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Toxicity of Copper(I)-NHC Against Human Tumor Cells: Induction of Cell Cycle Arrest, Apoptosis and DNA Cleavage.

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Compounds of wide structural diversity are used nowadays as therapeutic agents for cancer treatment. The most familiar is the metallo-drug cisplatin **1** that creates DNA intrastrand links and, in a lesser extent, interstrand links.[1] Cisplatin and its second generation analogues are effective on a narrow spectrum of tumour cells and are often associated to various toxicity issues such as neurotoxicity or nephrotoxicity.[2] Thus, discovering new organometallic complexes which are selectively active on cancerous cells in an anti-proliferative and/or pro-apoptotic manner remains a challenge.

Since the first synthesis of stable *N*-Heterocyclic Carbenes (NHC), a great interest in their preparation and applications has grown-up.[3] Although metal–NHCs are well recognized as outstanding catalysts; investigations in the therapeutic field remain limited. Most of the efforts have focused on the antimicrobials properties but studies as anti-cancer agents have recently been reported.[4] In this area, the series of dinuclear homoleptic gold(I) complexes such as **2** (Scheme 1) from the Baker and Berners-Price's group is probably the first example of pro–apoptotic metal–NHC ever reported.[4c-f] Since then, Panda and Ghosh have published a study devoted to the palladium(II)-NHC **3**. This complex proves to be superior to cisplatin as an anti-proliferative agent against the tumour cells HL60 (human promyelocytic leukaemia) and provokes a cell cycle arrest at the G2 phase.[4g] Very recently, Youngs has reported the properties of the first anti-cancer silver(I)-NHC **4** which displayed IC_{50} similar to cisplatin in OVCAR-3 (ovarian) and MB157 (breast) cell lines.[4h] This preliminary study also described **4** as active *in vivo*, provoking major cell death of the ovarian tumour while not affecting major organs.

Scheme 1. Metal-NHC complexes reported for their anti-proliferative properties.

Considerable efforts have been devoted to copper(I) complexes acting as Fenton–type reagents leading to DNA strand breaking.[5-7] These complexes may be roughly divided into two sets. The main one contains complexes of copper(II) that must be reduced at the vicinity of DNA by an external reagent, while the smallest set contains few examples of copper(II) complexes capable of cleaving DNA on their own (Tambjamine E, Prodigiosin and the synthetic [Cu^{II}(pyrimol)Cl], for example).[6] As an example of the first group, the natural compound Bleomycin binds copper(II) (and iron) which is further reduced in cells by glutathione (GSH).[7a] The same mechanism is evoked for 2/1 : 1,10-phenanthroline (Phen)/Cu^{I} complex and its clip-phen analogues.[7b-c] Once copper(I) is formed, it may either trap molecular oxygen to form hydrogen peroxide or react directly with H_2O_2 generated *in loco* by the cellular machinery leading to "oxo-copper" or "copper-hydroxyl" species. Oxidative attack mediated by these reactive oxygen species leads to the cleavage of DNA at several positions of the deoxyribose moiety. Unfortunately, the $Cu(Phen)^{2+}$ system presents an important drawback : the small association constant of the second phenanthroline limits its utilization in a physiological medium (and furthermore for therapy purpose).[8] We reasoned that metal-NHC complexes, recognized for their high stability and their good lipophilicity,[4d] could offer a excellent alternative to the copper-phenanthroline couple. Indeed, we presumed that NHC-copper(I) complexes would be stable enough to reach a biological target *in cellulo* and disrupt the cellular machinery in such a way that apoptosis would ensue.

For this purpose, we selected the copper(I)-NHC complex of heteroleptic nature [(SIMes)CuCl] (SIMes : 1,3-bis-(2,4,6-

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trimethylphenyl)imidazolin-2-ylidene) **5** that we compared with the benchmark metallo-drug cisplatin **1** (Scheme 2). We focused on the cytotoxic and apoptotic properties of **5** as well as its influence on the cell cycle. Effects of **5** on DNA were compared with other metal-NHC complexes differing in the nature of the metal and/or the number of carbene ligands (**6-8**).

Scheme 2. Cisplatin and selected metal-NHC complexes.

First, the effects of **5** and cisplatin on cancer cell growth were compared on five different human cancer cell lines (KB: oral carcinoma; HL60: promyelocytic leukaemia; MCF-7 and MCF-7R: breast cancer; LNCaP: prostatic cancer). The results are depicted in Figure 1 and Table 1.

Figure 1. Half-Maximum Inhibitory Concentration (IC_{50}) of **5** and cisplatin on a panel of human cancer cell lines.

Table 1. IC_{50}(μM) of compounds **1** and **5**.

Entry	Cells	**1**	**5**	**1/5**
1	KB	2.2 ± 0.2	0.12 ± 0.01	18
2	HL60	6.78 ± 0.08	0.04 ± 0.01	150
3	MCF-7R	4.49 ±0 .03	0.38 ± 0.03	27
4	MCF-7	10.4 ± 0.2	0.075 ± 0.002	140
5	LNCaP	2.9 ± 0.1	0.43 ± 0.01	7

To our delight, **5** exhibits higher cytotoxicity than the reference metallo-drug. This is best illustrated by an IC_{50} value of **5** being 150 fold lower than that of cisplatin in the HL60 cell line (Table 1, entry 2). This higher cytotoxicity is preserved regardless of the nature of the cell line. Also of importance is that **5** exhibits submicromolar cytotoxicities that compare well with the literature data for $[Cu^{II}(Pyrimol)Cl]$,[6c] $[Cu^{I}(Phen)_2Cl]$ and its clip-phen analogues,[7d] the gold(I) **2**,[4c] palladium(II) **3**,[4g] and silver(I)-NHC **4**[4h] complexes.

The nature of the cellular effects of **5** and **1** was then compared at the cell cycle and apoptotic levels with a focus on the breast tumour cell line MCF-7. In this view, for the cell cycle progression we paid particular attention to the fate of P21 and cyclin D1 which are two regulators of the G1 phase (Figure 2) and the phosphorylation of the protein cdc2 which indicates a G2 phase arrest (Figures 3).[9] Regarding apoptotic effects, we investigated the fate of PARP and P53 (Figure 4).[10,11] PARP − Poly-(ADP-ribose)-polymerase − is a highly conserved nuclear enzyme that recognizes DNA strand breaks and is implied in the apoptotic responses of cells and P53 is a protein involved in cell cycle regulation, apoptosis and DNA repair.

Figure 2. Cell cycle. Comparative western blot analysis of cisplatin and [(SIMes)CuCl] in MCF-7 cells.

The effect of **5** on the cell cycle is evidenced by the slight dose-dependent accumulation of P21 while the expression of cyclin D1 is strongly down–regulated. These two correlated effects indicate a stop at the G1 phase of the cell cycle which occurs at concentrations that are, at least, 10 times lower than those of cisplatin. It has been demonstrated that cisplatin induces a cell cycle arrest at the G2 phase.[9d] In order to definitively assess that [(SIMes)CuCl] differs from **1** in terms of its biological response, we investigated the phosphorylation of the protein kinase cdc2 (pcdc2), a marker of the G2 phase arrest (Figure 3).[9]

Figure 3. G2 phase. Comparative western blot analysis of cisplatin and [(SIMes)CuCl] in MCF-7 cells.

As expected, exposure of MCF-7 cells to **1** results in a dose–dependent pcdc2 production (Figure 3). Exposure to **5** does not reveal such expression. This enables us to definitely rule out a G2 phase arrest induced by **5**.

In light of the results demonstrating a lack of the expression of pcdc2 while a decrease of cyclin D1 concomitantly with an increase of P21 occurs, we conclude that the effects promoted by [(SIMes)CuCl] differ from that of cisplatin and provoke a G1 phase arrest of the cell cycle progression.

Figure 4. Apoptosis. Comparative western blot analysis of cisplatin and [(SIMes)CuCl] in MCF-7 cells.

Figure 4 shows that P53, accumulates rapidly in cisplatin treated cells but not with [(SIMes)CuCl]. Regarding PARP, both compounds induce its proteolytic cleavage in the characteristic inactive 85 kDa fragment. Importantly, [(SIMes)CuCl] induces the PARP cleavage with a greater efficiency than cisplatin.

The lack of correspondence of the marker pattern for the cell cycle progression and apoptosis induced by [(SIMes)CuCl] and cisplatin indicates different mechanisms.

Finally, to ensure that DNA is a possible target, we examined the genotoxicity *in vitro* using the pcDNA4TO plasmid.[12] The first experiments were conducted in aerobic conditions, without any reducing reagents, in a water-DMSO (9:1) mixture during 24 hours with complexes **1**, **5-8** (Figure 5).

Figure 5. Aerobic cleavage of plasmid DNA (1.0% agarose gel). Line 1: Supercoiled plasmid. Line 2: Linearized plasmid using the endonuclease BamH1. Line 3: Cisplatin. Line 4: [(SIMes)CuCl]. Line 5: [(SIMes)AgCl). Line 6: $[(SIMes)_2Cu]PF6$. Line 7: $[(IMes)_2PdCl_2]$. Concentration of all the complexes:10μM; supercoiled plasmid: 1.6μg.

Line 4 shows the conversion of the supercoiled form into an open circular conformation in the presence of **5**. Comparisons with linearized plasmid (line 2) and cisplatin action (line 3) enable us to rule out the possibility of a double strand break and a crosslink under aerobic conditions. Also of importance is the lack of activity displayed by [(SIMes)AgCl] (**6**), $[(SIMes)_2Cu]PF6$ (**7**) and $[(IMes)_2PdCl_2]$ (**8**) complexes (lines 5, 6 and 7). The passivity of the silver(I) and palladium(II)-NHC complexes highlights the necessity of a copper(I) atom for the nuclease activity and reinforces the hypothesis of a Fenton–type reaction. This copper aerobic activity is strictly restricted to a complex whose metal:

carbene ratio is 1/1 as demonstrated by the inactivity of the homoleptic copper(I)–NHC (**7**) (line 6).[13]

We then used a known inhibitor and reducing agents in order to ensure that the reaction involves a radical process (Figure 6).

Figure 5. Effect of additives on DNA cleavage. Line 1: Supercoiled plasmid. Line 2: linearized plasmid using the endonuclease BamH1. Line 3: [(SIMes)CuCl] (**5**), 3h. Line 4: **5**, 24h. Line 5: **5** + NaN_3, 24h. Line 6: **5** + ascorbic acid, 3h. Line 7: **5** + GSH, 3h. Line 8: $Cu(Phen)^{2+}$ + GSH, 3h. Line 9: **5** + GSH, 24h. Line 10: **5** + GSH, 48h. Concentration of all the complexes: 10μM; supercoiled plasmid: 1.6μg.

Lines 3 and 4 highlight the rate of the process under free reducing conditions. A small amount of supercoiled plasmid is converted into the open circular form in 3h, the process being complete in 24h. The addition of the singlet oxygen scavenger NaN_3 leads to a complete collapse of the nuclease activity (line 5).[14] Since all reactions are performed in the presence of the hydroxyl radical scavenger DMSO, [14] its inefficiency to inhibit the reaction argues against the involvement of a free, diffusible HO radical. These results suggest the activation of oxygen by **5** leading to the production of hydrogen peroxide.[5]

The reducing reagents GSH and ascorbic acid both reveal a pronounced accelerating effect (lines 6, 7 compared to lines 3, 4) and allow a complete conversion in 3h. Inspection of lines 6 and 7 also reveals the formation of a minor lower band below the open circular one. Comparison with $Cu(Phen)^{2+}$ (line 8) enables us to attribute this new band to the linearized plasmid (see also line 2). **5** operates more slowly than $Cu(Phen)^{2+}$: after 24h (line 9), the nuclease activity of **5** is still inferior to a 3h treatment of $Cu(Phen)^{2+}$ and a 48h exposure is necessary to achieve a significant linearization of the plasmid (line 10).

In conclusion, we have demonstrated that using [(SIMes)CuCl] as a source for copper(I) in human cancer cells is a valuable strategy. Its cytotoxicity compares well with that of cisplatin, copper(I)-phenanthroline complexes and other metal-NHCs. Unlike cisplatin, **5** arrests the cell cycle progression at the G1 phase and induces apoptosis at a lower concentration. We assume that an aerobic radical process, leading to a DNA strand break is responsible for the observed cytotoxicity. Importantly, the nuclease activity is considerably enhanced by the ubiquitous tripeptide GSH. Ongoing research in our laboratories focuses on completing the identification of the intimate biological mechanisms of action involving copper-carbene complexes.

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Keywords: Anticancer agent · Carbene · Apoptosis · Cell cycle

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Copper(I)-NHC as Anticancer Agent ----------

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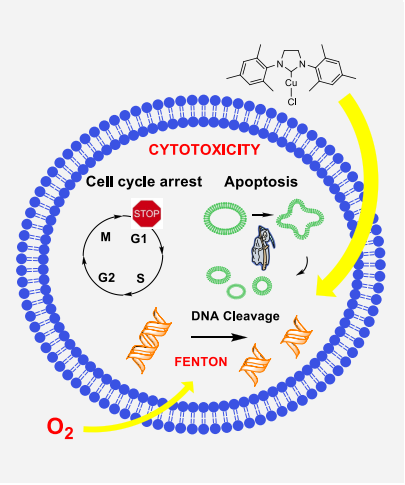
Toxicity of Copper(I)-NHC Against Human Tumor Cells: Induction of Cell Cycle Arrest, Apoptosis and DNA Cleavage.



Tumor cell killer: Copper(I)-NHC [(SIMes)CuCl] shows high cytotoxicity against human cancer. Up to 150 fold increase is observed compared to cisplatin. The complex causes arrest of the cell cycle progression at the G1 phase concomitantly with apoptosis induction at low concentration. This copper(I)-NHC also yield DNA strand breaks, demonstrating its value as Fenton-like reagent.

Abstract

Although NHC-complexes are renowned for their catalytic properties, studies of their anti-cancer properties are scarce. In our search for new potent metal-based drugs, we envisioned that copper(I)-NHC could serve as a stable analogue of copper(I)-phenantroline. Herein, we report the results of the biological evaluation of the anticancer properties of [(SIMes)CuCl] **5**. This complex showed impressive cytotoxicity against a panel of human tumor cell lines. Regarding IC_{50}, up to 150 fold decrease is observed compared to cisplatin. Cellular effects of [(SIMes)CuCl] were evaluated at the cell cycle and the apoptotic levels. **5** causes cell cycle arrest at the G1 phase as demonstrated by the concomitant accumulation of protein p21 and the strong down-regulation of cyclin D1. Monitoring of poly-(ADP-ribose)-polymerase, implied in apoptosis response of cells, showed that [(SIMes)CuCl] induces apoptosis at low concentration. Finally, we showed that [(SIMes)CuCl] targets DNA by *in vitro* studies. When inducing aerobic DNA cleavage, [(SIMes)CuCl] behaves analogously to copper(I)-phenantroline. Thus, our experiments evidenced that employing NHC to deliver active copper(I) species *in cellulo* is a valuable strategy in anticancer research.