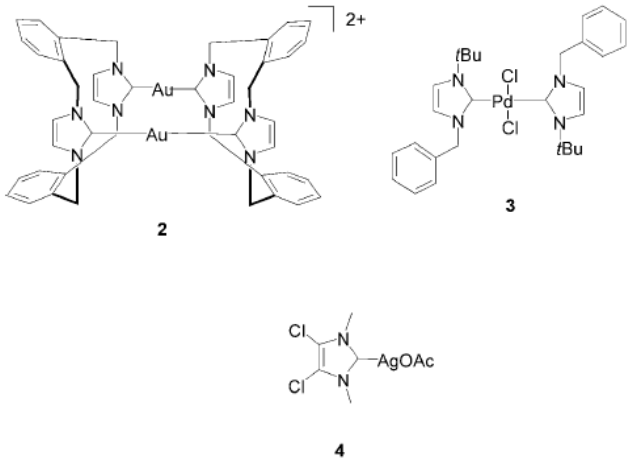
Toxicity of Copper(I)–NHC Complexes Against Human Tumor Cells: Induction of Cell Cycle Arrest, Apoptosis, and DNA Cleavage

Marie-Laure Teyssot,[a] Anne-Sophie Jarrousse,[b] Aurélien Chevry,[a] Angélique De Haze,[b] Claude Beaudoin,[b] Michèle Manin,[b] Steven P. Nolan,[c] Silvia Díez-González,[c] Laurent Morel,*[b] and Arnaud Gautier*[a]

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

Since the first synthesis of stable N-heterocyclic carbenes (NHC), a great interest in the preparation and applications of these compounds has grown up.[3] Although metal–NHCs are well recognized as outstanding catalysts, investigations in the therapeutic field remain limited. Most efforts have focused on the antimicrobial properties but studies of the anticancer properties have recently been reported.[4] In this area, a series of dinuclear homoleptic gold(I) complexes, such as **2** (Scheme 1) from Baker and Berners-Price's group, is probably the first example of pro-apoptotic metal–NHC compounds ever reported.[4e–f] Since then, Panda and Ghosh have published a study devoted to palladium(II)–NHC **3**. This complex proved to be superior to cisplatin as an antiproliferative agent against HL60 (human promyelocytic leukaemia) tumor cells, and provokes a cell cycle arrest at the G2 phase.[4g] Very recently, Youngs has reported the properties of the first anticancer silver(I)–NHC **4**, which displayed IC_{50} (half-maximum inhibitory concentration) values 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 tumor but not affecting major organs.



2 **3** **4**

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

Considerable efforts have been devoted to copper(I) complexes that act as Fenton-type reagents leading to DNA strand breaking.[5–7] These complexes may be roughly divided into two sets. The main set contains complexes of copper(II) that must be reduced in the vicinity of DNA by an external reagent, whereas the smaller set contains a few ex-

[a] Dr. M.-L. Teyssot, A. Chevry, Dr. A. Gautier
SEESIB, UMR 6504 CNRS, Clermont Université
24, avenue des Landais
63177 Aubière CEDEX (France)
Fax: (+33)473-40-77-17
E-mail: Arnaud.gautier@univ-bpclermont.fr

[b] Dr. A.-S. Jarrousse, A. De Haze, Dr. C. Beaudoin, Dr. M. Manin, Prof. L. Morel
GReD, UMR 6247 CNRS, INSERM U931, Clermont Université
24, avenue des Landais
63177 Aubière CEDEX (France)
Fax: (+33)473-40-70-42
E-mail: Laurent.MOREL@univ-bpclermont.fr

[c] Prof. S. P. Nolan, Dr. S. Díez-González
Institute of Chemical Research of Catalonia (ICIQ)
Av. Països Catalans 16
43007 Tarragona (Spain)

amples of copper(II) complexes capable of cleaving DNA on their own (Tambjamine E, Prodigiosin, and the synthetic [Cu^{II}Cl(pyrimol)] (pyrimol = 4-methyl-2-*N*-(2-pyridylmethylene)amino phenol), for example).[6] An example of the first group, the natural compound Bleomycin binds copper(II) (and iron) is further reduced in cells by glutathione (GSH).[7a] The same mechanism is evoked for the 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 situ by the cellular machinery, which leads to "oxo-copper" or "copper-hydroxyl" species. Oxidative attack mediated by these reactive oxygen species leads to the cleavage of DNA at several positions on the deoxyribose moiety. Unfortunately, the $Cu(phen)^{2+}$ system presents an important drawback. Indeed, the small association constant of the second phenanthroline limits its utilization in a physiological medium (and furthermore for therapeutic purposes).[8] We reasoned that metal–NHC complexes, recognized for their high stability and 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 a copper(I)–NHC complex with a heteroleptic nature, [CuCl(SIMes)] (SIMes: 1,3-bis-(2,4,6-trimethylphenyl)imidazolin-2-ylidene) **5**, that we compared with the benchmark metallodrug cisplatin **1**. We focused on the cytotoxic and apoptotic properties of **5** and 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**).

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.

To our delight, **5** exhibits higher cytotoxicity than the reference metallodrug. This is best illustrated by the IC_{50} value

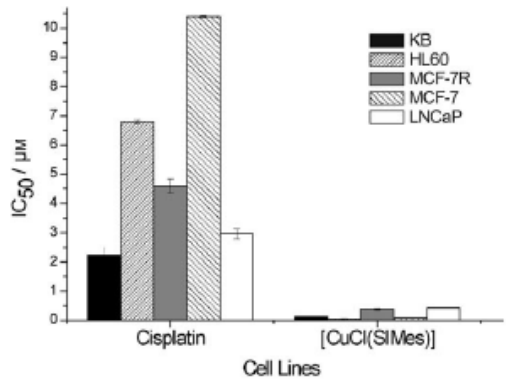


Figure 1. IC_{50} values for 5 and cisplatin on a range of human cancer cell lines.

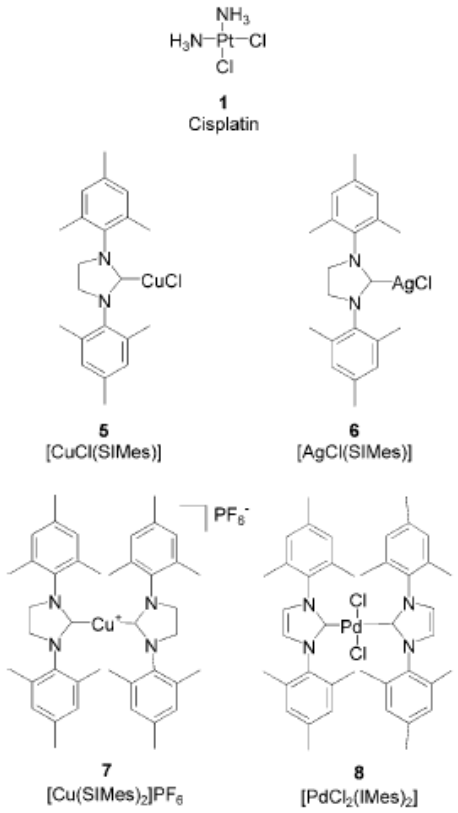


Table 1. IC_{50} [µM] of compounds **1** and **5**.

Entry	Cell line	**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

of **5** being 150 fold lower than that of cisplatin in the HL60 cell line (Table 1, entry 2). This higher cytotoxicity was 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}Cl(pyrimol)],[6c] [$Cu^{I}Cl(phen)_2$] and its clip-phen analogues,[7d] and the gold(I)–NHC **2**,[4c] palladium(II)–NHC **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 by focusing on the breast tumor cell line MCF-7. In this view of the cell cycle progression, we paid particular attention to the fates 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 (Figure 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 implicated in the apoptotic re-

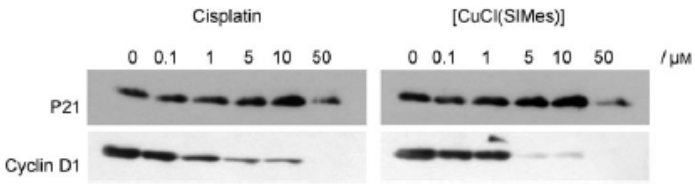


Figure 2. Cell cycle study: comparative western blot analysis of cisplatin and [CuCl(SIMes)] in MCF-7 cells.

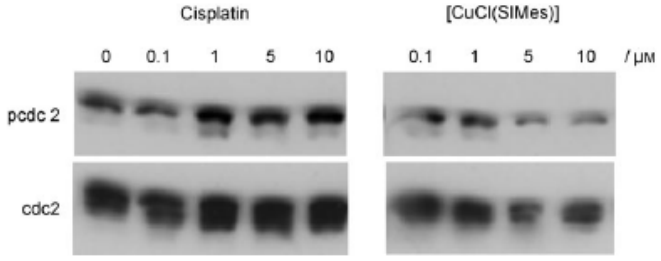


Figure 3. G2 phase study: comparative western blot analysis of cisplatin and [CuCl(SIMes)] in MCF-7 cells.

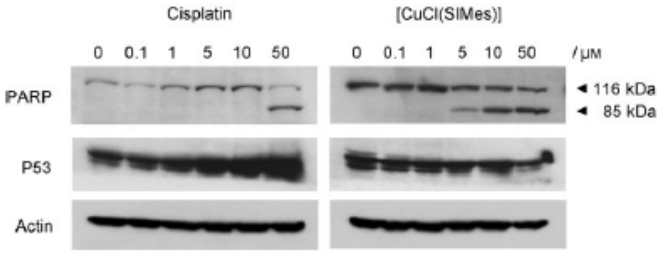


Figure 4. Apoptosis study: comparative western blot analysis of cisplatin and [CuCl(SIMes)] in MCF-7 cells.

sponses of cells and P53 is a protein involved in cell cycle regulation, apoptosis and DNA repair.

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 that occurs at concentrations that are at least ten times lower than those of cisplatin. It has been demonstrated that cisplatin induces a cell cycle arrest at the G2 phase.[9d] To definitively assess that [CuCl(SIMes)] differs from **1** in terms of its biological response, we investigated the phosphorylation of protein kinase cdc2 (pcdc2), a marker of the G2 phase arrest (Figure 3).[9]

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

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

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

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

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

In Figure 5, 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

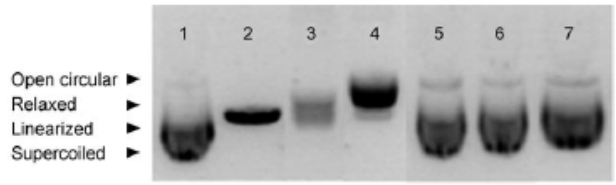


Figure 5. Aerobic cleavage of plasmid DNA (1.0% agarose gel). Line 1: supercoiled plasmid; line 2: plasmid linearized by using the endonuclease BamH1; line 3: cisplatin; line 4: [CuCl(SIMes)] **5**; line 5: [AgCl(SIMes)]: line 6: $[Cu(SIMes)_2]PF_6$; line 7: $[PdCl_2(IMes)_2]$. Concentration of all complexes: 10 μM; supercoiled plasmid: 1.6 μg.

action (Figure 5, 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 [AgCl(SIMes)] (**6**), $[Cu(SIMes)_2]PF_6$ (**7**), and $[PdCl_2(IMes)_2]$ (**8**) complexes (Figure 5, 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 nuclease activity and reinforces the hypothesis of a Fenton-type reaction. This copper aerobic activity is strictly restricted to a complex in which the metal/carbene ratio is 1:1, as demonstrated by the inactivity of the homoleptic copper(I)–NHC (**7**; Figure 5, line 6).[13]

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

In figure 6, lines 3 and 4 highlight the rate of the process under free reducing conditions. A small amount of supercoiled plasmid was converted into the open circular form in 3 h, the process being complete in 24 h. The addition of singlet oxygen scavenger NaN_3 led to a complete collapse of the nuclease activity (Figure 6, line 5).[14] All reactions were performed in the presence of hydroxyl radical scavenger DMSO[14] and its inability to inhibit the reaction argues against the involvement of a free, diffusible HO radical.

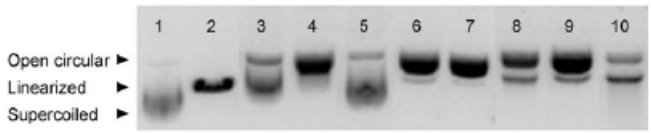


Figure 6. Effect of additives on DNA cleavage. Line 1: supercoiled plasmid; line 2: plasmid linearized by using the endonuclease BamH1; line 3: [CuCl(SIMes)] (**5**), 3 h; line 4: **5**, 24 h; line 5: **5** + NaN_3, 24 h; line 6: **5** + ascorbic acid, 3 h; line 7: **5** + GSH, 3 h; line 8: $Cu(phen)^{2+}$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

These results suggest that the activation of oxygen by **5** leads to the production of hydrogen peroxide.[5]

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

In conclusion, we have demonstrated that using [CuCl(SIMes)] as a source of copper(I) in human cancer cells is a valuable strategy. Its cytotoxicity compares well with that of cisplatin and 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 is focused on completing the identification of the intimate biological mechanisms of action that involve copper–carbene complexes.

Acknowledgements

The authors would like to thank Christian Provot for helpful discussions and Jean-Paul Sarut for western-blot analyses.

Keywords: antitumor agents · apoptosis · carbenes · cell cycle

[1] a) B. Rosenberg, L. van Camp, L. Krigas, *Nature* **1965**, *205*, 698–699; b) E. R. Jamieson, S. J. Lippard, *Chem. Rev.* **1999**, *99*, 2467–2498.

[2] For cisplatin, carboplatin, nedaplatin, and oxaliplatin, see: a) L. Kelland, *Nat. Rev. Cancer* **2007**, *7*, 573–584; b) M. A. Fuertes, C. Alonso, J. M. Pérez, *Chem. Rev.* **2003**, *103*, 645–662.

[3] a) K. J. Öfele, *J. Organomet. Chem.* **1968**, *12*, P42–P44; b) H. W. Wanzlick, H. J. Schönherr, *Angew. Chem.* **1968**, *80*, 154; *Angew. Chem. Int. Ed. Engl.* **1968**, *7*, 141–142; c) A. J. Arduengo, R. Krafczyk, R. Schmutzler, H. A. Craig, J. R. Goerlich, W. J. Marshall, M. Unverzagt, *Tetrahedron* **1999**, *55*, 14523–14534; d) *N-Heterocyclic Carbenes in Synthesis* (Ed.: S. P. Nolan), Wiley-VCH, Weinheim, **2006**; e) W. A. Herrmann, *Angew. Chem.* **2002**, *114*, 1342–1363; *Angew. Chem. Int. Ed.* **2002**, *41*, 1290–1309; f) N. Marion, S. Díez-González, S. P. Nolan, *Angew. Chem.* **2007**, *119*, 3046–3058; *Angew. Chem. Int. Ed.* **2007**, *46*, 2988–3000; g) D. Enders, O. Niemeier, A. Henseler, *Chem. Rev.* **2007**, *107*, 5606–5655.

[4] a) A. Melaiye, Z. Sun, K. Hindi, A. Milsted, D. Ely, D. H. Reneker, C. A. Tessier, W. J. Youngs, *J. Am. Chem. Soc.* **2005**, *127*, 2285–2291; b) A. Kascatan-Nebioglu, A. Melaiye, K. Hindi, S. Durmus, M. J. Panzner, L. A. Hogue, R. J. Mallett, C. E. Hovis, M. Coughenour, S. D. Crosby, A. Milsted, D. L. Ely, C. A. Tessier, C. L. Cannon, W. J. Youngs, *J. Med. Chem.* **2006**, *49*, 6811–6818; c) P. J. Barnard, L. E. Wedlock, M. V. Baker, S. J. Berners-Price, D. A. Joyce, B. W. Skelton, J. H. Steer, *Angew. Chem.* **2006**, *118*, 6112–6116; *Angew. Chem. Int. Ed.* **2006**, *45*, 5966–5970; d) M. V. Baker, P. J. Barnard, S. J. Berners-Price, S. K. Brayshaw, J. L. Hickey, B. W. Skelton, A. H. White, *Dalton Trans.* **2006**, 3708–3715; e) P. J. Barnard, S. J. Berners-Price, *Coord. Chem. Rev.* **2007**, *251*, 1889–1902 and references therein; f) J. L. Hickey, R. A. Ruhayel, P. J. Barnard, M. V. Baker, S. J. Berners-Price, A. Filipovska, *J. Am. Chem. Soc.* **2008**, *130*, 12570–12571; g) S. Ray, R. Mohan, J. K. Singh, M. K. Samantaray, M. M. Shaikh, D. Panda, P. Ghosh, *J. Am. Chem. Soc.* **2007**, *129*, 15042–15053; h) D. A. Medvetz, K. M. Hindi, M. J. Panzner, A. J. Ditto, Y. H. Yun, W. J. Youngs, *Met.-Based Drugs* **2008**, 7–14.

[5] a) D. S. Sigman, A. Mazumder, D. M. Perrin, *Chem. Rev.* **1993**, *93*, 2295–2316; b) D. S. Sigman, *Acc. Chem. Res.* **1986**, *19*, 180–186; c) D. S. Sigman, T. W. Bruice, A. Mazumder, C. L. Sutton, *Acc. Chem. Res.* **1999**, *32*, 2797–2816.

[6] a) M. S. Melvin, J. T. Tomlinson, G. R. Saluta, G. L. Kucera, N. Lindquist, R. A. Manderville, *J. Am. Chem. Soc.* **2000**, *122*, 6333–6334; b) S. Borah, M. S. Melvin, N. Lindquist, R. A. Manderville, *J. Am. Chem. Soc.* **1998**, *120*, 4557–4562; c) P. U. Maheswari, S. Roy, H. den Dulk, S. Barends, G. van Wezel, B. Kozlevcar, P. Gamez, J. Reedijk, *J. Am. Chem. Soc.* **2006**, *128*, 710–711.

[7] a) J. Chen, J. Stubbe, *Nat. Rev. Cancer* **2005**, *5*, 102–112; b) D. R. Graham, L. E. Marshall, K. A. Reich, D. S. Sigman, *J. Am. Chem. Soc.* **1980**, *102*, 5419–5421; c) M. Pitié, B. Sudres, B. Meunier, *Chem. Commun.* **1998**, 2597–2598; d) M. Pitié, A. Croisy, D. Carrez, C. Boldron, B. Meunier, *ChemBioChem* **2005**, *6*, 686–691.

[8] B. R. James, R. J. P. Williams, *J. Chem. Soc.* **1961**, 2007–2012.

[9] a) C. E. Caldon, R. J. Daly, R. N. Sutherland, E. A. Musgrove, *J. Cell. Biochem.* **2006**, *97*, 261–274; b) T. Waldman, K. W. Kinzler, B. Vogelstein, *Cancer Res.* **1995**, *55*, 5187–5190; c) J. W. Harper, P. D. Adams, *Chem. Rev.* **2001**, *101*, 2511–2526; d) S. Mueller, M. Schittenhelm, F. Honecker, E. Malenke, K. Lauber, S. Wesselborg, J. T. Hartmann, C. Bokemeyer, F. Mayer, *Int. J. Oncol.* **2006**, *29*, 471–479; **3** is also reported to arrest the cell cycle at the G2 phase, see ref. [4f].

[10] S. H. Kaufmann, S. Desnoyers, Y. Ottaviano, N. E. Davidson, G. G. Poirier, *Cancer Res.* **1993**, *53*, 3976–3984.

[11] a) V. J. Bykov, K. G. Wiman, *Ann. Med.* **2003**, *35*, 458–465; b) L. Römer, C. Klein, A. Dehner, H. Kessler, J. Buchner, *Angew. Chem.* **2006**, *118*, 6590–6611; *Angew. Chem. Int. Ed.* **2006**, *45*, 6440–6460.

[12] No reaction between [CuCl(SIMes)] with nucleosides (A, G, T) in a $[D_6]$DMSO/D_2O (1:1) solution was observed (^{1}H NMR spectroscopy).

[13] Activation of oxygen usually requires a two-electron transfer in a binuclear copper(I) complex, see: a) E. Kim, E. E. Chufán, K. Kamaraj, K. D. Karlin, *Chem. Rev.* **2004**, *104*, 1077–1133; b) K. D. Karlin, S. Kaderli, A. D. Zuberbuhler, *Acc. Chem. Res.* **1997**, *30*, 139–147; c) K. D. Karlin, Y. Gultneh, *Prog. Inorg. Chem.* **1987**, *35*, 219–327; d) I. E. Markó, P. R. Giles, M. Tsukazaki, S. M. Brown, C. J. Urch, *Science* **1996**, *274*, 2044; it is likely that either an electronic effect or the steric hindrance around the copper(I) atom inactivates **7**, for % V_{bur} factor (steric parameter), see: e) L. Cavallo, A. Correa, C. Costabile, H. Jacobsen, *J. Organomet. Chem.* **2005**, *690*, 5407–5413;

for X-ray structures of 7 and 5, see: f) H. Lebel, M. K. Janes, A. B. Charette, S. P. Nolan, *J. Am. Chem. Soc.* **2004**, *126*, 5046–5047 and g) P. de Frémont, N. M. Scott, E. D. Stevens, T. Ramnial, O. C. Lightbody, C. L. B. Macdonald, J. A. C. Clyburne, C. D. Abernethy, S. P. Nolan, *Organometallics* **2005**, *24*, 6301–6309, respectively.

[14] a) *Biochemical and Clinical Aspects of Oxygen* (Ed.: W. S. Caughey), Academic Press, New York **1979**, pp. 603–626; b) J. L. Sagripanti, K. H. Kraemer, *J. Biol. Chem.* **1989**, *264*, 1729–1734; c) Y. Li, P. Kuppusamy, J. L. Zweier, M. A. Trush, *Chem.-Biol. Interact.* **1995**, *94*, 101–120.