

Palladium(II)-Imidoyl Complexes: A New Piece in the Puzzle of Organopalladium Anticancer Agents

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Our search of new organopalladium compounds able to promote an effective antiproliferative action towards ovarian cancer cells continues. In this paper we have examined for the first time the anticancer activity of palladium imidoyl complexes, for which two different types of phosphines have been chosen as ancillary ligands: *i*) PTA and DAPTA to take advantage from their solubility in aqueous environment, and *ii*) dppf for combining the action of the Pd-imidoyl fragment with that, well-known, of ferrocene. The synthetic protocols as well as the exhaustive characterisation of the complexes through spectroscopic and diffractometric methods are described. *In vitro* tests

carried out to assess the cytotoxicity of the new compounds towards two ovarian cancer cell lines (one cisplatin sensitive and the other cisplatin resistant) have revealed an interesting effect of the halide coordinated to the palladium centre (halogen effect). Moreover, all complexes have shown the same activity against the cisplatin-sensitive (A2780) and cisplatin-resistant (A2780cis) cell lines, suggesting a different mode of action with respect to the "classical" platinum-based drugs. Finally, a selection of the most active compounds has shown an interesting selectivity towards ovarian cancer cells.

Introduction

Chemotherapy, despite its heavy impact on the organism and inherent limitations remains one of the indispensable weapons at our disposal in the battle against cancer.^[1,2] Among the development lines in this field, that concerning metallodrugs is becoming increasingly important, and several new products are flanking the classical platinum derivatives in the therapeutic protocols.^[3–5] Within this context, a promising upgrade is certainly represented by the arrival in the scene of organometallic compounds that can offer more possibilities of interaction with biological targets than coordination metal complexes.^[6,7] The reactivity of the latter are in fact generally limited to the exchange of ancillary ligands, which allows the metal centre to bind the target biomolecules, significantly

compromising their activity; the example of the interaction of cisplatin with DNA chains is, in this sense, paradigmatic. Instead, in the organometallic compounds the presence of the organic fragment can give biomolecules an additional site of attack. Moreover, the strength of the metal-carbon bond can ensure a particularly high stability to organometallic complexes, allowing them to act as structural-based drugs and potentially paving the way to computational docking approaches for their design. Some classes of transition metals organometallic compounds have already found an important place in medicinal chemistry and, among them, metallocenes,^[8–11] metallo-arenes,^[12–14] cyclo-metallated derivatives^[15–17] are particularly worthy of mention. Others are rapidly gaining ground and among them, organopalladium complexes are becoming increasingly important as evidenced by the numerous articles and reviews appeared on the subject in recent years.^[18–20] The chemical similarities with platinum complexes were the initial pretext to initiate studies on the antineoplastic activity of palladium compounds, although soon some significant differences in the biological behaviour were observed. Also our group has been involved in this topic, preparing and testing as anticancer agents, a large number of organopalladium complexes. The organometallic motifs by us so far examined included Pd(II)-allyls,^[21–27] palladacyclopentadienyls,^[27,28,29] Pd(0)-olefins^[27,30] and more recently Pd(II)-indenyls,^[31–33] while other research groups turned their attention to Pd(II)-alkynyls^[34] and an extensive panel of cyclopalladates.^[19,35–38] All these works indicate the primary importance of the organic fragment in determining the activity of the complex although it can be modulated by a careful choice of the ancillary ligands. It is therefore a good strategy to endeavour of expanding the library of palladium complexes tested as anti-proliferative agents towards cancer cells, although not being easily able to predict their effectiveness in advance. This is the logic that underlies our choice of describing

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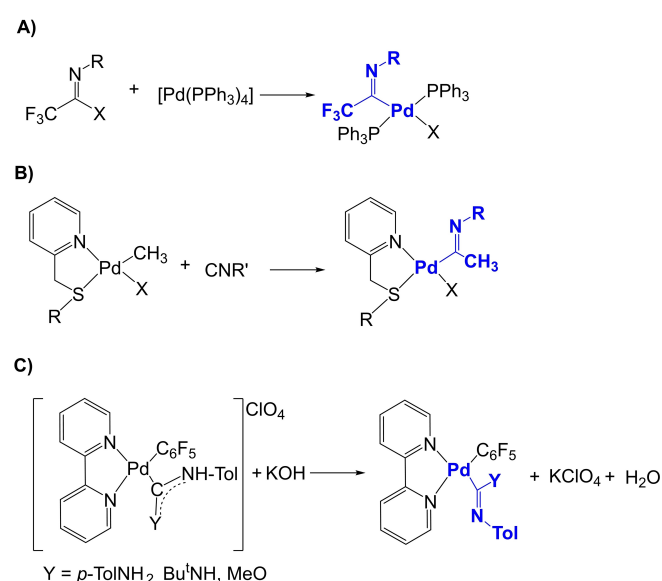
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in this paper the anticancer properties of a new class of organopalladium complexes such as Pd(II)-imidoyl derivatives. Pd(II)-imidoyl group assumed a certain importance in palladium-based homogeneous catalysis, being invoked as key-intermediate in many processes such as imido-lative couplings,^[39] imido-l halides homocoupling^[40] or the synthesis of α -imidoamides,^[41] just to mention some of the most significant examples. In contrast, till now, no study on the biological properties of palladium-imido-l derivatives is reported in the literature.

The synthetic procedures devised for the preparation of Pd-imido-l complexes can be summarised in three different approaches: (A) oxidative addition of imido-l halides to palladium(0) precursors;^[42] (B) insertion of isocyanides into Pd-alkyl or Pd-aryl bonds;^[43] (C) deprotonation of Pd-carbene complexes with strong bases.^[44] Some examples of the three methods are reported in Scheme 1. The entryway (B) is the most versatile, considering the high availability of commercial isocyanides and the relative ease of preparation of Pd-alkyl/aryl precursors.

The experience suggests that in the setting of an organometallic drug, a great importance must be reserved to the choice of the ancillary ligands which can reinforce the interaction of metal derivatives with the biological target, increase the cellular uptake and modulate the compatibility with the different cellular and extra-cellular compartments. In this paper we have planned to associate to the palladium-imido-l moiety two different types of supporting ligands: *i*) 1,3,5-triaza-7-phosphaadamantane (PTA) with its diacetyl derivative 3,7-diacetyl-1,3,5-triaza-5-phosphabicyclo[3.3.1]nonane (DAPTA)^[45] and *ii*) 1,1'-bis(diphenylphosphino)ferrocene (dppf). This choice is first supported by the well-known strength of the Pd-P bond, which should ensure a good stability of the compounds. Secondly, these ligands have already proven to have interesting properties in biological environment.



Scheme 1. General procedures for the preparation of Pd-imido-l complexes.

PTA is a cage-adamantane-like phosphine, relatively compact (cone angle = 103°), and characterized by a high water-solubility.^[46–48] Being this last feature partially maintained also by its metal complexes, these last have begun to be used in medicinal chemistry.^[46] Ruthenium complexes, collectively known as RAPTA (Ruthenium Arene PTA), represent the most successful example of PTA derivatives tested and studied as anticancer agents^[49–52] and, more recently, also some PTA-gold,^[25] -rhodium^[26] and -palladium^[23,24,26,30] complexes have given encouraging results.

Dppf is a bidentate phosphine with a large bite-angle (99°), containing in its core the ferrocenyl moiety. Derivatives of this popular organometallic function have been extensively studied as antibacterial, antifungal and anticancer agents,^[53–56] and therefore the coordination of the chelating dppf to a transition metal centre allows obtaining bimetallic systems with a possible synergic effect. Our research group has recently proposed a systematic study of the antiproliferative activity towards ovarian cancer cells of different organopalladium derivatives coordinating dppf, obtaining interesting responses.^[27]

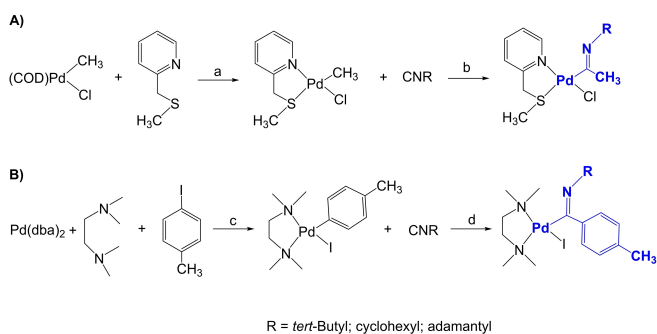
Ultimately, the aim of this work is the preparation of a series of new Pd-imido-l complexes coordinating PTA, DAPTA and dppf, with a preliminary study of their antiproliferative and pro-apoptotic activity towards ovarian cancer cells.

Results and Discussion

Synthesis of Pd-imido-l precursors

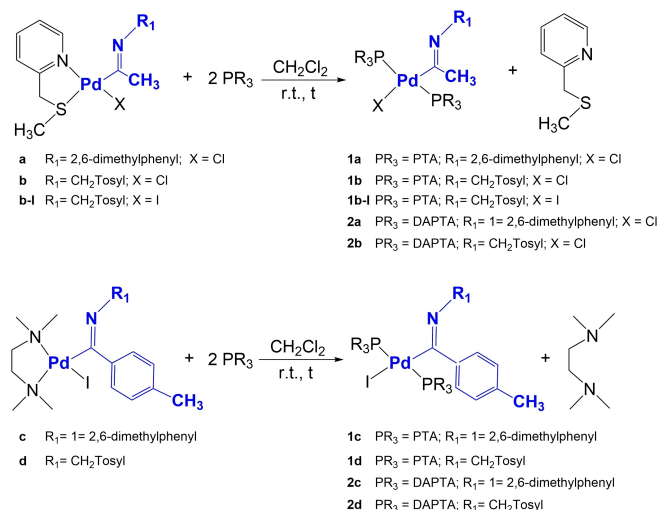
The most adaptable strategy to generate the Pd-imido-l fragment is to take advantage of the isocyanide insertion into a metal-alkyl/aryl bond. In this manner, it is possible to draw to the vast library of isocyanides commercially available and at the same time make use of the numerous synthetic procedures present in the literature for preparing palladium-alkyl and palladium-aryl derivatives. The best option for our purpose is therefore to prepare in this way suitable Pd-imido-l precursors, providing them with labile ligands easily replaceable with the phosphines of our interest. In this respect, in our previous work we have synthesized an array of palladium-imido-l complexes bearing pyridyl-thioethers as ancillary ligands.^[43] Reactions times and optimal operative conditions have been determined by a detailed kinetic study of the isocyanides insertion. Given this experience, we decided to adopt some of these compounds as precursors, preparing them with the multi-step synthesis described in Scheme 2A.

Actually, as precursor for aryl imido-l derivatives we have opted for [Pd(tmeda)(tolyl)]₂ (tmeda = tetramethylethylenediamine) which can be easily obtained from [Pd(dba)₂], by oxidative addition of 4-tolyl iodide (Scheme 2B). Preliminary tests carried out by ¹H NMR spectra have in fact shown that the successive insertion of DIC (2,6-dimethyl phenyl isocyanide) or TosMIC (toluenesulfonylmethyl isocyanide) takes place quickly and selectively at room temperature in dichloromethane. The only precaution to take it is to make the reaction in presence of two equivalents of free tmeda, to avoid that the isocyanide



Scheme 2. Synthetic routes to Pd-imidoyl precursors used in this work: a) in CH_2Cl_2 at r.t., $t = 15$ min; b) + 2 eq. 2-((methylthio)methyl)pyridine in CH_2Cl_2 , $t = 1$ h; c) in toluene $t = 50^\circ\text{C}$, 5 min; d) + 2 eq. TMEDA in CH_2Cl_2 , $t = 1$ h

replaces the diamine on the palladium centre. The same precaution has been adopted in the synthesis of methyl imidoyl precursors bearing 2-((methylthio)methyl)pyridine as ancillary ligand.



Scheme 3. Synthesis of Pd(II)-imidoyl complexes bearing PTA or DAPTA as spectator ligands.

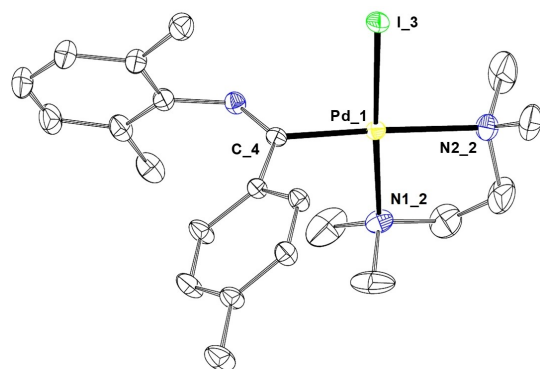


Figure 1. Molecular structure of **c** is shown with thermal displacement ellipsoids at the 50% probability level. Hydrogen atoms are omitted for clarity.

Palladium-imidoyl precursors **c** and **d** (see Scheme 3) are unpublished and for this reason have been entirely characterised. Their ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra are featured by the presence of the signals ascribable to the four methyl groups and two methylene groups of the tmeda, which are all distinguishable.

The insertion of isocyanides is testified by the presence of the signals belonging to the aryl substituents of the isocyanide (2,6-dimethyl phenyl and tosyl for complexes **c** and **d**, respectively) and those of the tolyl fragment, which are significantly shifted compared to those present in the starting complexes. Furthermore, the signal of imidoyl carbon, resonating at ca. 180 ppm, turns out particularly diagnostic in this context. For complex **c** we were also able to solve the X-ray structure, which is reported in Figure 1.

Synthesis of PTA/DAPTA palladium(II) imidoyl complexes

The ancillary ligands of the precursors are sufficiently labile to be replaced by the phosphines selected for this work, which have an high affinity for the soft Pd(II) metal centre. As evidence, the addition of two equivalents of monodentate phosphines (PTA or DAPTA) to a dichloromethane solution of precursors **a–d**, is sufficient to obtain in a few minutes at room temperature the target products which can be then easily isolated from the reaction mixture by precipitation with diethyl ether (Scheme 3).

Between the two possible geometric isomers, only the species with the two monodentate phosphines in mutual *trans* position is always isolated. In the case of PTA complexes, this appears particularly evident observing the presence of only one singlet in all $^{31}\text{P}\{^1\text{H}\}$ NMR spectra. This signal is located at chemical shifts ranging from -65 to -60 ppm, significantly downshifted with respect to the signal of the free PTA ($\Delta\delta \approx 40$ ppm), as a consequence of the coordination to the Pd(II) centre. Consistently, in the ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra, only two set of signals are always traceable for the two methylene groups of PTA (NCH_2N and NCH_2P), at ca. 4 and 4.5 ppm in the ^1H spectra, and at ca. 50 and 70 ppm in the carbon spectra, respectively. The integrity of the imidoyl fragment, is securely certified by the signal of the imidoyl carbon bonded to the palladium centre, which resonates at ca. 180 ppm for complexes **1a** and **1c**, (generated using DIC as isocyanide) and ca. 195 ppm for those deriving from TosMIC (**1b** and **1d**). A detailed analysis of the spectra (including HMQC, HMBC and NOESY) allows to identify and assign all the signals of the specific substituents of the imidoyl fragment. Moreover, the strong vibration bands located at ca. 1640 cm^{-1} , and characteristic of CN stretching of imidoyl group, is always present in the IR spectra of the synthesized compounds. Finally, crystals suitable for XRD analysis were obtained for complexes **1b**, **1c** and **1d** (Figure 2).

By analogy, complexes **2a–d** feature the same *trans* rearrangement, but in these cases the interpretation of NMR spectra is less foregone. The situation, in fact, is complicated by the possible presence of different conformers due to the *syn* or

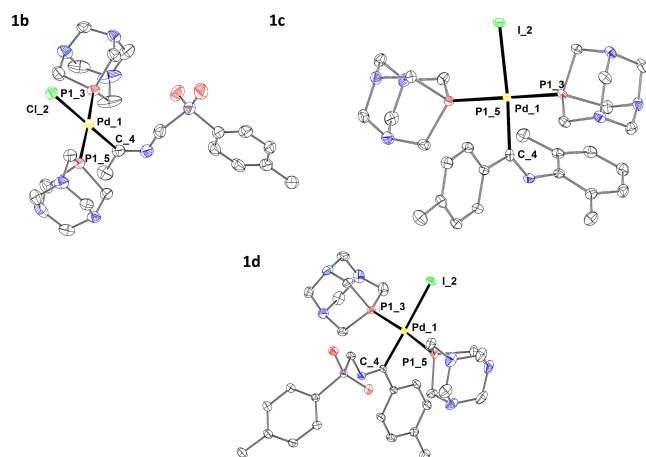


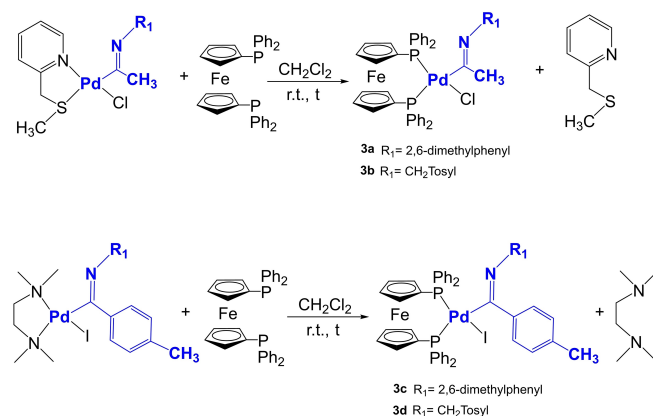
Figure 2. Molecular structures of **1b** (left), **1c** (right) and **1d** are showed with thermal displacement ellipsoids at the 50% probability level. Hydrogen atoms are omitted for clarity.

anti orientations that can take the acyl groups in the DAPTA ligands. This phenomenon, which had been previously reported for palladium and platinum complexes coordinating two DAPTA molecules,^[57–59] is highlighted by the presence in the NMR spectra of sets of signals attributable to more species. For instance, ³¹P{¹H} NMR spectra of complexes **2a–b** show three signals, whereas those ones of complexes **2c–d** show only two. Only in the case of the less encumbered complex **2a**, a fast interconversion of isomers at room temperature can be observed.

Synthesis of palladium(II) imidoyl complexes bearing dppf

Following the synthetic procedure described in the previous paragraph, we were able to prepare some Pd-imidoyl complexes equipped with the chelating phosphine dppf as supporting ligand (Scheme 4).

The compounds **3b** and **3d** (deriving by insertion of TosMIC) have been isolated with good yields, whereas com-



Scheme 4. Synthesis of Pd(II)-imidoyl complexes bearing dppf as spectator ligand.

plexes **3a** and **3c** (deriving by insertion of DIC) decompose rapidly in solution, generating a mixture of products difficult to identify.

The characterization of complexes **3b** and **3d** is quite straightforward, with the bidentate coordination of dppf that is highlighted by the differentiation of the two phosphorus donor atoms that generates in the ³¹P{¹H} NMR spectra a system of two doublets ($J_{PP} \approx 46$ Hz), both resonating at chemical shifts significantly higher than the signal of the free diphosphine. The structure of the obtained complexes also justifies the presence in the ¹H and ¹³C{¹H} NMR spectra of eight and ten different signals, respectively, which are ascribable to cyclopentadienyl protons and carbons of the dppf. Also in this case, the weak signal that falls to more than 190 ppm proves the presence of the imidoyl carbon coordinated on the palladium centre. The identification of all signals of the imidoyl moiety is a further confirmation of the identity of the products obtained. Among them, the couple of doublets due to CH₂SO₂ diastereotopic protons stands out. The identity of complex **3b** was also proven by XRD analysis on suitable crystals (see Figure 3).

Antiproliferative activity on ovarian cancer and normal cell lines

The antiproliferative activity of the synthesized palladium(II) imidoyl complexes was evaluated on A2780 (cisplatin-sensitive ovarian cancer) and A2780cis (cisplatin-resistant ovarian cancer) cell lines. The tested biological activity was compared with that of cisplatin, known to be one of the most commonly used antineoplastic drug for the treatment of ovarian cancer. Moreover, the best performing complexes (**1b–l**, **1c**, **1d**, **2c**, **3b**) were also tested in normal human keratinocyte HaCaT cells to demonstrate the selectivity that these derivatives show towards cancer cells (Table 1).

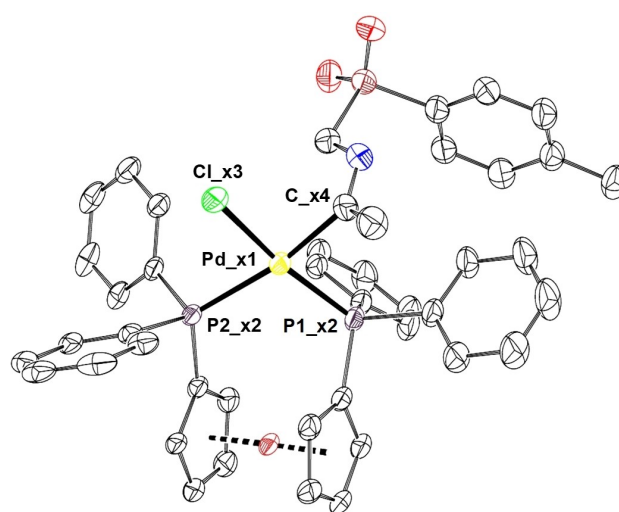


Figure 3. Molecular structure of complex **3b** is showed with thermal displacement ellipsoids at the 50% probability level. Hydrogen atoms are omitted for clarity.

Table 1. IC₅₀ (μM) of palladium(II) imidoyl complexes and cisplatin on cancer (A2780 and A2780cis) and normal (HaCaT) cell lines.

Complex	A2780	A2780cis	HaCaT
Cisplatin	0.65 ± 0.01	8.7 ± 0.2	6 ± 2
1a	28 ± 11	41 ± 5	/
1b	27 ± 7	57 ± 8	/
2a	26 ± 3	8.7 ± 0.4	/
2b	29 ± 4	76 ± 4	/
3b	4.6 ± 0.3	4.5 ± 0.3	72 ± 4
1c	0.9 ± 0.1	0.54 ± 0.02	2.9 ± 0.8
1d	3.2 ± 0.7	1.7 ± 0.7	6.84 ± 0.02
2c	5 ± 2	3.8 ± 0.1	58 ± 3
2d	8 ± 2	32 ± 7	/
3d	> 100	92 ± 17	/
1b-I	1.6 ± 0.2	5 ± 1	77 ± 13

Data after 48 h of incubation. Stock solutions in DMSO for all complexes; stock solutions in H₂O/NaCl for cisplatin. A2780, cisplatin-sensitive ovarian cancer cells; A2780cis, cisplatin-resistant ovarian cancer cells; HaCaT, normal keratinocytes.

The analysis of data reported in Table 1 offers interesting insights to define some correlations among the structures of the new complexes and their antiproliferative activity.

The first clear indication is represented by a sort of “halogen effect”. In fact, all complexes coordinating the iodide ligand and characterised by a *trans* geometry (**1c–d**, **2c–d** and **1b-I**) display a significant higher cytotoxicity than those bearing chloride (**1a–b** and **2a–b**), towards both ovarian cancer cell lines. This outcome is particularly evident by comparison between complexes **1b** and **1b-I**, which differ only in the coordinated halide. In this case, the IC₅₀ values differ by more than one order of magnitude. Intriguingly, this effect is reversed in the case of complexes equipped with the chelating phosphine dppf, where **3b** derivative (with chloride) is considerably more cytotoxic than **3d** (with iodide).

There appears to be some appreciable effects also passing from complexes coordinating PTA to those of DAPTA, being the former generally more active than the latter.

Another significant overall trend is the very similar level of cytotoxicity showed by most of complexes towards the two cancer cell lines (A2780 and A2780cis), which are cisplatin-sensitive and cisplatin-resistant, respectively. This fact seems to presuppose that our Pd-imidoyl complexes act with a mechanism of action different from that of cisplatin and therefore could be potentially applied against cisplatin resistant forms of ovarian cancer.

Finally, the determination of the cytotoxicity towards normal human cells (HaCaT) of those complexes that proved to be the most active against ovarian cancer cells, has revealed that a few of them show an interesting degree of selectivity. This is especially the case of compounds **1b-I**, **2c**, and **3b** for which the IC₅₀ values relating to normal cells are more than one order of magnitude higher than those obtained on ovarian cancer cells.

Pro-apoptotic effect on ovarian cancer cell lines

The Pd(II)-imidoyl complexes **3b**, **1c**, **1d**, **2c**, **1b-I** were more deeply investigated in order to understand if the found antiproliferative effects were correlated with apoptotic mechanisms, using the annexin V test on both A2780 and A2780cis ovarian cancer cells.

All the obtained results were summarized in Tables 2 and 3 and in Figures 4–6.

Only complex **1c** (1 μM) showed high pro-apoptotic activity (50% of apoptotic cells), in comparison to the DMSO vehicle (2.76%).

A2780cis cells, after treatment, as displayed in the following Table 3 and in the related Figure 5, are more sensitive to DMSO, the solvent (vehicle) used to dilute the analysed derivatives. Furthermore all the complexes, except **1c**, cause cell death, observable in the upper left quadrants, not due to apoptosis process. Conversely, derivative **1c**, also on this cell line, clearly induces apoptosis (28.27%) compared to the DMSO vehicle (16.36%).

1c was the only complex able to induce apoptosis in both A2780 and A2780cis cell lines; it is practically capable to fully stimulate the apoptosis process when the concentration was

Table 2. Induction of apoptosis of complexes **3b**, **1c**, **1d**, **2c**, **1b-I** and cisplatin on A2780 cancer cells.

Complex	Live %	Dead/Debris %	Total apoptosis %
Vehicle (H ₂ O)	97.90	1.35	0.75
Cisplatin	63.60	0.70	25.70
Vehicle (DMSO)	96.20	1.04	2.76
3b	93.94	1.42	4.64
1c	46.88	3.12	50.00
1d	89.70	4.55	6.45
2c	93.25	3.95	2.80
1b-I	88.20	7.85	3.95

The apoptosis level was detected on A2780 cells treated with the previously calculated IC₅₀ concentrations of complexes **3b** (4.6 μM), **1c** (1.0 μM), **1d** (3.0 μM), **2c** (5.0 μM), **1b-I** (1.6 μM), and cisplatin (0.7 μM).

Table 3. Induction of apoptosis of complexes **3b**, **1c**, **1d**, **2c**, **1b-I** and cisplatin on A2780cis cancer cells.

Complex	Live %	Dead/Debris %	Total apoptosis %
Vehicle (H ₂ O)	92.04	0.27	12.87
Cisplatin	88.56	0.44	11.00
Vehicle (DMSO)	71.05	12.59	16.36
3b	62.96	18.91	18.13
1c	58.36	13.37	28.27
1d	63.90	27.63	8.48
2c	62.45	26.20	11.35
1b-I	55.79	33.55	10.66

The apoptosis level was detected on A2780cis cells treated with the previously calculated IC₅₀ concentrations of complexes **3b** (4.5 μM), **1c** (0.5 μM), **1d** (1.5 μM), **2c** (4.0 μM), **1b-I** (5.0 μM), and cisplatin (8.5 μM).

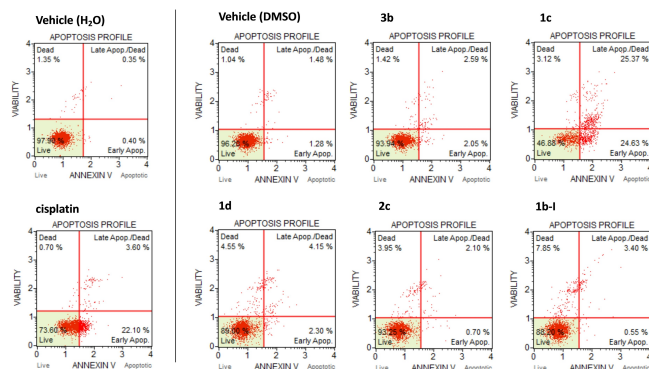


Figure 4. Induction of apoptosis of complexes **3b**, **1c**, **1d**, **2c**, **1b-I** and cisplatin in representative experiments. Annexin V/7-AAD assay was employed. A2780 cells were treated with the derivatives or cisplatin at IC_{50} concentration for 48 h and then assayed for apoptosis induction, in comparison with cells treated with vehicle (water or DMSO).

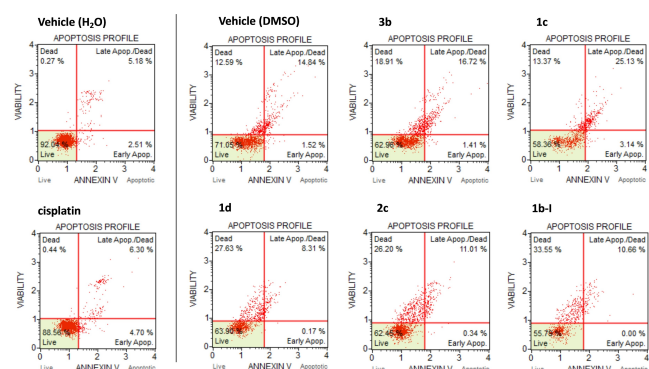


Figure 5. Induction of apoptosis of complexes **3b**, **1c**, **1d**, **2c**, **1b-I** and cisplatin in representative experiments. Annexin V/7-AAD assay was employed. A2780cis cells were treated with the derivatives or cisplatin at IC_{50} concentration for 48 h and then assayed for apoptosis induction, in comparison with cells treated with vehicle (water or DMSO).

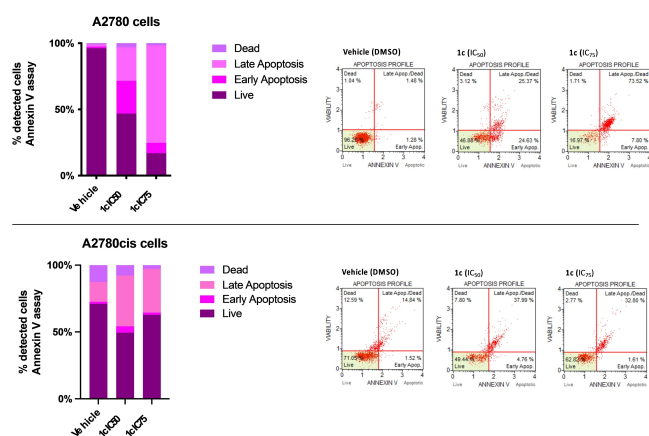


Figure 6. Induction of apoptosis of complex **1c** at different concentrations (IC_{50} and IC_{75}) on A2780 (upper) and A2780cis (lower) cells, in comparison with cells treated with vehicle (DMSO). Histograms highlight cells live (purple), in early apoptosis (fuchsia), when fluorescent annexin V binds the externalized phosphatidyl serine (PS) during the characteristic first steps of apoptosis, in late apoptosis (pink), when 7-AAD stains double stranded DNA.

increased to the IC_{75} value ($5 \mu\text{M}$) on A2780 cells and maintains cells in apoptosis (34.41%) at $1 \mu\text{M}$ (IC_{75} value) also on A2780cis cell line (Figure 6). In addition, in the right of the Figure 6, histograms report the values of early and late apoptosis, underlying, after 48 h of treatment, that A2780 cells were induced primarily in late apoptosis, while A2780cis cells were induced primarily in late apoptosis.

Conclusions

In this work a general synthetic protocol has been developed to prepare a selection of new Pd(II)-imidoyl derivatives. In particular, it has been found the suitable type of precursors, which must have ancillary ligands easily removable and being able to promote selectively the mono-insertion of isocyanide into Pd–Me and Pd–aryl bonds. The ligands chosen to prepare the target complexes have been the two monodentate phosphine PTA and DAPTA, which confer selectively a *trans* conformation and can contribute to increase the solubility of the metal derivatives in the cellular environment. Moreover, even two complexes bearing the chelating phosphine dppf have been synthesized, obtaining Pd–Fe bimetallic systems with the aim to exploit the combined effect of the two organometallic moieties. All complexes have been thoroughly characterized by NMR, IR and HRMS techniques, and in many cases, it has been also possible resolve their X-ray structures.

Practically, all complexes have resulted active towards the two ovarian cancer cell lines tested often with comparable or better efficiency than cisplatin. The most relevant contribute to the cytotoxicity of this kind of compounds seems to come from the presence of the iodide in place of chloride. This “halogen effect” that regularly appears for the complexes bearing PTA or DAPTA, is curiously reversed in those ones equipped with dppf, for which the compound **3b** (with chloride) has proven a higher antiproliferative activity than **3d** (with iodide).

Another important feature of the compounds reported in this work, is their very similar level of cytotoxicity on A2780 and A2780cis cell lines, to sanction that their mechanism of action is probably different from this of the classical Pt-based drugs. This evidence, together with the fact that some of our Pd-imidoyl complexes presents IC_{50} values lower than cisplatin, makes them interesting since they could be employed against the form of tumor cisplatin resistant. In this respect, it should be remembered that the first line of treatment for the ovarian cancer involves the use of the platinum-based drugs, but very often resistance phenomena are recorded against which these drugs become completely ineffective.

It is also of great significant that some complexes (especially **1b-I**, **2c**, and **3b**) have shown an interesting selectivity towards ovarian cancer cells, being the IC_{50} values for normal cells HaCaT more than one order of magnitude higher.

Furthermore, for what concerns the mechanism of action of the new palladium derivatives, it has been clearly demonstrated that at least the most active complex **1c** is able to promote effectively the cell death by apoptosis.

In the panorama of organopalladium compounds tested as anticancer agents, these new imidoyl derivatives displayed a significant activity. However, they are less active with respect to the most promising palladium-allyl derivatives previously described by our group.^[24,31] In particular the general trend according to which neutral palladium complexes are less active than cationic ones is confirmed.

In conclusion, this work allows us to select at least five compounds (**3b**, **1c**, **1d**, **2c**, **1b-l**) that deserve to be considered for further studies with the goal to better define their mechanism of action and test their activity on more complex biological systems than 2D cell cultures.

Experimental

Materials and methods

Dichloromethane, toluene and diethyl ether were anhydriated under molecular sieves (4 Å, 10%) and maintained under Argon atmosphere. The isocyanides dimethylphenyl isocyanide (DIC) and *p*-toluenesulfonylmethyl isocyanide (TosMIC), the amine tetramethylethylenediamine (TMEDA) and the phosphines 1,3,5-triaza-phosphadadamantane (PTA), 3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo[3.3.1]nonane (DAPTA) and 1,1'-ferrocenylbis(diphenylphosphine) (dppf) were used as purchased. Complexes **a** and **b** and the ligand 2-((methylthio)methyl)pyridine (NS-Me) were synthesized following the published procedures.^[43]

NMR spectra were obtained by Bruker 300 MHz spectrometer. For IR measurements a PerkinElmer Spectrum One spectrophotometer was employed. HRMS data were collected by a Bruker Compact Q-TOF.

General procedure for the synthesis of complexes

General procedure for the synthesis of complexes with NS ligands (a–b)

To a solution of one equivalent of [PdCl(NS-Me)(Me)] dissolved in anhydrous CH₂Cl₂, two equivalents of NS-Me and one equivalent of 2,6-dimethylphenyl isocyanide (DIC) or *p*-toluenesulfonylmethyl isocyanide (ToMIC) were added under inert atmosphere (Ar). The resulting yellow solution was stirred for 1 h at room temperature. The solvent was completely removed under vacuum, the title complex triturated by addition of diethyl ether and pentane, filter and dried under vacuum.^[43]

General procedure for the synthesis of complexes with NN ligands (c–d)

One equivalent of [Pd(TMEDA)(*p*-tolyl)] was dissolved in anhydrous CH₂Cl₂ under inert atmosphere. Two equivalents of tetramethylethylenediamine (TMEDA) and one equivalent of dimethylphenyl isocyanide (DIC) or *p*-toluenesulfonylmethyl isocyanide (TosMIC) were added. The dark orange solution was stirred for 20 min at room temperature and then was filter-off on a Millipore apparatus. The solvent removed under vacuum and the final compounds treated with diethyl ether and isolated on Gooch filter and dried under vacuum.

General procedure for the synthesis of PTA/DAPTA palladium(II) imidoyl complexes

Two equivalents of PTA or DAPTA were added to a solution of one equivalent of palladium NS (**a**, **b**) or NN precursor (**c**, **d**) in CH₂Cl₂ under inert atmosphere. After 15 min at room temperature the yellow solution was concentrated removing the solvent under vacuum. The final compound was precipitated by adding diethyl ether, isolated on Gooch filter and dried under vacuum.

General procedure for the synthesis of dppf palladium(II) imidoyl complexes **3b** and **3d**

One equivalent of dppf was added to a solution of one equivalent of palladium NS (**b**) or NN (**d**) precursor in CH₂Cl₂ under inert atmosphere. After 15 min at room temperature, the yellow solution was concentrated removing the solvent under vacuum. The final compound was precipitated by adding diethyl ether, isolated on Gooch filter and dried under vacuum.

Cell growth condition and treatments

Human ovarian cancer cisplatin-sensitive A2780 and cisplatin-resistant A2780cis cell lines were used to test the new palladium complexes.^[21] Cells were seeded in 24-well plates and maintained at standard conditions (37 °C in a humidified 5% CO₂ atmosphere) for 24 h, then treated and incubated for additional 48 h. Following 48 h of treatment, cells were detached from plates with Trypsin-EDTA counted using a BECKMAN COULTER Z2 cell counter (Beckman, Pasadena, CA, USA) and analyzed through Annexin V assays.

Antiproliferative activity

The antiproliferative effects of the new palladium complexes were investigated on A2780, A2780cis and HaCaT cell lines. For each sample, the antiproliferative activity has been evaluated working with different concentrations (0.1, 1, 10, 100 μM). 25,000 cells/mL were seeded in 24-well plates in RPMI complete medium. Twenty-four hours after seeding, compounds were added in serial dilutions. Cells were detached 48 h after and resuspended in fresh complete medium. Cells suspensions were thoroughly mixed and 50 μL from each well were taken to be diluted in 5 mL of physiological solution. Cell count was performed with the BECKMAN COULTER Z2 cell counter (Beckman, Pasadena, CA, USA). Each compound's IC₅₀ value (the compound concentration inhibiting cell proliferation of 50%) were determined.^[57–60]

Proapoptotic activity

Apoptosis was examined with Guava® Muse® Cell Analyzer instrument and the appropriate kit (Luminex Corporation, Austin, TX, USA) on A2780 and A2780cis cells according to the manufacturer's protocol. Cells, after the defined period of treatment, were washed twice with PBS, detached, and resuspended in fresh complete medium. 100 μL of cell suspension was incubated for 20 min, in the dark, at room temperature with an equal volume of Muse® Annexin V & 7-AAD (7-aminoactinomycin D) Dead Cell reagent. The bind of the fluorescent annexin V with the externalized phosphatidyl serine (PS), during the first step of apoptosis, highlights cells in early apoptosis, and the 7-AAD stains cells in late apoptosis. All the data were acquired and recorded exploiting the Annexin V and Dead Cell Software Module (Millipore, Billerica, MA, USA).

Crystal Structure Determination

Crystals data for complexes **c**, **1b**, **1c**, **1d** and **3b** (Tables S1–9 in Supporting Information) were collected at the Elettra Synchrotron, Trieste (Italy).^[61] The details of the procedure followed for the determinations of crystallographic data are reported in Supporting Information.

Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 2270640, 2270641, 2270642, 2270643, 2270646,

2270647, 2270645 and 2270648 for **1b** at 100 K, **1b** at 298 K, **1c** at 100 K, **1d** at 100 K, **c** in two enantiomorphic crystal packing at 100 K, **3b** polymorph α and polymorph β at 100 and 210 K respectively. These data can be obtained free of charge via <https://www.ccdc.cam.ac.uk/structures>.

Supporting Information

Additional references cited within the Supporting Info.^[62–72]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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