptake was quantified by ICP-MS (inductively coupled plasma mass spectrometry, NexION 2000, Perkin Elmer) on cellular pellets ( $5 \times 10^6$  cells), DNA extracts (using DNeasy kit from Qiagen) as previously described.<sup>45</sup> A2780 and H1299 cells were treated with 1.5µM **M1**, **M2**, **D2** or **D4** for 96 h. Trypan blue assay using the LUNA-II™ Automated Cell Counter showed that more than 96% of cells post NHC-Pt complexes' treatments remain viable. Around 20 µg DNA (quantified by nanodrop) was extracted from pellets using the DNeasy Blood & Tissue Kit from Qiagen. ICP-MS has been used also for monitoring the solubilization of platinum complexes in the aqueous solution. Prior to ICP-MS, the samples were digested with PlasmaPURE Plus HNO<sub>3</sub> (69%) at 95°C for cell pellets, and HNO<sub>3</sub> 0.1M for DNA. The Pt content was determined following a dose response curve established from known concentrations of cisplatin. The amount of platinum was then reported as ng of Pt/ $5 \times 10^6$  cells or pg Pt/µg DNA

### **Radiosensitization assay**

For one day or 10 min pretreatment, cells were seeded at a number allowing 7 days growth without reaching confluence in the untreated samples. They were then pretreated during the time indicated at the given **NHC-Pt** concentrations, irradiated at 1, 2, 3 and 4 Gy for A2780 cell lines and 2, 4, 6 and 8 Gy for H1299 and allowed to grow for 6 days. For 4 days pretreatment, cells were seeded in the conditions of cell cytotoxicity determination at the indicated **NHC-Pt** concentrations. They were trypsinized after 4 days treatment, seeded at a number allowing 6 days growth without reaching confluence in the untreated samples, treated for day and then irradiated as described before. Irradiation is carried out using GSR D1 irradiator (gamma-ray, 662 keV). The cells were left to grow for another 6 days to allow at least 5 population doublings. Cell count is carried out on each well and a graph is plotted with the percentage of cell survival as a function of Gys for each treatment. Percent cell survival is calculated based on the number of cells at 0 Gy for each treatment, thus giving 100% survival at 0 Gy regardless of treatment.

The survival plot is fitted in a KaleidaGraph software according to the linear quadratic model:  $S(D)/S(0) = \exp(-\alpha D - \beta D^2)$ <sup>31</sup> The D10 value (dose of Gy at which only 10% cells survive) is calculated using the  $\alpha$  and  $\beta$  parameters. A drop of the D10 values in the combined treatments, compared to ionizing radiation alone highlights a synergistic (radiosensitizing) effect between radiations and complexes.

### **Immunofluorescence Assays.**

A2780 were plated on 8-well labtek (Thermo fish scientific). To test the effects of the metallic complexes alone on the induction of  $\gamma$ -H2AX foci, cells were treated for 4 days at their respective IC80 concentrations (see main text). To test the radiosensitizing properties of NHC-Pt complexes in term of both  $\gamma$ -H2AX and 53BP1 foci induction, cells were firstly treated for 1 day with the NHC-Pt complexes at around their respective IC30 concentrations (see main text), and were irradiated then at 2 Gys using a GSR D1 irradiator (gamma-ray, 662 keV). After the indicated time post-IR, cells were treated for IF studies. Cells were washed with phosphate-buffered saline (PBS), then fixed 10 minutes in 2% Paraformaldehyde (PFA). After a wash with PBS, cells were permeabilized for 10 min at RT using 0.2% Triton X-100 and washed with PBS. The cells were incubated in blocking buffer (5% goat serum in PBS) for 60 min at RT before being incubated at 4 °C overnight in 1% BSA dissolved in PBS with the indicated primary mouse monoclonal antibody against  $\gamma$ -H2AX (clone JBW301, Millipore) or 53BP1(bethyl) respectively. On the second day, after three times wash with PBS, the cells were incubated for another 30 min with the Alexa Fluor 488-conjugated secondary antibody (Alexa Fluor 488 goat anti-mouse IgG; Life Technologies). Nuclei were labeled using DAPI and the cover slides were mounted with VectashieldTM. Acquisitions were performed on 3D-SIM by the microscopy platform of the Institut Curie. ImageJ software (NCBI) was used to project the *z-stacks* and count the number of  $\gamma$ -H2AX or 53BP1 spots, as well as their size in nucleus.

### **ASSOCIATED CONTENT**

#### **Supporting Information**

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/xxxxxxx>. <sup>1</sup>H and <sup>13</sup>C NMR spectra of compounds **2a-c**, **D1-D4**, **M1** and **M2** and HPLC chromatograms. Crystallographic data for compounds **M1** and **D2** (Figures S1-S3); Solubility (Figure S4) and Stability (Figure S5-S6) of **D2**; Radiosensitizing properties as function of treatment conditions for **D1**, **D3** and **D4** (Figure S7) and Kinetics study of DNA damage repair post irradiation by 53BP1 IF staining.(Figure S8); Crystal data for **M1** and **D2** (Table S1); Selected geometry parameters for **M1** and **D2** (Table S2); Cytotoxicity on normal cells (Table S3) (PDF)

Molecular formula strings file for compounds (**1a-c**, **2a-c**, **D1-4**, **M1** and **M2**) as data sheet in .csv format (CSV)



## Accession Codes

CCDC 2143695, 2144483 and 2144484 contain the supplementary X-ray crystallographic information for compounds M1, D2 (acetone) and D2 (THF). These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via URL [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif)

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## ABBREVIATIONS

NHC, (*N*-Heterocyclic Carbene); Pt, Platinum; Dvtms, Divinyltetramethylsiloxane; *t*-BuOK, Potassium tert-butoxide; DMSO, Dimethyl sulfoxide; UPLC-MS, Ultra Performance Liquid Chromatography Mass Spectrometry; ICP-MS, Inductively coupled plasma mass spectrometry; D10, Doses of ionizing radiation allowing 10 % survival; GSR, self-shielding gamma irradiator; Gy, Gray; NMR, Nuclear magnetic resonance spectroscopy; HRMS, High-resolution mass spectra; ELS detector, Evaporative light scattering detector; Karstedt's catalyst, Platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane

complex; PBS, Phosphate-buffered saline; IC30, IC50, IC80, Doses of complexes allowing 30 % 50% or 80 % cell proliferation inhibition, respectively; DMEM medium, Dulbecco's Modified.

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