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[Damnjan Pantic](#) , [Nikola Mirkovic](#) , [Tatjana Vulovic](#) <sup>\*</sup> , [Danijela Jovanovic](#) <sup>\*</sup> , [Stefan Jakovljevic](#) , [Petar Canovic](#) , [Milan M Zaric](#) , [Radica Zivkovic Zaric](#) , [Marina Kostic](#) , [Jovana Dragojevic](#) , [Vera Divac](#) , [Ziko Milanovic](#) , [Kristina Milisavljevic](#) , [Ivana Zelen](#)

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Article

# Evaluation of Newly Synthesized Schiff Base Pd(II) Complexes for Prostate Cancer Treatment Through In Vitro Cytotoxicity and Molecular Mechanistic Studies

Damnjan Pantic <sup>1,2,†</sup>, Nikola Mirkovic <sup>1,3,†</sup>, Tatjana Vulovic <sup>1,4</sup>, Danijela Jovanovic <sup>1,4</sup>, Stefan Jakovljevic <sup>1,5</sup>, Petar Canovic <sup>6</sup>, Milan Zaric <sup>6</sup>, Radica Zivkovic Zaric <sup>7,8</sup>, Marina Kostic <sup>9</sup>, Jovana Dragojevic <sup>10</sup>, Vera Divac <sup>10</sup>, Ziko Milanovic <sup>9</sup>, Kristina Milisavljevic <sup>9,10</sup> and Ivanka Zelen <sup>6</sup>

<sup>1</sup> Department of Surgery, Faculty of Medical Sciences, University of Kragujevac, Svetozar Markovic Street 69, 34000 Kragujevac, Serbia; damnjanpantic@yahoo.com (D.P.), drnikolamirkovic@gmail.com (N.M.), tatjana\_vulovic@yahoo.com (T.V.), danijeladrjovanovic@gmail.com (D.V.), stefan\_jakov87@yahoo.com (S.J.)

<sup>2</sup> Department of Urology, Clinic of Urology and Nephrology, University Clinical Center Kragujevac, Zmaj Jovina Street 30, 34000 Kragujevac, Serbia

<sup>3</sup> Department of Vascular Surgery Center, University Clinical Center Kragujevac, Zmaj Jovina Street 30, 34000 Kragujevac, Serbia

<sup>4</sup> Department of Anesthesia and Resuscitation, University Clinical Center Kragujevac, Zmaj Jovina Street 30, 34000 Kragujevac, Serbia

<sup>5</sup> Department of General Surgery, University Clinical Center Kragujevac, Zmaj Jovina Street 30, 34000 Kragujevac, Serbia

<sup>6</sup> Department of Biochemistry, Faculty of Medical Sciences, University of Kragujevac, Svetozar Markovic Street 69, 34000 Kragujevac, Serbia; petar.c89@gmail.com (P.C.), zaricmilan@gmail.com (M.Z.), ivankazelen@gmail.com (I.Z.)

<sup>7</sup> Department of Pharmacology and Toxicology, Faculty of Medical Sciences, University of Kragujevac, Svetozar Markovic Street 69, 34000 Kragujevac, Serbia; radica\_zivkovic@yahoo.com (R.Z.Z)

<sup>8</sup> Department of Clinical Pharmacology, University Clinical Center Kragujevac, Zmaj Jovina Street 30, 34000 Kragujevac, Serbia

<sup>9</sup> Department of Science, Institute for Information Technologies, University of Kragujevac, Jovana Cvijica Street bb, 34000 Kragujevac, Serbia; marinak@uni.kg.ac.rs (M.K.), ziko.milanovic@uni.kg.ac.rs (Z.M.), kristina.milisavljevic@uni.kg.ac.rs (K.M.)

<sup>10</sup> Department of Chemistry, Faculty of Sciences, University of Kragujevac, Radoje Domanovic Street 12, 34000 Kragujevac, Serbia; jovana.maranovic@pmf.kg.ac.rs (J.D.); vera.divac@pmf.kg.ac.rs (V.D.)

\* Correspondence: tatjana\_vulovic@yahoo.com (T.V.); danijeladrjovanovic@gmail.com (D.J.)

† These authors contributed equally to this work as the first author.

**Abstract: Background/Objectives:** Palladium(II) complexes are promising anticancer agents with potential advantages over platinum drugs. This study aimed to synthesize and characterize three novel Pd(II) complexes (2a-c) with Schiff base ligands derived from salicylic acid and amine scaffolds, and to evaluate their antitumor activity against prostate cancer cells. **Methods:** The Pd(II) complexes were synthesized and structurally characterized. Cytotoxicity was tested on two human prostate cancer cell lines (PC-3, DU-145) and healthy fibroblasts (MRC-5). Apoptosis induction was assessed by flow cytometry focusing on Bcl-2 and caspase proteins. Molecular docking examined binding to androgen receptor (AR) and apoptotic regulators (CASP3, BCL2, BAX). DNA and human serum albumin (HSA) binding were also investigated. **Results:** All complexes showed significant cytotoxicity, with complex 2c outperforming cisplatin (IC50: 7.1  $\mu$ M in DU-145; 8.6  $\mu$ M in PC-3). Apoptosis was confirmed as the main cytotoxic mechanism involving Bcl-2 and caspase activation.



Docking studies revealed complex 2c had the strongest binding affinity to AR and apoptotic proteins via hydrogen bonds,  $\pi$ - $\pi$  stacking, and hydrophobic interactions. DNA and HSA binding supported their biological relevance. **Conclusions:** Complex 2c exhibits potent anticancer activity through apoptosis induction and dual targeting of AR and apoptotic pathways, making it a promising candidate for further anticancer drug development.

**Keywords:** palladium; schiff bases; DNA; albumin; cytotoxicity; apoptosis; prostate cancer

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## 1. Introduction

The treatment of cancer is the most challenging issue of modern science, particularly due to the resistance of tumor cells to many utilized protocols and treatments, as well as due to serious side effects. Metal complexes have aroused as a suitable alternative for the treatment of different ailments due to their unique properties. Among them, platinum-based drugs still represent the most explored options for cancer treatment, but the novel studies concentrate on the other metal drugs with better efficacy [1–5]. The idea behind the design of novel metalotherapeutics with increased activity and lowered toxicity is based mainly on the elucidation of antitumor molecular mechanisms different from those expressed by platinum compounds [5]. The last two decades have brought increased interest in the design and synthesis of palladium complexes that could serve as alternative anticancer drugs [6–8] inspired by their coordination mode similar to that of platinum, but, according to some studies performed on rats, significantly lower toxicity [9]. As it undergoes redox reaction, the palladium is also capable of the formation of reactive oxygen species (ROS). Despite the obvious similarity of these two metalotherapeutics groups, the palladium complexes are much more reactive, therefore, imposing the need for their stabilization through the utilization of specific chelating ligands. The choice of the suitable ligand is a crucial step in the development of the promising antitumor candidate, knowing that finely tuned physicochemical properties of used ligand, its biological potential, and the strength of the ligand-metal bond directly influence the selectivity and mechanism of the antitumor activity at a molecular level.

The Schiff bases represent molecules widely utilized in many biological studies as pharmacophores capable of forming stable complexes with a variety of metals. Besides the nitrogen atom from imino groups, the molecular scaffold of Schiff bases can be easily manipulated to contain other donor atoms, such as oxygen and Sulphur and, in that way provide the desired physicochemical or pharmaceutical properties necessary for the establishment of targeted biological activity. There are numerous reports evidencing the pharmacological potential of these compounds reflected in pronounced antitubercular, anti-inflammatory, antiviral, anticholinesterase, antibacterial, anticancer, and antioxidant activities [10–17]. When it comes to the tumor cells, Schiff bases can be hydrolyzed by tumor cells in a selective manner or provide release of active amines as antimetabolites [18]. Other antitumor effects of Schiff bases include the cycle cell arrest by the production of free radicals and damage of proteins and nucleic acids [19–21]. As a consequence of above mentioned, the Schiff bases are also utilized as a ligand for the design of palladium complexes as promising candidates for photodynamic therapy [22], as well as effective anticancer agents against a variety of cancer cell lines through the different molecular pathways, such as inhibition of proteasomes, enzymes, and cancer protein markers [23].

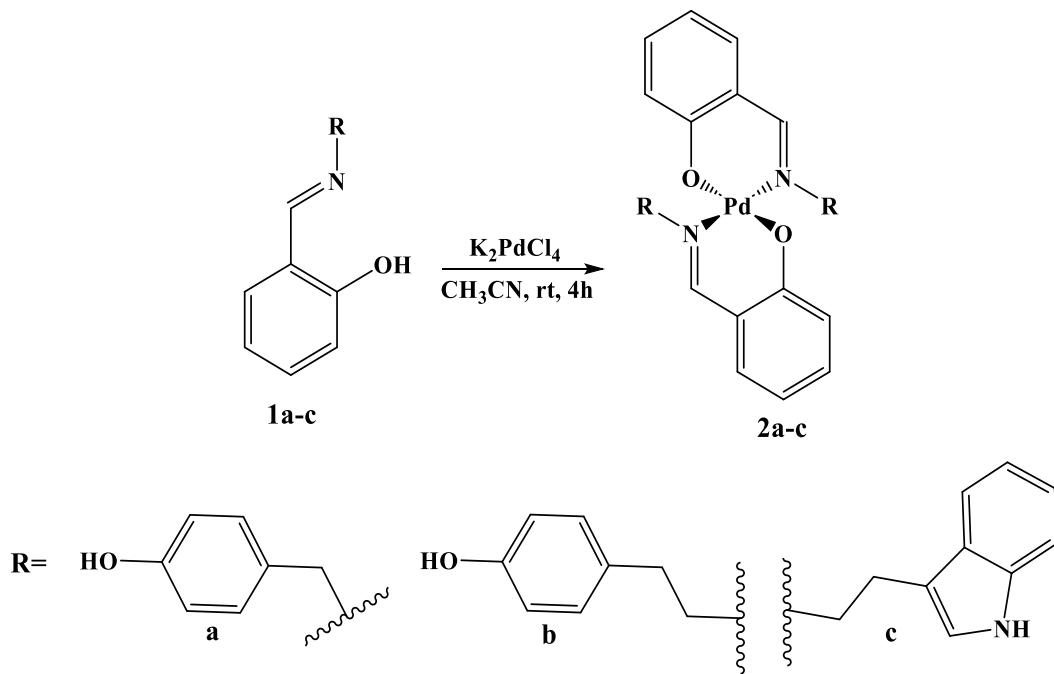
In the light of the foregoing, herein we report the synthesis, characterization, and results of in vitro cytotoxic assay of three novel palladium (II) complexes bearing Schiff base ligands combining salicylic aldehyde and tryptamine/tyramine and *p*-OH benzylamine structural motifs. While tryptamine and tyramine represent examples of biogenic amines, *p*-OH benzylamine was used due to the structural resemblance to the tyramine scaffold. The cytotoxic activity was assessed on two human prostate cancer cell lines PC-3 and DU-145 and the results were compared with those obtained with healthy fibroblast cell line MRC-5. It was of interest to compare the influence of the palladium metal ion coordination to the Schiff base ligand on their antitumor potential. To gain further insight

into their possible mode of action, we focused on one of the key molecular targets in prostate cancer – the androgen receptor (AR). This receptor plays a central role in disease progression by regulating the transcription of genes involved in cell growth, survival, and differentiation upon binding with androgens such as testosterone and dihydrotestosterone [24]. Notably, AR signaling remains active even under androgen-deprived conditions in tumor cells, facilitating continued tumor proliferation and the emergence of therapy resistance [25]. Given its critical role, a molecular docking study was undertaken to evaluate the interaction of the synthesized palladium complexes with AR, aiming to elucidate their potential as modulators of androgen receptor activity and their contribution to the observed cytotoxic effects.

## 2. Results and Discussion

### 2.1. Synthesis of Pd Complexes 2a-c

The targeted Pd(II) complexes **2a-c** have been prepared in one pot procedure comprising the reaction between corresponding imine **1a-c** as ligands and K<sub>2</sub>PdCl<sub>4</sub> as a metal source in acetonitrile, at room temperature in a 1:1 ratio (ligand vs. metal salt), as indicated in Scheme 1. Besides the salicylic part, the ligands were composed of imine patterns – tryptamine, tyramine, and *p*-OH benzyl moiety structurally related to the tyramine molecular scaffold. All complexes are new compounds obtained in the form of powders and fully characterized by the IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, mass analysis, and elemental analysis (Experimental part, Supplementary material).

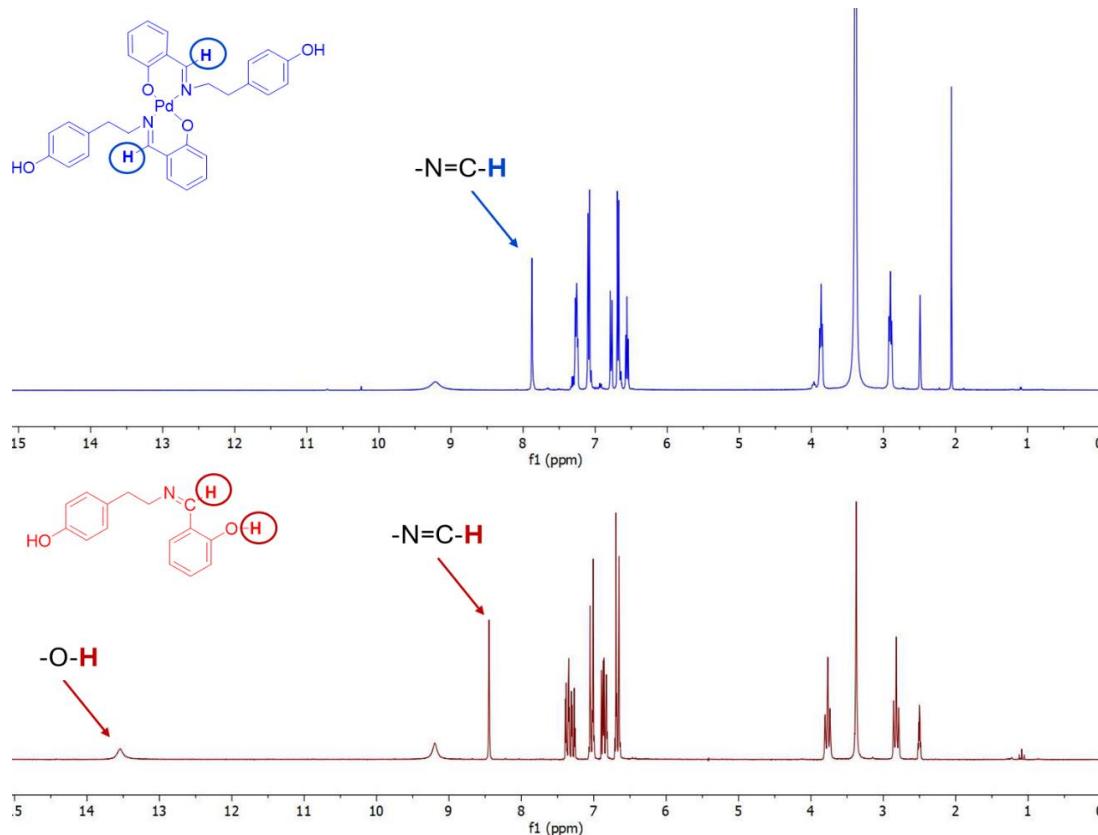


**Scheme 1.** Synthesis of Schiff base palladium (II) complexes **2a-c**.

### 2.2. NMR Studies

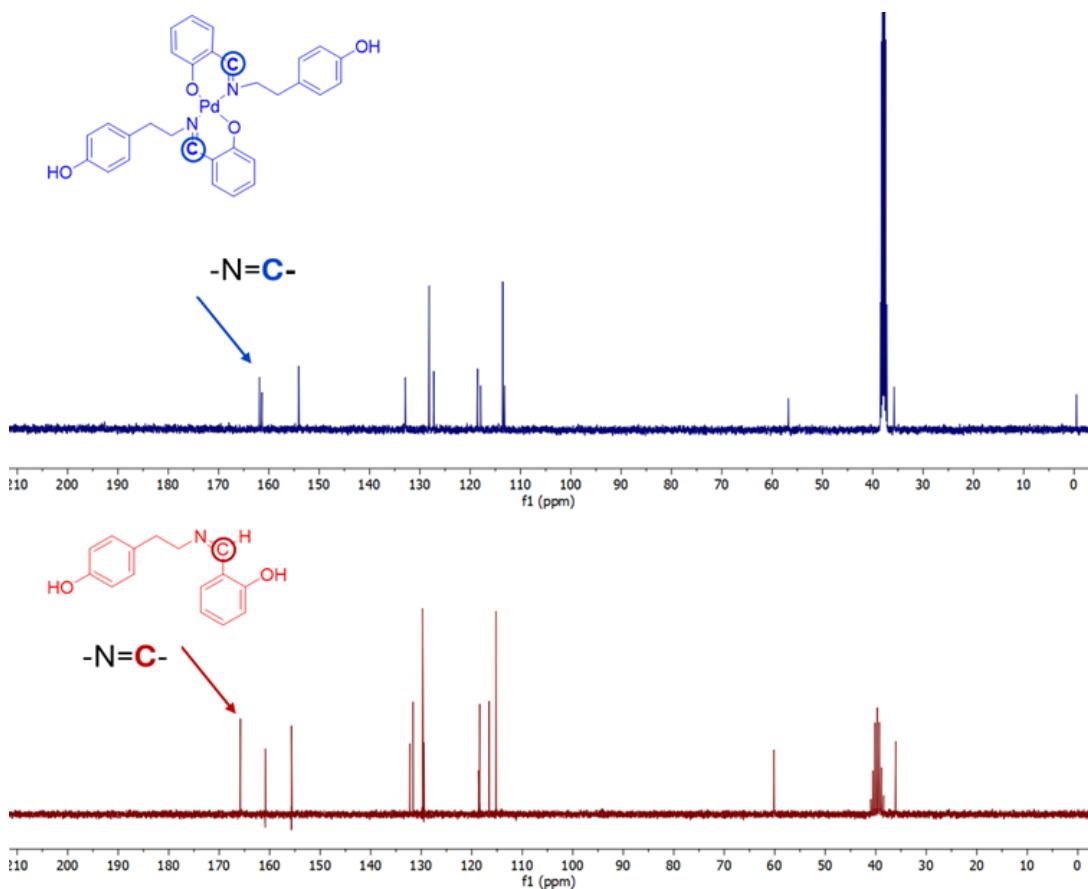
A detailed overview of the NMR characterization data for all synthesized compounds is provided in Supplementary Information (SI), while representative examples are discussed herein to highlight key structural changes upon complexation. The representative <sup>1</sup>H NMR spectra of palladium complex **2b** were analyzed by comparison with the spectra of ligand compound **1b**, as depicted in Figure 1, a). The azomethine proton –CH=N from the Schiff base located at 8.45 ppm is, in the case of the palladium complex, shifted to the 7.88 ppm, confirming the involvement of the imino group in the coordination with the palladium metal ion. The –OH proton peaks from salicylic moiety appearing at 13.54 ppm in the ligand spectra, completely disappear in the case of palladium

complex spectra, undoubtedly confirming the coordination of palladium ion with phenolate anion. Additionally, subtle changes in the aromatic region (6.5–8.0 ppm) further support coordination-induced electronic redistribution. The multiplet pattern of aromatic protons becomes slightly broadened and shifts marginally, which can be attributed to changes in magnetic environment and restricted rotation upon complexation. Taken together, these spectroscopic features strongly support the formation of a stable square planar Pd(II) complex, in which the ligand coordinates through both imine nitrogen and deprotonated phenolic oxygen, in agreement with the proposed structure shown in the figure.



**Figure 1.** Comparison of  $^1\text{H}$  NMR spectra of starting ligand **1b** and complex compound **2b**.

In the  $^{13}\text{C}$  NMR spectra, clear changes in the chemical shifts of characteristic carbon atoms were observed upon complexation with Pd(II), supporting the proposed coordination mode. In the free ligand **1b**, the signal for the azomethine carbon ( $-\text{CH}=\text{N}$ ) appears at 165.82 ppm, while the phenolic carbon bearing the  $-\text{OH}$  group ( $-\text{C}-\text{OH}$ ) resonates at 155.68 ppm (Figure 2). Upon complex formation, the azomethine carbon signal shifts slightly upfield to 163.54 ppm, indicating a decrease in electron density due to the coordination of the imine nitrogen to the palladium center, which is consistent with the formation of a Pd–N bond. The phenolic carbon signal shows a negligible shift (from 155.68 ppm to 155.72 ppm), which suggests that while deprotonation of the hydroxyl group occurs, the local electronic environment of the carbon remains largely stabilized, possibly due to resonance delocalization within the chelate ring and Pd–O bond formation. Additionally, several aromatic carbon signals appear in the 120–140 ppm region, with slight shifts compared to the free ligand, reflecting electronic redistribution upon coordination. These observations provide further evidence of ligand binding and the resulting structural reorganization. Overall, the  $^{13}\text{C}$  NMR data, in conjunction with the  $^1\text{H}$  NMR results, strongly support the successful coordination of ligand **1b** to Pd(II) through the imine nitrogen and phenolate oxygen atoms in complex **2b**.



**Figure 2.** Comparison of  $^{13}\text{C}$  NMR spectra of starting ligand **1b** and complex compound **2b**.

The corresponding  $^1\text{H}$  NMR spectral data for the  $\text{CH}=\text{N}$  and  $-\text{C}-\text{OH}$  protons, as most relevant shifts in complex structure determination for all complexes **2a-c** are given in Table 1.

**Table 1.**  $^1\text{H}$  NMR spectral data of ligands **1a-c** and complex compounds **2a-c**.

Compound	Imine Proton $-\text{HC}=\text{N}$	Salicylic OH Proton
1a	8.65 ppm, s	13.56 ppm, s
2a	8.20 ppm, s	missing
1b	8.45 ppm, s	13.54 ppm, s
2b	7.91 ppm, s	missing
1c	8.48 ppm, s	13.69 ppm, s
2c	7.96 ppm, s	missing

### 2.3. IR Analysis

The experimental IR spectra of all investigated ligands and their corresponding Pd(II) complexes are provided in the Supplementary Information (SI). IR spectral analysis offers valuable insights into the coordination behavior of ligands during complex formation with Pd(II) ions. One of the most significant vibrational bands in the FTIR spectra of Schiff base ligands is the imine stretching band ( $\nu\text{C}=\text{N}$ ), typically observed in the range of  $1610\text{--}1650\text{ cm}^{-1}$ . This functional group plays a key role in metal coordination, and any shift in its position upon complexation serves as strong evidence of such interaction.

In the IR spectra of the free ligands **1a-1c**, the  $\nu\text{C}=\text{N}$  stretching bands were observed at  $1641$ ,  $1631$ , and  $1630\text{ cm}^{-1}$ , respectively. After coordination with Pd(II), these signals were shifted to lower frequencies –  $1638\text{ cm}^{-1}$  for complex **2a**,  $1611\text{ cm}^{-1}$  for **2b**, and  $1618\text{ cm}^{-1}$  for **2c**. This redshift,

particularly pronounced in the case of complex **2b** ( $\Delta\nu = 20 \text{ cm}^{-1}$ ), indicates a partial loss of double bond character in the C=N bond as a result of electron donation from the imine nitrogen to the palladium ion.

The extent of the  $\nu\text{C}=\text{N}$  shift correlates with the strength of azomethine nitrogen coordination to the Pd(II) center, further supporting its direct involvement in complex formation. The uniform trend observed across all examined systems confirms that the ligands coordinate with the palladium ion via the imine nitrogen.

These IR findings are fully consistent with the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data previously discussed, providing complementary experimental evidence for the formation of bidentate N, O-chelated palladium(II) complexes.

### 2.3. Mass Analysis

The electrospray ionization mass spectrometry (ESI-MS) analysis was performed to confirm the molecular composition and stoichiometry of the synthesized palladium(II) complexes. In the mass spectrum of complex **2a**, a dominant signal was observed at  $m/z = 559.0857$ , which corresponds to the protonated molecular ion  $[\text{M} + \text{H}]^+$ , confirming the formation of the proposed bis-Schiff base Pd(II) complex (ESI, Figure SI2). Similarly, for complex **2c**, a strong peak at  $m/z = 633.1490$  was detected, also corresponding to the  $[\text{M} + \text{H}]^+$  species (ESI, Figure SI5). The presence of these molecular ion peaks, which match the calculated molecular masses of the respective complexes, confirms the proposed coordination of two Schiff base ligands to the Pd(II) ion in a 2:1 ligand-to-metal ratio. These results provide direct evidence for the successful formation of the target Pd(II) complexes and are in full agreement with the spectroscopic data (NMR and IR), thereby supporting the proposed coordination environment illustrated in the structural models.

### 2.4. In Vitro Cytotoxic Study

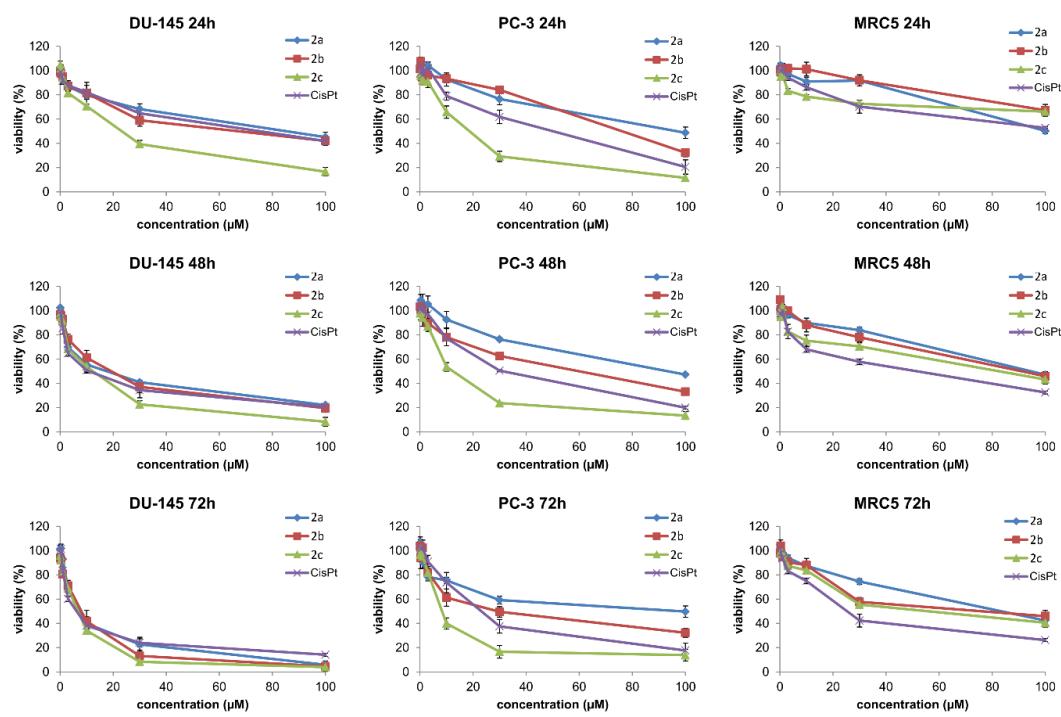
The cytotoxicity of the three synthesized Pd(II) complexes (**2a-c**) was assessed in vitro using human prostate carcinoma cells lacking prostate-specific antigen (PSA) and androgen receptor independence (DU-145), human prostate carcinoma cells (PC-3), and non-cancerous human fibroblasts (MRC-5). The evaluation was conducted via the MTT assay following 24, 48, and 72 hours of treatment. For comparative purposes, the cytotoxic profile of cisplatin was also examined. The findings unequivocally demonstrate that all Pd(II) complexes, as well as cisplatin, exhibit dose-dependent cytotoxic effects against DU-145 and PC-3 cell lines. The  $\text{IC}_{50}$  values for these Pd(II) complexes and cisplatin are summarized in Table 2, reinforcing their potential as anticancer agents (Figure 3).

**Table 2.**  $\text{IC}_{50}$  values in  $\mu\text{M}$  for Pd(II) complexes **2a-c** and cisplatin (CP) after 24, 48, and 72 h of drug exposure. Results are presented as mean  $\pm$  SD and determined from the results of MTT assay in three independent experiments.

Cell Line	Time	<b>2a</b>	<b>2b</b>	<b>2c</b>	CP
DU-145	24 h	83.6 $\pm$ 9.2	75.9 $\pm$ 7.3	48.4 $\pm$ 5.1	22.3 $\pm$ 2.4
	48 h	17.5 $\pm$ 1.6	19.2 $\pm$ 2.1	11.0 $\pm$ 1.2	11.4 $\pm$ 1.1
	72 h	9.3 $\pm$ 0.9	8.7 $\pm$ 0.8	7.1 $\pm$ 0.8	8.2 $\pm$ 0.8
PC-3	24 h	94.1 $\pm$ 9.1	75.2 $\pm$ 7.2	38.4 $\pm$ 3.6	15.6 $\pm$ 1.4
	48 h	89.8 $\pm$ 8.8	58.6 $\pm$ 5.6	12.5 $\pm$ 1.1	29.7 $\pm$ 3.1
	72 h	79.3 $\pm$ 7.7	29.1 $\pm$ 2.8	8.6 $\pm$ 0.9	21.9 $\pm$ 2.2
MRC-5	24 h	94.3 $\pm$ 9.7	87.7 $\pm$ 8.8	80.7 $\pm$ 8.1	90.1 $\pm$ 9.2
	48 h	92.4 $\pm$ 9.1	78.5 $\pm$ 7.7	75.6 $\pm$ 7.7	41.9 $\pm$ 4.4
	72 h	83.0 $\pm$ 8.1	49.2 $\pm$ 5.1	42.3 $\pm$ 4.1	24.4 $\pm$ 0.8

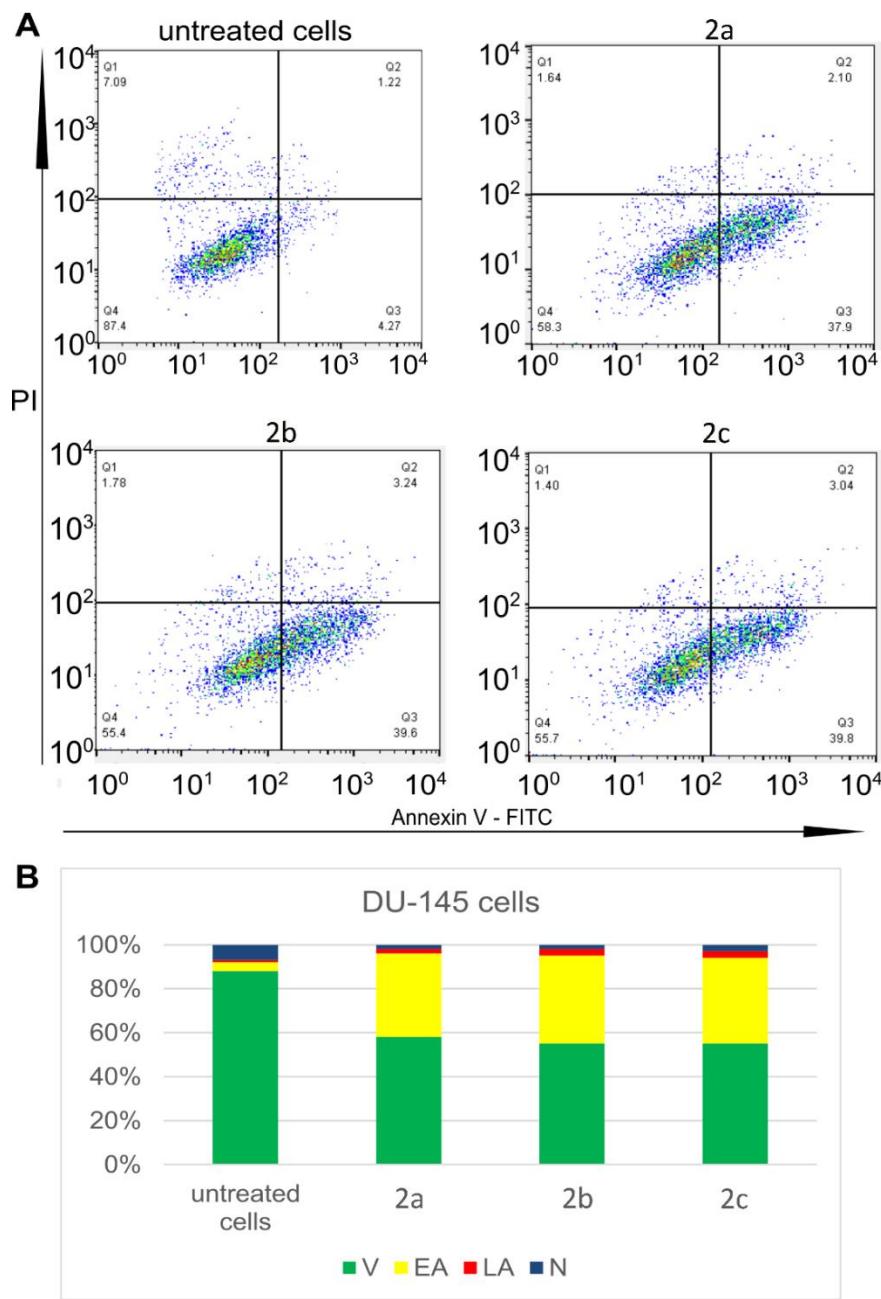
All tested Pd(II) complexes, as well as cisplatin, significantly reduced the viability of DU-145 and PC-3 prostate carcinoma cell lines following 24, 48, and 72 hours of treatment. Among the

synthesized complexes, compound **2c** exhibited the most pronounced cytotoxic activity across all exposure durations and against both cell lines. Notably, the cytotoxic effect of complex **2c** surpassed that of cisplatin after 48 and 72 hours of treatment under identical experimental conditions (Figure 3 and Table 3). Furthermore, while all Pd(II) complexes demonstrated a relatively lower cytotoxic impact on non-cancerous human fibroblasts (MRC-5) compared to cisplatin, exceptions were observed for complexes **2b** and **2c** at the 24-hour mark. These findings underscore the selective anticancer potential of the newly synthesized Pd(II) complexes (**2a-c**), particularly their preferential activity against human prostate cancer cell lines.

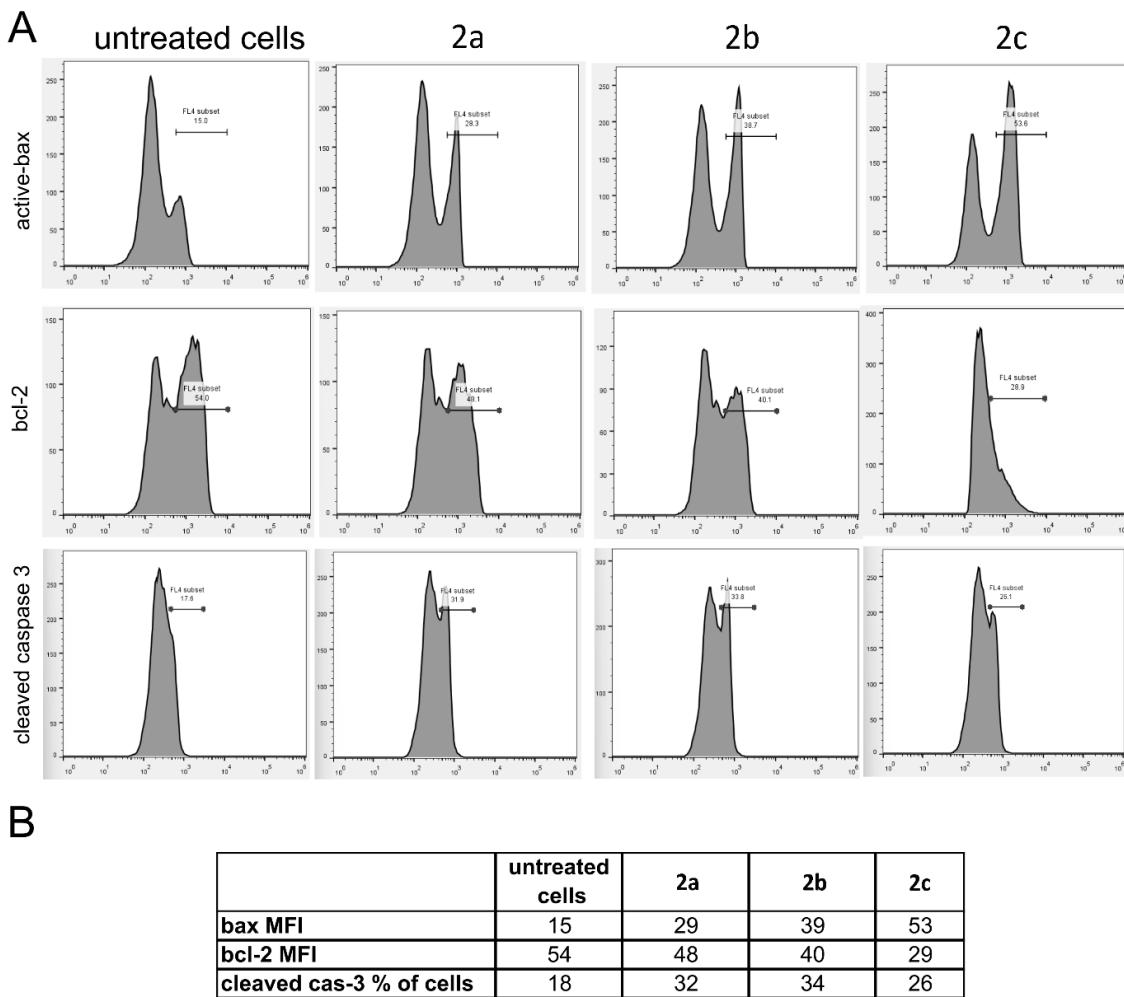


**Figure 3.** The effects of Pd(II) complexes (**2a-c**) and cisplatin (CisPt) on the viability of human prostate carcinoma cells without prostate-specific antigen (PSA) and androgen receptor-independent (DU-145), human prostate carcinoma cells (PC-3), and human non-tumor cells (MRC-5).

The subsequent phase of our investigation focused on determining the mode of cell death induced by the Pd(II) complexes in DU-145 and PC-3 prostate carcinoma cells. To achieve this, an annexin V/PI staining assay was employed. The results revealed that all tested Pd(II) complexes effectively triggered apoptosis (Figure 4). Notably, in DU-145 cells, treatment with an IC<sub>50</sub> dose of complex **2c** resulted in the highest proportion of necrotic cells. Further analyses were conducted to investigate whether these Pd(II) complexes affected the intracellular levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax. Additionally, caspase-3 activation was assessed in DU-145 cells exposed to the Pd(II) complexes. The findings demonstrated a significant reduction in Bcl-2 levels and a concomitant increase in BAX concentrations in cells treated with IC<sub>50</sub> doses of the Pd(II) complexes compared to untreated controls (Figure 4). Moreover, a marked elevation in the percentage of cells exhibiting active caspase-3 was observed following treatment with the Pd(II) complexes (Figure 5). Collectively, these results indicate that the Pd(II) complexes (**2a-c**) decreased the Bcl-2/BAX ratio, thereby promoting caspase-3 activation and inducing apoptosis via intrinsic apoptotic pathways.

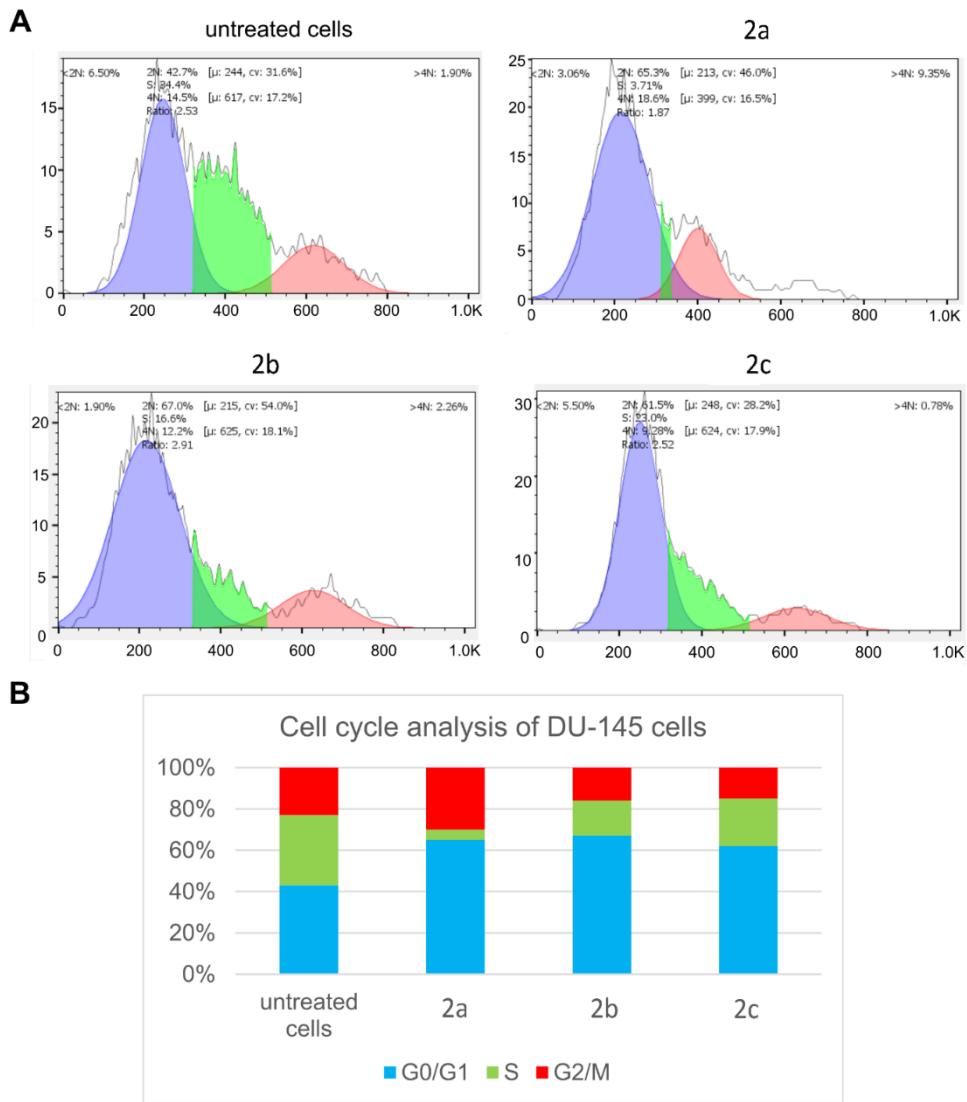


**Figure 4.** Three tested Pd(II) complexes (**2a-c**) decrease viability of treated human prostate carcinoma cells without prostate-specific antigen (PSA) and androgen receptor-independent (DU-145); (A) Representative flow cytometry plots using Annexin V-FITC/PI staining for apoptosis. (B) The average percentage of DU-145 viable cells (V), early apoptotic cells (EA), late apoptotic cells (LA) and necrotic cells (N) after 24h treatment with IC<sub>50</sub> of Pd(II) complexes (**2a-c**).



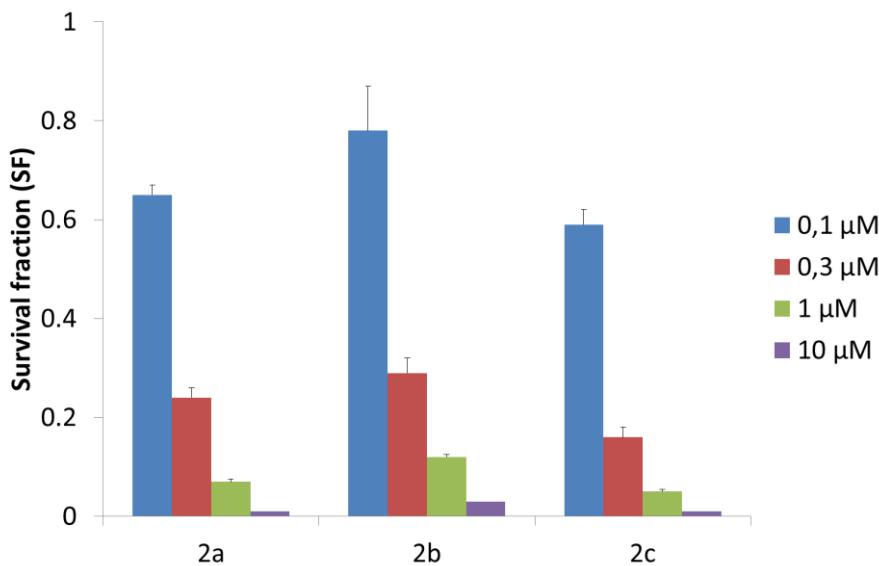
**Figure 5.** Pd(II) complexes **2a-c** induce apoptosis of human prostate carcinoma cells without prostate-specific antigen (PSA) and androgen receptor-independent (DU-145) via a caspase-dependent pathway. (A) MFI values (mean fluorescence intensity) for anti-apoptotic protein bcl-2, proapoptotic protein Bax and cleaved caspase 3 of DU-145 cells treated with IC<sub>50</sub> of Pd(II) complexes **2a-c**. (B) Overview of MFI values for pro-apoptotic protein active bax, antiapoptotic bcl-2 and active (cleaved) caspase-3 of DU-145 cells treated with IC<sub>50</sub> of Pd(II) complexes **2a-c**.

The induction of apoptosis and/or cell cycle arrest is a well-established mechanism for reducing cancer cell viability[26,27]. Notably, cancer cells exhibit a heightened reliance on DNA damage repair mechanisms and the abrogation of the G2 checkpoint. Arrest in the G0/G1 phase typically halts cellular proliferation while allowing for the repair of damage caused by anticancer agents. In contrast, G2/M phase arrest is often associated with the initiation of apoptosis. In our study, we investigated the effects of IC<sub>50</sub> concentrations of Pd(II) complexes **2a-c** on the cell cycle of DU-145 prostate cancer cells 24 hours post-treatment. Flow cytometric analysis was performed on propidium iodide (PI)-stained cells to evaluate cell cycle distribution (Figure 6). The results revealed that all Pd(II) complexes induced G0/G1 phase arrest in DU-145 cells, highlighting their ability to interfere with cell cycle progression. Furthermore, variations in the mechanisms of action among the Pd(II) complexes (**2a-c**) were observed, underscoring their distinct biological activities.



**Figure 6.** Pd(II) complexes (**2a-c**) induce G2/M cell cycle arrest of treated tumour DU-145 cells. (A) Representative flow-cytometry plots using PI staining for cell cycle phase detection. (B) The average percentage of DU-145 cells in G0/G1 cell cycle phase (blue), S cell cycle phase (green) and G2/M cell cycle phase (red) after 24h treatment with IC<sub>50</sub> of Pd(II) complexes (**2a-c**).

The long-term antiproliferative effect of Pd(II) complexes, was tested by using clonogenic assay. All tested compounds showed significant antiproliferative effect which was dose-dependend, and the most pronounced effect was detected after treatment with complex **2c** (Figure 7).



**Figure 7.** Survival fraction (SF) for Pd(II) complexes **2a-c** tested in four different concentrations in DU-145 cells. Results are presented as mean  $\pm$  standard deviation.

The query outlines the synthesis and evaluation of three novel Pd(II) complexes (**2a-2c**) for their cytotoxicity and mechanisms of action against human prostate carcinoma cells (DU-145 and PC-3) and non-cancerous fibroblasts (MRC-5). The findings demonstrate that all three complexes exhibit potent and selective cytotoxic effects on prostate carcinoma cells, with complex **2c** showing superior efficacy against DU-145 cells after 72 hours compared to cisplatin. The cytotoxic potency follows the order **2a** < **2b** < **2c**, potentially due to the presence of an indole ring in the ligand of complex **2c** [28,29]. Conversely, the selectivity increases in the reverse order (**2c** < **2b** < **2a**), with all Pd(II) complexes displaying greater selectivity than cisplatin.

The search results corroborate the potential of Pd(II) complexes as anticancer agents. For instance, studies have shown that Pd(II) complexes induce apoptosis through mechanisms such as mitochondrial membrane depolarization, Bax upregulation, and Bcl-2 downregulation in prostate cancer cells like DU-145 and PC-3 [30,31]. Additionally, some Pd(II) complexes demonstrate selective cytotoxicity against cancer cells while sparing non-cancerous cells. The observed differences in efficacy and selectivity among Pd(II) complexes may be attributed to structural variations in their ligands, as highlighted in other research on similar compounds. These findings underscore the therapeutic potential of Pd(II) complexes for prostate cancer treatment.

Pd(II) complexes exhibit substantial anticancer potential against DU-145 human prostate cancer cells, primarily through the induction of apoptosis, particularly early apoptosis [32]. This mechanism was confirmed in our study, where treatment with Pd(II) complexes resulted in a significant transition of cells from viability to the early apoptotic stage. These complexes demonstrate both cytotoxic and antiproliferative properties by disrupting cellular metabolism and inducing cell death [30]. Furthermore, Pd(II) complexes exert both short-term and long-term antiproliferative effects, significantly impairing cell growth and abolishing long-term proliferation. Their action also includes inhibition of cell migration and adhesion, induction of morphological changes such as cellular shrinkage, and interaction with DNA, leading to double-stranded DNA cleavage – a key contributor to their cytotoxic effects [33].

Studies corroborate these findings, highlighting that Pd(II) complexes induce apoptosis via mitochondrial membrane depolarization, Bax upregulation, and Bcl-2 downregulation [8]. Additionally, their ability to disrupt DNA integrity without activating DNA repair enzymes further emphasizes their therapeutic potential [34]. These results collectively position Pd(II) complexes as promising candidates for prostate cancer treatment due to their multifaceted anticancer mechanisms.

Research has demonstrated that Pd(II) complexes can induce apoptosis through multiple pathways, including the activation of cell death receptors and the generation of reactive oxygen species (ROS). These complexes have shown significant efficacy against DU-145 prostate cancer cells, highlighting their potential as anticancer agents. The metabolic effects triggered by Pd(II) complexes can be more rapid compared to those of cisplatin, which may result in faster patient recovery. This rapid metabolic response is advantageous as it could potentially reduce the duration and severity of side effects associated with chemotherapy.

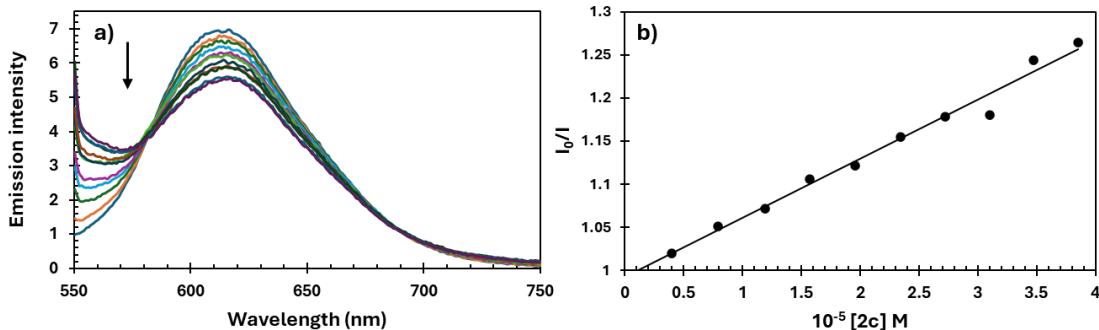
Studies on various Pd(II) complexes have revealed diverse mechanisms of action, including DNA interaction, ROS-mediated mitochondrial dysfunction, and the activation of intrinsic and extrinsic apoptotic pathways [8,35]. The ability of Pd(II) complexes to induce apoptosis without relying on DNA damage, as seen with cisplatin, suggests they may offer a distinct therapeutic approach with potentially fewer side effects [35]. Additionally, the activation of cell death receptors and the induction of apoptosis through ROS production underscore the versatility of Pd(II) complexes in targeting cancer cells [8,36].

Palladium(II) complexes have demonstrated significant cytotoxic and growth-inhibitory effects on the DU-145 human prostate adenocarcinoma cell line, primarily through mechanisms such as DNA interaction and apoptosis induction. These complexes induce cellular changes, including reduced cell numbers and morphological alterations, which are indicative of their cytotoxic effects. The formation of DNA adducts following the interaction of Pd(II) complexes with DNA is a key contributor to their anticancer activity. Studies highlight that complexation with thiosemicarbazone ligands enhances the antitumor potential of Pd(II) complexes, making this a promising strategy for developing novel anticancer agents. For instance, Pd(II)-thiosemicarbazone complexes have shown potent cytotoxicity against DU-145 cells, with  $IC_{50}$  values significantly lower than those of cisplatin [37]. Furthermore, Pd(II) complexes have been shown to modulate protein expression in DU-145 cells, potentially influencing pathways critical to cancer progression [38]. These findings underscore the therapeutic potential of Pd(II) complexes and the need for further research to optimize their efficacy and selectivity.

## 2.5. DNA Interaction Study

Many metal-based anticancer agents have DNA as a primary potential biological target. Accordingly, it is very important to understand the binding properties of different transition metal ion complexes. Two possible binding modes of the transition metal ion complexes toward DNA are defined as covalent and non-covalent types of interactions. The covalent bindings consider the replacement of the labile ligand of the complex by a nitrogen base of DNA and noncovalent are intercalation, electrostatic, or groove binding. The most employed method for determination of binding mode between complexes and DNA is fluorescence quenching [39]. For this investigation competitive studies with EB and Hoechst, 33258 (Hoe) were made using fluorescence quenching experiments.

Ethidium bromide (EB) is a classical intercalator that gives significant fluorescence emission intensity when it intercalates into the base pairs of DNA. The fluorescence titration of EB-DNA with increasing amounts of **2c** is shown in Figure 8. The decrease in the emission intensity of the band at 611 nm suggests that complex **2c** can replace EB from EB-DNA and interact with DNA by the interactive mode [40,41].



**Figure 8.** (a) Emission spectra of EB-DNA in the absence and presence of complex **2c**. pH = 7.4;  $\lambda_{\text{ex}} = 550$  nm; (b) plots of  $I_0/I$  versus concentration of quencher [**2c**].

The Stern-Volmer quenching constants ( $K_{\text{sv}}$ ) are calculated from the slopes of the plots of  $I_0/I$  vs. [**4**] from Equation 1 [42]

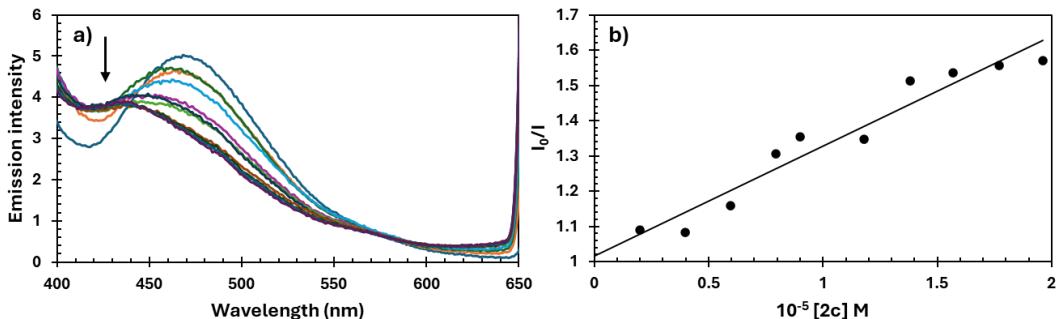
$$I_0/I = 1 + K_{\text{sv}}[Q] \quad (1)$$

and presented in Table 3. Quenching rate constants,  $k_q$  are calculated through the correlation (2), since its known that the average fluorescence lifetime of the DNA without a quencher ( $\tau_0$ ) is  $10^{-8}$  s. [43]

$$K_{\text{sv}} = k_q \times \tau_0 \quad (2)$$

The obtained values for  $K_{\text{sv}}$  suggest that **2c** can interact with DNA molecules in interactive mode [41].

In order to further elucidate the binding mode of complex **2c** with CT-DNA, competitive fluorescence quenching studies were also conducted using Hoechst 33258 (Hoe), a synthetic derivative of N-methylpiperazine known to bind specifically within the minor groove of DNA [7]. Minor groove binding represents a non-covalent interaction typical for small-molecule drugs and bioactive compounds, often associated with sequence-selective recognition [42]. The fluorescence titration of the pre-formed Hoe–DNA complex with increasing concentrations of complex **2c** is shown in Figure 9. A gradual decrease in fluorescence intensity suggests that complex **2c** can effectively displace Hoe from the minor groove, indicating a possible groove-binding mode of interaction with DNA.



**Figure 9.** (a) Emission spectra of Hoe-DNA in the absence and presence of complex **2c**. pH = 7.4;  $\lambda_{\text{ex}} = 346$  nm; (b) plots of  $I_0/I$  versus concentration of quencher [**2c**].

The decrease in fluorescence intensity, as shown in Figure 9, indicates that the palladium complex **2c** is capable of displacing Hoe from the Hoe–DNA complex. The Stern–Volmer quenching

constant ( $K_{sv}$ ), calculated and presented in Table 3, supports this observation and further confirms the proposed interaction between complex 2c and DNA through minor groove binding.

By comparing the  $K_{sv}$  values obtained from the EB and Hoe fluorescence quenching experiments (Table 3), it can be concluded that the complexes interact with double-stranded DNA through both binding modes – intercalation and minor groove binding. However, the higher Stern–Volmer constant observed for the Hoe displacement assay compared to the EB assay suggests that complex 2c exhibits a stronger preference for minor groove binding over intercalative interaction.

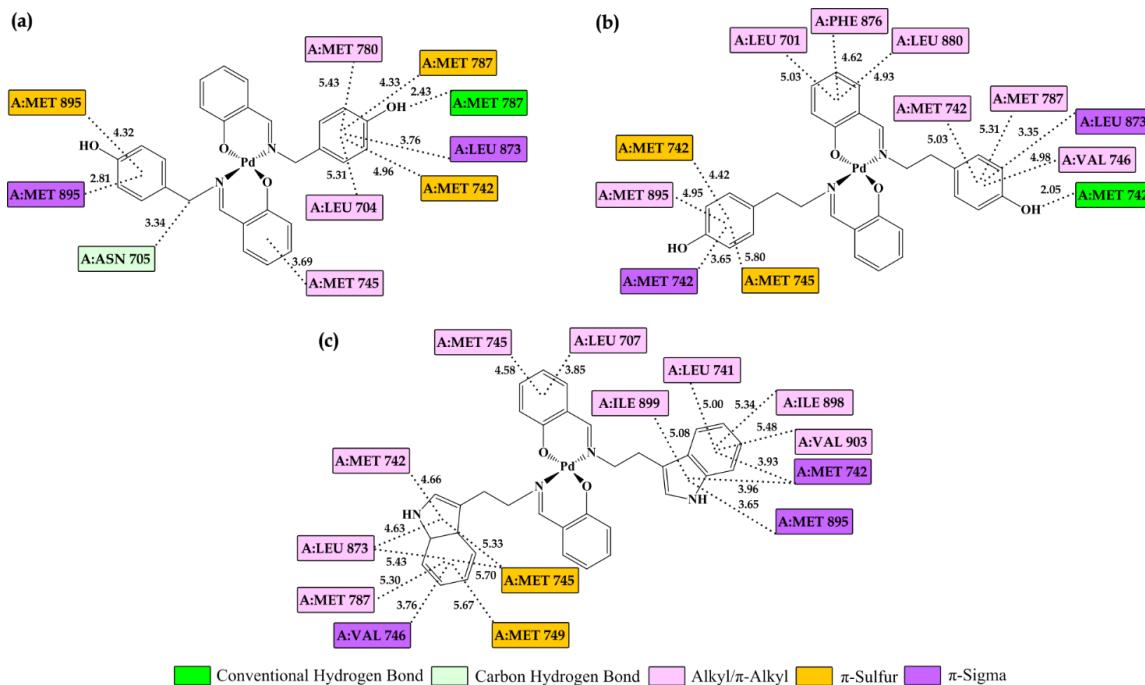
## 2.6. Molecular Docking Study

The inhibition of the AR plays a crucial role in the treatment of androgen-dependent diseases, particularly prostate cancer. Targeting AR with small molecules can modulate its activity, thereby disrupting androgen signaling pathways critical for tumor growth and progression. In this context, our molecular docking study aimed to evaluate the binding affinity of Pd-based complexes as potential AR inhibitors. As shown in Table 4, molecular docking results indicate that the **2c** complex exhibits the strongest interaction with the receptor, as evidenced by its lowest binding free energy value. Its inhibition constant, the smallest among the tested compounds, suggests a high potential for enzyme inhibition, making it the most promising candidate in this study. The **2b** complex also demonstrates significant inhibitory activity, albeit slightly weaker than **2c**, yet it still forms stable interactions with the receptor, highlighting its relevance as a potential inhibitor. In contrast, the **2a** complex shows the highest binding free energy value and the largest inhibition constant, indicating a weaker affinity for the enzyme's active site. For comparison, bicalutamide (**BIC**) was used as a reference inhibitor, and the results reveal that **2c** exhibits a stronger receptor binding affinity. These findings underscore the potential of **Pd** complexes as **AR** receptor inhibitors, with **2c** emerging as the most promising candidate for further investigation.

**Table 4.** Thermodynamic parameters (kcal/mol) of **Pd** complexes at the active site of Androgen Receptor (**AR**) determined after molecular docking simulation.

Complex	$\Delta G_{bind}$	$K_i$ ( $\mu\text{M}$ )	$\Delta G_{inter}$	$\Delta G_{vdw+hbond+desolv}$	$\Delta G_{elec}$	$\Delta G_{total}$	$\Delta G_{tor}$	$\Delta G_{unb}$
<b>AR</b>								
<b>2a</b>	-5.56	83.36	-7.21	-7.23	0.02	-1.29	1.65	-1.29
<b>2b</b>	-8.44	0.65	-10.64	-10.62	-0.02	-1.74	2.20	-1.74
<b>2c</b>	-10.51	0.02	-12.16	-12.21	0.05	-2.47	1.65	-2.47
<b>BIC</b>	-9.11	0.21	-10.76	-10.65	-0.11	-1.92	1.65	-1.92

The molecular interactions between Pd derivatives and **AR**, illustrated in Figure 10, offer valuable insights into the potential inhibitory properties of these compounds. Non-covalent interactions contribute significantly to the stability of the ligand-protein complex by reinforcing the binding affinity within the enzyme's active site. Hydrophobic interactions predominantly enhance the stability of the complex by facilitating favorable packing and minimizing solvent exposure, while conventional hydrogen bonds provide additional stabilization through directional electrostatic forces, further strengthening the ligand-enzyme interaction.



**Figure 10.** Visualization of key interactions between the Androgen Receptor (AR) and a) **2a**, b) **2b**, and c) **2c** in a 2D representation. Interatomic distances are measured in Å, with different colors indicating various interaction types, as described in the legend.

The hydrophobic segments of palladium derivatives enable interactions with various amino acids, such as A:LEU 704, A:VAL 746, and A:ILE 899, leading to the formation of alkyl/π-alkyl contacts. Additionally, other types of non-covalent interactions, including π-sigma and π-sulfur interactions, play a crucial role in ligand stabilization. π-sigma interactions arise due to the interaction between the electron cloud of aromatic rings and sigma bonds within the molecule [44], as observed in **2c** interacting with A:MET 742, A:VAL 746, and A:MET 895. Similarly, **2a** and **2b** also establish interactions with these amino acids, in addition to A:LEU 873. π-sulfur interactions, which involve interactions between sulfur atoms and the π-electron systems of aromatic rings [45], are characteristic of enzymes containing methionine residues, including A:MET 742, A:MET 745, A:MET 749, A:MET 787, and A:MET 895.

Conventional hydrogen bonds are essential for maintaining the stability of ligand–enzyme complexes [44,46]. These interactions arise between the hydroxyl groups of **2a** and **2b** derivatives and polar amino acid residues within the enzyme's active site, such as A:MET 742 and A:MET 787. Moreover, a distinct carbon-hydrogen bond is formed between the **2a** derivative and A:ASN 705, further stabilizing the complex and ensuring the proper alignment of the ligand within the catalytic pocket. All these interactions collectively play a crucial role in stabilizing the ligand within the enzyme complex.

The observed interactions with the androgen receptor (AR) suggest that **Pd** complexes can effectively modulate receptor activity. Overall, these diverse binding modes highlight the potential of palladium derivatives for therapeutic applications, particularly in targeting key regulatory proteins like AR. The molecular docking results indicate that all examined Pd complexes exhibit significant interactions with key regulators of apoptosis – caspase 3 (CASP3), Bcl-2-associated X protein (BAX), and the anti-apoptotic B-cell lymphoma 2 (BCL2) protein. The negative values of the binding free energy ( $\Delta G_{bind}$ ) suggest the stability of the formed complexes, while variations in the obtained parameters provide insight into the binding preferences of these compounds for pro-apoptotic (CASP3, BAX) and anti-apoptotic (BCL2) proteins.

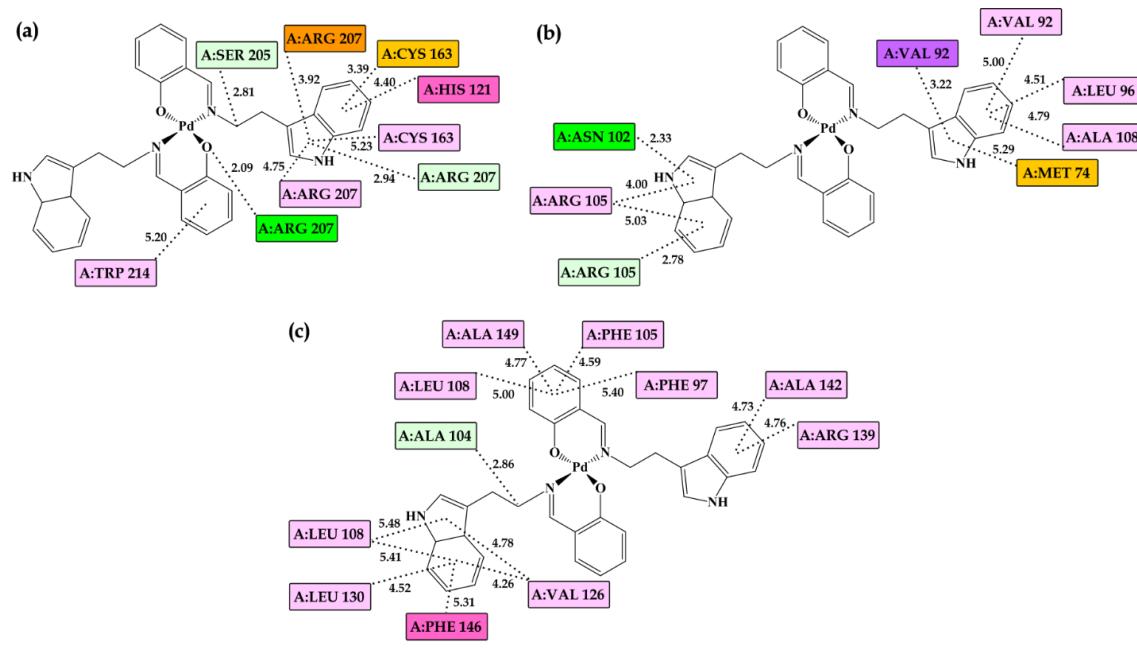
Analysis of the data in Table 5 highlights **2c** as the strongest binding ligand, exhibiting the lowest  $\Delta G_{bind}$  values for **CASP3** (-8.81 kcal/mol) and **BAX** (-8.44 kcal/mol). These results suggest that **Pd3**

preferentially targets pro-apoptotic proteins, which may indicate its potential to induce cell death by activating apoptosis. These findings suggest that **2c** exhibits selectivity toward pro-apoptotic proteins, highlighting its potential role as a pro-apoptotic modulator in anticancer strategies. In contrast, **2a** and **2b** display a more balanced binding affinity between pro-apoptotic and anti-apoptotic proteins, which may imply a different mechanism of action.

**Table 5.** Thermodynamic parameters (kcal/mol) of **Pd** complexes at the active sites of caspase 3 (**CASP3**), Bcl-2-associated X protein (**BCL2**), and anti-apoptotic B-cell lymphoma 2 (**BAX**) determined after molecular docking simulations.

Complex	$\Delta G_{bind}$	$K_i$ ( $\mu M$ )	$\Delta G_{inter}$	$\Delta G_{vdw+hbond+desolv}$	$\Delta G_{elec}$	$\Delta G_{total}$	$\Delta G_{tor}$	$\Delta G_{unb}$
<b>2a</b>								
<b>CASP3</b>	-7.88	1.66	-9.53	-9.49	-0.04	-1.18	1.65	-1.18
<b>BCL2</b>	-7.92	1.56	-9.57	-9.39	-0.18	-1.39	1.65	-1.39
<b>BAX</b>	-7.68	2.36	-9.32	-9.27	-0.05	-1.54	1.65	-1.54
<b>2b</b>								
<b>CASP3</b>	-7.98	1.42	-10.17	-9.92	-0.25	-1.90	2.20	-1.90
<b>BCL2</b>	-8.08	1.20	-10.27	-10.22	-0.06	-1.87	2.20	-1.87
<b>BAX</b>	-6.86	9.31	-9.06	-8.98	-0.08	-2.18	2.20	-2.18
<b>2c</b>								
<b>CASP3</b>	-8.81	0.35	-10.46	-10.41	-0.05	-2.89	1.65	-2.89
<b>BCL2</b>	-8.26	0.88	-9.91	-9.85	-0.06	-2.91	1.65	-2.91
<b>BAX</b>	-8.44	0.65	-10.09	-10.11	0.02	-2.72	1.65	-2.72

Since **2c** exhibited the most favorable binding results, it was selected to represent the intermolecular interactions with the active sites of **CASP3**, **BCL2**, and **BAX**, as shown in Figure 11. These interactions involve a combination of hydrophobic, electrostatic, and hydrogen bonding forces. Hydrophobic interactions play a central role in driving the binding process, while electrostatic forces aid in the precise alignment of **2c** within the enzyme's active site. Additionally, hydrogen bonds further stabilize the complex, enhancing both the strength and specificity of the binding. These findings underscore the molecular factors that contribute to **2c**'s potential as an effective modulator of apoptotic pathways.



Conventional Hydrogen Bond Carbon Hydrogen Bond  $\pi$ - $\pi$  T Shaped Alkyl/ $\pi$ -Alkyl  $\pi$ -Cation  $\pi$ -Sulfur  $\pi$ -Sigma

**Figure 11.** Visualization of key interactions between the **2c** and a) caspase 3 (**CASP3**), b) Bcl-2-associated X protein (**BCL2**), and c) anti-apoptotic B-cell lymphoma 2 (**BAX**) in a 2D representation. Interatomic distances are measured in Å, with different colors indicating various interaction types, as described in the legend.

In the context of **CASP3** (Figure 11a), **2c** engages in a conventional hydrogen bond with A:ARG 207, along with carbon-hydrogen interactions involving A:SER 205 and A:ARG 207. Additionally, alkyl/π-alkyl interactions are observed with A:CYS 163, A:ARG 207, and A:TRP 214. The π-π interactions can adopt different configurations, such as edge-to-face (T-shape) or offset stacking (shifted parallel alignment), depending on the local geometry and chemical environment of the binding site [45,47]. Notably, A:HIS 121 participates in one of these π-π interactions, adopting an edge-to-face (T-shape) configuration. **2c** also establishes a π-sulfur interaction with A:CYS 163, further stabilizing its binding. Moreover, electrostatic interactions play a critical role in ligand stabilization, particularly π-anion and π-cation interactions, which arise when the electron cloud of an aromatic ring interacts with an anion or cation [44,48]. In this context, **2c** forms a π-cation interaction with A:ARG 207, underscoring the selectivity of its binding. Together, these diverse interaction types contribute to a strong binding affinity, aligning with the observed activation of caspase-3. More broadly, they are essential for the stability and specificity of protein-ligand complexes, as they enhance charge complementarity between the ligand and active-site residues, ultimately strengthening overall binding affinity.

For **BCL2** (Figure 11b), **2c** establishes a conventional hydrogen bond with A:ASN 102 and a carbon-hydrogen bond with A:ARG 105. Additionally, alkyl/π-alkyl interactions are observed with A:VAL 92, A:LEU 96, A:ARG 105, and A:ALA 108, while π-sulfur hydrophobic interactions occur with A:MET 74. Notably, characteristic π-sigma interactions are also present with A:VAL 92. The inhibition of **BCL2** disrupts mitochondrial membrane stability, triggering the release of pro-apoptotic factors such as cytochrome c, a key molecule in the initiation of apoptosis.

In the case of **BAX** (Figure 11c), **2c** establishes a carbon-hydrogen bond with A:ALA 104 and engages in T-shaped π-π interactions with A:PHE 146. Additionally, alkyl/π-alkyl interactions are observed with multiple residues, including A:PHE 97, A:VAL 126, A:LEU 130, and A:ALA 149. These stabilizing interactions enhance **BAX** activation, promoting pore formation in the mitochondrial membrane, which facilitates the release of apoptotic factors and drives the process of programmed cell death.

The molecular interactions of **Pd**-based complexes with **CASP3**, **BCL2**, and **BAX** offer valuable insights into their potential role in modulating apoptotic pathways. Through diverse binding modes – including hydrogen bonding, π-π stacking, alkyl/π-alkyl interactions, π-sulfur interactions, and electrostatic forces – these complexes contribute to the stability and specificity of protein-ligand interactions. Collectively, these findings underscore the ability of **Pd**-based complexes to regulate key apoptotic proteins, highlighting their potential as therapeutic agents for apoptosis-related diseases. The specificity and strength of their interactions with critical residues further reinforce their significance in fine-tuning apoptotic signaling pathways.

### 3. Materials and Methods

The complete Methodology section is given in Supplementary Material

### 4. Conclusions

Three novel palladium complexes **2a-c**, derived from Schiff base ligands combining salicylic and tyramine/tryptamine or *p*-hydroxyl benzylamine scaffolds in structure, have been synthesized and characterized. The complexes displayed potent anti-proliferative activity against both human prostate cancer cell lines PC-3 and DU-145 and good selectivity between cancer and normal cells. Complex **2c**, combining salicylic and tryptamine structural motifs, has demonstrated even better activity than cisplatin for both tested cell lines, with IC<sub>50</sub> values of 7.1 μM (DU-145) and 8.6 μM (PC-3). The apoptotic assay has revealed the potential of complexes for apoptosis induction through the

Bcl-2 and caspase family activation pathways. Molecular docking studies revealed that complex **2c** exhibits the strongest binding affinity toward the androgen receptor (AR), surpassing the reference inhibitor bicalutamide. Additionally, **2c** showed selective interactions with pro-apoptotic proteins **CASP3** and **BAX**, involving stable non-covalent forces such as hydrophobic contacts,  $\pi$ - $\pi$  stacking,  $\pi$ -sulfur, and hydrogen bonds. These interactions suggest a dual mechanism of action – AR inhibition and pro-apoptotic modulation – supporting **2c**'s potential as a multifunctional anticancer agent. Taken together, these findings highlight the therapeutic potential of palladium complex **2c** as a promising lead compound for the development of novel, multitarget anticancer agents, particularly in the treatment of prostate cancer.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure SI1. IR spectra of 2a; Figure SI2. Mass spectra of 2a; Figure SI3. IR spectra of 2b; Figure SI4. IR spectra of 2c; Figure SI5. Mass spectra of 2c.

**Author Contributions:** Conceptualization, D.P. and P.C.; methodology, D.P., N.M., P.C., M.K., V.D., and Z.M.; software, S.J., M.Z., Z.M., and K.M.; validation, T.V., D.J., P.C., M.K., J. D., Z.M., and I.Z.; formal analysis, D.P., N.M., P.C., R.Z.Z., J.D., V.D., and K.M.; investigation, D.P., N.M., T.V., D.J., P.C., M.K., and I.Z.; resources, T.V., D.J., S.J., P.C., M.K., and I.Z.; data curation, P.C.; writing – original draft preparation, D.P., N.M., T.V., D.J., P.C., M.K., and I.Z.; writing – review and editing, D.P., N.M., T.V., D.J., P.C., M.K., and I.Z.; visualization, P.C., M.Z., M.K., and I.Z.; supervision, P.C.; project administration, D.P., N.M., and P.C.; funding acquisition, D.P., N.M., T.V., D.J., P.C., and I.Z. All authors have read and agreed to the published version of the manuscript.

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