RSC Advances

PAPER

Cite this: *RSC Adv.*, 2015, **5**, 86274

Chelate *N,O*-palladium(II) complexes: synthesis, characterization and biological activity†

Vladimir P. Petrović,[a] Marko N. Živanović,[b] Dušica Simijonović,[a] Jelena Đorović,[ac] Zorica D. Petrović*[a] and Snežana D. Marković[b]

Received 29th May 2015
Accepted 5th October 2015

DOI: 10.1039/c5ra10204a

www.rsc.org/advances

Four *trans* chelate *N,O*-palladium(II) complexes were synthetized starting from salicylaldehyde anil Schiff bases, as ligands. Their structures were elucidated using experimental and theoretical tools. The structures of the theoretically possible *cis* isomers are examined using the DFT method. The biological activity, *in vitro* cytotoxic and prooxidative effects against human breast carcinoma MDA-MB-231, human colon carcinoma HCT-116, and human fibroblast healthy MRC-5 cell lines of investigated compounds were determined. Schiff bases show a moderate or weak cytotoxic effect. On the other hand, complexes **Pd-1** and **Pd-6** show a significant cytotoxic effect on all three cell lines, with IC_{50} values in the range of 0.6 to 17.1 μM on HCT-116 cells, 7.2 to 55.6 μM on MDA-MB-231 cells and 34.5 to 48.1 μM on MRC-5 cells. Also, **Pd-1** and **Pd-6** induce extreme oxidative stress in the all treated cell lines. At this stage of investigation, **Pd-1** and **Pd-6** showed no selectivity towards cancer cells, *i.e.* they were also cytotoxic to MRC-5 cells to a similar extent. Taking into account these facts, it could be further investigated how the most active substances impact on the type of cell death (apoptotic and/or necrotic pathways).

Introduction

Azomethines, also known as Schiff bases, are an important class of organic compounds.[1] If these compounds contain an aniline moiety (or substituted aniline), they receive 'anil' as part of their names.[1] Some of these compounds have wide applications in organic synthesis, catalysis, analytical chemistry, the food industry, as well as the pigment and dye industries.[2] The azomethine group, as a structural fragment of these compounds, is present in various natural and synthetic products and it is responsible for a broad range of biological activities,[3] including antibacterial, antifungal, antimalarial, anti-inflammatory, antiviral, antiproliferative, and antipyretic.[3c,4]

Moreover, Schiff bases are a special class of ligands with a variety of donor atoms. If these compounds possess donor atoms such O, N or S, then they can act as chelating ligands in the design of many metal complexes. The high affinity of these compounds for complexation with transition metal ions is utilized for preparation of different complexes.[5] Phenolic Schiff bases as bidentate ligands play a very important role in coordination chemistry and their metal complexes have significant importance. It should be noted that these complexes possess a number of important properties such as, easy synthesis, stability and wide application.[6] A large number of Schiff base complexes have been reported so far, and their catalytic and biological properties have been studied intensively.[7] It is worth pointing out that complexation of the ligands with various metals ions results in an increasing biological activity.[8]

The antibacterial and antifungal activities of different transition metal complexes (Mn(II), Co(II), Ni(II), Cu(II) and Pd(II)) with Schiff bases ligands have been reported.[9] On the basis of the fact that there is structural and thermodynamic analogy between platinum(II) and palladium(II) complexes, the study of anticancer activity of palladium(II) complexes is of considerable importance. In addition, platinum based compounds had not entered clinical trial for more than a decade, which influenced development in research of other metal compounds.[10] A cytotoxic activities of different Schiff base Pd(II) complexes was evaluated on wide range of cancer cell lines.[11] However, the results related to the examination of anticancer activity of palladium(II) complexes with Schiff bases as ligands are limited. One of the possible causes may be the fact that many of them hydrolyse very fast, and that reaction produces reactive compounds unable to act as potential drugs.[10,12]

[a]*Faculty of Science, University of Kragujevac, Department of Chemistry, Radoja Domanovića 12, 34000 Kragujevac, Serbia. E-mail: zorica@kg.ac.rs; Fax: +381 34335040*

[b]*Faculty of Science, University of Kragujevac, Department of Biology and Ecology, Radoja Domanovića 12, 34000 Kragujevac, Serbia*

[c]*Bioengineering Research and Development Center, 34000 Kragujevac, Republic of Serbia*

† Electronic supplementary information (ESI) available: Characterization of Schiff bases **1**, **3**, **5**, **6** (^{13}C NMR spectra, Cartesian coordinates of the optimised structures) and of corresponding *trans* Pd(II) complexes **Pd-1**, **Pd-3**, **Pd-5**, and **Pd-6** (^{1}H and ^{13}C NMR spectra, Cartesian coordinates of the optimised structures). The optimized structures of the theoretically possible *cis* isomers Pd(II) complexes and their spectral data. Detailed oxidative stress status parameters for HCT-116, MDA-MB-231 and MRC-5 cells. See DOI: 10.1039/c5ra10204a

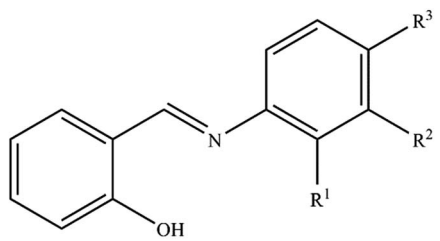
In the further course of our investigations of anil Schiff bases,[13] our advanced step was to evaluate their cytotoxic activity and oxidative stress status. Furthermore, we used four of them as *N*,*O*-bidentate ligands to synthetize stable Pd(II) complexes, and to test them for their cytotoxic activity and oxidative stress status, also. For this purpose we used human adherent colorectal cancer cell line (HCT-116), human metastatic mammary gland breast carcinoma cell line (MDA-MB-231) and human fibroblast healthy cell line (MRC-5). Moreover, we used cisplatin (**CisPt**) as positive control, while untreated cells were considered as negative control.

Results and discussion

In our previous work we presented antioxidative activity of some salicylaldehyde and vanillic anil Schiff bases.[13] In addition to this, we synthesized four palladium(II) complexes, starting from *N*-salicylidene aniline Schiff bases (**1**, **3**, **5**, and **6** from ref. 13) and palladium(II) acetate (molar ratio 2 : 1). Our efforts to synthetize pure chelate complexes with the Schiff bases **2**, **4**, and **7** were unsuccessful. However, the Schiff bases examined in this paper are presented on Fig. 1. The structure of the prepared complexes was elucidated using experimental and theoretical tools. Biological activity of these complexes and of their precursors was examined. It is worth pointing out that some structural characterizations of investigated complexes can be found in literature,[14] except for **Pd-3** which structural characterization was given first time now. Nevertheless, to our best knowledge, this kind of characterization for investigated complexes has not been reported until now.

Structural characterization of the investigated complexes

The optimized *trans* and *cis* geometries of investigated complexes (**Pd-1**, **Pd-3**, **Pd-5**, and **Pd-6**) are presented in Fig. 2 and S1.† Experimental and simulated IR spectra of palladium(II) complexes and of their Schiff base precursors are depicted in Fig. 3 and S2,† while ^{13}C NMR spectral characterization is presented in Tables 1 and S1.† Bond lengths, angles, and dihedral angles of all complexes calculated are listed in Tables S1–S4,† while corresponding atom labelling is depicted in Fig. S3.†



1 R^1, R^2=H, R^3=OH
2 R^1, R^2=H, R^3=NO_2
3 R^1, R^2=H, R^3=CH_3
4 R^1, R^2, R^3=H
5 R^1, R^2=H, R^3=F
6 R^1=H, R^2=OH, R^3=H
7 R^1=OH, R^2, R^3=H

Fig. 1 The Schiff bases examined.

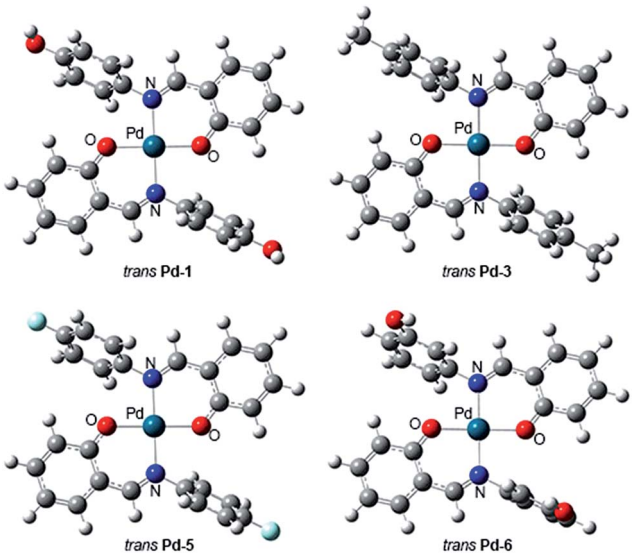


Fig. 2 The optimized geometries of *trans* complexes investigated.

All complexes exhibit nearly ideal square planar coordination, with angles around palladium close to 90° (Fig. 2 and Tables S2–S5†). Each ligand (one nitrogen and one oxygen donating atom) forms six-membered ring with palladium. It is worth pointing out that the optimized parameters (bond distances, angles, and dihedral angles) for **Pd-1** are in agreement with reported values for X-ray structure.[14]

The NBO analysis revealed that, in all cases, there are no covalent bonds of palladium with ligating atoms. Instead, there is strong donation of electron density from the donor atoms to palladium. Particularly, nitrogens delocalized their lone pairs from the sp^3 orbitals to the formally empty d orbital of palladium(II), while oxygens contribute with lone pairs from pure p orbitals. As a consequence, occupancies in the orbitals of the donor atoms are reduced (1.62 and 1.67 respectively), while the occupancy of palladium(II) formally empty d orbital is increased (0.99).

Next, we wanted to confirm that suggested structures correspond to the experimentally obtained complexes. For this purpose we applied IR and NMR spectroscopy, as well as density functional theory. Namely, experimental data (IR and ^{13}C NMR spectra) are compared to those theoretically obtained for *trans* and *cis* isomers. It is worth pointing out that in all cases *trans* isomers are more stable than the *cis* ones, Table 2.

On the basis of these facts, and experimental data (ref. 14), one can undoubtedly conclude that obtained complexes are *trans* isomers.

IR spectral characterization

At first glance, good agreement between experimental and calculated spectra is achieved, Fig. 3. In all calculated spectra, deviations from the experimental values are observed in the region above 3000 cm^{-1}. OH stretching vibrations are underestimated in case of ligands, while in the spectra of the complexes (where OH group is still present), these bands are overestimated. This can be attributed to the negligence of the

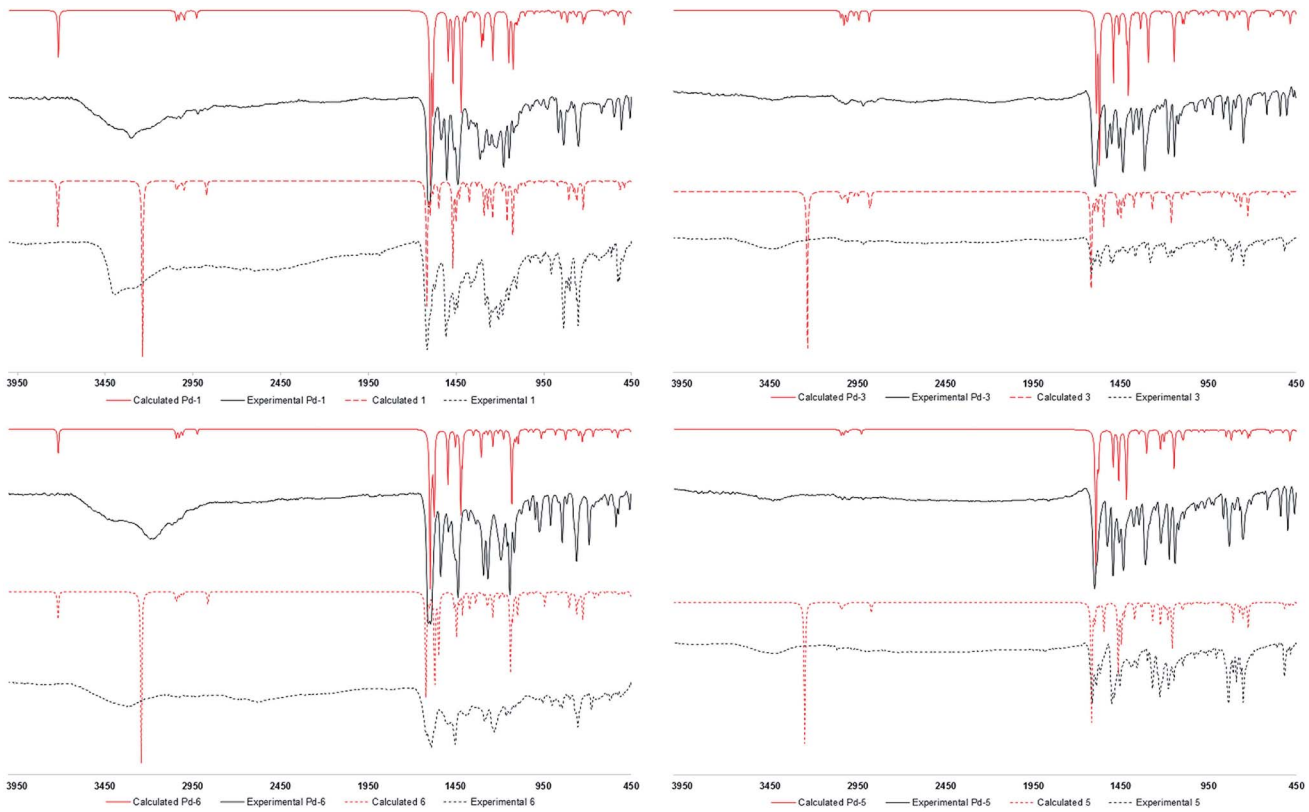


Fig. 3 Calculated and experimental IR spectra of *trans* complexes **Pd-1**, **Pd-3**, **Pd-5**, and **Pd-6**, and of Schiff base ligands **1**, **3**, **5**, and **6**.

intermolecular forces present in the solid state. Nevertheless, the calculated spectra of Schiff base ligands and corresponding palladium complexes reveal the difference in their structure. Namely, in the spectra of complexes **Pd-1** and **Pd-6** bands assigned to OH stretching vibrations are somewhat changed, while in cases of complexes **Pd-3** and **Pd-5**, these bands are completely absent from the spectra. This fact clearly shows that the salicylaldehyde originating oxygen (from the deprotonated phenolic group) became coordinated to palladium.

In the spectra of ligands the bands in region 1615–1620 cm^{-1} and 1620–1630 cm^{-1} (experimental and calculated values respectively) are assigned to the C═N stretching vibrations. In the spectra of the corresponding complexes (*trans* and *cis*), these vibrations are shifted to somewhat lower frequencies: 1600–1610 cm^{-1} and 1590–1600 cm^{-1}, respectively. These shifts are obviously a consequence of the formation of the palladium complexes, and are in agreement with the NBO analysis, which revealed donation of nitrogen's lone pair to the formally empty d orbital of palladium. Furthermore, comparison of the experimental spectra of ligands and analogous spectra of the complexes in the region of 505–540 cm^{-1} and 460–470 cm^{-1} showed that two new bands appeared in each spectrum of the complexes. These new bands are assigned to newly established Pd–O and Pd–N coordinative bonds, respectively. In addition, inspection of the calculated spectra confirmed this assumption, with the difference that these bands are slightly overestimated (525–560 cm^{-1} and 490–525 cm^{-1}). It is worth pointing out that on the basis of theoretical results for *cis* isomers (Fig. S3†) of investigated complexes, one can conclude that their IR spectra are very similar to the IR spectra of the *trans* isomers.

NMR spectral characterization

The summary of ^{13}C NMR data is presented in Table 1. The ^{13}C NMR properties of the ligands and corresponding complexes were predicted, and the chemical shifts for all carbon atoms were calculated relative to TMS. On the basis of the experimental and calculated shifts for the Schiff base ligands, one can conclude that theoretical model reproduced experimental NMR spectra with satisfactory accuracy. Namely, the Absolute Average Errors (AAE) for ^{13}C NMR amount to 2–6 ppm. In addition, the correlation coefficients (R) for the dependencies of the calculated chemical shifts on the experimental values are larger than 0.95. Theoretical model predicted the ^{13}C NMR spectra with high accuracy (AAE amount to 2–5, and R above 0.97), also.

Comparison of the chemical shifts in the Schiff bases and corresponding complexes (*trans* and *cis*) revealed that values for carbon atom from azomethine group, for the one from phenyl ring substituted with oxygen (salicylaldehyde originating oxygen), as well as for the carbon from phenyl ring bonded to nitrogen (aniline originating), are slightly elevated to higher values (Table 1). In accordance to these are predicted chemical shifts values. Similarly to the case of IR characterization, this clearly points out that the nitrogen from azomethine group and the salicylaldehyde originating oxygen became coordinated to palladium. It is worth pointing out that, as a consequence of Schiff bases coordination to the palladium, chemical shifts for

Table 1 ^{13}C NMR chemical shifts for the investigated Schiff bases and corresponding *trans* palladium complexes. *R* and AAE stand for correlation coefficient and Average Absolute Error

		C=N		Ar C–O$^-$		Ar C–N		Ar C				CH_3	
Compound		Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.		Calc.		Exp.	Calc.
1		160.28	164.31	157.07	161.25	139.38	^{15}4.85	132.62	119.08	140.71	117.89	—	—
								132.28	116.57	135.45	117.22		
R	AAE							122.72	116.10	134.85	116.20		
0.95	6							119.58		129.58			
Pd-1		164.26	169.56	163.22	164.36	155.88	154.13	140.92	120.43	142.59	119.59	—	—
								135.22	119.74	138.20	117.99		
R	AAE							134.84	114.70	130.07	112.47		
0.99	2							125.70	114.36	126.44	111.69		
3		161.69	166.86	161.16	162.75	145.97	147.42	136.86	120.97	137.20	117.80	21.00	22.42
								132.86	119.34	135.44	117.07		
R	AAE							132.08	118.94	130.06	116.79		
0.99	2							129.98	117.22	126.87	116.28		
Pd-3		165.26	168.19	162.68	162.37	147.12	146.03	136.07	124.40	137.76	124.99	21.10	21.81
								135.00	120.70	135.70	123.02		
R	AAE							134.38	120.33	134.93	116.43		
0.99	2							128.63	115.03	127.96	110.92		
5		164.13	166.97	162.46	161.56	161.09	160.56	159.23	122.66	145.82	118.04	—	—
								144.74	122.49	135.83	116.91		
R	AAE							133.20	119.12	135.41	116.10		
0.97	4							132.26	116.42	127.40	115.62		
Pd-5		165.20	170.68	163.66	164.38	163.07	161.90	158.78	126.23	146.80	120.61	—	—
								145.40	126.07	138.38	118.06		
R	AAE							135.49	120.31	130.16	114.73		
0.97	5							134.51	114.89	126.93	112.67		
6		163.23	167.46	160.43	160.67	158.43	156.87	149.42	119.22	151.52	116.95	—	—
								133.33	116.68	137.64	116.50		
								132.67	114.21	135.84	115.48		
R	AAE							130.29	112.15	131.29	111.99		
0.98	3							129.51	108.20	117.81	102.24		
Pd-6		164.28	168.97	163.31	162.06	157.19	155.23	150.23	120.04	151.73	116.97	—	—
								135.35	115.45	137.66	116.47		
								135.11	114.82	136.81	111.58		
R	AAE							128.78	113.37	128.64	109.37		
0.99	2							120.30	112.04	117.11	108.28		

Table 2 Difference in free energy (kJ mol^{-1}) of the corresponding *cis* and *trans* complexes

	ΔG_g (kJ mol^{-1})	$\Delta G_{solvent}$ (kJ mol^{-1})
Pd-1	14.56102	5.849614
Pd-3	9.646087	4.891306
Pd-5	16.75857	10.73304
Pd-6	16.5249	5.608068

all aromatic carbons are to some extent elevated, in both, experimental and theoretical spectra. Similarly to the case of IR spectral characterisation, on the basis of the obtained data, one can observe significant similarity between ^{13}C NMR spectra of the *cis* isomers and corresponding *trans* isomers.

Biological evaluation

Cytotoxic effects. The cytotoxicity of investigated substances was determined by MTT assay. The cytotoxic effects expressed as IC_{50} values for Schiff bases and their corresponding Pd(II) complexes on HCT-116 and MDA-MB-231 cancer cell lines and on human fibroblast healthy MRC-5 cell line are depicted in Table 3. It was shown that **Pd-1** and **Pd-6** show significant cytotoxic effects on all three cell lines, with IC_{50} values in range of 0.6 to 17.1 μM on HCT-116 cells, 7.2 to 55.6 μM on MDA-MB-231 cells (with exception of **Pd-1** after 24 h from treatment, IC_{50} = 276.9 μM) and 34.5 to 48.16 μM on MRC-5 cells. Bearing in mind that IC_{50} values obtained with **CisPt** on same cell lines are in range of 26.9 to >500 μM, results obtained with **Pd-1** and **Pd-6** are very promising. Also, **Pd(OAc)$_2$**, **Pd-3**, and **1** exerted higher cytotoxic effect in comparison to other Schiff bases and their complexes. One should take in consideration great influence of palladium(II) on cytotoxicity, especially in **Pd-1** and **Pd-6** complexes, whose ligands **1** and **6** do not exert such a significant cytotoxic effects. Investigation of **Pd(OAc)$_2$**, **Pd-3** and **Pd-5** complexes suggests that effects of investigated Pd(II) complexes primarily depend on chemical structure, rather than on possible hydrolyses of complexes. This assumption is supported by fact that IC_{50} values for **Pd(OAc)$_2$**, **Pd-3** and **Pd-5** are much higher than IC_{50} values of **Pd-1** and **Pd-6**. Hydrolysed complexes

Table 3 IC_{50} values (μM) of the investigated compounds

	IC_{50}, μM					
	HCT-116		MDA-MB-231		MRC-5	
	24 h	72 h	24 h	72 h	24 h	72 h
1	142.3	368.0	440.2	133.6	—	—
Pd-1	11.8	17.1	276.9	7.2	34.5	48.1
2	>500	>500	>500	>500	—	—
3	>500	>500	>500	383.4	—	—
Pd-3	135.7	>500	>500	>500	>500	>500
4	>500	295.3	>500	>500	—	—
5	>500	>500	>500	>500	—	—
Pd-5	>500	>500	>500	145.3	>500	>500
6	>500	277.6	>500	>500	—	—
Pd-6	5.8	0.6	55.6	40.7	36.6	42.5
7	>500	34.7	>500	>500	—	—
$Pd(OAc)_2$	111.7	91.2	>500	>500	—	—
CisPt	254.9	28.7	>500	57.7	200.4	26.9

should possess similar cytotoxic activity as **$Pd(OAc)_2$**, which under our measuring condition was not recorded. Results presented in this work indicate that HCT-116 cells are more sensitive than MDA-MB-231 cells, which is in agreement with our earlier findings.[15] It is important to point out that significant difference in the sensitivity of the examined cells arises from differences in origin of the tested cells. HCT-116 cells are of primary tumour origin, while MDA-MB-231 cells are metastatic, and thus more resistant cells. On the other hand, human fibroblast healthy MRC-5 cells are also sensitive to investigated substances.

Superoxide anion radical ($O_2^{\bullet-}$) content changes. Superoxide anion radical, important indicator of reactive oxygen species (ROS) level, was determined by spectrophotometric NBT assay. Results representing $O_2^{\bullet-}$ changes 24 h from treatment are summarized in Table S6.† Measurement on HCT-116 and MDA-MB-231 cells revealed that **1** induced significant increase of $O_2^{\bullet-}$. Compounds **3** and **5** also increased $O_2^{\bullet-}$, but in much reduced extent, while compound **6** even decreased $O_2^{\bullet-}$ content. Palladium(II) complexes of these ligands induced increasing of $O_2^{\bullet-}$. Although it appears that increase of $O_2^{\bullet-}$ for **Pd-1** and **Pd-6** is not extreme, we should bear in mind that produced $O_2^{\bullet-}$ was at significant level proportionally to the number of survived cells (Fig. S4†). Similarly, one bear in mind that **CisPt** decreased $O_2^{\bullet-}$ content in high concentrations, but relating to the number of survived cells, **CisPt** practically significantly increased $O_2^{\bullet-}$. We conclude that the cells affected by investigated complexes are under enormous oxidative stress.

Regarding the above described results (obtained 24 h from treatment), we found it appropriate to estimate whether the effect of the investigated substances is acute or permanent. Thus, we measured effects of substances 72 h from treatment. With regards to the cytotoxicity assay, which revealed greater cytotoxicity after 72 h, we expected that increasing of $O_2^{\bullet-}$ content could be greater when compared to 24 h, what we have confirmed (Table S7†). Toxic **Pd-1** and **Pd-6** induced significant increasing of $O_2^{\bullet-}$. Taking into account content of $O_2^{\bullet-}$ in relation to the number of survived cells, this increasing is even greater (Fig. S5†). Thus, we conclude that cells are under greater oxidative stress 72 h, compared to 24 h from treatment.

Significant cytotoxic effects of investigated substances on tumour cells turned our focus towards investigation on healthy MRC-5 cells. Similarly to cancer cells, it was found that toxic **Pd-1** and **Pd-6** also induce great oxidative stress in MRC-5 cells, causing significant cytotoxicity. Results of production of $O_2^{\bullet-}$ are represented in Tables S6 and S7 and in Fig. S4 and S5† for 24 h and 72 h from treatment respectively. **Pd-1**, **Pd-6** and **CisPt** (after 72 h) induced great production of superoxide anion radical. Considering the number of survived cells, we also concluded that MRC-5 cells are under enormous oxidative stress, especially in treatment with toxic **Pd-1** and **Pd-6**. Comparing these three cell lines, one could conclude that treatment with toxic **Pd-1**, **Pd-6** and **CisPt** significantly induced increasing of $O_2^{\bullet-}$. The greatest increasing is observed with MRC-5 and HCT-116. Chemically induced increase of production of free radicals is usually followed by increased cytotoxicity,[16] which was also shown in this investigation. Substances which increased $O_2^{\bullet-}$ content exerted increased cytotoxicity and *vice versa*. The greatest effect on increased production of $O_2^{\bullet-}$ was recorded for **1**, **Pd-1** and **Pd-6**. **Pd-1** and **Pd-6** show greater increasing of $O_2^{\bullet-}$, as well as the greater cytotoxicity on the tested cell lines than **CisPt**.

Nitrite (NO_2^-) content changes. Nitrite concentration may indicate the level of NO and other reactive nitrogen species (RNS) in cells. Results of nitrite level measurements 24 h from treatment are presented in Table S8.† On HCT-116 cells we observed that **1** and **3** significantly increased, while **5** and **6** decreased NO_2^- content. On the other hand, we observed increase of nitrites with Pd(II) complexes, with exception of **Pd-6**. On the other hand, considering the number of remained viable cells, we conclude that cells are under the extreme oxidative stress, *i.e.* cells increased nitrite content, especially in treatment with **Pd-1**, **Pd-6** and **$Pd(OAc)_2$** (Fig. S6†). On MDA-MB-231 cells all substances increased nitrite level. This effect is more obvious for **1** and **Pd-6**. Similarly to NBT assay, the great increasing of nitrite level 24 h from treatment opened the question whether the effect of the investigated substances is acute or permanent. Thus, we investigated substances also 72 h from treatment (Table S9†). Obtained results show more significant increasing in nitrite level, so we conclude that the effect of investigated substances is not acute. Considering these results in relation to the number of survived cells, we concluded that HCT-116 and MDA-MB-231 cells are under great oxidative stress (Fig. S7†). In addition to the tests on the tumour cells, it was estimated the impact of Pd(II) complexes and **CisPt** on healthy MRC-5 cells. It was found that toxic **Pd-1** and **Pd-6** significantly increased nitrites 24 and 72 h from treatment (Fig. S6 and S7†). Comparing investigated cell lines, we found that after 24 h **Pd-1** possess the greatest effect on MRC-5 and HCT-116 cells. **Pd-6** induces similar increasing of nitrites in all three cell lines. After 72 h, **Pd-1** affects all three cell lines in similar extent, while **Pd-6** shows the most significant effect on HCT-116 cell line.

Reduced glutathione (GSH) content changes. Glutathione is a tripeptide responsible for the defence of eukaryotic cells from the influence of ROS and RNS.[17] Table S10† represents the effects of tested compounds on change of content of GSH 24 h from treatment. It was observed that only **1** increased GSH level on HCT-116 cells, while on MDA-MB-231 cells **1** induced no changes. **3**, **5** and **6** mostly induced decrease of GSH level on both cell lines. Considering the production of remained survived cells, **1** actually significantly increased GSH level, especially on HCT-116 cells. Pd(II) complexes mostly induced significant increase of glutathione, especially when we take in consideration the number of viable cells (Fig. S8†). Compounds **1**, **Pd-1**, **Pd-6** and **Pd(OAc)$_2$** showed the greatest effect. Similarly as in NBT and Griess assays, we tested the GSH level 72 h from treatment (Table S11 and Fig. S9†). Similarly as after 24 h it was estimated significant increasing of GSH content 72 h from treatment, especially for toxic **Pd-1** and **Pd-6**. **CisPt** and **Pd(OAc)$_2$** also significantly increased GSH level.

Measuring of GSH level on healthy MRC-5 cells revealed that GSH also increased for toxic **Pd-1**, **Pd-6** and **CisPt** (Fig. S8 and S9†). Practically, all three cell lines possess similar positive feedback on GSH synthesis due to the influence of toxic substances, which introduced cells into enormous oxidative stress. Our assumption is that glutathione reacts with produced ROS/RNS reactive species and we may expect that some part of GSH reacted with applied substances.[18]

Conclusions

The results presented in this paper include the synthesis of the four *trans* chelate *N*,*O*-palladium(II) complexes, investigation of their structure using experimental and theoretical tools. The structures of the theoretically possible *cis* isomers are examined using DFT method, also. Study of biological activity of the complexes, as well as biological activity of the starting salicylaldehyde anil Schiff bases was performed. From the presented results it can be concluded that the investigated compounds act as prooxidants on the investigated cancer HCT-116, MDA-MB-231 and healthy MRC-5 cell lines, due to increased production of superoxide anion radical and nitrites. Greater production of ROS/RNS induced increasing in cytotoxicity, especially in treatment with **Pd-1** and **Pd-6**. Less cytotoxic ligands, and their complexes, taken as a relation to the number of survived cells, possess certain but not such a denominated prooxidative character as **Pd-1** and **Pd-6**. ROS/RNS induced disruption of redox equilibrium is related to the cell self-defence system, which influenced the enhanced production of the glutathione. The significant difference in activity of the investigated compounds may be attributed to the presence of phenolic OH groups in the complex ligands **1** and **6** and to the fact that these ligands are activated by the metal ion. Significant prooxidative and cytotoxic potential of the most active compounds open questions regarding estimation of the type of the cell death (apoptosis/necrosis cell pathways), and deserve further investigations.

Experimental

Materials and reagents

The compounds salicylaldehyde, vanillin, aniline, 4-fluoroaniline, 4-nitroaniline, toluidine, 2-hydroxyaniline, 3-hydroxyaniline, 4-hydroxyaniline, palladium(II) acetate and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Aldrich Chemical Co. The NMR spectra were run in DMSO and $CDCl_3$ on a Varian Gemini 200 MHz spectrometer. Melting points were determined on a Mel-Temp capillary melting points apparatus, model 1001. Elemental microanalysis for carbon, hydrogen, and nitrogen were performed at the Faculty of Chemistry, University of Belgrade. Dulbecco's Modified Eagle Medium (DMEM) and PBS were obtained from GIBCO, Invitrogen, USA. Foetal bovine serum (FBS) and trypsin–EDTA were from PAA (The Cell Culture Company, Pasching, Austria). Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and nitroblue tetrazolium (NBT) were obtained from SERVA, Heidelberg, Germany. *N*-1-Napthylethylenediamine dihydrochloride was purchased from Fluka chemie GMBH, Buchs, Switzerland. Sulfanilamide and sulphosalicylic acid were purchased from MP Hemija Belgrade, Serbia. All solvents and chemicals were of analytical grade.

Synthesis of Schiff bases. Schiff bases (**1–7**) were prepared according to procedure in our recently published paper.[13]

Synthesis of palladium(II) complexes. Palladium(II) acetate (0.5 mmol) was added to solution of corresponding Schiff base (**1**, **3**, **5**, **6**) (1 mmol) of ethanol (5 mL). The resulting mixture was heated at reflux for 3 h. After completion of the reaction, the solvent was evaporated and leaving powder was washed with ethanol (3 × 2 mL). Complexes were obtained in 75–80% yield. All complexes were characterized with melting point, elemental microanalysis, ^{1}H NMR and ^{13}C NMR spectra (Table 1 and ESI†).

Pd-1-orange crystals – mp > 250 °C; lit. 306 °C;[14a] $C_{26}H_{20}N_2O_4Pd$ (FW = 530.87): C, 58.82; N, 5.28; H, 3.80%; found: C, 58.11; N, 4.98; H, 3.91%.

Pd-3-orange crystals – mp 243–245 °C; $C_{28}H_{24}N_2O_2Pd$ (FW = 526.92): C, 63.82; N, 5.32; H, 4.59%: C, 63.73; N, 5.43; H, 4.70%.

Pd-5-yellow-orange powder – mp > 250 °C; lit. 335 °C;[14b] $C_{26}H_{18}F_2N_2O_2Pd$ (FW = 534.85): C, 58.39; N, 5.24; H, 3.39%; found: C, 58.09; N, 5.32; H, 3.22%.

Pd-6-yellow powder – mp 223–225 °C; lit. 223 °C;[14a] $C_{26}H_{20}N_2O_4Pd$ (FW = 530.87): C, 58.82; N, 5.28; H, 3.80%; found: C, 58.72; N, 5.34; H, 3.91%.

Computational methods

All calculations were performed with the Gaussian 09 software package.[19] M06 functional in combination with triple split valence basis set 6-311 + G(d,p) was used for all atoms (C, H, O, N, and F) excluding Pd, where LANL2DZ + ECP[20] was employed. M06 hybrid meta functional is "a method with good accuracy across-the-board for transition metals, main group thermochemistry, medium-range correlation energy, and barrier heights".[21] Hybrid meta-GGA M06, developed by Zhao and Truhlar, is characterized by the way it has been parameterized.

The structures of investigated compounds were fully optimised in the gas-phase, and in chloroform or dimethyl sulfoxide ($\varepsilon = 24.3$ and $\varepsilon = 46.8$ respectively), using the conductor-like solvation model (CPCM).[22] Frequency calculations were carried out to confirm that all structures are local minima (all positive eigenvalues). The gas-phase structures were used for examination of geometrical parameters, and predicting IR spectra. The computed frequencies were scaled by the factor of 0.955. The NMR properties of compounds investigated were predicted by calculating the NMR shifts for all carbon atoms relative to TMS. In this purpose, Gauge-Independent Atomic Orbital (GIAO) method was applied. The natural bond orbital analysis (Gaussian NBO version) was performed.

Cell preparation and culturing. The colon cancer cell line HCT-116, breast cancer cell line MDA-MB-231 and human fibroblast healthy cell line MRC-5 were purchased from the American Tissue Culture Collection (Manassas, VA, USA). The cells were propagated in a humidified atmosphere with 5% CO_2 at 37 °C and maintained in DMEM supplemented with 10% foetal bovine serum, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were grown in 75 cm^2 culture bottles until a confluence of 70–80% and after a few passages cells were seeded in assay plates. A number of 10^4 cells per well were seeded in a 96-well plate for MTT cell viability assay, determination of superoxide anion radical concentration (NBT assay) and NO_2^- (Griess assay) and 5×10^4 cells per well for determination of reduced glutathione concentration.

MTT assay for cell viability. The cell viability of the colon and breast cancer cells after exposure to the compounds was measured by MTT assay.[23] MTT assay is based on the colour reaction of mitochondrial dehydrogenase from living cells with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), and the fact that MTT is reduced to purple formazan in living cells. The absorbance of this coloured solution was quantified spectrophotometrically at 570 nm on microplate reader (ELISA 2100C, Hamburg, Germany). This assay was described in brief elsewhere.[15a] Cell proliferation was calculated as the ratio of absorbance of the treated group divided by the absorbance of the control group, multiplied by 100 to give a viability percentage. The absorbance of the control group of cells served as viability of 100%. A plot of percentage of cytotoxicity *versus* sample concentrations was used to calculate the concentration which showed 50% cytotoxicity (IC_{50}).

Determination of superoxide anion radical (NBT assay). This method involves estimation of the rate of the reduction of nitroblue tetrazolium (NBT) to nitroblue-formazan in the presence of $O_2^{\bullet -}$.[24] This assay was described in brief elsewhere,[15a] and the results were expressed as μM.

Determination of nitrites (Griess assay). The Griess coloured reaction represents the spectrophotometric determination of NO_2^- (indicator of the nitric oxide – NO level).[25] The Griess reaction is a process of diazotization in which the NO-derived nitrosating agent (*e.g.*, N_2O_3), generated from the acid-catalyzed formation of nitrous acid from NO_2^- (or the interaction of NO with oxygen), reacts with sulfanilic acid to produce a diazonium ion that is then coupled to *N*-(1-napthyl) ethylenediamine to form a chromophoric azo product that absorbs strongly at 550 nm. Griess assay is performed at room temperature. This assay was described in brief elsewhere,[15a] and the results were expressed in μM of NO_2^- from a standard curve established in each test, constituted of known molar concentrations of NO_2^-.

Determination of reduced glutathione (GSH). Glutathione assay is based on redox reaction of intracellular GSH with Ellmans reagent, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB),[26] forming yellow product of 5′-thio-2-nitrobenzoic acid (TNB) which strongly absorbs at 405 nm. Similarly to NBT and Griess assays, this assay was described in brief elsewhere,[15a] and the results were expressed in μM of GSH from a standard curve established in each test, constituted of known molar GSH concentrations.

Statistics. The data were expressed as mean ± standard error (SE). Biological activity was the result of 3 individual experiments, performed in triplicate for each dose. Statistical significance was determined using the Student's *t*-test or the one-way ANOVA test for multiple comparisons. A p value < 0.05 was considered as significant. The magnitude of correlation between variables was done using SPSS (Chicago, IL) statistical software package (SPSS for Windows, version 17, 2008). The IC_{50} values were calculated from the dose curves by a computer program (CalcuSyn).

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (projects no. 172016, III41010).

References

1 (*a*) H. Schiff and J. Liebigs, *Ann. Chem.*, 1864, **131**, 118–119; (*b*) P. A. Vigato and S. Tamburini, *Coord. Chem. Rev.*, 2004, **248**, 1717–2128; (*c*) C. M. da Silva, D. L. da Silva, L. V. Modolo, R. B. Alves, M. A. de Resende, C. V. B. Martins and Â. de Fátima, *J. Adv. Res.*, 2011, **2**, 1–8.

2 (*a*) C. T. Barboiu, M. Luca, C. Pop, E. Brewster and M. E. Dinculescu, *Eur. J. Med. Chem.*, 1996, **31**, 597–606; (*b*) S. Gaur, *Asian J. Chem.*, 2003, **15**, 250–254; (*c*) M. J. Gemi, C. Biles, B. J. Keiser, S. M. Poppe, S. M. Swaney, W. G. Tarapley, D. L. Romeso and Y. Yage, *J. Med. Chem.*, 2000, **43**, 1034–1040.

3 (*a*) G. Bringmann, M. Dreyer, J. H. Faber, P. W. Dalsgaard, D. Stærk, J. W. Jaroszewski, H. Ndangalasi, F. Mbago, R. Brun and S. B. Christensen, *J. Nat. Prod.*, 2004, **67**, 743–748; (*b*) A. O. de Souza, F. C. S. Galetti, C. L. Silva, B. Bicalho, M. M. Parma and S. F. Fonseca, *Quim. Nova*, 2007, **30**, 1563–1566; (*c*) Z. Guo, R. Xing, S. Liu, Z. Zhong, X. Ji, L. Wang and P. Li, *Carbohydr. Res.*, 2007, **342**, 1329–1332.

4 D. N. Dhar and C. L. Taploo, *J. Sci. Ind. Res.*, 1982, **41**, 501–506.

5 (*a*) B. K. Panda and A. Chakravorty, *J. Organomet. Chem.*, 2005, **690**, 3169–3175; (*b*) K. R. Krishnapriya and

M. Kandaswamy, *Polyhedron*, 2005, **24**, 113–120; (*c*) K. N. Kumar and R. Ramesh, *Polyhedron*, 1999, **18**, 1561–1568.

6 (*a*) K. Singh, M. S. Barwa and P. Tyagi, *Eur. J. Med. Chem.*, 2006, **41**, 147–153; (*b*) A. Majumder, G. M. Rosair, A. Mallick, N. Chattopadhyay and S. Mitra, *Polyhedron*, 2006, **25**, 1753–1762; (*c*) A. Freiria, R. Bastida, L. Valencia, A. Macias and C. Lodeiro, *Inorg. Chim. Acta*, 2006, **359**, 2383–2394.

7 (*a*) J. A. Faniran, K. S. Patel and J. C. Bailar, *J. Inorg. Nucl. Chem.*, 1974, **36**, 1547–1551; (*b*) H. D. Bian, J. Y. Xu, W. Gu, S. P. Yan, D. Z. Liao, Z. H. Jiang and P. Cheng, *Inorg. Chem. Commun.*, 2003, **6**, 573–576; (*c*) G. G. Mohamed, M. M. Omar and A. M. Hindy, *Turk. J. Chem.*, 2006, **30**, 361–382; (*d*) M. K. Bharty, A. K. Srivastava, R. Dulwere, R. J. Butcher and N. K. Singh, *Polyhedron*, 2011, **30**, 990–996.

8 (*a*) A. P. Mishra, *J. Indian Chem. Soc.*, 1999, **76**, 35–39; (*b*) M. Khare and A. P. Mishra, *J. Indian Chem. Soc.*, 2000, **77**, 256–258; (*c*) N. Raman, V. Muthury, S. Ravichandran and A. Kulandaisamy, *Proc. Indian Natl. Sci. Acad., Part A*, 2003, **115**, 161–167; (*d*) R. C. Sharma and V. K. Khar, *Asian J. Chem.*, 1998, **10**, 467; (*e*) R. Ramesh and M. S. Sundari, *Synth. React. Inorg. Met.-Org. Chem.*, 2003, **33**, 899–910; (*f*) L. Iqbal, M. Lateef, S. Ali, N. Riaz, G. M. Maharvi, M. Ashraf and N. Afza, *J. Chem. Soc. Pak.*, 2007, **29**, 51–54; (*g*) K. M. Khan, F. Rahim, N. Ambreen, M. Taha, S. Iqbal, S. M. Haider and S. Perveen, *J. Chem. Soc. Pak.*, 2012, **34**, 748–757.

9 (*a*) S. Kondaiah, G. N. R. Reddy, D. Rajesh and J. Joseph, *Indian J. Adv. Chem. Sci.*, 2013, **1**, 228–235; (*b*) E. Ispir, S. Toroglu and A. Kayraldiz, *Transition Met. Chem.*, 2008, **33**, 953–960; (*c*) A. Prakash and D. Adhikari, *Int. J. ChemTech Res.*, 2011, **3**, 1891–1896; (*d*) P. Kavitha and K. L. Reddy, *Arabian J. Chem.*, 2013, DOI: 10.1016/j.arabjc.2013.06.018; (*e*) O. E. Offiong, E. Nfor and A. A. Ayi, *Transition Met. Chem.*, 2000, **25**, 369–373.

10 N. Filipović, S. Grubišić, M. Jovanović, M. Dulović, I. Marković, O. Klisurić, A. Marinković, D. Mitić, K. Anđelković and T. Todorović, *Chem. Biol. Drug Des.*, 2014, **84**, 333–341.

11 (*a*) A. S. Abu-Surrah, K. A. Abu Safieh, I. M. Ahmad, M. Y. Abdalla, M. T. Ayoub, A. K. Qaroush and A. M. Abu-Mahtheieh, *Eur. J. Med. Chem.*, 2010, **45**, 471–475; (*b*) O. E. Offiong, E. Nfor and A. A. Ayi, *Transition Met. Chem.*, 2000, **25**, 369–373.

12 A. S. Abu-Surrah, H. H. Al-Sàdoni and M. Y. Abdalla, *Cancer Theranostics*, 2008, **6**, 1–10.

13 Z. D. Petrović, J. Đorović, D. Simijonović, V. P. Petrović and Z. Marković, *RSC Adv.*, 2015, **5**, 24094–24100.

14 (*a*) B. J. Tardiff, J. C. Smith, S. J. Duffy, C. M. Vogels, A. Decken and S. A. Westcott, *Can. J. Chem.*, 2007, **85**, 392–399; (*b*) L. Tao-Ping, C. Qiang, S. Wen-Jing, C. Liang-Zhen and T. Xiao-Chun, *Synlett*, 2012, **23**, 2333–2336.

15 (*a*) J. V. Košarić, D. M. Cvetković, M. N. Živanović, M. G. Ćurčić, D. S. Šeklić, Z. M. Bugarčić and S. D. Marković, *Journal of Balkan Union of Oncology*, 2014, **19**, 283–290; (*b*) V. P. Petrović, D. Simijonović, M. N. Živanović, J. V. Košarić, Z. D. Petrović, S. Marković and S. D. Marković, *RSC Adv.*, 2014, **4**, 24635–24644.

16 I. B. Afanasėv, ROS and RNS Signaling in Apoptosis, in *Signaling Mechanisms of Oxygen and Nitrogen Free Radicals*, CRC Press, Inc., Boca Raton, 2009, pp. 129–159.

17 A. Pompella, A. Visvikis, A. Paolicchi, V. de Tata and A. F. Casini, *Biochem. Pharmacol.*, 2003, **66**, 1499–1503.

18 G. K. Balendiran, R. Dabur and D. Fraser, *Cell Biochem. Funct.*, 2004, **22**, 343–352.

19 M. J. Frisch, W. G. Trucks, B. H. Schlegel, E. G. Scuseria, A. M. Robb, R. J. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, A. G. Petersson, H. Nakatsuji, M. Caricato, X. Li, P. H. Hratchian, F. A. Izmaylov, J. Bloino, G. Zheng, L. J. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, A. J. Montgomery Jr, E. J. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, N. K. Kudin, N. V. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, C. J. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, E. R. Stratmann, O. Yazyev, J. A. Austin, R. Cammi, C. Pomelli, W. J. Ochterski, L. R. Martin, K. Morokuma, G. V. Zakrzewski, A. G. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, D. A. Daniels, O. Farkas, B. J. Foresman, V. J. Ortiz, J. Cioslowski and J. D. Fox, *Gaussian 09, Rev A.1*, Gaussian Inc., Wallingford, 2009.

20 J. P. Hay and R. W. Wadt, *J. Chem. Phys.*, 1985, **82**, 270–283.

21 (*a*) Y. Zhao and D. G. Truhlar, *Theor. Chem. Acc.*, 2008, **120**, 215–241; (*b*) Y. Zhao and D. G. Truhlar, *Acc. Chem. Res.*, 2008, **41**, 157–167.

22 (*a*) V. Barone and M. Cossi, *J. Phys. Chem. A*, 1998, **102**, 1995–2001; (*b*) M. Cossi, N. Rega, G. Scalmani and V. Barone, *J. Comput. Chem.*, 2003, **24**, 669–681.

23 T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55–63.

24 C. Auclair and E. Voisin, Nitroblue tetrazolium reduction, in *Handbook of Methods for Oxygen Radical Research*, ed. R. A. Greenwald, CRC Press, Inc, Boka Raton, 1985, pp. 122–132.

25 P. Griess, *Ber. Dtsch. Chem. Ges.*, 1879, **12**, 426–428.

26 M. A. Baker, G. J. Cerniglia and A. Zaman, *Anal. Biochem.*, 1990, **190**, 360–365.