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The anticancer activity of an air-stable Pd(I)-NHC (NHC = N-heterocyclic carbene) dimer.

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A new dinuclear Pd(I) complex coordinating two bis(NHC) ligands revealed an unsuspected stability despite the unsaturation of the two metal centres. Even more surprisingly, the compound showed a high and selective antiproliferative activity on different cancer cell lines and ovarian cancer tumoroids, acting with a mechanism different from cisplatin.

The +1 oxidation state is mostly unusual for palladium[1,2] yet research carried out especially in the last twenty years have brought to light a considerable number of such compounds. These formally d^9 configuration species are generally dimeric and characterized by some degree of metal-metal interaction that renders them diamagnetic. Moreover, their chemical behavior, in many cases significantly different from that of well-recognized Pd(II)/(0) complexes, has allowed the development of several efficient catalytic systems. Among the most successful catalytic applications of bridging Pd(I) dimers are their role in classical Pd(II)/Pd(0) transformations such as in the Suzuki-Miyaura,[3] Negishi[4] and Sonogashira[5] couplings, Buchwald-Hartwig amination,[3a,c,6] α-arylation of carbonyls,[6] C-Se and C-S heterocoupling,[7] carboxylation of allylstannanes and allylboranes with CO_2[8] and even oxidative cross-coupling reactions involving aryl-boron and aryl-antimony substrates.[9] In all these cases, the Pd(I) derivatives are well-defined pre-catalysts with a 1:1 Pd to ligand ratio able to generate, under catalytic conditions, the active monoligated L-Pd(0) species. The most common patterns of Pd(I) dimers are presented in Figure 1 with the bridged ligands (X, L, LX or L_2) that are typically halides,[3a,10] hydride,[11] CO,[12] isoyanides,[13] η^3-allyls,[3d,8,14] η^3-Cp,[15] η^3-triazenido,[16] diphoshines,[17] phoshine-arene[18] and dienes.[19] The coordination sphere of each dimeric Pd(I) unit is generally completed by a terminal ligand (L or X) (Figure 1).

More recently, the great affinity of *N*-heterocyclic carbenes (NHCs) for transition metals[20] has been exploited for the preparation of dinuclear Pd(I) complexes both as terminal[3a,d,15] and as bridging ligands.[21] In particular, Gardiner and coworkers have described the synthesis of a terminal hydride dinuclear palladium complex stabilized by two bulky bridging bis(NHC) ligands (Figure 1).[21b,c] The mono-cationic species was prepared *via* a base-assisted reduction and is stable on anaerobic conditions.

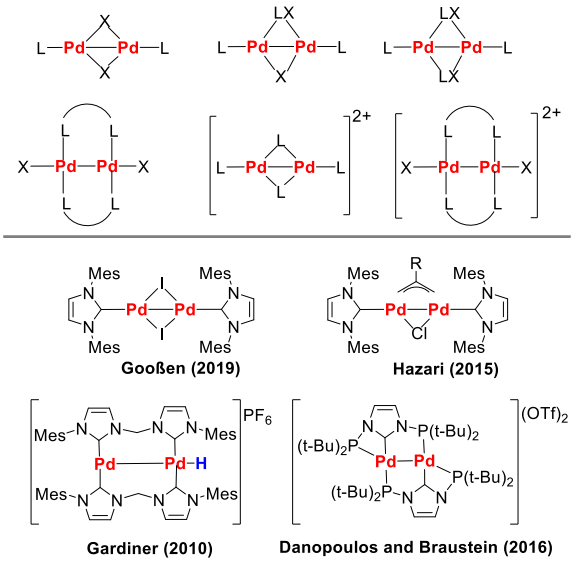

Figure 1. Examples of bridged palladium(I) dimer architectures.

In this contribution, we report the first Pd(I) dimer supported by only NHCs ligands which, in spite of the high unsaturation of the

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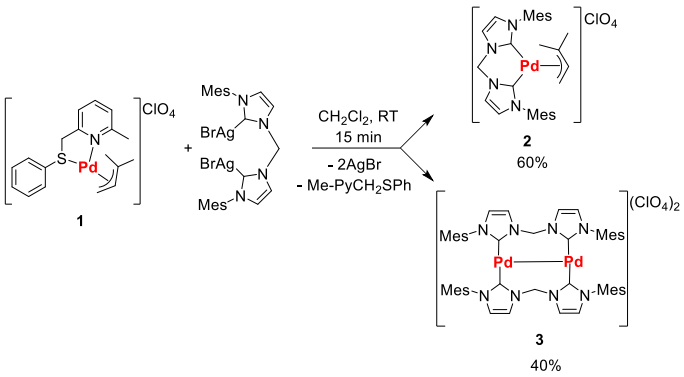
two metal centers shows a surprising bench-top stability with an elevated air and moisture tolerance in the solid-state and in solution. These features combined with its suitable solubility in water (μM range), have allowed us to test the anticancer properties of the complex. To the best of our knowledge, this is the first study of this kind carried out on palladium(I) species, which is in contrast to the more commonly encountered antitumor activity studies of Pd(II) and Pd(0) complexes.[22]

Our investigations are not limited to the determination of the antiproliferative activity on different cancer cell lines, but also include the identification of the cellular targets and *ex-vivo* experiments on human tumoroids derived from high grade serous ovarian cancer patients, which closely replicate the response of patients to therapy in clinical settings.[23]

The first isolation of the new Pd(I) complex **3** was serendipitously obtained as a minor byproduct during the synthesis of a desired bis(NHC) Pd(II)(η^3-1,1-dimethyl-allyl) complex **2** (Scheme 1 and Figs. S1-7).



Scheme 1. Formation of the Pd(II)-allyl complex **2** and the corresponding Pd(I) dimer **3.**

As depicted in Scheme 1, the two species are simultaneously present in the reaction mixture with a slight prevalence of the Pd-allyl derivative. In this procedure, we have taken advantage of the great lability of the 2-methyl-6-(phenylthiomethyl)pyridine ligand,[24] but exactly the same result can be obtained also starting from [Pd(η^3-1,1-dimethyl-allyl)Cl] dimer and using $AgClO_4$ as halide abstractor (Table S1, Entry 1 and Fig. S8). If the ratio between the two species appears to be independent by the synthetic procedure, it is instead heavily affected by the steric bulk of both allyl fragment and bisNHC ligand with the increase of steric strain that promote the Pd(I) dimers compared to Pd-allyl derivative. Thus, maintaining 1,1-dimethyl-allyl fragment, the use of less encumbered bisNHC, with benzyl substituents on external arms, produces exclusively the Pd-allyl compound (Table S1, Entries 2 and Fig. S9).

The same outcome is achieved when we involved allyl or 2-Me-allyl fragment regardless of the nature of the bisNHC ligand whereas 1-phenyl allyl (cinnamyl) fragment and mesityl-bisNHC lead to only 10% of the Pd(I) dimer (Table S1, Entries 3-5 and Figs. S10-12).

We observed that any significant steric repulsion between bisNHC side arms and terminal allyl substituents destabilizes the mononuclear chelate Pd(II) complex and favors the formation of the less sterically congested Pd(I) dinuclear species. Hazari has reported on the relative stability of the allyl moiety substitution as it affects Pd(I) bridging dimer formation.[8,15] The effects here are more complex as they also involve steric interactions with NHC side-arms. The details of the formation of this Pd(I) dimer are presently being examined and will be reported in due course.

Interestingly, in the case of 60:40 mixture of **2** and **3**, the Pd(I) dimer **3** can be easily separated from Pd allyl species **2** by its selective precipitation in a dichloromethane/diethyl ether mixture at 278 K. Surprisingly, considering its high electronic unsaturation, the pink product is stable as a solid and in solution of many organic solvents (methanol, chlorinated solvents, THF, DMSO) for an indefinite time (> 1 month) and can be handled in air without any special precautions. Moreover, complex **3** is stable in physiological solution for at least 96 h, as confirmed by UV-Vis, ESI-MS and NMR analyses (Figs. S13-15). ^1H and ^{13}C NMR spectra are rather simple showing the presence of signals diagnostic of the bis(NHC) system and confirming the high symmetry present of the structure of **3**. The atom connectivity and final structure was unambiguously established through X-ray diffraction studies on single crystal. The crystallographic data (Figs. S16-19 and Tables S2-4) clearly show a dinuclear structure in a planar coordination environment with a pseudo-C_2 symmetry in which the Pd-Pd (3.163(1) Å) distance is one of the longest recorded for palladium(I) dimers (Figure 2). Moreover, the Pd-carbene bond distances (~ 2.08-2.09 Å) are slightly longer than those normally observed.

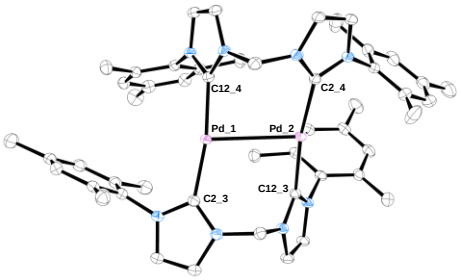


Figure 2. Ellipsoid representation of **3** (50% probability). Counterions and hydrogen atoms removed for clarity.

Analysis of the nature of that Pd-Pd bond was done by means of DFT calculations (See computational section in ESI). Inspection of all possible spin states for **3** including an open-shell singlet and a triplet states is carried out by two different approaches: *i)* fixing the Pd-Pd distance at 3.16 Å (experimentally reported) and relaxing everything else and *ii)* performing a relaxed geometry optimization permitting all atoms to be relaxed. The main reason for keeping the Pd-Pd distance at 3.16 Å is to preserve the biradical character of the open-shell singlet which otherwise vanishes upon Pd-Pd bond contraction and converges to a closed-shell singlet. Fixing the Pd-Pd distance during the optimization results in the open-shell singlet to be energetically lower than the closed-shell singlet and the triplet by 4.1 and 6.6 kcal/mol (See Table S5).

Differently, relaxing also the Pd-Pd distance in the optimization results in the closed-shell singlet becoming the most stable structure, with a Pd-Pd distance of 2.55 Å, the open-shell singlet converging into the closed-shell singlet while loosing the biradical character, and the triplet at 15.4 kcal/mol higher in energy, with a Pd-Pd distance of 2.83 Å (See Table S6). Considering the remarkable shortening of the Pd-Pd bond in the singlet state from the X-ray value upon full optimization, 0.61 Å, we believe that calculations with the Pd-Pd distance fixed at the X-ray value offer a better insight into the electronic structure of **3**, suggesting the complex having a biradical singlet character, with antiferromagnetically coupled Pd(I) species (Fig. S20).

With the aim of investigating the potential anticancer activity of the new palladium(I) dimer **3**, a group of eight different human tumor cell lines (ovarian cancers KURAMOCHI, OVCAR3, OVCAR5, A2780, with its cisplatin resistant clone A2780*cis*, lung cancer A549, cervical cancer HeLa and colon cancer DLD1) and MRC-5 normal cells (human lung fibroblasts) were treated for 96 hours with our compound and compared to cisplatin (positive control). The resulting half inhibitory concentrations (IC_{50}) values are reported in Table 1.

Table 1. Effects of **3** and cisplatin on the proliferation of several cancer and normal cell lines (after 96 h of incubation).

	IC_{50} (μM)	
Cell line	**Cisplatin[a]**	**Pd(I) dimer 3**
A2780	0.81 ± 0.06	0.025 ± 0.007
A2780*cis*	43 ± 5	1.9 ± 0.2
OVCAR5	5.2 ± 0.8	1.4 ± 0.3
OVCAR3	2.2 ± 0.1	3.0 ± 0.2
KURAMOCHI	1.7 ± 0.3	0.38 ± 0.09
HeLa	11 ± 2	3.5 ± 0.1
A549	6 ± 2	0.38 ± 0.02
DLD1	19 ± 4	2.8 ± 0.6
MRC-5	14 ± 1	>100

[a] The cisplatin values are taken from Ref. 21c

These results are very promising, with **3** showing activity, sometimes of an order of magnitude greater than cisplatin against all targeted tumor cell lines and at the same time a very low level of cytotoxicity toward normal fibroblast cells (Figs. S26-34). In this respect, it is important to point out that the selectivity toward cancer cells represents the first requirement to avoid a generalized toxicity of an anticancer drug.

With the aim of clarifying the primary biological effects generated by the treatment of cancer cells with the dimeric species **3**, a series of immunofluorescence experiments at different timepoints were carried out.

In order to specifically examine whether complex **3** triggers DNA damage, fluorescence analysis of phosphorylated histone γH2AX was performed. γH2AX is a well-established marker for double-strand-breaks, forming foci at the site of DNA damage.[25] Both HeLa and A2780 cells displayed green foci in the nucleus after treatment with cisplatin for 3-12 hours (see Figs. S21-22). In contrast, the cells treated with complex **3** do not show DNA damage after 12 hours. These results suggest that DNA is unlikely to be a major molecular target of Pd(I) dimer **3**.

In order to assess the damage at the mitochondrial level, we first determined by means of immunofluorescence techniques the release of cytochrome C.[26] The results summarized in Figs. S23-24 clearly show the release of cytochrome C already after 3 h in the case of A2780 and HeLa cells treated with **3**, even at the lowest concentration used. To confirm the early damage at the mitochondrial level, in Fig. S25 it is possible to observe a consistent depolarization of the mitochondrial membrane after the treatment of A2780 cells with compound **3** already after 30 minutes, using the JC-1 dye as fluorescent probe. Unlike untreated cells and those treated with cisplatin, which displayed red fluorescence, those treated with H_2O_2 (positive control) or Pd(I) dimer **3** displayed green/yellow fluorescence, indicating the alteration of the mitochondrial membrane potential.

All these experiments support, in a time sequence, that mitochondria are damaged before DNA by **3**.

In previous reports, we and others have demonstrated that cancer organoids could be a powerful tool to evaluate the efficacy[23] and toxicity[27,28] of drugs. In this context, three high grade serous ovarian cancer tumoroids previously characterized,[22c] were treated with Pd(I) dimer **3** and the IC_{50} values compared to carboplatin. Pd(I) dimer **3** is more effective than carboplatin in all three patients, suggesting a potential use in clinical settings.

Table 2. IC_{50} (μM) of **3** and carboplatin on ovarian cancer tumoroids after 96 h of incubation.

	IC_{50} (μM)	
Patient	**Carboplatin[a]**	**Pd(I) dimer**
OV-A	>100	2.7 ± 2.6
OV-B	>100	12.6 ± 7.5
OV-C	30 ± 8	20.8 ± 17.4

[a] The carboplatin values are taken from Ref. 21c

Moreover, the Pd dimer **3** demonstrated very low toxicity on normal liver organoids (IC_{50}> 100 μM), previously characterized by immunohistochemistry,[29] which is also very promising for future clinical applications.

In conclusion, we have isolated and characterised a new dinuclear Pd(I) complex, coordinating only two bis(NHC) ligands which, despite the high electronic unsaturation of the two metal centres, is remarkably stable both in the solid state and in solution. This feature has allowed us to carry out the first study of the anticancer activity of a palladium(I) derivative obtaining results particularly encouraging. As a matter of fact our complex proved to be (1) more cytotoxic than cisplatin, (2) selective toward tumor cells, (3) characterized by a mechanism of action different from cisplatin and (4) efficient also on more complex neoplastic structures as ovarian cancer tumoroids.

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Conflicts of interest

There are no conflicts to declare.

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